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Dopad rekurentních mutací na patogenezi a léčbu chronické lymfocytární leukémie

Habilitační práce

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Poděkování

Velké poděkování patří mým současným i bývalým spolupracovníkům z Fakultní nemocnice Brno za dlouhodobou spolupráci při výzkumu mutací, jejich funkčních dopadů a testování protinádorové terapie. Rovněž tak bych chtěl poděkovat lékařům z Interní hematologické a onkologické kliniky za vstřícnost a ochotu ohledně odběru vzorků a za mnohé užitečné klinickékonzultace. V neposlední řadě patří mé vřelé poděkování Prof. MUDr. Jiřímu Mayerovi, CSc. za jeho dlouhodobou podporu mého výzkumu jakož i celé naší laboratoře.

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1. Abstrakt

Habilitační práce využívá možnosti dané Lékařskou fakultou MU a je předkládána jako soubor komentovaných publikací aspiranta. Ten se v letech 2006-2015 spolupodílel na celkem dvaceti pěti publikacích spadajících pod vymezené téma habilitace, tedy "Dopad rekurentních mutací na potogenezi a léčbu chronické lymfocytární leukémie". Z tohoto počtu bylo do příloh zařazeno a v doprovodném textu okomentováno celkem patnáct prací: u dvanácti z nich je aspirant korespondujícím autorem (souhrnný IF přibližně 107) a ve třech případech je spoluautorem na mezinárodní publikaci s významným příspěvkem k danému tématu.

Z hlediska obsahového je práce rozdělena na tři okruhy, z nichž nejrozsáhlejší tvoří výzkum mutací v genu *TP53*. Lze říci, že na tomto poli má v současné době Centrum molekulární biologie a genové terapie FN Brno vskutku mezinárodní renomé. V komentářích k publikacím je nastíněn vývoj uvažování nad touto problematikou a jsou zmíněny výsledky experimentů od základních mutačních analýz tohoto genu, až po sofistikovanou analýzu pomocí sekvenování nové generace. Na problematiku *TP53* pak navazuje část věnovaná genům *ATM* a *SF3B1*. Zatímco v případě ATM jde o logický krok rozšiřující mapování dysfunkce v dráze okolo proteinu p53, u proteinu SF3B1 se jedná o jeho nečekané zapojení do regulace buněčné odpovědi na poškození DNA. Dvě komentované publikace jsou pak věnovány tématu *in vitro* testování současných terapeutik, fludarabinu a monoklonálních protilátek, na buňkách CLL s charakterizovanými aberacemi.

Dlouhodobý a systematický výzkum mutací a dalších genových defektů přispěl k prognostické a prediktivní stratifikaci pacientů s CLL léčených na IHOK FN Brno. Kromě toho postupně vedl k vytvoření unikátní kolekce vitálně zamražených primárních kultur CLL s dobře charakterizovanými genovými defekty. Logickým pokračováním výzkumu je tak v současné době probíhající částečný posun od mutačních analýz k vývoji a testování inovativní protinádorové terapie.

2. Základní patogeneze chronické lymfocytární leukémie

Chronická lymfocytární leukémie (CLL), nejčastější lymfoproliferativní onemocnění dospělých v západní civilizaci, se projevuje akumulací patologických B-lymfocytů o fenotypu CD5(+)CD19(+)CD23(+)sIgdim v periferní krvi, kostní dřeni, lymfatických uzlinách, játrech a slezině. V souhrnu se dá říci, že tato akumulace CLL buněk je stimulována třemi základními faktory působícími ve vzájemné součinnosti: (i) signalizací z B-buněčného receptoru (BCR) do nitra buňky navozují základní předpoklad pro její přežití (*falešnou* potřebnost v hematopoéze resp. imunitě), (ii) signalizací mikroprostředí skrze příslušné povrchové receptory B-buněk, rovněž navozující výraznou anti-apoptotickou ochranu a (iii) postupným hromaděním vnitřních genetických defektů podporujících maligní transformaci a progresi. Všem těmto třem zmíněným aspektům se bude věnovat následný stručný přehled patogeneze chronické lymfocytární leukémie.

B-buněčný receptor. CLL je principiálně (tj. u naprosté většiny pacientů) monoklonální onemocnění, což znamená, že maligní B-lymfocyty nesou jednu konkrétní přestavu V-, D-, a J-subgenů variabilní oblasti těžkého řetězce imunoglobulinového genu (IGHV). Absence či přítomnost somatických hypermutací v této oblasti pak determinuje dva základní subtypy CLL s výrazně odlišnou prognózou: pacienti bez somatických hypermutací (homologie s nejbližší zárodečnou linií IGHV ≥98%; v dalším textu U-CLL) vykazují nepříznivý průběh onemocnění s brzkou potřebou léčby a střední dobou přežití přibližně 8 let, zatímco pacienti s mutovaným IGHV (homologie s nejbližší zárodečnou linií <98%; v dalším textu M-CLL) léčbu většinou nepotřebují vůbec nebo až po delší době a medián přežití u nich dosahuje asi 25 let. Asociace mezi přítomností somatických hypermutací a prognózou CLL pacientů byla publikována v roce 1999 dvěma nezávislými pracovišti ve Velké Británii a USA (Hamblin et al., 1999, Damle et al., 1999) a dodnes patří k největším objevům na poli výzkumu CLL. Rozdělení pacientů na tyto dvě prognostické skupiny nebylo prakticky nikdy zpochybněno žádnou studií. Souvislost mezi statusem IGHV a prognózou je tedy zřejmě platná univerzálně a nejinak je tomu i na pracovišti *Interní hematologické a onkologické kliniky* (IHOK) FN Brno. Během následujících let se rovněž podařilo prokázat, že pacienti s U-CLL vykazují daleko častěji přítomnost dalších negativních prognostických faktorů jakými jsou např. cytogenetické aberace 11q- a 17p- nebo mutace v genu *ATM*. V principu se dá říci,

že špatná prognóza pacientů s U-CLL je těmito aberacemi ještě dále prohloubena (Oscier et al., 2002; Lin et al., 2002; Stankovic et al., 2002).

Na základě mutačního stavu IGHV vznikl model chronické lymfocytární leukémie, ve kterém U-CLL pochází z B-lymfocytu, který ještě neprošel germinálním centrem a nesetkal se tudíž s antigenem, zatímco M-CLL reprezentuje opačnou situaci. Nicméně tento model je zpochybněn jedním základním faktem: jak U-CLL tak i M-CLL preferenčně využívá určité V(H) subgeny a toto využití je ještě navíc spojeno s charakteristickým mutačním stavem (Klein and Dalla-Favera, 2010; Ghia and Caligaris-Cappio, 2006). Například u CLL nejčastěji používaný subgen vůbec, V(H)1-69, je spojen téměř výlučně s U-CLL, zatímco rovněž hojně používaný subgen V(H)4-34 se naopak typicky nachází u pacientů s M-CLL (Ghia and Caligaris-Cappio, 2006). Celkově se u každého subtypu CLL vyskytuje řádově několik desítek dominantně se opakujících V(H) subgenů a rovněž celý B-buněčný receptor (tedy těžký a lehký řetězec imunoglobulinu posuzovaný dohromady) vykazuje u značné části pacientů uniformní sestavu, neboli tzv. stereotypní motivy (Stamatopoulos et al., 2007). Rovněž proces somatické hypermutace samotné vykazuje u pacientů s M-CLL stereotypní znaky, tzn. vede u jednotlivých protilátek k přesnému cílení na určité aminokyseliny, aby tak zůstala zachována specificita vazby na antigen (Murray et al., Blood, 2008). Všechny tyto poznatky tedy v souhrnu silně indikují, že u obou subtypů CLL zřejmě dochází k selekci (stimulaci) leukemického klonu specifickými antigeny, možná autoantigeny (Messmer et al., 2004).

Opakující se motivy v IGHV vedly výzkumníky zabývající se CLL k vytvoření kategorie tzv. stereotypních subsetů. Jak se nakonec ukázalo při analýze více než sedmi tisíc pacientů, do těchto subsetů spadá celkově až 30 % z nich (Agathangelidis et al, 2012). Stereotypní subset se dá v podstatě představit jako užší, specifičtější podskupina pacientů nesoucích určitý V(H) subgen. Tak například existuje relativně početná skupina pacientů se subgenem V(H)3-21, která vykazuje nepříznivou prognózu bez ohledu na mutační stav IGHV, což je u CLL výjimka. Část těchto pacientů pak spadá do stereotypního subsetu označovaného jako subset #2 a jsou to ti, kdož nesou lehký řetězec IGLV3-21 a určitou specifickou oblast CDR3 (complementaritydetermining region 3); výmluvné přitom je, že přináležení do subsetu dále prohlubuje negativní dopad daného IGHV (Baliakas et al., 2015). Při výzkumu pacientů se stereotypními motivy IGHV se následně velmi překvapivě

ukázalo, že jednotlivé subsety jsou spojeny s určitými typickými genomickými aberacemi a/nebo rekurentními mutacemi. Tak například výše zmíněný klinicky agresivní subset #2 je asociován s abnormálně vysokou frekvencí mutací v genu *SF3B1* (Strefford et al., 2013). Naopak jen velmi zřídka vykazují příslušní pacienti abnormality v genu *TP53* (Malcikova et al., 2014). Naše vlastní výzkumná práce (myšleno aspiranta a jeho týmu) pak navázala na tyto poznatky a začlenila do subsetu #2 jako typický znak i mutace v genu *ATM* (viz podrobnější diskuze dále v textu).

Úroveň (síla) signalizace z BCR má přímý vztah ke klinické agresivitě CLL. Asi nejlépe je možné tuto situaci dokumentovat skrze aktivitu kinázy ZAP-70 (zeta-associated protein 70 kDa). Tato tyrosin-kináza je za normálních okolností exprimována T-lymfocyty a její zvýšená exprese (resp. aktivace) doprovázející signalizaci z imunoglobulinového komplexu byla překvapivě nalezena u významné části pacientů s CLL, dominantně těch patřících ke skupině U-CLL (Chen et al., 2002; Wiestner et al., 2003). Zvýšená exprese ZAP-70 byla dokonce navržena jako zástupný marker za mutační status IGHV (Crespo et al., 2003). Zřejmě přesnější vymezení je však takové, že zvýšená exprese genu resp. proteinu ZAP-70 je nezávislým negativním prognostickým faktorem u CLL pacientů bez ohledu na mutační stav IGHV (Durig et al., 2003).

Role mikroprostředí. CLL je prototypové onemocnění pokud se týče závislosti maligních lymfocytů na zdravých buňkách hematopoetického systému a prostředí krvetvorných tkání. V těsné součinnosti s nimi se odvíjí nejenom postupná progrese onemocnění, ale také se determinuje rezistence na terapii (ten Hacken a Burger, 2014). Rozhodující role mikroprostředí v podpoře přežívání CLL buněk může být odpozorována již z jednoduché skutečnosti, že osamocené CLL buňky nasazené do tkáňové kultury nejsou schopny proliferace a významná část buněk hyne poměrně rychle (v řádu dnů) spontánní apoptózou (Collins et al., 1989). Naopak podpora skrze patřičné mikroprostředí, vytvořené například prostřednictvím ko-kultivace s podpůrnými stromálními buňkami z kostní dřeně, nebo aplikací vybraných cytokinů (např. IL-4), vede k výrazně zvýšené viabilitě CLL buněk a jejich účinné ochraně před spontánní i léky indukovanou apoptózou (Danescu et al., 1992; Panayiotidis et al., 1996; Kurtova et al., 2009). K této ochraně je nezbytný přímý fyzický kontakt mezi buňkami stromatu a CLL lymfocyty, který je dominantně zprostředkován na straně

těch prvních chemokinovým ligandem 12 (CXCL12) a na povrchu CLL lymfocytů pakchemokinovým receptorem 4 (CXCR4) (Burger et al., 1999; ten Hacken a Burger, 2014). Základním vnitřním (v CLL buňkách) molekulárním mechanismem ochrany před apoptózou je zřejmě zvýšená hladina proteinů Mcl-1 (Kurtova et al., 2009) respektive Tcl-1 (Sivina et al., Leukemia 2012). Také z experimentů analyzujících mikroprostředí přímo u CLL pacientů jednoznačně vyplynulo, že na přežívání maligních buněk se spolupodílejí různé odlišné populace stromálních buněk i nemaligní lymfocyty (Burger a Peled, 2009).

Při použití určité kombinace ochranných a podpůrných faktorů je dokonce možné přimět CLL buňky ve tkáňové kultuře k částečné proliferaci (Pascutti et al., 2013). Proliferace CLL buněk v těle pacientů se pak odehrává zejména v kostní dřeni a lymfoidních tkáních jako jsou uzliny nebo slezina. Při důmyslném experimentu s tzv. těžkou vodou (^2H_2O; deuterium) schopnou inkorporace do nově syntetizované DNA a podávané pacientům po dobu 84 dnů se ukázalo, že v těchto proliferačních centrech dochází k obměně 0,1 % až >1 % CLL buněk za den, přičemž pacienti s vyšším *obratem* maligních lymfocytů vykazovali častěji progresi onemocnění a nepříznivou prognózu (Messmerat al., 2005). Tímto experimentem byla, mimo jiné, definitivně pohřbena původní hojně rozšířená představa, že CLL je onemocnění s pomalou akumulací lymfocytů *pouze* rezistentních na apoptózu, bez výraznější proliferace.

Bylo rovněž prokázáno, že mikroprostředí mění zásadním způsobem metabolismus CLL buněk, a to v rámci tzv. Warburgova efektu. Při tomto jevu si nádorové buňky vytvářejí jiný mechanismus tvorby ATP: od oxidativní fosforylace v mitochondriích přecházejí ke zvýšené akumulaci glukózy a její přeměně na laktát, a to i za podmínek dostatečného přísunu kyslíku (Samudio et al., 2009). U CLL bylo experimentálně prokázáno, že tomuto jevu opět výrazně napomáhá jejich ko-kultivace s podpůrnými stromálními buňkami, např. fibroblastovou linií HS-5 odvozenou z kostní dřeně, nebo primárními fibroblasty z kostní dřeně (Jitschin et al., 2015). Je zajímavé, že molekulárně je tento tzv. glykolytický přesmyk řízen signální dráhou Notch-c-Myc. Celá situace tak skýtá dvojí potenciální terapeutické využití: je možné uvažovat o aplikaci látky imitující glukózu, která by byla předložena CLL buňkám a narušila tak jejich metabolismus, nebo o blokování výše zmíněné molekulární dráhy, např.

pomocí tzv. γ-sekretázových inhibitorů vedoucích k přerušení aktivace proteinu Notch (Jitschin et al., 2015).

Vnitřní genetické defekty. CLL je charakteristická vysoce variabilním klinickým průběhem: toto se týká potřebnosti léčby, primární reakce na ni, délky období bez progrese (resp. do relapsu) i celkového přežití. Bylo tedy zřejmé, že stratifikace pacientů nemůže skončit u mutačního stavu IGHV. V roce 2000 vyšla průlomová německá studie, která vytvořila prognostický model celkového přežití pacientů s CLL na základě přítomnosti čtyř prominentních chromozomálních aberací: delecí 11q, 17p a 13q a trizomie chromozómu 12 (Dohner *et al.,* 2000). Protože tyto aberace se vyskytují u jednotlivých pacientů jak osamoceně, tak i v různých kombinacích, bylo potřeba vytvořit model hierarchický na základě biologické a klinické závažnosti jednotlivých defektů. To znamená, že pokud má pacient defekty dva (nebo více), spadá do kategorie dle nejzávažnějšího z nich. Tato hierarchická klasifikace přinesla poměrně jasné rozčlenění pacientů - dle nepříznivého dopadu se defekty seřadily takto: delece 17p (medián přežití 32 měsíců), delece 11q (79 měsíců), trizomie 12 (114 měsíců), normální karyotyp (111 měsíců) a delece 13q (133 měsíců). Zcela jasný byl také rozdíl v délce období bez progrese onemocnění po prodělané terapii - desetinásobný mezi nejhorším a nejpříznivějším defektem, tedy delecemi 17p a 13q (9 vs. 92 měsíců). V dalších letech pak většina světových pracovišť zabývajících se výzkumem a léčbou CLL tento hierarchický model potvrdila.

Z hlediska obecné onkogeneze je zřejmé, že cytogenetická delece určité chromozomální oblasti je pouze jednou částí patogenního procesu, který ve svém finálním výstupu směřuje k inaktivaci nějakého důležitého protinádorového genu skrze paralelní mutaci na druhé alele. Pokud budeme uvažovat neznámější tumor-supresorový gen vůbec, tedy *TP53* kódující protein p53, můžeme na situaci “delece vs. mutace” nahlížet také takto: z obecného modelu p53 mutageneze vyplývá, že záměnové mutace by měly mít větší dopad na výslednou aktivitu proteinu p53 v buňce než cytogenetická delece. Zatímco při cytogenetické deleci jedné alely se (teoreticky) sníží výsledné množství proteinu p53 v buňce na polovinu (pomineme-li možnou mírnou kompenzaci skrze zvýšenou expresi ze zachované alely), jedna záměnová mutace může, opět teoreticky (reálně spíše dle konkrétní mutace), narušit až 15 ze 16 finálních molekul p53. Tento protein totiž funguje ve výsledné podobě jako tetramer, na jehož složení se podílí polypeptidová vlákna

syntetizovaná dle obou alel genu (Weinberg,2007). Mutace v genu *TP53* se skutečně ukázaly jako velmi významný, na deleci 17p nezávislý prognostický faktor u pacientů s CLL. Situace bude podrobněji diskutována v další části práce v samostatné kapitole s komentářem k příspěvku aspiranta, a proto se jí dále v tomhle stručném přehledu patogeneze nevěnujeme. Totéž se dá říct i o mutacích v genu *ATM*, které se vyskytují zhruba u jedné třetiny pacientů s delecí 11q. ATM, klíčová regulační kináza proteinu p53, bude rovněž diskutována podrobněji dále. A do třetice to stejné zmiňujeme i pro gen *SF3B1*, který je jedním z nově objevených cílů mutageneze v CLL buňkách. Průlomové práce využívající celogenomové nebo celoexomové sekvenování, publikované postupně od roku 2011, přispěly zásadním způsobem k osvětlení některých aspektů patogeneze CLL a k dotvoření portfolia genových defektů determinujících toto onemocnění. Vedle *TP53*, *ATM* a *SF3B1* identifikovaly tyto práce jako významně rekurentně mutovaný gen také *NOTCH1* (Puente et al., 2011), u kterého se vyskytuje typická (*hot-spot*) mutace v podobě dvounukleotidové delece vedoucí ke zkrácenému, konstitutivně aktivovanému proteinu (Di Iani et al., 2009). To bylo velmi zajímavé zjištění, protože o proteinu Notch1 bylo již v tu dobu známo, že jeho aktivita koreluje s přežitím CLL buněk *in vitro* a s jejich rezistencí na apoptózu (Rosati et al., 2009). V souladu s tímto poznatkem byly následně aktivující mutace v *NOTCH1* asociovány s agresivitou CLL, rezistencí na chemoterapii, a dokonce i s transformací do Richterova syndromu (Fabbri et al., 2011; Rossi et al., 2012a).

Většina vědeckých prací diskutujících patogenezi CLL uvažuje až zralý B-lymfocyt, který tvoří základní buněčnou populaci tohoto onemocnění. Velmi překvapivé však bylo zjištění, že predispozice pro CLL se zřejmě ukrývá již v kmenových buňkách hematopoetického systému. Japonští autoři (Kikushige et al., 2011) xenotransplantovali hematopoetické kmenové buňky (HSC) od pacientů s CLL a zdravých kontrol do imunodeficientních myší a sledovali jejich schopnost vyvolat klonální expanzi B-lymfocytů. Na rozdíl od zdravých kontrol byly této (oligoklonální) expanze schopny pacientské HSC. Protože sekvence IGHV ani genomické aberace identifikované v této expandované populaci B-lymfocytů se nekryly s původní sestavou od jednotlivých pacientů, bylo zřejmé, že vývoj onemocnění silně připomínajícího CLL proběhl vlastně od počátku - právě díky nějaké dosud neobjasněné predispozici ukryté v HSC. Že se leccos důležitého z hlediska

molekulární patogeneze děje již dříve, než dojde k vytvoření finálního zralého maligního B-lymfocytu, podpořila i studie stopující známé patogenní mutace u CLL v progenitorových buňkách společných pro B-lymfocyty a jiné krevní řady (Damm et al., 2014). Bylo zjištěno, že se v těchto progenitorech nacházejí mj. známé mutace např. v genech *BRAF*, *NOTCH1* či *SF3B1*. Výskyt patogenních mutací mimo vlastní CLL lymfocyt byl prokázán také například u genu *ATM*, a to jak v podobě mutace ve společném progenitoru B- a T-lymfocytu, tak i v zárodečné formě (Stankovic et al., 2002; Navrkalova et al., 2013a).

V souhrnu se dá říci, že CLL je v současné době extrémně zajímavým modelem maligní transformace a progrese. Lze jen doufat, že exploze poznatků molekulární biologie povede v brzké době k překonání rezistence na terapii pozorované u značné části pacientů a tato leukémie přestane být uváděna s dovětkem “nevyléčitelné onemocnění”. Reálnou naději, zdá se, skytají inovativní malé molekuly navozující přerušení signalizace z BCR: *ibrutinib* cílený na Brutonovu tyrosinkinázu (BTK) nebo *idelalisib* blokující fosfoinositid 3-kinázu delta (PI3Kδ) (Crassini et al., 2015). Rovněž na našem pracovišti se budeme snažit testovat zajímavé inovativní molekuly s potenciálním protinádorovým účinkem, pokud budeme mít tu vzácnou přiležitost se k některým z nich dostat. Příkladem může být inhibitor OH209EN1 cílený na protein *checkpoint kinase 1* (Chk1), který vyvinul Dr. Kamil Paruch z Ústavu chemie Masarykovy univerzity a který vykazuje v dosavadních testech extrémně zajímavé účinky na primární buňky CLL (Zemanova et al., 2015).

3. Prognostická a prediktivní stratifikace pacientů s komentářem ohledně vlastního výzkumného přínosu k této problematice

Při založení *Centra molekulární biologie a genové terapie* (CMBGT) spadajícího pod IHOK FN Brno vyvstala otázka, jaké onemocnění zvolit pro hlavní výzkumnou činnost nově vzniklé laboratoře. Po intenzivní diskuzi s vedením kliniky byla nakonec vybrána chronická lymfocytární leukémie a čas ukázal, že to bylo dobré rozhodnutí. Málokteré hematoonkologické onemocnění prodělalo tak intenzivní výzkum a bezprecedentní akumulaci principiálně nových poznatků týkajících se i obecné onkogeneze. V tomto smyslu můžeme zmínit například roli mikroRNA, jejichž deregulace byla do kontextu onkogeneze zasazena primárně právě díky výzkumu CLL (Calin et al., 2002; Calin et al., 2004). Stěžejním pozitivním faktorem ohledně výzkumu CLL se ukázala rovněž skutečnost, že lze využít opakovaně odebírané vzorky periferní krve pacientů v průběhu onemocnění. Díky ochotnému a profesionálnímu přístupu lékařů z IHOK FN Brno se podařilo nastavit velmi kvalitní a propracovaný systém odběrů, který umožnil mj. analyzovat a částečně pochopit zákonitosti selekce nových mutací během vývoje CLL a terapie pacientů.

Vlastní výzkumná práce aspiranta a jeho nejbližších spolupracovníků na poli CLL započala v roce 2002 a od počátku se zaměřila zejména na analýzu defektů v genu *TP53* a jejich funkční dopad. Bylo tak činěno ze zřejmého důvodu: pacienti s CLL s delecí 17p vykazovali konzistentně špatnou prognózu napříč tehdejšími publikacemi a také bylo dobře známo, že dysfunkce proteinu p53 je spojena s agresivním klinickým průběhem u řady dalších hematoonkologických onemocnění (Robles and Harris, 2010). K úspěšnému rozvoji výzkumu přispělo navázání spolupráce s Ústavem patologie FN Brno, konkrétně s výzkumnou skupinou prof. Jany Šmardové, kde byla zavedena pro účely analýzy pacientů s CLL kvasinková funkční analýza FASAY. Ta umožnila efektivní predikci pacientů s mutací v genu *TP53*, přičemž konkrétní záměna v DNA pak byla následně identifikována v CMBGT sekvenačními metodikami. Společně s výsledky analýz delecí 17p prováděných pomocí fluorescentní *in situ* hybridizace (FISH; nejprve na Oddělení lékařské genetiky FN Brno a později v CMBGT) se podařilo vytvořit unikátní systém pro sledování obou alel *TP53* a vymezení jejich vzájemného vztahu. Později, konkrétně v roce 2009, jsme započali rovněž s výzkumem mutací v genu *ATM*, kódujícím kinázu se stěžejním významem pro aktivaci proteinu p53 po vzniku

dvouřetězcových zlomů v DNA. Sledování dvou genů zapojených do jedné signální kaskády nám umožnilo lépe pochopit některé zákonitosti selekce dysfunkce v buněčné odpovědi na poškození DNA a také rozšířit okruh rizikových pacientů z hlediska možné špatné reakce na terapii a negativní prognózy onemocnění. O dalších několik let později, zhruba od roku 2013, jsme pak začlenili do našeho výzkumu i gen *SF3B1.* Jeho mutace se hojně vyskytují u pacientů na naší klinice, v souladu s publikacemi predikujícími jejich výskyt na základě pilotních celogenomových resp. celoexomových analýz. Poslední oblastí výzkumu aspiranta a jeho pracovního týmu se pak stalo testování vybraných látek s antileukemickým účinkem na buňkách CLL a příbuzných modelech. Toto testování doprovázelo v některých případech základní mutační analýzy a stalo se tak součástí příslušných publikací. V některých případech pak testování protirakovinných látek tvořilo samostatný výzkumný projekt. K celkové filozofii budiž řečeno, že nevyužít potenciál mnoha set kultur CLL vitálně zamražených v tekutém dusíku s dobře chakterizovanými mutacemi a dalšími genovými defekty by byla ohromná škoda a nevyužitá šance. Proto se toto *in vitro* testování stává v současné době stěžejní částí výzkumu aspiranta a jeho týmu. Důležité bude rovněž začlenit do tohoto testování myší model, k čemuž již byly podniknuty patřičné kroky (Verner et al., 2015).

Dle výše nastíněné stručné geneze výzkumu na pracovišti je habilitační práce v dalším textu rozdělena na kapitoly pojednávající o výzkumu (a) mutací v genu *TP53* a jejich funkčním dopadu, (b) mutací v genech *ATM* a *SF3B1* a jejich funkčním dopadu a (c) testování látek s antileukemickým účinkem na buňkách CLL *in vitro*. Vlastní výzkum je vždy zasazen do kontextu celosvětového výzkumu. Do příloh jsou zařazeny publikace, u kterých je aspirant korespondujícím autorem a zaštiťoval tak daný projekt, a dále pak několik publikací mezinárodních, u kterých je aspirant spoluautorem, vždy však jen v případě, pokud byl jeho příspěvek k danému projektu opravdu významný. Všechny další spoluautorské práce, domácí i zahraniční, jsou uvedeny pouze v přehledu publikací.

3.1 Analýza mutací v genu *TP53* a jejich fukční dopad

Publikace aspiranta vztahující se k tématu (chronologicky 2006-2015)

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Komentář:

Z přehledu patogeneze CLL uvedeného výše je zřejmé, že pacienti s delecí jedné kopie genu *TP53* (17p-) vykazují jednoznačně nejhorší prognózu. A to v nejširším možném smyslu slova: terapii potřebují většinou brzy po diagnóze, téměř uniformně na ni reagují špatně a výsledkem je nejkratší střední doba přežití ze všech genetických skupin. Podle oficiálních doporučení (*guidelines*) pro diagnostiku a léčbu CLL, by mělo být pacientům s defekty *TP53* nabídnuto zařazení do klinických studií testujících alternativní léčebné přístupy, nebo by jim měla být nabídnuta allogenní transplantace kostní dřeně (Hallek et al., 2008).

Tato neuspokojivá situace vedla následně k otázce, jak je to s výskytem mutací v genu *TP53* u pacientů s CLL. Budou tyto mutace doprovázet deleci 17p a prohlubovat dále nepříznivou prognózu? Budou se tyto mutace vyskytovat i bez delece 17p jako monoalelické nebo bialelické defekty? Přispívat k odpovědi na tyto otázky jsme začali od roku 2006, kdy jsme publikovali v časopise *Leukemia* naši první významnější práci (letter to the editor) na souboru 168 pacientů (Trbusek et al., 2006) (**příloha 1**). Výzkum pak pokračoval popisem netradičních případů pacientů se somatickými hypermutacemi v genu *TP53*, vzniklými jako důsledek patologického působení aktivací-indukované cytidin deaminázy (AID), resp. opravných mechanismů po jejím působení (Malcikova et al., 2008; Stano-Kozubik et al., 2009) (**přílohy 2** a **3**).

Zásadní odpověď na otázku významu mutací v genu *TP53* však přinesl až paralelní výzkum několika evropských pracovišť včetně naší laboratoře publikovaný v letech 2008 a 2009. Tyto práce přinesly z větší části shodné a z menší části odlišné výstupy ohledně této problematiky. První prací byla německá studie (Zenz et al., 2008), která prokázala (na relativně malém souboru pacientů) na deleci 17p nezávislý negativní prognostický dopad mutací v genu *TP53*. Náš výzkum probíhal na souboru vzorků odebraných od 400 pacientů s CLL, z nichž 70 vykazovalo nějaký defekt v genu *TP53,* a byl publikován v časopise *Blood* (Malcikova et al., 2009) (**příloha 4**). Hlavní výstupy práce byly následující:

(a) Nejčastější variantou narušení *TP53* je společná inaktivace obou alel, a to dominantně v podobě cytogenetické delece jedné a bodové mutace druhé alely. Prognóza příslušných pacientů je velmi špatná.
(b) Samostatné cytogenetické delece se vyskytují spíše ojediněle (<1 % všech *TP53* defektů), jejich selekce v leukemických buňkách je slabá.
(c) Samostatné mutace se naopak vyskytují poměrně často (přibližně 20 % všech *TP53* defektů). Nejčastějším typem alterací jsou (podobně jako u jiných typů nádorů) záměnové mutace. Prognóza pacientů s monoalelickými mutacemi je rovněž špatná. Přežití této skupiny pacientů bylo výrazně zkrácené oproti wild-type (wt) skupině, i když na druhé straně přece jen o něco lepší než u skupiny s bialelickým defektem.

(d) Leukemické buňky příslušných pacientů reagují slabě, nebo nereagují vůbec na chemoterapii (v práci byl pro toto testování použit nukleosidový analog fludarabin).
(e) Konvenční terapie výrazně přispívá k selekci nových mutací v *TP53* u pacientů, kteří před léčbou vykazovali wt status.
(f) Samostatné mutace v p53 se zřejmě selektují společně s delecí 11q, tedy společně se ztrátou jedné alely *ATM*.

Téměř souběžně s touto naší publikací vyšly ještě dvě další práce na podobné téma (Rossi et al., 2009; Dicker et al., 2009). Na základě závažnosti poznatků o mutacích v p53 u pacientů s CLL vykrystalizovalo okolo roku 2009 přesvědčení, že mutační analýza *TP53* by měla doprovázet vyšetření delece 17p pomocí FISH. První relevantní výstup v tomto smyslu byl obsažen v závěrečném doporučení z XIII. Světového workshopu na chronickou lymfocytární leukémii (IWCLL) konaném v září 2009 v Barceloně. Bylo rovněž navrženo vymezení skupiny pacientů s extrémně vysokým rizikem (tzv. ultra high-risk group) (Stilgenbauer and Zenz, 2010), u které tvořili pacienti s abnormalitami *TP53* významný podíl. Skupina je charakteristická silnou rezistencí na jinak relativně efektivní léčebné režimy kombinující chemoterapii s monoklonálními protilátkami, včetně režimu “zlatého standardu”, tedy fludarabin, cyklofosfamid, rituximab (FCR). Později se ukázalo, že vedle defektů v genu *TP53* se budou u části pacientů na této rezistenci zřejmě podílet i mutace v jiných genech, například *BIRC3* (Rossi et al., 2012b).

Náš výzkum poté pokračoval mezinárodní publikací, ve které jsme - zejména ve spolupráci s německou skupinou - vymezili základní spektrum mutací v genu *TP53* u pacientů s CLL (Zenz et al., 2010) (**příloha 5**). Tato publikace byla zamýšlena jako referenční, tedy sloužící pro srovnání profilu *TP53* mutací u dalších publikací na dané téma. Vzhledem k citovanosti (počtu citací i jejich kontextu) lze říci, že naše společná publikace v časopise *Leukemia* tento účel splnila. Další stěžejní prací našeho výzkumu genu *TP53* u pacientů s CLL se pak stala publikace v časopise *Journal of Clinical Oncology* (Trbusek et al., 2011) (**příloha6**). V tu dobu jsme již měli k dispozici výsledky vyšetření genu *TP53* u 550 pacientů. To nám umožnilo přejít od základního srovnání „mutace vs. wt skupina“ k podrobnější analýze provedené dle typu jednotlivých mutací v proteinu p53. Provedli jsme několik rozčlenění mutací podle různých kritérií, z nichž jako naprosto nejlepší se nakonec ukázalo být

vyčlenění záměnových mutací v tzv. DNA-vazebných motivech (DBMs; DNA-bindingmotifs) proteinu p53. Tyto motivy byly definovány na základě krystalografické analýzy proteinu p53 jakožto místa, která zajišťují fyzický kontakt proteinu p53 s DNA (Cho et al., 1994). Při srovnání této skupiny (a) s ostatními záměnovými mutacemi a (b) se záměnami vedoucími k eliminaci proteinu p53 se ukázala dramaticky horší prognóza pacientů s mutacemi v DBMs. A to jak z hlediska času do první terapie, tak zejména co se týče celkového přežití. Tato analýza měla velkou vypovídací hodnotu zejména proto, že uvažovala pouze pacienty s nemutovaným IGHV. I v této již *a priori* nepříznivé prognostické kohortě se dopad jednotlivých typů mutací v proteinu p53 projevil s plnou přesvědčivostí. Důvodem pro výrazně horší prognózu pacientů s CLL s mutacemi v DBMs je zřejmě zisk nějaké onkogenní funkce příslušných mutantů (gain-of-function) (Oren and Rotter, 2010).

Výsledky na poli výzkumu *TP53* mutací nám následně umožnily spolupodílet se na vytvoření mezinárodních doporučení pro jejich analýzu vypracované v rámci Evropské společnosti pro chronickou lymfocytární leukémii (ERIC) (Pospisilova et al., 2012) (**příloha 7**) a rovněž sepsat vyžádané review (prof. Sami Malek, University of Michigan, USA) o tomto tématu do knihy *Advances in Chronic Lymphocytic Leukemia* (nakladatelství Springer, New York) (Trbusek and Malcikova, 2013) (**příloha 8**).

Naše dosavadní práce na poli výzkumu mutací *TP53* u pacientů s CLL byla prozatím završena recentní publikací v časopise *Leukemia* (Malcikova et al., 2015) (**příloha 9**). V této studii jsme již naplno využili všech předností technologie tzv. sekvenování nové generace (NGS), umožňující identifikovat záměny v DNA s extrémní citlivostí (až 0,2 % mutované DNA). Práce byla postavena na retrospektivní analýze vzorků odebraných před aplikací terapie u následujících skupin pacientů:

(a) Se selekcí mutace *TP53* léčbou (mutace byla jasně patrná v relapsu standardním sekvenováním).
(b) Bez selekce mutace *TP53* léčbou (vzorek vykazoval v relapsu status wt standardním sekvenováním).

U 18 ze 20 vzorků z první skupiny jsme prokázali pomocí NGS přítomnost mutace již před terapií, v podobě malého subklonu v rozmezí 0,2 - 3,7 % mutované DNA. Naproti tomu ve druhé skupině vykazoval jen 1 pacient ze 40 takovouto minoritní

mutaci. Tímto experimentem se nám podařilo prokázat, že tyto “malé” mutace představují značné riziko selekce léčbou do podoby dominantního klonu. V souladu s tímto poznatkem pak pacienti s minoritními mutacemi vykazovali zkrácené přežití ve srovnání se skupinou wt-*TP53* před terapií. V práci jsme ve finále zesumarizovali více a méně pravděpodobné scénáře toho, co se během terapie ohledně selekce *TP53* mutací odehrává. Snažili jsme se také pečlivě oddiskutovat, na jaké aspekty je potřeba brát zřetel při využívání technologie NGS v diagnostice *TP53* mutací. Celkově naše práce vhodně doplnila italskou studii na podobné téma, která byla publikována těsně před námi (Rossi et al., 2014).

V dalším výzkumu se nyní chceme zaměřit na analýzu selekce *TP53* defektů u pacientů léčených terapeutiky působícími (předpokládaně) nezávisle na proteinu p53, zejména ibrutinibem. Zajímá nás, zda dysfunkce p53 dráhy může přinášet CLL buňkám nějakou selekční výhodu i při léčbě cílené prokazatelně mimo protein p53 a bude se tak objevovat při relapsu onemocnění, podobně jako je tomu nyní u konvenční terapie.

Inactivation of p53 and deletion of *ATM* in B-CLL patients in relation to IgVH mutation status and previous treatment

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B-cell chronic lymphocytic leukemia (B-CLL) is characterized by a highly variable clinical course. At present, mutation status of the immunoglobulin heavy-chain variable region (IgVH) represents the most significant prognostic marker, with patients manifesting a high germline homology (⩾98%) having a significantly worse prognosis.[1,2] The unmutated IgVH locus is associated with the presence of unfavorable genomic aberrations: the 11q deletion (likely to involve the *ATM* kinase) is associated very strongly,[1,2] whereas the association for the 17p deletion (involving the *p53* gene) varies in different studies from strong[1] to that of borderline statistical significance.[2] Abnormalities in these two loci seem to be mutually exclusive in B-CLL cells[3] and they markedly deteriorate an already bad prognosis for IgVH-unmutated patients.[1,2] Two recent studies[4,5] also saw a strong association between the occurrence of p53 inactivation in B-CLL patients and the presence of previous treatment, indicating a possible involvement of DNA-damaging chemotherapy in the process of selection or even induction of *p53* mutations. These two studies did not, however, analyze the IgVH mutation status. It is well known that a much higher proportion of B-CLL patients bearing the germline IgVH sequence require treatment compared to IgVH-mutated patients. We therefore decided to correlate p53 inactivation with both the IgVH mutation status and the presence of previous therapy in a large cohort of B-CLL patients. The patient population was also studied for the presence of *ATM* locus deletions.

In order to achieve the maximal efficiency for *p53* mutation screening, we used a very sensitive functional yeast assay (FASAY).[6] The samples were also screened by Western blotting (WB) in order to search for an abnormal p53 protein level, which is known often to be underlined by a gene mutation in tumor cells. The data concerning *p53* and *ATM* deletions were obtained using interphase fluorescent *in situ* hybridization (I-FISH) with locus-specific probes. PCR and direct sequencing were used to analyze the IgVH rearrangements and mutation status. A summary of basic data regarding the patients evaluated is provided in Table 1 and a summary of the tests performed is listed in Table 2.

The blood and bone marrow samples were obtained with written informed consent and total RNA or proteins were isolated from mononuclear cells separated on histopaque gradient. As the FASAY is able to identify a mutation within a large fraction of cells bearing normal *p53*, it is especially suitable for B-CLL, where the *p53*-mutated cells often represent a clonal variant of the leukemic population. In this assay, the central part of the *p53* gene (amplified from cDNA between exons 4 and 10, i.e. codons 42–374) is introduced into an $ADE2^-$ yeast strain carrying a reporter with a p53-binding site upstream of the ADE2 gene. On the plates containing a low level of adenine, the p53 wild-type (wt-p53) samples then form large white colonies, whereas the colonies with the p53 mutations (mut-p53) are small and red, owing to limited growth and accumulation of a reddish product of adenine metabolism. Samples containing a wt-*p53* gene account for more than 90% of white colonies in the assay, whereas the remaining 10% represent background – a consequence of PCR-induced point mutations or a low-quality RNA.[6] We showed previously[7] that FASAY provides the expected numbers of red colonies when using serial dilutions of wt-*p53* and mut-*p53* plasmids for PCR amplification or when using leukemic cell lines with known *p53* status. We also observed that in properly handled clinical samples (which allow a standard PCR amplification), the background of FASAY is usually under 10%.

We performed the FASAY on 168 leukocyte samples derived from peripheral blood. Twenty-three cases (13.7%) provided an elevated portion of red colonies. In 10 samples, the *Split assay*, a modified version of FASAY analysis, which tests the 5′ and 3′ halves of the gene separately, was performed. This approach was used when the number of red colonies did not get over 20% and the PCR product used for transformation was normal, or did not get over 40% and the PCR product was too faint. Using this method, we confirmed the mutation identified by FASAY in six samples, whereas the remaining four samples bore the wt-p53. The other four samples, identified by FASAY as mutated ones, were later shown to harbor an alternative splicing (discussed later). Thus, in summary, 15 samples (9%) were confirmed as mutated after we completed the FASAY, Split assay and sequencing. The results of sequencing are provided in Table 3.

Table 1 Characterization of B-CLL patients

Patients	$n=168$
Median age at diagnosis	57 years
Females	$n=52$
Males	$n=116$
Low/intermediate stages of the disease (Rai 0, I, II)	$n=119$
Advanced stages of the disease (Rai III, IV)	$n=49$
Previously untreated	$n=119$
Previously treated[a]	$n=49$
Median of lymphocytes in tested samples	83%
Median of B-CLL cells within the lymphocytes	85%

Abbreviation: B-CLL, B-cell chronic lymphocytic leukemia.
[a]According to standard protocols at physician's discretion.

Table 2 Listing of the tests performed in B-CLL samples

Functional analysis of the p53 (FASAY); WB detection of the p53 protein level	$n=168$ (basic cohort)
I-FISH detection of the *p53* locus deletion	$n=141$ (84%)
I-FISH detection of the *ATM* locus deletion	$n=116$ (69%)
Mutation status of the IgVH locus	$n=147$ (88%)
Sequencing of DNA templates from red (i.e. *p53*-abnormal) yeast colonies	$n=23$ samples 4–6 colonies/sample 2 ts[a] colonies/sample

Abbreviations: B-CLL, B-cell chronic lymphocytic leukemia; FASAY, functional *yeast* assay; I-FISH, interphase fluorescent *in situ* hybridization; IgVH, immunoglobulin heavy-chain variable region.
A relationship between occurrence of p53 or *ATM* abnormalities and mutation status of IgVH was evaluated using relative risk (RR) with 95% CIs (confidence intervals) and odds ratios (ORs) with 95% CIs. The *P*-values (significance level) were calculated using the χ^2 test for association.
[a]ts – The temperature-sensitive p53 mutants.

Table 3 Summary of the p53 inactivation

Patient	*% of red colonies*	*Affected codon*	*Nucleotide change*	*Amino-acid change*	*Deletion 17p13.1 (%)*	*Protein level*	*Homology in IgVH (%)*
M1	92.9	—	del 511–547	del 126–137 in frame	67	+++	100
M2	35.0	278	CCT→CGT	Pro→Arg	50	++	100
M3	74.9	135	TGC→GGC	Cys→Gly	76	+++	100
M4	29.7	227	del CT	Ser→fr. stop at 227	93	–	100
M5	72.5	252	del CTC	del Leu	50	++	99.0
M6	56.0	132	AAG→AAC	Lys→Asn	10	ND	ND
M7	79.2	234	TAC→TGC	Tyr→Cys	83	+++	100
M8	83.9	236	TAC→TGC	Tyr→Cys	16	++	100
M9	86.7	173	GTG→ATG	Val→Met	96	+++	100
M10	76.5	282	CGG→CCG	Arg→Pro	33	++	100
M11	96.8	248	CGG→CAG	Arg→Gln	89	+++	100
M12	29.2	178	CAC→CAG	His→Gln	16	++	100
M13	94.4	220	TAT→TGT	Tyr→Cys	ND	+++	100
M14	52.6	216	GTG→ATG	Val→Met	Negative	ND	100
M15	48.8	281	GAC→GAG	Asp→Glu	34	+++	93.8
D1	7.7	—	—	—	21	–	99.3
D2^{ATM}	9.8	—	—	—	15	–	98.3
D3	1.4	—	—	—	10	–	91.6
D4	6.1	—	—	—	9	–	95.1
APL1	9.2	—	—	—	Negative	+++	ND

Abbreviation: IgVH, immunoglobulin heavy-chain variable region.
Correlation among mutations (M1–M15), single deletions 17p13.1 (D1–D4), abnormal wt-protein level (APL1) of the p53 tumor suppressor and IgVH mutation status (% of homology to the nearest germline sequence) in B-CLL samples. % of red colonies = in FASAY or Split assay. ND = not done; +++, ++, +, −, a level of the p53 protein as estimated from the Western blots; ^{ATM}, parallel deletion of *ATM* locus in 56% of the cells. The cutoff level for the detection of *p53*- and *ATM*-deleted cells by I-FISH (5% for both probes) was calculated in a series of 15 samples obtained from normal bone marrow donors (200 interphase nuclei per slide were evaluated) using the upper limit of binomial confidence interval. Sequences deviating more than 2% from the nearest germ line were considered to be IgVH-mutated. The closest germline VH was assigned using DNA plot or IgBlast databases. The mutations M3, M7, M13 and M14 were temperature-sensitive.

In all 15 *p53*-mutated samples, a corresponding base change was identified unambiguously. Surprisingly, none of the mutations were detected more than once and we therefore do not see the mutation hotspots emerging for the B-CLL in the p53 mutation database (codons 175, 248 and 273; www-p53.iarc.fr). The reason for these population differences in the frequency of *p53* mutations remains elusive, especially because we know little about functional consequences of different mutations (i.e. there may be different mutations reported in individual studies but leading to the same defective phenotype).

To our knowledge, we report for the first time in B-CLL an alternatively spliced variant of the p53 transcript, which we identified in four patients. The transcript includes a 133-bp insertion of intron 9 sequence, which results in a truncated protein of 341 amino acids. This alternative transcript was shown to be expressed in normal human cells, especially quiescent peripheral blood lymphocytes, where it was 'hidden' within 10% of background red colonies in FASAY.[8] The protein exhibits a defective tetramerization domain with the consequence of a transcriptional defect in mammalian cells *in vivo*. We detected this version of the p53 in four patients, giving 30, 30, 24 and 14% of red colonies, respectively. All of them were without a mutation in the *p53* gene, did not express a detectable level of protein on WB, were without cytogenetic defects in *p53* or *ATM* locuses, were not previously treated and all manifested mutated-IgVH genotype with supposed indolent course. The last correlation in particular is interesting. If proven in a larger number of patients, it could be another indicator of more quiescent state of the IgVH-mutated B-CLL cells. As Flaman *et al.*[8] identified this transcript in healthy human cells, we excluded this version of p53 abnormality from the set of mutations, or p53-inactivated samples, in our report.

In order to verify the mutations identified by FASAY and reveal other potential p53 abnormalities, not underlined by a mutation in the gene, we monitored the p53 protein level. We found a very good correlation between the high p53 protein level (detected by WB with DO-1 Mab) and a corresponding mutation (12/13, 92%; see also Table 3; figure of the WB's is not shown). There was also a good overlap between the deletion and accompanying mutation in the *p53* locus; however, this was not absolute (72%; see Table 3). In spite of using a complex approach and extremely sensitive FASAY methodology, the overall percentage of p53 inactivation (12%) is within the expected range. An interesting phenomenon is the remarkable difference in the percentage of red (*p53*-mutated) colonies and percentage of *p53*-deleted cells detected by I-FISH. As both tests provide at least a semiquantitative (i.e. not absolutely exact but, also, not to a substantial extent misleading) estimate of the percentage of defected cells, these differences might theoretically reflect the order of acquirement of *p53* mutation and deletion, respectively. These conclusions, however, require caution, and consecutive tests performed on samples derived during the course of the disease will be necessary to further clarify the response to this question.

The heterozygous *ATM* locus deletion was present in 21 samples (18%; extent 20–93% of the cells) and there was the only patient with the parallel abnormality in the *p53* locus (case D2 in Table 3). We therefore confirm, in a large cohort of patients, the previous finding[3] that the *ATM* abnormalities are highly likely an alternative to the p53 dysfunction ($P<0.001$ in our set of patients). The results also confirm[2] a very strong association between the presence of *ATM* deletion and the presence of the germline IgVH sequence (20/69 = 29% vs 0/35 = 0% in IgVH-mutated subgroup; $P<0.001$). We did not,

Table 4 Individual p53 abnormalities in relation to treatment and patient survival

Patient	Treatment before p53 diagnosis made	Treatment required after p53 status known	Survival (months)
M1	No	Yes	9 rd
M2	No	Yes	17 rd
M3	Yes	Yes	36 rd
M4	Yes	Yes	26 al
M5	Yes	Yes	89 rd
M6	Yes	Yes	17 rd
M7	No	No	41 al
M8	No	Yes	35 al
M9	No	Yes	21 rd
M10	Yes	Yes	35 al
M11	No	Yes	2 rd
M12	Yes	Yes	24 rd
M13	Yes	Yes	22 rd
M14	No	Yes	27 rd
M15	No	No	23 al
D1	Yes	Yes	18 ud
D2	Yes	Yes	67 al
D3	No	No	36 al
D4	No	Yes	49 al
APL1	No	Yes	2 ud

Survival time is considered from a diagnosis of B-CLL. In case of alive patients (al), the stated survival time reflects the period from diagnosis to the last date when a patient was examined. rd, B-CLL-related death; ud, B-CLL-unrelated death.

however, monitor the status of the second allele and it is hence not clear what proportion of deleted samples were really ATM-inactivated. The p53 abnormalities were also detected markedly more frequently in the unmutated-IgVH subtype compared to the mutated one (15/90 = 16.7% vs 3/57 = 5.3%; $P<0.05$). Moreover, two of the three IgVH-mutated cases harbored just a single *p53* deletion without an accompanying mutation.

The p53 abnormalities occurred more frequently in previously treated patients than in untreated ones (18.4 vs 9.2%) and a similar trend is also seen for the *ATM* deletion (18 vs 10%). There was an obvious difference in the proportion of treated patients between the IgVH-mutated and IgVH-unmutated subgroups. In the former, only 7% of patients were previously treated – none of them with the p53 abnormality or *ATM* deletion. By contrast, within the latter subgroup, 49% of patients were previously treated and the percentage with the p53 abnormality in both treated and untreated patients was similar (20 vs 17%), which was also the case for *ATM* deletion (20 vs 26%). The p53 inactivation therefore occurred in a proportion of patients who had not received any previous therapy, and in these cases the abnormalities likely originate from spontaneous mutagenesis. The possibility, however, that DNA-damaging chemotherapy may induce or select *p53* mutations in the other, previously treated, B-CLL cases cannot be rejected. This serious question warrants further detailed investigation, in particular a consecutive follow-up of the inactivation before and after treatment. Already, after a short follow-up, our study could also confirm an inferior prognosis for the patients having a p53 abnormality, as evidenced by an almost inevitable requirement for treatment and short survival time for many patients (see Table 4).

In addition, and in conclusion, we emphasize that FASAY may add a new quality to *p53* mutation screening in B-CLL patients and may appropriately supplement a routine cytogenetic screening.

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Short communication

Identification of somatic hypermutations in the *TP53* gene in B-cell chronic lymphocytic leukemia

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Abstract

Abnormalities of the *TP53* gene are associated with a particularly severe prognosis in patients with B-cell chronic lymphocytic leukemia (B-CLL). This tumor-suppressor is mostly inactivated by the deletion of one and point mutation of the other allele and has not been previously shown to be hypermutated in B-CLL. We identified two patients whose lymphocytes showed repeatedly an extensive proportion of *TP53* mutated cells by FASAY analysis (the yeast functional assay) and harbored various *TP53* mutations, mostly single-base substitutions, in individual cells. The mutation targeting exhibited characteristic traits of the somatic hypermutation process. In the first patient (harboring the unmutated *IgVH* locus) a significant bias to point mutations at CG pairs (21/25; $P=0.009$), their remarkable preference for the RGYW/WRCY motives (28%) and the highest expression of the activation-induced cytidine deaminase (*AID*) mRNA among the 34 tested B-CLL samples. In the second patient no CG bias was observed but the targeting of point mutations into the RGYW/WRCY motives was even more prominent here (7/16; 44%). Moreover, six out of eight point mutations affecting AT pairs were localized in the WA/TW motives, which are also characteristic for the somatic hypermutations. This patient, who was *IgVH*-mutated, already did not express any significant amount of the *AID* transcript. Our findings add a new aspect to the mosaic of the p53 mutability in B-CLL.
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Keywords: B-cell chronic lymphocytic leukemia; *TP53*; FASAY; Somatic hypermutation; Activation-induced cytidine deaminase

1. Introduction

B-CLL is an incurable disease characterized by a highly variable clinical course. The patients harboring abnormalities in the *TP53* gene are particularly at risk. Sporadic cases of B-CLL exhibit its aberrations in 10–15% of patients, predominantly those bearing the unmutated *IgVH* locus (Kröber et al., 2002). It is now widely accepted that defects in the *TP53* gene are strongly associated with the shorter survival rates and resistance to treatment (Montillo et al., 2005). The recently proposed model of the *TP53* mutability assumes that single-base substitutions, typical for this tumor-suppressor, are induced during transcription, when some bases in ssDNA secondary structures are prone to thermodynamic instability and intrinsic mutability (Wright et al., 2006). The hypermutational machinery targeting the *IgVH* locus through activation-induced cytidine deaminase (AID) (Muramatsu et al., 2000) has already been investigated as a potential source of mutations in the *TP53* gene *in vivo*. Instead of finding that, however, these reports noted characteristic traits of the hypermutation process in some other genes—the *BCL-6* in human tonsillar B cells (Yavuz et al., 2002), the *AID* itself, *BCL-6* and *C-MYC* in a B-lymphoma (Nilsen et al., 2005) and the *C-MYC*, *PIM-1*, *CD4* and *CD5* in T-lymphomas (Kotani et al., 2005). An ectopic, inducible expression of the AID had

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resulted, on the other hand, in the accrual of *TP53* mutations in human hepatoma HepG2 cell line (Kou et al., 2007). Some infectious agents seem to be very efficient inducers of the AID expression. The hepatitis C virus was shown to mutate the Ig-gene and *BCL-6* gene in a co-operation with this enzyme, while the targeting of mutations into the *TP53* gene was different, indicating another mechanism of action (Machida et al., 2004). Similar findings, an elevated expression of the AID enzyme and the targeting of cancer genes, have also been reported very recently for the EBV virus and Helicobacter pylori infections using *in vitro* systems (Epeldegui et al., 2007; Matsumoto et al., 2007). In these cases the nature of mutations in the *TP53* gene was consistent with the AID activity.

During our long-term screening of p53 functionality in B-CLL patients by FASAY analysis we unexpectedly identified two cases (among 350 investigated patients), who manifested the extensive mutation mosaic in the *TP53* gene. The analysis of affected nucleotides and targeted sequence motives strongly indicated an involvement of the somatic hypermutation process with contribution of the AID enzyme.

2. Material and methods

2.1. B-CLL patients with the TP53 hypermutations

Patient A—man, born in 1945, diagnosed with B-CLL in 2000, IgVH sequence unmutated (homology 100%, clone VH 1–18); patient B—woman, born in 1932, diagnosed with B-CLL in 1985, IgVH sequence mutated (homology 95.6%, clone VH 3–15). Both patients had been treated several times before the *TP53* mutations were identified and then also within the course of investigations. Their blood samples were obtained after informed written consent.

2.2. FASAY and split assay

In these functional assays the p53 *wild-type* (wt) samples form large white colonies, while the colonies with the p53 mutations are small and red (fully inactive mutants) or pink (partially inactive mutants). The FASAY was performed as described in Flaman et al. (1995), with some modifications published earlier (Smardova et al., 2001). The split assay is a modified version of FASAY allowing separate analyses of the 5′ and 3′ regions of the p53 cDNA. The assay was performed when the PCR product for FASAY was too faint (Smardova et al., 2002), using the protocol described by Waridel et al. (1997). The appropriate vectors were kindly provided by R. Iggo.

2.3. Recovery of p53-coding plasmid from yeast cells and DNA sequencing

The p53-coding plasmid was extracted from yeasts by using lysing enzymes of Trichoderma harzianum (Fluka). The *TP53* gene was amplified using the primers used for FASAY or split assay and sequenced with primers P5 (5′ cccaagcaatggatgatttgatgctg 3′), P6 (5′ ccatctacaagcagtcacagcaca 3′) and P7 (5′ gtggtaatctactgggacggaaca 3′), with ThermoSequenase™ Primer Cycle Sequencing Kit (Amersham Biosciences) on the desk automated sequencer ALF EXPRESS II (Amersham Biosciences).

2.4. Sequencing of TP53 cDNA and genomic DNA

The cDNA samples were PCR-amplified in three overlapping fragments with the following pairs of primers: F1 forward: 5′ cagccagactgccttccg 3′; R1 reverse: 5′gcaagtcacagacttggctg 3′; F2 forward: 5′ aacctaccagggcagctacg 3′; R2 reverse: 5′ggtggtacagtcagagccaac 3′; F3 forward: 5′ tcctcagcatcttatccgag 3′; R3 FASAY reverse: 5′accctttttggacttcaggtggctggagt 3′. The sequencing was performed using CEQ DTCS-Quick Starter Kit (Beckman Coulter) on CEQ8800 automatic sequencer (Beckman Coulter). Exons 2–10 of genomic DNA were amplified using primers described earlier (Sedlacek et al., 1998) and both DNA strands were sequenced with the BigDye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosysteme) on ABI PRISM 3130 automatic sequencer (Applied Biosystems).

2.5. Real-time quantitative PCR of AID gene

Total RNA from purified B-lymphocytes was isolated using RNeasy Mini Kit (Qiagen). The first-strand cDNA synthesis was performed using the SuperScript™ II with oligo$(dT)_{12-18}$ primer (Invitrogen). Five hundred nanograms of RNA was used for each 20 μl of RT-reaction. Each cDNA sample was analyzed in triplicates using TaqMan® Gene Expression Assay (Applied Biosystems), according to manufacturer's instructions. Amplification was detected using 7300 Real Time PCR System (Applied Biosystems). The TaqMan probe hybridised between exons 2–3 of the *AID* gene. Data were analyzed using Sequence Detection System (SDS) software, version 1.3.1. Results were obtained as the cycle number when the fluorescence reaches a set threshold (C_T). The relative expression of *AID* was quantified as a percentage of *GAPDH* expression. The difference in C_T values between the *AID* and *GAPDH* reactions (ΔC_T) was converted into relative AID expression using a formula $2^{-\Delta C_T} \times 100\%$.

3. Results and discussion

3.1. TP53 mutation screening by FASAY coupled to sequencing

During our partially published (Trbusek et al., 2006) and later extended screening of p53 functionality in B-CLL by FASAY analysis we reliably determined a corresponding clonal mutation in 45 affected samples. The two patients discussed here differed significantly, however, as their leukemic cells manifested various *TP53* mutations in individual red and pink yeast colonies.

The both patients exhibited a proportion of *TP53* affected cells in FASAY far above the background level (Table 1), with the different colour of colonies indicating a presence of more than one mutation (figure not shown). Consecutive sequencing revealed a completely heterogeneous pattern of mutations, mostly single-base substitutions (Table 2). The last analysis

Table 1
Proportion of mutated alleles of the *TP53* gene in patients *A* and *B* detected by FASAY or split assay

	B-CLL (%)	% of red and pink colonies	Comment
Patient A			
Analysis			
a	56	wt	
b	87	29	
c	ND	61	
d	94	69	
e	93	48	
f	90	70	Sorted B-cell fraction
	–	wt	Non-B-cell fraction
Patient B			
Analysis			
a	85	22/26	Split assay
b	80	45	

Investigations b–f of patient A cover a period of 2 years, investigations a and b in patient B were conducted 1 year apart. Ten percent of red colonies represent a standard background of the FASAY analysis in clinical samples (Smardova et al., 2002).

in patient A performed on sorted cells showed the p53-wt phenotype of non-B-cell fraction (selected by antibodies to CD2, CD3, CD14, CD16 and CD56 receptors), while the B-cells were proven to be substantially mutated (Table 1). This result has indicated a cell type-specific mechanism responsible for the multiple mutations.

The analysis of 45 point mutations identified in the background of three p53-wt clinical samples (data not shown) confirmed its different nature. In contrast to the background, the identified hypermutations affected several hot-spot codons (245 1×; 248 2×; 282 3×) and their incidence was on average remarkably higher in cancer (assessed using the database of the *International Agency for Research on Cancer*; www-p53.iarc.fr), referring to their biological selection and excluding their artificial introduction, i.e. through RT-PCR step.

3.2. Sequencing of cDNA and genomic DNA of the TP53 gene

The sequencing of three different cDNA samples and one genomic DNA sample in patient A always provided the *wild-type* output. It confirms that all the identified mutations represent minority fractions of B-CLL cells and cannot be detected without sub-cloning of the gene (i.e. cannot be detected with routine methodologies of mutation screening).

3.3. Analysis of the TP53 mutation target sites

The analysis of affected nucleotides and targeted sequence motives revealed some characteristic traits of the somatic hypermutation process in both patients. Patient A exhibited a significant bias ($P = 0.009$; χ-square test) for point mutations at CG pairs, accounting for 21/25 (84%) of detected single-base substitutions, while these two bases comprised only 58.5% of the analyzed sequence (960 bp). This corresponds to the expected activity of the AID enzyme (Petersen-Mahrt et al., 2002). A significant proportion of the identified point mutations (7/25; 28%) was localized within the sequence motif RGYW (R = A/G, Y = C/T, and W = A/T) or its inverse variant WRCY. As it was proven for the *IgVH* locus itself (Dörner et al., 1998), these motives accounted for 37% of all single-base substitutions in nonproductive V_HDJ_H rearrangements. Added to that, Kou et al. (2007) recently reported a finding of four mutations in the *TP53* gene after transient over-expression of AID in hepatoma cells, and one affected nucleotide – at position 623 (codon 208) – was mutated also in our patient.

Table 2
Individual mutations in the *TP53* gene assessed by FASAY or split assay coupled to sequencing in patients A and B

Patient	Investigation	Mutations	
A	**I** (b in Table 1)	**105** Asp → Gly [a]GGC → GAC; **178** His → Gln CAC → CAG (3x); **241** Ser → Ala [a]TCC → GCC	DelA in exon 8 + del 22N in exon 9; DelG in exon 8
	II (c)	**110** Arg → Leu CGT → CTT (2x); **139** Lys → Asn AAG → AAC; **176** Cys → Tyr [a]TGC → TAC; *178 His → Gln CAC → CAG*; **181** Arg → Cys CGC → TGC; **208** Asp → Val GAC → GTC; **215** Ser → Asn AGT → AAT; **245** Gly → Asp [a]GGC → GAC	**248** Arg → Trp [a]CGG → TGG; **277** Cys → Phe TGT → TTT; **278** Pro → Leu CCT → CTT; **282** Arg → Trp CGG → TGG; **282** Arg → Pro CGG → CCG; **285** Glu → Val GAG → GTG; Del 21N in exon 5
	III (e)	**135** Cys → Ser [a]TGC → TCC; **139** Lys → Asn AAG → AAT; **176** Cys → Ser [a]TGC → TCC	**259** Asp → Tyr GAC → TAC; **281** Asp → Tyr GAC → TAC
	IV (f)	*135 Cys → Ser [a]TGC → TCC; 139 Lys → Asn AAG → AAT* + **285** Glu → Lys GAG → AAG; **159** Ala → Thr GCC → ACC + **255** Ile → Phe ATC → TTC	*176 Cys → Ser [a]TGC → TCC; 248 Arg → Trp [a]CGG → TGG*; **258** Glu → Lys GAA → AAA; *259 Asp → Tyr GAC → TAC*
B	**I** (a)	**132** Lys → Arg [b]AAG → AGG; **132** Lys → Arg [b]AAG → AGG + 85 silent CCT → CCC; **135** Cys → Tyr [a]TGC → TAC; **136** Gln → Glu [a]CAA → GAA	**163** Tyr → Ser [b]TAC → TCC + **176** Cys → Phe[a]TGC → TTC; **176** Cys → Phe [a]TGC → TTC; **248** Arg → Gln CGG → CAG; Del 24N in exon 8
	II (b)	**126** Tyr → His [a,b]TAC → CAC; **214** His → Arg CAT → CGT; **234** Tyr → Cys [b]TAC → TGC; **247** Asn → Ile [a,b]AAC → ATC; **259** Asp → Glu GAC → GAA	**282** Arg → Gln CGG → CAG + **309** Pro → Ser [a]CCC → TCC; Del of exon 6; Del of 202N in exons 5 and 6; Insertion TGT after codon 261

[a] Mutation localized within the RGYW/WRCY motives.
[b] Mutation localized within the WA/TW motives. Italics = mutation was already detected in a previous analysis of the corresponding patient.

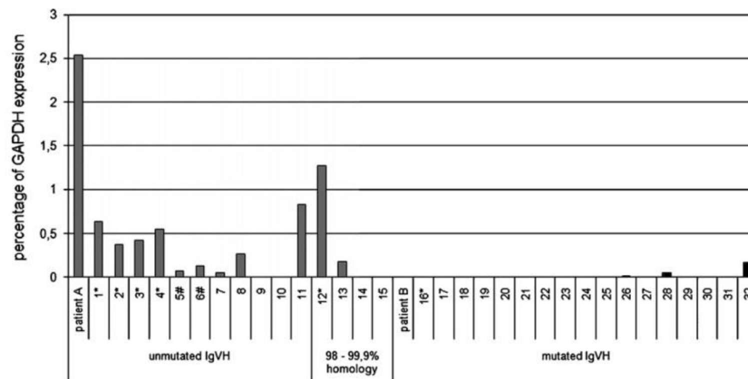

Fig. 1. Relative *AID* mRNA level in B-CLL cells. The relative expression of *AID* gene was quantified as a percentage of *GAPDH* expression. *Treated patients. #Patients with mutated *TP53* gene (bearing a single mutation).

In patient B we did not observe any bias for the *TP53* point mutations at CG pairs (50%). The frequency of RGYW/WRCY targeting was, however, more prominent here compared to patient A (7/16 point mutations; 44%). Moreover, six out of eight point mutations at AT-pairs were localized at WA/TW motives, with the A being affected five times in WA dinucleotide in 3′ position, on non-transcribed DNA strand. This is exactly expected for the somatic hypermutation process, in this case owing probably to the DNA polymerase eta (Rogozin et al., 2001; Mayorov et al., 2005). Interestingly, all the six mutated WA/TW motives were flanked by CG pairs on both sides.

Importantly, the clonal *TP53* point mutations ($n = 38$), which we have identified until now in 36 B-CLL patients (data provided as a Supplementary material), did not exhibit any signs of the hypermutation process. They manifest no CG bias (53% of point mutations affected this pair) and targeting of the RGYW/WRCY motives is absolutely negligible (3/38; 8%).

3.4. Assessment of activation-induced cytidine deaminase (AID) expression

It has already been reported (Nilsen et al., 2005), that the constitutive expression of AID may lead to mutagenesis outside of the *IgVH* locus in B-cells. Using a quantitative RT-PCR analysis we proved that patient A exhibited the highest expression of this gene among the 34 B-CLL patients tested, while the expression was undetectable in patient B (Fig. 1). This result fits in principle with the previous report (Heintel et al., 2004), which showed the association of detectable *AID* expression primarily with the unmutated *IgVH* (patient A). Since the AID seems to be necessary for the hypermutation process (Muramatsu et al., 2000), we assume that the machinery is turned off in patient B and therefore we did not search for the expression of other potential contributors (i.e. polymerases).

3.5. Clinical course and the therapy of hypermutated patients

Both patients manifested a phenotype characteristic for the p53 aberrations—frequent progressions with the requirement for treatment. In fact, we can only speculate what might be the reason for the observed hypermutations. Since both patients had been treated several times before we detected the *TP53* hypermutations, we may assume a potential role of the immunosuppression, which could lead to reactivation of some hypermutations-inducing viruses. Both patients suffered from hepatitis and therefore the HBV may be considered (Hussain et al., 2007), as well as the wide-spread EBV (Epeldegui et al., 2007). We emphasize that to study a role of chemotherapy in the process of *TP53* mutagenesis is fully warranted. In addition to patient A we actually identified another five cases in which the transition from originally *wild-type* to subsequently mutated *TP53* gene was detected and during this transition all the patients were treated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2007.08.017.

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Short communication

Inactivation of p53 and amplification of *MYCN* gene in a terminal lymphoblastic relapse in a chronic lymphocytic leukemia patient

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Abstract

B-cell chronic lymphocytic leukemia (CLL) is an incurable disease with a highly variable clinical course. A proportion of patients eventually progress to a higher stage of malignancy. A recent association has been observed between the presence of aberrant somatic hypermutations in leukemic cells (hypermutations occurring outside of the immunoglobulin locus) and the transformation to a diffuse large B-cell lymphoma or prolymphocytic leukemia. In this study, we report on the rarely observed blastic transformation in a CLL patient who had previously been shown to harbor aberrant somatic hypermutations in the *TP53* tumor-suppressor gene (Mol Immunol 2008;45:1525−29). The enzyme responsible, the activation-induced cytidine deaminase, was still active within the transformation, as evidenced by the ongoing class-switch recombination of cytoplasmic immunoglobulins. The transformation was accompanied by a complete p53 inactivation, as well as complex karyotype changes including prominent amplification of *MYCN* oncogene. Our case-study supports the view that the aberrant somatic hypermutation is associated with transformation of CLL to a more aggressive malignancy. © 2009 Elsevier Inc. All rights reserved.

1. Introduction

B-cell chronic lymphocytic leukemia (CLL) is an incurable disease characterized by accumulation of morphologically and phenotypically mature lymphocytes [1]. Its highly variable clinical course is mostly determined by two major biologic factors: mutation status of the immunoglobulin heavy-chain variable region (IgVH) [2], and four recurrent genomic aberrations − del(11)(q22-q23), del(13)(q14), del(17)(p13), and trisomy 12 [3]. An inferior prognosis is especially associated with inactivation of the *TP53* tumor-suppressor gene, which results in high resistance to chemotherapy [4]. A proportion of patients, often those with p53 inactivation and/or complex genomic aberrations, eventually progress to more aggressive malignancy, usually diffuse large B-cell lymphoma (DLBCL; Richter syndrome) [5] or prolymphocytic leukemia (PLL) [6]. Two recent reports [7,8] associated some cases of CLL transformation to DLBCL or PLL with the presence of aberrant somatic hypermutation, a pathologic process in which mutations are introduced into functional genes outside of the immunoglobulin locus.

In this study, we report on a rarely observed blastic transformation in a CLL patient whose *TP53* gene in original CLL lymphocytes had been affected by the aberrant somatic hypermutation.

2. Material and methods

2.1. Case history

A 55-year-old man was diagnosed with a classic $CD5^+$ CLL in March 2000. In 2003, his leukemic cells were shown to harbor the unmutated IgVH locus, del (13)(q14) in 30% of the cells and the wild-type *TP53* gene, as assessed by the yeast functional test FASAY [9]. Between

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2005 and 2007, the leukemic population manifested a high and progressively increasing proportion of *TP53* mutated cells. On the basis of point mutation targeting and prominent expression of activation-induced cytidine deaminase (*AID*) mRNA in CLL cells, we concluded and reported that the *TP53* gene had been affected by the aberrant somatic hypermutation [10].

From 2000 to 2007, the patient required repeated courses of chemo- and immunotherapy. In August 2007, he eventually underwent peripheral blood stem cell transplantation from a related donor as last salvage therapy. Despite the blood count normalization, the patient never achieved negative minimal residual disease (MRD), as evidenced by the sensitive "consensus MRD flow assay" and real-time quantitative allele-specific oligonucleotide polymerase chain reaction to IgVH sequence (RQ-ASO IgH-PCR) [11]. Although the lowest proportion of CLL cells achieved was 0.78% of leukocytes (limit of detection = 0.01% for FC and 0.001% for PCR) at the beginning of January 2008, the patient eventually developed fulminant relapse and died in March 2008. It was surprising that both flow-cytometric (Table 1) and morphologic (Fig. 1) analyses indicated a prominent phenotype of lymphoblastic cells, including the appearance of typical molecular markers CD10 [12], CD38 [13], and cytoplasmic CD22 [14]. Therefore, we made the assumption that either the original CLL underwent a blastic transformation leading to a phenotype mimicking acute lymphoblastic leukemia, or that de novo lymphoid malignancy developed. The transformation process was further analyzed.

Table 1
Flow-cytometric analyses of CLL and transformed cells

	CLL sample (% of leukocytes)	Transformed sample (% of leukocytes)
Lymphocytes	98	89
Monocytes	1	2
Granulocytes	1	9
	% of gated lymphocytes	% of gated lymphocytes
CD5	98.9	43.6
CD10	**0**	**33.9**
CD19	98.6	62.4
CD20	0.1	1.4
CD22	94	58
CD23	1.4	5.6
CD34	0	0,1
CD38	**0.1**	**87.9**
CD45	100	100
cCD22	**0**	**54**
cIgA	**0**	**5.2**
cIgD	**1.7**	**56.7**
cIgG	**0.6**	**9.4**
cIgM	**95.2**	**39.7**
c-kappa	16.3	7.4
c-lambda	0.7	6.2
CD5+CD19+	**97.9**	**9.3**
CD10+CD19+	**0.1**	**32.7**

A significant fraction of $CD19^{+}$ cells was associated with the CD10 antigen (a typical lymphoblastic marker) after transformation, while $CD5^{+}19^{+}$ B-lymphocytes; i.e. a typical major population of previously diagnosed CLL was comprised only 9% of lymphocytes. Two additional markers of acute leukemia also appeared in transformed cells (CD38 and cCD22). A remarkable immunoglobulin class-switch recombination to IgG and IgA classes reflects activity of the activation-induced cytidine deaminase.

2.2. Morphological analysis of the cells

Peripheral blood and bone marrow sections were fixed in May-Grünwald solution and stained by Giemsa-Romanowski. Morphology of the cells was evaluated at a magnification of 1,000 using BX41 (bone marrow) or Olympus BH2 (peripheral blood) microscopes.

2.3. Immunophenotyping of the transformed blast sample and corresponding CLL cells

Fresh peripheral blood sample (with EDTA) and cryopreserved, previously isolated CLL cells were analyzed by a Cytomics F500 flow cytometer (Beckman Coulter, Miami, FL). The cells were incubated with the monoclonal antibodies directed to surface antigens directly labeled with fluorochromes fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), or PE-Cy5 (Becton-Dickinson, San Jose, CA;

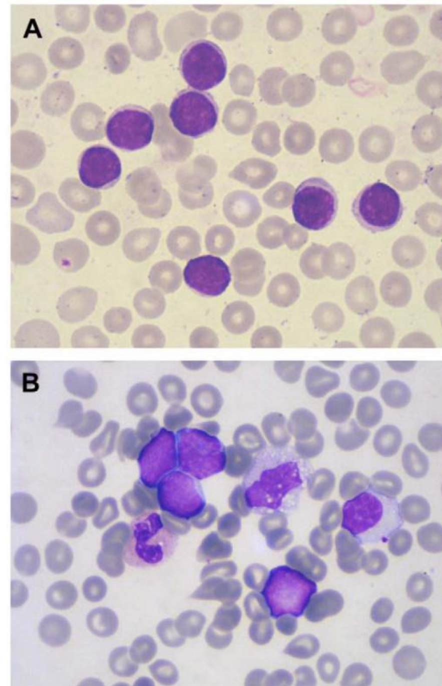

Fig. 1. (A) The bone marrow at CLL stage. CLL lymphocytes comprised about 98% of leukocytes. (B) The peripheral blood after lymphoblastic transformation. Undifferentiated B-lymphoblasts of medium size comprised approximately 23% of the cells. The blasts exhibited a high N/C ratio. Nuclei usually contained one to two nucleoli. The bone marrow was not examined because of the patient's death.

Caltag Laboratories, Burlingame, CA, Immunotech, Marselle, France). For detection of cytoplasmic molecules, the cells were permeabilized and labeled with the Fix & Perm Cell Permeabilization Kit (Caltag Laboratories). After permeabilization, monoclonal antibodies labeled with FITC and directed to cytoplasmic molecules were added (Caltag Laboratories).

2.4. Mutation analysis of the TP53 gene

Exons 5–9 were amplified from genomic DNA using primers described earlier [15]. The sequence of primers for exons 2–4 and 10 will be provided on request. Both DNA strands were sequenced with the BigDye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on ABI PRISM 3130 automatic sequencer (Applied Biosystems).

2.5. Array-based comparative genomic hybridization (arrayCGH)

DNA was extracted from peripheral blood leukocytes using DNeasy Blood and Tissue kit (Qiagen, Chatsworth, CA). Microarray analysis was performed using unrestricted oligonucleotide Human Genome CGH Microarray 4 $\times$ 44K (Agilent Technologies, Inc., Santa Clara, CA), according to manufacturer's instructions. Images were acquired with a microarray scanner from Agilent Technologies, Inc. The spots were analyzed with Feature Extraction Software version 9.5 and CGH Analytics Software (version 3.5; Agilent Technologies, Inc.).

2.6. Quantitative real-time PCR of the MYCN gene

The analysis was performed using the SYBR Green technology (Applied Biosystems). Two sets of primers that amplify two different regions of *MYCN* intron 2 were used: NMYC-F1: 5'cctggaaggaaacttggtga 3'; NMYC-R1: 5'tgag gctgttccagtctgtg 3'; NMYC-F2: cagactggaacagcctcaca 3'; NMYC-R2: 5'ctaccacgctggggtagaaa 3'. Primers used for the amplification of albumin gene (internal control) were the following: ALB-F: 5'gctgtcatctcttgtgggctgt 3'; ALB-R: 5'actcatgggagctgctggttc 3'. DNA amplification was detected using the 7300 Real Time PCR System (Applied Biosystems). Data were analyzed using Sequence Detection System (SDS) software version 1.3.1. The *MYCN* level normalized to the albumin level was compared with the normalized control genomic DNA.

3. Results

A clonal relationship between the transformed and original CLL cells was verified by RQ-ASO IgH-PCR, which disclosed a sharp increase in the original leukemic cells (approximately 50-fold) in comparison with the last MRD control (January 2008), confirming a clonality between the neoplasias. Therefore, for subsequent analyses, we looked at genes that had already been shown to be abnormal in CLL cells (*AID* and *TP53*). We also analyzed the presence of novel genomic aberrations.

Since we lacked material for any expression studies, we were unable to directly prove *AID* mRNA level in the transformed cells. In this respect, we can refer to the expression of cytoplasmic immunoglobulins (Table 1). The presence of a class-switch recombination — a process, which is fully dependent on AID enzyme [16] — confirmed that the protein was active during transformation. It remains unclear, however, whether AID further contributed to tumorigenesis in transformed cells. Theoretically, the enzyme could mutate other genes in addition to the *TP53*.

In the original CLL cells, the consecutive sequencing of individual DNA templates from yeast colonies disclosed twenty-five different mutations in the *TP53* gene, mostly single-base substitutions, while no prominent mutation was identified by the direct sequencing of genomic DNA. Therefore, we sequenced genomic DNA of the *TP53* gene in transformed cells to trace assumed mutation selection. Indeed, a single mutation *del T* in codon 212 was identified leading to a frame-shift and a subsequent stop-codon formation at position 246, with presumed complete loss of p53 function. The subcloning and sequencing of exons 6–8 confirmed that some previously identified mutations were co-selected.

To further elucidate the transformation process, we searched for other genomic aberrations in the lymphoblast cells. PCR analyses of the translocations that had already been reported in transformed CLL blasts [17], [i.e. t(8;14)(q24;q32), t(11;14)(q13;q32), and t(14;18)(q32;q21)], were all negative (data not shown). Some major deletions and amplifications in the transformed cells were, however, detected by the array CGH. The karyotype changes of original CLL cells at time preceding the transplantation were the following: del(13)(q14)x2, del(17)(q13.1), del(17)(q13.2p13.3), del(17)(q25.1), and del(17)(q25.3). It progressed in the transformed cells into the following: del(2)(p24.2p24.3), amp(2)(p24.3)(*MYCN*) (Fig. 2), del(5)(q14.3q33.2), del(6)(q14.3q22.1), del(13)(q14)x2, del(17)(11.1pter), del(17)(q24.3), del(18)(q21.2) (*SMAD4* tumor-suppressor gene), amp(18)(q21.2), dup(18)(q21.31q22.1) (anti-apoptotic *BCL-2* gene).

Amplification of the *MYCN* oncogene was by far the most prominent aberration in the transformed cells, owing to its clear definiteness. Therefore, we verified the presence of this abnormality by Q-PCR analysis (Fig. 3). The *MYCN* gene was amplified approximately 25-fold in the transformed cells compared to control cells (with two copies of *MYCN* gene).

4. Discussion

A blast crisis as a natural part of CLL history is rarely described [17,18]. In our hospital, we observed only one

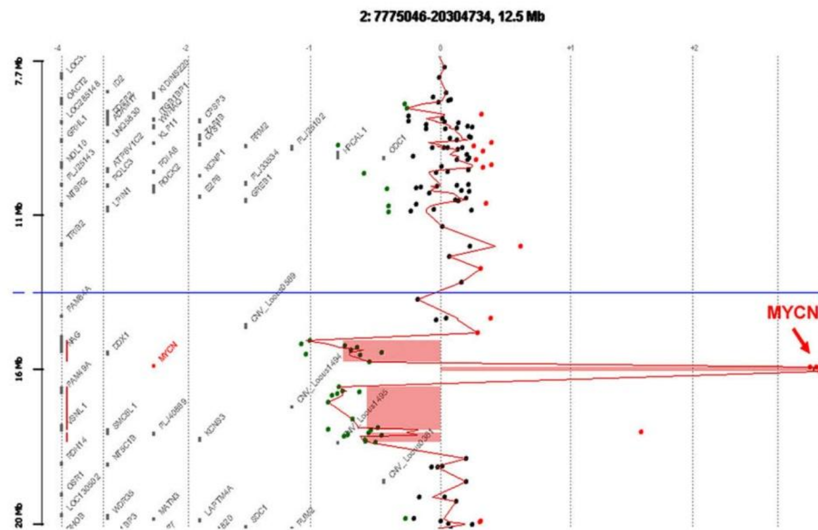

Fig. 2. Amplification of *MYCN* oncogene in the transformed lymphoblast cells assessed by arrayCGH. The figure depicts the 12.5 megabase of the short arm of chromosome 2 in region p24.2~p3. Deleted regions are visualized in green, amplified regions in red.

transformation to blastic stage in approximately 600 monitored patients. For seven years, the patient manifested typical CLL. In line with this phenotype, his leukemic cells harbored the IgVH sequence, which belongs to a restricted set of immunoglobulin gene rearrangement consensus motives identified recently in CLL patients, suggesting antigen selection [19]. It is comprised of the gene segments IGHV1-18, IGHD6-19, and IGHJ4, as well as the CDR3 region CARVQWLPQYYFDYW.

The association between aberrant somatic hypermutation (detected in *PIM-1*, *PAX-5*, *RhoH/TTF*, and *C-MYC* genes) and the transformation of CLL to DLBCL has been described recently [7]. Using an overlapping set of genes, this observation has also been extended to CLL transformation to PLL [8]. In this respect, our case-study supports the view that the aberrant somatic hypermutation is associated with CLL transformation, and further extends this observation to hypermutations in the *TP53* gene and the transformation of CLL to acute leukemia. In contradiction with the aforementioned observations [7,8], we have already detected massive aberrant somatic hypermutations in the nontransformed CLL cells.

The transformation process was accompanied by a broad range of genomic aberrations. In line with the data

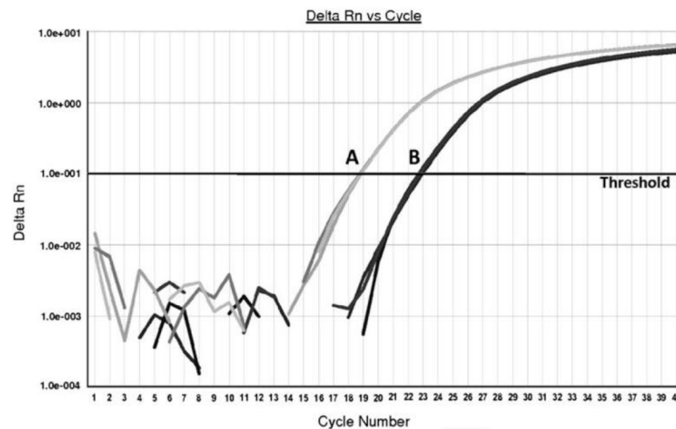

Fig. 3. Amplification of the *MYCN* gene was confirmed using Q-PCR. Amplification curves for the input of 10 ng of DNA are shown. (A) DNA sample isolated from transformed lymphoblastic cells (B) control DNA with two copies of *MYCN* gene. The 25-fold amplification was assessed after the conversion of values to the level of internal control (albumin gene).

published on relapsed acute leukemia [20], the *TP53* gene was completely abolished by deletion and inactivating mutation of the remaining allele. This inactivation was followed by the expected marked cytogenetic instability [20]. Amplification of the *MYCN* oncogene was the most prominent novel abnormality. Interestingly, gain of this oncogene with the corresponding elevation of the *MYCN* mRNA expression has already been identified as a recurrent alteration in CLL patients [21]. Its amplification has also been shown to occur in mice B-cell lymphoma model with p53 inactivation [22]. Very recently, the reported activity of noncoding microRNA miR-34a confirmed a direct link between the p53 protein and *MYCN* gene; miR-34a transcription is induced by the p53 protein and, conversely, can negatively regulate an overexpressed *MYCN* transcript by targeting its 3' UTR [23]. Based on these data, we assume that the *MYCN* amplification was uncontrolled in our patient. To our knowledge, amplification of this oncogene has not been observed in CLL transformation yet.

In summary, our case-report supports the previously published findings of the association between aberrant somatic hypermutation and transformation of CLL to more aggressive malignancy. It also indicates the potential of the *MYCN* gene amplification in CLL transformation.

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Monoallelic and biallelic inactivation of TP53 gene in chronic lymphocytic leukemia: selection, impact on survival, and response to DNA damage

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Monoallelic and biallelic inactivation of *TP53* gene in chronic lymphocytic leukemia: selection, impact on survival, and response to DNA damage

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Deletion of *TP53* gene, under routine assessment by fluorescence in situ hybridization analysis, connects with the worst prognosis in chronic lymphocytic leukemia (CLL). The presence of isolated *TP53* mutation (without deletion) is associated with reduced survival in CLL patients. It is unclear how these abnormalities are selected and what their mutual proportion is. We used methodologies with similar sensitivity for the detection of deletions (interphase fluorescence in situ hybridization) and mutations (yeast functional analysis) and analyzed a large consecutive series of 400 CLL patients; a subset of p53–wild-type cases (n = 132) was screened repeatedly during disease course. The most common type of *TP53* inactivation, ie, mutation accompanied by deletion of the remaining allele, occurred in 42 patients (10.5%). Among additional defects, the frequency of the isolated *TP53* mutation (n = 20; 5%) and the combination of 2 or more mutations on separate alleles (n = 5; 1.3%) greatly exceeded the sole deletion (n = 3; 0.8%). Twelve patients manifested defects during repeated investigation; in all circumstances the defects involved mutation and occurred after therapy. Monoallelic defects had a negative impact on survival and impaired in vitro response to fludarabine. Mutation analysis of the *TP53* should be performed before each treatment initiation because novel defects may be selected by previous therapies. (Blood. 2009;114:5307-5314)

Introduction

Chronic lymphocytic leukemia (CLL), the most frequent of all leukemias in the Western world, still represents an incurable disease.[1] Its highly variable clinical course is mostly determined by the combination of 2 major biologic factors that impact disease progression: mutation status of the immunoglobulin heavy-chain variable region (*IgVH*) and 4 prominent genomic aberrations, ie, deletions 11q22-23, 13q14, 17p13, and trisomy of chromosome 12.[2,3] The deletion at region 17p13, which harbors a tumor-suppressor gene *TP53* (coding for p53 protein), has been shown repeatedly and consistently to be associated with the worst prognosis in CLL patients.[4,5] On the contrary, a good prognosis requires, among other factors, an intact *TP53* gene,[6] although some rare exceptions exist.[7] A poor response to not only conventional DNA-damaging chemotherapy but also to its combination with rituximab is one of the major determinants for an inferior outcome in *TP53*-affected patients.[8,9] These patients are current candidates for alternative treatment approaches, which include submission to clinical trials that investigate drugs with p53-independent mechanisms of action, therapy by monoclonal antibody alemtuzumab, and stem cell transplantation.[10-12]

It is estimated that approximately half of all cancers lose their wild-type (wt)-p53 activity at a certain point in development.[13] The high pressure on the elimination of the p53 protein during tumor progression stems from its central role in several divergent yet interconnected processes, including the cell-cycle arrest, apoptosis, DNA repair, and senescence.[14] In addition to its well-established role in cancer, the p53 is currently recognized as an important player in some of the other types of cellular stress, eg, in ischemia disease or aging of an organism.[15]

The level of p53 protein in a cell is regulated through a direct binding to the E3 ubiquitin ligase MDM2, whose expression is induced by the p53.[16] This autoregulatory feedback loop is interrupted in the case of an initiation of DNA double-strand breaks by ATM and Chk2 kinases, which induce phosphorylation of the p53 at residues critical for MDM2 binding.[17] The ATM–Chk2–p53 axis has been proven as part of the fundamental DNA-damage response anticancer barrier, which is activated by impairment of the DNA in precancerous lesions and compromised later by mutations during a malignant conversion.[18]

Although the *TP53* was originally considered to be a recessive tumor suppressor, several precise studies have shown a profound effect of one allele loss or its inactivation by a sole mutation on tumorigenesis. An analysis of tumors in heterozygous ($p53^{+/-}$) mice showed that many of the tumors preserved a wt *TP53* allele, documenting the role of the p53 haploinsufficiency (gene dosage effect) in cancer progression.[19] However, the missense mutation analogous to human R175H hot-spot alteration introduced into a mouse led to a significantly greater metastatic potential of the developed tumors in comparison with the $p53(^{+/-})$ mice.[20] Besides

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the *TP53* defects, an attenuation of p53 activity and a predisposition to tumor formation also was proven to occur through enhanced activity of the negative regulatory protein MDM2, harboring a polymorphism in its promoter (SNP309), which shows how critical the standard level of the p53 protein is for cancer prevention.[21]

Recently, several research groups have analyzed the impact of an isolated *TP53* mutation (without accompanying deletion) on response to therapy, disease progression, and overall survival in CLL. Although the study by Grever et al[22] did not find any significant role of the sole *TP53* mutation in response to fludarabine-based therapy, 3 other reports correlated single *TP53* mutation with complex aberrant karyotype and rapid disease progression[23] and also with poor survival and chemorefractoriness.[24,25]

We analyzed the presence of *TP53* abnormalities in a consecutive series of 400 CLL patients, including the repeated monitoring of this gene in 132 of them. For the detection of chromosomal deletions and the identification of mutations, we used the methodologies with similar sensitivity, ie, interphase fluorescence in situ hybridization (I-FISH) and functional analysis of separated alleles in yeasts (FASAY), respectively. In our report we show that *TP53* mutation and not deletion is preferentially selected in CLL. We also present a strong association between the presence of therapy and the occurrence of novel *TP53* abnormalities and show that isolated *TP53* missense mutations impair the primary response of the CLL cells to DNA damage.

Table 1. Clinical and biologic characteristics of CLL patients

Characteristic	Value
Patients, n	400
Median age at diagnosis, y	60
Women/men, %	35/65
Stage at time of *TP53* and *ATM* examination (n = 365), %	
Low risk: Rai stage 0	30
Intermediate risk: Rai stage I/II	36
High risk: Rai stage III/IV	34
***IgVH*, % (n = 355)**	
Mutated	38
Unmutated	62
I-FISH according to the hierarchical classification of Döhner et al[26] (n = 400), %	
17p−	11
11q−	21
+12	11
13q−	34
Normal	23
Investigation of *TP53* and *ATM* status, %	
At time of diagnosis	48
During course of disease	52
Therapy before the first *TP53* and *ATM* examination, %	
No	73
Yes	27

CLL indicates chronic lymphocytic leukemia; and I-FISH, interphase fluorescence in situ hybridization.

Methods

CLL patients

The study was performed on blood samples of 400 CLL patients monitored and/or treated at the Department of Internal Medicine–Hematooncology, University Hospital Brno, during the years 2003 to 2008; a subset was obtained in collaboration with the Department of Hematology, J. G. Mendel Cancer Center at Novy Jicin, Czech Republic. All blood samples were processed with written informed consent in accordance with the Declaration of Helsinki under protocols approved by the Ethical Commission of the University Hospital Brno. Clinical and biologic characteristics of the patients are summarized in Table 1.

Identification of mutations in the *TP53* gene

All patients in the study (n = 400) were screened by the functional yeast analysis FASAY[27]; a subset (n = 132) was examined repeatedly. In this assay, the central part of the *TP53* gene (amplified from cDNA between exons 4 and 10, ie, codons 42 to 374) is introduced into an $ADE2^-$ yeast strain carrying a reporter with a p53-binding site upstream of the *ADE2* gene. On the plates containing a low level of adenine, the p53 wt samples form large white colonies, whereas the colonies with the p53 mutations (mut-p53) are small and red, which is attributable to limited growth and an accumulation of a reddish product of adenine metabolism. Samples containing a wt-*TP53* gene account for 90% or more of the white colonies in the assay; the remaining 10% represent background, a consequence of polymerase chain reaction (PCR)–induced mutations or a low-quality RNA. We have shown previously[28] that the use of FASAY provides the expected number of red colonies when serial dilutions of wt-*TP53* and mut-*TP53* plasmids for PCR amplification or when leukemic cell lines with known p53 status are used. We can also observe, on a long-term basis, that in properly handled clinical samples (which allow for standard PCR amplification) the background of the FASAY is less than 10%. In samples that provided more than 10% of red or pink colonies, we sequenced DNA templates isolated from 4 to 6 individual colonies to identify a clonal *TP53* mutation. The pink colonies are supposed to harbor a temperature-sensitive *TP53* mutation.[29] In the case of exon deletion, direct sequencing of genomic DNA was used to find the corresponding mutation. We found that, when using FASAY, we were not able to identify the CLL hot-spot 2-nt deletion in codon 209 (exon 6), probably because of nonsense-mediated mRNA decay of this particular molecule.[30] Therefore, we also supplemented the use of FASAY by high-resolution melting analysis of the exon 6 in 90 patients. The high-resolution melting was done by the use of Rotor-Gene6000 (QIAGEN). The exact conditions can be provided on request.

Other genetic characterizations of CLL cells

Deletions at the 11q22-q23 (*ATM*) and 17p13 (*TP53*) loci were detected by I-FISH by the use of locus-specific probes from Abott Vysis Inc, according to the manufacturer's instructions. At least 200 interphase nuclei per slide were evaluated with an Olympus BX61 fluorescence microscope equipped with a Vosskuhler 1300D digital camera and LUCIA-KARYO/FISH/CGH imaging system (Laboratory Imaging). The cut-off level for the detection of both *TP53* and *ATM* deletions (5%) was calculated in a series of 15 samples obtained from normal bone-marrow donors (200 interphase nuclei per slide were evaluated) by use of the upper limit of binomial confidence interval. PCR and direct sequencing were used to analyze the *IgVH* rearrangements and mutation status.

Western blot analysis of p53

The p53 protein was detected with anti-p53 antibody DO-1 (a gift from Dr Vojtesek, MMCI Brno); PCNA protein was used as an internal standard; antibody Mab424R (Chemicon). The procedure was as previously described.[31]

Viability testing after in vitro fludarabine administration

Samples, which had been vitally frozen in dimethyl sulfoxide and stored in liquid nitrogen, were exclusively used. Cells were kept in tissue culture under standard conditions for 24 hours (37°C, 5% CO_2) and then used for the metabolic WST-1 assay (Roche, CH). The procedure was as previously described.[31]

Real-time quantitative PCR of the p53 target genes

A quantitative reverse-transcription PCR assay was performed by use of TaqMan technology and the 7300 Real-Time PCR System (Applied

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Table 2. Summary of *TP53* abnormalities identified in CLL patients and their association with biologic and clinical variables

Abnormality	Monoallelic alterations	Biallelic alterations
Total	23	47
Type of defect (n)	del(17p) (3), mutation (20)	del(17p)/mutation (42), mutation/mutation (5)
Proportion of missense mutations, %	100	67*
High risk: Rai stage III/IV (at the time of *TP53* status examination), % (n)	48 (10/21)	67 (29/43)
P	.13	< .001
Unmutated *IgVH*, % (n)	86 (18/21)	93 (41/44)
P	.025	.001
11q deletion, % (n)	43 (10/23)	11 (5/47)
P	.014	.055

P = .003 (biallelic vs monoallelic alterations in relation to 11q deletion).
CLL indicates chronic lymphocytic leukemia.
*In the subgroup del(17p)/mutation.

Biosystems). The primer and probe sets were specific for the *BAX, BBC3* (PUMA), and *CDKN1A* (p21) genes (TaqMan Gene Expression Assay; Applied Biosystems). The geometric mean of *TBP* (TATAA-box binding protein) and *HPRT1* (hypoxanthine–guanine phosphoribosyltransferase) values served as an internal standard. The procedure was as previously described.[31]

Statistical evaluation

The χ^2 test was used to assess the association between genetic defects and (1) the Rai stage and (2) the mutation status of *IgVH*. The Fisher exact test was used for assessing the association among (1) *TP53* defects and *ATM* deletion; (2) the presence of therapy and novel *TP53* abnormalities; and (3) genetic abnormalities and requirement for therapy. Survival analysis was performed with the use of the Kaplan-Meier survival estimator. The response of CLL cells to fludarabine was evaluated by a 2-factor analysis of variance with the subsequent Tukey HSD post-hoc test.

Results

Characterization of the CLL cohort

The investigation of the *TP53* gene (mutation and deletion) and the *ATM* gene (deletion) from the same exact time of examination was performed in a consecutive series of 400 patients. The mutation status of the *IgVH* locus (Table 1) indicated a more unfavorable profile of the cohort in comparison with the other studies; 62% of the patients harbored the unmutated *IgVH* sequence (the reported ratio is usually 1:1 or that in favor of mutated *IgVH*). The reason for this bias is a local concentration of patients with inferior CLL at the University Hospital Brno; noncomplicated patients are monitored at regional hematologic centers in the Czech Republic.

Single *TP53* mutation but not single deletion is notably selected in CLL cells

The *TP53* gene was impaired in 70 patients (17.5%). A somewhat greater frequency than in other CLL studies (usually around 10%) should be assigned to (1) the previously discussed more-unfavorable profile of the cohort; (2) repeated investigation of *TP53* status in the subset of p53-wt patients (n = 132) during the course of the disease—this additional screening, which provided 12 novel abnormalities, is discussed later in the text; and (3) use of the highly sensitive FASAY analysis. The types of *TP53* defects are summarized in Table 2. Chromosomal deletions were present in 45 patients (11.3%), with the single deletion—without accompanying mutation on the remaining allele—being extremely rare (3 cases; 0.8%). In contradiction to this finding, a single *TP53* mutation was detected in 20 patients (5.0%), and 5 patients (1.3%) harbored at least 2 different mutations on separate alleles. The biallelic, but not monoallelic *TP53* abnormalities, were clearly associated with the high-risk (Rai III/IV) CLL (Table 2; $P < .001$ and $P = .13$, respectively); both biallelic and monoallelic *TP53* defects were associated with the unmutated *IgVH* locus (Table 2; $P < .001$ and $P = .025$, respectively). The identified mutations are listed in supplemental Table 1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). The alterations consisted mostly of missense mutations (77%), with the mutational hot-spots being codons 248 (5 cases), followed by codons 132, 175, and 234.

In contrast to biallelic *TP53* changes involving cytogenetic deletion, which contained on the other allele missense mutation (n = 28), frame-shift (n = 8) or in-frame deletion (n = 3), insertion (n = 1), and mutation affecting the exon/intron splicing site (n = 2), all 20 monoallelic mutational alterations represented missense substitutions. It points to their selection advantage, likely ensured by a dominant negative effect toward wt allele or a gain of function.[32] Moreover, in 2 cases in which the number of red colonies significantly exceeded 60% (complete inactivation of one allele plus background; supplemental Table 1), the presence of uniparental disomy (UPD) in the *TP53* locus could be considered. UPD has been observed recently in CLL patients as a frequent event and should lead to a phenotype comparable with the complete p53 inactivation.[33] Finite evidence for UPD would have to be provided, however, by the use of a single nucleotide polymorphism-based array. In 11 patients with a single mutation, we once again performed FASAY after a certain period (median, 9 months; range, 4-29 months) and confirmed in all cases the mutated phenotype; compatible FISH analyses were performed in 6 of the 11 cases and were always negative. Particular mutation was verified by sequencing in 9 cases and always confirmed.

Mutation of the *TP53* gene often leads to the stabilization of the p53 protein because of an inability of the mutated p53 to induce expression of its own negative regulator MDM2. To gain insight into the functionality of p53 in affected cells, we assessed the level of the p53 protein in samples with sole missense mutations versus the samples harboring biallelic inactivation consisting of cytogenetic deletion and missense mutation. Although only 2 (17%) of 12 cases in the former group manifested a clearly detectable level of p53, in the latter subset the stabilization was substantially more frequent (21/24; 88%); only 1 wt-p53 sample of 250 tested showed the p53 level comparable with mutated cases (supplemental Figure 1 and data not shown). It indicates that most of the cases with isolated missense mutations preserve a substantial p53 activity toward at least some target promoters.

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Table 3. Novel *TP53* abnormalities identified in CLL patients with originally intact gene (in investigation I)

Patient	Time between investigations, mo	*TP53* defect	Mutation	Therapy before mutation detection	Survival, mo: From time of abnormality detection	Survival, mo: From diagnosis
P1*	33	del/mut	del 1 nt in codon 294	R, CHOP, R-CHOP	1	129
P2	7	del/mut	R273H	CHOP, A, FC	7	28
P3*	51	del/mut	G244D	FCR	2	68
P4	37	del/mut	del 22 nt in intron 5 (splice mutation)	FC	3†	103†
P5*	10	mut/mut	K132R/del 14 nt in codon 194	A	16	85
P6	36	del/mut	T211I	FC, A	3	39
P7	11	wt/mut	I195T	FC, R-CHOP, CHOP, A	27†	48†
P8*	18	del/mut	R175H	FC	16†	102†
P9*	19	wt/mut	C275Y	A	3	148
P10*	19	del/mut	del 9 nt in codon 252	CHOP	32	52
P11*	24	del/mut	ASHM	R-CHOP	37	96
P12*	33	del/2 mut	K120M/del 2 nt in codon 209	FCR	12	92

A indicates alemtuzumab; ASHM, aberrant somatic hypermutations in the *TP53* gene[36]; C, cyclophosphamide; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone; F, fludarabin; and R, rituximab.

*Patient also received therapy before investigation I.

†Patient is alive.

Monoallelic *TP53* alterations are significantly associated with *ATM* deletion

Abnormalities of *TP53* and *ATM* genes are considered to be mutually exclusive in CLL cells because dysfunction of one has been proven to be an alternative to the defect of the other.[34] Therefore, we analyzed their relationship in our cohort, structured according to monoallelic versus biallelic *TP53* inactivation. In the *TP53*-wt patients, heterozygous *ATM* deletion was detected in 74 (22%) of 330 patients. Similarly to *TP53*, the *ATM* deletion was significantly associated with the unmutated *IgVH* gene (65/68, 96%; $P < .001$). Interestingly, in the *TP53*-affected patients, the presence of accompanying *ATM* deletion was significantly associated with monoallelic alterations (43%; $P = .014$; Table 2). The presence of complete p53 inactivation, on the other hand, resulted in the reduced frequency of *ATM* deletion (11%; $P = .055$; Table 2). This finding suggests that the monoallelic abnormalities in the *TP53* do not impair p53 protein completely and are coselected with accompanying defects in *ATM*.

Novel *TP53* abnormalities appeared after the administration of therapy and always included mutation

Identifying *TP53* changes early in their development may help to clear the selection preference with respect to deletion or mutation. Therefore, we repeatedly screened 132 patients with previously intact *TP53* genes by FASAY to reveal novel mutations. The FISH analysis for del(17p) and del(11q) was available in 83% of the samples (109/132). Because it has been reported that p53 inactivation may occur as a consequence of previous DNA-damaging chemotherapy in CLL,[35] we divided the cohort according to the presence of therapy in the period between the 2 investigations. Sixty-two patients received from 1 to 3 courses of chemo- (n = 22), chemoimmuno- (n = 30), or immunotherapy (n = 10). Seventy patients were devoid of any therapeutic intervention. The median of the proportion of CLL cells in the samples in investigation I was similar between the treated and untreated subgroups (87%, range, 20%-99% vs 88%, range, 25%-99%, respectively). The median time between investigations I and II was 33 months in the treated subgroup (range, 5-61 months) and 22 months in the untreated subgroup (range, 4-57 months). The median of CLL cells proportion in investigation II was also similar (83%, range, 21%-98% vs 89%, range, 47%-99%, respectively). However, the treated cohort manifested the following unfavorable factors: (1) a greater proportion of patients who received therapy before investigation I (44% vs 13%); (2) more patients in progression of the disease in investigation II (87% vs 57%); (3) more frequent unmutated status of *IgVH* (80% vs 42%); and (4) more cases with *ATM* deletion (n = 22 vs n = 10 in untreated cohort).

Novel *TP53* abnormalities were detected in 12 patients; all received therapy between investigations I and II ($P < .001$). The median time of their investigation was 22 months, fully comparable with the untreated cohort, excluding an influence of this parameter on mutation occurrence. In all instances the mutation was selected, accompanying the remaining allele by deletion (n = 9), another mutation (n = 1), or no alteration (n = 2; Table 3). This observation further indicates that *TP53* mutation and not deletion is a primary end point of selection in CLL cells. In 3 patients we detected a novel *ATM* deletion; 2 of the 3 patients were not treated between the investigations. It may show that ATM is not as closely associated with therapy as the p53; we emphasize, however, that we do not have information about the second *ATM* allele.

Monoallelic *TP53* abnormalities impair a survival of CLL patients

Although some *TP53* abnormalities are present at diagnosis, many of them develop later during the course of the disease. They are considered as clonal variants, which increase substantially an aggressiveness of CLL. In line with that, survival of CLL patients who developed a novel *TP53* defect in our study was dramatically shorter when assessed from the time of detection in comparison with survival from diagnosis (Table 3). We therefore created the survival curves from time of abnormality detection (*TP53*, *ATM*); date of investigation was used if a sample was wt. Median follow-up in living patients was 32.5 months. Both monoallelic and biallelic *TP53* abnormalities significantly ($P < .001$) deteriorated patients survival compared with all *TP53*-wt cases combined together regardless of the *ATM* and *IgVH* status (Figure 1). However, the unmutated *IgVH* itself had a negative influence on survival ($P < .001$; data not shown), and as discussed previously,

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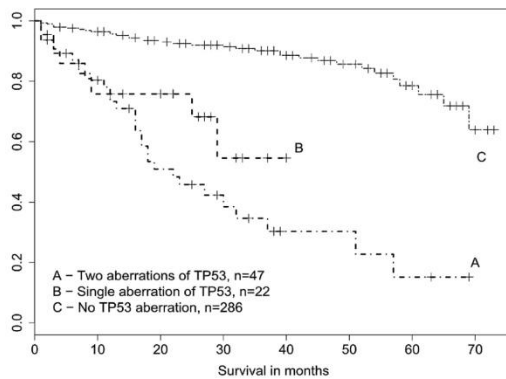

Figure 1. Survival of CLL patients structured according to the *TP53* defects. Survival was assessed from time of *TP53* investigation. Patients with both biallelic and monoallelic *TP53* defects showed significantly worse survival than p53 wt patients ($P < .001$).

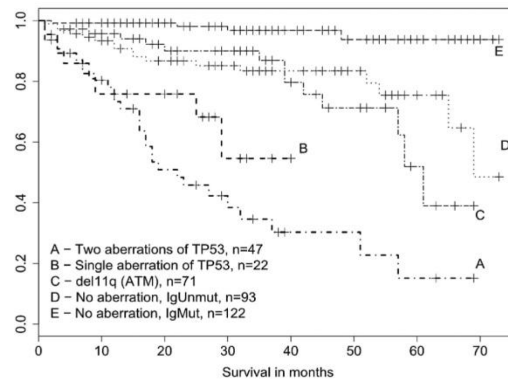

Figure 2. Survival of CLL patients structured according to the *TP53* defects, *ATM* deletion, and mutation status of *IgVH*. Survival was analyzed from time of *TP53*/*ATM* investigation. An effect of the abnormalities on survival was assessed in comparison with p53-wt/*ATM*-wt subgroup harboring the unmutated *IgVH* (curve D). Biallelic *TP53* changes showed strong effect ($P < .001$), monoallelic *TP53* abnormalities intermediate effect ($P = .021$), and *ATM* deletion no impact at all ($P = .55$).

the high-risk genetic abnormalities (in *TP53* or *ATM* genes) were derived almost exclusively from this subgroup.

Therefore, we assessed the survival also in relation to the unmutated *IgVH* (Figure 2). In this case the biallelic *TP53* defects showed a clear association with shorter survival ($P < .001$), whereas the monoallelic changes manifested a much weaker, albeit significant impact ($P = .021$) and *ATM* deletion had no effect at all ($P = .55$). The 2 patients with single *TP53* mutation, but supposed UPD, were alive at the time of analysis and therefore the reduced survival of the corresponding subgroup cannot be attributed to them. Patients with monoallelic *TP53* defects as a result constitute an independent prognostic subgroup, whose survival is substantially worse in comparison with patients possessing *ATM* deletion or unmutated *IgVH* with no accompanying high-risk abnormality.

In addition to survival, we also focused on the requirement for therapy within a 6-month period after the abnormality detection/wt investigation. The decision to use therapy in a particular patient was made according to the updated National Cancer Institute guidelines,[37] ie, it was not influenced by the knowledge of *TP53*, *ATM*, or *IgVH* status. The data are summarized in Table 4. Therapy was significantly ($P < .001$) more frequently required in all 3 aberrant subgroups compared with wt patients, unstructured according to *IgVH* status. Similarly to overall survival, the unmutated *IgVH* itself was significantly associated with requirement for therapy ($P = .001$). When the unmutated *IgVH* was used as a control group, the statistical significance ($P < .001$) remained only for biallelic *TP53* inactivation; there was only a trend ($P = .09$) for monoallelic *TP53* defects and no difference at all for *ATM* deletion.

Single *TP53* missense mutation impairs a response of CLL cells to fludarabine

A large body of evidence shows that the crucial impact of p53 on the fate of CLL patients is tightly connected with its role in response to therapy. The nucleoside analog fludarabine currently represents a key drug for CLL and has been confirmed to elicit p53-dependent up-regulation of downstream target genes in CLL cells.[38] Recently, Rossi et al[25] showed an association of *TP53* mutation, but not 17p deletion, with chemorefractoriness to fludarabine-based regimens. However, a direct comparison of primary in vitro response to fludarabine in CLL samples with monoallelic missense mutation versus biallelic *TP53* defects has never been, according to our knowledge, performed. We used 4 different concentrations of fludarabine (25, 6.25, 1.6, and 0.4 μg/mL), which has shown earlier to provide a concentration-dependent curve of viability after 48 hours of treatment in most of the CLL samples.[31] The testing of cellular viability is presented in Figure 3. The cells with both biallelic and monoallelic *TP53* inactivation were significantly ($P < .001$) more resistant than wt cells. At a concentration of 1.6 μg/mL, which is comparable with a clinical situation,[39] there was no difference between the biallelic and monoallelic *TP53* defect. However, when the greater concentration of fludarabine was used, the cells with monoallelic defect showed intermediate viability.

In addition to viability testing, we also assessed the induction of p53-downstream target genes *PUMA*, *BAX*, and *CDKN1A* (coding for p21 protein) after fludarabine exposure in a subset of samples. The results are presented in Figure 4. Although the samples harboring biallelic *TP53* inactivation lacked any induction, the response was quite heterogeneous in the cells with monoallelic

Table 4. Requirement for therapeutic intervention within the 6-month period after detection of *TP53*/ATM abnormalities or investigation-wt in individual patient subgroups

Status	Group	Treated	Untreated	Percentage of treated	P
TP53 biallelic inactivation	A	37	6	86	AF, < .001; AD, < .001
TP53 monoallelic inactivation	B	16	6	72	BF, < .001; BD, .09
Deletion of *ATM*	C	41	32	56	CF, < .001; CD, .47
wt unmutated *IgVH*	D	50	42	54	DE, < .001
wt mutated *IgVH*	E	18	107	14	
wt regardless of *IgVH*	F	68	149	31	

IgVH indicates immunoglobulin heavy-chain variable region; wt, wild-type; and AF, group A compared with group F, and so on.

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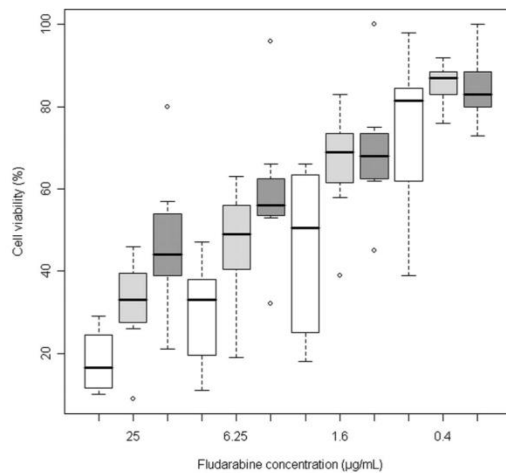

Figure 3. Viability of CLL cells after in vitro administration of fludarabine. Metabolic WST-1 assay was used. Dark gray bars indicate biallelic *TP53* defect (n = 7); Light gray bars, monoallelic *TP53* defect (n = 7); white bars, no *TP53* defect (n = 8); the last group consisted of 4 samples harboring *ATM* deletion and 4 purely wt samples with unmutated *IgVH*. The monoallelic *TP53* defects (missense mutations) impaired the response to fludarabine in comparison with the other high-risk CLL factors, ie, deletion of *ATM* or unmutated *IgVH* locus.

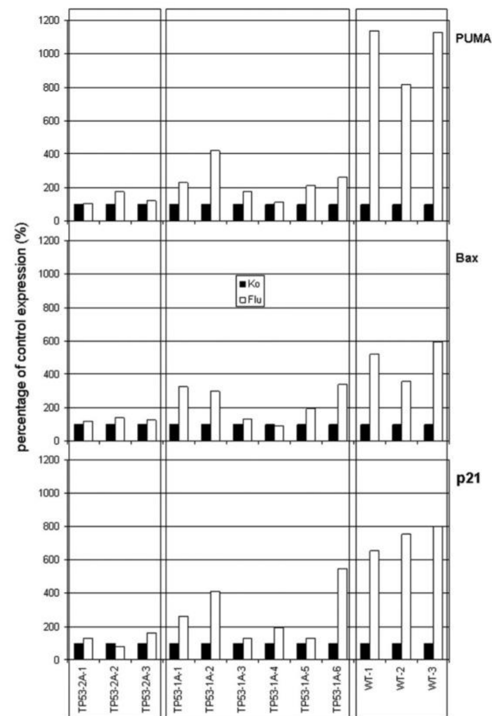

Figure 4. Induction of p53-downstream target genes *PUMA*, *BAX*, and *CDKN1A* (p21) after fludarabine administration. Quantitative PCR data show induction related to untreated control (set at 100%). Fludarabine was used in the concentration 3.6 µg/mL for 24 hours. TP53-2A indicates biallelic *TP53* inactivation; 1, del/R249G; 2, del/V216M; 3, del/L132R. TP53-1, monoallelic *TP53* inactivation; 1, wt/L194R; 2, wt/Y234N; 3, wt/D281N; 4, wt/C176W; 5, wt/C277F; 6, wt/A138P; and WT, no *TP53* abnormality.

defects: response to *PUMA* promoter was completely or substantially impaired in all of these samples (n = 6), whereas 3 cases induced expression of *BAX* and 1 case the *CDKN1A* gene in a manner similar to wt control.

Discussion

Defects in the *TP53* gene are decidedly associated with the worst prognosis in CLL patients. Although the p53 dysfunction is routinely assessed as a monoallelic gene deletion when I-FISH is used, 2 recent reports by Zenz et al[24] and Rossi et al[25] have clearly shown an independent prognostic value of single *TP53* mutation (without accompanying deletion) in CLL. These observations are important not only with respect to this leukemia but also for cancer research in general; the consequence of monoallelic *TP53* defects for tumor progression (gene dosage effect in the case of deletion and assumed dominant negative effect on wt allele or potential gain of function in the case of mutation) is unclear and remains a matter of intense debate.[32,40]

In our study we confirm the detrimental impact of monoallelic *TP53* aberrations on the survival of CLL patients, although survival was somewhat better than in patients with biallelic inactivation. In addition, our study shows that (1) single missense mutation but not single deletion is selected in CLL cells; (2) single *TP53* abnormalities are frequently accompanied by *ATM* deletion; (3) novel *TP53* abnormalities occur after therapy administration and always involve mutation; and (4) the single *TP53* missense mutation significantly impairs p53-dependent DNA-damage response in CLL cells.

The question of preferential selection of monoallelic *TP53* mutation versus 17p deletion was not addressed in the study by Zenz et al[24] because of the small number of corresponding cases. A more extensive study by Rossi et al[25] identified a large fraction of monoallelic *TP53* changes within the affected cohort (total, n = 44) and showed that a single deletion was more frequent (16/44) than a single mutation (10/44). This finding is in sharp contrast to our results, where deletion without an accompanying mutation was a very rare event (3/70 of the *TP53* affected patients), whereas missense *TP53* mutation was clearly the most commonly observed type of monoallelic abnormality. It is not easy to explain such a large discrepancy. One possibility is obviously to address the sensitivity of methodologies, especially to consider a more sensitive mutation screening in our study as the result of FASAY.

Although this possibility cannot be excluded, there is another potential explanation. The study by Rossi et al[25] was performed on a far more prognostically favorable patient cohort (unmutated *IgVH* locus in 38% of patients vs 62% in our study) and was done at diagnosis (whereas 52% of our patients were investigated at a later stage of disease). Therefore, it is possible that *TP53* missense mutation, which ought to be more harmful because of the resulting p53 activity than cytogenetic deletion,[41] is selected later in the course of the disease and is associated with an unfavorable profile of the cohort. In fact, we can also observe the preferential selection of *TP53* missense mutation as opposed to deletion through a proportion of affected cells in our study. Although all 3 isolated deletions were detected in only a fraction of the CLL cells (20%, 21%, and 24%), 9 of the 20 single missense mutations progressed to most or rather all CLL cells (≥ 50% of red or pink colonies in FASAY).

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Our findings support the view that *TP53* missense mutation represents a more severe defect than a deletion in this gene. The same conclusion was derived from a study[42] of Li-Fraumeni families, where the missense mutations resulted in tumor onset 9 years earlier than compared with the other *TP53* alterations and from the mouse model of Li-Fraumeni syndrome, where the mice harboring the equivalent of human hot-spot mutation R175H were burdened by a greater metastatic potential of tumor cells in comparison with $p53^{wt/-}$ mice.[43]

Our data concerning the repeated analysis of p53-wt patients during the course of CLL show that this gene is most commonly inactivated by a simultaneous impairment of both alleles. We must emphasize, however, that this selection was very likely to be accelerated by therapy and may not be the same as spontaneous *TP53* mutagenesis. This surely occurs in many CLL patients, eg, 32 patients in our study manifested *TP53* abnormality, when they have never been treated before. In fact, it is difficult to assign therapy as an unambiguous factor behind the novel mutations. The 2 study groups subjected to repeated investigation, treated and untreated patients, differed significantly from each other simply by the fact that the former required therapy because of a more advanced disease progression. Therefore, the more aggressive biologic behavior of the corresponding tumor cells, ie, faster proliferation or more advanced DNA damage could lead to mutation pressure on the *TP53*.

All novel mutations occurred in cases with the unmutated *IgVH* gene, which is associated with shorter telomeres and greater telomerase activity in CLL cells, ie, with their greater proliferation activity.[44] In addition, 8 of the 12 patients with novel *TP53* defect were treated before investigation I, ie, they manifested unfavorable disease on a long-term basis. In each case, however, the strict association between the newly acquired *TP53* defect and the presence of a closely preceding therapy is clear and unquestioned in our study. Theoretically, it is possible that some type of therapy may directly affect the DNA of the *TP53* gene, as suggested, eg, for alkylating agents used in CLL.[35] The most likely explanation for the observed association is that preexisting undetectable *TP53* abnormalities are simply selected in a vacant niche of blood compartments cleared by drug administration; the expansion of the clone would be more rapid under these circumstances than in blood compartments occupied by original CLL cells. This view is also in line with the observation that *TP53* mutations appeared in 2 patients treated only with alemtuzumab, an agent with supposedly p53-independent function and no direct effect on DNA damage. In the broadest sense our results support the view that therapeutic intervention in CLL should be performed with maximal caution and only if necessary.[37]

Our study also provides interesting data concerning the p53 protein activity in cells harboring sole missense mutation. Only a minority (17%) of corresponding samples showed a stabilization of the p53 protein by mutation. This observation is in line with the view that p53 stabilization cannot occur in the presence of the second wt allele.[45] Interestingly, the only 2 samples showing the p53 stabilization harbored mutations at frequently mutated codons (Y220C and D281N); the former mutation has been shown to lead to a marked destabilization of the p53 protein,[46] and the latter has been associated with the dominant-negative effect over wt allele.[47] Although most of missense mutations accompanied by the deletion of the remaining allele led to p53 accumulation (88%), there were some exceptions. Altogether our data show that approaches used for the identification of *TP53* mutations through the monitoring of the p53 protein level, ie, immunohistochemistry or western blot, lead to a significant bias in favor of only biallelic defects.

The fact that all tested monoallelic *TP53* mutants lost a substantial part of their activity toward the *PUMA* promoter (in comparison with the wt cells), with only some of them retaining the activity toward *BAX* or *CDKN1A* promoters, is interesting. It supports the view that Puma is a critical component of p53-mediated apoptotic response after fludarabine administration.[48] The impaired apoptosis in the subgroup of sole *TP53* missense mutations, as assessed by viability testing, may then be seen as a consequence of discriminative character in recognition and induction of target genes.[49,50]

In summary, our study confirms that most of the *TP53* defects in CLL result from a virtually simultaneous inactivation of both alleles. However, a significant proportion of affected patients harbor a sole *TP53* missense mutation. This abnormality significantly impairs prognosis (requirement for therapeutic intervention) and the survival of CLL patients. It also has a negative impact on a response to DNA damage. We support the view that mutation analysis of the *TP53* gene should be included into a prognostic stratification of CLL patients, in addition to common I-FISH screening. For this purpose, FASAY or denaturing high-performance liquid chromatography, both coupled to sequencing, may be considered as the most proper methodologies. They may offer a high rate of analyses and a similar cost-effectiveness. A major benefit of the former is its direct functional readout. FASAY can distinguish partially active (eg, temperature-sensitive) mutations and, because of subcloning, it can also disclose aberrant somatic hypermutations.[36] Because FASAY works with RNA, it may not identify some nonsense and frameshift mutations if they lead to nonsense-mediated mRNA decay. However, denaturing high-performance liquid chromatography is not able to detect large intragenic deletions. In each case, we recommend performing the mutation analysis before each treatment initiation because novel *TP53* defects may be selected by previous therapies.

Acknowledgments

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Authorship

Contributions: J. Malcikova, J.S., L.R., P.K., V.V., S.C., M.S., H.S.F., M.K., and S.P. performed experiments; Y.B., M.D., M.B., and J.Mayer provided samples and clinical data; B.T. performed statistical evaluation; J.S., J. Malcikova, and M.T. designed the study and evaluated results; and M.T. wrote the manuscript.

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ORIGINAL ARTICLE

TP53 mutation profile in chronic lymphocytic leukemia: evidence for a disease specific profile from a comprehensive analysis of 268 mutations

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The *TP53* mutation profile in chronic lymphocytic leukemia (CLL) and the correlation of *TP53* mutations with allele status or associated molecular genetics are currently unknown. We performed a large mutation analysis of *TP53* at four centers and characterized the pattern of *TP53* mutations in CLL. We report on 268 mutations in 254 patients with CLL. Missense mutations appeared in 74% of cases compared with deletions and insertions (20%), nonsense (4%) and splice site (2%) mutations. The majority (243 of 268) of mutations were located in the DNA-binding domain. Transitions were found in 131 of 268 mutations, with only 41 occurring at methylated CpG sites (15%), suggesting that transitions at CpGs are uncommon. The codons most frequently mutated were at positions 175, 179, 248 and 273; in addition, we detected a common 2-nt deletion in the codon 209. Most mutations (199 of 259) were accompanied by deletion of the other allele (17p–). Interestingly, trisomy 12 (without 17p–) was only found in one of 60 cases with *TP53* mutation (without 17p–) compared with 60 of 16 in the cohort without mutation ($P = 0.006$). The mutational profile was not different in the cohorts with and without previous therapy, suggesting that the mechanism underlying the development of mutations may be similar, independent of treatment.
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Keywords: CLL; genetics; 17p deletion; p53; TP53 mutation

Introduction

p53 plays a central role in multiple essential functions of the cell including cell cycle arrest, apoptosis and senescence. It is through these functions that p53 mediates its tumor suppressor activity by inducing or repressing a multitude of p53 target genes. Its importance in carcinogenesis is easily appreciated simply based on the fact that *TP53* is the gene most frequently mutated in cancer. The current release (R14) of the *TP53* mutation database of the IARC (International Agency for Research on Cancer) and UMD (Universal mutation database) (http://p53.free.fr/) holds almost 29 000 somatic mutations. The mutations are generally located in the DNA-binding domain and different types of hot spot mutations have been described. Contact mutations remove essential DNA-contact sites (R248Q, R248W, R273H and R273C), whereas structural mutations affect residues that are essential for the overall architecture (R175H, G245S, R249S and R282W).[1]

Different cancers have been found to exhibit disease-specific mutation profiles, which sometimes help to gain clues on the carcinogenesis or progression.[2,3] In addition, *TP53* mutations have been associated with poor prognosis in numerous cancers including lymphomas and chronic lymphocytic leukemia (CLL).[4–8]

Mutations of *TP53* are found in 4 to 37% of patients with CLL, and unselected cohorts of untreated patients can be expected to show TP53 mutations in the order of 10%.[4–10] The highest incidence of *TP53* mutation is seen in patients with fludarabine refractory CLL and much of the heterogeneity in mutation prevalence is explained by different patient cohorts.[7] The presence of mutations in *TP53* has been associated with poor prognosis in a number of retrospective studies, but the association of 17p deletion and *TP53* mutation has led to the pooling of mutations with 17p deletion (usually the majority) and cases without 17p deletion. Therefore, the exact prognostic relevance of *TP53* mutations (alone and in relation to 17p deletion) had not been documented until very recently. In more recent studies, a comprehensive molecular genetic characterization with mature follow-up including fluorescence *in situ* hybridization and immunoglobulin heavy chain variable region (IGHV) mutation status defined the clinical impact of *TP53* mutations in CLL (4–6).[8]

Because of the relatively low frequency of *TP53* mutations, few studies to date have assessed the particular mutation profile in CLL. The studies that have been reported included 13–46 patients with mutations and therefore these results are subject to bias.[11–14] In addition, detailed analysis of clinical or genetic characteristics was not possible and is also not possible from an analysis of the databases.

In order to establish a precise description of *TP53* mutations in CLL, with particular emphasis on the genetic background and the relation to previous therapy, we have compiled a large set of *TP53* mutations from CLL samples from different centers in Europe. We compared this profile with the currently available data from the *TP53* mutation databases and also included a matching control CLL cohort lacking *TP53* mutations to search for differences in the genetic profile.

Materials and methods

Patient cohort

In our effort to compile a large set of patients with *TP53* mutations, we combined four cohorts where mutation analysis

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was available. The first cohort consisted of patients analyzed at the University of Ulm using denaturing high-performance liquid chromatography (DHPLC) and sequencing ($n=128$ with 134 mutations (single-center cases, trial cohorts CLL4, CLL2H))[4] and a p53 resequencing CHIP (Roche Molecular Systems, Pleasanton, CA, USA; $n=26$ with mutation; exons 2–11). The cohort was formed by patients from different clinical studies and also included selected cases with 17p deletion. The second cohort was from the University of Liverpool, which was tested in Ulm using DHPLC/sequencing ($n=14$ with 17 mutations (out of 104 samples); exons 4–10). The third cohort was from Brno and this set was investigated by functional analysis of separated alleles in yeasts (FASAY) and sequencing ($n=58$ with 63 mutations; codons 67–346).[15] Finally, cases from Bournemouth were included, which were analyzed by sequencing ($n=28$ with mutations; 5–9). The control cohort consisted of 463 patients, who were shown to not have a *TP53* mutation using DHPLC from Ulm.

Mutation detection

Genetic analyses. Fluorescence *in situ* hybridization analysis and IGHV sequencing were performed in all cases as previously described.[16] To define the cutoff level for the presence of the *TP53* gene deletion, hybridization experiments of blood specimens from probands were performed. The cutoff level was defined by the mean plus three s.d. of the frequency of control cells exhibiting only one *TP53* signal. A germ line homology of 98% was used as the cutoff between IGHV mutated and IGHV unmutated cases. The genetic characterization was performed at each center separately.

TP53 *sequencing analysis.* We analyzed all samples by automated fluorescent sequencing using Big Dye Terminator Kit and ABI 3100 sequencer (Applied Biosystems, Carlsbad, CA, USA; exons 2–11 and 4–10, respectively). The primer sequences are available upon request. The primers were designed to cover all coding exons and intron–exon boundaries. Cases with 17p deletion where no mutation was identified by DHPLC were sequenced in all coding exons to confirm the absence of a mutation.

DHPLC. DHPLC was used to identify samples containing mutations in exons 2–11 (coding region of p53; $n=151$). In the cohort from Liverpool, analysis was confined to exons 4–10, because previous DHPLC analysis of samples from Ulm had shown the absence of mutations in exons 2, 3 and 11. DHPLC analysis is based on the temperature-dependent differences in column retention time of PCR products generated from homoduplex (wild-type) and heteroduplex (mutated) DNA, resulting in the presence of distorted or additional peaks when mutations are present. Details have been previously reported.[4,7] All mutations were confirmed by an independent PCR which was used for sequencing.

Amplichip resequencing

The AmpliChip p53 test assesses exons 2–11 of *TP53* and 2 bp of intronic sequence at the exon/intron boundaries. Exons are amplified in two multiplex PCR reactions from genomic DNA, fragmented, 3′-end labeled with a fluoresceinated dideoxynucleotide and hybridized to the AmpliChip surface. For each nucleotide position of *TP53*, specific oligonucleotides represent the potential mutations on the AmpliChip surface. The research assay was performed according to the instructions of the manufacturer (Roche Molecular Systems Inc.).

FASAY

In this functional assay, the *TP53* gene is introduced into yeast cells, where *TP53* wild-type samples form large white colonies, whereas the colonies with the *TP53* mutations are small and red (fully inactive mutants) or pink (partially inactive mutants). The FASAY was performed as described by Flaman *et al.*,[15] with some modifications published earlier.[17] The split assay is a modified version of FASAY, allowing separate analyses of the 5′ and 3′ regions of the p53 complementary DNA. The assay was performed when the PCR product for FASAY was too faint,[18] using the protocol described by Waridel *et al.*[19] The appropriate vectors and yeast strain $ADE2^-$ carrying a reporter with a p53-binding site upstream of the *ADE2* gene were kindly provided by R Iggo.

We noted that FASAY was not able to detect the hot spot deletion (2 nt) at position 209. Therefore, the deletion was detected in Czech cohort by high-resolution melting analysis of exon 6 performed on genomic DNA.

Databases and references

For comparison with the data generated in the current study, we compiled a cohort of *TP53* mutations as reported in different publications and the databases. To the number of 148 mutations taken from the IARC TP53 Mutation Database (version R12, November 2007) and the UMD_TP53 Mutation Database (2007_R1 release curated version), we added 38 mutations from five recently published studies not mentioned in the databases. The compiled cohort consisted of 186 *TP53* mutations in CLL.[11,20–23]

The CLL cohort without *TP53* mutations was from the same cohorts (Ulm: single center, first line treatment trial, refractory CLL trial). Cases with wild type were included in the matching control.

Meta-analysis of p53 mutant loss of function in tumors

All mutations were collected and analyzed using MUT-TP53, a spreadsheet that automatically assesses the biological activity and likelihood of each *p53* mutant.[24] Previous studies have shown that *TP53* mutations could be very heterogeneous depending on the strategy used for the analysis. The UMD TP53 database also includes functional data of the majority of missense mutants that allows comparison of each publication.[25] For functional analysis, the mean and 95% confidence interval of the remaining biological activity of all mutants were calculated using the residual transactivational activity for each mutant p53 towards waf-1 promoter. The reference value corresponds to the mean and 95% confidence interval of all studies for the specific cancer.[25] For CLL analysis, we used all publications describing more than five *TP53* mutations. For comparison of CLL results with other cancers, we downloaded the curated data set and again removed the known polymorphisms. The statistical analyses were performed with PRISM software (graphPad Software Inc.) on a Mac OS X platform.

All other statistical analyses were performed using the statistical software environment R, version 2.9.2 (R Development Core Team 2009; R Development Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2009.). For the analysis of contingency tables, Fisher's exact test was applied. A result was denoted as statistically significant at a P-value $<5\%$. No P-value adjustment owing to multiple testing was performed.

Results

For the current analysis, we have compiled a collection of 268 mutations in 254 patients with CLL (Table 1). In accordance

Table 1 Cohort of CLL patients with *TP53* mutation

	All mutations (n = 268)	
	Counts	*%*
Mutation type		
Transition (all)	131	48.9
At CpG	41	15.3
Transversion (all)	84	31.3
At CpG	15	5.6
Missense	198	73.9
Splice site	6	2.2
Nonsense	11	4.1
Inframe/frameshift		
Deletion	43	16.0
Insertion	10	3.7
IGHV status		
Mutated	51	21.1
Unmutated	191	78.9
NA	26	
Hierarchical cytogenetics		
Del 17p	199	76.8
Del 11q	14	5.4
Trisomy 12	1	0.4
Normal	21	8.1
Del 13q	24	9.3
NA	9	
Treatment		
Previous treatment	105	42.5
No previous treatment	142	57.5
NA	21	
Codons		
132	5	1.9
158	4	1.5
175	7	2.6
179	9	3.4
195	5	1.9
209	12	4.4
220	7	2.6
234	4	1.5
236	3	1.1
245	6	2.2
248	12	4.5
273	11	4.1
281	7	2.6

Abbreviations: CLL, chronic lymphocytic leukemia; NA, not available.

with the profound biological impact of a single *TP53* mutation, multiple mutations are very rare in CLL (14 patients with two mutations). The mutation profile showed mainly missense mutations (74%) but also a comparatively high incidence of insertions (4%) or deletions (16%; Figure 1) The mutations were mainly located in the DNA-binding domain and 90% of the mutations were located in exons 5–8 (Figure 2). The fraction of frameshift mutations was significantly higher in the non-DNA-binding regions (11 of 21 vs 42 of 239; $P = 0.0007$; Figure 2).

As shown in Figure 3, transitions were the most common events accounting for 49% of all alterations. Interestingly, transitions at CpG were relatively rare (41 of 131 (31% of all transitions)) compared with some other more common cancers such as colon (1518 of 2197 (69%; Fisher's exact test, $P < 0.001$)), lymphoma (206 of 492 (42%) $P = 0.04$) or breast cancer (542 of 1187 (45%) $P = 0.002$). The overall pattern of mutations was also different in comparison with these cancers ($P = 0.002$ (breast); $P = 0.04$ (non-hodgkin lymphoma); $P < 0.001$ (colorectal)).

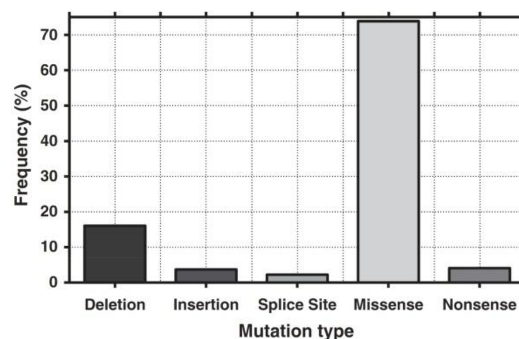

Figure 1 Mutation type profile in CLL ($n = 268$). The mutation profile of the cohort shows 73.9% missense, 4.1% nonsense and 2.2% splice site mutations (all single-base substitutions). The incidence of frame-shift mutations was high with 16.0% deletions and 3.7% insertions.

The amino acids most frequently mutated were at positions 175, 179, 248 and 273. This indicates that the classical hot spots are also commonly mutated in CLL. Codons 175, 179, 220, 248, 273 and 281 made up for 53 of 268 mutations (20%). In addition, we identified one other commonly mutated codon at position 209 (in all instances 2-nt deletion). This alteration was—together with the known hot spot codons 248 and 273—the single most common mutation (Figure 4).

Comparison of DHPLC and FASAY cohort

We performed a detailed analysis of the results obtained by DHPLC and FASAY. Although both methods will have their respective shortcomings, DHPLC is generally considered an unbiased approach. In contrast, FASAY has a functional read out and can be expected to pick up only mutants affecting p53 transcriptional activity. In addition, it may not detect frameshift mutations if they lead to nonsense-mediated mRNA decay (see Material and methods section). Consequently, splice site mutations and nonsense mutations were rather rare in the FASAY cohort, as opposed to an incidence of 12 of 151 in the cases assayed by DHPLC. However, the overall mutation pattern of the FASAY cohort was not significantly different from that generated by DHPLC (data not shown).

In addition, an analysis of functional data was performed for each of the four sets of mutations described in Material and methods (Figure 5). The analysis shows that for all cancers, the mean activity was situated around −1.2 with a narrow 95% confidence interval, demonstrating an apparent homogeneity of p53 mutant activity for all of the mutations included in the database (Figure 5). This value corresponds to a residual transactivational activity of about 10% compared with wild-type p53. Comparison of the four sets of p53 mutations used in the present study shows that they display a homogeneous distribution with a 95% confidence interval, which includes the global mean value of CLL. Comparison with all publications describing *TP53* mutations in CLL shows that all but one study have a similar profile.

Comparison with published data/database

When we compared our data with what is available from different publications and databases, we observed a number of differences. We especially found a lower proportion of transitions at CpGs (41 of 131 transitions vs 47 of 87; $P = 0.001$)

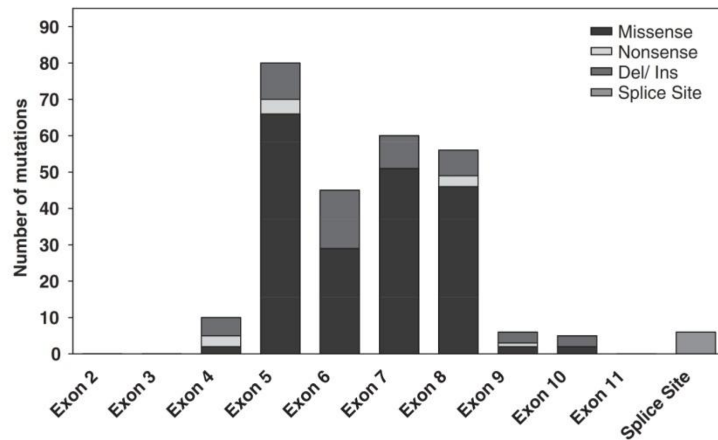

Figure 2 Exon distribution of *TP53* mutations in CLL. In the central core domain (exon 5–8), we found 89.9 of all mutations, mostly represented by missense mutations (80%). Outside this area (exon 2–4 and exon 9–11), nonsense (30 and 9.1%) and frameshift (50 and 54.5%) mutations were more frequent. del, deletion; ins, insertion.

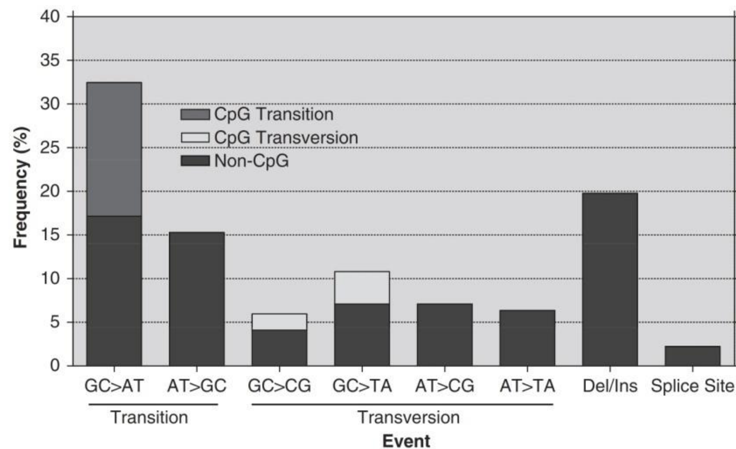

Figure 3 Mutational events in CLL. The most common events were transitions accounting for 48.9% of all events. Interestingly, transitions at CpG were relatively rare (41 of 131 (31% of all transitions)) compared with other more common cancers. In contrast, frameshift mutations are common in CLL. del, deletion; ins, insertion.

and fewer mutations at the hot spot codon 273 in our cohort (11 of 268 vs 20 of 186).

Comparison of the mutation profile in relation to the presence or absence of 17p deletion

In order to detect a potential difference in the *TP53* mutation profile of cases with and without 17p deletion, we studied the respective profiles (Supplementary Table 1). We found no difference in the proportion of transitions at CpGs in the two groups. There was an increased proportion of cases with insertions and deletions in the cohort with 17p deletion (23 vs 13%) and in turn a higher proportion of missense mutations in the cases without 17p– (82 vs 71%; Figure 6). A detailed comparison of mutational events showed no significant differences.

A comparison of the predicted residual activity of the mutated p53 towards a range of known targets showed no differences. In both groups, over 90% of all base substitutions were classified as non-functional. These findings suggest that the *TP53* mutations in CLL show a similar profile independent of the presence of 17p deletion.

Comparison of the mutation profile in relation to the presence or absence of previous therapy

TP53 mutations are more commonly observed with advanced disease and after previous therapy. Currently, we are lacking a precise picture of when these mutations occur during the disease and if the majority are selected or if *de novo* mutations occur more frequently after therapy. We therefore compared the mutation profile in our patients with ($n = 105$) and without

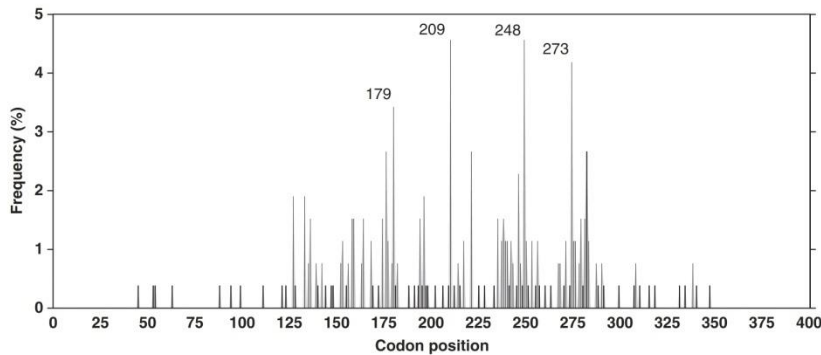

Figure 4 Codon distribution of *TP53* mutations in CLL. The amino acids most frequently mutated were at positions 175, 179, 209, 248 and 273. This indicates that the classical hot spots are also commonly mutated in CLL. Codons 175, 179, 220, 248, and 273, 281 made up for 53 of 268 of the mutations (20%), but we identified one other commonly mutated codon (209) that was—together with the known hot spot codon 248 and 273—the single most common mutation.

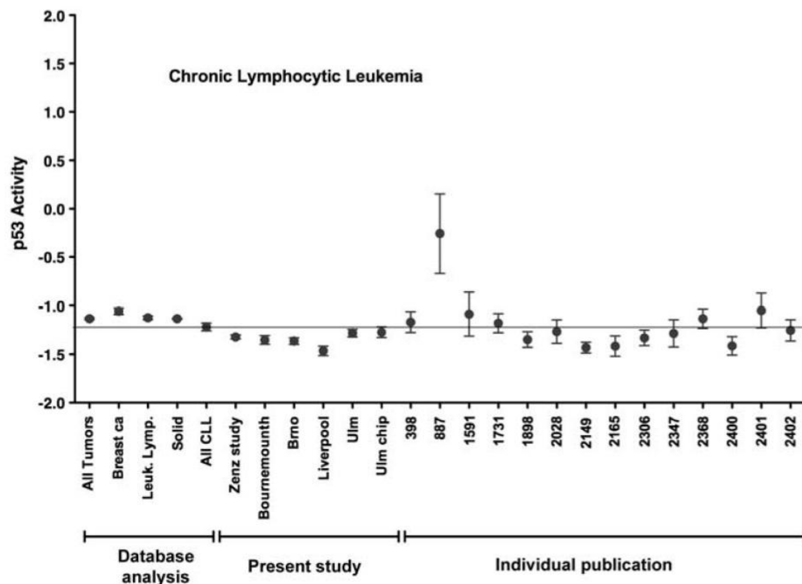

Figure 5 Analysis of p53 loss of function. Dot and bars; mean and 95% confidence interval (CI) of mean p53 activity as measured by transactivation with the waf promoter. The mean and 95% CI of p53 activity for all studies combined for a breast cancer and all cancer of the database is shown on the far left of the graph. The horizontal line shows the mean of the combined studies. The *y* axis corresponds to p53 transactivation activity, with a value of −1.5 for the negative control and a value of 2.5 for 100% of wild-type activity. All tumors: all tumors included in the database; breast ca: breast cancer; leuk lymp: all lymphoma and leukemia; solid: all non-hematological tumors; all CLL: all chronic lymphocytic leukemia; Zenz study: mutation from the present study; Bournemouth, Liverpool, Brno, Ulm and Ulm chip correspond to the various set of mutations described in Material and methods. Individual publication corresponds to each article describing *TP53* mutations in CLL.

($n = 142$) treatment before mutation detection. The mutation profile and analysis of residual function showed no significant differences suggesting that the mechanisms underlying mutation acquisition are identical regardless of chemotherapy. Alternatively, one might conclude that mutations might be selected by chemotherapy rather than being caused by it.

Comparison of the genetic profile of CLL with and without TP53 *mutation*

When we assessed the cytogenetic profile (fluorescence *in situ* hybridization) of the cohort with *TP53* mutations, we found an overrepresentation of cases with 17p deletion (199 of 259 (77%) vs 25 of 441 in the control group (5%); $P < 0.001$). Interestingly,

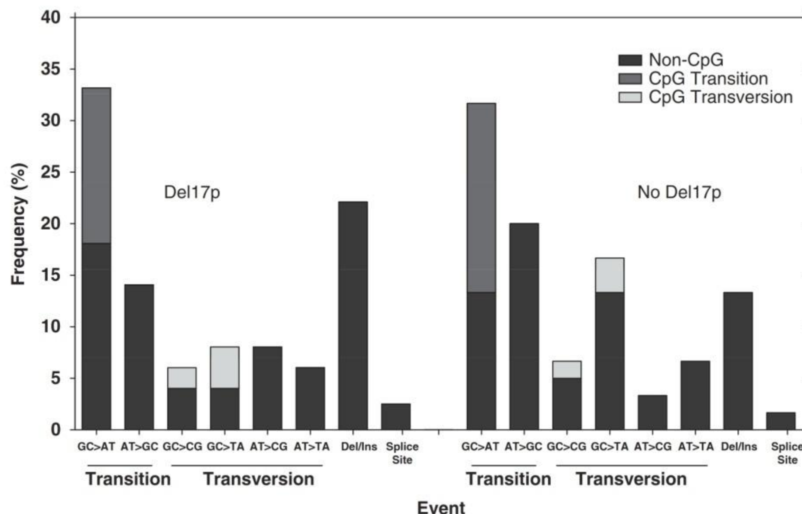

Figure 6 *TP53* mutation profile in CLL without ($n=60$) and with del17p ($n=199$). We found no difference in the proportion of transitions at CpGs in the two groups. There was an increased proportion of cases with frameshift mutations in the cohort with 17p deletion (22.6 vs 13.3%) and in turn a higher proportion of missense mutations in the cases without 17p– (81.7 vs 70.9%), without reaching statistical significance. del, deletion; ins, insertion.

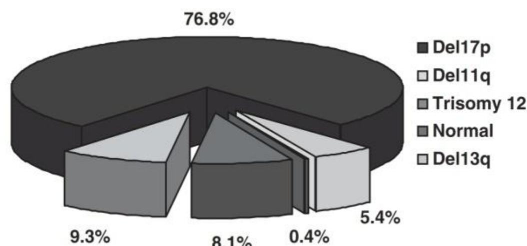

Figure 7 Hierarchical cytogenetics by fluorescence *in situ* hybridization in CLL with *TP53* mutation ($n=268$). In 76.8%, the mutations are accompanied by a deletion of the other allele (17p–). Deletion of 11q in the absence of deletion of 17p was found in 5.4% of the mutations. Deletion 13q in the absence of deletion 17p, 11q or trisomy 12 was seen in 9.3% of the cases. Trisomy 12 appeared just in one case in the absence of deletion 17p (0.4%).

the groups of patients with 11q deletion showed a similar frequency when excluding patients with 17p deletion (23 and 22% respectively). In contrast, trisomy 12 (in the absence of 17p deletion) was only seen once (1 of 60; 2%) in cases with *TP53* mutation compared with 60 of 416 (14%) when patients with 17p deletion were excluded ($P=0.006$; Figure 7).

The cases with *TP53* mutation mainly had an unmutated *IGVH* mutation status (191 of 242; 79%). There were five cases with V3-21 usage. The control cohort showed a significantly more balanced proportion of mutated and unmutated *IGVH* cases ($P<0.001$). An unmutated *IGVH* status was observed in 289 of 441 (66%) cases, whereas 152 of 441 (34%) had mutated *IGVH*. The *TP53* mutation profile did not differ in the groups with mutated or unmutated *IGHV*.

Discussion

Although the association of *TP53* mutations with poor prognosis has been proposed for many years,[16,21,26–31] the precise prognostic impact of *TP53* mutations in the absence of 17p deletion has only recently been shown.[4–6,8] Because of the profound clinical impact of *TP53* mutation in CLL, there is a continuing interest in its further characterization not only from a clinical and diagnostic but also a mechanistic perspective. In this respect, it is important to gain insight into the mutation profile of CLL. This has not been possible because of the limited size of previous studies. Although this problem may be approached by the compilation of mutation data in the IARC or other databases, the problem with the profile generated by databases is that it is usually compiled of different small and potentially biased studies.[25,32,33] The bias is usually imparted by different exon coverage, technique or patient cohort. Therefore, the correlation of *TP53* mutation results to database entries may be misleading.

The aim of our study therefore was to establish a reference dataset of *TP53* mutations in CLL. In order to achieve this, we have analyzed 268 *TP53* mutations from four different cohorts of CLL patients. Importantly, we have reanalysed the data comparing it to the current database information but also within the cohort by comparing the results from the DHPLC approach ($n=151$) and the results generated by FASAY ($n=63$). We intentionally avoided an analysis of clinical end points because the nature of recruitment into the cohorts/referral bias (clinical trial first line therapy, clinical trial refractory CLL, single-center cohorts) would have led to a bias.

The key findings of our study establish the disease-specific *TP53* mutation profile in CLL. Major outputs include the demonstration that the *TP53* mutation profile is independent of 17p deletion or previous therapy. These findings suggest that the *TP53* mutation, detectable after therapy, is selected rather than being caused by, for example, alkylating agents. The finding also supports the growing evidence that the clinical consequences of 17p deletion (and *TP53* mutation) are very similar to the *TP53* mutation in the absence of 17p–.

We found a low frequency of transitions at CpGs, a relatively high incidence of frameshift mutations (particularly in cases with 17p deletion) and confirmed the codon 209 frameshift mutation

as a 'hot spot' in CLL. Importantly, we did not find significant differences in the mutation profile of cases stratified by treatment history or 17p status suggesting that the underlying mechanisms are similar.

The first study investigating the mutation profile in CLL studied 42 mutations and found a CLL-specific profile when compared with the known *TP53* mutations in lymphoma at the time.[13] The study found a high incidence of codon 209 mutations (10%) and a high incidence of transversions in codon 273. This 'unusual' profile at CpG sites was suggested to implicate an exogenous carcinogen. In our study, we identify the codon 209 two base pair deletion as one of the most common mutations in CLL. Hot spot mutations are common in CLL. On the other hand, we found a comparatively small proportion of transitions at CpG sites compared with other cancers (for example, colon cancer). Interestingly, G→A and C→T transitions at the CpG sites were biased in favor of the G→A exchange in our study (27 vs 14; ratio 1.9:1). Similar data may be observed for CLL also in the IARC database, that is, 32 G→A mutations vs 15 C→T mutations (ratio 2.1:1). Other cancers showed a closer ratio (high grade lymphoma 128 vs 78 (ratio 1.6:1); breast cancer 335 vs 207 (ratio 1.6:1); colon cancer 864 vs 654 (ratio 1.3:1)). As the G→A transition detected at CpG site in the coding, non-transcribed DNA strand is considered to be a consequence of the cytosine deamination (and hence C→T mutation) on the opposite, transcribed DNA strand, it was suggested that these alterations are preferentially selected in quiescent non-dividing cells.[34]

There are a number of examples of disease-specific mutation profile of *TP53* in different cancers (reviewed in Soussi[3]). These include dinucleotide changes in UV-induced skin cancer, typical G→C to T→A transversions at codon 249[35] in liver cancer caused by aflatoxin B1 and, for example, a high incidence of G→T transversions (codon 157 of 158) in lung cancer.[36]

When we compared the genetic profiles of CLL cases with and without *TP53* mutation, some significant differences were observed. Although cases with 17p deletion show a lower incidence of concomitant 11q deletion (11%), the incidence of deletion 11q in cases with TP53 mutation only (23%) was not significantly different from the cohort without *TP53* mutation (22%). In contrast, there was a significant underrepresentation of trisomy 12 in the cohort of cases with *TP53* mutation in the absence of 17p deletion. Definite reasons for the imbalance are however currently unclear. Recent observations showing the very favorable outcome of patients with +12q after treatment with FC/R-FC may be partly imparted by the rarity of *TP53* mutations in the cohort.[37,38]

As the use of *TP53* mutational analysis to predict prognosis and define treatment strategies is likely to increase in CLL, the current study may serve as a reference point to the *TP53* mutation profile in CLL. Investigators assessing *TP53* mutations should preferentially cover exons 4–10 and detect the most common mutations including c.626_627delGA.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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JOURNAL OF CLINICAL ONCOLOGY — ORIGINAL REPORT

Missense Mutations Located in Structural p53 DNA-Binding Motifs Are Associated With Extremely Poor Survival in Chronic Lymphocytic Leukemia

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Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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A B S T R A C T

Purpose
There is a distinct connection between *TP53* defects and poor prognosis in chronic lymphocytic leukemia (CLL). It remains unclear whether patients harboring *TP53* mutations represent a homogenous prognostic group.

Patients and Methods
We evaluated the survival of patients with CLL and p53 defects identified at our institution by p53 yeast functional assay and complementary interphase fluorescence in situ hybridization analysis detecting del(17p) from 2003 to 2010.

Results
A defect of the *TP53* gene was identified in 100 of 550 patients. p53 mutations were strongly associated with the deletion of 17p and the unmutated *IgVH* locus (both $P < .001$). Survival assessed from the time of abnormality detection was significantly reduced in patients with both missense ($P < .001$) and nonmissense p53 mutations ($P = .004$). In addition, patients harboring missense mutation located in p53 DNA-binding motifs (DBMs), structurally well-defined parts of the DNA-binding domain, manifested a clearly shorter median survival (12 months) compared with patients having missense mutations outside DBMs (41 months; $P = .002$) or nonmissense alterations (36 months; $P = .005$). The difference in survival was similar in the analysis limited to patients harboring mutation accompanied by del(17p) and was also confirmed in a subgroup harboring *TP53* defect at diagnosis. The patients with p53 DBMs mutation (at diagnosis) also manifested a short median time to first therapy (TTFT; 1 month).

Conclusion
The substantially worse survival and the short TTFT suggest a strong mutated p53 gain-of-function phenotype in patients with CLL with DBMs mutations. The impact of p53 DBMs mutations on prognosis and response to therapy should be analyzed in investigative clinical trials.

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by a distinctively variable clinical course. Two major prognostic factors are mutational status of the immunoglobulin heavy-chain variable region (*IgVH*)[1,2] and the presence of cytogenetic aberrations.[3] A particularly poor prognosis is associated with the presence of del(17p).[4,5] This deletion is in nearly all cases of progressive leukemia accompanied by *TP53* gene mutation.[6] It has recently become clear that p53 mutation itself is responsible for an inferior prognosis in CLL, regardless of whether it is accompanied by del(17p).[6-9] The proportion of patients with a sole *TP53* mutation [without del(17p)] may vary from 3% to 4% among cohorts at diagnosis or before first therapy[8-10] to 12% in fludarabine-refractory CLL.[11] Patients with the p53 defect respond poorly to therapy involving DNA-damaging agents (eg, fludarabine, chlorambucil) and have a short response duration to chemoimmunotherapy or alemtuzumab.[12] According to the revised National Cancer Institute (NCI) Working Group/International Workshop on Chronic Lymphocytic Leukemia guidelines for CLL,[13] patients with del(17p) (p53 defect) should be offered investigative clinical protocols or should be appointed for allogeneic stem-cell transplantation. In this respect, it is critical to know whether all p53 mutations in CLL lead to a similar phenotype and prognosis.

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Prognostic stratification based on the type of mutation and its position in p53 protein was demonstrated as valuable in several cancers. For example, mutations at the residues, which are closely involved in the p53 binding to DNA, have been associated with a particularly severe phenotype in, for example, breast tumors[14] or diffuse large B-cell lymphoma.[15]

In collaboration with other groups, we recently reported that a majority of p53 mutations in CLL represent missense substitutions,[16] which occur in the DNA-binding domain (DBD) of p53 protein.[17] It is important to note that, in addition to a simple loss of protein function encoded from the affected allele, p53 missense mutations may result in a gain-of-function (GOF) phenotype reflecting a highly oncogenic activity of the altered protein.[18] A pivotal mechanism of the mutated p53 GOF seems to be an interference with the p53-related proteins (ie, p63 and p73).[19] Alternatively, or in parallel, some p53 mutants have been shown to upregulate genes that support cancer progression (eg, nuclear factor-κB)[20] or aggravate effective therapy (eg, multidrug resistance 1 gene [*MDR1*]).[21] *MDR1*, which codes for P-glycoprotein, is involved in a transport of certain drugs used in CLL therapy (eg, doxorubicin, vincristine).[22]

With this report, we show that patients with CLL harboring *TP53* mutation constitute two readily distinct prognostic subgroups. Thus, missense substitutions located in structural p53 DNA-binding motifs (DBMs) can be identified with clearly reduced survival rates compared with other p53 mutations. Our study clinically demonstrates the mutated p53 GOF phenotype.

PATIENTS AND METHODS

Patients

The analyzed cohort consisted of 550 patients with CLL monitored and/or treated at the Department of Internal Medicine–Hematooncology, University Hospital Brno (Brno, Czech Republic), between the years 2003 and 2010. CLL was diagnosed, and the patients were treated according to the 1996 NCI-sponsored CLL Working Group guidelines[23] or updated 2008 NCI Working Group/International Workshop on Chronic Lymphocytic Leukemia guidelines.[13] All blood samples were processed with written informed consent, and the study was approved by the Ethical Commission of the University Hospital Brno.

Our patient cohort is biased toward more severe CLL as evidenced by 65% of patients harboring the unmutated *IgVH* locus and almost one third of patients (30%) having been treated before the first *TP53* investigation. This bias emerged from a local concentration of patients with inferior CLL at the University Hospital Brno; noncomplicated patients are monitored at regional hematologic centers elsewhere in the Czech Republic.

Analysis of TP53 Mutations and Deletions

p53 mutations were identified by a yeast functional analysis (FASAY),[24] and cytogenetic deletions of the *TP53* (17p13.1) locus were detected by routine interphase fluorescence in situ hybridization analysis using a probe from Vysis-Abott (Chicago, IL). We have previously described the experimental conditions for both methodologies.[6] In addition to this earlier study, we performed a direct sequencing of genomic DNA (whole coding region, exons 2 to 11) in patients with del(17p) and wild-type p53 output by FASAY (n = 6). A mutation was identified in four patients (2-nt deletion in codon 209, n = 2; 1-nt insertion in codon 215, n = 1; and nonsense mutation in codon 317, n = 1). These mutations were not identified by FASAY, most likely because of the nonsense-mediated mRNA decay of corresponding molecules. In patients in whom the FASAY exceeded 50% of red colonies and we detected only one mutation and no del(17p), uniparental disomy (UPD) presence was considered.[11] These patients (n = 5) were analyzed using Affymetrix Cytogenetic 2.7M Array (Affymetrix, Santa Clara, CA) to confirm or exclude the UPD (Appendix Table A1, online only).

Classification of TP53 Defects and Mutations

Monoallelic defects were classified as either sole mutation or sole deletion. Biallelic defects were classified as deletion accompanied by mutation of the other allele, two or more mutations, or a mutation accompanied by loss of heterozygosity through the UPD. Nonmissense mutations were defined as any mutation other than missense (eg, nonsense mutation, in-frame or frameshift deletion, insertion or mutation leading to aberrant splicing). Missense mutations involved in the direct contact of p53 with DNA were adopted from the database of the International Agency for Research on Cancer[25] (in our study, codons 239, 241, 248, 273, 275, 277, and 280). Missense mutations in the structural p53 DBMs are mutations localized in the L2 and L3 loops involved in interaction with DNA in the minor groove (codons 164 to 194 and 237 to 250, respectively) and mutations localized in the loop-sheet-helix motif involved in interaction with DNA in the major groove (codons 119 to 135 and 272 to 287).[17] The DBMs are a part of the DBD (codons 102 to 292).[17]

Statistical Evaluation and Survival Analysis

The χ^2 test or Fisher's exact test were used to assess the association between *TP53* mutations and categorical variables. The unpaired t test was used to compare the age in individual groups. We previously reported[6] that survival is reduced dramatically in patients with novel p53 defects. Therefore, this survival analysis was performed from the time of p53 mutation detection/investigation showing wild-type p53, unless stated differently. For survival evaluation, only patients with one discrete mutation were considered to assess an impact of particular mutation. Survival analysis and the time to first treatment (TTFT) analysis were done using the Kaplan-Meier survival estimator. Median survival, median TTFT, and differences between the curves were evaluated by the log-rank test using the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Hazard ratios were determined by the univariate Cox proportional hazards model using MedCalc (MedCalc Sofware, Mariakerke, Belgium).

RESULTS

p53 Defects in Patients With CLL

An abnormality of the *TP53* gene was indentified in 100 of the 550 analyzed patients. Clinical and biologic characteristics of the patients are listed in Table 1. Types of p53 defects are listed in Table 2; and all mutations, detected in 96 patients, are listed in Appendix Table A1. A higher proportion of affected patients (18%) than is usually reported (10% to 15%) is in line with the unfavorable structure of our cohort (see Patients and Methods) and also reflects a repeated investigation in a proportion of patients (n = 195). This analysis disclosed 19 novel p53 defects, in all instances after previous therapy (97 of 195 patients were treated; median time to repeated investigation was 18 months). We previously discussed this negative impact.[6]

Missense substitutions accounted for 78% of mutations and were all located in the p53 DBD, specifically between amino acids 109 and 286. Thirteen of the 16 nonmissense mutations also directly affected the DBD, but the remaining three were located as far as in the C-terminal part of the protein. The p53 mutations were strongly associated with the unmutated *IgVH* locus and presence of del(17p) (both $P < .001$).

Survival in Relation to p53 Mutations

Two previous studies[26,27] have shown that survival of patients with CLL harboring *TP53* abnormalities is greatly influenced by the mutational status of the *IgVH* gene. Therefore, we first determined the impact of the *IgVH* status on the survival rate of p53-affected patients.

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Table 1. Demographics and Clinical and Biologic Characteristics of Patients With CLL

	No *TP53* Mutation				*TP53* Mutation				
	Mutated *IgVH* (n = 160)		Unmutated *IgVH* (n = 241)		Mutated *IgVH* (n = 11)		Unmutated *IgVH* (n = 82)		
Demographic or Characteristic	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	P*
Median age at diagnosis, years	59.6		61.7		55.2		59.0		.1889
Sex									
Male	108	67.5	161	66.8	3	27.3	56	68.3	
Female	52	32.5	80	33.2	8	72.7	26	31.7	.8919
Stage (at the time of *TP53* examination)	158		237		9		79		
Low risk, Rai 0	73	46.2	62	26.2	2	22.2	8	10.1	
Intermediate risk, Rai I/II	60	38.0	93	39.2	2	22.2	27	34.2	
High risk, Rai III/IV	25	15.8	82	34.6	5	55.6	44	55.7	< .001
Hierarchical cytogenetics (I-FISH)	159		240		10		81		
17p−	0	0	3	1.3	7	70.0	52	64.2	< .001
11q−	4	2.5	86	35.8	1	10.0	12	14.8	< .001
Trisomy 12	16	10.1	33	13.8	1	10.0	1	1.2	< .001
13q− sole	91	57.2	54	22.5	1	10.0	11	13.6	.0822
Normal	48	30.2	64	26.7	0	0	5	6.2	< .001

NOTE. Four patients with p53 defect and 52 patients with wild-type p53 had unknown status of *IgVH*.
Abbreviations: CLL, chronic lymphocytic leukemia; I-FISH, interphase fluorescence in situ hybridization.
*The statistical evaluation concerns a comparison of the following groups: no *TP53* mutation with the unmutated *IgVH* versus *TP53* mutation with the unmutated *IgVH*.

The data are presented in Figure 1. The patients who had p53 mutation but also the mutated *IgVH* gene (range of homology, 92.4% to 97.9%) had substantially better survival than p53-mutated patients with unmutated *IgVH* (homology ≥ 98%; P = .018). Therefore, we omitted the small subgroup (n = 11) of p53-affected patients with mutated *IgVH* gene from the subsequent analysis because their survival data would be misleading. Thus, only the wild-type p53 patients harboring the unmutated *IgVH* gene were used as a control group in all subsequent survival evaluations. In this respect, p53 mutations were clearly associated with a higher risk Rai stage and presence of del(17p). However, patients with p53 mutation less frequently exhibited del(11q), trisomy 12, and normal karyotype (Table 1).

Overall, p53 mutations were associated with an obviously reduced survival compared with wild-type p53 patients (median survival, 23 *v* 69 months, respectively; P < .001; data not shown). The analysis structured according to the type of p53 mutation showed that

Table 2. Summary of *TP53* Defects Identified in 550 Patients With CLL

Type of Defect	No. of Patients	Comment
Patients with defective p53	100	
p53 defect/mutated *IgVH*	11	Not analyzed further in this study*
p53 defect/*IgVH* not analyzed	4	Not analyzed further in this study
p53 defect/unmutated *IgVH*	85†	
Monoallelic alteration	22	
Missense mutation	17	
Nonmissense mutation	2	
Del(17p)	3	
Biallelic alteration	62	
Del(17p)/missense mutation	33	
Del(17p)/nonmissense mutation	13	
Missense mutation and UPD	4	
≥ 2 mutations‡	12	Not analyzed further in this study§

Abbreviations: CLL, chronic lymphocytic leukemia; UPD, uniparental disomy.
*Figure 1 shows a substantially better survival (P = .018) of these patients compared with patients with p53 mutation and unmutated *IgVH*.
†One patient with unavailable fluorescence in situ hybridization result (not analyzed further).
‡Six patients also harbored del(17p).
§It is impossible to assign the patients to individual mutation categories.

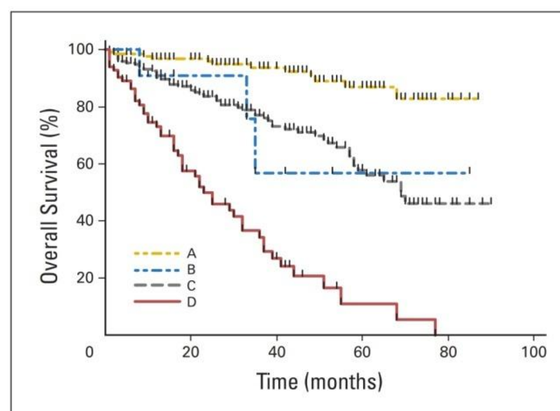

Fig 1. Survival of patients with p53 defect (mutation and/or 17p–) from the time of abnormality detection (or investigation showing wild-type p53) in relation to the *IgVH* mutational status: curve A, wild-type p53 and mutated *IgVH* (n = 131; median survival, not reached); curve B, p53 defect and mutated *IgVH* (n = 11; median survival, not reached); curve C, wild-type p53 and unmutated *IgVH* (n = 193; median survival, 69 months; 71 of 193 patients manifested a high-risk deletion of *ATM* [11q–]); and curve D, p53 defect and unmutated *IgVH* (n = 81; median survival, 23 months). Curve A *v* curve B, P = .016; curve A *v* curve C, P < .001; curve B *v* curve D, P = .018; curve C *v* curve D, P < .001.

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both missense substitutions and nonmissense alterations substantially reduced the survival compared with wild-type p53 patients ($P < .001$ and $P = .004$, respectively), but mutations did not differ significantly from each other ($P = .17$; data not shown).

An interaction with the target DNA is crucial to the activity of the p53 protein. Therefore, we focused on the structurally well-defined DBMs of p53 (see Patients and Methods), which ensure a proper contact with the DNA. Figure 2A shows the survival analysis irrespective of the del(17p) presence. Missense mutations in DBMs (n = 32) led to a clearly shorter survival (12 months) compared with both remaining missense mutations (n = 21; 41 months; $P = .002$) and nonmissense alterations (n = 15; 36 months; $P = .005$). There was no difference in the survival of patients affected in the L2 or L3 loops compared with patients with mutation in the loop-sheet-helix motif (median survival, 13 *v* 10 months, respectively; data not shown). This suggests that patients with DBMs mutation form one uniform group in terms of survival. The analysis limited to patients with p53 mutation and the accompanying del(17p) (n = 50) once again provided similar results (ie, a markedly reduced survival among patients with DBMs mutations; Fig 2B). In parallel, the analysis of a subgroup harboring a sole p53 mutation and intact remaining *TP53* allele (without 17p–) also noted a reduced survival rate for patients harboring DBMs mutation (n = 8; 9 months). Mutations outside DBMs, consisting of eight missense substitutions and two nonmissense alterations, resulted in a median survival time of 41 months (data not shown). However, this difference was not significant ($P = .36$), most likely because of the small number of patients studied.

We then specifically limited our subsequent analysis to mutations located at residues, which are in direct contact with DNA (see Patients and Methods). These patients (n = 13) also had a short median survival time (9 months; data not shown). This further supports the view that a modulation of p53-DNA interaction is critical in CLL.

A subset of p53 mutations in our study (n = 28) was already identified during diagnosis. Therefore, we verified the survival of this limited subgroup, divided again according to mutation presence in versus out of DBMs. In this analysis (Fig 3), the wild-type p53 subgroup showed a substantially longer survival (110 months) compared with the survival that had been observed from the time of p53 investigation (69 months). In contrast, patients with the p53 mutation in DBMs (n = 12) showed a short median survival of only 17 months ($P < .001$; hazard ratio compared with wild-type p53 patients, 20.8; 95% CI, 8.82 to 48.82), which was similar to the survival time measured from the time of abnormality detection (12 months). Mutations out of DBMs consisting of remaining missense substitutions (n = 9) and nonmissense alterations (n = 7) resulted in a median survival time of 51 months ($P < .001$; hazard ratio compared with wild-type p53 patients, 5.3; 95% CI, 2.41 to 11.69). Patients with mutations in versus out of the DBMs again differed significantly from each other in terms of survival ($P = .004$). The p53 mutations in DBMs compared with remaining p53 mutations were significantly associated with male sex ($P = .029$), whereas there was no correlation with age, Rai stage, or the hierarchical cytogenetics.[3]

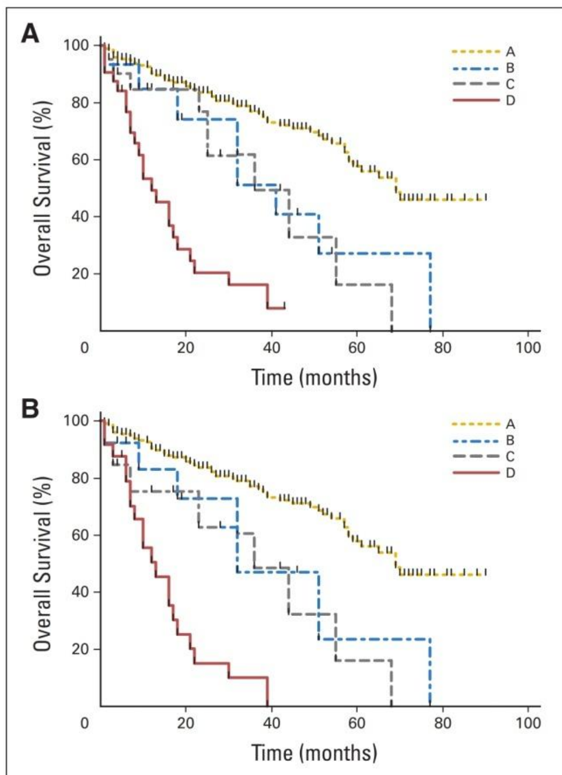

Fig 2. Survival of patients with p53 mutations in the DNA-binding motifs (DBMs) from the time of abnormality detection. (A) Analysis irrespective of del(17p) presence: curve A, wild-type p53 and unmutated *IgVH* (same as curve C in Fig 1); curve B, nonmissense p53 mutation (n = 15; median survival, 36 months); curve C, missense mutation outside DBMs (n = 21; median survival, 41 months); and curve D, missense mutation in DBMs (n = 32; median survival, 12 months). Curve D *v* curve C, $P = .002$; curve D *v* curve B, $P = .005$. (B) Only patients with accompanying del(17p): curve A, wild-type p53 and unmutated *IgVH* (same as curve C in Fig 1); curve B, nonmissense p53 mutation (n = 13; median survival, 36 months); curve C, missense mutation outside DBMs (n = 13; median survival, 32 months); and curve D, missense mutation in DBMs (n = 24; median survival, 13 months). Curve D *v* curve C, $P = .009$; curve D *v* curve B, $P = .002$.

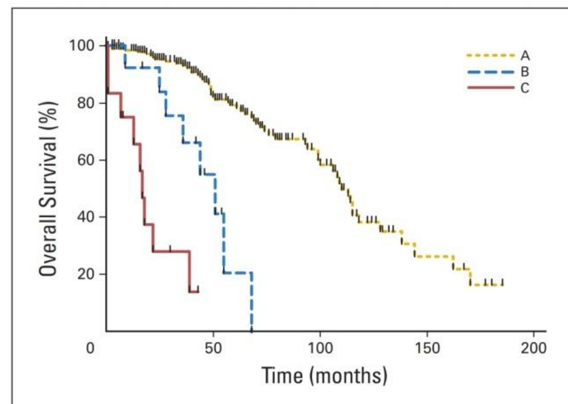

Fig 3. Survival of patients with p53 mutation in the DNA-binding motifs (DBMs) detected at diagnosis: curve A, wild-type p53 and unmutated *IgVH* (n = 193; median survival, 110 months); curve B, mutation outside DBMs (n = 16; median survival, 55 months); and curve C, missense mutation in DBMs (n = 12; median survival, 17 months). Curve A *v* curve B, $P < .001$; curve A *v* curve C, $P < .001$; curve B *v* curve C, $P = .004$.

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TTFT

Patients with the p53 mutation identified at diagnosis and all wild-type p53 patients were also analyzed for TTFT. Median TTFT was only 1 month for patients with p53 DBMs mutation (n = 12) and 6 months for patients with p53 mutation outside DBMs (n = 16; P = .042). Both groups differed significantly from wild-type p53 patients with the unmutated *IgVH* (n = 192, including 70 patients harboring the high-risk 11q–), with a median TTFT of 19 months ($P < .001$ and $P = .024$, respectively). Within the 6-month period from diagnosis, significantly more patients with p53 DBMs mutations (11 of 12 patients) required therapy compared with the remaining p53-affected patients (eight of 16 patients; $P = .039$). This further confirms a more severe disease course associated with a p53 missense mutation in DBMs.

DISCUSSION

The adverse prognostic impact of a p53 defect mirrored by the presence of del(17p) is unquestionable in CLL.[3-5,13] It is becoming clearer that a majority of affected patients harbor a mutation on the other *TP53* allele, and a subset of patients harbor a sole *TP53* mutation.[6-11] The p53 mutation has quite recently been shown to confer resistance to fludarabine-based therapeutic regimens in CLL.[10] p53 status is and will continue to be one of the most carefully examined factors in CLL clinical trials investigating conventional or experimental therapy.[28] In this sense, it is worth knowing whether one can expect similar biologic behavior of different p53 mutations and, hence, a similar prognostic consequence with studied patients.

In our report, we show that both missense and nonmissense p53 mutations reduce the survival rate of patients with CLL. In addition, we show that patients harboring missense mutations in structural p53 DBMs constitute a readily distinctive prognostic subgroup with a prominently reduced survival rate and extremely short TTFT. Although all mutations in our study led to a basic loss of p53 transactivation activity, because they would not otherwise be detected by FASAY, the DBMs mutations clearly behave differently than remaining p53 alterations in CLL. The most probable explanation for this observation is the mutated p53 GOF effect.[19] The GOF stems from a basic loss of p53 transactivation activity[29] and, therefore, should not be biased in the cohort screened by the FASAY, which is based on the detection of transactivation failure of mutants.

Although the mutated p53 GOF has not yet been tested directly in CLL cells, this effect can be anticipated. For example, two mutants detected in our study, R175H and R273H, have been recently shown to upregulate the mitogen-activated protein kinase kinase 3 (MAP2K3) through the involvement of the transcriptional cofactors NF-Y and NF-κB,[30] and these proteins are known to support the survival of CLL cells on bone marrow stromal cells.[31] Another described mechanism of the GOF effect predicts an interference with p53 homologues.[19] It is important to study this potential interference in CLL cells because there are innovative studies focusing on activation of p73 in patients with a p53 defect.[32-34] In this respect, it is advisable to compare a response of patients with absent p53 (eg, 17p– and frameshift mutation) versus patients with p53 missense mutation in DBMs and del(17p).

Our pivotal observation has been confirmed not only in sets of all identified mutations, but also in subgroups limited to patients with the accompanying del(17p). This analysis is crucial for the proof of clinically observed mutated p53 GOF because this aspect should be rigorously studied in the absence of wild-type p53.[19] The setting is unique in our CLL study because the status of other alleles was not considered in reports concerning other tumors.[14,15,35] Most importantly, we confirmed the negative prognostic role of DBMs mutations compared with other p53 alterations in patients investigated for p53 mutations at diagnosis. This confirms that the observation is not influenced by previous therapy and is not a result of bias imposed at the time of examination in samples analyzed during a disease course.

Remarkably, five of the six recently identified CLL-specific p53 mutation hot spots are located in the DBMs (codons 175, 179, 248, 273, and 281; the remaining hot-spot codon out of DBMs is 220).[16] The survival of a subgroup of patients with CLL-specific p53 hot-spot mutations (n = 11) was only 10 months in our study (data not shown). Altogether, this indicates that DBMs mutations are preferentially selected in patients with CLL and supports the view that the alterations in p53 binding to DNA might be critical with CLL progression. Interestingly, our data are markedly similar to those obtained recently with diffuse large B-cell lymphoma, in which p53 DNA-binding mutations were the strongest predictor of poor survival.[15] This observation indicates that the driving forces of p53 mutation selection might be similar in these closely related cancers.

A potential limitation with our study results from the unpredictable survival impact of diverse therapy (chemotherapy, chemoimmunotherapy, alemtuzumab, rituximab with glucocorticoids, and allogeneic stem-cell transplantation) given to patients with p53 defects. We cannot fully exclude that diverse therapy had an impact on some presented results. However, our data show how a strong variability in the p53 function is found in CLL cells, when differences between the mutant subgroups are clearly visible even in the heterogeneously treated cohort.

In summary, patients with p53 DBMs mutations seem to be the most critical subgroup of CLL. p53 is a transcription factor, in which subtle mutations lead to a markedly altered gene expression.[36] Hence, we propose that a genome-wide expression analysis might disclose whether there are any common coding genes or microRNAs[37] with altered expression in patients with p53 DBMs mutations. This analysis could indicate which processes might account for the hypothesized mutated p53 GOF anticipated in these patients. When prioritizing for allogeneic stem-cell transplantation,[13] patients with p53 DBMs mutations should be considered primary candidates, because their long-term survival is otherwise improbable. In conclusion, the effect of p53 DBMs mutations on survival and therapeutic response should be analyzed in ongoing or planned clinical trials to confirm or exclude their more aggressive nature under particular clinical settings.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

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Appendix

Table A1. All *TP53* Mutations Detected in 550 Patients With CLL

Patient No.	Codon	WT Codon	Mut Codon	WT aa	Mut aa	Mutation Type	Missense Mutation in DBMs (1 p53 mut/unmut *IgVH*)	17p− by FISH	*IgVH* Status
P1	109	TTC	TCC	Phe	Ser	Missense		Yes	Mut
P2	110	CGT	CCT	Arg	Pro	Missense	No	Yes	Unmut
P3	113	del 3 nt		Phe		Deletion in frame		No	Unmut
P4	120	AAG	ATG	Lys	Met	Missense		Yes	Unmut
P5	126	del 36 nt		Tyr		Deletion in frame		Yes	Unmut
P6	132	AAG	AGG	Lys	Arg	Missense	Yes	Yes	Unmut
P7	132	AAG	AAC	Lys	Asn	Missense		Yes	ND
P8	132	AAG	AGG	Lys	Arg	Missense		No	unmut
P9	134	TTT	ATT	Phe	Ile	Missense		No	Unmut
P10	134	TTT	TTG	Phe	Leu	Missense	Yes	No; UPD	Unmut
P11	135	TGC	GGC	Cys	Gly	Missense	Yes	Yes	Unmut
P12	138	GCC	CCC	Ala	Pro	Missense	No	No	Unmut
P13	143	GTG	ATG	Val	Met	Missense	No	No	Unmut
P14	155	ACC	ATC	Thr	Ile	Missense		No	Unmut
P15	157	GTC	TTC	Val	Phe	Missense		Yes	Unmut
P16	157	GTC	GGC	Val	Gly	Missense	No	Yes	ND
P9-M2	172	del 39 nt		Val		Deletion in frame			
P17	173	GTG	ATG	Val	Met	Missense	Yes	Yes	Unmut
P18	175	CGC	CAC	Arg	His	Missense	Yes	ND	Unmut
P19	175	CGC	CAC	Arg	His	Missense		No	Unmut
P20	175	CGC	CAC	Arg	His	Missense	Yes	Yes	Unmut
P21	176	TGC	TGG	Cys	Trp	Missense		No	Mut
P22	176	TGC	GGC	Cys	Gly	Missense		Yes	Mut
P23	178	CAC	CCC	His	Pro	Missense	Yes	Yes	Unmut
P24	179	CAT	CGT	His	Arg	Missense	Yes	Yes	Unmut
P25	181	CGC	TGC	Arg	Cys	Missense	Yes	Yes	Unmut
P26	184	ins 2 nt		Asp	Stop 247	Insertion frameshift		No; UPD	Unmut
P27	194	CTT	CGT	Leu	Arg	Missense	Yes	No	Unmut
P8-M2	194	del 14 nt		Leu	Stop 203	Deletion frameshift			
P28	195	ATC	ACC	Ile	Thr	Missense		Yes	Mut
P29	196	CGA	TGA	Arg	Stop	Nonsense		Yes	Mut
P30	196	CGA	GGA	Arg	Gly	Missense	No	No	Unmut
P31	205	TAT	CAT	Tyr	His	Missense	No	Yes	Unmut
P32	205	TAT	TGT	Tyr	Cys	Missense	No	Yes	Unmut
P33	209	del 2 nt		Arg	Stop 214	Deletion frameshift		Yes	Mut
P4-M2	209	del 2 nt		Arg	Stop 214	Deletion frameshift			
P34	211	ACT	ATT	Thr	Ile	Missense	No	Yes	Unmut
P35	213	CGA	TGA	Arg	Stop	Nonsense		Yes	Unmut
P36	215	AGT	AGA	Ser	Arg	Missense	No	Yes	Unmut
P37	215	ins 1 nt		Ser	Stop 221	Insertion frameshift	No	Yes	Unmut
P38	216	GTG	ATG	Val	Met	Missense	No	No	Unmut
P39	216	GTG	ATG	Val	Met	Missense	No	Yes	Unmut
P40	220	TAT	TCT	Tyr	Ser	Missense	No	Yes	Unmut
P41	220	TAT	TGT	Tyr	Cys	Missense		No	Mut
P42	220	TAT	TGT	Tyr	Cys	Missense	No	Yes	Unmut
P43	220	TAT	TGT	Tyr	Cys	Missense	No	Yes	Unmut
P44	226	del 2 nt		Gly	Stop 227	Deletion frameshift		Yes	Unmut
P45	234	TAC	AAC	Tyr	Asn	Missense	No	No	Unmut
P46	234	TAC	TGC	Tyr	Cys	Missense	No	No	Unmut
P47	234	TAC	TGC	Tyr	Cys	Missense	No	Yes	Unmut
P48	234	TAC	TGC	Tyr	Cys	Missense	No	No	Unmut
P49	236	TAC	TGC	Tyr	Cys	Missense	No	Yes	Unmut

(continued on following page)

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Table A1. All *TP53* Mutations Detected in 550 Patients With CLL (continued)

Patient No.	Codon	WT Codon	Mut Codon	WT aa	Mut aa	Mutation Type	Missense Mutation in DBMs (1 p53 mut/unmut *IgVH*)	17p− by FISH	*IgVH* Status
P50	236	TAC	TGC	Tyr	Cys	Missense	No	Yes	Unmut
P51	236	TAC	GAC	Tyr	Asp	Missense	No	No	Unmut
P52	237	ATG	ATA	Met	Ile	Missense	Yes	No	Unmut
P53	239	AAC	GAC	Asn	Asp	Missense	Yes	No	Unmut
P54	241	TCC	TAC	Ser	Tyr	Missense	Yes	Yes	Unmut
P55	244	GGC	GAC	Gly	Asp	Missense	Yes	Yes	Unmut
P56	246	ATG	GTG	Met	Val	Missense	Yes	No	Unmut
P57	248	CGG	CAG	Arg	Gln	Missense	Yes	Yes	Unmut
P58	248	CGG	CAG	Arg	Gln	Missense	Yes	Yes	Unmut
P59	248	CGG	TGG	Arg	Trp	Missense	Yes	Yes	Unmut
P60	248	CGG	CAG	Arg	Gln	Missense	Yes	Yes	Unmut
P61	248	CGG	CAG	Arg	Gln	Missense	Yes	Yes	Unmut
P62	249	AGG	ACG	Arg	Thr	Missense	Yes	No	Unmut
P63	249	AGG	GGG	Arg	Gly	Missense	Yes	No; UPD	Unmut
P64	249	del 3 nt		Arg		Deletion in frame		No	Unmut
P65	249	AGG	GGG	Arg	Gly	Missense	Yes	Yes	Unmut
P66	252	del 3 nt		Leu		Deletion in frame		Yes	Unmut
P67	252	del 9 nt		Ile		Deletion in frame		Yes	Unmut
P68	255	ATC	TTC	Ile	Phe	Missense	No	No; UPD	Unmut
P15-M2	256	ACA	CCA	Thr	Pro	Missense			
P19-M2	266	GGA	GTA	Gly	Val	Missense			
P69	272	GTG	ATG	Val	Met	Missense	Yes	Yes	Unmut
P35-M2	273	CGT	CTT	Arg	Leu	Missense			
P70	273	CGT	CAT	Arg	His	Missense	Yes	Yes	Unmut
P71	273	CGT	CAT	Arg	His	Missense		No	Unmut
P72	275	TGT	TAT	Cys	Tyr	Missense	Yes	No	Unmut
P73	275	TGT	TAT	Cys	Tyr	Missense	Yes	No	Unmut
P74	275	TGT	TTT	Cys	Phe	Missense	Yes	Yes	Unmut
P29-M2	277	TGT	TTT	Cys	Phe	Missense			
P71-M2	277	TGT	TTT	Cys	Phe	Missense			
P75	277	TGT	TTT	Cys	Phe	Missense	Yes	No	Unmut
P76	277	TGT	TTT	Cys	Phe	Missense	Yes	Yes	Unmut
P14-M2	278	CCT	TCT	Pro	Ser	Missense			
P77	278	CCT	CGT	Pro	Arg	Missense	Yes	Yes	Unmut
P78	278	CCT	TCT	Pro	Ser	Missense	Yes	Yes	Unmut
P79	278	del 3 nt		Pro		Deletion in frame		Yes	Unmut
P80	280	AGA	GGA	Arg	Gly	Missense	Yes	No	Unmut
P26-M2	281	GAC	AAC	Asp	Asn	Missense			
P81	281	GAC	GAG	Asp	Glu	Missense		Yes	Mut
P82	282	CGG	CCG	Arg	Pro	Missense	Yes	Yes	Unmut
P83	286	GAA	GTA	Glu	Val	Missense		Yes	ND
P84	289	del 2 nt		Leu	Stop 304	Deletion frameshift		Yes	Unmut
P85	294	del 1 nt		Glu	Stop 344	Deletion frameshift		Yes	Unmut
P86	314	del 14 nt		Ser	Stop 331	Deletion frameshift		Yes	Unmut
P87	317	CAG	TAG	Gln	Stop	Nonsense		Yes	Unmut
P88	346	ins 1 nt		Glu	Stop 346	Insertion frameshift		Yes	Unmut
P89	ASHM							No	Mut
P90	ASHM							No	Mut
P91	ASHM							ND	Mut
P92	ASHM							Yes	Unmut
P93	Multiple							Yes	Unmut
P94	Multiple							Yes	Unmut
P95	Intron 5	G-A in splice site			Stop 190	Splice		Yes	Unmut
P96	Intron 5	del 22 nt			Stop 190	Splice		Yes	Unmut

Abbreviations: aa, amino acid; ASHM, aberrant somatic hypermutations in the *TP53* gene; CLL, chronic lymphocytic leukemia; DBM, DNA-binding motif; FISH, fluorescence in situ hybridization; mut, mutated; ND, not determined; unmut, unmutated; UPD, uniparental disomy; WT, wild type.

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REVIEW

ERIC recommendations on *TP53* mutation analysis in chronic lymphocytic leukemia

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Recent evidence suggests that – in addition to 17p deletion – *TP53* mutation is an independent prognostic factor in chronic lymphocytic leukemia (CLL). Data from retrospective analyses and prospective clinical trials show that ~5% of untreated CLL patients with treatment indication have a *TP53* mutation in the absence of 17p deletion. These patients have a poor response and reduced progression-free survival and overall survival with standard treatment approaches. These data suggest that *TP53* mutation testing warrants integration into current diagnostic work up of patients with CLL. There are a number of assays to detect *TP53* mutations, which have respective advantages and shortcomings. Direct Sanger sequencing of exons 4–9 can be recommended as a suitable test to identify *TP53* mutations for centers with limited experience with alternative screening methods. Recommendations are provided on standard operating procedures, quality control, reporting and interpretation. Patients with treatment indications should be investigated for *TP53* mutations in addition to the work-up recommended by the International workshop on CLL guidelines. Patients with *TP53* mutation may be considered for allogeneic stem cell transplantation in first remission. Alemtuzumab-based regimens can yield a substantial proportion of complete responses, although of short duration. Ideally, patients should be treated within clinical trials exploring new therapeutic agents.

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Keywords: *TP53*; CLL; mutation

INTRODUCTION

The tumor suppressor p53 has a crucial role in cellular response to stress or DNA damage by induction of cell-cycle arrest, apoptosis or senescence.[1] Altered p53 function because of 17p deletion and/or *TP53* gene mutation is associated with poor prognosis in chronic lymphocytic leukemia (CLL) patients.[2–7] Aberrations of *TP53* gene occur on average in 10–15% of untreated CLL patients, but the incidence rises to 40–50% with fludarabine-refractory CLL.[8,9] Over 80% of cases with 17p deletion also carry *TP53* mutations in the remaining allele.[2,4–6] Recently it has been shown that *TP53* mutations in the absence of 17p deletion occur in a significant proportion of CLL patients (~5% in first line treatment situation) and are associated with significantly worse outcome.[3,5] Table 1 shows the impact of *TP53* mutations on outcome in recent prospective clinical trials.

The aim of this report is to provide recommendations on *TP53* mutation analysis in patients with CLL. Recommendations concerning several methodologies suitable for *TP53* analysis will be provided, including comments on their respective advantages, shortcomings and clinical utility. We will also discuss potential clinical consequences derived from the positive/negative results, although a full clinical review is beyond the scope of this article.

TP53 MUTATION IN CLL

Mutations represent the most frequent form of p53 inactivation in CLL and are frequently accompanied by the loss of the second allele (through 17p deletion, or more rarely copy number neutral loss of heterozygosity).[2,10–12] In total, 95% of mutations are localized within the central DNA-binding domain, impairing DNA binding and target gene transactivation.[13] Approximately 75% of all mutations represent missense mutations leading to amino-acid change. A significant proportion of all mutations are localized in classic hot-spot codons. These mutations either directly disrupt the p53-DNA interaction ('DNA-contact mutants'–e.g., residues 248 and 273) or cause conformational changes ('conformational mutants'–e.g., residues 175, 245, 249 and 282).[14] The vast majority of mutations lead to severely impaired p53 function.[15] However, not all missense mutations convey a complete loss of function and

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Table 1. Impact of TP53 mutations from prospective clinical trial cohorts

Reference	n	*Patients with TP53 mutation, n (%)*	*Treatment regimens*	*Overall response (mut vs wt)*	*PFS (median months) (mut vs wt)*	*OS (median months) (mut vs wt)*
Zenz *et al.*[3]	328	28 (8.5)	F vs FC	60% vs 91%	23 vs 62	29 vs not reached at 5 years
Gonzalez *et al.*[5]	529	40 (7.6)	Chl vs F v FC	27% vs 86%	6 vs 27	17 vs not reached at 5 years
Zenz *et al.*[29]	628	71 (11.9)	FC vs FCR	62% vs 95%	12 vs 45	39.3 vs not reached
Grever *et al.*[30]	235	25 (11)[a]	F vs FC	NA	NA	NA

Abbreviations: C, cyclophosphamide; Chl, chlorambucil; F, fludarabine; mut, mutated TP53; OS, overall survival; R, rituximab; wt: wild type. [a]This study included *TP53* mutations as well as known polymorphisms and unconfirmed mutations.

some of them may preserve partial activity that can be promoter selective and temperature sensitive.[16,17] In addition, mutated p53 protein may exert a dominant negative effect on wild-type p53. Some p53 mutants may also gain new functions, which can contribute to cancerogenesis. Preliminary data show that the mutated p53 gain-of-function phenotype may be present also in CLL.[18]

Currently, there is not sufficient evidence to consider specific mutations or clone size in the diagnostic process, but this information should be assessed and recorded. It is important that mutations should be compared with currently available databases (www-p53.iarc.fr). If residual function is preserved and the mutation has rarely or never been reported in cancer, the results should be reassessed and the investigation repeated for confirmation, as technical artifacts can sometimes cause spurious results.

WHEN TO ANALYZE 17p DELETION AND *TP53* MUTATION

It is important to emphasize that current recommendations are based on retrospective analyses and subgroup analyses. No prospective trial data has shown superiority of one treatment regimen/concept for patients with *TP53* mutations.

TP53 abnormalities should be assessed in:

(1) All patients included in clinical trials.
(2) Outside clinical trials in patients requiring therapy who would be eligible to an allogeneic stem cell transplantation or other intensive therapies (e.g., FCR and BR); *TP53* abnormalities should be investigated immediately before treatment decision (results at diagnosis can change over time because of the clonal evolution).
(3) Previously treated patients with wild-type *TP53* at the time of treatment, should be retested when further therapy is needed and results can be expected to influence choice of therapy.

HOW TO ANALYZE TP53 STATUS

The percentage of CLL cells in peripheral blood will influence the detection. Normally, in cases requiring therapy the majority of peripheral blood cells will belong to the CLL clone, but exceptions exist. If the proportion of leukemic cells in peripheral blood is lower than 50%, using bone marrow or lymph node tissue as source to investigate *TP53* status may be considered especially when using direct Sanger sequencing. Alternatively, usage of CD19+ cells can be considered.

Detection of *TP53* mutations

Although *TP53* mutation may be present in a small proportion of leukemic cells, the presence of minor subclones is relatively rare (unpublished observations). Several methods based on analysis of genomic DNA, complementary DNA or RNA may be used, and each method has both advantages and shortcomings. For the purpose of this review, we will discuss these as well as minimum requirements to obtain meaningful information. In this regard, it is important to stress that for all techniques, mutations must be confirmed on a separate PCR reaction and Sanger sequencing.

(1) Direct Sanger sequencing of genomic DNA or complementary DNA: Usage of genomic DNA is preferable, because some mutations may lead to RNA degradation because of nonsense-mediated mRNA decay.[19] Sequencing of (at least) exons 4–9 is recommended as the vast majority of mutations is located in this region (>95%).[13] In a recent analysis of 268 *TP53* mutations in CLL no mutations in exons 2, 3 and 11 were detected. Exon 10 harbored 4% of all *TP53* mutations.[13] Primers and reaction conditions can be found at www-p53.iarc.fr.

For centers planning *TP53* mutation testing, direct sequencing can be considered as relatively simple approach, available at most laboratories. However, the sensitivity of direct sequencing is limited and therefore small subclones with *TP53* mutation are unlikely to be detected. Sequencing analysis must be performed on forward and reverse reactions. Confirmation of mutations by a separate PCR reaction is mandatory.

Advantage: Provides direct information about the type of mutation.

Shortcomings: Relatively time consuming, low sensitivity (~25% of mutated alleles in the sample).

(2) Pre-screening techniques such as denaturing high-performance liquid chromatography or single-strand conformation analysis have the advantage of higher sensitivity and lower costs. Abnormal screening results must be confirmed by Sanger sequencing in an independent PCR to exclude analytical artifacts.

Advantages: Fast, simple, economic and highly sensitive.

Note: Does not provide data on specific mutation (requires direct sequencing for confirmation).

(3) Functional analysis of separated alleles in yeast: The principle of this methodology is based on the cloning of complementary DNA from tumor samples into modified yeast cells. Gene expression triggers transcription of a reporter gene (ADE2–enzyme for adenine synthesis). Non-functional p53 leads to accumulation of a red intermediate product of adenine metabolism. Sequencing of templates from red yeast colonies bearing mutant *TP53* for direct mutation identification is needed.

Advantages: Fast, cheap, no instrumentation required readout of transcriptional activity and sensitivity ~10% of mutated DNA in the sample.

Shortcomings: May not pick up mutations leading to RNA degradation because of nonsense-mediated RNA decay, that is, some nonsense or frame-shift mutations.

Note: Underlying mutation should always be determined by sequencing (functional analysis of separated alleles in yeast methodology is not sufficient by itself).

(4) Arrays: Two platforms are currently in use (but not widely available); Affymetrix/Roche (GeneChip Arrays and p53 AmpliChip) and arrayed primer extension.[20]

The GeneChip arrays are based on short immobilized nucleotides with central base changes representing all potential nucleotide exchanges characterized by significantly different levels of

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hybridization to analyzed DNA. This approach was used for the first generation of p53 sequencing chips–the Affymetrix p53 GeneChip (Affymetrix, Santa Clara, CA, USA). Further development resulted in Roche p53 AmpliChip (Roche, Pleasanton, CA, USA), which was designed as a diagnostic tool for detection of all single base pair substitutions and single nucleotide deletions in exons 2-11 and splice sites and could reach a detection limit 1-2% of mutated DNA. Arrayed primer extension is based on incorporation of one of four labeled dideoxyNTPs into immobilized oligonucleotide primers. The *TP53* arrayed primer extension array was designed to detect 95% of known *TP53* mutations in exons 2-9.[21]

Advantages: Amplichip–fast, user friendly; Affymetrix custom GeneChip arrays–mutational analysis of multiple genes in parallel is feasible.

Shortcomings: Detects only mutations with probes printed on the array (Amplichip–all single base pair substitutions and single nucleotide deletions in exons 2-11, and splice sites). These assays are currently not widely available.

(5) Sequencing with next-generation technology: With the advent of high-throughput sequencing approaches, it is foreseeable that larger laboratories with high-case throughput will use this technology as a fast and cost effective approach. The discussion of the different technologies is beyond the scope of this review, but a number of next generation sequencing machines for diagnostic purposes are in use or under development.

Advantages: Very high and variable sensitivity based on coverage of analyzed sequences.

Shortcomings: Initial upfront cost of instrumentation, useful and economic only with high throughput of samples or target genes.

In addition to the detection of 17p deletion or *TP53* mutation, there may be alternative lesions in the p53 pathway, which may contribute to the outcome of the disease.[22-24] For the time being, functional defects of the p53 pathway beyond *TP53* loss or mutation are a research topic and not considered for application in routine diagnostics. Although methods to detect the CLL cells' response to DNA damage or p53 activation may be suitable surrogates of *TP53* status (e.g., p53 target induction after DNA damage or interference of MDM2-p53 interaction), the current data is not sufficient to recommend the use of these techniques outside of the research scene.

ANALYSIS, REPORTING AND QUALITY CONTROL OF *TP53* MUTATIONS

General recommendations for sequencing methods include the analysis of both forward and reverse strands to confidently identify or exclude *TP53* mutations. Mutations should be confirmed in two separate PCR reactions because of the chances of polymerase errors leading to false positive results. This recommendation is highly dependent on the protocol used, as some proof-reading polymerases have significantly lower error rates than conventional Taq polymerases, and regional guidelines may differ from one country to another. It is strongly recommended that mutations that have not been previously characterized are confirmed in two independent reactions.

The report must include the parts of *TP53* analyzed and the methodology used, with corresponding caveats regarding sensitivity and specificity of the method used. In cases where a *TP53* mutation is found, this should be described according to the HGVS guidelines (http://www.hgvs.org/mutnomen) including both the nucleotide change at the DNA level as well as the amino-acid change at the protein level. The report should always include the reference *TP53* sequence accession and version number used for the analysis, where nucleotide 1 is always the A of the ATG-translation initiation codon.

External quality assurance is a requisite of all accreditation bodies for molecular genetics; therefore, all laboratories should ensure that they participate in relevant external quality assurance or sample exchange programs. In the absence of national or international external quality assurance schemes for *TP53* mutation analysis, it is recommended that all laboratories performing sequencing analysis are enrolled in a sequencing external quality assurance to ensure ongoing quality of data and interpretation (e.g., EMQN DNA-sequencing scheme, http://www.emqn.org/emqn/Schemes).

Irrespective of exchange programs, a simple assessment of test quality is the monitoring of the incidence of *TP53* mutation in cases with deletion of 17p, which should be above 70%. Incidences below 60% should lead to reassessment of the technique's performance and procedures.

CLINICAL CONSEQUENCES DERIVED FROM POSITIVE RESULT

It is widely accepted that patients with *TP53* abnormalities have an aggressive clinical course, require earlier intervention because of progressive disease and clinical symptoms, and respond poorly to therapy. This notion is based on data obtained in patients included in clinical trials, requiring therapy and thereby with poor prognosis. There are limited comprehensive studies of the natural history of CLL with *TP53* abnormalities in unselected patients.

At diagnosis, the incidence of *TP53* mutation has been reported to be 3.7%[25] and it is important to stress that there are a subgroups of patients with 17p deletion (and mostly mutated *IGHV* status) who are not progressing for years.[26,27] As disease progresses, incidence rises to 10-12% (first line treatment) and ~40% (F-refractory CLL).[8]

This emphasizes that treatment should only be initiated in the presence of active, symptomatic disease as recommended by the International workshop on CLL.[28]

For patients with *TP53* mutation and treatment indication current guidelines are mainly based on outcome data of retrospective analyses from clinical trials and single center cohorts. Although the consequences of *TP53* mutations as independent predictors of poor response and outcome are almost unequivocal, treatment recommendations will be based on cross-trial comparisons and not prospective randomized studies.

The authors hope that the careful but stringent integration of *TP53* mutation analysis as recommended in this paper will help to unfold the clinical impact of *TP53* abnormalities in CLL. Some recommendations are provided in Table 2.

Table 2. Indications for testing of *TP53* mutations

Disease stage	*Clinical trial*	*General practice*	*Comment*
Diagnosis	Recommended	Not indicated	Results of *TP53* mutation testing will not influence initial watch and wait strategy
First line treatment	Recommended	Desirable[a]	Patients with *TP53* mutation should be entered onto a clinical trial. May be considered for alemtuzumab-based treatment strategies and allogeneic SCT
>Second line treatment	Recommended	Desirable[a]	Patients with *TP53* mutation should be entered onto a clinical trial. May be considered for alemtuzumab-based treatment strategies and allogeneic SCT

Abbreviation: SCT, stem cell transplantation. [a]Analysis should be performed within a 1-2 months time frame of treatment initiation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Chapter 5
TP53 Aberrations in Chronic Lymphocytic Leukemia

Martin Trbusek and Jitka Malcikova

Abstract CLL patients harboring *TP53* defects remain the most challenging group in terms of designing rational and effective therapy. Irrespective of the treatment employed—chemotherapy, chemoimmunotherapy, or pure biological drugs—median survival of these patients does not exceed 3–4 years. This adverse outcome is caused by a less effective response to therapeutics acting through DNA damage induction and relying on the subsequent initiation of apoptosis as well as by virtually inevitable aggressive relapse. Patient proportions with *TP53* defects at diagnosis or before first therapy were reported within the range 5–15 %, but they increase dramatically in pretreated cohorts (reported up to 44 %), and also in patients with Richter transformation (50 % harbor *TP53* defects). Currently, most laboratories monitor *TP53* defect as presence of 17p deletion using I-FISH, but 23–45 % of *TP53*-affected patients were shown to harbor only mutation(s). In other patients with intact *TP53*, the p53 pathway may be impaired by mutations in *ATM* gene coding for the p53-regulatory kinase; however, prognosis of *ATM*-defective patients is not as poor as those with *TP53* abnormalities. Though many novel agents are under development, the monoclonal antibody alemtuzumab and allogeneic stem cell transplantation remain the basic treatment options for *TP53*-affected CLL patients.

Keywords *TP53*/p53 mutation • Deletion 17p • Apoptosis • Prognosis • Relapse • Chemo-refractoriness • Alemtuzumab • ATM

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Tumor Suppressor p53

p53: A Tumor Suppressor with Unique Properties

In 1979, two independent research groups [1, 2] published their reports noting a physical interaction between a large T-antigen of the SV40 virus and a cellular protein of approximately 53–54 kDa. This is when the fascinating story of p53 research commenced. Although this yet unknown protein attracted a lot of attention from the very beginning, determination of its principle role in tumor cells has not always been straightforward. Still, in 1984, p53 had been erroneously assigned among oncoproteins (for review see [3]). This incorrect classification was influenced by two basic factors: (a) protein accumulation was frequently observed in tumor cells, by contrast to normal cells, which resembled an oncogenic behavior, and (b) complementary DNA (cDNA) clones used for transfection experiments into human cells harbored missense mutation, and—as disclosed later—some missense mutations exert the ability to switch the p53 from a tumor suppressor to a powerful oncoprotein. Only later studies from the end of 1980s confirmed the tumor-suppressive behavior of wild-type p53 [4, 5] and, finally, 13 years from its discovery, the p53 protein was officially proclaimed as the “Guardian of the Genome” [6].

Although p53 activity was recently shown to impact the pathogenesis of several nonmalignant diseases, the p53 role in cancer prevention is substantially more elaborated. Currently, both experimental data and clinical observations recognize the p53 as the most important tumor-suppressor protein: (a) $TP53^{-/-}$ mice invariably develop tumors [7], (b) heterozygous inherited mutations predispose to the Li-Fraumeni cancer-prone syndrome in humans [8], and (c) somatic mutations are frequent in many different types of human tumors [9].

The p53 plays a critical role in an anti-cancer barrier preventing an organism from malignant cell proliferation [10–12]. During early cancerogenesis, tumor cells experience genotoxic stress, which elicits a DNA damage response (DDR) pathway—the hierarchically ordered machinery detecting DNA lesions and signaling their presence to protein complexes that either promptly repair the damaged DNA or arrest the cell cycle if DNA repair requires additional time. Alternatively—in case when DNA damage is too extensive and repair is not possible—the DDR pathway induces apoptosis or replicative senescence. For effective induction of the above-mentioned processes, p53 activity is crucial.

Considering these facts, it is not surprising that the central axis of the DDR pathway, involving both the p53 and its positive regulator, the ATM kinase (*A*taxia *T*elangiectasia *M*utated), is under enormous pressure to be impaired during malignant conversion [11, 12]. Indeed, *TP53* mutations in particular are frequent in many different tumors and most often observed in ovarian, colorectal, and esophageal cancer [13]. Even a low *TP53* mutation frequency, which is typical for some types of tumors, may not mean that p53 is irrelevant in prevention of their development. In cervical carcinoma, for instance, a typically non-mutated p53

protein is inactivated by direct physical interaction with an E6 oncoprotein encoded by the high-risk human papillomavirus (type 16 or 18) [14]. In other tumors with an intact *TP53* gene, the p53 pathway may also be abolished by enhanced activity of p53 inhibitors (e.g., MDM2) or defects in upstream p53 activators (such as ATM in CLL) or downstream target genes (e.g., inactivation of NOXA and others in different B-cell lymphomas [15]). Frequency of p53 inactivation in hematological malignancies is lower in comparison with solid tumors, usually reaching 10–15 % in unselected patient cohorts. However, in contrast with the solid tumor situation, virtually all relevant studies agree with a severe prognosis for patients with hematological malignancies and p53 inactivation [16].

p53 Protein Structure

The *TP53* gene is located at the short arm of chromosome 17 (17p13.1) and contains 11 exons, 10 of which (2–11) are coding. The full-length protein consists of 393 amino acids and harbors several structural domains: (a) the N-terminal domain, which ensures target gene transactivation; (b) the central DNA-binding domain, which directly interacts with consensus DNA sequence in the target promoters; (c) the oligomerization domain, through which the four monomeric polypeptide chains join together to form a final tetramer molecule; and (d) the C-terminal domain, which harbors important regulatory sites for the DNA-specific and also nonspecific p53 binding. Besides the basic full-length protein, analysis of *TP53* gene sequence also revealed 12 putative p53 isoforms (for review see [17]). Expression of individual isoforms is tissue-specific, and they differ in subcellular localization. In quiescent lymphocytes, isoform p53β is typically expressed [18].

The *TP53* gene contains many polymorphisms, with 11 of them being non-synonymous. The most frequent polymorphism P72R (changing the ancestral proline allele to arginine, which is notably more frequent in some populations) was described to have functional impact, and its role in cancer susceptibility, prognosis, and treatment response was studied in several malignancies with inconsistent results (for review see [19]). Similarly, in early CLL studies no relation between this polymorphism and clinical outcome was evident [20, 21]; in a more recent study, the proline allele in the homozygous state was associated with a shorter time to first treatment among the group of patients with mutated *IGHV* locus [22].

Regulation of p53 Protein

The p53 protein level in a cell is low under normal conditions—undetectable by western blot or immunohistochemistry. This is because the p53 induces—among other targets—expression of *MDM2* gene coding for a protein which serves as a negative regulator, targeting p53 protein for ubiquitin-mediated degradation

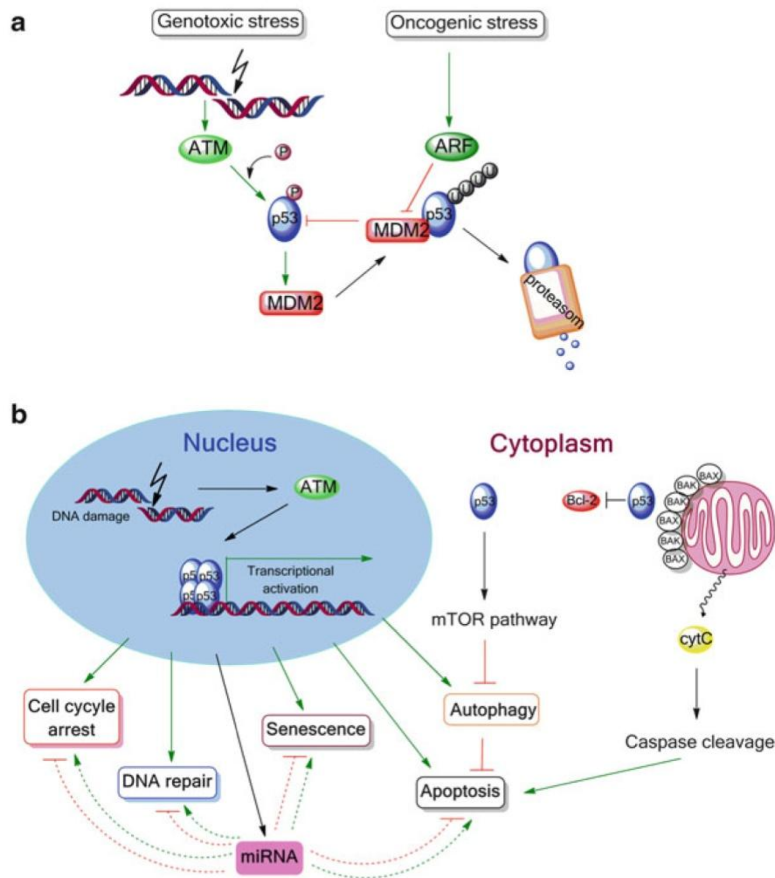

Fig. 5.1 Overview of p53 stabilization (**a**) and activity (**b**). In case of a genotoxic stress the p53 protein is phosphorylated by ATM kinase and thus prevented from MDM2-mediated degradation. Under oncogenic stress p53 is protected from degradation by p14^{ARF} protein. After stabilization the p53 protein regulates through transcriptional activation of target genes several divergent but interconnected processes, which are decisive for a cell fate. The p53 also localizes on mitochondria, where it interacts with Bcl2 protein family members. This translocation contributes to permeabilization of the mitochondrial membrane, cytochrome c release, and subsequent apoptosis

(Fig. 5.1a). p53 protein stabilization following genotoxic stress is then ensured through delicate posttranslational modifications namely involving phosphorylations, but also acetylations or sumoylations [23]. The p53 Ser-15 phosphorylation elicited by ATM kinase is critical for p53 protein stabilization after induction of DNA double-strand breaks (DSBs) [24, 25]; this phosphorylation prevents MDM2 binding to p53. Another situation arises in case of oncogenic stress (activation of oncogenes, e.g., BCR-ABL1), when p53 is stabilized by the product of a *CDKN2A* gene, i.e., p14^{ARF} protein that directly inhibits p53-MDM2 binding [26] (Fig. 5.1a).

Cellular Functions of p53

The p53 is a transcription factor with a consensus binding site consisting of two copies of the 10 base pair motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ separated by 0–13 base pairs [27]. It is estimated that p53 regulates more than 125 structural genes [28]. The expression of many p53-regulated proteins is critical for decisions made within the DDR (Fig. 5.1b). Regarding cell cycle arrest, the cyclin-dependent kinase inhibitor *CDKN1A* (coding for p21 protein) seems to be the most prominent target upregulated by p53, while *PUMA* (p53-upregulated modulator of apoptosis) is a key mediator of p53's apoptotic activity [28]. Transcriptional activity of p53 is tissue-specific [29] and is distinct in lymphocytes compared to other cells [30]. Among p53 target genes there are also numerous microRNAs, including miR-34a, with a proposed role in CLL pathogenesis [31, 32]. Recently, p53's role in posttranscriptional maturation of several microRNAs with growth-suppressive properties (including, e.g., miR-16) has been reported [33].

How p53 can discriminate between cell cycle arrest, senescence, or apoptosis still remains a matter of intense debate [34]. The p53 molecule conformation represents perhaps the most critical factor, and this conformation depends on two basic events: (a) posttranslational modifications, such as phosphorylations, methylations, or acetylations; and (b) DNA-binding itself that determines which cofactors will participate in target gene transactivation and thus influence transcription extent.

The p53 is also able to trigger apoptosis independently on transcription initiation. This mechanism facilitates a fast response to genotoxic stress, when p53 localizes on mitochondria, it interacts with Bcl2 protein family members, enabling oligomerization of bak and bax proteins and subsequent permeabilization of the mitochondrial membrane and cytochrome c release, which leads to caspase cleavage (Fig. 5.1b, for review see [35]). It was even suggested by Steele et al. [36] that transcriptional-independent apoptosis is a major route to cell death induction by p53 in CLL cells, as they showed that blocking of p53-mediated transcription paradoxically augmented apoptosis induction by chlorambucil and fludarabine through accelerating the proapoptotic conformation change of the Bax protein.

Another important p53 cytoplasmic function is inhibition of autophagy through mTOR pathway promotion. Interestingly, p53 plays an ambiguous role in autophagy control, as nuclear p53 can induce autophagy through transcriptional activation of positive autophagy regulators (including mTOR inhibitors) (for review see [37]). Autophagy is tightly connected with apoptosis, although their exact interplay remains a matter of debate. Autophagy inhibition facilitates cell death, yet on the other hand, its activation promotes the cell's attempt to cope with stress and to survive. However, with excessive autophagy, an autophagocytic death via necrosis may occur. Therefore, the p53 decision between autophagy induction and inhibition is crucial for cancer treatment, and autophagy was identified as an important mechanism of drug resistance. Early studies also suggested its relevance in CLL resistance to treatment by flavopiridol [38] or dasatinib [39].

p53 Mutagenesis: Dominant-Negative Effect and Gain-of-Function

In contrast with certain other tumor-suppressors, the *TP53* gene doesn't need to be inactivated on both alleles to eliminate p53 function. The p53 pathway is highly sensitive to p53 protein level changes, and the protein produced by only one allele may not be sufficient to ensure proper function. This effect, termed "haploinsufficiency," was documented on mouse models [40] and is assumed in patients with sole 17p deletion (del(17p)) or sole truncating mutation. However, simple loss of function (LOF) may not be the most grievous p53 defect contributing to tumor progression. This could have been predicted from the highly predominant occurrence of p53 missense substitutions (forming approximately 75 % of all mutations) leading to expression of aberrant protein in cancer patients. Indeed, besides LOF, another two effects clearly attributable to mutated p53 have been evidenced: (a) - dominant-negative effect (DNE) of monoallelic mutation towards the second intact (wild-type) allele, and (b) gain-of-function (GOF) effect probably acting independently on the allele status.

DNE is most likely caused by final p53 molecule inhibition through hetero-oligomerization of mutated and non-mutated p53 polypeptide chains. The p53 functions as a tetramer composed of a dimer of dimers with co-translational forming of individual dimers. Once one p53 allele is mutated in the DNA-binding region, half of the dimers would be active, the other half would be inactive, and final posttranslational tetramerisation would render inactive 75 % of p53 tetramers [41]. Accompanying del(17p), frequently observed in mutated patients, then probably eliminates the rest of p53 activity. By contrast, a sole del(17p) eliminates only 50 % of p53 molecules, and this may potentially explain why monoallelic missense mutation, but not sole del(17p), is frequently selected in CLL patients [42].

In addition to the DNE, it has also been well evidenced through numerous studies and several experimental systems that some missense mutations exert a strong GOF effect. This effect was initially demonstrated in cell lines lacking endogenous p53, when the mutated *TP53* gene had been expressed and the phenotype did not copy a simple loss of p53 function [43]. A pivotal mechanism of the mutated p53 GOF seems to be an interference with the p53-related proteins, i.e., p63 and p73, which prevents their tumor-suppressive functions [44]. In addition, some p53 mutants have been shown to upregulate genes and miRNAs, supporting cancer progression or precluding effective therapy. For instance, mutated p53 was shown to enhance the expression of multidrug-resistance gene 1 (*MDR1*) [45]. A number of other GOF mechanisms were described (for review see [46]) including direct protein–protein interactions, e.g., interaction with NF-κB leading to prominent enhancement of a cancer progression [47], or interaction with the nuclease Mre11, which suppresses the binding of the Mre11-Rad50-NBS1 (MRN) complex to DNA DSBs, leading to impaired ATM activation [48]. Mutated p53 has also been implicated in abrogation of the mitotic spindle checkpoint [49]. Both DNE and

GOF are mutant and cell-type-specific, and preliminary data suggests that a strong mutated p53 GOF effect may also be present in CLL patients with particular p53 mutations [50].

p53 Activity: More than Cancer Protection

Despite its indisputable role in protecting an organism from developing a tumor, p53 protein activity may not always be desirable. One example might be the adverse effect of anti-cancer therapy (namely chemotherapy and radiotherapy) on normal tissues; in this respect, a transient p53 inhibition during therapy could be a reasonable way how to protect healthy cells from unwanted apoptosis [51]. Recently, p53 protein contribution to the pathological elimination of nonmalignant cells is gradually being recognized. Specifically, p53 protein activation has been proven to result in cardiomyocyte necrosis during ischemic heart disease, and p53-dependent apoptosis then appears to lead to the pathological neurodegeneration in Alzheimer's, Parkinson's, and Huntington's diseases [28].

p53 Mutation Functional Impact Assessment

While some *TP53* mutations manifest obvious effects on p53 function (e.g., nonsense or frame-shift mutations which abolish the DNA-binding domain), identified missense substitutions should always be checked for severity and predicted clinical impact. Comprehensive analysis of individual *TP53* missense mutations is available via the web pages of the International Agency for Research on Cancer (IARC; http://www-p53.iarc.fr). The following statistics are available in this database: (a) mutated protein activity assessed towards eight selected target promoters (percentage in comparison with wild-type protein); this activity was analyzed using a yeast functional assay and is available for all potential 2 314 p53 missense mutations [52]; for some mutants, information about functionality in human cells is also available; (b) structural impact of mutations; (c) available data about DNE and GOF; (d) frequency of particular mutations in cancer patients and Li-Fraumeni families; (e) list of described p53 polymorphisms.

TP53 Gene Abnormalities in CLL

The frequency of *TP53* defects in hematological malignancies varies between 5 and 20 %, which is low in comparison with solid tumors, where it can reach up to 80 % (http://www-p53.iarc.fr); however, their impact on disease course is unequivocal, with p53 abnormalities having a well-documented role in chronic lymphocytic

leukemia. Within 1–2 years, patients with *TP53* defects almost uniformly require treatment, are often chemo-refractory, and their expected survival is distinctively reduced, with almost exclusive disease-related death. CLL patients with *TP53* gene defects were therefore assigned to a small but challenging subgroup of patients that was defined as "ultra high-risk CLL" [53].

History of TP53 Gene Defects Examination

Initial *TP53* gene defects in relation to CLL were reported as early as in 1991, when Gaidano et al. [54] identified *TP53* mutations in different human lymphoid malignancies and described their presence in late CLL stages. Subsequent studies showed the presence of *TP53* mutations in 10–15 % of patients, confirmed association with advanced stages [55], and delineated the association of mutations with chemo-refractoriness and poor clinical outcome [56]. Loss of the *TP53* locus (del(17p)) was not considered an important recurring event in CLL during early studies using conventional karyotyping, which most distinctively identified trisomy of chromosome 12 [57, 58]. Significance of del(17p) was proven only when interphase fluorescence in situ hybridization (I-FISH) was introduced [59]. Adverse prognostic impact of del(17p) was definitely confirmed in the year 2000 by Dohner et al. [60]. In this study, a comprehensive set of FISH probes was employed with del (17p) being the strongest predictor of poor survival and reduced time to treatment, followed by 11q deletion and trisomy 12; deletion 13q as the sole abnormality exhibited the best prognosis. Based on this observation, a hierarchical prognostic stratification model was suggested that is still referenced today, and assessment of del(17p) using I-FISH was introduced into general practice. Recommendation for a del(17p) examination, at least in clinical trials, was also included in a report from the International Workshop on Chronic Lymphocytic Leukemia (IWCLL) updating guidelines for the diagnosis and treatment of CLL [61].

The clinically relevant cut-off value for del(17p) presence has long been sought after. In the LRF CLL4 trial comparing Chlorambucil (Chl), Fludarabine (F) or Fludarabine, and Cyclophosphamide (FC), no difference in progression-free survival (PFS) or response duration was observed between the patients having 5–20 % of cells with del(17p) and those without this deletion. It was therefore suggested to use 20 % positivity as a clinically relevant cut-off for del(17p) [62]. However, there is still insufficient evidence to use the uniform clone size cut-off in diagnostic procedure.

While the assessment of the del(17p) presence is relatively easy, sensitive, and provides quantitative information on the proportion of affected cells, examination of *TP53* gene mutations is more complicated, and no standardized approach for CLL patients has been established. Initially, it was assumed that correlation between deletion and mutation of the second allele is high in cancer cells [63], although some reports noted a common presence of sole mutation in later stages of tumor development [64]. Currently, most laboratories still investigate the *TP53*

defect in CLL patients only as del(17p) by I-FISH both in routine clinical practice and in clinical trials according to official worldwide recommendations [61]. *TP53* mutations only became a renewed subject of interest after several studies showed that quite a large proportion of patients carry *TP53* mutations in the absence of del(17p) and that such mutations have an independent prognostic impact [42, 65–69]. Based on these observations, the European Research Initiative on CLL released the recommendations on *TP53* mutation analysis in CLL in 2012 [70]. Thus, examination of both mutations and deletions is currently recommended before any treatment initiation.

Prognostic Impact of 17p Deletions and TP53 Mutations

Despite rare cases with del(17p) and indolent disease course (usually manifesting a mutated *IGHV* status) [71, 72], a strong adverse impact of del(17p) was observed in numerous studies and clinical trials. With these patients both a short PFS and overall survival (OS) were documented [73, 74]. The p53-affected patients exhibit markedly poor responses to various chemotherapy-based regimens involving alkylating agents or purine analogues [75–78] as well as their combination with the anti-CD20 monoclonal antibody rituximab [79, 80]. Even chemoimmunotherapy involving rituximab in combination with fludarabine and cyclophosphamide (FCR), which is currently considered the first treatment option for physically fit CLL patients, did not abrogate the negative del(17p) prognostic effect. Although patients with this genetic abnormality enrolled in a CLL8 study comparing FC and FCR manifested a prolonged PFS in the FCR arm (FCR: 11.3 months vs. FC: 6.5 months; HR 0.47), only 5 % of patients achieved complete remission after FCR therapy, and OS was significantly shorter in comparison with all other cytogenetic subgroups, reaching a median of approximately 3 years [81].

Deletion 17p is usually accompanied by *TP53* mutation in CLL cells, but both sole del(17p) and sole *TP53* mutations occur. Their frequencies and mutual proportion vary among different studies (Fig. 5.2). In addition, a mutation in the absence of del(17p) may be accompanied by uniparental disomy (UPD) resulting in duplication of the mutant allele [82]. The first study which examined the impact of mutations in the absence of del(17p) was performed on patients enrolled in US Intergoup E2997 trial (F vs. FC), and in contrast to del(17p) presence, no independent impact on PFS was observed for *TP53* mutations. However, this output could be influenced by including polymorphisms and intronic as well as unconfirmed mutations in this study [76]. By contrast, later studies recorded a reduced time to first treatment [83], reduced PFS [68], and also adverse OS [42, 65, 68] in patients with sole *TP53* mutations. Independent negative prognostic impact of *TP53* mutations was also subsequently confirmed in prospective clinical trials [66, 69, 84].

A majority of mutations identified in CLL are missense substitutions localized in the DNA-binding domain with a mutation profile similar to other cancers, though several specific features were described (Fig. 5.3). A high incidence of an unusual

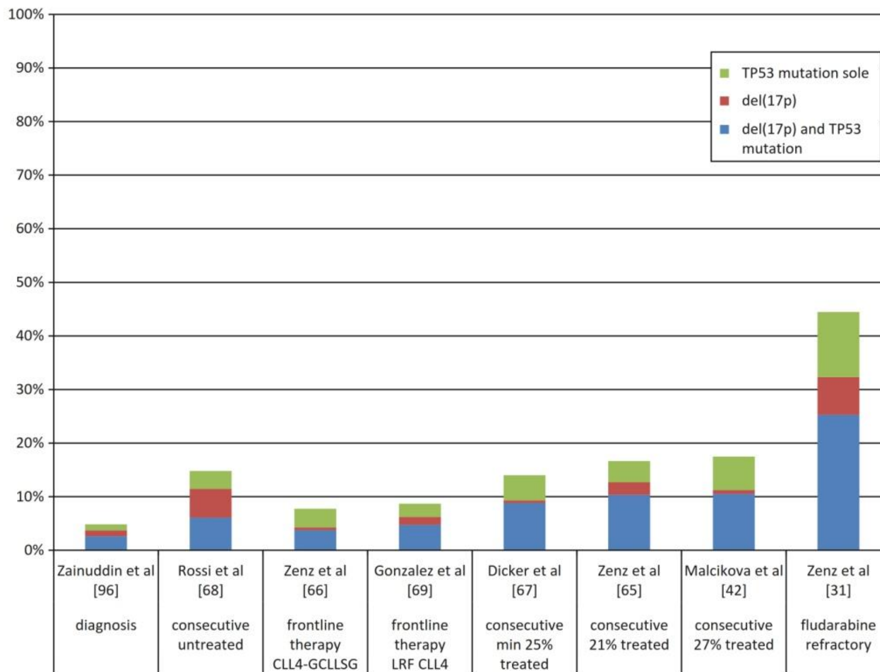

Fig. 5.2 Frequency of *TP53* defects and their composition in selected relevant CLL studies

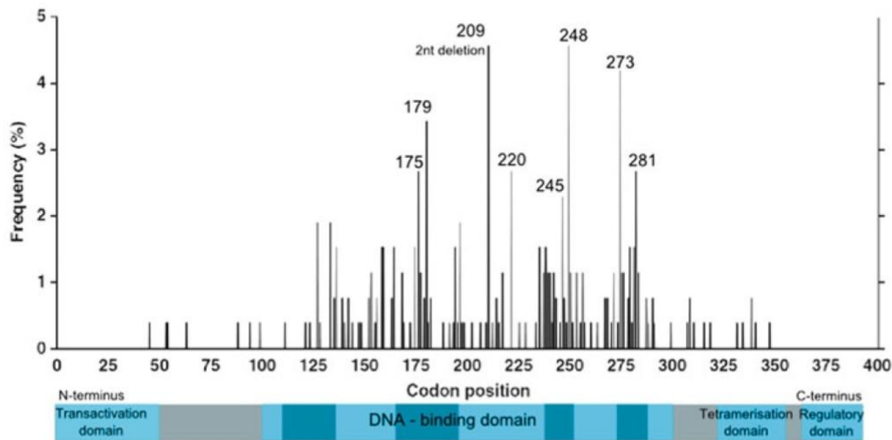

Fig. 5.3 Distribution of mutated codons in CLL patients based on the most comprehensive study by international collaborative group ($n = 268$ *TP53* mutations) [86]. Structure of p53 protein with highlighted DNA-binding motifs inside the DNA-binding domain

two-nucleotide deletion in codon 209 was documented [85] and later confirmed by an international collaborative study analyzing 268 mutations from four independent cohorts [86]. The same study also revealed a decreased percentage of transitions at CpG sites with bias favoring G-A exchange when compared to C-T exchange. Interestingly, G-A transitions were shown to be preferentially selected in quiescent, nondividing cells as a mirror reflection of cytosine deamination (C-T mutation) in the coding, non-transcribed DNA strand [87].

Data from in vitro studies suggest that not only the presence of a *TP53* mutation, but also the type of mutation and its position matter. Indeed, it was shown in other malignancies that specific *TP53* mutations are associated with either a poorer prognosis or a worse response to treatment than other *TP53* mutations; however, the results are often contradictory due to the complexity of p53 pathway defects, unpredictable other genetic context, and lack of prospective studies [88–92]. Preliminary data in CLL [50] showed that patients with mutations in p53 DNA binding motifs (DBMs) (codons directly involved in DNA binding localized in loops L2 and L3 and in the loop-sheet-helix motif) have clearly reduced survival rates compared with patients carrying other p53 mutations. All mutations included in this study led to a basic loss of p53 transactivation activity. Substantially worse survival of patients with DBMs mutations may, therefore, most likely be attributed to a strong mutated-p53 GOF. It is important to study this potential phenomenon in CLL cells since there are innovative studies focusing on activation of p53 homologs in patients with a *TP53* defect [93–95]. There might be a critical difference in utility of this approach between patients with absent p53 and those harboring p53 missense mutation connected to the GOF effect, which could potentially interfere with the activity of homologs.

Clonal Evolution of TP53 Defects

Frequency of mutations and deletions strongly varies depending on the disease stage and the cohort analyzed. At diagnosis, only 4.9 % of patients were reported to carry 17p deletion and/or *TP53* mutation [96]. In untreated cohorts and in patients analyzed before first therapy the frequency varies between 8.5 and 14.8 % [66, 68, 69], and occurrence sharply increases after treatment, where it can reach up to 44 % in fludarabine refractory disease [31] and 50 % after CLL transformation to Richter syndrome [97] or to prolymphocytic leukemia [98]. The increasing proportion of *TP53* abnormalities suggests that clonal selection of adverse genetic defects plays an important role during CLL progression. The Mayo Clinic report [99] illustrated the del(17p) selection during the CLL course and associated this selection with the high expression of zeta-associated protein (ZAP-70) and the presence of treatment. Another study [100] associated clonal evolution of del(17p) specifically with foregoing therapy presence. Several research groups analyzing p53 mutations then similarly documented their selection under therapy pressure [42, 65, 68, 101].

The higher frequency of *TP53* mutations after therapy administration and also the higher frequency of transversions as opposed to transitions led to the notion that these mutations might directly be induced by chemotherapy [85, 102], similarly to what was evidenced with the chemical compound aflatoxin or tobacco smoke in other types of cancer [103, 104]. However, the most extensive collaborative study involving 268 p53 mutations didn't record any differences in the mutation profiles of CLL patients with or without previous therapy [86], which indicated that treatment probably did not contribute to mutation origination. This view is currently supported by direct analyses of *TP53* mutation presence in samples taken before therapy administration from patients who were later (after therapy) shown to acquire clonal *TP53* mutation [65, 105].

Association of TP53 Defects with Other Genetic Variables

Abnormalities in the *TP53* gene mostly occur in patients with unfavorable unmutated *IGHV* locus [73, 86] and constitute an independent adverse prognostic factor within this subgroup [50]. In line with the role of p53 as the guardian of the genome (see above), *TP53* abnormalities were also associated with elevated genomic complexity in CLL patients [106, 107].

In 2001, Pettitt et al. [108] reported that CLL samples, which manifested p53 dysfunction (assessed through defective response to ionizing radiation) but did not harbor any *TP53* defect, have ATM kinase impaired by a mutation. Since ATM is a positive regulator of p53 protein, it has been suggested that ATM inactivation may represent an alternative to p53 dysfunction. This notion has subsequently been confirmed in other study [109] and is usually observed in routine clinical practice when investigating del(17p) and del(11p) presence, since these two deletions are rarely observed in the same patient. Importantly, with respect to p53 dysfunction, these two defects should not be considered as equivalent, as the prognosis of *ATM*-defective patients is not as poor as those with *TP53* abnormalities.

A polymorphism within the *p21* gene has also been linked to p53 pathway dysfunction [110]. In addition, impaired p21 up-regulation despite an intact p53 response, which is not connected to any known gene defect, has been associated with early relapse [111]. Recently, mutations in the *BIRC3* gene, a negative regulator of NF-κB signaling, have been shown as the cause of fludarabine chemo-refractoriness in CLL patients having an intact *TP53* gene, with patient prognosis being as poor as with *TP53*-defective group [112].

Current and Future Therapeutic Options for Patients with TP53 Defects

Chemotherapeutic regimens based on alkylation agents and/or nucleoside analogues act through DNA damage induction and therefore require functional

p53 for efficient triggering of apoptosis. Consequently, pure chemotherapy and also its combination with rituximab proved to be ineffective in p53-affected CLL patients (see above; reviewed in [113]).

Currently these patients are treated most often with the anti-CD52 monoclonal antibody alemtuzumab, which functions independently of p53. Initially, effectiveness of alemtuzumab was noted in relapsed/refractory CLL, with the patients with del(17p) responding similarly to other patients [114–117]. A CAM307 study comparing alemtuzumab and chlorambucil as frontline therapy also yielded promising results in relation to treatment response, although response duration was very short in all patients, i.e., also in the alemtuzumab arm [118].

Subsequent trials combined alemtuzumab with chemotherapy and also non-chemotherapy agents. Alemtuzumab with fludarabine alone (FluCam) [119, 120] or in combination with other agents (cyclophosphamide—FCCam [121, 122], or cyclophosphamide and rituximab—CFAR [123]) were reported to produce high response rates in del(17p) patients, but such regimens show extended risk of toxicity. On the other hand, the combination of alemtuzumab with glucocorticoids appears to be meaningful, as glucocorticoids act independently of the p53 pathway, and high dose methylprednisolone (HDMP) on its own was shown to induce remissions in patients with *TP53* abnormalities [124]. Glucocorticoids, by contrast to alemtuzumab, are effective in reducing lymphadenopathy and are able to wash out CLL cells from tissues into the bloodstream, where the cells can be more susceptible to alemtuzumab elimination. Accordingly, alemtuzumab in combination with methylprednisolone (CamPred) [125] or dexamethasone (CamDex) [126] was proven to be highly effective for del(17p) CLL patients. Glucocorticoids were combined not only with alemtuzumab but also with rituximab: R-HDMP [127, 128], *R*-dexamethasone [129], or humanized anti-CD20 antibody ofatumumab (*O*-dexamethasone); a phase II trial is ongoing and will be completed soon.

However, none of the currently approved therapeutic strategies noted above are able to attain long-term remissions in p53-defective patients. Therefore, ASCT still remains a viable option for young and physically fit p53-defective patients, with the potential to induce long-term disease-free survival [130, 131]. ASCT, however, is only available for a small subset of patients and is connected to nonrelapse mortality associated with development of graft-vs.-host disease (GVHD); both early and late relapses frequently occur.

A number of novel compounds are in various phases of clinical or preclinical testing. Biological targeted treatment functions via p53-independent triggering of apoptosis, which includes diverse mechanisms of action: (a) targeting of CLL cell surface molecules (CD20, CD23, CD37) by monoclonal antibodies or small modular immunopharmaceuticals (SMIP) [132]; (b) use of immunomodulatory and microenvironment modulating agents (IMiDs) [133]; (c) promoting the apoptotic pathway using, e.g., Bcl-2 antisense oligonucleotide, BH3 mimetics, or Bcl-2 inhibitors (for review see [134]); (d) altering histone modification by inhibition of histone deacetylases [135]; (e) targeting cell signaling through inhibition of cyclin-dependent kinases; [136] or by inhibition of the BCR signaling pathway—inhibition of NFκB and kinases PI3K-delta, SYK, AKT, Lyn, and mainly Bruton's

tyrosine kinase—this approach belongs among the most promising, as it directly impacts B-cell proliferation and survival (for review, see [137]). Another approach is activation of p53 homologs in patients with a *TP53* defect [93, 94] or direct targeting of mutant p53 protein and thus sensitizing the CLL cells to chemo/chemoimmunotherapy treatment using: (a) suppression of mutated p53 by antisense oligonucleotide [138]; (b) reactivation of p53 by specific compounds directed to wt or mutated p53 molecules [139]; (c) inhibition of heat-shock protein 90 that leads to destabilization of many tumor-promoting proteins, including mutant p53 molecules [140, 141] .

Notwithstanding progressive scientific achievements, there is currently no optimal treatment available for CLL patients with 17p deletions and/or *TP53* mutations. The patients still have dismal expectations and should be scheduled to participate in applicable trials whenever possible [61].

Techniques for *TP53* Mutation Analysis

No standardized methodology is currently used for *TP53* mutational analysis in CLL samples and individual centers utilize different approaches. A list of available methods with more detailed description is provided in ERIC recommendations on *TP53* mutation analysis, published in 2012 [70].

Direct Sequencing

Direct (Sanger) sequencing is still one of the most often used methods. Genomic DNA (gDNA) rather than cDNA is preferred, as RNA-based analysis may omit some mutations which lead to nonsense-mediated mRNA decay (i.e., some nonsense or frame-shift mutations) [142]. The detection limit of Sanger sequencing may not be sufficient to detect small subclones with *TP53* mutation, especially in cases without deletion of the second allele. Primers and reaction conditions can be found at the IARC p53 website (http://www-p53.iarc.fr/p53sequencing.html). Sequencing may be restricted to exons 4–9 or alternatively 4–10, as mutations in exons 2, 3, and 11 are very rare and exon 10 contains only about 4 % of all mutations [86].

Prescreening Methods

The usage of prescreening methods such as denaturing high performance liquid chromatography (DHPLC) or high resolution melting (HRM) makes the mutational screening faster, cheaper, and more sensitive. However, identification of the particular mutation by Sanger sequencing is always essential. Primers and conditions for

DHPLC can be also found at the IARC p53 website. DHPLC can reach a sensitivity of up to 5–10 % of mutated alleles (depending on the particular sequence); however, mutation confirmation by the less-sensitive Sanger sequencing method is not always possible.

Another screening option represents the yeast functional assay (FASAY—Functional Analysis of Separated Alleles in Yeast) that directly identifies inactivating mutations, thus distinguishing them from silent alterations, polymorphisms, and partially or fully functional mutations. In this assay, the *TP53* gene from patient cells is expressed in yeasts that function as reporter cells. FASAY is fast, cheap, and has a sufficient detection limit (10 %) [143, 144]. Underlying mutations should always be determined by sequencing. The mutation identification is based on DNA sequencing from yeast clones, which is more sensitive than direct sequencing of gDNA. However, since FASAY is a RNA-based method, it may not detect mutations leading to RNA degradation due to nonsense-mediated mRNA decay.

Microarrays

Microarray resequencing provides high sensitivity, with detection limits reaching up to 3 % of the mutated clone; however, this limit varies depending on the particular sequence. The Roche Amplichip p53 test, based on Affymetrix platform, is currently under development. The procedure is fast and user-friendly and is intended as in vitro diagnostic tool without need for confirmatory sequencing. This microarray was already tested in CLL studies [84, 145], but is not commercially available yet. The main shortcoming of microarray resequencing is the ability to detect only mutations for which the probes are printed on the array (for Amplichip, e.g., all single base pair substitutions and single nucleotide deletions in exons 2–11 and splicing sites).

Functional Tests of the p53 Pathway

Several partially modified tests have been suggested, based on DNA DSBs induction followed by the monitoring of p53 accumulation and subsequent p21 induction [108–111, 146, 147] or the induction of p21 together with other p53-downstream genes [146, 148]. An alternative approach utilized etoposide and nutlin-3a for efficient distinguishing of *TP53* and *ATM* defects [149]. Another alternative represents the measurement of miR34a base level. In this assay, no cell treatment is required since the miR34a basal level is decreased in patients with p53 defects in comparison with patients carrying functional p53 [31, 32]. Although a functional assessment of the p53 pathway seems to be an elegant means of identifying potential *TP53* and *ATM* defects, this testing is tricky and partially provides inconsistent results in relation to cell abnormalities [146] which still precludes its application in routine diagnostics.

Next-Generation Sequencing

Current rapid development of next-generation sequencing (NGS) technologies has allowed their utilization within a wider scientific community. Initial upfront cost of instrumentation is still very high, but with high throughput of samples or more screened target genes the methodology becomes cost-effective. Usage of highly sensitive technologies such as ultra-deep sequencing allows a detection of very small clones carrying *TP53* mutation, but the clinical impact and the relevance of these "minor" mutations for their subsequent selection is currently uncertain and under investigation.

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ORIGINAL ARTICLE

Detailed analysis of therapy-driven clonal evolution of *TP53* mutations in chronic lymphocytic leukemia

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In chronic lymphocytic leukemia (CLL), the worst prognosis is associated with *TP53* defects with the affected patients being potentially directed to alternative treatment. Therapy administration was shown to drive the selection of new *TP53* mutations in CLL. Using ultra-deep next-generation sequencing (NGS), we performed a detailed analysis of *TP53* mutations' clonal evolution. We retrospectively analyzed samples that were assessed as *TP53*-wild-type (wt) by FASAY from 20 patients with a new *TP53* mutation detected in relapse and 40 patients remaining *TP53*-wt in relapse. Minor *TP53*-mutated subclones were disclosed in 18/20 patients experiencing later mutation selection, while only one minor-clone mutation was observed in those patients remaining *TP53*-wt ($n=40$). We documented that (i) minor *TP53* mutations may be present before therapy and may occur in any relapse; (ii) the majority of *TP53*-mutated minor clones expand to dominant clone under the selective pressure of chemotherapy, while persistence of minor-clone mutations is rare; (iii) multiple minor-clone *TP53* mutations are common and may simultaneously expand. In conclusion, patients with minor-clone *TP53* mutations carry a high risk of mutation selection by therapy. Deep sequencing can shift *TP53* mutation identification to a period before therapy administration, which might be of particular importance for clinical trials.

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INTRODUCTION

In chronic lymphocytic leukemia (CLL), patients harboring *TP53* defects represent a major challenge concerning the effective treatment.[1] *TP53* mutation and/or 17p deletion severely impede response to chemotherapy,[2,3] and affected patients also manifest short clinical responses to its combination with rituximab.[4,5] Although alemtuzumab is supposed to act independently on p53, the response rates in monotherapy are far from satisfactory in chemorefractory patients.[6] The inability of mutated p53 protein to induce apoptosis properly seems to be a primary reason for the observed resistance to treatment.[7] The p53 dysfunction is also the major cause of genomic instability in CLL cells,[8] which leads to the acquisition of other genomic variants available for further selection.

TP53 gene defects have been observed as primarily subclonal events in CLL patients, often emerging at later disease stages.[9] The frequency of *TP53* defects at diagnosis or before first therapy is only between 5 and 15%,[2,3,10,11] but the proportion of affected patients is significantly higher after treatment and has been reported to be as high as 44% in a fludarabine-refractory cohort.[12] Clonal evolution of genetic abnormalities including *TP53* defects is well evidenced in CLL. Recent studies have illustrated the development of 17p and 11q deletions during the disease course, and associated clonal evolution of new 17p deletions with the presence of foregoing therapy.[13,14] Concerning *TP53* mutations, well-documented cases of their acquirement under the pressure of chemotherapy have also been reported by us and independently by others.[15–18] This led to the suggestion that *TP53* mutations should be investigated before each therapy in CLL patients.[19]

Next-generation sequencing (NGS) technologies currently enable mutation analyses in cancer patients with previously unattainable sensitivity, reaching as far as fractions of percentages. The clinical significance of minor-clone *TP53* mutations has recently been demonstrated by Rossi *et al.*[18] Therefore, we utilized this powerful tool to study the clonal evolution of *TP53* mutations in detail. We used an amplicon ultra-deep NGS approach with a high coverage to reach maximum sensitivity, and we used a highly accurate proof-reading polymerase to minimize the sequencing errors. The aims of this NGS-based study were to disclose (i) whether minor *TP53*-mutated clones had already been present before the preceding therapy, and if yes, (ii) whether some patients, who are *TP53*-wt after therapy, harbor minor *TP53* mutations that are not selected. These two issues should address whether NGS is capable of identifying patients at risk of *TP53* mutation selection by treatment.

MATERIALS AND METHODS

Patients' cohort

The study was performed on CLL patients' peripheral blood samples at the University Hospital Brno (with written informed consent provided in accordance with the Declaration of Helsinki). Four common cytogenetic aberrations including 17p deletions were detected by Interphase fluorescent *in situ* hybridization using probes from MetaSystems (Altlussheim, Germany), and were classified according to the hierarchical cytogenetic model.[20] *TP53* mutations were identified by yeast functional analysis (FASAY) coupled to Sanger sequencing of DNA templates from red

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colonies bearing non-functional p53.[16] Patient selection criteria for NGS analysis were: Cohort I: (i) *TP53* mutational status change from wild-type (wt) to mutated documented using FASAY; (ii) only one therapy applied between the last *TP53*-wt examination and new *TP53* mutation detection. This criterion was established to enable the tracking of clonal evolution during just one relapse; (iii) available DNA from the time when the sample was *TP53*-wt. Cohort II: (i) available results of consecutive FASAY analyses performed in relapse(s) with no *TP53* mutational status change; (ii) DNA available from the period preceding therapy (Supplementary Figure 1).

Statistical analyses

Fisher's exact test was used to assess the association between categorical variables. Mann–Whitney test was used to compare the continuous variables. Wilcoxon signed-rank test was used for paired comparison of mutation numbers. Survival analysis and time to mutation detection were calculated using the Kaplan–Meier survival estimator. Overall survival was assessed from the date of diagnosis; only disease-related death was considered as an event. Time to mutation detection was assessed from the date of diagnosis to the date of new *TP53* mutation detection (event) or the last *TP53*-wt examination (censored).

Median survival, time to mutation detection, differences between the curves, and hazard ratios were evaluated by the log-rank test using the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Ultra-deep NGS

NGS analysis was performed on MiSeq (Illumina, San Diego, CA, USA) using gDNA from cryopreserved peripheral blood separated CD19+ B-lymphocytes or mononuclear cells; the percentage of CLL cells (CD5+CD19+) was assessed using flow cytometry and was >80% in all cases. In all, 25 ng of patient DNA was amplified with highly accurate proof-reading Q5 Polymerase (New England Biolabs, Ipswich, MA, USA) using *TP53* exon-specific primers (Supplementary Table 1). The experimental design and reaction conditions followed the manufacturer recommendations. Briefly, PCR products were pooled, purified with Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA), and quantified using Qubit dsDNA HS Assay Kit (Life Technologies, Waltham, MA, USA). The purified amplicon mixes were diluted to a total amount of 1 ng. The indexed paired-end library was prepared with Nextera XT DNA Sample Preparation Kit (Illumina) and sequenced using MiSeq Reagent Kit v2 300 cycles (Illumina). To avoid cross-contamination, samples obtained from the same patient in different time periods were sequenced in separate runs. Amplicons and libraries for each run were prepared separately. The median coverage per base achieved was 31 599 reads (range 2601–177 021).

An in-house bioinformatics pipeline was established to call the sequencing variants. For read preprocessing and alignment, we used CLC Genomic Workbench (Qiagen, Hilden, Germany). Variant calling was performed using the deepSNV R-package[21] with a statistical approach applying the shearwater algorithm to compute Bayes classifiers based on a betabinomial model.[22,23] By the reproducibility test, we disclosed that we were able to reliably distinguish point mismatches and ⩾2 nt insertions/deletions (indels) at the level of 0.2% of variant reads, and 1-nucleotide deletions at the level of 1% of variant reads as these may be artificially introduced during the sequencing and alignment process. For further details, see Supplementary material. Moreover, to evaluate the established pipeline, 20 control samples (*TP53* exons 4–10) derived from healthy individuals were sequenced and no alteration in any sample was observed on the above-mentioned detection limits.

RESULTS

Consecutive *TP53* mutational analysis confirms the prominent impact of newly acquired *TP53* mutations on survival

Consecutive *TP53* mutation investigation using FASAY was performed in 330 patients in at least 2 serial samples. All patients harbored intact *TP53* gene at the time of the first analysis (for patients' characteristics, see Supplementary Table 2). Among 121 patients who did not receive any therapy during the follow-up, new *TP53* mutations were observed in only one patient (median follow-up of the group 50 months). In contrast, analyses performed at the time of relapse after one or several therapy lines ($n = 209$ patients; median follow-up of the group 61 months) identified new *TP53* mutation(s) in 43 patients. Altogether, the risk of *TP53* mutation acquisition at 5 years after diagnosis was 1% in untreated vs 17% in treated patients (hazard ratio 0.25 (95% CI 0.13–0.47; $P < 0.001$)) (Figure 1).

To assess the importance of *TP53* mutation status change from wt to mutated, we used our cohort analyzed using FASAY and compared the overall survival from diagnosis in patients who acquired a new dominant mutation in relapse with patients who remained *TP53*-wt in relapse, and patients who already harbored *TP53* mutations at diagnosis (Figure 2). The overall survival was significantly reduced in the group of patients who had selected *TP53* mutations compared with patients assessed as wt in relapse ($P = 0.03$). The shortest survival was noted for patients with *TP53* mutations already detected at diagnosis.

NGS analysis reveals the presence of minor mutated clones before their therapy-driven selection

In the first part of our retrospective study, we focused on 20 patients who had acquired a new *TP53* mutation in relapse, as

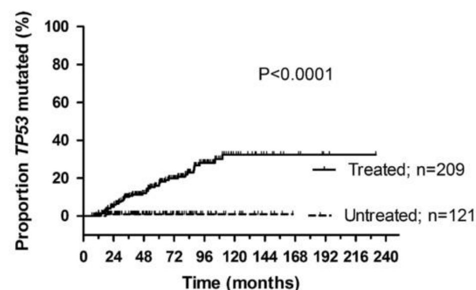

Figure 1. Time from diagnosis to *TP53* mutation acquisition. Patients with *TP53*-wt status at first investigation were repeatedly tested. Time to mutation detection was assessed from the date of diagnosis to the date of new *TP53* mutation detection (event) or the last *TP53*-wt examination (censored). Patients treated during the follow-up acquired new *TP53* mutation significantly more often than untreated patients.

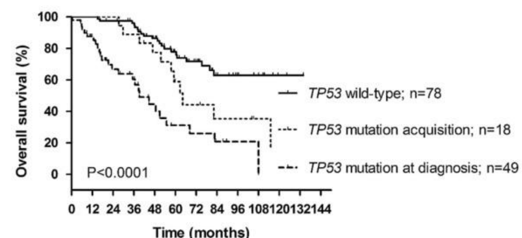

Figure 2. Overall survival according to the *TP53* mutational status in relapse. Overall survival from diagnosis in patients who acquired a new *TP53* mutation at relapse ($n = 18$; median survival 64 months) in comparison with patients who remained *TP53*-wt at relapse ($n = 78$; median survival undefined; pairwise comparison $P = 0.03$), and patients who harbored *TP53* mutation already at diagnosis ($n = 49$; median survival 39 months; pairwise comparison $P = 0.02$). Only patients with *TP53* status examined at diagnosis or 12 months thereafter were included. All patients included in the analysis underwent treatment and patients having *TP53*-wt status at diagnosis were repeatedly tested for *TP53* mutation presence at subsequent relapse(s).

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assessed by FASAY coupled to Sanger sequencing (Cohort I; Sample 2). In these patients, we used ultra-deep NGS to examine samples taken before the preceding therapy, which showed *TP53*-wt status using Sanger sequencing and FASAY (Sample 1). In 10 patients, these retrospective samples were treatment naïve (Cohort IA), while the remaining 10 patients had already been pretreated at the time of NGS analysis (Cohort IB) (Table 1). The schematic visualization of samples' inclusion criteria is shown in Supplementary Figure 1. The mutations analyzed consisted of 16 missense mutations, 2 non-sense mutations and 2 deletions and were hence representative of the p53 mutation profile in CLL.[24] To decipher *TP53* mutagenesis, we sequenced not only the affected regions but also all commonly mutated exons 4–9 (ref. 24) with a high median coverage for the positions containing mutations (25 709 reads; range 5245–64 979). We were able to detect minor-proportion mutations in 18/20 samples (90%), with a proportion of 0.20–3.71% of the reads showing mutations. In 2 of the 18 patients, we surprisingly detected other *TP53* mutations that had not undergone expansion. The results are summarized in Table 1, for details see Supplementary table 3.

Interestingly, in addition to the presumed retrospective mutations, we also identified other minor-proportion *TP53* mutations in both treatment-naïve and pretreated samples (10/20 patients; 2–6 mutations per patient) (Table 1). It indicates that in a proportion of patients, there is a pool of *TP53* mutations available for therapy-driven selection.

We next intended to investigate whether the minor *TP53*-mutated subclones detectable by NGS in pretreated samples and undergoing selection in subsequent relapse (Cohort IB) had already been present before first therapy. Therefore, we used NGS in four available treatment-naïve samples (patients no. 149, 365, 542 and 1043) and confirmed the presence of respective mutation in one of them (patient no. 1043—mutation c.844C > G (p.R282G) detected in 0.2% of NGS reads). This observation suggests that preexisting mutations may expand after the first but also after subsequent therapies at least in some patients.

Minor *TP53* mutations detectable before therapy are rare in patients remaining *TP53*-wt at relapse

As the next step, we analyzed 40 samples taken before first treatment in patients showing wt-*TP53* status at relapse after one or several therapy lines (Cohort II). These cases were selected from the cohort of relapsing patients, and the inclusion criteria were chosen to collect the cohort with biological and clinical characteristics matching Cohort I (Table 2; Supplementary Figure 1). In this experiment, besides exons 4–9, exon 10 was also sequenced as it may occasionally harbor mutations.[24]

We found *TP53* mutation in only 1 of the 40 patients (2.5%). Specifically, the mutation c.797G4A (p.G266E) was detected in 0.55% (148/32 973) of sequencing reads, and its presence was verified by an independent NGS run. This mutation did not undergo a clonal expansion during the disease course despite several treatment lines—the patient was treated consecutively with three distinct therapy lines (FCR, Alemtuzumab and Rituximab+Dexamethasone) and achieved two complete remissions. In the last available sample from the time of relapse after Rituximab+Dexamethasone treatment (follow-up 47 months) the same mutation was present in 1.4% of reads.

Clonal selection frequently affects multiple TP53 mutations simultaneously

As emerged from the previous analyses, multiple minor-clone *TP53* mutations are commonly observed in CLL patients. To further explore this phenomenon in relation to the expansion of major mutations, we performed ultra-deep NGS of *TP53* gene in samples taken at relapse(s) (Sample 2 in Supplementary Figure 1). For this analysis, we had chosen the following patients from Cohort I: (i) six patients with more than one mutation detected in sample 1; (ii) six patients with a single mutation detected in sample 1; and (iii) two patients with no mutation detected in sample 1. Furthermore, the patient with a single non-expanding mutation from Cohort II was also included. An increase in the number of mutations compared with the preceding samples was observed in 13/14 patients from Cohort I (Table 3; Figure 3a). In the paired analysis restricted to samples taken before the first therapy and in the first relapse (Cohort IA), a significant increase in the number of mutations per patient was observed (mean number of mutations per patient 2.1 vs 6.7; $P = 0.02$). In the patient from Cohort II, only one mutation was found in both samples.

Regarding the evolution of individual subclones, the most frequently observed event (7/14 patients) was the clear expansion of one mutation from minor to dominant clone accompanied with the occurrence of additional minor *TP53*-mutated clones. In addition to that, we also observed other specific situations: (i) in one case the consecutive selection of two different dominant *TP53* mutations at the first and then the subsequent relapse was noted (one mutation replaced by the other) (patient no. 820; Figure 3b); (ii) in four patients there was not a prominent clonal expansion of one mutation, but multiple clones expanded simultaneously (patients no. 8, 178, 354 and 485; Figure 3c); (iii) one patient underwent only a very slight expansion of a single minor-clone mutation in the first relapse (from 0.2 to 1.46% reads), in the second relapse the proportion of the mutation also increased only slightly (to 2.82% reads) and two other minor-proportion mutations appeared (patient no. 503; Table 3). The results summarizing the rise in the number of mutations in all performed NGS analyses are recapitulated in Supplementary Table 3.

Since the analysis of mutated patients disclosed an increased occurrence of minor *TP53* mutations after treatment, we further analyzed 15 randomly selected patients from Cohort II after 1–4 therapy lines using NGS. No *TP53* mutations were observed in any patient.

Molecular features of mutations

In total, we identified 148 mutations in 21 patients (Supplementary Table 3) in all the NGS analyses performed. The mutation profile is shown in Supplementary Figures 2 and 3. Compared with the reference study on *TP53* mutation profile in CLL[24] our results showed the following: (i) a similar proportion of missense mutations (79 vs 74%; $P = 0.4$) and non-sense mutations (both studies 4%); (ii) the same frequency of mutations at major hot spot codons (175, 179, 220, 248, 273 and 281) (20% of all mutations in both studies); (iii) a significantly higher proportion of splice-site mutations (9 vs 2%; $P = 0.005$) and, on the other hand (iv) a significantly lower frequency of indel mutations (7 vs 20%; $P = 0.0003$). Concerning point mutations, transitions represented 61% with only 29% of them (17% of all mutations) occurring at CpG sites. The G-A transitions at CpG predominated C-T transitions (G-A:C-T ratio 2:1). The lower proportion of CpG transitions and the prevalence of G-A exchanges coincided with the reference study.[24]

Comparison of *TP53* mutation profiles in cases with unmutated immunoglobulin heavy chain gene (*IGHV*; U-CLL) vs mutated *IGHV* (M-CLL) showed no difference in mutation frequency within sequence motif (RGYW/WRCY) recognized by activation-induced cytidin deaminase in U-CLL vs M-CLL (20 vs 18% of point mutations $P = 0.8$). In M-CLL, a significant prevalence of alterations in A:T pairs was found compared with U-CLL (56 vs 27% of point mutations; $P = 0.0008$). The A:T alteration predominance was the most prominent in case of A:T > C:G transversions (12% in M-CLL vs 1% in U-CLL; $P = 0.009$; Figure 4).

As the number of mutations increased after therapy, we also compared the molecular profile of mutations detected in pretherapy samples only ($n = 24$) with mutations that occurred

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Table 1. Summary of NGS analysis in patients acquiring a new *TP53* dominant mutation after treatment

Patient	*Mutation name*		*FASAY*		*Ultra-deep NGS—Sample 1*		*Time from dg to NGS (mo)*	*Time between sample 1 and sample 2 (mo)*	*Therapy before sample 1*	*Therapy between sample 1 and sample 2*	*Cytogenetic aberrations*	*IGHV*	*Disease status*
			Sample 1	*Sample 2*	*(%)*	*Total number of mutations*							
Cohort IA													
126	c.559+33_54del		neg	pos (40%)	0.52	4	77	23	–	FC	NA → 13q-,17p-	unmut	Alive
178	c.488A>G	p.Y163C	neg	pos (18%)	neg	0	45	66	–	FCR/R+D	neg → 13q-,17p-	unmut	Alive
199	c.548C>G	p.S183*	neg	pos (24%)	0.57	1	1	81	–	FCR	neg	unmut	Dead
227	c.536A>G	p.H179R	neg	pos (75%)	0.56	1	28	46	–	FC	11q-, 13q- → 13q-, 17p-	unmut	Dead
286	c.736A>G	p.M246V	neg	pos (56%)	2.38	1	29	47	–	FCO	13q-	unmut	Alive
503	c.838A>G	p.R280G	neg	pos (13%)	0.20	1	0	31	–	FCR	11q-, 13q-	unmut	Dead
618	c.745A>T	p.R249W	neg	pos (14%)	neg§	1	113	32	–	FCR	11q-, 13q-	unmut	Dead
812	c.817C>T	p.R273C	neg	pos (63%)	0.33	2	0	26	–	FCR	13q- → 13q-,17p-	mut	Alive
820	c.743_745del	p.R249del	neg	pos (17%)	2.40	6	0	17	–	FCR	11q- → 11q-, cn-LOH 17p	unmut	Dead
837	c.438G>A	p.W146*	neg	pos (17%)	0.85	6	0	24	–	FCR	11q- → 11q-, cn-LOH 17p	unmut	Alive
Cohort IB													
8	c.527G>T	p.C176F	neg	pos (22%)	1.10	4	92	30	Clb, FCR	F/RCHOP	13q- → neg	mut	Dead
149	c.814G>A	p.V272M	neg	pos (50%)	1.11	2	72	24	FCR	FCR	neg	unmut	Dead
161	c.731G>A	p.G244D	neg	pos (18%)	neg	0	14	48	A	FCR	neg → 13q-,17p-	unmut	Dead
280	c.818G>A	p.R273H	neg	pos (60%)	1.43	1	15	5	RCHOP	A	neg → 13q-,17p-	unmut	Dead
322	c.524G>A	p.R175H	neg	pos (67%)	0.25	1	68	18	Clb	FC	neg → 13q-,17p-	unmut	Dead
354	c.844C>T	p.R282W	neg	pos (22%)	0.76	1	77	18	FC	FCR	11q-, 13q-	unmut	Dead
365	c.329G>T	p.R110L	neg	pos (25%)	3.71	4	48	9	FCR/FC	FCR	13q- → 13q-,17p-	unmut	Dead
485	c.817C>T	p.R273C	neg	pos (18%)	neg§	2	115	33	Clb	FC	13q-	mut	Alive
542	c.814G>A	p.V272M	neg	pos (35%)	0.76	2	12	22	FCR	FCR	neg → 13q-	unmut	Dead
1043	c.844C>G	p.R282G	neg	pos (18%)	0.8	4	32	17	Clb	FCR	13q-	unmut	Alive

Abbreviations: A, alemtuzumab; Clb, chlorambucil; C, cyclophosphamide; F, fludarabine; R, rituximab; FC, fludarabine+cyclophosphamide; FCR, FC+rituximab; FCO, FC+ofatumumab; CHOP, cyclophosphamide; doxorubicin, vincristine, prednisone; RCHOP, CHOP with rituximab; R+D, rituximab+dexamethasone. Sample 1—sample showing *TP53*-wt status using FASAY; Sample 2—new *TP53* mutation in relapse assessed by FASAY; Cytogenetic aberrations—in case of change cytogenetic aberrations are listed in the format: Sample 1 → Sample 2; dg—diagnosis; mo—month; neg—negative; pos—positive; mut—mutated; unmut—unmutated; §—other than the later expanding *TP53* mutation detected (for details, see Supplementary Table 3); cn-LOH 17p—17p copy-neutral loss of heterozygosity involving *TP53* gene detected by Cytoscan Affymetrix arrays.

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Table 2. Clinical and biological characteristics of patients analyzed using NGS

	Cohort I		Cohort II		P
	Dominant TP53 mutation acquisition		No TP53 mutated dominant clone after therapy		
	Number	%	Number	%	
Number of patients	20		40		
Rai stage at diagnosis					
0	6	30	6	15	0.3049
I–II	10	50	23	58	0.5853
III–IV	4	20	11	28	0.7529
Age at diagnosis					
Median	56.8		59.2		0.3828
Range	45–76		38–77		
Gender					
Male	15	75	31	78	1.0000
Female	5	25	9	23	
IGHV status					
Mutated	3	15	4	10	0.6763
Unmutated	17	85	36	92	
I-FISH[a] before treatment					
Del(17p)	0	0	1	3	1.0000
Del(11q)	6	30	13	34	1.0000
+ 12	0	0	5	13	0.1578
Del(13q)	7	35	13	34	1.0000
Normal	7	35	8	21	0.2199
Follow-up[b] (months)					
Median	74.8		69.9		0.9808
Range	17–147		15–195		
Number of therapy lines during follow-up[b]					
Median	2		2		0.4768
Range	1–4		1–5		

Abbreviations: I-FISH, Interphase fluorescent *in situ* hybridization; NGS, next-generation sequencing. [a]According to the hierarchical cytogenetics.[20] [b]Follow-up: Cohort I—from diagnosis to dominant *TP53* mutation detection; Cohort II—from diagnosis to the last FASAY investigation.

exclusively after treatment ($n = 103$) and we did not observe any significant differences regarding the proportion of hot spot mutations, transversion-to-transition ratio, proportion of transitions at CpG sites and G:C to A:T ratio (data not shown).

DISCUSSION

The mechanisms leading to p53 mutation acquisition and accumulation in CLL are poorly understood. The direct induction of *TP53* mutations by DNA-damaging chemotherapy, namely alkylating agents, has been suggested.[25] In contrast, a large collaborative study involving 268 p53 mutations indirectly showed that the impact of therapy on *de novo* mutation induction is unlikely, as mutation spectra are similar in untreated and treated patients.[24] This observation, however, may not serve as definitive proof of the neutral impact of therapy on *TP53* mutagenesis, since similar mutations could evolve through different mechanisms. The current progress in highly sensitive techniques, specifically in ultra-deep NGS, allows the possibility to explore whether therapy merely selects *TP53* mutations present in minor CLL clones before drug administration. Moreover, identifying *TP53* defects as early as possible during their evolution may represent a significant achievement in the clinical management of high-risk CLL, since *TP53*-deffective patients could be offered alternative treatment.[1] The clinical impact of minor-proportion *TP53* defects is currently a matter of debate.[18,26,27] Their relevance for relapse development is supported by the actual number of mutated cells. For instance, at common pretherapy leukocytosis achieving $100 \times 10e9$ per liter with 90% CLL cells, a patient harbors approximately $4.5 \times 10e11$ CLL cells in peripheral blood, not considering other organs like the spleen. In this case, a 1% *TP53* mutation corresponds to $\sim 4.5 \times 10e9$ cells. Moreover, the clinical significance of small *TP53*-mutated clones under the detection limit of Sanger sequencing have very recently been manifested by the study of Rossi *et al.*[18] showing their similar unfavorable prognostic impact compared with clonal *TP53* defects.

With this report, we focused on two principal issues: (i) exploration of *TP53*-mutated clone evolution and (ii) assessment of NGS utilization in *TP53* mutation expansion prediction in clinical practice. Both these issues are important with respect to the clear negative impact of newly acquired *TP53* mutations on patients' prognosis, which was evidenced by Rossi *et al.*[28] using time-dependent Cox regression analysis, and is also confirmed here by survival analysis of patients with new mutations.

Concerning the clonal evolution, we documented that the risk of new *TP53* mutation acquisition at 5 years after diagnosis is 17% in patients requiring treatment, contrasting with 1% in untreated patients ($P < 0.001$), and we confirmed that selection of preexisting mutated clones by therapy is the predominant mechanism for *TP53* mutations' accumulation. Moreover, we showed that mutations expanding during relapse are detectable before the preceding therapy in the majority of patients. Admittedly, based on our study we cannot entirely exclude that at least some *TP53* mutations are the consequence of DNA damaging drugs[25] since many minor-proportion mutations were undetectable before first therapy despite using ultra-deep NGS. Although we have not observed any profound difference in the mutation profile of these mutations compared with the mutations present before treatment, they could be induced by therapeutic agents or spontaneous mutagenesis during relapse. Alternatively, they may be present in a very low proportion of leukemic cells under the NGS detection limit.

Our study independently confirms two recent reports[18,29] showing that in a proportion of patients there are multiple minor-clone *TP53* mutations (under the Sanger sequencing detection limit). These mutations may or may not accompany a major clonal mutation.[29] We noted the presence of multiple *TP53* mutations in patients with clonal selection of dominant *TP53* mutations, and also in patients with dominant *TP53* mutation detected at diagnosis (7/10 patients; data not shown). We further observed that selection may affect not only single *TP53*-mutated minor clone, but also in some patients multiple mutations simultaneously. In fact, at least some cases without prominent expansion of one mutation underwent a slight selection of a burden of different *TP53*-mutated clones that are not detectable by Sanger sequencing. Using FASAY, these patients were assessed as 'mutation acquisition' since with this methodology the overall percentage of red colonies equals the sum of all mutations present.

The striking aspect of our study is the actual number of multiple *TP53* mutations, as according to our observation even tens of mutations may be present in individual patients. Our conclusion that these multiple alterations are true mutations and not NGS artifacts is supported by the following: (i) the point mutations present in $\geqslant 0.2\%$ of NGS reads were confirmed in a reproducibility test; (ii) the same variants were often observed in consecutive samples, (iii) some of the minor-proportion mutations

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Table 3. Consecutive ultra-deep NGS analysis

Patient	FASAY		Mutation detected using FASAY—major mutation		Ultra-deep NGS						Time between samples 1 and 2 (mo)	Time between samples 2 and 3 (mo)	Therapy between samples 1 and 2	Therapy between samples 2 and 3
					Proportion of major mutation (%)			Total number of mutations						
	Sample 1	Sample 2			Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3				
Cohort IA														
178	neg	pos (18%)	c.488A > G	p.Y163C	neg	5.08		0	21		66		FCR/R+D	
199	neg	pos (24%)	c.548C > G	p.S183*	0.57	18.90		1	7		81		FCR	
227	neg	pos (75%)	c.536A > G	p.H179R	0.56	81.50		1	4		46		FC	
286	neg	pos (56%)	c.736A > G	p.M246V	2.38	48.70		1	1		47		FCO	
503	neg	pos (13%)	c.838A > G	p.R280G	0.20	1.46	2.82	1	1	3	31	10	FCR	A
618	neg	pos (14%)	c.745A > T	p.R249W	neg§	7.64		1	4		32		FCR	
812	neg	pos (63%)	c.817C > T	p.R273C	0.33	75.90		2	3		26		FCR	
820	neg	pos (17%)	c.743_745del	p.R249del	2.40	21.50	0.73	6	12	5	17	33	FCR	FCR, RCHOP, VAD
837	neg	pos (17%)	c.438G > A	p.W146*	0.85	64.3		6	7		24		FCR	
Cohort IB														
8	neg	pos (22%)	c.527G > T	p.C176F	1.10	1.00		4	23		30		F/RCHOP	
354	neg	pos (22%)	c.844C > T	p.R282W	0.76	3.89		1	9		18		FCR	
161	neg	pos (18%)	c.731G > A	p.G244D	neg	11.10		0	3		48		FCR	
485	neg	pos (18%)	c.817C > T	p.R273C	neg§	4.18	5.25	2	20	28	33	18	FC	R+D
542	neg	pos (35%)	c.814G > A	p.V272M	0.76	23.4		2	3		22		FCR	
Cohort II														
311	neg	neg	c.797G > A	p.G266E	0.55	1.40		1	1		47		FCR, A, R+D	

Abbreviations: A, alemtuzumab; Clb, chlorambucil; C, cyclophosphamide; F, fludarabine; R, rituximab; FC, fludarabine+cyclophosphamide; FCR, FC+rituximab; FCO, FC+ofatumumab; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone; RCHOP, CHOP+rituximab; R+D, rituximab+dexamethasone; VAD, vincristine, adriamycin, dexamethasone. Sample 1—sample showing *TP53*-wt status using FASAY; Sample 2—new *TP53* mutation in relapse assessed by FASAY; Sample 3—follow-up sample after mutation detection by FASAY; mo—month; neg—negative; pos—positive; mut—mutated; unmut—unmutated; §—other than the later expanding *TP53* mutation detected (for details, see Supplementary Table 3).

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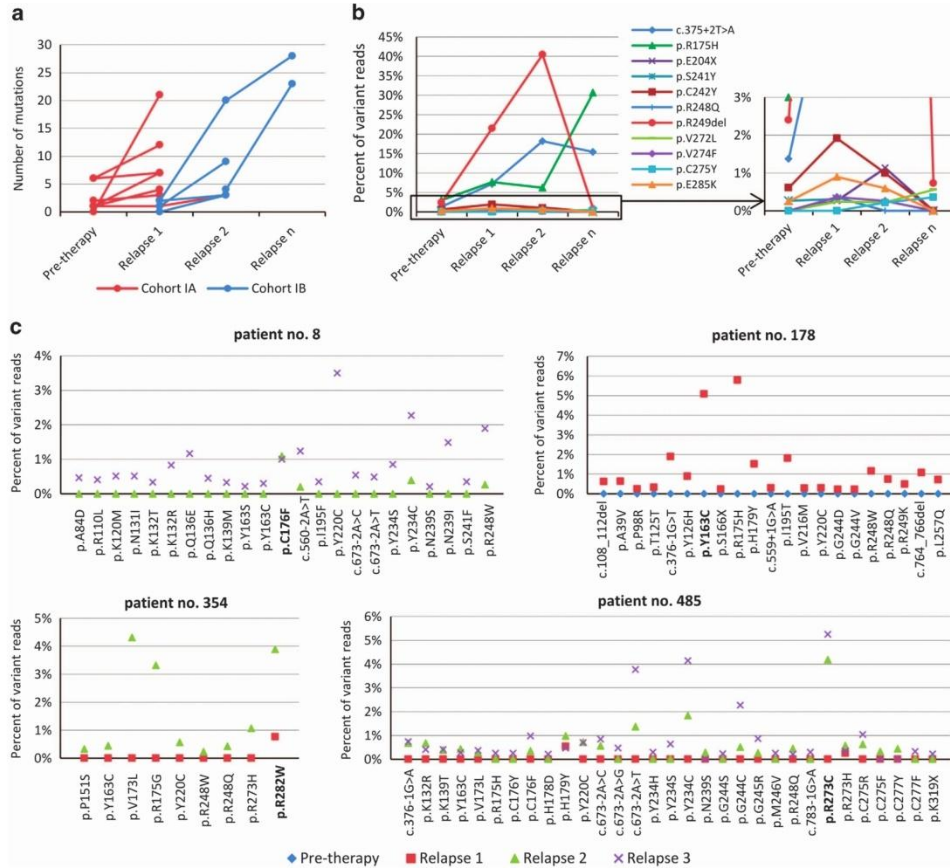

Figure 3. Kinetics of multiple mutations in subsequent samplings. (**a**) Increase in number of mutations detectable using NGS during the disease course. All patients form Cohort I with repeated NGS analysis are shown ($n = 14$). (**b**) Dynamics of clonal evolution in patient no. 820. Clone bearing mutation p.249del that was detected at Relapse 2 using FASAY first expanded and was later outgrown by another mutation p.R175H. Splicing mutation c.375+2T>A slightly expanded and coexisted as a minor subclone. Subclonal dynamics of additional minor clones present below 2% is shown in detail. (**c**) Examples of patients with no prominent expansion of one mutation is shown. Proportion of variant reads in individual disease time points is illustrated. Mutation detected using FASAY in the second sampling is highlighted in bold.

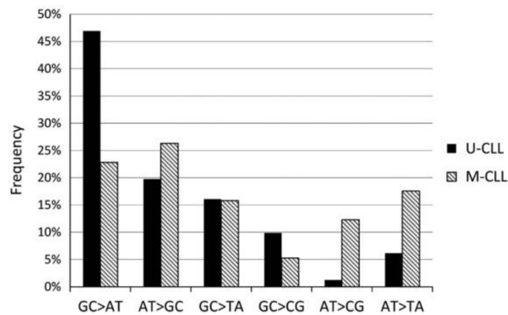

Figure 4. Comparison of *TP53* mutation profile in patients with unmutated *IGHV* (U-CLL) vs mutated *IGHV* (M-CLL). Percentage from all point mutations shown.

were also noted in individual colonies during FASAY analysis (Supplementary Table 3); this also shows that the mutations are present on separate alleles as FASAY is based on subcloning template molecules; (iv) only one mutation was detected in 56 samples from patients remaining *TP53*-wt throughout disease (40 pretherapy and 16 relapsed samples from Cohort II), and no mutation was observed in any healthy control sample ($n = 20$); and finally (v) the molecular profile of additional mutations was similar to that described for the reference cohort[24] with the common hot spots being the most prevalent mutations.

Despite the similarities between the mutation profile of additional mutations and the reference *TP53*-mutated CLL cohort, we noticed several specificities. The low number of indel mutations among additional mutations can likely be accounted to the NGS methodology itself as it is generally difficult to distinguish minor-proportion 1-nucleotide deletions from background. An interesting observation is the high number of

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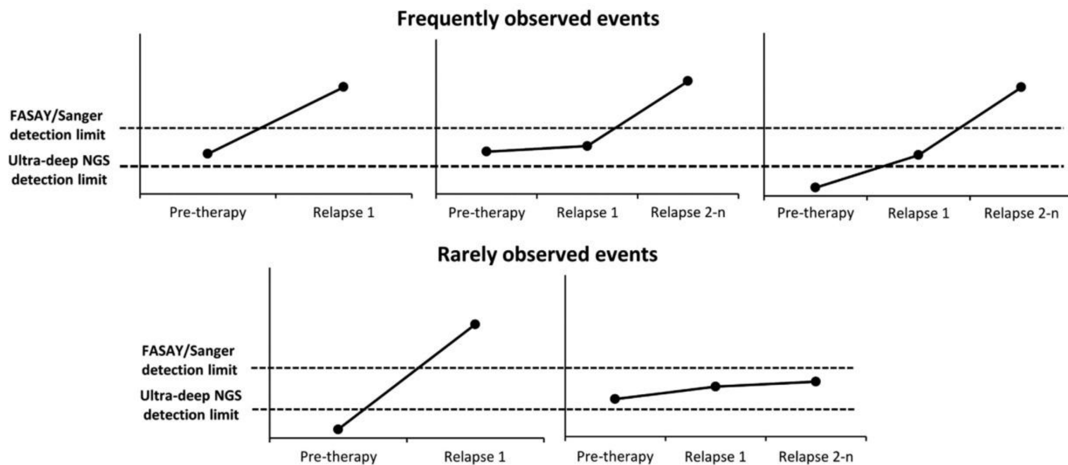

Figure 5. Schematic representation of different scenarios of *TP53*-mutated subclones clonal evolution.

minor-proportion splice-site mutations, predominantly in intron 6. These mutations are often present at the subclonal level; however for a yet unknown reason they only rarely expand to dominant clone. Apart from this, we were not able to find any rule concerning a preferential selection of distinct mutation types. For instance, we recorded patients in which a truncating mutation outgrew the clone carrying hot spot mutation with documented dominant-negative and gain-of-function effect. Therefore, there should be other factors contributing to the preferential selection of particular *TP53*-mutated subclones, for example, mutations in other genes or distinct stimulation by the microenvironment. In addition, an obvious important factor represents deletion 17p, since the wt allele absence may contribute to the selection advantage of a particular subclone. The new 17p deletion accompanying the new *TP53* mutation was found in 8/20 patients and in another two patients a new 17p copy-neutral loss of heterozygosity was noted. However, to determine the exact allele composition of minor subclones carrying different *TP53* mutations would require single-cell analysis, which was beyond the scope of this study.

The surprisingly large number of mutations led us to explore the mechanisms of *TP53* mutagenesis with regard to lymphoid-specific hypermutation machinery. No bias regarding mutations in sequence motifs recognized by activation-induced cytidin deaminase was found. Interestingly, we observed a prevalence of mutations in A:T pairs in patients with mutated *IGHV* compared with unmutated *IGHV*, which was most prominent in A:T > C:G transversions. A similar disproportion was found in a whole-genome sequencing study[30] and is most likely to be attributed to the operation of error-prone polymerase eta.[31]

The observation that the majority of new dominant mutations are already present before therapy offers the opportunity to predict their expansion later during the disease course and change the patients' care strategy. The obvious prerequisite for such clinical utilization is that persisting minor-proportion *TP53* mutations' existence is not a common phenomenon among patients who do not undergo massive mutation selection. Our long-term observation based on sensitive FASAY analyses in consecutive samples indicates that minor *TP53*-mutated clones may persist in occasional cases without significant expansion. Such a case was also documented here; in one patient we observed only a very slow increase of *TP53*-mutated subclone proportion in consecutive relapses. To explore the general incidence of non-selected mutations, we employed NGS and analyzed 40 pretherapy samples from patients remaining wt after treatment line(s) and observed that non-selected mutations are in fact rare since 39/40 patients were devoid of any mutation.

When considering the applicability of highly sensitive NGS in diagnostics, it is important to bear in mind that (i) the original clone size may be variable and under the detection limit of any method and (ii) the dynamics of the expansion process may vary among individual patients due to competition between CLL subclones and, potentially, also the type of therapy.

As we observed in our study, minor-clone mutations do not have to undergo selection after the first treatment. One may consider that the type of treatment could be critical for clonal selection, with the more intensive regimen being more likely to facilitate clonal selection as we indicated in the previous studies.[32,33] However, we document here that in some cases even administrating intensive chemo-immunotherapy resulting in complete remission does not necessarily result in clonal expansion. It is highly likely that there are other factors impacting the selection rate like other genomic defects present either in the *TP53*-mutated subclone itself or in the *TP53*-wt cells.

In conclusion, we show in our study that multiple *TP53* clonal evolution scenarios are possible, with some of them being more likely to occur (Figure 5). In cases when a minor-proportion *TP53*-mutated clone(s) is detected, the patient is at high risk of mutation selection by therapy in the first or subsequent relapse, and the presence of the new dominant mutation should be considered as a clearly negative factor impacting the patient's outcome. Moreover, our detailed analysis of *TP53* mutations at the subclonal level at different time points suggests that some patients are intrinsically prone to acquire *TP53* mutations and in the majority of these patients more than one clone carrying a different mutation with a different predisposition for expansion occur. Owing to deep sequencing, it is now technically possible to shift *TP53* mutation identification to time preceding therapy administration. It seems now especially interesting to explore whether similar rules drive the clonal evolution of other recurrently mutated genes in CLL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)

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3.2 Analýza mutací v genech *ATM* a *SF3B1* a jejich funkční dopad

Publikace aspiranta vztahující se k tématu (chronologicky 2013-2015)

ATM mutations uniformly lead to ATM dysfunction in chronic lymphocytic leukemia: application of functional test using doxorubicin. Navrkalova V, Sebejova L, Zemanova J, Kminkova J, Kubesova B, Malcikova J, Mraz M, Smardova J, Pavlova S, Doubek M, Brychtova Y, Potesil D, Nemethova V, Mayer J, Pospisilova S, Trbusek M. **Haematologica** 2013;98(7):1124-31. (M. Trbušek korespondující autor – **příloha 10**)
IF=5,9

The p53 pathway induction is not primarily dependent on Ataxia Telangiectasia Mutated (ATM) gene activity after fludarabine treatment in chronic lymphocytic leukemia cells. Navrkalova V, Sebejova L, Zemanova J, Jaskova Z, Trbusek M. **Leuk Lymphoma** 2013;54(8):1840-3. (M. Trbušek korespondující autor – **příloha 11**)
IF=2,8

The impact of SF3B1 mutations in CLL on the DNA-damage response. Te Raa GD, Derks IA, Navrkalova V, Skowronska A, Moerland PD, van Laar J, Oldreive C, Monsuur H, Trbusek M, Malcikova J, Lodén M, Geisler CH, Hüllein J, Jethwa A, Zenz T, Pospisilova S, Stankovic T, van Oers MH, Kater AP, Eldering E. **Leukemia** 2015;29(5):1133-42. (zahraniční práce s významným spoluautorským příspěvkem – **příloha 12**)
IF=10,4

Targeted next-generation sequencing in chronic lymphocytic leukemia: a high-throughput yet tailored approach will facilitate implementation in a clinical setting. Sutton LA, Ljungström V, Mansouri L, Young E, Cortese D, Navrkalova V, Malcikova J, Muggen AF, Trbusek M, Panagiotidis P, Davi F, Belessi C, Langerak AW, Ghia P, Pospisilova S, Stamatopoulos K, Rosenquist R. **Haematologica** 2015;100(3):370-6.
IF=5,9

ATM mutations in major stereotyped subsets of chronic lymphocytic leukemia: enrichment in subset #2 is associated with markedly short telomeres. Navrkalova V, Young E, Baliakas P, Radova L, Plevova K, Sutton LA, Mansouri L, Ljungström V,Ntoufa S, Davis Z, Juliusson G, Smedby KE, Belessi C, Panagiotidis P, Davi F, Langerak AW, Ghia P, Strefford JC, Oscier D, Mayer J, Touloumenidou T, Stamatopoulos K, Pospisilova S, Rosenquist R, Trbusek M. Přijato do časopisu **Haematologica** 24.5.2016. (M. Trbušek korespondující autor – **příloha 13**)

Komentář:

Kináza ATM hraje stěžejní roli v odpovědi buňky na poškození DNA v podobě dvouřetězcových zlomů (DNA DSBs), zejména během S-fáze buněčného cyklu resp. replikace (Johnson et al., 1999). Tyto zlomy představují nejnebezpečnější typ poškození, se kterým se buňka může setkat, a to jak z hlediska jejího vlastního přežití (s těmito zlomy se nelze dělit), tak i z hlediska možného vzniku nežádoucích aberací vedoucích naopak k její maligní transformaci (Bartek et al., 2007). Samotné

dvouřetězcové zlomy jsou nejprve rozpoznány proteinovým komplexem MRN (MRE11-Rad51-Nbs1) a poté je signalizace přenesena prostřednictvím proteinu ATM na další proteiny zastavující buněčný cyklus a navozující podmínky pro opravu DNA (Uziel et al., 2003). Klíčová signalizace od ATM se odehrává směrem k proteinu p53, a to prostřednictvím fosforylace p53 na Ser15 vedoucí k jeho stabilizaci (akumulaci v buňce) (Banin et al., 1998; Canman et al., 1998). Z tohoto pohledu je zřejmé, že identifikace mutací v genu *ATM* tvořila logické navazující pokračování výzkumu dysfunkce dráhy p53 u pacientů s CLL.

Identifikace pacientů s mutacemi *ATM* je ovšem komplikovaná a to ze dvou důvodů:

(a) i když minimálně deletovaná oblast 11q- zahrnuje gen *ATM* (lokus 11q22.3), není překryv mezi delecí 11q a mutací *ATM* velký: jen asi jedna třetina pacientů s 11q- nese mutaci na druhé alele. Naopak existují i pacienti s CLL bez této delece nesoucí mutaci *ATM* na jedné, nebo obou alelách (Austen et al., 2007);

(b) gen *ATM* je rozsáhlý (62 kódujících exonů) a vykazuje nepřítomnost preferenčních míst (*hot-spot* oblastí) pro vznik mutací.

Zaměřili jsme se tedy na podrobnou analýzu genu *ATM* s využitím těchto metodik:

1) I-FISH pro detekci chromozomálních delecí 11q;
2) resekvenační čip platformy Affymetrix pro identifikaci bodových záměn a krátkých delecí;
3) Western blotting proteinu ATM pro stanovení jeho celkové hladiny;
4) funkční testování dráhy ATM→p53 založené na sledování indukce genu *CDKN1A* (*p21*) po *in vitro* ošetření CLL buněk fludarabinem resp. doxorubicinem - v případě mutace *ATM* jsme předpokládali, že dojde k indukci *p21* po fludarabinu (vyvolává několik typů poškození DNA), nikoli však po doxorubicinu (vyvolává převážně DNA DSBs a měl by tedy vést k ATM-závislé odpovědi);
5) funkční testování ATM po ošetření CLL buněk γ-zářením se sledováním aktivace proteinu ATM (fosforylace na Ser1981) a indukce cílových genů proteinu p53 (*p21*, *PUMA*, *BAX* a *GADD45*);

6) přímé sekvenování vybraných exonů s mutacemi identifikovanými resekvenačním čipem, nebo celého genu *ATM* v případě dysfunkce v testu či nulové hladiny proteinu ATM na western blotu.

Jako stěžejní pro úspěch naší práce se ukázala skutečnost, že spolehlivě fungoval námi navržený funkční test, a to s vysokou specificitou a citlivostí. Komplexní práci na téma identifikace *ATM* mutací u pacientů s CLL jsme publikovali v časopise *Haematologica* (Navrkalova et al., 2013a) (**příloha 10**). Náš test nám umožnil (a) potvrdit funkční dopad mutací identifikovaných resekvenačním čipem a (b) identifikovat i několik dalších pacientů, u kterých resekvenační čip mutaci nenašel. Rovněž analýza proteinové hladiny pomocí western blotu se ukázala jako užitečná: takto jsme například potvrdili, že dysfukce v testu u pacienta SM spadala skutečně na vrub nefunkčnímu (tedy v tomto případě nepřítomnému) proteinu ATM. Žádnou mutaci v genu *ATM* jsme přitom u tohoto pacienta nenašli v několika následných odběrech. Přesnější molekulární účinky fludarabinu a doxorubicinu při indukci proteinu ATM jsme pak popsali v navazující technické práci (Navrkalova et al., 2013b) (**příloha 11**). Celkově naše studie doplnila existující portfolio funkčních testů pro predikci mutací v genu *ATM*, přičemž nespornou výhodou našeho testu oproti některým jiným přístupům bylo vyhnutí se ošetření CLL buněk γ-zářením (Carter et al., 2004; Johnson et al., 2009; Lin et al., 2012; te Raa et al., 2013; te Raa et al., 2014; te Raa et al., 2015a).

V roce 2012 se pak na nás obrátila výzkumná skupina z *Acamedic Medical Center Amsterdam* (Dr. Eric Eldering, Dr. Arnon Kater) s tím, že po poškození DNA pozorují u vzorků CLL s mutacemi v genu *SF3B1* (sestřihový faktor 3 podjednotka 1) podobnou dysfukci dráhy p53 jako u vzorků s mutacemi *ATM*. Tím byl dán základ naší zhruba dvouleté spolupráce, která byla úspěšně završena spoluautorskou publikací v časopise *Leukemia* (te Raa et al., 2015b) (**příloha 12**). Tato práce prostřednictvím několika paralelních experimentů prokázala, že u vzorků se samotnou mutací v genu *SF3B1* (bez doprovodných defektů v genech *ATM* či *TP53*) je skutečně narušena odpověď buňky na poškození DNA, a to zejména z hlediska snížené indukce genů regulovaných po poškození DNA proteinem p53. Protože klíčové experimenty ve výše zmíněné publikaci byly prováděny našimi zahraničními kolegy, ověřovali jsme posléze v naší laboratoři rozsah popsané dysfunkce. Prostřednictvím experimentu založeného na stejném designu jako ve zmíněné

publikaci (ošetření CLL buněk γ-zářením se sledováním indukce p53-závislých genů) jsme pozorovanou dysfunkci povrdili, a navíc jsme ukázali, že je nejvýraznější v případě genu *GADD45* (*growth arrest and DNA damage*). Ve výzkumu dopadu mutací vgenu *SF3B1* u pacientů s CLL v současné době intenzivně pokračujeme.

Poslední částí našeho výzkumu mutací v genu *ATM* je pak analýza jejich výskytu u pacientů s CLL spadajících do tzv. stereotypních subsetů IGHV (viz Úvod). Zde jsme byli požádáni koordinátorem výzkumných aktivit v této oblasti (Dr. Richard Rosenquist, University of Uppsala), abychom daný projekt vedli vzhledem k našim dosavadním zkušenostem s genem *ATM* u pacientů s CLL. Výsledkem práce je mezinárodní manuskript, který byl recentně přijat k publikaci v časopise *Haematologica* (Navrkalova et al., 2016) (**příloha 13**). Hlavní výstupy práce jsou následující:

a) Ze všech velkých stereotypních subsetů IGHV (#1-8) je výskyt *ATM* mutací nejvyšší v subsetu #2 (26 %). U tohoto klinicky agresivního subsetu byla již dříve prokázána vysoká frekvence mutací v genu *SF3B1*, a naopak spíše ojedinělý výskyt abnormalit v genu *TP53* (obě skutečnosti potvrdila i tato studie).
b) V subsetu #2 je bialelická inaktivace *ATM* (tedy dysfunkce proteinu ATM) spojena s přítomností extrémně krátkých telomér. To naznačuje jednak vysokou proliferaci CLL buněk, a také vysoké riziko vzniku chromozomálních aberací, které jsou s extrémně krátkými telomérami (tzv. *telomerecrisis*) obecně spojeny.
c) Aberace *ATM* (11q-, monoalelická mutace *ATM* i bialelický defekt) jsou spojeny se zkráceným přežitím pacientů s CLL ze subsetu #2.

Celkově naše práce vůbec poprvé asociuje výskyt *ATM* mutací s výskytem konkrétních stereotypních motivů IGHV a potvrzuje tak dosavadní náhled, že patogeneze CLL v sobě zahrnuje delikátní „kooperaci“ mezi signalizací z BCR a přítomností specifických genových defektů (Marincevic et al., 2010; Strefford et al., 2013).

ATM mutations uniformly lead to ATM dysfunction in chronic lymphocytic leukemia: application of functional test using doxorubicin

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ABSTRACT

ATM abnormalities are frequent in chronic lymphocytic leukemia and represent an important prognostic factor. Sole 11q deletion does not result in ATM inactivation by contrast to biallelic defects involving mutations. Therefore, the analysis of *ATM* mutations and their functional impact is crucial. In this study, we analyzed *ATM* mutations in predominantly high-risk patients using: i) resequencing microarray and direct sequencing; ii) Western blot for total ATM level; iii) functional test based on *p21* gene induction after parallel treatment of leukemic cells with fludarabine and doxorubicin. ATM dysfunction leads to impaired *p21* induction after doxorubicin exposure. We detected *ATM* mutation in 16% (22 of 140) of patients, and all mutated samples manifested demonstrable ATM defect (impaired *p21* upregulation after doxorubicin and/or null protein level). Loss of ATM function in mutated samples was also evidenced through defective p53 pathway activation after ionizing radiation exposure. *ATM* mutation frequency was 34% in patients with 11q deletion, 4% in the *TP53*-defected group, and 8% in wild-type patients. Our functional test, convenient for routine use, showed high sensitivity (80%) and specificity (97%) for *ATM* mutations prediction. Only cells with *ATM* mutation, but not those with sole 11q deletion, were resistant to doxorubicin. As far as fludarabine is concerned, this difference was not observed. Interestingly, patients from both these groups experienced nearly identical time to first treatment. In conclusion, *ATM* mutations either alone or in combination with 11q deletion uniformly led to demonstrable ATM dysfunction in patients with chronic lymphocytic leukemia and mutation presence can be predicted by the functional test using doxorubicin.

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by a poor curability and distinctively variable clinical course.[1] The mutation status of the immunoglobulin heavy-chain variable region (*IGHV*)[2] and the presence of recurrent cytogenetic aberrations[3] represent two major prognostic factors with clear clinical impact. The worst prognosis is associated with the occurrence of 17p deletion (17p-) and/or *TP53* mutation.[3–5] The heterozygous deletion at the locus 11q22-23 (11q-) affecting the Ataxia Telangiectasia-Mutated (*ATM*) gene may also significantly contribute to inferior patient outcome, especially in younger patients.[3,6,7] The pathogenic role of the *ATM* gene has been unambiguously proven by demonstrating the presence of somatic *ATM* mutations in a proportion of patients.[8–10] *ATM* abnormalities (deletions and mutations) are typically associated with extensive lymphadenopathy in CLL patients[11] and have recently been identified as the most common negative genetic defect at CLL diagnosis.[12] Nevertheless, the functional impact of *ATM* defects and their relevance in CLL still remains controversial[13–15] and further studies are needed.

Patients with both *ATM* alleles affected (deletion and mutation or two mutations) lack ATM activity, while patients with monoallelic mutation may have preserved ATM function.[16,17] Therefore, it is important to monitor status of both alleles, because *ATM* mutations only overlap poorly with 11q-.[12,17] In addition, various types of *ATM* mutations may have a different impact on the resulting ATM activity. In autosomal recessive disorder Ataxia-Telangiectasia (AT), characterized by progressive neurodegeneration, immunodeficiency, and predisposition to lymphoid malignancies,[18] the clinical heterogeneity can be attributed to different types of inherited *ATM* mutations. While truncating mutations are associated with full AT phenotype, splicing and missense mutations lead to milder clinical appearance due to partially preserved ATM expression and function.[19,20]

ATM plays a pivotal role in the DNA-damage response (DDR) pathway after DNA double-strand break (DSBs) recognition by Mre11/Rad50/Nbs1 (MRN) complex[21] through phosphorylation of many different targets, including p53 protein.[22] In CLL, Stankovic *et al.*[23] demonstrated that transcriptional response to DSBs is entirely dependent on ATM, where

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only part of this activity is transferred to the p53 pathway, leading to pro-apoptotic signaling. Monitoring of ATM function may be a feasible tool for disclosing *ATM* mutations. Several slightly modified tests have been suggested, based on monitoring p53 and p21 accumulation after cell exposure to ionizing radiation (IR).[17,24-26] An alternative approach utilizing etoposide and nutlin-3a was also used which enabled efficient differentiation of *TP53* and *ATM* defects.[27]

Despite undeniable progress, *ATM* mutation identification in CLL remains challenging due to: i) an extreme gene size (62 coding exons) with lack of well-characterized (hot-spot) mutations; ii) the difficult interpretation of polymorphisms and pathogenic mutations resulting from only vague information about their functional consequences. Therefore, relevant information is lacking about the clinical impact of ATM mutations, including the response of affected patients to chemoimmunotherapy.

In our CLL patient cohort, we found that all *ATM* defects involving mutation(s) resulted in disruption of ATM activity towards p53 pathway activation. In addition, we present a novel functional test based on monitoring *p21* induction after parallel treatment of CLL cells with doxorubicin and fludarabine that have different DNA damage mechanisms. This test proved to be an effective means to search for *ATM* mutations, which had been selected in a dominant proportion of leukemic cells.

Design and Methods

Patients' samples

Peripheral blood mononuclear cells (PBMNC) of 140 CLL patients were processed after obtaining informed consent in accordance with the Declaration of Helsinki under protocols of the University Hospital Brno (Ethics Committee approval NS10439-3). The proportion of leukemic cells ($CD5^+/CD19^+$) exceeded 80% in all samples. Basic characteristics of the cohort are summarized in Table 1. The cohort consisted of predominantly high-risk CLL patients (harboring *TP53* defect and/or 11q- and/or unmutated IGHV), and 45% of patients were treated with various regimens before *ATM* mutation analysis (*Online Supplementary Table S1*).

ATM mutation analysis

Custom resequencing microarray (Affymetrix, CA, USA) was used to detect 1-nt substitutions in all 62 coding exons and splicing sites of the *ATM* gene. The resequencing procedure was carried out according to the manufacturer's protocol (Affymetrix GeneChip Custom Resequencing Array Protocol). The resequencing principle is based on allele-specific hybridization. The hybridization of fluorescently labeled DNA fragments to particular positions determined the nucleotides in sequence with the ability to distinguish between homozygous and heterozygous state. Final sequence data was acquired using The GeneChip Sequence Analysis Software (GSEQ) processing fluorescence intensity files.

Direct Sanger sequencing (3130xl Genetic Analyzer, Applied Biosystems) was used to: i) confirm *ATM* alterations detected by microarray analysis; ii) identify other mutations indicated by functional test and/or Western blot (WB) through screening of all 62 coding exons and splicing sites. A search was made for all identified sequence variations in appropriate databases of single nucleotide polymorphisms (SNPs) and mutations.

Variations detected by the resequencing array but not confirmed by Sanger sequencing were evaluated by next-generation deep sequencing technology (GS Junior, Roche) to distinguish subclonal *ATM* alterations occurring under direct sequencing detection limit from the array false positivity.

Western blotting

Protein level analysis was performed using the following antibodies (Cell Signaling Technology): anti-ATM (mAb D2E2), anti-ATM-Ser1981 (mAb D6H9), anti-p53-Ser15 (mAb 16G8), and anti-β-actin (mAb 13E5). Total p53 level was detected by DO-1 mAb (a gift from Dr. Vojtesek, MMCI, Brno). For ATM analysis PBMNC were lysed in RIPA buffer and protein lysates (50 ug for ATM-Ser1981, 100 μg for total ATM) were run on NuPAGE Novex 3-8 % Tris-Acetate Gel (Invitrogen).

Induction of p53-downstream target gene expression after DNA damage

Primary CLL cells were subjected to IR (5 Gy in total, 0.3 Gy/min) or treated with fludarabine (Bayer-Schering, 3.6 μg/mL) or doxorubicin (Teva, 0.25 μg/mL). Cells were seeded in 6-well plates (2.5×10^7 cells per well, volume 5 mL) and harvested after 2, 10 and 24 h in case of IR or after 24 h drug exposure. Real-time polymerase chain reaction (PCR) was performed using TaqMan technology and 7300 Real-Time PCR System (Applied Biosystems). Primer and probe set was specific for the *CDKN1A* (*p21*), *BBC3 (PUMA)*, *BAX*, and *GADD45* genes (Applied Biosystems).

Cell viability after doxorubicin and fludarabine administration

CLL cells were seeded in quadruplicates using 96-well plates (5×10^5 cells per well) and treated for 48 h with chemotherapeutics at four different concentrations. The viability was assessed by the metabolic WST-1 assay (Roche) using spectrophotometer 1420 Multilabel Counter Victor (PerkinElmer).

Table 1. Clinical and biological characteristics of CLL patients.

Patients (n)	Cohort I (107)	Cohort II (33)
Age at diagnosis: median, range (years)	61 (34-79)	62 (38-81)
Sex (M/F)	65/42	24/9
Clinical stage* (%)		
Low risk: Rai 0	20	30
Intermediate risk: Rai I, II	36	30
High risk: Rai III, IV	44	40
Therapy status* (%)		
Untreated	51	67
Treated	49	33
IGHV status (%)		
Unmutated	83	75
Mutated	17	25
Hierarchical I-FISH* (%)		
17p-	21	9
11q-	47	24
+12	3	12
13q- sole	17	43
Normal	12	12
TP53 defect (%)[#]	44	12

*Cohort I: patients investigated by resequencing array, functional test and WB; Cohort II: patients investigated only by functional test and WB. *Assessed at the time of ATM mutation examination. #Mutation and/or deletion.*

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Results

ATM mutations identification by resequencing microarray

Chronic lymphocytic leukemia samples from 107 patients were investigated for *ATM* mutations using the resequencing array as a pre-screening method. This cohort (Cohort I) was intentionally selected and biased towards high-risk CLL patients (Table 1). In total, 46 different *ATM* alterations were identified in a variable number of patients, and 29 of these variants were readily confirmed by direct Sanger sequencing. Next-generation deep sequencing applied to unconfirmed variants revealed only two real substitutions that were present in 16% and 10% of molecules, which is under the direct sequencing detection limit. The rest represented array false positivity (*Online Supplementary Table S2*).

In total, only 29 properly selected and clearly confirmed alterations were further considered as real *ATM* substitutions. Among them 13 represented different known SNPs, and their frequencies in our cohort are listed in the *Online Supplementary Table S3*. The remaining 16 alterations detected in 13 patients (P1-P13 in Table 2) included two nonsense mutations, one known splicing mutation[28] leading to null expression of normal transcript (*data not shown*), and missense mutations.

With regards to allele composition, 10 of 13 mutated patients (77%) manifested 11q- in addition to *ATM* mutation. Another patient (P2) having homozygous mutation and no 11q- demonstrated uniparental disomy of 11q arm (detected by CytoScan HD array, Affymetrix) (*data not shown*). Two remaining patients exhibited heterozygous mutations, and we searched for a second potential mutation by direct sequencing. Such a mutation was disclosed in patient P12, while the last patient (P13) harboring a well known pathogenic mutation Arg3008Cys in heterozygous state did not exhibit another mutation. This mutation may, therefore, have a predicted dominant-negative effect (DNE) on wt allele.[18] Interestingly, patient P12 with two mutations recorded during the original investigation manifested only mutation Arg3008His in a sample obtained 12 months later. This further supports the notion that alterations in this mutable codon are connected with DNE.

Table 2. *ATM* mutations identified in CLL patients.

Patient	*ATM* gene mutation					ATM analysis		11q- (%)
	Nucleotide change	Amino acid change	Type	Origin	Reported previously	ATM level	ATM function	
P1	c.875C>G c.7375C>T	p.Pro292Arg p.Arg2459Cys	ms ms	ND ND	no no	null	norm	97
P2	c.902-1G>T	p.Ala302fs	spl	ND	AT	null	def	no[1]
P3	c.2950C>G c.4724G>A c.6820G>A	p.Gln984Glu p.Arg1575His p.Ala2274Thr	ms ms ms	som germ germ	no no CLL	null	def	94
P4	c.3075T>G	p.Phe1025Leu	ms	som	no	pos	def	93
P5	c.3250C>T	p.Gln1084*	ns	ND	no	pos	norm	99
P6	c.5789A>T	p.Asp1930Val	ms	som	no	null	norm	88
P7	c.6101G>C	p.Arg2034Pro	ms	som	no	null	NA	95
P8	c.6559G>T	p.Glu2187*	ns	som	no	null	def	96
P9	c.7280T>C	p.Leu2427Pro	ms	ND	no	pos	def	94
P10	c.7463G>A	p.Cys2488Tyr	ms	germ	no	pos	def	90
P11	c.8194T>G	p.Phe2732Val	ms	som	no	pos	def	98
P12#	c.8668C>G c.9023G>A	p.Leu2890Val p.Arg3008His	ms ms	som som	MCL, PLL, CLL CLL, MCL, DLBCL	pos	def	no
P13	c.9022C>T	p.Arg3008Cys	ms	som	CLL, MCL, PLL	pos	def	no
P14	c.3_21del c.2329_2332del	p.Met1? p.Arg777fs	fs fs	som som	no no	pos	def	55
P15	c.877A>T	p.Lys293*	ms	som	no	null	def	92
P16	c.1402_1403del	p.Lys468fs	fs	som	AT, CLL	null	norm	91
P17	c.3802del c.5748_5750del	p.Val1268* p.Met1916delinsIle	ns if	ND ND	AT no	null	NA	no
P18	c.7996A>G	p.Thr2666Ala	ms	som	LC	null	def	91
P19	c.5044G>C	p.Asp1682His	ms	som	PLL	pos	def	93
P20	c.6185C>T	p.Ala2062Val	ms	som	no	pos	def	87
P21	c.7089+1del	p.Asn2326_Lys2363del	spl	som	no	pos	def	98
P22	c.7515+1_2del	p.Arg2506_Asn2543del	spl	som	no	null	def	95

Cohort I: patients P1-P18. Cohort II: patients P19-P22. Mutations in P1-P13 were detected by microarray; ms: missense; spl: splicing (skipping of exon confirmed by cDNA analysis); ns: nonsense; fs: frameshift; if: in-frame; ND: not determined; som: somatic; germ: germline; AT: Ataxia Telangiectasia; MCL: mantle cell lymphoma; PLL: prolymphocytic leukemia; DLBCL: diffuse large B-cell lymphoma; LC: lung cancer; norm: normal; def: defective; NA: not applicable due to presence of TP53 defect; pos: positive. #Only mutation Arg3008His was detected 12 months since the original investigation (mutation Leu2890Val disappeared), 1 uniparental disomy of 11q.

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Functional test and Western blot reveal ATM protein inactivation in mutated patients

In order to directly assess overall ATM status in affected patients, samples from all 13 patients (P1-P13) with array-identified mutation were subjected to the ATM protein functional analysis and to ATM protein level assessment.

We designed the functional test based on parallel treatment of CLL cells with doxorubicin and fludarabine followed by *CDKN1A* (*p21*) gene expression monitoring. The impaired *p21* induction as an ATM dysfunction marker was selected on the basis of its demonstrated ATM-dependent upregulation following DSBs after IR.[25] We presumed that: i) doxorubicin as a radiomimetic drug has a similar effect to IR; ii) mutations leading to ATM protein dysfunction should impair *p21* induction during response to DSBs imposed by doxorubicin,[29] while *p21* activation should be preserved after fludarabine, creating a broader spectrum of DNA (and also RNA) damage;[30] iii) *TP53* defects should result in reduced *p21* induction after exposure to both drugs. The proposed principle of our functional test was proven by testing 3 TP53-wt/ATM-wt samples with and without the presence of ATM-specific inhibitor KU55933[31] (Figure 1).

All samples with an array-identified mutation excepting one (n=12) showed ATM dysfunction using the described functional test and/or manifested null ATM protein level on Western blot (WB) (Table 2 and *Online Supplementary Figure S1*); the last sample (P5) harboring nonsense mutation and 11q- exhibited no defect in these tests due to incomplete mutation selection (*data not shown*). Therefore, all patients with ATM mutation identified by resequencing microarray (which is neutral regarding identified mutation function) can be considered as having disturbed ATM activity. Furthermore, in 2 samples with detectable ATM level (P9 and P13), we performed additional Western blots analyzing ATM function. ATM-Ser1981 autophosphorylation was obviously diminished in one sample, while p53-Ser15 phosphorylation and p53 accumulation was completely lost in both samples, confirming the elimination of ATM activity towards p53 pathway activation (*Online Supplementary Figure S2*).

By contrast, all tested samples with only *ATM* polymorphism and no mutation (11 of 13 detected polymorphisms were tested) (*Online Supplementary Table S3*) displayed normal profile in the functional test.

Functional test and Western blot disclosed other patients with ATM mutation

In order to identify other potential causal mutations undetectable by resequencing microarray (nonmissense mutations), we employed the functional test and WB analysis on samples from 64 patients with no detected mutation and with material available. Either one or both of these tests indicated a mutation presence in 7 patients (11%), and in 5 cases the mutation(s) were indeed found by direct sequencing. In 2 patients (P14, P17) there were two short deletions, and another (P16) harbored one short deletion accompanied by 11q-. Two other patients (P15, P18) manifested nonsense and missense mutation that was not recognized by array, again together with 11q-. The situation of the last 2 patients remains unclear as no

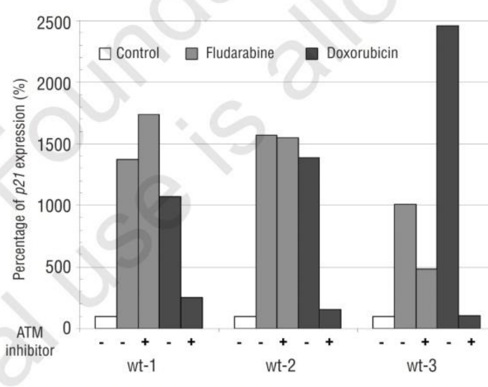

Figure 1. The *p21* expression induction after ATM inhibition. CLL cells were pre-treated with ATM inhibitor (KU55933; 10 μM) for 1 h and exposed to fludarabine (3.6 μg/mL) or doxorubicin (0.25 μg/mL) for 24 h. The level of *p21* expression was determined by real-time PCR in comparison with untreated control set to 100%. Sample's C_t values were subjected to $2^{-\Delta\Delta Ct}$ analysis.

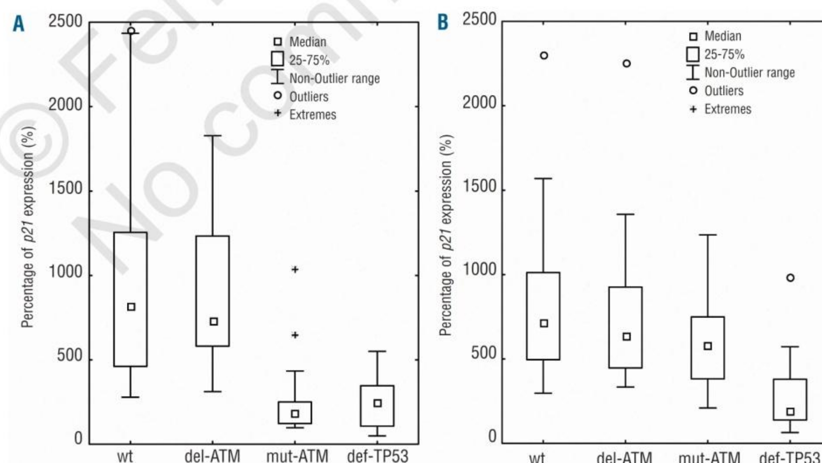

Figure 2. The *p21* expression induction in individual genetic groups. Medians of *p21* expression and the significance (Kruskal-Wallis ANOVA test) related to wt group were following: (A) after doxorubicin treatment – wt: 815%; del-ATM: 731%; non-significant (NS); mut-ATM: 182%, *P*<0.001; def-TP53: 250%, *P*<0.001. (B) after fludarabine treatment – wt: 718%; del-ATM: 639%, NS; mut-ATM: 617%, NS; def-TP53: 190%, *P*<0.001.

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mutation was found. In total, using resequencing microarray, functional test, and WB, we identified 18 patients (17%) in Cohort I having *ATM* mutation confirmed by Sanger sequencing and exhibiting ATM dysfunction.

The additional analysis performed on 33 randomly selected samples (Cohort II; Table 1) was carried out to evaluate the efficiency of our functional test. The test indicated *ATM* mutation in 4 patients, and in all cases a mutation was identified by direct sequencing (P19-P22) (Table 2). The final cut-off value for ATM dysfunction was determined using receiver operation characteristic analysis (*Online Supplementary Figure S3*) applied on selected samples with known *ATM* mutation status (omitting the TP53-defective group). The *p21* expression induction of less than 300% in comparison with untreated control (100%) after doxorubicin treatment defined dysfunctional cases. The functional test showed 80% sensitivity and 97% specificity in this setting. Using a dilution series of wt cells and *ATM* mutated cells, we determined that this test is able to detect mutation, if present in at least 80% of cells (*Online Supplementary Figure S4*); our test is, therefore, suitable for detection of properly selected *ATM* mutations.

Based on our retrospective analysis, the sample should be screened for *TP53* defect if *p21* induction does not exceed 400% after exposure to each drug.

ATM mutations are unevenly distributed in genetic groups and frequently occur upon CLL diagnosis

Collectively in Cohorts I and II, we detected *ATM* mutation(s) in 16% (22 of 140) of CLL patients. Mutated patient proportions in groups divided according to high-risk genetic features were as follows: 34% (17 of 50) in patients with 11q-; 4% (2 of 51) in patients having *TP53* mutation and/or deletion 17p; and 8% (3 of 39) in patients without these defects. The association between *ATM* mutation presence and *IGHV* mutation status was not assessed because of predominant 11q- occurrence in IGHV-unmutated samples.

We did not observe any tendency towards *ATM* mutation accumulation in previously treated patients; the frequency of *ATM* mutations was 18% (14 of 77) in untreated and 13% (8 of 63) in treated patients. The retrospective analysis of 9 mutated patients disclosed a mutation presence at the time of diagnosis or up to one year after in 8 cases. Moreover, the *ATM* mutation germline origin was disclosed in 2 (P3, P10) of 17 analyzed patients with available material (buccal swab). These observations all indicate that the genesis of a substantial proportion of *ATM* mutations can be considered primarily as an early event in CLL pathogenesis.

Cells with ATM mutation exhibit impaired overall response to doxorubicin

The strong association between presence of *ATM* mutation and dysfunction status prompted us to compare p53-downstream gene induction and overall cell viability after doxorubicin and fludarabine exposure in the following genetic categories: cells without any *TP53* or *ATM* abnormality ("wt"), with sole 11q- ("del-ATM"), with *ATM* mutation regardless of 11q- presence or absence ("mut-ATM"), and TP53 defect ("def-TP53").

Data concerning *p21* induction after cell exposure to doxorubicin and fludarabine is summarized in Figure 2A and B, respectively. It is evident that mut-ATM (n=20) and def-TP53 (n=30), but not del-ATM (n=23) samples exhibited significantly impaired induction after doxorubicin exposure in comparison with wt cells (n=31). By contrast, after treating the same samples with fludarabine, we observed that mut-ATM samples manifested similar p21 induction compared to wt and del-ATM groups. The def-TP53 samples exhibited diminished induction similarly to doxorubicin exposure.

In addition to impaired p21 induction, ATM mutated samples displayed also identically disturbed induction of pro-apoptotic genes *BBC3 (PUMA)* and *BAX*, and DNA damage response gene *GADD45* after doxorubicin exposure (*Online Supplementary Figure S5*). Similarly to doxorubicin, a series of the samples with different *ATM* mutations also exhibited defective p53-downstream gene induction after IR exposure, which is commonly used for DSBs induction and for ATM function testing. However, the difference in gene expression was obvious only for three out of the four studied genes (i.e. *p21, PUMA, GADD45*) at certain times after radiation (*Online Supplementary Figure S6*).

In agreement with the data regarding p53-downstream gene induction, we observed significantly higher resistance of mut-ATM (n=8) and def-TP53 (n=5) samples to doxorubicin when compared with wt samples (n=5); del-ATM samples (n=9) behaved similarly to the wt group (Figure 3A). The contrasting negligible effect of *ATM* mutations on p53-downstream gene induction elicited by fludarabine was also in compliance with cell viability in the tested groups (Figure 3B). Only def-TP53 (n=13), but neither mut-ATM (n=14) nor del-ATM samples (n=8), showed higher resistance to fludarabine compared with the wt group (n=8).

Patients having ATM mutation and those with sole 11q- have identical time to first treatment

The above data confirm that cells with sole 11q- ("del-ATM") have preserved ATM activity,[17] while defects involving *ATM* mutation ("mut-ATM") in our study exclusively led to ATM dysfunction. We, therefore, focused on a correlation between these defects and the time to first treatment (TTFT). We limited our analysis to patients with unmutated *IGHV* locus (n=41) because of the strong association with ATM defects and to those with ATM abnormality observed at the diagnosis or up to one year after. TTFT medians in the group mut-ATM (n=8), del-ATM (n=15), and wt (n=18) were as follows: 7, 9.5, and 27 months (Figure 4). Both defective groups exhibited significantly reduced TTFT in comparison with wt patients (both *P*=0.04) and did not differ mutually (*P*=0.6).

We also performed a progression-free survival (PFS) analysis after the first therapy consisting of chemoimmunotherapy (76%, 25 of 33 patients) and chemotherapy (24%, 8 of 33 patients) with a similar proportion in individual genetic groups. PFS medians were 9, 16 and 16.5 months in the mut-ATM, del-ATM and wt group, respectively, with no statistical significance among tested groups (*Online Supplementary Figure S7*). Overall survival analysis was not performed due to short median follow up.

Discussion

ATM defects are commonly assessed as 11q- presence, but sole deletion does not mean ATM inactivation if the other allele remains intact.[17,24] Resulting ATM activity

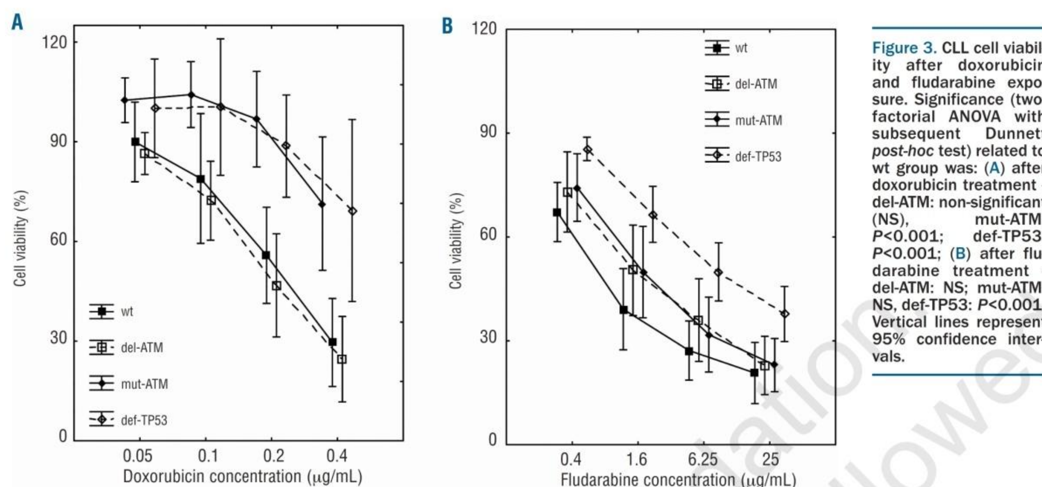

Figure 3. CLL cell viability after doxorubicin and fludarabine exposure. Significance (two-factorial ANOVA with subsequent Dunnett *post-hoc* test) related to wt group was: (A) after doxorubicin treatment - del-ATM: non-significant (NS), mut-ATM: $P<0.001$; def-TP53: $P<0.001$; (B) after fludarabine treatment - del-ATM: NS; mut-ATM: NS, def-TP53: $P<0.001$. Vertical lines represent 95% confidence intervals.

depends on the defect composition (monoallelic *vs.* biallelic) and, additionally, on functional consequences of individual *ATM* mutations, which have a decisive impact according to type and position.[19,20] Thus, it is difficult to properly distinguish patients with clear ATM dysfunction. With the advent of next-generation sequencing technologies that provide a more sensitive test for *ATM* mutation, a function assessment will be crucial to set aside real pathogenic mutations.

We detected *ATM* mutation(s) in 16% (22 of 140) of the patients. Since we analyzed predominantly high-risk CLL patients, this frequency is not representative of CLL cohorts in general. The occurrence of *ATM* mutated patients in individual genetic groups matched previously reported observations. The highest frequency was noted in the 11q- group (34%), which is nearly identical to the 36% presented in the study by Austen *et al.*[17] Mutations were rare in patients with *TP53* defects (4%), confirming mutual exclusivity of p53 and ATM inactivation.[24,25] Interestingly, in 3 patients experiencing progression after therapy (P10, P17, P19), during routine *TP53* examinations we observed an emergence of new mutations accompanying ATM dysfunction. This suggests that *TP53* mutation may provide further advantage to an *ATM* mutated clone under therapy pressure.

Using several complementary methodologies proved to be beneficial for effective ATM status assessment. The functionally neutral resequencing array enabled fast analysis of point mutations, but false positivity reached 30% and false negativity 11%. Also, its inability to detect other mutations is a clear drawback[32] since the proportion of short deletions detected by functional approach was high (25%, 7 of 28 mutations). In our study, all mutated patients exhibited obvious ATM dysfunction, which was predominantly caused by biallelic defects (95%, 21 of 22) but also in one case by sole heterozygous mutation in codon 3008 (P13). In addition, alteration in the same codon was found as the only selected mutation in the second investigation of patient P12, who originally harbored two mutations. Interestingly, both of these samples with sole mutation in codon 3008 showed null p53 downstream gene induction after both IR and doxorubicin, confirming their presumed DNE. Our proposed functional test is, therefore, suitable for common identification of all mutation types that lead to ATM dysfunction. By contrast, none of the SNPs detected in our study disturbed ATM activity, supporting the view they do not contribute to adverse prognosis in CLL patients.[33] Our observations conclusively suggest that complete disruption of ATM function in DDR is prominently selected among high-risk CLL patients.

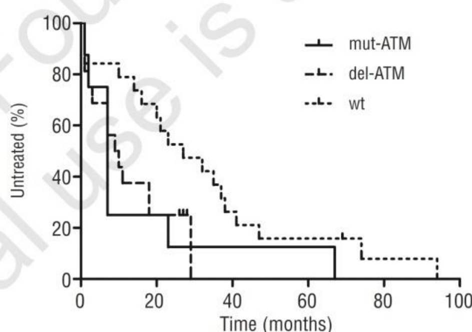

Figure 4. Time to first treatment analysis limited to patients with unmutated *IGHV*. Mut-ATM and del-ATM groups related to wt patients: both $P=0.04$. Kaplan-Meier curves were created and statistically evaluated using the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

ATM mutation occurrence during the disease course is an important issue to clarify. ATM activation in DDR is a critical defensive mechanism already in early cancerogenesis when genomic instability begins.[34] The recurrent presence of 11q- in patients with monoclonal B-cell lymphocytosis[35] can indicate a similar ATM role in early CLL development. *ATM* mutations may already be present in germline[8,10] or in hematopoietic precursor cells,[24] and may contribute to rapid disease progression through loss of the

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other allele.[36] At the same time, *ATM* mutations have been observed as clonal variants contributing further to the 11q- subclone expansion.[17] The considerable proportion of *ATM* mutations in our study can be regarded as an early event in the CLL course, which is supported by their frequent presence before first therapy, at the time of diagnosis or even in germline form. Nonetheless, Landau *et al.*[37] recently reported that *ATM* mutations also frequently occur in subclonal form, contributing later to disease progression.

The defects in ATM-p53-p21 pathway integrating response to DNA damage have shown independent prognostic value in CLL,[38] and effective functional testing of this pathway is, therefore, desirable. Initially, two basic defect types after IR exposure were defined as "type A", which associates with *TP53* mutation, and "type B" connected with *ATM* mutation.[25] Later, "type C" that was correlated with polymorphism in *p21* gene[39] and "type D", having unclear association with any gene defect[38] were described. The functional test based on IR may not, however, be convenient for all laboratories, and use of radiomimetic drugs seems to be a reasonable alternative approach.[27] In our study, we show that CLL cells with *ATM* mutation(s) have an analogously poor response to IR and doxorubicin; both agents should primarily create DNA DSBs.[25,40] Our functional test using doxorubicin and fludarabine in parallel enables effective identification of *ATM* mutations (sensitivity 80%, specificity 97%) and distinguishing between *ATM* and *TP53* defects based on different DNA damaging mechanisms of these drugs (*data not shown*). In our study, this test failed in several cases for unknown reasons. We also assume that samples without *ATM* mutation that were approaching the cut-off value could potentially harbor other defect types in the ATM-p53-p21 pathway, e.g. "type C" defect.

ATM dysfunction might play a role in patient response to the conventionally used DNA damaging drugs. In this respect, our data suggest that doxorubicin, which is included in CHOP and R-CHOP regimens employed in lymphoproliferative disorders,[41] is most probably ineffective in *ATM* mutated patients. Notably, low ATM expression level was associated with resistance to doxorubicin in breast cancer patients.[42] With regards to fludarabine, the situation remains to some extent more elusive. It was reported that ATM deficiency leads to impaired ATM-mediated phosphorylation[17] and higher *in vitro* resistance of CLL cells to this drug.[43] Despite recorded observations, we have demonstrated that cells with obvious ATM dysfunction had preserved response to fludarabine; i.e. normal p53-downstream gene induction and similar *in vitro* sensitivity as wt and 11q- cells. We propose that overall response to fludarabine could be mediated through other proteins involved in DDR in ATM-deficient patients, although our findings are not conclusive. Thus, primary response to fludarabine seems to be less influenced by ATM inactivation than generally anticipated.

Although patients with sole 11q- have preserved ATM function,[13,17] it has been reported that this defect is associated with reduced TTFT[44] and distinctive gene expression profile.[12] Our analysis recorded almost identically reduced TTFT in groups with sole 11q- and *ATM* mutation in comparison with wt patients. A potential explanation for the observed clinical impact of sole 11q- may possibly result from a gene dosage effect of *ATM*[12,45,46] or other genes located in deleted region at 11q,[13] or from mutations affecting genes like *BIRC3* located at 11q. This gene could be an interesting candidate because its mutations are recurrent in CLL and have been recognized as mutually exclusive with *TP53* defects,[47] similarly to *ATM* mutations.[25]

In conclusion, *ATM* defects involving mutation uniformly resulted in obvious ATM dysfunction throughout our study. By contrast, sole 11q- does not affect ATM function, and simultaneous *ATM* mutation analysis is, therefore, warranted. Currently, prediction of mutation presence is feasible through functional testing using IR or based on our study using doxorubicin, and this will be critical for distinguishing CLL patients with unambiguously inactive ATM. Indeed, this has potential predictive value and may also offer novel therapeutic strategies utilizing the synthetic lethality concept based on DDR inhibitor application in ATM-defective patients.[48] Furthermore, knowledge of *ATM* mutations will be important to delineate their association with mutations in newly described CLL-related genes, e.g. *SF3B1*.[49]

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The p53 pathway induction is not primarily dependent on Ataxia Telangiectasia Mutated (ATM) gene activity after fludarabine treatment in chronic lymphocytic leukemia cells

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Abstract

The prognostic role of *ATM* defects is well documented in chronic lymphocytic leukemia. However, the predictive value of ATM inactivation is much less understood, even in response to common drugs like fludarabine. It has been demonstrated that CLL cells having inactive ATM exhibit defective phosphorylation of its downstream targets after fludarabine treatment. We performed alternative analysis focusing on fludarabine-induced p53 accumulation and induction of p53-downstream genes after artificial ATM inhibition and, in parallel, using cells with endogenous ATM inactivation. We show that after 24h fludarabine exposure: (i) 5 out of 8 ATM-deficient samples (63%) normally accumulated p53 protein, and (ii) all analyzed ATM-deficient samples (n = 7) manifested clear induction of *p21*, *PUMA*, *BAX*, and *GADD45* genes. In all experiments, doxorubicin was used as a confined ATM inductor and confirmed effective ATM inactivation. In conclusion, CLL cells lacking functional ATM appear to have normal response to fludarabine regarding the p53 pathway activation.

Keywords: ATM, fludarabine, DSBs, p53 pathway, CLL

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Introduction

Biallelic inactivation of the Ataxia Telangiectasia-Mutated (*ATM)* gene results in development of the autosomal recessive disorder Ataxia telangiectasia characterized by neurodegeneration, immunodeficiency and predisposition to lymphoid malignancies [1]. *ATM* codes for a kinase, which is crucial in signaling after recognition of DNA double-strand breaks (DSBs) by Mre11/Rad50/Nbs1 (MRN) complex [2]. This response leads towards the activation of many targets including p53 protein [3], which induces transcription of cell cycle regulatory, proapoptotic and DNA repair genes.

In chronic lymphocytic leukemia (CLL), *ATM* gene defects represent one of the most frequent negative genetic abnormalities during the disease course. Both heterozygous 11q22-23 deletion (11q-) and somatic *ATM* mutations lead to inferior patient outcome and poor prognosis [4]. Cells with sole 11q-, however, manifest preserved ATM function, while biallelic defects abrogate ATM function [5]. Overall ATM activity influences DNA damage response (DDR) to DSBs originating spontaneously or as a consequence of DNA-damaging chemotherapeutic drugs. It was observed that *ATM* mutations are associated with poor response to these drugs [5,6] and, specifically, with reduced progression-free and overall survival after fludarabine treatment [4].

The dependence of fludarabine-induced response on ATM activity is ambiguous in CLL cells. Austen *et al.* [5] demonstrated that fludarabine causes DSBs, and cells with biallelic *ATM* defects have reduced or absent ATM autophosphorylation and p53 Ser15 phosphorylation when treated with this drug. The low ATM level was associated with reduced apoptosis after 24h fludarabine exposure, but after 72h this effect disappeared, indicating early ATM involvement in this response [7]. Altogether this suggests that ATM participates in the primary response to fludarabine. On the other hand, fludarabine has multiple action mechanisms including inhibition of enzymes participating in DNA synthesis, and incorporation into RNA resulting in transcription failure [8]. Fludarabine can therefore lead to various kinds of DNA damage that are processed by signaling with other kinases in addition to ATM.

In our study, we show that ATM inactivation does not abolish early *in vitro* response of CLL cells to fludarabine. Both p53 accumulation and activation of p53-downstream target gene expression is preserved on ATM-deficient background.

Materials and Methods

CLL patients and sample characterization

The study was performed on peripheral blood samples of CLL patients followed at the Department of Internal Medicine – Hematology and Oncology, University Hospital Brno.

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All samples were processed with written informed consent in accordance with the Declaration of Helsinki.

Peripheral blood mononuclear cells (PBMNC) were isolated using Histopaque-1077 (Sigma) and all samples were vitally frozen and stored in liquid nitrogen. The leukemic cells proportion (CD5+/CD19+cells) was determined by flow cytometry and exceeded 80% in all samples. Cytogenetic characterization was done by interphase fluorescence in situ hybridization (I-FISH) using specific probes (Abbott Vysis, Inc.) for 11q22-23 (*ATM*) and 17p13 (*TP53*) loci. Mutation analysis was performed by direct sequencing of all *ATM* coding exons or *TP53* exons 4-10 in order to select *ATM*-mutated, *TP53*-mutated or *ATM/TP53*-wild-type (wt) samples for assumed experiments.

Assessment of γH2AX and PCNA level after fludarabine treatment

Vitally frozen CLL cells were thawed and cultured for 24 h. Cells were seeded in 6-well plates (6.25×10^6 cells per well, volume 5 ml) and treated with fludarabine (Bayer-Schering, 3.6 μg/ml), doxorubicin (Teva, 0.25 μg/ml) or ionizing radiation (IR) (5 Gy; 0.3 Gy/min) and harvested after 24h drug administration or after 1, 5 and 24h in case of IR. Histone extraction was performed using an EpiQuik Total Histone Extraction Kit (Epigentek). For each sample, 5 μg of histone lysate was subjected to electrophoresis on 15% polyacrylamide gel and transferred to nitrocellulose membrane (0.2 μm pores, Bio-Rad). Equal protein lysate loading was controlled using total histone H2AX. The membrane was blocked with 5% nonfat milk in TBS buffer containing 0.1% Tween (TBS-T) for 1h and then incubated overnight at 4°C with rabbit Phospho-Histone H2AX (Ser139) monoclonal antibody (Cell Signaling Technology).

Proliferating cell nuclear antigen (PCNA) level was analyzed on cells seeded in 6-well plates (2.5×10^7 cells per well, volume 5 ml) and treated with identical fludarabine and doxorubicin concentrations as in the case of γH2AX or left as untreated control. Western blot (WB) was performed using 30 μg of protein lysate and PCNA was detected by incubation with PC10 monoclonal antibody (Millipore, CA) for 2h at room temperature.

Analysis of total p53 and Ser15 phosphorylation level after ATM inhibition

Cells were pre-incubated with ATM-specific inhibitor KU55933 (10 μM, cat# 118500, Merck) for 1h. The same conditions as in the case of PCNA were then used for treatment with fludarabine and doxorubicin. Total p53 level was analyzed by WB using 30 μg of protein lysate and monoclonal antibody DO-1 (gift from Dr. Vojtesek, MMCI Brno). The same lysates were used for Ser15 phosphorylation detection using Phospho-p53 (Ser15) rabbit antibody (Cell Signaling Technology) diluted in TBS-T buffer containing 5% BSA at 4°C overnight.

The p53-downstream gene induction after fludarabine treatment

The cells were processed identically to the treatment before p53 WB, i.e., seeded in 6-well plates (2.5×10^7 cells per well, volume 5 ml), exposed to fludarabine (3.6 μg/ml) or doxorubicin (0.25 μg/ml) and harvested after 24h. Cells were subsequently lysed using TriReagent (Molecular Research Center, Inc.), and total RNA was isolated by the RNeasy Mini Kit with the DNase I digestion (Qiagen). RNA was reverse-transcribed using Superscript II RT (Invitrogen). Real-time PCR assay was performed using primer and probe set specific for the *CDKN1A* (*p21*), *BBC3* (*PUMA*), *BAX,* and *GADD45* genes (TaqMan Gene Expression Assays; Applied Biosystems) and 7300 Real-Time PCR System (Applied Biosystems). *TBP* and *HPRT1* genes were not influenced by fludarabine (data not shown) and their geometric mean of cycle threshold (C_t) values served as an internal standard. The fluorescence emission data were subjected to Sequence Detection Software (version 1.3.1; Applied Biosystems) analysis and sample's C_t values to $2^{-\Delta\Delta Ct}$ analysis in order to evaluate gene expression in relation to untreated control.

Results

Fludarabine creates DSBs and reduces PCNA level

We presumed that response of CLL cells to fludarabine, which causes a wider spectrum of DNA damage [8], is not exactly the same as response to DSBs confined inductors, e.g., IR and doxorubicin.

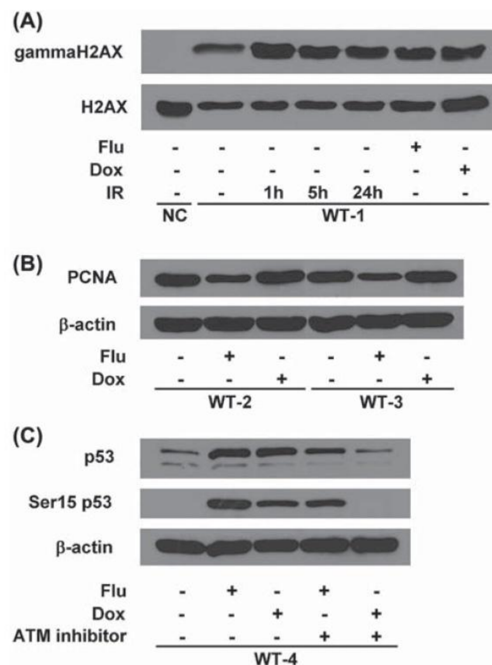

Figure 1. Impact of fludarabine-induced DNA damage on gammaH2AX, PCNA and p53 level in wt CLL cells. (A) Phosphorylated H2AX level after 24h fludarabine treatment in comparison with confined DSBs inductors (IR/1h, 5h, 24h; doxorubicin/24h). NC: negative control (Roche, cat# 10223565001). (B) Different PCNA level after 24h drug treatment. (C) Total p53 and Ser15-phosphorylation level in ATM inhibitor absence or presence after 24h drug exposure.

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We first confirmed previously reported data that fludarabine induces DSBs [5]. Analysis of histone H2AX phosphorylation at Ser139 (γH2AX) was performed on two wt CLL samples. Accumulation of γH2AX after 24h fludarabine treatment in comparison with untreated control was noted in both tested samples and this accumulation was similar to that of confined DSBs inductors as illustrated in Figure 1A.

Nevertheless, a principally distinct DNA damaging effect of fludarabine compared with doxorubicin can be evidenced by different level of PCNA protein, a part of DNA polymerase delta involved in DNA synthesis and repair. The PCNA level was reduced after 24h fludarabine treatment in 68% (21/31) of tested samples, while its level remained stable after 24h doxorubicin exposure in all cases. Two typical samples are illustrated in Figure 1B.

ATM activity is not necessary for p53 accumulation after fludarabine treatment

Since ATM is a critical molecule in signaling after DSBs, four *TP53*-wt/*ATM*-wt samples treated with fludarabine and ATM-specific inhibitor were tested for p53 accumulation after 24h. The p53 protein was readily accumulated without inhibitor in all cases, and ATM blockade did not prevent p53 accumulation in two samples. Phosphorylation on Ser15 accompanied p53 accumulation after fludarabine and inhibitor exposure (Figure 1C). p53 accumulation was absent after doxorubicin treatment in case of ATM inhibition in each sample proving the effective ATM elimination in our setting.

p53 accumulation was also analyzed using four *ATM* mutated samples to consider natural *ATM* inactivation. The p53 stabilization (comparable with wt cells) was observed in three samples and partial stabilization in one case after 24h fludarabine exposure (figure not shown).

Fludarabine induces p53-downstream gene expression irrespective of ATM inactivation

Induction of *p21*, *PUMA*, *BAX*, and *GADD45* genes after fludarabine treatment was explored on the background of ATM inhibition in three *TP53*-wt/*ATM*-wt patient samples. In all of them, gene induction was present in both settings with and without ATM specific inhibitor. Parallel application of doxorubicin was included to verify again presumed ATM inhibition and expression induction was obviously prevented in all studied genes. The gene induction after both drugs is illustrated for one representative wt sample in Figure 2A.

p53-downstream gene analysis was also performed using four *ATM* mutated samples harboring biallelic defect. All these samples had 11q- (range 55-98%, median 96%) accompanied by non-missense mutation leading to presumed ATM elimination. We observed behavior identical to the case of artificial ATM inhibition, i.e., clear induction of p53-downstream genes after fludarabine but not doxorubicin

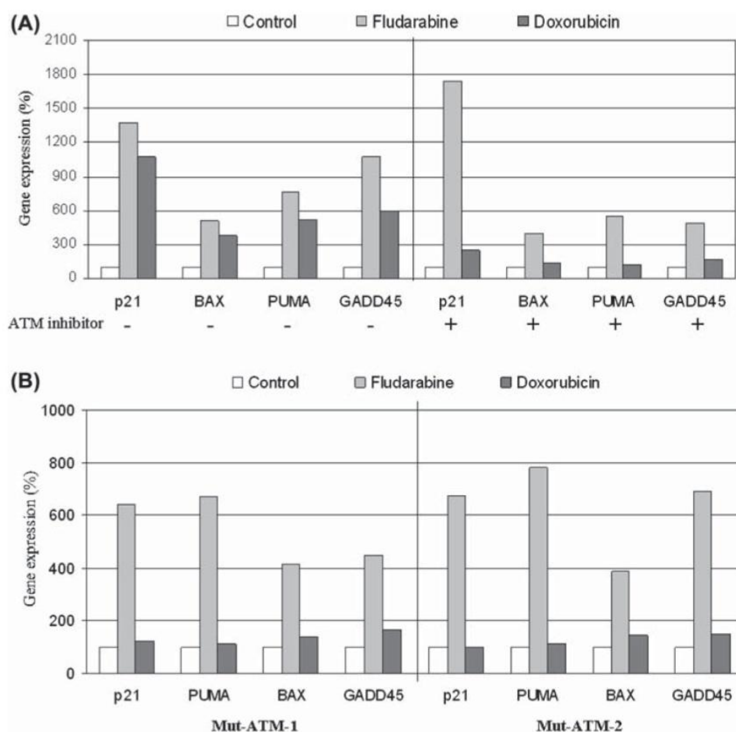

Figure 2. Induction of p53-downstream gene expression on background of ATM inactivation. Cells were treated 24h with fludarabine and doxorubicin. ATM inactivation generated: (A) artificially using ATM-specific inhibitor; (B) through endogenous biallelic *ATM* defect; sample Mut-ATM-1: 11q- (98%) and splicing mutation of exon 50, sample Mut-ATM-2: 11q- (55%) and frame-shift mutation. Gene expression was related to untreated control using $2^{-\Delta\Delta Ct}$ analysis.

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(Figure 2B). Three other control wt samples showed evident induction of the studied genes after both drugs (as in Figure 2A without ATM inhibitor), while three *TP53*-mutated samples were devoid of any induction (data not shown).

Discussion

ATM defects are being considered as common negative prognostic factor in CLL for a long time, but their predictive value for particular therapeutic regimens is still disputed. In terms of response to DNA damaging agents, ATM activity within the ATM-p53-p21 pathway seems to be critical. With fludarabine, it has been distinctly demonstrated that this drug elicits activity of this kinase as evidenced by defective phosphorylation of ATM targets in CLL cells with complete ATM inactivation [5].

In our study, we focused on an alternative analysis of ATM role in the ATM-p53-p21 pathway during *in vitro* response of CLL cells to fludarabine. We show unambiguously that induction of key p53-downstream target genes does not require ATM activity. This was demonstrated using both artificial blocking of ATM protein with specific inhibitor and samples with natural pathogenic *ATM* inactivation. The uniformly preserved induction of *p21*, *PUMA*, *BAX*, and *GADD45* genes following fludarabine administration was in contrast with uniformly diminished gene induction after doxorubicin, which is a known inductor of ATM-directed response to DSBs [9].

Our data concerning p53 accumulation after fludarabine does not show obligatory ATM recruitment in fludarabine response. In relevant proportion of cases with artificially or naturally inactivated ATM (5/8; 63%), fludarabine-induced p53 stabilization was present. The lack of this stabilization in the remaining three samples suggests that ATM could be required in certain circumstances.

Altogether, our observations show subtle ATM impact on studied response to fludarabine. It is therefore apparent that there must be other signaling kinase(s) involved in p53 pathway activation. Since Ataxia telangiectasia and Rad3-related (ATR) kinase is switched off in CLL lymphocytes [10], DNA-dependent protein kinase (DNA-PK) is the most probable candidate. Fludarabine-induced DSBs lead to non-homologous end joining (NHEJ) repair triggered by DNA-PK [11], which subsequently stabilizes p53 by phosphorylation on Ser15 [12]. Basic DNA-PK level and activity varies significantly in CLL cells [13], and the impact of chemotherapeutic drugs on these parameters remains unclear. This variable activity could be the reason for p53 accumulation absence in some cases with ATM inactivation (3/8). In any case, if DNA-PK was the responsible kinase, it would remain unclear why the p53 accumulation is not present after doxorubicin. Alternatively to DNA-PK hypothesis, ATM-independent p53 stabilization can also be executed through phosphorylation on Ser20 mediated by other kinases than ATM and ATR [14].

In CLL, four different defect types in the ATM-p53-p21 pathway were identified through defective response to IR and associated with abnormalities in *TP53*, *ATM*, and *p21* genes [15]. This categorization would be useful if these heterogeneous defects could be associated with chemotherapy response. Our data shows that *in vitro* response to fludarabine is not entirely dependent on ATM pointing out that *TP53* and *ATM* defect cannot be uniformly associated with impaired primary response to fludarabine. The p53 upstream mechanisms involved in response to different kinds of DNA-damaging therapy are decisive and need to be studied further.

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ORIGINAL ARTICLE

The impact of *SF3B1* mutations in CLL on the DNA-damage response

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Mutations or deletions in *TP53* or *ATM* are well-known determinants of poor prognosis in chronic lymphocytic leukemia (CLL), but only account for approximately 40% of chemo-resistant patients. Genome-wide sequencing has uncovered novel mutations in the splicing factor sf3b1, that were in part associated with *ATM* aberrations, suggesting functional synergy. We first performed detailed genetic analyses in a CLL cohort ($n = 110$) containing *ATM*, *SF3B1* and *TP53* gene defects. Next, we applied a newly developed multiplex assay for p53/ATM target gene induction and measured apoptotic responses to DNA damage. Interestingly, *SF3B1* mutated samples without concurrent *ATM* and *TP53* aberrations (sole *SF3B1*) displayed partially defective ATM/p53 transcriptional and apoptotic responses to various DNA-damaging regimens. In contrast, *NOTCH1* or *K/N-RAS* mutated CLL displayed normal responses in p53/ATM target gene induction and apoptosis. In sole *SF3B1* mutated cases, ATM kinase function remained intact, and γH2AX formation, a marker for DNA damage, was increased at baseline and upon irradiation. Our data demonstrate that single mutations in sf3b1 are associated with increased DNA damage and/or an aberrant response to DNA damage. Together, our observations may offer an explanation for the poor prognosis associated with *SF3B1* mutations.

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by an extremely heterogeneous clinical course, ranging from an indolent disease without progression towards overt disease requiring immediate treatment. The variable course of CLL is driven, at least in part, by molecular heterogeneity. The most important genetic defects thus far associated with adverse prognosis and chemo-resistance are aberrations in the pivotal regulators of the DNA-damage response pathway, *ATM* and *TP53*.[1,2] However, in around 60% of the patients, the molecular basis for chemo resistance is not linked to *ATM* or *TP53* defects.[3]

In recent years, a number of novel recurrent somatic mutations in various genes, including but not limited to *SF3B1*, *NOTCH1*, *KRAS* and *NRAS* have been uncovered by genome and exome-wide sequencing studies[4–8] in CLL. Relatively high frequencies for *SF3B1* mutations[6,7,9] (10–15%) and *NOTCH1* mutations[4–7,10] (4–16%) were found, whereas mutations in *K-/N-RAS* occur only occasionally in CLL.[11] Both *SF3B1* and *NOTCH1* mutations correspond to inferior prognosis.[4,6,7,9,10,12] In addition, *SF3B1* and *NOTCH1* were frequently mutated in fludarabine-refractory CLL, which might indicate selection due to chemotherapy.[4,9]

Notch1 and K- and N-ras are well known (proto-) oncogenes,[11,13] whereas *SF3B1* encodes a splicing factor. Interestingly, mutations in *SF3B1* have also been found in other hematological disorders[14–16] and in solid tumors.[17] In addition, mutations in other genes encoding for proteins involved in the splicing machinery were found in hematological malignancies.[6,7,15,18,19] Moreover, alterations in splicing have been related to tumorigenesis.[20–22] This indicates that spliceosomal defects constitute an important and ubiquitous pathway in malignant transformation. Although it is tempting to speculate that mutations in *SF3B1* might lead to aberrant splicing of specific transcripts that affect the pathogenesis of CLL, the underlying mechanism of dysfunction in *SF3B1* mutated patients remains elusive thus far.

SF3B1 mutations often occur together with 11q23 deletions (52–57%),[7,23] the locus for the *ATM* gene. In addition, patients with combined *SF3B1* and *ATM* mutation without 11q deletion were identified.[7] This suggests that *SF3B1* mutations might be functionally synergistic with *ATM* loss. Furthermore, *SF3B1* mutations were initially reported as mutually exclusive with *TP53* disruption,[9] which suggested similarity in biological consequences. Therefore, we hypothesized that *SF3B1* defects may impact on the ATM and/or p53 pathways, either in conjunction with or independently of *ATM* aberrations.

The goal of this study was first to characterize the genetic landscape of *SF3B1* mutations in relation to *ATM* and *TP53* aberrations (mutations and deletions) in CLL. Second, we investigated the functional consequences of *SF3B1* mutations in response to DNA damage, in comparison with other recurrent somatic mutations recently found in CLL. Our data indicate that *SF3B1* mutations in CLL independently lead to defects in the DNA-damage response.

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MATERIALS AND METHODS

Patient and samples

CLL patients ($n = 110$) from four different cohorts were enrolled: (1) 'AMC cohort'; both untreated and treated patients, (2) 'H68 cohort'; treatment-naïve patients with high-risk CLL (defined by 17p deletion, 11q deletion, or trisomy 12, or unmutated IGHV and/or VH3-21) included in the HOVON68 clinical trial,[24] (3) 'd'Accord cohort'; fludarabine-refractory patients included in D'accord clinical trial[25] and (4) 'Brno cohort', enriched for 11q deletion and/or *ATM* mutations. The study was conducted in accordance with the Declaration of Helsinki and informed consent was obtained from all patients. Diagnosis of CLL was according to IWCLL-NCI Working Group criteria. Peripheral blood mononuclear cells were isolated and frozen as earlier described.[26] After thawing, CLL cells were enriched, in case CD19/CD5 purity was below 90%, *via* negative depletion using α-CD3, α-CD14 and α-CD16 (CLB, Amsterdam, The Netherlands) as described previously.[26]

Mutational analyses

Sequence analysis was performed on a set of genes (*TP53, NOTCH1, SF3B1, NRAS, KRAS, MYD88, BRAF, EZH2* and *PIK3CA*) by next-generation sequencing on samples from the 'H68 cohort' using a 454-based platform (Roche Diagnostics Corporation, Indianapolis, IN, USA)[27] or by Sanger sequencing[2] on the remaining cohorts (Supplementary Table 1). Samples containing a sole *SF3B1* mutation were also sequenced for *TP53* (ex2-11), which revealed no additional mutations. *ATM* (ex4-65) was analyzed by either Sanger sequencing or using resequencing microarray and direct sequencing as described previously.[1,28]

P53 and ATM target gene induction analyzed by reverse transcriptase multiplex ligation-dependent probe amplification

CLL cells were treated with or without irradiation (IR) (5Gy), fludarabine or doxorubicin (Sigma-Aldrich, St Louis, MO, USA), in the presence or absence of 10 μM Ku-55933 (ATM inhibitor, Selleckchem, Houston, TX, USA) and cultured for 16 h at 37 °C. For some experiments, CLL cells were co-cultured with CD40L-expressing (3T40) or control 3T3 fibroblasts for 48 h as previously described.[26,29] Reverse transcriptase multiplex ligation-dependent probe amplification (RT-MLPA) (MRC-Holland, Amsterdam, The Netherlands) was performed as described before,[26] using a newly designed RT-MLPA probe set (R016-X2, te Raa *et al*., manuscript in preparation), which included several p53 and ATM target genes (*CD95, Bax, Puma, p21, FDXR, PCNA, NME1, ACSM3, MYC and PYCR1*).[30] Expression of these genes was normalized to a set of housekeeping genes included in the probeset. See also Supplemental Methods. Representative wild type (WT) and *TP53* mutated samples were included as controls in all functional experiments. WT samples were defined as: the absence of *TP53* aberrations, 11q deletion, trisomy 12 and *SF3B1* mutation, and fludarabine sensitive *in vitro*. *TP53* mutated cases harbored both a *TP53* mutation and a 17p deletion and were fludarabine resistant *in vitro*.

Western blot and CD95 determination by flow cytometry

CLL cells were irradiated (5Gy), cultured for 45 min or 16 h and lysed in Laemmli sample buffer and western blotting was performed using standard conditions as described previously.[26] The following antibodies were used: puma, p53, phosphorylated-p53 (p-p53-ser15), ATM, KAP (TIF1ß), phosphorylated-KAP (phospho-TIF1ß-ser824; Cell Signaling, Danvers, MA, USA), sf3b1 (Sigma-Aldrich), p21 and β-actin (Santa Cruz Biotechnology, Dallas, TX, USA). Protein expression was quantified using the Licor Odyssey software (Li-Cor, Lincoln, NE, USA) and corrected for the expression of actin. CD95 expression on CLL cells was determined by flow cytometry using CD5-PE, CD19-APC (eBioscience, San Diego, CA, USA) and CD95-FITC (BD Biosciences, San Jose, CA, USA). Data were normalized for isotype control.

Apoptosis induction by fludarabine, doxorubicine, IR and nutlin-3a

Thawed CLL cells were cultured (1.5×10^6/ml) in the presence of fludarabine, nutlin-3a (Sigma-Aldrich) or doxorubicin at indicated concentrations or following exposure to IR (5 Gy), for 48 h at 37 °C. Apoptosis was measured by flow cytometry as previously described.[26] Specific cell death was calculated as $((\%\text{apoptosis}_{\text{treated cells}} - \%\ \text{apoptosis}_{\text{untreated cells}})/\%\ \text{viable}_{\text{untreated cells}}) \times 100$.

γH2AX expression by flow cytometry

Thawed CLL cells were incubated for 30 min in the presence or absence of 1 μM NU7441 (DNA-PK inhibitor; Selleckchem) at 37 °C, followed by IR (5 Gy). At the indicated times, CLL cells were permeabilized (Foxp3 staining kit; eBioscience) and stained using the following antibodies: CD5-PE, CD19-APC (eBioscience) and isotype-AF488 or γH2AX-AF488 (phosphorylated-H2AX-ser-139; Cell Signaling). Expression of γH2AX was measured using flow cytometry. Data were normalized for isotype control.

Statistical analysis

Non-parametric Mann–Whitney-*U* test was used for comparison of two independent groups. For RT-MLPA and apoptotic responses, one-way ANOVA with Dunnett's *post hoc* test was used for multiple comparison correction. To test the effect of Nu7441 on γH2AX expression, a non-parametric paired test, Wilcoxon signed-rank test, was used. Correlations were analyzed by Spearman's rank correlation test. A *P*-value of < 0.05 was considered as statistically significant.

RESULTS

Genetic landscape of SF3B1 mutations in relation to ATM and TP53 aberrations

In a cohort of 110 patients enriched for *ATM* mutations and 11q deletions, *SF3B1*, *ATM* and *TP53* mutational status and FISH analysis were determined. In total, 32 (29%) *SF3B1* mutations and 29 (26%) *ATM* mutations were identified (Figure 1a). The K700E

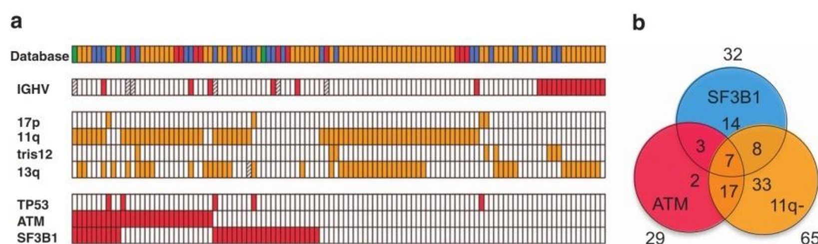

Figure 1. Genetic landscape of *SF3B1* mutations and *TP53* and *ATM* aberrations. *SF3B1* mutational analysis was performed on a cohort of $n = 110$ CLL patients enriched for *ATM* aberrations. (**a**) Rows represent mutated genes and columns represent individual patients. Upper row: color coding is based on the type of cohort (red, 'AMC-cohort'; blue, 'H68-cohort'; green, 'D'accord cohort'; orange, 'Brno-cohort'). Second row: IGHV mutation status (white, IGHV unmutated; red, IGHV mutated), Third row: FISH analysis for deletions in 17p and 11q, trisomy 12 and deletions in 13q (white, the absence of aberration; orange, the presence of aberration) and lower row: gene mutation status for *TP53*, *ATM* and *SF3B1* (white, the absence of mutation; red, the presence of mutation). Hatched boxes indicate analysis not performed. (**b**) Overlap in *SF3B1* mutations, *ATM* mutations and 11q deletions is presented in a Venn diagram. Only patients with an *SF3B1* mutation and/or *ATM* aberration are shown ($n = 84$). Numbers of patients are mentioned. Note: 14 instead of 13 sole *SF3B1* mutated cases are mentioned, since one patient also had a concurrent *TP53* mutation.

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mutation in *SF3B1* was the most prevalent mutation detected, in line with the previous analyses.[6,7,9] Mutations in *ATM* were found across the full-length transcript (ex4-65) without hotspots, as described.[1,31] In total, 56% of *SF3B1* mutated cases carried concurrent *ATM* aberrations (11q deletion and/or *ATM* mutation) (18/32; Figures 1a and b).[7,23] A concurrent *TP53* defect was found in 9% (3/32) of *SF3B1* mutated cases. Although there was considerable overlap especially between *ATM* and *SF3B1* lesions, 13 patients were identified with an *SF3B1* mutation and intact *ATM* and *TP53* genes (sole *SF3B1*) (Figure 1b; note that here 14 instead of 13 sole *SF3B1* mutated cases are mentioned, since one patient also had a concurrent *TP53* mutation).

Impaired ATM/p53 transcriptional responses in SF3B1 mutated patients, comparable to ATM mutated samples

We investigated whether *SF3B1* mutations functionally affect the ATM/p53 axis. Viable CLL cells derived from WT ($n=15$), *TP53* ($n=9$), *ATM* ($n=14$) and *SF3B1* ($n=24$) mutated cases were analyzed (Supplementary Table 2). We analyzed *SF3B1* cases with a combination of *SF3B1+TP53* mutation ($n=3$), *SF3B1+ATM* mutation ($n=6$), *SF3B1* mutation+11q deletion ($n=4$) and sole *SF3B1* mutation ($n=11$). Represented in Figures 2a–c are the sole *SF3B1* cases without concurrent *TP53* or *ATM* aberrations. All represented *ATM* mutated cases harbored an 11q deletion and all *TP53* mutated cases carried a 17p deletion in the remaining allele. We applied a newly designed RT-MLPA assay that quantifies mRNA expression of six ATM/p53 target genes; *CD95*, *p21*, *Bax*, *Puma*, *FDXR* and *PCNA*. As expected, after IR WT samples showed an upregulation of all these genes and *TP53* mutated samples displayed a negligible response as described also previously.[26] *ATM* mutated samples showed an impaired but not absent upregulation of these genes. Interestingly, sole *SF3B1* mutated cases as a group ($n=11$) also displayed a partial decreased response to IR for all six ATM/p53-target genes with a pattern intermediate between WT and TP53 mutated cases (Figure 2a).

To further compare *SF3B1* mutated cases with *ATM* and *TP53* mutated cases, expression levels of *NME1* and *ACSM3* were measured. *NME1* and *ACSM3* show a distinct response to IR in *TP53* versus *ATM* mutated CLL cells.[30] Sole *SF3B1* mutated CLL cells showed clearly decreased upregulation of *NME1*, comparable to *ATM* mutated cases (Figure 2b), but a significant difference was not found for *ACSM3*.

To determine whether the RT-MLPA assay could distinguish samples according to their mutational status, we performed a multidimensional scaling analysis,[32] a statistical method for exploring similarities or dissimilarities in data. Multidimensional scaling analysis showed clear separation between the WT, *ATM* mutated and *TP53* mutated cases, indicating that the 8-gene panel captures changes in gene expression associated with mutational status (Figure 2c). Intriguingly, the sole *SF3B1* mutated cases clustered close together, in an area overlapping both *ATM* mutated and WT cases. No difference in gene induction or clustering was found between IGHV mutated and unmutated CLL cases (data not shown).

The variation in the responses observed in the sole *SF3B1* mutated cases following IR was not correlated with a specific type of mutation or the mutant allele burden (range: 20–50%) (Supplementary Figure 1). An overview of the statistical differences between WT samples and the various mutated groups, including all *SF3B1* mutated subgroups, is presented in Supplementary Table 3. This demonstrates that either solely or in combination with *ATM* or *TP53*, *SF3B1* defects show a defective response in at least seven out of eight tested genes ($P<0.05$ compared with WT). Furthermore, the ATM/p53 response in WT, *TP53*, *ATM* and sole *SF3B1* mutated cases following exposure to DNA-damaging agents fludarabine and doxorubicin was evaluated and also revealed decreased upregulation of several genes including *p21* and *Puma* in sole *SF3B1* mutated samples (Supplementary Figure 2), corroborating the observed transcriptional defect following IR.

Theoretically, transcriptional pathways in general could be affected by *SF3B1* or *ATM* mutations. Therefore, we checked whether the NF-κB pathway was intact. CLL cells were stimulated *via* CD40, which results in strong NF-κB activity and induction of Bfl-1, Bcl-X_L and Bid[29] genes. Bfl-1, Bcl-X_L and Bid were normally upregulated in *SF3B1* and *ATM* mutated CLL cells, indicating that at least the CD40-mediated NF-κB response is not affected (Figure 2d).

In conclusion, *SF3B1* mutated CLL cells display a functional defect in p53 and ATM target gene induction, in a pattern more comparable to *ATM* rather than *TP53* mutation. Although the majority of *SF3B1* mutated cases analysed also carried *ATM* defects, the observed impaired responses clearly occurred also independently of *ATM* aberrations.

ATM/p53 responses in SF3B1 mutated CLL cells on protein level

The observed ATM/p53 responses on mRNA level in the various genotypes showed considerable variation, therefore the p53 targets, p21 and puma, as well as p53 itself and phosphorylated-p53 (p-p53) protein levels were determined after IR by western blotting. As expected, *TP53* and *ATM* mutated CLL cells showed significantly impaired upregulation of both p21 and puma, also reflected by decreased p-p53 and p53 protein levels after IR. Sole *SF3B1* mutated cases also displayed defects of the ATM/p53 response at the protein level. The quantified results over multiple samples reached statistical significance only for p21 (Figures 3a and b, Supplementary Figure 3), but a trend toward impaired induction could also be observed for puma and p53 induction in sole *SF3B1* mutated cases. Furthermore, using flow cytometry, a decreased CD95 upregulation after IR was observed in two out of three tested sole *SF3B1* mutated samples (Figure 3c), which reflected the RNA levels in these samples. In summary, the defects in transcriptional induction of ATM/p53 targets were also observed at the protein level in sole *SF3B1* mutated CLL samples.

Sole SF3B1 mutated CLL cells show defective apoptotic responses to DNA-damaging agents

Next, apoptosis responses following fludarabine, doxorubicin or IR (5 Gy) *in vitro* treatment were assessed in cells from variously mutated CLL patients. In line with the previous reports,[28,31,33] *TP53* mutated cells were completely resistant and *ATM* mutated cells were partially resistant to fludarabine and IR, whereas both were resistant to doxorubicin. Importantly, sole *SF3B1* mutated CLL samples displayed also partial but significant resistance to fludarabine and IR and almost complete resistance to doxorubicin (Figure 4a).

Nutlin-3a is an mdm2 inhibitor that can overcome ATM-mediated resistance to fludarabine.[34] Sole *SF3B1* mutated CLL cells displayed intact sensitivity to nutlin-3a and the combination of nutlin-3a and fludarabine (Figure 4b), indicating that the defect is localized upstream of p53. Overall, these results show that a sole *SF3B1* mutation affects apoptotic responses upon exposure to various DNA-damaging agents in a manner comparable to *ATM* mutated rather than to *TP53* mutated cases.

NOTCH1 or K-/N-RAS mutation in CLL do not lead to overt ATM/p53 dysfunction

In our CLL cohort, also mutations in *NOTCH1*, *KRAS* and *NRAS* were found (Supplementary Table 2), and the ATM/p53 target responses were analyzed as described above. No impaired mRNA responses after IR were found (Supplementary Figures 4a and b). In line with these virtually normal mRNA responses in *NOTCH1* mutated CLL cells, no differences were found for p21 and puma protein induction following IR. Apoptotic responses following fludarabine and nutlin-3a treatment were comparable to WT CLL cells (Supplementary Figures 4c–e). In summary, mutations in

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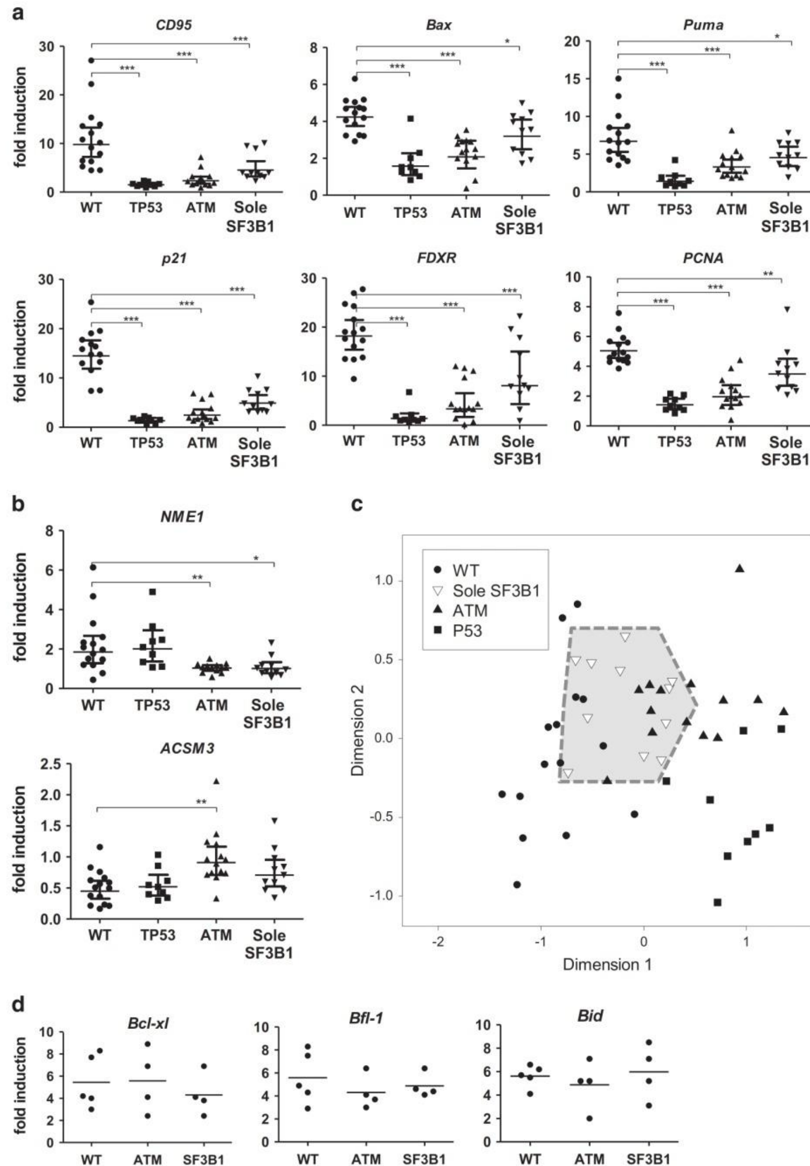

Figure 2. *SF3B1* mutated CLL cells display defective ATM/p53 transcriptional responses. (**a**–**c**) CLL cells of 15 WT (nrs. 61–75), 9 *TP53* (nrs. 4–12), 14 *ATM* (nrs. 14–27) and 11 sole *SF3B1* (nrs. 41–48; 76–78) mutated patients from whom viable cells were available for *in vitro* culture were included in the analysis and treated with IR (5 Gy) followed by measurement of mRNA expression levels using RT-MLPA of (**a**) six ATM/p53 target genes and (**b**) two ATM/p53 discriminative genes. Gene expression following IR was related to non-irradiated cells and is depicted as fold induction. Symbols represent individual patients. Geometric mean ± 95%CI within each group is shown. Significant changes in fold inductions compared with WT are presented as $*0.01 \leqslant P < 0.05$; $**0.001 \leqslant P < 0.01$; $***P < 0.001$ (one-way ANOVA with Dunnett's *post hoc* test). (**c**) Projection of the pairwise relationships among 49 patient samples ($n = 15$ WT, 9 *TP53*, 14 *ATM*, 11 sole *SF3B1*) based on the fold induction of mRNA expression levels following IR and generated using multidimensional scaling analysis. Each patient is represented by a symbol, with the spatial proximity between any two symbols indicating the degree of similarity between the induction profiles of eight selected genes (*CD95*, *Bax*, *Puma*, *p21*, *FDXR*, *PCNA*, *NME1* and *ACSM3*) for the two patients. The area surrounding the sole SF3B1 mutated samples has been indicated. (**d**) CLL cells of five WT (nrs. 64, 65, 67, 72, 73), four *ATM* (nrs. 23–26) and four *SF3B1* (nrs. 34, 36, 39, 46) mutated cases were stimulated *via* CD40 followed by measurement of mRNA expression levels of *Bfl-1*, *Bcl-X_L* and *Bid* using RT-MLPA. Gene expression following CD40 (3T40) stimulation was related to gene expression in 3T3 (control) and depicted as fold induction. Dots represent individual patients and mean within each group is shown.

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known oncogenes in CLL do not confer overt defects in the DNA-damage response.

SF3B1 mutated cases display normal ATM protein and intact ATM-kinase activity

ATM and its function were further evaluated by measuring ATM protein levels in variously mutated CLL cells (as indicated in legends to Figure 5). Decreased ATM protein levels were found in *ATM* mutated cases as expected, whereas levels were unaffected in sole *SF3B1* mutated cases (Figures 5a and b). In addition, sf3b1 protein levels were also unaffected in *SF3B1* mutated cases (Supplementary Figure 5). Subsequently, the functionality of ATM was assessed in two ways; by measuring the phosphorylation of KAP, following exposure to IR[35] and by evaluating ATM/p53-target gene induction in the presence of an ATM inhibitor (epistasis experiment).[36] As expected, *ATM* mutated cases displayed decreased phosphorylation of KAP, whereas in sole *SF3B1* mutated cases it remained intact (Figures 5a and c). Furthermore, addition of an ATM inhibitor further reduced the ATM/p53-transcriptional defects in sole *SF3B1* mutated cases (Figure 5d, data shown for *p21* only). Also, sole *SF3B1* mutated cases displayed a further decrease in the ATM-target gene *NME1* and an increase in the ATM-target gene *ACSM3* (Figure 5d). In summary, these data indicate that the function of ATM *per se* is not affected in sole *SF3B1* mutated cases.

SF3B1 mutated CLL cells displayed increased phosphorylation of H2AX (γH2AX)

The variant histone H2AX is phosphorylated by the PI3K-related kinases ATM and DNA-PK, in response to DNA strand breaks and this serves as an early mark of DNA damage.[37] We applied a flow cytometry-based assay that measures γH2AX in response to IR in CLL ($CD5^+CD19^+$) and T ($CD5^+CD19^-$) cells within one sample (Figure 6a). The peak of phosphorylation occurred at 2 h following IR, and subsequently declined and resolved after 24 h. As expected, significantly decreased γH2AX formation was observed in *ATM* mutated ($n=6$), but not in *TP53* mutated ($n=6$) CLL cells (Figure 6b). Of note, also in ATM defective CLL, γH2AX formation was largely resolved after 24 h. This pattern differed from the strongly delayed resolution observed in cycling ATM-defective cancer cell lines.[38] Remarkably, sole *SF3B1* ($n=6$) mutated cases displayed increased γH2AX levels compared with WT following IR. In addition, increased γH2AX levels were already present at baseline without exposure to IR (Figure 6c). The observed differences in γH2AX formation in *ATM* and sole *SF3B1* mutated cases were CLL specific, since they were only present in the CLL cells and not in the T lymphocytes (Figure 6a). Phosphorylated H2AX forms large nuclear foci at sites of DNA strand breaks that can be detected by immunofluorescence.[37] We compared a sole *SF3B1* mutated and WT CLL sample for γH2AX foci using immunofluorescence and indeed observed increased foci at

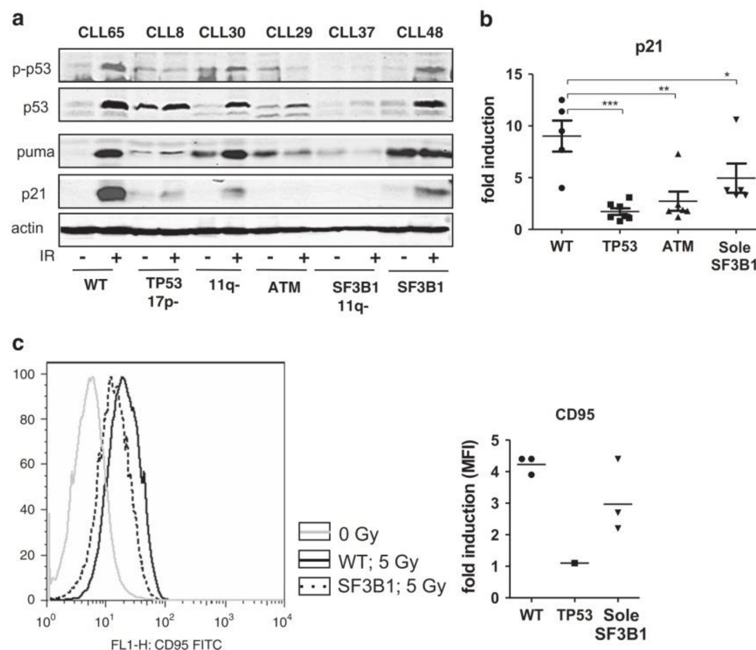

Figure 3. ATM/p53 responses in *SF3B1* mutated CLL cells on protein level. (**a**, **b**) CLL cells from five WT (five different experiments, four different patients, nrs. 62, 64, 65 and 67), seven *TP53* (seven different experiments, four different patients, nrs. 4, 5, 8, 12), six *ATM* (nrs. 14, 16, 21, 22, 25, 26), two sole *ATM* (patient nrs. 28, 29), one sole 11q-deleted (nr. 30) and five sole *SF3B1* (nrs. 43–46, 48) mutated patients were treated with or without IR (5 Gy) followed by measurement of protein levels of p21, puma, p-p53 and p53 using western blot after 16 h of culturing. Protein expression levels following IR were related to protein expression in non-irradiated cells and depicted as fold induction. (**a**) A representative blot of seven independent experiments is shown. (**b**) Summarized quantified data of p21 from all tested WT, *ATM*, *TP53* and sole *SF3B1* mutated samples. The mean ± s.e.m. within each group is shown. Significant changes in expression compared with WT are presented as $*0.01 \leqslant P < 0.05$; $**0.001 \leqslant P < 0.01$; $***P < 0.001$ (Mann–Whitney *U*-test). (**c**) CLL cells of three WT (nrs. 67, 74, 75), one *TP53* (nr. 8) and three sole *SF3B1* (nrs. 76–78) mutated patients were treated with or without IR (5 Gy) followed by measurement of CD95 expression using flow cytometry at 24 h. CD95 expression following IR was related to non-irradiated cells and is depicted as fold induction. For all three sole *SF3B1* mutated cases the CD95 protein expression was in accordance with CD95 mRNA measured by RT-MLPA. Representative FACS plots of CD95 staining and summarized data of CD95 expression levels of all tested WT, *TP53* and sole *SF3B1* mutated samples are shown. Bars represent mean ± s.e.m.

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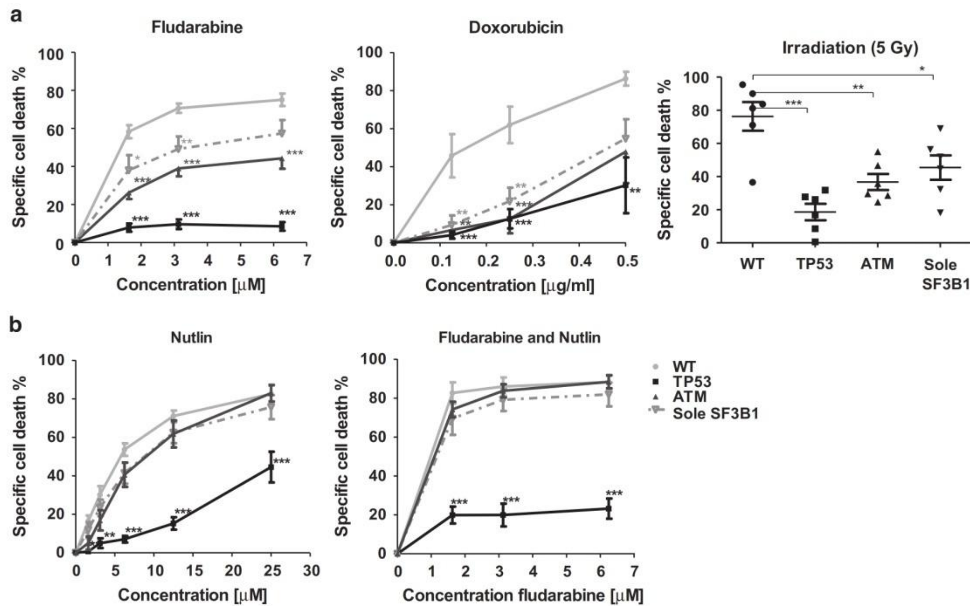

Figure 4. *SF3B1* mutated CLL cells show defective apoptotic responses to various DNA-damaging agents, but not to nutlin-3a. (**a**, **b**) CLL cells of seven WT (nrs. 61–65, 67, 68), nine *TP53* (nrs. 4–12), nine *ATM* (nrs. 14, 16, 21–27) and six sole *SF3B1* (nrs. 43–48) mutated patients were treated with fludarabine or nutin-3a at increasing concentrations as indicated. In addition, CLL cells of six WT (nrs. 61–64, 67, 68), six *TP53* (nrs. 4, 5, 7–9, 12), six *ATM* (nrs. 14, 90, 20, 24, 26+27) and six sole *SF3B1* (nrs. 43–48) mutated patients were treated with doxorubicin at increasing concentrations as indicated or IR (5Gy). And, CLL cells of four WT (nrs. 61, 62, 64, 67), four *TP53* (nrs. 4, 5, 8, 12), six *ATM* (nrs. 14, 16, 21, 22, 25, 27) and six sole *SF3B1* (nrs. 43–48) mutated patients were treated with fludarabine at increasing concentrations combined with 3.1 μM nutlin-3a. After 48 h, cell death was assessed by $DIOC_6$/PI staining and specific cell death was calculated as described in Materials and methods section. Presented is mean ± s.e.m. Significant responses compared with WT are presented as $*0.01 \leqslant P < 0.05$; $**0.001 \leqslant P < 0.01$; $***P < 0.001$ (one-way ANOVA with Dunnett's *post hoc* test). (**a**) Plots from responses to fludarabine, doxorubicin or IR are shown. (**b**) Plots from responses either to nutlin-3a or to the combination of fludarabine and nutlin-3a are shown.

baseline and following IR in the sole *SF3B1* mutated CLL cells, confirming the observed results measured by flow cytometry (Supplementary Figures 6a and b).

To further probe whether *SF3B1* mutations acted independently from ATM dysfunction, a chemical inhibitor of DNA-PK, NU7441, was used. As expected, decreased γH2AX formation in *ATM* mutated ($n = 6$) and not in WT ($n = 6$) CLL cells in the presence of NU7441 following IR was observed. As a group, sole *SF3B1* mutated ($n = 6$) CLL cells did not display a significant decrease of γH2AX formation in exposure to NU7441 (Figure 6d), suggesting that ATM kinase activity remains intact and that *SF3B1* mutations lead to the observed defects independently from ATM-kinase dysfunction.

DISCUSSION

Systematic studies of cancer genomes have yielded numerous novel cancer genes in recent years. An intriguing finding in CLL was that *SF3B1*, encoding a splicing factor, is frequently mutated, which is linked with unfavorable prognosis. This study represents the first combined genetic and functional analysis of *SF3B1* mutations in CLL in relation to DNA-damage responses. We investigated both ATM/p53 target gene and protein induction and apoptotic responses upon various DNA-damaging agents. Clearly, sole *SF3B1* mutations affected all these responses with partial impairment, in a pattern resembling *ATM* dysfunction rather than *TP53* dysfunction. Importantly, sole *SF3B1* mutated cases were sensitive to nutlin-3a, which indicates that the defect in DDR is localized upstream of p53.[34] ATM kinase activity was however unaffected with intact phosphorylation of KAP and as determined by epistasis experiment using an ATM inhibitor. Intriguingly, sole *SF3B1* mutated cases displayed increased γH2AX levels at baseline and upon IR. On the basis of our data, we propose that *SF3B1* mutations lead to a defective DNA-damage response in the presence of intact ATM kinase activity. In contrast, we did not find global defects in ATM/p53 target responses in *NOTCH1* and/or *K-/N-RAS* mutated CLL.

Thus far, attempts at unraveling functional consequences of *SF3B1* mutations used deep sequencing of mRNA[18] or exon arrays,[6] to search for genes affected by defective splicing and consequently leading to abnormal function.[6,7,18] This option is appealing and reinforced by the notion that aberrant splicing may underlie the development of certain tumors.[20,39] Several genes reportedly were affected by mutations in *SF3B1*, including *RUNX1*,[18] *BRD2* and *RIOK3*,[40] and *FOXP1*,[6] encoding a forkhead transcription factor whose altered expression has been linked to diffuse large B-cell lymphoma.[41] Yet, until now no candidate aberrantly spliced gene could be functionally linked to CLL pathogenesis or chemoresistance. Interestingly, a novel splice junction in the *ATM* gene in *SF3B1* mutated cases was recently reported,[42] which might explain the observed functional overlap between *SF3B1* and *ATM* mutations in CLL. We have examined potential splicing defects in the *ATM* gene by PCR analysis of cDNA fragments (data not shown) and by western blot (Figure 5), but found no apparent changes in *SF3B1* mutated cases. In addition, although the alternatively spliced *ATM* mRNA product was indeed detectable by qPCR, its levels were considerably lower than the normal *ATM* mRNA (Supplementary Figure 7). Thus,

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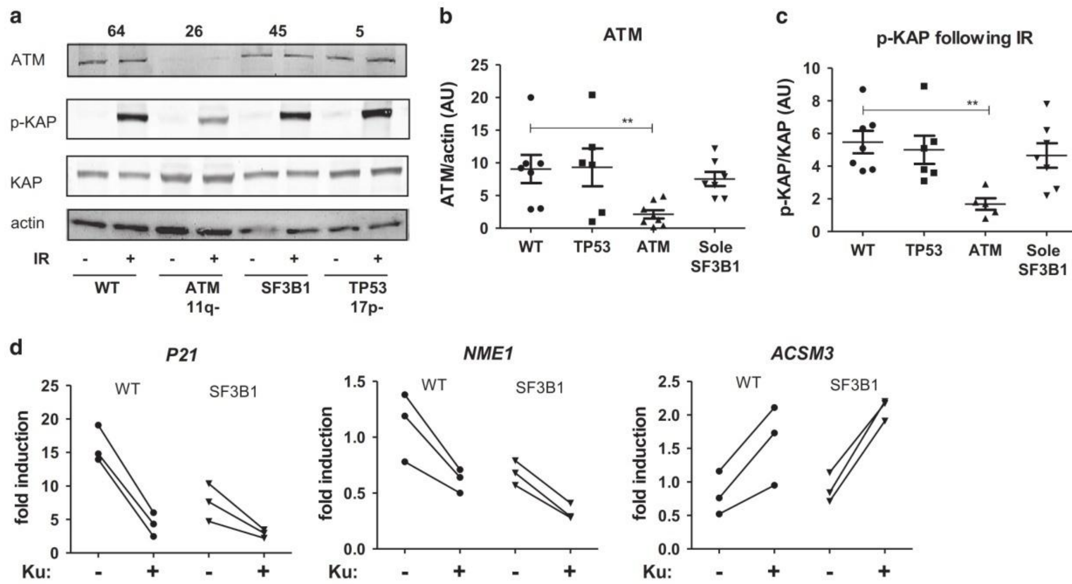

Figure 5. *SF3B1* mutated CLL showed intact ATM protein expression and intact ATM function. (**a**, **b**) Unstimulated CLL cells of seven WT (nrs. 61–64, 66–68), six *TP53* (nrs. 4, 5, 7–9, 12), eight *ATM* (nrs. 14, 16, 19–22, 25, 26) and seven sole *SF3B1* (nrs. 42–48) mutated samples were lysed and ATM levels were measured by western blot. (**a**) A representative blot is shown. (**b**) Summarized quantified data of ATM protein expression levels of all tested WT, *TP53*, *ATM* and sole *SF3B1* mutated samples. Protein expression was corrected for the expression of actin. (**a**, **c**) CLL cells of seven WT (nrs. 61–64, 66–68), six *TP53* (nrs. 4, 5, 7–9, 12), five *ATM* (nrs. 14, 19, 20, 25, 26) and seven sole *SF3B1* (nrs. 42–48) mutated patients were treated with or without IR (5 Gy) followed by measurement of protein levels of KAP and phosphorylated-KAP (p-KAP) using western blot after 45 min. (**a**) A representative blot is shown. (**c**) Summarized quantified data of phosphorylated-KAP expression in irradiated samples of all tested WT, *TP53*, *ATM* and sole *SF3B1* mutated samples. Protein expression was corrected for the expression of KAP. For (**b**, **c**), Symbols represent individual patients. Mean ± s.e.m. is shown. Significant changes in expression compared with WT are presented as $^{**}0.001 \leqslant P < 0.01$ (Mann–Whitney *U*-test). (**d**) CLL cells of three WT (nrs. 67, 74, 75) and three sole *SF3B1* (nrs. 76–78) mutated patients were treated with or without IR (5 Gy) in the presence (+) or absence (−) of 10 μM Ku-55933 (ATM inhibitor) followed by measurement of mRNA expression levels of ATM/p53 target genes and ATM/p53 discriminative genes, using RT-MLPA. Gene expression following IR was related to non-irradiated cells and is depicted as fold induction. Symbols represent individual patients.

alternative splicing of *ATM* seems unlikely to explain the observed defects.

Phosphorylation of histone H2AX is a hallmark for chromatin-modification associated with DNA strand breaks,[37] and is defective in *ATM* mutated cells. In contrast to this, we observed increased levels of γH2AX in *SF3B1* mutated CLL cells. Intriguingly, combined *SF3B1* and *ATM* mutated CLL samples display H2AX phosphorylation at normal level (Supplementary Figure 8), indicating that at this level the mutations act independently. Together with the epistasis experiments and considering the intact phosphorylation of KAP in sole *SF3B1* mutated cases, this, again suggests that the roles of ATM and sf3b1 in the DNA damage response are not overlapping.

Mutations in *SF3B1* might either contribute to increased DNA-damage or alternatively might indirectly lead to an aberrant response to DNA damage. Corroborating our findings, various studies have in recent years yielded clues for involvement of the spliceosomal machinery, or splicing factors, in maintaining genome stability, in the replication stress response or the DDR. First, several recently performed unbiased functional screens showed that components of the spliceosomal machinery, including sf3b1, are required for maintaining genome stability in mammalian cells.[43–45] Second, recent publications demonstrated involvement of splicing factors in sensing and signaling of DNA damage.[46–48] Finally, splicing (factors) appear to be important for resolving R-loops in chromatin, which are at the basis of increased genomic instability.[49]

Although we find evidence for a functional link between sf3b1 and the DDR, further research is required to explore the mechanism and causality of this association. Unfortunately, attempts at recombinant expression of *SF3B1* mutants are hampered by difficulties in bacterial cloning of *SF3B1*.[50,51] In the context of MDS, *SF3B1* manipulation by RNA interference was used and this showed increased ring sideroblast formation.[52] We also performed RNA interference experiments of *SF3B1* in B-cell lines, resulting in decreased overall cell viability without impaired ATM/p53 response (data not shown). This might be explained by the fact that reducing the level of sf3b1 does not reflect the functional consequences of heterozygous missense mutations affecting specific protein domains, which suggests a gained or altered function.[7,9,18,51]

The variation observed in the ATM/p53 response could not be associated with the mutant allele burden, suggesting that additional factors or as yet unknown genetic modifications might have a role. Overall, the mutant allele burden was usually close to 50% as determined by next-generation sequencing, suggesting that the majority of samples studied harbored in fact a heterozygous mutation. Examination of the individual sole *SF3B1* mutated cases revealed that some samples had in fact near normal responses in most of our experiments (Supplementary Table 4). This might suggest a causative role for specific amino-acid mutations in the observed defective responses.

Whereas *TP53*-disruptive cases exhibit complete absence of DNA-damage induced responses *in vitro* and show an aggressive clinical course *in vivo*, *SF3B1* and *ATM* mutated cases show

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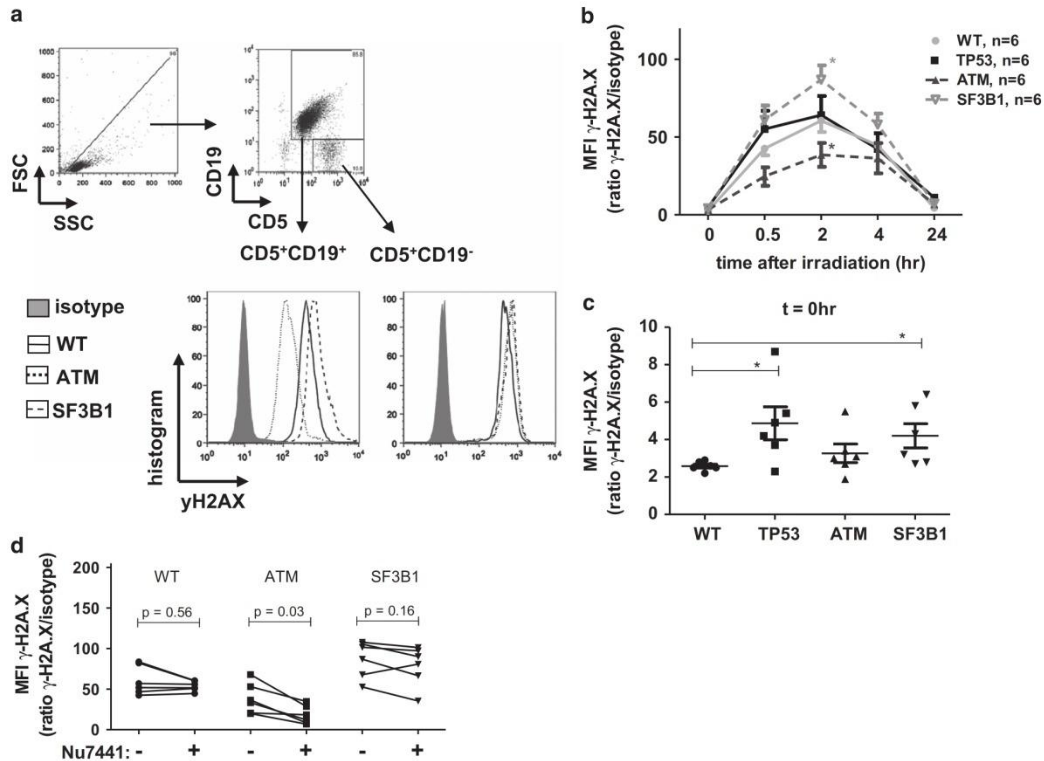

Figure 6. Sole *SF3B1* mutated CLL cells showed increased phosphorylation of H2AX (yH2AX). (**a**–**d**) CLL cells of six WT (nrs. 61, 63, 64, 66–68), six *TP53* (nrs. 4, 5, 7–9, 12), six *ATM* (nrs.14, 19, 20, 24–26) and six sole *SF3B1* (nrs. 43–48) mutated patients were (**a**–**c**) treated with or without IR (5Gy) followed by measurement of yH2AX using flow cytometry at the indicated time points or (**d**) treated with or without 1μM NU7441 (DNA-PK inhibitor) followed by IR (5 Gy) and measured at 2 h using flow cytometry. (**a**) Representative FACS plots of yH2AX staining. (**b, c**) Summarized data of yH2AX expression levels of all tested WT, *TP53*, *ATM* and sole *SF3B1* mutated samples are shown (**b**) at different time points following IR and (**c**) at baseline. For (**c**) Symbols represent individual patients. For (**b, c**), Data were normalized for isotype control. Mean ± s.e.m. is shown. Significant responses compared with WT are presented as $*0.01 \leqslant P < 0.05$ (Mann–Whitney *U*-test). (**d**) Summarized data of all tested samples are shown. Data were normalized for isotype control. To test the effect of Nu7441 on γH2AX expression, a non-parametric paired test was used (Wilcoxon signed-rank test). *P*-values are mentioned.

intermediate responses with residual capacity for DNA-damage induced transcriptional and apoptotic responses *in vitro*, and a more prolonged survival *in vivo*.[53,54] Interestingly, we do not observe overt further impairment of DNA-damage responses *in vitro* to the level of a p53 defect in cases harboring concurrent *ATM* and *SF3B1* mutations. Currently, it is unknown whether the clinical outcome for patients with combined or sole *SF3B1* mutations is distinct, but it would be interesting to elucidate whether the clinical course reflects the *in vitro* data.

In conclusion, the recently described mutations in *SF3B1*, encoding a splicing factor, can be genetically linked with *ATM* defects, and functionally linked with a defective DDR, suggesting an explanation for the poor clinical prognosis of CLL patients with *SF3B1* mutations. We propose that sf3b1 might be involved in other processes besides splicing, and/or that splicing factors are functionally involved in the DDR. In addition, the newly elucidated link between sf3b1 and the DDR offers opportunities for screening and exploring novel treatment options for affected CLL patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

GDR designed and performed experiments, analyzed data and wrote the paper; IAMD, JL, CO and HM performed experiments; VN performed experiments and analyzed data; AS, JH and AJ performed experiments and analyzed data; PDM performed data analysis; JM and MT analyzed data; ML coordinated the development of the RT-MLPA probe mix; CG managed patient cohorts; TZ supervised 454 sequencing and analyzed data; SP and TS reviewed the manuscript and contributed to the design of experiments; MHO and APK managed patient cohorts and contributed to the design of experiments; EE designed the study, performed data analysis and wrote the paper. All authors reviewed and corrected the manuscript.

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***ATM* mutations in major stereotyped subsets of chronic lymphocytic leukemia: enrichment in subset #2 is associated with markedly short telomeres**

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Running head: *ATM* mutations in CLL subsets

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The online version of this article contains a supplementary appendix.

Letter to the editor

In chronic lymphocytic leukemia (CLL), antigenic stimulation through the B-cell receptor (BcR) and the accumulation of genetic defects critically affect the natural history of the disease[1]. The importance of antigen involvement is underscored by the existence of stereotyped BcR in approximately 30% of CLL cases, where patients belonging to different stereotyped subsets share similar biological profile and clinical course[2,3]. *ATM* defects have been associated with CLL evolution and outcome[4], however, their contribution to the pathobiology of individual subsets remains unexplored. Therefore, we decided to use targeted next-generation sequencing (NGS) to detect variants in the entire coding region of the *ATM* gene in well-characterized CLL patients assigned to one of eight major subsets (#1-8). Since ATM is employed in signaling of telomere erosion, we next sought to investigate the potential correlation between *ATM* defects and telomere length in selected subsets as well as the clinical impact of these parameters.

A total of 249 CLL patients from a large European multicenter cohort, assigned to major subsets #1-8 according to established criteria[2], were included in this study. All patients were diagnosed in accordance with the 2008 iwCLL guidelines[5], and informed consent was obtained according to the Declaration of Helsinki with local review committees granting ethical approval. Clinical and biological characteristic of the cohort are summarized in *Online Supplementary Table 1*. The entire coding region (62 exons) and adjacent splicing sites of the *ATM* gene were investigated using targeted deep-sequencing (n=237 samples) or Sanger sequencing (n=12 samples) with details described in *Online Supplementary methods*. *ATM* variants listed only in dbSNP and not in mutation databases COSMIC or HGMD were regarded as non-pathogenic polymorphisms and excluded from subsequent analyses. A conservative 10% variant allele frequency cut-off was applied to avoid false-positives; all mutations (range 10.4% - 99.1%) were confirmed by Sanger sequencing.

Within the evaluated cohort of 249 CLL patients, the most populated subset was #2 (n=81, comprising 47 IGHV-mutated (M-CLL) and 34 IGHV-unmutated (U-CLL) cases, followed by the U-CLL subsets #1 (n=68), #7 (n=31), #6 (n=16), #8 (n=14), #5 (n=12) and #3 (n=12). Subset #4 (n=15) was,

with a single exception, composed of M-CLL patients. Heat-maps detailing the overall distribution of genomic aberrations and/or recurrent mutations within each subset are illustrated in Figure 1A. The proportions of cytogenetic defects and gene mutations in subsets are shown in *Online Supplementary Table 2* and *Online Supplementary Figure 1*, respectively. Overall, our data corresponded well to recent reports showing enrichment of *TP53* aberrations in subsets #1, *SF3B1* mutations in subset #2, and trisomy 12 in subset #8[3,6,7].

We detected 61 *ATM* mutations in 47/249 (19%) patients across all subsets; the mutational spectrum in visualized in Figure 1B and mutations are listed in *Online Supplementary Table 3*. Concerning the functional impact, 54/61 (88%) of identified mutations were deemed presumably deleterious based on either mutation type (nonsense, frameshift, abolishing splice sites) or variant effect evaluation using the PredictSNP tool[8]. The remaining 7 variants were assessed as neutral; however, their negative impact on ATM function cannot be completely excluded as this may depend on the status of the other allele (2/4 samples with available data harbored 11q-) or ATM properties not included in *in silico* evaluation. Moreover, PredictSNP confirmed that all presumable polymorphisms were functionally neutral. The highest mutation frequency was observed in subset #2 (21/81, 26%) with a significant enrichment in U-CLL versus M-CLL patients (13/34 vs. 8/47, respectively; p=0.041). Within poor-prognostic U-CLL subsets, *ATM* mutations predominated in subsets #6 (4/16, 25%) and #7 (7/31, 23%), while the remaining subsets #3, #5, #1, and #8 showed lower frequencies (2/12, 17%; 2/12, 17%; 9/68, 13%; and 1/14, 7%, respectively) (*Online Supplementary Figure 1A*). When comparing the two most populated subsets #1 and #2, *ATM* mutations were overrepresented in the latter. This association did not reach statistical significance (p=0.086), however, when restricting the analysis only to U-CLL subset #2 cases a significantly higher *ATM* mutation frequency was seen compared to subset #1 [13/34 (38%) vs. 9/68 (13%); p=0.005] (Figure 1C). Importantly, this difference remained significant also in a more stringent analysis involving only potentially deleterious *ATM* mutations (10/34 vs. 7/68; p=0.023).

Considering that (a) telomere length (TL) reflects the proliferation history of cells, and (b) ATM is necessary for launching cellular response to critically short telomeres, we next evaluated the impact of *ATM* mutations on TL in the context of stereotyped subsets. The prognostic relevance of TL in CLL has been repeatedly reported[9,10] and CLL cells with *ATM* mutations were recently shown to display extreme telomere shortening, allowing telomere fusions and subsequent large-scale genomic rearrangements facilitating disease progression through increased genomic instability[11,12]. Relative telomere length (RTL) was investigated by real-time quantitative PCR as originally described by Cawthon *et al*.[13] with certain modifications (*Online Supplementary methods*). The median RTL value 0.4 (range 0.05 to 2.25) was subsequently used to distinguish between 'short' and 'long' telomeres.

The RTL was assessed in 213/249 patients analyzed for *ATM* mutations. As expected, indolent M-CLL subset #4 patients had the longest telomeres (median RTL 0.86), which was markedly different not only from U-CLL subsets (range 0.29 to 0.51) but also from subset #2 (0.45) (*Online Supplementary Figure 2*), even when restricting the comparison to subset #2 M-CLL cases. In fact, no difference in RTL was noted between M-CLL and U-CLL patients in subset #2 (median 0.45 and 0.37, p=0.51; *Online Supplementary Figure 3*); well in line with the previous observation that both subgroups have poor outcome independent of IGHV mutation status[14].

In order to evaluate the impact of *ATM* defects on RTL, we decided to focus on the most populated subsets in our study, i.e. #1, and #2. Considering the expected negative impact of *TP53* aberrations on RTL[9,15], the samples were divided into five genetic groups based on the hierarchical presence of *TP53* and/or *ATM* defects: 'Def-*TP53*' (*TP53* defect(s): 17p- and/or *TP53* mutation), 'Def-*ATM*' (biallelic *ATM* defect: 11q-/mutation or two *ATM* mutations), 'Sole 11q-' (only 11q- with the other *ATM* allele intact), 'Sole *ATM*-mut' (single *ATM* mutation without 11q-), and 'WT' (wild-type: no *TP53* or *ATM* defect) (patient numbers in *Online Supplementary Table* 4).

Within subset #1, we observed particularly short telomeres in the 'Def-*TP53*' group (median RTL 0.2 vs. 0.46 in the WT group; p<0.001), while there was no significant impact of *ATM* defects. In contrast, the few subset #2 cases harboring *TP53* defects showed RTL values similar to the WT group (0.4 vs.

0.49, p=0.47; Figure 2A). Intriguingly, the 'Def-*ATM*' group demonstrated the shortest RTL of all subset #2 patients (0.23, p=0.003 compared to WT; Figure 2A); a similar impact of *ATM* inactivation was observed when analyzing only U-CLL subset #2 cases (0.19 vs. 0.49 in WT, p=0.02; *Online Supplementary Figure 4*). Since subset #2 is enriched for *SF3B1* mutations, we evaluated the impact of mutations within *SF3B1* on TL in patients lacking *TP53/ATM* mutations; however, both *SF3B1*-mutated and *SF3B1*-WT cases showed similar RTLs (median 0.48 and 0.49, respectively; p=0.48; Figure 2B).

To gain insight into the clinical relevance of our observations, we focused on the impact of RTL on TTFT and OS in subset #2 patients. The short telomeres were significantly associated with both reduced TTFT (medians 18 vs. 42 months for long telomeres; p=0.039; Figure 3A) and OS (medians 88 months vs. not reached for long telomeres; p=0.016; Figure 3B) in subset #2. Although a previous report showed relatively stable TL after treatment in CLL patients[15], we cannot fully exclude an impact of therapy administration on survival analysis since a proportion of samples (24%) was collected after therapy. Regarding genetic defects, we also assessed their impact on OS. We divided *ATM* defects according to their type as in the aforementioned RTL analysis; all *ATM* abnormalities resulted in reduced median OS (71, 82, 89 months in 'Def-*ATM*' (n=11), 'Sole *ATM*-mut' (n=6) and 'Sole 11q-' (n=7) groups, respectively) compared to WT patients (121 months, n=40) *(Online Supplementary Figure 5)*, however none of these comparisons were statistically significant in univariate or multivariate analyses (data not shown). Interestingly, the rare subset #2 patients with *TP53* defects had a similar survival (127 months, n=6) as the WT group, underscoring previous observations that *TP53* dysfunction plays a minor role in this subset[7]. Thus, the very short telomeres in subset #2 patients with biallelic *ATM* defects imply a synergistic proliferative effect of a distinctive BcR signaling combined with impaired telomere length maintenance. By contrast, the impact of *ATM* defects themselves seems to be less prominent, possibly due to heterogeneous nature of mutations affecting distinct parts of ATM protein, their variable association with 11q- and probably few patients in each subgroup.

To summarize, we demonstrate that *ATM* mutations can be added to the list of genetic defects with a biased distribution in stereotyped subsets. The enrichment of *ATM* defects in subset #2 was associated with particularly short telomeres, proposing a role for *ATM* inactivation in shaping the aggressive phenotype of this subset. This study further reinforces the recent suggestion that CLL development is driven by antigenic selection linked with preferential acquisition of specific genetic defects during disease evolution.

Authorship and Disclosures

VN and MT were the principal investigators, designed the study, and wrote the manuscript; VN and EY performed the experiments; LR performed statistical analyses; PB, LAS, VL, KP, LM, KS and SP edited the manuscript; SN, ZD, GJ, KES, CB, PP, TT, FD, AWL, PG, JCS, DO and JM provided samples; RR co-designed the study and edited the manuscript. The authors report no potential conflicts of interest.

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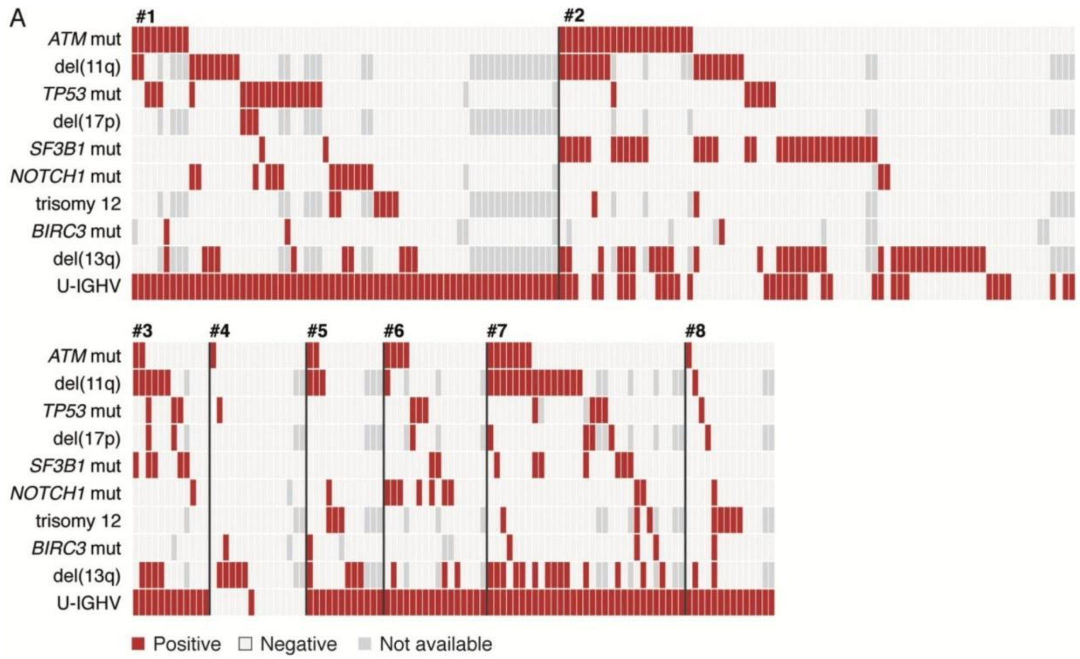
Legends to Figures

Figure 1: *ATM* mutations in major stereotyped BcR subsets. A) Heat map showing incidence of *ATM* mutations and main genetic defects, IGHV mutational status and their associations in individual patients assigned to subsets #1-8 (rows correspond to aberrations, columns represent individual patients). B) Schematic localization of identified mutations along the *ATM* gene displaying different mutation types, affected protein domains and subset #2 cases (framed symbols). C) Frequency of *ATM* mutations in the most populated subsets #1 and #2, also considering U-CLL and M-CLL #2 cases separately (red: positive, grey: negative).

Figure 2: Relative telomere length according to genetic defects presence in subset #2. A) Impact of *TP53* and *ATM* defects (hierarchical order). B) Impact of *SF3B1* mutation in WT group (without *TP53* and *ATM* defects). Statistically significant differences are marked in the graphs.

Figure 3: Clinical outcome of subset #2 patients according to telomere length. A) TTFT and B) OS for patients with short and long telomeres.

Figure 1



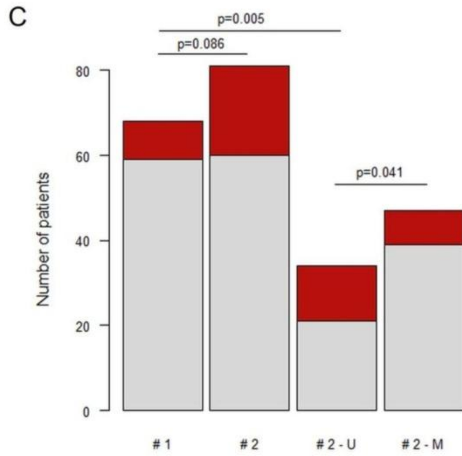


Figure 2

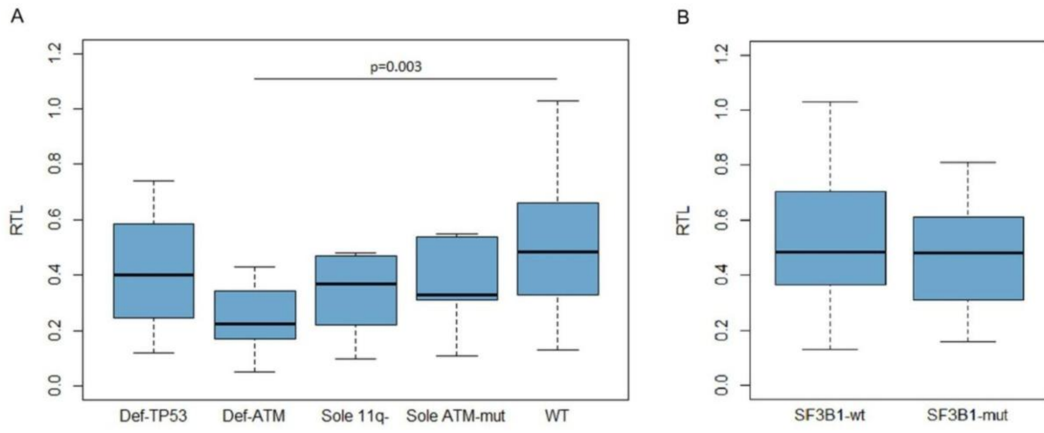
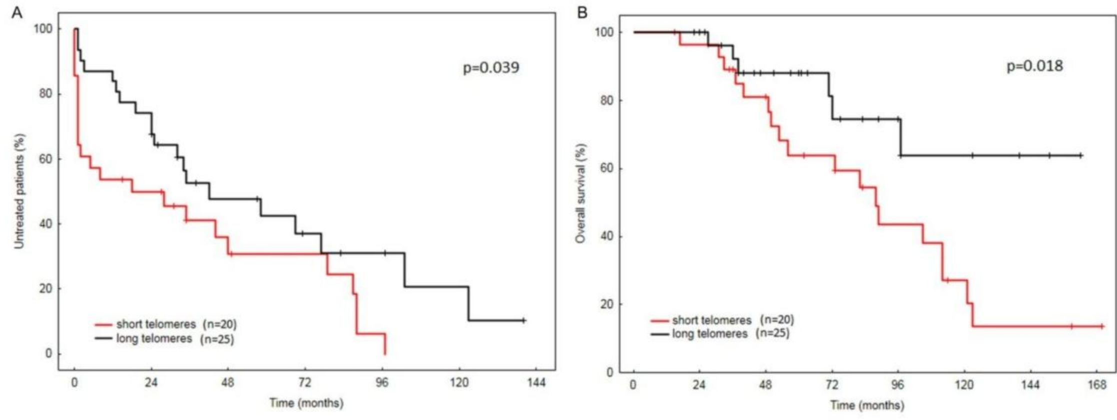


Figure 3



3.3 Testování látek s protinádorovým účinkem na buňkách CLL s charakterizovanými aberacemi

Publikace aspiranta vztahující se k tématu

Presence of heterozygous ATM deletion may not be critical in the primary response of chronic lymphocytic leukemia cells to fludarabine. Cejkova S, Rocnova L, Potesil D, Smardova J, Novakova V, Chumchalova J, Zezulkova D, Borsky M, Doubek M, Brychtova Y, Pospisilova S, Klabusay M, Mayer J, Trbusek M. **Eur J Haematol** 2009;82(2):133-42. (M. Trbušek korespondující autor – **příloha 14**)
IF=2,4

Distinct in vitro sensitivity of p53-mutated and ATM-mutated chronic lymphocytic leukemia cells to ofatumumab and rituximab. Sebejova L, Borsky M, Jaskova Z, Potesil D, Navrkalova V, Malcikova J, Sramek M, Doubek M, Loja T, Pospisilova S, Mayer J, Trbusek M. **Exp Hematol** 2014;42(10):867-74. (M. Trbušek korespondující autor – **příloha 15**)
IF=2,4

Komentář:

Jak je patrné z předchozího textu, mutační analýzy genů klíčových pro patogenezi CLL by nebyly možné (resp. byly by značně nekompletní) bez ověření funkčního dopadu identifikovaných mutací. Analýza celkové viability CLL buněk po jejich ošetření cytostatiky se ukázala být jedním z vhodných nástrojů pro sledování účinku mutací. Takto se nám například podařilo prokázat, že monoalelické a bialelické defekty *TP53* se liší v citlivosti na fludarabin (Malcikova et al., 2009) nebo že celková citlivost CLL buněk na fludarabin a doxorubicin velmi dobře koreluje se schopností indukovat p53 dráhu (Navrkalova et al., 2013a, 2013b).

Během období, kdy stěžejní náplní práce aspiranta a jeho výzkumné skupiny byly mutační analýzy pacientů a záležitosti s nimi spojené (2002-2014), se podařilo naší výzkumné skupině publikovat rovněž dvě práce primárně zaměřené na testování citlivosti CLL buněk na terapeutika. V první práci (Cejkova et al., 2009) (**příloha 14**) jsme ukázali, že samotná delece 11q (bez mutace *ATM* na druhé alele) nevede k rezistenci CLL buněk na nukleosidový analog fludarabin. Tento výsledek byl v souladu s faktem, že zachovaná jedna alela by měla být dostatečná pro pokrytí funkce proteinu ATM (Pettit et al., 2001; Austen et al., 2007). Kromě toho naše práce potvrdila, že CLL buňky s defekty *TP53* reagují na fludarabin podstatně hůře než vzorky *TP53*-wt (Rosenwald et al., 2004).

V naší recentní práci publikované v časopise *Experimental Hematology* (Sebejova et al., 2014) (**příloha 15**) jsme se pak zaměřili na *in vitro* analýzu citlivosti CLL buněk s mutacemi v genech *TP53* a *ATM* na monoklonální protilátky ofatumumab a rituximab. I když se před započetím experimentů nezdálo pravděpodobné, že by mutace v předmětných genech mohly nějak výrazněji ovlivňovat reakci na monokonální protilátky - vzhledem k tomu, že jejich mechanismus účinku leží primárně mimo indukci apoptózy (Villamor et al., 2003; Wang and Weiner 2008) - realita byla nakonec jiná. Zatímco vzorky s mutacemi *TP53* odpovídaly na protilátky slabě nebo neodpovídaly vůbec, kultury s inaktivním proteinem ATM vykazovaly velmi překvapivě značnou citlivost. Tato odlišná reakce byla podložena odpovídajícími rozdíly v hladině cílového antigenu CD20 i klíčových inhibitorů komplementu, molekul CD55 a CD59. Naše práce byla celosvětově druhá, která ukázala, že hladina CD20 asociuje s nějakou rekurentní genetickou abnormalitou v CLL buňkách a tím pádem i s *in vitro* odpovědí na anti-CD20 protilátky -tedy vedle dříve popsané souvislosti mezi trizomií chromozómu 12 a vysokou hladinou CD20 (Tam et al., 2008). Později jsme rovněž zjistili, že špatná primární reakce na testované monoklonální protilátky se pojí i s mutacemi v genu *NOTCH1* (manuskript v přípravě). Další rozkrývání vztahu mezi rekurentními mutacemi CLL buněk a jejich reakcí na anti-CD20 monoklonální protilátky tak může ještě přinést mnohé zajímavé poznatky a zejména (snad) přispět k lepší prognostické stratifikaci pacientů.

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ORIGINAL ARTICLE

Presence of heterozygous ATM deletion may not be critical in the primary response of chronic lymphocytic leukemia cells to fludarabine

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Abstract

Objectives: Abnormalities of the *TP53* or *ATM*, cooperating tumor-suppressor genes, significantly worsen the treatment options for chronic lymphocytic leukemia (CLL) patients. Although the aberrations seem to be mutually exclusive in this leukemia, inactivation of the former gene leads to worse prognosis. We tested the *in vitro* sensitivity of the CLL samples with heterozygous *ATM* deletion to fludarabine and combination of fludarabine and rituximab; the responses were compared with the *TP53*-abnormal and wild-type (wt) cells to delimitate relative significance of *ATM* deletion. *Methods: In vitro* analysis was performed on fifty-nine characterized CLL samples using viability assay WST-1. Western blot and real-time RT-PCR were used to monitor the activation of the ATM/p53 pathway. *Results and conclusions:* At the clinically relevant concentration of fludarabine, *TP53*-abnormal samples exhibited markedly higher resistance to fludarabine than the remaining CLL samples ($P = 0.012$); cohort with *ATM* deletion was not more resistant than wt cells. A similar induction of the p53 protein and its downstream target genes *PUMA* and *BAX* in *ATM*-deleted and wt cells confirmed that the former subgroup has preserved a critical pro-apoptotic response. Proportions of the samples, which had been sensitized to fludarabine by rituximab pretreatment, were insignificantly lower ($P = 0.22$) in the *TP53*-abnormal and *ATM*-deleted subgroups compared to the wt cases (30%; 29%; 50%, respectively). The presence of *ATM* (11q22–23) deletion in the CLL cells should not be considered an indication of resistance to fludarabine or its combination with rituximab.

Key words chronic lymphocytic leukemia; ATM; TP53/p53; fludarabine; rituximab

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A highly variable clinical course of B-cell chronic lymphocytic leukemia (CLL) is mostly determined by two major biological factors: mutation status of the immunoglobulin heavy-chain variable region (IgVH) (1), and four recurrent genomic aberrations (2). Among the latter, deletions 11q22–q23 and especially 17p13, confer an inferior prognosis. The inactivated tumor-suppressor genes at corresponding loci, i.e. *ATM* in a proportion of cases and *TP53*, code for the proteins, which closely cooperate in the cell during DNA damage response (3). As *ATM* and *TP53* abnormalities seem to be mutually exclusive in CLL patients, it has been proposed that the inactivation of one is an alternative to dysfunction of the other (4). Microarray analysis of gene expression further confirmed the cooperative role of ATM and p53 in response to DNA damage in the CLL cells, although this study emphasized that the ATM and p53 pathways overlap, but are not congruent (5). In line with this observation, the inactivation of *TP53* leads to a substantially higher resistance of CLL cells to apoptosis (induced by ionizing radiation) compared with *ATM*-mutated cells (4, 6).

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CLL still constitutes an incurable disease. Nucleoside analogue fludarabine has undoubtedly become one of its key therapeutics (7). This drug exerts its activity primarily through inhibition of DNA synthesis and, to a lesser extent, by transcription interference (8). The efficacy of fludarabine has been significantly reduced in a proportion of patients (7), presumably those harboring a mutated or deleted *TP53* gene (9, 10). A microarray analysis has confirmed p53-dependent up-regulation of the downstream target genes in the CLL cells after fludarabine treatment (11). Despite observed connection between p53 and ATM inactivation (4), the role of ATM kinase after drug administration still remains to be more precisely determined. The heterozygous 11q22–q23 (*ATM*) deletion analyzed by interphase I-FISH occurs in about 18% of unselected CLL patients (12, 13) and these patients manifest a consistent disease progression and reduced survival (12, 14). A substantially decreased phosphorylation of the p53 protein at critical serine-15 residue (3) was shown in ATM-inactivated CLL samples after fludarabine treatment (15). The same residue may also be phosphorylated by another kinase, DNA-PK, in response to nucleoside analogues (16) or an alkylator agent chlorambucil (17).

The introduction of rituximab into clinical practice has significantly potentiated treatment options in CLL (18). This antibody, which targets CD20 receptor on the surface of leukemic cells, exerts at least three different mechanisms of action, namely complement-dependent cellular cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and induction of apoptosis (19, 20). Importantly, rituximab potentiates fludarabine in non-Hodgkin's lymphoma (21) and also in CLL cells (22). A retrospective analysis of CLL patients receiving rituximab with fludarabine as opposed to those given only fludarabine as monotherapy has not only shown a significantly longer progression-free survival but also overall survival, using combined treatment (23). Further analysis of the high-risk genetic factors, i.e. mutation status of IgVH and the presence of cytogenetic aberrations del 11q22–q23 or del 17p13, confirmed the association of the former and especially the latter factor to a worsened prognosis (24).

Altogether, aberrations in the *TP53* and *ATM* seem to have an effect on the response of CLL cells to fludarabine or its combination with rituximab. It must be noted, however, that mutation analysis of *ATM* gene is not commonly available in routine clinics, while the presence of heterozygous *ATM* deletion in CLL cells commonly constitutes the only criteria for decision-making. In our study, we tested CLL cultures harboring heterozygous *ATM* deletion for sensitivity to fludarabine and also its combination with rituximab. The response was compared with *TP53*-abnormal and wt cases, to delimitate its relative significance. At the same time, we analyzed the activation of the p53 apoptotic pathway in corresponding samples.

Material and methods

Patient samples

Peripheral blood samples from CLL patients were obtained with informed written consent. Mononuclear cells were separated on the Histopaque gradient. Samples, which had been vitally frozen in DMSO and stored in liquid nitrogen, were exclusively used. In all cases, the proportion of CLL lymphocytes ($CD5^+$ $CD19^+$) in the sample exceeded 85%. The male to female ratio of the samples was 1.6 : 1.

Characterization of the ATM and TP53 abnormalities and IgVH status

Deletions at the 11q22–q23 (*ATM*) and 17p13 (*TP53*) loci were detected by I-FISH. Mutations in the *TP53* gene were identified by the yeast functional analysis (FASAY) coupled to sequencing. PCR and direct sequencing were used to analyze the IgVH rearrangements and mutation status. The procedures are as previously described (13). The *ATM* gene was directly sequenced from genomic DNA in two selected samples. Sixty-two coding exons were amplified according to previous report (25) with several modifications, which will be provided on request. Sequencing was performed on ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA, USA). The whole coding sequence and exon/intron splicing sites were analyzed.

Metabolic assay WST-1

Cells with viability exceeding 85% (trypan-blue staining) were seeded in 96-well plates in quadruplicates (5×10^5 cells per well, volume 200 μL); half of these cells was treated with a standard *in vitro* dose (10 μg/mL) of rituximab (Roche, Basel, Switzerland). Fludarabine (Bayer-Schering, Berlin, Germany) was administered after 72 hrs at four different concentrations (25, 6.25, 1.6, and 0.4 μg/mL) in small volume (20 μL) and each well was mixed thoroughly. The cell viability was assessed by the metabolic WST-1 assay (tetrazolium salt) (Roche, CH). 10 μL of this reagent was added per well 4 hrs before the end of cultivation. Absorbance at 450 nm was read on the SLT SPECTRA reader (SLT Instruments, Salzberg, Austria). Neither rituximab nor fludarabine alone had any effect on WST-1 reagent.

Western blot analysis

CLL cells were lysed for 30 min in an ice-cold NP40 buffer (150mM NaCl, 1% NP-40, 50mM Tris pH 8.0, 5mM EDTA pH 8.0) supplemented with protease and phosphatase inhibitors (Sigma, St. Louis, MO, USA). Proteins (30 μg) were run on 10% SDS-PAGE, and subsequently

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transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). After 2 hrs blocking in 5% non-fat milk or 5% BSA (in the case of anti-serine-15 p53 antibody) supplemented with 0.1% Tween 20 (Sigma), the membranes were probed with anti-p53 (DO-1, gift from Dr Vojtesek, MMCI Brno) or anti-PCNA (Chemicon, Billerica, MA, USA) antibodies for 2 hrs. The anti-serine-15 phosphorylated p53 antibody (Cell Signalling Technology, Danvers, MA, USA) was applied O/N at 4°C, with gentle shaking. After incubation with the HRP-conjugated secondary antibodies (DakoCytomation, Glostrup, Denmark) reactions were developed with chemiluminescent substrate (Lumi-Light Western Blotting Substrate; Roche) and visualized on chemiluminescent-detection film (Lumi-Film; Roche). The films were scanned using densitometer GS-800 (Bio-Rad) and quantity of the bands was assessed by the PDQUEST software (Bio-Rad).

Real-time quantitative PCR of the p53 target genes

Total RNA was isolated by the RNeasy Mini Kit with the DNase I digestion (Qiagen GmbH, Hilden, Germany). RNA (500 ng) was reverse transcribed in 20 μL volume using Superscript II RT (Invitrogen, Carlsbad, CA, USA). After adding 80 μL of water and mixing, 3 μL of cDNA were used for each TaqMan reaction. Q-RT-PCR assay was performed using a TaqMan technology and 7300 Real-Time PCR System (Applied Biosystems). The primer and probe sets were specific for the *BAX* and *BBC3* (*PUMA*) genes (TaqMan Gene Expression Assay; Applied Biosystems). Geometric mean of *TBP* (TATAA-box Binding Protein) and *HPRT1* (Hypoxanthine-guanine phosphoribosyltransferase) values served as an internal standard. These two transcripts (Pre-Developed TaqMan Assay Reagents; Applied Biosystems) were chosen as the most stable among the seven housekeeping genes. The Q-RT-PCR itself consisted of the following steps: incubation at 95°C for 15 min, and cDNA amplification for 45 cycles (denaturation at 95°C for 15 s and combined annealing/extension at 60°C for 1 min). Each sample was analyzed in duplicate. Sequence Detection Software (version 1.3.1; Applied Biosystems) was used to analyze the fluorescence emission data after PCR. The threshold cycle (Ct) values of each sample were exported to the Microsoft Excel for $2^{-\Delta\Delta Ct}$ analysis.

Determination of the CD20 receptor densities

The cells were washed in PBS and incubated for 15 min at 4°C with the following monoclonal antibodies: anti-CD20 conjugated with fluorescein isothyocyanate (FITC), anti-CD5 conjugated with phycoerythrine Cy-5 (PE-Cy5) (both from Caltag Laboratories, Inc., Burlingame, CA, USA) and anti-CD19 conjugated with phycoerythrine (R-PE) (Beckman-Coulter, Fullerton, CA, USA). Negative controls consisted of cells stained with isotype-matched FITC-labeled antibodies. The antibody-binding fluorescent microparticles Quantum™ FITC high level (Bangs Laboratories, Inc., Fishers, IN, USA) were added to each sample. The samples were analyzed at the flow-cytometer Cytomics™ FC500 (Beckman-Coulter) according to a protocol provided by the manufacturer of the micro-element particles (Bangs Laboratories). At least 20 000 cells in the lymphocyte gate (CD5+; CD19+) were evaluated in each sample. The median values of fluorescence intensity were converted to MESF using QUICK-CAL software and subsequently converted to ABC units using Simply Cellular Microbeads (both from Bangs Laboratories, Inc., IN, USA).

Statistical evaluation

The repeatability of the WST-1 assay was verified by performing a complete experiment with one wt sample four times. The results were compared using three-factor analysis of variance (for fludarabine, rituximab, and repetition) and the factor of repetition was not significant ($P = 0.39$). An impact of the *ATM* and *TP53* aberrations on the sensitivity of samples to clinical concentration of fludarabine was evaluated by Mann–Whitney *U*-test. The impact at different concentrations of fludarabine was assessed by two factorial ANOVA with the subsequent Tukey HSD *post-hoc* tests. Because of the non-homologous variances at different concentrations of fludarabine (tested by Levene's test), the statistical evaluation of rituximab pretreatment was performed with a non-parametric alternative to the two-factor analysis of variance, namely the two-factor analysis of variance using the rank transform property and ANOVA type of statistics (26). The proportions of sensitized samples, in different genetic subgroups, were compared by the chi-square test.

Results

Detection of TP53 and ATM abnormalities in CLL cells

Basic characterization of our samples, which was performed by FISH analysis and the yeast p53 functional assay, is summarized in Table 1. Other important biological variable, i.e. the mutation status of IgVH, closely mimicked the expected proportions in tested subgroups (6, 13) (Table 1).

CLL cells with aberrant ATM readily activate p53 protein after induction of DNA double-strand breaks

To get insight into functionality of ATM kinase in deleted samples, we analyzed a proportion of tested

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Table 1 Characterization of CLL samples

	TP53-abnormal $n = 20$	ATM-deleted $n = 21$	Wild-type $n = 18$
Type of the defect	del/mut: 13× mut/mut: 1× wt/mut: 6×	Heterozygous deletion in >50% cells (median 85%)	None
Stage of the disease	Rai 0, I, II: 8 Rai III, IV: 12	Rai 0, I, II: 11 Rai III, IV: 10	Rai 0, I, II: 12 Rai III, IV: 6
Proportion of IgVH unmutated /mutated samples (%)	78/22	100/0	50/50
Proportion of samples from previously treated patients (%)	45	48	28

cultures for p53 induction and phosphorylation on serine-15 residue after initiation of DNA double-strand (ds) breaks (3). Such a probing was shown to effectively set aside CLL samples with ATM dysfunction (4, 27, 28). Doxorubicin is a known inducer of DNA ds-breaks and has been previously proven to elicit ATM-dependent response (29). As it is evident from Fig. 1, all tested CLL cultures, two wt, and six with *ATM* deletion, clearly induced p53 protein. Densitometric analysis showed that one sample manifested partially (ATM-3) and one sample substantially (ATM-6) reduced phosphorylation on serine 15 residue: ratio of serine-15 p53 level to total p53 level achieved < 50% of the values assessed for wt samples (36% and 11%, respectively). This result suggests that only a minority of our *ATM*-deleted samples manifests a complete inactivation of this kinase. Importantly, this blot shows that a reduced ATM protein level, which should be expected in CLL cells with 11q22–23 loss of heterozygosity (LOH) (30), does not interfere with normal p53 induction after DNA-damage. Moreover, absence of the p53 serine-15 phosphorylation in ATM-6 sample together with clear induction of p53 protein surprisingly suggest that ATM may not be critical for p53 stabilization in CLL cells. To verify the presumed ATM inactivation in this sample, we sequenced all 62 coding exons of this gene. Indeed, three missense mutations, i.e. 875C > G, 2119T > C and 7375C > T, were identified in the remaining allele of ATM-6 sample (Fig. 2); the control sample with clearly detectable serine-15 phosphorylation (ATM-4) showed no DNA alteration within the coding sequence and splicing sites. The first identified mutation (P292R) occurred at a CpG site, which has already been reported to be mutated in A-T patient (25) the second one (P707S) is a well-known mutation associated with breast and thyroid cancers (25, 31, 32) and the last one (R2459C) is a C-T transition at a CpG site, which has not been, according to our knowledge, reported before. Although it is unusual to find three different mutations within one *ATM* allele, we assume that these DNA alterations were selected stepwise during intensive chemotherapy of the patient. Altogether, our western blot and sequencing data suggest that intact both copies of *ATM* are not required for p53 induction in CLL cells after administration of certain chemotherapeutics.

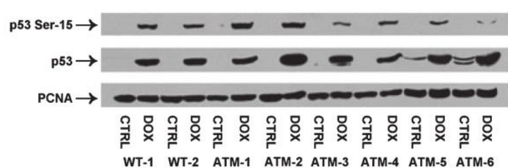

Figure 1 CLL cells with *ATM* deletion readily stabilize the p53 protein after induction of DNA-damage. Western-blot shows that all tested samples harboring *ATM* deletion ($n = 6$) induced the p53 protein after 16 hrs administration of doxorubicine (0.5 μg/mL), a known inducer of dsDNA breaks, although the sample ATM-3 exhibited partially and sample ATM-6 substantially reduced phosphorylation on p53 serine-15 residue. The latter sample was subsequently shown to harbor mutations in the remaining *ATM* allele (see text and Fig. 2). With the exception of one case (ATM-2), the proportion of CLL lymphocytes exceeded 91% in the samples.

TP53-abnormal but not ATM-deleted subgroup exhibited high resistance to fludarabine

Fludarabine, applied at four different concentrations (25, 6.3, 1.6, and 0.4 μg/mL), provided a clear concentration-dependent curve of viability after 48 hrs of cultivation in most of the 59 tested CLL samples, with the exception

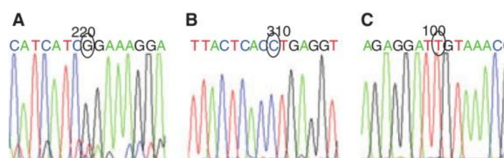

Figure 2 Mutation analysis of *ATM* gene in cells with aberrant serine-15 phosphorylation on p53 protein (sample ATM-6; see Fig. 1.). Mutations 875C≥G (A), 2119T≥C (B) and 7375C≥T (C) identified in exons 9, 15, and 52, respectively, are shown. Mutated nucleotides are circled.

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of some strongly resistant cases bearing *TP53* abnormalities. The basic sensitivity was assessed at a concentration of 1.6 μg/mL, as it had provided the most significant differences among individual cases. This selected concentration closely mimicked an *in vivo* situation, as a standard therapeutic dose of fludarabine (25 to 30 mg/m^2/d given over 30 min for 5 d) results in a plasma concentration of about 3 μmol/L (approximately 1μg/mL) at the end of each infusion (8). The viability of samples in individual tested subgroups is presented in Fig. 3. Samples harboring *TP53*-abnormalities were remarkably more resistant than remaining CLL samples ($P = 0.012$), while the *ATM*-deleted cells were even slightly more sensitive than wt cells (without statistical significance). However, when the average sensitivity of these two subgroups was assessed in all four tested concentrations of fludarabine, the difference in favor of the higher sensitivity of *ATM*-deleted cells was observed ($P = 0.019$) (Fig. 4). High resistance of the *TP53*-abnormal cohort was also noted in this setting (*TP53*-abnormal vs. wt $P = 0.006$; *TP53*-abnormal vs. del-*ATM* $P = 0.00002$). Importantly, while ATM-3 sample with partially reduced serine-15 phosphorylation on p53 protein reacted similarly to the other *ATM*-deleted cells, sample ATM-6, which showed clearly aberrant phosphorylation and was shown to harbor mutations in the remaining allele, was the most resistant case from the whole *ATM*-deleted tested series (Fig. 1). In fact, it was the most resistant sample from the whole *ATM*-deleted cohort with 85% viability (1.6 μg/mL F); the other five tested samples had an average viability of 59% (range 35–78%). In line with the previous study (15), we confirm that the status of non-deleted *ATM* allele influences the response of CLL cells to fludarabine.

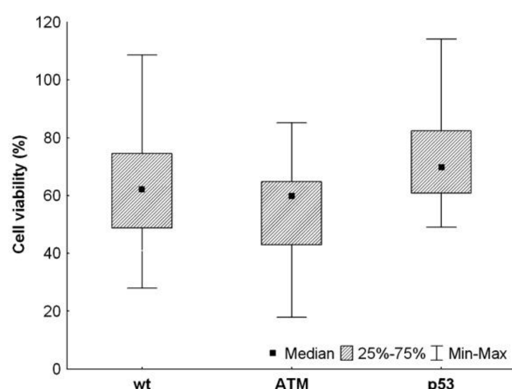

Figure 3 Viabilities of tested subgroups after administration of a clinically relevant concentration of fludarabine (1.6 μg/mL). The graph summarizes all tested samples (*TP53*-abnormal: $n = 20$; *ATM*-deleted: $n = 21$; wt: $n = 18$).

Fludarabine-induced expression of p53-downstream genes confirmed a borderline between the TP53-abnormal samples and remaining CLL

Fludarabine elicits p53-dependent apoptotic response in CLL cells both *in vivo* and *in vitro* (11); we focused on this pathway aiming again towards the basic observation of whether there is any significant difference between the *ATM*-deleted and wt-samples. p53 protein level was clearly stabilized after fludarabine administration in all tested wt ($n = 4$) and *ATM*-deleted ($n = 6$) samples. A remarkable induction was usually noted (Fig. 5), except for one sample from each subgroup. Subsequently, we assessed (via Q-RT-PCR) expression changes of two proapoptotic genes, i.e. *PUMA* and *BAX*; recently, their induction has been proven to be prominent and p53-dependent after *in vitro* treatment of CLL cells by fludarabine (33). The Q-RT-PCR data are summarized in Fig. 6. All wt-samples ($n = 12$) and all except one (ATM-12) *ATM*-deleted samples ($n = 14$) exhibited clear

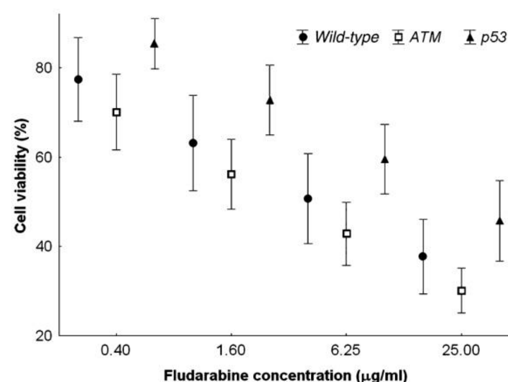

Figure 4 Viabilities (average ± 95% confidence interval) of tested subgroups after the administration of four different concentrations of fludarabine. The graph summarizes all tested samples as stated in Fig. 3.

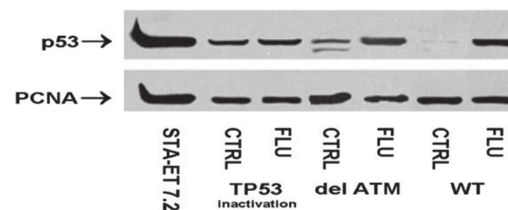

Figure 5 Fludarabine stabilizes p53 protein in *ATM*-deleted samples in a similar manner to wt cells. Western blot of an illustrative case is shown. The samples were treated for 24 hrs as follows: CTRL: no treatment; FLU: 3.6 μg/mL of fludarabine; STA-ET 7.2: neuroblastoma cell line with mutated *TP53* (control for WB).

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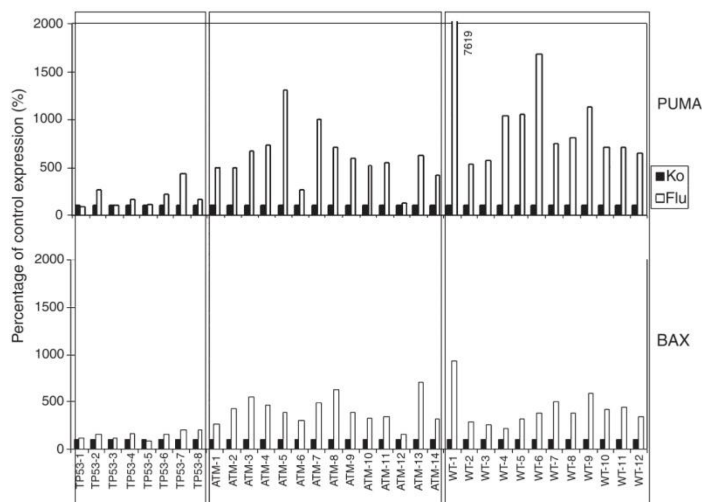

Figure 6 *ATM*-deleted and wt cells induce the p53-downstream target genes in a similar manner after fludarabine exposure. Expression values assessed by Q-RT-PCR were related to untreated control (set up as 100%) in every sample. Two-fold increase in the expression (one cycle-change in Q-RT-PCR) was considered to be positive induction. The cells were treated as in the WB experiment in Fig. 4.

induction of these genes after fludarabine exposure. Defective ATM-12 sample belonged among three the most resistant *ATM*-deleted samples. As we lacked frozen cells from the corresponding patient, any other analyses could not be performed in this case. The previously discussed sample ATM-6, which harbors mutations in remaining *ATM* allele and exhibits defective serine-15 phosphorylation on p53, induced both genes, although *PUMA* only weakly. It further confirms that functional ATM may not be required for induction of p53 and (at least some) critical pro-apoptotic downstream genes. Induction of these genes in *TP53*-defected samples was substantially impaired, although not completely absent in all samples (Fig. 6).

A proportion of samples sensitized to fludarabine by rituximab was insignificantly decreased in both aberrant subgroups

The whole cohort of 59 CLL cultures was also tested for rituximab-mediated sensitization to fludarabine. As expected (34), in our setting, which omits the complement pathway, the rituximab alone used at standard *in vitro* concentration 10 μg/mL (19) had virtually no significant effect on the viability of CLL cells, which usually varied within the range of $\pm 15\%$ in individual samples by the end of experiment (day +5). The effect of rituximab pretreatment on fludarabine activity was examined in all four concentrations of fludarabine. Statistical evaluation was performed when at least two neighboring concentrations had been potentiated. By using this approach, a significant ($P < 0.01$) sensitization effect was noted in 30% (6/20) of *TP53*-abnormal, 29% (6/21), of *ATM*-deleted and 50% (9/18) of wt samples. The examples of positively sensitized samples are presented in Fig. 7. Intriguingly, rituximab slightly induced metabolic activity in many samples albeit they were simultaneously sensitized for fludarabine (Fig. 7B, C, D). Although aberrant samples were less frequently sensitized, this difference was not significant ($P = 0.22$). Primary reaction to fludarabine influenced the presence of sensitization effect elicited by rituximab; sensitization was stronger when samples were more resistant to fludarabine ($P = 0.002$; Spearman rank order correlation). Sensitization was more frequently observed among samples from previously untreated (42%) rather than treated (26%) patients ($P = 0.077$; chi-square test). Wt samples, which were evenly distributed between the *IgVH*-unmutated and mutated cohorts, did not manifest any trend in response to fludarabine or sensitization by rituximab.

Density of the CD20 receptor has not determined the sensitization effect

Theoretically, the observed sensitization effect could be derived from an activity related to the CD20 receptor or could depend on some general apoptotic pathway. The receptor density had been assessed in twenty-three samples (eight of them were sensitized by rituximab and fifteen were sensitization-refractory) and was shown to be quite heterogenous among the samples (maximum 42 423 and minimum 4898 ABC units). However, the medians of both subgroups were almost identical (14 288 units vs. 14 077, respectively) indicating no impact of this parameter on the sensitization process.

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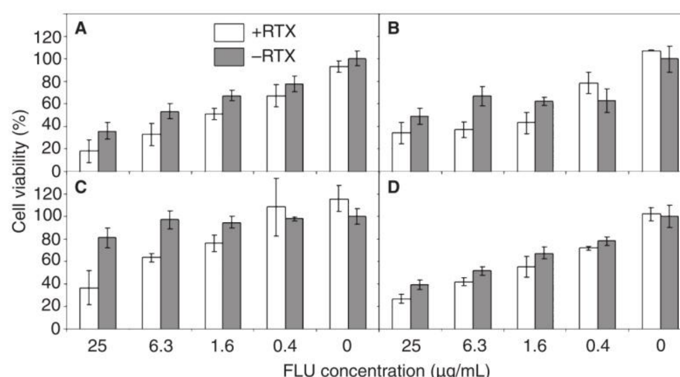

Figure 7 Examples of rituximab-mediated sensitization to fludarabine in *ATM*-deleted (A, B) and wt samples (C, D). The cellular viability was assessed by WST-1 assay. The values were set against the untreated control (RTX−/FLU−). Each measurement was performed in quadruplicates, standard deviations are provided. Statistical significance was achieved for all four concentrations together in samples (A) and (D); for the three highest concentrations in samples (B) and (C).

Discussion

It is now widely accepted that defects in the *TP53* gene confer significant resistance to CLL cells in their response to chemotherapy (4, 5, 7, 9, 10, 24, 35). However, situation concerning ATM kinase, the p53 partner acting in response to DNA-damage, is less clear. This protein is mutated in autosomal recessive disorder ataxia-telangiectasia, which is characterized by prominent radiosensitivity and predisposition to lymphoid tumors, among other symptoms (36). Although it has been proven that mutations in the *ATM* gene lead to impaired up-regulation of the p53 protein in CLL cells (and may hence represent an alternative to its dysfunction) (4, 27, 28), the *ATM*-affected cells usually exhibit a better response to chemotherapy than cells with *TP53* aberrations (4, 12). Several studies also consistently confirm a substantially better survival in patients harboring 11q22–q23 (*ATM*) deletion compared with those having deletion 17p13 (*TP53*) (2, 12, 24). To complicate the situation further, not every 11q deletion can be associated with ATM inactivation in the CLL cells, as a mutation of the remaining allele was reported to occur in only 23% (12) to 36% of patients (15). In each case, however, the heterozygous 11q22–q23 status obtained by I-FISH is the only information available in routine clinical practice. Therefore, our study aimed to delineate its relative significance in primary response to fludarabine and its combination with rituximab, comparing to *TP53*-abnormal and wt cells.

Concerning fludarabine itself, we may readily confirm (35) that the *TP53*-abnormal subgroup is – as a whole – significantly more resistant to this drug than remaining CLL cells *in vitro*. In spite of this and in line with the previous *in vivo* observation (37), many *TP53*-abnormal samples somehow reacted to fludarabine confirming an alternative pathway in resting lymphocytes (38). In fact, there were just two samples, which were absolutely resistant to fludarabine (showing viability >90% even for the highest concentration used, i.e. 25 µg/mL): one harbored LOH and mutation Y220C and the second harbored LOH and mutation Y234C. Interestingly, in collaboration with another laboratory, we have recently reported (39) that these two codons are among prominent mutation hotspots in CLL. Regarding the other samples, some of them (namely the three *ATM*-deleted and four wt) were also highly resistant to fludarabine, with viability over 75% after 48 hrs of fludarabine exposure (clinically relevant dose of 1.6 µg/mL). As our complex mutation and deletion analysis of the *TP53* gene had been very thorough including a very sensitive functional assay, it is highly unlikely that some aberrations in this gene were overlooked; therefore, the observed resistance is likely caused by other defects.

We did not observe higher resistance of *ATM*-deleted samples in comparison to wt cells. On the contrary, *ATM*-deleted samples were even more sensitive. Although ATM defects in CLL cells are commonly thought to be associated with increased resistance to radiation or chemotherapeutic agents, our observation fits with some other studies showing that the inhibition of ATM kinase leads to sensitization of cancer cells to ionizing irradiation and DNA-damaging agents (40, 41). In CLL, a marked resistance of the cells with *ATM* inactivation (deletion with mutation) (15), *ATM* mutation (42), and low level of ATM protein (43) to fludarabine has already been reported. Although it has been observed that LOH at the 11q22–q23 locus leads to an almost complete absence of ATM protein in CLL cells (30), our data do not support the view that the heterozygous *ATM* deletion enhances resistance to fludarabine. Standard activation of the p53-regulated genes *PUMA* and *BAX* in 12/14 *ATM*-deleted samples suggests that majority of our cases preserved critical pro-apoptotic pathway. As we selected cases with *ATM* deletions and

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only their minority harbors mutation of the remaining allele (15), our observation is partially in line with the report by Austen *et al.* (44), which showed that 11q-deleted tumors with the second wt allele have normal DNA-damage response. In addition, our example of normal p53 stabilization in the cells with impairment of both *ATM* alleles and absent serine-15 p53 phosphorylation indicates that this key pro-apoptotic activity does not depend entirely on ATM in CLL cells. Indeed, previous comprehensive research has confirmed that a wide range of chemotherapeutic drugs does not depend on intact ATM and utilizes other kinases (45). Moreover, the very recent paper by Gine *et al.* (46) shows that some apoptotic mechanisms, which operate in CLL cells after administration of fludarabine, namely the histone H1.2 cytosolic release, depends on p53, but not ATM.

Our study also brought up some interesting observations concerning the sensitization of CLL cells to fludarabine by rituximab. This sensitization was tested in plasma-free medium, which enabled us to monitor rituximab-mediated support to fludarabine in apoptosis; previously studied complement pathway (22) was omitted in our setting. Therefore, instead of observing an approximately additive cytotoxic effect of these two agents (22), we noted that rituximab somehow supported fludarabine, without being directly cytotoxic to the cells. It suggests that the combined effect may not only be derived from the complementary mechanisms of action. As the CD20 level had no effect on rituximab-mediated support to fludarabine, it seems that the receptor pathway had a slight role in our study. A proportion of the sensitized samples was somewhat lower in aberrant subgroups, but not significantly. It suggests that some apoptotic mechanisms out of the central p53 pathway were probably employed.

In summary, our *in vitro* data suggests that the presence of heterozygous *ATM* deletion does not mean *per se* that the cells are resistant to fludarabine or its combination with rituximab. For clinical consideration it must be emphasized, however, that we analyzed only an immediate response of the cells, while the therapeutical benefit depends obviously on the long-lasting effects of the therapy. In this sense, we propose that an inferior outcome of CLL patients harboring *ATM* deletion after administration of therapy may be derived from a higher capacity of these cells to repopulate blood compartments from a remaining malignant clone, as opposed to being a consequence of an ineffective initial response. This view is also supported by our observation that only one out of nine *ATM*-deleted patients from our *in vitro* cohort was refractory to fludarabine-based chemotherapy *in vivo*; the regimens F, FC (F + cyclophosphamide), and RFC (FC + rituximab) were considered; time period of six months before/after the testing; evaluation was performed according to the up-dated NCI criteria (47). Also the cells from the refractory patient reacted normally to fludarabine *in vitro* (in a concentration-dependent manner). It was in a contrast to the *TP53*-abnormal cohort, which reacted poorly both *in vitro* and *in vivo*; 5/10 patients showed a stable disease or progression after administration of fludarabine-based regimens. Although not directly refractory, the 11q22–23-deleted CLL cells were reported to manifest cell cycle and apoptosis deregulation because of other missing genes, in addition to *ATM* (48). In this respect, it might be worth considering consolidation and maintenance regimen for high-risk CLL patients treated with fludarabine. Moreover, such a regimen utilizing rituximab for this purpose, has already been successfully tested and very recently reported (49).

Acknowledgements

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Conflict of interest

M.D., Y.B., S.P., J.M. and M.T. have had the costs of participating in scientific meetings reimbursed by the pharmaceutical industry.

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Distinct in vitro sensitivity of p53-mutated and ATM-mutated chronic lymphocytic leukemia cells to ofatumumab and rituximab

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Abnormalities in *ATM* and *TP53* genes represent important predictive factors in chronic lymphocytic leukemia (CLL); however, the efficacy of CD20 targeting immunotherapy is only poorly defined in the affected patients. Therefore, we tested the *in vitro* response to ofatumumab (OFA) and rituximab (RTX) in 75 CLL samples with clearly defined p53 or ATM inactivation. Using standard conditions allowing complement-dependent cytotoxicity, i.e., 10 μg/mL of antibodies and 20% active human serum, we observed clear differences among the tested genetic categories: *ATM*-mutated samples (n = 17) represented the most sensitive, wild-type samples (n = 31) intermediate, and *TP53*-mutated samples (n = 27) the most resistant group (*ATM*-mut vs. *TP53*-mut: $P = 0.0005$ for OFA and $P = 0.01$ for RTX). The response correlated with distinct levels of CD20 and critical complement inhibitors CD55 and CD59; CD20 level median was the highest in *ATM*-mutated and the lowest in *TP53*-mutated samples (difference between the groups $P < 0.01$), while the total level of complement inhibitors (CD55 plus CD59) was distributed in the opposite manner ($P < 0.01$). Negligible response to both OFA and RTX was noted in all cultures (n = 10) tested in the absence of active serum, which strongly indicated that complement-dependent cytotoxicity was a principal cell death mechanism. Our study shows that (1) common genetic defects in CLL cells significantly impact a primary response to anti-CD20 monoclonal antibodies and (2) *ATM*-mutated patients with currently poor prognosis may potentially benefit from immunotherapy targeting CD20. **© 2014 ISEH - International Society for Experimental Hematology. Published by Elsevier Inc.**

Introduction

Chronic lymphocytic leukemia (CLL) represents the most frequent leukemia in the western world and still remains an incurable disease. The worst prognosis is associated with the presence of 17p deletion (17p-) and/or *TP53* mutation [1,2]. The inability of dysfunctional p53 protein to induce apoptosis properly seems to be a primary reason for the observed treatment resistance [3]. Respective patients respond poorly to DNA damage-based therapy [4] but also have the worse response rates and response duration to chemoimmunotherapy [5,6]. Heterozygous 11q22-23 deletion (11q-) involving *ATM* (Ataxia-Telangiectasia Mutated) gene frequently occurs in younger individuals, and also negatively affects patients' outcome [1,7]. *ATM* abnormalities (11q- and/or mutations) are associated with extensive lymphadenopathies and have been identified as the most common unfavorable defect at CLL diagnosis [8]. Interestingly, two recent clinical studies dealing with the chemoimmunotherapy regimens noted 11q- affected patients' favorable response to this kind of treatment [9,10]. From the point of view of genetic defects it is important to consider that approximately one-third of patients with 11q- harbor *ATM* mutation on the other allele and only these patients manifest real ATM inactivation [11,12]. Owing to cooperative activity of ATM and p53 proteins in DNA-damage response (DDR) pathway [13], defects in respective genes are commonly mutually exclusive in CLL [3].

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Ofatumumab (OFA) and rituximab (RTX) are type I anti-CD20 monoclonal antibodies (Mabs) eliminating tumor cells through complex mechanisms principally involving [14]: (a) complement-dependent cytotoxicity (CDC), which has a major effect in type I Mabs [15]; (b) antibody-dependent cellular cytotoxicity mediated by NK cells and macrophages; (c) direct cell death induced either by cross-linking of Mab with CD20 or via homotypic adhesion. RTX is a chimeric human-mouse Mab selectively binding large loop of CD20 antigen on the B-cell surface [16]. OFA is fully humanized Mab binding a different epitope than RTX (both large and small loop of CD20 molecule, more closely to the cellular membrane) [17]. This contributes to better translocation of CD20 molecules into membrane lipid rafts resulting in greater CDC compared to RTX [18]. Currently, RTX is involved in the most potent therapeutic regimen in CLL, i.e., FCR (fludarabine, cyclophosphamide, RTX); this regimen seems to be effective in all genetic groups except patients with 17p- [6,9]. OFA shows good efficacy in patients with CLL resistant to fludarabine and alemtuzumab or manifesting with bulky lymphadenopathy [19], and exhibits satisfactory effect in combination with chemotherapy compared to historical trials evaluating RTX with chemotherapy [20].

Despite the reported observation [9] that 17p- and 11q-affected CLL patients show a different response to chemoimmunotherapy involving monoclonal antibodies neither OFA nor RTX have been tested on their own on CLL cells *in vitro* or in patients with clearly defined p53 and ATM dysfunction. In our study, we assessed *in vitro* response of well-characterized CLL cells with *TP53* or *ATM* mutations to these Mabs. The testing was performed under conditions enabling CDC to resemble *in vivo* mechanism of Mabs action.

Materials and methods

Patients' samples

The study was performed using samples from 75 CLL patients. Basic patients' characteristics are summarized in Table 1. Peripheral blood mononuclear cells (PBMNC) were obtained with written informed consent; leukemic cells' proportion (CD5+/CD19+) was assessed by flow cytometry and exceeded 90% in all samples. PBMNC were isolated using gradient centrifugation with Histopaque-1077 (Sigma, Steinheim, Germany) and stored in liquid nitrogen.

Healthy donors' samples

B-cells from healthy donors, which have substantially higher CD20 density than CLL cells, were used to demonstrate the activity of OFA and RTX in our experimental system. The cells were separated from buffy coats using gradient centrifugation with Ficoll-Paque™ Plus (GE Healthcare, Uppsala, Sweden) and immunomagnetic separation according to manufacturer's instructions (B Cell Isolation Kit II, human and LD columns; Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated B-cells were vitally frozen in liquid nitrogen.

Table 1. Biological and clinical characteristics of CLL patients

Total number of patients: n = 75	
Age at diagnosis: median, range (years)	62 (38–81)
Sex (M/F)	49/26
Clinical stage at the time of blood collection (%)	
Low risk: Rai 0	15
Intermediate risk: Rai I, II	32
High risk: Rai III, IV	53
Therapy status (at the time of blood collection; %)	
Treated	43
Untreated	57
IgVH status (%)	
Mutated	15
Unmutated	85
Hierarchical I-FISH at the time of blood collection (%)	
17p-	24
11q-	39
12+	1
Sole 13q-	27
Normal	9
TP53 mutation (%)	36
ATM mutation (%)	23

Samples' distribution into individual genetic groups

All CLL samples were previously characterized for *TP53* and *ATM* mutations using functional screening and sequencing [12,21]. Tested samples were divided into three functionally distinct groups according to the presence of (1) p53 dysfunction defined as functionally-proven inactivating *TP53* mutation regardless of 17p- occurrence (n = 27) (2) ATM dysfunction defined as functionally-proven inactivating *ATM* mutation regardless of 11q- occurrence (n = 17), and (3) functional ATM/p53 pathway (wt) (n = 31). The last category consisted of samples with sole 11q- and intact second *ATM* allele and completely *ATM*/*TP53*-wt samples. Samples from previously treated patients were more frequent in *TP53*-mutated group (59%) than in *ATM*-mutated (29%) or wt group (35%).

Ofatumumab and rituximab in vitro testing

Vitally frozen cells were uniformly used for the testing. For metabolic WST-1 assay measuring cellular viability (Roche, Mannheim, Germany), cells were seeded in 96-well plates in quadruplicates (500,000 cells per well) and cultivated 48 h in the presence of 10, 20, and 30 µg/mL of OFA (GlaxoSmithKline, Brentford, Middlesex, UK), RTX (Roche, Basel, Switzerland) and nonspecific immunoglobulin (IgG; Instituto Grifols, Barcelona, Spain) as a negative control. Twenty percent active human serum was added to allow CDC and the longer cultivation time was chosen to employ potential other cell death mechanisms. Final cell viability was assessed at 450 nm on SLT. Spectra reader (SLT Lab Instruments, Salzburg, Austria). The testing not involving active human serum was performed in the same manner.

Propidium iodide (PI; Exbio, Prague, Czech Republic) was used to evaluate cell death using flow cytometry. Cells were treated with 10 µg/mL Mabs in the same experimental setting as for WST-1 assay. PI binds to double stranded DNA, but is excluded from viable cells with intact plasma membranes. PBMNC, 300,000 per tube, were stained according to manufacturer's instructions. Data acquisition with subsequent analysis was performed on BD

FACSAria III (BD, Franklin Lakes, NJ) using the BD FACSDiva software (BD Biosciences).

CD20, CD55, and CD59 level determination

Flow cytometry analysis was done using BD FACSAria III (BD, Franklin Lakes, NJ). 300,000 PBMNC cells were collected for each reaction. Cells were stained with the following combination of Mabs: CD19/CD20, CD19/CD55/CD59 [mouse anti-CD59 FITC conjugated antibody (Invitrogen, Camarillo, CA), anti-CD55 PE-Cy7 conjugated antibody (BioLegend, San Diego, CA), anti-CD20 PE conjugated antibody (BD Quantibrite, San Jose, CA), anti-CD19 APC-eFluor780 conjugated antibody (eBiosciences, San Diego, CA)]. Mean fluorescence of tested antigens on viable CD19+ cells was used for Antibody Binding Capacity (ABC) calculation using a pipeline by Bangs Laboratories (http://www.bangslabs.com/products/quickcal).

Statistical analysis

Mann-Whitney U-test was used to compare (a) CLL cells' viability after treatment with Mabs in the tested genetic groups, and (b) CD20, CD55, and CD59 levels in the tested genetic groups. Nonparametric Spearman statistical evaluation was used to correlate the effect of Mabs with the CD20 density in individual samples. Nonparametric Kruskal-Wallis ANOVA test was used to compare cell death in individual genetic groups in the testing based on PI staining.

Results

ATM-mutated CLL cells exhibit high sensitivity to ofatumumab and rituximab

Overall viability analysis (WST-1 assay) was used to cover all possible cell death mechanisms evoked by Mabs. The following principal observations obtained on 75 CLL cultures were recorded: (1) Concentration 10 μg/mL was sufficient for both OFA and RTX testing, higher concentrations produced very similar effect in virtually all cultures (data not shown); (2) Viability after 48 h treatment with Mabs (10 μg/mL) in individual samples was highly variable and ranged from 21–100% for OFA and from 31% to 100% for RTX (untreated control set as 100% in each sample); (3) There were clear differences in sensitivity to antibodies among the tested genetic groups (Fig. 1). In the case of OFA, median viability was 92% in *TP53*-mutated group, 58% in *ATM*-mutated, and 79% in wild-type (wt) samples; significantly, this difference was also obvious in analysis limited to samples from untreated patients, i.e., 99%, 52%, and 79% among the above mentioned groups, respectively. Regarding RTX, the median viability in all samples was 95% in *TP53*-mutated group, 71% in *ATM*-mutated, and 83% in wt samples. Again, the output was very similar in analysis limited to untreated patients: 88%, 65%, and

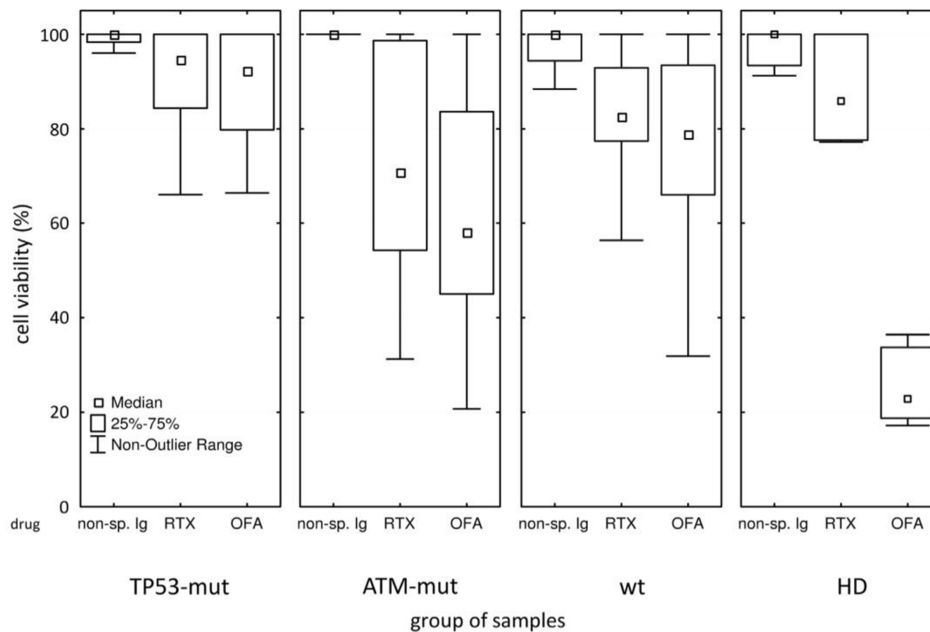

Figure 1. Viability of CLL cells after 48 h treatment with antibodies. The graph summarizes 75 CLL samples tested for antibodies' concentration 10 μg/mL in the presence of 20% active human serum. The cell viability was related to fully untreated control (set as 100%) in all cultures. HD: healthy donors (n = 10); TP53-mut: samples with p53 dysfunction (n = 27); ATM-mut: samples with ATM dysfunction (n = 17); wt: samples with functional p53 and ATM (n = 31); nonsp. Ig: nonspecific immunoglobulin (negative control); RTX: rituximab; OFA: ofatumumab.

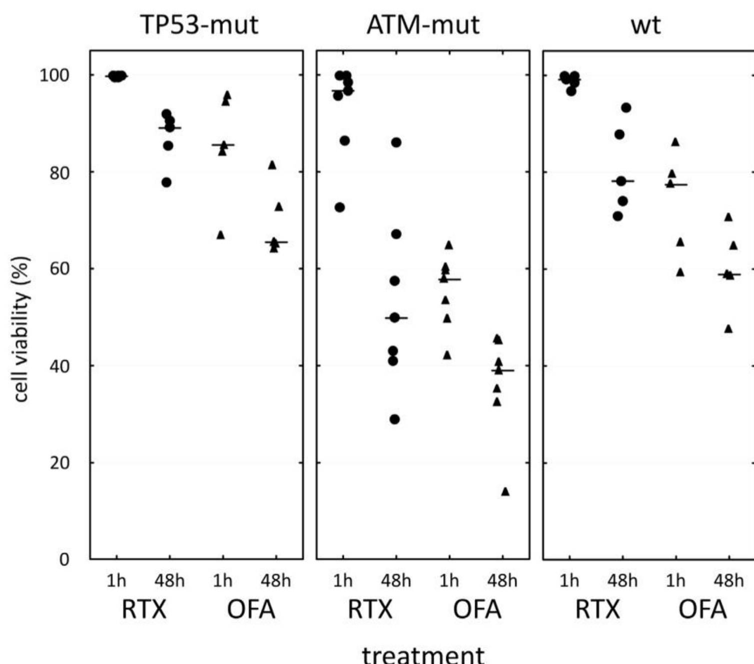

Figure 2. Cell death assessed by PI staining using flow-cytometry. The graph shows individual response in 17 CLL samples. TP53-mut: samples with p53 dysfunction (n = 5); ATM-mut: samples with ATM dysfunction (n = 7); wt: samples with functional p53 and ATM (n = 5). Mabs concentration: 10 µg/mL. Treatment was performed in the presence of 20% active serum. RTX = rituximab; OFA = ofatumumab.

84%, respectively. Thus, the *TP53*-mutated group was substantially more resistant than both wt samples ($P = 0.01$ for OFA and 0.006 for RTX) and particularly than *ATM*-mutated samples ($P = 0.0005$ for OFA and 0.01 for RTX). Essentially, when we separately analyzed samples with 11q- but intact second *ATM* allele (n = 14), the effect was quite poor and similar to completely wt samples (median viability 83% for OFA and 82% for RTX) (Supplementary Fig. E1). Therefore, of all the tested groups, considerable cell death induction was present only in the one with *ATM* mutations (ATM dysfunction).

To ascertain whether the observed differences in sensitivity cannot be partially accounted to different propensity to spontaneous *in vitro* cell death, we compared the basic samples' viability (PI staining using flow-cytometry) assessed immediately before treatment with antibodies. The median viability was very similar in the tested genetic groups, i.e., 73%, 74%, and 77% in *ATM*-mutated, wt, and *TP53*-mutated samples, respectively. Thus, this parameter should not have an impact on the noted differences.

CDC constituted a major cell death mechanism after OFA and RTX administration

To gain an insight into the basic cell death mechanism evoked by Mabs, we employed the strict cell death assay (PI staining) in a subset of cultures (n = 17) and analyzed two distinct times of treatment: 1 h presumably allowing only CDC and 48 h enabling also any other potential cell death mechanisms like apoptosis. The results are summarized in Figure 2. In cases of OFA, the cell response was already clearly evident after 1 h, with markedly different sensitivity of *ATM*-mutated and *TP53*-mutated cultures ($P = 0.0036$). After 48 h, the distribution of viability in individual genetic groups was very similar to WST-1 assay, with obviously sensitive *ATM*-mutated samples (*ATM*-mutated vs. wt $P = 0.064$; *ATM*-mutated vs. *TP53*-mutated $P = 0.0017$). Concerning RTX, the response was virtually none after 1 h. After 48 h, the distribution again resembled WST-1 assay, with a significant difference between the sensitive *ATM*-mutated and resistant *TP53*-mutated samples ($P = 0.015$). Taken together, the PI assay strongly suggested that direct lysis by a complement is the primary cell death mechanism in the case of OFA.

Since CDC requires the presence of a functional complement, we still tested 10 cultures exhibiting good response to Mabs with active serum (final viability ~50% in WST-1 assay), presently in the absence of active serum. Under this condition, all 10 cultures displayed viability >90% after 48 h treatment with OFA and RTX, which further indicated CDC as a principal cell death mechanism for both

antibodies. In addition, there was a trend towards greater effect of OFA on cell viability compared to RTX, which was noted in all comparisons we performed, i.e., in all tested concentrations of Mabs and in all genetic groups (data not shown); this observation again points to CDC, as OFA is known to induce more efficient lysis than RTX [22].

Concerning the cross-sensitivity of individual samples to OFA and RTX, we mostly observed either a response or complete resistance to both antibodies. There were, however, some rare samples that responded to OFA, but not to RTX; the opposite variant did not occur in our testing.

CD20, CD55, and CD59 levels impacted the response to antibodies

The efficacy of antibody-mediated CDC has been previously associated with CD20 expression in CLL cells [23]. Therefore, we analyzed the CD20 level in relation to (a) the presence of genetic defects, and (b) efficacy of lysis in individual samples. Besides the CD20 expression, we tested the levels of major complement inhibitors CD55 and CD59 also substantially impacting the CDC [24].

The CD20 density varied substantially among individual samples (n = 54) (range 2,450–115,627 ABC units). CD20 level medians in the tested groups were the following: 34,281 in *TP53*-mutated samples (n = 15), 72,001 in *ATM*-mutated (n = 14), and 57,694 in wt samples (n = 25) (Fig. 3). Thus, the CD20 level was on average substantially lower in the *TP53*-mutated group in comparison with both *ATM*-mutated and wt samples ($P < 0.01$). Concerning the analysis in all individual samples (n = 54) regardless of their genetics, we observed a weak, albeit highly significant correlation between the better cell lysis and higher CD20 density (R = −0.555 for OFA and −0.350 for RTX; P = 0.000013 and 0.0094, respectively) (Fig. 4).

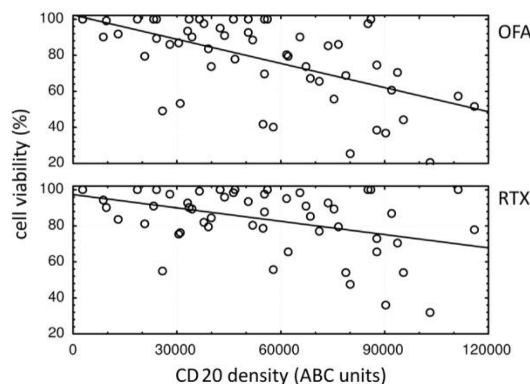

Figure 4. Correlation between CD20 density and cell viability. The graph summarizes 54 CLL cultures. RTX = rituximab; OFA = ofatumumab.

In line with the observed good, intermediate, and poor lysis in the tested groups, the expression of complement inhibitors (sum of CD55 and CD59) was the lowest in *ATM*-mutated samples (n = 7; median 76,939 ABC units), intermediate in wt group (n = 13; 94,758), and the highest in *TP53*-mutated samples (n = 9; 120,148) (*TP53*-mut vs. wt P = 0.013 and vs. *ATM*-mut P = 0.0043) (Fig. 5). The same trend was noted for both receptors analyzed separately (data not shown).

Discussion

Several factors determining the effectiveness of OFA and RTX in killing CLL cells have been described. Among them, eliminating CD20 molecules by the immune system after the exposure of a patient to the antibody seems to be

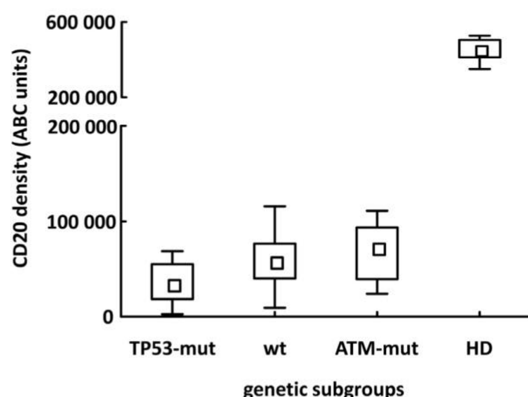

Figure 3. CD20 level (ABC units) in the tested genetic groups. HD: healthy donors (n = 10); TP53-mut: samples with p53 dysfunction (n = 15); ATM-mut: samples with ATM dysfunction (n = 14); wt: samples with functional p53 and ATM (n = 25).

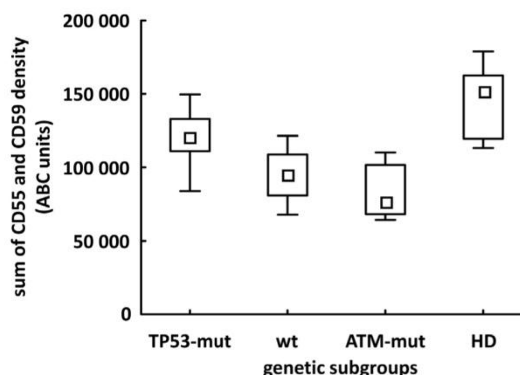

Figure 5. Sum of CD55 and CD59 density (ABC units) in the tested genetic groups. HD: healthy donors (n = 10); TP53-mut: samples with p53 dysfunction (n = 9); ATM-mut: samples with ATM dysfunction (n = 7); wt: samples with functional p53 and ATM (n = 13).

critical [25,26]. Although the recurrent genetic defects in CLL cells presumably also have an effect on patient outcome after CD20-based chemoimmunotherapy [5,6], their contribution to a primary CLL cells' resistance has not been extensively studied. Therefore, we assessed *in vitro* sensitivity to OFA and RTX in our series of well-characterized samples harboring high risk p53 or ATM dysfunction.

Using two different approaches for cell death assessment, we noted high resistance of *TP53*-mutated CLL cells to both antibodies. By contrast, the marked sensitivity of ATM-inactive samples was intriguing. Although ATM is tightly connected with the regulation of apoptosis and does not have an established role in cell lysis by complement, we demonstrated that CDC is highly likely to be a primary cell death mechanism in our study. We showed it more clearly for OFA, in which considerable cell death was very quick (readily detectable already 1 h after treatment), which is in line with the expected direct cell lysis by complement. In the case of RTX, the cellular response was much slower, and we demonstrated the critical role of complement primarily through the null response if the active serum is absent. This difference between the active complement-mediated CLL cells' cytotoxicity and no cytotoxic response when the serum is omitted has been repeatedly reported for RTX [23,24,27].

The active serum initiated CDC in most of the cultures, but generally we noted rather limited effects of both OFA and RTX on CLL cells' viability. Altogether in 75 cultures tested using WST-1 assay, a median viability at the end of the testing (48 h, 10 μg/mL Mabs) was 79% for OFA and 83% for RTX compared to the untreated control. This mild effect was in line with the responses observed when CLL cells had been tested with RTX in the presence of 25% active serum [24] or using the whole blood assay involving both active serum and effector cells able to develop antibody-dependent cellular cytotoxicity [23].

The response to Mabs was particularly weak in *TP53*-mutated cells, as 67% (18/27; OFA) or 59% of respective samples (16/27; RTX) showed final viability >90%. Thus, the poor primary CLL cells' response to anti-CD20 monoclonal antibodies is another type of resistance resulting from p53 inactivation, in addition to the previously reported resistance to chemotherapy involving alkylating agents or nucleoside analogs [28], to chemoimmunotherapy combining rituximab with fludarabine [5] and to a highly effective (in *TP53*-wt patients) FCR regimen [6,9]. While there are numerous papers explaining the resistance of *TP53*-mutated CLL cells to DNA-damaging chemotherapy, the hypothetical link between p53 activity and complement regulation or CDC is elusive. Indeed, in this respect, up-to-date knowledge comprises only a potential regulation of CD59 expression by p53; the gene coding for CD59 contains two consensus binding sites for p53 protein [29,30]. Based on such sparse information, it is currently impossible to speculate more about potential mechanisms accounting for the resistance of p53-mutated CLL cells to OFA and RTX.

On the other hand, the samples harboring ATM dysfunction owing to inactivating *ATM* mutation were, on average, by far the best responding group; 53% (9/17; OFA) or 47% of samples (8/17; RTX) showed a final viability reduction >40%. Interestingly, our results could potentially help to explain why patients with 11q- (in contrast with those having normal karyotype) benefited from rituximab when treated with FCR in the clinical trial comparing this regimen to FC [9]. The significantly better outcome could potentially be attributed to patients having *ATM* mutation in addition to 11q-. In this respect we can suggest analogical extrapolation to the study by Tsimberidou *et al.* [10], who also noted good prognosis after RTX-based chemotherapy in patients with 11q-.

In our study, *ATM*-mutated CLL cells' sensitivity to OFA and RTX could logically be viewed as a consequence of a relatively (considering CLL) high CD20 level together with the low level of complement inhibitors, when compared to the remaining samples studied. To the best of our knowledge, there are no published data on the ATM's role in CD20 level regulation or on ATM participation in the complement system, including its inhibition. Therefore, it is currently difficult to hypothesize relevantly about a reason for these quite surprising associations. One theoretical possibility would be that ATM dysfunction and the higher CD20 level are somehow attracted to one another due to their mutual, positively selected contribution to enhanced cell proliferation. Inactivation of ATM protein, one of the critical cell cycle regulators, supposedly results in deregulated proliferation [31]; CD20 is mechanistically a channel regulating a calcium flux and, consequently, B-lymphocyte proliferation [32]. Another option would be to consider a process that would enhance CD20 as a consequence of ATM absence. One such possibility could be the level of oxidative stress in a cell; it has been reported that CD20 expression is elevated by oxidative stress [33] and, at the same time, it is well known that cells derived from patients having inactive ATM consistently manifest increased oxidative stress [34,35]. However, we must also admit the weaknesses of this hypothesis which are the following: (a) the oxidative stress can also rise with p53 deficiency [36] and—as is clear from our study—there is a low CD20 level in corresponding samples, and (b) it is not entirely clear whether CD20 is indeed enhanced in ATM deficient CLL cells; since CD20 is severely shifted (reduced) from normality in all CLL samples (see healthy B-lymphocytes in Fig. 3), it is difficult to be sure that CD20 is really up-regulated in ATM deficient CLL and not e.g., further down-regulated in wt and *TP53*-mutated samples.

In summary, our key results are: (1) Effects of Mabs on CLL cells' viability were caused primarily by CDC; (2) CDC was influenced by the level of surface molecules CD20, CD55, and CD59 and median levels of these critical

molecules differed in tested categories; and (3) Lysis was distinct in individual genetic groups, clearly the most prominent in *ATM*-mutated samples and almost absent in *TP53*-mutated cells. It has been already reported [37] that the presence of particular genetic defect, namely +12, predisposes CLL patients to a good response to RTX-based therapy, presumably due to the associated high CD20 level. In this respect, our present study shows that *ATM* mutation is another genetic defect in CLL cells connected with high CD20 density (and moreover low CD55 and CD59 density) and hence, hypothetically, with a favorable response to therapy. We assume that *ATM* mutations will be more intensely analyzed in CLL patients in the near future—owing to next-generation sequencing technologies—and our study demonstrates that it could also be important with respect to CD20-targeted immunotherapy.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared for any author.

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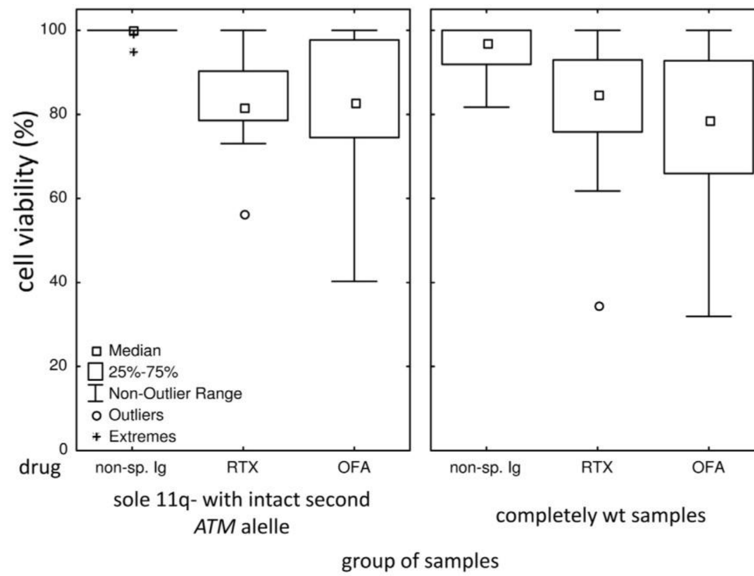

Supplementary Figure E1. Response of CLL cells to monoclonal antibodies in the groups with sole 11q- and complete *ATM*/*TP53*-wt status. The graph summarizes 31 CLL samples (14 with 11q- and intact second *ATM* allele) tested for antibodies concentrations 10 μg/mL in the presence of 20% active serum. Nonsp. Ig = nonspecific immunoglobulin (negative control); RTX = rituximab; OFA = ofatumumab.

4. Závěr

Práce na analýzách rekurentních mutací u pacientů s CLL sledovaných a/nebo léčených na IHOK FN Brno přinesly za 14 let výzkumu řadu zajímavých poznatků a přispěly svým dílem k prognostické a prediktivní stratifikaci nemocných. To se týká zejména defektů v genu *TP53*, kde je situace přehledná a jednoznačná: prakticky bez výjimek se všechna světová pracoviště shodují v názoru, že dotyční pacienti mají velmi špatnou prognózu – budou reagovat slabě, nebo nebudou reagovat vůbec na terapii navozující apoptózu skrze poškození DNA a při použití konvenčních přístupů tak prakticky nemají šanci na delší remisi natož vyléčení. I díky naší práci se podařilo vymezit v mezinárodních doporučeních, jak k těmto pacientům přistupovat z hlediska identifikace a validace *TP53* mutací a také jakým způsobem přistoupit k jejich léčení.

Mutace v genech *ATM* a *SF3B1* jsou u našich pacientů rovněž velmi časté a na základě mnoha našich experimentů můžeme zodpovědně říci, že významně mění biologii CLL buněk. Jak s nimi však naložit v klinickém kontextu zůstává otázkou. Léčba pacientů s CLL je ve své podstatě komplexní, zahrnuje tedy povětšinou několik léků s rozdílnými mechanismy účinku, přičemž z hlediska přítomnosti rekurentních mutací mohou být některé tyto účinky příznivé zatímco jiné nepříznivé. Pokud budeme uvažovat režim "zlatého standardu", tedy fludarabin, cyklofosfamid, rituximab, můžeme se například ptát, jakým způsobem budou ovlivňovat výsledek terapie mutace v genu *ATM*: na základě našich analýz víme, že jsou tyto mutace asociovány s vyšší hladinou CD20, což by mělo vést k dobré reakci na rituximab; na druhou stranu se však dá předpokládat, že chemoterapie poškozující DNA bude mít u *ATM*-mutovaných CLL buněk účinnost sníženou. Těžko tedy soudit, na kterou stranu se výsledný efekt převáží. V tomto smyslu hodláme v nejbližší době provést analýzu klinických dat u našich pacientů nesoucích mutace v *ATM* a léčených režimy FCR, Q-FCR (snížené dávky léků) a RB (rituximab, bendamustin)..

Z hlediska našich (myšleno aspiranta a jeho skupiny) dalších výzkumných plánů a strategií lze říci, že budou i nadále zahrnovat analýzy rekurentních mutací s ohledem na jejich dopad na osud pacientů s CLL. Vedle toho se však hodláme intenzivně věnovat i vývoji a testování nových terapeutických strategií, což by mělo v optimálním případě směřovat k návrhu inovativní terapie pro pacienty s CLL. Prvním krokem v tomto smyslu je již nyní běžící grant Agentury zdravotnického

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