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FÚZNÍ GENY TEL/AML1, BCR/ABL A TEL/ABL U DĚTSKÝCH AKUTNÍCH LYMFOBLASTICKÝCH LEUKÉMIÍ

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Obsah

1. Úvod

1.1 Akutní lymfoblastická leukémie u dětí

1.1.1 *Heterogenita dětských akutních lymfoblastických leukémií*

Leukémie představují skupinu maligních onemocnění charakterizovaných klonální expanzí transformovaných hematopoetických buněk s poruchou diferenciace a deregulací buněčné proliferace. Dle liniového původu dělíme leukémie na lymfoidní a myeloidní, dle průběhu onemocnění na akutní a chronické. Na rozdíl od situace u dospělých převažují v dětském věku leukémie akutní. Nejčastějším typem, se kterým se setkáváme v 80% všech případů dětských leukémií, je akutní lymfoblastická leukémie (ALL). Samotná ALL pak představuje 25% ze všech malignit v dětství a je tak vůbec nejčastější malignitou tohoto věkového období. Incidence ALL v České republice je přibližně 3 nové případy na 100 000 dětí (tj. ve věku 0-18 let) ročně s vrcholem výskytu mezi 1. a 5. rokem života.

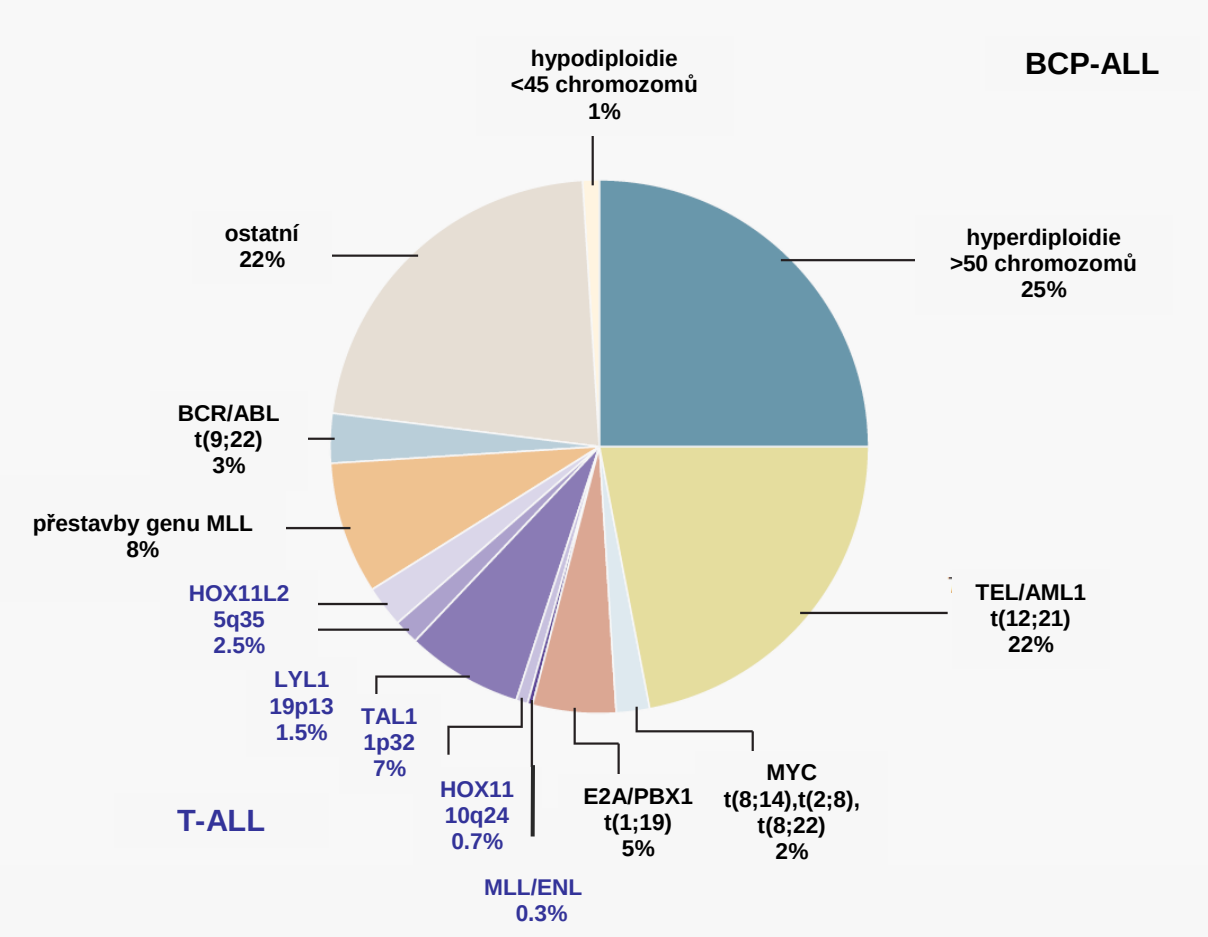
Dětská ALL zahrnuje skupinu značně heterogenních onemocnění s odlišným biologickým a klinickým chováním. Akutní lymfoblastické leukémie klasifikujeme pomocí morfologických, imunofenotypových a genetických znaků. Na základě morfologie lymfoblastů určuje tradiční FAB klasifikační systém 3 podtypy ALL: L1 (nejčastější podskupina, 70-80% dětských ALL), L2 a L3. Dle liniově příslušných znaků rozlišujeme ALL B-buněčného a T-buněčného původu. Přibližně 80% všech dětských ALL pochází z nezralých B-buněčných prekurzorů (B-cell precursor; BCP-ALL) a tyto se dále dělí dle stupně zralosti do následujících imunofenotypových podskupin: pro-B ALL, cALL (“common“ ALL, nejčastější podskupina) a pre-B ALL. Přibližně 15% dětských ALL představuje skupina T-ALL a vzácně se setkáváme s ALL ze zralých B buněk.

Za klinicky i biologicky nejrelevantnější se považuje klasifikace dětských ALL do genotypových podskupin podle přítomnosti rekurentních chromozomálních aberací či jejich molekulárních protějšků. U dětských ALL nalézáme chromozomální aberace numerické, jako jsou hyperdiploidie a hypodiploidie, a dále aberace strukturní, především chromozomální translokace (nejčastější genetické aberace dětské ALL a jejich frekvence jsou uvedeny na obrázku 1). Ty zastávají mezi genetickými aberacemi

u dětských leukémií v porování se situací u ostatních malignit významné místo, jinými slovy, častý výskyt chromozomálních translokací je považován za typický znak dětských ALL. Rekurentní genetické aberace dětských leukémií jsou aberace získané a hrají roli v etiopatogenezi těchto onemocnění. Jejich následkem dochází obecně k aktivaci protoonkogenů a/nebo inaktivaci tumor supresorových genů, avšak zvláště v případě aberací numerických nejsou všechny geny, které se účastní procesu maligní transformace, dosud zcela známy. Následkem chromozomálních translokací vznikají často fúzní geny, které kódují chimerické transkripční faktory a chimerické kinázy s aberantní aktivitou. Fúzními geny TEL/AML1, BCR/ABL a TEL/ABL a jimi vymezenými podskupinami dětských BCP-ALL jsem se zabývala ve své práci a podrobně se jim věnují další kapitoly úvodu.

Jednotlivé genotypové podskupiny ALL představují biologicky i klinicky odlišná onemocnění. Na podkladě společného biologického základu se leukémie daného genotypu vyznačující do určité míry společným fenotypem a chováním a to včetně klinicky významných vlastností jako jsou agresivita onemocnění či citlivosti k terapeutikům. Některé genetické aberace tak mají prognostický význam a genotyp leukémií je jedním z faktorů, které se používají při řazení pacientů do rizikových skupin a řízení terapie. Z aberací vyskytujících se u dětských BCP-ALL byly jako prognosticky příznivé identifikovány hyperdiploidie nad 50 chromozomů, fúzní gen TEL/AML1 a naopak za prognosticky nepříznivé fúzní gen BCR/ABL, přestavby genu MLL či hypodiploidie [1, 2].

Nověji se také začíná uplatňovat molekulární klasifikace dětských ALL pomocí expresního profilování. Již několik rozsáhlých studií ukázalo, že celogenomovým expresním profilováním pomocí DNA čipů je možné velmi dobře identifikovat podskupiny ALL odpovídající známým prognosticky významným genotypovým skupinám [3-6]. Tyto práce identifikovaly geny, jejichž exprese (ať už vysoká či nízká) je vysoce specifická pro určitý genotyp. Tyto geny lze využít jako nové diagnostické a klasifikační markery [7], a potenciálně i jako markery pro monitorování minimální reziduální nemoci. Dále byla pomocí expresních profilů mezi heterogenními geneticky nedefinovanými leukémiemi identifikována zcela nová podskupina BCP-ALL [3]. Jde o skupinu se špatnou prognózou a na základě značné podobnosti s expresním profilem BCR/ABL pozitivních leukémií získala pojmenování “BCR/ABL-like“.



Obrázek 1 – Frekvence specifických genetických aberací u dětských ALL (upraveno z Pui CH et al., 2004)

Jak již bylo uvedeno, leukémie jsou značně heterogenní onemocnění a s určitou mírou heterogenity se setkáváme i v rámci definovaných genotypových podskupin, kde se jednotlivé případy leukémií liší citlivostí na chemoterapii, odpovědí na léčbu, rizikem relapsu. V expresních profilech byly nalezeny nové prognosticky významné geny, které umožňují detailněji než dosud klasifikovat leukémie v rámci i vně známých genotypových podskupin do nových rizikových podskupin, u kterých bude dále možné přizpůsobovat terapii k dosažení lepších léčebných výsledků [6, 8-10]. Expresními profily nově identifikované geny potenciálně zahrnují i nové cíle pro specifickou terapii

a v neposlední řadě jsou novými východisky pro studium molekulární patogeneze leukémií.

Také profilování genetické a epigenetické (zde zatím především profilování DNA metylace) nabývají v posledních letech na významu a obdobným způsobem přispívají ke klasifikaci leukémií do rizikových skupin, k poznání biologické podstaty leukémií a k identifikaci potenciálních cílů pro nová terapeutika. Genetickým profilováním pomocí celogenomových CGH a SNP čipů byla odhalena řada nových získaných genetických aberací, které nebyly nalezeny standardními cytogenetickými a molekulárně genetickými metodami [11-14]. Ukázalo se, že genotyp leukémií je mnohem komplexnější, než se dříve jevilo. Na jednu BCP-ALL bylo nalezeno průměrně 6,6 genetických aberací [12]. Řada těchto aberací je opět rekurentní a bez pochyby se podílí na vzniku onemocnění. Zastoupení jednotlivých aberací a stejně tak jejich celkový počet se liší mezi tradičně definovanými genotypovými podskupinami. Nejméně aberací (v průměru 1) se vyskytuje u leukémií s přestavbami genu MLL a naopak nejvíce v podskupině leukémií s hyperdiploidií nad 50 chromozomů (v průměru 11,1) [12]. Jednou z nově nalezených je aberace genu IKZF1, která se velmi často vyskytuje u BCR/ABL pozitivních a „BCR/ABL-like“ ALL a byla identifikována jako nový prognosticky nepříznivý marker [15]. Také aberantní DNA metylace, především hypermetylace, je u leukémií častá, vede k inaktivaci genů a je tak jedním z mechanizmů podílejících se na vzniku leukémií [16]. Velmi recentní práce na rozsáhlých skupinách pacientů ukázaly, že obdobně jako pomocí expresních profilů lze na základě profilů DNA metylace klasifikovat ALL do známých genotypových podskupin a na základě nově identifikovaných prognostických markerů i do dalších nových rizikových podskupin [17-20].

1.1.2 *Etiopatogeneze ALL*

Příčiny vzniku ALL nejsou dosud navzdory intenzivnímu výzkumu plně objasněny. Na základě více či méně podložených populačních studií byly za možné rizikové faktory pro vznik leukémií označeny například zvýšená expozice ionizujícímu záření či elektromagnetickému poli a některým chemikáliím, některá virová onemocnění a konstitutivní genetické defekty [21]. Z epidemiologických dat vycházejí dvě blízce příbuzné a všeobecně přijmané teorie o “infekčním původu“ leukémií. Obě tyto teorie

předpokládají, že riziko vzniku leukémie je zvýšeno během aberantní imunitní reakce při styku vnímavých jedinců s jinak běžnými patogeny, vůči nimž nezískali tito jedinci imunitu v obvyklém věkovém období důsledkem předchozí populační [22] či socio-ekonomické [23] izolace.

Jako u ostatních maligních onemocnění jsou obecnou molekulárně biologickou podstatou vzniku leukémií aktivace genů s protoonkogenním a/nebo inaktivace genů s tumor supresivním účinkem. K těmto změnám u leukémií dochází především následkem získaných genetických a epigenetických aberací. U leukémií bylo popsáno již poměrně velké množství takto postižených genů. Jak bylo zmíněno v předchozí podkapitole, klasické cytogenetické a molekulárně genetické metody identifikovaly u ALL skupinu rekurentních genetických aberací, mezi nimiž dominují chromozomální translokace. Ty postihují nejčastěji geny pro transkripční faktory (TEL, AML1, MLL, E2A, PBX1) a geny s kinázovou aktivitou (BCR, ABL1) a ve většině případů vedou ke vzniku fúzních genů [1]. Ačkoliv přesná funkce produktů fúzních genů - chimerických transkripčních faktorů a kináz - není ještě do detailů objasněna a je zřejmě velmi komplexní, obecně lze říci, že jejich působením dochází k narušení základních biologických procesů, jako jsou kontrola sebeobnovy, diferenciace, řízení buněčného cyklu, proliferace či buněčné smrti. Genetickým mapováním pomocí DNA čipů byla popsána další početná skupina rekurentních genetických aberací [24], ve které jsou nejvíce zastoupeny aberace genů účastnících se B buněčné diferenciace (transkripčních faktorů PAX5, E2A, EBF1, IKZF1, IKZF3; genů pro rekombinázy RAG1, RAG2). Tyto studie navíc potvrdily již dříve popsaný velmi častý výskyt delecí genů pro regulátory buněčného cyklu (CDKN2A, CDKN2B) [25].

I pro leukemogenezi platí obecná teorie popisující maligní transformaci jako vícestupňový proces, při kterém je nezbytná kooperace více (minimálně dvou) aberací, tzv. zásahů [26]. Dochází nejprve k vytvoření preleukemického klonu a poté k vývoji klonu leukemického [27]. Ke vzniku leukemických fúzních genů a započetí procesu leukemogeneze může docházet již prenatálně. Svědčí o tom studie leukémií u dvojčat a přítomnost preleukemických buněk v pupečnkových krvích (viz dále) i pozitivní výsledky tzv. backtrackingu fúzních genů a dalších leukemických markerů na Guthrieho kartičkách s novorozeneckou krví [28-34]. Platnost dvouzásahové teorie dokazují u BCP-ALL výsledky několika typů studií. Za prvé jsou to výsledky studií

myších modelů BCP-ALL, které ukázaly, že jednotlivé genetické aberace indukují preleukemické změny, ale samy o sobě nevedou (či vedou jen s dlouhou latencí) ke vzniku leukémií. Riziko vzniku leukémie v těchto modelech se zvyšuje při spolupůsobení dalších genetických aberací, vnesených, indukovaných čí získaných během dlouhé doby latence před vznikem leukémie [35-39]. Obdobné závěry vyplývají z analýzy frekvence výskytu fúzních genů v pupečníkových krvích zdravých dětí, která je nejméně 100-násobně vyšší, než je riziko onemocnění leukémií [40]. Třetím důkazem jsou výsledky studia konkordance leukémií u monozygotních dvojčat. Preleukemické buňky jsou díky propojení krevních oběhů *in utero* u monozygotních dvojčat sdíleny, konkordance BCP-ALL u těchto dvojčat je však pouze 10-15% [41-44]. Souhrnně jsou tedy fúzní geny považovány za primární aberace (1.zásah) vznikající prenatálně a indukující vznik preleukemického klonu, který může perzistovat i několik let [45]. Vývoj leukémie je doprovázen ziskem dalších genetických aberací (dalších zásahů) a může při něm docházet ke vzniku více subklonů [46-48]. Jednotlivé subklony se liší zastoupením získaných genetických aberací a mohou mít i rozdílné biologické vlastnosti, např. odlišnou citlivost na chemoterapii. Preexistující subklony (a to i minoritně zastoupené v době diagnózy) pak mohou být zdrojem relapsu onemocnění [49]. Od diagnózy k relapsu onemocnění zpravidla dochází k dalším změnám genomu leukemické populace [50-53]. Dle Mullighana et al. je pouze 8% relapsů geneticky identických s leukémií z doby diagnózy. Ve 34% případů dochází k zisku nových genetických aberací (a následkem nových delecí i ke ztrátě některých původních změn), přibližně polovina relapsů vzniká zcela novým vývojem z preleukemického klonu a 6% relapsů nejsou ve skutečnosti relapsy původního onemocnění ale nově vzniklé “nepříbuzné“ leukémie [54].

Předmětem intenzivního studia je také další velmi zajímavá otázka: z buňky jakého hematopoetického vývojového stádia dětské ALL vycházejí. Ve skutečnosti jde spíše o dvě podotázky: 1) v buňce jakého typu vzniká primární genetická aberace a 2) z buňky jakého typu pak vzniká leukemický klon. Tyto otázky nebyly dosud jasně zodpovězeny. V některých studiích byly primární genetické aberace (TEL/AML1, MLL/AF4, hyperdiploidie) nalezeny v liniově nezadaných a tedy potenciálně multipotentních progenitorech [55-57]. U části BCR/ABL pozitivních ALL byl fúzní gen BCR/ABL nalezen i v buňkách myeloidní řady, což přímo dokazuje jeho původ v multipotentní

progenitorové buňce [58-60]. Buňky, ze kterých vychází vlastní leukemický klon (tzv. leukémii iniciující buňky) však nemusejí být s buňkou vzniku primární aberace totožné a zdá se, že u většiny dětských ALL vychází leukemický klon z lymfoidně zadaných progenitorů [61, 62]. Proč se však některé genetické aberace pojí výhradně s lymfoidním typem leukémie, je-li primárním místem jejich vzniku multipotentní progenitor? Dle Greavese jde velmi pravděpodobně o důsledek selektivního vlivu genetické aberace na buňky určité linie a diferenciačního stádia [26].

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1.1.3 *Terapie ALL*

Prognóza dětí s ALL se během několika posledních desetiletí výrazně zlepšila a v současné době je úspěšně vyléčeno přibližně 80-90% dětí [63, 64]. Za tímto úspěchem stojí zavedení nových léčebných postupů a stratifikace pacientů do rizikových skupin umožňující volbu vhodné terapie, tj. na jedné straně dostatečně intenzívní a na druhé straně s přijatelnou mírou možných nežádoucích komplikací. Pacienti jsou stratifikováni do rizikových skupin při zahájení terapie a dále v jejím průběhu. Kritéria používaná ke stratifikaci pacientů při zahájení terapie jsou klinická a laboratorní (věk, počet leukocytů, imunofenotyp, postižení CNS) a přítomnost již zmíněných prognosticky významných genetických aberací; v průběhu terapie jsou dále pacienti stratifikováni dle odpovědi na léčbu [2, 64]. Současné léčebné protokoly sestávají ze tří hlavních fází - indukce remise, fáze konsolidace a reindukce (pozdní intenzifikace) - a z fáze udržovací. Ty jsou navíc doprovázeny terapií cílenou na CNS. Cílem první fáze, která obvykle trvá 4-6 týdnů, je dosažení remise onemocnění a obnovení normální hematopoézy. Stěžejními léky této fáze jsou glukokortikoidy, L-asparagináza, vinkristin a antracykliny. Rychlost a stupeň odpovědi na indukční fázi stanovené dle poklesu blastů v kostní dřeni a periferní krvi jsou významnými prediktory výsledku terapie [65-73]. Cílem konsolidace a reindukce je eradikace reziduální nemoci, intenzita této fáze léčby se značně řídí typem leukémie a mírou rizika. Udržovací léčba, která trvá 1,5-3 roky, by měla nadále potlačovat případné reziduální blasty a z nich potenciálně vznikající rezistentní klony a dlouhodobě tak stabilizovat remisi onemocnění. Při terapii některých vysoce rizikových skupin

dětských ALL se uplatňuje také transplantace hematopoetických kmenových buněk (haematopoietic stem cell transplantation, HSCT).

Přes výrazný pokrok v léčbě dětských ALL nadále přetrvává skupina pacientů (představuje přibližně 10-20% pacientů) u nichž není dosaženo remise nebo dochází k relapsu onemocnění [74, 75]. Zdrojem relapsu mohou být buňky leukemického klonu či subklonu, ale také buňky preleukemického klonu u kterých došlo k vývoji do nového leukemického klonu [54]. Pro zlepšení léčebných výsledků u relabujících či na terapii špatně odpovídajících pacientů je potřeba najít prognostická kriteria, která identifikují leukémie vyššího rizika již před zahájením terapie a umožní zařadit tyto pacienty do adekvátních léčebných protokolů. K identifikaci nových prognostických markerů významně přispívají genetické, expresní a epigenetické profilovací studie, jak již bylo zmíněno výše. Tyto nové prognostické markery umožňují podrobnější stratifikaci pacientů do menších biologicky a klinicky homogennějších skupin s přesněji definovaným rizikem se snahou zvolit jednotlivým podskupinám terapii "ušitou na míru" a maximalizovat léčebné úspěchy.

Pokroky v terapii dětských ALL mohou v nejbližší době přinést také cílená terapeutika, například monoklonální protilátky a inhibitory aberantních signálních drah [76]. Mezi těmi zaujímají významné místo inhibitory kinázy BCR/ABL (imatinib mesylát a tyrosin-kinázové inhibitory dalších generací), které jsou již řadu let úspěšně používány v léčbě chronické myeloidní leukémie (CML) dospělých a byly zařazeny i do nejnovějších protokolů pro léčbu dětských BCR/ABL pozitivních ALL [77, 78]. Prohlubující se poznání biologické podstaty dětských leukémií, identifikace nových genů a drah účastnících se procesu maligní transformace a pochopení mechanizmu jejich funkce přináší stále nové potenciální cíle pro vývoj specificky cílených molekulárních terapeutik. V cílené terapii dětských ALL by mohlo nalézt uplatnění také umlčování leukemických fúzních genů pomocí RNA interference (RNAi). Zjednodušeně je RNA interference proces využívající endogenní buněčný aparát k posttranskripčnímu umlčení genové exprese. Ke spouštění tohoto procesu dochází pomocí tzv. siRNA molekul. Ty jsou v buňce endogenním aparátem rozpoznány, navázány a použity ke specifické vazbě na mRNA obsahující sekvenci komplementární se sekvencí siRNA; mRNA je poté v místě vazby štěpena a je tak bráněno syntéze produktu umlčovaného genu. Jako taková má obrovské využití jako metoda výzkumná

a má i potenciál terapeutický, a to teoreticky u všech onemocnění, kde existuje patogeneticky významný cíl s jedinečnou sekvencí mRNA. Jedinečnost sekvence je nezbytným předpokladem pro to, aby docházelo ke specifickému umlčování pouze vybraného cíle a nikoliv dalších genů s fyziologickou aktivitou. Vhodnými cíli u dětských ALL by mohly být fúzní geny, nesoucí takovou unikátní sekvenci v místě fúze partnerských genů. Bylo již publikováno několik prací popisujících následky umlčení některých leukemických fúzních genů touto metodou. Umlčení fúzních genů BCR/ABL, E2A/PBX1 a MLL/AF4 významně oslabilo maligní potenciál leukemických buněk a ukázalo, že zde tedy je předpoklad pro úspěch na fúzní geny cílené terapie pomocí RNAi [79-81]. Nejen u dětských ALL však tato metoda zatím zůstává především nástrojem výzkumným a existují velmi skeptické názory na její reálné využití terapeutické. Další skupinu potenciálních terapeutik představují tzv. epigenetická terapeutika – DNA demetylační látky a inhibitory deacetyláz histonů (HDACi). Demetylační terapeutika se již používají v léčbě některých myeloproliferací [82]. Protinádorový efekt HDACi byl prokázán in vitro i in vivo a jsou nyní testovány pro léčbu vybraných hematologických malignit v několika klinických studiích [82]. Některé leukemické fúzní geny (u BCP-ALL konkrétně fúzní gen TEL/AML1, u akutní myeloidní leukémie fúzní gen AML1/ETO) coby aberantní transkripční faktory přispívají k patogenezi leukémií zřejmě deregulací genové exprese. K té dochází na základě jejich interakce s deacetylázami histonů, které pak modifikují chromatin do neaktivní podoby [83, 84]. U těchto leukémií se předpokládá, že by HDACi mohly blokovat účinky fúzních genů a vykazovat specifický terapeutický efekt.

1.1.4 *Minimální reziduální nemoc*

O minimální reziduální nemoci (MRN) hovoříme, pokud u pacienta přetrvávají leukemické blasty v množství, které je pod detekčním prahem běžných morfologických metod. Monitorování MRN umožňuje hodnotit odpověď na léčbu a predikovat její výsledek. Řada studií demonstrovala, že detekovatelná MRN během terapie (především na konci indukční fáze, ale i v dalších časových bodech) a také před HSCT je velmi silným a nezávislým prediktorem relapsu [65-73, 85-92]. Kromě své prediktivní hodnoty navíc monitorování MRN umožňuje rozpoznat nastupující relaps onemocnění dříve, než může být detekován morfologickými metodami. Monitorování MRN se

využívá především ke stratifikaci pacientů v léčebných protokolech, ale i k řízení intenzity předtransplantační chemoterapie a potransplantační imunoterapie u transplantovaných pacientů [93].

K měření MRN u dětských ALL se používají metody založené na průtokové cytometrii a kvantitativní reverzně-transkriptázové polymerázové řetězové reakci (qRT-PCR). Průtokovou cytometrií jsou leukemické blasty detekovány na základě specifického imunofenotypu, který je odlišuje od zdravých hematopoetických buněk. Specificita leukemického imunofenotypu je dána abnormální kombinací imunofenotypových znaků, ke které může docházet zvýšenou, sníženou, asynchronní či liniově nepříslušnou expresí [94]. Výhodou metod založených na imunofenotypizaci je široká aplikovatelnost, nevýhodami jsou především nižší senzitivita oproti molekulárně genetickým metodám a nestabilita exprese některých imunofenotypových znaků během chemoterapie [95-97]. Pro měření MRN pomocí qRT-PCR jsou používány v zásadě dva typy leukemických markerů: transkripty fúzních genů a klonální přestavby imunoreceptorových genů. Fúzní transkripty představují vysoce specifické a s leukémií stabilně asociované markery, jejich detekce je metodicky méně náročná a relativně levnější, neboť pro podskupinu pacientů s daným fúzním genem lze použít univerzální detekční systém. To na druhou stranu přináší i určitou nevýhodu - riziko vzájemné kontaminace vzorků jednotlivých pacientů. Za další nevýhody jsou považovány rozdílné hladiny fúzních transkriptů mezi blasty jednotlivých pacientů a změny exprese vyvolané chemoterapií či degradací vzorku před zpracováním. Tyto nevýhody odpadají při detekci fúze na genomické úrovni, která je pro jednotlivé pacienty specifická, přítomná obvykle v jedné kopii na buňku a vůči degradaci odolnější. Identifikace místa genomické fúze je však metodicky značně náročná a z tohoto důvodu se příliš nepoužívá. Monitorování MRN pomocí detekce fúzních genů či transkriptů navíc není aplikovatelné u všech BCP-ALL, neboť fúzní geny se nacházejí jen u přibližně 40% všech případů [93]. Standardně se k monitorování MRN u BCP-ALL používá kvantitativní detekce klonálních přestaveb genů pro imunoreceptory. Stejně jako u fyziologických protějšků dochází u blastů BCP-ALL k přestavbám genových segmentů (V, D, J) genů pro těžké a lehké řetězce imunoglobulinů a také genů pro T-buněčné receptory. Při přestavování segmentů těchto genů vznikají v místech jejich spojování unikátní sekvence, které mohou sloužit jako specifické markery leukemického klonu. U

více než 95% všech dětských BCP-ALL jsou přestavěny geny pro imunoglobuliny a u většiny také geny pro T-buněčný receptor [98, 99]. Tato metoda měření MRN je tak aplikovatelná téměř u všech BCP-ALL. Díky přítomnosti jedné kopie imunoreceptorového cíle na buňku umožňuje přesně stanovit počet blastů. Navíc jsou imunoreceptorové cíle unikátní pro jednotlivé pacienty, čímž mizí riziko vzájemné kontaminace. Na druhou stranu je však nutné u každého jednotlivého pacienta tyto klonální markery identifikovat a vytvořit pro ně unikátní detekční systémy, což je metodicky, finančně i časově náročné. Pokračující či sekundární přestavování imunoreceptorových genů může vést k vytvoření subklonů a změnám jejich proporcionálního zastoupení během terapie či mezi diagnózou a relapsem, proto se doporučuje pokud možno vždy monitorovat alespoň dva imunoreceptorové cíle současně [100-106]. K standardizaci a vysoké reproducibilitě této metody výrazně přispělo vytvoření jednotných protokolů pro identifikaci imunoreceptorových cílů a pravidel pro kvantifikaci MRN a interpretaci výsledků [99, 107, 108]. V současné době jsou oba základní přístupy – kvantifikace přestaveb imunoreceptorových genů a cytometrická analýza – využívány ve většině moderních léčebných protokolů včetně protokolů BFM používaných v České republice.

1.2 **Fúzní gen TEL/AML1**

1.2.1 *TEL*

Gen TEL (podle oficiální nomenklatury ETV6) se nachází na chromozomu 12 v oblasti 12p13 [109]. Kóduje protein, který byl na základě přítomnosti vysoce konzervované domény ETS zařazen do rodiny ETS transkripčních faktorů. Alternativním sestřihem u něj vzniká více izoforem mRNA, některé z nich potenciálně kódují protein s dominantně negativní funkcí [110]. Majoritní izoforma mRNA zahrnující všech 8 exonů genu TEL obsahuje dva alternativní translační počátky uvnitř jediného čtecího rámce a jsou jí kódovány dvě hlavní izoformy proteinu o velikosti 50 a 57 kDa, které se liší pouze přítomností prvních 42 aminokyselin [111].

Protein TEL obsahuje dvě konzervované funkční domény: N-koncovou HLH (pointed, SAM) doménu a C-koncovou ETS doménu. Oblast mezi oběma doménami kódovaná z velké části exonem 5 se nazývá centrální oblast (centrální represorová oblast/doména, centrální doména). HLH doména zprostředkovává dimerizaci (a to homo- i hetero-) [112, 113] a interakci s dalšími proteiny [114-117], ETS doména nese jaderný lokalizační signál a zprostředkovává vazbu na DNA [111], centrální oblast interaguje s několika korepresory [115, 118, 119]. Protein TEL je lokalizován převážně v jádře [111]. Jeho funkce je regulována prostřednictvím posttranslačních modifikací, jako jsou sumoylace a fosforylace, které současně s funkcí regulují i export z jádra do cytoplazmy [114, 120-122]. TEL je zřejmě sekvenčně specifický transkripční represor a pro tuto jeho funkci je nezbytná dimerizace zprostředkovaná HLH doménou i přítomnost centrální oblasti kódované exonem 5 (a pochopitelně přítomnost DNA vazebné domény ETS) [118, 123]. Lopez et al. identifikovali v proteinu TEL dvě autonomní represivní domény. Jedna se nachází zcela v centrální oblasti, druhou tvoří koncová část centrální oblasti a ETS doména [123]. Transkripční represe je zřejmě zprostředkována interakcí s korepresory a s histon deacetylázami (HDAC) vedoucí k inaktivaci chromatinu následkem jeho těsnější vazby k deacetylovaným histonům. Bylo prokázáno, že HLH doména interaguje s korepresorem mSin3A a centrální oblast s korepresory SMRT, mSin3A, N-CoR a s deacetylázou histonů 3 [115, 118]. Boccuni et al. demonstrovali, že TEL interaguje také s proteinem H-L(3)MBT ze skupiny

polycomb a mohl by tak reprimovat transkripci i nezávisle na deacetylaci histonů [116]. Dosud nebyly identifikovány téměř žádné cílové geny tohoto transkripčního represoru. Jedinými cílovými geny, u nichž byla potvrzena přímá vazba proteinu TEL na promotor, zůstávají gen pro anti-apoptotický faktor BCL-X_L a gen pro matrixovou metaloproteinázu MMP3 (stromelysin-1) [124, 125]. Geny, jejichž expresi v modelových systémech TEL svou přítomností ovlivňuje (a mezi nimiž by mohly být další přímé cíle), byly popsány pomocí expresního profilování ve dvou experimentálních studiích [126, 127].

Fyziologická role genu TEL není příliš známa. Je ubiquitně exprimován a to i během embryonálního vývoje [128]. S použitím myších modelů se pokusili objasnit jeho roli během embryogeneze Wang et al. [128, 129]. Zjistili, že myši se zcela vyřazeným genem (TEL *knock-out* myši) umírají kolem 11. dne embryonálního vývoje. Tyto myši mají sice zachovanou hematopoézu ve žloutkovém váčku, je zde však významná porucha angiogeneze, a dochází i k apoptóze mezenchymálních a neurálních buněk. U chimerické myši, kde je gen TEL vyřazen v hematopoetické linii, probíhá fetální hematopoéza (která se odehrává nejprve ve žloutkovém váčku a později v játrech) normálně. Krátce po narození, kdy se hematopoéza přesouvá do kostní dřeně, však dochází k jejímu narušení a kompletní absenci všech hematopoetických linií. TEL je tedy nepostradatelný pro založení definitivní hematopoézy, konkrétně zřejmě pro usídlení a přežívání hematopoetických buněk v kostní dřeni. Inducibilní liniově specifický *knock-out* později umožnil studovat roli genu TEL i v hematopoéze dospělých myší [130]. Ukázalo se, že i v dospělosti, po úspěšném založení definitivní hematopoézy v kostní dřeni, je TEL pro její udržení nadále nezbytný. Byla vyslovena hypotéza, že zprostředkovává kontinuální signalizaci esenciální pro přežití hematopoetických kmenových buněk (HSC), která je zřejmě spouštěna interakcí specifických receptorů s ligandy v mikroprostředí kostní dřeně.

TEL je velice často deletován či jinak narušen u hematologických malignit (viz níže) ale i u jiných typů nádorových onemocnění [131, 132]. Je tedy vysoce pravděpodobné, že by mohl mít nádorově supresivní vlastnosti. To dokládá i několik experimentálních studií, ve kterých byl TEL schopen potlačovat proliferaci různých typů primárních i transformovaných buněk, snižovat invazivitu transformovaných fibroblastů in vitro i in

vivo, redukovat tvorbu solidních nádorů u myši či indukovat apoptózu represí antiapoptotického proteinu Bcl-X_L [124, 125, 133-135].

U hematologických malignit je tento gen aberován mnoha způsoby. U ALL je často deletován, zejména (avšak nejen) u pacientů s fúzním genem TEL/AML1 [12, 136-140] - tato kombinace tak vede k inaktivaci obou kopií TEL. V některých případech ALL, přestože je na genomické úrovni zachován, je porušena jeho exprese [141, 142]. Dalším typem aberací postihujících tento gen u ALL jsou různé chromozomální translokace, které vedou ke vzniku fúzních genů [143-146]. Suverénně nejčastěji se vyskytuje fúzní gen TEL/AML1 (viz samostatná podkapitola). U akutních myeloidních leukémií (AML) nejsou přestavby genu TEL časté, pouze vzácně se vyskytují některé fúzní geny [147]. Bylo však zjištěno, že TEL může být postižen bodovými mutacemi, které mají za následek dominantně negativní funkci proteinu, a až u 1/3 pacientů s AML je porušena jeho exprese [148]. Aberace genu TEL se vyskytují také u chronické myelo-monocytární leukémie (CMMoL) a u myelodysplastického syndromu (MDS) [147].

TEL má mnoho fúzních partnerů, je to jeden z vůbec nejčastěji přestavovaných a nejvíce "promiskuitních" genů u hematologických malignit. Fúzními partnery genu TEL jsou jednak geny kódující tyrosin kinázy (PDGFRB, ABL1, ABL2, JAK2, NTRK3, SYK a další), jednak geny transkripčních faktorů (AML1, MN1, PAX5, CDX2, EVI1 a další) [147]. Fúzí tak vznikají chimerické kinázy, které jsou konstitutivně aktivovány na základě dimerizace zprostředkované HLH doménou genu TEL, či chimerické transkripční faktory s aberantní funkcí.

1.2.2 *AML1*

Gen AML1 (také CBFa2, podle oficiální nomenklatury RUNX1) leží na chromozomu 21 v oblasti 21q22 [149]. Kóduje jeden ze tří členů rodiny RUNX, charakteristické přítomností konzervované domény RHD (runt homology domain) vysoce homologní s proteinem *runt* Drosofily [150, 151]. Jde o velký gen rozsahu přibližně 260 kb obsahující 9 exonů, je přepisován ze dvou promotorů a transkripty podléhají alternativnímu setřihu. Vzniká tak řada izoforem mRNA [152-155]. Transkripce, sestřih i translace jsou tkáňově regulovány [154].

Translací vznikají dva majoritní proteiny - AML1b a AML1c - a méně zastoupená kratší izoforma AML1a. Proteiny AML1b a AML1c jsou dlouhé 453 respektive 480 aminokyselin a liší se svými N-konci. AML1a je téměř o polovinu kratší a postrádá C-koncovou oblast [153, 154]. Doména RHD zprostředkovává proteinové interakce a vazbu na DNA a to specificky na motiv TGT/cGGT [156-158]. Na C-konci proteinu bývá popisována transaktivační doména [154] - jde o poněkud zavádějící označení, neboť celá oblast ležící C-koncově od RHD domény zprostředkovává řadu interakcí a to nejen s koaktivátory, ale i s korepresory (viz níže). Prostřednictvím domény RHD váže AML1 protein CBFb a vytváří s ním heterodimerní transkripční faktor CBF [157, 158]. Transkripční faktor CBF má dvě další podoby - obsahují stejnou CBFb podjednotku a jeden z dalších dvou členů rodiny proteinů RUNX, AML2 (RUNX2, CBFa3) či AML3 (RUNX3, CBFa1), coby CBFa podjednotku. CBFb se v rámci heterodimeru neúčastní vazby na DNA, avšak zvyšuje afinitu AML1 k DNA a stabilizuje jej tím, že brání jeho ubiquitinylaci a následné degradaci [159, 160]. AML1 se nachází v jádře, na jeho C-konci je jaderný lokalizační signál, při jehož mutaci je porušena distribuce a tím i funkce AML1 [161, 162].

AML1 (coby součást CBF) různými způsoby reguluje transkripci prostřednictvím interakcí s řadou partnerů. Interaguje s koaktivátory p300, CBP a MOZ, které mají vlastní histon acetyl transferázovou aktivitu, a dále s koaktivátory ALY a YAP [163-165]. Je tedy schopen aktivovat transkripci. Na druhou stranu interaguje rovněž s korepresory mSin3A, N-CoR a TLE, s deacetylázami histonů a s metyltransferázou histonů SUV39H1 a může tedy transkripci také reprimovat [166-171]. Při regulaci transkripce dále interaguje a kooperuje s řadou liniově specifických transkripčních faktorů, jako jsou např. ETS1, PU1, c-MYB, C/EBPa, LEF1, GATA1, PAX5 [172-181]. Předpokládá se tedy, že by mohl plnit funkci organizátoru, který umožňuje sestavení transkripčně regulačního komplexu [182]. Při regulaci některých cílů je zřejmě nezbytná i jeho homodimerizace [183]. Výsledná povaha regulace (ve smyslu aktivace či transkripce) pak závisí na struktuře konkrétního promotoru a buněčném kontextu. Kratší formy AML1, jako AML1a, mají zvýšenou afinitu k DNA, ale nemají C-koncovou oblast odpovědnou za řadu výše zmíněných interakcí a mohly by tak působit dominantně negativně vůči majoritním izoformám [184, 185]. Mezi známými cíly AML1 figurují především liniově specificky exprimované geny, jako jsou IL3,

TCR, GM-CSF, M-CSFR, CD11a, CR1, myeloperoxidáza, neutrofilní elastáza, BLK, GZMB či PKC. AML1 se podílí na aktivaci jejich transkripce [181, 186-196]. Jedním z mála známých cílů, jejichž transkripci AML1 reprimuje, je p21 [197].

Aktivita AML1 je regulována prostřednictvím několika typů posttranslačních modifikací. Pokud je AML1 ubiquitinylován, dochází k jeho degradaci. Fosforylace prostřednictvím MAPK dráhy vede k uvolnění vazby na mSin3A a aktivaci transaktivační funkce, ale zároveň navozuje degradaci AML1, neboť mSin3A stejně jako CBFb chrání AML1 před ubiquitinylací [198]. AML1 je dále acetylován proteinem p300, čímž se zvyšuje jeho afinita k DNA [199].

Vzhledem k velmi četným a častým aberacím AML1 u hematologických malignit je tento transkripční faktor jistě významným regulátorem hematopoézy. Z jeho cílových genů vyplývá, že se uplatňuje při realizaci liniově specifických transkripčních programů při vývoji jednotlivých hematopoetických linií. Během embryonálního vývoje je AML1 silně exprimován v hemangioblastech, společných prekurzorech pro primitivní hematopoézu a vaskulogenezi, ale i v jiných tkáních [200]. U myši s vyřazeným genem AML1 (*knock-out* myš) dochází k normální morfogenezi i primitivní hematopoéze ve žloutkovém váčku, avšak zcela chybí definitivní hematopoéza (tedy hematopoéza všech linií) v játrech [201, 202]. Myší embrya umírají kolem 12. dne intrauterinního vývoje na krvácení do CNS. Stejný dopad na embryonální hematopoézu byl pozorován také u inaktivace CBFb, je tedy evidentní, že pro hematopoézu je nezbytná přítomnost obou složek CBF transkripčního faktoru [203, 204]. Aby bylo možno obejít embryonální letalitu při úplném vyřazení AML1, byly dále vytvořeny chimerické myši. Bylo prokázáno, že u těchto myší nejsou AML1 deficientní buňky schopny podílet se na definitivní hematopoéze [201]. K narušení embryonální hematopoézy, a to především k narušení jejího časoprostorového průběhu a počtu HSC a nezralých progenitorů, dochází také u haploinsuficientních myší [205]. AML1 je tedy pro embryonální hematopoézu zcela esenciální. Na rozdíl od genu TEL, který je nezbytný pro přežívání HSC v dospělosti, však inaktivace AML1 v dospělosti funkci a přežívání HSC neovlivňuje [206]. V prvním popsaném dospělém myším modelu měla inaktivace AML1 celkově poměrně omezený dopad, vedla “pouze“ k poruchám vyzrávání megakaryocytů a vývoje B a T řady. Při dlouhodobém sledování však byl u dospělých myší s inaktivovaným AML1 pozorován vznik T-buněčných

lymfomů, splenomegalie a změny podobné myelodysplastickému syndromu [207]. Pro významnou roli AML1 v postnatální hematopoéze u člověka svědčí navíc řada hematologických poruch spojených s jeho aberací (viz níže).

Několik prací ukázalo, že AML1 se podílí také na regulaci buněčného cyklu. AML1 zkracuje G1 fázi a podporuje přechod z G1 do S fáze [208, 209]. Jedním z mechanizmů této regulace může být popsaná přímá aktivace transkripce cyklinu D3 [210]. Ten pak zřejmě negativní zpětnou vazebou reguluje hladinu AML1 a ta se v průběhu buněčného cyklu specificky mění [211]. Funkce AML1 v regulaci buněčného cyklu je inhibována dominantně negativně působícími izoformami AML1 a fúzními geny (CBFb/SMMHC, AML1/ETO), což je zřejmě podkladem jimi indukované inhibice proliferace [210, 212-215].

Gen AML1 je u hematologických malignit aberován několika mechanizmy. Patří mezi ně chromozomální translokace vedoucí ke vzniku fúzních genů, amplifikace i bodové mutace vedoucí k hyperaktivaci, ztrátě funkce či zisku dominantně negativní funkce [216]. Funkce AML1 může být negativně ovlivněna i aberacemi druhé podjednotky transkripčního faktoru CBF, CBFb. V experimentálních pracích jsou AML1 přičítány jak onkogenní tak nádorově supresivní vlastnosti. AML1 je schopen transformovat fibroblasty, na druhou stranu ztráta AML1 v HSC vede k poruše senescence indukované onkogenem RAS, která obecně představuje důležitý obranný mechanizmus vůči onkogenním inzultům [217-219]. Z výše vyjmenovaného souboru aberací vyplývá, že k transformaci hematopoetických buněk vede jak změna genové dávky a nadbytečná exprese "*wild type*" (*wt)* AML1, tak ztráta funkce či zisk aberantní funkce, tedy že u hematologických malignit lze AML1 chápat jako nádorový supresor i onkogen současně. Nejčastějším typem aberace tohoto genu je chromozomální translokace t(12;21)(p13;q22), vedoucí ke vzniku fúzního genu TEL/AML1 a vyskytující se u dětských BCP-ALL. Dále se u dětských BCP-ALL vyskytují amplifikace tohoto genu (přibližně u 3% pacientů), které nejsou doprovázeny mutacemi [220-223]. U některých pacientů byla popsána aberantně zvýšená exprese AML1, přestože u nich není gen amplifikován [224, 225]. Přibližně u 15% AML nacházíme translokaci t(8;21)(q22;q22), na jejímž podkladě vzniká fúzní gen AML1/ETO [149]. AML1/ETO je schopen heterodimerizovat s CBFb, vázat se na DNA a reprimovat transkripci některých genů fyziologicky transaktivovaných AML1 [188, 226, 227]. Během

embryogeneze vyvolává AML1/ETO podobné změny jako inaktivace AML1, nejde však o úplnou fenokopii, neboť ve fetálních játrech AML1/ETO pozitivních embryí se nacházejí dysplastické multiliniové progenitory, jejichž *in vitro* sebeobnovovací schopnost je aberantně zvýšena [228]. AML1/ETO tedy zřejmě nejen inhibuje funkci *wt* AML1, ale má i vlastní aberantní funkci. Ve shodě s myším modelem AML1/ETO transdukovaný do lidských CD34 pozitivních progenitorů zvyšuje schopnost jejich sebeobnovy, avšak snižuje schopnost tvorby kolonií u liniově zadaných progenitorů [229]. K maligní transformaci může přispívat tím, že blokuje diferenciaci myeloidní řady [230-232]. Některé práce však popisují, že AML1/ETO paradoxně také inhibuje buněčnou proliferaci [233]. AML1/ETO sám o sobě není schopen vyvolat AML [215]. Pro transformaci je potřeba kooperace s dalšími zásahy [234], ty mohou přispívat k transformaci právě odstraněním proliferačního bloku. V souladu s touto hypotézou byl popsán zvýšený onkogenní potenciál u C terminálně zkrácené varianty proteinu AML1/ETO, která na základě ztráty dvou C-koncových domén kódovaných fúzním partnerem ETO nemá inhibiční vliv na buněčnou proliferaci [235]. U AML a MDS se vzácněji nacházejí i další translokace, např. t(3;21)(q26;q22) vedoucí ke vzniku fúzních genů AML1/MDS1, AML1/EAP a AML1/EVI1 [182]. Stejně jako u ALL i u AML se objevují amplifikace AML1, avšak mnohem vzácněji [236]. Naopak mnohem častější jsou bodové mutace, které se objevují u 5-10% de-novo vzniklých a u 16-40% sekundárních AML/MDS [237-241]. V neposlední řadě u AML také často nacházíme inverzi chromozomu 16, která sice nepostihuje přímo gen AML1, ale druhou část transkripčního faktoru CBF - gen CBFb. Na podkladě inv(16)(p13;q22) či o něco vzácnější translokace t(16;16)(p13;q22) vzniká fúzní gen CBFb/SMMHC a ten podobně jako AML1/ETO interferuje s funkcí *wt* AML1 [242].

1.2.3 *TEL/AML1*

Kryptická translokace t(12;21)(p13;q22), na jejímž podkladě vzniká fúzní gen TEL/AML1, byla poprvé popsána v roce 1994 [143]. V následujících letech se ukázalo, že jde o vůbec nejčastější strukturální chromozomální aberaci dětských leukémií vyskytující se přibližně u 25% BCP-ALL [243-246]. Ke zlomům DNA při vzniku této translokace dochází v rozsáhlé oblasti (≈160 kb) tvořené introny 1 a 2 genu AML1 a téměř výhradně v relativně krátkém (≈15 kb) intronu 5 genu TEL. Translokace tak

typicky spojuje exon 5 genu TEL k exonům 2 nebo 3 genu AML1 [247-249]. Co je příčinou vzniku této chromozomální translokace není zcela jasné. Obecně bylo popsáno několik mechanizmů, které mohou při vzniku chromozomálních translokací hrát roli: 1) aberantní aktivita rekombináz se může podílet na přestavbách genů, které obsahují sekvence totožné (alespoň částečně) s rozpoznávacími sekvencemi pro V-(D)-J rekombinaci imunoreceptorových genů [250, 251], 2) některé translokace mohou vznikat homologní rekombinací mezi Alu sekvencemi [252], 3) vznik DNA zlomů a následně translokací mohou indukovat inhibitory topoizomerázy II (např. etoposid) [253]. Druhý a třetí z těchto mechanizmů se uplatňují při vzniku přestaveb genu MLL. Metaanalýza relativně velkého souboru genomických fúzí TEL/AML1 ukázala, že v intronu 1 genu AML1 se nachází poměrně široký shluk obsahující přibližně 60% všech identifikovaných zlomů, a dále že v intronu 5 genu TEL se nenachází žádné shluky zlomů. Ani u jednoho z genů navíc v místě zlomů nebyly nalezeny žádné specifické sekvenční motivy, které by indikovaly roli místně specifické rekombinace při vzniku fúzního genu TEL/AML1 [254]. *In vitro* experimenty ukázaly, že u buněčných linií může vlivem apoptotických stimulů (odnětí séra, etoposid, kyselina salicylová) docházet k indukci dvouvláknových zlomů DNA v intronu 5 genu TEL a v intronu 1 genu AML1 a k následnému vzniku fúzního genu TEL/AML1 [255]. K opravě dvouvláknových zlomů a vzniku fúzního genu zřejmě dochází spojováním nehomologních konců [254].

Translokace t(12;21) je reciproká a na jejím podkladě zpravidla vzniká nejen fúzní gen TEL/AML1, ale i fúzní gen AML1/TEL. Zatímco TEL/AML1 je exprimován vždy, transkript AML1/TEL je přítomen u velké části, avšak ne u všech případů [256, 257]. Předpokládá se tedy, že na rozdíl od TEL/AML1 nehraje AML1/TEL kauzální roli při vzniku leukémií. Hybridní protein TEL/AML1 zahrnuje HLH doménu a centrální oblast proteinu TEL a téměř celý protein AML1 včetně RHD domény a C-koncové oblasti. TEL/AML1 tak získává řadu fukčních a interakčních schopností, které jsou vlastní *wt* proteinům TEL a AML1. Prostřednictvím HLH domény TEL/AML1 homodimerizuje a prostřednictvím RHD domény se váže na DNA [244, 258]. Dále si udržuje schopnost interagovat s CBFb pomocí RHD domény [226]. HLH doména, centrální oblast proteinu TEL, ale i C-koncová oblast AML1 umožňují interakci s korepresorem mSin3A [259], HLH doména a centrální oblast interagují i

s korepresorem N-CoR [260]. Použitím reporterových esejí několik autorů demonstrovalo, že TEL/AML1 je schopen vázat se do promotorových oblastí cílových genů AML1 a reprimovat jejich transkripci [258, 259, 261, 262]. TEL/AML1 je tak zřejmě transkripčním represorem a dereguluje transkripci cílů AML1 inaktivací chromatinu prostřednictvím korepresorů a s nimi interagujících deacetyláz histonů [263]. Tento mechanizmus by mohl být podkladem jeho leukemogenní aktivity. To naznačují i studie TEL/AML1 transfomovaných progenitorů, u kterých není TEL/AML1 schopen navodit charakteristické změny, pokud je mutován a neschopen vázat se na DNA či interagovat s CBFb [226, 264]. Dosud však nejsou známy žádné přímé cílové geny TEL/AML1 v leukemických buňkách. Leukemogenní aktivita TEL/AML1 může být podložena i dalšími mechanizmy. Gunji et al. demonstrovali, že TEL/AML1 heterodimerizuje s *wt* proteinem TEL a působí vůči němu dominantně negativně [265]. TEL/AML1 brání schopnosti genu TEL reprimovat transkripci a suprimovat růst transformovaných fibroblastů. Ve stejném modelu se TEL/AML1 chová dominantně negativně i vůči AML1, kdy naopak inhibuje schopnost AML1 fibroblasty transformovat. Další skupina autorů demonstrovala schopnost TEL/AML1 interagovat s koaktivátorem p300, sekvestrovat jej do cytoplazmy a bránit tak transaktivační funkci *wt* AML1 [266, 267]. To však jistě nebude hlavní mechanizmus funkce TEL/AML1, neboť tyto práce jsou v rozporu s pozorováním, že protein TEL/AML1 je v leukemických buňkách lokalizován v jádře [111].

Jsou tedy navrženy potenciální mechanizmy, zatím ale není objasněno, jaké dráhy a procesy TEL/AML1 narušuje a jak přesně ke vzniku leukémie přispívá. Dosavadní poznatky z experimentálních modelů, dvojčecích studií, “backtrackingu“ TEL/AML1 na Gutrieho kartičkách s novorozeneckou krví a z analýzy pupečníkových krví ukazují, že TEL/AML1 je primární genetickou aberací (prvním zásahem), k jejímuž vzniku dochází často již prenatálně. Sám o sobě však není pro vznik leukémie dostatečný a je pouze jedním z několika zásahů, které jsou pro maligní transformaci nezbytné (viz první kapitola úvodu). V prvním vytvořeném transgenním myším modelu nevyvolala exprese TEL/AML1 u myši pod kontrolou enhanceru specificky aktivního v B řadě žádné patologické změny fenotypu [35]. V dalším modelu byly myši transplantovány TEL/AML1-transdukovanými buňkami kostní dřeně [36]. U *wt* myší došlo ke vzniku leukémií s nízkou frekvencí a s dlouhou latencí, kdežto u myší s inaktivovaným

lokusem pro regulátory buněčného cyklu p16 a p19 se objevila leukémie s vysokou frekvencí a kratší latencí. TEL/AML1 tedy vyvolával leukémii ve spolupráci s dalšími zásahy, ať už preexistujícími, či během dlouhé latence získanými. Pomocí tří obdobných "transdukčně-transplantačních" myších modelů byly později popsány preleukemické změny, které TEL/AML1 u myší vyvolává. Působením TEL/AML1 dochází k expanzi HSC a časných progenitorů, ke zvýšení jejich schopnosti kompetitivně rekonstituovat hematopoézu *in vivo* a sebeobnovovat se *in vitro*, ale i k částečnému bloku vývoje B řady [37, 268, 269]. *In vitro* preleukemické vlastnosti (tj. zvýšená sebeobnova) jsou závislé na přítomnosti HLH domény a centrální oblasti genu TEL i RHD domény genu AML1 [264].

Schindler et al. vytvořili nedávno nový transgenní myší model, ve kterém demonstrovali, že funkce TEL/AML1 je zřejmě vysoce závislá na daném buněčném kontextu [270]. Tento model věrněji imituje situaci u TEL/AML1 pozitivních leukémií, neboť na rozdíl od předchozího arteficiálnějšího modelu byl zde gen TEL/AML1 cíleně vnesen pod kontrolu endogenního promotoru genu TEL. Na rozdíl od myší s vyřazenými geny TEL či AML1, nedocházelo u TEL/AML1 transgenních myší k poruchám embryonálního vývoje. TEL/AML1 tedy nepůsobil jako dominantně negativní inhibitor genu TEL a na rozdíl od fúzního genu AML1/ETO (u něhož se předpokládá obdobný molekulární mechanizmus funkce) ani jako dominantně negativní inhibitor AML1. Během embryonálního vývoje nedocházelo u těchto myší k poruchám vývoje B řady, pouze mírně narůstal počet a schopnost sebeobnovy B progenitorů, avšak tento efekt TEL/AML1 byl tranzientní a vymizel po narození. K poruchám vývoje B řady docházelo, pokud byly fetální jaterní TEL/AML1 pozitivní HSC transplantovány dospělým myším či pokud byla indukována exprese TEL/AML1 u myší až v dospělosti. Vývoj B řady byl blokován už ve stadiu velmi časného B-lymfoidního progenitoru. Zatímco TEL/AML1 na jedné straně značně oslabil potenciál HSC diferencovat do B-lymfoidní řady, na druhé straně znatelně zvýšil celkový počet HSC a jejich klidovou frakci. U myší nedocházelo k progresivní akumulaci HSC ani ke vzniku leukémie. Pokud byly TEL/AML1 pozitivní HSC transplantovány sekundárním příjemcům, dlouhodobě přežívaly, aniž by vytlačily TEL/AML1 negativní protějšky. Hladiny známých regulátorů byly u TEL/AML1 pozitivních HSC na normální úrovni, což nasvědčuje tomu, že byly pod kontrolou fyziologických regulačních mechanizmů.

TEL/AML1 u dospělých myší výrazně zvyšoval riziko vzniku leukémie při expozici mutagenu. Tento efekt TEL/AML1 byl však zcela podmíněn expresí TEL/AML1 v HSC. Riziko vzniku leukémie nebylo zvýšeno, pokud byl TEL/AML1 exprimován z B-lymfoidního promotoru. TEL/AML1 tedy indukuje vznik mírně expandovaného dlouhodobě přežívajícího rezervoáru HSC, které sice zůstávají pod vlivem normálních regulačních mechanizmů, ale jsou "zranitelné" a zvýšeně náchylné ke vzniku leukémie. Na základě popsaných výsledků byla dále vyslovena hypotéza, že ke vzniku leukémie z TEL/AML1 pozitivních HSC dochází pouze před nástupem dospělé hematopoézy, neboť v dospělosti je již vývoj B řady blokován a nemohou vznikat B-lymfoidní preleukemické či leukemické buňky. Tato hypotéza by mohla být vysvětlením velmi raritního výskytu TEL/AML1 pozitivních leukémií v dospělosti [139].

Uznávaná "infekční" teorie vzniku leukémií (viz první kapitola úvodu) předpokládá, že k rozvoji leukémie dochází následkem aberantní imunitní reakce. Práce publikovaná v loňském roce zkoumala, jakým způsobem by během takové imunitní reakce mohlo docházet k rozvoji leukémie z preexistujícího TEL/AML1 pozitivního preleukemického klonu [271]. Bylo zjištěno, že TEL/AML1 pozitivní buňky mají *in vitro* i *in vivo* značnou růstovou výhodu a tím schopnost expanze v přítomnosti inhibičně působícího cytokinu TGFβ, který je během imunitní reakce produkován aktivovanými T lymfocyty.

TEL/AML1 pozitivní leukémie představují relativně homogenní skupinu leukémií. Postihují nejčastěji děti ve věku 2-5 let a prakticky se nevyskytují v kojeneckém věku ani v dospělosti [244, 246]. Svým imunofenotypem se řadí mezi BCP-ALL, od ostatních BCP-ALL je lze velmi dobře odlišit pomocí dvou povrchových imunofenotypových znaků: CD27 a CD44 [7]. TEL/AML1 leukémie jsou CD27 pozitivní a CD44 negativní; při ideálním nastavení "cut-off" hladin je lze pomocí těchto znaků identifikovat coby TEL/AML1 pozitivní se 100% specifitou a 85% senzitivitou. TEL/AML1 leukémie jsou dále charakterizovány nízkou iniciální leukocytózou, výbornou odpovědí na léčbu a příznivou prognózou [243-245, 272-274]. Mají charakteristický expresní profil (nejen profil exprese kódujících genů ale i microRNA), který je odlišuje od ostatních podskupin BCP-ALL [4, 5, 275]. V expresních profilech byla u TEL/AML1 pozitivních leukémií nalezena specificky vysoká exprese receptoru pro erythropoetin [4]. Následná *in vitro* funkční studie u TEL/AML1 pozitivních linií

demonstrovala pozitivní vliv signalizace přes tento receptor na proliferaci a schopnost čelit apoptotickým signálům [276]. Dále byla v profilech identifikována skupina genů, jejichž exprese odlišuje TEL/AML1 pozitivní ALL od TEL/AML1 negativních ALL, a biologické dráhy, jichž se tyto geny účastní [277]. Vztah mezi TEL/AML1 a těmito geny však zatím nebyl objasněn. Nejčastější přídatnou genetickou aberací TEL/AML1 pozitivních leukémií je delece druhé alely genu TEL, která byla genetickým profilováním pomocí SNP čipů nalezena u 70% TEL/AML1 pozitivních ALL [12, 136, 138, 278]. Z cytogenetických nálezů, kde bývá tato delece často popisována jako subklonální, a z dvojčecích studií vyplývá, že se jedná o sekundární změnu, tedy jeden z dalších zásahů vedoucích ke vzniku leukémie [43, 62, 136, 138]. Delece genu TEL může hrát roli v leukemogenezi jednak tím, že vede ke ztrátě funkce genu TEL, jemuž jsou připisovány tumor supresorové účinky, nebo naopak tím, že se jí uvolňuje genem TEL navozený blok leukemogenní funkce TEL/AML1, zprostředkovaný vzájemnou heterodimerizací [263]. Dalšími velmi častými sekundárními aberacemi jsou delece lokusu zahrnujícího geny PAX5 a CDKN2A, které byly nalezeny přibližně u čtvrtiny TEL/AML1 pozitivních leukémií a potenciálně vedou k deregulaci vývoje B řady a buněčného cyklu [12]. Již méně častou, na druhé straně však vysoce specifickou aberací, je delece genu TBL1XR1, která vede k narušení genové exprese řízené receptory hormonů [12, 279]. Tyto i další nově popsané aberace představují další výchozí body pro navazující studie na cestě k lepšímu pochopení vzniku a biologie TEL/AML1 pozitivních leukémií.

1.3 **Fúzní gen BCR/ABL**

1.3.1 *BCR*

Gen BCR leží na chromozomu 22 v oblasti 22q11, obsahuje dva alternativní exony 1 (e1, e1') a exony 2 (e2, e2') a dalších 21 exonů (e3-e23). BCR je ubiquitně exprimován a kóduje protein o velikosti 160 kDa, který obsahuje několik funkčních domén. Na N-konci se nachází oligomerizační *coiled-coil* doména, následují serin/threonin kinázová, SH2 vázající, *dbl-like* a *plecstrin-homology* domény [280-283]. Jediným známým subtrátem, který je fosforylován prostřednictvím kinázové domény, je protein Bap-1a z rodiny proteinů 14-3-3, které se zřejmě podílejí na regulaci buněčného cyklu [284]. *Dbl-like* a *plecstrin-homology* domény stimulují výměnu GDP za GTP u *guanidin exchange* faktorů Rho a ty mohou mimo jiné aktivovat transkripční faktor NF-κB [285]. Mezi doménami SH2 a *dbl-like* může být BCR fosforylován na pozici 177, čímž získává schopnost vázat GRB2 a potenciálně tak interagovat s dráhou Ras [286]. C-konec BCR aktivuje GTPázovou funkci proteinu Rac, který patří do nadrodiny proteinů Ras a podílí se na regulaci polymerizace aktinu a aktivity NADPH oxidázy u fagocytů [287]. Přítomnost všech těchto funkčních domén naznačuje, že protein BCR se účastní transdukce signálů, jeho přesná funkce však není dosud známá. Myši s vyřazeným genem BCR (*knock-out* myši) se vyvíjejí zcela normálně, jediným patologickým nálezem u těchto myší je porucha oxidativního vzplanutí neutrofilů [288].

1.3.2 *ABL*

230 kb velký gen ABL leží na chromozomu 9 v oblasti 9q34 a včetně dvou alternativních exonů 1 (1a, 1b) obsahuje 12 exonů [289]. Je exprimován ubiquitně a kóduje protein o velikosti 145 kDa, který má díky alternativním exonům 1a a 1b dvě izoformy [290]. Směrem od N-konce obsahuje 3 *Src homology* (SH) domény. SH1 doména má tyrosin-kinázovou aktivitu, SH2 a SH3 domény zprostředkovávají interakce s řadou proteinů vazbou jejich fosfotyrosinů, respektive oblastí bohatých na prolin [291]. Prostřednictvím intramolekulárních interakcí regulují N-konec proteinu a SH3 doména tyrosin-kinázovou aktivitu proteinu [292]. Dále ABL obsahuje oblast bohatou na prolin, prostřednictvím níž interaguje s dalšími proteiny, např. adaptorovým

proteinem Crk [293]. Směrem k C-konci se nacházejí tři nukleární lokalizační signály a DNA vazebné regiony, doména vážící F-aktin a signál pro nukleární export [294-297]. Pokud je N-konec kódován exonem 1a, protein je lokalizován v jádře, pokud exonem 1b, nachází se v cytoplazmě asociován s plazmatickou membránou [295, 297, 298]. Funkce proteinu ABL není ještě do všech detailů objasněna, v cytoplazmě se podílí na přenosu signálů z extracelulárního prostředí přes receptory integrinů, v jádře zřejmě na regulaci buněčného cyklu a buněčné odpovědi na genotoxický stres [299-303]. Díky četným interakcím, schopnosti fosforylovat řadu proteinů a pohybovat se mezi jádrem a cytoplazmou je tak zřejmě schopen integrovat signály z extracelulárního i intracelulárního prostředí a ovlivňovat buněčnou odpověď v rámci buněčného cyklu či apoptózy [304, 305]. Jeho role v hematopoéze není známa. Myši s vyřazeným genem ABL se rodí zakrslé a umírají brzy po narození [306, 307]. U některých byla nalezena atrofie sleziny i thymu a lymfopenie, ty jsou však zřejmě vyvolány sekundárně vysokými hladinami kortikosteroidů.

1.3.3 *BCR/ABL*

Fúzní gen BCR/ABL vzniká na podkladě reciproké chromozomální translokace t(9;22)(q34;q11) [308, 309]. Derivovaný chromozom 22, na kterém leží, je nazýván filadelfským chromosomem (Philadelphia chromosome, Ph). Tato genetická abnormalita se vyskytuje přibližně u 3-5% dětských BCP-ALL [310-312], daleko častěji se s ní však setkáváme u hematologických malignit v dospělosti, a to jednak u zhruba jedné třetiny ALL a především téměř u všech CML [313, 314]. Ke zlomům genu ABL dochází v poměrně rozsáhlé, 300 kb dlouhé oblasti zahrnující oba alternativní exony 1 a přilehlé intronové oblasti, přičemž nejčastěji se zlomy vyskytují právě mezi oběma alternativními exony [315]. U dětských BCP-ALL se setkáváme se dvěma variantami fúzního genu: u 80-90% případů se vyskytuje minor BCR/ABL (m-BCR/ABL), u 10-20% Major BCR/ABL (M-BCR/ABL) [311, 316]. Při vzniku m-BCR/ABL dochází ke zlomům BCR v 55 kb dlouhé oblasti (m-BCR) mezi oběma alternativními exony 2 [317]. Alternativní exony 1‘ a 2‘ genu BCR a 1a genu ABL jsou z primárních transkriptů vystřiženy, v transkriptu m-BCR/ABL je tedy fúzován první exon genu BCR k druhému exonu genu ABL [318]. Kromě dětských BCP-ALL se minor varianta vyskytuje u většiny BCR/ABL pozitivních BCP-ALL dospělých a velmi

raritně u CML dopělých [313, 319]. Při vzniku M-BCR/ABL se uplatňuje menší oblast zlomů o velikosti 5,8 kb (M-BCR) zahrnující exony 12-16 genu BCR. Na úrovni transkriptu M-BCR/ABL jsou nejčastěji fúzovány exony 13 či 14 genu BCR k druhému exonu genu ABL [320]. Díky alternativnímu sestřihu je u leukémií s genomickou fúzí M-BCR/ABL exprimován také m-BCR/ABL transkript a jím kódovaný protein [321, 322]. M-BCR/ABL se vyskytuje u menšiny dětských a dospělých BCR/ABL pozitivních ALL a prakticky u všech BCR/ABL pozitivních CML [311, 313, 314].

Fúzní transkript m-BCR/ABL kóduje protein o velikosti 190 kDa ($p190^{BCR/ABL}$), který zahrnuje oligomerizační doménu, SH2 vazebnou doménu, serin/threonin kinázovou doménu a vazebné místo pro GRB2 kódované prvním exonem BCR a prakticky celý ABL počínaje SH1 tyrosin-kinázovou doménou. M-BCR/ABL kóduje protein o velikosti 210 kDa ($p210^{BCR/ABL}$), který oproti předchozí variantě zahrnuje navíc domény *dbl-like* a *plecstrin-homolgy*. Oba proteiny, $p190^{BCR/ABL}$ i $p210^{BCR/ABL}$, jsou lokalizovány cytoplazmaticky a u obou je konstitutivně aktivována tyrosin-kinázová doména, a to zřejmě na podkladě homodimerizace zprostředkované oligomerizační doménou BCR [282, 323, 324]. Substráty tyrosin-kinázové aktivity BCR/ABL jsou jednak hybridní protein sám, jednak řada dalších proteinů zahrnující adaptorové proteiny (např. $p62^{DOK}$, Crk, Crkl, Shc), proteiny asociované s cytoskeletem a s membránou (např. talin, paxillin, Fak) a proteiny s vlastní katalytickou funkcí (např. PI3K, PLCγ, fosfatáza Syp) [304]. Výběr substrátu je ovlivněn cytoplazmatickou lokalizací a zřejmě závisí i na buněčném kontextu, neboť zatímco hlavním substrátem v BCR/ABL pozitivních neutrofilech u CML je adaptor Crkl, v časných progenitorech CML je hlavním substrátem adaptor $p62^{DOK}$ [325, 326]. Díky konstitutivně aktivní tyrosin-kinázové funkci a prostřednictvím interakcí přes další funkční domény BCR/ABL aberantně aktivuje několik důležitých signalizačních drah a proteinů (Ras/MAPK, PI3K/Akt, JAK/STAT, NF-κB, Myc), jejichž prostřednictvím aktivuje mitogenní signalizaci a inhibuje apoptózu [304, 327]. Těmito mechanizmy a zřejmě také dalšími (např. alterací buněčné adheze a deregulací signalizace přes integriny) přispívá BCR/ABL k maligní transformaci [304].

Řada prací demonstrovala, že *in vitro* je BCR/ABL schopen transformovat fibroblasty i primární hematopoetické buňky a udělit hematopoetickým liniím (lymfoidním i myeloidním) nezávislost na růstových faktorech [282, 283, 295, 323, 324, 328-333].

Tyto práce se také zabývaly otázkou, které domény a jimi regulované dráhy jsou pro transformaci nezbytné. Zdá se, že jediné domény, které jsou absolutně nezbytné pro efekt BCR/ABL v hematopoetických buňkách, jsou tyrosin-kinázová doména ABL a ji aktivující oligomerizační doména BCR, kdežto pro transformaci fibroblastů jsou nezbytné i další domény. To může být způsobeno zčásti tím, že k aktivaci týchž (či funkčně ekvivalentních) drah dochází v hematopoetických buňkách na rozdíl od fibroblastů vícero mechanizmy a přes několik různých domén najednou; funkce domén nezbytných pro transformaci fibroblastů je tedy u hematopoetických buněk nahrazena jinými doménami [327].

Za účelem studia *in vivo* efektu BCR/ABL bylo vytvořeno několik transgenních a "transdukčně-transplantačních" myších modelů [38, 39, 334-343]. Bylo zjištěno, že u myši vyvolává BCR/ABL různé typy hematologických malignit včetně myeloproliferací podobných CML a akutních lymfoidních leukémií, ale i např. akutní myeloidní leukémie či tumory z makrofágů. Z těchto modelů vyplynulo, že obecně jsou u myši obě varianty BCR/ABL (minor i Major) schopny vyvolat jak chronické myeloproliferace, tak BCP-ALL, avšak existují mezi nimi určité rozdíly. U transgenní myši vytvořené Vonckenem et al. vyvolával M-BCR/ABL akutní leukémie B i T původu i myeloproliferace, kdežto m-BCR/ABL vyvolával pouze BCP-ALL a to s kratší latencí než M-BCR/ABL [338]. Li et al. pozorovali v "transdukčně-transplantačním" modelu stejnou schopnost obou variant vyvolávat myeloproliferaci podobnou CML, kdežto ke vzniku ALL docházelo u m-BCR/ABL s kratší latencí [39]. Li et al. také u m-BCR/ABL popsali vyšší schopnost transformovat lymfoidní linie *in vitro* a tato schopnost korelovala s jeho vyšší tyrosin-kinázovou aktivitou oproti M-BCR/ABL [39]. Obdobně dvě další práce ukázaly, že m-BCR/ABL má vyšší schopnost *in vitro* transformovat primární lymfoidní prekurzory [333, 344]. Tyto výsledky z myších modelů částečně korespondují se situací u člověka, kdy u většiny BCP-ALL dětí i dospělých nalézáme m-BCR/ABL variantu. Schopnost vyvolat akutní leukémii by mohla být u M-BCR/ABL spojena se získáním většího transformačního potenciálů při vyšší expresi [305]. V souladu s touto teorií dochází u myší ke vzniku BCP-ALL se 100% penetrancí při vysoké expresi M-BCR/ABL z poměrně silného promotoru virového původu [343]. Stejně tak byly nalezeny vyšší hladiny M-BCR/ABL u BCP-ALL a u CML při progresi do blastické krize oproti chronické fázi CML [305, 345].

Popsaná nižší tyrosin-kinázová aktivita M-BCR/ABL a zřejmě i přítomnost domén *dbl-like* a *plecstrin-homology*, které se nevyskytují u m-BCR/ABL, by naopak mohly hrát roli v preferenční indukci CML u člověka [305]. Otázkou zůstává, proč mBCR/ABL oproti myším modelům prakticky nezpůsobuje CML u člověka. Důvodem by mohl být jednak preferenční transformační účinek na lidské lymfoidní hematopoetické buňky či specifický výskyt m-BCR/ABL v lymfoidních buňkách (oproti popsanému multiliniovému výskytu M-BCR/ABL u CML) [305]. Výsledky studií zaměřených na původ BCR/ABL pozitivních ALL se různí, ale minimálně u některých m-BCR/ABL pozitivních ALL (a také u M-BCR/ABL pozitivních ALL) byl fúzní gen nalezen nejen v lymfoidních, ale i v dalších liniích [58-60, 346-348]. BCR/ABL pozitivní ALL tak mohou zřejmě být nejen lymfoidního, ale i multiliniového původu.

Z analýzy klonality BCR/ABL pozitivních akutních leukémií u myších "transdukčně-transplantačních" modelů a především z opakovaně popsané přítomnosti BCR/ABL u zdravých jedinců vyplývá, že pro vznik leukémie zřejmě není BCR/ABL sám o sobě dostačující a ke vzniku klinicky aktivní malignity je potřeba dalších leukemogenních zásahů [38, 39, 349, 350]. Nejčastějším přídatným zásahem vyskytujícím se u více než 80% dětských BCR/ABL pozitivních ALL je delece genu IKZF1, který kóduje transkripční faktor Ikaros nezbytný pro vývoj lymfoidních linií. Dalšími častými zásahy jsou delece PAX5 a CDKN2A. Samotný BCR/ABL zřejmě hraje roli nejen v indukci, ale i v udržení leukémie. U transgenní myši s represibilním BCR/ABL dochází při vypnutí BCR/ABL ke kompletní remisi leukémie a stejně tak umlčení BCR/ABL pomocí RNAi vede ke ztrátě maligního fenotypu u leukemických linií [79, 343, 351].

U dětí je BCR/ABL pozitivní ALL onemocněním se špatnou prognózou [310, 312, 352-355]. Samotný BCR/ABL fúzní gen je nepříznivým prognostickým faktorem, navíc se u dětí s BCR/ABL pozitivní ALL často vyskytují i další rizikové faktory jako starší věk, vyšší iniciální leukocytóza či infiltrace CNS. Děti s BCR/ABL pozitivní ALL tak mají zvýšené riziko selhání terapie a následného relapsu [310, 352, 354, 355]. Při jejich léčbě se uplatňují protokoly pro vysoce rizikové ALL, zahrnující intenzivní chemoterapii, transplantaci kostní dřeně a nejnověji také tyrosin-kinázové inhibitory (imatinib, dasatinib a další). I v rámci této podskupiny BCP-ALL však existují pacienti s relativně lepší prognózou, a to pacienti s nízkou iniciální leukocytózou či s dobrou odpovědí na iniciální terapii [311, 316, 356, 357]. U těchto pacientů by k vyléčení

mohla stačit intenzivní chemoterapie, zatímco pro vyléčení pacientů zařazených do vysokého rizika se zatím zdá být nezbytná alternativní forma terapie zahrnující například alogenní transplantaci kostní dřeně v první kompletní remisi [311, 356, 358-360]. Role imatinibu či novějších kinázových inhibitorů v terapii dětských BCR/ABL pozitivních ALL se zatím vyhodnocuje, v recentní studii vedlo kontinuální podávání imatinibu k významnému zlepšení léčebných výsledků [78]. Stejně jako u dětských BCP-ALL obecně, i při terapii BCR/ABL pozitivních ALL zaujímá velmi významné místo monitorování MRN. V první řadě umožňuje posoudit odpověď na léčbu a identifikovat výše zmíněné pacienty s dobrou odpovědí a lepší prognózou, cenné informace o vývoji onemocnění však přináší i v dalších fázích léčby. Standardní metodou je i zde monitorování klonálně specifických přestaveb genů pro imunoreceptory pomocí qPCR, ale práce Cazzanigy et al. ukázala, že k monitorování odpovědi na terapii lze využít také kvalitativní detekci fúzního transkriptu BCR/ABL a že i touto metodou lze identifikovat pacienty s vyšším rizikem relapsu [361].

1.4 **Fúzní gen TEL/ABL**

Fúzní gen TEL/ABL byl poprvé nalezen v roce 1995 u kojence s BCP-ALL [144]. V průběhu následujících let se ukázalo, že se jedná o velmi vzácně se vyskytující genetickou aberaci [362, 363], kdy do dnešního dne bylo celkově popsáno pouze 19 případů hematologických malignit s tímto fúzním genem [112, 144, 364-378]. Jedná se o značně heterogenní skupinu zahrnující 9 případů CML, 3 případy jiných myeloproliferativních onemocnění, 4 případy AML a 3 případy ALL (z toho jeden T-ALL). V tomto souboru figurují pouze 2 děti, u obou byla nalezena TEL/ABL pozitivní ALL [144, 377]. K vzácnosti této aberace může částečně přispívat skutečnost, že k jejímu vytvoření je vzhledem k obrácené orientaci obou genů potřeba nejméně tří zlomů DNA. Na rozdíl od fúzních genů BCR/ABL a TEL/AML1 tedy tato aberace nemůže vznikat na základě prosté reciproké translokace a z tohoto důvodu zřejmě není přítomen ani reciproký fúzní transkript [144, 379]. U většiny případů jsou popsány poměrně složité přestavby zahrnující kromě chromozomů 9 a 12, na kterých se nacházejí fúzní partneři, i další chromozomy. Na rozdíl od častého výskytu u TEL/AML1 pozitivních leukémií [380] není u TEL/ABL pozitivních případů nalézána delece druhé alely genu TEL. U jednoho pacienta byla nalezena delece zahrnující geny pro regulátory buněčného cyklu CDKN2A a CDKN2B, se kterou se u leukémií setkáváme poměrně často a která je považována za jeden ze zásahů přispívajících k maligní transformaci [25]. Za charakteristický znak TEL/ABL pozitivních hematologických malignit byla označena eosinofilie, ta se však objevuje i u dalších hematologických malignit s aberacemi genu TEL [381, 382].

Experimentální práce ukázaly, že chimerická kináza TEL/ABL má velmi podobné funkční vlastnosti jako mnohem častěji se vyskytující chimerická kináza BCR/ABL. Stejně jako v případě BCR/ABL lze *in vitro* inhibovat její kinázovou aktivitu a na ní závislý proliferační potenciál leukemických buněk imatinib mesylátem [383, 384]. Ten byl použit v léčbě některých pacientů [366-368, 374-376], u poloviny z nich však byla odpověď na terapii pouze krátkodobá [366, 368, 375]. Při progresi klasické CML do blastického zvratu narůstá hladina fúzního transkriptu BCR/ABL [345]. Obdobný jev však nebyl pozorován v případě fúzního transkriptu TEL/ABL, jehož hladina byla sledována u jediného pacienta při progresi CML z první blastické krize do druhé [366].

Překvapivě došlo u tohoto pacienta s progresí onemocnění k poklesu hladiny *wt* transkriptu TEL, přestože na základě absence delecí tohoto genu (viz výše) není ztráta aktivity *wt* alely považována za jeden z mechanizmů účastnících se patogeneze TEL/ABL leukémií.

Hybridní protein TEL/ABL je lokalizován v cytoplazmě, kde zřejmě interaguje s aktinovými filamenty [112, 385, 386]. Kinázová funkce ABL části hybridního proteinu je konstitutivně aktivována a tato aktivace, stejně jako cytoplazmatická lokalizace proteinu, je závislá na přítomnosti HLH domény TEL, která umožňuje dimerizaci [112, 385]. TEL/ABL transformuje fibroblasty (získávají schopnost růstu nezávisle na kontaktu s podložkou) i primární buňky kostní dřeně u myší [112] a hematopoetickým buněčným liniím udílí schopnost proliferace nezávisle na přítomnosti růstových faktorů [385, 386]. Substráty kinázy TEL/ABL se značně shodují se substráty kinázy BCR/ABL, přičemž jsou odlišné od substrátů *wt* ABL [386]. Obě chimerické kinázy tak aktivují tytéž signál transdukujících dráhy, jako jsou Ras/MAPK dráha, PI3K/Akt dráha či dráhy zahrnující signalizaci přes STAT5 či NF-κB, a mají zřejmě i velmi podobné biologické účinky [386-389]. Mezi ty patří mimo jiné zrychlená oprava DNA poškozené působením exogenních genotoxických faktorů, ale i stimulace vzniku dvouvláknových zlomů DNA a s tím spojené zvyšování chromozomální nestability [390, 391].

U myši vyvolává TEL/ABL fúzní gen dva typy onemocnění: 1) stejně jako fúzní gen BCR/ABL vyvolává meyloproliferaci podobnou CML, 2) vyvolává nový syndrom, který nebyl popsán u BCR/ABL, zahrnující meyloidní infiltraci tenkého střeva, vysokou hladinu cirkulujících endotoxinů a TNFα a fulminantní selhání jater a ledvin [392]. Pro vznik obou onemocnění jsou nezbytné HLH doména TEL a ABL-kinázová aktivita. Myeloproliferativní onemocnění je multiliniového původu a na rozdíl od myeloproliferace vyvolané fúzním genem BCR/ABL není přenositelné do sekundárního příjemce. U pacientů byly popsány dva typy fúzních transkriptů – v prvním je exon 4 genu TEL fúzován na druhý exon genu ABL, ve druhém je navíc obsažen exon 5 genu TEL. U některých pacientů byl popsán pouze jeden typ transkriptu [112, 144, 364, 366, 370, 376], u dalších jsou následkem alternativního sestřihu přítomny oba typy [365, 367, 371, 372, 375, 377]. V experimentálních modelech není exon 5 genu TEL potřebný pro konstitutivní kinázovou aktivaci ani pro

udělení nezávislosti na růstových faktorech hematopoetickým liniím [385]. Nepřítomnost exonu 5 však znamená ztrátu schopnosti transformovat fibroblasty a oslabuje schopnost vyvolávat myeloproliferativní onemocnění u myši [393]. To může být zapříčiněno ztrátou interakce s GRB2, kterou zprostředkovává právě oblast kódovaná exonem 5 genu TEL, neboť stejné následky má ztráta vazebného místa pro GRB2 u BCR/ABL fúzního genu [394].

2. **Cíle a výsledky**

V naší práci jsme se zaměřili na studium biologických a klinických aspektů fúzních genů TEL/AML1, BCR/ABL a TEL/ABL a jimi vymezených genotypových podskupin dětských BCP-ALL. Veškeré výsledky jsou podrobně zpracovány v šesti přiložených publikacích, zde uvádím pouze stručný přehled cílů a hlavních výsledků.

U fúzního genu TEL/AML1 jsme se zabývali několika otázkami. Nejprve jsme se pokusili identifikovat přímé cíle TEL/AML1 analýzou expresního profilu TEL/AML1 pozitivní leukemické linie REH ovlivněné inhibitorem deacetyláz histonů valproátem (VPA). Vycházeli jsme z částečně prokázané hypotézy, že TEL/AML1 coby hybridní transkripční faktor aberantně reprimuje transkripci genů (dosud neznámých), jež jsou za fyziologických podmínek v lymfoidní vývojové linii regulovány proteinem AML1, a to prostřednictvím inaktivace chromatinu navozené deacetylací histonů. Předpokládali jsme, že funkci TEL/AML1 bude možné specificky blokovat pomocí VPA, čímž zvrátíme aberantní represi těchto neznámých cílových genů a budeme je moci identifikovat v expresním profilu na základě zvýšení jejich exprese. Vytvořili jsme modelovou *in vitro* reportérovou esej v linii HeLa, ve které jsme demonstrovali schopnost TEL/AML1 reprimovat transkripci z promotoru granzymu B, známého cílového genu AML1, a potvrdili jsme schopnost VPA tuto funkci TEL/AML1 blokovat. Dále jsme aplikovali VPA *in vitro* na TEL/AML1 pozitivní leukemickou linii REH a sledovali změny fenotypu a především expresního profilu, které VPA vyvolal. Abychom zjistili, zda VPA může prostřednictvím inhibice TEL/AML1 navodit změny fenotypu specifické pro TEL/AML1 pozitivní buňky, porovnali jsme jeho efekt na TEL/AML1 pozitivní a TEL/AML1 negativní leukemické linie. Na rozdíl od TEL/AML1 negativních linií vedla aplikace VPA u TEL/AML1 pozitivní linie ke změnám imunofenotypových markerů odpovídajícím mírnému diferenciačnímu posunu a k zástavě buněčného cyklu v G1/G0 fázi. Dále jsme u TEL/AML1 pozitivní linie pomocí DNA čipu analyzovali změny exprese vyvolané aplikací VPA. Analýzu jsme cílili na soubor genů, u nichž jsme v dostupných expresních profilech dětských ALL nalezli specificky nižší expresi u TEL/AML1 pozitivní podskupiny a u nichž se zároveň exprese u TEL/AML1 pozitivních ALL významně nelišila od studované linie REH. Mezi těmito geny jsme identifikovali ty, jejichž exprese se u linie REH statisticky

významně zvýšila po podání VPA a které by tak mohly být přímými cíly TEL/AML1. Výsledky jsme publikovali v původním článku v časopise *Clinical Cancer Research* v roce 2007.

Dále jsme se zabývali otázkou, jakou roli hraje fúzní gen TEL/AML1 v leukemických buňkách pro udržení jejich maligního potenciálu. Rozhodli jsme se specificky umlčet TEL/AML1 pomocí RNAi a studovat dopad tohoto umlčení na viabilitu a proliferační aktivitu leukemických buněk a také na genovou expresi. Navrhli jsme sadu 11 možných různých siRNA cílených na místo fúze obou partnerů tak, aby tyto siRNA byly cíleny pouze na fúzní transkript TEL/AML1 a nikoliv na *wt* transkripty TEL a AML1 přepisované z netranslokovaných alel těchto genů. Jelikož se hybridní protein TEL/AML1 v přirozených leukemických buňkách (buněčných linií i pacientů) poměrně nesnadno detekuje, pro testování účinnosti siRNA jsme si vytvořili náhradní model – transgenní reportérovou linii HeLa$^{TEL/AML1\text{-}ires\text{-}EGFP}$. V této linii je z trvale vneseného konstruktu přepisována bicistronická mRNA a z ní jsou translatovány proteiny TEL/AML1 a EGFP. Účinnost siRNA cílených proti fúzi TEL/AML1 jsme vyhodnocovali dle poklesu intenzity fluorescence EGFP proteinu (reportéru) měřené průtokovou cytometrií. Nejúčinnější siRNA jsme dále testovali na úrovni mRNA v TEL/AML1 pozitivní leukemické linii REH. K maximálnímu poklesu mRNA TEL/AML1 docházelo 48 hodin po transfekci, použití dvou nejúčinnějších siRNA vedlo ke snížení hladiny fúzního transkriptu o 57% a 58%. Směs těchto siRNA jsme dále použili k umlčení fúzního genu TEL/AML1 v TEL/AML1 pozitivních leukemických liniích REH a UOC-B6. Dvojí transfekcí siRNA jsme dosáhli značného poklesu hladiny TEL/AML1 proteinu v obou liniích, kdy množství proteinu kleslo v průměru o 74% v linii REH a o 86% v linii UOC-B6. Následkem umlčení TEL/AML1 nedocházelo k indukci apoptózy či poklesu viability a proliferace. Naopak v obou liniích jsme pozorovali mírný, ale vysoce reprodukcibilní a signifikantní nárůst S fáze a zvýšení frakce proliferujících buněk. Vyslovili jsme hypotézu, že k tomu dochází následkem odstranění inhibice funkce proteinu AML1, který podporuje přechod z G1 do S fáze buněčného cyklu. Pro ověření této hypotézy jsme provedli experiment opačného charakteru, pomocí RNAi jsme umlčeli *wt* AML1. V obou leukemických liniích, REH i UOC-B6, došlo ke změnám buněčného cyklu a proliferace opačného charakteru než v případě umlčení TEL/AML1, tedy k poklesu S fáze a k poklesu

proliferační aktivity. Dále jsme analyzovali změny expresního profilu při umlčení TEL/AML1 v linii REH. Naším cílem bylo pokusit se ověřit předchozí prací identifikované potenciální cílové geny TEL/AML1. Nenalezli jsme však žádné signifikantní změny exprese. Výsledky této práce jsme sepsali do původního článku, který je nyní posuzován pro publikaci.

Ve spolupráci se zahraničními kolegy jsme dále zkoumali roli microRNA 125b2 u TEL/AML1 pozitivních leukémií. Zjistili jsme, že tato microRNA spolu se dvěma dalšími, micro RNA let-7c a microRNA 99a, jsou vysoce exprimovány specificky u TEL/AML1 pozitivních ALL a mohly by tak hrát roli v patogenezi těchto leukémií. Studovali jsme, zda při snížení či nadprodukci proteinu TEL/AML1 dochází ke změně exprese těchto microRNA. K umlčení TEL/AML1 jsme použili výše popsaný postup založený na RNAi. Snížení množství TEL/AML1 proteinu v leukemické linii REH nevedlo ke změně exprese sledovaných microRNA. Stejně tak nevedla ke změně jejich exprese nadprodukce TEL/AML1 v myších progenitorových hematopoetických buňkách. Chromatinovou imunoprecipitací jsme dále ukázali, že TEL/AML1 se neváže do promotorové oblasti těchto microRNA a že ke zvýšení jejich exprese u TEL/AML1 pozitivních leukémií tedy dochází nezávisle na fúzním genu TEL/AML1. Abychom posoudili biologický význam zvýšené exprese microRNA 125b2 u TEL/AML1 pozitivních leukémií, studovali jsme jednak následky nadprodukce této microRNA v myší progenitorové hematopoetické linii, jednak následky její suprese v TEL/AML1 pozitivní leukemické linii REH. Zjistili jsme, že zvýšená exprese microRNA 125b2 poskytuje leukemické buňce zvýšenou schopnost přežití v přítomnosti růst inhibujících signálů. Výsledky této práce byly publikovány v původním článku v časopise *Leukemia* v roce 2009.

Ve skupině 71 TEL/AML pozitivních ALL diagnostikovaných v České republice v období od roku 2005 do roku 2007 jsme objevili dva případy s variantním fúzním genem TEL/AML1. U obou případů došlo následkem raritního zlomu genu TEL v intronu 4 k vytvoření variantní fúze a k expresi variantního transkriptu, který na rozdíl od typického fúzního transkriptu TEL/AML1 neobsahuje exon 5 genu TEL. Kvůli absenci tohoto exonu jsme u obou případů získali při rutinním testování přítomnosti fúzního transkriptu TEL/AML1 pomocí RT-PCR falešně negativní výsledek, který byl v rozporu s pozitivním nálezem translokace t(12;21) metodou FISH.

Analýza genomické fúze a hledání potenciálně přítomných netypických transkriptů TEL/AML1 i AML1/TEL následně vedly k identifikaci výše popsané variantní fúze. Variantní fúzní gen TEL/AML1 kóduje hybridní protein, který na rozdíl od typického proteinu TEL/AML1 postrádá centrální oblast kódovanou exonem 5 genu TEL. Tato centrální část genu TEL byla podle některých studií dosud považována za nezbytnou pro leukemickou transformaci řízenou genem TEL/AML1. V této oblasti se nacházejí autonomní transkripčně represivní domény proteinu TEL, jejichž ztráta by mohla modifikovat funkci proteinu TEL/AML1 a případně fenotyp leukémií. Zaklonovali jsme variantní fúzi TEL/AML1 do expresního plasmidu a pomocí výše popsané reportérové eseje jsme studovali, zda u variantního proteinu TEL/AML1 dochází k ovlivnění jeho transkripčně represivní schopnosti. Zjistili jsme, že transkripčně represivní schopnost zůstává u variantního proteinu TEL/AML1 plně zachována. Dále jsme porovnali klinické a biologické znaky leukémií s typickou versus variantní fúzí TEL/AML1, konkrétně klinické znaky při manifestaci onemocnění (věk, počet leukocytů, proporce blastů v kostní dřeni), odpověď na terapii, imunofenotyp a expresní profil. V těchto znacích se případy s variantní fúzí nelišily od případů s typickou fúzí TEL/AML1. Výsledky této práce jsme sepsali do původního článku, který je nyní posuzován pro publikaci.

V práci zabývající se BCR/ABL pozitivními ALL jsme studovali možnosti využití fúzního transkriptu BCR/ABL coby cíle pro monitorování MRN. V souboru 218 vzorků kostní dřeně od 17 dětí s BCR/ABL pozitivní ALL jsme retrospektivně určovali hladiny MRN pomocí kvantitativní detekce fúzního transkriptu BCR/ABL a současně i pomocí standardní metody, kterou je u dětských ALL kvantitativní detekce klonálně specifických přestaveb genů pro imunoreceptory. Zjistili jsme, že hladiny MRN naměřené těmito dvěma způsoby korelují pouze částečně a míra korelace se u jednotlivých pacientů výrazně liší, od výborné po velmi špatnou. U jedné pětiny všech vzorků z vyšetřeného souboru jsme pomocí metody založené na detekci imunoreceptorových cílů nedetekovali přítomnost MRN, přestože obdobně citlivou metodou založenou na detekci fúzního transkriptu byla v těchto vzorcích reziduální nemoc nalezena (a to i na relativně vysokých hladinách). Zaměřili jsme se na možný klinický význam přítomnosti MRN zachycené detekcí fúzního transkiptu. Zjistili jsme, že monitorováním MRN pomocí fúzního transkriptu BCR/ABL lze v průběhu

dlouhodobého sledování pacientů lépe a dříve predikovat relaps onemocnění než při monitorování MRN standardní metodou. Vyslovili jsme hypotézu, že jednou z příčin diskordance obou metod monitorování MRN by mohla být přítomnost fúzního transkriptu BCR/ABL v buňkách mimo dominantní leukemický klon, které na rozdíl od dominantního klonu neobsahují specifické přestavby genů pro imunoreceptory. Pro ověření této hypotézy jsme u pacientky s velmi špatnou korelací obou metod a s dlouhodobě vysokými hladinami fúzního transkriptu sortovali subpopulace hematopoetických buněk a vyšetřili v nich přítomnost jak fúzního transktiptu tak imunoreceptorových cílů. Zjistili jsme, že na rozdíl od imunoreceptorových cílů je fúzní transkript BCR/ABL přítomen nejen v plně leukemických buňkách, ale i mimo dominantní leukemický klon - v buňkách B řady i myeloidní řady. Výsledky této práce jsme publikovali v původním článku v časopise *Leukemia* v roce 2009.

Mezi diagnostickými leukemickými vzorky vyšetřenými na našem pracovišti přibližně od konce roku 2003 do poloviny roku 2009 jsme zachytili 3 případy (2 děti a 1 dospělý) ALL s velmi vzácně se vyskytujícím fúzním genem TEL/ABL. Detailně jsme studovali klinický a biologický charakter těchto případů, abychom ověřili dosud popsané či nalezli nové znaky této raritní podskupiny leukémií. Zjistili jsme, že u všech tří případů se vyskytují oba dosud popsané fúzní transkripty TEL/ABL následkem alternativního setřihu. Pomocí array CGH jsme studovali přítomnost a typ přídatných genomických aberací a u dvou ze tří pacientů jsme nalezli deleci lokusu zahrnující geny CDKN2A a CDKN2B či přilehlou oblast. U obou dětských pacientů jsme použili fúzní transkript TEL/ABL jako cíl pro monitorování MRN a porovnali jsme takto naměřenou reziduální nemoc s MRN naměřenou standardním způsobem založeným na detekci imunoreceptorových cílů. U obou pacientů obě metody velmi dobře korelovaly. Dále jsme u jednoho z dětských pacientů nalezli místo genomické fúze obou genů a využili jsme jej coby marker pro “backtracking“ leukemického klonu na Guthrieho kartičce s novorozeneckou krví. Zjistili jsme, že u tohoto pacienta byl fúzní gen TEL/ABL přítomen již při narození. Tyto výsledky zasazené do detailního přehledu všech do současné doby popsaných TEL/ABL pozitivních případů a jejich klinických a biologických znaků jsou obsahem původního článku, který je nyní posuzován pro publikaci.

3. **Přiložené publikace**

The Identification of (ETV6)/RUNX1-Regulated Genes in Lymphopoiesis Using Histone Deacetylase Inhibitors in ETV6/RUNX1-Positive Lymphoid Leukemic Cells

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Abstract

Purpose: Chimeric transcription factor ETV6/RUNX1 (TEL/AML1) is believed to cause pathologic block in lymphoid cell development via interaction with corepressor complex and histone deacetylase. We wanted to show the regulatory effect of ETV6/RUNX1 and its reversibility by histone deacetylase inhibitors (HDACi), as well as to identify potential ETV6/RUNX1-regulated genes.

Experimental Design: We used luciferase assay to show the interaction of ETV6/RUNX1 protein, ETV6/RUNX1-regulated gene, and HDACi. To identify ETV6/RUNX1-regulated genes, we used expression profiling and HDACi in lymphoid cells. Next, using the flow cytometry and quantitative reverse transcription-PCR, we measured differentiation changes in gene and protein expression after HDACi treatment.

Results: Luciferase assay showed repression of granzyme B expression by ETV6/RUNX1 protein and the reversibility of this effect by HDACi. Proving this regulatory role of ETV6/RUNX1, we identified, using complex statistical analysis, 25 genes that are potentially regulated by ETV6/RUNX1 protein. In four selected genes with known role in the cell cycle regulation (*JunD*, *ACK1*, *PDGFRB*, and *TCF4*), we confirmed expression changes after HDACi by quantitative analysis. After HDACi treatment, ETV6/RUNX1-positive cells showed immunophenotype changes resembling differentiation process compared with other leukemic cells (BCR/ABL, ETV6/PDGFRB positive). Moreover, ETV6/RUNX1-positive leukemic cells accumulated in G_1-G_0 phase after HDACi whereas other B-lineage leukemic cell lines showed rather unspecific changes including induction of apoptosis and decreased proliferation.

Conclusions: Presented data support the hypothesis that HDACi affect ETV6/RUNX1-positive cells via direct interaction with ETV6/RUNX1 protein and that treatment with HDACi may release aberrant transcription activity caused by ETV6/RUNX1 chimeric transcription factor.

Neoplastic transformation is characterized by inadequate proliferation and changes in the mechanisms of differentiation and apoptosis (1). Aberrant transcription factors, frequently present at the diagnosis of leukemias, are probably responsible for one of the steps in leukemogenic transformation in these particular cases. The expression of chimeric transcription factors results in a block of differentiation and apoptosis by interfering with the function of their wild-type counterparts. The role for one of these chimeric transcription factors, ETV6/RUNX1 (TEL/AML1), in leukemogenesis is still not completely understood despite the frequency of t(12;21)-positive acute lymphoblastic leukemia (ALL; up to 25%; refs. 2–4).

In normal hematopoietic cells, the erythroblast transformation–specific (ETS) family transcription factor ETV6 (TEL) is composed of two major functional domains: an NH_2-terminal pointed (PNT) domain and a COOH-terminal ETS domain (5–7). RUNX1 [AML1, core binding factor (CBF)-α2], a member of the RUNX protein family (8), represents an α-subunit of CBF and displays high homology with a segment of the *Drosophila* gene *runt*. RUNX1 is composed of some characteristic domains. The Runt domain is responsible for the binding with DNA and protein-protein interaction, and the transactivation domain is located at the COOH-terminal site (9, 10). The ETV6/RUNX1 fusion protein contains the NH_2-terminal part of the ETV6 protein with the dimerization PNT domain and almost the complete RUNX1 protein with all functional domains. Deficiency in RUNX1 expression leads to

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an early block in hematopoietic differentiation. Therefore, RUNX1 knockout animals completely lack mature hematopoiesis and die embryonically (11, 12). RUNX1 seems to have a dual role in promoting cell cycle progression and differentiation, presumably depending on the presence of different factors that interact with it in different stages of development (13). RUNX proteins are able to either increase or actively inhibit the transcriptional activity of target genes, most likely depending on the specific cell type as well as the particular target gene (14). ETV6 function is essential for the establishment of hematopoiesis of all lineages in the bone marrow (15). ETV6$^{-/-}$ mice are embryonic lethal because of a yolk sac angiogenic defect.

As the DNA-binding domain of RUNX1 is retained in the ETV6/RUNX1 fusion, it is expected that this fusion protein binds to DNA through this domain and functions in a dominant-negative fashion. Several theories propose a mechanism of repressional activity of ETV6/RUNX1. Siu et al. describe heterodimerization of ETV6/RUNX1 and ETV6 that prevents normal ETV6 activity. Another study suggests that the dominant-negative effect of ETV6/RUNX1 is caused by the binding with RUNX1 coactivators (e.g., p300) and sequestering them into a complex localized in the cytoplasm (16, 17). Another theory proceeds from a previously described association of the ETV6 part of the chimeric protein with the nuclear corepressors mSin3A, N-CoR, and histone deacetylase (HDAC)-3, which led to the assumption that ETV6/RUNX1 works as a transcriptional repressor by changing chromatin pattern (7, 18, 19). Published results confirmed a direct negative effect of the ETV6/RUNX1 protein by using reporter constructs driven by regulatory regions derived from hematopoiesis-specific genes including the lymphoid-specific T-cell receptor-β enhancer and interleukin-3 promoter (10, 20). However, no other target genes of ETV6/RUNX1 expressed in hematopoietic cells have been identified thus far. The proposed mechanism of action via chromatin remodeling using HDACs has not been proved.

HDACs are characterized by their capability to cleave acetyl groups from the lysine residues localized at the ends of histones. The role of HDACs in hematological diseases was described for the first time in a study on acute promyelocytic leukemia. Retinoic acid receptor-α receptor is able to bind the corepressor complex including HDACs, and this function is extended by binding with its fusion partner promyelocytic leukemia in t(15;17). Another fusion partner of retinoic acid receptor-α, promyelocytic leukemia zinc finger protein in t(11;17), also binds the corepressors and inhibits transcription of the target genes, consequently blocking myeloid differentiation. The aberrant repression in both *in vivo* and *in vitro* models of acute promyelocytic leukemia was counteracted only by HDAC inhibitors (HDACi) in combination with retinoic acid (21). A number of HDACi have been characterized that inhibit the deacetylation of histones, which is associated with the reactivation of gene expression, leading to differentiation and thus abolishing tumor growth (1, 22, 23).

We attempted to identify genes regulated by ETV6/RUNX1 in lymphoblasts using expression profiling and studied whether their expression can vary depending on the acetylation/deacetylation of histones. We compared different genetically characterized subgroups (ETV6/RUNX1, BCR/ABL, and MLL/AF4) most frequently present in children with ALL to identify specific genes of ETV6/RUNX1 genotype. It was proposed that chromatin remodeling is responsible for the leukemogenic role of ETV6/RUNX1 fusion protein and that this effect could be reversible by the application of HDACi (24). The present work aimed to verify this assumption by using HDACi trichostatin A (TSA) and valproic acid (VPA). We tested the hypothesis that aberrant chromatin remodeling affects expression of genes originally transactivated by RUNX1 (25–27). We tested whether HDACi can release repression activity of ETV6/RUNX1 and induce differentiation in lymphoid leukemic cells by comparison with other B-lymphoid leukemic cells with different mechanisms of leukemogenesis (BCR/ABL, ETV6/PDGFRB).

Materials and Methods

Cell cultivation. REH, a B-cell precursor leukemia cell line with translocation (12;21), kindly provided by R. Pieters (Erasmus Medical Center Rotterdam), Nalm-6 B-precursor leukemic cell line with translocation (5;12), Nalm-24 B-precursor leukemic cell line with translocation (9;22), and NC-NC, normal lymphoblastoid cells immortalized by EBV transformation, were used for experiments. Cell cultures except for REH were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures; Braunschweig, Germany). Cells were cultured at the Roswell Park Memorial Institute (RPMI) 1640 with 2 mmol/L L-glutamine with 10% fetal calf serum and 10 mL/L antibiotic solution (100 units/mL penicillin, 100 μg/mL streptomycin). All suspension cultures were maintained at 37°C in 5% CO_2. Cells were collected by centrifugation for 10 min at 240 × *g* and resuspended at a density of 5×10^5/mL in fresh medium 24 h before all experiments. Cell lines were treated in six-well plates for 24 and 48 h with either VPA at concentrations of 0.5 and 1.0 mmol/L or TSA at concentrations of 120 and 240 nmol/L. The doses of VPA were chosen according to the optimum serum concentrations in neurologic patients (0.35-0.7 mmol/L).

Isolation of mRNA and cDNA conversion. Total RNA was extracted from a standardized amount of mononuclear cells isolated from cell lines using a modified method described by Chomczynski and Sacchi (28) and from patient samples as previously described (29). The total extraction volume of RNA was adjusted to the number of processed cells and converted into cDNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Carlsbad, TX) according to the manufacturer's instructions.

Flow cytometry. Immunophenotype measurement of treated cells was done with a combination of fluorescent monoclonal antibodies. Particular antibodies were chosen to cover specific stages of studied B-cell development. The selected immunostainings were measured by multicolored combinations: surface antigens—CD10 FITC/CD20 phycoerythrin/CD71 A1633/4′,6-diamidino-2-phenylindole; intracellular antigens—iTdT FITC/iIgM phycoerythrin/20 PC5 and iRAG-1 A1633. For measurement, we used a BD FACSAria Cell Sorting System (Becton Dickinson, San Jose, CA) and data acquisition analyses were done by FlowJo (TreeStar, Ashland, OR) and by CELLQuest (Becton Dickinson) software applications. CycleTEST PLUS DNA Reagent Kit (Becton Dickinson) was used for DNA analysis. Proliferation activity was assessed as a percentage of cells in S and G_2-M phases of cell cycle. All individual cultivations and cell measurements were done in independent triplicates. Statistics was calculated by ANOVA test.

Plasmids. *Myc*-tagged ETV6/RUNX1 in pcDNA3.1 vector (pcDNA3.1Myc-ETV6/RUNX1) for transient ETV6/RUNX1 model and pGZMB-luc for luciferase assay were kindly provided by A. Ford (Institute of Cancer Research, LRF, London, United Kingdom). Control vector pcDNA3.1-empty was prepared by *Eco*RI restriction and pGL3-basic and pRL-CMV were purchased from Promega (Madison, WI).

Luciferase assay. The human carcinoma cell line HeLa was seeded at 1.5×10^5 per well and transfected with pcDNA3.1Myc-ETV6/RUNX1 or pcDNA3.1-empty (1.6 μg) by Lipofectamine (2 μL) in 600 μL of serum-free medium 24 h before transfection with pGZMB-luc. DNA fragment of granzyme B (GZMB) promoter was subcloned into the *Sma*I site of pGL3-basic luciferase reporter gene construct to form pGZMB-luc. Cells were transfected by pGL3-basic to normalize the luciferase activity (pGZMB-luc/pGL3-basic). pRL-CMV was transfected to each sample for normalizing transfection efficiency.

To test the effect of HDACi on ETV6/RUNX1, cotransfected HeLa cells were treated with VPA or TSA and luciferase activity was monitored. Subsequently, differences between untreated and treated cells were calculated. Luciferase activity was measured by a luminometer Microplate TLX2 using Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions. Luciferase activity of untreated cells was arbitrarily set to 100%. All experiments were done in triplicate. Data were expressed as relative luciferase activity (RLU).

Quantitative reverse transcription-PCR. Real-time quantitative reverse transcription-PCR (RT-PCR) was done in the LightCycler rapid thermal cycler system (Roche Diagnostic GmbH, Basel, Switzerland) and Q-Cycler (Bio-Rad, Hercules, CA) according to manufacturers' instructions. Oligonucleotide hydrolyzation probes were used in systems for quantification of genes selected from expression profiling, SYBR Green DNA-binding dye for *RAG-1* and *TdT* genes, and oligonucleotide hybridization probes for *β_2-microglobulin* (housekeeping gene; ref. 4).

Gene expression measurements and analyses. Spotted cDNA microarrays were used that contain more than 43,000 features representing ~30,000 genes (Stanford Functional Genomics Facility, Stanford, CA). The reference RNA used for all the arrays was Universal Human Reference RNA (Stratagene Europe, Amsterdam, the Netherlands). We labeled each of the sample RNA (RNA of untreated REH cells, TSA-treated REH cells, VPA-treated REH cells) with a red fluorescent dye (Cy5-dUTP, Amersham Pharmacia Biotech Europe, Freiburg, Germany) and the reference RNA with a green fluorescent dye (Cy3-dUTP, Amersham Pharmacia Biotech Europe) and comparatively hybridized sample RNA and reference RNA to an array. Each experiment was done in triplicates.

The fluorescence intensities of Cy5 and Cy3 were measured using a GenePix 4000 scanner (Axon Instruments, Foster City, CA) and analyzed using GenePix Pro 4.1 software (Axon Instruments). Subsequent analysis and normalization of data was done as previously described (29, 30).

Statistical analysis. To analyze the data of REH samples treated with HDACi in the supervised analysis, we used significance analysis of microarrays (31). A list of significantly differentially expressed genes was obtained by carrying out a 1,000-fold permutation test and considering a false discovery rate of 5% and a fold change of >2. Analysis of ALL patient expression data was done following our previously described method (32).

Results

Functional proof of ETV6/RUNX1 regulation of granzyme B

The transcription of granzyme B is known to be directly regulated by the RUNX1 protein (33, 34). Therefore, the granzyme B promoter region was cloned into the luciferase construct to determine whether ETV6/RUNX1 represses granzyme B expression on the basis of an existing RUNX1-binding site. Luciferase activity was measured in HeLa cells transfected with pcDNA3.1 *Myc*-ETV6/RUNX1 and compared with HeLa cells transfected with pcDNA3.1 empty vector. The mouse DNA fragment of granzyme B (bp −324 to −43) has two RUNX1-binding sites (−278 to −273 and −219 to −214) and is identical to the human gene (whole inserted sequence of granzyme B has 84% homology with human gene fragment). Cells were transfected with pGZMB-luc or pGL3-basic to normalize the luciferase activity. A decrease to 33% in ETV6/RUNX1 cells versus control cells with empty vector (100%) indicated that granzyme B was down-regulated by ETV6/RUNX1. Twenty-four hours of cultivation with HDACi released this expression block and increased luciferase activity in ETV6/RUNX1 cells by >180%. Values were calculated as relative luciferase units (RLU) of ETV6/RUNX1 cells divided by RLU of control cells (Fig. 1).

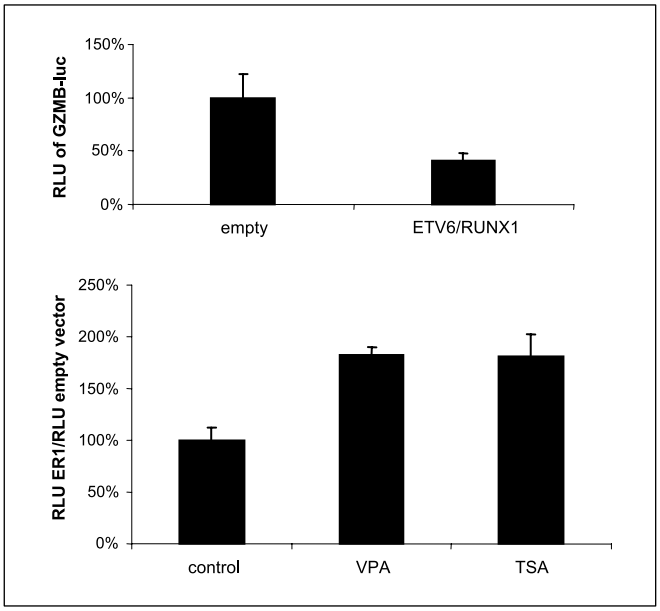


Fig. 1. Granzyme B expression regulation by ETV6/RUNX1 chimeric transcription factor. Normalized luciferase activities of GZMB-luc detected in HeLa cells with empty vector (assigned as 100%) and cells transiently transfected with ETV6/RUNX1. The ratio of RLU in HeLa GZMB-luc/ETV6/RUNX1-transfected cells to RLU of HeLa control cells (GZMB-luc/empty vector-transfected) was assigned to a value of 100%. Both control and ETV6/RUNX1 cells were then treated with HDACi (VPA 1 mmol/L, TSA 240 nmol/L). Increase of measured luciferase activity due to released repression activity of ETV6/RUNX1 protein was recalculated as a percentage.

Screening strategy for identification of downstream genes

Step I. Recalculation of expression profiling data from ALL patients was done by analyzing the data published by Fine et al. (29, 30), which are available through the Stanford Microarray Database[4] as previously described (32). Differences between genotypic subgroups were calculated using data from patients with B-cell precursor ALL. For graphical comparison of the predictive value of genes correlating with a genotype assessed by different methods, the probe set–specific optimal cutoff value was determined using statistical software R.[5] For each probe set, the frequencies of cases above all possible cutoff values were computed. The level that led to the largest distance

[4] http://genome-www.stanford.edu/microarray
[5] http://www.r-project.org

from the diagonal of equality was used as the optimum cutoff value for each probe set (32).

This approach was applied to ETV6/RUNX1-positive versus ETV6/RUNX1-negative, genotypically defined patients (BCR/ABL and MLL/F4 positive) to select genes specific for ETV6/RUNX1-positive phenotype. Expecting the repression effect of ETV6/RUNX1 fusion protein, we searched for genes down-regulated in the ETV6/RUNX1-positive group. The scale of cutoff values for each probe set was from 0 to 100. This meant that genes selected for further analysis had to have a value of difference between txx (cutoff value of a single gene in ETV6/RUNX1-positive patients) and tyy (cutoff value of a single gene in ETV6/RUNX1-negative patients) ≤ -33.3. This approach allowed us to identify 2,539 down-regulated genes specific for the ETV6/RUNX1 phenotype in ascending order (order of importance).

Step II. Then we asked whether an ETV6/RUNX1-positive cell line REH could serve as a model in the next intended *in vitro* experiments. Therefore, we compared data from two independent expression profiling experiments, one on ALL patients and the other using REH samples. It was essential for our future strategy to choose only those genes from step one of our analyses that had similar relative expressions in both tested sets. Mathematically, genes selected in step two had to have differences ("closeness") between their expression in ETV6/RUNX1-positive patients and that in REH cells closer than the minimum difference in expression between ETV6/RUNX1-positive and ETV6/RUNX1-negative patients. The output of the second step of analysis resulted in 927 genes. This set of down-regulated genes characterized equally ETV6/RUNX1-positive patient samples and the REH cell line. For exact algorithm, see Fig. 2.

Step III. In the next step of analysis, we selected genes that changed their expression after VPA treatment in samples of REH cells. Again, only genes with increased expression after VPA were chosen, thus corresponding with the expected underlying mechanism of ETV6/RUNX1 action. The difference between median gene expression in treated samples (with VPA) and median gene expression in untreated samples (median VPA − median C) had to be higher than zero. This third step limited our list to 759 genes.

Step IV. In the last step, we selected genes with a significantly changed expression pattern after VPA treatment ($P \leq 0.05$) and these genes were listed in descending order. This fourth step

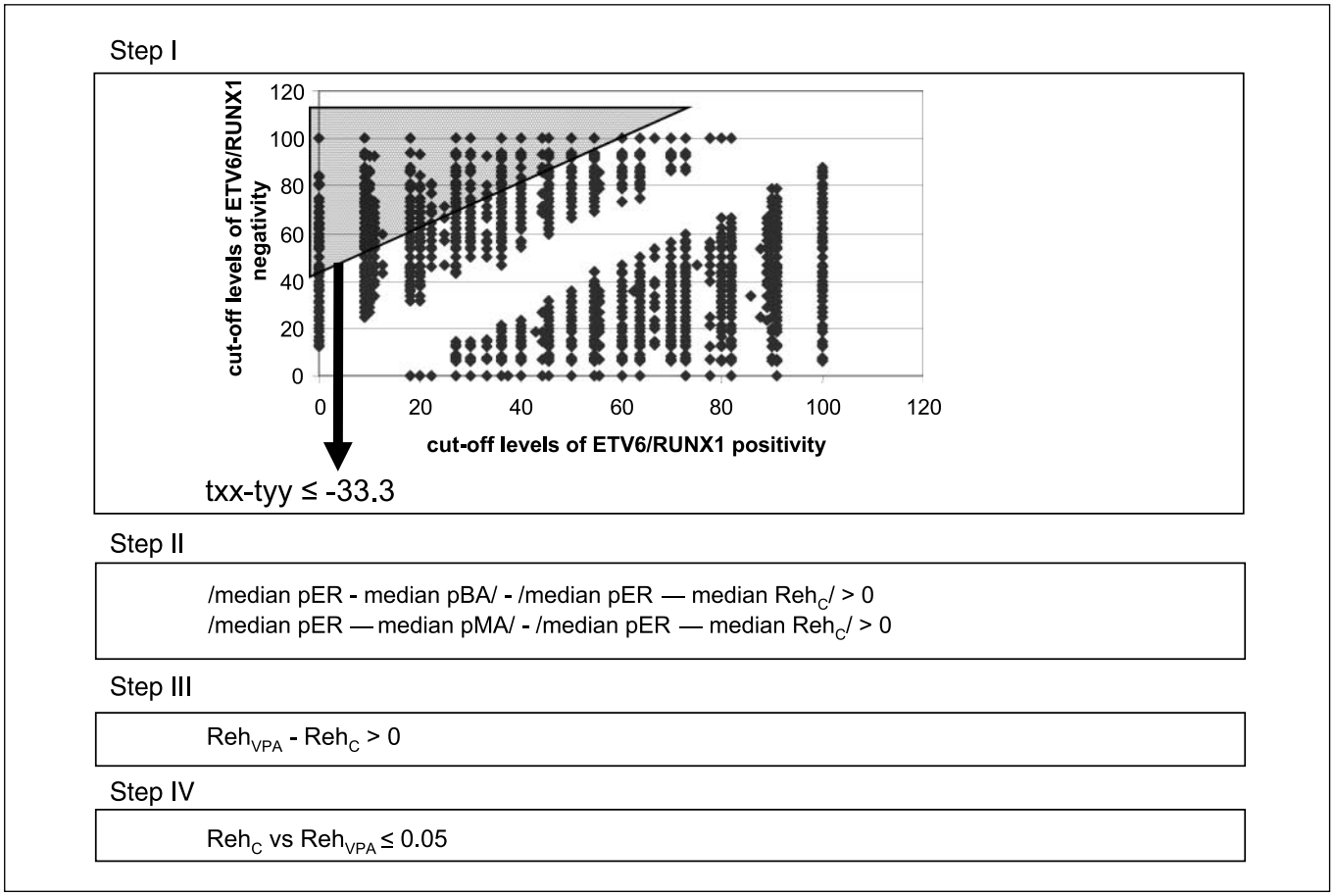


Fig. 2. Diagrammatic representation of four steps of expression profiling data analysis for identification of genes regulated by ETV6/RUNX1. Step I: Identification of genes discriminating subgroup of ETV6/RUNX1-positive patients. Selected range characterized only by down-regulated genes. txx, cutoff value of each probe set in ETV6/RUNX1-positive patients; tyy, cutoff value of each probe set in ETV6/RUNX1-negative patients. Step II: Selection of the genes on the basis of similarity (closeness) of REH (ETV6/RUNX-positive cell line) cells and ETV6/RUNX1-positive patients. Median pER, median of relative expression of screened gene in all ETV6/RUNX1-positive patients; median pBA, median of relative expression of screened gene in all BCR/ABL-positive patients; median pMA, median of relative expression of screened gene in all MLL/AF4-positive patients. Step III: Selection made on the basis of direction of change in the expression level of selected genes after VPA treatment. Step IV: Selection of genes with significantly changed expression ($\leq$0.05) after VPA treatment.

Table 1. Selection of genes with significantly changed expression (≤0.05) after the treatment with VPA

Name	txx	tyy	Closeness	Median C	Median VPA	VPA-C	p(VPA-C)
PAK1 || 5058	0.00	56.25	0.36	−0.28	1.06	1.34	0.0012
Similar to peptidylprolyl isomerase A (LOC402673), mRNA || 402673	54.55	100.00	0.10	−0.32	0.45	0.77	0.0017
NID67 || 85027	18.18	73.33	0.49	−1.06	1.26	2.32	0.0018
FLJ00012 || 89849	20.00	68.75	0.62	−1.32	0.62	1.94	0.0024
HRMT1L1 || 3275	36.36	75.00	0.47	−0.34	0.34	0.68	0.0032
AA252394	50.00	93.33	0.24	−0.24	0.49	0.73	0.0054
ACK1 || 10188	27.27	100.00	0.20	−0.79	0.25	1.04	0.0054
CXXC5 || 51523	18.18	81.25	0.12	−0.02	0.96	0.98	0.0063
BSPRY || 54836	9.09	92.86	0.60	−0.82	0.41	1.23	0.0074
TNFRSF5 || 958	20.00	53.33	0.28	−0.05	0.32	0.37	0.0079
RNF144 || 9781	10.00	62.50	0.06	−0.03	0.54	0.57	0.0079
AHI1 || 54806	9.09	71.43	0.13	−0.20	0.72	0.92	0.0097
C14orf101 || 54916	45.45	93.75	0.22	−0.60	0.01	0.61	0.0103
CDNA FLJ45905 fis, clone OCBBF3026576	45.45	84.62	0.08	−0.31	0.61	0.92	0.0103
CDNA FLJ10641 fis, clone NT2RP2005748	22.22	64.29	0.32	−0.42	0.65	1.07	0.0104
PDGFRB || 5159	0.00	53.33	0.05	−0.81	1.58	2.39	0.0112
ARD1 || 8260	9.09	68.75	0.30	−0.49	0.25	0.74	0.0114
AI302216	18.18	62.50	0.18	−0.24	0.43	0.67	0.0117
MGMT || 4255	0.00	33.33	0.12	−0.36	0.70	1.06	0.0123
N24645	9.09	50.00	0.06	−0.26	0.47	0.73	0.0125
ANTXR2 || 118429	10.00	81.25	0.32	−0.25	1.33	1.58	0.0140
ITM2C || 81618	0.00	68.75	0.57	−1.50	0.52	2.02	0.0141
WDR7 || 23335	45.45	84.62	0.43	−0.91	−0.16	0.75	0.0150
STARD9 || 57519	30.00	81.25	0.37	−0.34	0.33	0.67	0.0154
LOC125893 || 125893	0.00	35.71	0.12	−0.11	0.46	0.57	0.0165

NOTE: txx, cutoff for each gene in ETV6/RUNX1-positive patients; tyy, cutoff for each gene in ETV6/RUNX1-negative patients; closeness, relative gene expression similarity between ETV6/RUNX1-positive patients' samples and ETV6/RUNX1-positive cell line (see also text); median C, median expression level in untreated ETV6/RUNX1-positive cells; median VPA, median expression level in VPA-treated ETV6/RUNX1-positive cells; VPA-C, difference between median C and median VPA with a *P* value.

separated only 72 genes. We present these top 25 genes in Table 1.

Expression profiling versus quantitative RT-PCR

For further analysis, we chose genes with a known proliferative or cell cycle regulative function. Using quantitative RT-PCR, we confirmed these expression changes after the treatment with HDACi (VPA, TSA) in four genes, three of them down-regulated and one up-regulated in ETV6/RUNX1-positive leukemia cells. First, *ACK1* (alias *TNK2*, activated p21cdc42Hs kinase) and *PDGFRB* (platelet derived growth factor receptor β) were chosen from our list of 25 genes (see above). Moreover, differential expression in ETV6/RUNX1-positive and ETV6/RUNX1-negative patients was confirmed by comparison with other publicly available expression profiling data[6] (35, 36). Gene *JunD* (*junD* proto-oncogene) was chosen from the more extensive list of the first 72 genes, and gene transcription factor 4 (*TCF4*) was selected as an example of genes up-regulated in ETV6/RUNX1-positive cells. Expression of *JunD*, *ACK1*, and *PDGFRB* in ETV6/RUNX1-positive patients as well as in the cell lines was low with a clear increase after HDACi treatment. *TCF4* gene expression was high and the treatment with HDACi led to a decrease. We confirmed changes of expression levels by quantitative RT-PCR between treated and untreated cells: *JunD*, TSA $P = 0.013$, VPA $P = 0.0008$; *ACK1*, VPA $P = 0.07$, TSA $P = 0.5$; *PDGFRB*, TSA $P < 0.0001$, VPA $P = 0.016$; *TCF4*, TSA $P < 0.0001$, VPA $P = 0.0002$ (Fig. 3).

Effect of HDACi on proliferation activity and apoptosis

We tested cell cycle progress and apoptosis rate after HDACi treatment in ETV6/RUNX1-positive cells and compared them with pre–B-leukemic cells with different leukemogenic mechanism (ETV6/PDGFRB) to look if there are any differences in drug effect. EBV-transformed mature lymphocytes were used as a control to test the toxicity of the compounds in nonleukemic B-lymphoid cells. An increase in the number of apoptotic cells was expressed as the ratio of apoptotic fraction in treated cells to apoptotic fraction in untreated cells. In the REH cell line, the apoptosis rate was 8% using 1 mmol/L VPA and 26% using 240 nmol/L TSA, in contrast to 3.7% and 1.7% in control lymphocytes (NC-NC cell line). Similarly, in Nalm-6, the apoptosis rate reached 17% with VPA and 41% with TSA. The Nalm-6 and REH cell lines reduced their proliferation activity (S + G_2-M) by 19% (VPA), 28% (TSA) and 7% (VPA), 15% (TSA), respectively. These data suggest that treatment with TSA preferentially leads to apoptosis in both ETV6/RUNX1-positive and ETV6/RUNX1-negative leukemic cell lines. However, this effect was clearly seen after VPA treatment only in Nalm-6 cells, whereas in REH cells, we observed a relative increase in G_1-G_0. In contrast, in NC-NC cells the proportion of retained cell cycle phases was almost unchanged (Fig. 4).

[6] Available at: http://www.stjuderesearch.org/ALL1 and http://www.stjuderesearch.org/ALL3.

Detection of differentiation antigen shift

Three leukemic cell lines with known fusion genes, REH (ETV6/RUNX1), Nalm-24 (BCR/ABL), and Nalm-6 (ETV6/PDGFRB), were selected for monitoring of HDACi-driven changes in immunophenotype. Initially, CD10 and CD20 levels were detected 24 and 48 h after HDACi (VPA, TSA) treatment. Due to different developmental stages of selected cell lines, mean fluorescence area intensity instead of genuine cell count was measured. This allowed a simple comparison of differentiation pattern shift. The pattern of expression for native REH cells was $CD10^{++}/CD20^{-}$. After cultivation with HDACi, the CD10 hyperexpression decreased ($CD10^{+}$; VPA $P = 0.0004$, TSA $P = 0.0002$) and CD20 expression increased to low positivity ($CD20^{dim/+}$; VPA $P = 0.005$, TSA $P = 0.01$). The expression pattern for native Nalm-24 cells was $CD10^{+}/CD20^{+}$. After treatment, expression of both studied antigens decreased (VPA $P = 0.0008$, TSA $P < 0.0001$; VPA $P = 0.08$, TSA $P = 0.0004$, respectively). The expression pattern for native Nalm-6 cells was $CD10^{++}/CD20^{-}$. Expression of CD10 was dysregulated in an up-and-down manner depending on the time of acquisition and dose and type of HDACi. Although its level oscillated, its expression tended to be slightly higher. Surface antigen CD20 maintained its initial negativity under treatment with HDACi (Fig. 5).

Whereas Nalm-24 and Nalm-6 cell lines did not display evident differentiation drift, the REH cell line expressed CD10 and CD20 in a pattern similar to more mature normal blasts (37). We used another differentiation marker of B-cell development, TdT. After VPA administration, the expression of TdT in the REH cell line significantly decreased (VPA $P = 0.0001$), whereas in Nalm-24 cells, the TdT level decrease was not significant (VPA $P = 0.24$). RAG-1 expression in REH cell line paralleled that of TdT (VPA $P = 0.006$). Lower doses (0.5 mmol/L VPA, 120 nmol/L TSA) of VPA and TSA were less effective; however, the same trends were observed. After 48 h, the effect of HDACi was less pronounced.

The expression decrease of RAG-1 and TdT antigen was confirmed at the mRNA level by quantitative RT-PCR. The transcription levels of RAG-1 and TdT were normalized to the expression of the housekeeping gene *β2-microglobulin*. Normalized expression data of treated and untreated samples were compared.

The mRNA level of *RAG-1* gene rapidly decreased after 24 h from the initial level 3.30 ± 0.321 to 1.004 ± 0.053 after VPA ($P = 0.0002$) and 0.197 ± 0.046 after TSA ($P < 0.0001$) administration. Similarly, the mRNA level of *TdT* significantly decreased within 24 h after TSA or VPA treatment from initial RNA level 1.767 ± 0.178 to 0.888 ± 0.110 ($P = 0.01$) and 0.072 ± 0.020 ($P = 0.0001$), respectively (Fig. 6).

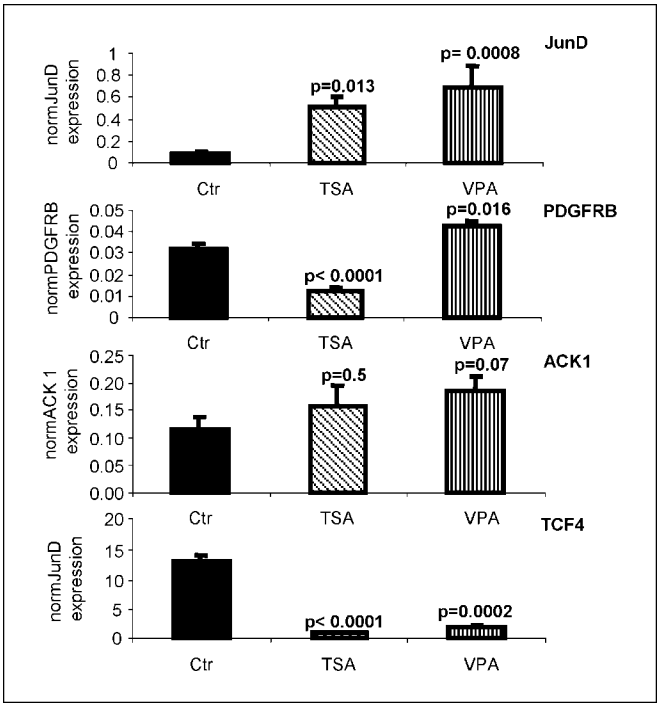


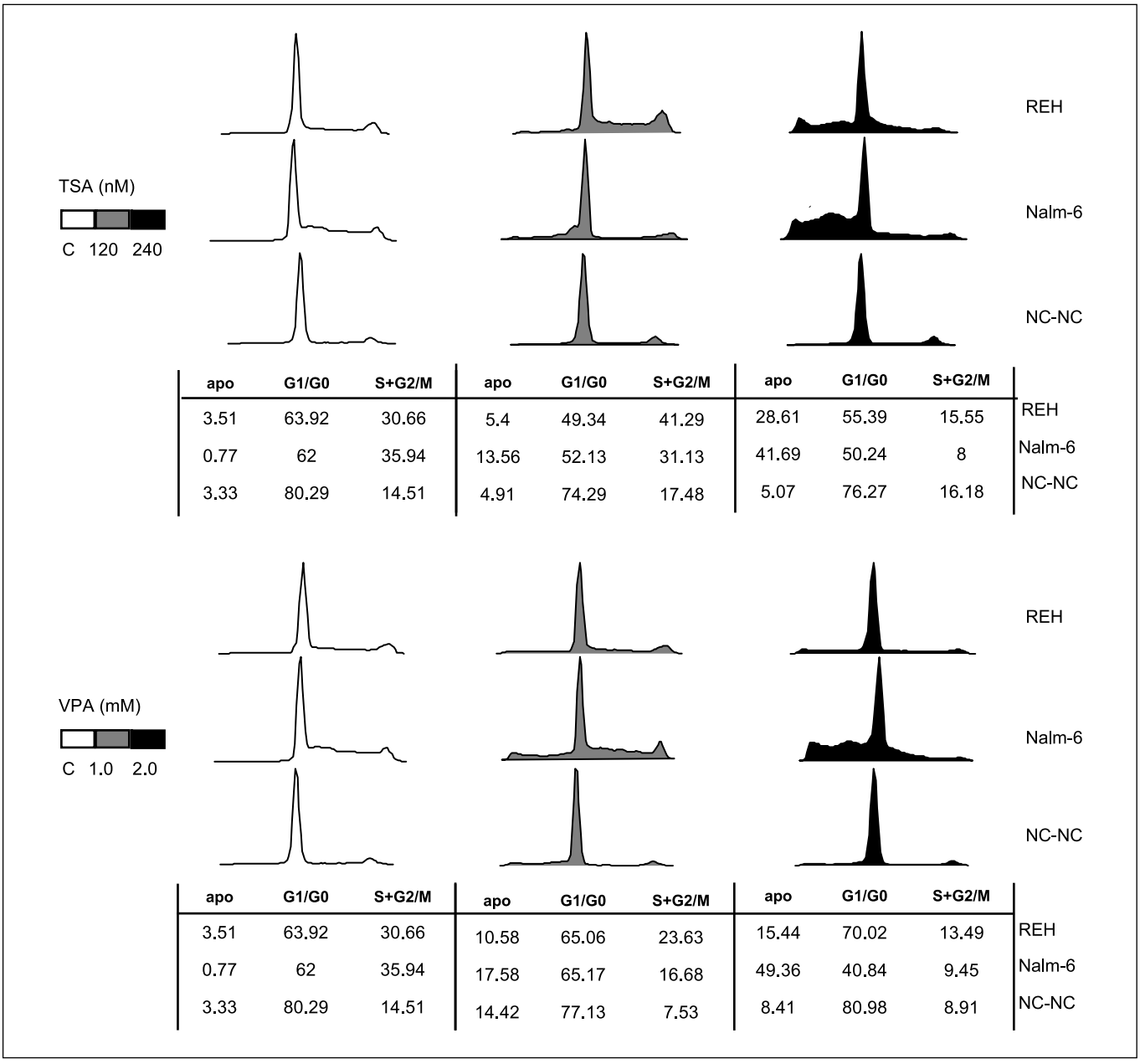
Fig. 3. Analysis of selected genes expression using quantitative RT-PCR. Quantification of four genes selected from microarray data was done by quantitative RT-PCR comparing untreated and treated (VPA, TSA) REH cells. Gene expression of target was normalized to β_2-*microglobulin* as a housekeeping gene. All samples were measured in triplicates and gene expression of each replicate was detected in duplicate. *P* value was calculated from the mean of ratio of selected gene to β_2-*microglobulin* for all replicates with absolute error.

Discussion

The functional consequences of fusion gene formation are either constitutive kinase activation (e.g., BCR/ABL) or altered transcriptional regulation. Published studies suggest that RUNX1 fusion proteins may act as negative regulators of genes that, in normal hematopoiesis, are transactivated by the RUNX1 transcription factor (18, 38–40). RUNX1 is known to regulate promoters of various genes involved in myeloid differentiation, such as macrophage colony-stimulating factor receptor, granulocyte-macrophage colony-stimulating factor, interleukin-3, neutrophil elastase, myeloperoxidase, and others (38, 41). RUNX1 regulates hematopoietic myeloid cell differentiation and transcriptional activation but its role in lymphoid development is not yet fully understood (42). Only a few RUNX1 target genes or regions have been described in lymphoid cells (i.e., B-lymphoid kinase, complement receptor 1, enhancers of T-cell receptor β, and immunoglobin α; refs. 10, 17, 43).

We hypothesized that the normal role of RUNX1 in B-cell precursors is compromised in a dominant-negative fashion by the ETV6/RUNX1 fusion. ETV6/RUNX1 recruits a corepressor complex including HDAC, which likely induces chromatin remodeling and consequently blocks the transcription of genes normally transactivated by RUNX1. To prove the direct involvement of HDACs in ETV6/RUNX1 function, we used HDACi to overcome this presumptive block. Despite expected broad effects of HDACi treatment on cellular transcriptional regulation, these compounds act very selectively to alter the transcription of fewer than 2% of expressed genes (44).

To prove the direct effect of HDACi on ETV6/RUNX1 in cells *in vitro*, we chose a previously identified target gene of RUNX1, granzyme B. To our knowledge, this is the first time that a direct effect of the interaction between ETV6/RUNX1 and any potential target genes of RUNX1 has been shown. For this purpose, we have established a transient ETV6/RUNX1 expressing model to show the down-regulation of granzyme B in the presence of the ETV6/RUNX1 protein compared with ETV6/RUNX1-negative controls. Next, we



apo	G1/G0	S+G2/M	apo	G1/G0	S+G2/M	apo	G1/G0	S+G2/M	
3.51	63.92	30.66	5.4	49.34	41.29	28.61	55.39	15.55	REH
0.77	62	35.94	13.56	52.13	31.13	41.69	50.24	8	Nalm-6
3.33	80.29	14.51	4.91	74.29	17.48	5.07	76.27	16.18	NC-NC

apo	G1/G0	S+G2/M	apo	G1/G0	S+G2/M	apo	G1/G0	S+G2/M	
3.51	63.92	30.66	10.58	65.06	23.63	15.44	70.02	13.49	REH
0.77	62	35.94	17.58	65.17	16.68	49.36	40.84	9.45	Nalm-6
3.33	80.29	14.51	14.42	77.13	7.53	8.41	80.98	8.91	NC-NC

Fig. 4. Detection of changes in cell cycle progress. Cell cycle was monitored 48 h after HDACi treatment of REH, Nalm-6, and NC-NC cell lines. Cell cycle fractions (apoptosis, G_1-G_0, S + G_2-M phases) are presented as a percentage of all cells.

showed that HDACi treatment relieves the repression observed in the presence of the ETV6/RUNX1 fusion protein. This experiment confirms the hypothesis that the ETV6/RUNX1 fusion protein can act as a transcriptional repressor and its activity is reversible by HDACi. Therefore, HDACi can be used as a tool for the identification of genes potentially regulated by ETV6/RUNX1.

Childhood ALL subgroups known thus far are characterized by the presence of specific genetic aberrations, mainly chromosomal translocations leading to the fusion genes formation. They are part of different pathways, thus contributing to leukemogenesis by different mechanisms. To study neoplastic transformation in the presence of ETV6/RUNX1 and to identify potential ETV6/RUNX1 target genes, we compared expression data of ALL patients of three genotypically defined groups: ETV6/RUNX1, BCR/ABL, and MLL/AF4. Identification of specific genes of the ETV6/RUNX1-positive phenotype was based on the analysis of particular cutoffs; a selection of genes specifically overexpressed or underexpressed in the cells of ETV6/RUNX1-positive patients was made. This group of genes was compared with the genes whose expression patterns changed significantly after treatment with HDACi. From the resulting pool of genes that fulfilled all of these criteria of analysis, we selected four genes (*JunD*, *ACK1*, *PDGFRB*, and *TCF4*) to compare expression data from expression profiling and data from quantitative RT-PCR. These four genes were

selected from the extensive list on the basis of their previously described fundamental role in cell proliferation and cell cycle regulation (45–48). Our analysis pinpointed top 25 genes that either are directly regulated by the ETV6/RUNX1 fusion protein or are in ETV6/RUNX1-regulated pathways. Most likely, they represent target genes of the RUNX1 transcription factor involved in B-lineage lymphopoiesis. We assume that these genes (albeit not exclusively) are likely to be involved in ETV6/RUNX1-driven leukemogenesis.

We also investigated the influence of HDACi on cell cycle progress and differentiation in ETV6/RUNX1-positive cells. As the morphologic shift was hardly to be expected in lymphoblasts, we concentrated on flow cytometry analysis. These immunophenotype changes differed between ETV6/RUNX1-positive and ETV6/RUNX1-negative cells. ETV6/RUNX1-positive cells showed indications of differentiation (decrease of CD10, TdT, and RAG-1 expression and up-regulation of CD20) compared with cell lines derived from leukemias with aberrant tyrosine kinase fusion genes (BCR/ABL, ETV6/PDGFRβ), which underwent unspecific immunophenotype changes without a differentiation effect.

We are aware of the effect of HDACi on apoptotic pathways in malignant cells that can complicate analysis of apoptosis in our model cell lines. Indeed, a proapoptotic effect was observed in both ETV6/RUNX1-positive and ETV6/RUNX1-negative leukemic cells. In contrast to leukemic cells, the administration of HDACi did not change the progress of the cell cycle in normal mature lymphocytes. HDACi administration decreased the number of proliferating cells in the leukemic cell lines. TSA had a more pronounced effect with a significant shift towards apoptosis in both ETV6/RUNX1-positive and ETV6/RUNX1-negative cells, whereas VPA acted more selectively, arresting ETV6/RUNX1-positive cells in the G_1-G_0 phase of the cell cycle instead of driving them to apoptosis. This result is in agreement with the previously published data suggesting that VPA preferentially acts on corepressor-associated HDACs and inhibits class I HDACs more efficiently than class II enzymes. Previous studies also showed lower toxicity of VPA to normal hematopoietic precursors (25).

HDACi are currently among the most promising compounds in drug development for cancer therapy, and first-generation HDACi are currently being tested in phase I/II clinical trials (49). ALL patients carrying the ETV6/RUNX1 fusion have a good prognosis compared with other subgroups of childhood ALL, but relapses still occur in this group. And although generally the prognosis after relapse tends to be good, second relapses also occur. As in all subgroups with overall superior

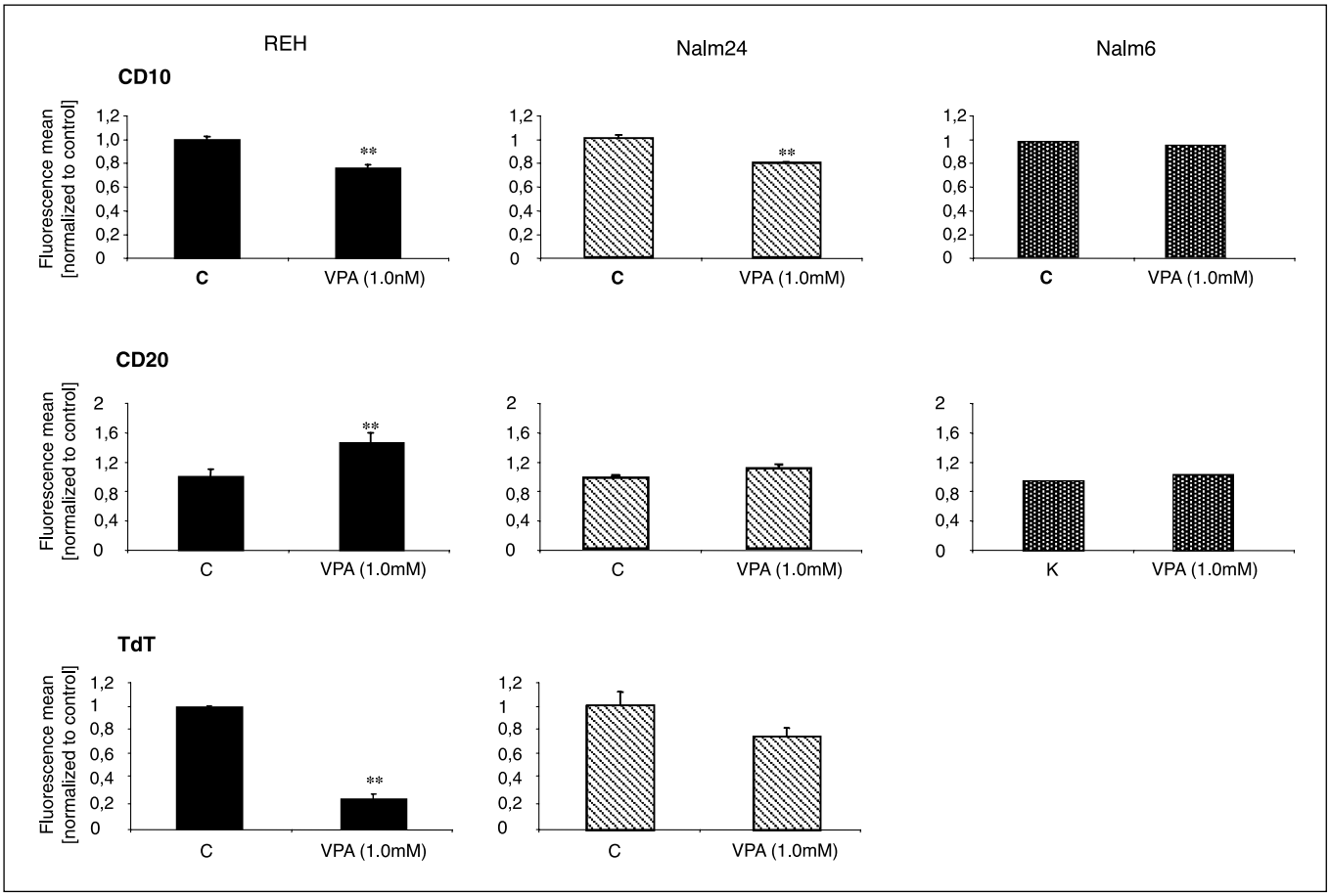


Fig. 5. Expression of differentiation markers monitored in three types of leukemias. Typical B-lineage differentiation markers (CD10, CD20, and TdT) were detected in three lymphoid leukemia cell lines, REH (ETV6/RUNX1 positive), Nalm-24 (BCR/ABL positive), and Nalm-6 (ETV6/PDGFRB positive), by flow cytometry. Changes of expression of individual markers after the treatment with VPA (1.0 mmol/L) are given as the fluorescence mean normalized to control (untreated cells). *, $P \leq 0.05$; **, $P \leq 0.01$ (ANOVA test).

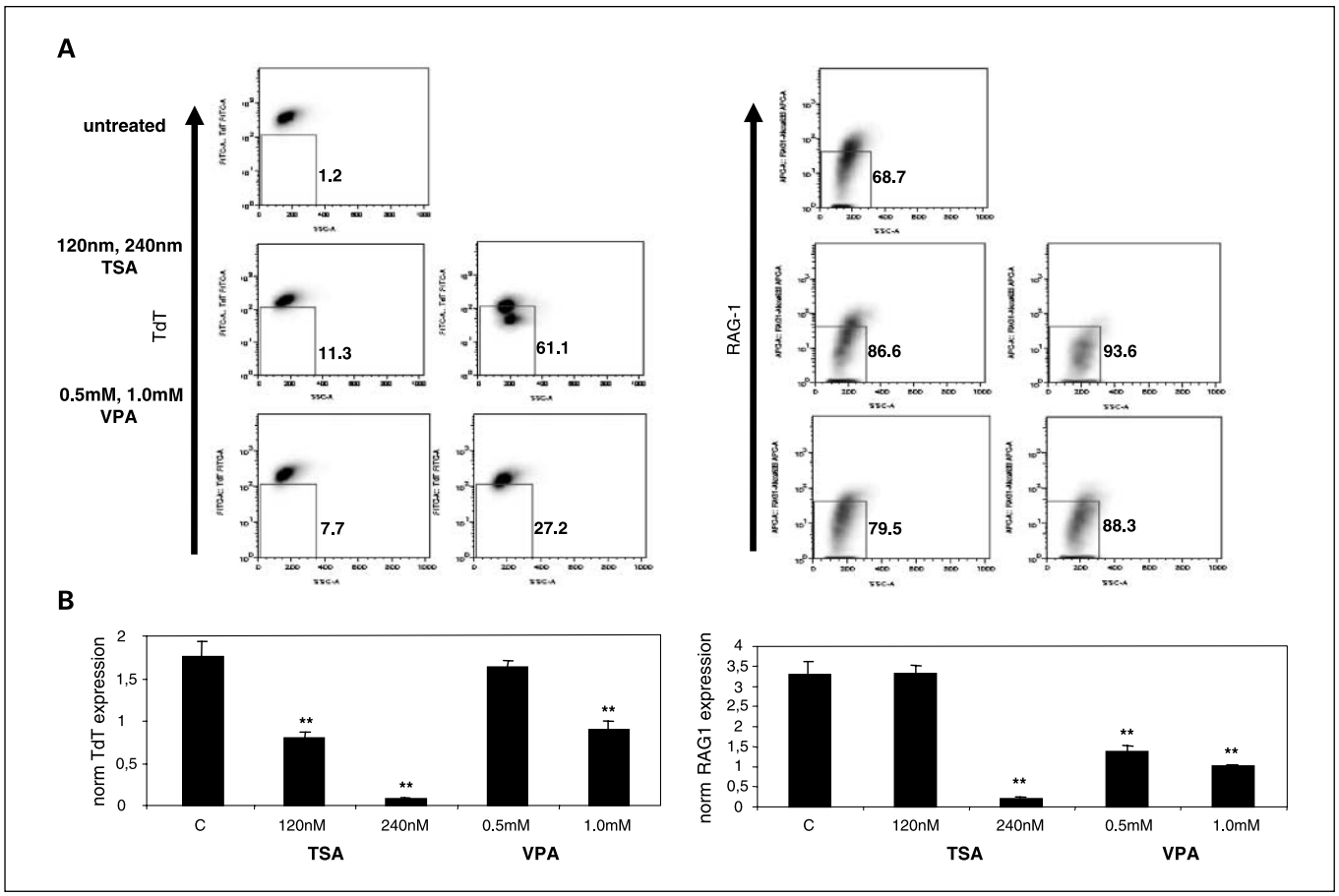


Fig. 6. Comparison of changes in gene expression and protein expression levels of TdT and RAG-1 after HDACi treatment. The expression of differentiation markers was measured using flow cytometry 24 and 48 h after the treatment with HDACi in REH cells. *A,* dot blot images show effect of TSA (120 and 240 nmol/L) and VPA (0.5 and 1.0 mmol/L) on the expression of TdT and RAG-1 48 h after the treatment. Data are presented as a percentage of cells of specific quadrants. *B,* normalized gene expression level by quantitative RT-PCR 24 h after incubation of REH cells with HDACi. *, $P \leq 0.05$; **, $P \leq 0.01$ (Mann-Whitney test).

prognosis, the main challenge is to identify the sporadic high-risk patients. Minimal residual disease quantitation represents an available tool for the identification of slow responders to the initial phase of treatment as well as patients with adverse dynamics of the leukemic clone. As recently showed, minimal residual disease assessment can even help to test the efficacy of individual drugs in the multidrug induction therapy in ETV6/RUNX1-positive patients (50).

We presume that HDACi can potentially be used in the treatment of patients with detectable residual disease or with molecular relapses in the relapse treatment and after the transplantation in this genotypically defined subgroup of patients. The doses of VPA we used in our study correspond to the serum concentrations of this agent in neurologic patients. The use of this agent for human medicine predisposes VPA for testing in high-risk ETV6/RUNX1-positive leukemia patients.

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Title page

Manuscript title:
Revealing the role of TEL/AML1 for leukemic clone survival by RNAi mediated silencing

Running title:
TEL/AML1 silencing

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Abstract
Translocation (12;21), the most frequent chromosomal aberration in childhood ALL creates TEL/AML1 fusion gene. Resulting hybrid protein was shown to play a role in preleukemia establishment. To address its role for leukemia persistence and to explore the possibilities of potential TEL/AML1-targeted therapy we applied RNA interference to silence TEL/AML1 in leukaemic cells. We designed and tested eleven different oligonucleotides targeting the TEL/AML1 fusion site. Using most efficient siRNAs we achieved an average of 74% and 86% TEL/AML1 protein knock-down in REH and UOCB-6 leukemic cells, respectively. TEL/AML1 silencing neither decreased cell viability, nor induced apoptosis. On the contrary, it resulted in the modest but significant increase in the S-phase fraction and in higher proliferation rate. Opposite effects on cell cycle distribution and proliferation were induced by AML1 silencing, thus supporting our hypothesis that TEL/AML1 may block AML1-mediated promotion of G1/S progression through the cell cycle. In line with the lack of major effect on phenotype we found no significant changes in global gene expression pattern upon TEL/AML1 depletion. Our data indicate, that though TEL/AML1 is important for the (pre)leukemic clone development, it is dispensable for leukemia persistence and would not be a suitable target for gene specific therapy.

Introduction

Wild type (wt) TEL (ETV6) and AML1 (RUNX1) genes encode transcription factors, that play important roles in hematopoiesis, and are frequently rearranged in human lymphoid and myeloid leukemia (1, 2). Both genes are affected by the most common genetic abnormality of childhood B-cell precursor acute lymphoblastic leukemia (ALL), the cryptic chromosomal translocation t(12;21)(p13;q22), that fuses TEL on chromosome 12 to AML1 on chromosome 21 (3, 4). Resulting TEL/AML1 fusion gene encodes hybrid protein composed of dimerization domain and the central region of TEL fused to almost entire AML1 including the DNA binding runt homology domain.

Despite intensive studies the leukemogenic role of TEL/AML1 is not fully understood. In general, TEL/AML1 is believed to interfere with the normal function of its wt counterparts. Most likely, TEL/AML1 acts as an aberrant transcription factor and deregulates AML1-dependent transcription (5). This theory is strongly supported by the finding that TEL/AML1 *in vitro* activity is abrogated upon loss of DNA binding as a result of a single point mutation within the runt homology domain (6). As a further evidence, TEL/AML1 was shown to interact with corepressors and histon deacetylases and to repress transcription of AML1 targets through the direct binding to AML1 consensus binding sites. The promoter regions employed in these reporter assays mainly belonged to the AML1 targets in myeloid (7) or T lymphoid cells (5, 8, 9) that do not represent the physiological counterparts of TEL/AML1 leukemic cells. Only little is known so far about the direct targets of TEL/AML1 in leukemic cells that are of B cell origin.

In vivo studies showed, that TEL/AML1 is not able to trigger leukemia as a single event and that additional cooperating events are needed for the development of full blown leukemia. Depending on the experimental model used, presence of TEL/AML1 protein itself either does not lead to any observable changes (10) at all or it induces a “preleukemic” state characterized by the expansion of multipotent or B-cell precursors with enhanced self-renewal and slightly impaired differentiation to more mature B-cell stages (11-13).

Unlike its role in (pre)leukemic clone origin, the significance of TEL/AML1 protein for leukemia persistence has not been studied enough. To evaluate the contribution of TEL/AML1 to fully malignant phenotype and also to assess the potential of TEL/AML1-targeted therapy we applied RNA interference (RNAi) to knock-down TEL/AML1 in definitive leukemic cells. RNAi is a complex endogenous mechanism resulting in a sequence specific gene silencing. It can be triggered by introduction of small interfering RNA molecules (siRNAs), double strand RNA oligonucleotides sequentially homologous to the targeted gene. As such, RNAi has a great potential not only for the analysis of gene function but also as a gene-specific therapeutic approach.

In our study we aimed to analyse the impact of TEL/AML1 depletion in the fully malignant leukemic clone on cell viability, proliferation and global gene expression pattern. As a study model we used the only two accessible cell lines established from TEL/AML1-positive patients’ leukemias - REH and UOC-B6. Our results indicate that TEL/AML1 fusion gene is not necessary for the maintenance of leukemia phenotype and as such would not be a suitable target for specific therapy via RNA interference.

Materials and Methods

Cell cultivation and transfection

REH (human B cell precursor leukemia, TEL/AML1-positive) and HELA (human cervix carcinoma) cell lines were purchased from German Collection of Microorganisms and Cell Cultures (DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and cultivated according to producer's instructions. UOC-B6 cell line (human B cell precursor leukemia, TEL/AML1-positive) (14) was kindly provided by Dr. Ondrej Krejci (Massachusetts General Hospital, Boston) and cultivated under the same conditions as REH cell line.
For the transfection experiments HELA cells were seeded at 160 000 cells per well ($2cm^2$) one day prior to transfection. Adherent cultures were transfected using Lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA) reagent according to manufacturer's instructions: 1.6μg plasmid DNA with 2μL Lipofectamine or 50pmol siRNA with 1μL Lipofectamine in 600μL serum free medium per well.
REH and UOC-B6 cells were transfected via electroporation performed on EPI2500 electroporator (Dr. L. Fischer, Heidelberg, Germany) using 0.4 cm gap Gene Pulser/MicroPulser Cuvettes (Bio-Rad, Hercules, CA, USA) under following conditions: 1 000 000 of cells/100μl of culture medium, 500nM siRNA, 1 pulse of 10ms, 350V and 1200μF. The siRNA transfection efficiency was monitored by flow cytometry.

HELA-TEL/AML1_ires2_EGFP permanent cell line
Using restriction endonuclease EcoRI (Fermentas, Burlington, Canada) TEL/AML1 coding sequence was cut from pcDNA3.1 plasmid (kindly provided by Dr. Anthony Ford, Institute of Cancer Research, UK) and cloned to pIRES2-EGFP plasmid (BD Bioscience Clontech, Palo Alto, CA). TEL/AML1_ires2_EGFP plasmid was transfected to HELA cells using Lipofectamine as described above and stable transfectants were selected with geneticin. The stable EGFP expression was verified by flow cytometry, the stable expression of TEL/AML1 protein was analysed by western blot.

siRNAs
Eleven TEL/AML1 targeting custom designed siRNAs (TA-1)-(TA-11) (Stealth™ siRNAs, Invitrogen) were tested for TEL/AML1 knock-down efficiency in HELA-TEL/AML1_ires2_EGFP cells. Non-T1 was used as a negative control (Stealth RNAi™ siRNA Negative Control Lo GC Duplex #2; Invitrogen). Mix of fluorescein-labeled TA-1 and TA-5 (TA-1+5) was used for TEL/AML1 knock-down in REH and UOC-B6 cells with Non-T1 as a negative control.
C1 siRNA used for TEL/AML1 silecing by Diakos et al (15) with set of 4 siRNAs targeting Tel portion of TEL/AML1 (Tel-set, M-010510-02; Dharmacon, Lafayette, CO, USA) as a positive control and Non-T2 (siGENOME® Non-Targeting siRNA Pool, D-001206-13; Dharmacon) as a negative control were also tested for knock-down efficiency and specificity in REH cells.
AML1 targeting siRNAs pool (AML1-pool, ON-TARGETplus SMARTpool, L-003926-00; Dharmacon) was used for AML1 knock-down in REH cells with Non-T3 (ON-TARGETplus Non-targeting Pool, D-001810-10; Dharmacon) as a negative control.
All 3 negative controls (Non-T1-3) are designed by producers to have the minimal sequence homology to any known human transcript and have the same chemical structure as the corresponding targeting siRNAs.

RNA isolation, qRT-PCR
Total RNA was extracted using a modification of the method described by Chomczynski and Sacchi (16) or using RNeasy Plus Mini kit (QIAGEN GmbH, Hilden, Germany)) according to the manufacturer's instructions. Total RNA was reverse transcribed into complementary

DNA using MoMLV Reverse Transcriptase (Gibco BRL, Carlsbad, TX, USA) according to the manufacturer's instructions.
The TEL/AML1 and β2M transcripts were detected as described previously (17, 18).
The AML1 transcript was amplified using following primers: forward primer (5'-GCATATTTGAGTCATTTCCTTCGTAC-3') and reverse primer (5'-CTCGTGCTGGCATCGTGG-3'). qRT-PCR was performed on LightCycler™ instrument (Roche Diagnostic GmbH, Basel, Switzerland) in a final volume of 20µl 1xQuantiTect SYBR Green PCR Master Mix (QIAGEN GmbH, Hilden, Germany) containing 0.8µmol/L of each primer and 1µl template cDNA under the following conditions: 94°C for 15 minutes; 50 cycles of 94°C for 5 seconds, 60°C for 15 seconds.
The standard curves were generated using serial dilutions of plasmid calibrators containing the sequences of measured transcripts. The β2M transcript was used as an endogenous control to normalise cDNA concentration and quality.

Protein isolation, western blotting
Nuclear protein lysates were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA) supplemented with Complete™ Protease Inhibitor Cocktail (Roche, Basel, Switzerland) according to manufacturer's instructions. 100ug of protein were resolved by 8% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Nonspecific binding on the membrane was blocked with PBS containing 5% dry milk. The membrane was probed with anti-TEL (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-AML1 (Abcam, Cambridge, MA, USA) or anti-LAMIN B (Santa Cruz Biotechnology) antibodies in 5% dry milk overnight. The bound antibody was detected with the appropriate secondary antibody conjugated with horseradish peroxidase and visualized using Immun-Star HRP Substrate Kit (Bio-Rad) followed by exposition to X-ray film.
To quantify proteins detected by western blot TIFF images of scanned X-ray films were analysed using ImageJ software (19).The LAMIN B protein was used an endogenous control to normalise the protein concentration and quality.

Flow cytometry
Flow cytometry was performed on a BD FACSAria Cell Sorting System instrument (Becton Dickinson, San Jose, CA, USA).
Immediately after electroporation of fluorescein-labeled siRNA transfection efficiency was directly assessed as a percentage of positively-fluorescent cells from total transfected cell population.
The intensity of EGFP fluorescence in HELA-TEL/AML1_ires2_EGFP cells was measured directly after the cell trypsinization and PBS (phosphate buffered saline) wash. The acquired data were analysed using FlowJo software (TreeStar, Ashland, OR, USA).
DNA content in REH and UOC-B6 cells was measured using Cycletest™ Plus DNA Reagent Kit (Becton Dickinson) according to manufacturer's instructions. ModFit LT™ software (Verity Software House, Topsham, ME, USA) was used for data analysis.
To analyse the rate of apoptosis 100 000 REH and UOC-B6 cells were washed in PBS, resuspended in 50µL of Annexin-V binding buffer and stained on ice with 2.5µL of propidium iodide and 2.5µL of Dy647-conjugated Annexin-V in dark. After 30 minutes cells were washed with Annexin-V binding buffer, resuspended in 100µL of Annexin-V binding buffer and analysed by flow cytometry. The acquired data were analysed using FlowJo software.
Proliferation rate was assessed as DNA synthesis rate in REH cells measured using Click-iT™ EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Invitrogen) according to

manufacturer's protocol. Cells were pulsed with EdU for 12 hours and subsequently analysed. As a part of the manufacturer's protocol DNA content was measured by Click-iT^{TM} EdU Cell Cycle 488-red dye (Invitrogen). The acquired data were analysed using FlowJo software. Mann-Whitney test was used for statistical analysis of cytometric data.

Gene Expression Analysis

Spotted cDNA microarrays were used that contain more than 43,000 features representing about 30,000 genes (Stanford Functional Genomics Facility, Stanford, CA). The reference RNA used for all the arrays was Universal Human Reference RNA (Stratagene Europe, Amsterdam, the Netherlands). We labeled each of the sample with a red fluorescent dye (Cy5-dUTP, Amersham Pharmacia Biotech Europe, Freiburg, Germany) and the reference RNA with a green fluorescent dye (Cy3-dUTP, Amersham Pharmacia Biotech Europe) and comparatively hybridized sample RNA and reference RNA to an array. Each experiment was done in triplicates. The fluorescence intensities of Cy5 and Cy3 were measured using a GenePix 4000 scanner (Axon Instruments, Foster City, CA) and analyzed using GenePix Pro 4.1 software (Axon Instruments). Subsequent analysis and normalization of data was done as previously described (Cario et al., Blood 2005). To analyze the data in a supervised approach, we used significance analysis of microarrays (SAM) with a 1,000-fold permutation and considering a false discovery rate (FDR) of 5% as significant (Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A 2001;98: 5116 ^ 21).

Results

Identification of the efficient and specific siRNAs targeting the TEL/AML1 fusion

We designed eleven different Stealth siRNAs (TA-1)-(TA-11) targeting the transcriptomic TEL/AML1 fusion site. The Stealth siRNAs synthesized by Invitrogen are 25 nucleotides long blunt-ended duplexes that are chemically modified in order to minimize the off-target effects associated with induction of cellular stress response and sense strand guided silencing. Our siRNAs were designed with target sites moved by one nucleotide each, all of them spanning the fusion point and, thus, lacking the total sequence homology to wt TEL and AML1 genes to avoid their silencing (Fig.1). Our siRNAs also do not have sequence homology to any other known human transcript further diminishing the potential risk of the off-target silencing.

To effectively test the efficiency of siRNAs we employed the HELA-TEL/AML1_ires2_EGFP model. HELA- TEL/AML1_ires2_EGFP cells are permanently transfected with TEL/AML1_ires2_EGFP construct and express a single bicistronic mRNA from which both TEL/AML1 fusion protein and enhanced green fluorescent protein (EGFP) are translated. siRNA targeting the TEL/AML1 fusion region of the bicistronic mRNA triggers silencing of both TEL/AML1 and EGFP and, thus, we could highly accurately and reproducibly compare the efficiency of our siRNAs by measuring decrease of EGFP fluorescence by flow cytometry. The efficiency of individual siRNAs assessed 48 hours post siRNA transfection varied from 58% to 6% (i.e. EGFP fluorescence was reduced to 42%-94% of its level in control, Figure 2A).

The 5 most efficient siRNAs (TA-1)-(TA-5), that induced the reduction of EGFP fluorescence to at least 50% of its level in control were further tested in TEL/AML1-positive leukemic REH cells. Electroporation by rectangular pulses was used to deliver siRNAs into REH cells. Transfection efficiency monitored as incorporation of fluorescein-labeled siRNAs by flow cytometry reached 90% and more. We compared the TEL/AML1 knock-down efficiency of

individual tested siRNAs at the mRNA level 24h post transfection (analysis of time points 12h, 24h and 48h post transfection revealed, that at the transcript level silencing reaches its maximum 24h post transfection). We found the siRNAs TA-1 and TA-5 to be the most efficient ones inducing in average 58% and 57% transcript knock-down, respectively (Figure 2B).
We pooled fluorescein-labeled TA-1 and TA-5 (TA-1+5) siRNAs and further compared their efficiency with C1 siRNA used for TEL/AML1 silencing by Diakos et al. (15). Commercially available set of 4 siRNAs (TEL-set) with validated efficiency targeting the TEL portion of TEL/AML1 was used as a positive control. The efficiency of TA-1+5 in REH cells was comparable to TEL-set (61% and 64% knock-down, respectively) and higher than the efficiency of C1 siRNA (48% knock-down) (Figure 2C). Although our siRNAs were designed to be strictly TEL/AML1 specific we wanted to directly proof that they do not influence the level of wt allele transcripts. Like in the majority of TEL/AML1-positive ALLs the wt TEL is deleted in both cell lines used in this study and thus the potential risk of its silencing is abolished. However, this is not the case of wt AML1. We analysed the AML1 transcript level 24h after siRNA transfection and found that C1 siRNA caused 46% AML1 knock-down while our TA-1+5 siRNA pool caused only a negligible reduction (14%) of AML1 transcript level (Figure 2D). Thus, we found our TA-1+5 siRNAs pool to be more efficient and far more specific than previously published C1 RNAi oligo.

TEL/AML1 knock-down in REH and UOC-B6 cells
Having identified the most efficient and specific siRNAs we analysed their potential to silence TEL/AML1 protein. We found no effect at the protein level after the single siRNA transfection into REH cells at the time points 24h, 48h and 72h and only a slight reduction of TEL/AML1 protein 96h post transfection (data not shown). This potentially indicates a longer half-life of TEL/AML1 protein, so to achieve its knock-down we had to further optimize the experimental design to prolong and maximize the transcript silencing. Finally we performed two rounds of siRNA transfection in 48h interval with the evaluation of transfection efficiency and transcript reduction after each transfection and the protein analysis 48h after the second transfection (see experimental scheme in figure 3). Using this scheme we reached a substantial depletion of TEL/AML1 protein in REH cells in all 3 experimental replicates: 68%, 73% and 81% knock-down, respectively (Figure 4A). Using the identical approach the similar results were obtained also in another TEL/AML1-positive leukemia cell line, UOC-B6: 75%, 89% and 93% TEL/AML1 protein knock-down, respectively (Figure 4B).

The biological consequences of TEL/AML1 knock-down in REH and UOC-B6 cells
Next we studied the impact of TEL/AML1 knock-down on cell viability and cell cycle distribution. Utilizing trypan blue staining followed by optical microscopy we did not observe any decrease in viability of TEL/AML1 depleted cells. In agreement with this observation we did not detect the increased apoptosis rate assessed by annexin V and propidium iodide staining. We further analysed the cell cycle distribution by measuring the DNA content using flow cytometry. We performed several measurements in 24h intervals from the start of the experiment to the time point 144h (and beyond). As shown in Fig. 6A, we found highly reproducible changes of the cell cycle distribution with the maximum intensity 24h post TEL/AML1 depletion in both REH and UOC-B6 cell lines used in this study: The fraction of S-phase cells increased (from 24.5% to 28.8% in REH and from 28.4% to 34.1 % in UOC-B6; P=0.0495) with a corresponding decrease in the fraction of G0/G1- phase cells within TA-1+5 treated population compared to controls (from 68.0% to. 64.1% in REH and from 66.5% to 59.8 % in UOC-B; P=0.0495). To address the question whether the observed increase in S

phase is accompanied by the higher proliferation rate, we pulsed the TEL/AML1-depleted REH cells with EdU nucleoside analogue. After 12h-pulse we measured the percentage of cells that incorporated EdU, and thus entered or passed the S phase of the cell cycle. Upon TA1+5 siRNA treatment, more cells incorporated EdU compared to control siRNA treated cells (63.7% vs. 61.0%, P=0.0495; Table 1) giving evidence of higher proliferation rate. The proportion of cells in S and G2M phases within the total EdU-positive population measured by DNA staining did not differ between TA1+5 and control treated cells thus indicating a normal passage through the S-phase in TEL/AML1-depleted cells (Supplementary figure1). To assess the impact of TEL/AML1 on the global gene expression pattern we performed genome-wide gene expression profiling of REH cells 24h after TEL/AML1 protein depletion (TP120h). No significantly differentially expressed genes were identified comparing treated and untreated cells (SAM, 1000 permutations, FDR<5%).

The biological consequences of AML1 knock-down in REH cells

It was previously demonstrated, that AML1 promotes the transition from the G1 to the S phase of cell cycle (20, 21). We hypothesized that TEL/AML1, at least partially, interferes with this process and that the cell cycle changes found after TEL/AML1 depletion resulted from the restoration of AML1 function. Therefore, we performed an opposite experiment – we silenced AML1 and analysed the effect of this on the cell cycle. As the TEL/AML1 fusion includes almost entire AML1, the commercially available validated siRNA pool (AML1-pool) used for AML1 knock-down targets both wt AML1 and TEL/AML1. We used the same experimental scheme with double transfection as described above and we achieved an excellent suppression of AML1 protein in REH cells in all 3 experimental replicates: 91%, 92% and 94%, respectively (Figure 5). The concomitant suppression of TEL/AML1 protein by AML1- pool reached 30%, 59% and 73%, respectively. In agreement with our hypothesis, AML1 depletion had an opposite effect on REH cell cycle distribution compared to TEL/AML1 depletion. With the maximum intensity 48 hours post depletion the fraction of S-phase cells significantly decreased (from 26.0% to. 22.1%; P=0.0495) while the fraction of G0/G1- phase cells significantly increased in AML1-pool treated cells (from 63.5% to 68.7%; P=0.0495; Figure 6B). In line with the changes of cell cycle distribution, EdU pulse-chase experiments demonstrated a reduction of proliferation rate upon AML1 silencing (50.4% vs. 55.5%, P=0.0495; Table 1).

Discussion

In our study, we tested the effect of TEL/AML1 knock-down in human leukaemic TEL/AML1-positive cells. Knowing that highly efficient and specific siRNA would be the key step to perform successful RNAi, we designed a set of all possible Stealth siRNA's (n=11) specifically targeting the TEL/AML1 fusion region without targeting the wt TEL and AML1. We used an EGFP-reporter system in HeLa cells to check their efficiency and after an arduous process of optimising the transfection we subsequently tested selected siRNAs in human TEL/AML1-positive cell line. The two most efficient siRNAs were than pooled for further experiments. We reached adequate TEL/AML1 silencing on both mRNA and protein level reproducible in two TEL/AML1-positive cell lines.

One would expect that silencing of TEL/AML1, a fusion protein which is the hallmark of nearly one fourth of all childhood B-cell precursor leukaemias, leads to major changes in the cell survival, cell cycle and/or expression profile of the target genes. Such an effect was described for other chimearic genes in leukaemias, including BCR/ABL and MLL/AF4 (22-

24) . However, in our experiments we observed rather subtle changes in the cell cycle and neither changes in survival characteristics of leukaemic cell nor the change in the gene expression pattern. Nevertheless, we are convinced that our results are in line with the current knowledge of the natural history of childhood ALL carrying TEL/AML1 fusion.
TEL/AML1 fusion is known to originate during the prenatal period of life (25-27). This single genetic aberration leads to the generation of an covert preleukaemic clone (found in up to 1% of normal physiological newborns) with a low probability of conversion to a definitive full-blown leukaemia. This process requires further genetic events and takes typically 2 to 6 years, given the age distribution of TEL/AML1-positive ALL (28). In accord with these epidemiological data, in vitro and in vivo experiments confirm the insufficiency of TEL/AML1 fusion alone for the malignant transformation. In some of the murine models, TEL/AML1 transduction did not lead to any phenotypic changes (10); in others to the increase of multipotent or B-cell progenitors numbers with an incomplete differentiation block (11-13). In none of these models TEL/AML1 expression had led to the leukaemia origin without other additional genetic changes. Genomic profiling showed that TEL/AML1-positive leukemic blasts carry in average 6.7 additional copy number alterations (29). Most recent twin study by Bateman et al. documents that these secondary ("second-hit") changes affect both genes regulating differentiation and proliferation (PAX5, EBF1, CDKN2A/B, RB1 deletions) suggesting both these processes may be deregulated in leukemic cells independently of TEL/AML1 (30). In addition, we demonstrated that aberrant expression of specific miRNAs in TEL/AML1-positive blasts provides survival advantage to growth inhibitory signals and represents another event independent of TEL/AML1 (31).
Rrecent work of Schindler et al. demonstrated that only TEL/AML1 expression in HCS's has the potential for leukaemia initiation in contrast to TEL/AML1 expressing committed lymphoid progenitors (32). They also showed that TEL/AML1-positive HSC's did not progressively accumulate over time, did not outcompete the wt HSC's, were more quiescent than wt HSC's and did not exhibit deregulated expression of major known regulators of HSC's. Thus, when translating this knowledge to the clinical setting, TEL/AML1-positive HSC's can survive in the patient's organism for a long period of time waiting for the secondary event or the series of them. One potential mechanism for the long term survival and propagation of the preleukaemic clone was proposed by Ford et al. In the reaction to TGFB signalling, TEL/AML1-positive preleukaemic cells can exert their proliferative advantage and expand (33).
Ford et al also analysed the cell cycle changes after induced expression of TEL/AML1 in the murine BaF3 cell line. They clearly demonstrated that TEL/AML1 expressing cells elevated the G0/G1 fraction, decreased S/G2/M fraction and slowed down the proliferation. This is in agreement with the subtle but significant opposite changes in the cell cycle observed in our experiments after TEL/AML1 silencing. We also show that REH cells with TEL/AML1 knock-down tend to higher proliferation rate. It was previously demonstrated, that AML1 stimulates G1 to S progression and shortens the G1 phase of the cell cycle (20, 21). This function of AML1 seems to be inhibited by AML1/ETO fusion protein (34). We hypothesised, that TEL/AML1 also partially inhibits AML1 function and that the observed cell cycle changes occurred due to the functional restoration of AML1 after TEL/AML1 depletion. To prove this hypothesis we performed the opposite experiment – RNAi-mediated AML1 knock-down – and analysed the cell cycle and proliferation. AML1 silencing indeed led to the increase in G1/G0 phase and decrease in S phase and to slowing the overall proliferation rate.This shows that the functional block of AML1 by TEL/AML1 is incomplete and thus we observe enhancement of the changes typical for TEL/AML1 presence when AML1 is totally depleted.

One of the obvious effects of silencing a potential leukaemogenic fusion gene to be tested would be the changes in the expression profile. The pattern of changes may point to the functional pathways to be disrupted and help to identify the target genes of the culprit. So far, no direct target gene of AML1 and/or TEL/AML1 proteins was identified and only handful were proposed in the lymphoid lineage (35, 36). We have previously published the list of potential TEL/AML1-regulated genes, identified among upregulated genes in the expression profile of REH cells treated with HDACi (thus targeting the functional pathway of TEL/AML1 protein function) (8). Given the subtle effect of TEL/AML1 silencing on cell cycle and survival, we did not expect major changes in GEP performed 24 hours after RNAi-mediated TEL/AML1 silencing. Indeed, no detectable changes were recorded by GEP analysis. This finding, though seemingly counterintuitive is in fact in agreement with the findings of Fischer et al, who also found very subtle changes in the GEP of TEL/AML1-transduced HSC's (11). Clearly, the potential of RNAi in the search for potential target genes of the "first hit" aberrations in leukemia is rather limited.

During our work on the current project, contradictory data on TEL/AML1 silencing were published(15). It was described, that TEL/AML1 silencing has an proaptotic effect and leads to profound changes in gene expression profiles. However, we found that the siRNA oligo used in that study (C1) is not exclusively specific for the TEL/AML1 fusion but is fully complementary to the wt AML1 sequence (see Fig. 1 for sequence comparison). This different silencing pattern might – at least in part –explain the differences between our and these data. Our experiments confirmed that AML1 was efficiently silenced using the oligo C1, however, the TEL/AML1-targeted efficacy was lower than the combination of oligos we designed and used in the current study (TA-1+5). Therefore, the lack of apoptosis we observed using our TA-1+5 oligos clearly cannot be caused by the insufficient knock-down. This notion is further supported by the fact that more efficient silencing in UOC-B6 cell line (average of 86% vs. 74% in REH) resulted in more profound changes in the cell cycle (S-phase increase: 5.7% in UOC-B6 vs. 4.3% in REH; G1 decrease: 6.7% in UOC-B6 vs. 3.9% in REH). Additional increase of the silencing efficacy would very likely intensify these changes, but would not lead to a reverse effect. Interestingly, in contrast to other studies working with the identical model cell system, Diakos and colleagues showed that the ectopic expression of TEL/AML1 leads to the growth factor independence in BaF3 cell. (10, 33) Here, further models are needed to find the real effect of an TEL/AML1 ectopic expression. In summary, in line with the majority of recent reports, our data support the hypothesis that the TEL/AML1 protein is essential for the formation and preservation of the preleukaemic clone, but may be dispensable for the definitive leukaemic cells.

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Disclosure of Conflicts of Interest
The authors have no conflicts of interests and no competing financial interests to disclose.

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Table 1
Proliferation rate

TEL/AML1 knock-down in REH

	EdU-positive	*Mean* / *SD*	*P*
TA-1+5	62.8% 63.9% 64.5%	63.7% 0.7%	0.0495
Non-T1	59.1% 61.6% 62.1%	61.0% 1.3%	

AML1 knock-down in REH

	EdU-positive	*Mean* / *SD*	*P*
AML1-pool	49.3% 52.0% 49.8%	50.4% 1.2%	0.0495
Non-T3	54.4% 53.9% 58.2%	55.5% 1.9%	

Figures Legends

Table 1 – Proliferation rate
Proliferation rate, e.i. the fraction of de novo DNA synthesizing cells, was assessed by flow cytometry as a percentage of the cells that incorporated EdU (EdU-positive) from the total EdU pulsed population; Mean =mean of EdU-positive cells; SD = standard deviation

Figure 1 – Sequences and target sites of siRNAs within the TEL/AML1 fusion
Partial sequences of TEL/AML1 fusion and wt AML1 and TEL transcripts are shown. Black color indicates TEL and TEL part of TEL/AML1, grey color indicates AML1 and AML1 part of TEL/AML1; dashed line runs through the fusion site; 3 base-pair region of sequence homology between both wt trancripts surrounding the fusion site is underlined.
The siRNAs (TA-1) –(TA-11) and C1 sequences are positioned to indicate the targeted region within the TEL/AML1 fusion.

Figure 2 – siRNA efficiency in HELA-TEL/AML1-ires2-EGFP and REH cells
A- the efficiency of (TA-1) – (TA-11) siRNAs in HELA-TEL/AML1-ires2-EGFP cells measured as a decrease of EGFP fluorescence intensity by flow cytometry; y axis – mean fluorescence intensity (MFI) normalized to control (Non-T1); standard deviation refer to experimental hexaplicate
B,C – the TEL/AML1 knock-down efficiency of selected siRNAs in REH cells at the transcript level; the TEL/AML1 transcript level was normalized to the β2M transcript level
D – the level of AML1 transcript knock-down induced by selected siRNA in REH cells; the AML1 transcript level was normalized to the β2M transcript level
B,C and D - standard deviation refer to experimental triplicate

Figure 3 – Schema of experimantal design
Timeline of individual experimental steps.
1st and 2nd Transfection – electroporation of siRNAs; mRNA – evaluation of the knock-down at the transcript level; Protein - evaluation of the knock-down at the protein level; GEP – gene expression profiling; Cell cycle distribution/Apoptosis – flow cytometric analysis of cell cycle distribution, proliferation and apoptosis rate

Figure 4 - TEL/AML1 protein knock-down in REH and UOC-B6 cells
TEL/AML1 protein detected by western blot at TP 96h in TA-1+5 or control (Non-T1) siRNA treated REH (A) and UOC-B6 (B) cells in three independent experimental replicates (Exp.1-3). LAMIN B is shown as nuclear protein loading control. Graphs show the relative quantity of TEL/AML1 protein normalised to LAMIN B and further to (TEL/AML1)/LAMIN B ration of control for each individual experiment.

Figure 5 - AML1 and TEL/AML1 proteins knock-down in REH cells
TEL/AML1 and AML1 proteins detected by western blot at TP 96h in AML1-pool or control (Non-T3) siRNA treated REH cells in three independent experimental replicates (Exp.1-3). LAMIN B is shown as nuclear protein loading control. Graphs show the relative quantity of TEL/AML1 and AML1 proteins normalised to LAMIN B and further to (TEL/AML1)/LAMIN B or AML1/LAMIN B ration of control for each individual experiment.

Figure 6 – Cell-cycle distribution

The proportion of G1 and S phases of cell cycle after TEL/AML1 knock-down in REH and UOC-B6 cells (A) and after AML1 knock-down in REH cells (B) versus controls (Non-T1 and Non-T3, respectively); y axis – percentage of cells from the total population; *P= 0.0495

Figure 1 siRNAs sequences and positions within TEL/AML1 fusion

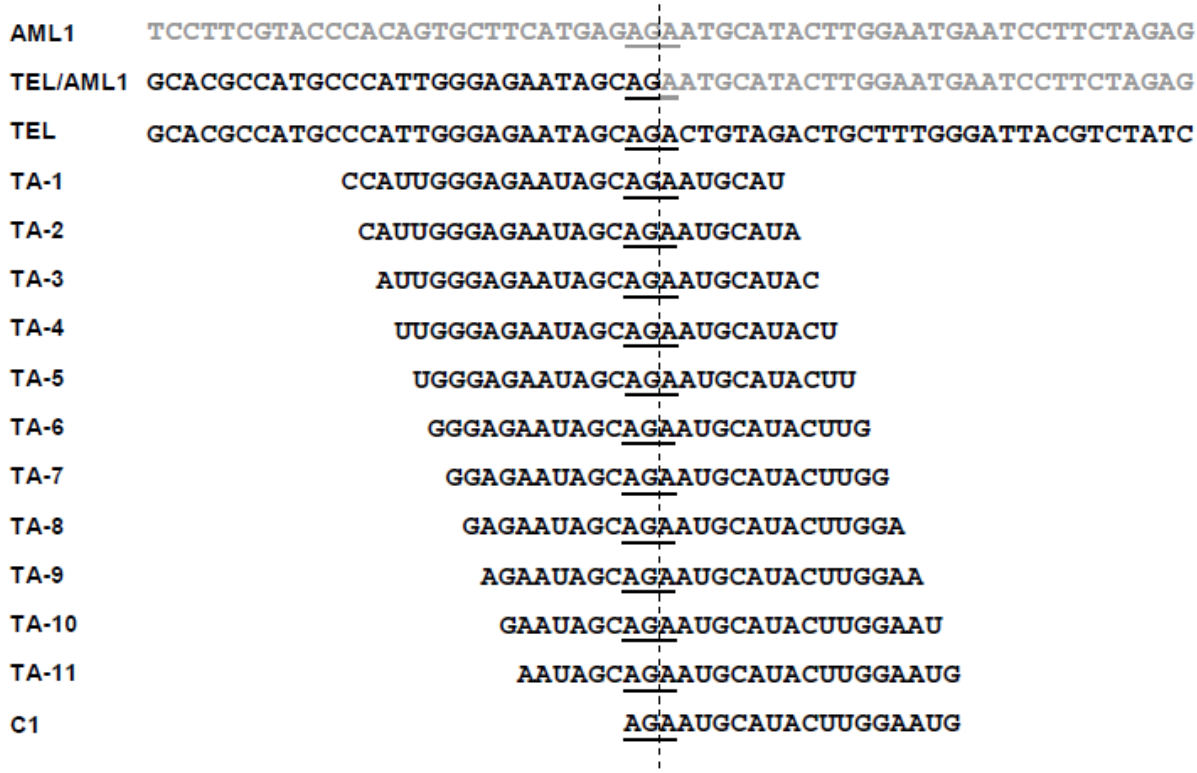


Figure 2 siRNA testing in HELA-TEL/AML1_ires2_EGFP and REH cells

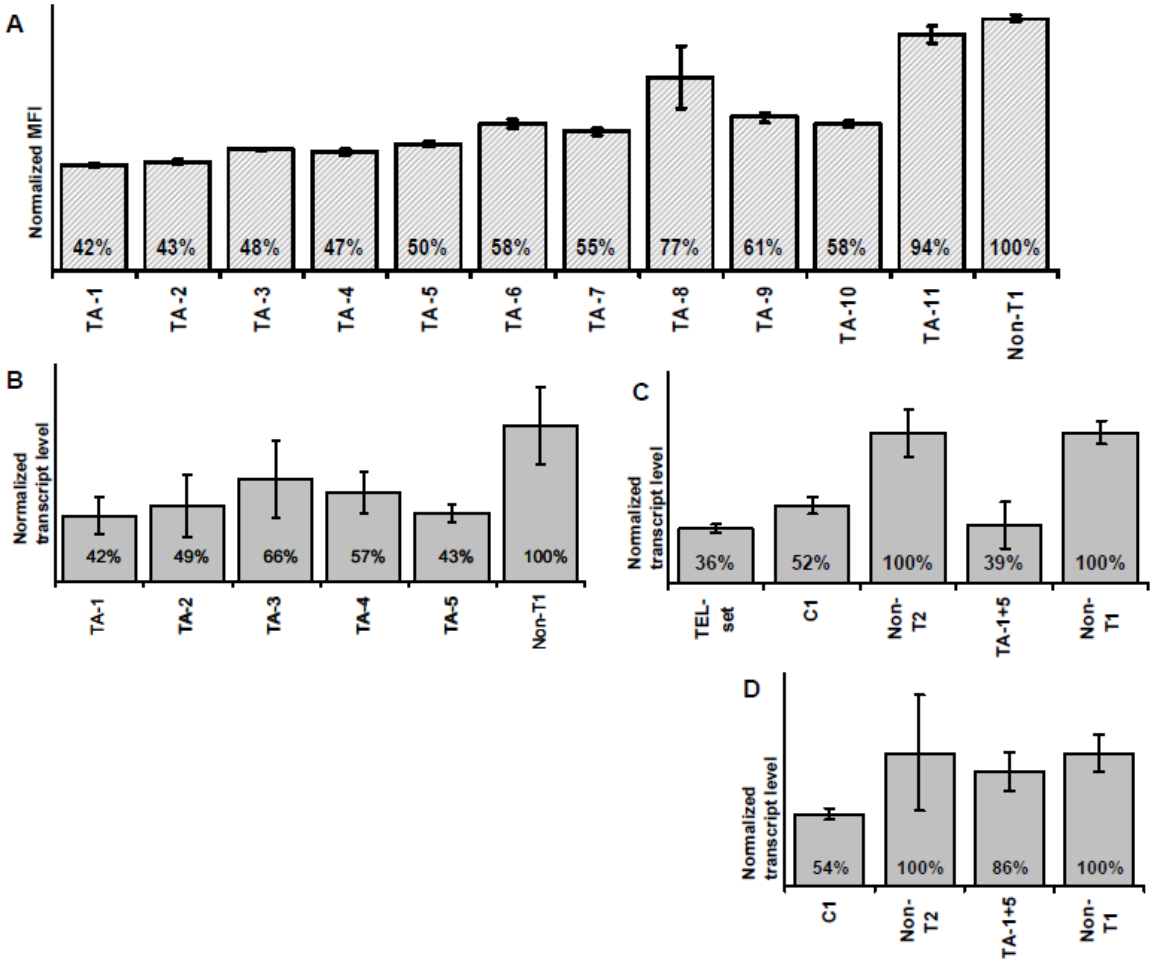


Figure 3 Schema of experimental design

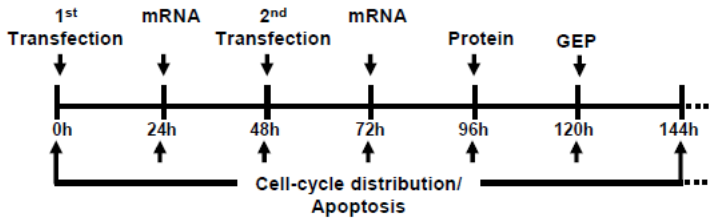


Figure 4 TEL/AML1 protein knock-down in REH and UOC-B6 cells

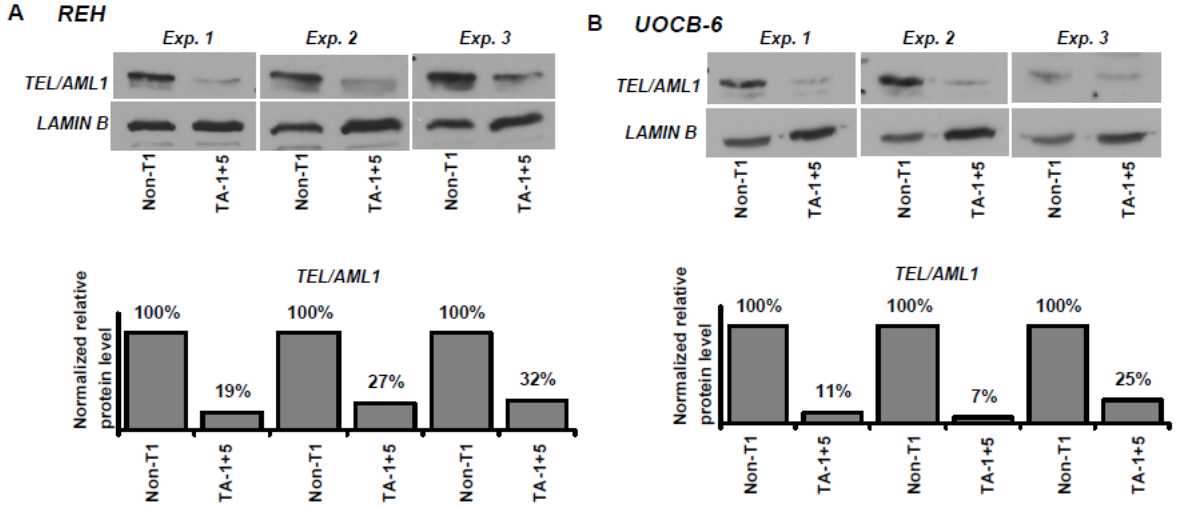


Figure 5 AML1 and TEL/AML1 proteins knock-down in REH cells

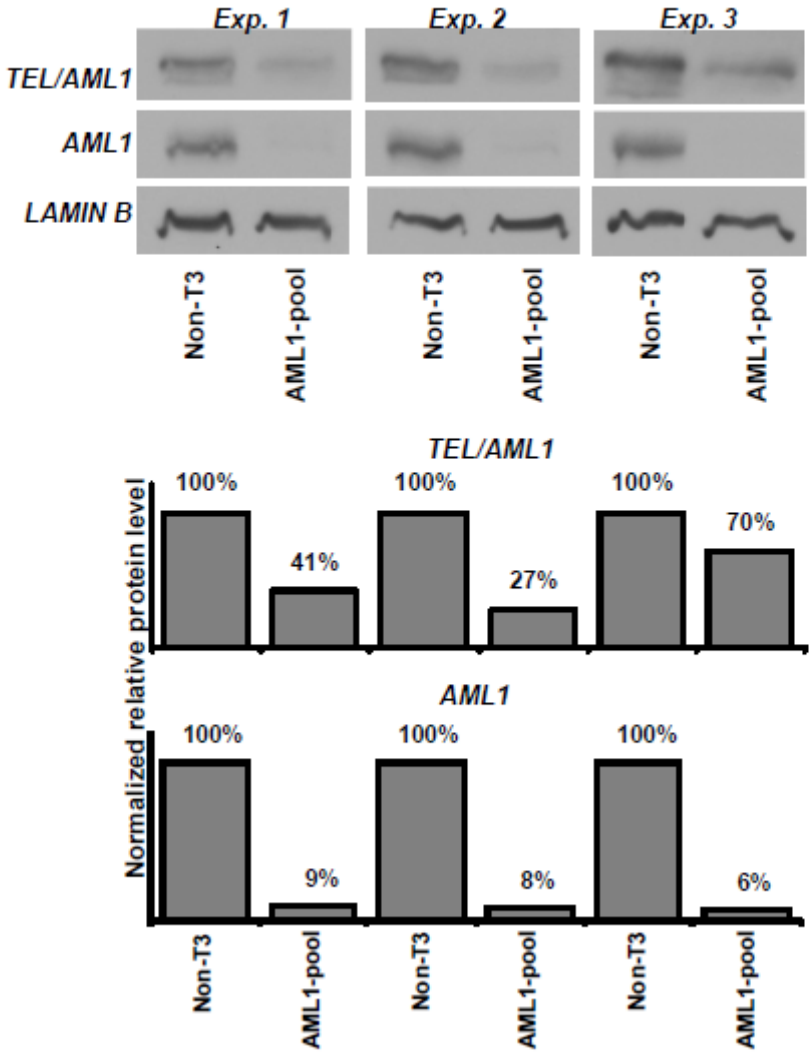
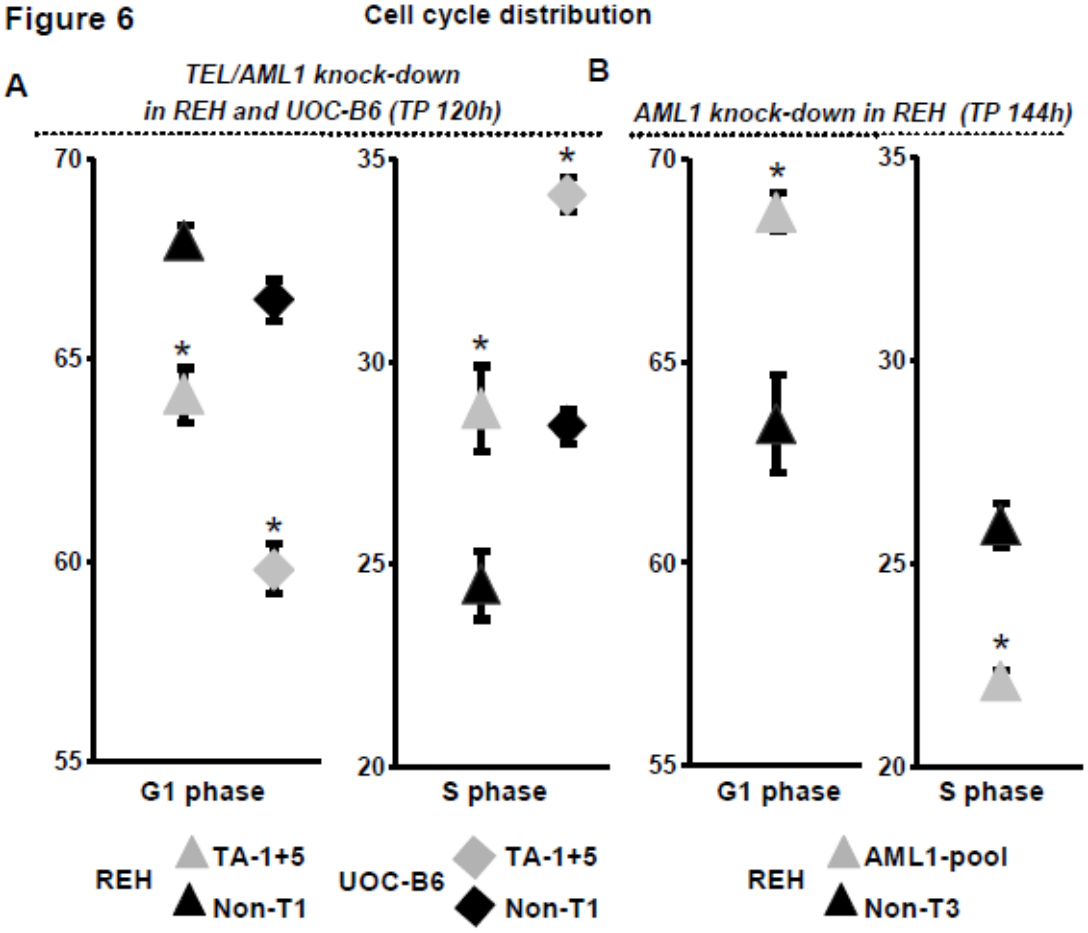
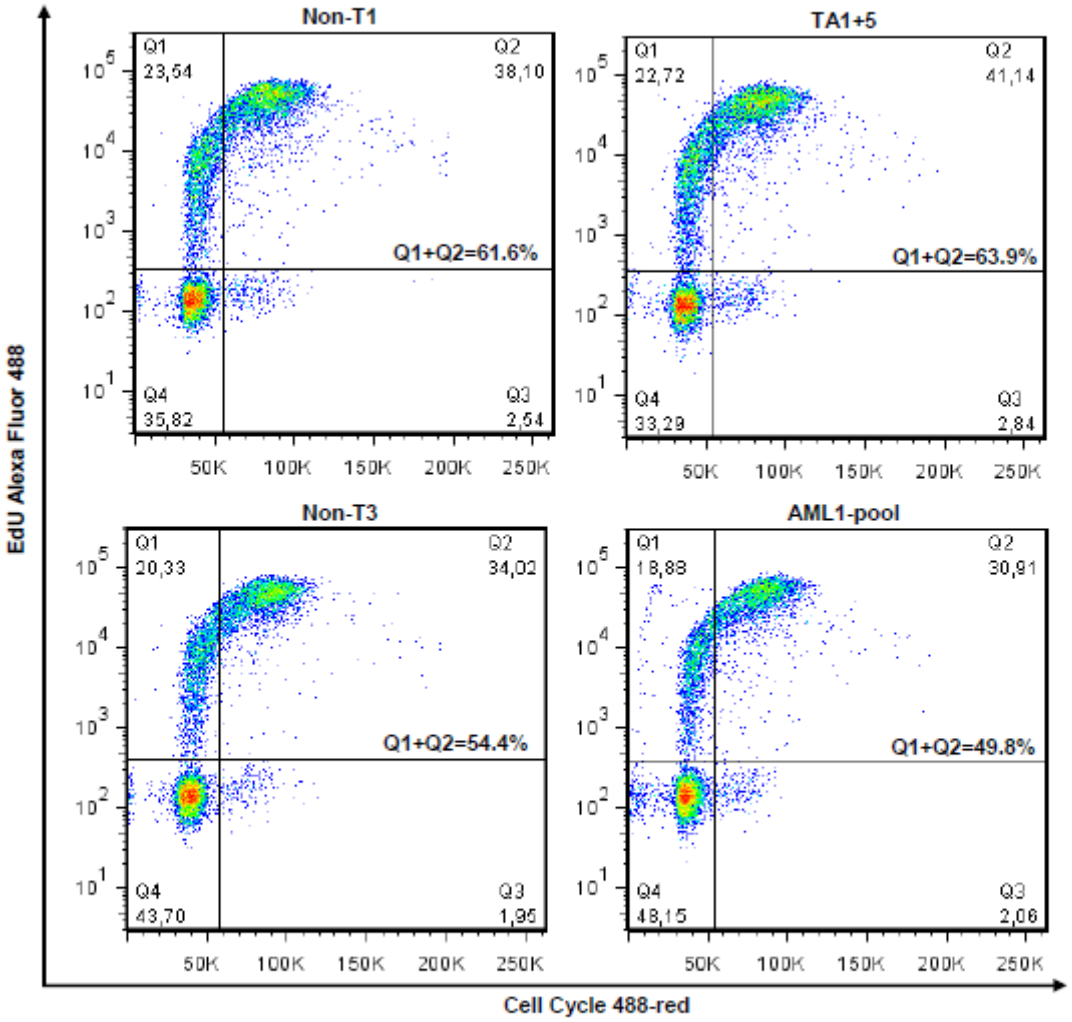


Figure 6


Cell cycle distribution
A
TEL/AML1 knock-down
in REH and UOC-B6 (TP 120h)
B
AML1 knock-down in REH (TP 144h)
70
65
60
55
35
30
25
20
*
G1 phase
S phase
REH
TA-1+5
Non-T1
UOC-B6
TA-1+5
Non-T1
REH
AML1-pool
Non-T3

Supplementary Figure 1 Proliferation rate assesssed by EdU incorporation



Proliferation rate after TEL/AML1 and AML1 depletion, respectively, was assessed as de novo DNA synthesis rate measured by EdU incorporation using fow cytometry. After EdU pulse cells were stained for incorporated EdU (EdU Alexa Fluor 488) and for DNA content (Cell Cycle 488-red). Quandrant 1 represents S-phase cells and quandrant 2 represents G2M-phase cells within the total fraction of EdU-positive cells (Q1+Q2). Data are shown for 1 of 3 representative experiments.

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ORIGINAL ARTICLE

Hsa-mir-125b-2 is highly expressed in childhood *ETV6/RUNX1* (TEL/AML1) leukemias and confers survival advantage to growth inhibitory signals independent of p53

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MicroRNAs (miRNAs) regulate the expression of multiple proteins in a dose-dependent manner. We hypothesized that increased expression of miRNAs encoded on chromosome 21 (chr 21) contribute to the leukemogenic function of trisomy 21. The levels of chr 21 miRNAs were quantified by qRT–PCR in four types of childhood acute lymphoblastic leukemia (ALL) characterized by either numerical (trisomy or tetrasomy) or structural abnormalities of chr 21. Suprisingly, high expression of the *hsa-mir-125b-2* cluster, consisting of three miRNAs, was identified in leukemias with the structural *ETV6/RUNX1* abnormality and not in ALLs with trisomy 21. Manipulation of *ETV6/RUNX1* expression and chromatin immunoprecipitation studies showed that the high expression of the miRNA cluster is an event independent of the *ETV6/RUNX1* fusion protein. Overexpression of *hsa-mir-125b-2* conferred a survival advantage to Ba/F3 cells after IL-3 withdrawal or a broad spectrum of apoptotic stimuli through inhibition of caspase 3 activation. Conversely, knockdown of the *endogenous* miR-125b in the *ETV6/RUNX1* leukemia cell line REH increased apoptosis after Doxorubicin and Staurosporine treatments. P53 protein levels were not altered by miR-125b. Together, these results suggest that the expression of *hsa-mir-125b-2* in *ETV6/RUNX1* ALL provides survival advantage to growth inhibitory signals in a p53-independent manner.
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Keywords: acute lymphoblastic leukemia (ALL); *ETV6/RUNX1*; TEL/AML1; microRNA

Introduction

Aberrations in chromosome 21 (chr 21) are commonly found in childhood acute lymphoblastic leukemia (ALL). The high-hyperdiploid ALL (HHD ALL),[1,2] the most common type of childhood ALL with *numerical* chromosomal aberrations, is characterized by 3–4 copies of chr 21 and a variable presence of other specific chromosomal trisomies. The markedly increased incidence of ALL in children with Down's syndrome (DS)[3] strongly suggests that trisomy 21 is leukemogenic, but the chr 21 genes involved in these leukemias are presently unknown. A newly discovered rare subtype of childhood ALL is characterized by an intrachromosomal amplification of a small region within the long arm of chr 21 (iAMP21) around the RUNX1 locus.[4] In contrast to *ETV6/RUNX1* and HHD ALL, it is associated with poor prognosis. The most common *structural* chromosomal aberration in childhood ALL occurring in approximately 25% of pediatric B-cell precursor ALL fuses the RUNX1 (AML1) gene on chr 21 with the ETV6 (TEL) gene on chr 12.[5,6] Although *ETV6/RUNX1* causes a prenatal preleukemic clonal expansion, additional genetic events are required for evolution of leukemia.[7,8]

MicroRNAs (miRNAs) are 20–25 nucleotide long non-coding RNAs that have a vital function in the regulation of protein expression.[9] Currently, there are six validated mature miRNAs encoded on human chr 21 (http://microrna.sanger.ac.uk Figure 1a). *Hsa-mir-99a, hsa-let-7c* and *hsa-mir-125b-2* are clustered together in the same intron of the *C21ORF34* gene on 21q.21.1 (NC_000021.7) (*hsa-mir-125b-2* cluster). *Hsa-mir-125b-1*, the homolog of *Caenorhabditis elegans* lin-4, has been shown to be expressed in solid tumors and associated with enhanced cell proliferation and survival.[10–12] An insertion of *hsa-mir-125b-1* into the IGH locus was described in a patient with B-cell precursor ALL suggesting its involvement in the leukemic process.[13] It has been shown to be expressed in myeloid malignancies and to block myeloid differentiation.[14] Recently, it was also reported to protect from apoptosis through negative regulation of p53 in zebrafish, human neuroblastoma cells and lung fibroblasts, but not in mouse cells.[15] *Hsa-mir-155* has been linked to B-cell development. Others and we have shown its increased expression in lymphomas.[16–19] Strikingly, transgenic expression of miR-155 induced pre-B-cell leukemias and lymphomas in mice,[20] thus miR-155 seems to be a potent oncogene.

The mechanisms of miRNA function suggest that their activity should be *correlated with their dosage* and that each miRNA may regulate multiple targets.[21] Thus, we hypothesized that miRNAs contribute to the oncogenic effects of chromosomal trisomies, a situation in which a small change ($\times 1.5$) in genomic dosage results in profound effects. This hypothesis is strengthened by our recent observations showing a general increase in expression of multiple genes from the trisomic chromosomes.[22] Thus, we expected that miRNAs from chr 21 would be overexpressed in HHD and DS ALL (3–4 copies of chr 21) relative to *ETV6/RUNX1* and iAMP21 leukemias.

Here, we report the surprising observations that unlike most of the genes encoded on chr 21, the expression of the *hsa-mir-125b-2* cluster does not correlate with gene copy number, but

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[8]These two authors contributed equally to this work.
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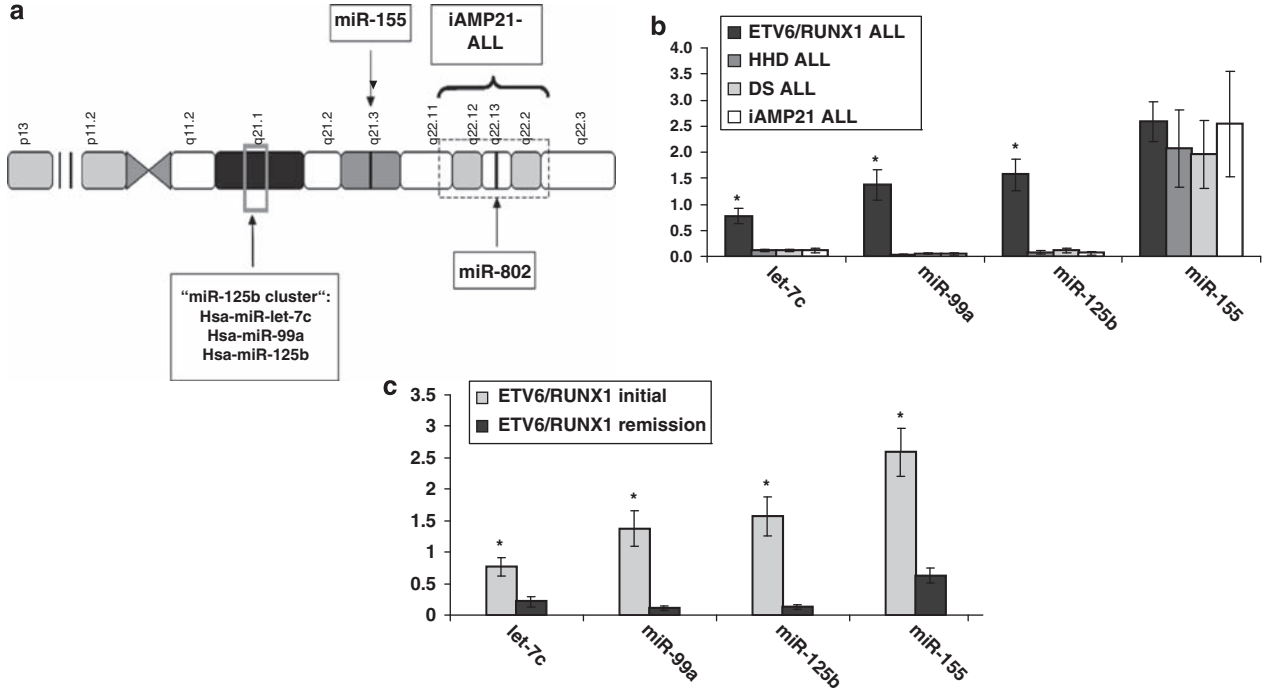

Figure 1 Expression of miRNAs encoded on chr 21 in different leukemia samples. (**a**) Schematic representation of the location of miRNAs encoded on chr 21. The level of the mature *hsa-miR-802* could not be measured because of the lack of an appropriate qRT–PCR-based assay. (**b**, **c**) MiRNA expression was determined by qRT–PCR using the TaqMan *MicroRNA Assays* from *Applied Biosystems.* The numbers on the *y* axis represent relative expression levels of mature miRNA normalized to an internal control (RNU43). Standard errors are indicated. Asterisk marks significance. (**b**) Expression of the chr 21 miRNAs in diagnostic bone marrow samples of different ALL subtypes: *ETV6/RUNX1* (24 patients), DS (10 patients), high hyperdiploid (10 patients), iAMP21 (7 patients) $P<0.05$ (ANOVA). (**c**) Comparison of miRNA expression between diagnosis and remission samples of *ETV6/RUNX1* leukemias $P<0.005$ (*T*-test).

rather is highly expressed in *ETV6/RUNX1* ALL. Additional research suggests that the expression of the *hsa-miR-125b-2* cluster is an independent event in *ETV6/RUNX1* ALL, conferring survival advantage under growth inhibitory and apoptotic conditions in a p53-independent manner.

Materials and methods

Patients

RNA was derived from diagnostic or remission bone marrow samples of childhood ALL patients obtained with informed consent. Diagnostic bone marrow samples contained at least 80% lymphoblasts. The samples were anonymized before shipping except for the information on the genetic subgroup. The study was approved by the IRB of the Israeli Health Ministry and Sheba Medical Center.

The rest of the material and methods are detailed in a supplementary file.

Results

Hsa-mir-125b-2 cluster is highly expressed in ETV6/RUNX1 leukemias

We measured the expression of the mature forms of four miRNAs encoded on chr 21 *(hsa-mir-99a*; *hsa-let-7c*; *hsa-mir-125b-2* and *hsa-mir-155)* by qRT–PCR in RNA derived from diagnostic bone marrow samples of four diverse subtypes of ALL with structural or numerical aberrations of chr 21: *ETV6/RUNX1* ALL ($n=24$), HHD ALL ($n=10$), DS ALL ($n=10$) and iAMP21 ALL ($n=7$). Surprisingly, extra copies of chr 21 did not contribute to the expression level of these miRNAs (Figure 1b). Although *hsa-mir-155* is similarly expressed between the different ALLs, the other miRNAs *hsa-mir-99a*, *hsa-let-7c* and *hsa-mir-125b-2* are up-regulated in the *ETV6/RUNX1* ALL, the subtype of leukemia with a *structural* aberration of chr 21. The expression was derived from the leukemic blasts, as it was not observed in remission samples (Figure 1c). To ensure that the observed results were not because of the normalization to a single internal control (RNU 43), we have extended the miRNA profiling using TaqMan Low Density miRNA arrays (TLDA, ABI) normalizing to two additional internal controls (RNU6 and RNU48). As can be seen in Supplementary Figure 1, the TLDA results are similar to the results obtained with the singleplex miRNA expression profiling.

Hsa-mir-125b-2 cluster is not a target of the ETV6/RUNX1 fusion protein

To test whether *mir-125b-2* is a target of ETV6/RUNX1, we performed overexpression experiments in two mouse hematopoietic progenitor cell systems: Ba/F3 cells and primary mouse embryonic fetal liver progenitors. It has been earlier shown that ectopic expression of *ETV6/RUNX1* in these progenitors induces proliferation of B-cell progenitors, similar to the preleukemia observed in human beings[23] (Supplementary Figure 2). In neither of these cells, the expression level of the *mmu-mir-125b-2* cluster was affected by the fusion protein (Supplementary Figure 3). As overexpression in mouse cells may not represent the relevant model, we have knocked down *ETV6/RUNX1* in the human REH ALL cell line with siRNA

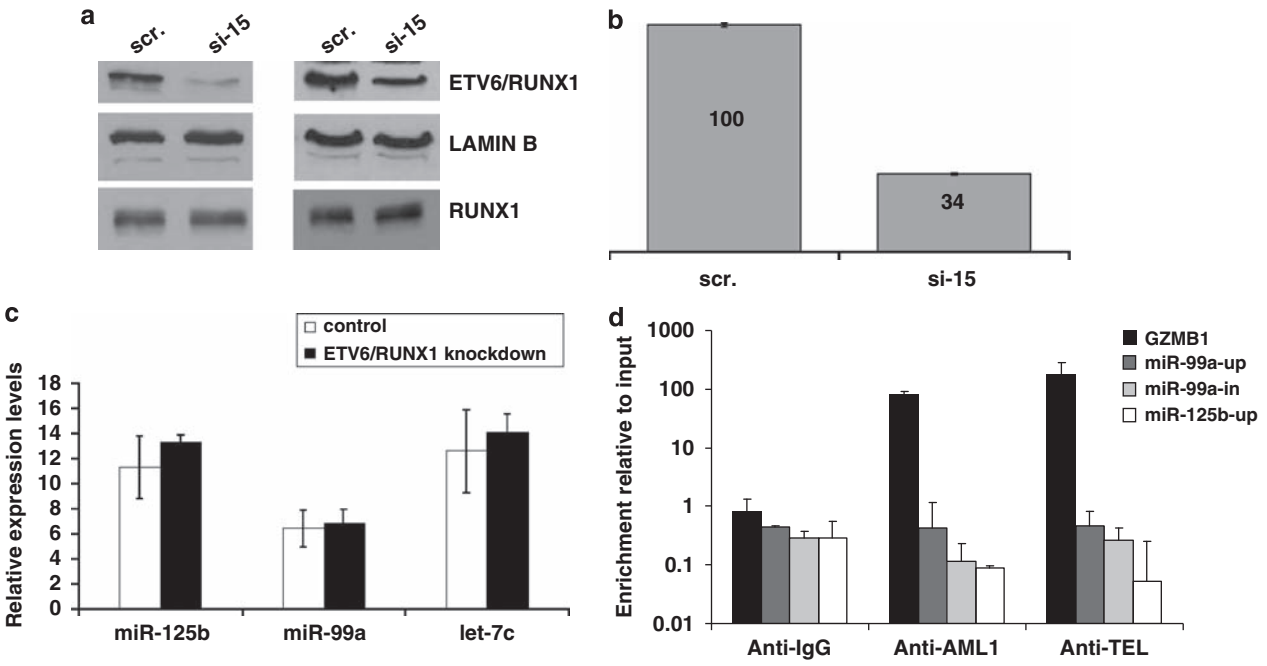

Figure 2 The *hsa-mir-125b-2* cluster is not a target of the *ETV6/RUNX1* fusion protein shown by knockdown of *ETV6/RUNX1* in REH cells. REH cells were transfected with siRNA directed against the fusion part of the ETV6 and RUNX1 genes (see Materials and methods). (**a**) Western blot analysis of *ETV6/RUNX1* knockdown in REH cells: Scr, scramble siRNA; si-15, mixture of siRNAs 1 and 5. Two independent experiments are shown. (**b**) A histogram showing quantification of the average *ETV6/RUNX1* protein level as detected by two independent western blots, determined by the ImageJ software. (**c**) No significant change in expression levels of the *hsa-mir-125b-2* cluster could be observed in REH cells after knockdown of *ETV6/RUNX1* as determined by qRT–PCR. An average of three independent experiments is shown. Expression levels are normalized to an internal control (RNU43). (**d**) qRT–PCR of chromatin immunoprecipitation analysis with anti-ETV6, anti-RUNX1, anti-IgG (served as negative control) or anti-TBP (served as positive control—see Materials and methods). Granzyme B (GZMB1) was used as positive control for the binding of RUNX1. The *y* axis indicates the enrichment normalized to GAPDH from sample and input. Three independent experiments were performed. Standard errors are indicated.

oligonucleotides (oligos) directed against the fusion part of this translocation (Supplementary Figure 4). Two-third silencing efficiency of *ETV6/RUNX1*, but not RUNX1, was observed by qRT–PCR and by western blots 5 days after the first round of transfection (Supplementary Figure 5; Figure 2a and b, respectively). Figure 2c shows that the expression of the *hsa-mir-125b-2* cluster was not influenced by the knockdown of the *ETV6/RUNX1* fusion protein. To further examine whether *ETV6/RUNX1* protein binds to RUNX1 sites on the vicinity of the *hsa-mir-125b-2* cluster, we performed chromatin immunoprecipitation analysis. There are three potential RUNX1 binding sites in the *hsa-mir-125b-2* cluster, one upstream of each miRNA (Supplementary Figure 6). As a positive control, we examined the promoter of granzyme B (GZMB), a RUNX1-regulated protein.[24] Figure 2d clearly shows that while the RUNX1 binding site upstream granzyme B (GZMB) is occupied by the *ETV6/RUNX1* and RUNX1 proteins, the putative RUNX1 binding sites of the *hsa-mir-125b-2* cluster are not bound by the fusion protein.

Taken together these experiments suggest that the *hsa-mir-125b-2* cluster is *not* a direct target of *ETV6/RUNX1*, but rather an independent event occurring during leukemogenesis.

Hsa-miR-125b-2 has a pro-survival effect under growth inhibitory conditions

To test the hypothesis that the expression of the *hsa-mir-125b-2* cluster contributes to survival and growth of lymphoid progenitors, we performed forced expression experiments in IL-3-dependent Ba/F3 cells. Transformation of these pro-B cells is commonly used to identify activating mutations of kinases.[25] We decided to focus on *hsa-mir-125b-2* because of earlier studies implicating a function of this miRNA in human cancers.[10,14,26,27] In addition, the endogenous mmu-miR-99a and let-7c are expressed in Ba/F3 cells, whereas miR-125b is not expressed. Furthermore, the sequence of the mature miR-125b is identical in human and mouse. We transduced Ba/F3 cells with an empty retroviral vector, a vector expressing *hsa-mir-125b-2* or a construct encoding *hsa-mir-125b-2* mutated in the seed region (see Materials and methods; Supplementary Figure 7) and confirmed the expression of the mature miRNAs by northern blot analysis (Figure 3a). No differences in growth were observed under steady-state conditions (not shown). However, Ba/F3 cells expressing *hsa-mir-125b-2* were highly resistant to a transient removal of IL-3. On reintroduction of IL-3 to the growth medium only *hsa-mir-125b2*-transduced Ba/F3 cells, but not cells transduced with an empty vector or a mutated *hsa-mir-125b-2,* resumed their normal growth (Figure 3b). To examine the mechanism of resistance to IL-3 withdrawal, we performed a cell cycle analysis of Ba/F3 cells transduced with the different constructs before and after IL-3 withdrawal; 24 h after IL-3 deprivation most of the *hsa-mir-125b-2* transfected cells were arrested in G1 (71.7%), whereas most of the control Ba/F3 cells were apoptotic (73% in subG1 phase; Figure 4a). This relative protection from apoptosis conferred by *hsa-mir-125b-2* was evident at different time points during the first 24 h of IL-3 deprivation (Figure 4b). The anti-apoptotic activity of *hsa-mir-125b-2* was associated with a marked inhibition of caspase 3 activation and the cleavage of its substrate PARP (Figure 4c).

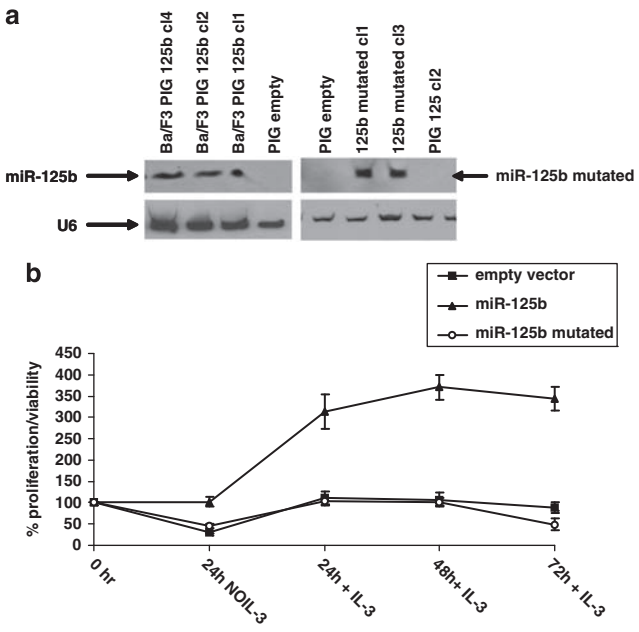

Figure 3 *Hsa-mir-125b-2* confers growth advantage in Ba/F3 cells after transient IL-3 deprivation. Ba/F3 cells were transduced with an empty vector, a vector expressing *mir-125b-2* or mutated *mir-125b-2*. (**a**) Northern blot analysis showing representative clones overexpressing *hsa-mir-125b-2* or mutated *hsa-mir-125b-2*. (**b**) Ba/F3 cells transduced with either empty vector, a vector expressing *hsa-mir-125b-2* or mutated *hsa-mir-125b-2* were cultured in the absence of IL-3 for 24 h. After 24 h, IL-3 was added to the medium and cell proliferation was measured by MTT every 24 h. The graph shows an average of at least three different clones and three experiments. Standard errors are indicated.

To examine how broad the anti-apoptotic phenotype observed in Ba/F3 cells overexpressing miR-125b is beyond the growth factor weaning, we exposed the cells to different apoptotic stimuli. Supplementary Figure 8 summarizes the percentage of living cells as has been quantified by FACS analysis of Annexin negative and 7AAD negative staining of four different treatments. As cytokine survival pathways involve activation of multiple kinases, we examined the effect of miR 125b on apoptosis induced by three kinase inhibitors with decreasing specificity: JAK Inhibitor I, AG490 and Staurosporine. Ba/F3 transduced with *hsa-mir-125b-2* were markedly resistant to each of these inhibitors in comparison with Ba/F3 cells transduced with mutated miR or with empty vector. Mild, but statistically significant resistance was also observed after treatment with Doxorubicin. Thus, miR125b provides survival advantage in response to multiple pro-apoptotic stimuli.

To test whether the survival advantage observed in Ba/F3 cells overexpressing miR-125b is relevant to *ETV6/RUNX1* leukemia, we used REH cells, a cell line derived from a patient with *ETV6/RUNX1* leukemia that highly expresses miR-125b. We knocked down the endogenous miR-125b by using LNA oligos labeled with FAM (Figure 5a) and measured the level of living cells of the FAM positive population (transfected with the LNA oligo) after treatment with either Doxorubicin or Staurosporine (see Materials and methods). Figure 5b and c shows that on knocking down miR-125b, the REH cells become more sensitive to Staurosporine and Doxorubicin treatments, respectively. These results indicate that the endogenous *mir-125b-2* provides partial protection from apoptosis induced by those agents similar to our observation in Ba/F3 cells.

A recent publication by Le *et al*.[15] suggested that miR-125b is a negative regulator of zebrafish and human (but not mouse) p53. We, therefore, asked whether the effects of miR-125b on apoptosis of Ba/F3 and REH cells could be explained by its regulation of p53 protein levels. Thus, we checked the levels of p53 and its main cellular protein target, p21, both under steady-state conditions and during apoptotic stress in both human and mouse models. The binding site of miR-125b on the 3′UTR of p53 is different between mouse and human particularly in the 'seed' area, an area considered to be the most significant for the recognition of miRNAs and their targets (Figure 6a). Therefore, it was not surprising that in the mouse Ba/F3 model, we have not observed any changes in the level of p53 neither under steady-state conditions nor under apoptotic conditions of IL-3 withdrawal (data not shown) or Doxorubicin treatment (Figure 6b and c). Surprisingly, knocking down miR-125b in the human REH cells did not alter p53 protein levels or p21 levels. Furthermore, treating the REH cells with Doxorubicin resulted in similar elevation of p53 levels in the cells transfected with a control LNA oligo or a miR-125b antisense LNA oligo, suggesting that p53 is not a target of miR-125b in these cells. This is further substantiated in the lack of alteration in the levels of p21 (Figure 6d and f). Similarly, there was no effect of miR-125b knockdown on p53 or p21 levels on treatment with Staurosporine (Figure 6e and f). Thus, p53 levels are not regulated by miR-125b in REH cells.

Discussion

Here, we describe that expression of the *hsa-mir-125b-2* cluster residing on chr 21 characterizes *ETV6/RUNX1* leukemias compared with other 'chr 21' leukemias. We show that this miRNA cluster is not regulated by the *ETV6/RUNX1* fusion protein and, therefore, hypothesize that its expression is an independent event occurring during the evolution of these leukemias. We further show by overexpression and knockdown studies that *mir-125b-2* provides a survival advantage by suppressing apoptosis and caspase 3 activation in response to growth inhibitory conditions.

This study was prompted by the hypothesis that increased expression of chr 21 miRNAs may explain the leukemogenic function of trisomy 21 in ALL of DS and in sporadic HHD leukemias (which uniformly contain either 3 or 4 copies of chr 21). This hypothesis was on the basis of our earlier observations that trisomies are usually associated with increased expression of multiple genes from the trisomic chromosomes[22] and by increased expression in DS AML of the miRNAs belonging to the *hsa-mir-125b-2* cluster.[28] Furthermore, increased expression of all chr 21 miRNAs has been recently reported in fetal heart and fetal hippocampus of DS patients.[29] Our findings that the expression of those miRNAs in B-cell precursor ALLs was *not* correlated to gene dosage but rather to the leukemia subtype were, therefore, unexpected. Increased expression levels of the *hsa-mir-125b-2* cluster were observed in *ETV6/RUNX1* ALL, whereas *hsa-miR-155* was similarly expressed in all subtypes. Furthermore, the expression of the *hsa-mir-125b-2* cluster was specific to the leukemic cells, as it was not observed in remission samples.

ETV6/RUNX1 is the most common translocation in childhood ALL. Similar to HHD ALL, these are B-cell precursor leukemias with excellent prognosis on contemporary treatment protocols.[30] The translocation is necessary for the initiation of a preleukemic clone, but insufficient for the evolution of leukemia.[7,8,31] Little is known about the acquired somatic

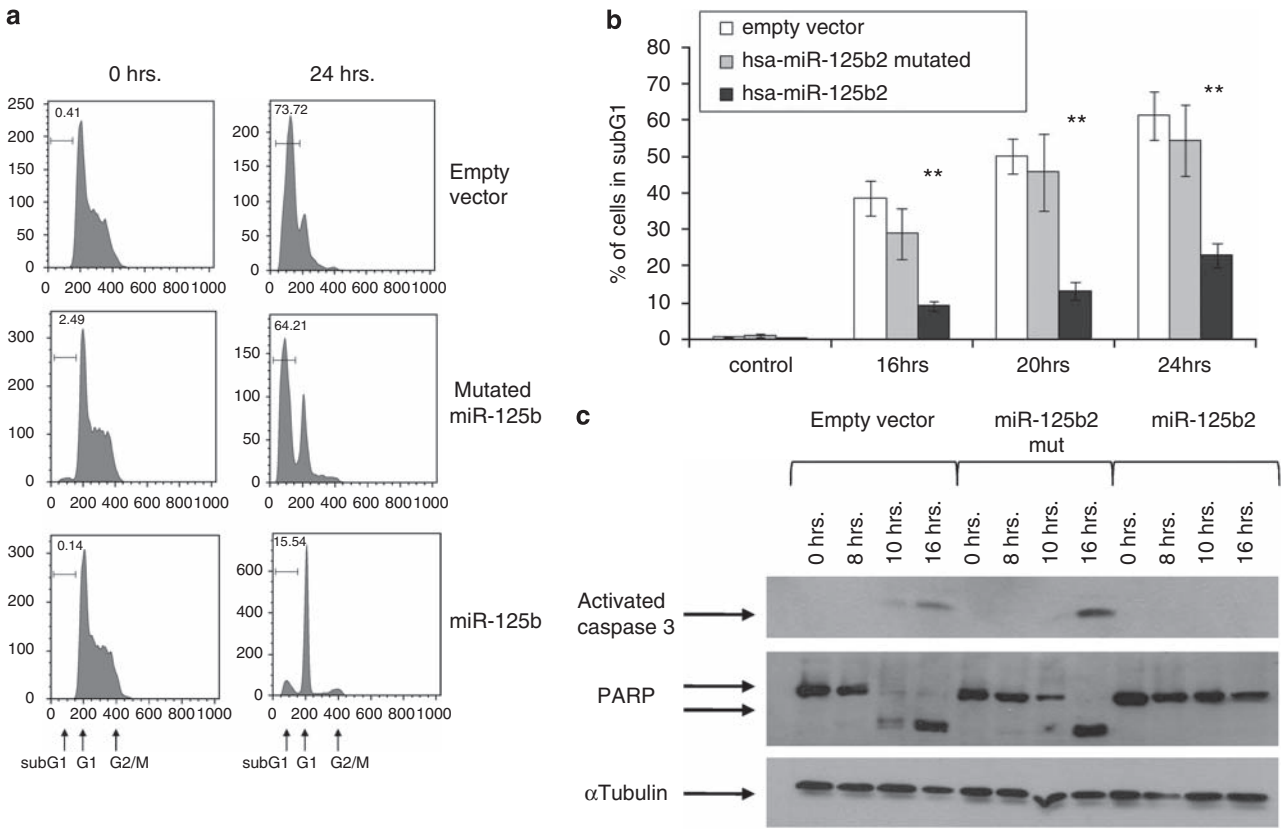

Figure 4 *Hsa-mir-125b-2* has an anti-apoptotic effect in Ba/F3 cells. (**a**, **b**) Quantification of apoptosis by measuring the subG1 fraction of Ba/F3 cells stained with propidum iodide, comparing wild-type cells, cells overexpressing *hsa-mir-125b-2* and cells overexpressing mutated *mir-125b-2*. (**a**) A representative example before (time 0) and 24 h after IL-3 withdrawal. (**b**) Kinetics of apoptosis with time after IL-3 withdrawal. Asterisks marks significance of $P<0.005$ as calculated by ANOVA. Standard errors are indicated. (**c**) Western blot analysis showing lack of activation of caspase 3 and cleavage of PARP after removal of IL-3 in Ba/F3 cells overexpressing *hsa-mir-125b-2* in comparison to wild-type Ba/F3 and those overexpressing mutated *hsa-mir-125b-2*. Experiments were repeated five times.

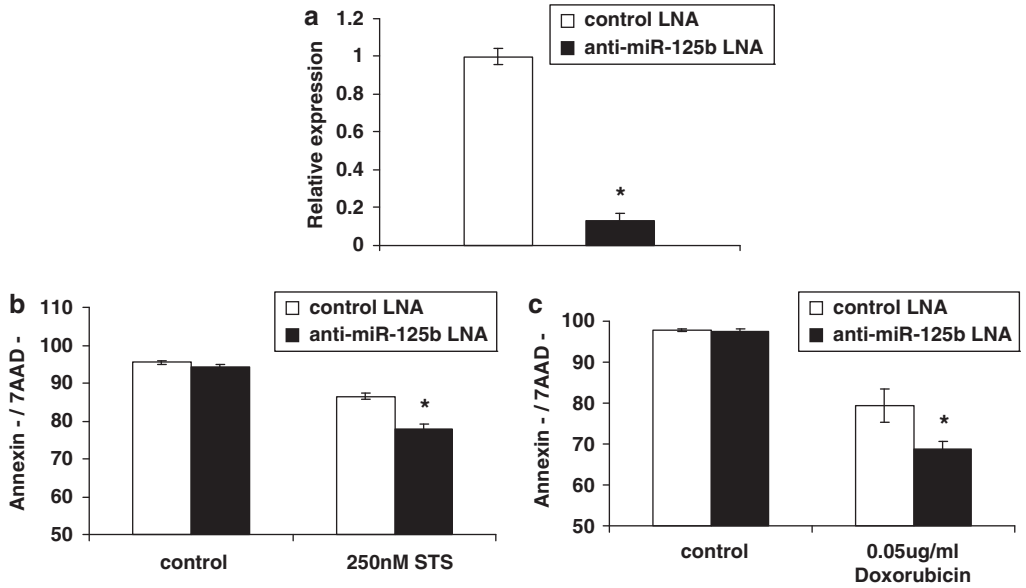

Figure 5 The endogenous *hsa-miR-125b* protects *ETV6/RUNX1* human leukemia cells from apoptosis. (**a**) qRT–PCR analysis of the knockdown efficiency of *hsa-miR-125b* in REH cells using a specific miR-125b antisense LNA oligo or a control oligo; *y* axis represents relative expression to the internal control RNU43. Eight independent experiments are shown. (**b, c**) Knockdown of miR-125b in REH cells increases the sensitivity to Staurosporine or Doxorubicin treatments, respectively, as indicated by decreased percentage of living cells (Annexin/7AAD negative). Data are means ± s.d. of four independent experiments for each treatment. *$P<0.05$ as calculated by *T*-test.

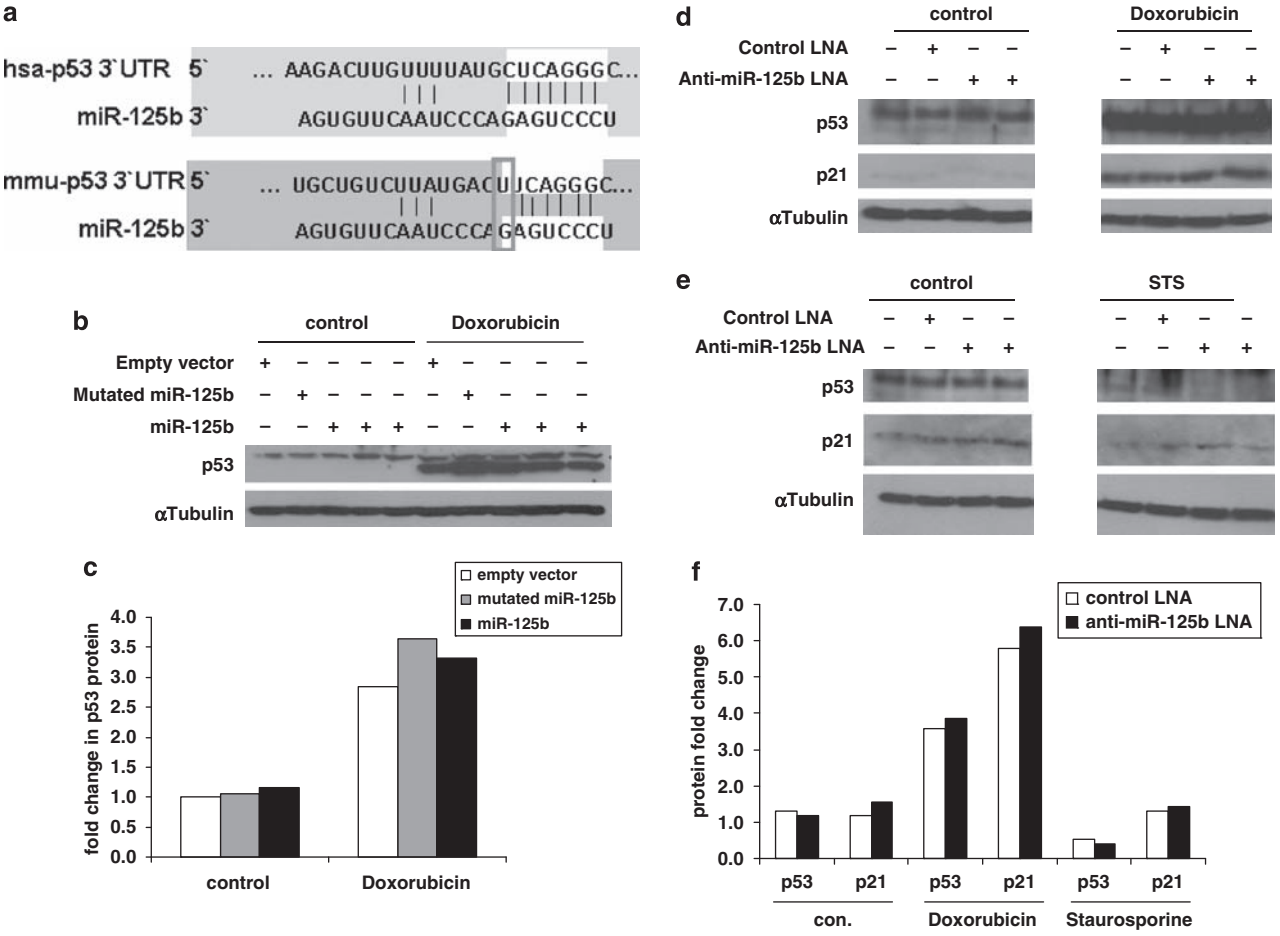

Figure 6 P53 is not a target of miR-125b in Ba/F3 and REH cells. (**a**) MiR-125b-binding site in the 3′UTR of the human and mouse p53 as predicted by TargetScan. Marked in white is the 'seed' area of miR-125b, also marked is the single different nucleotide in the potential miR-125b-binding site in the 3′UTR of the human and mouse p53. (**b**) Overexpression of miR-125b in mouse Ba/F3 cells does not down-regulate *endogenous* p53 levels. A representative western blot of *endogenous* p53 in Ba/F3 cells transduced with either the empty vector or the vector expressing mutated miR-125b or miR-125b. The cells were treated for 4 h with 0.2 μg/ml Doxorubicin. (**c**) Quantification of p53 protein level determined by western blot using the ImageJ software, normalized to αTubulin and presented as fold change relative to the level of p53 in Ba/F3 cells transduced with an empty vector. (**d**, **e**) *Endogenous* human p53 and p21 level after *endogenous* miR-125b knockdown in the human REH cells. After the knockdown, the cells were treated with either 0.05 μg/ml Doxorubicin (**d**) or Staurosporine (**e**). (**f**) Quantification of p53 and p21 protein levels determined by western blot using the ImageJ software, normalized to αTubulin level and presented as fold change relative to p53 or p21 levels, respectively, in REH cells transfected with a control LNA oligo.

oncogenic events that promote the progression of an *ETV6/RUNX1* preleukemic clone into a frank leukemia. The observation that the *hsa-mir-125b-2* cluster is not regulated by the *ETV6/RUNX1* fusion protein, suggests that its over-expression might be such an independent progression event.

MiRNAs have been shown to be dysregulated in cancer in tissue and cancer type-specific patterns. Although one miRNA acts as an oncogene in one type of cancer, the same miRNA serves as a tumor suppressor in another. In breast cancer, miR-125b has been found to suppress *ERBB2 (HER2)* and *ERBB3 (HER3)* expression[26] and thus functions as tumor suppressor. Conversely, a recent study suggested that miR-125b enhances growth and survival of prostate cancer and glioma cells.[12,27] To test the hypothesis that the increased expression of *mir-125b-2* in *ETV6/RUNX1* leukemia has a similar pro-survival function in hematopoietic cells, we used the IL-3-dependent pro-B Ba/F3 cells. These cells are considered as a standard screening tool to identify leukemia kinase oncogenes.[25,32–34] Functional analysis of *hsa-mir-125b-2* in Ba/F3 cells showed that this miRNA conferred growth factor independence by blocking apoptosis induced by IL3 withdrawal through delayed activation of caspase 3.

Cytokines such as IL3 promote cell survival through activation of JAK/STAT and related signal transduction pathways.[35–38] The function of mir-125b in protecting from death caused by silencing of pro-survival kinase regulated pathways is shown by the relative resistance it endowed Ba/F3 cells to three kinase inhibitors in decreasing specificity from JAK inhibitor 1 to Staurosporine. However, the anti-apoptotic effect of miR-125b is not limited to kinases as modest, but consistent and statistically significant resistance to apoptosis was also conferred to cells treated by the DNA damaging drug Doxorubicin. The anti-apoptotic phenotype was not an artifact of miRNA overexpression in Ba/F3 cells. Its relevance to *ETV6/RUNX1* leukemias is suggested by the sensitization to apoptosis induced by Staurosporine and Doxorubicin after knockdown of the endogenous miR-125b in REH cells.

Like most of the miRNAs, the precise targets of miR-125b are unknown. MiRNAs are believed to regulate many targets and currently bioinformatic target prediction algorithms are rather

limited.[39] A recent study suggested that miR-125b reduces the level of the pro-apoptotic protein BAK1,[27] thus blocking apoptosis. Though, we have not seen any reduction in BAK1 in either Ba/F3 or HEK293T cells overexpressing *hsa-mir-125b-2* (data not shown). We also have not detected any observable changes in several related pro-apoptotic proteins including BIM, BID, BAX and MCL-1 (data not shown). The recent report showing that p53 is negatively regulated by miR-125b[15] is intriguing as p53 is a major regulator of apoptosis in response to a variety of stresses including Doxorubicin. However, our experiments do not support that report. Similar to the observations by Le *et al*., and consistent with the lack of conservation of the miR-125b seed region in the 3′UTR region of mouse p53, we have not seen any alteration of the mouse p53 protein levels in Ba/F3 cells overexpressing miR-125b. Moreover, the very efficient knockdown conferred by specific LNA oligos in REH cells did not lead to any alterations in the human p53 levels or its target p21 before or after treatment with Doxorubicin. Thus, at least in these hematopoietic cells, p53 is not regulated by miR-25b.

Two provoking recent proteomic studies show that single miRNAs induce very small alterations in a large number of proteins and affect functional pathways by acting as sensitive 'Rheostats'.[40,41] Although such small alterations putatively induced by miR-125b in proteins that regulate cell survival may not be significant under steady-state conditions, they could provide growth advantage during periods of growth factor deprivation.

The pathogenesis of *ETV6/RUNX1* leukemias has been a mystery. *In vivo* modeling of *ETV6/RUNX1* leukemias by many laboratories has been proven experimentally challenging and resulting at most in a preleukemic condition.[31] A recent analysis of a 'knock-in' mouse model in which *ETV6/RUNX1* was expressed from the ETV6 promoter has shown an increase in hematopoietic stem cells. Intriguingly, the hematopoietic stem cells expressing the fusion protein were markedly more sensitive to apoptosis induced by cytokine withdrawal.[42] We propose that *hsa-mir-125b-2* and possibly the two other miRNAs in this cluster may collaborate with *ETV6/RUNX1* in the leukemogenic process by providing survival advantage under growth inhibitory conditions. It would be interesting to test this hypothesis in that mouse model.

Conflict of interest

The authors declare no conflict of interest.

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TEL/AML1-positive patients lacking TEL exon 5 resemble canonical TEL/AML1 cases: central region of TEL is not essential for TEL/AML1 driven leukemogenesis.

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Abstract

Background: The TEL/AML1 fusion gene which represents the most frequent genetic abnormality in childhood ALL, usually results from genomic breakpoints in TEL intron 5 and AML1 intron 1 or 2. At the protein level the helix-loop-helix domain and exon 5-coded central region of TEL are typically fused to almost entire AML1 including DNA binding domain.
Procedure: We identified two ALL patients with genomic breakpoints within TEL intron 4 resulting in variant TEL/AML1 fusion lacking the TEL exon 5-coded central region. This region was supposed to play an important role in TEL/AML1 function, particularly in TEL/AML1-mediated transcriptional repression of AML1 targets. We aimed at investigating the impact of the loss of this region on disease behavior and TEL/AML1 function. We compared clinical and biological characteristics, treatment response and outcome of the variant vs. classical TEL/AML1 cases, analyzed genome wide gene expression profiles and performed reporter gene assay.
Results: No distinct differences between variant and classical TEL/AML1 cases were observed including gene expression profiling and detailed immunophenotyping. By using reporter gene assay we showed that the loss of the central region does not influence the TEL/AML1-mediated transcriptional repression.
Conclusions: We had the opportunity to study leukemic characteristics in unmanipulated natural acute leukemias. The deletion of the central region did not affect the TEL/AML1-specific phenotype and comparing clinical and biological features of the variant vs. classical TEL/AML1-positive cases, we did not find any relevant differences in its characteristics. Thus, the central region seems to be dispensable for TEL/AML1 leukemogenesis.

Introduction

The TEL/AML1 (ETV6/RUNX1) fusion gene, resulting from the cryptic chromosomal translocation t(12;21)(p13;q22), is the most frequent genetic abnormality in childhood acute lymphoblastic leukemia (ALL) occurring in approximately 20-25% of B-cell precursor (BCP) ALL [1-3]. The TEL/AML1-positive cases form a relatively homogeneous ALL subgroup with typical onset of the disease in the pre-school age, low white blood cell count (WBC), characteristic immunophenotype and excellent prognosis [4,5]. Genomic breakpoints are found within the long (> 160 kB) area of AML1 introns 1 and 2, but cluster almost exclusively to the relatively short intron 5 (< 15 kB) of the TEL gene. Thus, the translocation typically fuses the TEL exon 5 to the AML1 exon 2 or 3 (classical" TEL/AML1) [6-9]. Both TEL/AML1 and the reciprocal AML1/TEL transcripts were described in leukemias carrying the TEL/AML1 fusion. However, the AML1/TEL transcript is not detected in all cases while the TEL/AML1 is expressed consistently; thus, only the TEL/AML1 is supposed to play an important (although not fully understood yet) role in leukemogenesis [6]. AML1 and TEL genes are frequently rearranged in human lymphoid and myeloid leukemias [10,11]. Both genes encode transcription factors important in hematopoiesis [12-15] and both are attributed with tumor suppressor properties [16,17].

The TEL/AML1 fusion as a single event is not sufficient to induce overt disease and further cooperating events are needed for leukemia development [18-20]. The TEL/AML1 itself induces a preleukemic state characterized by expansion of B-cell precursors with enhanced self-renewal and impaired differentiation to more mature B-cell stages [21-23]. At the protein level, the TEL/AML1 contains the helix-loop-helix (HLH) domain and central region of TEL, fused to almost entire AML1, including runt homology domain (RHD) responsible for DNA binding. Morrow et al. demonstrated that *in vit*ro TEL/AML1 preleukemic activity requires multiple functional domains: HLH domain, central repressor domain and DNA binding domain [24].

How does TEL/AML1 contribute to the pathogenesis of ALL? In general, the TEL/AML1 probably interferes with normal function of its wild type counterparts. According to the favored hypothesis TEL/AML1 acts as an aberrant transcription factor and deregulates the AML1 target genes [25]. Several *in vit*ro studies described the ability of TEL/AML1 chimeric protein to interact with corepressors and to bind to the AML1 binding sites within the promoter regions of AML1 targets and repress transcription [26-31]. This observation was further supported by the finding, that single point mutation within the DNA binding RHD domain abrogates the TEL/AML1 induced preleukemia [24]. An alternative mechanism of the TEL/AML1 interference with the AML1 function is the homodimerization of the TEL/AML1 and subsequent formation of complexes with coactivators. The resulting sequestration of the coactivators into cytoplasm finally prevents AML1-mediated transactivation [32,33]. Through the HLH domain TEL/AML1 is not only able to homodimerize but also to heterodimerize with TEL and by this inhibits the TEL-induced transcriptional repression and tumor suppressor function [34]. However, recently published data suggest that the effect of TEL/AML1 does not result from acting dominant negative over AML1, at least not in hematopoietic stem cell model [35].

Here we report two cases of TEL/AML1-positive leukemia with, so far, extremely rare genomic breakpoints within the TEL intron 4. To our knowledge only one such case has been already mentioned [1]. The TEL/AML1 protein encoded by the identified variant transcript lacks the TEL exon 5-coded amino acids corresponding to the TEL central region, which seemed to play essential role in the TEL-repressive and TEL/AML1 preleukemic activities so far [24,36]. However, we demonstrate that in our two patients this variant TEL/AML1 does not result in distinct clinical or biological features as compared to classical TEL/AML1 cases.

Moreover, utilizing reporter assay we show that loss of the TEL exon 5 coded central region within the TEL/AML1 does not hamper its ability to repress transcription from the AML1 target gene promoter. Thus, our study demonstrates that the central region of TEL seems not to be required for the TEL/AML1 (pre)leukemic activity.

Methods

Patients

Two patients (UPN916 and UPN1078) diagnosed with B-cell precursor ALL in 2005 and 2007, respectively, are reported. Both children were treated according to the ALL IC-2002 BFM protocol. This treatment protocol was used for all children (1 - 18 years) diagnosed in the Czech Republic with BCR/ABL-negative ALL between November 2002 and November 2007. We analyzed diagnostic bone marrow (BM) samples from all 305 patients diagnosed in the Czech Republic during this period for the presence of TEL/AML1 fusion by both RT-PCR and fluorescence in-situ hybridization (FISH). Moreover, 20 diagnostic bone-marrow cDNA samples from patients with TEL/AML1-positive ALL were analyzed for the expression of the TEL/AML1 transcripts missing the TEL exon 5.

All experiments were conducted according to the principles expressed in the Declaration of Helsinki. Ethical committee and Institutional review board approved the project and all samples were analyzed with written informed consent of subjects or their guardians.

PCR analysis

The TEL/AML1 fusion transcript in patients UPN916 and UPN1078 was amplified using touchdown RT-PCR with primers Tel-F (5'-ATGTCTGAGACTCCTGCTCAGTG-3') and TA-R (5'-GCGGCAACGCCTCGC3'). To detect TEL/AML1 transcripts with outspliced TEL exon 5 in 20 TEL/AML1-positive patients we amplified TEL/AML1 fusion using Tel-ex4F (5'-GGTGATGTGCTCTATGAACTCCTTCAGCATATTC -3') and TA-R primers.

The TEL/AML1 and AML1/TEL genomic fusion sites in leukemic cells of patients UPN916 and UPN1078 were identified with an asymmetric multiplex PCR and long-distance PCR.

Gene expression profiling

Genome wide gene expression was analyzed as described previously [39]. Unsupervised cluster analysis [40] was performed using gene expression profiles (GEP) of initial leukemic samples of the variant samples UPN916 and UPN1078 and those of TEL/AML1-, MLL/AF4- and BCR/ABL-positive samples published recently [41].

Cloning, Western blotting

The TEL-exon 4/AML1-exon 3 transcript was cloned into the pcDNA3.1 expression plasmid resulting in the TELΔex5/AML1-pcDNA3.1 construct. The TELΔex5/AML1-pcDNA3.1 was transfected into HeLa cell line. The TELΔex5/AML1 protein expression was checked 48 hours post transfection by Western blot.

Luciferase assay

The HeLa cells were seeded at 150.000 cells per 1 well of 24-well plate and cotransfected with 0.8μg of expression vectors (pcDNA3.1, TELΔex5/AML1-pcDNA3.1, TEL/AML1-pcDNA3.1 or AML1-pcDNA3.1), 0.75μg of luciferase reporter (GZMB-pGL3 or $GZMB^{m}$-pGL3) and 0.05μg of renilla reporter (pRL-CMV5) per well ($2cm^2$) using 2μl Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) in 600μl of serum free medium.

Firefly and renilla luciferase activity were measured 48hours post transfection by a luminometer Microplate TLX2 using Dual-Luciferase Reporter Assay System (Promega,

Madison, WI, USA) according to the manufacturer's instructions. The firefly luciferase activity was normalized to renilla luciferase activity. All experiments were performed in triplicate. Data are expressed as relative luciferase activity (RLU). The AML1- pcDNA3.1, TEL/AML1-pcDNA3.1 and GZMB-pGL3 containing partial sequence of granzyme B (GZMB) promoter with AML1 consensus binding sites were kindly provided by Dr. Anthony Ford, Institute of Cancer Research, UK.
In $GZMB^{m}$-pGL3 both AML1 consensus binding sites were mutated from TGTGGT to TGCAGT using PCR-mediated site-directed mutagenesis.

Results

Fusion transcripts analysis

Among the 305 children diagnosed in the Czech Republic between November 2002 and November 2007 the TEL/AML1 fusion gene was detected by FISH in 71 children (23%). By standardized RT-PCR screening we determined the TEL/AML1-positivity in 69/305 cases; in two FISH-positive cases (patients UPN916 and UPN1078) the RT-PCR produced negative results. As the vast majority of other standardized protocols for RT-PCR screening [42], our RT-PCR system for TEL/AML1 detection utilizes primers annealing to the TEL exon 5 and AML1 exon 3 to detect both common types of the TEL/AML1 fusion transcripts (TEL-exon 5/AML1-exon 2 and TEL-exon 5/AML1-exon 3). To test the presence of an atypical TEL/AML1 transcript in patients UPN916 and UPN1078 we performed RT-PCR with primers in other exons of TEL and AML1. Using the primers located in TEL exon 1 and AML1 exon 3 we detected specific PCR products in both patients (Figure 1A and B). The PCR products were sequenced and the analysis revealed the presence of TEL/AML1 transcripts fusing TEL-exon 4 and AML1-exon 3 in patient UPN916 (Figure1A) and TEL-exon 4 and AML1-exon 2 in patient UPN1078 (Figure 1B).
Such TEL/AML1 transcripts may result from either alternative splicing of TEL exon 5 or from genomic TEL/AML1 fusion with the breakpoint in TEL intron 4. To our knowledge, the alternative splicing of TEL exon 5 within TEL/AML1 fusion was not described yet. However, we amplified the TEL/AML1 transcripts with outspliced TEL exon 5 in 16 diagnostic bone marrow samples from 20 patients positive for the classical TEL/AML1 (data not shown). Thus, alternative splicing of TEL exon 5 from TEL/AML1 fusion is a common phenomenon. To investigate whether the transcripts in patients UPN916 and UPN1078 result from an alternative splicing of the TEL exon 5 or from genomic breakpoint within the TEL intron 4 we further analyzed reciprocal AML1/TEL fusion transcripts. Using the RT-PCR system described by Nakao et al.[38] specific products were obtained in both patients (Figure 1A and B). In patient UPN1078 sequencing revealed presence of the AML1-exon 1/TEL-exon 5 transcript pointing to the genomic breakpoint within the TEL intron 4 (Figure 1B). Surprisingly, in patient UPN916 we detected AML1-exon 2/TEL-exon 6 fusion at the cDNA level (Figure 1A). Both the TEL/AML1 and AML1/TEL transcripts of patient UPN916 lacked the TEL exon 5. Thus, the localization of the genomic breakpoint within the TEL gene (intron 4 or intron 5) could not be elucidated by the analysis of transcripts in this sample.

Genomic fusion sites analysis

Using the LD-PCR we amplified the genomic TEL/AML1 fusion in both patients. Sequencing revealed TEL-intron 4/AML1-intron 2 fusion in UPN 916 and TEL-intron 4/AML1-intron 1 fusion in UPN1078, corresponding to the transcripts TEL-exon 4/AML1-exon 3 and TEL-exon 4/AML1-exon 2, respectively (Figure 2A and C).
While the genomic DNA analysis corresponds with the RT-PCR data in UPN1078 confirming the breakpoints in the TEL intron 4 and AML1 intron 1 the data from UPN916 were still

ambiguous. To uncover whether the reciprocal AML1-exon 2/TEL-exon 6 transcript in UPN916 is a result of a breakpoint located in the TEL intron 5 or of an alternative splicing of the TEL exon 5 we further utilized the LD-PCR to locate the reciprocal AML1/TEL genomic breakpoint. The analysis confirmed the genomic fusion of AML1 intron 2 to TEL intron 5 (Figure2) revealing the loss of approximately 15kb of genomic region within the TEL gene, including the TEL exon 5 (see schematic presentation of TEL/AML1 and AML1/TEL genomic fusions and transcripts of both patients in Figure 3).
We utilized the identified genomic fusion sequences as clonotypic markers for (pre-)leukemia 'backtracking' on the archived Guthrie cards (neonatal blood spots) that were available for both patients. However, negative (and thus uninformative) results in both cases (data not shown) were obtained.

Biological and clinical features of patients with variant TEL/AML1 fusion
The two patients with the variant TEL/AML1 fusion lacking the exon 5 of TEL presented with clinical characteristics similar to and typical for other TEL/AML1-positive cases (Table 1). Deletion of the second TEL allele was shown by FISH in one of the patients (UPN 916). The immunophenotypic characteristics were typical for the TEL/AML1 subgroup as well: CD27-positivity (above 30%) and CD44-negativity (below 40%) characterize the TEL/AML1 subgroup with 100% specificity and > 85% sensitivity ([43] and our unpublished updated data) and both the patients with the variant fusion fall into this well-defined immunophenotypic subgroup (with CD27 expression > 40% and CD44 expression < 20% in both patients).
Moreover, we analyzed GEP in primary leukemic cells of both patients and compared them to those of childhood ALL samples positive for TEL/AML1, MLL/AF4 and BCR/ABL rearrangements, respectively, by performing unsupervised hierarchical cluster analysis.[41] Although analyzed in different batches, the variant samples clustered within the group of classical TEL/AML1-positive patients indicating the similarity of GEP of variant and classical TEL/AML1 positive ALL (Figure 4).
The patient UPN916 was stratified into the standard risk arm of the treatment protocol, the patient UPN1078 into the intermediate risk (on the basis of age > 6 years and WBC > 20,000 x 10^9/L). Both patients responded well to the initial prednisone pre-phase (prednisone good responders) and reached remission by the day +15 of the treatment. They are still in the first continuous complete remission with the follow-up of 43 and 22 months, respectively.

Luciferase-reporter assays revealed no functional difference between classical and variant TEL/AML1 fusion protein at the granzyme B promoter
The protein resulting from the TEL/AML1 rearrangement in patients UPN916 and UPN1078 lacks 182 amino acids encoded by the TEL exon 5 (TELΔex5/AML1 protein) corresponding to central region of TEL (see Figure 3A). Therefore, we asked whether the loss of TEL exon 5-coded amino acids might influence TEL/AML1 repressive activity. The TEL/AML1 coding sequence from UPN916 was cloned to expression plasmid. After the transfection to HeLa cell line the TELΔex5/AML1 protein expression was confirmed by Western blot showing product of smaller molecular weight compared to the classical" TEL/AML1 (Figure 5A). We compared the repressive activity of the TELΔex5/AML1 protein with the classical" TEL/AML1 protein using luciferase reporter assay with the granzyme B (GZMB) promoter established previously [27]. We found no significant differences between the two variants of the fusion protein: both TELΔex5/AML1 and classical" TEL/AML1 proteins had similar repressive activity at the GZMB promoter (Figure 5B). While AML1 expression induced major increase in the luciferase activity, both TEL/AML1 variants decreased the basal activity to the same level (66% and 63% for the TEL/AML1 and TELΔex5/AML1 proteins,

respectively). Moreover, the repressive effect of both TEL/AML1 and TELΔex5/AML1 proteins was dependent on the presence of the AML1 consensus binding sites within the GZMB promoter region - mutation of both binding motifs resulted in elimination of the repressive effect (99% and 112% of the basal activity, respectively). The transactivation by AML1 was also significantly reduced by mutation of AML1 binding sites. (However, it was not abolished completely as the AML1 binding was probably partly preserved, either to the mutated sites or to other parts of the promoter.) The repressive effect was visible also after co-transfection of TEL/AML1 and TELΔex5/AML1 proteins with wild type AML1 (repression of the AML1-induced activation to 78% and 82%, respectively; data not shown). Again, the effect of both TEL/AML1 variants was identical.

Discussion

The 305 children diagnosed with the ALL in the Czech Republic between 2005 and 2007 were routinely screened for the presence of TEL/AML1 fusion gene by both FISH and RT-PCR methods. Discrepant results (FISH positivity vs. RT-PCR negativity) were obtained in two patients. Transcript and genomic fusion site analysis showed that both leukemias express variant TEL/AML1 fusion lacking the TEL exon 5 as a result of a genomic breakpoint within the TEL intron 4. Interestingly, we found that due to an alternative splicing of the TEL exon 5 the majority of classical-TEL/AML1-positive patients (16/20, i.e. 80%) also express the variant TEL/AML1 mRNA (together with the classical transcript).

We would have failed to identify the variant TEL/AML1 fusions in our patients when having applied only one of the methods. Both patients were treated according to the ALL IC-2002 BFM protocol that does not consider TEL/AML1-positivity for risk group stratification. Thus, a false negative result would not have impeded the proper stratification. However, some treatment protocols currently use the TEL/AML1 status for treatment stratification. As discussed above, variant patients treated on these protocols would be stratified as TEL/AML1-positive or TEL/AML1-negative depending on the detection method used. From this point of view, we find the parallel screening by both methods helpful, as it enables identification of the variant fusion, which prognostic value needs to be further evaluated.

To our best knowledge, the TEL/AML1 fusion lacking TEL exon 5 was only mentioned once so far [1] (and thus could be considered extremely rare). Here, the variant transcript was described in one patient and no further analysis was performed to confirm a genomic breakpoint in TEL intron 4 instead of intron 5. Our two patients with variant TEL/AML1 fusion represent 2.8% (2/71) of TEL/AML1 cohort on ALL IC-2002 BFM protocol in the Czech Republic. The overall frequency of this aberration may be underestimated due to the fact that only single method for routine TEL/AML1 screening (either FISH or RT-PCR) is often employed for diagnostics, hampering a detection of the variant cases as discussed above.

Despite considerable effort to elucidate the TEL/AML1 function and its role in leukemogenesis, it is still not fully understood. Animal and in-vitro models showed, that TEL/AML1 as a single event is not able to confer full leukemic transformation but rather induces a preleukemic state characterized by expansion of early B-cell precursors with enhanced self-renewal and impaired differentiation to more mature B-cell stages. Employing deletion mutants, Morrow et al [24] demonstrated that the central domain of Tel is required for TEL/AML1 preleukemic activity. This is in contrast to our data describing ALLs driven by TEL/AML1 lacking this region. At the molecular level, the best described mechanism of the TEL/AML1 function is transcriptional repression of AML1 targets. The TEL/AML1 transcriptional repression is believed to be mediated through interaction with corepressors. Both, mSin3A and N-CoR corepressors, interaction partners of the wild type TEL [44,45], were shown to interact also with TEL/AML1 [29,30]. In accordance with the studies on the

wild type TEL the same regions were found to be responsible for interaction with these corepressors within the TEL/AML1: the HLH domain interacts with mSin3A [30] and the central region interacts with N-CoR [29]. The central region of TEL was further shown to recruit also SMRT corepressor [45] and finally one of the two functionally autonomous repression domains essential for the TEL-induced transcriptional repression was mapped to the exon 5-coded amino acid residues 171-215 [36]. This indicates that central region of TEL may play an important role also in the TEL/AML1-mediated transcriptional repression. To test whether the loss of central region attenuates the ability of TEL/AML1 to repress the transcription of AML1 targets we utilized the reporter gene assay. Direct target genes of AML1 (TEL/AML1) in lymphoid cells have never been identified, therefore, we used the reporter system with granzyme B promoter, that was successfully used in previous studies. We saw the same level of transcriptional repression on granzyme B promoter mediated by the variant TEL/AML1 compared to the classical TEL/AML 1. Thus the repressive ability of TEL/AML1 seems not to be attenuated by the loss of the central region in the variant fusion.

The TEL gene is frequently rearranged in leukemias and has several fusion partners. The genomic breakpoints of TEL usually occur in introns 4 and 5. Interestingly, both types of breakpoint were described in fusions with some partners (ABL1, JAK2) whereas some selectivity exists in fusions with other genes: breakpoints in intron 4 exclusively occur in fusions with PDGFRB and breakpoints in intron 5 are preferentially employed in fusions with AML1 [10]. The nature of this selectivity is unclear. In TEL/ABL1 mouse model it was demonstrated, that the ability of this fusion gene to induce CML-like disease in mouse is attenuated in the absence of the TEL exon 5 [46]. Thus although the TEL exon 5 is not essential for the TEL/ABL1 leukemogenesis its loss modulates the TEL/ABL1 functional properties and disease phenotype at least in the animal model. In another TEL fusion gene - TEL/TRKC - the presence/absence of the TEL exon 5 is associated with 2 unrelated types of cancer: TEL/TRKC including the TEL exon 5 occurs in solid tumors (e.g. congenital fibrosarcoma) while TEL/TRKC lacking the exon 5 was found in AML. This suggests that the central region of TEL possibly confers cell type specific functional properties of TEL/TRKC fusion gene [47]. Furthermore, the loss of a protein domain with interaction properties similar to the central region of TEL within the AML1/ETO fusion also has an important impact on its function. The AML1/ETO fusion gene (occurring in childhood AML) is also believed to block the AML1 function. In AML1/ETO9a, a C-terminal domain, which (correspondingly to the TEL exon 5-coded corepressor region) interacts with N-CoR/SMRT corepressors, forms complexes with histone deacetylases and deregulates AML1 targets expression, is lost. As a result, the AML1/ETO-induced inhibition of cell proliferation is abrogated which probably confers the enhanced leukemogenic potential of AML1/ETO9a in a mouse model [48].

Using a reporter gene assay, we did not observe any impact of loss of the Tel central region on the repressive TEL/AML1 transcriptional potential. We can not exclude that other TEL/AML1 functional properties could have been influenced and that the variant patients form a specific subgroup within the TEL/AML1-positive leukemias. However, here we had the opportunity to study leukemic characteristics in unmanipulated natural acute leukemias that are - in terms of defining a leukemogenic properties - superior to any animal or in-vitro model. Importantly, the deletion of the exon 5 coded central region did not affect the TEL/AML1-specific phenotype and comparing clinical and biological features of the two variant TEL/AML1-positive leukemias with the classical TEL/AML1-positive cases, we didn’t find any relevant differences in its characteristics. The same holds true for immunophenotyping and GEP. Thus, we conclude that the central region of TEL encoded by the exon 5 is dispensable for TEL/AML1 leukemogenesis.

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Figure Legends

Fig. 1. PCR products with amplified TEL/AML1 and AML1/TEL fusion transcripts on agarose gels with the length markers and transcript sequences with solid lines representing the site of fusion are shown for patient UPN 916 (**A**) and patient UPN 1078 (**B**).

Fig. 2. PCR products with amplified TEL/AML1 genomic fusion in patient UPN 916 (**A**), AML1/TEL genomic fusion in patient UPN 916(**B**) and TEL/AML1 genomic fusion in patient UPN 1078 (**C**) on agarose gels with the length markers and corresponding PCR product sequences are shown. Dashed lines border the regions with sequences that are identically found in both fusion partners.

Fig. 3. Schematic presentation of TEL/AML1 protein including in scale position of key functional domains and TEL exon 5 coded region (**A**): HLH = helix-loop-helix domain, CR = central region, RHD = runt homology domain. Schematic presentation of wt TEL and AML1 genes (**B**), genomic fusions and fusion transcripts in patients UPN 916 (**C**) and UPN 1078 (**D**): numbered boxes represent exons, numbered lines represent introns, complete coding region was analyzed in both patients; note the loss of 15kb genomic region including TEL exon 5 bordered with dashed lines in patient UPN 916.

Fig. 4. Unsupervised hierarchical cluster analysis of gene expression data in leukemic samples positive for variant-TEL/AML1, classical-TEL/AML1, MLL/AF4 and BCR/ABL rearrangements, respectively, was carried out considering those clones that had a 2-fold difference in expression from the mean on at least 6 arrays (1271 clones). Each column represents one sample, each row one clone. Gene expression is displayed as a variation in color as indicated in the left lower corner. Gray represents data that were omitted because they were not well measured.

Fig. 5. Western Blot analysis demonstrated the stable expression of the variant TEL/AML1 fusion protein (**A**): classical TEL/AML1 protein with molecular weight approximately 97 kDa was detected in nuclear lysate of TEL/AML1-positive leukemic cell line Reh; TELΔex5/AML1 with a molecular weight of approximately 75 kDa is the variant TEL/AML1 protein identified in nuclear lysates of HeLa cell line, transiently transfected with TELΔex5/AML1-pcDNA3.1 construct. Luciferase assay comparing ability of classical (TEL/AML1-pcDNA3.1) and variant (TELΔex5/AML1-pcDNA3.1) TEL/AML1 proteins to repress transcription from Granzyme B promoter (**B**): relative luciferase activity was normalized to empty vector (pcDNA3.1) and fold activation on wt Granzyme B promoter (GZMB-pGL3) and Granzyme B promoter with mutated AML1 binding sites ($GZMB^{m}$-pGL3) is shown.

Supplemental Appendix

Sample processing, nucleic acids isolation, reverse transcription
Mononuclear cells from diagnostic bone marrow samples were isolated by Ficoll-Paque (density 1.077g/mL; Pharmacia, Uppsala, Sweden) density centrifugation. Aliquoted cells were stored at –80°C prior to the DNA/RNA extraction.
Genomic DNA was isolated by the QIAamp® DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany).
Total RNA was extracted using a modification of the method described by Chomczynski and Sacchi [37]. Total RNA was reverse transcribed into complementary DNA (cDNA) using MoMLV Reverse Transcriptase (Gibco BRL, Carlsbad, TX, USA) according to the manufacturer's instructions.

RT-PCR and analysis of PCR products
The TEL/AML1 fusion transcript in patients UPN916 and UPN1078 was amplified using touchdown RT-PCR with primers Tel-F (5'-ATGTCTGAGACTCCTGCTCAGTG-3') and TA-R (5'-GCGGCAACGCCTCGC3'). The RT-PCR was performed in a final volume of 25 µl containing 2.5µl 10x HotMaster Taq Buffer, 0.3µl HotMaster Taq DNA Polymerase (Eppendorf); 0.4µM each primer; 200µM each dNTP; 1µl template cDNA. PCR was performed on a Gene Amp PCR System 2400 (Applied Biosystems, Foster City, CA, USA) under the following conditions: 94°C for 5 minutes; 10 cycles of 94°C for 30 seconds, 68°C minus 1°C per each cycle for 20 seconds, 72°C for 1 minute; 30 cycles of 94°C for 30 seconds, 58°C for 20 seconds, 72°C for 1 minute; 72°C for 7 minutes. The AML1/TEL transcript was amplified as described previously [38]. PCR products were analyzed on agarose gel containing ethidium bromide and directly sequenced. Sequencing was performed on ABI PRISM® 3100 Avant Genetic Analyzer with BigDye™ Primer v3.0 Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences were analyzed using Chromas version 1.5 software (Technelysium, Queensland, Australia).
To detect TEL/AML1 transcripts with outspliced TEL exon 5 in 20 TEL/AML1-positive patients we amplified TEL/AML1 fusion using Tel-ex4F (5'-GGTGATGTGCTCTATGAACTCCTTCAGCATATTC -3') and TA-R primers. The RT-PCR reaction was set as described above and the same cycling conditions were used except for annealing and elongation times which were shortened to 15 seconds per each. The PCR products were analyzed on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using a DNA Series II Kit (DNA 1000 Assay) and Agilent 2100 Expert software according to the manufacturer's instructions.

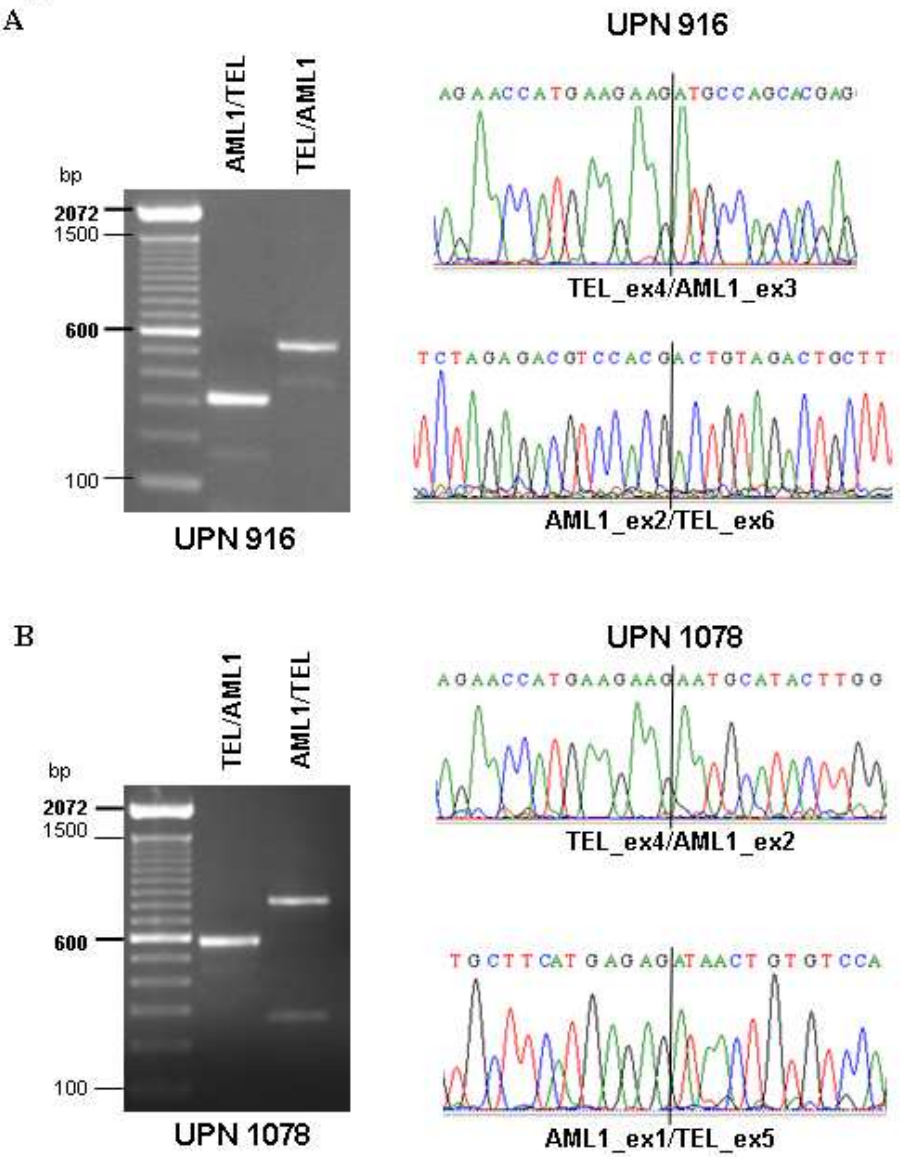
Gene expression profiling
To reduce potential bias resulting from the use of two different array batches, data were mean-centered within each of the 2 batch runs. Only those clones were considered with data evaluable on at least 70% of arrays and that had a 2-fold difference in expression from the mean on at least six arrays. The primary data and the image files are stored in and are publicly available through the Stanford Microarray Database (http://smd.stanford.edu).

Genomic fusion sites analysis
The TEL-AML1 genomic fusion sites in leukemic cells of patients UPN916 and UPN1078 were identified with an asymmetric multiplex PCR. With the primer pairs TEL.I4.F4-AML.I2.R54B (5'- TTTGTAGTGTAGGCGCCCTGAAAATAGAGC -3'+5'-CGGGAAGAACTAGCGTTCGAGGATAAAAGA-3') and TEL.E4.F1-AML.I1.R49 (5'-TTCACCATTCTTCCACCCTGGAAACTCTAT -3'+5'-

CTCAGGCATATCCAGGCTAGCAATTTTCAT-3') a 2.7 kb and 8.5kb big fragments were amplified in patients UPN 916 and UPN1078, respectively. PCR products were analyzed on agarose gel and directly sequenced as described above. The long distance PCR (LD-PCR) was performed on a GeneAmp® PCR SYSTEM 9700 (Applied Biosystems, Foster City, CA, USA), using the PCR Extender System (5 PRIME Inc. Gaitherburg, MD, USA) under following conditions: 93°C for 3 minutes; 10 cycles of 93°C for 15 seconds, 62°C for 30 seconds, 68°C for 18 minutes; 27 cycles of 93°C for 15 seconds, 62°C for 30 seconds, 68°C for 18 minutes + 20 seconds.
The AML1/TEL genomic fusion site in patient UPN916 was amplified using primers TA-F (5'-TGCATACTTGGAATGAATCCTTCTAGA-3') and ETS (5'-TTGTAGTAGTGGCGCAGGGC3')
The long distance PCR (LD-PCR) was performed on a Gene Amp PCR System 2400 (Applied Biosystems, Foster City, CA, USA) using Elongase® Enzyme Mix (Invitrogen, Carlsbad, CA, USA) under the following conditions: 92°C for 2 minutes; 40 cycles of 92°C for 30 seconds, 65° for 30 seconds, 68°C for 19 minutes 30 seconds.
PCR products were analyzed on agarose gel and directly sequenced as described above.

Cloning, Western blotting
The TEL-exon 4/AML1-exon 3 transcript from diagnostic BM cDNA of patient UPN916 was amplified using following primers: forward primer (5'-TAGAATTCCCTGATCTCTCTCGCTGTGA-3') and reverse primer (5'-TAGAATTCTCAGTAGGGCCTCCACACGGCCT-3').
The RT-PCR was performed in a volume of 50µl containing: 5µl 10x Pfx Amplification Buffer, 10µl 10x PCRx Enhancer Solution, 0.4µl Platinum® Pfx DNA Polymerase (Invitrogen, Carlsbad, CA, USA); 0.3µM of each primer; 300µM of each dNTP; 1µl template cDNA. The PCR was performed on a Gene Amp PCR System 2400 (Applied Biosystems, Foster City, CA, USA) under the following conditions: 94°C for 5 minutes; 10 cycles of 94°C for 30 seconds, 60°C minus 0,5°C per each cycle for 30 seconds, 68°C for 2 minutes; 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 2 minutes; 68°C for 7 minutes.
PCR product was subcloned into the PCR®2.1-TOPO vector using TOPO® TA Cloning® Kit (invitrogen, Carlsbad, CA, USA), the insert was cut from PCR®2.1-TOPO vector with EcoRI restriction endonuclease (Fermentas, St. Leon-Rot, Germany) and cloned into the pcDNA3.1 expression plasmid resulting in the TELΔex5/AML1-pcDNA3.1 construct.
The TELΔex5/AML1-pcDNA3.1 was transfected into HeLa cell line (Human epithelial carcinoma cell line) using Lipofectamine™ 2000 (Invitrogen**,** Carlsbad, CA, USA) according to the manufacturer's instructions. The TELΔex5/AML1 protein expression was checked 48 hours post transfection by Western blot. Nuclear lysates were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA) supplemented with Complete™ Protease Inhibitor Cocktail (Roche, Basel, Switzerland). Proteins were resolved by 8% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Nonspecific binding on the membrane was blocked with PBS containing 5% dry milk. The membrane was probed with anti TEL (N-19) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in 5% dry milk overnight. The bound antibody was detected with the appropriate secondary antibody conjugated with horseradish peroxidase and visualized using Immun-Star HRP Substrate Kit (Bio-Rad, Hercules, CA, USA).

Figure 1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100
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AML1_ex2/TEL_ex6
B
UPN 1078
TEL/AML1
AML1/TEL
bp
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1500
600
100
UPN 1078
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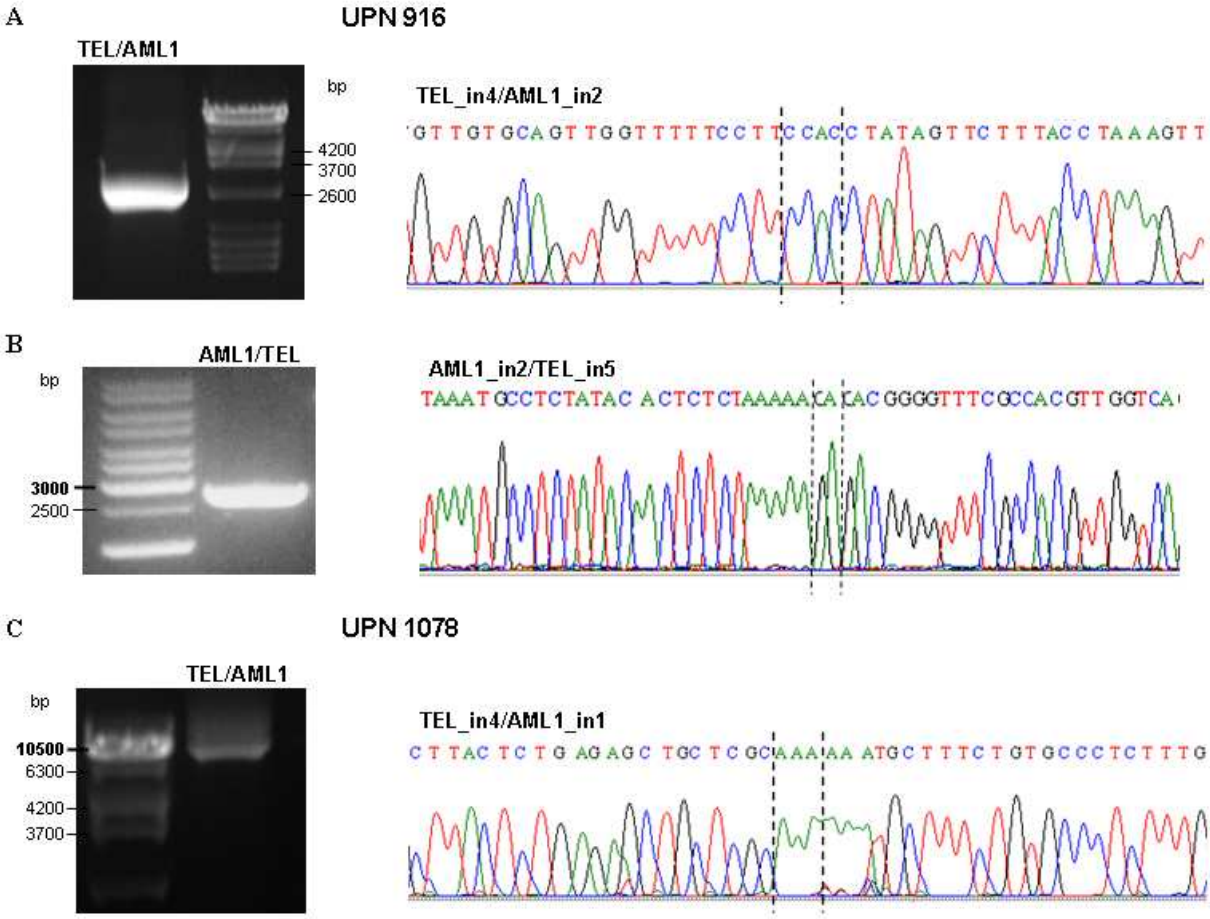
AML1_ex1/TEL_ex5

Figure 2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AML1_in2/TEL_in5
C
UPN 1078
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bp
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6300
4200
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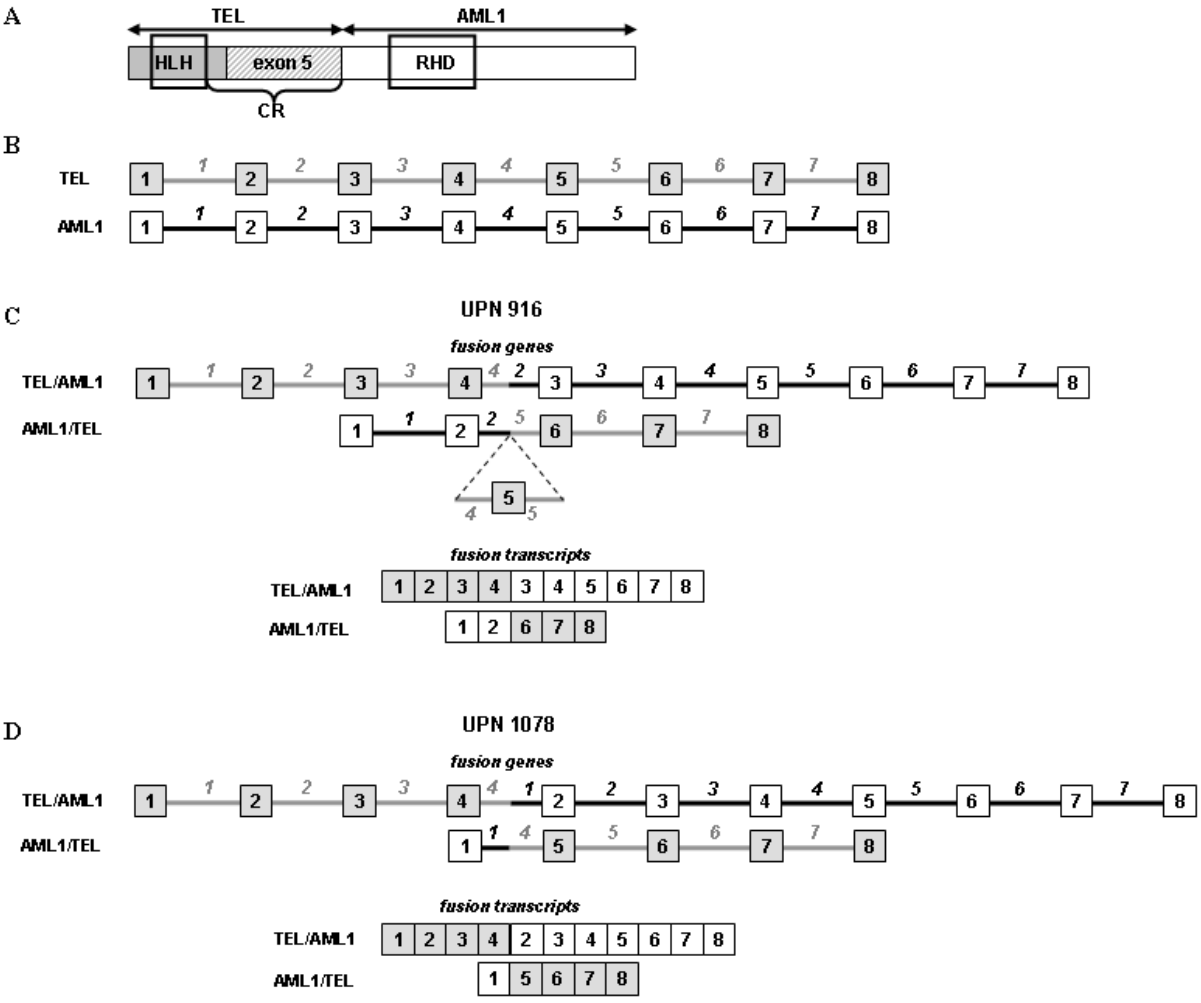
TEL_in4/AML1_in1

Figure 3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exon 5
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CR
B
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AML1
C
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fusion genes
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fusion transcripts
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fusion transcripts
TEL/AML1
AML1/TEL

Figure 4

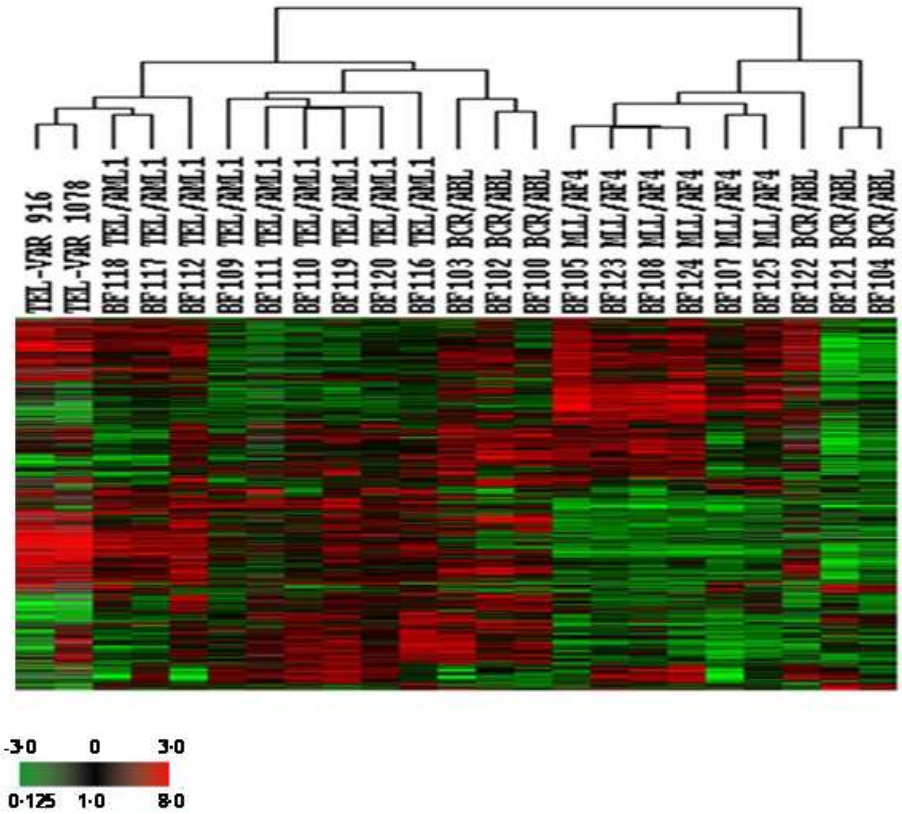
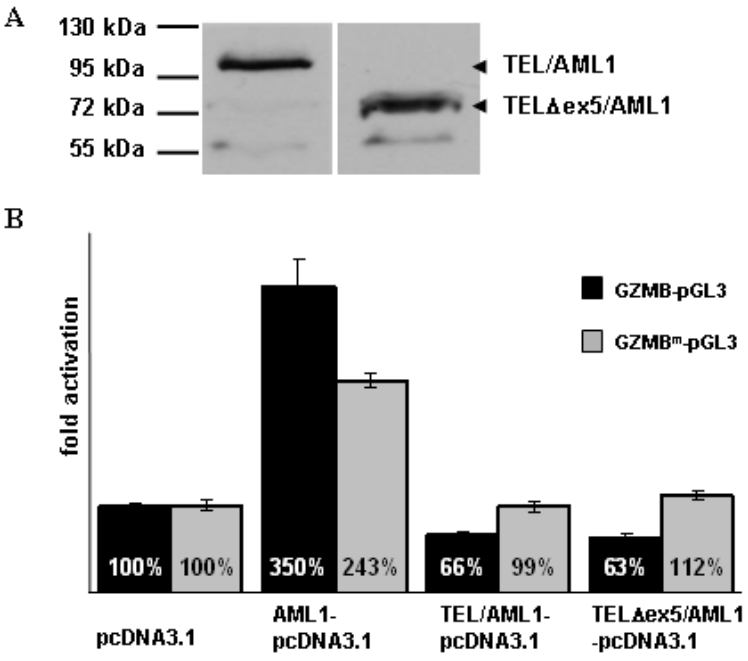


Figure 5



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ORIGINAL ARTICLE

Quantification of fusion transcript reveals a subgroup with distinct biological properties and predicts relapse in BCR/ABL-positive ALL: implications for residual disease monitoring

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Minimal residual disease (MRD) monitoring is an essential tool for risk group stratification in current treatment protocols for childhood acute lymphoblastic leukaemia (ALL). Although quantitative detection of clonal immunoglobulin (*Ig*) and T-cell receptor (*TCR*) gene rearrangements is currently considered to be the standard method, leukaemia fusion genes provide other possible targets for MRD follow-up, as already demonstrated in *TEL/AML1*-positive ALLs. We analysed and compared MRD levels quantified by *BCR/ABL* transcript detection and by the standard *Ig/TCR*-based method in 218 bone marrow specimens from 17 children with *BCR/ABL*-positive ALL. We found only a limited overall correlation of MRD levels as assessed by the two methods (correlation coefficient $R^2 = 0.64$). The correlation varied among patients from excellent ($R^2 = 0.99$) to very poor ($R^2 = 0.17$). Despite identical sensitivity of the approaches, 20% of the samples were negative by the *Ig/TCR* approach whereas positive by the *BCR/ABL* method. We show that multilineage involvement is at least partly responsible for the discrepancy. Moreover, our data demonstrate that *BCR/ABL* monitoring enables better and earlier prediction of relapse compared to the standard *Ig/TCR* methodology. We conclude that *BCR/ABL*-based MRD monitoring of childhood ALL is a clinically relevant tool and should be performed in parallel with the standard *Ig/TCR* follow-up.

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Keywords: acute lymphoblastic leukaemia; *BCR/ABL*; *Ig/TCR*; minimal residual disease; relapse

Introduction

The chromosomal translocation t(9;22) resulting in the Philadelphia chromosome (Ph) is found in 3–5% of all children with acute lymphoblastic leukaemia (ALL).[1] Two types of this chromosomal rearrangement occur in childhood ALL. In the majority of ALL cases (~90%), the chromosome 22 breakpoint is located in the minor breakpoint cluster region (*m-BCR*), and translocation to the *ABL* gene on chromosome 9 generates the *m-BCR/ABL* fusion gene, encoding a 190 kDa hybrid protein (p190). The breakpoint within the Major breakpoint cluster region (*M-BCR*) of the *BCR* gene is typical for chronic myeloid leukaemia (CML) but it is less frequent (~10% of cases) in ALL.[2,3] The latter variant results in the *M-BCR/ABL* fusion gene, encoding a 210 kDa hybrid protein, p210.

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The presence of the *BCR/ABL* fusion gene in ALL is an adverse prognostic factor associated with a high risk of therapeutic failure.[4–6] Although the prognosis of *BCR/ABL*-positive childhood ALL is generally poor, certain heterogeneity in clinical outcomes reflecting the biological heterogeneity of this disease has been reported.[4,7–9] The key components of the modern high-risk *BCR/ABL*-positive childhood ALL therapy are intensive chemotherapy (including bcr/abl kinase domain inhibitors) and haematopoietic stem-cell transplantation (HSCT).

Minimal residual disease (MRD) monitoring is used for the evaluation of treatment response, to stratify patients into risk groups on treatment protocols, and it also significantly influences clinical decisions in transplanted and off-protocol-treated patients. The highly significant prognostic value of MRD level at specific time points during treatment (both on frontline protocols and after HSCT) has been clearly documented in several studies.[10–16]

Real-time quantitative polymerase chain reaction (RQ-PCR)-based detection of clonal immunoglobulin (*Ig*) and T-cell receptor (*TCR*) gene rearrangements is the only standard method for MRD monitoring in childhood ALL. This method is applicable in virtually all (~95%) ALL patients. Use of the European BIOMED-1 and BIOMED-2 standardized protocols for target identification and the ESG-MRD-ALL (European Study Group for MRD detection in ALL—recently renamed to ESLHO, European Scientific Foundation for Laboratory Haematology and Oncology) standardized principles for MRD quantification and interpretation provides an excellent basis for the substantial reproducibility of this method.[17–19] On the other hand, due to the necessity of individual target identification and RQ-PCR optimization, this MRD method is extremely labourious and time consuming and relatively expensive. Moreover, in a subset of patients, a clonal evolution of *Ig/TCR* targets during the follow-up period can hamper the accuracy of MRD monitoring.[20–23] In Ph-positive (Ph+) ALL cases, quantitative detection of the *BCR/ABL* fusion transcript provides an alternative option for follow-up. MRD monitoring based on fusion transcript detection possesses some disadvantages,[24] but the substantial advantage of this method is the opportunity to use a universal system for fusion transcript identification and monitoring, which makes this method relatively easier, faster and cheaper than the *Ig/TCR* approach. We have previously shown that in *TEL/AML1*-positive childhood ALL, fusion transcript-based MRD monitoring is clinically relevant and closely correlates with the *Ig/TCR* approach.[25,26] The clinical relevance of fusion transcript-based MRD monitoring in *BCR/ABL*-positive childhood ALL was shown in a study by Cazzaniga *et al.*,[7] demonstrating that prospective qualitative monitoring of *BCR/ABL* by reverse transcription-PCR reveals the heterogeneity in treatment response and risk of therapy failure. However, there are no

published data regarding quantitative *BCR/ABL* follow-up in childhood ALL and, importantly, no comparison of quantitative *BCR/ABL* fusion transcript detection and standard *Ig/TCR*-based MRD monitoring has been ever provided.

In our study we aimed to determine the correlation between two independent MRD approaches in *BCR/ABL*-positive childhood ALL. We retrospectively analysed 218 samples from 17 children with Ph+ ALL and compared MRD levels by quantitative detection of *Ig/TCR* and *BCR/ABL* fusion transcript. Our data show that there is only a limited correlation between both methods and a relatively high subset of samples with discordant MRD information and that multilineage involvement is at least partly responsible for the discrepancy. We demonstrate that parallel MRD monitoring based on *BCR/ABL* transcript detection brings additional clinically relevant information to *Ig/TCR*-based follow-up and, thus, should be preformed in all children with *BCR/ABL*-positive ALL.

Patients and methods

Patients and samples

A total of 17 children (6 girls and 11 boys, age 4–17 years) with Ph+ ALL diagnosed in the Czech Republic between 1994 and 2007 were enroled in our retrospective study. Depending on the time of diagnosis, they were treated according to one of four consecutive BFM-based treatment protocols. Of the 17 children, 13 suffered from one or more relapses during the treatment (bone marrow relapse: $n=13$; isolated central nervous system relapse: $n=2$; isolated testicular relapse: $n=1$), 4 patients are in the first complete remission (CR; median follow-up of these 4 patients is 62 months). Of the children, 15 underwent HSCT (8 in the first CR, 7 in the second CR). For the MRD analysis, 218 bone marrow (BM) follow-up samples were available. The informed consent of the patients' parents or guardians was obtained in all cases.

Cell processing, DNA and RNA extraction, reverse transcription

Mononuclear cells from diagnostic and relapse BM/peripheral blood (PB) samples were isolated by Ficoll–Paque (density 1077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. Follow-up samples were processed by erythrocyte lysis. Aliquoted cells were stored at −80 °C before DNA/RNA extraction.

Genomic DNA was isolated by one of the following methods: the QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany); BioRobot EZ1 with the EZ1 DNA Blood Kit (QIAGEN GmbH); ethanol precipitation from phenol phase after RNA isolation; the salting out method.[27]

Total RNA was extracted using a modification of the method described by Chomczynski and Sacchi.[28] Total RNA was reverse transcribed into cDNA using MoMLV Reverse Transcriptase (Gibco BRL, Carlsbad, TX, USA) according to the manufacturer's instructions.

BCR/ABL *RQ-PCR detection*

BCR/ABL and β2 microglobulin (*β2M*) transcript levels in the cDNA samples were determined by RQ-PCR in the LightCycler system (Roche Diagnostic GmbH, Basel, Switzerland) according to manufacturer's instructions. The *β2M* transcript was used as an endogenous control to normalize cDNA concentration and quality as described previously.[26] The *BCR/ABL* Major and minor variants were detected by two separate systems with identical reverse primers and hydrolysation probes but two different forward primers (*ABL* reverse primer: GGTTTGGGCTTCACACCATTC; probe: 6FAM-CCATTGTGAT TATAGCCTAAGACCCGGAGCTXT-PH; *m-BCR* forward primer: GCCCAACGATGGCGAGG; *M-BCR* forward primer: TTCCGCTGACCATCAATAAGG). The PCR amplification was carried out in 1 × PCR buffer (supplied with Platinum Taq DNA polymerase) containing 200 μmol/l of each dNTP, 0.5 μmol/l of each primer, 0.2 μmol/l of probe, 5 μg of bovine serum albumin and 1 U of Platinum Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA, USA) per reaction in a final reaction volume of 20 μl. The cycling conditions were 150 s at 95 °C followed by 45 cycles at 95 °C/3 s, 62 °C/10 s and 72 °C/5 s. The standard curves were generated using serial dilutions of plasmid calibrators containing the sequences of measured transcript. The standard curve parameters were obtained automatically by the LightCycler software using calculations of the second derivative maximum. Concentrations of the target (*BCR/ABL*) and control (*β2M*) were measured in each sample, and the relative copy number of the target (target level) was calculated as a ratio between *BCR/ABL* and *β2M* concentrations. Experiments were performed in duplicate, except for 12 samples where the RQ-PCR reactions were run just once due to a lack of cDNA material. Correlation of MRD levels between the *Ig/TCR* and *BCR/ABL* approaches did not differ significantly in these 12 samples compared to the rest of the sample cohort.

Ig/TCR *detection*

Rearrangements of immunoglobulin heavy chain (*IGH*), immunoglobulin light chain kappa (*IGK*) and T-cell receptors delta (*TCRD*), gamma (*TCRG*) and beta (*TCRB*) were identified using single or multiplex PCR.[18,19] Quantification of clone-specific antigen-specific receptor gene rearrangements was performed using germline probes and reverse primers[29–33] as described previously.[13] The albumin gene was used to normalize DNA concentration and quality.[34] We monitored three *Ig/TCR* targets in 1 patient, two *Ig/TCR* targets in 13 patients and one *Ig/TCR* target in 3 patients. The highest *Ig/TCR* target level was used for MRD assessment.

MRD assessment

The ESG-MRD-ALL criteria for RQ-PCR sensitivity, quantitative range and MRD interpretation were applied to both *Ig/TCR* and *BCR/ABL* approaches.[17] The RQ-PCR sensitivity was at least 10^{-4} for *Ig/TCR* and 10 transcript copies for *BCR/ABL*. Strict cut-off values for the minimal control gene/transcript concentrations were applied to eliminate samples with inadequate DNA/cDNA quality. The main cut off (1% of 'reference value') was applied to all samples. To preclude the possibility of false-negative results we applied an even stricter second cut off (10% of 'reference value') to the samples with immeasurable *Ig/TCR* or *BCR/ABL* MRD levels. The 'reference value' for the DNA experiments was the level of albumin assessed in 200 ng of buffy coat DNA. The 'reference value' for the cDNA experiments was set individually in each patient as a *β2M* concentration assessed in the diagnostic cDNA containing 10^5 copies of *BCR/ABL* transcript; this strict approach ensures comparable sensitivity of *BCR/ABL* quantitative monitoring up to the MRD level 10^{-5} in all patients. The samples with a DNA/cDNA concentration under the cut-off values were excluded from further analysis. Thus, we refer to 17 diagnostic/relapse samples and 201 follow-up specimens. MRD in follow-up samples was expressed as a

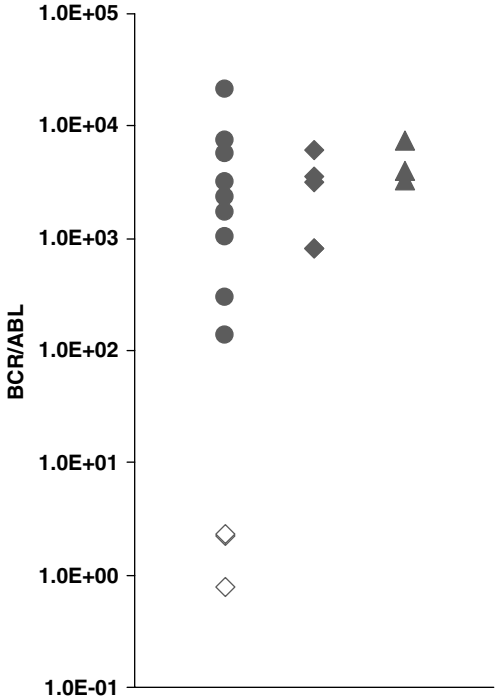

Figure 1 Quantitative *BCR/ABL* levels (logarithmic scale) in diagnostic/relapse samples. ●: *m-BCR/ABL* in diagnostic samples of patients expressing only *m-BCR/ABL*; ◇: *m-BCR/ABL* in diagnostic samples of patients expressing both *m-BCR/ABL* and *M-BCR/ABL*; ▲: *M-BCR/ABL* in diagnostic samples of patients expressing both *m-BCR/ABL* and *M-BCR/ABL*; ◆: *m-BCR/ABL* in relapse samples of patients expressing only *m-BCR/ABL*.

ratio of target level in a particular sample to target level in a diagnosis or relapse (in the 5 patients with unavailable material from diagnostic BM aspiration).

Cell sorting

In one of the recent cases with sufficient amount of material we were able to sort the BM cells from one of the follow-up samples according to the presence of surface antigens into three different subpopulations: malignant B-cell precursors (CD19+, CD20−, CD34+), non-malignant B-lineage (CD19+, CD20+, CD34−, CD13−, CD14−) and myeloid cells (CD13+ or CD14+, CD19−). The sorting was performed on high speed cell sorter FACS Aria (BD, San Jose, CA, USA).The purity of the sorted subpopulations was >99%.

Results

The BCR/ABL *level in the diagnostic/relapse samples*

We detected *m-BCR/ABL* expression in all 17 patients and *M-BCR/ABL* in 3 cases, expressing both transcripts simultaneously. The relative *m-BCR/ABL* copy number in nine diagnostic BM samples from patients expressing only the *m-BCR/ABL* transcript ranged from 134 to 20,909, spanning >1.5 logs (Figure 1). The relative *M-BCR/ABL* copy number in three diagnostic samples from patients expressing both variants ranged from 3276 to 7453 spanning less than 0.5 log (Figure 1). The relative *m-BCR/ABL* copy number in diagnostic samples from these three patients ranged from 0.8 to 2.3, being on average 10^3 times lower than in patients expressing *m-BCR/ABL* only (Figure 1). White blood cell (WBC) count, age, immunophenotype and blast proportion in BM showed no relation to the initial *BCR/ABL* level (data not shown). Due to the observed interpatient differences in initial *BCR/ABL* expression, the MRD level was expressed as a ratio of the target level in a particular follow-up sample to the target level in a diagnostic sample. This approach is analogous to ESG-MRD-ALL principles, where the diagnostic sample is assigned a value of 1 (=100%).

The *M-BCR/ABL* was used for MRD assessment in the follow-up samples of all patients expressing both *BCR/ABL* variants. In five patients expressing only m-BCR/ABL, we had no material from the diagnostic BM. In these patients, we used relapse as the reference value for MRD follow-up. The relative m-BCR/ABL copy number in these five relapse samples ranged from 820 to 6040, matching the range of the diagnostic samples (Figure 1).

Correlation of BCR/ABL- *and* Ig/TCR-*based MRD*

Figure 2 shows the overall correlation between *BCR/ABL*- and *Ig/TCR*-based MRD ($R^2=0.6429$; $n=201$). In total, 70 (35%) and 84 (42%) samples were MRD-negative or -positive by both methods ('double-negative' and 'double-positive'), respectively. The double-positive samples with *Ig/TCR* and *BCR/ABL* levels within 1 log (as well as all double-negative samples) are considered to be well correlating. The quantitative levels differed by >1 log in 25 (30%) double-positive samples, being higher by *Ig/TCR* method in 8 (10%) samples and higher by the *BCR/ABL* method in 17 (20%) samples. Of the samples, 47 (23%) were positive by one method only ('single-positive'): 7 (3%) *Ig/TCR* single-positive and 40 (20%) *BCR/ABL* single-positive. The highest MRD level in the *Ig/TCR* single-positive samples did not exceed 5×10^{-4}. However, in 19 (48%) *BCR/ABL* single-positive samples, the MRD was higher than 5×10^{-4} (MRD$>5\times10^{-4}$: $n=2$; MRD$>10^{-3}$: $n=10$; MRD$>10^{-2}$: $n=4$; MRD$>10^{-1}$: $n=3$). We compared the correlation of *M-BCR/ABL* and *m-BCR/ABL* with *Ig/TCR*. Our data showed a better correlation of *Ig/TCR* with *M-BCR/ABL* than *m-BCR/ABL* ($R^2=0.8114$; $n=33$ vs $R^2=0.6103$; $n=168$).

The m-BCR/ABL *transcript is present also in* Ig/TCR-*negative non-leukaemic cells*

In one of the patients with *m-BCR/ABL* transcript and poor *BCR/ABL* vs *Ig/TCR* correlation (UPN1092) we were able to analyse one of the early follow-up samples for the presence of clonal markers in three sorted cell populations: malignant B-cell precursors, non-malignant B-lineage and myeloid cells. As expected, the levels of both *Ig/TCR* targets (*IGH* and *IGK*) and *m-BCR/ABL* found in pure malignant B-cell precursor subpopulation were higher compared to the unsorted diagnostic sample. For the comparison of subpopulations the levels in the leukaemic cells were stated as 100%. Although the *m-BCR/ABL* expression was high also in the non-malignant B-lineage and in myeloid cells (>20% and >10% of the 'leukaemic cells expression', respectively) the level of both *Ig/TCR* markers was very low in non-malignant subpopulations (0.02–0.1%) and corresponded to the level of sort purity (Figure 3). The *Ig/TCR* vs *BCR/ABL* ratio was 1.0 (malignant B-cell precursors), 0.005 (non-malignant B-lineage) and 0.004 (myeloid lineage).

The level of BCR/ABL *transcript is better and earlier predictor of relapse than* Ig/TCR

Altogether, 14 relapses with at least 1 preceding BM sample occurred during the follow-up of our cohort. We aimed to assess the value of both MRD-monitoring approaches in terms of

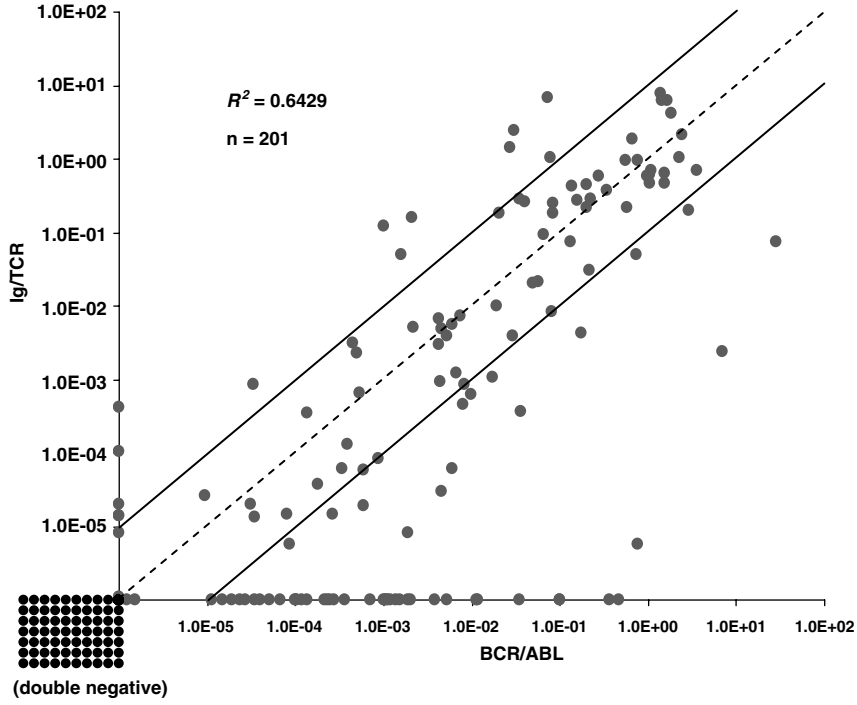

Figure 2 Correlation scatterplot of *BCR/ABL*- and *Ig/TCR*-based minimal residual disease (MRD; logarithmic scales). Dashed line shows theoretical optimal correlation, area between the full lines shows acceptable correlation with less than 1 log difference between both methods. R^2 = correlation coefficient. n = number of samples. For the mathematical analysis, positive samples with MRD below the quantitative range (otherwise referred as 'positive' only) were set numerically.

prediction of subsequent relapse and to determine whether the discordant *BCR/ABL* vs *Ig/TCR* MRD levels in BM samples have any clinical significance. The predictive value of MRD positivity in the early time points is very limited as virtually all the samples show high MRD levels. Among 35 BM samples taken within the first 12 weeks of the frontline treatment (samples used for treatment stratification of patients according to the current MRD-based childhood ALL protocols) 32 were double-positive, 1 double-negative and 2 *BCR/ABL* single-positive (both at a high level $>10^{-1}$). The level of positivity was $>10^{-3}$ by both methods in 29/32 double positive samples. Thus, to analyse predictive value of the two approaches we compared number of *BCR/ABL* and *Ig/TCR*-positive (MRD level $>10^{-5}$) samples among all BM specimens taken 6 and 12 months before relapse. The distribution of the positive samples was significantly different. Although the majority of samples preceding relapse were *BCR/ABL*-positive (17/23 and 29/45 6 and 12 months before relapse) only a minority of samples were *Ig/TCR*-positive (8/23 and 15/45, respectively). The non-equal distribution of the *BCR/ABL* and *Ig/TCR*-positive samples was statistically significant (Fisher's exact test, $P=0.017$ and $P=0.0058$ for the two time points). The difference was also substantial when only the last 1 (in total 14 samples) or the last 2 (27 samples) BM specimens before relapse were taken into account (*BCR/ABL*-positive, 10/14 and 20/27; *Ig/TCR*-positive, 4/14 and 7/27; $P=0.057$ and $P=0.0009$ for the last one and the last two samples, respectively). On the contrary, the distribution of MRD positivity in the BM samples taken more than 1 year before relapse was comparable using both approaches ($P>0.3$ for samples taken between 1 and 2 years before relapse and also for all BM samples preceding relapse by >1 year).

Discussion

We present the first study evaluating the use of quantitative *BCR/ABL*-based MRD monitoring in childhood ALL. Moreover, we show its direct correlation with the *Ig/TCR*-based approach, the only standard method for the MRD monitoring in ALL. We analysed quantitative levels of the *BCR/ABL* fusion transcript in 17 BM diagnostic/relapse samples and in 201 follow-up samples from patients with Ph+ ALL. The level of *BCR/ABL* transcript expression at the time of diagnosis of childhood ALL has not been studied so far. In a study by Yokota *et al.*,[35] the initial levels of *m-BCR/ABL* in 11 adults with ALL differed by 0.56 orders of magnitude. Substantial differences in the levels of *M-BCR/ABL* between patients in specified stages of CML were also described (>1.5 logs and >1 log in chronic phase and blast crisis, respectively)[36] as well as an increase in *M-BCR/ABL* expression level corresponding to disease progression from the chronic phase to the blast crisis of CML.[37] However, the significance of the different *BCR/ABL* expression levels between individual CML (at the same disease stage) and ALL patients remains unknown. In our cohort, the levels of m-*BCR/ABL* transcript at diagnosis varied significantly between patients, spanning >1.5 logs.

We report three patients with simultaneous expression of minor- and Major-*BCR/ABL* transcripts. In all of them, the quantitative level of *M-BCR/ABL* in the diagnostic sample was significantly higher than the level of *m-BCR/ABL* and remained dominant in all follow-up samples. Low levels of *m-BCR/ABL* transcript were also described in adult *M-BCR/ABL*-positive CML patients, where they are believed to arise through alternative splicing or missplicing of the Major variant.[38] The number of patients expressing both *BCR/ABL* variants in our

study was too small to draw any broad conclusions. However, our data show that all children with Ph+ ALL should be initially screened for both *BCR/ABL* variants, and in case they are expressed simultaneously, both transcripts should be analysed in all follow-up samples. In each particular follow-up sample, the dominant transcript should then be used for MRD assessment.

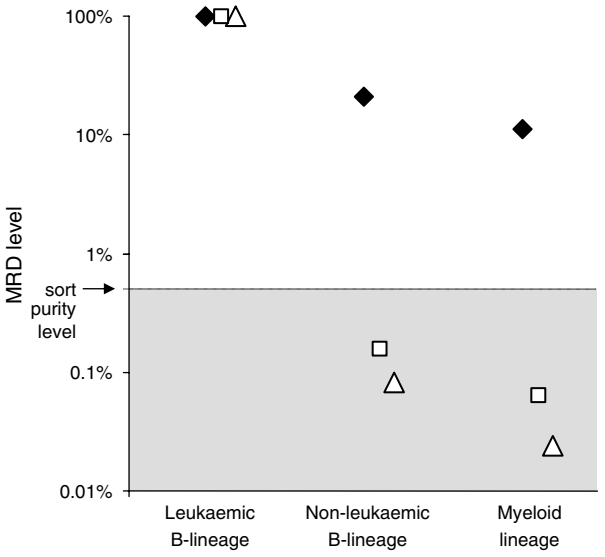

Figure 3 *Ig/TCR* and *m-BCR/ABL* levels in sorted cell populations in a follow-up sample from patient UPN1092. ♦: *m-BCR/ABL*; □: *IGH*=immunoglobulin heavy chain rearrangements; △: *IGK*=immunoglobulin light chain kappa. Purity of the sorted subpopulations is >99% (shown by 'sort purity level' dashed line).

In our study, we assessed MRD by two independent methods: quantitative detection of the *BCR/ABL* transcript at the mRNA level and quantitative detection of *Ig/TCR* rearrangements at the DNA level. We applied ESG-MRD-ALL principles for MRD quantification to both approaches in an attempt to provide the highest possible reproducibility, objectivity and comparability. In our previous study, we compared these two approaches in a biologically distinct subtype of childhood leukaemia—*TEL/AML1*-positive ALL.[25] In that study we compared MRD levels assessed by Ig/TCR monitoring and by fusion transcript-based RQ-PCR in 117 samples from TEL/AML1-positive patients. We demonstrated an excellent correlation of the two methods in the TEL/AML1-positive ALL ($R^2=0.9032$) with only <7% of samples differing by >1 log. In contrast to that study, we found only a limited overall correlation of both methods in *BCR/ABL*-positive cases ($R^2=0.6429$) using exactly the same methodology as described in the TEL/AML1 report.

The distribution of discordant samples between BCR/ABL-positive patients was non-random. Although the concordance in some patients was excellent (for example, patient UPN861: $R^2=0.99$, 13 samples), in most of the cases the correlation was unsatisfactory (the lowest concordance found in patients UPN1092: $R^2=0.17$, 10 samples; UPN4: $R^2=0.35$, 28 samples). Some patients were consistently MRD-positive by the *BCR/ABL* method and negative by the *Ig/TCR* approach during long period before relapse. In Figure 4, the levels of MRD by both methods are shown for two such patients (Figures 4a and b) and for one patient with excellent overall correlation (Figure 4c). Although our data suggested better correlation of

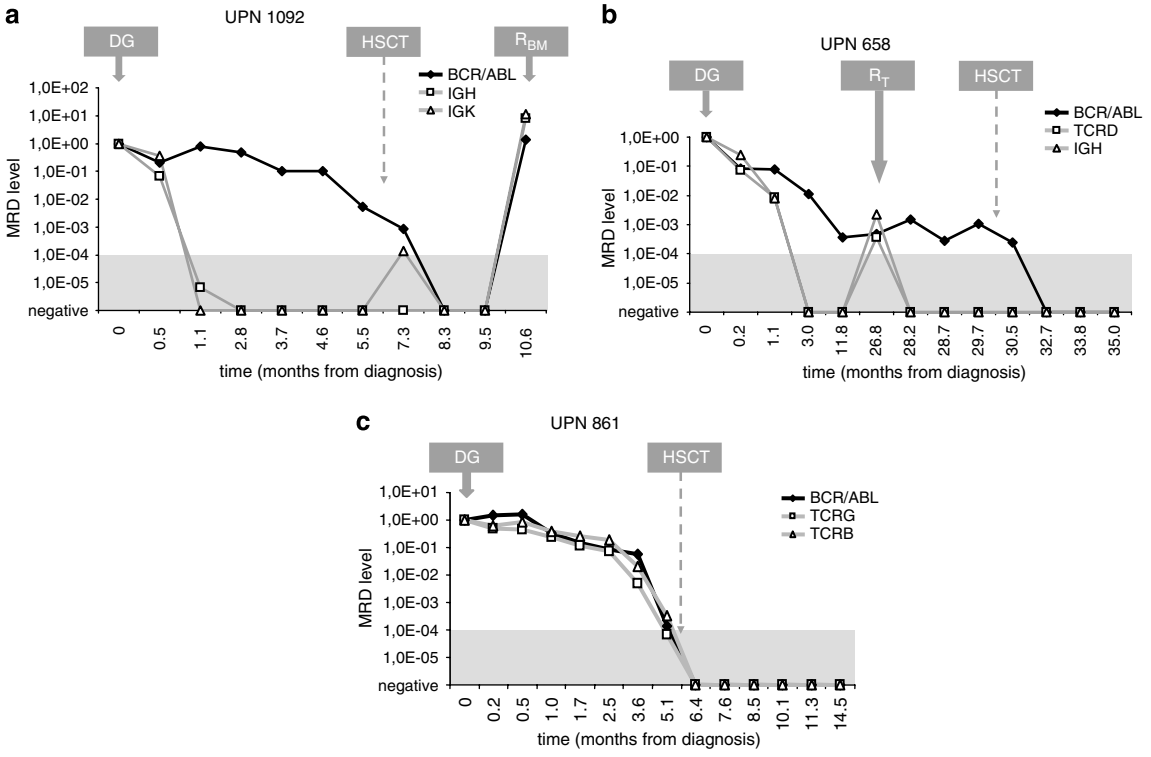

Figure 4 Minimal residual disease (MRD) follow-up of patients UPN1092 (**a**), UPN658 (**b**) and UPN861 (**c**). MRD levels of *BCR/ABL* and two *Ig/TCR* targets are shown. *IGH*=immunoglobulin heavy chain rearrangements; *IGK*=immunoglobulin light chain kappa rearrangement; *TCRD*=T-cell receptor delta rearrangement; *TCRG*=T-cell receptor gamma rearrangement; *TCRB*=T-cell receptor beta rearrangement; DG=diagnosis; R_{BM}=bone marrow relapse; R_T=testicular relapse; HSCT=haematopoietic stem cell transplantation. Grey area represents MRD levels below the quantitative range (for the graphs, positive samples with MRD below the quantitative range were set numerically).

M-BCR/ABL expression with the *Ig/TCR* approach (2/3 patients showed excellent correlation, $R^2 = 0.99$ and $R^2 = 0.93$), the low number of *M-BCR/ABL*-positive patients ($n = 3$) precluded any reasonable conclusions.

During the follow-up of our patients we also examined 138 PB samples (data not shown). We found a significant discrepancy between the correlation levels in BM vs PB samples (PB: $R^2 = 0.5389$). Our results demonstrate that leukaemic cells have lower *BCR/ABL* expression in PB compared to BM, despite being positive for clonal *Ig/TCR* markers in both compartments (data not shown). Thus, we strongly recommend preferential use of BM rather than PB specimens for *BCR/ABL*-based MRD monitoring and we report only to BM samples in this study.

Six of our patients received the specific *BCR/ABL* inhibitor, imatinib, during the follow-up period. Although this compound became a crucial component of CML treatment, publications analysing its role in the management of childhood ALL are so far extremely rare.[39] The correlation of the two analysed MRD methods is very similar in the 'imatinib' vs 'non-imatinib' BM samples and our data do not demonstrate any tendency to lower BCR/ABL expression in Ig/TCR-positive cells during the imatinib treatment. Prospective studies with imatinib in childhood ALL are in progress worldwide; these studies will definitely show whether imatinib has a specific effect on *BCR/ABL* expression and the correlation of the DNA vs mRNA-based MRD approach.

We consider the high frequency of *BCR/ABL* single positivity to be the most significant result of our study. Among all samples, 20% were *BCR/ABL*-positive and *Ig/TCR*-negative. There are several possible explanations of this discrepancy: (1) false *BCR/ABL* positivity due to sample cross-contamination, (2) false *Ig/TCR* negativity (for example, due to the subclonal *Ig/TCR* target or loss of *Ig/TCR* positivity during clonal evolution), (3) higher sensitivity of the fusion transcript-based method, (4) the presence of the *BCR/ABL* fusion in *Ig/TCR*-negative cells (for example, due to a multilineage *BCR/ABL* involvement) or (5) very high *BCR/ABL* expression in a very small cell population. Although we cannot completely rule out the reasons listed under points 1–3, we believe they are very unlikely explanations for the discrepancy between the two approaches. Sample cross-contamination is considered to be a general disadvantage of fusion transcript-based MRD monitoring, and to prevent this, we followed strict rules for sample processing. In some patients the *BCR/ABL* single positivity was present at a high level, and *BCR/ABL* was continuously single-positive in many subsequent samples processed separately, over the course of years. Thus, we consider cross-contamination to be a highly improbable explanation of this phenomenon. In a majority of patients, we monitored $\geqslant 2$ clonal *Ig/TCR* targets, and in all cases we repeated *Ig/TCR* rearrangement screening upon relapse of the disease. We found no loss of *Ig/TCR* target due to the clonal evolution in our cohort of patients. According to our experimental data, the sensitivity of both methods was identical. Moreover, in the patients with concordant results, the MRD data correlated well even at low levels.

We believe that both of the reasons listed as (4) and (5) could play a role in the overall unsatisfactory correlation of the two methods. Although multilineage/stem cell involvement has been proven in *M-BCR/ABL*-positive CML, there is still dispute about its role in *BCR/ABL*-positive ALL. The published studies usually refer to a limited number of patients and draw different conclusions, but at least in a subset of patients, multilineage involvement has been clearly demonstrated.[40–45] We were able to analyse sorted cell populations from a follow-up sample in one of the recent cases with *m-BCR/ABL* expression (UPN1092) and we conclusively show that although the clonal *Ig/TCR* rearrangements are present only in the leukaemic cells, the *BCR/ABL* fusion gene is found also in a subset of non-malignant B cells and in myeloid lineage. The data demonstrate that *BCR/ABL* fusion as the first or early hit in leukaemogenesis arises in a progenitor cell with ability to differentiate into multiple haematopoietic lineages. This also opens an important question of a boundary between ALL and lymphoid blast crisis of CML; however, speculations about optimal treatment of these cases go beyond the scope of this study.

Different levels of *BCR/ABL* expression in various Ph+ subsets have been demonstrated as already mentioned above. The data show higher *BCR/ABL* expression in less mature cells in CML.[46,47] Theoretically, these less mature cells (if also present in ALL cases) could cause a discrepancy in the *BCR/ABL* and *Ig/TCR MRD* approach by very high *BCR/ABL* expression in a limited subpopulation—perhaps potentiated by combination with *Ig/TCR* negativity.

The important question is: does *BCR/ABL* positivity in *Ig/TCR*-negative samples have clinical relevance, or is it a meaningless finding? Our data clearly show that the *BCR/ABL* positivity during the long-term follow-up is an earlier and better predictor of subsequent relapse of the disease compared to the *Ig/TCR*; thus, we believe *BCR/ABL* positivity reflects either the remaining leukaemic blast population, persistence of vulnerable cells with a significant leukaemic potential (leukaemic stem cells?) or—most likely—combination of both.

In conclusion, we strongly recommend monitoring of both MRD targets (*BCR/ABL* and *Ig/TCR*) in all Ph+ ALLs. The higher MRD level—obtained by either approach—should then be considered clinically relevant.

Acknowledgements

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Acute leukaemias with *TEL/ABL* (*ETV6/ABL1*) fusion: poor prognosis and prenatal origin

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Short title: *TEL/ABL*-positive leukaemias

Abstract

The *TEL/ABL* (*ETV6/ABL1*) fusion gene is a rare aberration in human oncology. Only nineteen cases of *TEL/ABL*-positive haematological malignancy have been published, diagnosed with chronic myeloid leukaemia or another type of chronic myeloproliferative neoplasm, acute myeloid leukaemia or acute lymphoblastic leukaemia (ALL). The present study reports 3 new cases (aged 8 months, 5 years and 33 years) of ALL with the *TEL/ABL* fusion found by screening 392 newly diagnosed ALL patients (335 children and 57 adults). A thorough review of the literature and an analysis of all published data, including the 3 new cases, reveal poor prognosis of *TEL/ABL*-positive acute leukaemias. The course of the disease in the two paediatric patients is characterised by minimal residual disease monitoring, using quantification of both the *TEL/ABL* transcript and immunoreceptor gene rearrangements. Eosinophilia can not be confirmed as a hallmark of the *TEL/ABL*-positive disease. Scrutiny of the neonatal blood spot demonstrates that, in the child diagnosed at 5 years, the *TEL/ABL* fusion initiating the ALL originated prenatally.

Keywords: *ETV6, ABL1*, leukaemia, aetiology, prognostic factor, minimal residual disease

Introduction

The *TEL/ABL* (*ETV6/ABL1*) fusion gene, first described in 1995 (Papadopoulos et al., 1995), is thought to be a very rare aberration in human haematopoietic malignancies. A thorough search of the literature reveals 19 cases published so far, diagnosed with chronic myeloid leukaemia (CML, 9 patients) or another type of chronic myeloproliferative neoplasm (cMPN, 3 patients), acute myeloid leukaemia (AML, 4 patients) or acute lymphoblastic leukaemia (ALL, 3 patients) (Papadopoulos et al., 1995; Brunel et al., 1996; Golub et al., 1996; Andreasson et al., 1997; Van Limbergen et al., 2001; Keung et al., 2002; La Starza et al., 2002; Lin et al., 2002; O'Brien et al., 2002; Barbouti et al., 2003; Meyer-Monard et al., 2005; Tirado et al., 2005; Mozziconacci et al., 2007; Baeumler et al., 2008; Kawamata et al., 2008; Kelly et al., 2009; Nand et al., 2009). The list of cases indicates a preference of myeloid over lymphoid lineage (16 vs. 3 cases), a male to female ratio 2.6:1 and a median age of 48 years at diagnosis. The review of the literature is summarised in Table 1.
Inverse orientation of the *TEL* (located at 12p13) and *ABL* (9q34) genes requires at least three DNA breaks to form functional *TEL/ABL* fusion - this fact is probably at least partly responsible for the rareness of this aberration and for the fact that the reciprocal *ABL*/*TEL* fusion has never been described so far in parallel with the *TEL/ABL*. At the mRNA level, two types of the fusion transcript were described - the type A transcript, composed of the first 4 exons of *TEL* fused to the second exon of *ABL*, and the type B transcript, where the *TEL* exon 5 is included as well. As demonstrated in the mouse model, the ability of *TEL/ABL* to induce CML-like disease in mice is attenuated in the absence of the *TEL* exon 5, and the *TEL/ABL* variant missing this exon is defective for B-lymphoid transformation in vitro and lymphoid leukemogenesis in vivo (Million et al., 2004). Thus, although the *TEL* exon 5 is not essential for the *TEL/ABL* driven leukaemogenesis, its presence modulates the *TEL/ABL* functional properties and disease phenotype at least in the animal model.
Eosinophilia was suggested as a clinical hallmark of the *TEL/ABL*-positive leukaemia (La Starza et al., 2002) – 5/19 published case-reports do not specify the number of eosinophils; the remaining 14 cases presented eosinophilia.
The biological consequences of *TEL/ABL* show a significant similarity with the far more frequent fusion in human leukaemia, *BCR/ABL*. In vitro studies demonstrated that both aberrations lead to constitutive activation of the non-receptor tyrosine kinase ABL with similar downstream effects associated to cellular growth, survival, growth factor independence and transforming capacity (Okuda et al., 1996; Malinge et al., 2006; Pecquet et al., 2007). However, differences between the two fusions have been described, for example, in the substrate preferences (Voss et al., 2000). The similarity with the *BCR/ABL*-induced transformation is also reflected in the fact that tyrosine kinase inhibitor imatinib can inhibit the growth of *TEL/ABL*-positive cells in vitro (Carroll et al., 1997; Okuda et al., 2001), and six published case reports also suggest transient (O'Brien et al., 2002; Barbouti et al., 2003; Kelly et al., 2009) or prolonged (Tirado et al., 2005; Kawamata et al., 2008; Nand et al., 2009) treatment response of *TEL/ABL*-positive leukaemia to imatinib or second-generation kinase inhibitors.
The vast majority of infant leukaemias (diagnosed before 1 year of age) are thought to originate already during in-utero development. Studies on monozygotic twins/triplets with acute leukaemia (Ford et al., 1993; Zuna et al., 2003) and retrospective analysis of neonatal blood spots (Guthrie cards) (Gale et al., 1997) revealed that acute leukaemias are initiated prenatally also in a substantial number of older paediatric patients, often by formation of a leukaemogenic fusion gene (e.g. *TEL/AML1* or *AML1/ETO*) (Ford et al., 1993; Ford et al., 1998; Wiemels et al., 2002). Prenatal initiation of leukaemia with clinical onset delayed typically by several years clearly demonstrates the need for an additional, post-natal hit(s) in the pathogenesis of the disease.
Our study describes 3 new *TEL/ABL*-positive patients (one adult and two children) uncovered in a population-based study, all diagnosed with ALL. We show here clinical and biological features of these leukaemias, including evidence for the prenatal origin of the *TEL/ABL* fusion and the long

latency period (>5 years) before the clinical onset. Our data provide new insights into the biology of this rare but remarkable subgroup of haematological malignancies.

Materials and Methods

Patients and samples

Between November 2003 and June 2009, 392 diagnostic BM samples from patients diagnosed in the Czech Republic with ALL were screened for the presence of *TEL/AML1*, *BCR/ABL*, *MLL/AF4*, *E2A/PBX1* and *TEL/ABL* fusion genes using the multiplex RT-PCR system. The cohort was composed of 335 children (187 tested prospectively (all newly diagnosed children in the Czech Republic between December 2006 and June 2009) and 148 retrospectively (all newly diagnosed children in the Czech Republic between November 2003 and November 2006 except for 50 cases with already known *TEL/AML1*, *BCR/ABL*, *MLL/AF4* or *E2A/PBX1* positivity)) and 57 adults (all tested prospectively).

Twenty-four follow-up samples from the two *TEL/ABL*-positive paediatric patients (UPN1014 and UPN1168) were available for minimal residual disease (MRD) analysis. A Guthrie card from patient UPN1168 was obtained from the central repositories in the Czech Republic. A Guthrie card from an age-matched healthy child was used as a control for Guthrie card analyses.

The informed consent of the patient or patient's parents/guardians was obtained in all cases.

Cell processing, DNA and RNA extraction, reverse transcription

Mononuclear cells from diagnostic BM samples were isolated by Ficoll-Paque (density 1,077g/mL; Pharmacia, Uppsala, Sweden) density centrifugation. Aliquoted cells were stored at –80°C prior to DNA/RNA extraction.

Genomic DNA was isolated by one of the following methods: the QIAamp® DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany); BioRobot® EZ1 with the EZ1 DNA Blood Kit (QIAGEN GmbH, Hilden, Germany); ethanol precipitation from phenol phase after RNA isolation; the salting out method.

Total RNA was extracted using a modification of the method described by Chomczynski and Sacchi (Chomczynski et al., 1987). Total RNA was reverse transcribed into cDNA using MoMLV Reverse Transcriptase (Gibco BRL, Carlsbad, TX, USA) according to the manufacturer's instructions.

RT-PCR, cloning and sequencing

The primers and conditions used in the diagnostic multiplex RT-PCR were adapted from BIOMED-1 paper (van Dongen et al., 1999) with primers TEL-C (5'-AAGCCCATCAACCTCTCTCATC-3') located in the *TEL* exon 5 and ABL-a3-B hybridizing to the *ABL* exon 3 (5'-GTTTGGGCTTCACACCATTCC-3'). To further analyse the presence of two described variants of the *TEL/ABL* transcript, we performed the touchdown RT-PCR with the forward primer TEL-ex4-F hybridizing to the *TEL* exon 4 (5'-GTGATGTGCTCTATGAACTCCTTCAGCATATTC-3').

The PCR products were analysed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using a DNA Series II Kit (DNA 1000 Assay) and Agilent 2100 Expert software, according to the manufacturer's instructions.

The PCR products were cloned using a TOPO® TA Cloning Kit for Sequencing (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions and subsequently sequenced. Sequencing was performed on an ABI PRISM® 3100 Avant Genetic Analyzer with a BigDye™ Primer v3.0 Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences were analyzed using Chromas version 1.5 software (Technelysium, Queensland, Australia).

Conventional cytogenetics

Conventional G-banded chromosomal analysis was carried out on bone marrow samples cultured for 24 hours without stimulation, prepared using classical techniques. Where possible, G-banded

analysis was performed on a minimum of 20 metaphases. Karyotypes were described according to the International System of Human Cytogenetic Nomenclature (ISCN, 1995).

Fluorescence in situ hybridization
For the molecular cytogenetic analysis, we performed FISH with locus-specific (Locus Specific Identifier (LSI) ETV6 Break Apart (BA), LSI BCR/ABL Extra Signal (ES), LSI BCR/ABL + 9q34 Dual Fusion (DF), LSI TEL/AML1 ES), centromeric (Chromosome Enumeration Probe (CEP) 12) and subtelomeric (TelVysion 9p/9q, TelVysion 12p/12q) commercially available DNA probes (Abbott Vysis, Des Plaines, IL, USA). Cells were counterstained with DAPI (4,6-diamidino-2-phenylindole). At least 20 mitoses and 200 interphase nuclei were analyzed per hybridisation probe mix, using an AXIOPLAN 2 Imaging microscope (Zeiss) and ISIS computer analysis system (MetaSystems). Structural and/or complex chromosomal aberrations and chromosomal breakpoints were identified by multicolor FISH (mFISH) and multicolor banding (mBAND), using a 24*X*Cyte color kit and an *X*Cyte color kit, respectively (MetaSystems, Altlussheim, Germany). FISH assays were made according to manufacturer´s protocols

Array CGH
The array CGH was performed using CytoChip Focus Haematology arrays (BlueGnome, Cambridge, UK), following the manufacturer's instructions (including the dye-swap experiments in all three cases). Array scanning was performed on an Axon GenePix® Professional 4200A instrument (Molecular Devices, Sunnyvale, CA, USA). The CGH data were analysed using BlueFuse for Microarrays software (BlueGnome, Cambridge, UK).

Genomic fusion sites analysis
The *TEL/ABL* genomic fusion site in patient UPN1168 was identified with an asymmetric multiplex PCR. With the primer pair ETV6.F5-ABL1.I1.R14 (5'-GGCTTCAAGGACTGGGAGAAGTCACTGTAT -3' and 5'-GTTGAGACGTCATAAGTCAGGGACCATCTG-3') a 2.2 kb fragment was amplified. The PCR products were analysed on agarose gel and directly sequenced as described above. The long distance PCR (LD-PCR) was performed on a GeneAmp® PCR SYSTEM 9700 (Applied Biosystems, Foster City, CA, USA), using the PCR Extender System (5 PRIME Inc. Gaitherburg, MD, USA) under the following conditions: 93°C for 3 minutes; 10 cycles at 93°C for 15 seconds, 62°C for 30 seconds, and 68°C for 18 minutes; 27 cycles at 93°C for 15 seconds, 62°C for 30 seconds, and 68°C for 18 minutes + 20 seconds.

Guthrie card PCR
The following PCR primers were used for the specific detection of the *TEL/ABL1* genomic fusion: forward primer (5'-GCCCTTCTGTAGACCTCTGTAGCC-3'), reverse primer (5'-AAGTGATCCTCCTAATCCACCTCC-3'). To achieve and accurately determine the maximum PCR sensitivity, the qPCR system was optimised, using serial dilutions of patients' diagnostic DNA sample mixed with the DNA of a healthy donor. The qPCR amplification was carried out in 1xQuantiTect SYBR Green PCR Master Mix (QIAGEN GmbH, Hilden, Germany), containing 1.75µmol/L of each primer in a final reaction volume of 25µL. The cycling conditions were 15 minutes at 95°C followed by 50 cycles at 95°C/5s and 62°C/30s.
Guthrie cards were processed using INSTAGENE™ DRY BLOOD kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. Guthrie card PCR was performed using the conditions specified above in a final volume of 50µL; a segment of Guthrie card (1/8 to 1/16) was added directly to the 200µl PCR tube. It was prepared in a separate room and in a separate PCR box, and all other common anti-contamination procedures were followed to avoid any potential risk of contamination. A random Guthrie card stored for the same time in the same conditions was used as a negative control.

PCR products were analysed on an Agilent 2100 Bioanalyzer as described above. All positive products from the Guthrie card were directly sequenced (as described above) to confirm the specificity of the product.

MRD monitoring
In patients UPN1014 and UPN1168, the MRD was monitored using quantitative detection of the *TEL/ABL* transcript at the mRNA (cDNA) level and immunoglobulin (*Ig*) and T-cell receptor (*TCR*) gene rearrangements at the DNA level. For the quantitative *TEL/ABL* detection the TEL-C primer was used together with the reverse primer (ENR561) and the probe (ENP541) both located in *ABL* and described previously (Gabert et al., 2003). The *beta-2-microglobulin* gene was used to normalise the cDNA concentration and quality. Quantification of clone-specific *Ig/TCR* gene rearrangements was performed using germline probes and reverse primers as described previously (Sramkova et al., 2007). The *albumin* gene was used to normalise DNA concentration and quality.
The ESG-MRD-ALL criteria for RQ-PCR sensitivity, quantitative range and MRD interpretation were applied to both the *Ig/TCR* and *TEL/ABL* approaches (van der Velden et al., 2007; Zaliova et al., 2009). The qPCR sensitivity was at least 10^{-4} for all *Ig/TCR* targets as well as for the *TEL/ABL* transcript. MRD in follow-up samples was expressed as a ratio of target level in a particular sample to target level in the diagnostic sample.

Results

Clinical features of the TEL/ABL-positive patients
We screened 90% of all children diagnosed with ALL between November 2003 and June 2009 in the Czech Republic by multiplex RT-PCR and found two patients bearing the *TEL/ABL* fusion gene (overall incidence 0.5%). Moreover, 1/57 (1.8%) of the adults with the same diagnosis examined in our lab was also found to be positive for the *TEL/ABL* fusion. A more detailed description of the 3 positive cases follows:

Infant patient UPN1014. At the age of 8 months, the boy was diagnosed with common ALL. His bone marrow was uniformly infiltrated by lymphoblasts with L1 morphology and common ALL immunophenotype with aberrant expression of CD15 and euploid DNA content. Peripheral blood count showed 565 $x10^9$/L WBC, 89% blasts and 1% (i.e. $5.6x10^9$/L) eosinophils. He had no blasts in the cerebrospinal fluid (CSF). Conventional cytogenetics showed 46,XY,der(1)t(1;?)(p?;?)[14]/46,XY[5]; FISH analysis revealed normal configuration of the *MLL* gene. The treatment was started according to the Interfant 2006 protocol, low risk arm. He was a good prednisone responder and reached haematological remission at day +33 of the treatment. Eight months from diagnosis and 24 days after cessation of intensive chemotherapy, a very early isolated CNS relapse was diagnosed with massive infiltration of the CSF with blasts (6400/μL) while still in haematological remission in bone marrow and lacking any clinical symptoms. After intrathecal application of methotrexate, he started the ALL REZ 2002 protocol, S2 arm, and was scheduled for stem cell transplantation (SCT) (Bader et al., 2009). Before SCT he received 5 blocks of chemotherapy (F1-F2-R2-R1-R2), the last four followed by two-week intervals of imatinib mesylate (300mg/m^2/day). Out of the 48 days of peroral imatinib, the dose of the drug was reduced for 29 days (leukopaenia) and skipped once. Unrelated cord blood was transplanted 4 months after the first relapse. The second relapse of ALL with infiltration of bone marrow, testes and CNS was diagnosed on D+105 after the SCT, and the boy died 1 month later.

Paediatric patient UPN1168. The 5½ year old boy was diagnosed with common ALL, L1 morphology, euploid with aberrant expression of CD15, CD66c, CD33 and low levels of CD38 and CD34. Hyperleukocytosis in peripheral blood reached $184.1x10^9$/L with 67% of blasts; eosinophilia was present (8%, i.e. $15x10^9$/L). Three suspect blasts were present in the CSF. Conventional

cytogenetics showed a normal karyotype (46,XY[20]). He started treatment according to the Interim AIEOP BFM 2000 protocol; due to poor response to the prednisone prephase (absolute blast count D+8: 1421/mm^3), he was advanced to the high-risk arm. Complete haematological remission in bone marrow was reached at D+33. The patient received no imatinib treatment. As this report is written, the patient is still in the first complete remission, 24 months after diagnosis.

Adult patient UPN1146. The 33 year old woman first displayed leukocytosis ($45.3x10^9$/L). Two days later, her WBC spontaneously decreased to $21.8x10^9$/L, and after another 5 days (i.e. one week after the first examination) she had only mild leukocytosis (WBC $16.0x10^9$/L), still without corticoids or any other treatment. She had no eosinophilia (maximum $0.05x10^9$/L, i.e. <1%); the bone marrow aspiration showed infiltration by lymphoblasts (63%), and she was diagnosed with common ALL (CD34/CD19/CD10-positive with coexpression of CD117). The karyotype of the blasts was 46,XX,t(8;9;12)(p12;q34;p13)[22]. The patient moved away from the Czech Republic before the start of the treatment protocol, and we have no additional data on the continued course of the disease. She reached haematological remission but subsequently died 1.5 years after the diagnosis.

Molecular genetics and minimal residual disease follow-up

Multiplex RT-PCR screening revealed the "type B" *TEL/ABL* transcript (*TEL* exon 5 - *ABL* exon 2) in all three cases, suggesting a genomic breakpoint in intron 5 of *TEL* and intron 1 of *ABL*. To determine whether the "type A" transcript is also expressed, we performed RT-PCR with a forward primer in the *TEL* exon 4. All three diagnostic samples proved to be positive for the expression of both types of the *TEL/ABL* transcript. On the other hand, we did not detect the reciprocal *ABL/TEL* mRNA.

During the follow-up of the two paediatric patients, we monitored MRD using the standard method, quantifying *Ig/TCR* gene rearrangements (two unrelated immunoglobulin heavy chain (*IGH*) targets in patient UPN1168 and three targets (two unrelated immunoglobulin kappa (*IGK*) rearrangements and one *IGH* target) in patient UPN1014), and, moreover, we also quantified the levels of *TEL/ABL* transcript. Patient UPN1014 responded favourably to the initial treatment and became MRD-negative in all 4 targets at the end of induction therapy; however, at another prognostically important timepoint (before OCTADA protocol (Van der Velden et al., 2009)) his MRD rose above 10^{-4}. Despite administration of the combined chemotherapy with kinase inhibitor imatinib after the subsequent CNS relapse, he did not reach the complete MRD-negativity before the SCT and relapsed 15 weeks after the transplantation. The initial response of patient UPN1168 was slower, and he was MRD-positive in all targets (although below the quantitative range of the analysis) at the end of induction. In addition, he was still positive in 1 out of the 3 targets at week +12 of treatment. However, he became completely negative 4 weeks later after the first high risk block and remained negative thereafter.

The follow-up of the two patients is shown in the Figure 1.

Cytogenetics, FISH, array CGH

The rearrangement between chromosomes 9 and 12 was not revealed by conventional cytogenetics in the two paediatric patients, original karyotypes being 46,XY,der(1)t(1;?)(p?;?)[14]/46,XY[5] and 46,XY[20] in patients UPN1014 and UPN1168, respectively. The examination of the adult case (UPN 1146) revealed a 3 way translocation 46,XX,t(8;9;12)(p12;q34;p13)[22]. FISH with LSI BCR/ABL ES, TEL/AML1 and CEP12 probes showed insertion of part of the *ABL* gene into the 12p13 region in patients UPN1146 and UPN1168, without rearrangement of subtelomeric regions of 9q and 12p. No abnormality of the *ABL* and *TEL* genes was found by FISH in patient UPN1014 using the LSI BCR/ABL ES and TEL/AML1 probes. However, a complex aberration between chromosomes 1, 9 and 12 resulting in insertion of the subtelomeric region 12p13.3 into the 9q34 locus - probably together with the telomeric part of *TEL* - was detected using mFISH/mBAND. The complex karyotype after mFISH/mBAND experiments was concluded to be 46,XY,

der(1)inv(1)(p11p34.2)t(1;9)(p11;p21)del(1)(q41),der(9)t(9;12)(q34.3;p13.3),der(9)t(1;9)(p11;p21),der(12)t(1;9;12)(q41?;q34.3;p13.3)[14]/46,XY)[5] (Figure 2).
In all three patients, array-CGH analysis was performed. Apart from the DNA copy number changes caused by the physiological rearrangements of immunoglobulin genes (chromosome 2, Ig-kappa; chromosome 14, Ig-heavy chain gene; chromosome 22, Ig-lambda), we detected a short deletion in the CDKN2A/CDKN2B (p16/p15) locus at 9p21 in patients UPN1014 and UPN1146.

Prenatal origin of the TEL/ABL fusion in the paediatric patient
To get insight into the aetiology of *TEL/ABL*-positive ALL, we traced Guthrie cards (archived neonatal blood spots) from both paediatric cases. Unfortunately, we were not able to define the intronic *TEL/ABL* breakpoint at the genomic DNA level in the case of infant ALL (UPN1014). Thus, we analysed only the preschool boy's (UPN1168) Guthrie card for the presence of the *TEL/ABL* fusion. The sensitivity of the patient-specific system was >= 3.3×10^{-5}, i.e. 1 positive cell among ~30 000 *TEL/ABL*-negative cells. Our analysis showed that the *TEL/ABL* fusion originated prenatally as it was present (in 3/4 segments) already at birth, more than 5 years before clinical symptoms of the leukaemia. In all positive segments, the genuine *TEL/ABL* genomic fusion, identical to that found at diagnosis, was confirmed by sequencing of the PCR product. The results of the backtracking are shown in the Figure 3.

Discussion

The number of the *TEL/ABL*-positive cases published so far (22 including our three patients since the first report in 1995) suggests that this translocation is extremely rare. However, the RT-PCR targeted to this fusion transcript is only seldom performed routinely, and routine cytogenetics fail to detect the fusion in most cases. The translocation between chromosomes 9 and 12 is usually cryptic, below the sensitivity threshold of conventional karyotyping. The fusion could be revealed using FISH; however, a probe designed for the *TEL/ABL* detection is not commercially available. Thus, only a mixture of standard probes in combination with metaphasic chromosomes can give clues to unravel the fusion. Our systematic PCR screening of newly diagnosed patients with ALL revealed 3/392 positive cases, i.e. between 0.5 and 1%. This data implies that the incidence of this aberration in human haematological malignancies might be underestimated.
The *TEL/ABL* fusion gene was described in various haematological malignancies, chronic and acute, lymphoid and myeloid. Eosinophilia, typical for haematological neoplasms with aberrant activation of *PDGFRA/B* kinases, was suggested also as a hallmark of the *TEL/ABL*-positivity. Interestingly, we can not confirm a100% correlation between *TEL/ABL* and eosinophilia. Only 1 out of 3 patients described in this report displayed elevated eosinophils at diagnosis (UPN1168; 8% eosinophils, i.e. 15×10^9/L). The other child (UPN1014) had only 1% eosinophils in the peripheral blood at the beginning of the treatment; no eosinophils (<1%) were described in the differential count two days before the start of the therapy, during the first hospital admission (with leukocytosis 563.8×10^9/L). Eosinophilia is usually defined by the absolute number of eosinophils in the blood (> 0.5×10^9/L). Despite this, the patient exceeded this level due to a prominent hyperleukocytosis; we do not think this condition with a proportional increase of eosinophils (accompanied by an elevation of segments and lymphocytes) should be considered a typical eosinophilia. The adult patient (UPN1146) did not fulfil any criteria for eosinophilia (0.05×10^9/L, <1%).
Two types of the fusion transcript were described so far, differing in presence/absence of the *TEL* exon 5. Only two reports detected solely the type A transcript (lacking the *TEL* exon 5) (Papadopoulos et al., 1995; La Starza et al., 2002), while in the remaining 13 cases reporting the RT-PCR data, either solely type B transcript (6 patients) or both types (7 patients) were reported (see Table 1). It is not clear whether, in the two cases without the type B variant, this transcript was absent indeed, or whether the system used for the detection was not sensitive enough. In the original study by Papadopoulos et al. (Papadopoulos et al., 1995), the size of the type A RT-PCR product,

with the primers used in the nested approach for the transcript detection, exceeded 1200 bp in the 1st round and 1000bp in the 2nd round. Thus, this product might have been easily missed in the analysis. In the paper by La Starza et al. (La Starza et al., 2002), the RACE experiments showed the presence of *TEL* exon 5, however, the 5' splicing site of intron 5 was probably impaired by the break, and thus, exon 5 was perhaps outspliced. Information indicating whether an RT-PCR system with the forward primer in *TEL* exon 5 was used for the type B transcript detection is missing. We looked for both transcripts and in all three of our patients we detected the presence of both type A and type B variants. Taking the data together, the published results suggest that in the vast majority of (if not all) cases, the genomic breakpoints are localised in *TEL* intron 5 and *ABL* intron 2 (between exons 1a and 2), and alternative splicing produces both described transcript variants in a majority of the cases.
In two patients (UPN1146, UPN1168) the mechanism of the *TEL/ABL* fusion was probably identical - part of the 9q34 locus including the C-terminus of the *ABL* gene was inserted (in inverse orientation) into the *TEL* gene at the 12p13 locus. The mechanism of cytogenetic changes was probably more complicated in the third patient (UPN1014). Our data suggest that in this case part of the *TEL* gene was inserted into *ABL* and, moreover, both chromosomes 9 and one chromosome 1 were included in the complex rearrangement. Various additional chromosomal aberrations were described in the published case reports regarding the *TEL/ABL*-positive patients, which probably correspond with the more complicated process of the *TEL/ABL* fusion generation requiring 3 or more breakpoints. Similar aberrations to those described in our study were already shown in previous reports – a translocation involving the same locus of chromosome 8 (8p12) but with a different partner (12p13 vs. 9q34) was described by La Starza et al. (La Starza et al., 2002). The cytogenetic result of our patient UPN1014 and of the case published by Baeumler et al. (Baeumler et al., 2008) involves the same locus of chromosome 1 (1q41). Moreover, the deletion of 9p21, including the *CDKN2A/CDKN2B* region - a feature present in 2 out of 3 patients in our study (UPN1014 and UPN1168) - was previously described as well (Baeumler et al., 2008). However, more cytogenetic/molecular data need to be collected to reveal whether p15/p16 silencing is a more common mechanism of *TEL/ABL* driven leukaemogenesis.
The *TEL/ABL* fusion results in the constitutive activation of the abl tyrosine kinase. This effect is identical to the far more frequent *BCR/ABL* fusion. Notably, the immunophenotype of our two *TEL/ABL* paediatric cases resembles *BCR/ABL*-positive ALL, and these patients immunophenotypically cluster among the *BCR/ABL* cases (e.g. high expression of CD66c, CD34 and CD22; low/negative CD38 on a subpopulation of blasts). Also the adult case reported here generally fits to this "*BCR/ABL*-like" picture; however, the lack of detailed immunophenotypic data prevents a thorough analysis.
Like the *BCR/ABL*, the *TEL/ABL*-positive cells show very good in-vitro sensitivity to the kinase inhibitor imatinib as well (Carroll et al., 1997; Okuda et al., 2001). The real clinical effect of this drug in the *TEL/ABL*-positive leukaemia is, however, still to be determined, as only 7 patients (including one case from this report) received this treatment. Moreover, these patients were treated for various diseases (CML, AML, MPN, ALL) at various stages of therapy protocols and by various dosages of the drug. In all published cases, at least a temporary response to imatinib (or nilotinib) was demonstrated. However, 4 of them (including our patient UPN1014) subsequently died; in 2 cases, the last published response durations were 7 and 12 months (Tirado et al., 2005; Kawamata et al., 2008). In the last case, the imatinib treatment failed after 17 months; the patient was switched to the second generation inhibitor nilotinib and was still in remission after another 11 months (Nand et al., 2009). Thus, we can conclude that kinase inhibitors might be potent drugs in the *TEL/ABL*-positive malignancies - but an optimal treatment schedule is yet to be determined.
Also the actual prognostic impact of the *TEL/ABL* fusion is indistinct - again, different types of malignancy, different treatment protocols and highly variable age in a small cohort of positive patients preclude an accurate analysis. Despite this fact, we tried to pool all the available published data, separate chronic leukaemias from AML and ALL, and the result of this "meta-analysis" is shown in the Figure 4. The resulting picture suggests that the prognosis of *TEL/ABL*-positive acute

leukaemias is very poor with a survival rate of only 15%. In particular, four patients (including two in the present study) were children (0-5 years old), all of them diagnosed with ALL. Despite the relatively high survival rate of childhood ALL (approximately 80-90%), compared to other haematological malignancies, only 1 out of the 4 reported paediatric cases survived more than 13 months after diagnosis (as this report is written, patient UPN1168 from the present study has been in complete remission for 24 months). Thus, the *TEL/ABL* is probably a poor prognostic factor in childhood ALL and perhaps in acute leukaemias in general.

Prenatal initiation of leukaemia was already sufficiently evidenced in a substantial number of genetically defined subgroups of ALL and AML. In-utero origin of various leukaemogenic aberrations (*MLL* rearrangements, *TEL/AML1*, *AML1/ETO*, *PML/RARalpha*, *CBFbeta/MYH11* fusion genes, *NOTCH1* mutation, hyperdiploidy) was demonstrated using scrutiny of Guthrie cards or banked cord bloods in cases of paediatric leukaemia (reviewed in (Greaves et al., 2003; Burjanivova et al., 2006; Zuna et al., 2009)). We were able to prove the in-utero origin of the *TEL/ABL* fusion in a boy (UPN1168) diagnosed with ALL at the age of 5½ years. The relatively long latency period suggests that at least one secondary genetic hit was required for the overt disease in this patient. Though we suppose that *TEL/ABL* fusion occurred prenatally in the infant case (UPN1014) as well, we were not able to find the genomic breakpoint between the two genes needed to prove this hypothesis via the neonatal blood spot scrutiny. We did not find an additional aberration that could be considered the second hit in the Guthrie card-positive case using cytogenetic and molecular-cytogenetic methods. However, the deletion at the *CDKN2A/CDKN2B* region in the remaining two cases (also described previously in *TEL/ABL*-positive ALL (Baeumler et al., 2008)) suggests that loss of function in this region or in other tumour-suppressor loci (not necessarily detectable by cytogenetics) might contribute to leukaemogenesis of the primary aberration.

Interestingly, the non-translocated allele of the *TEL* gene (playing an important role in the *TEL/AML1*-positive leukaemias (Zuna et al., 2004)) does not seem to be crucial in the *TEL/ABL* pathogenesis. Its deletion/silencing was described only twice so far (Golub et al., 1996; Barbouti et al., 2003) and, correspondingly, none of the three cases from our study show any aberration of the non-translocated *TEL* allele. Taking into account all the published cases, the median age at diagnosis of all *TEL/ABL*-positive leukaemias (including the 3 from this report) is over 40 years. So far, four children with this aberration were diagnosed (including the two cases from the present study), all as ALL and all under six years of age. There are no published *TEL/ABL*-positive leukaemias in patients aged 6 to 24 years. All the adult cases are then relatively evenly distributed in the 3rd to 8th decades.

Considering the overall low number of patients, we can only speculate on two different mechanisms of the *TEL/ABL* fusion origin - childhood leukaemias with prenatally generated *TEL/ABL* fusion (possibly preferring the lymphoid phenotype of resulting leukaemia) and postnatally initiated cases with increasing prevalence in adulthood (corresponding to the *BCR/ABL*-positive leukaemias).

In this report, we present three new cases of leukaemia characterised by the *TEL/ABL* fusion gene together with a thorough review of the literature, and we integrate the new patients into already published data. For the first time, we show detailed characterisation of the course of the disease, including MRD data in two paediatric cases, and in one of them we demonstrate the prenatal origin of the *TEL/ABL* fusion.

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Figure legends:

Figure 1:

Monitoring of minimal residual disease during the course of ALL in the two paediatric patients (UPN1014 (A) and UPN1168 (B)). Filled squares (■) represent levels of the *TEL/ABL* m-RNA transcript, open symbols stand for *Ig/TCR* rearrangements quantified at the genomic DNA level. Three (*IGK-1* (△), *IGK-2* (□) and *IGH* (◇)) and two (*IGH-1* (△) and *IGH-2* (◇)) unrelated *Ig/TCR* targets were monitored in the patients UPN1014 and UPN1168, respectively. Sensitivity of all targets was 10^{-4}. For the graphical display, all the samples positive below this level (i.e. positive, not quantifiable) were assigned the level 5×10^{-5}. SCT - stem cell transplantation; IM - imatininib mesylate.

Figure 2:

The mFISH karyotype of the patient UPN1014 revealing complex chromosomal rearrangements 46,XY,der(1)inv(1)(p11p34.2)t(1;9)(p11;p21)del(1)(q41),der(9)t(9;12)(q34.3;p13.3),der(9)t(1;9)(p11;p21),der(12)t(1;9;12)(q41?;q34.3;p13.3) (A). Precise breakpoints were analysed with the "XCyte" probe kits for chromosome 1 (B), 9 (C) and 12 (D), respectively. Translocation of the 12p13.3 region into the 9q34.3 locus was proved also by FISH with subtelomeric probe TelVysion 12p/12q (E).

Figure 3:

Results of backtracking PCR with Guthrie card of patient UPN1168. The PCR products were run on Agilent 2100 Bioanalyzer. Guthrie card of patient UPN1168 (lanes 1-4) showing PCR products of the expected size (163 base pairs) in lines 1, 2 and 4. Control negative Guthrie card (lanes 5-8) and positive controls diluted to 10^{-4} (lanes 9 and 10) and 3.3×10^{-5} (lanes 11 and 12). The positive controls were analysed in a separate PCR run. M - molecular weight markers.

Figure 4:

Kaplan-Meier graph showing overall survival curves for all *TEL/ABL*-positive patients published so far, including the three cases from the present study. In some cases the time to failure had to be estimated (+/- 3 months). The patients were divided into three groups - chronic leukaemias (CML/cMPN, dotted line; cases 3-5, 8-9, 12-15, 17-19 as indicated in the Table 1) acute myeloid leukaemias (AML, dashed line; cases 2, 7, 10, 11) and acute lymphoblastic leukaemias (ALL, continuous line; cases 1, 6, 16 and 20-22).

Table 1
Review of the literature regarding the *TEL/ABL*-positive leukaemias. ALL - acute lymphoblastic leukaemia; AML - acute myeloid leukaemia; CML - chronic myeloid leukaemia; LBC - lymphoid blast crisis; MBC - myeloid blast crisis; cMPN - chronic myeloproliferative neoplasm; RAEB - refractory anaemia with excess blasts; TKI - tyrosine-kinase inhibitors; CR - complete remission; SCT - stem cell transplantation; n.r. - not reported. In the two youngest patients also the age at diagnosis in months is shown in parentheses.

Case number	Diagnosis	Gender	Age at diagnosis (years)	TKI treatment	Outcome	Eosinophilia	Type of transcript	Reference
1	ALL	F	1 (22M)	no	died	n.r.	A	Papadopoulos
2	AML-M6	M	81	no	died	n.r.	B	Golub
3	CML (atypical)	n.r.	49	no	died	yes	B	Brunel
4	CML	M	32	no	CR (>3Y)	yes	B	Andreasson
5	CML	M	59	no	died	yes	A, B	Van Limbergen
6	ALL (T-lineage)	M	4	no	died	yes	A, B	Van Limbergen
7	AML-M6 (or CML-MBC?)	M	38	yes	died	n.r.	A, B	O'Brien
8	CML	M	53	no	CR (>6Y)	yes	A, B	Lin
9	CML	F	44	no	CR (>6M)	yes	n.r.	Keung
10	AML-M2	M	29	no	CR (>20M post SCT)	yes	A	La Starza
11	AML-M1 (after RAEB)	M	48	no	died	yes	B	La Starza
12	CML-MBC	M	36	yes	died	yes	B	Barbouti
13	CML-LBC	M	72	yes	CR (>12M)	n.r.	B	Tirado
14	cMPN - myeloid sarcoma	F	65	no	died	yes	A, B	Meyer-Monard
15	cMPN	M	57	no	CR (>15Y)	yes	n.r.	Mozziconacci
16	ALL	M	30	n.r.	died	n.r.	A, B	Baeumler
17	CML	F	24	yes	CR (>7M)	yes	A, B	Kawamata
18	cMPN	F	61	yes	CR (>3Y)	yes	n.r.	Nand
19	CML (atypical)	M	79	yes	died	yes	n.r.	Kelly
20	ALL	F	33	n.r.	died	no	A, B	present study
21	ALL	M	5	no	CR (>24M)	yes	A, B	present study
22	ALL	M	0 (8M)	yes	died	no	A, B	present study

Figure 1

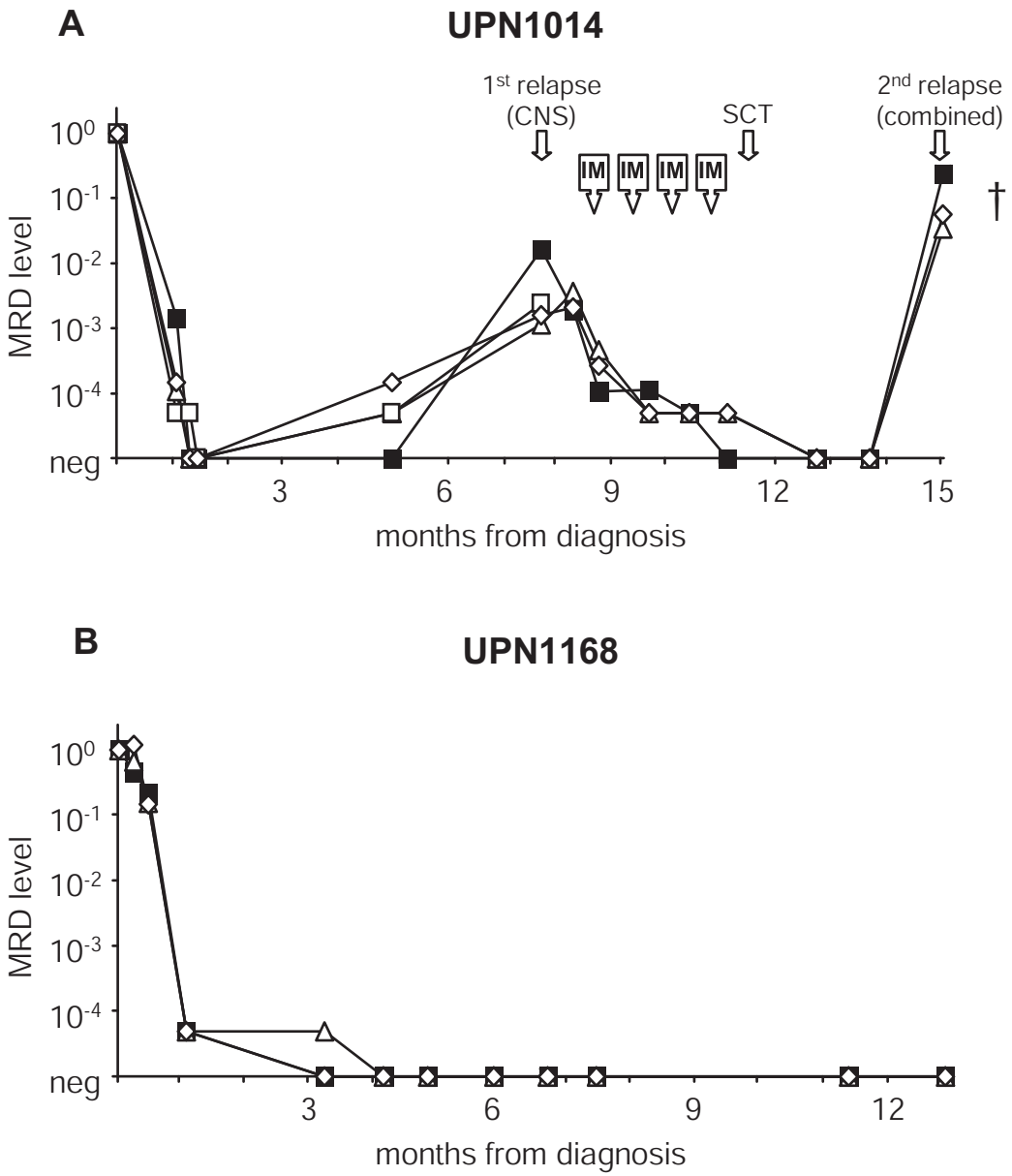

A
UPN1014
1st relapse
(CNS)
SCT
2nd relapse
(combined)
IM
IM
IM
IM
†
MRD level
10^0
10^-1
10^-2
10^-3
10^-4
neg
3
6
9
12
15
months from diagnosis
B
UPN1168
MRD level
10^0
10^-1
10^-2
10^-3
10^-4
neg
3
6
9
12
months from diagnosis

Figure 2

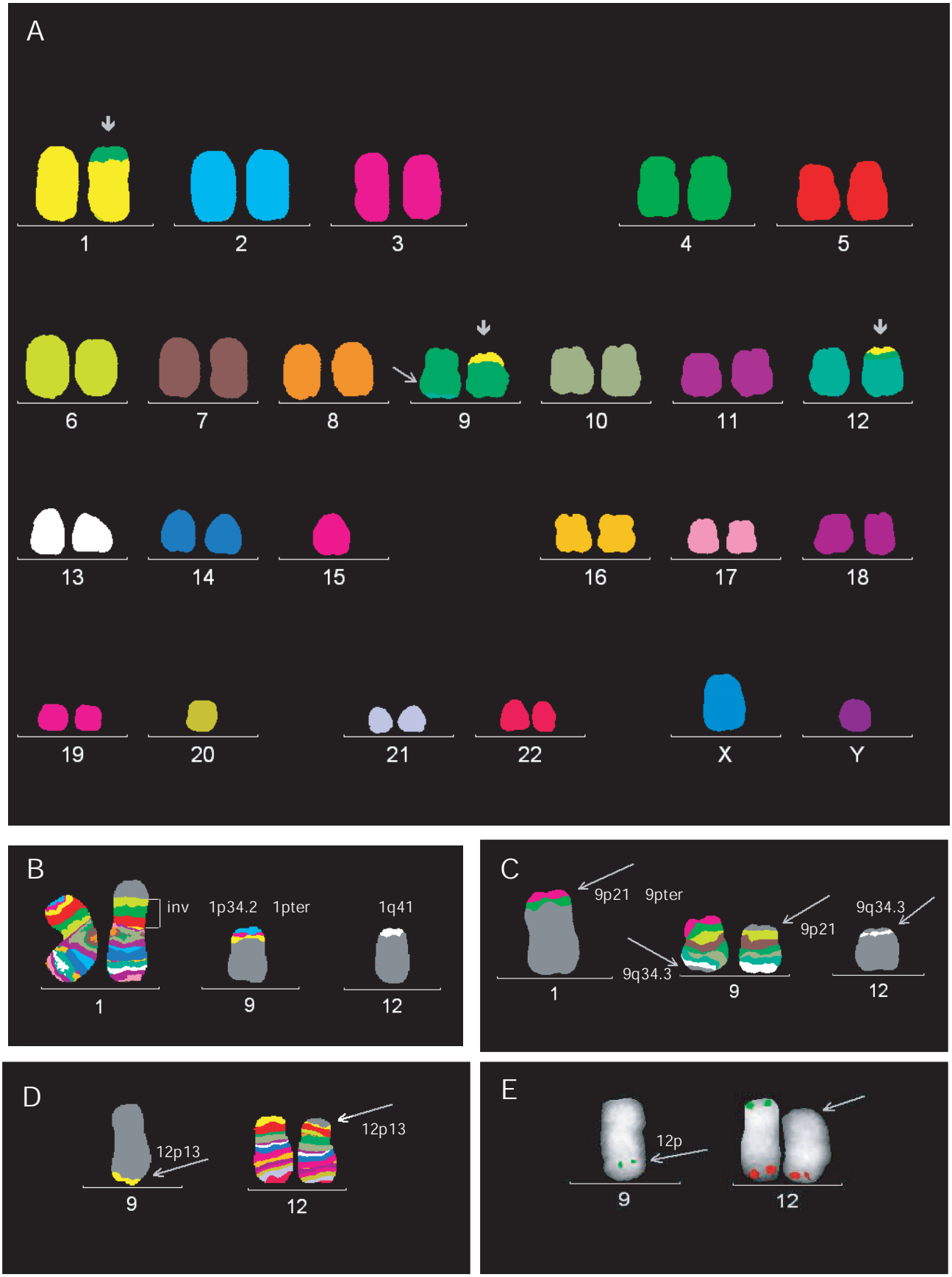

A
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y
B
inv
1p34.2
1pter
1q41
1
9
12
C
9p21
9pter
9q34.3
9p21
9q34.3
1
9
12
D
12p13
12p13
9
12
E
12p
9
12

Figure 3

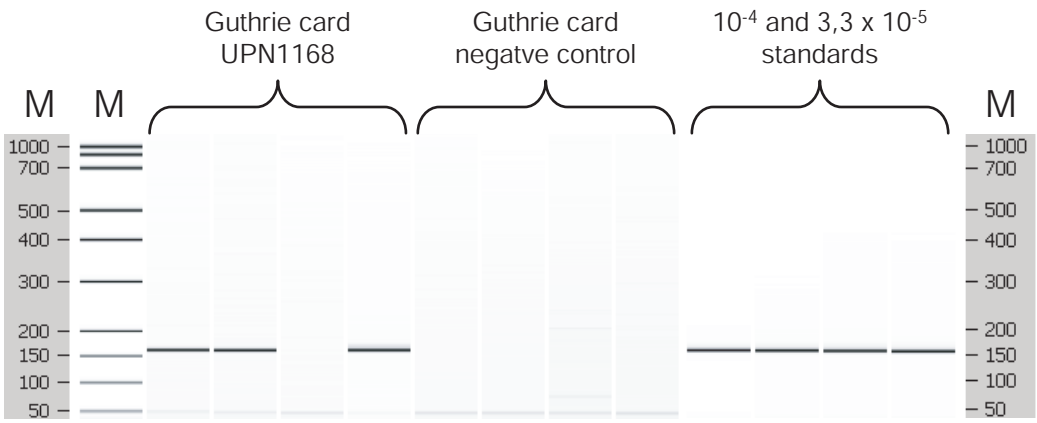
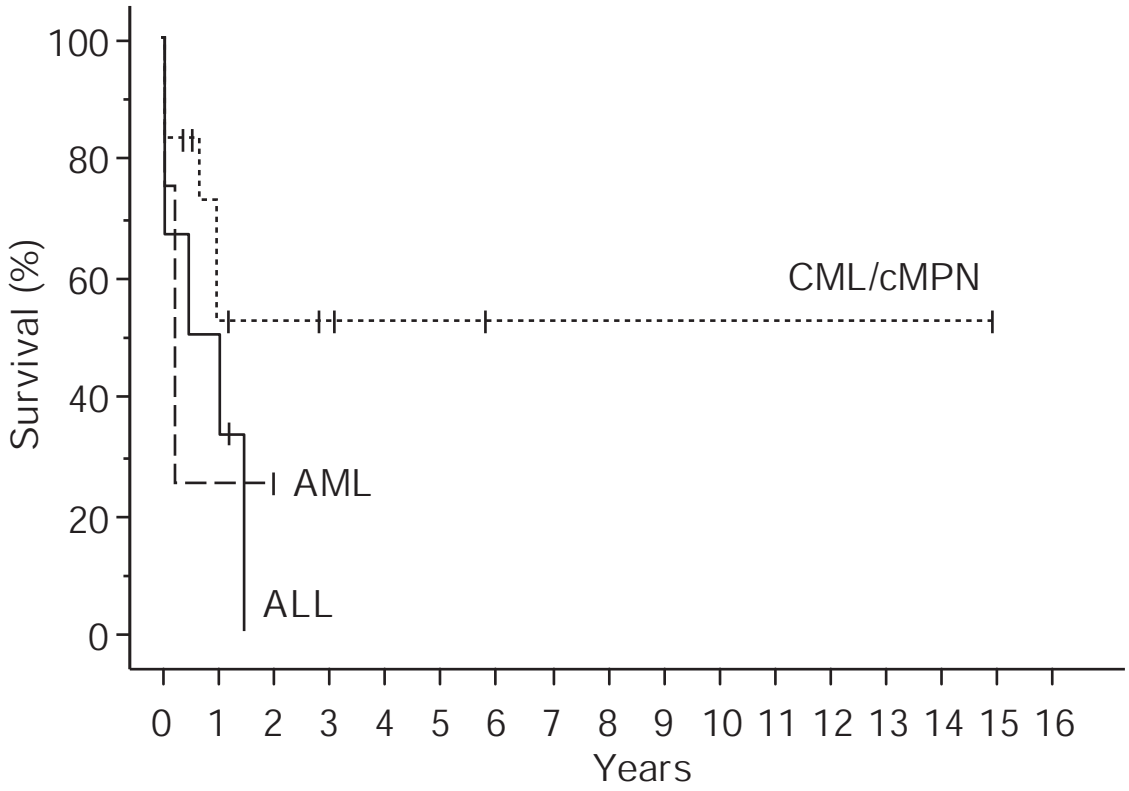

Guthrie card
UPN1168
Guthrie card
negatve control
10-4 and 3,3 x 10-5
standards
M
M
M
1000
700
500
400
300
200
150
100
50

Figure 4


100
80
60
40
20
0
Survival (%)
CML/cMPN
AML
ALL
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Years

4. Závěr a diskuze

Intenzívní výzkum během uplynulých 15 let od identifikace fúzního genu TEL/AML1 a jeho častého výskytu u dětských ALL přinesl mnoho poznatků o molekulárním mechanizmu funkce TEL/AML1 a jeho roli při vzniku leukémií. Některé klíčové otázky však zůstávají nezodpovězeny. Působí-li TEL/AML1 jako aberantní transkripční faktor, které geny a tedy které biologické procesy jsou jím deregulovány? Hraje fúzní gen TEL/AML1 roli nejen při vzniku leukémie, ale také při její perzistenci? K zodpovězení těchto otázek jsme se pokusili přispět naší prací. V *in vitro* reportérové eseji jsme potvrdili schopnost fúzního proteinu TEL/AML1 reprimovat transkripci z promotoru AML1-cílového genu a navíc jsme ukázali, že tuto funkci TEL/AML1 lze inhibovat VPA. Naše data tak potvrzují hypotézu, že TEL/AML1 by mohl v leukemických buňkách reprimovat transkripci prostřednictvím interakce s korepresory a deacetylázami histonů. Inhibici transkripčně represivní aktivity TEL/AML1 pomocí VPA jsme využili k identifikaci přímých cílů TEL/AML1 v leukemických buňkách. Potenciální cílové geny identifikované tímto přístupem se nám však v další práci nepodařilo potvrdit, neboť umlčení TEL/AML1 fúzního genu v leukemických buňkách pomocí RNAi nevedlo k žádným signifikantním změnám exprese. Přestože jsme použitím několikastupňového algoritmu směrovali hledání přímých cílů TEL/AML1 po aplikaci VPA do specifické podskupiny genů, nemůžeme vyloučit, že ke změně exprese námi identifikovaných potenciálních cílů dochází buď zprostředkovaným a nikoliv přímým působením TEL/AML1, nebo následkem působení VPA na jiné transkripční faktory a procesy. Také se domníváme, že změny exprese indukované TEL/AML1 mohou být relativně malé, mohou se týkat relativně omezeného počtu genů, nebo mohou být časově omezeny na iniciální fázi leukemogeneze. Nemusely tak být při umlčení TEL/AML1 pomocí RNAi zachyceny na jedné straně robustním, na druhé straně omezeně reproducibilním expresním profilováním pomocí DNA čipů. Cílové geny TEL/AML1 v leukemických buňkách tak nadále zůstávají neznámé. Myslíme si, že jsme však přispěli k zodpovězení druhé otázky. Ukázali jsme, že umlčení TEL/AML1 nevede k oslabení maligního potenciálu leukemických buněk a tedy že funkce tohoto fúzního genu je v již definitivních leukemických buňkách zřejmě postradatelná. Naše výsledky jsou v rozporu s nedávno

publikovanou studií, ve které po umlčení TEL/AML1 pomocí RNA interference v leukemických buňkách docházelo k buněčné smrti apoptózou [395]. Ve zmiňované práci byla vyslovena hypotéza, že na tomto jevu by se mohlo podílet snížení hladiny apoptotického inhibitoru survivinu vyvolané TEL/AML1 deplecí. Podporou této hypotézy byl nález opačného efektu, tj. zvýšení hladiny survivinu a zisk na růstových faktorech nezávislé proliferace a rezistence k apoptotickým stimulům při expresi TEL/AML1 v myší hematopoetické linii. Hodnotu této práce však snižují zásadní nedostatky. Pro umlčení TEL/AML1 byla použita siRNA, která zároveň cílí i na *wt* AML1. Dále se fenotyp myší linie exprimující TEL/AML1 významně lišil od fenotypu popsaného minimálně dvěma dalšími pracemi, v nichž TEL/AML1 závislost této linie na růstových faktorech neodstranil [35, 271]. Naše výsledky navíc zcela korespondují s myšími modely, kde se TEL/AML1 chová jako slabý onkogen, který není schopen vyvolat leukémii bez spolupráce s dalšími zásahy [35-37, 268]. V další práci na poli TEL/AML1 pozitivních leukémií jsme ukázali, že jedním z kooperujících zásahů vedoucích ke vzniku těchto leukémií, by mohla být na TEL/AML1 zřejmě nezávislá nadprodukce microRNA 125b2, která udílí hematopoetické buňce rezistenci k signálům inhibujícím růst. Častým námětem studia fúzního genu TEL/AML1 je rovněž otázka, které funkční domény TEL/AML1 (a odvozeně tedy jaké molekulární mechanizmy) jsou nezbytné pro jeho leukemogenní funkci. Studium arteficiálně vytvořených delečních mutant ukázalo, že pro *in vitro* preleukemickou aktivitu TEL/AML1 jsou nezbytné HLH doména a centrální oblast TEL a DNA vazebná doména AML1 [264]. My jsme identifikovali přirozeně se vyskytující variantu fúzního genu TEL/AML1, která kóduje hybridní protein postrádající centrální oblast genu TEL kódovanou exonem 5. Z toho jednoznačně vyplývá, že v rozporu se zmíněnou *in vitro* studií není centrální oblast pro leukemogenní funkci TEL/AML1 *in vivo* nezbytná. Zajímavé rovněž je, že zatímco přítomnost centrální oblasti kódované exonem 5 genu TEL modifikuje charakter onemocnění vyvolaných fúzními geny TEL/ABL či TEL/NTRK3 [393, 396], fenotyp TEL/AML1 pozitivních leukémií není přítomností/absencí této domény modifikován. Přestože se centrální oblast podílí na interakci s korepresory a obsahuje autonomní represivní domény proteinu TEL, v námi vytvořené modelové reportérové eseji nevedla její ztráta k oslabení transkripčně represivní schopnosti fúzního proteinu TEL/AML1.

Monitorování MRN je v současné době nepostradatelnou součástí terapeutických protokolů dětských ALL. Na základě měření hladin MRN lze u BCR/ABL pozitivních ALL stanovit časnou odpověď na terapii a identifikovat podskupiny pacientů s odlišnou prognózou [361]. Zpřesnění odhadu rizika napomáhá stanovení vhodného terapeutického postupu a zlepšení léčebných výsledků této prognosticky nepříznivé podskupiny ALL. V naší práci jsme ukázali, že oproti dosud používané standardní metodě založené na detekci imunoreceptorových cílů umožňuje monitorování MRN pomocí detekce fúzního transkriptu BCR/ABL lépe a dříve identifikovat pacienty se zvýšeným rizikem relapsu i v průběhu dlouhodobého sledování. Na rozdíl od situace u TEL/AML1 pozitivních leukémií, kde MRN založená na detekci fúzního transkriptu přináší prakticky totožnou informaci jako standardně měřená MRN [397] a hraje tak roli především u případů, kde nejsou k dipozici vhodné imunoreceptorové cíle, u BCR/ABL pozitivních pacientů přináší tento způsob monitorování MRN nové, klinicky relevantní informace. Proto doporučujeme, aby u BCR/ABL pozitivních pacientů byl společně s imunoreceptorovými cíly vždy monitorován také fúzní transkript. Biologická podstata pozorované diskrepance obou přístupů měření MRN u BCR/ABL pozitivních pacientů má teoreticky několik možných vysvětlení. Jedním z nich je vznik fúzního genu BCR/ABL v buňce s multiliniovým potenciálem, který byl již dříve popsán u několika pacientů a který jsme nyní prokázali u pacientky se značnou diskrepancí obou metod. Jaký je celkový podíl této a dalších teoreticky možných příčin však zůstává nejasné.

Fúzní gen TEL/ABL se vzácně vyskytuje u různých typů hematologických malignit. Tři nové námi identifikované případy TEL/ABL pozitivních ALL přinesly nové klinické a biologické poznatky a doplnily současný stav znalostí těchto malignit. Zjistili jsme, že TEL/ABL pozitivní ALL u dětí mohou být prenatálního původu a že na vzniku těchto leukémií by se mohla podílet inaktivace regulátorů buněčného cyklu CDKN2A/CDKN2B. Z klinického hlediska by se mělo k TEL/ABL pozitivním ALL přistupovat jako k onemocněním se špatnou prognózou, v jejichž terapii by se mohly uplatnit tytéž přístupy jako u BCR/ABL pozitivních ALL, s nimiž, zdá se, tyto leukémie sdílí nejen biologickou podstatu, ale právě i nepříznivou prognózu.

5. Přehled publikací, abstrakt a přednášek

Seznam publikací:

Publikované původní články

1. Starkova J, Madzo J, Cario G, Kalina T, Ford A, Zaliova M, Hrusak O, Trka J: The identification of (ETV6)/RUNX1-regulated genes in lymphopoiesis using histone deacetylase inhibitors in ETV6/RUNX1-positive lymphoid leukemic cells. Clin Cancer Res. 2007 Mar 15;13(6):1726-35.
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1. Žaliová M, Trka J, Madžo J: Knock-down fúzního genu ETV6/RUNX1 (TEL/AML1) prostřednictvím RNA interference. XVth Working Conference of Pediatric Hematologists of Czech and Slovak republic, České Budějovice, 2005 (in czech)
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6. **Seznam použitých zkratek**

ALL	akutní lymfoblastická leukémie
AML	akutní myeloidní leukémie
B-ALL	akutní lymfoblastická leukémie z B-buněk
BCP-ALL	akutní lymfoblastická leukémie z B-buněčných prekurzorů
BFM	pracovní skupina Berlin-Frankfurt-Münster
CGH	komparativní genomická hybridizace
CML	chronická myeloidní leukémie
CMMoL	chronická myelo-monocytární leukémie
CNS	centrální nervový systém
FAB	French-American-British Cooperative Group
FISH	fluorescenční in situ hybridizace
HDAC	histon deacetylázy
HDACi	inhibitor histon deacetyláz
HLH	helix-loop-helix
HSC	hematopoetická kmenová buňka
HSCT	transplantace hematopoetických kmenových buněk
MDS	myelodysplastický syndrom
MRN	minimální reziduální nemoc
qRT-PCR	kvantitativní reverzně-transkriptázová polymerázová řetězová reakce
RHD	runt homology domain
SH1	src homology domain 1
SH2	src homology domain 2
SH3	src homology domain 3
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
T-ALL	akutní lymfoblastická leukémie z T-buněk
VPA	valproát
wt	wild type

7. **Použitá literatura**

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