Imunologická diagnostika a monitorování léčebné odpovědi u nádorových onemocnění krvetvorby v dětském věku

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1. Úvod

Mezi základní podtypy maligních onemocnění krvetvorby u dětí patří především akutní leukémie. U dětí leukémie tvoří nejčastější maligní onemocnění. Leukémii lze definovat jako skupinu chorob, u kterých genetické změny v nezralé krvetvorné buňce vedou k růstové výhodě a/nebo poruše apoptózy. Nejčastějším podtypem u dětí je leukémie z lymfocytárních prekurzorů (ALL), která tvoří asi 77% ze všech leukémií. Další podtypy maligních onemocnění krvetvorby u dětí jsou: akutní myeloidní leukémie (AML), která tvoří asi 11%, chronická myeloidní leukémie (2 – 3%) a myelodysplastický syndrom (1 – 2%). Léčebný úspěch u dětské ALL představuje jednu z nejúspěšnějších kapitol medicíny minulého století, v průběhu 40 let, kdy na počátku byla prakticky nulová šance na vyléčení, se daří v současnosti více než 80% pacientů dlouhodobě vyléčit. Již od počátku léčby ALL je snaha pacienty co nejvíce stratifikovat podle míry rizika selhání léčby. AML je na rozdíl od dospělých u dětí vzácná a výsledky jsou přes podstatně agresivnější léčbu oproti ALL stále neuspokojivé. Myelodysplastický syndrom (MDS) je v dětském věku velmi vzácné onemocnění, s odlišnou biologií nemoci od dospělých a v léčbě u dětí se uplatňuje především alogenní transplantace kostní dřeně (SCT). V rámci diferenciální diagnózy MDS a akutních leukémií je nutné zmínit i aplastickou anémii, onemocnění vznikající na podkladě autoimunitního poškození kmenových buněk kostní dřeně [1]. Sledování redukce nádorové masy (reziduální nemoci) metodikami přesnějšími a citlivějšími, než je klasická optická mikroskopie, je čím dál důležitějším parametrem stratifikace pacientů v moderních léčebných protokolech. Nejčastěji používanými metodikami jsou u ALL sledování klonálních přestaveb genů pro imunoglobuliny a T-buněčné receptory (Ig/TCR) pomocí kvantitativní PCR (RQ PCR) a průtoková cytometrie. U AML lze u části pacientů použít pro detekci MRN fúzní geny (AML1/ETO, PML/RARα, CBFβ/MYH11) nebo průtokovou cytometrii. Přes svoji relativní ekonomickou nenáročnost a dostupnost je ale cytometrie stále metodou používanou pro MRN poměrně málo, všechny dosud publikované práce jsou buď omezené na jednu instituci, či jednu centrální laboratoř. Jedním z důvodů je i nedostatečná standardizace této komplexní metodiky. V rámci svého postgraduálního studia jsem se zabývala zapojením

průtokové cytometrie a její standardizací v kontextu dalších metod u hematologických malignit v dětském věku.

1.1. Akutní lymfoblastická leukémie

V České republice onemocní ročně přibližně 65-70 dětí. Prognóza dětí v ČR je srovnatelná se světovými výsledky. U naprosté většiny pacientů neznáme přesně etiologii nemoci, vzhledem ke kumulaci onemocnění u dětí v předškolním věku lze očekávat, že významnou úlohu v leukemogenezi hraje postupné setkávání se s infekcemi a získávání adaptivní imunity závislé na lymfocytech [2]. U menšího počtu pacientů jsme schopni identifikovat genetickou poruchu, která je spojená s vyšší incidencí ALL. Typicky se jedná o geneticky podmíněné poruchy kontroly buněčného cyklu (např. Bloomův syndrom, ataxia teleangiectasia, Nijmegen breakage syndrome) [3, 4]. ALL rovněž může vzniknout jako sekundární onemocnění po onkologické léčbě pro jinou malignitu (včetně ALL) (sekundární ALL po léčbě ALL shrnujeme v **příloze 9**) [5, 6]. Za postupným zlepšováním prognózy dětí s ALL stály především randomizované studie. Hlavní podíl na nynější úspěšnosti mají přitom léky objevené před rokem 1980. V současnosti se postupně do léčby dostávají nová léčiva, často cíleně namířená na struktury leukemických buněk (monoklonální protilátky, inhibitory tyrosinkináz). Tato léčiva v současnosti se ale používají u specifických podtypů leukémie (např. BCR/ABL pozitivní ALL) nebo u nemoci refrakterní na klasickou léčbu.

1.1.1. Prognostické faktory a klasifikace ALL

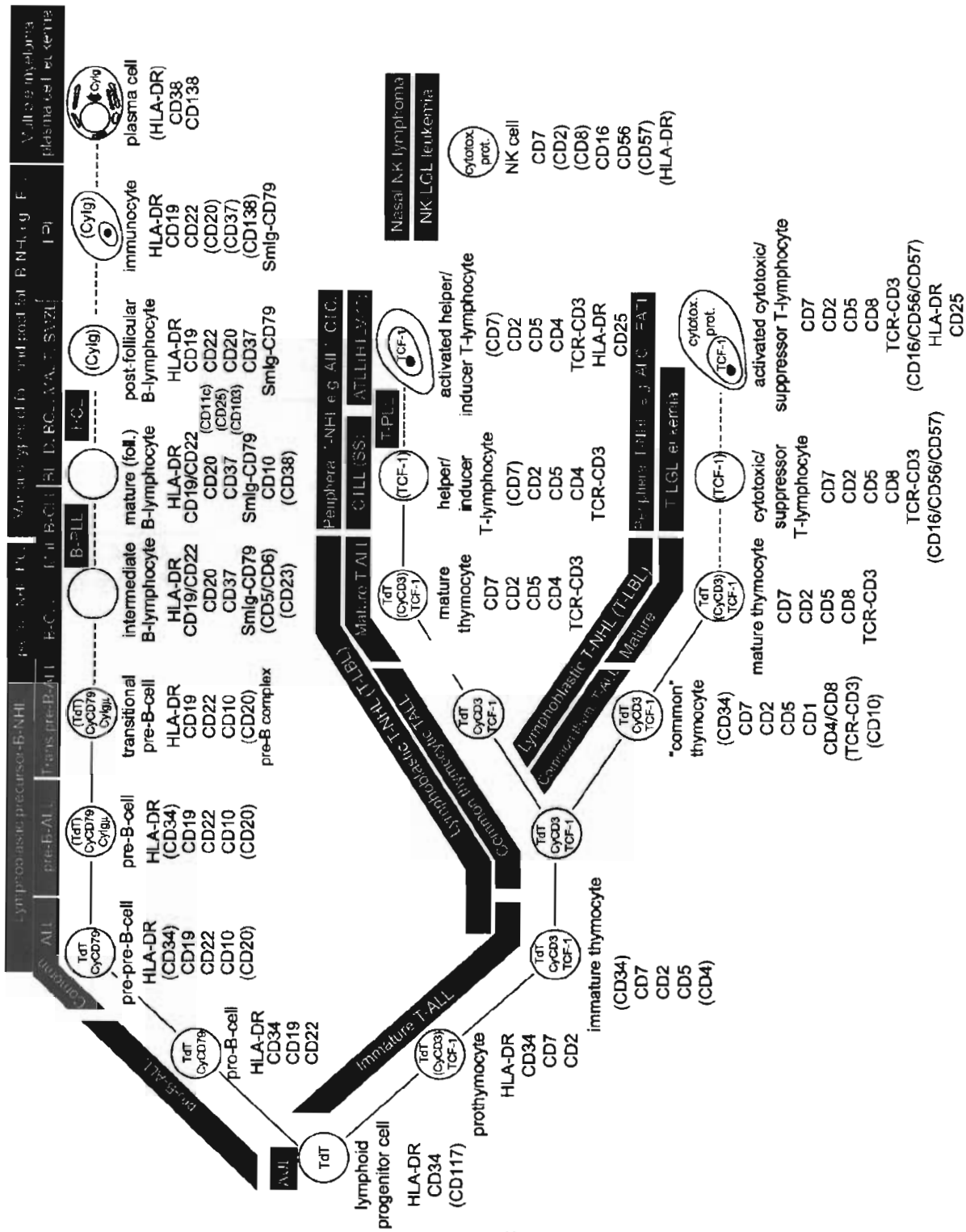
Morfologická klasifikace – rozlišení zralé a prekurzorové B ALL

Nejstarší dělení je na L1, L2 a L3 subtyp (L1 – homogenní malé blasty, L2 - blasty různé velikosti, L3 – typická je vakuolizace s intenzivní bazofilní cytoplazmou při barvení podle Wright - Giemsy). Diagnosticky a léčebně je důležitý zejména podtyp L3, který odpovídá imunologicky zralé B leukémii (leukemizovaný non hodgkinský lymfom (NHL) Burkittova typu s více než 25% infiltrací kostní dřeně nebo periferní krve nádorovými buňkami). U zralé B ALL se používá jiná léčebná strategie oproti typické ALL, s použitím krátkých intenzivních bloků chemoterapie, nesprávné zařazení může vést k selhání léčby a následnému relapsu onemocnění [7]. Cytogeneticky asi u 85% pacientů se zralou B leukémií nacházíme typickou translokaci t(8;14)(q24;q32), u zbývajících pacientů nacházíme translokace t(2;8)(p11;p24) a t(8;22)(q24;q11) [8, 9]. Translokace mají společné to, že se protoonkogen MYC přesouvá do oblasti genů, včetně jejich promotorů, pro těžký nebo lehké řetězce imunoglobulinů. Diagnosticky svízelný může být nález tzv. „transitional" praeB ALL s pozitivitou

povrchového IgM (μ řetězec) [10] a někdy i s expresí jednoho z lehkých řetězců (kapa nebo lambda), zpravidla však ne na všech blastech. Někdy současně morfologicky nacházíme i vzhled připomínající L3. Publikovaných dat je málo, nicméně cytogeneticky nenacházíme výše zmíněné přestavby MYC protoonkogenu typické pro zralou B ALL [11, 12]. U takto sporných případů je klíčová informace z cytogenetického vyšetření a případná léčba onemocnění je indikována podle ní. Současná pozitivita lehkého řetězce kapa a lambda je zpravidla artefaktem, způsobeným nízkou teplotou při transportu vzorku. Diagnostické rozpaky při podezření na leukémii může rovněž přinést zvýšený počet tzv. hematogonů v kostní dřeni u pacientů s regenerující hematopoézou (např. při virovém infektu nebo po chemoterapii), které mohou připomínat blasty ALL. Tyto hematogony v podstatě představují zmnožené prekurzory B lymfocytů, imunofenotypizace je v těchto situacích dokáže správně odlišit od fenotypu leukemických buněk [13]. Nejzralejší stádia těchto B prekurzorů (zpravidla slabě $CD10^{poz}$ a jasně $CD20^{poz}$) lze nalézt v malém počtu i v periferní krvi [14], částečně tyto buňky odpovídají populaci tzv. „transitional" B lymfocytů [15].

1.1.1.1. Imunologická klasifikace ALL

Leukemické buňky jsou částečně imunofenotypově podobné svým nemaligním protějškům, částečně nacházíme v expresi jednotlivých antigenů četné asynchronie a aberace odlišující je od nemaligních buněk. Klasifikaci, kterou v současné chvíli používáme u dětských ALL vychází z upravené a aktualizované klasifikace EGIL (European Group for the Immunological Characterization of Acute Leukemias)[16, 17]. Diagnosticky důležité je rovněž správné imunologické odlišení od AML.



Obrázek 1 (převzato z review Szczepanskeho et al. [18]***).*** *Schematický teoretický přehled vývoje nemaligních lymfocytů s nejdůležitějšími antigeny exprimovanými v jednotlivých stádiích diferenciace. Zároveň je schematicky naznačen vztah vývoje lymfocytů k nejčastějším podtypům lymfoidních malignit.*

1.1.1.1.1. ALL z prekurzorů B lymfocytů (BCP ALL)

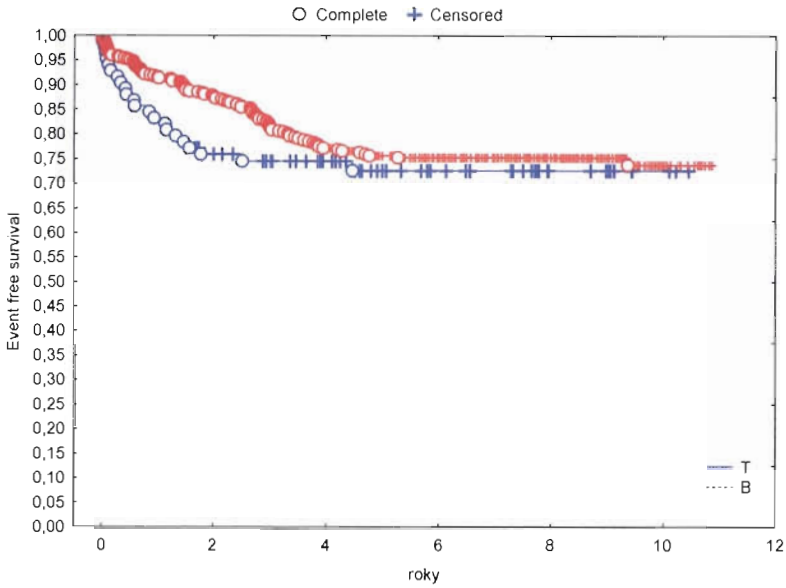
B prekurzorová leukémie je dominujícím podtypem ALL jak u dětí, tak i u dospělých. Základní imunofenotypové rozdělení je v tabulce 1, vlastní výsledky a podrobnější rozbor tohoto podtypu uvádím v kapitole 3.1.1.

Kategorie	Kritéria	Podtřída	Kritéria
B prekurzorová	• 2 nebo 3 z následujících: $CD19^{poz}$, (intra)$CD79a^{poz}$ a $CD22^{poz}$ • $CD3^{neg}$ • $intraCD3^{neg}$ • κ^{neg} a λ^{neg}	proB ALL	$CD10^{neg}$ $CD20^{neg}$
		cALL	$CD10^{poz}$ $intraIgM^{neg}$
		praeB ALL	$intraIgM^{poz}$
zralá B	• 2 nebo 3 z následujících: $CD19^{poz}$, (intra)$CD79a^{poz}$ a $CD22^{poz}$ • $CD3^{neg}$ • $intraCD3^{neg}$ • κ^{poz} nebo λ^{poz}	*Bez další subklasifikace*	

Tabulka 1. Klasifikace ALL B řady, adaptovaná podle EGIL [16]***, která je používána v rámci protokolů skupiny BFM (Berlin – Frankfurt – Münster) pro léčbu dětské ALL.***

1.1.1.1.2. ALL z prekurzorů T lymfocytů (T ALL)

T ALL jsou méně častým podtypem, z dětských leukémií tvoří asi 10 až 15%. Často při diagnóze nacházíme nádorovou infiltraci thymu, vedoucí k tumoru mediastina, který může způsobovat syndrom horní duté žíly, respektive dušnost na podkladě obstrukce dýchacích cest [19]. Tato nádorová infiltrace v sobě odráží normální vývoj T lymfocytů, který právě z větší části probíhá v thymu. Není neobvyklé, že u těchto pacientů při prvním kontaktu s lékařem nacházíme ještě normální krevní obraz a během krátkého intervalu dochází k tzv. leukemizaci do periferní krve a kostní dřeně. Hranice mezi lymfoblastickým lymfomem a ALL je tak dána arbitrárně procentem 25% blastů v kostní dřeni. Terapeutický přístup je ale v současné době k oběma jednotkám velmi podobný [20]. Retrospektivní analýzou přežití dětí diagnostikovaných s de novo ALL mezi zářím 1996 a srpnem 2006 nevidíme v ČR signifikantní rozdíl mezi T a BCP ALL (obrázek 2). Postupné zlepšování je například dokumentováno v analýze provedené kolegy z Německa u vysoce rizikových T ALL, kde se mezi protokoly ALL BFM 90 a ALL BFM 95 došlo zlepšilo přežití bez události v 5 letech od diagnózy o 20% [21]. Podle imunofenotypu lze T ALL dělit podle EGIL klasifikace [16, 22] na proT, praeT, intermediární T a zralou T (tabulka 2)[23], toto členění má částečně i prognostický význam.



Obrázek. 2. *Analýza přežití dětí diagnostikovaných mezi lety 1996 až 2006. Rozdíl mezi přežitím pacientů bez události s T ALL a BCP ALL není signifikantní (Cox-Mantelův test). Celkem do analýzy zahrnuto 582 pacientů (83 T ALL, 499 BCP ALL). Událost je definována jako relaps, sekundární malignita nebo smrt. Medián sledování kohorty pacientů s T ALL je 3,9 let a BCP ALL 4,4 let. Pravděpodobnost přežití (pEFS) pacientů s T ALL v 5 letech je 72%±5%, s BCP ALL 75%±2,1%.*

Kategorie	***Kritéria***	***Podtřída***	***Kritéria***
T ALL	(intra)CD3^{poz} a CD7^{poz}	proT ALL	CD2^{neg} CD5^{neg} CD8^{neg}
		preT ALL	CD2^{poz} a/nebo CD5^{poz} a/nebo CD8^{poz}
		intermediární T ALL	CD1a^{poz}
		zralá T ALL	CD3^{poz} CD1a^{neg}
		TCRαβ^{poz} T ALL	TCRαβ^{poz}
		TCRγδ^{poz} T ALL	TCRγδ^{poz}

Tabulka 2. Klasifikace ALL T řady, adaptovaná podle EGIL [16]***,*** *která je používána v rámci protokolů skupiny BFM pro léčbu dětské ALL.*

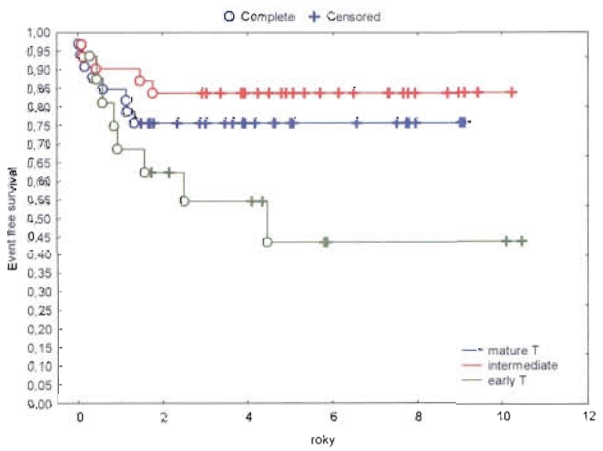
T ALL lze rovněž členit podle typu genetických změn (viz kapitola Genetické podtypy T ALL) [24-26]. Lepší prognóza u dětí i u dospělých je spojena s podtypem intermediární, resp. tzv. „thymic" T ALL [27, 28]. Srovnání přežití s pacienty s BCP ALL a rozdělení podle zralosti T imunofenotypu u českých dětí je na obrázku 2 a 3. Studie z konce 80. let v rámci studie POG 7865 a 8035 neprokázala asociaci zralosti podle imunofenotypu s prognózou, ukázala však, že pacienti s nezralou formou T ALL častěji nedosáhnou kompletní remise [29]. Naše data dokumentují prognostický význam zralosti T ALL (obrázek 3), i když jsou omezena velikostí

souboru. V rámci protokolů GMALL pro dospělou ALL (Německo) jsou je imunofenotyp intermediární T ALL jedním z kritérií standardního rizika [30]. Alternativní imunofenotypovou klasifikaci, která lépe odráží vývoj T řady z pohledu přestaveb T buněčného receptoru podle našich současných znalostí, navrhla skupina pod vedením profesorky Macintyrové (tabulka 3) [31-34].

Podtyp T ALL	*Exprese antigenů*
nezralá T ALL	Cyt-β^{neg}, povrchová $CD3^{neg}$, $TCR\alpha\beta^{neg}$ a $TCR\gamma\delta^{neg}$
pre-αβ T ALL	Cyt-β^{poz}, povrchová $CD3^{neg}$, $TCR\alpha\beta^{neg}$ a $TCR\gamma\delta^{neg}$
TCR-αβ T ALL	povrchová $CD3^{poz}$, TCR-$\alpha\beta^{poz}$
TCR-γδ T ALL	povrchová $CD3^{poz}$, TCR- $\gamma\delta^{poz}$

Tabulka 3. *Klasifikace T ALL podle Macintyrové*

Skupina profesorky Macintyrové rovněž prokázala, že s věkem až do pozdní dospělosti přibývá nezralých T ALL a ubývá zralých prekurzorových forem T ALL, zejména TCR-αβ [33]. Tento jev pravděpodobně souvisí s postupnou involucí thymu v průběhu života.



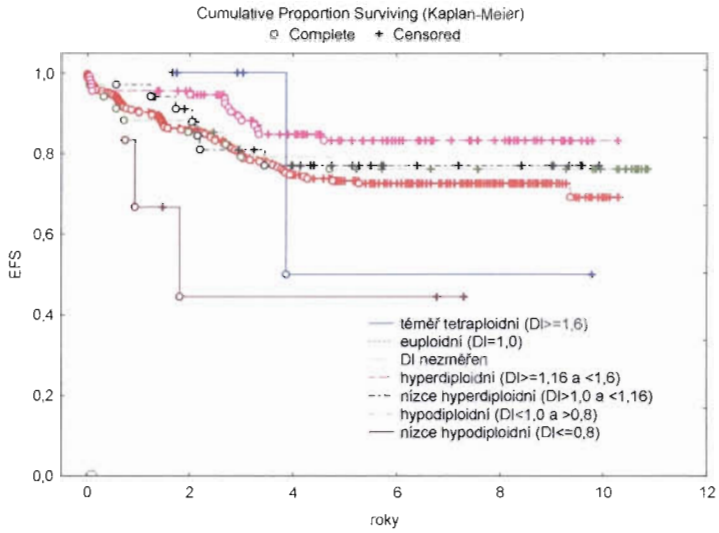
Obrázek 3. *Prognóza dětí s de novo T ALL* (n=80); podtypy *zralá [mature] T* n=33, *intermediární [intermediary] T* n=31 *a nezralá [early] T* n=17 *(shrnující: praeT* n=16 *a pro T* n=1*). Je zřetelná signifikantně horší prognóza skupiny s nezralou T ALL (pEFS v 5 letech 44%±14%) oproti skupině s intermediární T ALL (Cox-Mantel, p=0.01, pEFS skupiny s intermediární T v 5 letech 84%±6,6%). Rozdíly mezi skupinou nezralou T a zralou T, resp. mezi zralou a intermediární signifikantní nejsou (pEFS skupiny se zralou T v 5 letech 76%±7,5%).*

1.1.1.2.1. Genetické podtypy BCP ALL

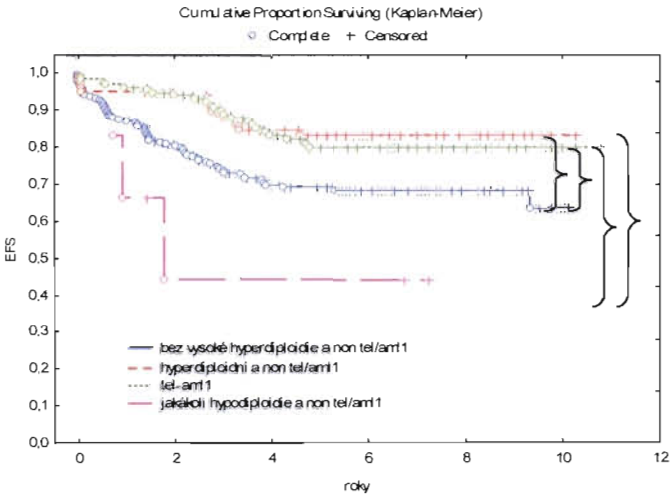
Nejčastějšími změnami genotypu u dětských leukémií je hyperdiploidie a přítomnost fúzního genu TEL/AML1. Tyto dvě aberace jsou přítomny celkem asi u 50% dětských BCP ALL a korelují s dobrou prognózou [36-38]. Podle některých studií jsou pro dobrou prognózu hyperdiploidní ALL specificky významné trizómie chromozómů 4, 10 a 17, což může být zohledněno i léčebně [39].

Hyperdiploidii, respektive hypodiploidii lze kromě klasických cytogenetických metod rovněž stanovit pomocí fluorescenčních barviv, schopných stechiometricky vázat nukleové kyseliny (např. propidium jodid, DAPI nebo HOECHST). Zmnožení DNA vyjadřujeme tzv. DNA indexem (poměr modu G01 fáze aneuploidní populace ku modu G01 fáze euploidní kontroly). Korelace cytometrického DNA indexu s cytogenetickým vyšetřením není stoprocentní. Důvodem ojedinělých diskrepancí může být jak horší senzitivita cytometrického vyšetření u méně výrazného zmnožení genetického materiálu, tak i poměrně špatná schopnost leukemických buněk proliferovat in vitro, která často vede k selhání cytogenetického vyšetření. Hodnota DNA indexu nad 1,16 a pod 1,6 koreluje s dobrou prognózou u BCP ALL [40] (obrázek 5 a 6). Naopak hypodiploidie pod 0,8 je spojena s horší prognózou, (hypodiploidie (<45 chromozómů) až haploidie (23 – 29 chromozómů) [41]. Nález signifikantní hypodiploidie (<44 chromozómů nebo DNA index <0,81) kvalifikuje příslušného pacienta v některých léčebných protokolech do vysokého rizika [39]. U části pacientů dochází ke zdvojení genetického materiálu části hypodiploidního klonu a nacházíme pak současně jak hypodiploidní tak hyperdiploidní klon. Ve studii Nachmana et al. se toto zdvojení častěji děje u pacientů s počtem chromozómů 24 až 29 a méně často u pacientů s počtem chromozómů 33 až 39 [41]. Prognóza pacientů se zdvojeným hypodiploidním klonem je stejně špatná jako u pacientů bez tohoto zdvojení. Správná identifikace hypodiploidního klonu je důležitá pro správnou stratifikaci pacientů [42]. Nález velmi vysoké hyperdiploidie až téměř tetraploidie je velmi vzácná (incidence ze všech ALL asi 1% až 2%), u BCP ALL koreluje s fúzním genem TEL/AML1 [43-45]. Špatná prognóza je rovněž spojena s přestavbami genu MLL (11q23) zejména u dětí mladších 1 roku [46], u starších dětí s MLL přestavbou je zřejmá nižší kumulativní incidence relapsů oproti kojencům v rámci BFM protokolů [47]. Negativní vliv na prognózu má translokace (9;22) vedoucí ke vzniku fúzního genu BCR/ABL [48, 49]. U dětí, narozdíl od dospělých, je tento fúzní gen poměrně vzácný. Přestože je znakem špatné prognózy i u dětí, neprojevuje se pro celkovou vzácnost významně na celkové prognóze u

ALL. U dospělých tvoří až 30% (obrázek 4) a podílí se na významně horší prognóze ALL v této věkové skupině.



Obrázek 5. *Přežití českých pacientů s BCP ALL podle obsahu DNA. Kategorie jsme definovali pomocí DNA indexu (DI). (euploidní* n=307, *hyperdiploidní* n=112, *nízce hyperdiploidní* n=35, *hypodiploidní* n=1, *nízce hypodiploidní* n=6 *a téměř tetraploidní* n=6. *Analýza všech podskupin najednou potvrzuje signifikantní rozdíly (p=0,017). Pro úplnost je ukázáno přežití bez události i u pacientů, kde pro nedostatek materiálu nebo technickou závadu na přístroji nebyl DNA index změřen* (n=34). *Rozdíl mezi podskupinou euploidní a hyperdiploidní je signifikantní (p = 0,023). Prognóza ostatních podskupin se od euploidní BCP ALL neliší.*



Obrázek 6. *Prognostický význam DNA indexu v kontextu TEL/AML1 genotypu: vysoká hyperdiploidie (DNA index ≥1,16 a <1,6)* n=112, *pacienti s jakoukoli hypodiploidií* n=6, *TEL/AML1^{poz} pacientů* n=141 *a ostatní* n=218. *Rozdíly jsou celkově signifikantní (p=0,00007). Všechny svorky znázorňují signifikantní rozdíly mezi dvěma podtypy (p<0,01). Z obrázku je zřejmá podobná prognóza pacientů s TEL/AML a pacientů s vysokou hyperdiploidií.*

1.1.1.2.2. Genetické podtypy T ALL

Pro relativní vzácnost T ALL je vztah genotypu a prognózy stále poněkud méně objasněný než u BCP ALL. Zhruba u 5% T ALL nacházíme téměř tetraplodii, která nemá prognostický význam. Zhruba u 50% dětí s T ALL nacházíme rekurentní chromozomální translokace [50]. Do většiny těchto translokací je zavzat T-buněčný receptor TCR-α/δ nebo TCR-β s předpokladem, že se jedná o chybu vzniklou při fyziologickém přestavování genů pro TCR. Další geny často zapojené do přestaveb jsou MYC, TAL1, TAL2, LYL1, bHLHB1, LMO1, LMO2, homeoboxové geny (HOX11/TLX1, HOX11L2/TLX3 nebo HOXA cluster). Vzácnou translokací u T ALL, která demonstruje složitý vztah genotypu a fenotypu, je fúzní gen CALM/AF10, vzniklý na podkladě translokace (10;11). U T ALL se fúzní gen CALM/AF10 pojí s přestavbami TCR γδ [51]. Tato translokace však způsobuje i AML nebo např. histiocytární lymfom [52].

Zhruba u poloviny pacientů s T ALL nacházíme mutace v genu NOTCH1, které vedou k upregulaci signalizační dráhy, do níž je NOTCH1 zapojen [53]. Důležité pro terapii T ALL je, že existují inhibitory této dráhy (inhibitory γ-sekretázy) [54].

Nejeví se zatím jednoznačná asociace genotypových změn a zralosti dle imunofenotypu [26].

1.1.1.3. Klinické a biologické projevy ALL při diagnóze

Leukémie se klinicky při diagnóze nemoci projevuje především příznaky ze selhání nemaligní krvetvorby, tzv. leukemickou trias: anémií, krvácivostí, zejména na podkladě trombocytopenie a imunodeficitem na podkladě redukce počtu normálních imunokompetentních buněk. Jak již bylo zmíněno výše, T ALL často infiltruje thymus a způsobuje kompresi fyziologických anatomických struktur přítomných v mediastinu s typickými klinickými důsledky (syndrom horní duté žíly, obstrukce dýchacích cest apod.). Rovněž při leukocytóze může nastat leukostáza (porucha mikrocirkulace na podkladě zvýšené viskozity krve). Typicky u ALL dále nacházíme infiltraci jater a sleziny (hepatosplenomegalii), případně lymfatických uzlin. Dále u části pacientů je zřetelná bolestivost kostí a subfebrilie až febrilie, část těchto příznaků je způsobená i cytokiny, které pravděpodobně vyplavují jak leukemické buňky, tak i aktivované buňky imunitního systému.

1.1.1.4. Biologické projevy ALL

Věk a leukocytóza při diagnóze jsou již dlouhou dobu známým důležitým prognostickým faktorem. Poměrně často jsou zmiňována tzv. NCI (National Cancer Institute) kritéria [39, 55]. Podle těchto NCI kritérií standardní riziko splňuje pacient 1 až 10 let věku a s iniciální

leukocytózou nižší než 50000/μL, ostatní pacienty řadí NCI klasifikace do vysokého rizika. Kojenci (děti mladší 1 roku) jsou naproti tomu velmi rizikovou skupinou ALL s často nepříznivou cytogenetikou a špatnou odpovědí na léčbu. O adolescentech je známo, že částečně hůře odpovídají na léčbu a zejména dívky trpí vyšší toxicitou léčby; oba tyto faktory se podílejí na horší prognóze dětí mezi 15 až 18 lety [56]. Postižení CNS při diagnóze ALL je rovněž nepříznivým faktorem, který ale lze překonat u těchto pacientů intenzivnější cílenou léčbou, jak chemoterapií, tak i radioterapií [3].

1.1.1.5. Farmakogenetika ALL

Snížená akumulace aktivních metabolitů cytostatik v leukemické buňce, ať už je způsobena zvýšenou clearence, inaktivací či jinými důvody, je spojena s horší prognózou. Nedávno byla publikována řada studií zabývající se odlišnou expresí genů důležitých pro metabolizaci léků. Současné podání některých antiepileptik (např. fenytoin, fenobarbital nebo karbamazepin) významně zvyšuje clearence některých cytostatik produkcí cytochromu P-450. Polymorfismy genů, které kódují metabolizující enzymy, transportéry, receptory jsou zodpovědné za významné rozdíly v odpovědi na jednotlivé léky. Tyto polymorfismy se ne vždy odrážejí v léčebných výsledcích [3]. Jedinou významnou výjimkou je vztah polymorfismů thiopurin methyltransferázy. Pacienti s vrozenou homozygotní či heterozygotní deficiencí enzymu thiopurin methyltransferázy, který katalyzuje S-metylaci (inaktivaci) merkaptopurinu, mají signifikantně vyšší riziko toxických nežádoucích účinků, na druhou stranu mají lepší odpověď na léčbu v protokolech obsahujících tento lék [57].

1.1.1.6. Princip terapie ALL

Většina léků používaných v léčbě leukémií interferuje s buněčným cyklem. Léky postihující syntézu DNA nazýváme **antimetabolity** a poškozují především dělící se buňky. Patří sem např. metotrexát blokující enzym dihydrofolát reduktázu, která je zodpovědná za redukci kyseliny listové na kyselinu tetrahydrolistovou, která hraje klíčovou v syntéze purinů. Dalším důležitým antimetabolitem je cytosin arabinosid (ara-C), 6-merkaptopurin a 6-thioguanin. **Alkylační cytostatika** poškozují více dělící se buňky, narušují ale i strukturu DNA v nedělící se buňce (např. odloučení purinových bází, zlomy v jednom či obou vláknech DNA). Patří sem např. cyklofosfamid a ifosfamid. **Antracyklinová antibiotika** (např. doxorubicin a daunorubicin) blokují především enzym topoizomerázu II. **Rostlinné alkaloidy** vinkristin, vinblastin a vinorelbin se především váží na mikrotubuly dělícího se vřeténka. Významným lékem v léčbě ALL je **asparagináza**, která necílí na syntézu či strukturu DNA, ale způsobuje

depleci extracelulární aminokyseliny asparaginu, což postihuje především proteosyntézu leukemických buněk, které nedokáží kompenzovat nedostatek této aminokyseliny. **Kortikoidy** obecně po pasivní difúzi buněčnou membránou se váží na specifický kortikoidový receptor a s ním jsou transportovány do jádra. Glukokortikoidy mají mimo jiné výrazný účinek na buňky imunitního systému zejména na lymfoidní buňky, u kterých ve farmakologických dávkách indukují apoptózu.

Léčba a dodržení léčebného schématu bez dlouhých prodlev v terapeutickém schématu je velmi důležitým prognostickým faktorem. Velkým zdrojem poučení jsou studie srovnávající prognózu adolescentů a mladých dospělých léčených pediatrickým protokolem nebo protokolem pro dospělé. Podle publikovaných studií je prognóza pacientů léčených protokolem pro dospělé horší [58-60]. Za tímto rozdílem může stát nižší intenzita protokolů pro dospělé i vyšší protokolární compliance a úspěšnější podpůrná léčba na pediatrických pracovištích.

Současná léčba ALL v jednotlivých protokolech je postavena na několika léčebných principech:

1) **indukční léčba ALL** má za cíl dosáhnout kompletní remise (<5% blastů v KD a normálně fungující hematopoéza, redukce extramedulárního postižení např. tumoru mediastina, sanace mozkomíšním moku od blastů při iniciální CNS infiltraci apod.). V rámci indukce se zpravidla uplatňuje kombinace kortikoidů (prednisonu nebo dexamethasonu) s vinkristinem a dále s asparaginázou nebo antracykliny (resp. s oběma). V rámci BFM protokolů je strategie v indukci u všech pacientů stejná (stejná kortikoidová předfáze s prednisonem a jednou dávkou intrathekálního metotrexátu, pokračování v kortikoterapii spolu s vinkristinem, asparaginázou a daunorubicinem.
2) velmi časně podaná **léčba** CNS, jak prokazatelného, tak i subklinického postižení (např. pomocí intrathekálně podaného metotrexátu)
3) **konsolidace remise a reindukční léčba** v době, kdy pacient již má dobře fungující normální krvetvorbu s cílem léčit zbytky resistentních leukemických buněk
4) **udržovací léčba**, např. v rámci BFM protokolů s perorálním 6-merkaptopurinem a metotrexátem. Přesný mechanismus účinku udržovací léčby není znám, ale její zkrácení např. v průběhu studie ALL BFM 1990 vedlo k signifikantnímu nárůstu relapsů ve skupině SR rizika [49]. Je možné, že dlouhodobé podávání cytostatik po skončení intensivní části léčby ničí leukemické buňky s pomalejším dělením. Naopak intenzivnější prvek v podobě pulsů vinkristinu a dexamethasonu v průběhu udržovací

léčby se ukázal jako neúčinný v rámci současného typu léčby (v protokolu ALL BFM 95)[61, 62]. Historický význam těchto pulsů mohl vyplývat z obecně nižší intenzity předchozí léčby.

5) **ozařování CNS** je v současné době používáno jen u podskupiny s největším rizikem pro CNS relaps (např. pacienti s T ALL, vysoké riziko ALL, infiltrace CNS při diagnóze).

1.1.1.6.1. Odpověď na léčbu ALL

1.1.1.6.1.1. Kompletní remise

Stav, kdy nacházíme v kostní dřeni optickou mikroskopií méně než 5% blastů a kdy došlo k rekonstituci fyziologické hematopoézy a v případě extramedulárního postižení (CNS, mediastinum) je zřetelná sanace od blastů, resp. redukce tumoru mediastina na zobrazovacích metodách o více než třetinu od iniciální velikosti, nazýváme termínem **kompletní remise**. Standardně se daří kompletní remise dosáhnout u více než 98% dětských pacientů s ALL. Nedosažení kompletní remise po prvním indukčním bloku je vzácné a je spojeno s velmi špatnou prognózou, tito pacienti jsou vždy řazeni do nejvyššího rizika a v řadě léčebných protokolů jsou indikováni k alogenní transplantaci kostní dřeně od příbuzného i nepříbuzného dárce[39, 63, 64].

1.1.1.6.1.2. Korelace počtu blastů v den 8 v periferní krvi po kortikoidové předfázi - prednisonová odpověď

Dobře známé je hodnocení tzv. prednisonové odpovědi, které se používá v protokolech skupiny BFM. Tento parametr zavedl profesor Riehm a spočívá v hodnocení úbytku blastů v periferní krvi po týdnu léčby prednisonem a jedné dávky intrathekálního metotrexátu [65]. Pacienti s více než 1000 blastů/μL v periferní krvi v den 8 mají signifikantně horší prognózu a v protokolech BFM skupiny jsou řazeni do vysokého rizika. Špatná prednisonová odpověď vzhledem k vysokému riziku relapsu těchto pacientů tak neukazuje jen farmakorezistenci na prednison, ale i vlastně na další cytostatika použitá následně v léčbě ALL. Špatná prednisonová odpověď predikuje horší prognózu i u nepříznivých podskupin ALL, jakou je například kojenecká ALL [66] nebo ALL s fúzním genem BCR/ABL [67]. Studie Gajjara et al. prokázala horší prognózu pacientů s prokazatelnými blasty v periferní krvi po týdnu léčby kombinací cytostatik a kortikoidů [68].

1.1.1.6.1.3. Hodnocení poklesu blastů v kostní dřeni před dosažením remise

Další parametry, které se buď používaly, nebo používají v léčebných protokolech (např. protokoly Children Cancer Group), jsou odvozené od hodnocení odpovědi v kostní dřeni před vlastním dosažením kompletní remise [39, 69, 70]

1.1.1.6.1.4. Minimální reziduální nemoc (MRN)

Problematice MRN je věnována kapitola ve Výsledcích a diskusi (3.2 - Léčba leukémie, monitorování účinnosti léčby)

1.1.2. Přístup k pacientům s relapsem ALL a k primárně vysoce rizikové ALL

1.1.2.1. Indikace transplantace kostní dřeně (SCT) u pacientů v první kompletní remisi ALL

V rámci ALL je alogenní transplantace krvetvorných kmenových buněk indikována u podskupiny pacientů s velmi vysokým rizikem selhání léčby s předpokládaným přežitím bez události nižším než 50%. Studie Balduzziové et al. prokázala lepší prognózu dětí transplantovaných od příbuzných shodných dárců s velmi vysokým rizikem na rozdíl od dětí léčených pouze chemoterapií [71]. Velmi vysoké riziko bylo v této studii definováno jako nedosažení remise po indukčním bloku nebo přítomnost fúzního genu BCR/ABL nebo MLL/AF4, špatná odpověď na prednison spojená s T imunofenotypem a/nebo s leukocytózou nad 100.10^9/L [71]. Jedinou nadějí pacienta s vysoce rizikovou formou AL je někdy nepříbuzenská transplantace kostní dřeně s neúplnou shodou (**příloha 10**).

Autologní transplantace není u ALL indikována.

1.1.2.2. Přístup k pacientům s fúzním genem BCR/ABL

Prognóza těchto pacientů je velmi nepříznivá a ve většině léčebných protokolů jsou řazeni do vysokého rizika [67]. V současné době lze těmto pacientů přidat ke standardní chemoterapii specifickou terapii inhibitorem BCR/ABL kinázy imatinib mesylátem (*imatinib*). V současné době jsou dostupné na trhu i další inhibitory BCR/ABL kinázy, které se testují pro použití i u dětí s rezistencí či intolerancí imatinibu. V současné době existuje několik přístupů, jak kombinovat chemoterapii s léčbou imatinibem [72]. Časné podání imatinibu do indukční léčby zvyšuje signifikantně procento dosažených kompletních remisí po iniciální léčbě u dospělých pacientů, nicméně dopad na celkové přežití je omezený [73]. Přestože i samotný imatinib může navodit remisi onemocnění, tyto remise jsou jen dočasné a velmi rychle dochází k rozvoji

resistence na tuto léčbu [74]. Mechanismus vzniku resistence není zcela objasněn, ale klíčovou roli hrají bodové mutace v tyrosinkinázové doméně, resp. ve vazebné doméně pro inhibitor tyrosinkinázy. Tyto mutace jsou často přítomny v subklonu leukémie a během léčby tyrosinkinázovým inhibitorem dochází k jejich selektivní expanzi [75]. Od září 2004 jsou děti s nově diagnostikovanou ALL s prokázaným fúzním genem BCR/ABL od dne 33 přeřazeny do protokolu EsPhALL (European Intergroup Study on Post Induction Treatment of Philadelphia Positive Acute Lymphoblastic Leukaemia with Imatinib). Pacienti s vysokým rizikem dostávají v protokolu EsPhALL ke standardní chemoterapii ještě imatinib a SCT. Pacienti s lepší prognózou jsou randomizováni do ramene s a bez imatinibu, a podle hladin MRN a dostupnosti dárce rovněž SCT. I přes léčbu kombinací imatinibem, chemoterapií a SCT je prognóza části pacientů s tímto fúzním genem velmi špatná. Příčin selhání léčby je řada, jednou z nejdůležitějších je již výše popsaný dynamický vznik resistence BCR/ABL na podávaný inhibitor na podkladě mutace v ABL kinázové doméně. Dále je u pokročilých forem CML popisována kompenzační hyperexprese BCR/ABL na úrovni mRNA, přesný podíl tohoto jevu na klinické resistenci na imatinib není znám [76], pravděpodobně hraje i roli v resistenci na *imatinib* u Ph^{poz}ALL. Další typy resistence označujeme jako BCR/ABL nezávislé, jedná se zejména o konstitutivní aktivaci kináz z rodiny SRC [76]. Postupně se dostávají na trh další inhibitory tyrosin kináz (např. dasatinib, nilotinib) s větší terapeutickou schopností inhibovat BCR/ABL tyrosinkinázu a překonávat část mutací, způsobujících resistenci na imatinib [77]. U dasatinibu na rozdíl od imatinibu je navíc popisována i schopnost pronikat hematoencefalickou bariérou do CNS při systémovém podání [78] a schopnost inhibovat kinázy z rodiny SRC [76]. Zatím není zcela objasněn vliv působení, zdá se že inhibice SRC kináz může vést k poruše funkce zejména T lymfocytů a vést tak k sekundárnímu imunodeficitu. Výzvou pro výzkum nových inhibitorů tyrosinkináz je mutace T315I, která vede k rezistenci na všechny dosud používané inhibitory (imatinib, dasatinib, nilotinib) jak u CML tak i ALL.

1.1.2.3. Přístup k pacientům s vysoce rizikovou T ALL

Prognózu části pacientů se špatnou odpovědí na prednisonovou předfázi a T imunofenotypem zlepší SCT [21]. Vývoj léčby T ALL dobře demonstruje nutnost stavět nové protokoly na datech MRN. Pro relativní vzácnost T ALL srovnávala Schrauderova studie efekt SCT u pacientů s vysoce rizikovou T ALL léčených podle protokolů ALL BFM 90 a 95. Nutno zmínit, že protokol ALL BFM 90 znamenal zhoršení prognózy u všech vysoce rizikových ALL oproti předchozímu protokolu ALL BFM 86, pravděpodobně z důvodu snížení celkové dávky

alkylačních cytostatik a z důvodu léčby krátkými rotačními bloky chemoterapie po indukci [49] bez delšího protokolu II s prolongovaným působením cytostatik. Toto zhoršení léčebných výsledků bylo reflektováno v protokolu ALL BFM 95, který přinesl signifikantní zlepšení prognózy vysoce rizikové T ALL oproti předchozímu protokolu. Ze studie Willemse et al. je zřejmé, že pacienti s T ALL obecně odpovídají na léčbu pomaleji, nicméně pacienti s T ALL, kteří jsou 3 měsíce od zahájení léčby v rámci BFM protokolu MRN negativní, prakticky nerelabují [79]. Předběžné výsledky z ALL-BFM 2000 protokolu, které zatím nebyly publikovány pro krátkou dobu sledování celé kohorty, ukazují, že negativita 3 měsíce od diagnózy znamená výbornou prognózu i u pacientů se špatnou odpovědí na prednisonovou předfázi. Na základě těchto výsledků v současné době u těchto pacientů není indikována transplantace kostní dřeně v první remisi, shodně se postupuje i u dětí v ČR. Naopak pacienti s vysokou hladinou MRN 3 měsíce od diagnózy jsou ohroženi vysokým rizikem časného relapsu a je u nich indikována SCT i od nepříbuzného dárce. Ještě před SCT tito pacienti dostanou vysoce intenzivní léčbu s monitorováním MRN. Při nedostatečném poklesu MRN během následných bloků chemoterapie, kdy už je zřejmé, že nemoc je velmi chemorezistentní, jsou takoví pacienti kandidáty pro změnu léčby včetně experimentální léčby. V současné době se na trh dostávají nové léky namířené nejen na léčbu T ALL [80]. Příklady těchto léku jsou nelarabin (ARA-G) a forodesin, inhibitor purin nucleosid fosforylázy (PNP). Forodesin navodí situaci podobnou vrozenému poškození PNP. Mutace genu pro PNP vedoucí k jejímu vrozenému poškození je příčinou varianty těžkého kombinovaného imunodeficitu. Absence funkční PNP vede ke zvýšené apoptóze T lymfocytů na podkladě zvýšené kumulace metabolitů purinů. Zajímavá je myšlenka kombinovat u refrakterní T ALL/LBL nelarabin s forodesinem, a prohloubit tak vzájemně jejich antileukemický účinek. Inhibitory gama-sekretáz nejen blokují zvýšenou aktivaci dráhy spuštěné aktivační mutací v proteinu NOTCH 1 (přítomno asi u 50% T ALL), ale i zatím podle in vitro dat dokáží zvýšit senzitivitu na dexamethason u T ALL buněčných linií [81, 82].

1.1.2.4. Přístup k pacientům s relapsem ALL

Prognóza relapsu ALL je závislá na několika faktorech a dlouhodobě se daří vyléčit přibližně 30 – 40% pacientů [83]. Prognóza relapsů je mimo jiné závislá na efektivitě front line protokolů. S vyšší efektivitou léčby klesá celkový počet relapsů a kumulují se rizikovější a resistentnější pacienti. Mění se i skladba pacientů v relapsových protokolech (zpravidla je kritériem pro zařazení do protokolu 1. relaps ALL po chemoterapii bez předchozí alogenní SCT) související s rozšiřující se indikací alogenní transplantace v 1. kompletní remisi (Ph^{poz} ALL, již zmíněná

vysoká hladina MRN ($\geq 10^{-3}$) 3 měsíce od diagnózy apod.). Nejdůležitější prognostické faktory pro úspěch léčby relapsu jsou doba od diagnózy primárního onemocnění (**velmi časný** méně než 18 měsíců od zahájení léčby, **časný** méně než 6 měsíců od konce léčby a více než 18 měsíců od diagnózy a **pozdní** více než 6 měsíců od konce léčby) , imunofenotyp (B/T) a místo relapsu onemocnění (v kostní dřeni nebo mimo kostní dřeň (extramedulární) nebo kombinovaný). Podle těchto tří faktorů v rámci protokolu ALL REZ BFM 2002, podle kterého se léčí pacienti i v České Republice, se pacienti rozdělují do 4 skupin S1 až S4 (rozdělení tabulka 4).

lokalizace / časový bod	BCP ALL			T ALL	
	izolovaný mimodřeňový	kombinovaný dřeňový	izolovaný dřeňový	izolovaný mimodřeňový	jakýkoliv dřeňový
velmi časný	S2	S4	S4	S2	S4
časný	S2	S2	S3	S2	S4
pozdní	S1	S2	S2	S1	S4

Tabulka 4. *Přehled prognostických podskupin relapsu ALL.*

S1 skupina

Pacienti v S1 skupině splňují kritéria pozdního izolovaného extramedulárního relapsu B nebo T imunofenotypu, mají nejlepší prognózu a dosahují přežití bez události vyšší 75% bez použití transplantace kostní dřeně.

S2 skupina

U této skupiny (zařazení v tabulce 4) je pravděpodobnost přežití v 5 letech od relapsu asi 45%. Indikace k transplantaci kostní dřeně se v této skupině řídí hladinou reziduální nemoci po dvou blocích chemoterapie. Publikace Eckertové et al. prokázala u pacientů léčených podle protokolů ALL REZ BFM 90, 95 a 96 signifikantně lepší prognózu pacientů s hladinou MRN nižší než 10^{-3} po dvou blocích chemoterapie (pravděpodobnost přežití v 6 letech od relapsu skupiny s nízkou až negativní MRN bylo 86%, pacienti s vysokou MRN všichni zrelabovali) [84]. Na základě studie Eckertové et al. byl designován protokol ALL REZ BFM 2002, kde v podskupině S2 transplantace kostní dřeně je indikována u pacientů s hladinou MRN $\geq 10^{-3}$ po dvou blocích chemoterapie.

S3/S4 skupina

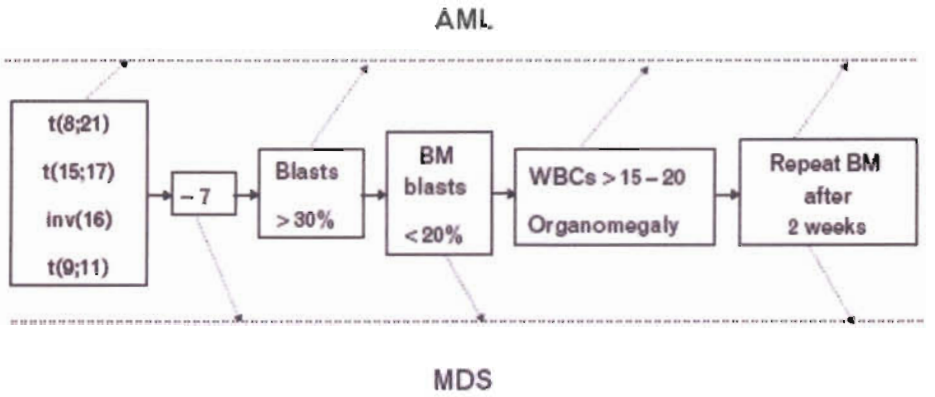
Do této skupiny jsou řazeni pacienti s nejvíce rizikovým typem relapsu ALL (zařazení v tabulce 4). Remise se daří dosáhnou u 80% pacientů ze skupiny S3 a asi jen u poloviny pacientů z S4 podskupiny, medián trvání remise je ale jen asi 8 měsíců (S3), případně pouze 3 měsíce (S4). Do S4 skupiny jsou řazeni pacienti s velmi časným relapsem B prekurzorové leukémie a jakýmkoli dřeňovým relapsem T leukémie. U těchto dětí se remise daří dosáhnout

jen asi v 50 až 60% případů. U těchto pacientů, pokud se podaří dosáhnout kompletní remise, je jednoznačně indikována transplantace kostní dřeně.

1.2. Akutní myeloidní leukémie (AML)

AML je především onemocněním starších dospělých. V dětské populaci je incidence přibližně 8×10^{-6}. Analýza incidence AML podle věku ukazuje kumulaci pacientů do 2 let (12×10^{-6}), dále mírný pokles incidence do 9 let věku ($3,8 \times 10^{-6}$) a dále kontinuální vzestup incidence s každým rokem věku s určitou kumulací incidence kolem 16. roku (9×10^{-6}) [85]. Vyšší incidence AML M3 je popisována např. v Itálii a v Latinské Americe [86, 87]. Zajímavým zjištěním o incidenci M3 podtypu u českých dětí je její dlouhodobě vysoké (13%) zastoupení mezi AML, sice nedosahující hodnot výskytu u dětí v Itálii, Španělsku či Latinské Americe, ale významně vyšší než v SRN, Skandinávii či Velké Británii (~6%) (Starý et al., publikace v přípravě).

Významnou podskupinou jsou pacienti se sekundární AML po léčbě nádorového onemocnění chemoterapií nebo radioterapií, problematika se překrývá se sekundárním MDS [88-95]. Z cytostatik se na vzniku sekundárních AML podílejí nejvíce inhibitory topoizomeráz a alkylační cytostatika. K sekundárním AML řadíme rovněž AML vzniklé jako komplikace u pacientů s definovaným kongenitálním selháním kostní dřeně (např. Kostmannova agranulocytóza, Fanconiho anémie, Diamond Blackfanova anémie, Shwachman-Diamondův syndrom, amegakaryocytární trombocytopénie s radioulnární synostózou) [96]. V České republice je každý rok nově diagnostikováno přibližně 10 - 18 dětí mladších 18 let a dále je asi u 4 dětí ročně diagnostikován relaps onemocnění. V současné době přežívá dlouhodobě více než polovina dětí [97]. Zlepšení prognózy u dětí bylo stejně jako u ALL dosaženo pomocí randomizovaných studií. Za selhání léčby jsou zodpovědné jak relapsy, tak i agresivnější biologické chování nemoci a toxicita vlastní léčby, která je významně intenzivnější než u ALL [98, 99]. Důležité pro prognózu pacienta je rovněž správné zhodnocení všech vstupních vyšetření, zejména při hraničním počtu blastů a negativitě fúzních genů AML1/ETO a CBFβ/MYH11, zda se nejedná o pokročilou formu myelodysplastického syndromu (RAEB), u kterého je u dětí jednoznačně indikována alogenní transplantace kostní dřeně a u kterého intenzivní léčba není zcela jednoznačně indikována [100, 101]. Algoritmus pro odlišení těchto jednotek u sporných případů je uveden na obrázku 7. Při pozitivitě již zmíněných rekurentních genetických abnormit AML1/ETO a CBFβ/MYH11 je pacient léčen jako AML při libovolném procentu blastů v KD.



Obrázek 7. *(převzato z review Hasleho et al. [102]). Schéma diagnostického algoritmu k rozlišení AML a MDS u pacientů s hraničním počtem blastů v kostní dřeni. Schéma nepřímo demonstruje blízkost obou onemocnění, zejména při absenci typických cytogenetických změn.*

1.2.1. Prognostické faktory a klasifikace AML

Morfologická klasifikace

Na rozdíl od ALL, kde morfologie blastů kromě L3 podtypu prakticky nekoreluje s imunologickým nálezem, u AML má morfologické rozdělení stále své významné místo [103]. Nejznámějším a dosud používaným rozdělením je tzv. FAB klasifikace (Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group) [104], která hodnotí morfologickou a cytochemickou zralost leukémie (M0 – s minimální diferenciací, M1 – bez vyzrávání, M2 – s vyzráváním, M3 - promyelocytární, M4 – myelomonocytární, M5 – monocytární, M6 – erytroleukémie, M7 – megakaryoblastická). Podtyp M2 koreluje s přítomností fúzního genu AML1/ETO. Podtyp M4eo koreluje s fúzním genem CBFβ/MYH11. Podtyp M7 specificky v dětském věku nacházíme u malých dětí s m. Down. U dětí s AML M7 a bez Downova syndromu nejčastější strukturální abnormitou je t(1;22)(p13;q13), která je asociována s velmi nízkým mediánem věku (4,2 měsíce) [105]. M6 a M7 u starších dětí bez m. Down se částečně překrývá s problematikou pokročilých forem MDS [106]. U podtypu M4 a M5 často nacházíme přestavby MLL genu. Později FAB klasifikace zahrnula i hodnocení imunofenotypu [107], zejména pro definici podtypu AML M7 a M0. Současná klasifikace WHO[108] hodnotí nález podle přítomnosti cytogenetických abnormit (t(8;21)(q22;q22) (AML1/ETO), t(15;17)(q22;q12) (PML/RARα), inv(16) (CBFβ/MYH11), AML s abnormitami 11q23 lokusu, komplexní karyotyp), podle morfologie (AML s multilineární dysplázií, rovněž předchozí FAB kategorie jsou ponechány pro cytogeneticky

neklasifikované AML) a podle geneze vzniku (sekundární AML po léčbě pro jiné nádorové onemocnění nebo po předchozím myelodysplastickém syndromu).

1.2.1.1. Imunologická klasifikace AML

Dosud nejrozsáhlejší studie zabývající se hodnocením korelace morfologie s imunofenotypem a prognostickým významem exprese některých antigenů u dětské AML je práce profesorky Creutzigové z roku 1995 [109]. Tato práce nenašla prognostický význam žádného z vyšetřených antigenů. Imunofenotyp koreluje dobře se specifickými genotypovými podskupinami AML, jakými jsou například AML1/ETO, CBFβ/MYH11 a PML/RARα [110].

1.2.1.2. Genotypová klasifikace, epigenetické změny u AML a jejich význam pro terapii

Genotypová klasifikace v sobě odráží heterogenitu AML. V současné době často zmiňovaná hypotéza definuje tzv. leukemickou kmenovou buňku jako buňku se schopností sebeobnovy. V této leukemické kmenové buňce jsou nakumulované genetické změny ovlivňující jejich diferenciaci a proliferaci. Tyto genetické změny podle současných znalostí dělíme do 3 skupin [111]:

a) mutace typu I indukují proliferaci a vytvářejí růstovou výhodu proti buňkám bez mutace (např. FLT3/ITD, resp. FLT3 mutace, c-KIT, RAS, PTPN11, JAK2).
b) mutace typu II blokují myeloidní diferenciaci a dávají buňkám schopnost sebeobnovy (např. AML1, CEBPα, WT1, PML-RARα)
c) mutace v genech zapojených do kontroly buněčného cyklu a apoptózy (např. NPM1 (nukleofosmin), TP53)

Mutace typu I a II často nacházíme u pacientů s AML současně, což částečně podporuje hypotézu vzniku AML pomocí dvou zásahů do genomu [112].

V současné době jsou pro léčbu pacientů důležité tzv. epigenetické změny. Epigenetické změny jsou změny, které nejsou důsledkem změny sekvence DNA, ale ovlivňují regulaci exprese genů, typicky posttranslační modifikace histonů, metylace cytosinových bazí. Acetylace histonů zpravidla vede k expresi genů, deacetylace vede zpravidla k utlumení exprese genů.

1.2.1.2.1. AML s fúzním genem AML1/ETO, CBFβ/MYH11 a PML/RARα

Přítomnost fúzních genů AML1/ETO a CBFβ/MYH11 je spojena s lepší prognózou onemocnění a celkem tyto dvě translokace nacházíme asi u 7 až 8% dětí s AML [113].

Gen AML1 kóduje protein CBFα2, který je součástí CBF (core binding factor) heterodimeru zodpovědného za regulaci velkého počtu genů. Součástí CBF komplexu je rovněž CBFβ. Fúzní protein způsobí špatnou funkci CBF a nepřímo tak represi velkého počtu genů [114]. Tyto leukémie jsou někdy označovány jako tzv. **core binding factor leukémie** [115]. Tyto leukémie jsou v řadě studií řazeny do lepšího rizika a obecně se u nich neindikuje transplantace kostní dřeně v první kompletní remisi. U AML s fúzním genem AML1/ETO nacházíme imunologicky často diferenciaci leukemických buněk, která koreluje s morfologickou heterogenitou blastů, a typicky aberantní expresi CD19, někdy nacházíme i pozitivitu intra-TdT a intra-CD79a. Tyto pozitivity lymfoidních antigenů mohou někdy vést k diagnostickým rozpakům [116, 117], ale podle našich zkušeností jsou často přítomny jen v menší subpopulaci blastů a zpravidla ani nevedou k překročení AHL skóre podle EGIL klasifikace. Imunohistochemicky je často u tohoto podtypu prokazatelný B lymfoidní transkripční faktor PAX5 [118-120]. AML s fúzním genem CBFβ/MYH11 typicky spadají do morfologické podskupiny AML M4eo, imunologicky je prokazatelná diferenciace blastů s častou pozitivitou aberantní CD2 a CD7, rovněž je zřetelná i podle imunofenotypu atypická eosinofilie.

Přítomnost fúzního genu PML/RARα velmi těsně koreluje s AML M3 podtypem a tato leukémie je dnes léčena spolu s klasickou chemoterapií derivátem all – trans retinové kyseliny (ATRA), která indukuje diferenciaci blastů [121]. Imunofenotypově blasty odpovídají částečně promyelocytům (pozitivita CD117, CD15, jasná CD33), časté jsou aberantní exprese CD2 (především u variantního M3 podtypu) a CD19. Typická je negativita HLA DR a CD34. Tento podtyp AML je dnes nejlépe léčitelný [122].

1.2.1.2.2. AML/MDS u pacientů s konstitutivní trizómií 21 (m. Down)

Zvláštní kapitolu představuje AML u pacientů s konstitutivní trizómií 21. chromozómu – s Downovým syndromem. U těchto pacientů je známa vyšší incidence leukémií zejména AML a MDS [123]. AML/MDS u pacientů s Downovým syndromem mladších 4 let má charakteristický fenotyp (AML M7) [124]. Pacienti s touto formou leukémie mají výbornou prognózu, častěji jsou ale ohroženi toxicitou léčby, je tedy snaha u nich léčbu redukovat [125, 126]. I přes redukci intenzity léčby je prognóza těchto pacientů velmi dobrá. Tyto leukémie mají specifický imunofenotyp s kombinovanou diferenciací do megakaryocytární a erytroidní linie [127]. Typicky prokazujeme aberantní expresi CD7 a CD56, typicky je dále pozitivní molekula CD4 na CD33 pozitivních blastech. Součástí diferenciace do megakaryocytární linie je pozitivita trombocytárních antigenů CD41, CD42 a CD61. Pro diferenciaci do erytroidní

linie svědčí pozitivita molekuly CD36 a CD71 (zpravidla s vyšší intenzitou exprese CD71 než mají proliferující buňky obecně). Nemaligní $CD33^{poz}$ myeloidní buňky pacientů s m. Down v regenerující fázi kostní dřeně významně exprimují aberantní molekulu CD56 (71%±6% u pacientů s m. Down versus 4%±1% u pacientů bez Downova syndromu a léčených pro AML [128]). Vysoká exprese CD56 (NCAM) korelovala v této studii s vysokou expresí genu RUNX1 [128], který je lokalizovaný na dlouhém raménku 21. chromozómu (21q22.3). Tuto odlišnost těchto pacientů v regeneraci kostní dřeně v podmínkách stresové hematopoézy je nutné mít na paměti při hodnocení MRN u pacientů s m. Down. Biologie AML u pacientů s m.Down starších 4 let se již podobá více sporadické formě tohoto onemocnění [129].

1.2.1.2.3. AML s přestavbami genu MLL (11q23)

AML s přestavbami 11q23 jsou časté spíše u menších dětí [130]. Morfologicky jsou zpravidla klasifikovány jako podtypy M4 či M5 dle FAB klasifikace, čili mají naznačenou či úplnou monocytární diferenciaci. Tomu většinou odpovídá i imunofenotyp. Navíc často aberantně exprimují NG2 (molekulu chondroitin sulfátu korelující s přestavbami MLL genu). Vztah k prognóze je v literatuře rozporný, současný názor je nestratifikovat tyto pacienty do horšího rizika jen na podkladě přestavby 11q23 [131], v některých studiích dokonce tato skupina měla lepší prognózu [132].

1.2.1.2.4. AML s FLT3 interní tandemovou duplikací (ITD) nebo aktivační mutací FLT3(D835)

AML s FLT3 mutacemi odpovídají horší prognóze a u dětí jsou přítomny asi v 15% případů, s horší prognózou jsou spojeny především mutace vedoucí ke konstitutivní aktivaci signalizace spuštěné přes FLT3 [133, 134]. V protokolu AML BFM 2004 jsou tito pacienti řazeni do vyššího rizika.

1.2.1.3. Klinické a biologické projevy AML, primárně extramedulární leukémie

Mezi projevy nemoci nacházíme příznaky zmíněné u ALL, vyplývající jak z útlaku nemaligní hematopoézy, tak i projevy způsobené zmnožením leukemických buněk. Obecně je AML velmi agresivní onemocnění vyžadující rychlé stanovení diagnózy a co nejčasnější zahájení intenzivní léčby. Mezi faktory ovlivňující časnou prognózu patří koagulopatie, zejména diseminovaná intravaskulární koagulopatie, která může být poměrně rychle fatální a leukostáza. Mezi faktory spojené s koagulopatií patří především M3, M4, M5 podtyp,

hyperleukocytóza a infekce [98, 99]. Při diagnóze onemocnění někdy nacházíme extramedulární leukemické infiltráty (kůže, CNS apod.). Vzácně jsou diagnostikovány AML s primární extramedulární infiltrací bez infiltrace kostní dřeně – tzv. myelosarkom [135]. Nejčastěji infiltrace postihuje kůži, prakticky ale může být infiltrováno jakékoli místo v těle (měkké tkáně, CNS, orbita atd.). Pacienti mohou být na rozdíl od pacientů s AML primárně infiltrující kostní dřeň zpravidla v lepším klinickém stavu a často se primárně myslí na jiný původ nádoru. Interpretace biopsie může být někdy obtížná, průtoková cytometrie v těchto situacích může vést rychle ke správné diagnóze. U dětí se často se nachází myelomonocytární diferenciace, ze studie profesora Reinhardta a profesorky Creutzigové 73% pacientů bylo podle FAB klasifikováno jako M5 podtyp [135, 136]. Cytogeneticky, resp. molekulárně geneticky nejčastější identifikovanou abnormitou byl nález translokace t(9;11) a (8;21), většina pacientů neměla ale zachycenou žádnou odchylku v genotypu [135, 136]. Pro vzácnost myelosarkomu je obtížné dělat závěry o prognóze, z retrospektivních dat se ukazovala horší prognóza, která mohla být způsobena odkladem léčby nebo nižší intenzitou léčby nejčastěji na podkladě špatné diagnózy (např. za záměnou za NHL) [135]. V současné době by měli pacienti s myelosarkomem být zcela léčeni v rámci AML protokolů.

1.2.1.4. Farmakogenetika AML

Podobně jako u ALL nacházíme individuální odchylky v citlivosti leukemických buněk a v toxických účincích na normální tkáně u jednotlivých cytostatik. Podkladem jsou polymorfismy nejrůznějších genů podílejících se mimo jiné na metabolizmu těchto látek [137]. Studie CCG prokázaly horší výsledky léčby u dětí hispánského a černošského původu, pravděpodobným podkladem je rozdílná farmakogenomika. Dosud byly publikovány spíše jednotlivé polymorfismy a jejich vztah k prognóze, např. byl publikován vztah ztráty funkce glutathion S-transferázy theta 1 (GSTT1) a častějších toxických komplikací a horším přežitím v porovnání s pacienty s alespoň jednou funkční alelou [138-140].

1.2.1.5. Princip léčby AML, perspektivní nová léčiva

Léčba AML je velmi intenzivní a je postavena na limitovaném počtu bloků chemoterapie s použitím cytosin arabinosidu a antracyklinů (obrázek 8). Většina skupin zabývajících se léčbou AML v Evropě neindikuje v iniciální léčbě alogenní transplantaci kostní dřeně, jiné léčebné skupiny např. COG (Children´s Oncology Group, USA) indikují alogenní transplantaci v první remisi u všech pacientů kromě pacientů s nejlepší prognózou s fúzním genem AML1/ETO a CBFβ/MYH11. Velkým problémem léčby AML je velký počet smrtí

v souvislosti s léčbou (tzv. „treatment related deaths") a nabízí se hledání specifické biologické léčby s menším počtem nežádoucích účinků [137]. Velký počet nových léků je v současné době vyvíjen a testován, ale jejich testování probíhá především ve studiích zahrnující dospělé pacienty. Rozšíření těchto léku do léčebných protokolů pro děti je obtížné pro celkově malý počet pacientů. Podobně jako u ALL hraje v léčbě AML významnou roli léčba subklinického postižení CNS chemoterapií, část léčebných studií u pacientů indikuje i radioterapii CNS (např. protokoly BFM s výjimkou pacientů mladších 1 roku) [97, 141]. Nicméně podobně jako u ALL je zřetelný ústup od preventivního ozařování CNS.

1.2.1.5.1. Léčba AML M3 s fúzním genem PML/RARα

Příkladem již používané léčby se specifickým účinkem na myeloidní buňky je použití ATRy (all trans retinoic acid) u pacientů s fúzním genem PML/RARα, které vede k diferenciaci leukemických buněk. V současné době se u tohoto podtypu AML začíná zatím především u dospělých používat oxid arsenitý (As_2O_3) v kombinaci s ATRou [142, 143]. Probíhají studie s indukční léčbou postavenou pouze na ATře a na As_2O_3 [144]. Otázka, zda je možné u tohoto podtypu v budoucnu dosáhnout dlouhodobého vyléčení bez intenzivní chemoterapie, se v současné době řeší v rámci randomizovaných studií. U dětí jsou zkušenosti s As_2O_3 ještě více limitované, v ČR zatím byl léčen pouze jeden dětský pacient s relapsem AML M3, který na této léčbě nedosáhl molekulární remise a vyvinul další hematologický relaps, který byl léčen intenzivní chemoterapií a alogenní transplantací.

1.2.1.5.2. Imunoterapie AML

Molekula CD33 je exprimována prakticky na všech blastech u AML. Anti- CD33 léčba s konjugovaným cytostatikem calicheamicinem **gemtuzumab ozogamicin (GO)** byla původně designována pro léčbu starších pacientů s AML, netolerujících standardní chemoterapii. Postupně je tento lék používán již v iniciální léčbě v kombinaci s ostatními cytostatiky, zatím především u dospělých pacientů [145]. Jsou už publikovány i zkušenosti s tímto lékem u dětí jak jednotlivě[146, 147], tak i v kombinaci s ostatními cytostatiky [148]. U dětí je zatím GO spíše využíván jako záchranná léčba při selhání standardních bloků chemoterapie. Nevýhodou tohoto léku je možná komplikace ve formě venookluzivní nemoci (VOD) [149, 150] a opožděné rekonstituce počtu trombocytů [151]. Za VOD pravděpodobně stojí interakce GO s Kupfferovými buňkami jater, které jsou rovněž $CD33^{poz}$. Při použití analogického konjugátu calicheamicinu s jinou protilátkou (anti-CD22) se sice opožďuje rekonstituce trombocytů, ale VOD zatím nebyla pozorována. Pravděpodobné jsou i určité

imunopatologické mechanismy, vzhledem k publikované úspěšné léčbě trombocytopénie po GO intravenózními imunoglobuliny [152]. U AML M3 je rovněž dokumentována i excelentní odpověď na imunoterapii pomocí jiné anti-CD33 protilátky i bez konjugovaného cytostatika [153]. Na AML blastech se dále nachází i řada dalších vhodných cílů pro imunoterapii (např. anti-CD52, anti-CD45 apod.) [154-156]. Důležitou otázkou, pokud lze akceptovat hypotézu leukemické kmenové buňky, je přítomnost cílové struktury právě na této populaci buněk. Zdá se, že molekula CD33 je přítomna i na populaci leukemických kmenových buněk [157, 158]. Jiným vhodným cílem na leukemických kmenových buňkách se zdá být molekula CD44 [159].

1.2.1.5.3. Inhibitory kináz

Zvýšená kinázová aktivita hraje významnou roli v patogenezi AML (např. signalizace přes c kit či flt3). Tuto aktivitu lze například blokovat **imatinibem** nebo tzv. duálním inhibitorem **dasatinibem** (je prokázána i účinnost u pacientů s mutací *C-KIT* [160]) (viz léčba BCR/ABL^{poz} ALL) [161]. Jako perspektivní se jeví rovněž inhibice flt3 pomocí např. **PKC412** [162, 163]. Snahou je tyto léky v současné době zapojovat do standardních léčebných protokolů, podobně jako u Ph^{poz} ALL je důležité i vyřešit vhodné načasování této léčby v kontextu ostatní chemoterapie (např. jeden lék může fungovat jako senzitizátor pro lék druhý, v opačném případě kombinace nemusí být vůbec účinná, někdy může být vhodnější léky dávat současně). Na tomto místě je rovněž nutné zmínit blokátory farnesyl transferázy, které vedou k blokádě RAS signalizační dráhy.

1.2.1.5.4. Inhibitory histon deacetyláz, demetylující látky, terapeutické ovlivnění epigenetických změn u leukemických buněk

Jak již bylo zmíněno výše, jedna ze změn regulujících expresi genu je acetylace resp. deacetylace histonů. Typicky u již zmíněných tzv."core binding leukémií" (AML1/ETO nebo CBFβ/MYH11) může léčba **inhibitory histon deacetyláz (HDAC)** podobně jako ATRA u PML/RARα pozitivní AML indukovat diferenciaci a apoptózu [164]. Nejznámějším inhibitorem je kyselina valproová, používaná v humánní medicíně jako antiepileptikum, což významně usnadňuje zařazení tohoto léku do léčby leukémií [165]. Dalším známým inhibitorem HDAC je trichostatin nebo depsipeptide[166, 167], postupně jsou vyvíjeny i nové inhibitory [168].

Dalším mechanismem, kterým jsou regulovány exprese jednotlivých genů, je již zmíněná **metylace, resp. demetylace** cytozinových bazí v promotorových CpG oblastech. V současné době jsou dostupné léky, které jsou schopné demetylovat metylové skupiny na cytozinech. Hypermetylace nebo aberantní metylace jsou častou změnou u AML/MDS [169-171]. Tyto léky

jsou primárně určené pro léčbu MDS v dospělém věku a patří sem např. 5-aza-2´-deoxycytidine (decitabine) [172]. Léčba vede k lepšímu vyzrávání jednotlivých linií v kostní dřeni, snižuje potřebu transfúzí a redukuje počet infekčních komplikací.

1.2.1.5.5. Nová cytostatika

Rezistence na cytosin arabinosid je jedním z nejdůležitějších faktorů selhání léčby AML, a proto je snaha vyvíjet nové analogy nukleotidů nukleových kyselin.

2-chlorodeoxyadenosin (cladribine) podobně jako cytosin arabinosid je fosforylován na trifosfát a je tak inkorporován do DNA během její syntézy, postupně vede k zástavě buněčného dělení a k buněčné smrti. Dokáže indukovat apoptózu u neproliferujících buněk a je rezistentní vůči inaktivaci deaminací. Je prokázána větší senzitivita podtypu FAB M5 [173]. V rámci protokolu AML BFM 2004 je cladribine zařazen pro léčbu AML vysokého rizika, do kterého jsou řazeni i pacienti s AML M5 podtypem.

Clofarabine je podobně jako cladribine analog nukleosidů a kombinuje farmakokinetické vlastnosti fludarabinu a cladribinu. U pacientů s relapsem/refrakterní AML byla prokázána jen částečná odpověď na toto cytostatikum [174].

1.2.1.5.6. Odpověď na léčbu AML, terapeutické ovlivnění leukemické kmenové buňky

1.2.1.5.6.1. Odpověď na léčbu definovaná morfologicky

Na rozdíl od ALL jsou pacienti s AML léčeni podstatně agresivnější chemoterapií s vyšší toxicitou až úmrtností v souvislosti s terapií. S agresivnější chemoterapií souvisí i nemožnost sledování bližší dynamiky poklesu leukémie, protože naprostá většina pacientů rychle upadá do aplázie (schéma léčebných protokolů BFM98 a 2004 je na obrázku 8). Léčebná stratifikace v jednotlivých protokolech zahrnuje hodnocení blastů po jednom až dvou blocích chemoterapie. V protokolech BFM skupiny je pacient s četností blastů >5% po 1. bloku zařazen do vyššího rizika [175]. Podobně jako u ALL většina pacientů dosáhne kompletní remise po 1. indukčním bloku, výjimkou jsou pacienti s AML M3, kteří remise v klasickém smyslu slova často nedosáhnou, přesto je prognóza obecně dobrá.

1.2.1.5.6.2. Molekulárně genetické a cytogenetické cíle detekce MRN

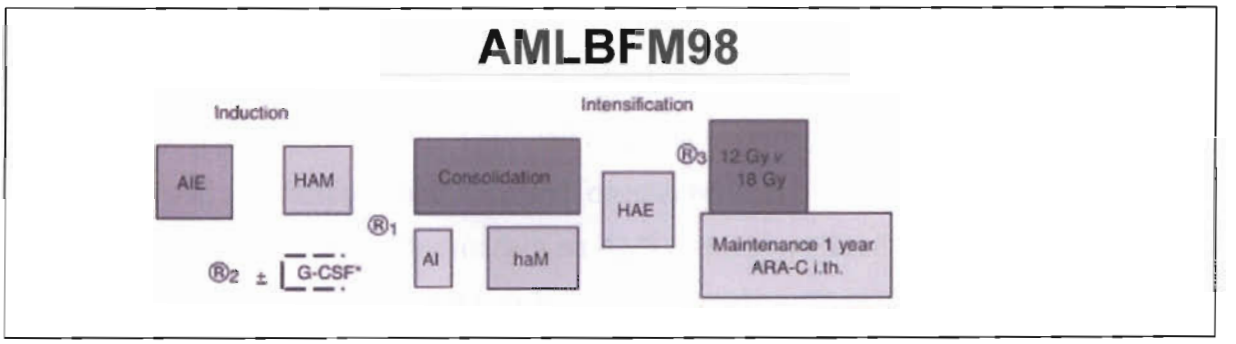
Detekce minimální reziduální nemoci nemá na rozdíl od ALL obecně přijímaný zlatý standard. Jen u malé části pacientů lze použít molekulárně genetický cíl (např. AML1/ETO, CBFβ/MYH11, PML/RARα, přestavby 11q23 s identifikovaným fúzním partnerem) [176-178].

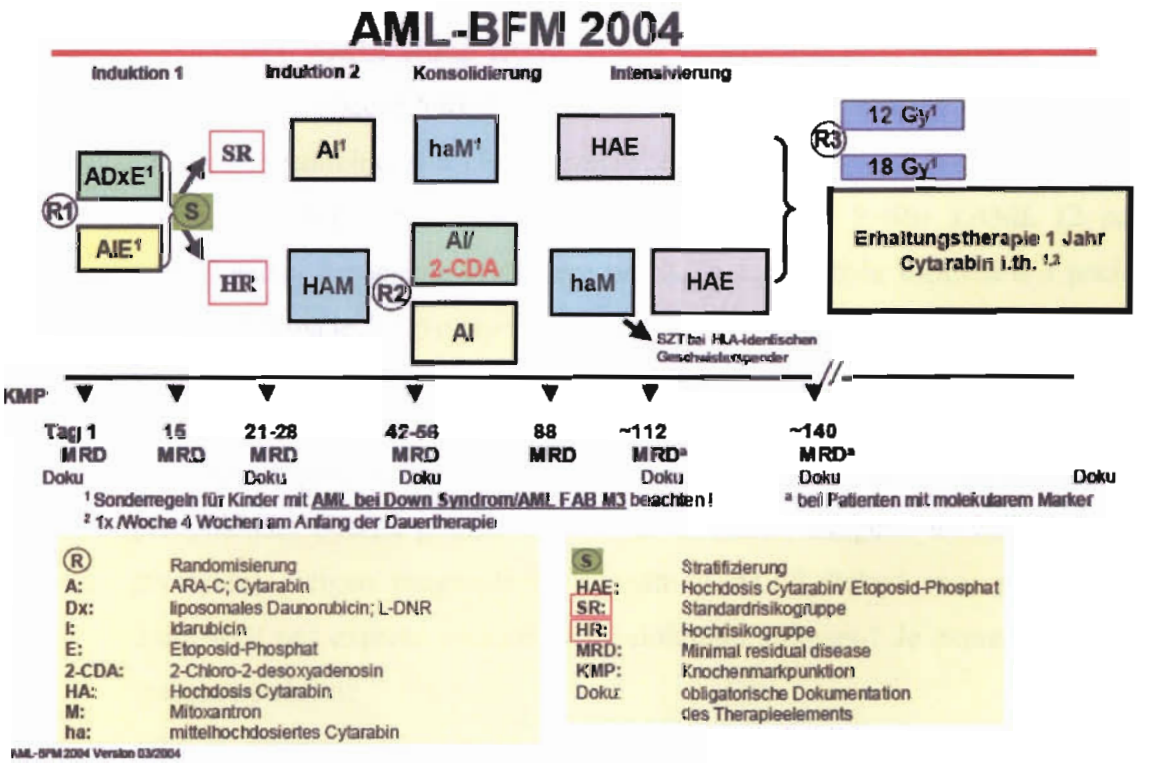
Kromě pacientů s fúzním genem PML/RARα není přesně definován význam molekulární remise ve smyslu úpravy léčebné strategie. U pacientů se znovuobjevenou PML/RARα pozitivitou je indikována léčba pomocí ATRy, resp. As_2O_3. Naopak jsou publikovány případy pacientů s několikaletou pozitivitou fúzních genů AML1/ETO nebo CBFβ/MYH11 v kompletní remisi [179]. Nejen z našich zkušeností spíše tato pozitivita vedla v různém časovém intervalu k relapsu onemocnění a dosažení molekulární remise je zpravidla podmínkou vyléčení [180]. Publikované práce s perzistující pozitivitou AML1/ETO ne vždy dostatečně dlouho sledovaly pozitivní pacienty, jejichž MRN může přetrvávat i řadu let [179]. Zprvu slibný marker AML, gen WT-1, lze použít ke sledování jen s obtížemi – často je poměrně slabě exprimován ve srovnání s nemaligním pozadím [181-183]. Mutace v transkripčním faktoru GATA-1 jsou typické pro myeloproliferace charakteristické pro Downův syndrom (TMD a AML-M7) a lze je využít jako případné cíle pro sledování MRN [184-186]. Pro detekci MRN lze rovněž u některých pacientů s vhodnou genetickou změnou použít metody cytogenetické zejména fluorescenční in situ hybridizaci (FISH) [187]

1.2.1.5.6.3. Princip detekce MRN cytometricky u AML

Vývoj fyziologické myeloidní řady je podstatně méně imunologicky definovaný. Zajímají nás především antigeny asynchronně exprimované (antigeny asociované s progenitory a antigeny charakteristicky exprimované na zralejších myeloidních buňkách, např. exprese myeloidního antigenu CD15 na $CD34^{poz}$ blastech), antigeny aberantně exprimované (např. CD19, CD56, CD7, CD2 nebo NG2) nebo hyperexprese či snížená exprese antigenů oproti fyziologickým buňkám. Pro AML je typická imunofenotypová heterogenita blastů a je zpravidla nutné MRN detekovat více kombinacemi protilátek [188, 189] (**příloha 4**). Langebrake et al. rovněž prokázala srovnáním imunofenotypu AML u dětí při diagnóze a při relapsu, že změnu v expresi alespoň jednoho antigenu má 88% sledovaných pacientů [190]. Je tedy zřejmé, že v rámci detekce AML je potřeba hledat univerzální odchylky leukemických buněk od nemaligní kostní dřeně bez ohledu na iniciální imunofenotyp pacienta. Průtoková cytometrie se na rozdíl od molekulárně genetických metod nabízí jako metodika dostupná pro prakticky všechny pacienty. Jak u dospělých [191], tak u dětí v regenerující KD nacházíme normální prekurzorové buňky splňující kritéria leukemického imunofenotypu v některých časových bodech až v řádu procent [192]. V rámci mezinárodní studie, kterou koordinovali kolegové z Německa, jsme prokázali prognostický význam reziduální nemoci u AML pouze v univariantních analýzách, nikoli v rámci multivariantní analýzy zahrnující všechna kritéria použitá pro stratifikaci pacientů[189]. Dosud publikované studie se zabývaly sledováním MRN průtokovou cytometrií u AML

především u dospělých pacientů [178, 191, 193-196]. I přes vzácnost AML u dětí byly publikovány studie u dětí ukazující na horší prognózu dětí s prokazatelnou MRN podle průtokové cytometrie [197-199]. Interpretaci MRN u AML komplikují dvě okolností: již zmíněná nestabilita imunofenotypu leukemických buněk a „nestandardní chování" regenerujících nemaligních prekurzorů, které mohou exprimovat i molekuly považované za asynchronní či aberantní. Proto je vhodné definovat normy buď v jednotlivých časových bodech [192], nebo nalézt vzorce exprese antigenů, které neprokazujeme na regenerujících prekurzorech [200-202]. Studie van Rhenen et al. se zabývala především detekcí imunofenotypových odlišností $CD34^{poz}CD38^{neg}$ leukemických buněk a $CD34^{poz}CD38^{neg}$ prekurzorů. V rámci fyziologické kostní dřeně jsou $CD34^{poz}CD38^{neg}$ Lin^{neg} prekurzory považované za nejvíce kmenové a s největším potenciálem repopulace [203]. Analogicky u AML je tato subpopulace blastů považována za nejvíce kmenovou [204, 205]. Tato subpopulace může z celé populace blastů tvořit variabilní část, častěji tento imunofenotyp je přítomen u nediferencovaných AML (AML M0) [206].





Obrázek 8. Léčebný protokol AML BFM 98 a 2004 *(AML BFM 98 schéma převzato z publikace [141], AML BFM 2004 převzato z protokolu). Je patrné, že léčba AML je již od začátku velmi intenzivní, takže většina pacientů velmi rychle upadá do aplázie. Časné hodnocení odpovědi v den 15 slouží jako přídatné kritérium pro stanovení rizika (≥5% vysoké riziko).*

1.2.2. Přístup k pacientům s relapsem AML a k primárně vysoce rizikové AML

Jak již bylo zmíněno výše, různé pracovní skupiny zařazují alogenní SCT pro rozdílnou část pacientů. Obecně panuje shoda neindikovat SCT v první kompletní remisi u pacientů s příznivou cytogenetikou (AML1/ETO^{poz}, CBFβ/MYH11^{poz}). Přístup BFM skupiny, podle jejíchž protokolů se léčí i děti v ČR, je velmi rezervovaný k SCT v první kompletní remisi u

de novo AML a obecně není indikována. V případě relapsu AML je SCT důležitou léčebnou modalitou. Obecně prognóza relapsu závisí na několika faktorech, jedním z nejdůležitějších je délka první remise (<1 rok, >1 rok). Další významnou podskupinou pacientů indikovaných k SCT jsou pacienti se sekundární AML, jak na podkladě kongenitálního selhání kostní dřeně, tak v souvislosti s léčbou pro jiné nádorové onemocnění. Pacienti s AML vzniklou po léčbě pro jiné nádorové onemocnění lze rozdělit na AML vzniklé po léčbě inhibitory topoizomeráz, které mají krátký odstup vzniku od této léčby (1 až 3 roky) a typicky nesou přestavby MLL genu, a na AML po alkylujících cytostaticích, které vznikají později (4 až 6 let po primární léčbě), pro něž jsou typické změny na 7. a 5. chromozómu, fáze sekundárního MDS může předcházet sekundární AML. Nová léčiva včetně monoklonálních protilátek jsou u dětí v porovnání s dospělými obecně hůře dostupná. Již zmíněná kombinovaná imuno (anti-CD33, konjugovaná s calicheamicinem) a chemoterapie je u dětí indikována v případě rezistentní nemoci nebo relapsu. V ČR byla tato léčba použita dosud u 5 dětí s AML (2 pacienti s rezistentním relapsem, 3 pacienti s relapsem po SCT). Léčba byla úspěšná u 3 pacientů a umožnila SCT (ve 2 případech šlo o retransplantaci).

2. Cíle

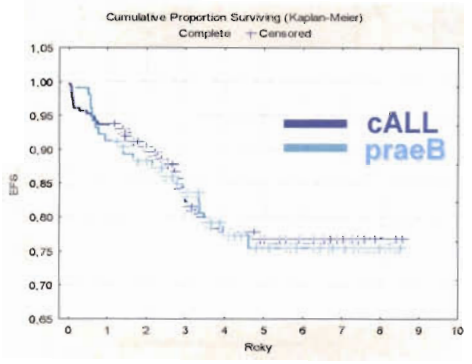
1) Je mezi myeloidními antigeny exprimovanými u ALL souvislost? Dá se předpokládat obecná příčina exprese myeloidních antigenů u ALL? Má některý myeloidní antigen prognostický význam u ALL? Pokud ano, je tato exprese důležitější než exprese jakéhokoli myeloidního antigenu? Je exprese myeloidních antigenů stabilní?
2) Jaký je prognostický význam diferenciačního antigenu CD10 u B prekurzorové ALL?
3) Jaký je význam nových kritérií definovaných podle MRN? Význam průtokové cytometrie v monitorování časné léčebné odpovědi.
4) Význam MRN u transplantovaných dětí s ALL. Ovlivňuje rekonstituce nemaligní B řady specificitu MRN podle přestaveb imunoglobulinových genů?
5) Zjistit četnost, biologický charakter a prognózu sekundární ALL po léčbě ALL.
6) Prognostický význam 4barevné cytometrie v monitorování MRN u dětí s AML.

3. Výsledky a Diskuse

3.1. Imunologická diagnostika ALL

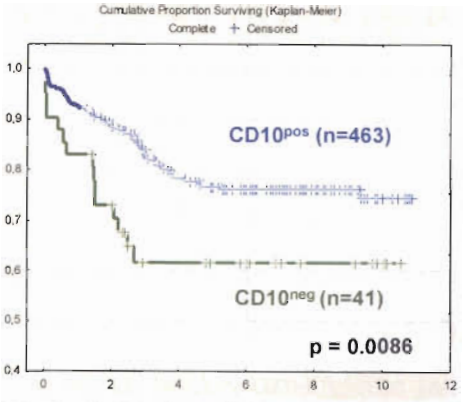
3.1.1. ALL z prekurzorů B lymfocytů (BCP ALL)

B prekurzorové ALL imunologicky členíme na proB, common a praeB [23] podle exprese antigenu CD10 a intracelulárního IgM. Funkce molekuly CD10 na leukemických a na fyziologických prekurzorech není známa. Prognosticky nepříznivý význam má především podtyp proB ALL (CD10 negativní a intra-IgM negativní), který koreluje s přestavbami genu MLL (11q23) a je častý u kojenců. Naopak v podstatě žádný prognostický význam nemá klasifikace podle pozitivity intracelulárního IgM na podskupinu praeB a cALL (Obrázek 9). Data jak z dospělých, tak i dětských ALL navíc ukazují, že ani pro korelaci s přestavbami genu MLL není intracelulární exprese IgM důležitá [207, 208].

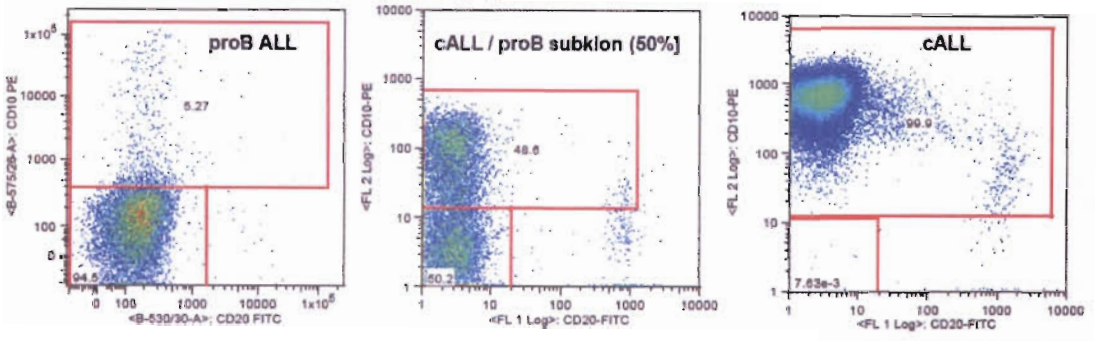


Obrázek 9. *Obrázek ukazuje stejné přežití u pacientů diagnostikovaných jako cALL nebo praeB ALL. V analýze jsou zahrnuti všichni pacienti s námi určenou diagnosou cALL nebo praeB ALL v letech 1999 až 2006,* n=356, *p=ns.*

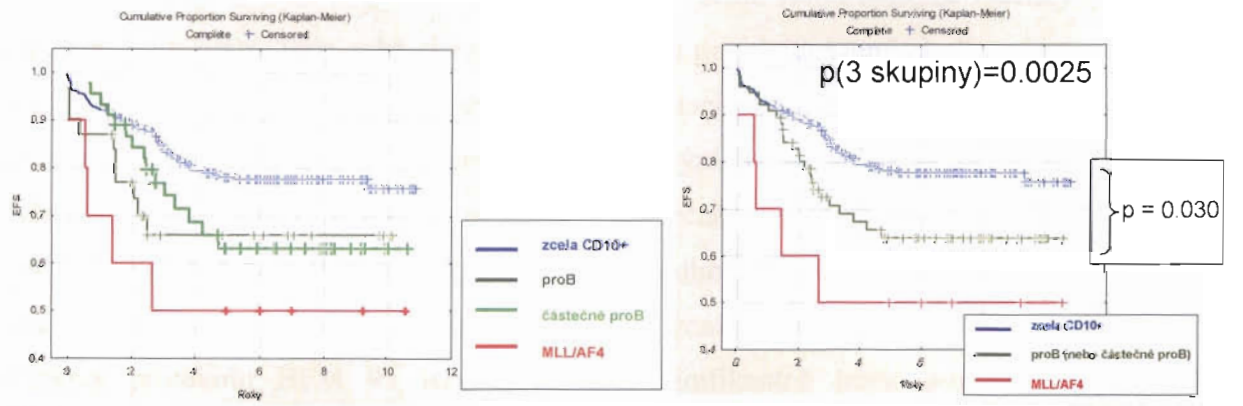
Na obrázku 10 je znázorněna analýza BCP ALL klasifikovaných do dvou skupin podle exprese antigenu na $CD10^{poz}$ (≥20%) a $CD10^{neg}$, ukazující signifikantní rozdíl v pravděpodobnosti v přežití. Exprese antigenu CD10 i u pacientů, které hodnotíme jako pozitivní, se u jednotlivých pacientů mohou významně lišit (od pozitivity hraničního počtu blastů s většinou blastů negativních až po typickou hyperexpresi všech buněk, obrázek 11). Proto jsme se rozhodli testovat hypotézu, že i pacienti s cALL/praeB ALL s významným podílem $CD10^{neg}$ blastů mají horší prognózu.



Obrázek 10. *Signifikantně horší přežití pacientů s negativitou CD10 (<20%).*



Obrázek 11. *Různá exprese antigenu CD10 při diagnóze BCP ALL, obrázek uprostřed ukazuje typického pacienta s proB subklonem a vpravo je pacientka s typickou hyperexpresí CD10.*



Obrázek 12. Vlevo: *Rozdělení pacientů podle hladiny exprese CD10 (zcela CD10+ jsou pacienti s méně než 20% $CD10^{neg}$ blasty, proB ALL exprimují CD10 na méně než 20% blastů, částečně proB exprimují CD10 (≥20%), ale současně obsahují významný podíl (≥20%) $CD10^{neg}$ blastů, všichni MLL $AF4^{poz}$ pacienti měli expresi CD10 nižší než 20% a nejsou zahrnuti do křivky proB.* ***Vpravo:*** *Stejná analýza se spojenou skupinou proB a částečné proB.*

Signifikantně horší prognóza klasicky definované proB ALL, ukazovaná na obrázku 10, je do značné míry dána nepříznivou MLL/$AF4^{poz}$ ALL; oddělíme-li MLL/$AF4^{poz}$ ALL, přestane být rozdíl mezi proB a ostatními ALL významný. Námi definovaná podskupina „částečně proB“

má ale podobnou prognózu jako proB ALL (obrázek 12) Populační analýza tedy ukazuje, že významná přítomnost $CD10^{neg}$ blastů určuje špatnou prognózu – bez ohledu na to, zda jsou přítomny i $CD10^{poz}$ buňky.

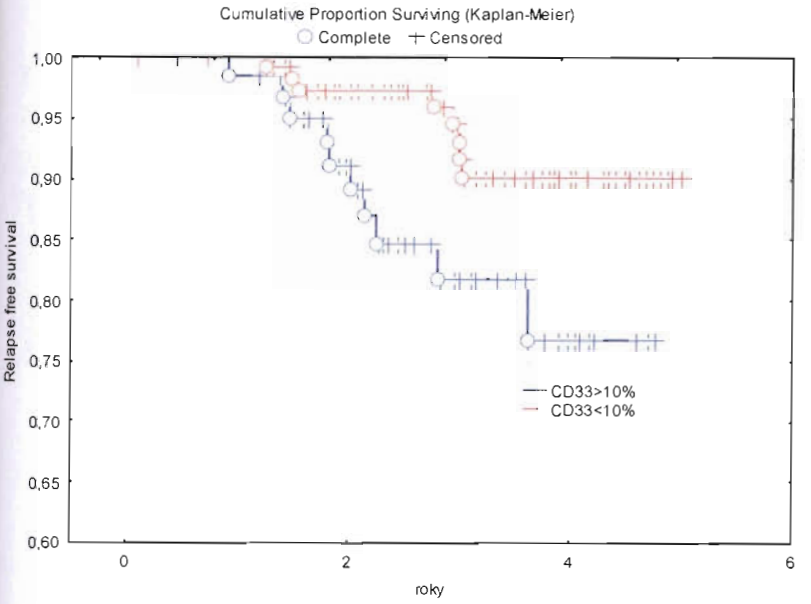
3.1.2. Aberantní exprese antigenů

Jak již bylo zmíněno v Úvodu, leukémie napodobují svým imunofenotypem své nemaligní protějšky v kostní dřeni, resp. v thymu. Expresi antigenů z jiné linie,než z které vycházejí leukemické buňky, označujeme jako aberantní. Přesnou příčinu aberantní exprese u ALL a AML neznáme. V zásadě existují 2 základní hypotézy:

a) aberantní exprese jsou důsledkem deregulované exprese genů v důsledku leukemogeneze (např. mechanismus aberantní exprese CD2 u mikrogranulární varianty AML) [209].

b) aberantní exprese je důsledkem nezralosti leukemických buněk nebo může odpovídat stádiu fyziologických prekurzorů s potenciálem diferencovat do různých linií [210].

Exprese myeloidních antigenů u ALL byla považována na základě prvních studií za marker horší prognózy. Zároveň známou podskupinou exprimující myeloidní antigeny u dětí s ALL jsou pacienti s fúzním genem TEL/AML1 [211, 212] asociovaným s dobrou prognózou. Prognostický význam exprese těchto antigenů v jednotlivých studiích vedl v průběhu let k protichůdným závěrům [212-217]. Slabé místo většiny těchto studií bylo, že všechny myeloidní antigeny byly brány jako sobě si rovné a zpravidla pozitivita kteréhokoli antigenu pacienta klasifikovala jako tzv. MyAg pozitivního. V situaci, kdy neznáme biologický podklad aberantní exprese antigenů, není správné je v analýzách hodnotit společně. Další slabinou některých studií byla analýza B a T ALL společně nebo krátká doba sledování, která nezachytila pacienty s pozdním relapsem ALL. Souhrn charakteristik jednotlivých studií je uveden v tabulce, předložené v rámci recenzního řízení **k příloze 1 (tabulka na straně 81)**. V rámci protokolu BFM 95 jsme prokázali signifikantně horší prognózu BCP ALL s aberantní expresí molekuly CD33 **v příloze 1**. Tuto horší prognózu jsme potvrdili i v multivariantní analýze **v příloze 1**. Horší prognózu jsme potvrdili i u pacientů léčených v další studii (ALL IC BFM 2002) s kratší dobou sledování (BCP ALL: medián 2,9 let, minimum 0,09, maximum 4,99 let) (obrázek 13).



Obrázek 13. *Zřetelně horší přežití bez relapsu u pacientů s BCP ALL a expresí CD33 na více než 10% blastů léčených v rámci protokolu ALL IC BFM 2002 (Cox-Mantelův test, p=0,0204, pRFS ve 4 letech pro $CD33^{neg}$ 90%±3,4%, pro $CD33^{poz}$ 77%±7,2%, pEFS ve 4 letech pro $CD33^{neg}$ 88%±3,5%, pro $CD33^{poz}$ 74%±7,2%). Výsledek nezávisle potvrzuje naše zjištění v příloze 1.*

3.1.3. Akutní hybridní leukémie

Leukémie se signifikantním vzhledem blastů, jak z lymfoidní, tak i myeloidní linie, nazýváme hybridní. Podle typu je lze členit na leukémie s primárně definovanou linií a významnou koexpresí antigenů z jiné linie, dále na leukémie s dvěma liniově různými populacemi definované imunologicky a/nebo morfologicky, a nakonec na leukémie, které signifikantně změní svůj fenotyp do jiné linie před dosažením kompletní remise. BCP ALL je definována podle adaptované EGIL klasifikace pozitivitou alespoň dvou ze tří antigenů: CD19, (intra)CD79a a/nebo, CD22, zároveň musí být negativní všechny tyto molekuly: intra CD3, CD3, lehké řetězce imunoglobulinu kapa a lambda. T ALL je definována pozitivitou (intra)CD3 a pozitivitou CD7. AML je definována splněním alespoň dvou následujících kritérií: $intraMPO^{poz}$, $CD13^{poz}$, $CD33^{poz}$, $CD65^{poz}$ a/nebo $CD117^{poz}$ a současně úplnou (intracelulární, příp. i povrchovou) negativitou CD3, CD79a a CD22.

Imunologicky jsou AHL s primárně definovatelnou linií a signifikantní koexpresí antigenů z jiné linie nejčastějším podtypem AHL a pro její definici lze např. použít již zmíněnou EGIL klasifikaci [16], ve které jednotlivým antigenům je přiřazeno skóre podle jejich příslušnosti

k lymfoidní, resp. myeloidní linii (tabulka 5, viz též příloha 12). V případě překročení skóre nad 2 je nález klasifikován jako AHL.

skóre	*B řada*	*T řada*	*myeloidní linie*
2	(intra)CD79a, intra IgM, CD22	(intra)CD3, TCRαβ, TCRγδ	intraMPO
1	CD19, CD10, CD20	CD2, CD5, CD8, CD10	CD13, CD33, CD65, CD117
0.5	intraTdT, CD24	intraTdT, CD7, CD1a	CD14, CD15, CD64

Tabulka 5. *Skóre jednotlivých antigenů pro diagnostiku AHL. Splní-li blasty definici jedné z řad ALL a současně překročí myeloidní skóre 2 nebo splní-li definici AML a překročí-li skóre B nebo T řady 2, je nález klasifikován jako AHL.*

Pro porovnání uvádím alternativní klasifikaci AL a AHL, která je používána v St. Jude's Hospital v Memphisu (USA) a definuje jednotlivé podtypy takto [10]:

- **B-lineage My+ALL** splňují všechna následující kritéria:

a) Blasty jsou $CD19^{poz}$ a ($CD22^{poz}$ nebo intra-$CD79a^{poz}$ nebo intra-IgM (μ řetězec))

b) Blasty jsou intra-$CD3^{neg}$

c) Blasty jsou intra-MPO^{neg}

d) Blasty exprimují jeden nebo více myeloidních antigenů (CD13, CD15, CD33 nebo CD65)

- **T-lineage My+ALL** splňují všechna následující kritéria:

a) Blasty jsou $CD7^{poz}$ a intra-$CD3^{poz}$

b) Blasty jsou $CD22^{neg}$

c) Blasty jsou intra-MPO^{neg}

d) Blasty exprimují jeden nebo více myeloidních antigenů (CD13, CD15, CD33 nebo CD65)

- **Ly^{poz} AML** splňují všechna následující kritéria:

a) Blasty jsou intra-MPO^{poz} (nebo je pozitivní nespecifická esteráza u AML M5)

b) Blasty jsou intra-$CD3^{neg}$

c) Blasty jsou intra-IgM(μ řetězec)^{neg} a neexprimují současně CD22 a intra-CD79a

d) Blasty exprimují jeden a více z následujících antigenů: CD2, CD5, CD7, CD19, CD22, CD56 nebo intra-CD79a.

- **Bifenotypické leukémie** splňují jedno z následujících kritérií

a) Myeloid/B – lineage bifenotypové leukémie koexprimují intra-MPO a CD22 spolu s CD19 nebo intra-CD79a.

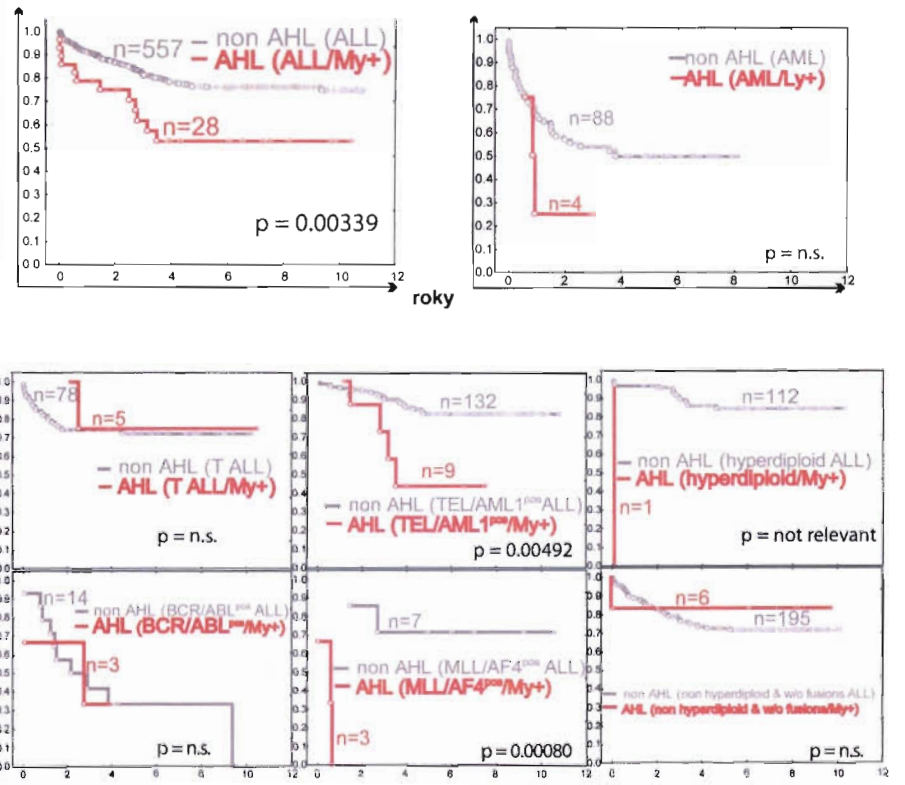
b) Myeloid/T – lineage bifenotypové leukémie koexprimují intra-MPO a intra-CD3

c) Mixed B/T lineage bifenotypové leukémie koexprimují intra-CD3 a intra-IgM(μ řetězec) nebo intra-CD3 spolu s intra-CD79a.

V rámci diagnostiky považujeme za důležité, aby osoba hodnotící cytometrický nález se vyjádřila k primární linii a které znaky hodnotí jako aberantní. EGIL ani jiné klasifikace nejsou v současné době zcela vyhovující a v druhé polovině roku 2008 je očekávána nová WHO klasifikace hematologických malignit, kde bude část věnována i hybridním leukémiím. Většina doposud publikovaných studií hodnotila buď dospělé pacienty [218], nebo kohorty dětí dohromady s dospělými [219-221] Poměrně málo byl v těchto studiích zohledněn typ léčby a její výsledky na celkovou prognózu. Z našich dat je zřejmé, že pokud striktně aplikujeme EGIL klasifikaci na čistě pediatrickou kohortu, zdaleka nejčastější podskupinou definovanou imuno-genotypově jsou pacienti s fúzním genem TEL/AML1 (viz tabulka 8) vzhledem k častému výskytu tohoto podtypu, ale není rozdíl signifikantní oproti skupině klasifikované jako non AHL. TEL/AML1 genotyp je známý svoji afinitou exprimovat myeloidní antigeny [211, 212]a tato koexprese může vést až k překročení AHL skóre, přestože z pohledu diagnostiky a klinického průběhu se jinak jedná o typické ALL (i když je jejich prognóza signifikantně horší – obrázek 14). Podobně jako v předchozích studiích jsme potvrdili vyšší incidenci u AHL genotypu BCR/ABL a MLL/AF4 (tabulka 6) a s tím související celkovou horší prognózu dětí klasifikovaných jako AHL. V analýze v rámci jednotlivých imuno/genotypových podskupin (obrázek 14) prokazujeme signifikantně horší přežití u dětí klasifikovaných jako AHL v rámci TEL/AML1 a MLL/AF4 podskupiny. Jen pro úplnost retrospektivního hodnocení AHL u dětí, v uvedeném rozpětí (září 1996 – srpen 2006): 4 další děti jsme klasifikovali jako AHL primárně AML s lymfoidními znaky. Všechny 4 děti přesáhly AHL skóre expresí T lymfocytárních znaků, prognóza této podskupiny byla extrémně špatná (v 1. roce od diagnózy žil a dosud žije pouze 1 pacient po alogenní transplantaci od HLA identického sourozence), u žádného z těchto dětí jsme neprokázali klonální přestavby Ig/TCR.

	AHL	Non AHL	p hodnota
T ALL	18%	14%	n.s.
TEL/AML1	32%	24%	n.s.
Hyperdiploidní	4%	21%	p=0,026
BCR/ABL	9,4%	2,6%	p=0,044
MLL/AF4	10,7%	1,3%	p=00098
Ostatní BCP ALL	21%	35%	n.s

Tabulka 6. *Četnost jednotlivých imunologických a molekulárně genetických podskupin u pacientů s AHL podle skóre EGIL a u nehybridních ALL. Statistická významnost rozdílu v četnosti jednotlivých podskupin je hodnocena Fisherovým exaktním testem. Červeně znázorněny signifikantní rozdíly v zastoupení AL klasifikovaných jako AHL.*



Obrázek 14. ***Nahoře vlevo:*** *Křivky přežití ukazují signifikantní rozdíl (Cox-Mantelův test) mezi skupinou AHL* n=28 *a non AHL* n=557. ***Nahoře vpravo:*** *V rámci AML nevidíme signifikantní rozdíl.* ***Dole:*** *Pokud hodnotíme přežití v jednotlivých imuno-genotypových podskupinách, signifikantní rozdíl v přežití nacházíme v podskupinách MLL/AF4 a TEL/AML1.*

3.1.4. Leukémie s nálezem blastů z různých linií a liniový přesmyk během časné fáze léčby před dosažením kompletní remise.

Přesmyk z jedné linie do druhé vyžadující reklasifikaci leukémie nebo nález dvou oddělených populací při diagnóze leukémie s nemožností nález jednoznačně zařadit jako lymfoidní, respektive myeloidní leukémii, jsou obecně v léčbě leukémií velmi vzácnou situací. Literárně lze najít spíše jednotlivá kasuistická sdělení než epidemiologická data. Leukémie s nálezem více různých populací blastů například shrnul Weir et al [222]. V jednotlivých kasuistikách je přesmyk z jedné linie do druhé popisován např. u ALL/AML s přestavbou MLL genu [223-225], u akutních leukémií s monozómii 7. chromozómu [226, 227] nebo u pacientů fúzním genem BCR/ABL [228]. Bierings et al. popsala případ pacientky s přesmykem [229] z BCP ALL během indukční léčby do myelomonocytární leukémie a v relapsu zpět do BCP ALL se stále stejnými klonálními přestavbami Ig-TCR bez prokázané přestavby MLL genu. Případná plasticita fenotypu leukemických blastů na počátku léčby by mohla zásadně ovlivnit spolehlivost diagnostiky, zejména imunologické. Proto jsme si položili otázku, zda k přesmyku nedochází častěji než se předpokládalo. Velkou roli v identifikaci přesmyku linie hraje častost a precisnost sledování dynamiky nádorové nálože před dosažením kompletní remise. Je totiž možné, že část pacientů nakonec dosáhne kompletní remise, a fenotypový přesmyk leukémie proběhne během indukční léčby bez povšimnutí. Leukemické buňky obecně mění během léčby transkripci řady genů, což lze identifikovat jak na základě expresního profilování [230], tak i na úrovni imunofenotypových změn [231]. Část těchto změn pravděpodobně přímo způsobují podané léky [232]. Typicky tyto změny u BCP ALL postihují např. tyto antigeny: pokles intenzity až vymizení pozitivity CD10, TdT (lze pozorovat i u T ALL) , vzestup exprese – CD20, CD45. Změny v expresi těchto antigenů částečně kopírují vyzrávání během nemaligního vývoje B řady [233-235]. Tyto změny zpravidla nevedou ke změně klasifikace vlastní leukémie, jsou však velmi důležité především pro vlastní detekci a správnou interpretaci MRN.

V rámci protokolu ALL IC BFM 2002 bylo jedním z nejdůležitějších výzkumných cílů zodpovědět otázku, zda časná odpověď v den 8 a den 15 ukazuje prognózu pacientů (přílohy 7 a 11). Součástí protokolu tak byla i centrální evaluace dne 8 a dne 15 morfologicky, imunologicky i molekulárně geneticky. Morfologické vyšetření bylo prováděno centrálně v Praze a v Olomouci. Celkem u 4 dětí s BCP ALL (charakteristika pacientů je v tabulce 3) s BCP ALL (3x uniformní infiltrace KD blasty, u pacienta DH signifikantní koexistence BCP ALL blastů (57%) a myelomonocytárních blastů (15%) jsme identifikovali v den 8

signifikantní změnu fenotypu (pacienti PM, LD a DK) směrem k myeloidní linii (pokles B lymfocytárních antigenů, vzestup myeloidních antigenů včetně CD33, CD14 a intracelulární myeloperoxidázy). V průběhu indukční léčby postupně tyto buňky změnily svůj fenotyp natolik, že v podstatě imunologicky a morfologicky vypadaly jako normální monocyty. V těchto buňkách jsme ale sortováním prokázali identické klonální přestavby s původním lymfoidním klonem při diagnóze. Artefakt způsobený příměsí nechtěných buněk jsme vyloučili jednak vysokou četností leukemických přestaveb v „monocytech", jednak vnitřní kontrolou (nemaligní T lymfocyty), která byla negativní. Na počátku léčby mohou tedy leukemické buňky dosud neobjasněným mechanismem změnit nejen imunofenotyp, ale dokonce i mikroskopický vzhled natolik, že připomínají nemaligní myeloidní či monocytární buňky. Tuto skutečnost jsme pozorovali hlavně u dětí se špatnou odpovědí na prednison. Incidence mezi pacienty se špatnou odpovědí na prednison a BCP ALL na protokolu ALL IC BFM 2002 (11/2002 - 10/2007) byla 19%. Ukázali jsme, že tento biologický jev může být příčinou rozporných výsledků stanovení procenta blastů mezi molekulární genetikou, imunofenotypizací a morfologií. Všechny pacienty s pozdějším přesmykem spojovala již při diagnose BCP ALL signifikantní exprese molekuly CD2, která jinak nepatří mezi časté aberantní antigeny. Přesmyk z lymfoidní linie do myeloidní linie u části pacientů ukazuje dosud nedoceněnou plasticitu leukemických buněk a alespoň u části těchto pacientů očekáváme shodné dosud nepoznané genetické pozadí.

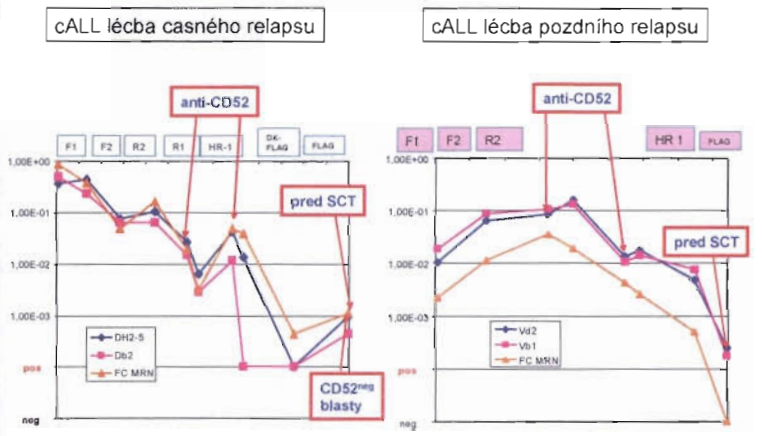
Iniciály /věk(r)	Imuno fenotyp/ Morfologie	Identické klon. přestavby Ig-TCR ve srovnání s leukemickými blasty	Leukocyty (10^3/ µL)	Genotypové změny	Prednisonová odpověď	Změna léčby po standardním protokolu ALL léčby	Prognóza
DH /2	cALL (60%) a myelomono (40%)	myelomono: ano (současně i FLT3ITD+)	190	FLT3 ITD delece 11q23 včetně MLL	špatná	Interfant, SCT	Relaps ALL po SCT
PM /15	proB L1, aberantní CD2	mono ano	15	nenalezeny	špatná	Interfant, SCT	CR1 2,5r
LD /17	cALL L1 aberantní CD2	mono ano	6,9	nenalezeny	špatná	Interfant, SCT	CR 1,2r
DK /5	cALL L1 aberantní CD2	mono ano	45	nenalezeny	dobrá	0	CR1 8m
IS /16	cALL L1 aberantní CD2	„sek"AML ano	138	nenalezeny	špatná	smrt v relapsu	sek AML 6m po dg, smrt v progresi

Tabulka 7. *Přehled základních charakteristik pacientů s přesmykem směrem k myeloidní linii v rámci protokolu ALL IC BFM 2002, pro úplnost uvedena i pacientka IS z retrospektivní analýzy protokolu ALL BFM 95.*

3.1.5. Imunologická diagnostika terapeutických cílů na leukemické buňce. Anti-CD33 léčba, perspektiva využití dalších monoklonálních protilátek v léčbě dětské ALL

Imunofenotypizace rovněž přináší informace o případných molekulárních cílech pro eventuální specifickou léčbu – zejména monoklonálními protilátkami. Vzhledem k horší prognóze pacientů s aberantní expresí CD33, kterou jsme prokázali na kohortě pacientů léčených v rámci protokolu ALL BFM 95, se nabízí otázka, zda by se u této podskupiny nedala využít cílená anti-CD33 terapie **(Příloha 1).** Terapie humanizovanou monoklonální protilátkou anti-CD33 (klon hp67.6) konjugovanou s cytostatikem calicheamicinem (gemtuzumab ozogamicin - GO) je používána především u dospělých pacientů s AML, kde naprostá většina leukemických buněk exprimuje molekulu CD33. Původně bylo léčivo testováno v monoterapii u pacientů netolerujících konvenční indukční léčbu AML (zejména pacienti starší 65 let s významnými komorbiditami znemožňujícími klasickou intenzivní chemoterapii). V současné době již byly publikovány a probíhají další studie kombinující GO s další chemoterapií u dospělých pacientů s AML. U dětí byly publikovány zkušenosti s GO v terapii jak u AML [146], tak i u ALL [236-238]. Studie Zwaana et al. prokázala signifikantně nižší

hodnotu LC_{50} samotného calicheamicinu in vitro u pacientských vzorků s ALL oproti AML, u kterých Goemansová et al. prokázala významnou inter-individuální variabilitu v citlivosti na calicheamicin [239]. Zdá se, že určitou roli v odpovědi na tento lék u AML (u ALL nebylo dosud zkoumáno) hrají i polymorfismy molekuly CD33 [240]. Širšímu použití u ALL brání v současnosti omezení na $CD33^{poz}$ ALL a literárně počet publikovaných pacientů nepřesahuje maximálně několik málo desítek pacientů, zpravidla s relapsem, resp. refrakterní nemocí. Naše zkušenosti s léčbou monoklonálními protilátkami u ALL jsou omezené na pacienty s refrakterním onemocněním, resp. relapsem po SCT. U všech pacientů před podáním monoklonální protilátky byla ověřena pozitivita příslušné molekuly (u všech byla pozitivita na hladině nejméně 40%). Celkem byla monoklonální protilátka v léčbě ALL podána u 6 pacientů s relapsem ALL. Pacient s 2. relapsem BCP ALL a s částečnou expresí CD33 byl léčen kombinací anti-CD33 (GO) s chemoterapií bez efektu, pacient s refrakterním velmi časným 1. relapsem praeT ALL byl léčen anti-CD33 (GO) bez efektu, pacient s 1. relapsem sekundární AHL (proB/My+) po alogenní transplantaci dosáhl kompletní remise a kompletní dárcovské krvetvorby po dvou dávkách samotné anti-CD33, dva pacienti s 1. relapsem BCP ALL byli léčeni kombinací anti-CD52 (Campath) a chemoterapií pouze s částečným efektem bez dosažení molekulární remise a pacient s 1. relapsem BCP ALL s vysokou pozitivitou CD20 na reziduálních leukemických blastech byl léčen anti-CD20 (rituximab) spolu s chemoterapií a dosáhl molekulární remise.



Obrázek 15. *Ukázka monitorování léčebného efektu kombinace chemoterapie a anti-CD52 (Campath™). MRN byla detekována pomocí kvantitativního sledování klonálních přestaveb Ig-TCR a pomocí 8-barevné průtokové cytometrie (CD58 FITC/CD66c PE/CD10 ECD/CD45PerCp/CD34 APC/CD20 PB/CD38 A700). U pacienta s časným relapsem jsme při pozitivitě MRN podle průtokové cytometrie před alogenní transplantací prokázali selekci CD52 negativních blastů (ověřeno klonem s jiným cílovým epitopem než má anti-CD52 v Campathu). DH2-5, Db2, Vd2, Vb1- Ig/TCR jsou cíle pro sledování MRN.*

V současné době se již do klinické praxe dostává humanizovaná anti-CD22 protilátka konjugovaná s calicheamicinem - **inotuzumab ozogamicin.** První perspektivní výsledky již byly ukázány u dospělých pacientů s lymfomy a na zvířecím modelu ALL [241, 242]. Perspektiva tohoto léku u ALL je veliká, prakticky všechny BCP ALL tuto molekulu exprimují. Podobný je i mechanismus fungování jako u gemtuzumab ozogamicinu, po navázání protilátky dochází k internalizaci komplexu, v lysozymu dojde k oddělení cytostatika, které poškozuje DNA cílové buňky podobným mechanismem jako ionizující záření. Toxicita jak gemtuzumab tak i inotuzumab ozogamicinu není zanedbatelná a nelze je přidat navíc ke standardní protokolární léčbě bez redukce a/nebo změny ostatních léků a nelze tak očekávat v dohledné době zapojení do indukční léčby ALL, když procento dosažených kompletních remisí v uznávaných léčebných protokolech je vyšší než 95%. Monoklonální protilátky fungující samostatně bez navázaného cytostatika lze zpravidla do stávajících léčebných protokolů přidávat snadněji (např. anti-CD22 epratuzumab [243], anti-CD20 rituximab, anti-CD52 campath). V současné době protokol COG pro relaps BCP ALL přidává anti-CD22 protilátky (epratuzumab) přímo do indukční léčby relapsu ALL [244]. Podobná strategie se v současné době zvažuje i v novém léčebném protokolu pro recidivy dětské ALL v rámci BFM skupiny. Převažující zkušenosti jsou tedy především z léčby refrakterní ALL, kde již zpravidla testujeme efekt na polyrezistentní leukemické buňky. Za zmínku stojí ještě občasné použití monoklonální protilátky OKT-3 (anti-CD3) v léčbě refrakterního relapsu T ALL [245].

3.2. *Léčba leukémie, monitorování účinnosti léčby*

3.2.1. Hodnocení minimální reziduální nemoci

Základními metodikami pro sledování MRN je kvantitativní sledování přestaveb genů Ig/TCR a průtoková cytometrie (detailní charakteristika metodik je rozvedena dále). Zlatým standardem pro monitorování ALL je v současnosti RQ PCR pro Ig/TCR. Reziduální nemoc se postupně zapojuje do léčebných protokolů nejen dětské ALL. Díky sledování MRN pomocí PCR je tak definován nový pojem - molekulární remise. Obecně pacienti s BCP ALL, kteří dosáhnou v rámci protokolů BFM molekulární remise po prvním indukčním bloku, jsou pacienti s velmi dobrou prognózou [246]. Pacienti s T ALL mají jiný charakter odpovědi, obecně odpovídají na léčbu pomaleji, platí ale, že pacienti negativní tři měsíce od diagnózy prakticky nerelabují [79].

V současné době je sledování MRN pomocí RQ PCR Ig/TCR pro řadu zejména ekonomicky méně vyspělých zemí metodikou nedostupnou. Naproti tomu průtoková cytometrie je rozšířená i v zemích s horším ekonomickým zázemím. Vzhledem k nedostatečné standardizaci cytometrie, především její interpretace, jsou všechny dosavadní cytometrické studie MRN omezené buď na jednu instituci nebo laboratoř [39, 69, 247-249].

3.2.1.1. Imunofenotypová detekce MRN u ALL

Imunofenotyp ALL blastů odpovídá více či méně imunofenotypu normálně se vyvíjejících prekurzorů. Nemaligní protějšky prekurzorů T lymfocytů se fyziologicky vyskytují pouze v thymu, pro sledování reziduální nemoci v periferní krvi a kostní dřeni u T ALL by teoreticky mělo stačit detekovat blasty podle imunofenotypu nezralých T prekurzorů (exprese intracelulárního TdT, negativita povrchové CD3 v rámci $CD7^{poz}CD5^{poz}$ T lymfocytů [250]. Dále je známá typická hyperexprese molekuly CD99 u významné části T ALL [251]. Naproti tomu nemaligní B prekurzory jsou ve variabilním počtu prakticky vždy v kostní dřeni zastoupeny [13]. Známý je tzv. rebound fenomén, kdy nacházíme v regenerující kostní dřeni B prekurzory např. po chemoterapii až v desítkách procent. Hledání rozdílů v imunofenotypu blastů od nemaligních B prekurzorů je zásadní problém reziduální nemoci u BCP ALL [250].

Pro odlišení leukemických B prekurzorů od nemaligních protějšků využíváme několik typických vlastností leukemických blastů:

snížená nebo naopak zvýšená exprese některých molekul (např. snížená exprese až negativita molekuly CD38, hyperexprese CD10, CD58)

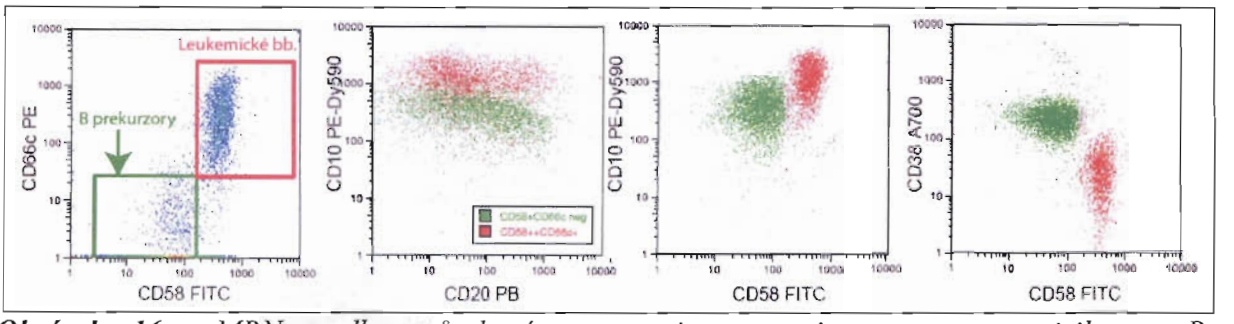
exprese aberantních molekul z jiné linie než lymfoidní (např. exprese CD66c, CD33, CD13, CD15)

exprese molekul v rámci normální hematopoézy se vůbec nevyskytující (NG2 – molekula chondroitin sulfátu, která se exprimuje typicky u ALL i AML s přestavbami MLL genu)

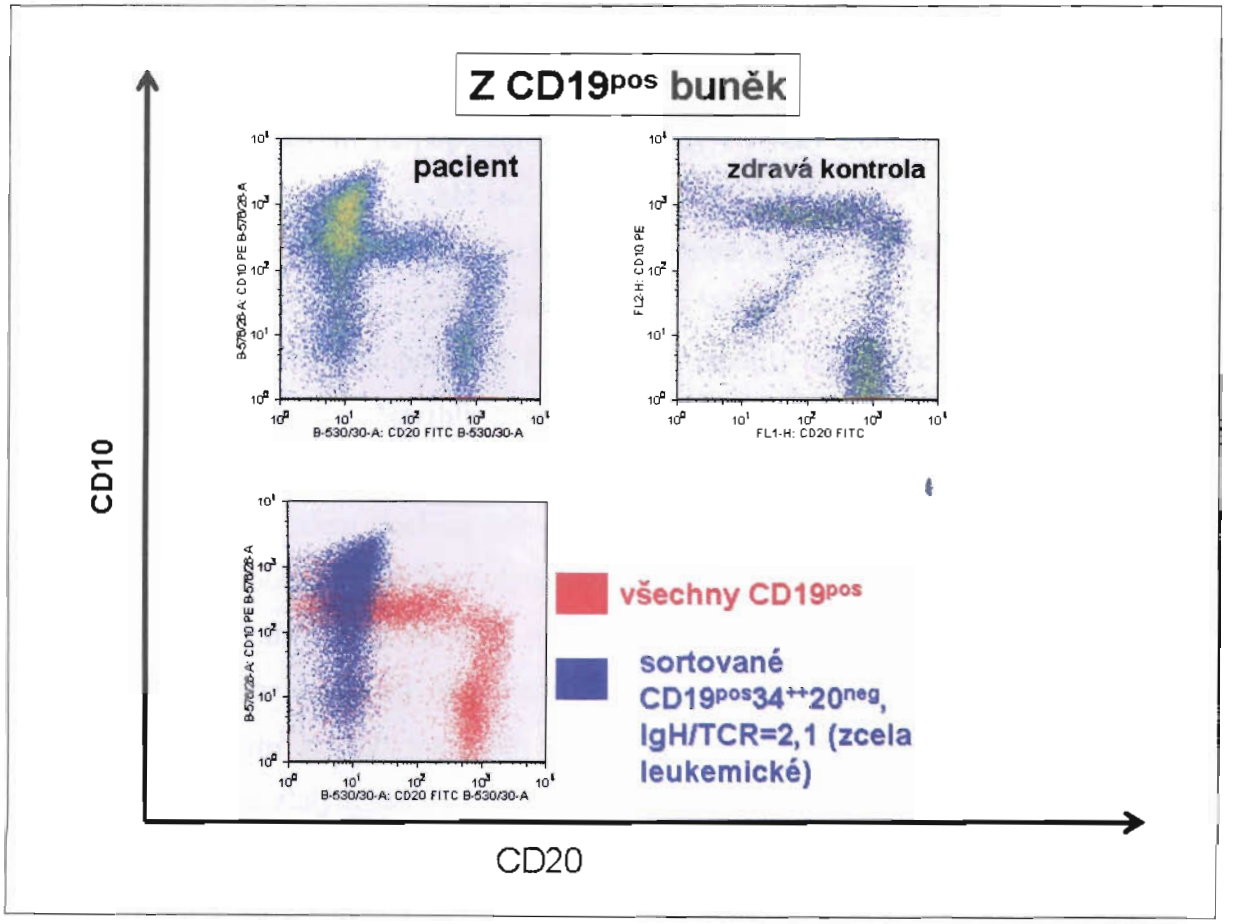
asynchronní exprese molekul (např. intracelulární exprese TdT na $CD34^{neg}$ blastech, pozitivita CD21 na $CD34^{poz}$ blastech)

Je nutné zmínit i změny imunofenotypu u dětské ALL během léčby. V rámci indukce ALL, kdy významnou část terapie tvoří kortikosteroidy, je časté potlačení exprese některých molekul, které při diagnóze hodnotíme jako hyperexprimované (např. CD10, CD34) nebo naopak upregulace molekul, které hodnotíme při diagnóze jako sníženě exprimované (např. CD45) [231, 232]. Mezi diagnózou a relapsem často nacházíme nestabilitu exprese aberantních molekul, jako je např. CD33, CD13 a CD15 [252], naopak molekula CD66c ukazuje významnou stabilitu [253]. Prokázali jsme ale stabilitu myeloidního antigenu CD33 v časné fázi léčby [252].

V současné době se postupně stává dostupná 9 a více-barevná cytometrie (příklad na obrázku 16), umožňující analýzu více než 11 parametrů na jednotlivé buňce. Očekáváme s tímto přístupem zvýšení senzitivity a specificity průtokové cytometrie zejména v časových bodech s regenerací v KD.



Obrázek 16a. *MRN podle průtokové cytometrie u pacienta s vysoce rizikovou B prekurzorovou ALL 3 měsíce od zahájení léčby. Pacient s iniciální hyperleukocytózou až 200000 WBC/uL, dobrou odpovědí na prednisom a více než 25% blastů (M3) v KD v den 15, což pacienta kvalifikovalo do vysokého rizika. Analýza kombinace CD58/CD66c/CD10/CD19/CD45/CD34/CD38/CD20. Všechny obrázky jsou z gatu $CD19^{poz}$ buněk. V kostní dřeni jsou zřetelné jak nemaligní B prekurzory ($CD66c^{neg}$ $CD58^{poz(bez\ hyperexprese)}$ $CD38^{poz}$), tak i leukemické buňky (aberantní exprese CD66c, hyperexprese CD58, nízká až negativní CD38, vyšší intenzita CD10 oproti nemalignim B prekurzorům). Celková hladina MRN je 1,2% ze všech jaderných buněk a tato hodnota by pacienta podle nejnovějších BFM kritérii kvalifikovala pro maximální terapii včetně alogenní SCT i od nepříbuzného dárce (pacient žije v 1. kompletní remisi 22 měsíců od iniciální diagnózy na udržovací terapii dle protokolu ALL IC BFM 2002).*



Obrázek 16b. *Vysoká hladina MRN (modře) na hladině 5,8% s koexistencí nemaligní B řady (červeně) u pacienta s Ph^{poz} ALL 180 dní po SCT. Po neúspěšné indukční terapii bylo remise dosaženo až po přidání imatinibu. Pacient byl transplantován v molekulární remisi podle přestaveb Ig/TCR (pacient číslo 36 z* **přílohy 5**), *přesto velmi časně narostla MRN a následně nemoc zrelabovala (jedná se o jediného pacienta s negativní MRN před SCT, který zrelaboval). Pacient zemřel v léčbě relapsu na systémovou mykotickou infekci. Leukemický původ blastů znázorněných na tomto obrázku jsme ověřili přímým sortováním a následným ověřením přítomnosti klonálních přestaveb Ig/TCR.*

3.2.1.1.1. Cytometrická reziduální nemoc v mezinárodní studii Mini-Mini

Jak již bylo zmíněno, všechny dosud publikované i aktuálně probíhající studie využívající MRN průtokovou cytometrií jsou zpravidla omezeny na jednu laboratoř, respektive jednu instituci [39, 69, 247, 254, 255]. Je zřejmé, že detekce MRN ve studii tohoto rozsahu je možná jen multicentricky. Průtoková cytometrie je považována za metodiku obecně dostupnější i v zemích s horším ekonomickým zázemím. Asi u 5 až 10% pacientů se nepodaří zavést PCR systém, buď z důvodu nenalezení vhodných dostatečně specifických a senzitivních klonálních přestaveb, nebo z důvodů logisticko - biologických (nemožnost získat dostatečné množství materiálu při diagnóze onemocnění, nezaslání rozhodujících vzorků do laboratoře). U těchto pacientů logicky vzniká otázka použitelnosti klasifikace MRN podle průtokové cytometrie. Dosud ale nebyla publikována jednoznačná kritéria pro hodnocení cytometrické MRN.

V rámci studie Mini Mini jsme navrhli jednotný panel monoklonálních protilátek s použitelností prakticky pro všechny pacienty s ALL. V rámci hodnocení MRN jsme definovali široké spektrum subpopulací rámci B řady i T řady s cílem postihnout i případné imunofenotypové odchylky vzniklé jak v časné fázi léčby [231, 256-258]. V rámci B řady jsme definovali podle pacientů z pilotní fáze (děti s relapsem ALL a sledovanou MRN podle RQ PCR a pacienti s regenerující kostní dřeni v průběhu léčby a po SCT) celkem 29 subpopulací a v rámci T řady celkem 5 subpopulací. Do studie se zapojily laboratoře z Chorvatska, Izraele, Maďarska a Hong Kongu. Navrhli jsme jednotné templáty pro interpretaci dat v softwaru Cellquest (BD San Jose) a FlowJo (TreeStar, Oregon). Léčba ALL představuje komplexní situaci, kdy v kostní dřeni dochází postupně k redukci nádorového klonu a znovuobjevení nemaligní hematopoézy. Oba tyto dynamické aspekty je průtoková cytometrie schopná obsáhnout. Vzhledem k tomu, že podle protokolu ALL IC BFM-2002 se léčí od října 2002 a medián sledování celé kohorty stále není dostatečný pro korelaci s prognózou, bylo nutné najít v první fázi jiný způsob, jak ověřit tento způsob hodnocení cytometrických dat. Jak již bylo zmíněno výše, zlatým standardem v současné době je detekce MRN podle RQ PCR Ig/TCR. Podle náhodné poloviny negativních vzorků v daný časový bod a podle tzv. „cross lineage" kontrol (pacienti s T ALL změření kombinacemi proB řadu a naopak) jsme definovali pozadí v kostní dřeni specifické pro každý časový bod zvlášť. Hodnoty subpopulací, které přesáhli trojnásobek 98. percentilu subpopulací „cross lineage" kontrol a RQ PCR negativních vzorků, jsme definovali jako MRN, pokud bylo více hodnot subpopulací hodnoceno jako MRN, jako MRN jsme označili nejvyšší z nich. Hodnocení senzitivity a specificity MRN podle průtokové cytometrie bylo tedy vztaženo k hladině MRN podle RQ PCR (**příloha 11**).

Zjistili jsme, že předem definované subpopulace mohou poskytnout relevantní informaci i v platformě 4barevné cytometrie. Jak rozvádím v **příloze 11**, standardně definované subpopulace rozdělí v den 15 pacienty s BCP ALL (ale nikoliv s T ALL) podle pravděpodobnosti MRN podle citlivé PCR v den 33 a v týden 12. Tyto závěry mají bezprostřední význam pro plánování dalších léčebných protokolů.

3.2.1.2. Molekulárně genetická detekce MRN u ALL

Obecně u ALL je možné nalézt molekulárně genetické cíle u většiny pacientů (minimálně u 80%). Jednak využíváme jak přestavby genů Ig/TCR, u pacientů s přítomným fúzním genem (např. TEL/AML1 nebo BCR/ABL) lze použít i sledování jejich transkriptů. Většina

léčebných stratifikací v současné době vychází u ALL především z kvantitativního sledování přestaveb Ig/TCR nebo průtokové cytometrie [247, 249, 259].

3.2.1.2.1. Sledování MRN u ALL pomocí přestaveb imunoreceptorových genů (Ig/TCR)

Detekce MRN pomocí kvantifikace klonálních přestaveb genů pro imunoglobuliny (Ig) a T-buněčné receptory (TCR) vychází z předpokladu, že buňky ALL jsou maligními protějšky nezralých lymfoidních buněk [210]. Pokud maligní zásah postihne lymfoidní prekurzor, který již zahájil proces V-(D)-J rekombinace, mají všechny jeho dceřiné buňky stejné sekvence přechodových oblastí podjednotek antigenových receptorů specifické pro leukemický klon daného pacienta. Mezi nejčastěji detekované klonální přestavby u leukémií z B řady patří přestavby těžkých řetězců imunoglobulinů, které lze detekovat u více než 95% dětských ALL z B řady. Většinu z nich tvoří kompletní přestavby V-D-J, v přibližně 20% lze nalézt nekompletní D-J přestavby. U T-ALL lze sledovat přestavby T buněčného receptoru gama (TCRG), delta (TCRD), a beta (TCRB), přestavby podjednotky alfa se pro přílišnou složitost tohoto vyšetření rutinně nevyšetřují. Zvláštním fenoménem u leukémií je tzv. liniová promiskuita, která způsobuje přestavování genů pro receptory T lymfocytů u B prekurzorové ALL (TCRG, TCRB a nekompletní přestavby TCRD) a naopak výskyt nekompletních přestaveb IgH u T ALL [260].

Prvním krokem metodiky je určení klonality přestaveb pomocí screeningového panelu, který zahrnuje (podle postupu používaného naší laboratoří) 27 PCR reakcí pro jednotlivé rodiny segmentů těžkých (IGH) a lehkých (IGK) řetězců imunoglobulinů a pro nejčastěji přestavované segmenty TCRB, TCRG a TCRD [261]. Monoklonální produkty PCR ověřené pomocí analýzy heteroduplexů [261] jsou sekvenovány a na základě přechodových V-(D)-J sekvencí jsou navrženy specifické primery. Pomocí těchto primerů a fluorescenčně značených sond je pak zavedeno pacient-specifické RQ-PCR [46, 262, 263]. MRN je kvantifikována s použitím DNA z diagnostického vzorku kostní dřeně jako standardu. Takto je možné v laboratořích s dostatečnou zkušeností zavést systém pro sledování MRN až u 95% pacientů s ALL, ve skutečnosti je vždy celková úspěšnost nakonec nižší, vstupuje do toho často faktor i logistický (nedostatečné množství materiálu, nedodání vzorku do laboratoře apod.). Tato univerzálnost je však vykoupena velkou finanční, časovou a metodickou náročností této metody. Dalším negativem je možnost nespecifického nasedání primerů na přestavěné sekvence Ig/TCR ve zdravých lymfocytech s nebezpečím falešné pozitivity vyšetření. Proto byla Evropskou skupinou pro reziduální nemoc u ALL (ESG-MRD-ALL) navržena pravidla

pro vyhodnocování MRN pomocí této metody, která zahrnují mimo jiné použití multiplikátu DNA z lymfocytů zdravých dárců jako negativní kontroly [264]. Prokázali jsme, že regenerace B řady po transplantaci kostní dřeně je spojená s falešnou pozitivitou přestaveb imunoreceptorových genů v cílech zahrnující některé typy přestaveb těžkého i lehkého řetězce, přestože vzorky byly vyhodnoceny jako pozitivní podle kritérií ESG. Míra této regenerace především závisí na souběžně podávané imunosupresi: při podání kortikoidů nacházíme významnou redukci B prekurzorů až jejich absenci, s čímž je spojen významně nižší počet falešných pozitivit v systémech pro detekci MRN podle Ig/TCR přestaveb (příloha 6).

3.2.1.2.2. Sledování MRN u ALL pomocí fúzních genů

U části pacientů s ALL jsme schopni identifikovat tzv. fúzní geny. Tyto fúzní geny lze současnými metodikami detekovat v některých případech jak na úrovni mRNA, tak i na úrovni DNA (například fúzní geny zahrnující gen MLL), některé fúzní geny se z technických důvodů detekují pouze na RNA úrovni (BCR/ABL). Prokázána je dobrá korelace hladiny transkriptu TEL/AML1 a přestaveb Ig/TCR [265]. TEL/AML1 se tedy nabízí jako vhodný cíl při nemožnosti sledování specifických cílů podle přestaveb Ig/TCR. Dosud není publikována práce, která by technicky a prognosticky srovnala detekci MRN podle přestaveb Ig/TCR a pomocí fúzního genu BCR/ABL. U korelace fúzního genu BCR/ABL s přestavbami Ig/TCR je třeba mít na paměti, že fúzní gen BCR/ABL může být u ALL přítomen (podobně jako u blastického zvratu CML) i v jiných liniích než v lymfoidní [266-268]. Nevýhodou sledování MRN pomocí fúzních genů na RNA úrovni je možnost kontaminace s rizikem falešné pozitivity a dále celkově vyšší požadavky na kvalitu a stáří vzorku.

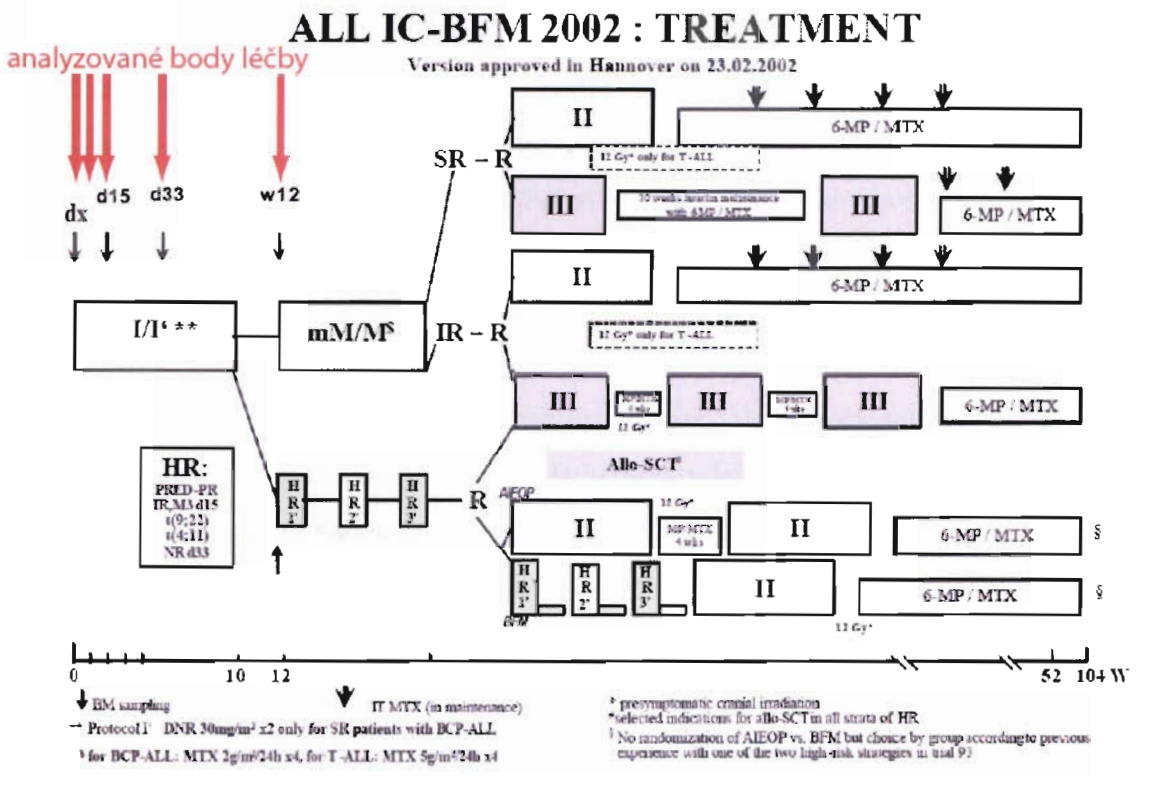
3.2.1.3. Nepřímé sledování MRN pomocí chimérismu po alogenní SCT

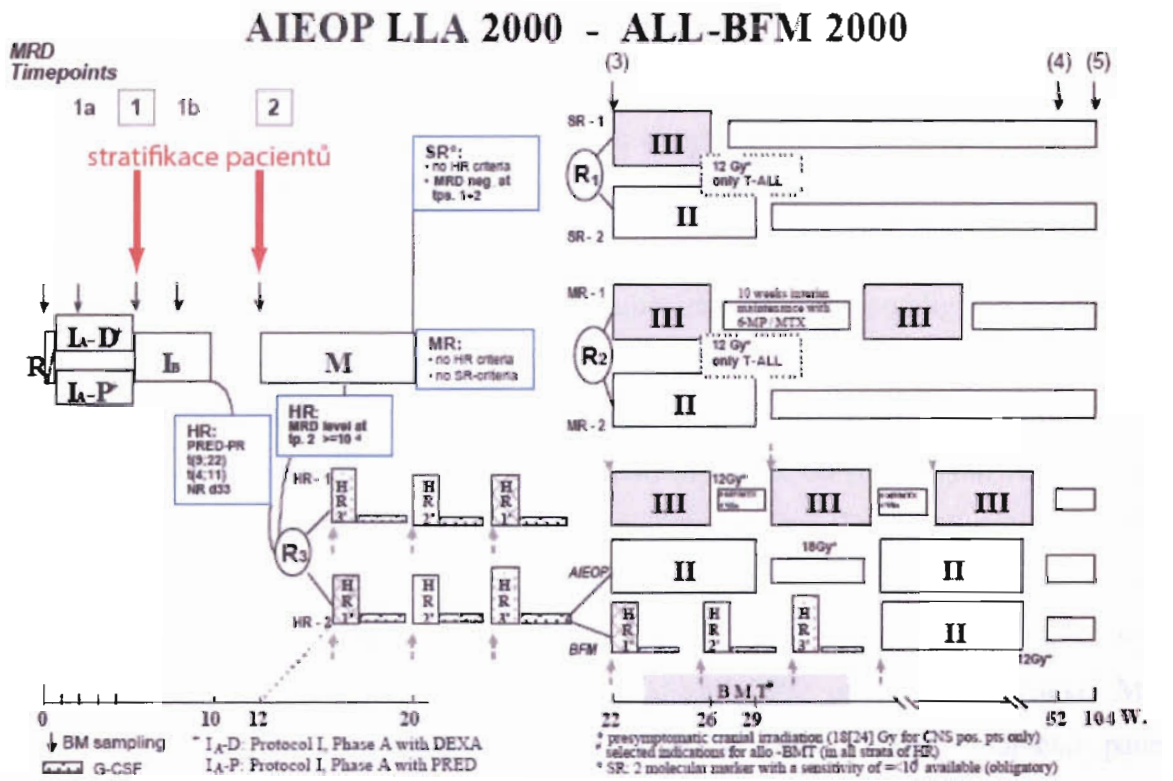
Pro úplnost je třeba ještě zmínit sledování případných reziduálních leukemických buněk obecně u hematologických malignit, jak akutních, tak i chronických, pomocí chimérismu s přibližnou citlivostí 10^{-2}. Zlatým standardem v současné době je sledování pacienta a dárce pomocí specifických STR (short tandem repeats) sekvencí [269-272]. Samozřejmě signál příjemce nemusí vždy znamenat nutně relaps malignity. Populaci, ve které detekujeme genotyp příjemce, lze ozřejmit sortováním [269].

3.2.2. Léčebný protokol ALL IC-BFM 2002 pro primární léčbu de novo ALL

Studie ALL IC-BFM 2002 je léčebný protokol, který byl koordinován Českou republikou a jeho vznik byl motivován snahou zlepšit léčebné výsledky v zemích s dostatečnou zkušeností s protokoly BFM skupiny, ale nedostatečně zkušených v monitorování MRN. Jedním z cílů bylo srovnat stratifikaci pacientů podle klinických a biologických vlastností se stratifikací protokolu ALL-BFM 2000, ve kterém byli pacienti stratifikováni především podle hladin MRN. Základní schéma obou protokolů je na obrázku 17. V ALL-BFM 2000 se zásadně změnila stratifikace, některá klasická kritéria, která se používala v předcházejících léčebných protokolech, jako věk a leukocytóza, nebyla zahrnuta do stratifikace pacientů [259, 273] (příloha 7). Tato kritéria byla nahrazena parametrem MRN podle přestaveb Ig/TCR ve dvou časových bodech – v den 33 a týden 12 od diagnózy ALL. Tyto časové body pro detekci MRN byly podloženy retrospektivní analýzou MRN publikovanou v roce 1998[246]. Pacienti s negativní reziduální nemocí v den 33 a v týdnu 12, s dobrou odpovědí na prednison a bez fúzního genu BCR/ABL a MLL/AF4 jsou řazeni do standardního rizika. Pacienti se špatnou odpovědí na prednison, s prokázaným fúzním genem BCR/ABL nebo MLL/AF4, nedosažením remise v den 33 nebo MRN vyšší než 10^{-3} v týden 12 jsou řazeni do vysokého rizika. Ostatní pacienti, včetně těch, u kterých není možné zavést systém na sledování přestaveb imunoreceptorových genů, jsou řazeni do středního rizika. Léčebný protokol ALL IC-BFM 2002 zahrnuje země ze všech kontinentů a v současné době bylo do tohoto protokolu od října 2002 zařazeno více jak 4000 dětí s nově diagnostikovanou ALL. Základní otázkou tohoto protokolu je porovnání klasických stratifikačních kritérií s novou stratifikací zohledňující MRN v rámci ALL-BFM 2000. Pacienti standardního rizika v protokolu ALL IC-BFM 2002 splňují následující kritéria: jsou mladší 6 let, iniciální počet bílých krvinek je nižší než 20000/μL, v den 33 je dosaženo kompletní remise, v den 15 v kostní dřeni je méně než 25% blastů, mají dobrou odpověď na prednison a nemají prokázaný fúzní gen BCR/ABL ani MLL/AF4. Pacienti vysokého rizika splňují alespoň jednu podmínku z následujících: špatná odpověď na prednison, nedosažení kompletní remise v den 33, prokázaný fúzní gen BCR/ABL nebo MLL/AF4, více než 25% blastů v den 15 u pacientů jinak nesplňujících standardní riziko. Ostatní pacienti jsou řazeni do středního rizika. V rámci protokolu probíhají dvě výzkumné studie srovnávající výslednou stratifikaci v obou protokolech: studie Mini Risk sleduje, zda pacienti standardního rizika by splnili kritéria pro zařazení do nízkého rizika i v protokolu ALL BFM 2000, studie Mini Mini (viz kapitolu 3.2.1.1.1, str. 4) řeší otázky

detekce MRN průtokovou cytometrií. Prokázali jsme, že skupina standardního rizika se liší v obou protokolech [273](příloha 7).





Obrázek 17. *Schéma léčebného protokolu ALL IC-BFM 2002 a AIEOP BFM 2000. Zdůrazněny jsou časové body, v kterých byla monitorována MRN v protokolu ALL IC BFM 2002 a v kterých časových bodech se stratifikuje v léčebném protokolu ALL BFM 2000.*

3.2.3. Detekce MRN po relapsu ALL a u pacientů indikovaných k alogenní SCT

Pacienti, kteří jsou zahrnuti v příloze 5 [274], jsou vůbec prvními, u kterých byla v České republice sledována léčebná odpověď podle přestaveb imunoreceptorových genů. Tito pacienti představovali první kohortu pacientů pro ověření senzitivity a specificity MRN podle průtokové cytometrie, která byla v pilotní studii používána v naší laboratoři již od roku 1996. V rámci studie jsme prokázali, že negativita MRN podle přestaveb imunoreceptorových genů před SCT (vzorek odebraný těsně před zahájením přípravného režimu – tzv. „conditioningu") je podle našich dat významným prediktorem přežití bez události jak v univariantní, tak i multivariantní analýze. Do studie byli zahrnuti pacienti jak s relapsem ALL, tak i pacienti s vysoce rizikovou formou ALL, u kterých byla indikována SCT v 1. kompletní remisi.

Dosud jedinou publikovanou studií o prognostickém významu MRN podle průtokové cytometrie u relapsu ALL je práce Coustan – Smith et al. [275]. V této studii byli hodnoceni všichni pacienti, včetně pacientů s extramedulárním relapsem, kteří měli vyšetřenou hladinu MRN po skončení indukce a měli vhodný imunofenotyp pro sledování. Tato studie v rámci multivariantní analýzy ukázala jako negativní prognostický faktor relaps vzniklý v průběhu léčby a MRN ≥0,01% na konci indukce. Tato publikace potvrzuje zkušenost i jiných pracovních skupin na světě, že časný relaps v kostní dřeni na léčbě je pouhou chemoterapií prakticky nevyléčitelný [276].

U pacientů zahrnutých v článku Šrámkové et al. byla sledována MRN rovněž průtokovou cytometrií, v letech 1996 až 2002 detekce byla prováděna měřením jedné nebo dvou kombinací protilátek, které reflektovaly nejvhodnější imunofenotypové aberace (tzv. LAIP – Leukemia Associated Immunophenotype) odlišující maligní od nemaligních buněk. Tato strategie je v detekci MRN velmi častá [254, 277, 278]. Pro pokrytí eventuálních imunofenotypových shiftů se doporučuje použít pro detekci MRN alespoň dvě imunofenotypové aberace [279]. Rozlišení leukemických buněk od případných regenerujících prekurzorů je postaveno na expertním hodnocení cytometristy. Tento přístup neumožňuje komplexní přístup k reziduální nemoci průtokovou cytometrií, např. není možné tímto přístupem definovat, jaké populace jsou v daný časový bod normou a jaká hranice jejich procentuálního zastoupení již svědčí pro MRN. Od září 2002 jsme zahájili detekci MRN v rámci studie Mini Mini v protokolu ALL IC-BFM 2002 podle jednotného panelu čtyřbarevných kombinací zvlášť pro BCP ALL (SYTO16/CD19/CD45, CD20/CD10/CD19/CD34, CD10/CD66c/CD19/CD45, CD58/CD10/CD19/CD34) a pro T

ALL (SYTO16/CD19/CD45, CD99/CD7/CD5/CD3 a intra-TdT/CD7/CD5/intra-CD3) (**příloha 11**). Tyto kombinace jsme ve stejné podobě začali používat pro detekci MRN i u pacientů sledovaných po relapsu či po transplantaci. V podstatě nám výše uvedené kombinace umožňují definovat dostatečný počet imunofenotypových aberací vhodných pro sledování MRN.

3.2.4. Detekce MRN u pacientů s AML

Detekce MRN u AML byla první aplikace, kde jsme začali používat 4-barevnou průtokovou cytometrii a v rámci této studie jsme definovali řadu principů použitelných i pro detekci MRN u ALL. Celá studie probíhala v rámci protokolu AML BFM 98, který byl koordinována kolegy z Münsteru. V naší studii jsme definovali jako základní 2 antigeny CD34 a CD33 („páteř (backbone)"), na nichž se detekovaly jednotlivé imunofenotypové aberace. Pro kvantifikaci MRN z jaderných buněk jsme zavedli měření fluorescenční barvičky SYTO-16, které volně prochází buněčnou membránou a značí nukleové kyseliny a tím pomáhá k odlišení jaderných buněk od nejaderné drti. Medián vzniku relapsů u dětských AML je okolo 1 roku [102, 280], což umožňuje rychlé hodnocení vlivu MRN na výsledky léčby. Detekce MRN probíhala ve dvou časově oddělených periodách, přičemž na pacientech z první kohorty (2000 až 2001) se stanovily prahové hodnoty („cut offy") jednotlivých subpopulací v definovaných časových bodech (den 15, 21 až 28, 42 – 56, 70 až 84) pro predikci relapsu onemocnění [192]. Na společných datech jsme prokázali, že pacienti s prokazatelnou imunologickou přítomností blastů ve 3 a více časových bodech měli signifikantně horší přežití bez události (**příloha 4**) [189]. V rámci multivariantní analýzy jsme ale neprokázali přídatnou hodnotu MRN podle průtokové cytometrie ke kritériím, která se používala ve stratifikaci podle protokolu AML BFM 98 [141, 175].

4. Závěr

4.1. Myeloidní antigeny u ALL

Objevili jsme nové souvislosti mezi aberantními myeloidními antigeny a jejich vztah k prognóze a ke genotypu (**přílohy 1, 3**). Objevili jsme silný prognostický význam exprese CD33 u ALL celkově i u důležitých podskupin pacientů (příloha 1). Naše výsledky ukazují, že jednotlivé myeloidní antigeny mohou být regulovány rozdílně a že tedy dosavadní praxe jejich směšování byla nesprávná. Demonstrovali jsme nestabilitu exprese myeloidních

antigenů zejména při primární léčbě ALL (**přílohy 1, 3**), přičemž některé antigeny (CD66c) mají srovnatelnou expresi při relapsu ALL, jiné (např. CD33) nikoliv.

4.2. Význam diferenciačního antigenu CD10

Naše data ukazují, že exprese jednoho z nejdůležitějších antigenů u leukémií, CD10, může být v rámci klasifikace B prekursorové ALL použita k lepšímu prognostickému rozdělení ALL.

4.3. Minimální reziduální nemoc

Průtoková cytometrie při primární léčbě rozdělí pacienty s B prekurzorovou ALL podle hladiny MRN. Spolehlivé použití 4 barevné cytometrie s předdefinovanými gaty je limitováno na prvních 15 dnů protokolu ALL-IC BFM2002, přičemž tato hodnota spolehlivě určí zařazení do skupin MRN měřených PCR. Analogické využití časné odpovědi na léčbu T ALL není možné pro jinou kinetiku léčebné odpovědi (**přílohy 7, 11**).

U nepříznivých forem ALL lze cytometricky detekovat MRN i v pozdějších fázích léčby (**příloha 5**), pravděpodobně díky menší fenotypové plasticitě maligních blastů. Rekonstituce B řady je potenciálním zdrojem falešných pozitivit i při detekci MRN pomocí přestaveb genů Ig (**příloha 6**)

Cytometrie detekuje MRN u AML, ale není schopna prognosticky rozdělit pacienty v rámci existujících rizikových skupin (**příloha 4**).

5. Seznam zkratek

ABL	gen V-abl Abelson murine leukemia viral oncogene homolog, lokalizace 9q34.1
AEIOP	Associazonie Italiana Ematologia Oncologia Pediatrica (pracovní skupina)
AF10	gen ALL1 fused gene from chromosome 10, lokalizace 10p12
AHL	akutní hybridní leukémie
ALL	akutní lymfoblastická leukémie
AML	akutní myeloidní leukémie
AML M4eo	akutní myeloidní leukémie varianta M4 s eosinofilií
AML1	gen Acute myeloid leukemia 1 (synonymum RUNX1 (runt-related transcription factor 1), CBFA2 (core binding factor A2)), lokalizace 21q22.3
ara-C	cytosin arabinosid
ARA-G	arabinosyl guanin
ATRA	all-trans retinová kyselina (retionoic acid)
BCP	B prekurzorová (B cell precursor)
BCR	Breakpoint cluster region, lokalizace 22q11.2 (součást fúzního genu BCR/ABL)
BFM	Berlin-Frankfurt-Münster (pracovní skupina)

bHLHB1	gen Basic helix-loop-helix domain containing, class B, 2, lokalizace 21q22.11
CALM	gen calmodulin 1 (phosphorylase kinase, delta), lokalizace 14q24-q31
CBF	core binding factor
CBFβ	gen podjednotky β CBF, lokalizace 16q22
CCG	Childhood Cancer Group (pracovní skupina)
CD	cluster of differentiation (nomenklaturní systém antigenů)
CEBPα	gen pro CCAAT enhancer binding protein alpha, lokalizace 19q13.1
C-KIT	gen receptoru pro stem cell faktor, lokalizace 4q12
CNS	centrální nervový systém
COG	Childhood Oncology Group (pracovní skupina)
DAPI	4",6 - diamidino - 2 - phenylindole, dihydrochloride (fluorescenční barvivo značící stechiometricky DNA)
DNA	deoxyribonykleová kyselina (deoxyribonucleic acid)
EFS	přežití bez události („událostí" je smrt, relaps nebo sekundární malignita, event free survival)
EGIL	European Group for the Immunological Characterization of Acute Leukemias
ESG-MRD-ALL	European Study Group on MRD detection in ALL (pracovní skupina)
EsPhALL	European Intergroup Study on Post Induction Treatment of Philadelphia Positive Acute Lymphoblastic Leukaemia with Imatinib
ETO	gen Eigth twenty one (synonymum RUNX1T1 (runt-related transcription factor 1; translocated to, 1 (cyclin D-related)), lokalizace 8q22
FAB	French-American-British (klasifikace leukémií)
FLT3	gen FMS-like tyrosine kinase 3, lokalizace 13q12.2
FLT3/ITD	interní tandemová duplikace FLT3
GMALL	German Multicenter study group on Adult acute Lymphoblastic Leukemia (pracovní skupina a protokol)
GO	gemtuzumab ozogamicin, Mylotarg
GSTT1	enzym glutathion S-transferáza theta 1
HDAC	histon deacetyláza (histon deacetylase)
HLA	human leucocyte antigen
HOX11	rodina genů Homebox11 (TLX1,2,3)
HOX11L2	gen Homebox11L2, lokalizace 7p15-7p14.2
Ig	imunoglobulin
ITD	interní tandemová duplikace
JMML	juvenilní myelomonocytární leukémie
L	litr
LAIP	leukemia associated immunophenotype
LMO1	gen (LIM domain only 1 (rhombotin-like 1)), lokalizace 11p15
LMO2	gen (LIM domain only 2 (rhombotin-like 2)), lokalizace 11p13
LYL1	gen Lymphoblastic leukemia derived sequence 1, lokalizace 19p13.2
MDS	myelodysplastický syndrom
MLL	gen Myeloid/lymphoid or mixed lineage leukemia, lokalizce 11q23
MRN	minimální reziduální nemoc
MYC	protooncogen MYC, lokalizace 8q24
MYH11	gen Myosin heavy chain, lokalizace 16p13
NCI	National Cancer Institute
NG2	molekula chondroitin sulfátu

NHL	Non Hodginský lymfom
NOTCH1	gen Notch homolog 1, translocation-associated (Drosophila), lokalizace 9q34.3
NPM1	gen nucleophosmin, lokalizace 5q35
OKT-3	klon anti-CD3 protilátky
PAX5	Paired box gene 5, lokalizace 9p13
PCR	polymerázová řetězová reakce (polymerase chain reaction)
pEFS	pravděpodobnost přežití bez události („událostí" je smrt, relaps nebo sekundární malignita, event free survival)
Ph	Filadelfský (Ph chromozóm na podkladě translokace BCR/ABL (9;22)
PML	gen promyelocytární leukémie
PNP	purine nucleoside fosforyláza
POG	Pediatric Oncology Group (pracovní skupina)
pRFS	pravděpodobnost přežití bez relapsu
RAEB	refrakterní anémie s excesem blastů (refractory anemia with excess of blasts)
RAEB-t	refrakterní anémie s excesem blastů v transformaci (refractory anemia with excess of blasts in transformation)
RARα	gen receptoru pro retinovou kyselinu alfa (retinoic acid receptor alpha)
RC	refrakterní cytopénie
RFS	přežití bez relapsu onemocnění (relapse free survival)
RNA	ribonukleová kyselina (ribonucleic acid)
RQ PCR	kvantitativní polymerázová řetězová rekace
SCT	transplantace krvetvorných buněk (stem cell transplantation)
STR	short tandem repeats
SYTO-16	zelené fluorescenční barvivo značící nukleové kyseliny a volně procházející buněčnou membránou
TAL1	gen T-cell acute leukemia 1, lokalizace 1p32
TAL2	gen T-cell acute lymphoblastic leukemia 2, lokalizace 9q31
TCR	T buněčný receptor (T cell receptor)
TdT	terminální deoxynukleotidyl transferáza
TEL	gen Translocation ets leukemia, lokalizace 12p13.1
TLX1	gen T cell leukemia, gen z rodiny homebox genů, lokalizace 10q24
TLX3	gen T-cell leukemia, gen z rodiny homebox genů, lokalizace 5q35.1
TMD	tranzitorní myeloproliferativní nemoc (transitory myeloproliferative disease)
TP53	gen proteinu p53 (o velikosti 53 kDa), lokalizace 17p13
VOD	venookluzivní nemoc (venooclusive disease)
WT1	tumor supresorový gen Wilmsova nádoru, lokalizace 11p13

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6.2. *Přiložené publikace s impact faktorem*

Příloha 1

Correlation of CD33 with poorer prognosis in childhood ALL implicates a potential of anti-CD33 frontline therapy

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Correlation of CD33 with poorer prognosis in childhood ALL implicates a potential of anti-CD33 frontline therapy

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TO THE EDITOR

There has been a controversy about the prognostic impact of myeloid antigens (MyAgs) in ALL. The issue has now regained significance since an anti-CD33 monoclonal antibody (mAb) is available for clinical treatment, which is considered for AML as well as for $CD33^{pos}$ ALL.[1–3] We evaluated the prognostic impact of MyAgs in uniformly treated patients. From 9/1996 to 10/2002, 343 children with ALL were enrolled in a Czech nationwide study ALL-BFM95. In 327 patients (96%) immunophenotyping and molecular genetics were performed centrally in our reference labs. Prednisone good responders (PGR) with WBC at diagnosis $<20000/\mu L$ and age 1–5 years were assigned into standard risk group (SRG). High RG (HRG) corresponded to prednisone poor response (PPR) or no remission at day 33 or BCR/ABL or MLL/AF4 fusions. The remaining patients were in the intermediate RG (IRG). Full statistics details are in the Supplementary Information.

Immunophenotyping was performed on bone marrow (BM) samples at diagnosis on flow cytometry (FC) using FACSCalibur (BD San Jose, CA, USA). Informed consent was obtained from the patients and/or their guardians. A standard panel of mAbs was used for FC immunodiagnostics, including: CD13 (SJ1D1 Immunotech, only in B-cell precursor (BCP) ALL), CD15 (MMA BD), CD33 (D3HL60.251 Immunotech) and CD65 (88H7 Immunotech), unvaryingly throughout the study.

In a multivariate analysis of BCP ALL patients (Table 1), CD33 was the only MyAg with an independent prognostic impact. A similar analysis on T ALL ($n=45$) did not show a prognostic impact of listed MyAgs, therefore, we excluded T ALL cases from further analyses. Three cases (0.92%) were excluded due to the absence of a representative leukemic population by FC and one case was excluded since CD33 was not assessed. RFS was significantly worse in BCP ALL (total = 278) patients with higher $CD33^{pos}$ percentage (patients with higher and lower CD33 percentage: 5-year RFS 57 ± 6 *vs* $87\pm3\%$, median follow-up 3.3 and 4.0 years, $n=98$ and $n=180$, respectively; cutoff level: 10%; Figure 1). A similar analysis with a cutoff level of 20% would reveal significant difference ($P=0.0073$, not shown). When analyzed separately by RG, this difference remains apparent and remains significant for SRG and IRG (5-year RFS in cases with lower and higher CD33 percentage, cutoff 10%: SRG, 88 ± 5 *vs* $59\pm11\%$, $n=70$ and $n=36$, $P=0.01$; IRG, 92 ± 3 *vs* $60\pm9\%$, $n=88$ and $n=49$, $P=0.00073$; HRG, 58 ± 13 *vs* $38\pm16\%$, $n=22$ and $n=13$, $P=0.076$). The difference in IRG was significant at any cutoff level from 10 to 70% (always $P<0.02$, data not shown). Thus, CD33 is prognostically important especially in IRG, which is the most abundant RG among relapsed cases but patients at risk are otherwise difficult to discern. The BCP ALL cases with an intermediate percentage of $CD33^{pos}$ blasts (10–50%) had a poorer outcome (5-year RFS: $61\pm7.9\%$) than the $CD33^{neg}$ cases ($P=0.00043$), but not different than the cases with high $CD33^{pos}$ percentage ($\geqslant 50\%$ blasts; $P=0.32$). The difference in the EFS of cases with higher $CD33^{pos}$ cell frequency was less profound (BCP ALL: $P=0.0022$ and $P>0.05$, for cutoff 10 and 20%, respectively; data not shown) compared to RFS ($P=0.00002$ and $P=0.0073$, Figure 1a). A significant difference was noted in separate analyses for PGR (total = 252), TEL/$AML1^{pos}$ (total = 82) and hyperdiploid (total = 62) ($P<0.00001$, $P=0.0051$ and $P=0.019$, respectively; Table 2). In PPR, we failed to prove prognostic significance of CD33 expression ($P>0.05$, data not shown).

The striking prognostic importance of CD33 contrasts with several studies from the 1990s,[4–6] which failed to find a different prognosis of $MyAg^{pos}$ ALL. These papers followed a period of controversy between studies showing significantly worse prognosis of $MyAg^{pos}$ ALL[7–9] or no difference.[10] However, recent *in vitro* data showed a higher resistance for ALL blasts with MyAgs.[11] Although the aberrant MyAg expression has been known for at least 40 years, little is known about its regulation and cell biological relevance. The mechanisms that lead to the coexpression of several MyAgs in some cases are obscure; it is also not proven whether the mechanisms causing MyAg expression are comparable in T-ALL and in BCP ALL. Therefore, it is inappropriate to combine several MyAgs into composite criteria or to analyze the significance of MyAgs in BCP ALL together with T-ALL cases. A shorter follow-up is among possible reasons for a nonsignificant outcome; analysis of our data by the criteria used by Uckun *et al*[4] (CD33 and/or CD13 at a 30% cutoff value) does reveal a significant difference in outcome (5-year EFS 52 ± 9.2 and $77\pm3.4\%$, $P=0.0052$, compared to 4-year EFS 77 ± 4 and $76\pm1.8\%$, n.s.[4] in $MyAg^{pos}$ and $MyAg^{neg}$ BCP ALL, respectively). The median follow-up was 3.6 and 2.7 years in this and the previous[4] study, respectively. Since toxic deaths are less likely to correlate significantly with the immunophenotype, impact on RFS should always be analyzed. The difference in EFS may become insignificant when smaller cohorts of patients are analyzed. Another obscuring

Table 1 Cox multivariate analysis in B-cell precursor ALL: correlation of variables with RFS

Variables	P
CD65%	0.85
CD33%	0.0020
CD13%	0.44
CD15%	0.51
WBC at diagnosis	0.84
Response to prednisone	0.079
Age at diagnosis	0.088
bcr/abl or mll/af4	0.000013

The independent prognostic significance of myeloid antigens (MyAgs) together with the protocol-defined risk criteria is assessed by the Cox's proportional hazard regression model. All patients in whom all listed MyAgs were investigated, $n=255$, are included.

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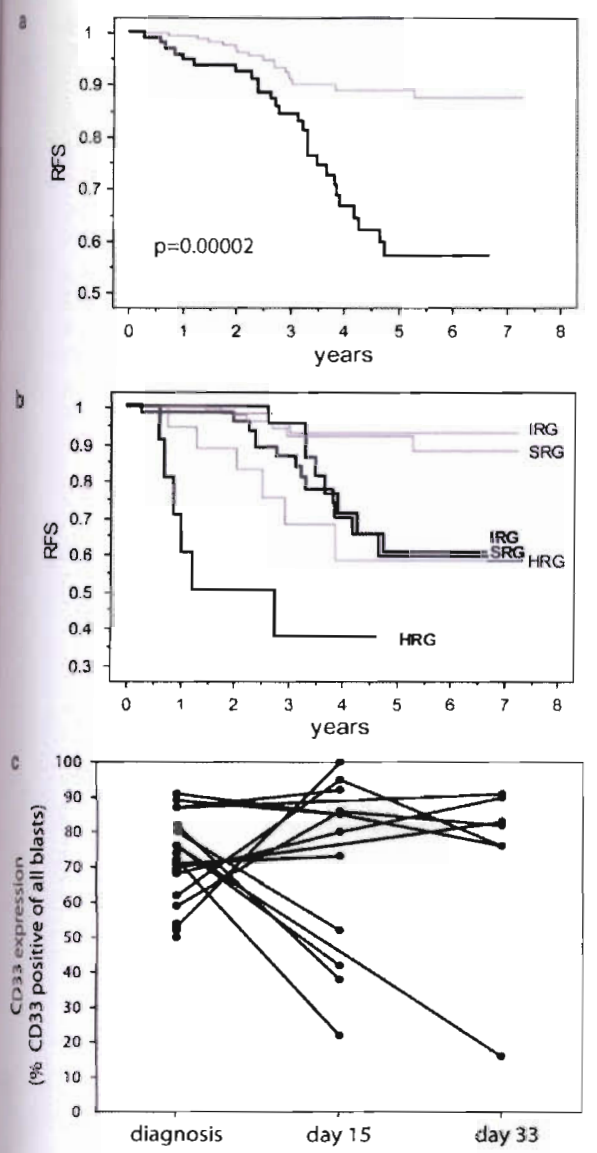

Figure 1 Outcome significance of CD33 in B-cell precursor (BCP) ALL. (a) Cases with higher percentage of $CD33^{pos}$ cells (bold black lines) are compared to those with lower $CD33^{pos}$ percentage (thin gray lines). Cutoff 10% is represented in these plots, analyses on other values are described in text; all BCP ALL cases ($n=278$); (b) RFS in BCP ALL split by risk groups (P-values are 0.011, 0.00073 and 0.076 for SRG, IRG and HRG, respectively). The cutoff value and the line colors are identical to the panel (a). (c) Percentage of leukemic cells expressing CD33 during the induction therapy. Cases with cytomorphologically detectable minimal residual disease were evaluated for CD33 expression. Cells were gated according to optical scatter properties and by the expression of CD10 and CD19 in three-color flow cytometric measurements.

variable is the difference in mAb clones used in the study – various mAb clones differ in the sensitivity towards the respective MyAgs.[12] This may have influenced especially multicentric analyses (Baruchel, personal communication). Therefore, it is appropriate to analyze RFS of BCP and T-ALL separately, the MyAgs should be assessed by identical mAb clones preferably in a single laboratory and the cohort should be treated by a single chemotherapy protocol. The presented population-based analysis contains all these features.

Antigen shift represents one of the possible reasons of the immunotherapy failure. Since the anti-CD33 immunotherapy is a potential extension of the presented analysis, we checked for the stability of CD33 expression at relapse. In 39 of 51 relapsed patients, we could compare the levels of CD33 expression at diagnosis and at relapse. In line with previous studies,[13] the expression among relapsed patients was rather unstable (17 of 39 switched between positivity and negativity, cutoff 20%, data not shown), and included also cases whose blasts completely lost CD33 expression at relapse. The expression of CD13 and CD15 was also unstable, contrasting with the stability of a MyAg CD66c.[14]

We next asked whether a substantial shift in CD33 expression could be observed already during the induction treatment. We selected patients whose BM specimens contained at least 50% $CD33^{pos}$ blasts at diagnosis. Of these, specimens were selected with a clear minimal residual disease at days 15 and/or 33 by FC. CD33 expression was ascertained with a three-color combination of CD10 (ALB2), CD33 (D3HL60.251) and CD19 (J4.119, all from Immunotech). Percentage of $CD33^{pos}$ cells was assessed in gated cells according to optical scatter and CD10 and CD19 expression. The data show that despite fluctuations (Figure 1c), most patients retain the high CD33 percentage during the induction treatment (9/12 at day 15 and 7/8 at day 33). Loss of CD33 expression thus usually does not occur at an early phase of treatment.

So far, anti-CD33 treatment in ALL has been considered only for patients with high percentage of $CD33^{pos}$ blasts and no other realistic hope for cure.[1–3] The minimum percentage of $CD33^{pos}$ blasts suitable for anti-CD33 immunotherapy is unknown. In the presented cohort, there are 18 patients (5.5%) who meet the arbitrary criteria of having BCP ALL with CD33 detectable on majority (greater than 50%) of blasts and being in IRG or HRG. The 5-year RFS of these patients was $40\pm15\%$ (the 5-year RFS of the remaining patients from the same subset was $80\pm4\%$, $P=0.0011$). In total, eight of these patients relapsed and one developed a secondary AML. Three of them underwent allogeneic BM transplantation in second or third complete remission. Economic reckoning of anti-CD33 immunotherapy should consider that a proportion of the patients might be rescued from an intensive relapse treatment including BM transplantation.

Setting the anti-CD33 immunotherapy to the induction phase of treatment seems to overcome the issue of antigen shift (Figure 1c). In addition, peripheral nonmalignant $CD33^{pos}$ cells are reduced by the intensive initial chemotherapy. This limits the risk of therapy failure due to peripheral consumption of the anti-CD33 mAb[15] and the effective dose may be lower. Clinical experience is cumulating with single anti-CD33 immunotherapy as well as in combination with chemotherapy. If the presented results are to be taken into clinical trials, a dose escalation study early during treatment is preferable to select a safe dose that does not cause a significant delay in chemotherapy but significantly reduces the leukemic clone. The fact that CD33 is not expressed on all blasts in some cases may appear a problem for the immunotherapy since $CD33^{neg}$ cells may remain unaffected. However, this is not different from the contemporary treatment philosophy; most single agents can kill just a portion of the leukemic cells by themselves and the final success is achieved by combining them. Whereas standard chemotherapy targets mostly dividing cells, the suggested immunotherapy would aim at cells based on partly different characteristics.

Table 2 Incidence of CD33^{pos} cases in ALL subtypes using two cutoff values for positivity (percent CD33^{pos}, number of CD33^{pos} cases/total per subset)

	CD33 at 10%	P-value	CD33 at 20%	P-value
BCP ALL	35% (98/278)	0.0003	23% (65/278)	0.007
T ALL	13% (6/45)		8.9% (4/45)	
PGR (BCP ALL)	35% (89/252)	NS	23% (57/252)	NS
PPR (BCP ALL)	35% (9/26)		31% (8/26)	
TEL/AML1+	59% (48/82)	0.0003	40% (33/82)	0.0001
hyperdiploid	26% (16/62)		9.7% (6/62)	
BCR/ABL+	50% (4/8)		38% (3/8)	
Nonhyperdiploid, BCR/ABL, MLL/AF4, TEL/AML1 negative (BCP ALL)	27% (28/105)		22% (23/105)	

Statistics show differences in respective subgroups (chi-square).

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Supplementary Information

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

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Příloha 2

Transfer of genomics information to flow cytometry: expression of CD27 and CD44 discriminates subtypes of acute lymphoblastic leukemia

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Leukemia 2005 (IF 6,924)

Table for referees only, Mejstrikova et al, Leukemia, rev I.

age group	lab diagnosis	Reference number	Definition of MyAg	Cutoff for positivity [%]	No. of patients	% of positive patients (B and T)	% of positive BCP ALL	% CD33 above cutoff (BCP and T)	% CD33 above cutoff (BCP ALL)	multivariate analysis	univariate analysis	CD33 clone	method	protocols	Treatment results (EFS of entire cohort) [%]	MyAg+ treatment results (EFS of entire cohort) [%]	MyAg- treatment results (EFS of entire cohort) [%]	Treatment results (EFS of BCP) [%]	MyAg+ treatment results (EFS of BCP) [%]	MyAg- treatment results (EFS of BCP) [%]	Median follow up [years]	EFS at [years]
Childhood	single lab	(1)	CD11b, 13, 33, 36, 14, 15, w12. Grouped as: 0,1 and >1 MyAgs	25	267	16 (out of 372)	nd	3.2	nd	nd	n.s.	My9	microscopy or 1FC/2FC	St. Jude protocol	not shown	nd	nd	nd	Graph approx.: 80	Graph approx.: 75	2.5	4
Childhood	single lab	(2)	CD13 or CD33 or CD14	30	236	22	24	8.8	9.7	significant	significant	My9	1FC/2FC	CCG	nd	nd	nd	nd	39+-13	78+-5	nd	3.3
Childhood	single lab	(3)	CD65,CD13,CD 15, CD33	20	206	12	13	1.9	2.3	significant	significant	My9	microscopy	ALL-A-84 and ALL-BFM-86	not shown	nd	nd	nd	38+-13	75+-4	3.3	5
Childhood	single lab	(4)	CD33 or CD13	30	1557	nd	14	nd	nd	nd	n.s.	not shown	FC	CCG	nd	nd	nd	nd	77+-4	76+-1.8	2.7	4
Childhood	single lab	(5)	One of: CD33, CD11b, 13, 14, 15, and 65	20	908	32	33	14	15	n.s.	n.s.	not shown	FC	AIEOP ALL 88 AND 91	66+-1.6	69+-2.3	65+-2.3	nd	nd	nd	4	6
Adult	multi-centric	(6)	CD33 and/or CD13	20	562	9.3	8.9	7.3	6	nd	n.s.	not shown, varied	usually by FC	LALA87	nd	nd	nd	20	nd	nd	2.8	3
Adult	single lab	(7)	CD33 and/or CD13	10	259	30	33	2.7	3.0	nd	n.s.	not shown	FC	CALBG (8811,9111,9311,9511)	nd	nd	nd	39+-8	Graph approx.: 45	Graph approx.: 45	3.8	3
Childhood	single lab	this analysis, Cox's multivariate	CD33, CD13, CD15, CD65	N/A	255	N/A				CD33 significant	N/A	D3HL60 .251	2FC/3FC	ALL BFM 95	70+-3	N/A		72+-3	N/A		3.6	5
Childhood	single lab	this analysis, univariate	CD33	10	324	32	35	32	35	N/A	significant	D3HL60 .251	2FC/3FC	ALL BFM 95	70+-3	55+-6	78+-3	72+-3	54+-6.5	82+-3	3.6	5
			CD33	20		21	24	21	23		significant					53+-8	76+-3		51+-9	78+-3		
			CD33	30		18	20	18	20		significant					53+-8.8	74+-3.2		52+-9	77+-3.3		
			CD33 or CD13	30		24	25	18	20		significant					53+-8.7	74+-3.2		52+-9.2	77+-3.4		

Abbreviations as listed in the paper, nd = not done (not provided), n.s. = not significant, (1,2,3)FC = (1,2,3)-color flow cytometry,
The data demonstrate the points mentioned in the paper, including:

- Criteria for MyAg positivity differ, making comparisons cumbersome.
- Variable percentage of $MyAg^{pos}$ or of $CD33^{pos}$, clones frequently unspecified.
- As newly added to the paper, short follow up possibly causes some of the differences (compare ref 4 and the last line).
- Important parts of survival analyses are often missing.

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Příloha 2

Transfer of genomics information to flow cytometry: expression of CD27 and CD44 discriminates subtypes of acute lymphoblastic leukemia

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CORRESPONDENCE

Transfer of genomics information to flow cytometry: expression of CD27 and CD44 discriminates subtypes of acute lymphoblastic leukemia

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TO THE EDITOR

Expression profiling studies have provided data on an unprecedented number of genes that are expressed in malignant cells.[1–4] The critical number of genes which can predict a particular genotype is a matter of discussion. Downing pointed out that as few as 20 genes may be necessary for an accurate prediction of subsets and prognosis; these genes should be specifically studied, perhaps by methods like reverse transcription polymerase chain reaction (RT-PCR) or flow cytometry (FC) (Downing in Carroll *et al*[5]). FC, which shows the expression of molecules in mutual context on individual cells, appears optimal for such analysis.

The present study (*Microarray-guided FC*) is designed to systematically screen for genes within the existing expression profiling studies on childhood acute lymphoblastic leukemia (ALL).[1,3] The genes, which are identified as best correlating with the pediatric ALL subgroups (E2A/PBX1, MLL, TEL/AML1, BCR/ABL, 'novel' and hyperdiploid genotypes; patients who later relapsed and those who developed therapy-induced acute myeloblastic leukemia), are selected. After recalculation just for the B precursor ALL cases, we select the genes in which the difference in expression is likely to be observed at the protein level. Next, we select molecules with suitable cellular localization (nonsecreted proteins) and with an available mAb. Reactivity and specificity of mAbs are tested in healthy peripheral blood cells and/or in cell lines. The respective molecules are investigated by four-color FC in diagnostic bone marrow (BM) samples. Five molecules have proceeded into this step (CD44, CD27, CD49f, CD247 and CD103). We present here the results of CD44 and CD27 expression, which are investigated in the largest cohort.

A total of 66 patients with B-cell precursor ALL and 14 patients with T lineage ALL were considered to enter CD44 and CD27 investigation. These patients represent all Czech children (age lower than 18 years) diagnosed with ALL between 03/2003 and 02/2004. Five patients (four B-cell precursor and one T lineage ALL) were excluded due to low sample volume. CD44 expression was investigated in 62 B-cell precursor ALL patients (21 TEL/AML1^{pos}, 18 hyperdiploid, four BCR/ABL^{pos}, two MLLR^{pos} and 17 with none of the above-mentioned genotype) and CD27 expression in 56 B-cell precursor ALL patients (21 TEL/AML1^{pos}, 15 hyperdiploid, four BCR/ABL^{pos}, one MLLR^{pos} and 15 with none of the above-mentioned genotype). Informed consent was obtained from patients and/or their guardians. Patients were treated according to ALLIC BFM 2002 or Interfant 99 protocols. The presence of TEL/AML1, BCR/ABL fusion genes and MLL gene rearrangement (MLLR) was detected by two-round nested PCR; hyperdiploidy was assessed using DNA index flow-cytometric measurement. CD44 and CD27 antigens were stained with an anti-CD44 FITC and anti-CD27 FITC (BD, San Jose, CA, USA) in four-color combinations with antigens from a standard panel (anti-CD10 PE, anti-CD19 PC5 or PC7, anti-CD34 APC, Immunotech, Marseille, France). Data were acquired using a FACS Calibur flow cytometer (BD). Antigen positivity was analyzed on gated malignant cells according to the isotype control. To evaluate differences of expression between the subgroups, a nonparametric Mann–Whitney test was performed using StatView software (SAS Institute, Cary, NC, USA).

Expression profiling found the gene for CD44 (Hermes, Pgp-1) to be one of the best correlating with the MLL genotype and with the subgroup of T-ALL patients who later developed hematological relapse.[1,3] Although MLLR^{pos} blasts in our cohort did show CD44 positivity, so far we have not observed a higher CD44 expression compared to other CD44^{pos} B-cell precursor ALL cases (Figures 1a, 2a). In addition, CD44 expression significantly correlated with higher risk T-ALL ($P=0.032$) (Figure 2b). This also indicates that the current risk stratification of the T-ALL patients within the ALL-IC BFM2002 protocol (based on age, leukocyte count at presentation, early treatment response and unfavorable molecular genetics, as in other major frontline therapy protocols) corresponds to the true biological risk. Furthermore, CD44 expression was significantly lower in TEL/AML1^{pos} ALL ($P<0.0001$), which is in line with the observation of one of the two expression profiling studies.[3]

The association with TEL/AML1 genotype was also found in CD27 (TNFRSF7) gene expression,[1] but another expression profiling study[3] showed only a correlation with BCR/ABL genotype. We found a strong correlation of CD27 with TEL/AML1 positivity. CD27^{pos} blasts above 30% were detected in 20/21 and 2/35 patients with TEL/AML1^{pos} and TEL/AML1^{neg} ALL, respectively ($P<0.0001$) (Figure 1a). Since the opposite correlations with TEL/AML1 were observed for CD27 and CD44, we analyzed the composite picture of the expression of these two molecules simultaneously (Figure 1a, b). Most cases in both expression profiling studies and in our cytometric study can be considered either CD44^{pos} or CD27^{pos}. Dual CD27^{pos}CD44^{pos} blasts are typically seen in BCR/ABL^{pos} ALL and a subset of TEL/AML1^{pos} patients exists with CD44^{pos}CD27^{pos} blasts. In our cohort, the TEL/AML1^{pos} case with 51% CD44^{pos} cells presented with an unusually high blast count (peripheral leukocytosis 109×10^6 per ml) and higher percentage CD66c^{pos} blasts (25%) compared to the typical TEL/AML1^{pos} patients[6] – other presentation parameters corresponded well with the genotype.

CD27 has been considered to be a general marker for memory B-cells in humans and so far its expression in human B-cell precursors has not been reported. Here we show that this antigen is indeed expressed in malignant B precursors at a protein level. Moreover, an experiment with seven-color FC on nonmalignant BM (FACS Aria, BD, data not shown) did confirm

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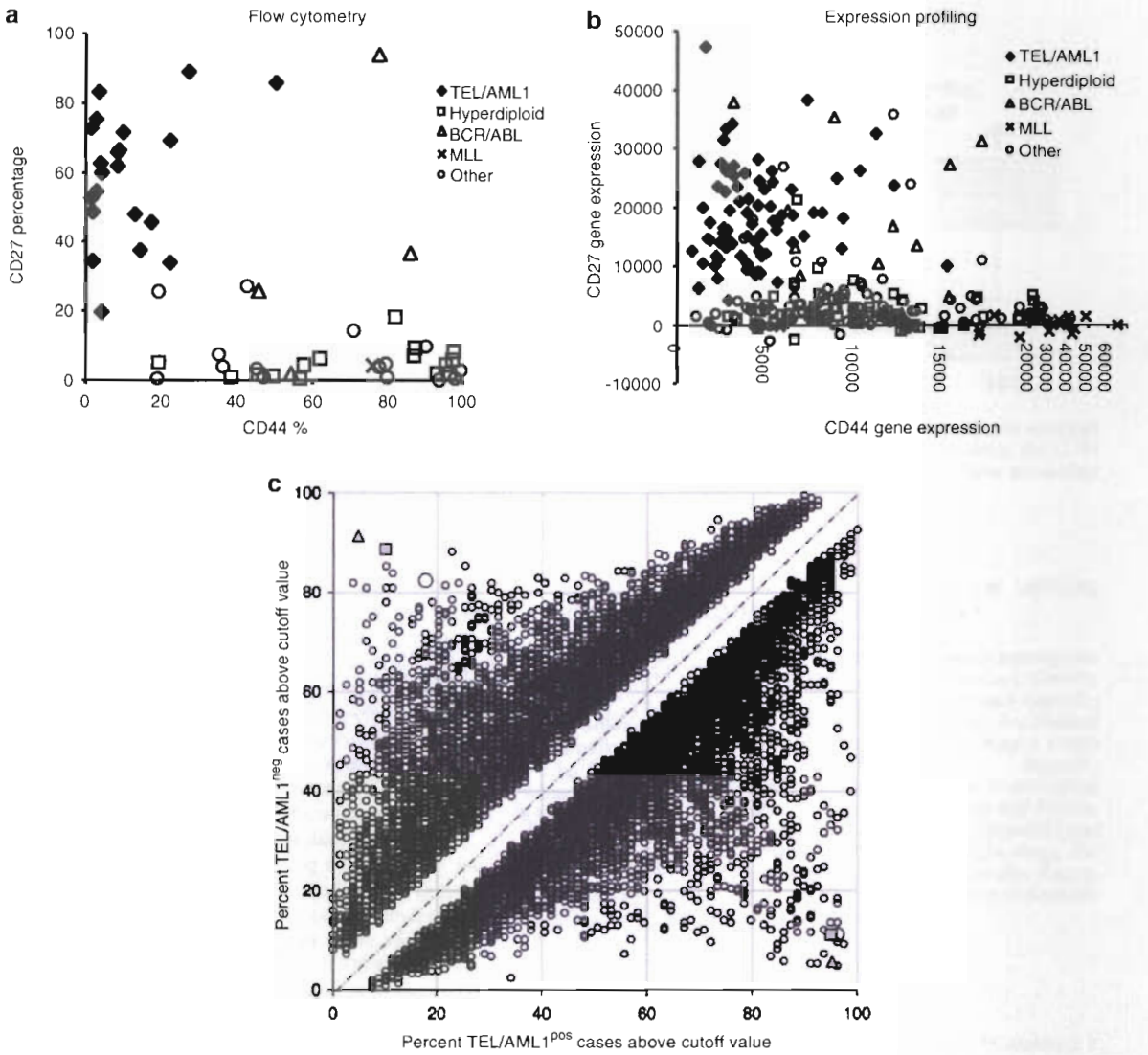

Figure 1 Clustering of ALL genotypic subgroups according to CD44 and CD27 expression. (a) FC data. (b) Expression profiling data from Yeoh *et al.*[1] Probe set numbers 40493_at (CD44) and 38578_at (CD27). (c) The TEL/AML1 predictive value of CD44 and CD27, detected by FC or in two expression profiling studies. Comparison to the other 12 623 probe sets analyzed in Yeoh *et al.*[1] Noncorrelating genes are near the hatched diagonal. An optimal cutoff value was found for each probe set based on the best discrimination of TEL/AML1^{pos} from other B-precursor ALL cases (largest distance from the noncorrelating diagonal). Circles represent individual probe sets (values corresponding to the best CD44 and CD27 probe sets are enlarged). Squares represent CD44 and CD27 from Ross *et al*[3] (probe set numbers 212063_at for CD44 and 206150_at for CD27), triangles show CD44 and CD27 by FC, this cohort. In expression profiling studies, the best correlating probe sets were used in both instances.

CD27^{pos} cells in all four specimens (12±2.2% among DAPI^{neg}CD19^{pos}CD10^{pos}CD20^{neg} cells, among these CD27^{pos} cells 45±13% were CD34^{pos}). The dynamics of CD44 expression on developing B-cells during hematopoiesis was already reported. We are going to analyze the recombination status of Ig genes in subpopulations of precursor B-cells with CD44 and CD27 expression corresponding to lymphoblasts.

To compare the value of CD44 and CD27 with the other expression profiling data for the prediction of TEL/AML1 status, we used the same plot as described previously in the FC metaanalyses[6] (Figure 1c). This format depicts graphically the predictive value of each probe set or molecule for the TEL/AML1 status. Each probe set or molecule is separately compared to its optimal cutoff value in all BCP ALL patients of the respective cohort. The probe-set-specific optimal cutoff value was determined using a statistical software R (http://www.r-project.org). The optimal cutoff value is the one that leads to the best resolution between TEL/AML1^{pos} and TEL/AML1^{neg} subsets, judging by the distance from a noncorrelating diagonal. The percentage of TEL/AML1^{neg} patients above the cutoff value is compared to the respective value in TEL/AML1^{pos} patients. The overall predictive values for CD44 and CD27 are 93 and 95% in FC, 82 and 91% in Yeoh,[1] and 90 and 90% in Ross,[3] respectively. The difference in predictive values is in compli-

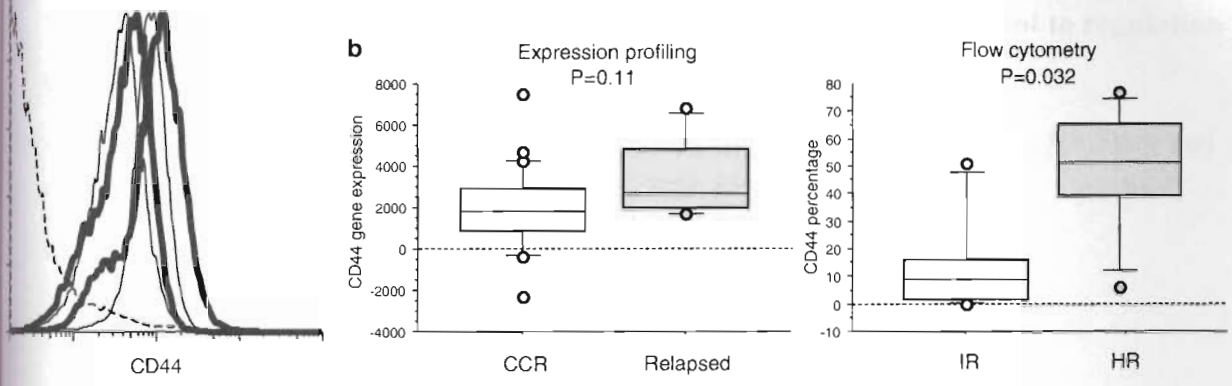

Figure 2 Significance of CD44 expression in specific subsets. (a) Intensity of CD44 expression in B-cell precursor ALL. Histograms represent MLLR^{pos} (thick lines), together with typical hyperdiploid (thin lines) and TEL/AML1^{pos} (dashed line) cases. Y-axis shows frequency. (b) CD44 expression in T-ALL patients. Expression profiling data of patients in clinical complete remission (CCR) and relapsed patients.[1] Probe set number 1126_at is shown (left panel). FC data of patients in the intermediate (IR) and high-risk (HR) groups (right panel).

ance with the fact that FC can investigate the expression of tested molecules on pure ALL cells. We studied the other molecules (CD49f, CD247 and CD103) in specimens of fewer patients and thus it would be too early to establish their predictive values.

The principle that class-defining genes may be selected within microarray data has been suggested previously (Downing in Carroll *et al*[5]). The results of the presented screening strategy prove this principle. Lack of information on protein expression appeared to be the most limiting factor reducing the number of candidate genes in the final FC testing. Improbability of the cell-bound form also excluded molecules during the screening for FC – these secreted molecules may be studied by protein biochemistry. Although cytometric studies on molecules that came from a systematic screening strategy in microarrays have not been presented yet, one new molecule (CD58) has been introduced into FC testing based on expression profiling.[7] The presented data not only show that information from microarrays can be transferred to cell-based investigation by FC, but also that the composite microarray information can be successfully replaced by strong predictors like CD44 and CD27. The ongoing project *Microarray-guided FC* tests whether other molecules can be found with comparable or better predictive values for ALL genotype and prognosis.

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Příloha 3

Myeloid antigens in childhood lymphoblastic leukemia:clinical data point to regulation of CD66c distinct from other myeloid antigens

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Myeloid antigens in childhood lymphoblastic leukemia:clinical data point to regulation of CD66c distinct from other myeloid antigens

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Abstract

Background: Aberrant expression of myeloid antigens (MyAgs) on acute lymphoblastic leukemia (ALL) cells is a well-documented phenomenon, although its regulating mechanisms are unclear. MyAgs in ALL are interpreted e.g. as hallmarks of early differentiation stage and/or lineage indecisiveness. Granulocytic marker CD66c – Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is aberrantly expressed on ALL with strong correlation to genotype (negative in TEL/AML1 and MLL/AF4, positive in BCR/ABL and hyperdiploid cases).

Methods: In a cohort of 365 consecutively diagnosed Czech B-precursor ALL patients, we analyze distribution of MyAg+ cases and mutual relationship among CD13, CD15, CD33, CD65 and CD66c. The most frequent MyAg (CD66c) is studied further regarding its stability from diagnosis to relapse, prognostic significance and regulation of surface expression. For the latter, flow cytometry, Western blot and quantitative RT-PCR on sorted cells is used.

Results: We show CD66c is expressed in 43% patients, which is more frequent than other MyAgs studied. In addition, CD66c expression negatively correlates with CD13 ($p < 0.0001$), CD33 ($p = 0.002$) and/or CD65 ($p = 0.029$). Our data show that different myeloid antigens often differ in biological importance, which may be obscured by combining them into "MyAg positive ALL". We show that unlike other MyAgs, CD66c expression is not shifted from the onset of ALL to relapse ($n = 39$, time to relapse 0.3–5.3 years). Although opposite has previously been suggested, we show that CEACAM6 transcription is invariably followed by surface expression (by quantitative RT-PCR on sorted cells) and that malignant cells containing CD66c in cytoplasm without surface expression are not found by flow cytometry nor by Western blot in vivo. We report no prognostic significance of CD66c, globally or separately in genotype subsets of B-precursor ALL, nor an association with known risk factors ($n = 254$).

Conclusion: In contrast to general notion we show that different MyAgs in lymphoblastic leukemia represent different biological circumstances. We chose the most frequent and tightly genotype-associated MyAg CD66c to show its stabile expression in patients from diagnosis to relapse, which differs from what is known on the other MyAgs. Surface expression of CD66c is regulated at the gene transcription level, in contrast to previous reports.

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Background

Although expression of surface markers in acute lymphoblastic leukemia (ALL) parallels that of normal hematopoietic precursors, several markers of myeloid lineage are found on ALL lymphoblasts. This phenomenon is referred to as "aberrant expression". The issue of the regulatory mechanisms that allow it has been addressed repeatedly throughout the recent 40 years [1,2]. Although several hypotheses stressing either possible lineage indecisiveness or genetic misprogramming have been raised, the phenomenon is still not fully understood. We and others have shown that the myeloid antigen CD66c is very frequently aberrantly expressed in B-precursor ALL, however, a large study showing its frequency in the light of other myeloid antigens has been missing. CD66c expression was found on cases of childhood and adult ALL in strong correlation with nonrandom genetic changes (BCR/ABL positivity [3], hyperdiploidy and TEL/AML1 negativity [4], reviewed in [5]).

CD66c (CEACAM6, previously called Nonspecific cross-reacting antigen, NCA 90/50 and KOR-SA3544 antigen) is a member of the carcinoembryonic antigen family. This heavily glycosylated molecule consists of two constant Ig-like domains and one variable Ig-like domain and it is anchored to the membrane via its glycosylphosphatidylinositol (GPI). Within the hematopoietic system, CD66c expression is limited to granulocytes and its precursors [3,6], where it serves homotypic and heterotypic adhesion [7], Ca2+ mediated signaling [8] and is markedly upregulated from intracellular stores after activation [9].

It is also found in epithelia of various organs [7]. Upregulation of CD66c is an early molecular event in transformation leading to colorectal tumors [10]. It was also confirmed to inhibit anoikis (apoptotic response induced in normal cells by inadequate or inappropriate adhesion to substrate) in the in vitro model of carcinoma of colon [11] and specific silencing of this gene led to decreased metastatic potential in pancreatic adenocarcinoma [12].

Surprisingly, Sugita et al [13] reported intracellular presence of CD66c in all leukemic cell lines examined, regardless of surface presence or absence, with a different antigen distribution in cytoplasm that determined surface expression. They speculated that presence of an undisclosed transporter would target this molecule to granules and for surface expression, whereas surface $CD66c^{neg}$ cell lines lack this transporter. This intriguing hypothesis prompted us to test whether transcription of CEACAM6 gene and/or intracellular CD66c expression is always followed by surface expression.

Uniqueness of aberrant expression of CD66c on malignant lymphoblast is exploited for diagnosis of ALL and follow-up of a minimal residual disease (MRD) using flow cytometry [14,15]. To use a marker for a MRD assessment a critical question must be addressed, whether the aberrant expression is a stable property of the malignant clone or whether it can be subject to immunophenotype shift.

In the present study we set out to address the frequency of CD66c molecule expression in childhood ALL, the regulation of CD66c expression from gene transcription to cytoplasmic and surface expression, and we follow immunophenotype stability from diagnosis to relapse. We also discuss relevance of CD66c for prognosis prediction.

Methods

Patients

The cohort of all Czech children (<18 years) diagnosed with B-precursor ALL investigated in our reference laboratory from 1.5.1997 to 23.7.2004 was used for current study (n = 381). Informed consent was obtained from patients and/or their guardians. The presence of TEL/AML1, BCR/ABL and MLL/AF4 fusion genes was detected by two-round nested PCR, hyperdiploidy was assessed using DNA index flow cytometric measurement as described previously [4]. Patients' genotype and corresponding surface CD66c expression is shown in Figure 1 (genotype available in 98% of patients). For intracellular staining and FACS sorting, only samples with enough material were selected.

Cell lines

Surface CD66c negative cell lines with typical translocation found in childhood ALL: TEL/AML1pos (REH) was kindly provided by R. Pieters (University Hospital Rotterdam), MLL/AF4pos (RS4;11) translocation and with no fusion (NALM-6) were obtained from German Cell Line collection (DSMZ, Braunschweig, Germany)

Flow Cytometry

Flow cytometry immunophenotyping of bone marrow (BM) aspirates was performed in at diagnosis and at relapse. Routine immunophenotypic classification using panel of monoclonal antibodies (moAbs) was performed as described previously [4]. Briefly, BM samples were stained with 2-, 3- and 4-color combinations of moAbs for 15 min in darkness, erythrocytes were lysed with NH_4Cl-containing lysing solution for 15 min, washed and data were acquired using single FACS Calibur instrument throughout the study (BD Biosciences, San Jose, CA, USA) flow cytometer. Anti-CD66c (CEACAM6) moAb used in all diagnostic and relapse measurements in this study was clone KOR-SA3544 directly labeled to FITC (Immunotech, Marseille, France). Intracellular staining was performed using Fix & Perm kit (Caltag, Burlingame, CA,

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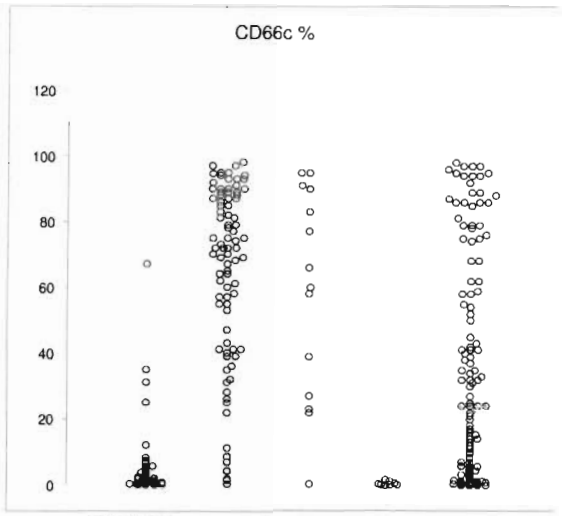

Median	0.55	72	60	0.33	11.5
n	105	91	15	8	154

Figure 1
Correlation of ALL genotype categories and percentage of CD66c positivity. Median percentage of $CD66c^{pos}$ blasts is listed below each genotype group. Data of consecutive unselected patients with BCP ALL (n = 373) are shown.

USA) according to manufacturer's protocol. Acquired data was analyzed with Cell Quest (BD Biosciences) or Flow Jo (Tree Star, Ashland, OR, USA) software, lymphoblast gate was drawn based on optical scatter and $CD19^{pos}$ blast population was selected for further analysis.

Value of 20% was chosen as a threshold of positivity as recommended by EGIL [16]. For robust prognostic significance testing, other threshold values were also tested as indicated in results.

Cross-blocking of CD66c moAbs

Bone marrow samples of CD66c positive blasts were stained with anti-CD66c moAb clone 9A6 (Genovac, Freiburg, Germany) moAb for 15 min, erythrocytes were lysed with NH_4Cl-containing lysing solution for 15 min, washed and sample was incubated with anti-CD66c moAb KOR-SA3544 PE moAb conjugate.

Western blot

Samples containing 5 × 10^6 cells were lysed for 30 min at 4 °C in 100 µl lysis buffer containing 20 mM Tris-HCl (pH 8.2), 100 mM NaCl, 50 mM NaF, 10 mM EDTA, 10 mM pyrophosphate ($Na_4P_2O_7$) and Complete Mini EDTA-Free (protease inhibitor cocktail tablets, Roche Diagnostics, Mannheim, Germany). Debris was sedimented by centrifugation for 3 min at 13000 rpm, 0 °C. Supernatants were mixed with 100 µl 2× Laemmli's SDS-polyacrylamide gel electrophoresis (PAGE) sample loading buffer, and heated for 5 min at 100 °C. Proteins were fractionated by SDS-PAGE on 12.5% gels and electrophoretically transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked for 1 h in PBS (pH 7.4) containing 0.5% Tween-20 and 5% nonfat dried milk. Blots were then incubated for 1 h at room temperature with anti-KOR-SA3544 (Immunotech, Marseille, France) or anti-beta-actin (Sigma-Aldrich, Saint Louis, MO, USA) moAbs and then developed using goat anti-mouse IgG (H+L)-HRP conjugate (Bio-Rad). Immunoreactive material was then revealed by enhanced chemiluminescence (ECL, Amersham, Little Chalfont Buckinghamshire, UK) according to the manufacturer's instructions.

Isolation of RNA and Real-Time Quantitative PCR analysis (RQ-PCR)

For RQ- PCR analysis, leukemic blasts were FACS sorted using sorting option on FACS Calibur or on FACS Aria instrument (1.1 × 10^4 - 4.7 × 10^5 cells from one patient). Isolation of RNA from FACS-sorted cells was performed using Trizol-reagent (Gibco BRL, Carlsbad, CA, USA) according to manufacturer's instructions [17]. Complementary DNA was prepared using M-MLV Reverse Transcriptase (Gibco) according to manufacturer instructions. Glycogen (Gibco) 250 µg /mL was added when initial cell number was lower than 10^5. Quality of cDNA was verified by PCR on beta-2-microglobulin (B2M) housekeeping gene.

RQ-PCR was performed in the LightCycler™ rapid thermal cycler system (Roche Diagnostic GmbH, Mannheim, Germany), according to manufacturer's instructions, using SYBR green intercalating dye. CEACAM6 specific primers 3'-CGCCTTTGTACCAGCTGTAA and 5'-GCATGTCCCCT-GGAAGGA designed by Baranov [18] were used for CEACAM6 amplification and B2M specific primers 3'-GATGCTGCTTACATGTCTCG 5'-CCAGCAGAGAAT-GGAAAGTC [19]were used for total cDNA quantification.

PCR amplification was carried out in 1× reaction buffer (20 mmol/L Tris-HCl, pH 8.4; 50 mmol/L KCl); and 2.0 mmol $MgCl_2$ containing 200 µmol/L of each dNTP, 0.2 µmol/L of each primer, 5 µg bovine serum albumin per reaction, and 1 U of Platinum Taq DNA polymerase (all from Gibco) in a final reaction volume of 20 µL. For each PCR reaction, 2 µL of cDNA template and 2 µl of SYBR Green 5 × 10^{-4} (FMC BioProducts, Rockland, MA, USA) fluorescent dye was included. The cycling conditions were 2.0 minutes at 95 °C followed by 45 cycles of

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Table 1: Frequency of CD66c and myeloid antigen expression. Cases with >20% blasts are regarded positive, coexpression of CD66c and other MyAg is tested by Fisher's exact test.

Molecule	No of cases (total = 365)	Proportion [%]	Coexpression with CD66c	
CD66c	156	43		
CD33	85	23		
CD15	72	20		
CD13	57	16		
CD65	14	3.8		
CD66c and CD33	21	5.8	mutually exclusive	p = 0.002
CD66c and CD15	30	8.2	random	NS
CD66c and CD13	9	2.5	mutually exclusive	P < 0.0001
CD66c and CD65	2	0.55	mutually exclusive	p = 0.029

denaturation at 94°C for 5 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 15 seconds. CEACAM6 and B2M gene were amplified separately from the same cDNA, and all experiments were performed in duplicate. Melting curve analysis was performed after each run; in case of peak melting temperature shift, PCR products were verified on agarose gel electrophoresis.

Normalized CEACAM6 Expression (CEACAM6n)

Amplification and calibration curves were generated by using affiliated software (LightCycler 3 data-analysis software; version 3.5.28; Idaho Technology Inc., Salt Lake City, UT, USA). A calibration curve for the B2M and CEACAM6 housekeeping gene was generated using the series of 10× diluted cDNA from peripheral blood granulocytes as a standard for both reactions. Crossing point (Cp) value was calculated with LightCycler 3 software using second derivative maximum method. CEACAM6n value is relative and represents a ratio of CEACAM6 to B2M (CEACAM6n = CEACAM6/ B2M). Standard cDNA from granulocytes was assigned CEACAM6n value of 1, the same aliquot of granulocytes cDNA was used throughout of study.

Statistics

Statistical evaluation was done with Statview software, (SAS Institute Inc, NC, USA). We used Fisher's exact test, regression coefficient, Mann-Whitney test and Logrank (Mantel-Cox) test as described in text.

Results

Frequency of CD66c and myeloid antigen (MyAg) expression

We selected 365 patient's samples obtained at diagnosis of B-precursor ALL with available information on the expression of MyAg CD13, CD15, CD33, CD65 and CD66c. This subcohort represents 96% of all B-precursor ALL diagnosed in the study period. The CD66c molecule was expressed on 43% cases (Table 1, cases with >20% positive blasts were considered positive). For the fraction of positive cells and correlation with genotype see [5], of note, 29% of patients expressed CD66c on more then 50% blasts. Comparison with other MyAg showed that CD66c is more frequently expressed. Coexpression of CD66c with other MyAg was not a usual finding (Table 1, Figure 2). Expression of CD13, CD33 and CD65 tended to be non-random (mutually exclusive) with CD66c (Table 1). Coexpression of CD66c with any 2 of the other MyAg was found in fewer than 4 cases in each combination. Interestingly, mutual relationship of other MyAg was random, with the exception of CD13 and CD33 coexpression ($p < 0.0001$) and CD15 and CD65 coexpression ($p = 0.0002$). The analysis was performed also at different cutoff values (10, 30 and 50 %; data not shown). The same or less significant correlations were also observed at different cutoff values.

Cross-blocking of KOR-SA3544 clone with 9A6 clone

The moAb clone KOR-SA3544 was not included in Human Leukocyte Differentiation Antigens workshop, but was characterized by Sugita et al [13]. To prevent ambiguous interpretation of our data we extended characterization of KOR-SA3544 clone of CD66c moAb by blocking experiments on $CD66c^{pos}$ blasts. Pretreatment of cells with workshop-typed clone 9A6 moAb completely blocked binding of KOR-SA3544 clone in all 9 leukemic specimens and in granulocytes (data not shown).

Cytoplasmic presence of CD66c in ALL blasts

We have studied surface and cytoplasmic expression of CD66c in 20 ALL diagnostic samples by flow cytometry. In contrast to findings of Sugita et al [13], we have detected CD66c exclusively in all 8 surface positive cases. None of the 12 surface negative cases stained in cytoplasm

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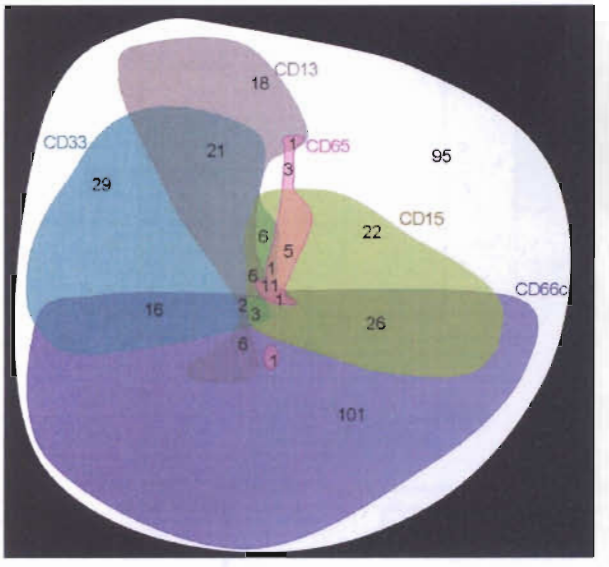

Figure 2
Graphical illustration of myeloid antigen positivity in childhood B-precursor ALL. For each antigen, positive cases are represented by a colored form. The areas of the forms roughly correspond to the frequency of positive cases (observed numbers of patients are marked in red) while the shapes are constructed to illustrate the respective coexpressions. An arbitrary cutoff value of 20% is used for all antigens. The CD66c positivity correlates with negativity of any of the following: CD33 ($p = 0.002$), CD13 ($p < 0.0001$) and CD65 ($p = 0.029$). There was a significant correlation between CD33 and CD13 positivity ($p < 0.0001$) and between CD15 and CD65 positivity ($p = 0.0002$) whereas the positivity of no other two antigens of the ones shown correlated significantly with each other. Total number of B-precursor cases illustrated is 365.

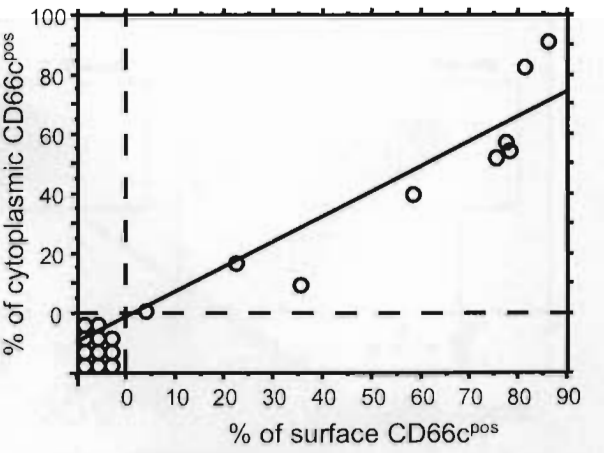

Figure 3
Relationship of surface and cytoplasmic expression of CD66c. Percentage of surface expression of CD66c in ALL blasts is plotted against cytoplasmic expression (after cell membrane permeabilization). Samples of 20 patients at ALL diagnosis are shown, 12 CD66c negative and 8 CD66c positive. Regression coefficient $R^2 = 0.927$

(Figure 3). The probable cause of the opposite finding in several cases (lower percentage after permeabilization than on surface) is a higher background after permeabilization (isotypic control mean fluorescence intensity was 4.3 ± 2.0 and 9.7 ± 3.7 for surface and permeabilized staining, respectively), which covers borderline events.

Transcription of CEACAM6 gene

To extend the above findings, we used Real-Time Quantitative Reverse Transcription-PCR (RQ-RT-PCR) to quantitatively assess presence of specific CEACAM6 mRNA. We FACS-sorted CD19^{pos}CD66c^{neg} or CD19^{pos}CD66c^{pos} blast cells for RQ-RT-PCR analysis. We didn't find significant amount of CEACAM6 transcript in surface CD66c^{neg}lymphoblasts, whereas CD66c^{pos} cells contained CEACAM6. When CD66c^{neg} and positive fraction was FACS-sorted of heterogeneous specimens (lymphoblasts partly positive for CD66c) the level of CEACAM6 was observed higher in CD66c^{neg} cells and lower in CD66c^{pos} cells as compared to uniform populations (Figure 4). In one specimen (ALL patient with Down syndrome), CEACAM6 wasn't increased in CD66c^{pos} fraction.

Western blot

We further question the intracellular CD66c positivity in surface CD66c negative cell lines. We performed Western blot as described by Sugita et al. [13] on REH (TEL/AML1^{pos}) and RS4;11 (MLL/AF4^{pos}) cell lines and found no CD66c protein (Figure 5). Furthermore we found NALM-6 (surface CD66c^{neg}, no translocation) cell line negative. Two BCR/ABL and four hyperdiploid (all surface CD66c^{pos}) diagnostic samples used as positive controls were positive, with the similarly narrow band contrasting to broad band detected in granulocytes (Figure 5), suggesting different glycosylation in keeping with report by Sugita.

Stability of surface expression from diagnosis to relapse

All relapsed patients up till 12/2003 with available information on CD66c expression at diagnosis and at relapse were used to assess stability of CD66c expression. Comparison of CD66c expression in 39 cases of relapsed childhood ALL cases to their immunophenotype at diagnosis

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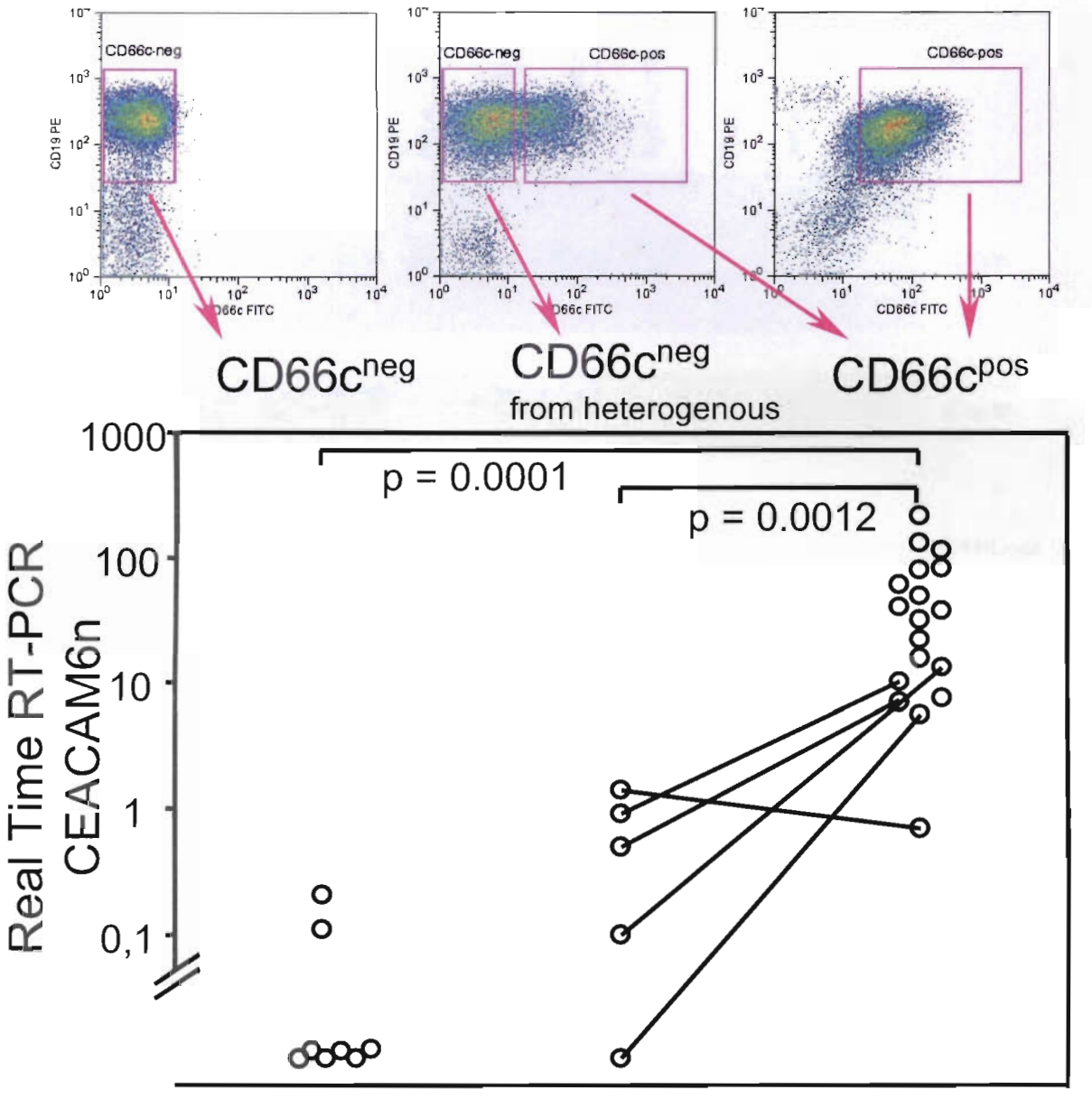

Figure 4

Transcription of CEACAM6 versus surface CD66c expression on sorted cells. FACSsorted CD66c surface negative (CD66c^{neg}) or positive (CD66c^{pos}) ALL lymphoblasts, five patients with heterogeneous CD66c expression were sorted into both CD66c negative and CD66c positive fraction (lines connect sorted fractions from the same specimen). Mann-Whitney test was used to compare groups (n = 32). CEACAM6n value is normalized to beta-2-microglobulin (see Methods).

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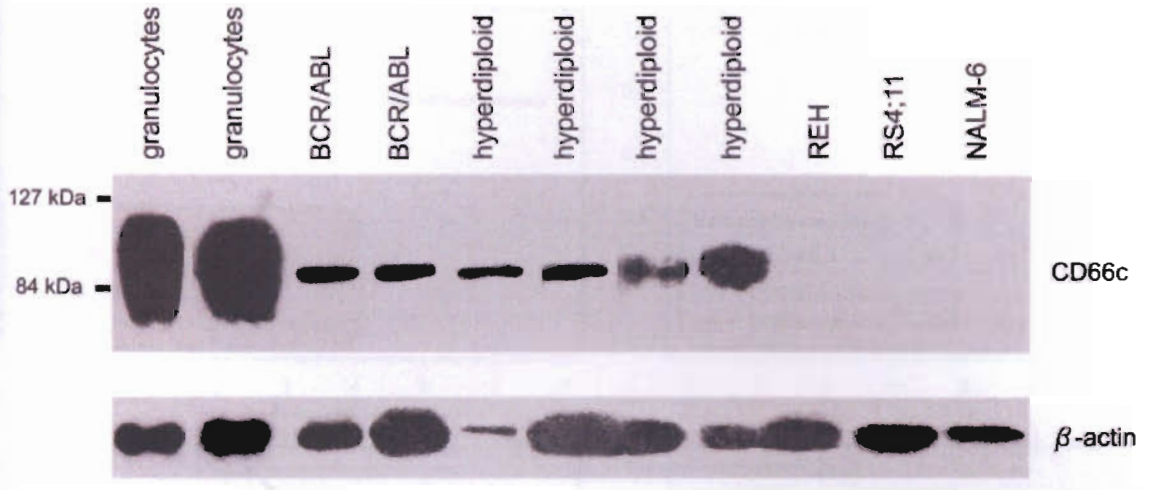

Figure 5
Western blot of granulocytes, ALL samples of CD66c positive cases and surface CD66c^{neg} cell lines with TEL/AML1pos (REH), MLL/AF4pos (RS4;11) translocation and with no fusion (NALM-6).

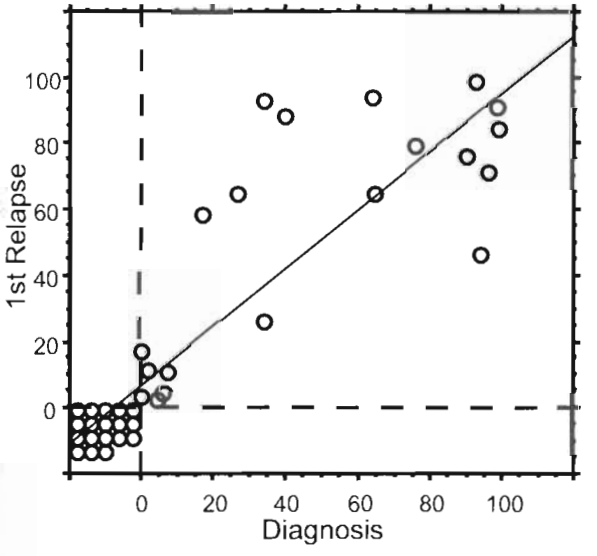

Figure 6
Stability of CD66c from diagnosis to relapse. Each circle represents one patient (n = 39). Percentage of CD66c^{pos} blasts at diagnosis is plotted against percentage of CD66c^{pos} blasts at relapse. Regression line with 95% confidence R^2 = 0.755

revealed that both negativity and positivity of this antigen was retained from diagnosis to relapse (Figure 6; median time to relapse 2.5y min 0.3y, max 5.3y). Although the quantitative levels of CD66c expression differed in some patients (median difference 0.0%, standard deviation 21%), no case of CD66c complete loss or gain was found in our cohort.

Prognostic significance of CD66c expression

Only B-precursor ALL patients treated on the same ALL BFM 95 treatment protocol [20] (n = 254) were evaluated for prognostic impact. The prognosis did not differ for cases with either CD66c^{pos} blasts exceeding either 20% (Figure 7) or any other cutoff value tested (5%, 10% and 50%, data not shown).

Next, we asked whether CD66c expression correlated with the risk factors used in ALL BFM-95 protocol for stratification into risk groups [21]. No difference in relapse free survival (RFS) was noted when analyzed separately for each risk group or higher and lower initial leukocytosis (cutoff value: 2×10^4 cells per ml), age group or response to prednisone (groups as in Table 2).

When analyzed with respect to a genotype, we found no prognostic value of CD66c in any defined group (BCR/ABL^{pos}, TEL/AML1^{pos}, hyperdiploid ALL and none of the above-mentioned genetic changes, Figure 7 and Table 2).

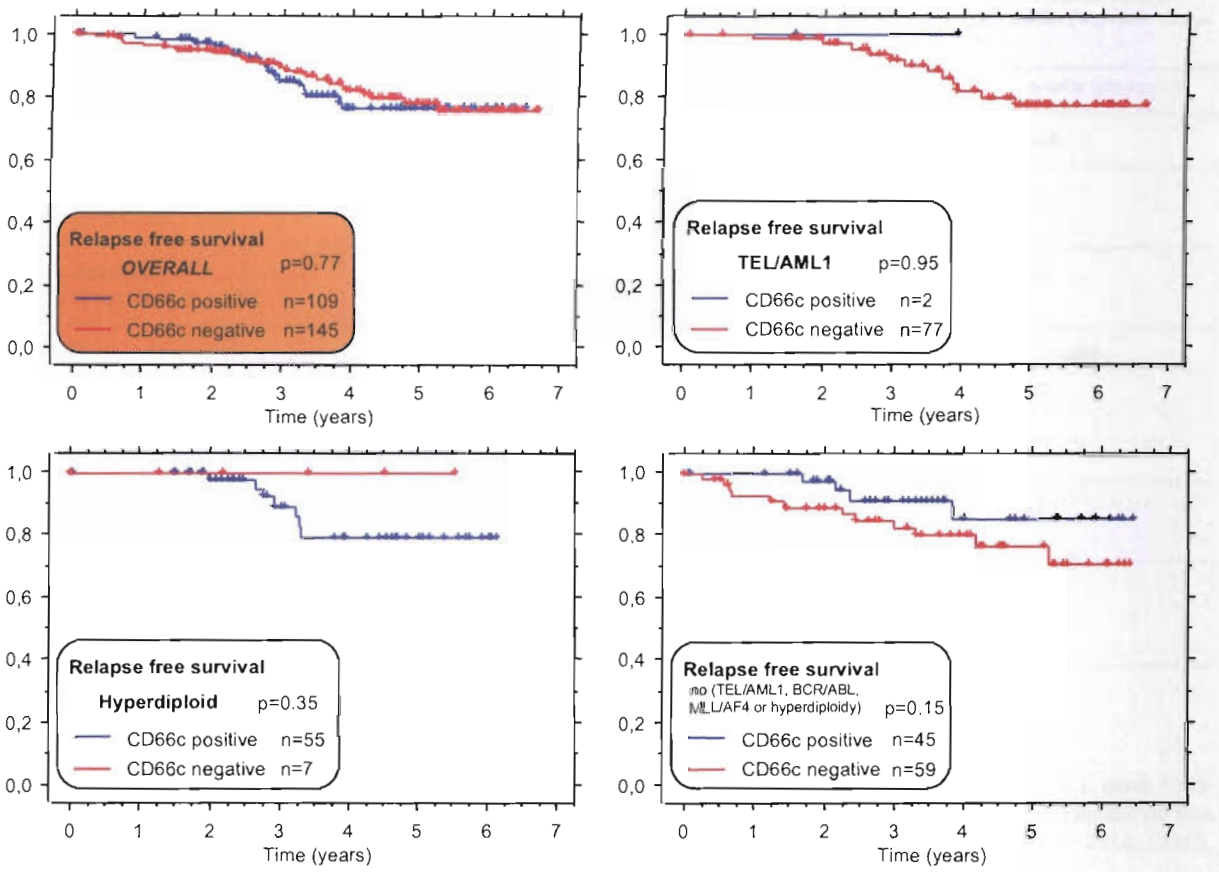

Figure 7
Relapse free survival of cases with CD66c ^{pos} (blue line) or CD66c^{neg}(red line) B-precursor ALL. Unselected consecutive patients treated on ALL BFM95 protocol (median follow up 3.64 years). Since surface CD66c associates with genotype, separate analyses for distinct genotype subgroups are shown.

In contrast to the study by Hanenberg et al [22], there was no correlation between initial leukocytosis and CD66c in our cohort (Table 2).

Discussion

Our data on childhood B-precursor ALL show that CD66c is more frequently expressed than the myeloid antigens included in the standard immunophenotyping panels for ALL. To our knowledge, CD66c is the most frequent myeloid marker in childhood ALL. This, together with the tight correlations between CD66c and genotype [5], makes CD66c a pertinent object of research on aberrant expression regulation.

In line with the data from Sugita, we confirm the specificity of KOR-SA3544 clone moAb for CD66c by CEACAM6 mRNA detection and by cross-blocking of KOR-SA3544 binding by representative 9A6 clone, that suggests a spatial proximity of the two epitopes recognized. Furthermore we show that all CD66c^{pos} ALL specimens show a similar extent of glycosylation as cell lines analyzed by Sugita, which differs from the extent of glycosylation in granulocytes.

Since there is a strong correlation of ALL genotype and CD66c expression, we hypothesized that surface CD66c expression would be controlled by gene transcription rather than by targeting to surface from intracellular stores as proposed by Sugita [13]. In accordance with this, both intracellular staining and Western blot failed to identify cytoplasmic CD66c protein in any surface CD66c^{neg} cells. Down the same line, no CEACAM6 transcript was

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Table 2: Correlation between risk factors and CD66c expression. The distribution of CD66c^{pos} and CD66c^{neg} cases (cutoff 20%) is shown. In addition, no difference was observed in the RFS of the risk-defined subsets based on the CD66c expression (log-rank test p-value > 0.05 in all analyses). Only patients treated by a single ALL BFM-95 protocol are shown here (n = 254).

	CD66c^{pos} cases	CD66c^{neg} cases	p-value (chi-square)
All patients	109	145	N/A
Prednisone poor responder	9	12	n.s.
Prednisone good responder	100	133	
Initial leukocytosis = > 20 × 10^9/L	28	44	n.s.
Initial leukocytosis < 20 × 10^9/L	81	101	
TEL/AML1	2	77	P < 0.0001
BCR/ABL	7	1	
MLL/AF4	0	1	
Hyperdiploid	55	7	
Other genotype (not TEL/AML1, BCR/ABL, MLL/AF4 or hyperdiploidy)	45	59	
Age 1–5	59	88	n.s.
Age >5	50	57	
Standard risk group	40	58	n.s.
Intermediate risk group	54	72	
High risk group	15	15	

detected in surface CD66c^{neg} lymphoblasts. Overall our data suggest that transcription is the checkpoint that leads to surface expression, rather then the former model, which proposed that all malignant lymphoblasts generate the CD66c molecule but only some of them target it for the cell membrane.

Interestingly, importance of this molecule was shown in a model of colorectal carcinoma where transfection with CEACAM6 inhibited anoikis (10), high CEACAM6 predicted high risk patients with resectable colorectal cancer (9) and CEACAM6 gene silencing decreased resistance to anoikis in vitro leading to inhibition of metastatic ability in mouse model (11). Although the function of CEACAM6 in ALL blasts is still unknown, this molecule's function has been recently associated with pathogenesis of other types of cancer in man [10-12,23,24]. Study of anti-CEACAM6 immunotoxin-based therapy in mouse model of pancreatic carcinoma was published recently [25].

So far, prognostic significance of expression of myeloid antigens CD13, CD14, CD33, CD65w, CD11b and CD15 has been studied with conflicting results (summarized in [26]). As determined in our large cohort of patients treated on ALL BFM 95 protocol, no prognostic significance of CD66c could be revealed in general, nor when we analyzed separate risk groups or TEL/AML1^{pos}, BCR/ABL^{pos}, hyperdiploid and other B-precursor ALL cases separately. Furthermore, instability of aberrant expression was reported for most myeloid markers (CD13, CD14, CD15, CD33 and CD65).

Stability of expression is a major concern of flow cytometric studies of MRD. In present, use of multiple CD markers is widely recommended to prevent MRD underestimation due to the immunophenotype shift (discussed in [15,27]). In current study we show for the first time that CD66c expression stays qualitatively stable from diagnosis to relapse in all relapsed cases studied. This finding, together with high frequency of CD66c^{pos} cases, supports inclusion of CD66c into a moAbs panels for MRD detection in patients positive for this CD marker at diagnosis. However, anecdotal downregulation of CD66c expression during chemotherapy has been observed [15], but has not been methodically studied yet. Any temporary downregulation might lead to falsely lower values of MRD measurement – thus, it would be worthwhile to disclose whether this phenomenon occurs regularly at certain points of chemotherapy.

Mutual exclusiveness of MyAg expression as well as different stability of CD66c compared to other MyAgs [28] challenges the general practice of prognostic evaluation of MyAg^{pos} ALL cases as a group [26] and favors individual

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evaluation of contribution/regulation of each MyAg for blast cell.

Conclusion

CD66c presents some of the tightest associations with ALL genotype. Although our findings indicate that CD66c is unlikely to gain a practical importance as a prognosis predictor, there are several reasons to focus on it in diagnostic and MRD studies. CD66c, apparently the most frequently expressed aberrant antigen in childhood ALL, is very useful in discriminating leukemic blasts from non-malignant cells. Aberrant expression remains a puzzling phenomenon that warrants further investigation. If it is confirmed by techniques sensitive enough that the so called "aberrant markers" are truly not expressed on any subtle population of lymphoid precursors, there will be an opportunity to find new targets for specific ALL therapy (e.g. monoclonal antibodies against differently glycosylated form of CD66c) that will spare the non-leukemic precursors, thus reducing the treatment toxicity.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

TK performed flow cytometry, cell sorting, RQ-RT-PCR study and drafted the manuscript, MV carried out the Western blot study, EM acquired and analyzed patients flow cytometry data and performed the statistical analysis, JM designed and assisted to the RQ-RT-PCR study, JT designed RT-PCR, did the genotype detection and critically discussed the manuscript, JS contributed to the study design and organization and OH conceived of the study, analyzed data and drafted the manuscript. All authors read and approved the final manuscript.

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Příloha 4

Residual disease monitoring in childhood acute myeloid leukemia by multiparameter flow cytometry: the MRD-AML-BFM study group

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Residual Disease Monitoring in Childhood Acute Myeloid Leukemia by Multiparameter Flow Cytometry: The MRD-AML-BFM Study Group

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ABSTRACT

Purpose
Monitoring of residual disease (RD) by flow cytometry in childhood acute myeloid leukemia (AML) may predict outcome. However, the optimal time points for investigation, the best antibody combinations, and most importantly, the clinical impact of RD analysis remain unclear.

Patients and Methods
Five hundred forty-two specimens of 150 children enrolled in the AML-Berlin-Frankfurt-Muenster (BFM) 98 study were analyzed by four-color immunophenotyping at up to four predefined time points during treatment. For each of the 12 leukemia-associated immunophenotypes and time points, a threshold level based on a previous retrospective analysis of another cohort of children with AML and on control bone marrows was determined.

Results
Regarding all four time points, there is a statistically significant difference in the 3-year event-free survival (EFS) in those children presenting with immunologically detectable blasts at 3 or more time points. The levels at bone marrow puncture (BMP) 1 and BMP2 turned out to have the most significant predictive value for 3-year-EFS: 71% ± 6% versus 48% ± 9%, $P_{\text{Log-Rank}} = .029$ and 70% ± 6% versus 50% ± 7%, $P_{\text{Log-Rank}} = .033$), resulting in a more than two-fold risk of relapse. In a multivariate analysis, using a combined risk classification based on morphologically determined blasts at BMP1 and BMP2, French-American-British classification, and cytogenetics, the influence of immunologically determined RD was no longer statistically significant.

Conclusion
RD monitoring before second induction has the same predictive value as examining levels at four different time points during intensive chemotherapy. Compared with commonly defined risk factors in the AML-BFM studies, flow cytometry does not provide additional information for outcome prediction, but may be helpful to evaluate the remission status at day 28.

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INTRODUCTION

Minimal residual disease (MRD) monitoring in childhood and adult acute myeloid leukemia (AML) using flow cytometry is still under discussion in terms of the prognostic impact, the optimal time points for analysis, and the best antibody combinations. AML blast cells do not express specific antigens that could serve as single and unambiguous markers for RD in regenerating bone marrow. It is therefore necessary to carefully characterize combinations of antigens that are able to sensitively detect residual blast cells among normal hematopoietic cells during treatment. Another obstacle for MRD monitoring in AML is the instability of the blast cell antigen expression pattern. As previously shown by us[1] and others,[2-4] the vast majority of AML cases undergo a shift of antigen expression pattern between diagnosis and eventual relapse. It is therefore indispensable for MRD evaluation to monitor a wide range of leukemia-associated immunophenotypes (LAIP).

Until now, there are only a few reports about the prognostic relevance of RD monitoring in pediatric[5,6] and adult[7-10] AML. These investigations were based on different therapy regimens and have employed divergent technical approaches in terms of utilized LAIP, time points of analysis during therapy, and the positivity thresholds used for outcome prediction.

The objective of this study was to determine the following assessment criteria for the AML-Berlin-Frankfurt-Muenster (BFM) treatment strategy: the

sensitivity of specific LAIP based on a retrospective analysis of children with AML; the appropriateness of RD assessment by multidimensional flow cytometry for outcome prediction in children with AML; the most predictive time point during therapy; and the additional value of flow cytometric assays for outcome prediction in comparison to known risk factors.

PATIENTS AND METHODS

Study Design

The AML-BFM MRD study is composed of two phases. Phase A includes the establishment and standardization of a consensus panel for four-color immunophenotyping. The focus was to define the sensitivity and specificity of different LAIP in normal and regenerating bone marrow specimens[11] and to identify clinically relevant threshold-levels for each LAIP at defined time points during treatment in a retrospective approach. Phase B was designed to prospectively apply these thresholds to evaluate the impact of RD monitoring for outcome prediction.

Patients

Phase A. Retrospectively, 65 children with de novo AML (continuous complete remission: n = 40; relapse: n = 25) and enrolled in the AML-BFM 98 study (Table 1) at a median follow-up of 1.5 years have been studied for the occurrence of LAIP in regenerating bone marrow.

Phase B. Children enrolled in the AML-BFM 98 study and diagnosed for de novo AML between January 1, 2002 and July 31, 2004 were eligible for the prospective evaluation. Excluded were children with acute promyelocytic leukemia (AML French-American-British [FAB] M3), with t(15;17)/PML-RARα and children with trisomy 21 because they exhibit biologically different leukemias and receive slightly different chemotherapy. Altogether, 542 samples from 150 children (Table 1) were available. This cohort is representative as compared with the overall study collective in terms of age, sex, WBC, FAB classification, and risk-group allocation.

From these 150 children, five children with detectable blast cells by flow cytometry, but an antigen expression pattern that was not covered by the LAIPs used for RD monitoring (CD33/7/117/56, CD33/56, CD7/33) were excluded.

The standard risk group (SR) definition that is generally used for stratification in the AML-BFM studies comprises FAB subtype (M1/M2+ Auer rods, M3, M4eo), favorable cytogenetics, and morphologically determined bone marrow blasts of less than 5% at day 15 (not required for FAB M3).[12] For this analysis, the morphologic evaluation at day 28 of treatment (< 5% blasts in bone marrow) was included as an additional parameter for the SR group (herein referred to as extended AML-BFM risk).

The date of each bone marrow puncture was correlated to the courses of intensive chemotherapy of the AML-BFM 98 study (Fig 1). Only specimens that were obtained at one of the first four scheduled time points (bone marrow puncture [BMP]1: day 15; BMP2: before second induction; BMP3: before third therapy course; BMP4: before fourth therapy course) were included in the study (Table 2).

Bone marrow specimens were obtained after informed consent from each patient or each patient's guardian. All children were treated according to the German AML-BFM 98 study (as to the treatment schedules see Creutzig et al[13]). All investigations performed had been approved by the local ethics committees and were in accordance with an assurance filed with and approved by the Department of Health and Human Services.

Diagnosis

Diagnosis and classification were established according to the criteria of the FAB[14-16] group by the reference laboratory of the AML-BFM studies in Muenster and were reviewed by an expert group of independent hematologists. In addition to the Pappenheim stained bone marrow and blood smears the following cytochemical stainings were performed: periodic acid Schiff, myeloperoxidase, alpha-naphtyl-acetate esterase, and acid phosphatase. The diagnoses of M0 and M7 subtypes were always confirmed by immunological methods.

Cytogenetic and molecular genetic data were obtained from the reference laboratory of the AML-BFM study (J. Harbott, Giessen, Germany).

Multiparameter Flow Cytometry

Four-color flow cytometry—according to the consensus panel of the AML-BFM MRD study group as previously described in detail[11]—was performed at the immunology laboratories at the University Children's Hospital, Muenster, Germany, the St Anna Children's Hospital, Vienna, Austria, the

Table 1. Patient Characteristics at Diagnosis for Both Parts of the Study

Characteristic	Phase A (retrospective) No.	%	Phase B (prospective) No.	%
Age at diagnosis, years				
Median	7.59		9.98	
Range	0.2-17.7		0.06-20.0	
Leukocytes, 1/μL				
Median	15,400		12,800	
Range	200-500,000		500-550,000	
Bone marrow blasts, %*				
Median	69		62	
Range	18-98		15-99	
Sex (male/female)	34/31	52/48	75/75	50/50
FAB				
M0	6	9	6	4
M1/M2	20	31	61	41
M4/M5	22	34	62	41
M6	5	8	4	3
M7	8	12	12	8
Other/not classifiable	4	6	5	3
Karyotype				
Normal	20	31	24	16
t(8;21)	5	8	22	15
inv(16)	2	3	16	11
11q23	15	23	22	15
Risk group (SR/HR)	16/49	33/67	48/102	32/68
Total	65		150	

Abbreviations: FAB, French-American-British; SR, standard risk; HR, high risk.
*Three children presented with < 20% blasts at diagnosis: two children (17% and 18%) with M2 and t(8;21) and one child with M7 (15% in the smear; no bone marrow punch biopsy available) and myelofibrosis.

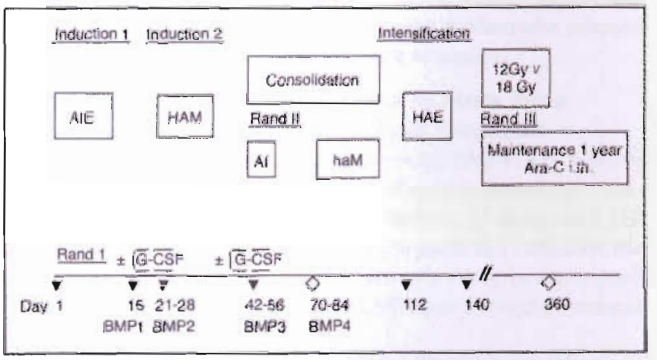


Fig 1. Acute Myeloid Leukemia-Berlin-Frankfurt-Muenster study 98 treatment schedule with bone marrow puncture (BMP) time points. AIE, Ara-C (cytarabine), idarubicin, and etoposide; HAM, high-dose Ara-C and mitoxantrone; AI, Ara-C and idarubicin; haM, medium-high-dose Ara-C and mitoxantrone; HAE, high-dose Ara-C and etoposide; Ara-C i.th., intrathekal Ara-C; G-CSF, granulocyte colony stimulating factor; Rand, randomization.

Table 2. Distribution of Eligible Specimens and Time Points

Time Point	No. of Specimens	Days From Start of Chemotherapy		
		Median	Range	Protocol
BMP1	97	15	13-27	15
BMP2	123	29	14-56	21-28
BMP3	120	60	31-101	42-56
BMP4	62	96	59-146	70-84

Abbreviation: BMP, bone marrow puncture.

Charles University, Prague, Czech Republic, and at the University Hospital, Goettingen, Germany, at initial diagnosis and during intensive chemotherapy. Anticoagulated bone marrow samples were sent to one of the laboratories by overnight mail.

A wide antibody panel based on a CD33/CD34 backbone, independent of the initial immunophenotype including fluorescence conjugated myeloid markers CD13-PE (SJ1D1; Immunotech, Krefeld, Germany), CD15-FITC (MMA; Becton Dickinson, Heidelberg, Germany), CD33-PC5 (D3HL60.251; Immunotech), CD33-APC (D3HL60.251; Immunotech), and HLA-DR-FITC (L243, Becton Dickinson), lymphoid markers CD7-PE (8H8.1; Immunotech), CD10-FITC (ALB2; Immunotech), CD19-FITC (J4.119; Immunotech), CD56-PE (NCAM 16.2; Becton Dickinson), the activation and proliferation marker CD38-PE (HB7; Becton Dickinson) as well as the progenitor-associated markers CD34-APC (8G12; Becton Dickinson), CD34-PC7 (581; Immunotech) and CD117-FITC (95C3; Immunotech) was applied (Table 3). Syto 16 (Molecular Probes, Eugene, OR) was used for staining nucleated cells to exclude debris and not completely lysed erythrocytes from analysis.

After incubating the bone marrow samples with monoclonal antibodies for 15 minutes, erythrocytes were lysed for 7 minutes using FACS Lysing Solution (Becton Dickinson) or Versa Lyse (Beckman Coulter, Krefeld, Germany). Afterwards, the specimens were washed twice with 2 mL phosphate-buffered saline (PBS)-buffer (pH 7.4) and centrifuged (5 minutes, 20°C, 600 g) to remove excess antibodies and lysed RBCs. Specimens were measured using the FACS-Calibur (Becton Dickinson) or EPICS (Beckman Coulter), analyzing at least 30,000 events. Extensive interinstrumental comparisons were performed to ensure that either site could detect similar percentages of positivity.

Data Analysis/Gating Strategy

The specimens were analyzed using the Paint-a-gate PRO-Software (Becton Dickinson) or Cytomics RXP Software (Beckman Coulter). All data were reviewed centrally in the immunology laboratory of the University Children's Hospital Muenster, in order to warrant homogeneous data analysis and interpretation. Residual malignant cells among normal hematopoietic cells were identified by cluster analysis using six parameters (forward and side scatter properties as well as four antigens simultaneously). Antigen positivity was inferred if the fluorescence intensity could be clearly separated from negative controls (isotypic controls or negative cell populations for the examined antigen). In a three-step analysis the residual blast cells were determined roughly by their forward scatter/side scatter properties followed by a precise gating according to the LAIP to be investigated (for details see Langebrake et al[1]). To avoid bias, all samples were evaluated by at least two investigators.

Table 3. Consensus Antibody Panel Used for RD Monitoring Within the AML-BFM MRD Study Group

Tube No.	Antibody			
	FITC	PE	PC5 or APC	APC or PC7
1	Syto 16	CD7	CD45	CD34
2	HLA-DR	CD38	CD33	CD34
3	CD15	CD13	CD33	CD34
4	CD2	CD7	CD33	CD34
5	CD15	CD117	CD33	CD34
6	CD19	CD56	CD33	CD34

Abbreviations: RD, residual disease; AML-BFM, Acute Myeloid Leukemia Berlin-Frankfurt-Muenster; MRD, minimal residual disease; FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin; PC5, phycoerythrin-cyanine 5; PC7, phycoerythrin-cyanine 7.

Statistics

Event-free survival (EFS) was calculated from date of diagnosis to last follow-up or first event (failure to achieve remission, resistant leukemia, relapse, second malignancy, or death of any cause). Failure-free survival (FFS) was calculated from date of diagnosis to last follow-up or failure (relapse, nonresponse). Patients who did not attain a complete remission (according to the Cancer and Leukemia Group B criteria[17]) were considered failures at time zero. Survival was calculated from date of diagnosis to death of any cause or to last follow-up. Univariate analysis was conducted by the Wilcoxon test for quantitative variables and Fisher's exact test for qualitative variables. When frequencies were sufficiently large, χ^2 statistic was used. Probabilities of survival were estimated using the Kaplan-Meier method, with SEs according to Greenwood, and were compared with the log-rank test. Cumulative incidence functions of relapse and death in continuous complete remission were constructed by the method of Kalbfleisch and Prentice. Computations were performed using SAS version 6.12 (SAS Institute Inc, Cary, NC).

RESULTS

Definition of Cut Off Levels by Retrospective Analysis

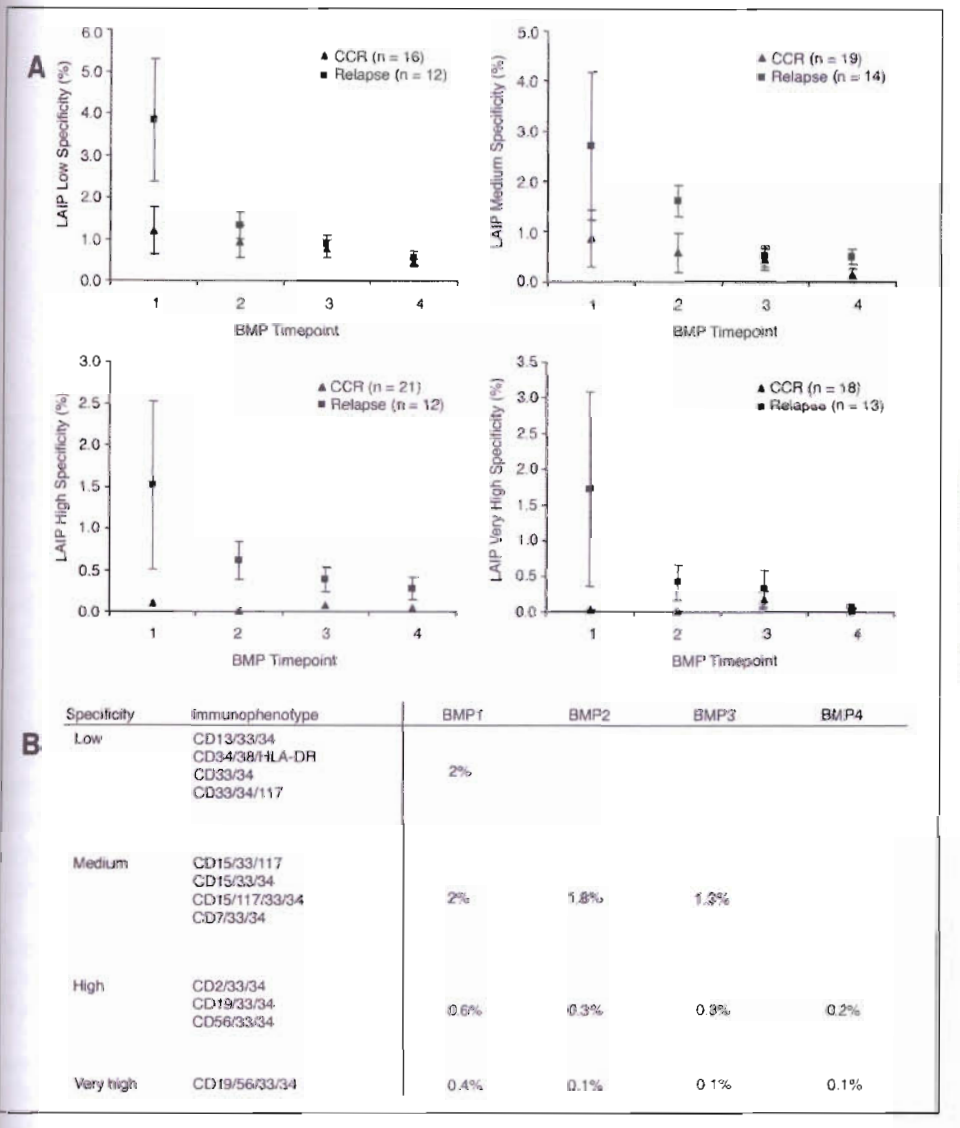
The determination of LAIP specificity has been described in detail previously.[11] In brief, bone marrow specimens of 39 children with acute lymphoblastic leukemia, Ewing sarcoma, non-Hodgkin's lymphoma, or without a malignant disease were evaluated for the presence and amount of different LAIP. Three groups of specificity could be defined according to the median percentage of LAIP in regenerating bone marrow: low specificity with 1.0% or more, medium specificity from 0.1 to 1.0%, and high specificity with less than 0.1%.

Based on these results, clinically prognostic relevant thresholds for each specificity group and time points were defined by retrospectively analyzing 25 children with relapse and 40 children without relapse at a median follow-up of 1.5 years. Low specificity LAIP are only informative at BMP1, while high and very high specificity LAIP can be utilized for discrimination at all four time points. According to these data, the thresholds given in Figure 2 were calculated that are able to unambiguously discriminate between those children who relapsed and those children in continuous complete remission.

Serial Assessment of RD at Three or More Time Points Identifies Children With Poor Prognosis

Children with at least three specimens until BMP4 ($n = 95$) have been evaluated to investigate the impact of serial immunologic monitoring for outcome prediction. In 34 children, all measured LAIP levels were below the threshold at each time point. In 13 children, the measured LAIPs in at least three specimens were above the determined threshold. In 48 children, one or more LAIPs were above the threshold at one or two time points (Table 4).

Using this approach, it was possible to identify a poor-risk group, characterized by positive flow cytometric assays at three or more consecutive time points during chemotherapy, with an EFS of 31% ± 11% (Fig 3). However, EFS was not significantly different between children who were negative by flow cytometry at all analyzed time



B

Specificity	Immunophenotype	BMP1	BMP2	BMP3	BMP4
Low	CD13/33/34 CD34/38/HLA-DR CD33/34 CD33/34/117	2%			
Medium	CD15/33/117 CD15/33/34 CD15/117/33/34 CD7/33/34	2%	1.8%	1.3%	
High	CD2/33/34 CD19/33/34 CD56/33/34	0.6%	0.3%	0.3%	0.2%
Very high	CD19/56/33/34	0.4%	0.1%	0.1%	0.1%

Fig 2. (A) Median percentage of leukemia-associated immunophenotypes (LAIP ± SE) in bone marrow according to specificity and time point. Data are obtained by a retrospective analysis of children with acute myeloid leukemia suffering from relapse versus continuous complete remission. (B) Cutoff level (as percentage of nucleated cells) according to LAIP specificity and time point utilized for residual disease classification. CCR, continuous complete remission; BMP, bone marrow puncture.

points and those with one or two positive time points (73% ± 8% *v* 61% ± 7%, $P = .43$). Combining the good and the intermediate group, the difference in contrast to the poor group was statistically significant: P_{EFS} 65% ± 5% *v* 31% ± 13%; $P = .02$. Shifting the cut off levels, which were used for group allocation, did not result in a better distinction in terms of EFS (data not shown).

Single Time Point RD Assessment Before Second Induction Is Most Informative for Outcome Prediction

When investigating the four different time points separately, we found that only at BMP1 and BMP2, there is a statistically significant difference between RD-positive and RD-negative children in the 3-year EFS (Fig 4; 71% ± 6% *v* 48% ± 9%; $P_{Log\text{-}Rank} = .029$; 70% ±

Table 4. Risk Groups and Outcome According to Residual Disease Determined in Children With Three or More Eligible Time Points

Risk Group	Time Points	No. of Patients	CCR	Relapse	NR	Death in CCR	Secondary Leukemia
Good	All negative	34	25	7	0	2	0
Intermediate	1-2 above threshold	48	30	16	1	0	1
Poor	≥ 3 above threshold	13	4	6	2	1	0

Abbreviations: CCR, continuous complete remission; NR, nonresponse.

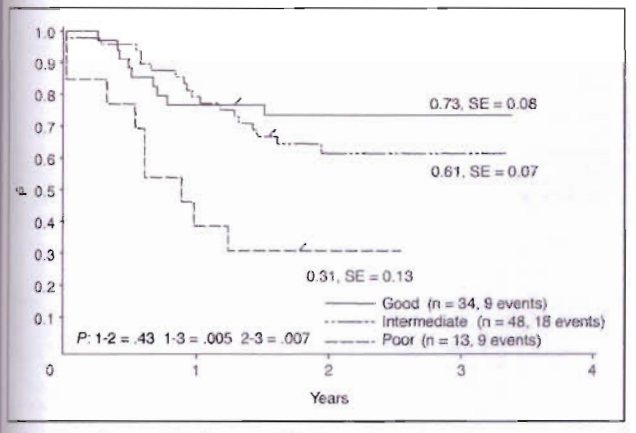


Fig 3. Event-free survival (3 years) of children with at least three specimens until BMP4 (good: all time points negative; intermediate: 1-2 time points positive; poor: ≥ 3 time points positive; SE, standard error probability of event-free survival).

6% *v* 50% ± 7%; $P_{\text{Log-Rank}} = .033$). This is also true, if only high and very high specificity LAIPs are regarded (70% ± 6% *v* 37% ± 11%; $P_{\text{Log-Rank}} = .031$; 66% ± 6% *v* 45% ± 8%; $P_{\text{Log-Rank}} = .045$). Similarily, only at BMP1 and BMP2 the RD-positive children differed significantly from the RD-negative children regarding their cumulative nonresponse and relapse incidence: 48% ± 10% *v* 25% ± 6%; $P_{\text{Gray}} = .02$; 48% ± 7% *v* 25% ± 6%; $P_{\text{Gray}} = .01$. Regarding 3-year overall survival, only BMP2 turned out to be a statistically significant discriminator ($P = .036$).

MRD Monitoring Has No Additional Prognostic Impact Compared With Known Risk Factors

Using the univariate COX regression model for FFS risk assessment, both BMP1 and BMP2 had statistically significant impact: RR, 2.35 (95% CI, 1.13 to 4.89; $P = .021$) and risk ratio, 2.21 (95% CI, 1.18 to 4.14; $P = .013$), respectively.

Regarding only those children, who responded to treatment as determined by morphology at day 15, there is a statistically significant difference in 3-year EFS between MRD-negative and MRD-positive children: 69% ± 6% *v* 40% ± 15% ($P < .05$).

A multivariate analysis controlling for AML-BFM risk classification, including FAB subtype, cytogenetics, and morphologically determined blasts at day 15, was performed. At BMP1, both flow cytometry and AML-BFM risk show almost the same risk ratio for FFS with similar 95% CIs (2.09; 1.00 to 4.39; 2.06; 0.87 to 4.88). The influence of BMP2 on the risk of failure is less than the impact of the AML-BFM risk, however, the differences are not significant. Using an extended risk group classification including morphologically determined blasts at day 28 as a covariate, this turned out to have more impact on FFS with a RR of 2.8 for both time points (Table 5).

We further analyzed, whether the inclusion of flow cytometry in the risk group assessment could help to define more precisely risk groups for additional treatment stratification. Therefore, the 3-year EFS according to immunologically determined RD was calculated separately for the SR and high-risk group. There was no difference in both groups at BMP1 or BMP2 (Table 6).

DISCUSSION

Due to the fact that the antigen expression pattern of AML blasts differs significantly between diagnosis and relapse[1-4] and that no specific antigens exist which clearly can identify leukemic blasts, we have developed an antibody panel that allows us to detect residual blast cells independently of the initial immunophenotype. Based on a CD33/CD34 basis, the LAIPs utilized for MRD assessment comprise the commonly accepted antigen expression patterns in AML.[18-20] We are the first group applying time-dependent prognostically relevant cut off levels that have been determined by the retrospective analysis of children treated within the AML-BFM studies for the occurrence of 12 different LAIPs at defined time points, instead of empirically defining cut off level.

In our international prospective study, we were able to show that the detection of residual blast cells by flow cytometry at early time points of follow-up (until day 84) is a significant predictor of treatment outcome regarding 3-year EFS. Especially in the very early course of therapy—before the start of the second induction, at day 28 from diagnosis—multidimensional flow cytometry can help to differentiate between children with good and poor prognosis. Similar results were obtained by the exclusive analysis of LAIPs with high or very high specificity, indicating that antigene combinations with low or medium specificity are not relevant for RD monitoring. Furthermore, flow cytometry is also able to detect "minimal" RD in those children with morphologically undetectable blasts after first induction.

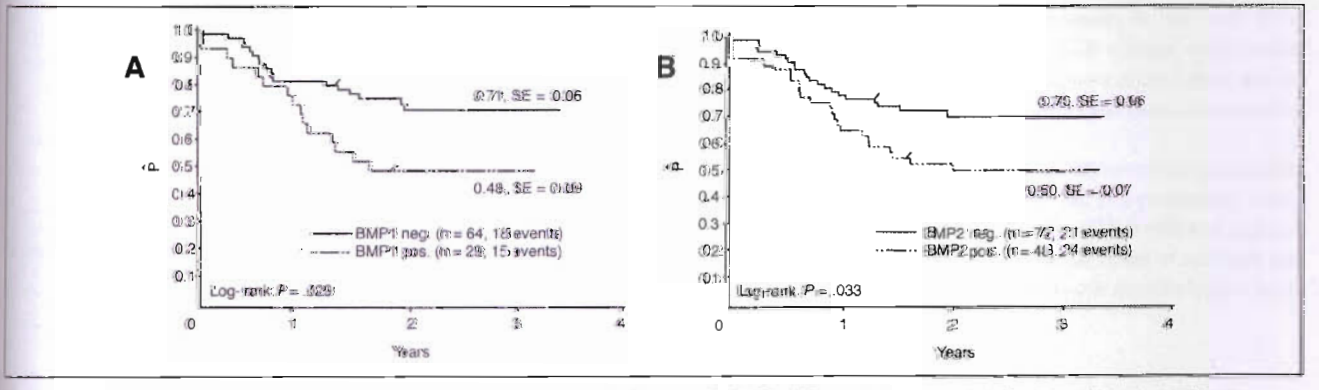


Fig 4. Event-free survival (3 years) of (A) bone marrow puncture (BMP) 1 and (B) BMP2 (SE, standard error probability of event-free survival).

Table 5. Univariate and Multivariate Cox Models for Failure-Free Survival

Cox Model	Time Point: BMP 1 RR	BMP 1 95% CI	BMP 1 P	BMP 2 RR	BMP 2 95% CI	BMP 2 P
Univariate						
Flow cytometry	2.35	1.13 to 4.89	.02	2.21	1.18 to 4.14	.01
Multivariate						
Flow cytometry	2.09	1.00 to 4.39	.05	1.84	0.97 to 3.51	.06
AML-BFM risk	2.06	0.87 to 4.88	.10	2.24	1.01 to 4.97	.05
Multivariate						
Flow cytometry	1.98	0.95 to 4.16	.07	1.79	0.94 to 3.41	.08
Extended AML-BFM risk	2.80	1.05 to 7.46	.04	2.80	1.15 to 6.80	.02

Abbreviations: BMP, bone marrow puncture; RR, risk ratio; MRD, minimal residual disease; AML-BFM, Acute Myeloid Leukemia Berlin-Frankfurt-Muenster.

Our results are consistent with reports on pediatric and adult AML regarding the prognostic impact of immunological blast detection after first induction.[5,7,9] The series of Coustan-Smith et al[5] comprises the analysis of residual blasts at the end of remission induction therapy by defined marker combinations dependent on the initial immunophenotype. In the RD-negative group, their results show less than 0.1% residual AML cells: six children (21%) relapsed, and three children (10%) died in CR, whereas in the RD-positive group the proportion of children who relapsed and of those in CR is equal (both relapse and CCR, n = 5; 38%; death in CR, n = 3). The probability of 2-year overall survival is statistically different, but the assimilation of the two curves after that time indicates that a stable situation has not yet been achieved.

Other investigator groups found that only the monitoring at later time points[6,8,10] (after consolidation therapy) is significant for outcome prediction, which may limit the clinical usefulness of these data for risk-adapted therapy tailoring. The most recent report by Kern et al[21] even revealed that only time points longer than 1 year after diagnosis were independently related to EFS and overall survival. During this period of therapy follow-up, RD positivity may rather have represented resurgent leukemia (occult relapse) than a surrogate marker of initial therapy response usable for treatment stratification. The individual differences in the kinetics of leukemic recurrences may therefore impede a prospective application of this approach for the early diagnosis of relapse.

Table 6. 3-Year EFS According to Risk Group Stratification and Flow Cytometric Determined Residual Disease Level at Time Points BMP1 and BMP2

Risk Group	No. of Patients	No. of Events*	3-Year EFS	SE	P
BMP1					
SR					
Negative	28	4	0.86	0.07	.31
Positive	6	2	0.67	0.19	
HR					
Negative	36	14	0.60	0.08	.16
Positive	23	13	0.43	0.10	
BMP2					
SR					
Negative	31	8	0.84	0.07	.66
Positive	8	2	0.75	0.15	
HR					
Negative	41	16	0.58	0.08	.22
Positive	40	22	0.44	0.08	

Abbreviations: EFS, event-free survival; BMP, bone marrow puncture; SR, standard risk; HR, high risk.
*An event is the failure to achieve remission, resistant leukemia, relapse, second malignancy, or death of any cause.

Although we could show that flow cytometry is a reliable and objective method to detect residual blast cells in regenerating bone marrow specimens, and that it is therefore appropriate for outcome prediction, we further wanted to know whether these results bear additive values for treatment stratification as compared with conventional risk factors.

Notably, the AML-BFM risk group classification[12] is based on initial cytogenetics, FAB classification, and morphologically detectable blast cells of more or less than 5% at day 15. Applying this binary risk group classification to the children analyzed, a highly significant difference in terms of 3-year EFS is achieved without using immunological information: 78% ± 6% versus 49% ± 6% ($P = .0025$). When including information on morphological blast counts from day 28 (BMP2) in addition to the conventional risk classification (extended AML-BFM risk classification), the difference in 3-year EFS between the two risk groups even increased to 85% ± 5% versus 48% ± 5% ($P = .0002$).

The impact of flow cytometry results on FFS at either time point was found equivalent to that of the AML-BFM risk in terms of risk ratio and 95% CIs. When including the extended AML-BFM risk classification in the COX-model, it turned out that immunological RD as a covariate does not contribute to a better risk group separation. It has to be mentioned that none of the recently published studies of other groups included information of RD monitoring as compared with morphologically determined blast cells at the analyzed time points as part of the risk classification system. Considering our results, this comparison has to be recommended in order to interpret potential additional values of flow cytometric investigations correctly. Applying the covariates used by other groups (age at diagnosis, leukocyte count at diagnosis, karyotype) to our data, we obtain similar significant results for the influence of BMP1 and BMP2 on outcome. However, in our study the more sophisticated AML-BFM risk group classification has been taken as the basis to determine the true additional value of MRD monitoring. In conclusion, for the AML-BFM studies, risk group stratification based on FAB subtype, cytogenetics, and morphologically determined bone marrow blasts before second induction does not benefit from inclusion of RD data, as assessed by multidimensional flow cytometry.

Further investigations will focus on an improved risk group stratification including blast percentage at day 28. In a prospective study, we will evaluate whether the discrimination of blast cells and regenerating bone marrow cells can be improved in terms of accuracy and objectivity by flow cytometry as compared with morphological interpretation alone.

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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Příloha 5

Detectable minimal residual disease before allogeneic hematopoietic stem cell transplantation predicts extremely poor prognosis in children with acute lymphoblastic leukemia

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Pediatr Blood Cancer

Detectable Minimal Residual Disease Before Allogeneic Hematopoietic Stem Cell Transplantation Predicts Extremely Poor Prognosis in Children With Acute Lymphoblastic Leukemia

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Background. The level of minimal residual disease (MRD) prior to allogeneic hematopoietic stem cell transplantation (HSCT) has been shown to be an independent prognostic factor for outcome of pediatric patients with high-risk acute lymphoblastic leukemia (ALL). Retrospective studies which used (semi-) quantitation of clone-specific immunoglobulin/T-cell receptor (Ig/TCR) rearrangements have documented the feasibility and practicality of this technique. This approach has also been disputed due to the occurrence of clonal evolution and generally high MRD levels prior to HSCT. ***Procedure.*** In our prospective study, MRD before and after HSCT was monitored using quantitative real-time PCR in a cohort of 36 children with ALL consecutively transplanted in our center between VIII/2000 and VII/2004. ***Results.*** In 25 of 36 patients, MRD level prior HSCT was assessed. Seventeen patients were classified as MRD-negative and eight were MRD-positive up to 9×10^{-2}. In MRD-positive subgroup, seven events (six relapses) occurred post-transplant in striking contrast to only one relapse in MRD-negative subgroup (event-free survival (EFS) log-rank $P < 0.0001$). MRD proved to be the only significant prognostic factor in a multivariate analysis ($P < 0.0001$). Adoptive immunotherapy including donor lymphocyte infusions in patients with adverse dynamics of MRD after HSCT had only limited and/or temporary effect. Clonal evolution did not present a problem precluding MRD monitoring in any of patients suffering a post-transplant relapse. ***Conclusions.*** We show that MRD quantitation using clonal Ig/TCR rearrangements successfully assesses the risk in pediatric ALL patients undergoing allogeneic HSCT. As our ability to treat detectable MRD levels after HSCT is very limited, alternative strategies for MRD-positive patients prior HSCT are necessary. Pediatr Blood Cancer

Key words: acute lymphoblastic leukemia; childhood; hematopoietic stem cell transplantation; immunoglobulin and T-cell receptor gene rearrangements; minimal residual disease

INTRODUCTION

Despite the overall improvement in the chemotherapy-based front-line treatment of the childhood acute lymphoblastic leukemia (ALL), the hematopoietic stem cell transplantation (HSCT) remains an important treatment option for the patients with resistant, very high-risk, and/or relapsed disease. However, the curative effect of allogeneic HSCT is hampered by a relapse occurrence that represents a major cause of the HSCT failure. Already in 1998, Knechtli et al. showed that the level of minimal residual disease (MRD) prior HSCT represents an important prognostic factor [1]. They used a semi-quantitative approach for the detection of immunoreceptor gene—immunoglobulin and T-cell receptor genes (Ig/TCR) rearrangements. In their cohort, all children entering the pre-transplant conditioning with a high-level MRD suffered a post-transplant event and children with low-level MRD had significantly poorer outcome compared to MRD-negative subgroup. Similar data were obtained in the subsequent studies, partly using the new technique of real-time quantitative PCR (RQ-PCR) [2,3].

When this quantitative technique for MRD detection was completely introduced and progressively standardized, the international *Pre-BMT MRD Study Group* (part of the *European Study Group on Minimal Residual Disease in ALL—ESG–MRD–ALL*) [4] retrospectively analyzed the pre-transplant MRD levels and the post-transplant outcome in a cohort of 140 pediatric ALL patients [5]. MRD proved to be a highly significant ($P < 0.001$) independent factor to influence event-free survival (EFS) of this group. High MRD burden, together with a shorter duration of the first complete remission (CR), MLL gene rearrangements, and pro-B immunophenotype proved to be the only negative risk factors.

This large multicenter, retrospective study was recently disputed by Imashuku et al. [6]. Their analysis, based on a group of 95 transplanted patients (age <20 years), showed no correlation between the pre-transplant MRD burden and the post-transplant relapse. Imashuku and colleagues made a couple of rather surprising observations: first, they found 96% of their patients to be MRD-positive prior HSCT.

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Second, their ability to predict the relapse after HSCT was hampered by the clonal evolution of Ig/TCR rearrangements. Eleven of 16 patients having relapse after HSCT showed a totally different rearrangement pattern at relapse when compared to the initial screening. This is in striking contrast to previously published data that show more than 95% of relapsed patients have preserved rearrangements allowing the MRD follow-up [7]. On the basis of their results, Imashuku et al. dispute the practicality of the Ig/TCR-based approach.

Here, we present a series of 36 pediatric ALL patients who consecutively underwent allogeneic HSCT using unmanipulated grafts at our institution. In 25 of them, we were able to assess MRD level prior and after the transplant using RQ-PCR-based detection of Ig/TCR rearrangements. We show that detectable MRD before HSCT is a very strong negative prognostic factor and that the clonal evolution of the Ig/TCR rearrangements does not hamper the relapse prediction. Therefore, our study clearly demonstrates for one thing the feasibility of the method and for another a very strong clinical value of this approach for the identification of patients at the risk of relapse after allogeneic HSCT.

MATERIALS AND METHODS

Patients and Treatment

Between August 2000 and September 2004, 36 consecutive pediatric patients (age 1.1–19 years) with ALL indicated to HSCT were enrolled to our study (Table I). This cohort comprised all such pediatric patients from the Czech Republic in the given period of time and all transplants were

TABLE I. Transplanted Patients With ALL

No.	Age/sex	Immunophenotype	Fusion gene	Remission	Conditioning	Donor (HLA match)	GVHD grade	Follow-up (months)
1	11/m	T-ALL	Not detected	CR1	TBI 12 Gy, VP16	MSD	III, cGVHD	61
2	12/m	pro-B	ETV6/RUNX1	PR3	TBI 12 Gy, VP16	MSD	II	15*
3	10/m	cALL	ETV6/RUNX1	CR3	TBI 14.4 Gy,VP16	UD (10/10)	II	55
4	8/m	T-ALL	Not detected	CR1	TBI 12 Gy, VP16	MSD	II	52
5	7/m	T-ALL	Not detected	CR2	TBI 14.4, Cy	UCB (5/6)	II	4*
6	15/m	pro-B	MLL/AF4	CR1	TBI 12 Gy, VP16	MSD	I, cGVHD	46
7	10/m	cALL	ETV6/RUNX1	CR2	TBI 12 Gy, VP16	MSD	II	44
8	10/m	AHL/cALL	BCR/ABL	CR1	TBI 12 Gy, VP16	UD (10/10)	II	21*
9	11/m	cALL	BCR/ABL	CR2	TBI 12 Gy, VP16	UD (10/10)	—	24*
10	15/f	praeB/cALL	BCR/ABL	CR2	TBI 12 Gy, VP16	UD (9/10)	—	6*
11	12/m	praeB/cALL	BCR/ABL	CR1	TBI 14.4 Gy, Cy	UD (9/10)	—	4*
12	19/m	praeB/cALL	Not detected	CR3	TBI 12 Gy, VP16	UD (10/10)	II	38
13	16/m	cALL	Not detected	CR2	TBI 12 Gy, VP16	UD (10/10)	II	2*
14	10/m	cALL	ETV6/RUNX1	CR2	TBI 12 Gy, VP16	UD (7/10)	II	9*
15	8/f	cALL	ETV6/RUNX1	CR2	TBI 12 Gy, VP16	MSD	—	32
16	7/f	cALL	Not detected	CR2	TBI 12 Gy, VP16	MSD	II	32
17	3/m	T-ALL	Not detected	CR1	TBI 12 Gy, VP16	MSD	II	31
18	10/f	cALL	BCR/ABL	CR2	TBI 12 Gy, VP16	UD (10/10)	—	30
19	2/m	pro-B	MLL rearrangement	CR2	BuCy, VP16	UD (10/10)	—	0*
20	8/m	AHL/pro-B	BCR/ABL	CR2	TBI 12 Gy, VP16	UD (9/10)	II	26
21	8/m	cALL	Not detected	CR2	TBI 12 Gy, VP16	UD (9/10)	II	26
22	1/f	T-ALL	Not detected	CR1	BuCy, VP16	UD (10/10)	II	12*
23	8/m	T-ALL	SIL/TAL1	CR1	TBI 12 Gy, VP16	MFD (10/10)	II	23
24	10/f	cALL/pro-B	ETV6/RUNX1	CR2	TBI 12 Gy, VP16	UD (9/10)	II	22
25	6/m	cALL	Not detected	PR3	TBI 12 Gy, VP16	UD (10/10)	II	19
26	2/m	pro-B	MLL/AF9	CR2	BuCy, Mel	MSD	II	18
27	12/m	cALL	BCR/ABL	CR1	TBI 12 Gy, VP16	UD (8/10)	II	18
28	9/m	praeB	Not detected	CR2	TBI 12 Gy, VP16	UD (9/10)	IV	1*
29	5/m	cALL	BCR/ABL	CR1	TBI 12 Gy, VP16	UD (10/10)	II	17
30	8/m	cALL	ETV6/RUNX1	CR2	TBI 12 Gy, VP16	UD (10/10)	II	17
31	12/m	pro-B/cALL	Not detected	PR3	TBI 12 Gy, VP16	UD (10/10)	II	16
32	16/m	praeB	Not detected	PR3	TBI 12 Gy, VP16	UD (10/10)	—	1*
33	9/m	cALL	Not detected	CR2	TBI 12 Gy, VP16	MSD	II	15
34	17/f	AHL	BCR/ABL	CR1	TBI 12 Gy, VP16	UD (7/10)	II	14
35	7/m	cALL/praeB	ETV6/RUNX1	CR3	TBI 12 Gy, VP16	UD (9/10)	II	12
36	14/m	cALL	BCR/ABL	CR1	TBI 12 Gy, VP16	UD (10/10)	—	10*

Bu, busulphan; Cy, cyclophosphamide; Mel, melphalan; VP16, vepesid; CR, complete remission; PR, partial remission; TBI, total body irradiation; MSD, matched sibling donor; UD, unrelated donor; UCB, unrelated cord blood; MFD, matched family donor; GVHD, graft-versus-host disease; cGVHD, chronic graft-versus-host disease.
*— time to event.

performed at our institution. The group of patients consisted of 30 children with B-cell precursor (BCP) ALL and 6 children with T-cell ALL. Twelve children were transplanted in the first CR, all of them due to high-risk ALL (T-ALL and prednisone poor response (4), BCR–ABL-positive (7), MLL rearranged (1)); 20 children were transplanted in the second or higher remission; and 4 children in partial remission (without increased number of blast cells in the bone marrow but without recovery of hematopoiesis). Donors of hematopoietic stem cells were HLA-identical siblings in 10 cases, unrelated donor from BMT registries with variable rate of HLA match (from 7 to 10/10 antigen match on high resolution PCR level) in 24 cases; one patient was transplanted using unrelated cord blood and one patient from phenotypically identical mother.

In the majority of transplants (n = 32), we used a similar pre-transplant conditioning regimen based on the total body irradiation (TBI) in the dose 12 or 14.4 Gy and etoposide 60 mg/m^2, in the cord blood transplantation TBI plus cyclophosphamide 2 × 60 mg/kg. In three children under 2 years of age a busulphan-based conditioning (2× busulphan, cyclophosphamide, and melphalan, 1× busulphan, cyclophosphamide, and etoposide) was used. In the majority of unrelated donor transplants (n = 19) we used rabbit antithymocytic globulin (ATG, Fresenius) at 10 mg/kg for 4 days.

Graft-versus-host disease (GVHD) prophylaxis consisted of intravenous (i.v.) cyclosporin A (CsA) in the dose 3 mg/kg/day in HLA-identical sibling donors and using the combination of CsA 5 mg/kg/day and methotrexate (MTX) administered days +1, +3, and +6 in unrelated donor transplants, always with transition to oral CsA in the adequate dosage. Since February 2003, we started reduced GVHD prophylaxis according to ALL SCT–BFM 2002 protocol where only targeted dose of CsA with required serum levels between 80 and 130 μg/l (Fluorescence Polarization Immunoassay method) was given. Only in one case (the cord blood transplant) the combination of CsA and methylprednisolone was used. Incidence of acute GVHD was low in our group with 1 child developing acute GVHD Grade I, 24 children experiencing acute GVHD grade II, and 7 children having no acute GVHD. We have registered only two cases of acute GVHD grade III–IV. Follow-up of the whole group ranges from 12 to 61 months with median 26 months after HSCT.

MRD Assessment

For the MRD assessment we examined BM samples from both diagnosis and relapse, 1 week before the start of the pre-transplant conditioning and then after HSCT, on a regular basis: days +28, +60, +100, +180, and later 9, 12, 18, and 24 months after HSCT (or more frequently in MRD-positive patients when adoptive immunotherapy was considered). In children with a very high-risk of relapse, additional peripheral blood (PB) samples were taken every month during first 6 months after HSCT and every 3 months later on. Mononuclear cells from the diagnostic or relapse BM samples were isolated by Ficoll–Paque (density 1.077 g/ml, Pharmacia, Uppsala, Sweden) density centrifugation. Follow-up BM or PB samples were processed by erythrocyte lysis.

Genomic DNA was isolated by QIAamp® DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany). DNA was stored at −20°C before processing. Primers and protocols for immunoglobulin heavy chain (IGH), immunoglobulin light chain kappa (IGK), T-cell receptor gamma (TCRG), T-cell receptor delta (TCRD) gene rearrangements, and TAL1 deletions detection were described previously [8,9]. Clonality of PCR products was confirmed by the heteroduplex analysis [10]. Monoclonal PCR products were cut from the gel, reamplified with the same set of primers, and purified by QIAquick PCR Purification Kit (QIAGEN). Sequencing was performed in the ABI PRISM® 310 Genetic Analyzer with BigDye™ Primer v3.0 Sequencing Kit (Applied Biosystems, Foster City, CA). Variable (V), diversity (D), and joining (J) regions of the immunoreceptor gene were identified by comparison with sequences in GenBank using the Im Muno Gene Tics (IMGT) Database (http://imgt.cines.fr, IMGT, European Bioinformatics Institute, Montepellier, France) and the IGBlast search (http://www.ncbi.nlm.nih.gov/igblast/, National Center for Biotechnology Information, Bethesda, MD).

Patient-specific forward primers for RQ-PCR were designed using the VECTOR NTI 8 Suite Software (Informax, Bethesda, MD). Family-specific reverse primers and probes for IGH, IGK, TCRD, and TCRG were described previously [11–14]. Ig/TCR RQ-PCR was performed in the iCycler IQTM Real-Time PCR Detection System (BIO-RAD, Hercules, CA) and in the ABI PRISM® 7700 Real-Time PCR System (Applied Biosystems). Standard curves were prepared by diluting the diagnostic (HSCT in CR1) or relapse DNA samples in polyclonal DNA from healthy donors. The albumin gene was used to normalize the DNA concentration and quality [15]. The ESG–MRD–ALL criteria for RQ-PCR sensitivity and quantitative range (QR) interpretation were used [4]. In six patients who suffered from relapse after HSCT, BM samples were re-analyzed for the presence of clonal Ig/TCR rearrangements to evaluate the extent of clonal evolution.

RESULTS

Feasibility of the Approach

We were able to evaluate MRD level prior HSCT using Ig/TCR rearrangements in 25 of 36 patients. In two cases, the pre-transplant sample was not available due to severe bone marrow aplasia, and in nine cases we did not find a target with adequate sensitivity and specificity. All 25 patients were regularly monitored using Ig/TCR rearrangements (two

targets in 14 patients, one target in 9). At least one target with sensitivity 10^{-4} was available for all but one patient (No. 12), where only one target with sensitivity 10^{-3} was found.

Clonal Evolution of Ig/TCR Rearrangements

In our cohort, clonal evolution did not preclude MRD monitoring in any of the patients. All post-transplant relapse samples when compared to the diagnosis/first relapse specimen showed at least one stable Ig/TCR rearrangement.

MRD Level Prior HSCT Predicts Post-transplant Outcome

According to MRD level in BM prior the conditioning regimen we divided our patients into two groups (Table II). The first group (MRD-positive) consisted of patients with MRD detectable within the QR of the method. The second group (MRD-negative) included patients with undetectable or very low MRD positivity (below the QR; $n = 2$) prior to the transplant.

The MRD-positive subgroup consisted of eight patients (Nos. 2, 8, 9, 10, 11, 13, 22, and 35). One of them died due to post-transplant complications (day + 66—multiorgan failure after gram-negative sepsis), six children experienced a hematological relapse, although all of them achieved transient post-transplant MRD negativity, and one is alive in continuous hematological remission. However, the latter patient (No. 35) suffered from molecular genetic relapse and was treated with the adoptive immunotherapy with a follow-up of 12 months.

The group of MRD-negative patients included 17 children (Nos. 1, 4, 12, 15, 16, 17, 18, 20, 21, 23, 24, 26, 27, 29, 30, 31, and 36), all of whom are alive except one and in complete hematological remission with a follow-up of 16–61 months (median 26 months). One patient of this group (No. 36) suffered a relapse 10 months after HSCT. Interestingly, although MRD-negative by Ig/TCR approach, BCR/ABL fusion gene reverse-transcriptase PCR analysis prior HSCT showed borderline positivity (data not shown).

EFS analysis clearly supports the hypothesis that MRD positivity prior transplant is a significant adverse prognostic factor (log-rank $P < 0.0001$; Fig. 1). Further division into the subgroups with high MRD positivity ($\geq 10^{-3}$; n = 4) and low MRD positivity ($>10^{-4}$ and $<10^{-3}$; n = 4) did not show any effect, evidently due to a low number of patients and a high frequency of events (data not shown). Multivariate analysis (including also sex, age at diagnosis, first CR duration, type of donor, and fusion gene—BCR/ABL, MLL/AF4, and ETV6/RUNX1—presence) identified pre-transplant MRD as the only significant risk factor ($P < 0.0001$).

TABLE II. MRD Levels in the Bone Marrow Before Conditioning and Survival of the Patients

Patient no.	ALL subtype	Number of Ig/TCR targets	MRD level	Relapse after HSCT	Comments
2	ETV6/RUNX1	1	1.47×10^{-4}	Yes	Died in remission (systemic fungal infection, severe induced GVHD after DLI)
8	BCR/ABL	2	5.3×10^{-2}, 9.2×10^{-2}	Yes	Died in remission (systemic fungal infection)
9	BCR/ABL	2	Negative, 1.3×10^{-4}	Yes	Died in progression of disease (on treatment of relapse)
10	BCR/ABL	1	1.2×10^{-3}	Yes	Died in progression of disease
11	BCR/ABL	2	2.3×10^{-4}, 6.2×10^{-4}	Yes	Died in remission (systemic fungal infection, severe induced GVHD after DLI)
13	BCP	1	2.8×10^{-2}	No	Died due to multiorgan failure in gram-negative sepsis day + 66
22	T-ALL	1	6.4×10^{-3}	Yes	Alive in CR2; after 2nd HSCT
35	ETV6/RUNX1	2	4.1×10^{-4}, 3.5×10^{-4}	No	Alive in CCR
1	T	2	Negative, negative	No	Alive in CCR
4	T	1	Positive (below QR)	No	Alive in CCR
12	BCP	1	Negative	No	Alive in CCR
15	ETV6/RUNX1	2	Negative, negative	No	Alive in CCR
16	BCP	1	Negative	No	Alive in CCR
17	T-ALL	2	Negative, negative	No	Alive in CCR
18	BCR/ABL	2	Negative, negative	No	Alive in CCR
20	BCR/ABL	1	Negative	No	Alive in CCR
21	BCP	2	Positive (below QR)	No	Alive in CCR
23	T-ALL	2	Negative, negative	No	Alive in CCR
24	ETV6/RUNX1	1	Negative	No	Alive in CCR
26	MLL/AF9	2	Negative, negative	No	Alive in CCR
27	BCR/ABL	2	Negative, negative	No	Alive in CCR
29	BCR/ABL	2	Negative, negative	No	Alive in CCR
30	ETV6/RUNX1	2	Negative, negative	No	Alive in CCR
31	BCP	2	Negative, negative	No	Alive in CCR
36	BCR-ABL	1	Negative	Yes	Died, systemic fungal infection

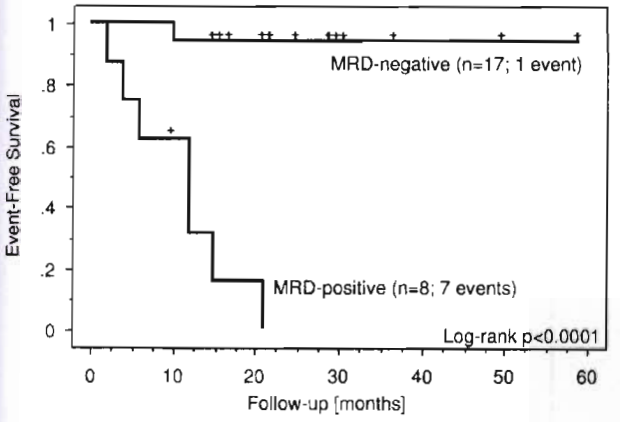

Fig. 1. EFS in MRD-positive and MRD-negative patients. Median follow in MRD-negative group = 25 months.

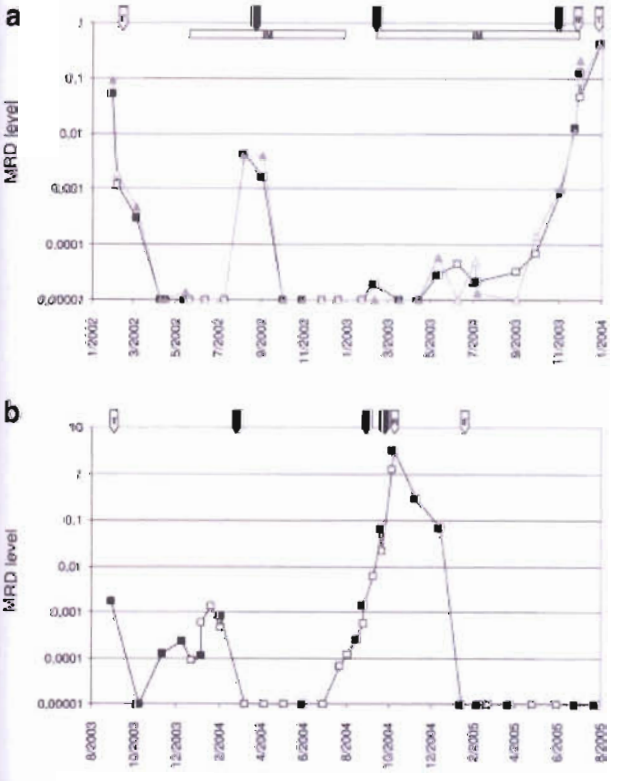

Fig. 2. Disease course and MRD follow-up in Patients 8 and 22. **a**: Disease progression after HSCT in BCR/ABL-positive patient (No. 8) was only temporarily retarded by imatinib treatment and DLI. Squares = Ig rearrangement target No. 1; triangles = Ig rearrangement target No. 2 (full symbols = BM samples; open symbols = PB samples); black arrows = DLI; IM = imatinib mesylate; R = relapse; T = allogeneic HSCT. **b**: Pre-transplant MRD status determines post-transplant course of the disease in T-ALL infant patient. Squares = TCR delta incomplete rearrangement target (full symbols = BM samples; open symbols = PB samples).

MRD Monitoring and Treatment Strategies After HSCT

Post-transplant MRD dynamics was monitored in all patients with detectable targets. In the group of pre-transplant MRD-positive patients, five displayed minimally one MRD-positive sample before the emergence of relapse. In three patients, there was a time-frame for an attempt to avert the relapse manifestation, but despite our effort all three patients subsequently relapsed (Fig. 2). Period from the first MRD positivity after transplantation to the diagnosis of hematological relapse was 0–486 days (0, 30, 36, 330, 365, and 486 days). Treatment of post-transplant hematological relapse was heterogeneous and it is summarized in Table III.

DISCUSSION

Detection of MRD levels has already become an integral part of treatment of childhood ALL patients including those undergoing HSCT. Ongoing front-line treatment trials, based on previous retrospective studies [16,17], aim to demonstrate the benefit of MRD-based stratification in the prospective setting. Retrospective single- and multicenter analyses of MRD in transplanted pediatric ALL children showed clearly the significant impact of pre-transplant MRD on outcome [1–3,5]. Imashuku et al. questioned these data, showing a surprisingly high proportion of MRD-positive patients at the start of a conditioning regimen (96%) and strikingly high frequency of the clonal evolution hampering MRD detection itself [6]. Although they did not use an up-to-date methodology (specific probe hybridization was employed instead of RQ-PCR) and a full spectrum of Ig/TCR rearrangements, their results cast doubt upon the practicality of the whole approach.

In this study, we concentrated on Ig/TCR quantitation only, despite the fact that significant proportion of patients bear the fusion genes (BCR/ABL, ETV6/RUNX1, MLL/AF4, MLL/AF9) as potential targets for MRD detection as well. As already mentioned, the clonal Ig/TCR quantitation methodology has been increasingly standardized throughout the last years within the ESG–MRD–ALL. This standardization process and also newly developed interpretation criteria improved significantly its clinical value. When properly applied, this method provides a reliable, clinically useful tool, as it was proved by numerous international quality controls [4]. However, this is not the case for the quantitative analysis of the fusion genes expression in pediatric ALL. We have recently shown a very good correlation between ETV6/RUNX1 transcript levels and Ig/TCR quantitation but in the cohort that consisted dominantly from the front-line treated patients [18]. In the current study, we observed a minor but significant discrepancy between BCR/ABL and Ig/TCR MRD levels in Patient 36. He displayed borderline positivity in nested qualitative PCR for BCR/ABL prior to the transplant and he was the only patient

TABLE III. Patients With Post-Transplant Hematological Relapse and Their Treatment

Patient no.	Molecular genetic relapse (day)	Treatment	Secondary GVHD	Molecular remission achieved	Hematological relapse (day)	Treatment	CR achieved	Secondary GVHD	Cause of death
2	D + 480	—	—	—	D + 480	Reduced chemotherapy DLI	Yes	Yes, Grade IV	Systemic fungal infection
8	D + 180	Imatinib mesylate, three doses of DLI, IFN alfa	No	Yes	D + 660	Reduced chemotherapy DLI	Yes	No	Systemic fungal infection
9	D + 360	Immunosuppression tapering, imatinib mesylate	Yes, Grade II	Yes	D + 640	Imatinib mesylate reduced chemotherapy	No	No	Disease progression
10	D + 180	—	—	—	D + 192	No	—	—	Disease progression
11	D + 130	—	—	—	D + 139	Immunosuppression tapering DLI imatinib mesylate	Yes	Yes, Grade IV	Systemic fungal infection
22	D + 60	Immunosuppression reducing, three doses of DLI	Yes, Grade II	Yes	D + 395	High dose chemotherapy 2nd HSCT	Yes	No	Alive in CR2
36	D + 90	Immunosuppression tapering, three doses of DLI, imatinib mesylate	No	Yes	D + 317	Chemotherapy	Yes	No	Systemic fungal infection

in the Ig/TCR MRD-negative subgroup who developed relapse after HSCT.

Our prospective single-center study clearly documents the feasibility and applicability of the Ig/TCR quantitation technique of MRD detection in the transplant setting. The efficacy of this approach, in terms of the identification of at least one target with adequate specificity and sensitivity per patient, increased steadily throughout the study: it was 61% in the first half of the cohort and 80% in the latter. Our results are in agreement with the previously published data [1–3,5,19] and in striking contrast to the study by Imashuku et al. [6]. Both survival and multivariate analyses demonstrate the significance of MRD level before HSCT. Patients who enter the transplant conditioning phase of treatment with MRD level higher than 10^{-4} are at high-risk of post-transplant relapse.

Treatment of these relapses has been very disappointing so far. Three different approaches may lead to a potential solution of this problem: (1) reduction of the malignant clone prior to the transplant using intensified or additional treatment, (2) modification of the HSCT procedure, and (3) post-transplant treatment modifications based on the close follow-up of MRD levels. Pre-transplant treatment intensification is complicated by the fact that vast majority of patients have been heavily pre-treated. The idea of employment of some drugs that are not normally used in the front-line treatment failed to show a significant effect. Potentially, introduction of new agents (e.g., kinase inhibitors, monoclonal antibodies, new antimetabolites, such as clofarabine) might be an option [20]. However, no convincing data are available thus far.

Modification of transplant procedure based on MRD positivity prior to HSCT aims for the reduction of GVHD prophylaxis, thus boosting the graft-versus-leukemia effect. Removing ATG, targeted dose of CsA and rapid immunosuppression tapering may lead to this effect. Although partly encouraging, results of an ongoing Dutch study have thus far been inconclusive [19]. In some patients in our cohort, we used targeted dose of CsA. In patients younger than 16 years of age transplanted from matched sibling, we did not use MTX in GVHD prophylaxis and in some patients transplanted from well-matched unrelated donor even ATG was omitted. Due to the small numbers and heterogeneity of patients with ALL, it is difficult to prove any noticeable effect of this strategy in terms of relapse prevention. We should be cautious as this strategy may significantly increase the risk of transplant-related morbidity and mortality without clear evidence of efficacy.

Adoptive immunotherapy after the transplantation was generally not successful in our cohort. In our hands, interventions, such as early and rapid discontinuation of immunosuppression, infusion of DLI in 4–6 weeks interval, and/or use of imatinib mesylate in BCR/ABL-positive ALL were not sufficient enough to prevent onset of relapse. Application of these approaches in patients with imminent

relapse have led to extended remission but often at a price of severe, uncontrolled GVHD, and life-threatening invasive fungal infections. Moreover, no permanent effect was seen in our group of patients. Heavily pre-treated patients bearing a chemoresistant leukemia have an extreme morbidity and mortality. Therefore, an early initiation of adoptive therapy might at least postpone, if not prevent, relapse, and facilitate further efficacious chemotherapy; if subsequent remission is reached, these patients should be indicated for re-transplantation. A second HSCT may be curative in such settings. Our results demonstrate that the possibilities of treatment of the post-transplant relapse are extremely limited. Introduction of new treatment modalities is desirable for the patients with molecular genetic relapse after HSCT. These should include not only those mentioned above (kinase inhibitors, monoclonal antibodies, new antimetabolites) but also the inhibitors of enzymes controlling epigenetic modifications, specifically DNA methyltransferases and histone deacetylases [21].

We demonstrate feasibility of MRD quantitation using clonal Ig/TCR rearrangements as an approach for the pre-transplant risk assessment in pediatric ALL patients undergoing allogeneic HSCT. We show that, although, we are able to identify the patients in an almost certain risk of relapse, our ability to respond and to avert an impending relapse is very limited. In spite of the use of currently available set of treatment approaches after HSCT, we failed to permanently avert a predicted relapse. The change of the approach to MRD-positive patients prior to HSCT is necessary because of very questionable benefit of HSCT in these children. We are confident that all efforts should be aimed to better control pre-transplant MRD levels.

Note added in proof

Patient No. 35 suffered from hematological relapse 14 months after HSCT and died due to disease progression.

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Příloha 6

B-cell reconstitution after allogeneic stem cell transplantation impairs minimal residual disease (MRD) monitoring in children with ALL

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ORIGINAL ARTICLE

B-cell reconstitution after allogeneic SCT impairs minimal residual disease monitoring in children with ALL

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Minimal residual disease (MRD) detection using quantification of clone-specific Ig or TCR rearrangements before and after transplantation in children with high-risk ALL is an important predictor of outcome. The method and guidelines for its interpretation are very precise to avoid both false-negative and -positive results. In a group of 21 patients following transplantation, we observed detectable MRD positivities in Ig/TCR-based real-time quantitative PCR (RQ-PCR) leading to no further progression of the disease (11 of 100 (11%) total samples). We hypothesized that these positivities were mostly the result of nonspecific amplification despite the application of strict internationally agreed-upon measures. We applied two non-self-specific Ig heavy chain assays and received a similar number of positivities (20 and 15%). Nonspecific products amplified in these RQ-PCR systems differed from specific products in length and sequence. Statistical analysis proved that there was an excellent correlation of this phenomenon with B-cell regeneration in BM as measured by flow cytometry and Ig light chain-κ excision circle quantification. We conclude that although Ig/TCR quantification is a reliable method for post transplant MRD detection, isolated positivities in Ig-based RQ-PCR systems at the time of intense B-cell regeneration must be viewed with caution to avoid the wrong indication of treatment.

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Keywords: ALL; SCT; minimal residual disease; KREC; B-cell reconstitution; donor lymphocyte infusion

Introduction

During the past decade, minimal residual disease (MRD) monitoring using patient-specific antigen receptor gene rearrangements has been incorporated into major frontline and relapse treatment protocols for childhood ALL.[1–4] Several groups, including ours, reported the unfavorable prognostic significance of high MRD levels before transplant in children with high-risk ALL.[5–8] Studies exploring the significance of post transplant MRD were based on the detection of mixed chimerism,[9,10] flow cytometry (FC),[11] the fusion gene PCR in case of Ph+ ALL,[12] PCR using clone-specific Ig or TCR V- (D)-J sequences[13–16] and, most recently, real-time quantitative PCR (RQ-PCR) detection of clonal Ig/TCR rearrangements.[17] Consistently, all studies showed that detectable MRD at any time after SCT represents a substantial risk of post transplant relapse, both in children and in adults. Several measures exist to avert hematological relapse after SCT when molecular relapse is detected. The first option is the reinforcement of the GVL effect by immunosuppression reduction or donor lymphocyte infusion (DLI); the second is the application of further cytoreductive therapy, including MoAb or specific tyrosine kinase inhibitors, in the case of BCR/ABL-positive ALL.[18–26] The benefit from such therapy has been described in a small proportion of ALL patients. To date, there is no general consent regarding the choice and timing of therapy in case of post transplant MRD positivity.

The ESG-MRD-ALL (European Study Group on Minimal Residual Disease in ALL) set guidelines for the interpretation of quantitative Ig/TCR-based MRD that include the use of polyclonal DNA from the peripheral blood (PB) of healthy donors in multiplicate as a negative control and a strict definition of MRD positivity.[27] This definition is even stricter when aimed at therapy intensification (for example, DLI) to prevent false-positive results. So far, this method has proved to be reliable, leaving the potential nonspecific amplification in regenerating nonmalignant lymphocytes only a theoretical possibility.

Starting 140 days and later after SCT, we observed MRD positivities in accordance with all ESG-MRD-ALL criteria in patients who subsequently turned MRD-negative without any antileukemic treatment and had been in complete remission for several years after SCT. We hypothesized that

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the B-cell regeneration after SCT caused an unspecific binding of clone-specific primers, which bypassed the otherwise strict ESG-MRD-ALL criteria for MRD positivity. MRD detection using non-self patient-specific assays revealed false MRD positivity in a substantial portion of post-SCT BM specimens. This false positivity was limited to the samples containing high numbers of B-cell progenitors as measured by immunophenotyping and by recently described B-cell recombination (κ deleting) excision circles (KREC) detection. Moreover, using high-resolution capillary electrophoresis and sequencing, we observed that the size of such nonspecific RQ-PCR products differed from the specific products. We therefore conclude that MRD results post-SCT should be approached with extreme caution, and offer recommendations for avoiding MRD misinterpretation.

Patients and methods

Patients

A total of 38 children with ALL (aged 1–18 years) underwent allogeneic SCT in the Czech Republic from January 2003 to October 2006. Of them 21 patients with B-precursor ALL were selected for the study based on following criteria: leukemia-free survival with a follow-up of at least 12 months after transplant (median follow-up 46 months, range 17–62 months) and the availability of MRD results and residual DNA samples from at least three (3–7) of the following time points after SCT: days +30, +60, +100, +140, +180, 1 year, 2 years and 3 years. In total, 100 BM DNA samples (leftover material after MRD detection) were investigated. Washed leftover material from tubings and the transfusion bag following the complete administration of the BM graft was used as a control for KREC detection. Informed consent for the use of residual material after protocol-based examination for research purposes was obtained from patients or their guardians.

Transplants

The patients were transplanted due to relapsed or high-risk ALL in their first (7), second (11) or third (3) remission. Donors of hematopoietic stem cells were HLA-identical siblings in 5 cases, a sibling with 9/10 HLA antigen match in 1 case, and unrelated donors from BMT registries in 15 cases. BM was transplanted in ten cases, PBSC in nine cases, umbilical cord blood (UCB) in one case and both BM and UCB (sibling) in one case. In the majority of transplants ($n=19$), we used a similar pre-transplant conditioning regimen based on TBI at a dose of 12 Gy and etoposide 60 mg/kg, and in two children, a BU-based conditioning (1 × BU, CY and melphalan, 1 × BU, CY and etoposide) was used. In the majority of unrelated donor transplants ($n=13$), we used the rabbit antithymocytic globulin (Fresenius, Bad Homburg, Germany) at a dose of 10 mg/kg for 4 days. GVHD prophylaxis consisted of CsA in HLA-identical sibling transplants and a combination of CsA and MTX administered on days +1, +3 and +6 in unrelated donor transplants, respectively. In one patient (UCB transplant), a combination of CsA and methylprednisolone was used. No patient received any antileukemic treatment (including adoptive immunotherapy) during the post transplant period.

Detection of residual disease

Mononuclear cells from the diagnostic or relapse BM samples were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density centrifugation and stored in liquid nitrogen. Follow-up BM samples were processed by erythrocyte lysis and stored at −80 °C. Genomic DNA was isolated using a QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). Primers and protocols for the detection of Ig heavy chain (IGH) rearrangements, Ig light chain-κ deletions (KDE), TCR-γ (TCRG), TCR-δ (TCRD) gene rearrangements and TAL1 deletions have been described previously.[28,29] Clonality of PCR products was confirmed by the heteroduplex analysis.[29] Sequencing was performed in the ABI Prism 310 Genetic Analyzer with BigDye Primer v3.0 Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Variable (V), diversity (D) and joining (J) regions of the immunoreceptor gene were identified by comparison with sequences in GenBank using the ImMunoGeneTics database (http://imgt.cines.fr, European Bioinformatics Institute, Montepellier, France) and the IGBlast search (http://www.ncbi.nlm.nih.gov/igblast/, National Center for Biotechnology Information, Bethesda, MD, USA). Patient-specific forward primers for RQ-PCR were designed using the Vector NTI 8 Suite Software (InforMax, Bethesda, MD, USA). Family-specific reverse primers and probes for IGH, IGK, TCRD and TCRG have been described previously.[30–33] Ig/TCR RQ-PCR was performed in the iCycler IQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Standard curves were prepared by diluting the diagnostic samples in pooled polyclonal DNA from the PB of five healthy donors, which was also used as negative controls. MRD using patient-specific as well as non-self-specific systems was measured in triplicate, with 2.5 μl of DNA per reaction. The albumin gene was used to normalize the DNA concentration and quality.[34] The ESG-MRD-ALL criteria for RQ-PCR sensitivity, quantitative range and MRD interpretation were used.[27]

DNA analysis by on-chip electrophoresis

RQ-PCR product (1 μl) was analyzed in 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.

Flow cytometry

K3 EDTA BM aspirate specimens were stained within 12 h from sample collection using a whole-blood lysis technique with ammonium chloride. About 50–100 μl of sample (according to cellularity) was incubated with MoAb at dark for 15 min, and then sample was incubated with lysing solution for 15 min at dark and centrifuged (400 *g*). After supernatant removal, the sample was resuspended in 200 μl of PBS and analyzed on flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA, USA or CyAn; Dako, Glostrup,

Denmark). For B- and T-cell regeneration/FC MRD after SCT, the following MoAb combinations were used: CD20/CD10/CD19/CD34, SYTO16/CD19/CD45 and CD3/CD16-56/CD45/CD19. The following antibody clones were used: CD20 FITC (cloneL27; BD Biosciences), CD10 PE (clone SS2/36; Dako), CD19 PC7/PC5 (clone J4.119; Immunotech, Marseilles, France), CD34 APC (clone LIQ; Immunotech), CD19 PE (clone SJ25C1; Immunotech), CD45 PerCP (clone 2D1; BD Biosciences), CD3 FITC (clone Sk7; BD Biosciences), CD16 PE (clone B73.1; BD Biosciences), CD56 PE (clone B73.1; BD Biosciences) and CD19 APC (clone SJ25C1; BD Biosciences). SYTO-16 (green fluorescent nucleic acid stain) was purchased from Invitrogen-Molecular Probes (Carlsbad, CA, USA).

SYTO-16 green fluorescent nucleic acid stain exhibits bright green fluorescence upon binding to DNA and RNA. SYTO-16 was used for reporting the percentage of $CD19^{pos}$ cells out of all nucleated cells ($SYTO16^{pos}$). The following subpopulations were reported: $CD19^{pos}$, $CD19^{pos}CD45^{dim}$, $CD10^{+\,+}CD19^{pos}$, $CD10^{pos}CD19^{pos}$, $CD34^{pos}CD19^{pos}$ and $CD3^{pos}$. The B cells with a lower expression of CD45 (dim) correspond to immature cells.[35]

Detection of B-cell receptor excision circles

We used the method of KREC detection,[36] slightly modified as follows. We did not employ the simultaneous detection of intron-Kde rearrangements as suggested. Instead, we used a ΔC_t method with a calibrator sample—the donor DNA extracted from the residuum after BM transplantation. We considered the differences in DNA concentration of the samples and used RQ-PCR for the albumin gene as described previously.[34] The final amount of KREC relative to the calibrator was expressed as: $2^{C_t\text{calibrator}-C_t\text{sample}+\log2(\text{DNAconc.calibrator}/\text{DNAconc.sample})}$.

Statistical analyses

The distribution of frequencies between groups with positive and negative MRD was assessed using Fisher's exact test. The Mann–Whitney test was used to estimate the significance of differences concerning continuous MRD values. The statistical analyses were performed using StatView version 5.0 (StatView Software, Cary, NC, USA). Trend analysis in Figure 4 was calculated using GraphPad Prism Software version 5 (GraphPad Software, San Diego, CA, USA). First, means of individual variables in individual time points were compared using one-way analysis of variance (ANOVA), then post-hoc linear trend analysis was used to test decrease or increase in naturally ordered groups. Simultaneously, Kruskal–Wallis test was used for comparison of means among individual time points.

Results

Frequency of hypothetically nonspecific amplification using patients' clone-specific systems in the course of prospective post-SCT monitoring

A patient-specific RQ-PCR system with minimal sensitivity of 10^{-4} was designed for all patients, and 17 out of 21 patients were monitored using two independent Ig/TCR targets. Figure 1 shows the results of post transplant MRD monitoring. In total, 16 of 151 (11%) samples from nine patients were MRD positive according to ESG-MRD-ALL criteria in at least one target (the C_t value of at least one of the three replicates was within 4.0 from the highest C_t value of the sensitivity and the C_t value of at least one of the three replicates was $\geqslant 1.0$ lower than the lowest C_t of the background).[27] Eight samples fulfilled ESG-MRD-ALL criteria for positivity that aim at therapy intensification (the C_t value of at least one of the three replicates was $\geqslant 3.0$ lower than the lowest C_t of the background). All samples were evaluated as 'positive, not quantifiable'. Fifteen of sixteen positive samples were examined by two targets; one sample was positive in both of them. The RQ-PCR targets with positive samples used IGH ($7\times$), KDE ($5\times$), TCRD ($4\times$) and TCRG ($1\times$) rearrangements for MRD detection. Nine of sixteen positive samples turned negative in the following BM examinations; three patients were still positive at the end of follow-up. Only 1 of 16 positive samples was taken during corticosteroid treatment of GVHD, compared to 51 of 135 negative samples ($P=0.01$).

On the basis of following, we hypothesized that most of the positive results were false positives and were most likely caused by unspecific binding of patient-specific primers to similar V- (D)-J sequences of nonmalignant lymphocytes in BM. First, all patients with at least one positive sample remained free of leukemia with a median follow-up of 31 months (range 17–61; not significantly different from patients with no positive sample, $P=0.17$, Mann–Whitney). Also, all patients who entered the study were MRD negative ($n=16$) or low positive ($<5\times10^{-4}$, $n=5$) before the transplant. Finally, simultaneous RQ-RT-PCR detection of the respective fusion gene (BCR/ABL and TEL/AML1) in 10 relevant samples tested gave a negative MRD result.

Frequency of amplification using non-self clone-specific systems

Because of the suspicion of false positivity, we used different RQ-PCR systems specific for unique V- (D)-J sequences of two other patients (not specific for the leukemic cells of the given patients) to test whether the V- (D)-J sequences would even be amplified in such circumstances. A total of 100 post-SCT samples with sufficient leftover DNA were tested. The frequency of MRD-positive samples in the selected cohort did not differ from the whole cohort (11%).

We used two patient-specific systems based on the VH3-JH4 rearrangement. Both the VH3 family and JH4 represent the most frequently rearranged segments in physiological B-cell development.[37,38] To maintain the reproducibility of the data, we used assays with no background amplification of polyclonal DNA from PB of healthy donors ('buffy coat' cells). The assays were originally designed for two patients with ALL (not included in the investigated cohort) using patient-specific primers situated with their 5′ end in the VH3 segment and the last seven nucleotides spanning N segments. In total, 20 out of 100 (20%) and 15 out of 100 (15%) samples were positive according to the ESG-MRD-ALL criteria using those

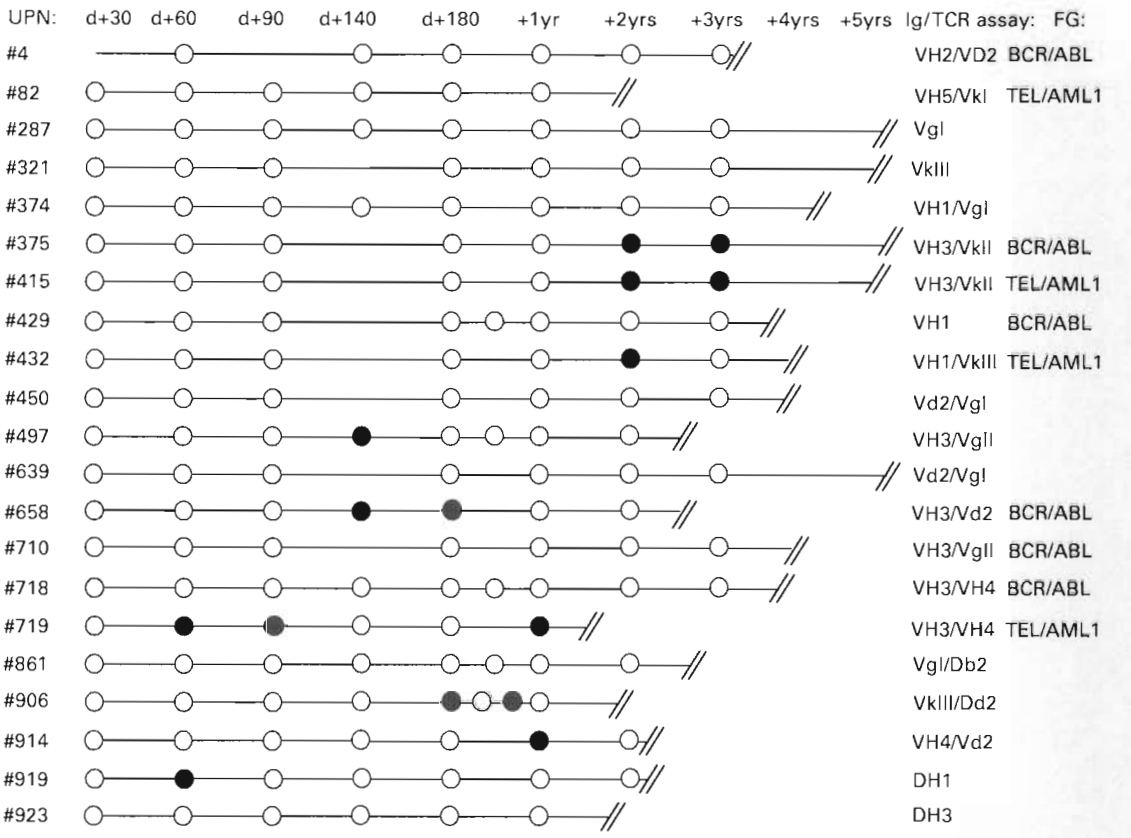


Figure 1 Post transplant minimal residual disease (MRD) results (Ig/TCR) from 21 patients included in the study. All positive samples were evaluated as 'positive, not quantifiable'. ○, MRD negative; ●, MRD positive; UPN, unique patient number; FG, fusion gene.

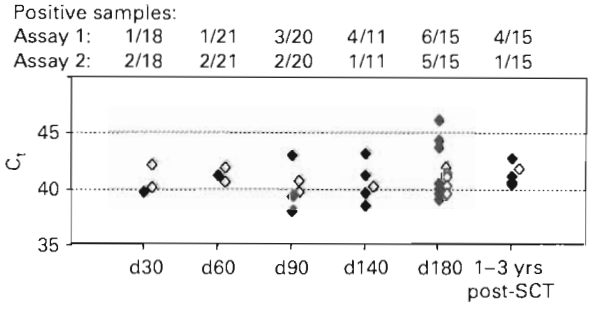


Figure 2 C_t values of false minimal residual disease (MRD) positive samples using two non-self-specific Ig/TCR assays. Filled and empty diamonds represent the two different assays. In the case that more members of the examined triplicate were positive, the highest C_t value was used.

assays. Of the eleven samples positive in their own patient-specific systems, five and four were also positive in the two non-self clone-specific assays, respectively.

Figure 2 shows C_t values of false-positive samples in both assays. As the onset of positive signal was different between the two assays, their C_t values are not mutually comparable. Given the fact that no background amplification was present, all samples would fulfill the criteria for therapy intensification.[27]

Nonspecific RQ-PCR products differ from the specific products in length and sequence

As we observed no difference in the length of most nonspecific RQ-PCR products on 8% polyacrylamide gel (data not shown), we employed a more sensitive Agilent DNA analysis by on-chip electrophoresis. Figure 3 shows an electrophoretogram of the diagnostic sample (1:10 000 dilution in buffy coat) of the patient whose assay was used for amplification compared to three different false-positive samples from different patients that differ in size and/or pattern.

Sequencing of 10 false-positive RQ-PCR products revealed the use of different N nucleotides downstream from the matching sequence of the patient-specific primer.

Assessment of B-cell reconstitution in the BM using KREC detection

In the course of V- (D)-J recombination, Ig (TCR) gene segments are assembled, leaving nonreplicable circular DNA fragments as 'by-products'. T-cell recombination circles detection has been commonly used as a quantitative marker of thymic output.[39] Detection of B-cell recombination circles has not been widely employed due to the complicated structure of Ig genes. Recently, detection of excision circles originating in the deletions of Ig light chain-κ (KREC) was described.[36] As IGK deletion occurs in all B

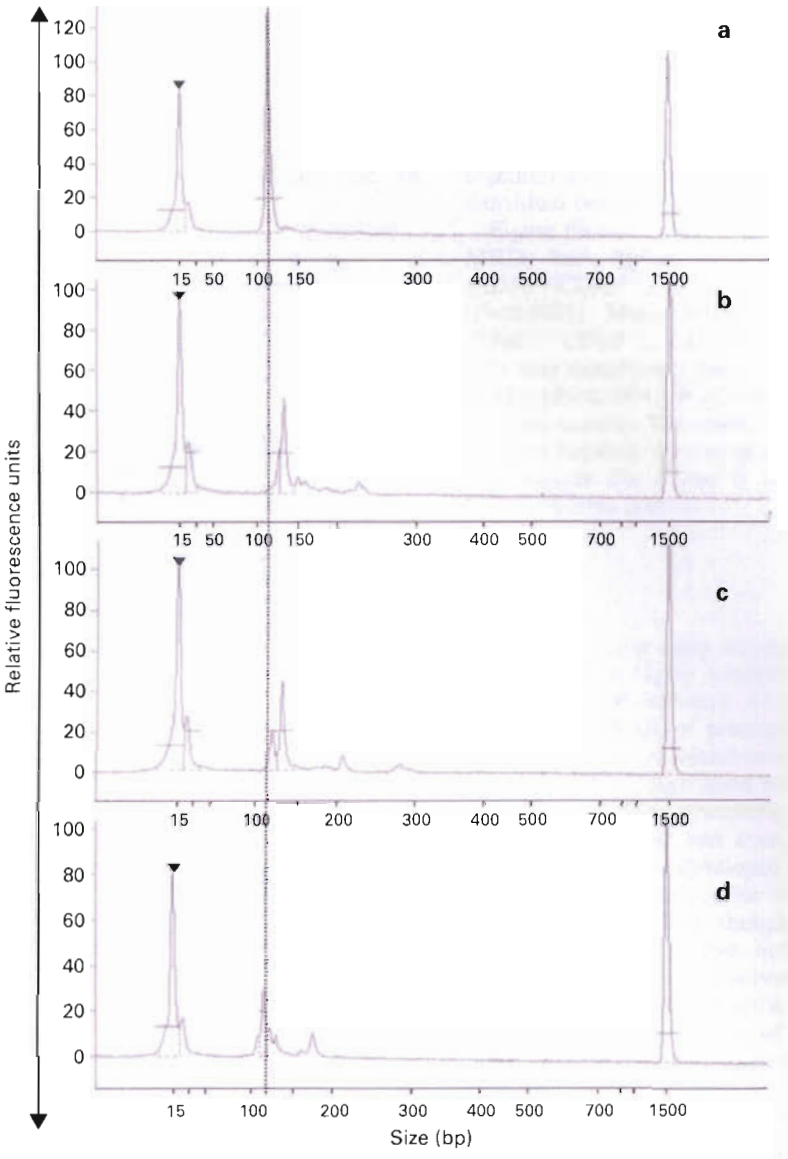


Figure 3 Agilent DNA chip electrophoretogram of specific and unspecific real-time quantitative PCR (RQ-PCR) products. Both 15 and 1500 bp peaks represent lower and upper DNA markers, and *y* axis values are expressed as relative fluorescence units. (**a**) The product of patient-specific RQ-PCR. The diagnostic ALL sample used for amplification was 1:10 000 diluted in polyclonal DNA from healthy donors. (**b–d**) Nonspecific RQ-PCR products amplified from three different patients' follow-up samples using the non-self-specific assay of the patient from (**a**).

lymphocytes that fail to rearrange IGK productively on one or both alleles, the number of KRECs in the BM reflects the number of developing B lymphocytes.

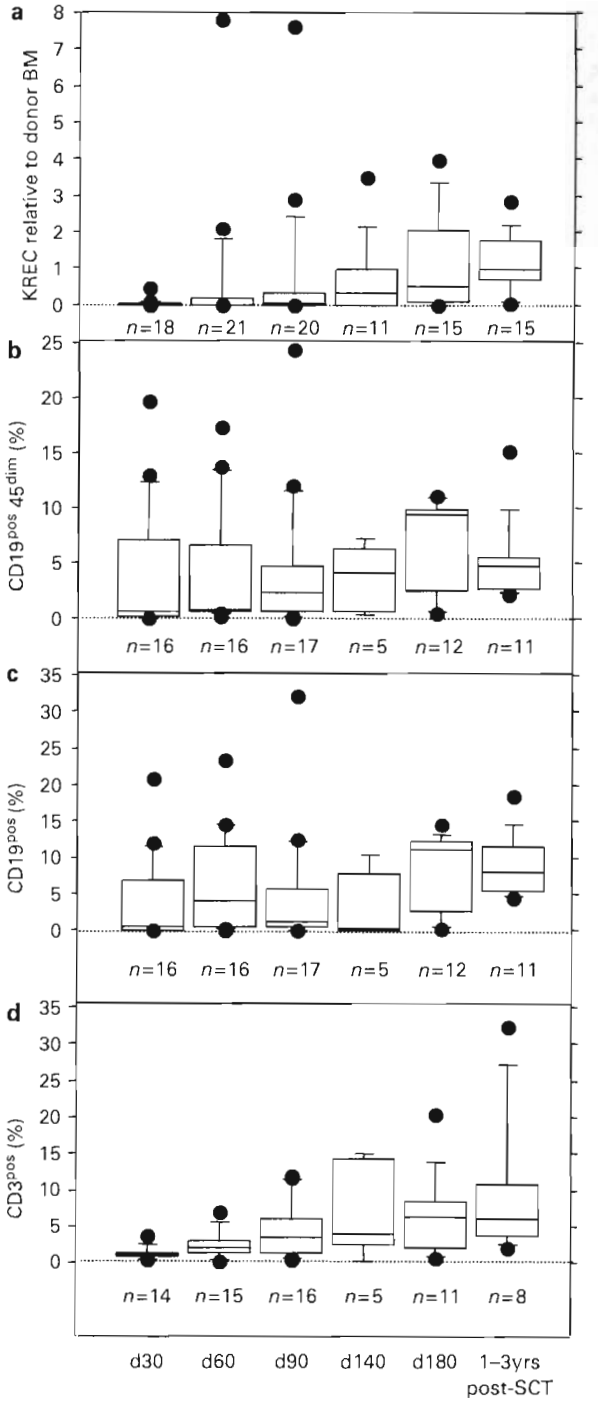
Figure 4a shows the number of KRECs relative to the DNA from the BM donor sample during the post transplant period in 100 BM samples. With few exceptions, the KREC levels rose continuously, remaining lower than those of the control until day 90 and having a median value higher than the control 1–3 years post-SCT. This result was in concordance with the lower intensity of immunosuppression in later time points. Figure 5a shows the impact of immunosuppression taken at the time of sample collection on the number of KRECs for all time points together. Supplementary Figure 1 shows the same analysis for individual time points.

KREC number correlated well with the false positivities observed in non-self-specific Ig/TCR assays. Figure 6a shows the difference in the number of KRECs between the samples with negative and false-positive MRD. False MRD positivity was more frequent in samples with higher KREC number than in the negative ones ($P<0.0001$, Mann–Whitney). This trend was also significant for all but one (day 140) post-SCT time points tested individually (Supplementary Figure 2a).

Assessment of lymphocyte reconstitution in the BM samples by immunophenotyping

Evaluation of B-cell subsets and T cells was available in 77 out of 100 and 69 out of 100 samples, respectively. Consistent with the results of KREC detection, immunophenotyping showed a trend toward higher total B-cell ($CD19^{pos}$) numbers ($P=0.06$, Kruskal–Wallis) and an increase in T-cell numbers during the post transplant period ($P=0.02$, one-way ANOVA; Figures 4c and d). The difference in B-cell progenitor ($CD19^{pos}CD45^{dim}$) numbers was not significant (Figure 4b). The impact of immunosuppression on the number of B-cell progenitors, B and T cells is shown in Figures 5b, c and d (for all time points together) and in Supplementary Figures 1b, c and d (for individual time points).

Figure 6b and c shows that samples with false-positive MRD had higher numbers of immature B cells ($CD19^{pos}CD45^{dim}$) and total B cells than negative ones ($P<0.0001$, Mann–Whitney). Also, the numbers of $CD10^{+}{}^{+}CD19^{pos}$, $CD10^{pos}CD19^{pos}$and $CD34^{pos}CD19^{pos}$ cells were significantly higher in samples with false-positive MRD ($P<0.0001$, $P=0.0002$ and 0.0001, respectively, data not shown). The number of T cells ($CD3^{pos}$) was not different between the two groups (Figure 6d). Supplementary Figures 2b, c and d show the same analysis for individual time points.



Discussion

MRD monitoring using antigen receptor gene rearrangements offers a highly sensitive tool for post transplant management of high-risk ALL. On the basis of MRD positivity, methods of preemptive immunotherapy (DLI, immunosuppression withdrawal) or chemotherapy can be employed earlier than when using the less sensitive mixed chimerism or flow cytometric detection. However, our study demonstrated that even when all the criteria for MRD interpretation developed during the past decade are met, there is a considerable risk of false-positive MRD results after SCT. Even though all such results would be classified only as 'positive, not quantifiable', they would mean at best the necessity of repeated examination together with putting more stress on the patients and their parents, and at worst the application of preemptive treatment.

In our cohort, 9 of 21 patients were MRD-positive at least once during the first 3 years post-SCT using clone-specific Ig/TCR RQ-PCR assays. No patient has relapsed so far, with a median follow-up of 31 months. This number does not reflect the exact number of positive results in all transplanted patients, as our cohort was selected based on

Figure 4 Lymphocyte reconstitution during the post-SCT period was described by the following: (**a**) κ-deletion excision circles (KREC) numbers expressed relative to the donor BM sample (KREC number = 1). One-way analysis of variance (ANOVA), not significant difference; Kruskal–Wallis, $P=0.0011$ (significantly different level between time points 1 and 5, 1 and 6, 2 and 5). (**b**) Percentage of B-cell precursors ($CD19^{pos}CD45^{dim}$) out of the total BM cell number during the post-SCT period. One-way ANOVA, not significant difference; Kruskal–Wallis, not significant difference. (**c**) Percentage of B cells ($CD19^{pos}$) out of the total BM cell number during the post-SCT period. One-way ANOVA, not significant difference; Kruskal Wallis, not significant difference ($P=0.06$). (**d**) Percentage of T cells ($CD3^{pos}$) out of the total BM cell number during the post-SCT period. One-way ANOVA, significant difference ($P=0.0020$); linear trend, significant increase among time points (slope 0.68, $P<0.0001$); Kruskal–Wallis, $P=0.0015$ (significantly different level between time points 1 and 5, 1 and 6).

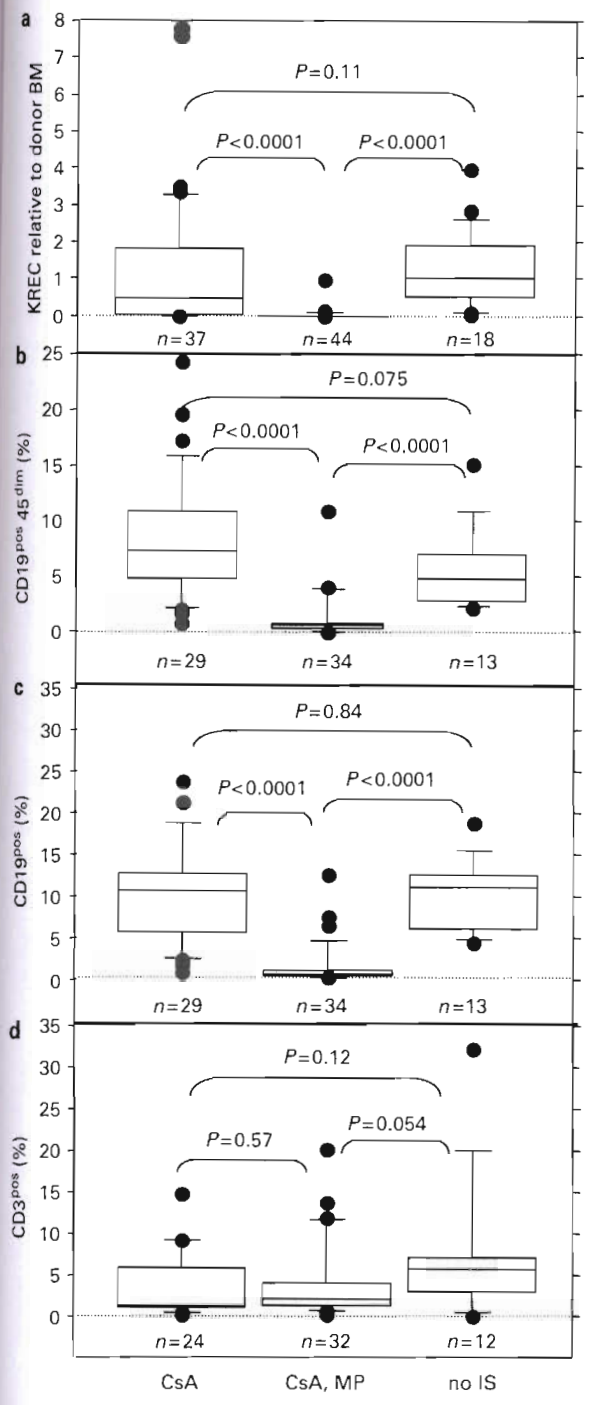


Figure 5 The impact of immunosuppressive treatment taken at the time of sample collection on lymphocyte subsets (all time points together). Expressed as κ-deletion excision circles (KREC) numbers relative to the donor BM sample (**a**), and as a percentage of B-cell precursors ($CD19^{pos}CD45^{dim}$, **b**), B cells ($CD19^{pos}$, **c**) and T cells ($CD\ 3^{pos}$, **d**) out of the total BM cell number. The Mann–Whitney test was used for statistical analysis. MP, methylprednisolone; IS, immunosuppression, CsA, cyclosporin A. One patient received mycophenolate mofetil plus methylprednisolone 1 year post-SCT (not shown).

the leukemia-free survival and on the availability of post transplant material. Knechtli *et al.*[13] observed post transplant MRD positivity in 8 of 36 patients who remained in continuing complete remission. All MRD-positive samples in their study had been followed by negative ones, compared to 9 of 16 positive samples in this study. On the basis of the interim results, we already considered the last three positive samples in our study to be false positive and did not invite patients to undergo repeated examination out of schedule, so we have not received the MRD results so far. One can speculate that those positivities were real and reflected GVL effect preventing a hematological relapse. All of the patients selected for this study were MRD negative or low positive before the transplant, which has been shown to denote a low risk of post transplant relapse in several studies.[5–8,17] Moreover, 11 of 16 positive samples coming from patients with fusion genes were negative in simultaneous fusion transcript detection. We employed two non-self-specific RQ-PCR assays and observed a substantial percentage of false-positive amplifications in our cohort of samples. MRD monitoring based on antigen receptor gene rearrangements utilizes 'fingerprint-like' V- (D)-J sequences of leukemic clones for primer design. Nonspecific amplification in polyclonal lymphocytes with similar rearrangements is usually determined using pooled PB DNA from 5–10 healthy donors, and its occurrence depends on the type of Ig/TCR target and the number of inserted N nucleotides (about 30–40% in IGH whereas 90% in TCRG targets).[40] Recently, van der Velden *et al.*[41] reported that the level of nonspecific amplification in IGH targets depends on the time point during the induction treatment and is the highest in the post-maintenance period due to the prevalence of $CD10^{pos}TdT^{neg}$ precursor B cells with complete VH-JH rearrangements in the B-cell compartment. The authors concluded, however, that ESG-MRD-ALL guidelines for interpretation of RQ-PCR data were sufficient for frontline ALL therapy monitoring, with less than 2% false-positive results. As we were limited in sample size, so we could only analyze two IGH targets representing the same type (VH3 and JH4) of rearrangement to minimize assay-specific variations. Both assays had no background in PB buffy coat; still, we observed 20 and 15% false-positive results using regenerating post-SCT BM samples. Regarding immune cell reconstitution, SCT constitutes a unique situation. According to the KREC levels representing in our setting the output of B cells from BM, the median level remains lower than that of the control group until 1-year post-SCT. This result is in concordance with the study of Kook *et al.*,[42] who observed a depression of total B-lymphocyte count until 18 months post-SCT. However, a fraction of patients had higher values than healthy BM donor starting in a few cases as soon as day 60 post-SCT, and creating more than half of the cohort 1–3 years post-SCT. Higher numbers of total B cells were also observed in a study of patients surviving 20–30 years after transplantation.[43]

The problem of extensive regeneration causing unspecific primer binding could be theoretically overcome by using regenerating BM DNA instead of DNA from buffy coats as a negative control; however, the availability of such

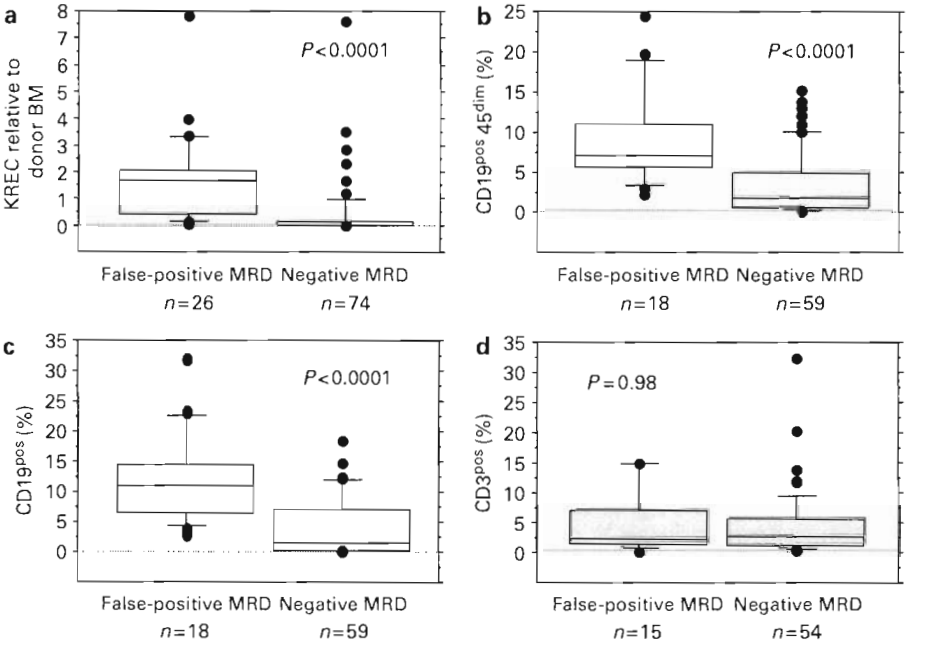


Figure 6 Lymphocyte reconstitution in minimal residual disease (MRD)-negative vs MRD false-positive samples (all time points together). (**a**) Expressed as κ-deletion excision circles (KREC) numbers relative to the donor BM sample. (**b**) Expressed as a percentage of B-cell precursors ($CD19^{pos}CD45^{dim}$) and (**c**) B-cell percentage ($CD19^{pos}$) out of the total BM cell number. (**d**) The number of T cells ($CD\ 3^{pos}$) in the two groups was not different. The Mann–Whitney test was used for statistical analysis. The sample was assessed as MRD false positive if positivity according to European Study Group on Minimal Residual Disease in ALL (ESG-MRD-ALL) criteria was detected in at least one of the two non-self-specific assays.

material in practice is very limited. Our recommendation is to continue using PB buffy coats while carefully judging cases of not quantifiable post-SCT MRD positivity. As all the RQ-PCR products we sequenced and analyzed by on-chip electrophoresis differed from the specific ones, performing at least one of the two techniques would pay off in such cases.

The impact of preemptive therapy based on post transplant MRD or chimerism detection has not been proven so far on a larger cohort of patients. In our previous study, all attempts used to avert post-SCT relapse showed only a limited or temporary effect.[8] On the basis of these results, we are now working on pre-transplant MRD monitoring to enable adding treatment to minimize MRD level before the transplant and thus the risk of relapse. However, post transplant MRD monitoring still remains an extremely useful tool for therapy management, if judged with caution.

Acknowledgements

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Supplementary Information accompanies the paper on Bone Marrow Transplantation website (http://www.nature.com/bmt)

Příloha 7

Minimal residual disease (MRD) analysis in the non-MRD-based ALL IC-BFM 2002 protocol for childhood ALL: is it possible to avoid MRD testing?

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ORIGINAL ARTICLE

Minimal residual disease (MRD) analysis in the non-MRD-based ALL IC-BFM 2002 protocol for childhood ALL: is it possible to avoid MRD testing?

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The ALL IC-BFM 2002 protocol was created as an alternative to the MRD-based AIEOP-BFM ALL 2000 study, to integrate early response criteria into risk-group stratification in countries not performing routine PCR-based MRD testing. ALL IC stratification comprises the response to prednisone, bone marrow (BM) morphology at days 15 and 33, age, WBC and BCR/ABL or MLL/AF4 presence. Here, we compared this stratification to the MRD-based criteria using MRD evaluation in 163 patients from four ALL IC member countries at days 8, 15 and 33 and week 12. MRD negativity at day 33 was associated with an age of 1–5 years, WBC $<20\,000\,\mu l^{-1}$, non-T immunophenotype, good prednisone response and non-M3 morphology at day 15. There were no significant associations with gender or hyperdiploidy in the study group, or with *TEL/AML1* fusion within BCP-ALL. Patients with M1/2 BM at day 8 tended to be MRD negative at week 12. Patients stratified into the standard-risk group had a better response than intermediate-risk group patients. However, 34% of them were MRD positive at day 33 and/or week 12. Our findings revealed that morphology-based ALL IC risk-group stratification allows the identification of most MRD high-risk patients, but fails to discriminate the MRD low-risk group assigned to therapy reduction.

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Keywords: acute lymphoblastic leukemia; minimal residual disease; risk-group stratification; immunoglobulin and T-cell receptor gene rearrangements; bone marrow morphology

Introduction

Several retrospective studies from the late 1990s have shown the clinical significance of minimal residual disease (MRD) in childhood acute lymphoblastic leukemia (ALL).[1–3] Based on these results, MRD testing has become a part of the risk-group stratification procedure in several of the most progressive ALL treatment protocols.[4–7] In 2000, the International Berlin–Frankfurt–Münster Study Group (I-BFM-SG) incorporated MRD testing into risk-group stratification in the AIEOP-BFM ALL 2000 trial. Almost all 'classical' risk features (except prednisone response, t(4;11), t(9;22) and induction failure) were omitted in this risk-group stratification. MRD negativity at day 33 (d33, end of induction phase 1) and at week 12 (w12, before consolidation treatment) stratified patients to the standard- (low) risk group, while high MRD level at w12 was an additional contribution to the classical high-risk features.[8]

Minimal residual disease monitoring in the AIEOP-BFM ALL 2000 trial is based on real-time quantitative PCR (RQ-PCR) detection of patient-specific immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements, which is currently considered the most reliable tool for MRD diagnostics. However, this method is time-consuming, logistically demanding and relatively expensive. Moreover, to guarantee the credibility of the results, it requires regular inter-laboratory quality controls.[9] For these reasons, this method is still inapplicable in many laboratories around the world.

In 2000, many countries within I-BFM-SG were not able to apply routine MRD testing to clinical practice; therefore, a new protocol, ALL IC-BFM 2002, was designed in parallel to AIEOP-BFM ALL 2000. Countries that joined the ALL IC consortium wanted to test the possibility of (at least partially) gaining additional prognostic information by non-MRD-based methods. The patients were stratified into standard-risk, intermediate-risk and high-risk groups (SRG, IRG and HRG, respectively), according to age, white blood cell (WBC) count, blast proportion in peripheral blood after 7 days of prednisone and a single intrathecal dose of methotrexate (prednisone response) and the presence of t(9;22) and t(4;11) fusions (Figure 1). As the morphological assessment of bone marrow (BM) at day 15 (d15) provided additional prognostic information,[10] d15 BM evaluation was newly added to the stratification scheme. A slow response to treatment detected as M3 BM at d15 re-stratified patients to higher risk groups. More than 800 children a year from 12 countries all around the world (including Argentina, Chile, Croatia, Czech Republic, Hong Kong, Hungary, Israel, Poland, Serbia, Slovakia, Slovenia and Uruguay) were treated according to the ALL IC protocol. Apart from the treatment questions, one of the goals of this study was to compare this risk-group assessment to the MRD-based criteria used in AIEOP-BFM ALL 2000. Therefore, this study asked the question whether it is possible to avoid MRD testing in some subgroups of patients. The study was called 'Mini Risk' and consisted of MRD evaluation in 163 patients treated in four member countries of the ALL IC consortium (Czech Republic, Israel, Hong Kong and Uruguay).

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Patients, materials and methods

Patients

A total of 207 children with ALL (age 1–18 years) treated in the Czech Republic (November 2002 to February 2004, $n=105$), Israel (January 2004 to December 2004, $n=48$), Hong Kong (January 2003 to March 2004, $n=36$) and Uruguay (April 2004 to March 2005, $n=18$) were included in the Mini Risk study. All children were treated according to the ALL IC-BFM 2002 protocol. As the Mini Risk study was mainly aimed at SRG, sampling of SRG patients in the Czech Republic continued to October 2004. Moreover, Czech HRG and T-cell ALL (T-ALL) patients consecutively diagnosed up to December 2004 were enrolled in the study to acquire sufficient numbers of patients in the analyzed subgroups. Twenty patients were not analyzed for MRD: 3 because of early death during the induction phase and 17 because of poor quality or missing diagnostic DNA. The BM sampling was performed at up to six fixed time points along the course of the treatment protocol: d8 (Czech Republic only) and d15 of induction, end of induction phase 1 (d33), pre-consolidation (w12), pre-reinduction (w22) and at the end of the first year of treatment (w52). BM was classified as M1 (<5% blasts), M2 (5–24% blasts) and M3 (≥25% blasts) by national reference laboratories using standard morphological criteria. All patients or their parents or guardians gave informed consent for the treatment study.

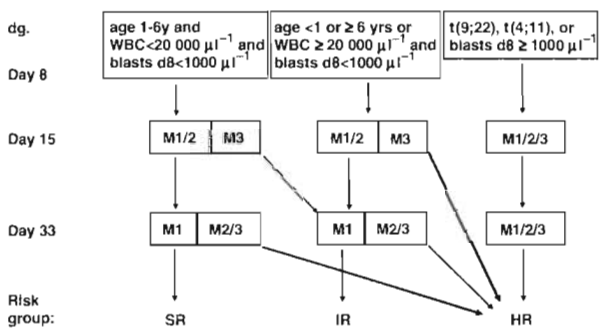

Figure 1 The risk-group stratification scheme in the ALL-IC BFM 2002 trial. M1/2/3, BM status according to morphology, M1 (<5% blasts), M2 (5–24% blasts) and M3 (≥25% blasts). BM, bone marrow.

Treatment

Figure 2 shows the treatment scheme of the ALL IC-BFM 2002 protocol. In brief, after 7 days of steroid prephase and one injection of i.t. MTX (intrathecal methotrexate), all patients received the 8-agent, 8-week induction therapy (protocol I). Consolidation therapy (protocol mM/M) consisted of four courses of high-dose (HD) MTX 2 g m^{-2} (5 g m^{-2} for T-ALL) for SRG and IRG patients. Cranial irradiation was applied in non-transplanted HRG patients, T-ALL patients and in patients with CNS status 3. At the beginning of delayed re-induction, patients were randomly assigned to receive one protocol II vs two protocol III (using less dexamethasone, vincristine, cyclophosphamide and anthracyclines) treatments for SRG and one protocol II vs three protocol III treatments for IRG. HRG therapy consisted of three HR-blocks and triple re-intensification with three protocol III treatments (HR-1) vs six HR-blocks plus protocol II treatment (HR-2B) vs three HR-blocks

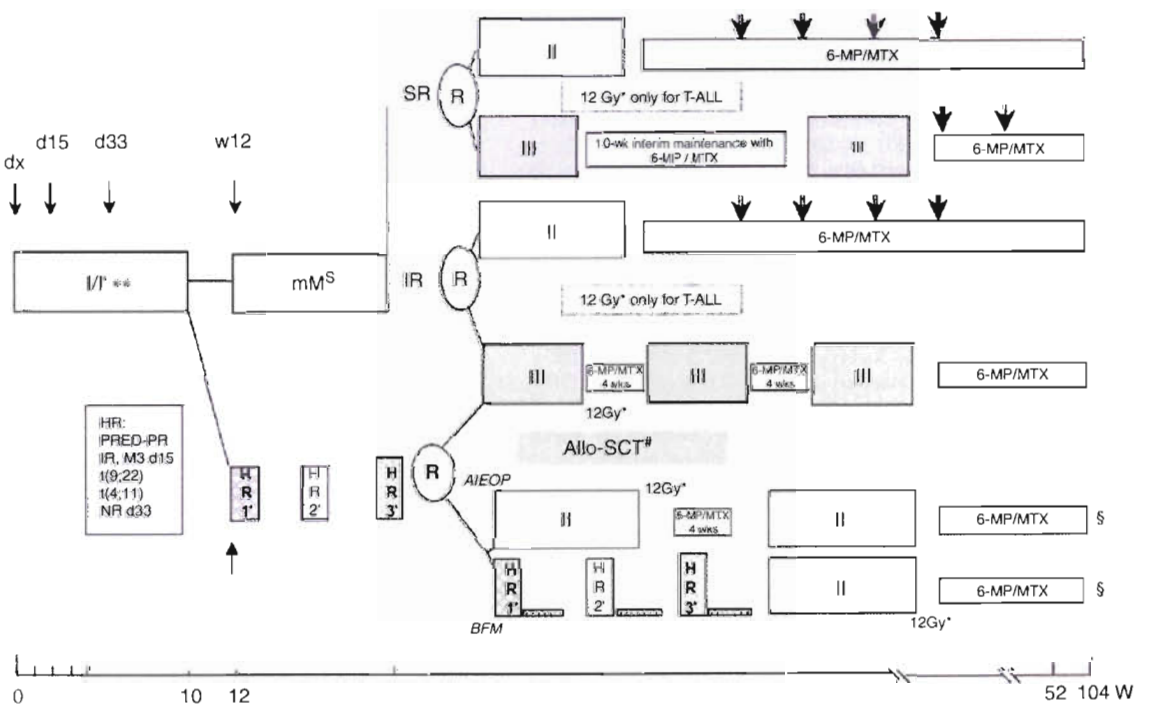

Figure 2 ALL IC-BFM 2002 treatment. ↓ BM sampling; ⇓ i.t. MTX in maintenance therapy; **Prot. I', DNR 30 mg m^{-2} only for SR patients with BCP-ALL; §BCP-ALL, MTX 2 g m^{-2} per 24 h × 4; T-ALL, MTX 5 g m^{-2} per 24 h × 4; *CNS status 1/2, pCRT – 12 Gy; CNS status 3, tCRT:12/18 - dosage by age at treatment delivery, infants <1 year of age had neither pCRT nor tCRT; $^{\#}$no randomization of AIEOP vs BFM but choice by group according to previous experience with one of the two high-risk strategies. i.t. MTX, intrathecal methotrexate.

and two protocol II treatments (HR-2A). Maintenance therapy for a total treatment duration of 24 months consisted of 6-mercaptopurine daily, MTX weekly and extended i.t. MTX for children receiving HD MTX 2 g m^{-2} in consolidation. All elements of this BFM-type therapy have already been published.[11,12]

Flow cytometry

Flow cytometry immunophenotyping of BM aspirates was performed at diagnosis using a panel of mAbs recommended by the European Group for the Immunological Characterization of Leukemias.[13]

Detection of fusion genes

The presence of *TEL/AML1*, *BCR/ABL* and *MLL/AF4* fusion genes was examined as a part of routine ALL diagnostics by real-time PCR in the Czech Republic and by fluorescence *in situ* hybridization in Israel, Hong Kong and Uruguay.

DNA index

The DNA index was assessed routinely at diagnosis using the CycleTestPlus DNA Reagent kit (BD, San Jose, CA, USA) as described previously.[14] The DNA index was defined as a ratio of the mode of fluorescence of cells in the G0/G1 phase and the mode of fluorescence of normal peripheral blood in G0/G1. Prognostically relevant hyperdiploidy was assessed as having a DNA index $\geqslant$1.16 and $\leqslant$1.6.

Detection of residual disease

Mononuclear cells from the diagnostic BM samples were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density centrifugation and stored in liquid nitrogen. Follow-up BM or peripheral blood samples were processed by erythrocyte lysis and stored at −80 °C.

Genomic DNA was isolated by the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). DNA was stored at −20 °C before processing. Primers and protocols for detection of immunoglobulin heavy chain, immunoglobulin light chain κ, T-cell receptor γ, T-cell receptor δ gene rearrangements and TAL1 deletions were described previously.[15,16] Clonality of the PCR products was confirmed by heteroduplex analysis.[17] Sequencing was performed using the ABI PRISM 310 Genetic Analyzer with the BigDye Primer v3.0 Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Variable (V), diversity (D) and joining (J) regions of the immunoreceptor gene were identified by comparison with sequences in GenBank using the ImMunoGeneTics (IMGT) Database (http://imgt.cines.fr, IMGT, European Bioinformatics Institute, Montepellier, France) and the IGBlast search (http://www.ncbi.nlm.nih.gov/igblast/, National Center for Biotechnology Information, Bethesda, MD, USA).

Patient-specific forward primers for RQ-PCR were designed using the Vector NTI 8 Suite Software (Informax, Bethesda, MD, USA). Family-specific reverse primers and probes for immunoglobulin heavy chain, immunoglobulin light chain κ, T-cell receptor δ and T-cell receptor γ were described previously.[18–21] Ig/TCR RQ-PCR was performed using the iCycler IQ™ real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) and the ABI Prism 7700 real-time PCR System (Applied Biosystems, Foster City, CA, USA). Standard curves were prepared by diluting the diagnostic samples in polyclonal DNA from healthy donors. The albumin gene was used to normalize the DNA concentration and quality.[22] The ESG-MRD-ALL (European Study Group on Minimal Residual Disease in ALL) criteria for RQ-PCR sensitivity and quantitative range interpretation were used.[23]

Feasibility of MRD monitoring

A total of 187 patients were investigated for the presence of clonal Ig/TCR rearrangements. In 179 (96%) patients, at least one monoclonal marker was found. Patient-specific RQ-PCR assay with minimal sensitivity of 10^{-4} was established for 163 (87%) patients. The main reason for inadequate sensitivity was early amplification of control DNA from healthy donors. One hundred and four patients (53%) had two sensitive Ig/TCR targets. We have previously shown a good correlation between two independent Ig/TCR targets during the ALL IC-BFM 2002 induction treatment.[24] Higher values were used for MRD analysis in patients with two Ig/TCR targets.

Statistical analyses

Distribution of variables between groups with positive and negative MRD was assessed using Fisher's exact test. The Mann–Whitney and Kruskal–Wallis tests were used to estimate significance of differences concerning continuous MRD values. The statistical analyses were performed using StatView version 5.0 (StatView Software, Cary, NC, USA).

Results

For intergroup analysis, a consecutively recruited cohort of patients was analyzed, with MRD values available in 69 (d8), 87 (d15), 133 (d33) and 133 (w12) samples. For internal SRG, HRG and T-ALL group analyses, augmented consecutively recruited groups were used as described in the Patients section.

MRD and ALL IC-BFM 2002 risk groups

Table 1 shows the distribution of MRD positivity and negativity at d33 and w12. Overall, 68 of 133 (51%) consecutively diagnosed patients had, at d33, a BM that was negative for MRD (d33 MRD^{neg}) and 107 of 135 (79%) were MRD negative at w12 (w12 MRD^{neg}). Twenty-five of 133 (19%) patients had, at d33, MRD levels that were higher than 10^{-3}. MRD correlated significantly with the ALL IC-BFM 2002 risk groups. Median MRD level at d33 was 1.0×10^{-3} in HRG patients compared to 1.9×10^{-5} in IRG patients and 0 in SRG patients (Figure 3a).

Standard-risk group patients responded better in terms of MRD negativity at d33 than IRG patients ($P = 0.0018$). However, 21 of 62 (33.9%) SRG patients for whom both d33 and w12 follow-up samples were available were d33 and/or w12 MRD^{pos} (ranging from borderline positivity to 1.5×10^{-2} at d33 and to 1.2×10^{-4} at w12). These patients would not qualify as MRD-based SRG in AIEOP-BFM ALL 2000. Figure 3b shows MRD clearance during the initial phase of treatment in the ALL IC SRG, highlighting patients with slow molecular response. They did not differ significantly from those with rapid molecular response with respect to gender, WBC, BM morphology at d15 or presence of hyperdiploidy or *TEL/AML1* fusion. The only difference was in a higher proportion of M3 BM at d8 in MRD slow responders ($P = 0.016$, see below).

Conversely, 26 of 64 (40.6%) consecutively diagnosed ALL IC IRG patients were MRD negative at both d33 and w12 (15 patients by 2 Ig/TCR targets, thus fulfilling AIEOP-BFM ALL 2000 criterion for SRG).

Table 1 Impact of clinical and biological factors on MRD status at d33 and w12

	Age		*WBC (× 1000 µl^{-1})*		*Immunophenotype*		*PRED response*		*Gender*		*TEL/AML1*		*TEL/AML1 BCP-ALL*		*Hyperdiploidy*[a]	
	< 6 years	*> 6 years*	*< 20*	*> 20*	*BCP-ALL*	*T-ALL*	*PGR*	*PPR*	*M*	*F*	*No*	*Yes*	*No*	*Yes*	*Yes*	*No*
d33 neg.	52 (63%)	16 (32%)	52 (62%)	16 (33%)	65 (57%)	3 (17%)	68 (54%)	0	36 (37%)	32 (57%)	47 (46%)	21 (68%)	44 (52%)	21 (68%)	17 (63%)	42 (49%)
d33 pos.	31 (37%)	34 (68%)	32 (38%)	33 (67%)	50 (43%)	15 (83%)	57 (46%)	8 (100%)	41 (53%)	24 (43%)	55 (54%)	10 (32%)	40 (48%)	10 (32%)	10 (37%)	44 (51%)
		P = 0.0007		**P = 0.0013**		**P = 0.0019**		**P = 0.0026**		P = 0.514		**P = 0.0412**		P = 0.2031		P = 0.270
w12 neg.	70 (81%)	36 (77%)	77 (88%)	29 (64%)	91 (79%)	15 (83%)	103 (82%)	3 (43%)	60 (77%)	46 (84%)	80 (79%)	26 (81%)	65 (78%)	26 (81%)	18 (72%)	62 (79%)
w12 pos.	16 (19%)	11 (23%)	11 (12%)	16 (36%)	24 (21%)	3 (17%)	23 (18%)	4 (57%)	18 (23%)	9 (16%)	21 (21%)	6 (19%)	18 (22%)	6 (19%)	7 (28%)	16 (21%)
	P = 0.508		**P = 0.0028**		P > 0.999		**P = 0.0311**		P = 0.389		P > 0.999		P = 0.8033		P = 0.423	

	Risk group		*d8 BM morphology*[b]			*d8 BM morphology-BCP-ALL*			*d15 BM morphology*[c]		
	SRG	*IRG*	*HRG*	*M1*	*M2*	*M3*	*M1*	*M2*	*M3*	*M1*	*M2*	*M3*
d33 neg.	39 (71%)	28 (42%)	1 (9%)	6 (67%)	12 (67%)	24 (49%)	6 (86%)	12 (75%)	23 (53%)	51 (54%)	14 (54%)	1 (12%)
d33 pos.	16 (29%)	39 (58%)	10 (91%)	3 (33%)	6 (33%)	25 (51%)	1 (14%)	4 (25%)	20 (47%)	44 (46%)	12 (46%)	7 (88%)
		P = 0.0018			P > 0.999		P > 0.999			P > 0.999		
		P = 0.0039			P = 1561			P = 0.0640			**P = 0.0303**	
w12 neg.	51 (88%)	50 (76%)	5 (56%)	9 (100%)	17 (94%)	34 (71%)	7 (100%)	15 (94%)	29 (71%)	79 (81%)	21 (81%)	3 (50%)
w12 pos.	7 (12%)	16 (24%)	4 (44%)	0	1 (6%)	13 (29%)	0	1 (6%)	12 (29%)	18 (19%)	5 (19%)	3 (50%)
		P = 0.106			P > 0.999			P > 0.999			P > 0.999	
		P = 0.0824			**P = 0.0129**			**P = 0.0225**			P = 0.0959	

Abbreviations: BM, bone marrow; d33, day 33; HRG, high-risk groups; IRG, intermediate-risk group; neg, negative; pos, positive; PGR, prednisone good responders; PPR, prednisone poor responders; SRG, standard-risk group; WBC, white blood cell; w12, week 12.
[a]Hyperdiploidy assessed in Czech, Hong Kong and Uruguay patients.
[b]d8 BM assessed in Czech patients.
[c]d15 BM not evaluable in four patients.

MRD and clinical/biological factors

Age. Using a cutoff of 6 years applied in the ALL IC-BFM 2002 stratification, patients older than 6 years were more likely to be d33 MRD^{pos} than patients 1–5 years of age ($P=0.0007$). Quantitative MRD levels differed significantly at d15 ($P=0.045$) and d33 ($P=0.0025$) between the two groups and showed no significant difference at d8 and w12 (Figure 4a). In w12, neither a 6-year nor 10-year cutoff significantly distinguished MRD negative patients from those who were MRD positive.

White blood cell count. Patients with a presenting leukocyte count of less than 20 000 µl^{-1} at diagnosis, corresponding to the cutoff used in the ALL IC-BFM 2002 stratification, were more likely to be d33 MRD^{neg} than those with WBC > 20 000 µl^{-1} ($P=0.0013$). The difference was still present at w12 ($P=0.0028$). Figure 4b shows box-plots representing quantitative MRD levels for WBC lower or higher than 20 000 µl^{-1} during the initial phase of therapy, showing a significant difference between the two groups at d33 ($P=0.0009$) and w12 ($P=0.018$).

Gender. There was no difference in molecular response between boys and girls, neither in achieving d33 or w12 MRD negativity, nor in MRD levels at respective time points during therapy (Table 1).

Immunophenotype. Patients with BCP-ALL were more likely to achieve d33 MRD negativity than those with T-ALL ($P=0.0019$, Table 1). However, this difference was not distinct at w12. When comparing quantitative MRD levels, patients with BCP-ALL had significantly lower MRD levels at d15 ($P=0.023$) and d33 ($P=0.002$) than T-ALL patients; the difference was not significant at w12 (Figure 4c). A multivariate analysis including age, WBC and B vs T immunophenotype did not show a significant impact of immunophenotype on d33 MRD status ($P=0.26$), which was caused by its correlation with WBC (WBC: $P=0.029$; age: $P=0.039$).

TEL/AML1. Although median MRD level in d33 MRD^{pos} patients was 2.3×10^{-4} in *TEL/AML1*-positive compared to 3.0×10^{-3} in *TEL/AML1*-negative BCP-ALL patients, there was no significant difference in achieving d33 MRD negativity between patients with and without the *TEL/AML1* fusion (Table 1). Within the consecutively diagnosed *TEL/AML1* group, 10 of 31 (32.3%) patients had detectable MRD at the end of induction. There was no impact of the *TEL/AML1* fusion on w12 MRD status.

Other chromosomal translocations. As the patients with t(9;22) have been treated according to the EsPhALL protocol since 2004 in the Czech Republic and Israel, there were only four patients with the *BCR/ABL* fusion in our cohort, thus precluding statistical analysis. Similarly, there were no children with t(4;11) in the cohort.

DNA ploidy. The data concerning DNA ploidy were only available in Czech, Hong Kong and Uruguay patients. There was no significant difference in molecular response between patients with a hyperdiploid phenotype and those with a normal karyotype, neither in achieving MRD negativity (Table 1), nor in MRD levels at respective time points during therapy (data not shown).

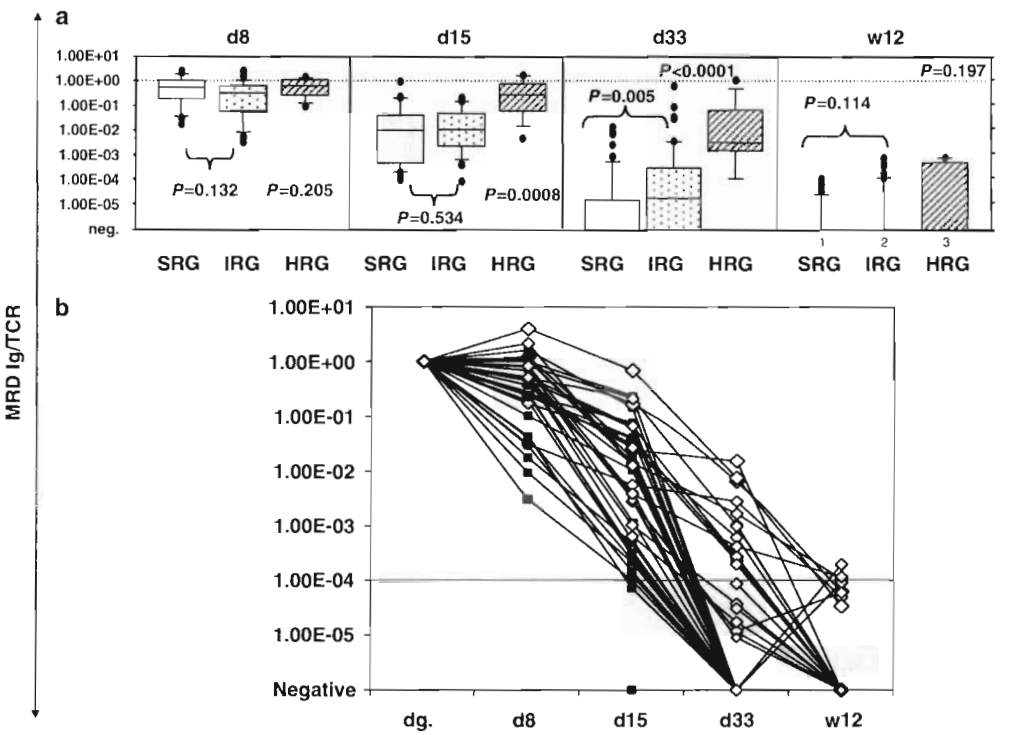

Figure 3 MRD and ALL IC-BFM 2002 risk-group stratification. (**a**) Quantitative MRD levels (logarithmic scale) at d8, d15, d33 and w12 according to the ALL IC-BFM 2002 risk groups. (**b**) MRD clearance in patients stratified to the ALL IC-BFM 2002 standard-risk group. Empty diamonds indicate patients with MRD positivity at d33 and/or w12, who would not qualify as SRG in the MRD-based AIEOP-BFM ALL 2000 trial. MRD, minimal residual disease; SRG, standard-risk group.

MRD and early response to therapy

Prednisone response. None of the patients with more than 1000 blasts μl^{-1} in peripheral blood after 1 week of prednisone and one i.t. MTX (poor prednisone response) achieved negativity at d33 ($P=0.0026$, Table 1). The difference remained significant at w12 ($P=0.031$). Quantitative MRD levels differed significantly between prednisone poor and good responders at d15 ($P=0.037$), d33 ($P<0.0001$) and w12 ($P=0.032$, Figure 5a).

Day 15 bone marrow morphology (BM d15). The d15 morphology evaluation was performed centrally in the ALL IC-BFM 2002 protocol due to BM d15 implementation in risk-group stratification. In the whole cohort, only two patients were re-stratified from SRG to IRG and two patients from IRG to HRG. Two IRG patients with M3 BM at d15 were not treated as HRG due to treatment intolerance. Patients with M3 BM d15 were more likely to be d33 MRD^{pos} than patients with M1 and M2 ($P=0.03$, Table 1). Figure 5c shows the comparison of d15 BM status with MRD levels at d15 and its impact on quantitative d33 and w12 MRD levels. There was no difference in MRD status between patients with M1 and M2 BM on d15 at any time point.

Figure 6 shows the correlation of morphological- and PCR-based d15 BM assessment, with 28 of 95 (29%) samples evaluated differently by each method. Three cases were assessed as M1 by morphology and M3 by PCR, and, vice versa, one case was evaluated as M3 by morphology and M1 by PCR.

We then evaluated the impact of PCR-based MRD at d15 on PCR-based MRD at d33 (Figure 7). Using the MRD cutoff level 5×10^{-4}, 15 (14%) patients had an MRD lower than this value. None of these patients were d33 MRD^{pos}, compared to 53 (60%) patients with d15 MRD higher than 5×10^{-4}. Similar results were observed using an MRD d15 cutoff of 1×10^{-3}. The MRD low-risk group defined by this cutoff comprised 19 patients (18%) and only 1 of these patients was MRD positive at d33, compared to 51 (59%) in the second group.

Day 8 bone marrow morphology (BM d8). Day 8 BM morphology is assessed as a part of the ALL IC-BFM 2002 in the Czech Republic. The proportions of M1, M2 and M3 d8 BM in the consecutively recruited group were 12, 24 and 64%, respectively. BM d8 status had no impact on d33 MRD positivity (Table 1). However, this was mainly due to a higher percentage of M1 BM at d8 (7 of 21, 33%, $P=0.035$) in T-ALL patients, who tended to have slower MRD response during the induction phase. Interestingly, only 1 of 14 SRG patients with d8 M1 or M2 BM was positive at d33, compared to 13 of 30 patients with d8 M3 BM ($P=0.019$). The impact of d8 BM status on MRD status is still evident at w12, even when analyzing the whole cohort, including T-ALL patients (Table 1). Figure 5b shows the quantitative MRD levels according to d8 BM status, with significantly higher levels at d15 in patients with M3 BM at d8. We observed no significant correlation between d8 and d33 MRD levels (data not shown). The overall results of d8 BM evaluation and its impact on survival in a larger cohort will be reported after sufficient follow-up.

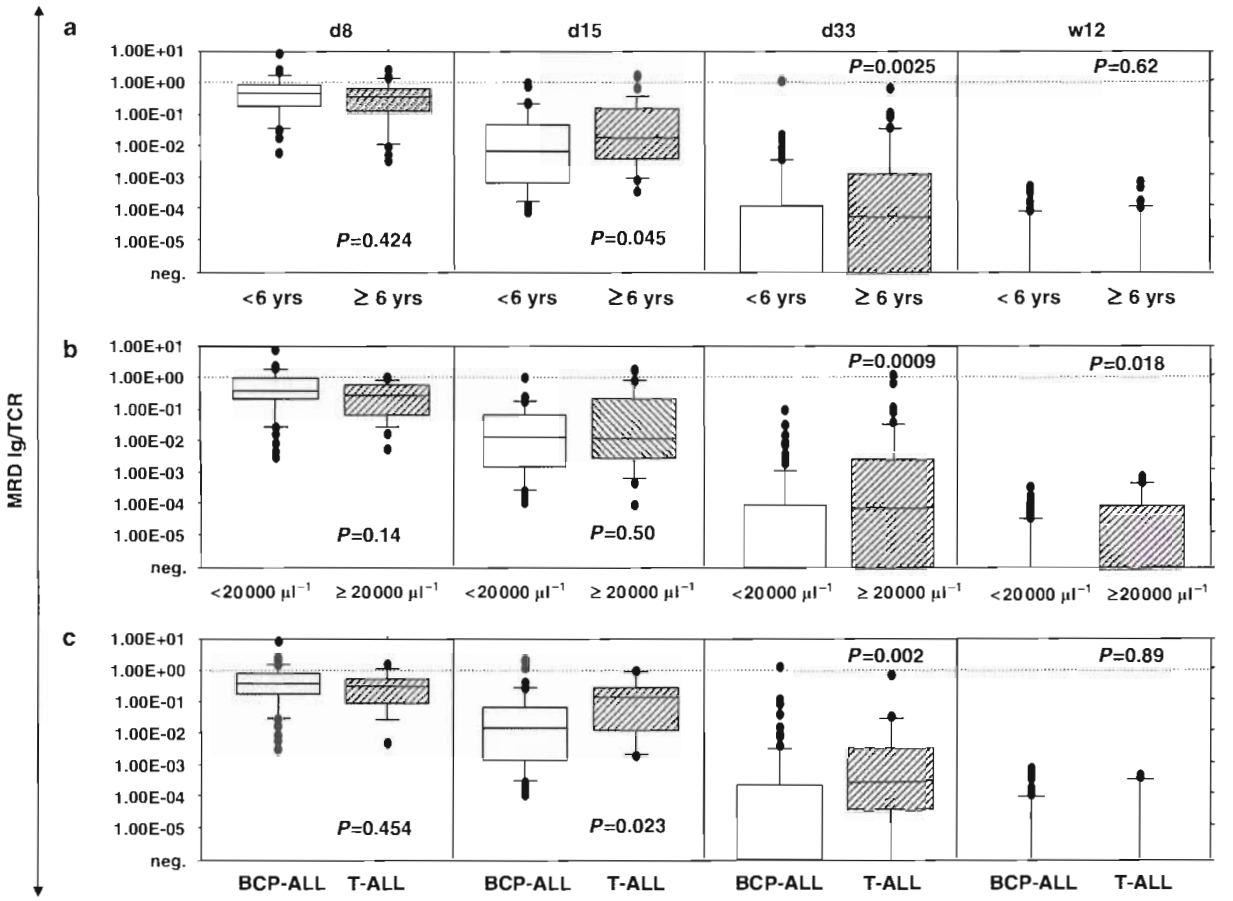

Figure 4 Relation of clinical features to MRD at d8, d15, d33 and w12. Quantitative MRD levels (logarithmic scale) were related to (**a**) age at diagnosis, (**b**) presenting white blood cell count at diagnosis and (**c**) B vs T immunophenotype. MRD, minimal residual disease; WBC, white blood cell.

Discussion

Most modern treatment protocols for childhood ALL have implemented some sort of MRD assessment into their risk-group stratification, aimed at both treatment intensification and reduction. Using MRD with the aim of therapy reduction requires a highly sensitive method (at least 10^{-4}), which so far has not been commonly accessible for many countries worldwide. The ALL IC protocol was designed to span the period during which the involved countries established routine MRD testing. One of the goals of the design was to implement d15 BM morphology evaluation to re-stratify early slow responders to treatment intensification. Antigen receptor-based MRD evaluation was used in our study to determine the correlation of ALL IC risk-group assignment with molecular response during the initial phase of therapy. MRD correlated strongly with ALL IC risk groups. As expected, HRG patients showed slower molecular response than IRG and SRG patients. None of the analyzed patients would be stratified into HRG by AIEOP-BFM ALL 2000 criteria, solely based on high MRD level at w12. All such patients were also prednisone poor responders. IRG patients had higher MRD levels at d33 than SRG patients. However, about one-third of ALL-IC SRG patients were positive at the end of induction and/or at w12. Based on the previous findings, these patients have a higher risk of relapse.[3] Consequently, no treatment reduction would be justified in a protocol based on ALL IC stratification criteria.

Next to age and WBC as classical risk factors, we observed a significant difference in molecular response between BCP-ALL and T-ALL patients. BCP-ALL patients showed a better response at d15 and d33, while the difference between the two groups was no longer distinct at w12. This finding is in agreement with a previous study comparing BCP-ALL and T-ALL patients treated according to the ALL-BFM 90 protocol[25] and implies the potential benefit available to T-ALL patients from treatment modalities used later in the treatment. As most of the T-ALL patients were positive at d33 and negative at w12, another time point in between the two might be beneficial for risk stratification of T-ALL patients in BFM-based protocols.

BM evaluation at d15 has been an integral part of the ALL IC stratification and M3 BM at d15 showed a negative impact on d33 MRD in our study. Only four patients in our cohort were re-stratified into therapy intensification based on M3 BM d15. In all four cases, the morphological evaluation was concordant with the PCR-based evaluation. In general, the correlation between morphology and PCR was far from ideal. It could be explained by a difficult BM evaluation at this time point. On the other hand, RQ-PCR also has limitations, mainly in the exact determination of high MRD levels: one PCR cycle difference means a twofold difference in MRD value. Using a cutoff of

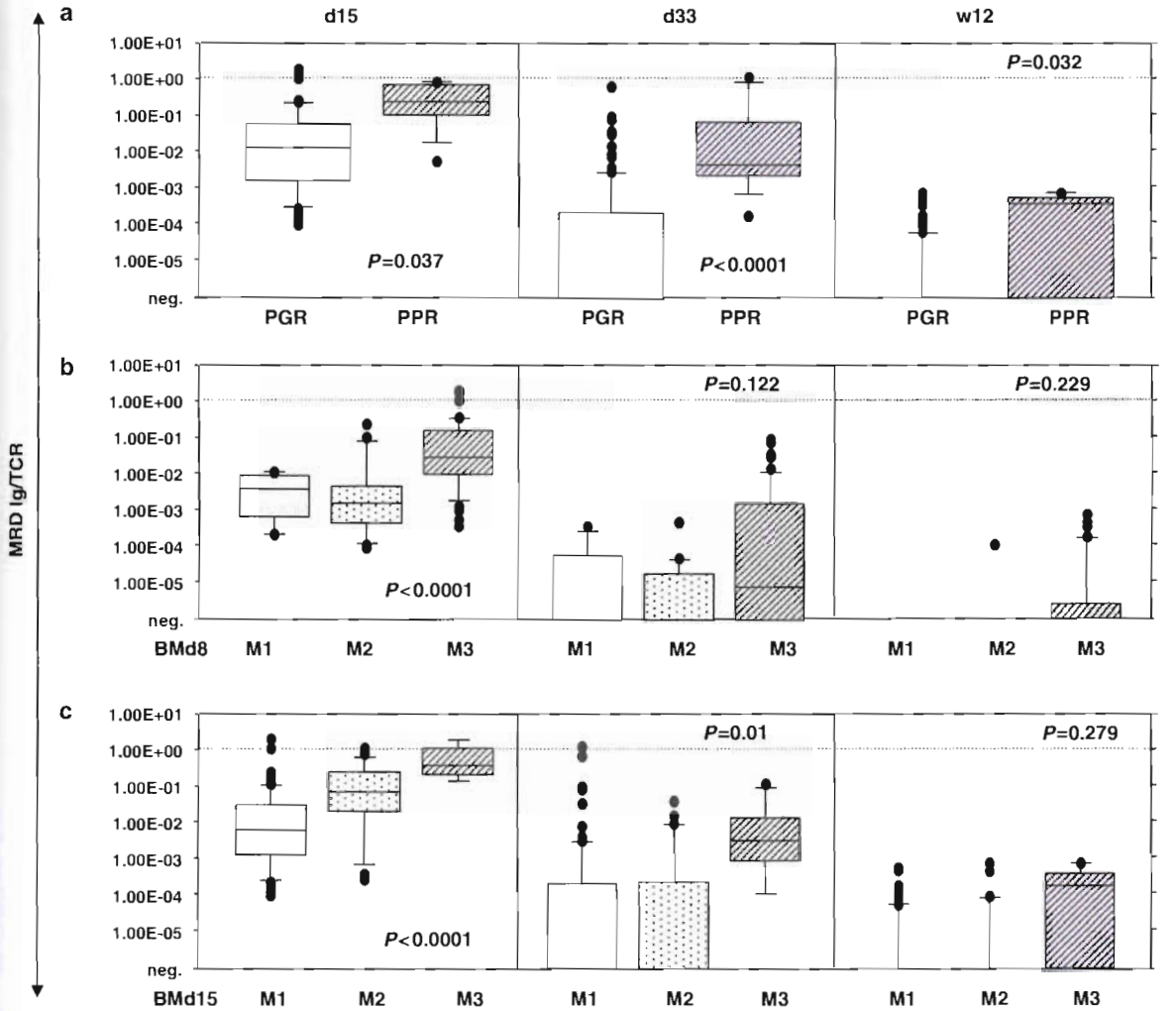

Figure 5 Relation of early treatment response in PB (**a**) and BM (**b**, **c**) to MRD at d15, d33 and w12. Quantitative MRD levels (logarithmic scale) were related to (**a**) blast count in PB after 7 days of prednisone and one i.t. MTX (prednisone response) of less than $1000\,\mu l^{-1}$ (PGR) vs more than $1000\,\mu l^{-1}$ (PPR), (**b**) BM status at d8 (BCP-ALL only) and (**c**) BM status at d15. BM, bone marrow; i.t. MTX, intrathecal methotrexate; MRD, minimal residual disease; PB, peripheral blood; PGR, prednisone good responders; PPR, prednisone poor responders.

5×10^{-4} for PCR at d15, we could identify a small group of patients (14%) who achieved MRD negativity at d33. This is in agreement with the previous study of Panzer-Grumayer *et al.*,[26] who observed an excellent outcome in a similar group of patients treated according to the ALL-BFM 90 protocol. However, the use of Ig/TCR-based MRD monitoring for treatment assignment in such an early time point is limited by the extreme logistical requirements of this method. Flow cytometry seems to be a more convenient tool for the early identification of patients with excellent prognosis.[27,28]

BM d8 evaluation on the BFM-based protocol has not been reported so far. The Children's Oncology Group study concerning BM d8 reflects a different treatment approach (without a prednisone pre-phase). With regard to this, it is not surprising that the proportion of M1 and M2 marrows in the Children's Oncology Group study is significantly higher.[29] In our study, BCP-ALL patients with M3 BM d8 were more likely to be MRD positive at both d33 and w12. This trend was even preserved in SRG. However, 67% of SRG patients in our cohort had M3 BM d8 and more than half of them were subsequently d33 MRD negative, which impairs the practical use of this factor. Our results indicate that M1 BM d8 might potentially identify a small group (11%) of BCP-ALL patients with excellent prognosis. However, it will be necessary to extend the cohort and perform a follow-up to confirm this hypothesis.

Patients with *TEL/AML1* translocation did not differ from the other BCP-ALL patients in molecular response. About one-third of *TEL/AML1*-positive patients had detectable MRD at d33. According to a previous study, those patients have a higher risk of subsequent relapse.[30]

In contrast to the well-documented favorable prognostic impact of hyperdiploidy,[31] about half of the hyperdiploid cases were MRD positive at d33. This could potentially mean that those patients have a greater risk of relapse. Unfortunately, the original BFM MRD study[3] lacked information on DNA ploidy. Borowitz *et al.*[29] reported a surprisingly high percentage of MRD positive cases at the end of induction among patients with favorable trisomies, despite the excellent prognosis of this group.

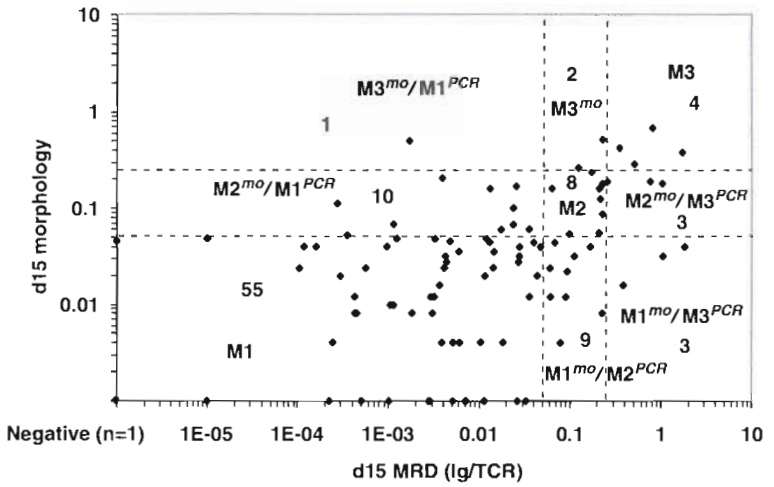

Figure 6 Correlation of morphological (mo) and PCR-based BM assessment at d15. Percentage of blasts according to Ig/TCR-based RQ-PCR (*x* axis) vs percentage of blasts according to morphology (*y* axis) in 95 BM samples. BM, bone marrow; RQ-PCR, real-time quantitative PCR.

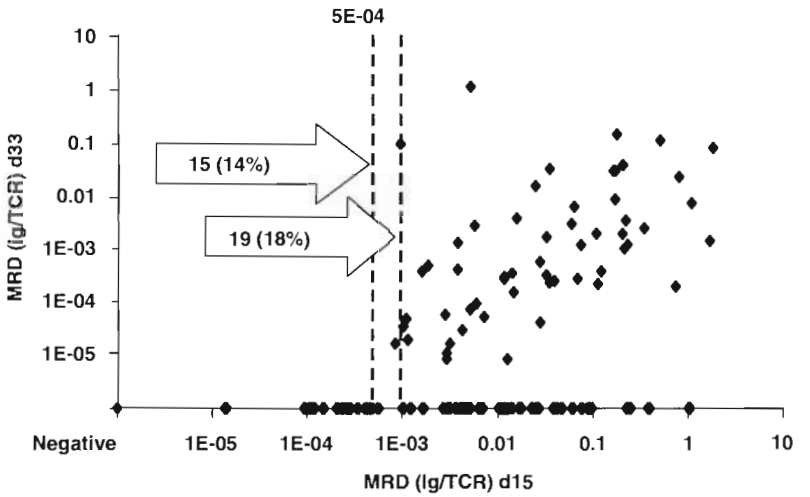

Figure 7 Impact of PCR-based MRD at d15 on PCR-based MRD at d33. Percentage of blasts at d15 (*x* axis) vs percentage of blasts at d33 (*y* axis) in 104 BM samples. Arrows indicate the respective d15 cutoffs of 1×10^{-03} and 5×10^{-04} that define the MRD low-risk groups. BM, bone marrow; MRD, minimal residual disease.

Our study was aimed at correlation of clinical, morphological and biological stratification criteria used in the ALL IC-BFM 2002 protocol with MRD clearance. Generally, MRD reduction during the first 3 months of therapy correlated with ALL IC-BFM 2002 risk groups. This was most distinct in the high-risk group, implying that the 'classical' criteria used in ALL-IC are able to identify most HRG patients. However, there was a large overlap between the intermediate- and standard-risk groups concerning MRD negativity at the end of induction. Thus, the ALL IC criteria are not able to reliably define the low-risk group potentially assigned to therapy reduction, which is the challenge of modern leukemia treatment. During our study, the countries involved implemented the methodology of PCR-based MRD testing and are now prepared to use the MRD-based protocol. Still, great effort should be made to the identification of simpler stratification criteria (for example, flow cytometry MRD assessment), since PCR-based MRD testing is still unavailable in many countries.

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Příloha 8

CD44 and CD27 delineate B-precursor stages with different recombination status and with an uneven distribution in nonmalignant and malignant hematopoiesis

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CD44 and CD27 delineate B-precursor stages with different recombination status and with an uneven distribution in nonmalignant and malignant hematopoiesis

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Key words
acute lymphoblastic leukemia; B-cell development; CD27; CD44

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Abstract

The expression of CD27 and CD44 correlate with the genotype of B-precursor acute lymphoblastic leukemia (ALL). Based on the expression of these antigens, we identified counterparts of *TEL/AML1*^{pos} and *TEL/AML1*^{neg} leukemic cells in nonmalignant bone marrow. Although CD27 is known as a marker of mature memory B cells, we recently showed that CD27 is also expressed by malignant and nonmalignant B precursors. Here, we show that CD27 and CD44 delineate stages of B-precursor development. Well-established differentiation markers showed that the developmental sequence starts from undetermined progenitors, expressing CD44. Upon B-lineage commitment, cells gain CD27 and lose CD44. The CD27^{pos}CD44^{neg} (CD27 single positive, 27SP) cells are the earliest stage within CD10^{pos}CD19^{pos} B precursors and express *RAG-1* and *TDT*. These cells correspond to *TEL/AML1*^{pos} ALL (1/4 pediatric B-precursor ALL). The development follows to CD27/CD44 double-positive (27/44DP) stage, 44SP stage and CD27/CD44 double-negative (27/44DN) stage. Before exit to periphery, CD44 is reexpressed. The 27/44DP cells are mostly large and profoundly suppress RAG-1. Despite their presumably high proliferation potential, 27/44DP cells rarely dominate in leukemia. At 44SP stage, which corresponds to *TEL/AML1*^{neg} leukemias, *RAG-1* is reexpressed and Ig light chain gene starts to be rearranged.

Introduction

By making the lineage decision, human progenitor cells start the B-lineage development in the bone marrow (BM) [reviewed in (1, 2)]. This early stage is accompanied by the upregulation of B-cell-specific transcriptional regulators Pax-5 and very likely also early B-cell factor. Since the earliest stage, the B-lineage-committed cells start to rearrange the genes for Ig heavy chain (*IGH*). Upon successful *IGH* rearrangement, the recombination machinery is suppressed and cells quickly proliferate. In the following stage, Ig light-chain (*IGL*) genes are rearranged. Cells that complete both *IGH* and *IGL* are ready to be functionally competent and develop into naïve mature B cells. Such cells leave the BM environment.

On cell surface level, the first B-committed cells express CD34, which disappears in the course of maturation. Very early after B-lineage decision, CD10 is expressed and continues to be present on the cell surface until *IGH* and *IGL* are rearranged. An important surface marker of B lineage, CD19, is expressed since the very early stage when *IGH* rearranges. Experimental evidence shows that CD10 precedes the expression of CD19, perhaps in majority of cells. The CD10^{pos}CD34^{pos}CD19^{neg} cells are mostly committed to B lineage, although they can develop also to other lineages (3). However, the most immature B-precursor leukemia stage called pro-B is defined as CD19^{pos}CD34^{pos}CD10^{neg} (4, 5). Leukemia nomenclature, reflecting the current understanding of malignant transformation during lymphopoiesis, therefore assumes CD19 expression before CD10. At CD10^{pos}CD19^{pos} stages of development, important events occur including *IGH* rearrangement completion, cell expansion and *IGL* rearrangement. This

CD10^{pos}CD19^{pos} immunophenotype is found in the majority of B-cell-precursor acute lymphoblastic leukemias (BCP ALLs). Our previous study showed that major genotypic BCP ALL subsets strictly correlate with the surface CD27 and CD44 expression (6) (updated results are in http://clip.lf2.cuni.cz/files/archiv/CD44_and_CD27_updated_results.pdf), thus confirming RNA genomics data on protein level (7). Interestingly, *TEL/AML1*^{pos} BCP ALL corresponds to CD27^{pos} phenotype, in the absence of CD44. Although CD27 was considered to be a general marker for memory B cells in humans, we showed its expression also on CD10^{pos}CD19^{pos} B-cell precursors (6). Its expression in BCP ALL and in nonmalignant B precursors was then confirmed in another study (8). CD27 (TNFRSF7, T-cell activation antigen) is a 110-kDa transmembrane glycoprotein composed of disulfide-linked 55-kDa monomers, and it is a member of the tumor necrosis factor receptor family. In humans, it is expressed on the large subset of peripheral T cells, on most medullary thymocytes (9) and on natural killer cells (10). In more mature stages of B-lineage development, it is expressed on somatically mutated B cells (11).

CD44 (Hermes, Pgp-1) is a cell surface glycoprotein coded by 10 standard exons found in all CD44 isoforms; the other 10 are variably spliced (12). CD44 was originally identified on hematopoietic cells (13), but it was also found on a wide range of other tissues (14–16). The CD44 mediates cellular adhesion. It is a receptor for hyaluronate (17), but it binds other compounds of extracellular matrix also. It is involved in the process of lymphocyte activation (18), and its interaction with stromal cells is important during lymphopoiesis and myeolopoiesis (19, 20). The CD44v6 splice variant is often associated with metastatic potential of nonhematopoietic neoplasias, for example, in gastric adenocarcinoma (21). Its expression also correlates with shorter survival of patients with acute myeloblastic leukemia (22) and is more frequently expressed in medium- or high-risk group in pediatric ALL (23). We searched for cells with CD27 and CD44 expression corresponding to leukemia subtypes in nonmalignant BM. Among CD10^{pos}CD19^{pos} cells, we found CD27^{pos}CD44^{neg} B precursors (CD27 single positive, 27SP) corresponding to *TEL/AML1*^{pos} leukemia and CD27^{neg}CD44^{pos} (44SP) cells that correspond to most other ALL subtypes and double-positive (27/44DP) and double-negative (27/44DN) cells, which are rarely seen in BCP ALL. We tested the hypothesis that the expression of CD44 and CD27 nonrandomly correlates with the differentiation stage of B precursors. Therefore, we investigated differentiation markers with known dynamics during B-cell development [CD10, CD34, intracellular IgM, intracellular VpreB (CD179a), terminal deoxyribonucleotidyl transferase (TdT), RAG-1 and Ig gene rearrangements] in immunophenotypic subpopulations defined by CD44 and CD27.

Materials and methods

Patients

BM specimens of children without any evidence of malignant or residual malignant disease (patients 1 and more years after BM transplant, after the end of the ALL therapy and patients investigated to exclude hematological malignancy) were used. Specimens were collected according to the Czech law and institutional regulations and with informed consent. Only leftover material from specimens after completed diagnostic investigations was used.

Flow cytometry

Cell suspension of the unseparated BM or peripheral blood (PB) was stained with four- to eight-color combinations of monoclonal antibodies (mAbs). The following fluorochrome-labeled mAbs were used: anti-CD44 fluorescein isothiocyanate (FITC) (reacting with the standard isoform of CD44) and anti-CD27 phycoerythrin (PE) (BD, San Jose, CA), anti-CD10 ECD, anti-CD179a PC5, anti-CD19 PC7 and anti-CD34 allophycocyanin (APC) (Immunotech, Marseille, France), anti-TdT FITC (Dako, Glostrup, Denmark), anti-CD20 Alexa405 (Serotec, Kidlington, Oxford, UK) and anti-IgM Dyomics647 (Exbio, Prague, Czech Republic). In addition, DAPI (4,6-diamidino-2-phenylindole) (Molecular Probes, Leiden, The Netherlands) and FIX&PERM cell permeabilization kit (An der Grub, Vienna, Austria) were used. Nonmalignant BM samples were analyzed using FACS Aria (BD) and BD LSRII (BD) flow cytometers and sorted using FACS Aria flow cytometer (BD). For polychromatic flow cytometry experiments, photomultiplier (PMT) voltage was set above electronic noise threshold and automated compensation matrix calculation was performed using single-color-stained tubes (DIVA 4.1.2 or SUMMIT 4.3). Gating strategy of compensated data was determined using Fluorescence Minus One controls (24). Analysis was performed using FLOWJO 8.1.1 software (Treestar, Ashland, OR) using the same strategy of positivity determination. For each sample, 4×10^5 to 4×10^6 events were recorded. Gating strategy was used for the analysis and sorting of the cells with a given immunophenotype as follows: CD10^{pos} cells were selected from the gate of CD19^{pos}DAPI^{neg} cells (live B-lineage cells). Four subpopulations based on CD44 and CD27 positivity and negativity were identified according to Fluorescence Minus One controls among these cells (Figure 1). The cells falling above or below a threshold of fluorescence intensity set by these controls are called *positive* or *negative subpopulations* throughout the paper. The experiments with sorted subpopulations were performed in duplicates or triplicates. The purity of sorted subpopulations was always more than 95%. Therefore, the

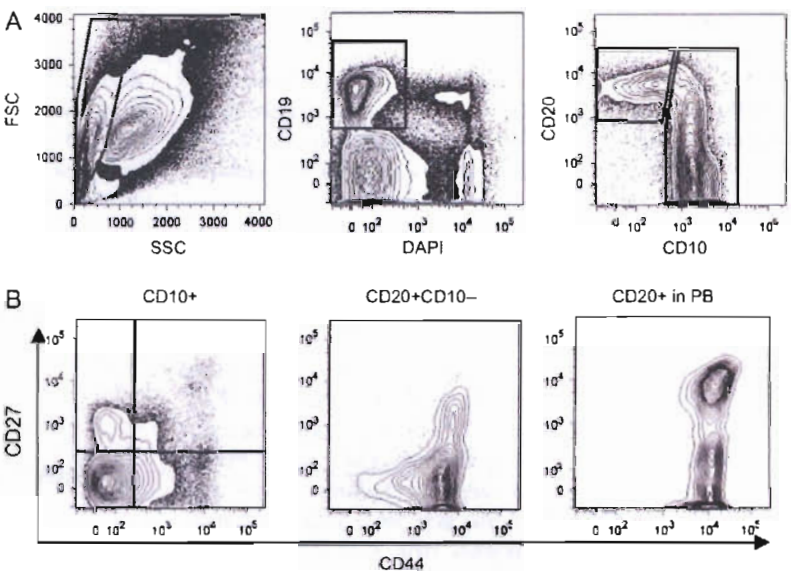

Figure 1 CD27 and CD44 expression in non-malignant BM and PB. Gating strategy used in seven-color staining of one BM specimen for analysis of the differentiation markers in particular subpopulations and for their sorting. (A) $CD10^{pos}$ cells were selected from the gate of $CD19^{pos}$ $DAPI^{neg}$ cells (live B-lineage cells). (B) CD27 and CD44 expression in $CD10^{pos}CD19^{pos}$ cells in BM, in $CD19^{pos}CD20^{pos}CD10^{neg}$ cells in BM and in $CD20^{pos}$ cells in PB of different patients. BM, bone marrow; DAPI, 4,6-diamidino-2-phenylindole; FSC, forward scatter; PB, peripheral blood; SSC, side scatter.

results of the mRNA and DNA analyses indeed describe the sorted subpopulations.

Real-time quantitative reverse transcription–polymerase chain reaction

Complete RNA from sorted cells was isolated by RNeasy Micro Kit (Qiagen GmBH, Hilden, Germany) according to manufacturer's instructions. RNA was converted into complementary DNA (cDNA) using MoMLV Reverse Transcriptase (Gibco BRL, Carlsbad, TX). Real-time quantitative reverse transcription–polymerase chain reaction (PCR) analyses were performed using LightCycler™ rapid thermal cycler system (Roche Diagnostics GmbH, Mannheim, Germany). Fluorescent DNA-binding dye SYBR Green (FMC BioProducts, Rockland, MA) was used for quantification of *RAG-1* and *TDT* gene expression. Control gene beta-2-microglobulin (b2m) was measured using hybridization probes as described previously (25). The primer sequences were as follows – for *TDT*: forward 5′-gTCgTgCCTTTgCCCTgTT-3′, reverse 5′-TCCgCTCATgTgTggCATAg-3′ and for *RAG-1*: forward 5′-TgAgTAATATCAACCAAATTgCAgACA-3′, reverse 5′-ggATCTCACCCggAACAgC-3′. The composition of PCR mix was as follows: 1 U of Platinum *Taq* DNA polymerase in the PCR buffer provided by the manufacturer (Gibco BRL – Life Technologies Inc., Gaithersburg, MD), $MgCl_2$ 3 mM (for *RAG-1*) or 2 mM (for *TDT*), deoxynucleotide triphosphate 0.2 mM each, bovine serum albumin 0.25 μg/μl, primers 0.5 μM each; fluorescent signal was generated using 0.2 μl SYBR Green (2×10^{-4} of the stock concentration, diluted by dimethyl sulfoxide; for *RAG-1* and *TDT*). One microliter of cDNA was added in a final volume of 20 μl. The LIGHTCYCLER program for *RAG-1* and *TDT* consisted of the initial denaturing at 94°C for 90 s, followed by 40 PCR cycles: 95°C for 5 s, 62°C for 40 s (single fluorescence measurement), 72°C for 12 s. The melting curve analysis was performed to confirm specific amplification and to identify nonspecific templates after each run. RAG-1 and TdT mRNA expression was shown as a ratio to *b2m* expression; this value was normalized to 1 in the most immature subset investigated.

Real-time quantitative PCR analysis of immunoglobulin gene rearrangements

DNA from sorted cells was isolated by QIAamp DNA Blood Micro Kit (Qiagen GmbH). Multiplex real-time quantitative PCR (RQ-PCR) for *IGH* detecting virtually all complete *IGH* rearrangements and RQ-PCR for intron recombination signal sequence to kappa deleting element (RSS-Kde) was performed using family-specific V segment forward primers and J segment-specific reverse primers and probes as described previously (26, 27) in the iCycler IQ™ thermal cycler system (Bio-Rad, Hercules, CA). The starting concentration of template was measured against the dilution series of positive-control DNA in germ-line (unrearranged) Hela DNA. REH cell line served as a positive control for intron RSS-Kde recombination. Samples of patients with ALL containing 87% and 97% of clonal cells with monoallelic rearrangements were used for standard curve preparation in *VH1-3–JH1-6* and *VH4-7–JH1-6* assays, respectively. The cycling conditions were as follows: initial denaturing at 95°C for 10 min, 50 cycles of

denaturing at 94°C for 15 s, annealing extension at 64°C (*IGH*) or 62°C (intron RSS-Kde) for 1 min; DNA at a concentration of 0.01–0.2 μg/μl was used for each PCR reaction. All the assays reached sensitivity of at least 1% of rearranged alleles in the germ-line background. Each sample was run in duplicate, and a mean value was used for further analysis. Albumin gene was used to normalize the DNA concentration and quality (28). ICYCLER IQ Optical System software version 3.0a was used for quantification, and a final value was shown as a percentage of rearranged alleles relative to the respective clonal control DNA, which was set as 100%.

Results

CD27 and CD44 expression define phenotypic stages in B precursor development

As CD44 negativity in combination with CD27 positivity is found exclusively in one subtype of leukemia (*TEL/AML1*^{pos}) (6), we searched for such cells among B precursors in nonmalignant BM. Within CD10^{pos}CD19^{pos} cells, such 27SP cells were present in all 14 specimens. In addition, 44SP cells as well as 27/44DP and 27/44DN cells were found (Figure 2). Next, markers of B-precursor differentiation were investigated by polychromatic flow cytometry. The expression of CD44, CD27 and a B precursor defining the combination of CD19 and CD10 was studied together with the differentiation markers CD34, TdT, cytoplasmic IgM and cytoplasmic VpreB (CD179a). The percentage of CD34^{pos} cells is the highest in 27SP and decreases gradually in 27/44DP, 44SP and 27/44DN subpopulations (Figure 3A,B). A similar trend is found in the percentage of CD10^{bright} cells, which become virtually missing in CD27^{neg} B-precursor stages (Figure 3C,D). This sequence of developmental stages was further supported by

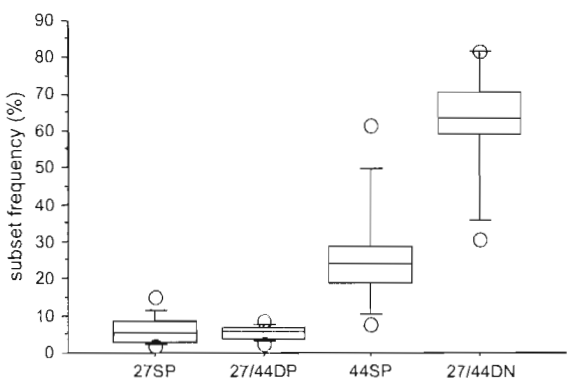

Figure 2 Frequencies of subpopulations defined by CD27 and CD44 expression within CD10^{pos}CD19^{pos} B precursors. Fourteen specimens were used for this analysis. DN, double negative; DP, double positive; SP, single positive.

a gradual loss of intracellular TdT and VpreB (Figure 4A,B) and by the increase of intracellular IgM^{pos} cells (Figure 4C). The observed sequence of developmental stages of B precursors is thus: 27SP, 27/44DP, 44SP and 27/44DN. We fit these subpopulations into two well-established models of B-cell development (Table 1) (2, 29).

27/44DP cells downregulate the key recombination enzymes

We sorted subpopulations based on the CD44 and CD27 expression to compare their recombination potential by measuring TdT and RAG-1 mRNA expression by RQ-PCR. Similar to the protein level, TdT mRNA expression decreases in concordance with the suggested developmental stages (Figure 5A). Interestingly, the 27/44DP cells express the lowest amount of RAG-1 transcripts (Figure 5B), suggesting that these cells are in the stage of suppressed RAG-1 expression after completed *IGH* rearrangement. RAG-1 is reexpressed during *IGL* rearrangement, as proven by the reappearance in the 44SP cells. Because the cells with a downregulated *RAG-1* are known to be frequent among the large proliferating cells, we analyzed the percentage of large cells (estimated by cytometry as the proportion of cells with a higher forward scatter). The 27/44DP subpopulation appears to be composed mostly by the large cells, based on the highest percentage of cells with high forward scatter (Figure 6A,B).

Immunoglobulin gene rearrangement

After a successful rearrangement of *IGH* genes in early B precursors, cells proliferate and *IGL* genes start to rearrange. In all four subpopulations, heavy-chain genes (both segments *VH1-3–JH* and *VH4-7–JH*) were rearranged (data not shown), proving that heavy chains start to rearrange at or before the earliest stage of differentiation among the analyzed subpopulations. The low quantitative range of the system for *VH1-3–JH* and *VH4-7–JH* detection did not enable the exact quantification, mainly because of a limited DNA concentration obtained from sorted cells. Next, we investigated the *IGL* rearrangement. The system detected the intron RSS-Kde rearrangements, which appear in the late phase of *IGL* rearrangement. As shown in Figure 7, *IGL* genes begin to be rearranged at the 44SP stage, whereas intron RSS-Kde rearrangements are virtually missing at earlier stages.

Both CD27 and CD44 reappear at CD20^{pos}CD10^{neg} stages

As reported earlier, mature PB B lymphocytes are CD44^{pos} (15). Our data show that majority of CD20^{pos}CD10^{neg} cells express CD44 already in BM, while PB B lymphocytes are almost exclusively CD44^{pos} (Figure 1B), contrasting with BM CD10^{pos} cells, which contain a greater fraction of

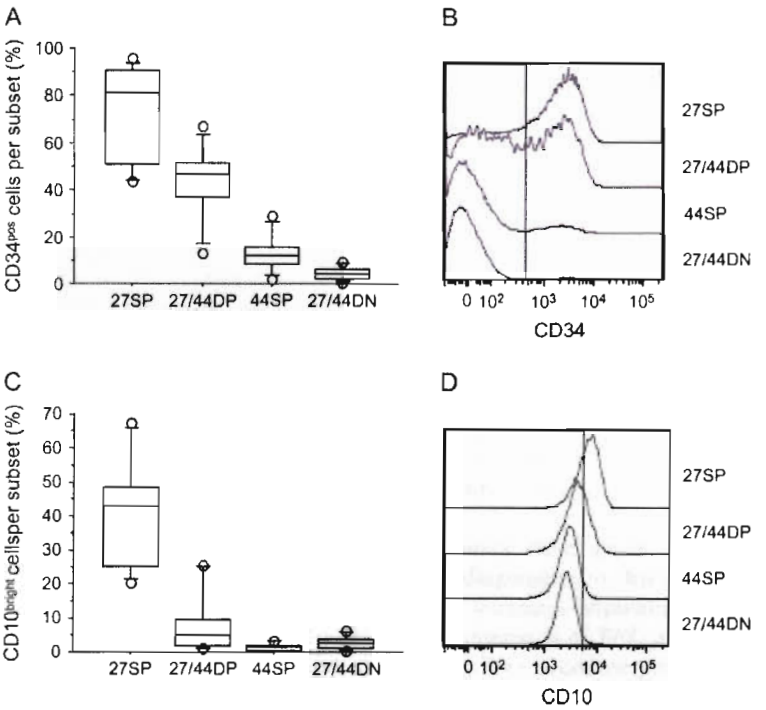

Figure 3 Expression of CD34 and CD10 within CD27- and CD44-defined subsets. The percentage of $CD34^{pos}$ cells (A) and CD34 fluorescence intensity (B). The percentage of $CD10^{bright}$ cells (C) and CD10 fluorescence intensity (D). Fourteen specimens were used for this analysis. The vertical lines distinguish $CD34^{pos}$ and $CD34^{neg}$ cells or $CD10^{bright}$ and $CD10^{dim}$ cells. DN, double negative; DP, double positive; SP, single positive; TdT, terminal deoxyribonucleotidyl transferase.

$CD44^{neg}$ cells. Thus, immature, naïve B cells reexpress CD44 in BM, before they exit to periphery.

Lack of CD27 expression in stem cells

Within $CD34^{pos}$ cells, Nilsson et al. reported higher proportion of $CD27^{pos}$ cells in $CD19^{neg}$ subpopulation compared with $CD19^{pos}$ subpopulation, which was interpreted that B-lineage-committed $CD34^{pos}$ cells express less CD27 (8). Although we used fluorochrome (PE) with higher fluorescence intensity and a higher resolution than FITC used in Nilsson's study, we found only $1.9 \pm 1.7\%$ of $CD27^{pos}$ cells ($n = 14$) among $CD19^{neg}CD34^{pos}$ cells.

As we used a reliable cytometric system acquiring millions of events and selecting exclusively viable cells for the analysis, we could further divide these $CD19^{neg}CD34^{pos}$ cells. We used CD10 for finer division of the $CD19^{neg}CD34^{pos}$ cells because it is known that the $CD19^{neg}CD10^{pos}CD34^{pos}$ cells are biased towards B lineage, although they can develop also into other lineages (30, 31). The $CD10^{neg}$ and $CD10^{pos}$ fractions of $CD19^{neg}CD34^{pos}$ cells contained $0.23 \pm 0.21\%$ and $38.8 \pm 26.3\%$ $CD27^{pos}$ cells ($n = 14$), respectively. In comparison, $CD19^{pos}CD34^{pos}$ cells contain $67 \pm 14.1\%$ $CD27^{pos}$ cells ($n = 14$). In $CD34^{pos}CD19^{neg}$ subpopulation, the acquisition of CD10 is accompanied by CD44

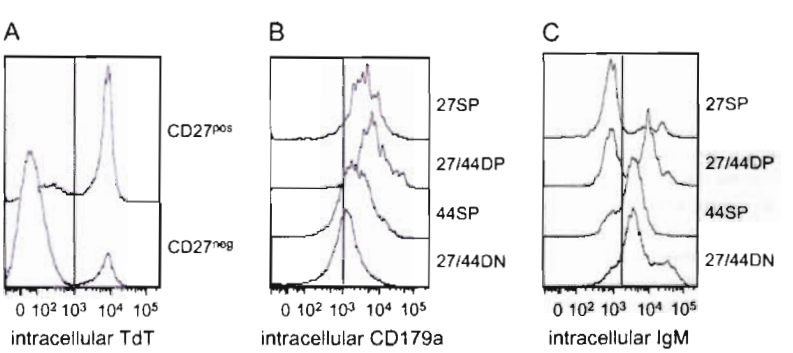

Figure 4 The intensities of intracellular TdT (A), intracellular CD179a (B) and intracellular IgM (C) within CD27- and CD44-defined subsets. Representative samples of five measurements are shown. The vertical lines distinguish positive and negative cells. DN, double negative; DP, double positive; SP, single positive; TdT, terminal deoxyribonucleotidyl transferase.

Table 1 The models of B-cell development[a]

	LeBien (2)	Loken et al. (29)	CD27 and CD44
Pro-B (I)	CD19, CD10, CD34, CD24, IL-7R, RAG, VpreB, Ig-α, VDJ_H	$CD10^{high}$, CD34, TdT	27SP
Pre-BI (II)	CD19, CD10, CD24, pre-B receptor, low RAG, IgM HC	CD10	27/44DP
Pre-BII (III)	CD19, CD10, CD24, pre-B receptor, RAG, IgM HC, VJ_K		44SP, 27/44DN
Immature B (III) (IV)	CD19, CD10, CD20, CD21, CD22, CD24, CD40, IgM	CD10+, IgM IgM	27/44DN

DN, double negative; DP, double positive; HC, heavy chain; SP, single positive; TdT, terminal deoxyribonucleotidyl transferase

[a] We show CD27- and CD44-defined differentiation stages in the context of two published models. The stages in the model by Loken et al. (29) and LeBien (2) are assigned by numerals and their names, respectively, in the first column.

downregulation (Figure 8), which is in line with our results that CD44 negativity together with CD27 positivity is found in the earliest $CD19^{pos}$ cells.

Discussion

We previously reported that CD27 and CD44 define ALL subtypes (6). The data presented here show that the expression of these molecules correlates nonrandomly with several independent markers of B-precursor differentiation. Among $CD10^{pos}CD19^{pos}$ cells, both maturity of *IG* rearrangements and the expression of differentiation markers prove the following developmental sequence: 27SP, 27/44DP, 44SP and 27/44DN. The data showing heavy chain gene rearrangements in 27SP, 27/44DP, 44SP as well as 27/44DN cells indicate that *IGH* rearrange at stages up to 27SP. Upon transition to the 27/44DP stage, in which the large cells dominate, *RAG-1* expression is suppressed. Afterwards, CD27 disappears and cells enter the next stage, 44SP. This stage is characterized by restarting the recombination machinery (shown by *RAG-1* reexpression). Only at this stage, cells start to contain completed *IGL* rearrangements (shown by *KDE* detection). Cells apparently attain smaller size at 44SP stage. This process is finished by loss of CD44. The resulting 27/44DN phenotype dominates among the $CD10^{pos}$ B precursors in human BM. The 27/44DN cells complete the differentiation process before the cells lose CD10, regain CD44 and are allowed to exit to periphery.

Interestingly, whereas *RAG-1* is profoundly suppressed at 27/44DP stage, TdT expression decreases gradually throughout the differentiation. The suppression of *RAG-1* may be important during the cell proliferation as RAG-1 (unlike TdT) may cause unwanted DNA recombination during replication. This may explain the differences between TdT and RAG-1 expression at the 27/44DP stage, containing large proliferating cells.

The knowledge on B-precursor development can be combined with the leukemia diagnostics to discover the counterparts of the dominant leukemia population. The 27SP cells are physiological counterparts of $TEL/AML1^{pos}$ ALL, and the 44SP cells are the principal counterparts of all other B-precursor ALL subtypes. The 44/27DP and 44/27DN immunophenotype is rarely seen among B-precursor leukemias. This contradicts a logical expectation that the proliferating compartment (large pre-B, mostly found in 44/27DP stage) might be more likely to transform into malignancy.

Because the nonmalignant counterparts of $TEL/AML1^{pos}$ ALL are less mature than the counterparts of $TEL/AML1^{neg}$ ALL, it is possible to speculate that the differentiation of $TEL/AML1^{pos}$ leukemic cells is blocked in earlier stage than in other leukemias.

After exit to periphery, a subset of B cells reexpresses CD27 during the process of somatic hypermutation and it is then constitutively present as a marker of memory cells (32). In line with this, we observed a $CD27^{pos}$ subpopulation among $CD20^{pos}CD10^{neg}$ cells not only in PB but also in BM. The BM $CD27^{pos}CD20^{pos}CD10^{neg}$ B cells are probably recirculating memory cells. Migration of memory cells to BM was proven by the analysis of BM cells with preferential

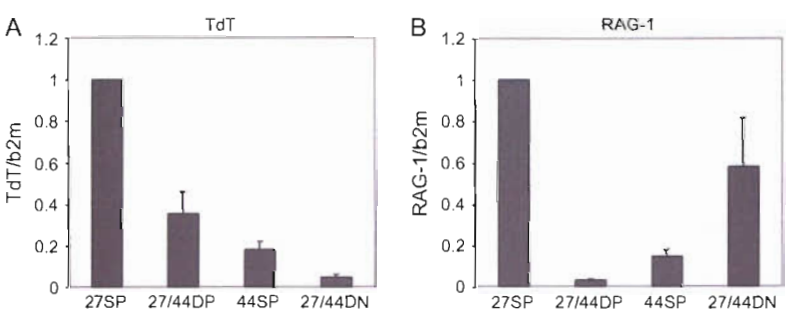

Figure 5 Recombination enzyme terminal deoxyribonucleotidyl transferase (A) and RAG-1 (B) mRNA expression. Expression levels are shown as ratio to control gene expression; this value was normalized to 1 in the most immature subset investigated. Experiments were performed in duplicates. DN, double negative; DP, double positive; SP, single positive; TdT, terminal deoxyribonucleotidyl transferase.

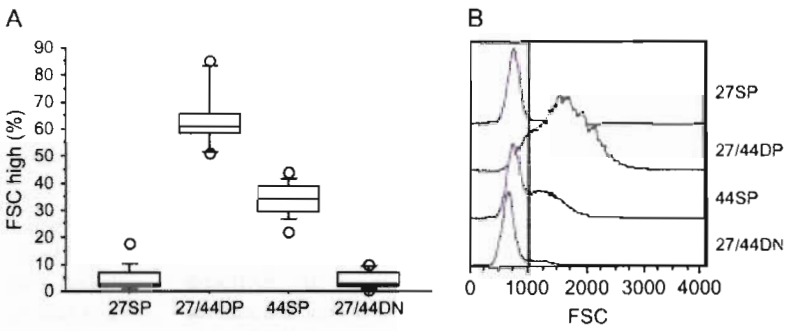

Figure 6 Frequencies of large cells defined as the subpopulation with the high FSC (A) and representative sample of FSC intensity. Fourteen specimens were used for this analysis. DN, double negative; DP, double positive; SP, single positive. DN, double negative; DP, double positive; FSC, forward scatter; SP, single positive.

mutations in the complementarity determining regions (CDR) (33) of the V(D)J regions, which is the pattern that evolves during the antigen selection in germinal centers. Interaction of CD27 with its ligand CD70 expressed on activated T and B cells regulates B-cell growth and differentiation to IgG- and IgM-producing cells (34, 35).

At the early developmental stages, CD27 expression was reported on murine hematopoietic stem cells (36), and Nilsson et al. reported a high CD27 expression in human $CD34^{pos}$ cells even before CD19, similar to mice (8). Although we did observe such cells, they were almost always $CD10^{pos}$ and their percentage increases only after B-lineage commitment. Because the CD27 and CD44 are known to play a role in apoptotic or adhesion processes, it can be speculated that their expression merely reflects the functional status of the precursor B cell, regardless of its developmental stage. However, our data clearly show that CD27 and CD44 are expressed in an organized fashion during the precursor development. Consequently, this forms a basis for a further research into functional similarities between normal precursor and the leukemia cell.

Although the straightforward explanation of the presented data is that the observed subpopulations reflect consequent developmental stages, alternative scenarios may be provided. We may hypothesize that the following phenomena may not represent regular features in B-precursor differentiation at $CD10^{pos}CD19^{pos}$ stage: (i) CD27 expression, (ii) the decrease in CD44 expression or (iii) the reappearance of CD44 seen in 27/44DP and 44SP cells. The scenario (i) is definitely possible in transgenic animals because B cells do develop in $CD27^{-/-}$ mice (37). However, our data do not support this scenario as a major pathway in healthy humans. The $CD10^{pos}CD19^{pos}$ B precursors would start from the 27/44DN phenotype, which contains only few $CD34^{pos}$ and TdT^{pos} cells – to the contrary, most cells have a more mature phenotype with rearranged *IGL*. Such immature cells would differentiate into the 44SP phenotype, which contains at least some large pre-B cells. The explanation of the fact that we do see majority of immature ($CD34^{pos}$, TdT^{pos}, *IGL*-unrearranged, i-IgM^{neg}, i-$VpreB^{pos}$ and $CD10^{bright}$) cells among $CD27^{pos}$ cells would require a complicated assumption that some immature cells may express CD27 for a prolonged time, thus increasing the apparent frequency. According to the

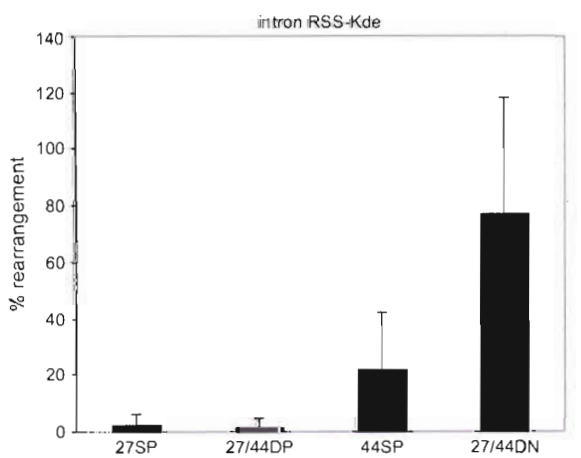

Figure 7 Immunoglobulin light-chain gene rearrangement. The percentage of intron RSS-Kde rearrangements is shown. The experiment was performed in triplicate. DN, double negative; DP, double positive; SP, single positive.

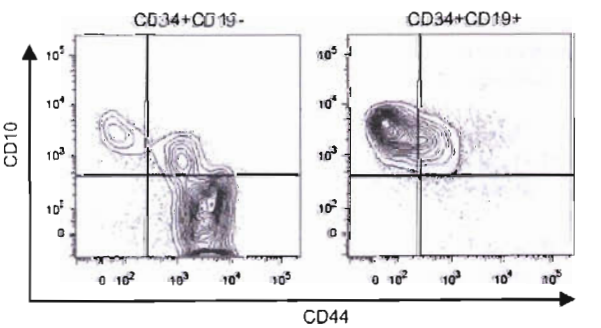

Figure 8 CD44 expression in $CD10^{pos}CD34^{pos}CD19^{neg}$ and in $CD19^{pos}CD34^{pos}$cells.

scenario (ii), the most immature cells would enter the 27/44DP stage. Therefore, the cells would need to have completed *IGH* prior to the $CD10^{pos}CD19^{pos}$ stage. Analogically to the scenario (i), we would need to accept that some cells might still lose CD44 and acquire the 27SP phenotype. Our observation of high RAG-1 expression at the 27SP cells makes this scenario very unlikely as we would assume that the cells with completely rearranged *IGH* reexpress RAG-1 upon losing CD44 before returning back to the $RAG\text{-}1^{neg}$ 27/44DP phenotype. The scenario (iii) also appears unlikely as differentiating from 27SP directly to 27/44DN phenotype would circumvent the large pre-B stage with suppressed RAG-1. If this scenario occurred in a significant fraction of cells, it would contradict the current understanding of B-precursor differentiation. A possible role of CD27 is the regulation of apoptosis because association with apoptotic cascades was reported. Data from different models illustrate that the net effect of CD27 may be either negative or positive. Nolte et al. showed that murine $CD27^{-/-}$ progenitors proliferated more rapidly, suggesting a regulatory role of CD27 in the growth of these cells. They propose that such a regulatory role may be triggered by the interaction of CD27 on B precursors with CD70 on activated T cells. This could be beneficial during infection as the presence of foreign antigen in the BM could induce an unwanted selective tolerance (37). CD27 triggering on primary plasma cell leukemia has antiapoptotic effect (38). Overexpression of CD27-binding protein Siva induces apoptosis in cell lines (39). It was also shown that CD27 associates with tumor necrosis factor-receptor-associated factor (TRAF)2 and TRAF5 signal transducers responsible for NF-κB activation (40). Apoptosis plays an important role during B-cell development when cells unsuccessfully rearranging Ig genes die by apoptosis. The question is whether CD27 on B precursors could mediate proapoptotic or antiapoptotic signal because the published data concerning CD27 role in apoptosis are diverse in different cell types. Although our data do not answer this question, it is obvious that among B precursors, CD27 identifies mainly the earliest stages at or before the start of *IG* rearrangement. The specificity of the early-stage detection is strengthened when CD27 positivity is combined with CD44 negativity.

Our data show that during B-cell development, CD44 undergoes two waves of downregulation. If the uncommitted $CD34^{pos}$ cells are mostly $CD44^{pos}$, then CD44 expression decreases together with the two-step acquisition of CD10 (Figure 7). The $CD44^{neg}$ cells have been described previously among $CD34^{pos}$ BM cells and have been, in line with our results, shown as $CD10^{pos}CD19^{pos}$ B precursors (41). These $CD44^{neg}$ cells are 27SP. The following reemergence of CD44, resulting in 27/44DP stage corresponds with one of its supposed functions, which is regulation of cell proliferation. While suppressing *RAG-1* expression, cells proliferate at the 27/44DP. The expression of CD44 again ceases only two stages down, at the 27/44DN stage, in which *IGL* are fully rearranged and the cells do not proliferate and are not dependent on CD44-mediated contact with stromal cells. The role of CD44 during hematopoiesis was experimentally shown by the addition of anti-CD44 mAbs that inhibit or enhance stromal cell-dependent hematopoiesis (42).

There are more models of B-lymphocyte development, and the nomenclature of B-cell developmental stages is still unsettled. Any nomenclature should be based on fitting surface markers with *IG* gene rearrangement status. CD27 and CD44 extend the B-cell development model because their expression also reflects not only the *IG* rearrangements status but also a different likelihood to transform into leukemia and/or to block the differentiation in different genetic subsets of ALL.

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Příloha 9

Childhood secondary ALL after ALL treatment

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ORIGINAL ARTICLE

Childhood secondary ALL after ALL treatment

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Data on secondary acute lymphoblastic leukaemia (sALL) following ALL treatment are very rare. However, the incidence might be underestimated as sALLs without a significant lineage shift might automatically be diagnosed as relapses. Examination of immunoglobulin and T-cell receptor gene rearrangements brought a new tool that can help in discrimination between relapse and sALL. We focused on the recurrences of childhood ALL to discover the real frequency of the sALL after ALL treatment. We compared clonal markers in matched presentation and recurrence samples of 366 patients treated according to the Berlin–Frankfurt–Munster (BFM)-based protocols. We found two cases of sALL and another three, where the recurrence is suspicious of being sALL rather than relapse. Our proposal for the 'secondary ALL after ALL' diagnostic criteria is as follows: (A) No clonal relationship between diagnosis and recurrence; (B) significant immunophenotypic shift – significant cytogenetic shift – gain/loss of a fusion gene. For the sALL (A) plus at least one (B) criterion should be fulfilled. With these criteria, the estimated frequency of the sALL after ALL is according to our data 0.5–1.5% of ALL recurrences on BFM-based protocols. Finally, we propose a treatment strategy for the patients with secondary disease.

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Introduction

Secondary or treatment-related acute leukaemia is a well-known complication of previous cancer therapy. The vast majority of cases in paediatric patients comprise secondary acute myeloid leukaemia (AML), whereas secondary acute lymphoblastic leukaemia (ALL) is considered to be a rare disease. Case reports of secondary ALL (sALL) in children have been described following treatment of various malignant diseases (Wilm's tumour, Hodgkin's disease, neuroblastoma, Ewing's sarcoma, osteosarcoma, medulloblastoma, retinoblastoma, ependymoma and so on)[1,2] However, although ALL is the most common malignancy in childhood, the data on childhood sALL following ALL treatment are scarce.

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In four large studies describing the incidence of secondary neoplasms in children treated for ALL, no secondary ALL was diagnosed among the total of more than 25 000 children developing altogether 171 secondary malignancies.[3–6] The data demonstrate two facts (1) ALL treatment bears a relatively low risk in terms of secondary tumours compared with treatment of other frequent childhood malignancies.[7] (2) Diagnosis of secondary ALL after ALL treatment is extremely rare. This rareness might be caused by the fact that ALL recurrence after ALL treatment is usually automatically diagnosed as a relapse of the original leukaemia. Comparative analysis of immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements brought a new tool that can help in discrimination between real relapse and sALL clonally unrelated to the original disease.[8]

Only very few case reports have been published admitting that a supposed ALL relapse might represent a secondary malignancy but no comprehensive study aimed at determining the frequency of secondary ALL after ALL treatment has been presented so far.

In our study, we aimed to answer the question what is the actual frequency of this phenomenon and to show that it might be underestimated. By comparison of Ig/TCR rearrangements and other markers of the malignant clone in matched presentation and recurrence samples, we screened a series of 366 patients consecutively diagnosed in four centres and treated according to the Berlin–Frankfurt–Munster (BFM)-based protocols. We found two cases with secondary ALL and another three in which the second malignancy is suspicious of being sALL rather than relapse. On the basis of our results, we propose guidelines for defining the secondary ALL after ALL treatment.

Materials and methods

Patients

A total of 366 childhood patients with relapsed ALL were analysed both at diagnosis and recurrence of the disease in four centres (Prague, Paris, Berlin, Rotterdam). All children were treated according to the BFM or BFM-related protocols (BFM ($n=81$), (European Organisation of Research and Treatment of Cancer) EORTC ($n=199$), (Dutch Childhood Leukemia Study Group) DCLSG ($n=86$)). The patients are consecutive unselected cases from given protocols in whom marker stability for all recurrences of childhood ALL was assessed at respective centres. Informed consent for the therapy and joint research examination was obtained from patients or their guardians, and

protocols were reviewed by Institutional Review Boards (IRB) or Research Ethics Committees (REC) of respective centres. Patient UPN5 was reported previously in the study concerning Ig/TCR rearrangements.[9]

Immunoreceptor gene rearrangements

We examined the Ig/TCR gene rearrangement patterns in bone marrow samples of ALL patients using PCR primer sets covering the vast majority (>90%) of Ig-heavy chain (*IGH*), Ig-κ (*IGK*), TCR-δ (*TCRD*) and TCR-γ (*TCRG*) rearrangements in B-cell precursor (BCP) ALL and the set of reactions covering the T-cell acute lymphoblastic leukaemia (T-ALL) rearrangements (*TCRD*, *TCRG* and *SIL-TAL1* rearrangements). In cases with no clonal relationship between the samples from diagnosis and recurrence in these loci, we added examination of incomplete *IGH* rearrangements, and (except for the patient UPN4, where the low amount of available DNA precluded the analysis) both complete and incomplete TCR-β (*TCRB*) rearrangements. All detected rearrangements were sequenced and compared between the primary and secondary leukaemia to distinguish completely new rearrangements from related rearrangements with secondary changes. In the discordant patients, the diagnostic samples were analysed for the presence of all new 'recurrence rearrangements' to exclude their presence even at lower level in the original leukaemic population. The sensitivity of this analysis was 10^{-4}–10^{-5} in the patients UPN1–4 and 10^{-2} in patient UPN5.

Sequences of primers and PCR conditions were specified elsewhere.[10–13] To reliably distinguish clonal PCR products from polyclonal, we performed heteroduplex analysis of fragments using polyacrylamide gel.[14]

Fusion gene determination and cytogenetics

Presence of *TEL-AML1*, *BCR-ABL* and *MLL-AF4* fusion genes was examined as a part of routine ALL diagnostics at original diagnosis and recurrence. *MLL* fusion sequence in patient UPN1 was established at the Diagnostic Centre for Acute Leukaemia (DCAL) in Frankfurt. A long-distance inverse PCR method was used to determine the genomic fusion break point.[15] Routine karyotyping was performed at diagnosis and recurrence of the disease. In some cases, fluorescence *in situ* hybridization analysis targeted to *MLL* gene rearrangements was added at the time of recurrence.

Flow cytometry

Flow cytometry immunophenotyping of bone marrow aspirates was routinely performed at diagnosis and at relapse using panel of mAbs recommended by the European Group for the Immunological Characterization of Leukemias.[16]

Genetic identity confirmation

In three patients (UPN2, UPN3 and UPN4), suspect from the secondary ALL, a patient identity confirmation of diagnostic and recurrence samples was performed to rule out the possibility of sample confusion. Microsatellite testing using AmpFlSTR Profilerplus Kit (PE Applied Biosystems, Darmstadt, Germany)[17] or by PromegaPowerplex 16 kit (Promega, Madison, WI, USA) was used for the affirmation.

Results

Among 366 relapses analysed in our study, we found five cases without any clonal relationship between diagnosis and recurrence of the disease. We aimed to verify the discordance of clonal markers in these patients at different levels – by the analysis of immunophenotype, cytogenetics, fusion genes and Ig/TCR rearrangements. The Ig/TCR rearrangements were present as specific clonal markers in virtually all ALL patients. Thus, we focused on detailed analysis of the rearrangements in the five patients and besides routine Ig/TCR screening, we compared sequences of the rearrangements at diagnosis and recurrence; and we also attempted to backtrack all new rearrangements from the recurrence of the disease back to the original diagnosis. Neither the comparison of sequences nor the backtracking showed any clonal relationship between the primary and secondary leukaemia in these five cases. The patient's characteristics are summarized in Table 1. In addition to the completely new pattern of Ig/TCR rearrangements at the time of ALL recurrence, 2/5 patients (UPN1 and UPN2) showed additional immunophenotypic (lineage switch from BCP to T-cell leukaemia) and genetic (occurrence of a new *MLL* fusion gene, loss of *TEL-AML1* fusion gene) features supporting designation as the secondary leukaemia. In the patient UPN1, the recurrence referred here is the second recurrence; the first recurrence of this child bore all clonal signs of a genuine relapse with immunophenotype, cytogenetics and Ig/TCR rearrangements corresponding to the original diagnosis. At the second recurrence this patient showed complete immunophenotypic switch from BCP to T-ALL, the blast cells lost their hyperdiploid character (DNA index was 1.17–1.21 and 1.00 at diagnosis, the first recurrence and the second recurrence, respectively) and a novel type of *MLL* gene rearrangement appeared with a fusion to the *MAML2* gene at 11q21.

Discussion

To our knowledge, only six cases of ALL recurrence that might be considered as being secondary rather than relapsing leukaemia have been described so far. The review of the literature is summarized in Table 2. The list of cases includes three children with 'late developing' t(4;11),[18,19] and two cases where other translocations involving *MLL* gene arose (t(11;14)[20] and t(11;16)[21]). In the remaining case (as well as in the t(11;14) case), the assumption that the recurrence is rather a secondary leukaemia came from the fact that no common marker was found after examination of Ig/TCR rearrangements at diagnosis and recurrence of ALL[9,20]

We are aware that definite diagnosis of an indisputable sALL is intricate. In most cases, there is a hypothetical possibility that the disease has originated in a very early progenitor with ability to differentiate into very dissimilar cell populations with seemingly no clonal relationship to each other. Unless the leukaemic stem cell is defined, it is virtually unfeasible to rule out the possibility of a biphenotypic/biclonal disease at diagnosis with a small, undetected subclone outwardly unrelated to the predominant diagnostic clone. Such cells could emerge after an effective treatment of the major clone and give rise to a dominant relapse population. Thus, the only virtually indisputable sALLs are cases where a fusion gene, which is thought to be the first hit in leukaemogenesis (for example, TEL-AML1 or MLL-AF4), is lost at the recurrence. To preclude or at least to minimize the risk of a hidden biphenotypic/biclonal disease at diagnosis, absence of all the recurrence-specific rearrangements should be verified in the diagnostic sample.

However, the probability of a recurrence constituted by cells with completely unrelated clonal characteristics but still originated in the same leukaemic stem cell as the original

Table 1 Characteristics of the 5 patients with possible secondary ALL after ALL treatment

Patient	*UPN1*	*UPN2*	*UPN3*	*UPN4*	*UPN5*
Age at diagnosis (years)	5.1	5.8	3.0	7.8	8.2
Gender	F	M	M	F	F
Immunophenotype (P/R)	BCP/BCP/T	BCP/T	T/T	BCP/BCP	BCP/BCP
Fusion genes (P/R)	neg/neg/MLL-MAML2	TEL AML1/neg	Neg/neg	Neg/neg	NA/NA
Ig/TCR (P/R)	ABC/ABC/DEF	ABCD/EFGH	AB/C	AB/CDE	ABCDE/FGH
Treatment preceding the sALL	ALL-BFM95-SR/ALL-Rez BFM96-S2	EORTC-58951-VHR	ALL-BFM 2000-MR	EORTC-58881-S	DCLSG-ALL-8-HR
CR preceding the sALL (years)	2.9/2.7	2.2	1.7	6.5	3.5
CR after sALL	No	Yes	Yes	Yes	No
Outcome (months in CR)	Toxic death in the recurrence	Death due to extramedullary (thymic) progression	Second CR (32) after alloBMT	Second CR (72)	Death in the recurrence (progressive disease)
Radiation (Gy)[a]	18	No	12	No	No
Etoposide (mg/m^2)[a]	2000	1350	No	No	1350
Daunorubicin (mg/m^2)[a]	225	220	120	240	270
Cyclophosphamide/ Ifosfamide (g/m^2)[a]	10.2	4	3	3	7

Abbreviations: alloBMT, allogeneic bone marrow transplantation; BCP, B-cell precursor; CR, complete remission; Ig/TCR, immunoglobulin and T-cell receptor; NA, not available; P, presentation; R, recurrence; sALL, secondary acute lymphoblastic leukaemia.
Ig/TCR: in each patient, different letters stand for different and unrelated rearrangements.
In patient UPN1, we refer to presentation and both first (genuine relapse) and second (sALL) recurrences.
[a]Cumulative doses of selected drugs preceding the sALL.

Table 2 Review of the literature – cases of possible sALL after ALL described so far

Reference	*Original diagnosis*	*Secondary ALL*	*Ig/TCR rearrangements*	*Outcome*
Szczepanski *et al.*[20]	T-ALL	T-ALL	Unrelated	Not reported
	T-ALL	T-ALL with t(11;14)	Unrelated	Not reported
Millot *et al.*[19]	Mature B-ALL with t(8;14)	BCP-ALL with t(4;11)	Unrelated	CR achieved
Hunger *et al.*[21]	T-ALL	BCP-ALL with t(11;16)	Unrelated (only TCR-β tested)	Not reported
Brizard *et al.*[18]	ALL	BCP-ALL with t(4;11)	Not done	CR achieved, alloBMT performed
	ALL	BCP-ALL with t(4;11)	Not done	CR achieved, alloBMT performed

Abbreviations: alloBMT, allogeneic bone marrow transplantation; ALL, acute lymphoblastic leukaemia; B-ALL, B-cell acute lymphoblastic leukaemia; BCP, B-cell precursor; CR, complete remission; Ig/TCR, immunoglobulin and T-cell receptor; sALL, secondary acute lymphoblastic leukaemia; T-ALL, T-cell acute lymphoblastic leukaemia; TCR-β, T-cell receptor-β.

diagnosis is low. Moreover, this dilemma is rather academic as from the practical point of view (for example, treatment purposes) such case should not be considered as a standard relapse anyway.

Our multicentre study is the first to investigate systematically the clonal relationship between presentation and recurrence in childhood ALL. We present a large cohort of patients examined on running trials and analysed subsequently with regard to reveal incidence of possible sALL after ALL treatment. As reports in the literature are extremely rare, no standards defining this issue have been postulated so far. Our proposal for the diagnostic criteria of 'secondary ALL after ALL treatment' is as follows:

(A) No clonal relationship between diagnosis and recurrence (Ig/TCR, fusion genes at DNA level, cytogenetic marker).
(B)
- significant immunophenotypic shift (typically lineage switch)
- significant cytogenetic shift
- gain or loss of a fusion gene

For the diagnosis of secondary leukaemia, (A) plus at least one (B) criterion should be fulfilled.

In our study, two patients (UPN1 and UPN2) meet our criteria for sALL. The patients fulfilling only the (A) criterion (patients UPN3, UPN4 and UPN5 in our study) are 'possible' secondary ALLs, but without additional evidence the diagnosis of secondary leukaemia could be challenged.

The number of Ig/TCR markers changed between diagnosis and recurrence (the (A) criterion) might also have its significance – providing no identical or related marker is maintained between the two time points, the more changes detected the higher is the probability of secondary ALL. Thus, in our case UPN5 with eight such changes, the sALL is highly possible even without any (B) criterion fulfilled. On the other hand, in case UPN3 (only three changes and no (B) criterion), the diagnosis of sALL could be questioned more easily.

Although the longer remission duration could be considered as a supporting evidence for secondary rather than relapsed ALL, we did not include the criterion of the time to recurrence into our proposal. Studies on very late relapses show that even recurrence more than 20 years from diagnosis are clonally related to the original leukaemic cells (13/13 very late relapses 5–24 years from diagnosis[22,23]). On the contrary, very early recurrences (less than 1 year from the original diagnosis) are certainly more likely to be genuine relapses.

Occurrence of a new fusion gene is not a guarantee *per se* that the recurrence is a secondary leukaemia. For example, the t(9;22), associated with the BCR-ABL rearrangement, could be a late appearing, therapy-related secondary event in the evolution of the primary clone.[24–28]

ALL is the most common childhood malignancy, and thus it can be supposed that sALL after ALL treatment is more common than reported – sALLs without a significant lineage shift have probably been automatically diagnosed as relapses; in cases where an immunophenotypic shift is considerable (such as in our patients UPN1 and UPN2), the flow cytometry is the first method that draws our attention to a possible secondary disease.

The main oncogenic factors increasing the risk of subsequent neoplasms are a genetic susceptibility and a previous therapy, particularly radiotherapy, topoisomerase inhibitors (etoposide, doxorubicin), alkylating agents (cyclophosphamide) and some antimetabolites (6-thioguanine). More intensive use of some of these treatment options in certain older ALL protocols led to increased frequency of secondary malignancies to almost 5%.[29] Although these components are also used in current BFM-based ALL protocols, the overall extent of the use is relatively low compared to the treatment of other paediatric malignancies and some are applied only in subgroups of patients (T-cell ALL and high-risk ALL). Also the dosing schedule is adapted to cause as little late effects as possible. Only five patients in our study are suspect of suffering treatment-related secondary leukaemia, and all of them were treated according to the standard protocols as well as the rest of the children in this report. Thus, we cannot draw any reasonable conclusion regarding the relationship of primary treatment and the risk of sALL. Nevertheless, it is of note that 3/5 cases in our report (patients UPN1, UPN2 and UPN5) underwent a very intensive therapy before sALL. Patient UPN1 was stratified to standard risk treatment but suffered relapse and received another protocol of intensive chemotherapy before her second recurrence identified as a secondary ALL. Patients UPN2 and UPN5 responded poorly to initial treatment and were re-stratified to the very high-risk arm of the EORTC protocol and high-risk arm of the DCLSG protocol, respectively.

Previous treatment can play a role in the risk of sALL. However, while the link between specific drugs and the risk of secondary AML is very strong, in secondary ALL the effect of previous treatment is less pronounced. This fact suggests that other mechanisms – particularly genetic susceptibility – might be also involved. Polymorphisms of several detoxification genes (*NQO1*, *CYP3A* and *GST*) have been shown to be related to the increased risk of secondary leukaemia, mutations of ATM gene have been linked specifically to T-ALL.[7,30] Notably, 3/5 patients described here suffered ALL recurrence from T-lineage when 2 of these 3 T-ALLs represented a lineage shift from BCP-ALL. Detailed polymorphism and mutational analysis of these genes might be helpful in unmasking the sALL pathogenesis.

On the basis of current knowledge on childhood ALL, there are probably three types of disease recurrence:

1. genuine relapse from a resistant diagnostic (sub)clone,
2. secondary leukaemia arising from the original pre-leukaemic clone (with some clonal markers maintained and some changed) as demonstrated in late relapses of *TEL-AML1* positive cases[31] and
3. pure sALL, clonally unrelated to the original leukaemia.

It is very difficult to distinguish between the first and second type of recurrence using current standard techniques as in both of these events some clonal markers are maintained between the original diagnosis and recurrence but other can be altered. The only method described so far to distinguish at least some 'secondary leukaemias from pre-leukaemic clone' from 'genuine relapses' is the analysis of the non-translocated *TEL* gene deletions in the subgroup of patients with the *TEL-AML1* fusion gene. Despite the number of published cases where the recurrence is believed to be a 'secondary leukaemia from the original pre-leukaemic clone' is less than 10,[31,32] the published data suggest that the frequency of this type of recurrence can be as high as 20%, particularly in late relapses.[31]

The estimated frequency of the pure sALL after ALL treatment is low but not null – according to our data 0.5–1.5% of ALL recurrences on BFM-based protocols. In our study at least two patients belong to the category of pure sALL.

In the 'pure sALL' and the 'sALL from the same pre-leukaemic clone' cases both the previous and the subsequent treatment strategies should be considered. The frequency of these types of recurrence (together possibly even more than 20% of late events) should be considered in discussions regarding an intensity of preceding frontline treatment strategies – these failures might, in fact, occur not because of low intensity of therapy but can be triggered due to overtreatment. As for the adequate subsequent treatment, it should be stressed that we deal in fact with new diseases and not with resistant clones selected by a previous therapy.

On the one hand, the 'sALL from the same pre-leukaemic clone' cases might be candidates for standard frontline treatment rather than intensified relapse protocol; however, as mentioned above, disclosure of these cases and their distinction from genuine relapses in current routine practice is intricate. On the other hand, the 'pure sALLs' represent second independent malignancies of haematopoietic cells and thus some (possibly inherited) susceptibility to the disease must be taken into account. In such cases, haematopoietic stem cell transplantation (SCT) should be considered to replace the predisposed haematopoietic cells. As the 'pure sALLs' (unlike the 'sALLs from pre-leukaemic clone') can be revealed in time to adjust their treatment (immunophenotyping, fusion genes detection and Ig/TCR analysis can all be done within a few days), we suggest an approach applying a frontline therapy followed by SCT for all the indisputable cases. For the 'possible' sALL cases (fulfilling only the (A) criterion from the above sALL diagnostic proposal), we would rather recommend a standard relapse therapy including stratification according to a protocol. However, in the low-risk groups (where there is only a limited SCT indication in most of the current protocols), an SCT should also be discussed based on a deeper understanding and evidence of biological origin of sALL. Therefore, we strongly advocate that all treatment decisions should be handled with caution and should be guided via study centres to ensure a harmonized clinical approach.

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Příloha 10

Allogeneic stem cell transplantation in children with leukemia using human leukocyte antigen-mismatched unrelated donors

Sedlacek P, Formankova R, **Mejstrikova E**, Keslova P, Hubacek P, Dobrovolna M, Vrana M, Kupkova L, Pittrova H, Stary J.

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Allogeneic stem cell transplantation in children with leukemia using human leukocyte antigen-mismatched unrelated donors

Sedlacek P, Formankova R, Mejstrikova E, Keslova P, Hubacek P, Dobrovolna M, Vrana M, Kupkova L, Pittrova H, Stary J. Allogeneic stem cell transplantation in children with leukemia using human leukocyte antigen-mismatched unrelated donors
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Abstract: Allogeneic HSCT is a curative treatment, when chemotherapy fails, for certain malignant diseases. In Europe, only 15% of the indicated children have an HLA-matched sibling available; in 65–70% of others, HLA allele-matched (9–10/10) UDs can be identified. For the rest, it is necessary to identify other alternative donors (HLA-mismatched family or unrelated cord blood). We present our data of HSCT using HLA partially allele-mismatched (7–8/10) UDs in 24 children with leukemia. Uniform GvHD prophylaxis was used (rATG, CsA and MTX). Acute GvHD grade II was diagnosed in 70.8% of the patients and grade III–IV in 12.5%. Overall incidence of chronic GvHD was 38.7% (extensive in 30%). The probability of EFS was 60.3% (95% CI 35.5–78.1) and OS was 74.9 (95% CI 49.1–88.9). No difference in survival between PBSC and BM recipients was observed. TRM at day + 100 was 4%, and overall was 12.5%. We conclude that used combination of drugs for GvHD prophylaxis is efficient even for patients transplanted with grafts from a HLA-mismatched UDs. It enables stable engraftment, good control of GvHD, full reconstitution of immunity, and is not connected with unacceptable transplant-related mortality.

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Key words: allogeneic hematopoietic stem cell transplantation – human leukocyte antigen-mismatched – leukemia – unrelated donor – children

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Allogeneic HSCT is a potentially curative treatment for certain malignant diseases. It is often indicated in patients with leukemia where response to chemotherapy is inadequate. Unfortunately, in about 15–20% of the patients, we are not able to find a HLA-matched RD or UD. Infusion of a graft from a HLA partially mismatched donor, unrelated cord blood, or

Abbreviations: *, with 12 men and 12 women; $\frac{1}{24}$ pair, one pair out of 24 pairs (donor-recipient); ABiL, acute biphenotypic leukemia; ABLC, amphotericin B lipid complex injections; AdV, adenovirus; AML, acute myelogenous leukemia; ANC, absolute neutrophil count; ATG, antithymocyte globulin; BKV, BK virus; BM, bone marrow; cGvHD, chronic graft vs. host disease; CML, chronic myelogenous leukemia; CMV, cytomegalovirus; CsA, cyclosporine A; DLI, donor lymphocyte infusion; EBV, Epstein–Barr virus; EFS, event-free survival; ESG-MRD-ALL, European study group on detection of MRD in acute lymphoblastic leukemia; FPIA, fluorescence polarization immunoassay; GvHD, graft vs. host disease; HHV6, human herpesvirus 6; HHV7, human herpesvirus 7; HLA, human leukocyte antigen; HLA-A,B,DR, human leukocyte antigens-A,B,DR; HSCT, hematopoietic stem cell transplantation; HSV, herpes simplex virus; IFI, invasive fungal infection; IgH/TCR, immunoglobulin heavy chain rearrangement/T cell receptor; IST, immunosuppressive therapy; MRD, minimal residual disease; MTX, methotrexate; OS, overall survival; PBSC, peripheral blood stem cells; PCR, polymerase chain reaction; PCR-SSP, polymerase chain reaction-sequence specific primer; QoL, quality of life; STR, short tandem repeats; rATG, rabbit antithymocyte globulin; RD, related donor; TRM, transplant-related mortality; UD, unrelated donor; VNTR, variable number tandem repeats; VZV, Varicella zoster virus.

haploidentical family donor with graft manipulation is then considered with preference mostly based on local experience and/or availability (1). However, all these alternatives have an increased risk of post-transplant morbidity/mortality because of an increased risk of GvHD, graft failure, and slow immune reconstitution (2, 3). Furthermore, in patients with malignancies, manipulation of the graft may adversely affect a favorable alloreactive effect directed against residual disease (4). Since the beginning of 1999, HLA typing using PCR methods was routinely available at our institution enabling high-resolution (allele) typing of class I (A*, B*, Cw*) and class II HLA antigens (DRB1*, DQB1*) in all patient–donor pairs. Based on level of HLA allele match between donor and recipient, we were able to identify an acceptable HLA partially mismatched donor (7–8/10) for a majority of patients lacking a fully matched (9–10 out of 10 alleles) donor. Here, we report our clinical experience with uniform GvHD prophylaxis using a combination of CsA, MTX, and rATG prior to unmanipulated graft administration in children with leukemia (5, 6).

Patients and methods

Between January 1999 and December 2006, in our unit, 24 consecutive children with leukemia (detailed diagnoses are shown in Table 1) underwent 26 allogeneic HSCT using an HLA-mismatched UD because we failed to identify a suitable HLA-matched donor in the time frame available. The interval between search and day of HSCT ranged from 98 to 492 days, median 147 days.

These 24 patients (age range 2.2–18.1 yr; median 11.3) were transplanted with unmanipulated grafts from UD mismatched in two (13 patients) or three (11 patients) HLA alleles. The locations of the various allele mismatches prospectively identified by PCR-SSP were as follows (A* -2x, B* -13x, Cw* -27x, DRB1* -4x, DQB1* -13x). Six out of 24 donor–recipient pairs were matched in six out of six alleles in loci A*, B*, or DRB1*. Another 17/24 (71%) pairs were mismatched in five out of six alleles, 1/24 pair was mismatched in B*, DRB1* (and Cw*) alleles (four out of six alleles); details in Table 1. The median age of UD was 34 yr (age range 22–49) with 12 males and 12 females. Conditioning regimens used for first HSCT were fully myeloablative in 23/24 patients (Table 2).

Uniform GvHD prophylaxis consisted of combination of CsA, MTX, and rATG. CsA starting at day 1 before infusion of the graft was given in two to three daily infusions (over two h) to maintain the trough serum levels of 200–250 μg/L (method of detection – FPIA). MTX was given on day + 1 (15 mg/m^2 or only 10 mg/m^2 in more advanced disease), day + 3 (10 mg/m^2), and day + 6 (10 mg/m^2) with leucovorine rescue (15 mg/m^2) in a single dose 24 h following every dose of MTX. Thymoglobuline was used in four patients in a daily dose 3.75–4 mg/kg for days −4 through −1 (total 15–16 mg/kg). Later on, within further 20 patients, ATG Fresenius S was used in a daily dose 10 mg/kg for days −4 through −1 (total 40 mg/kg).

Primary grafts were PBSC (n = 13) and BM (n = 11). The final decision about type of graft was made by the donor and/or local harvest center based on transplant center preference. Characteristics of grafts are shown in Table 3. Two patients later received a second graft (PBSC) from the same donor, both for leukemia relapse.

Acute and chronic GvHD were diagnosed and graded using established criteria (7, 8) and were primarily treated with prednisone, CsA, tacrolimus, sirolimus, or MMF in standard doses (9–11).

Day of neutrophil engraftment was defined as the first out of three consecutive days when the ANC reached 0.5 × 10^9/L or more. Platelet engraftment was defined as platelet count 20(50) × 10^9/L or more for seven consecutive days without transfusion. Chimerism was assessed by using PCR-based analyses of polymorphic VNTR/STR on recipient from unseparated peripheral blood frequently starting on day + 14 and then once weekly until day + 100, later less frequently in patients with stable full donor chimerism, to confirm efficient engraftment and to rule out risk of late graft failure/rejection or relapse (12). PCR assay of specific fusion genes and IgH/TCR receptors according to the type of leukemia was used for MRD monitoring pre and after HSCT. The testing was proceeded and evaluated according to the criteria of the ESG-MRD-ALL (13, 14). A marrow sample was taken routinely in patients with any defined leukemia target two to three wk before HSCT, at days + 28, + 60, + 100, 6 and 12 months after transplantation, or in case of decreasing overall chimerism or positive MRD in previous samples. A complete hematological remission was defined as less than 5% blasts in the marrow aspirate and functional hematopoiesis.

Surveillance of viral infections as a common cause of transplant-related morbidity and mortality was based on quantitative real-time PCR technique on DNA from whole blood. EBV and CMV were screened routinely as part of weekly testing. Other viruses, HSV, VZV, HHV6, HHV7, adenoviruses group A–C, and BK virus (15–17), were tested only in case of clinical suspicion. Results for leukotrophic viruses (CMV, HHV6, HHV7, and EBV) were normalized to 100 000 human genomic equivalents assessed by quantification of the albumin gene.

All these studies were approved by local ethical committee and all parents signed informed consents.

Results

Hematopoietic engraftment and chimerism

Full trilineage-stable primary engraftment was achieved in all 24 children (100%). Engraftment characteristics are listed in Table 3. Complete donor chimerism was observed in all 24 patients, and could be documented after a median of 21 days (range 14–98) with no difference between PBSC and BM. Reappearance of mixed chimerism was detected only in patients with emerging leukemia relapse.

GvHD incidence and severity

Acute GvHD grade II was diagnosed in 17 (70.8%) patients; grade III–IV in three (12.5%). Overall incidence of chronic GvHD in 23 evalu-

Table 1.

UPN	Dg at SCT	Stage	Date of SCT	1st MM	2nd MM	3rd MM	rATG brand	Graft	(DLI) CD3 + (kg) bw	AGVHD Grade	cGVHD grade	Event	Outcome	IST in survivors
114	CML	AP	X-99	Cw	Cw	DQB1	Thymo 16 mg/kg	BM	(1) 10/6	III	Extensive	Cytog.relapse	Alive/well	None
116	MDS	RAEBt/AML	XI-99	Cw	Cw	DQB1	Thymo 16 mg/kg	BM		0	None		Alive/well	None
122	ALL	CR2	III-00	DQB1	DQB1		Thymo 16 mg/kg	BM		III	Not eval	EBV PTLD	Died	***
142	CML	CP1	III-01	B	Cw		Fresenius	BM		II	Extensive		Alive/well	None
143	CML	CP1	IV-01	B	Cw	DQB1	Fresenius	PBSC		II	Extensive		Alive/well	None
148	CML	CP1	VI-01	Cw	DQB1	DQB1	Fresenius	BM		II	Limited		Alive/well	None
153	MDS	RC	IX-01	Cw	Cw		Fresenius	BM		0	None		Alive/well	None
159	NHL	CR3	XII-01	B	Cw	DQB1	Fresenius	BM		II	None		Alive/well	None
173	ALL	CR2	X-02	A	Cw	Cw	Fresenius	BM		II	None	CMV pneumonia	Died	***
183	CML	CP1	III–03	B	Cw	DQB1	Fresenius	PBSC		II	Limited		Alive/well	None
200	AML	PR1	IX-03	DRB1	Cw	Cw	Fresenius	PBSC	(1) 10/6	II	None	Relapse	***	***
200	***	CR2	I-05				Fresenius 50 mg/kg	PBSC		II	None		Alive/well	None
211	Ph+ALL	CR1	III-04	B	Cw		Fresenius	PBSC		II	Extensive		Alive/well	None
216	AML	CR3	IV-04	DRB1	Cw		Fresenius	PBSC	(4) 10/5; 5 × 10/6;10/7; 5 × 10/6	0	None	MRD positive	Alive in relapse	None
223	Ph+ALL	CR1	VII-04	B	Cw	DQB1	Fresenius	PBSC		II	None		Alive/well	None
233	MDS	RAEBt	XI-04	B	Cw		Fresenius	PBSC		IV	Extensive	Ext. cGVHD	Died	***
244	Ph+ALL	CR1	II-05	A	DQB1		Fresenius	BM		II	None	Relapse	Died	***
245	ABiL	CR1	VI-05	B	Cw		Fresenius	PBSC	(2) 10/6; 5 × 10/6	II	None	Relapse	***	***
245	***	CR2	VII-06				Fresenius	PBSC		III-IV	***	MDF	Died	***
251	AML	CR2	VI-05	B	Cw	Cw	Fresenius	PBSC		II	None		Alive/well	None
253	ALL	CR3	VI-05	DRB1	B	Cw	Thymo 16 mg/kg	PBSC		II	Extensive	Relapse	Alive in relapse	None
258	Ph+ALL	CR2	IX-05	B	Cw		Fresenius	PBSC		II	None		Alive/well	None
266	sMDS	RAEB	I-06	B	Cw		Fresenius	PBSC		0	None		Alive/well	None
268	ALL switched to AML	CR1	I-06	DRB1	DQB1		Fresenius	PBSC		II	Extensive		Alive/well	CsA taper
282	sAML	CR1	VII-06	Cw	DQB1		Fresenius	BM		II	None		Alive/well	None
285	MDS	RC/mon. 7	IX-06	B	Cw		Fresenius	BM		II	none		Alive/well	MMF

Not eval., not evaluable.

Table 2.

Characteristics of conditioning regimens used prior first HSCT (n = 24 patients)			
	Fractionated total body irradiation (fTBI)		
	fTBI 14.4 Gy	Cyclophosphamide 120 mg/kg	7
	fTBI 12 Gy	Etoposide 60 mg/kg	7
	Oral busulphan (Bu)		
	Bu 16 mg/kg	Cyclophosphamide 120 mg/kg	
		Melphalan 140 mg/m^2	9
UPN 153	Fludarabine (Flu)		
	Flu 160 mg/m^2	Thiotepa 15 mg/kg	1

Characteristics of conditioning regimens used prior first HSCT (n = 2 patients)			
UPN 200	Fludarabine	150 mg/m^2	(D–7...D–3)
	Melphalan	140 mg/m^2	(D–2)
	Cyclosporine A	3 mg/kg i.v.	(start D–1)
	Methotrexate	10 mg/m^2	(D+1, +3, +6)
	rATG Fresenius	50 mg/kg	(D–2...D+2)
UPN 245	fTBI	12 Gy	(D–4...D–2)
	Etoposide	60 mg/kg	(D–1)
	Cyclosporine A	3 mg/kg i.v.	(start D–1)
	Methotrexate	10 mg/m^2	(D+1, +3, +6)
	rATG Fresenius	40 mg/kg	(D–4...D–1)

Table 3. Characteristics of primary grafts, engraftment and graft versus host disease

	PBSC (n = 13)	BM (n = 11)	Total (n = 24)
NC/kg bw × 10^8 median/range	12 (5.7–29)	4.3 (2.6–6.6)	6.3
CD34+ cells/kg bw × 10^6 median/range	8 (5–16)	5 (2.4–11.8)	6
CD3+ cells/kg bw × 10^8 median/range	3.7 (0.6–15.8)	0.3 (0.2–0.7)	1.15
engraftment of ANC >0.5 × 10^9/l median/range	16 (12–22)	22 (19–27)	19.5
engraftment of platelets >20 × 10^9/l median/range	23 (16–94)	28 (19–41)	24
engraftment of platelets >50 × 10^9/l median/range	23 (16–166)	28 (19–100)	24
aGVHD none or I	2 (11)	2 (9)	4
aGVHD II/IV			20/24 (83%)
aGVHD none or limited	9 (5)	8 (2)	17
aGVHD extensive			7/23 (30%)
alive/well	11	8	19
alive without event	9	7	16

able patients was 38.7%; 8.7% experienced limited and 30% extensive, respectively (Tables 2 and 3).

Relapse rate, prevention, and treatment

Leukemia relapse occurred in five (hematological in 4, cytogenetic in 1) out of 24 (20.8%) patients 309–544 days after HSCT (median 410 days). Until now, two patients died as a consequence of leukemia relapse at a median 134 days after relapse confirmation. Eight donor lymphocyte infusions directed according to the level of MRD post-transplant or mixed and increasing chimerism were given to four patients (CML, AML, AML, ABiL) 120–505 days after HSCT (median 246 days) in doses of 1×10^5 to 5×10^7/kg CD3 + cells (median 2.5×10^6). In two patients (AML, ABiL), it failed to prevent hematological relapse (18–20). These two patients, subsequently, underwent high-dose chemotherapy, achieved CR, and were retransplanted with PBSC from the same donors as before (111 and 155 days following 1st HSCT) (Table 2). One patient remains in remission; the other died of regimen and early GvHD-related toxicity. Two other (AML and CML) continue in complete remission 26 and 72 months following the last dose of DLI.

Infectious complications

In 16 out of 24 patients (67%), reactivation of CMV, EBV, BKV, or AdV was detected. The common pathogens include CMV in 11 patients (46%) with one patient who died due to CMV pneumonia; EBV in eight patients (33%) with one patient who died as a consequence of EBV lymphoproliferative disease; hemorrhagic cystitis in four patients where BKV was detected in urine in all four, and three patients, where AdV (serotype 31) was detected in blood, but none of them developed clinical symptoms despite no therapy was given.

Fungal infections were not frequent in this cohort with very high risk of developing IFI. Mycotic pneumonia was proved (*Aspergillus* species) in one and probable in second patient, in both only pretransplant. Both were treated during early post-transplant period with ABLC,

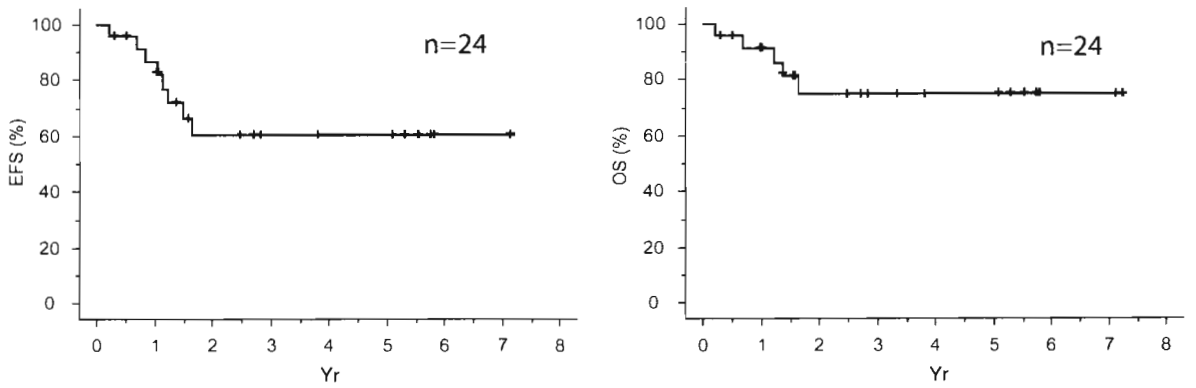

Fig. 1. Probability of overall survival and event-free survival in entire cohort of patients.

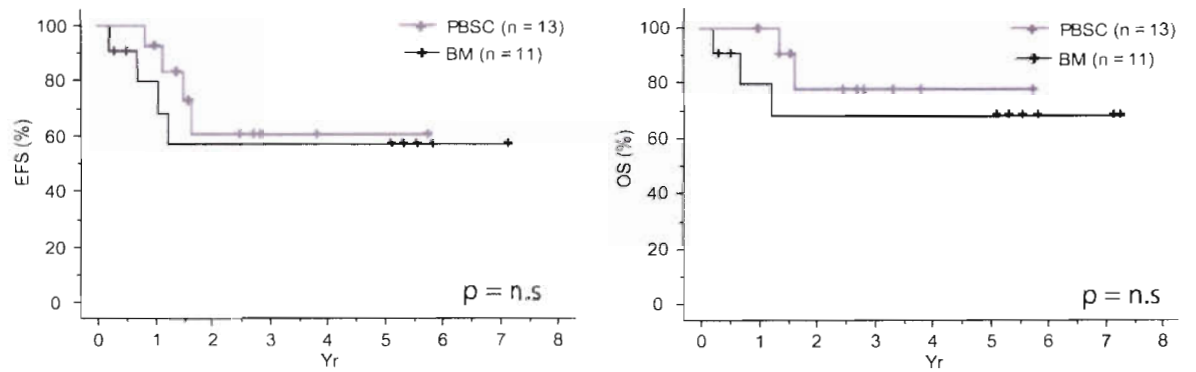

Fig. 2. Event-free survival and overall survival in patients transplanted using bone marrow and peripheral blood stem cells.

which was later switched to oral voriconazole. None of them suffered from reactivation of IFI. All other patients received prophylactic oral suspension of itraconazole. In patients with extensive chronic GvHD, we usually used prophylaxis with oral voriconazole. No patient in this cohort died as a consequence of fungal infection and that was also confirmed on autopsies.

QoL following HSCT

Patients on no continuous IST are usually fully active, have no limitations, and continue with normal life. Out of seven patients who did suffer from chronic extensive GvHD, one died with active extensive cGvHD, one is on CsA tapering with no signs of cGvHD and five are off any IST (Table 1). So far, it appears that there is no difference in QoL when comparing this group with the other patients transplanted at our institution using 9–10/10 HLA-matched UDs.

Overall outcome

EFS is calculated from the date of transplantation to the last follow-up or first event (death or relapse of the primary disease whatever occurred first). Probabilities of EFS and OS are estimated using the Kaplan–Meier method. Median follow-up till first event or last follow-up of total cohort is 1.57 yr (range 0.2–7.2 yr). According to the type of graft, median follow-up is 1.65 for PBMC (range 0.85–5.8 yr) and 1.24 for BM (range 0.2–7.2 yr). The probability of EFS was 60.3% (95% CI 35.5–78.1) and OS was 74.9 (95% CI 49.1–88.9) (Fig. 1). EFS of BM group was 56.8 (95% CI 21.3–81.3) and OS was 68.2% (95% CI 29.7–88.6). EFS of PBMC group was 60.6 (95% CI 24.7–83.5) and OS was 77% (95% CI 31.2–94.3). Difference between PBMC and BM groups in EFS and OS is not statistically significant (Cox–Mantel test) (Fig. 2).

TRM at day + 100 was 4% with overall TRM 12.5%. Altogether, five patients died at a median of 1.54 yr post-transplant (range 0.2–1.65), two (8.4%) died as a consequence of leukemia relapse, one of CMV pneumonia (0.68 yrs), one of EBV lymphoproliferative disease (0.2 yrs), and one of gastrointestinal bleeding because of extensive GvHD (1.65 yr).

Discussion

Improvements in HLA typing at the allele level, wider spectrum of efficient drugs for GvHD prophylaxis and therapy, and prospective PCR quantitative monitoring of viral load have led to decrease in TRM in patients transplanted from HLA-matched UDs. Which alternative donor is better in the case of no available HLA-matched donor remains to be resolved. Different centers have different preferences mostly based on local experience.

Many centers successfully use unrelated cord blood where the naïve immune system permits reduced stringency of HLA and, therefore, within the acceptable level of mismatch, it is possible to find a suitable donor for the majority of children. Its wide use although is limited by the efficient cell dose available for older children and adults (21, 22). This can be overcome by using double cord transplants (23). Other disadvantages include naivete of immunity against viruses and unavailability of cord blood for potential adoptive immunotherapy or re-transplantation.

Use of haploidentical family donors is possible, but large and frequent experience of cooperating laboratories for preparation of T-cell-depleted graft is essential to limit the risks of non-engraftment and GvHD. High incidence of viral infections in the early post-transplant period increases the risk of TRM. Therefore, this method is more restricted to several centers with large experience. New techniques of depletion may improve the immune reconstitution and graft vs. leukemia effect without the enormous risk of serious GvHD (24).

We present our experience with another alternative. HLA-mismatched UDs were prospectively selected based on level of allele match. We and others speculate that allele or antigen mismatch is equally adverse to survival. In our clinical experience, UD with upto three allele mismatches (no more than one in loci A*, B*, or DRB1*) could be used for a patient with malignant disease with acceptable risk for toxicity if adequate serotherapy is given together with a myeloablative conditioning regimen. On the contrary, we speculate that the practice still used in many centers to select donors based on HLA match in A*, B*, or DRB1* loci, with no respect to numbers of potential mismatches in Cw* and/or DQB1* loci, is not efficient. Such attitude may explain inferior outcome results compared with those achieved in our cohort. It is not rare to have many Cw* and/or DQB1* mismatches even in donor–recipient pairs, other vice allele matched in 5–6/6 in standard A*, B*, and DRB1* loci.

In our series we did not observe engraftment problems. All patients experienced primary and stable engraftment with full donor chimerism. Reappearance of mixed chimerism was detected only as a consequence of emerging relapse of leukemia.

Highly incident acute GvHD mostly of grade II was manageable by standard corticosteroids. Rate of leukemia relapse as well as the incidence of fatal viral infections was low. We speculate that the dose of rATG given in our cohort of patients is safe in preserving the graft vs. leukemia effect (high incidence of acute GvHD, low incidence of relapses), and is efficient in protecting the patient against moderate to severe GvHD without increasing the risk of post-transplant fatal infections (very low TRM).

There is consensus that matching of UDs and patients for HLA class II alleles improves the outcome of HSCT. However, the significance of HLA class I allelic mismatches for transplant outcome is under ongoing discussion, and reports on long-term effects like chronic GvHD are rare. Some studies, especially published earlier, are biased by the fact that HLA typing was not performed by PCR methods at high-resolution level (four digits) in all typed alleles. Other studies are biased by different proportion of minorities relevant to the different incidence of certain HLA alleles among patients and/or recipients (25). Serologically undisclosed HLA disparities account for the increased rate of post-transplant complications. Whereas, a HLA-ABDR-serologically identical donor can be identified in the International Registry for >90% of the patients, only upto half of them can benefit of a highly compatible donor if donor selection is based on allele level matching for HLA-A/B/Cw/DRB1/B3/B5/DQB1 loci among the Caucasian population. Most of the incompatibilities are clustered in a limited number of serotypes that can be targeted first during the searches. Because of linkage disequilibrium (e.g., B-Cw or DRB1-DQB1), incompatibilities at a given locus are often associated with disparities at adjacent loci (26).

Schaffer et al. have published an analysis of outcome in 104 donor–recipient pairs, transplanted in between 1988 and 1999, retrospectively typed for HLA class I and class II by PCR-SSP. They concluded that genomic HLA class I and class II typing may improve the outcome after unrelated HSCT and also that the awareness of HLA class I and II mismatches, not detected by older methods, in a recipient–donor pair makes it possible to give appropriate pre- and post-transplantation treatment (27). In addition, others investigated the association of HLA class I allele

mismatches and outcome. In cohort of 144 patients given a HSCT from an UD who were matched for HLA-DRB1, DRB3/4/5, and DQB1 alleles the risk of chronic GvHD was significantly increased in patients with class I-mismatched donors (mismatch either detected by low- or high-resolution typing). A single HLA class I allele mismatch significantly increased the risk of chronic GvHD in multivariate analysis. OS was significantly reduced in patient–donor pairs with more than one allele class I mismatch (28).

On the contrary, Duggan et al. reported 57 patients receiving UD HSCT and matched for the disease and stage with other 57 recipients of genotypically matched RD HSCT. All UD recipients were matched serologically for A and B and by high resolution for DR and DQ antigens. All patients received CsA and short-term MTX. UD HSCT recipients also received rATG (Thymoglobulin) over three days pretransplant. They concluded that UD HSCT recipients matched as above, and given pretransplant ATG have similar outcomes to recipients of matched RD HSCT using conventional drug prophylaxis (5).

Based on our results and together with information published so far (29), we also conclude that high resolution of HLA alleles, both class I and class II plays an important role in the selection of a suitable UD. However, when a fully matched donor is not available, we show that GvHD prophylaxis with use of rATG enables the use of an unmanipulated, partially mismatched donor without excessive risk of poor outcome because of severe acute GvHD.

Several different brands of ATG are available, and therefore, when using ATG in conditioning regimen, one needs to consider the ATG brand, the adequate dose and the proper timing. Exact correlation between different brands is not clear yet as they have different activity against different populations of cells. Also the dose may vary based on the type of donor and conditioning regimen. Lower doses (Thymoglobulin 6–10 mg/kg total; rATG Fresenius 20–25 mg/kg total) are currently used in reduced intensity conditioning regimen when fully matched donor is used (30) or in patients who underwent SCT using T cell highly depleted graft from haploidentical donor (31). Much higher doses (Thymoglobulin 15 mg/kg total; rATG Fresenius 60 mg/kg total and more) are used in transplants using mismatched donors and unmanipulated graft (32, 33).

Conclusions

Our study shows that combination of CsA, short-term MTX and rATG in GvHD prophylaxis prior to HSCT using unmanipulated grafts of HLA-mismatched UDs is efficient to prevent occurrence of very severe acute GvHD grade III–IV. Serotherapy (rATG Fresenius; Fresenius-Biotech) was well tolerated and in doses given (40 mg/kg total) did not increase post-transplant mortality by long-lasting depression of immunity or increase risk of leukemia relapse. Incidence of chronic GvHD was not increased compared with series where fully matched UDs were used, more over in majority of patients it disappeared within the time without serious consequences (Table 1). Overall outcome is satisfactory, and therefore, it is possible to use such alternative donors in patients with advanced leukemia lacking a HLA-matched donor as a reasonable alternative to unrelated cord blood or haploidentical family donor. We strongly recommend to extend HLA typing for clinically relevant Cw* and DQB1* loci in centers still using only A*, B* and DRB1* for selection of suitable donor.

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Detection of residual B precursor lymphoblastic leukemia by uniform gating flow cytometry

Running title: Leukemia early response by flow cytometry

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Abstract

Residual disease (RD) is an important prognostic factor in acute lymphoblastic leukemia (ALL). Flow cytometry (FC)-based RD detection is easy to perform, but interpretation requires expert analysis due to individual differences among patients. We focused at the design of standardized and reproducible RD monitoring in ALL. RD was investigated by a uniform gating strategy, which was designed internationally and tested in one center by Ig/TCR rearrangements. For each gate, positivity cut-off value was assigned using quantification of non-leukemic background. Comparing to Ig/TCR at 0.1% level, 83 of 103 specimens were correctly diagnosed by FC. The predictive value of FC RD at day 15 was then analyzed. In B lineage ALL, day 15 FC significantly correlated with Ig/TCR results at day 33 and/or week 12 ($p<0.01$). No significant correlation was found in T lineage ALL. Thus, FC with preset uniform gating at day 15 predicts PCR-detectable MRD in B precursor ALL.

Introduction

The speed of leukemia clearance during therapy is a major prognostic factor. Residual disease (RD) during the early phase of therapy can be assessed by microscopy (1) or by more sensitive techniques, such as flow cytometry (FC) or polymerase chain reaction (PCR) of the rearrangements of immunoglobulin (Ig) or T cell receptor (TCR) gene segments (2-4). The strong predictive value of RD is also maintained at the level of minimal RD (MRD) during complete remission (CR) (5-7). Several strategies are used when utilizing information from previous retrospective studies: stratification is based on MRD level from either a single time-point (8, 9) or consecutive time-points (7, 10). Real-time quantitative (RQ) PCR-based detection of specific Ig and TCR gene rearrangements represents the current gold standard for MRD. Standardized RQ PCR detects MRD both specifically and reproducibly, with a detection limit of 10^{-4} to 10^{-5}. Nevertheless, such PCR methods are laborious and costly. Flow cytometry is a method with acceptable sensitivity and wide availability in many centers/countries for hematological and immunological investigations. While FC investigates antigens on intact cells, in large trials covering several continents, it is impossible to concentrate MRD diagnostics into one lab. The interpretation of FC data is complicated and requires highly experienced and skilled experts. Therefore, it is important to set clear definitions and standards, especially for inter-lab studies. The unfortunate reality is that current published papers usually lack detailed information on gating strategies due to differences in interpretation among patients. Only one published study by Coustan-Smith et al. evaluated two simple predefined subpopulations, $CD10^{pos}CD19^{pos}$ and $CD19^{pos}CD34^{pos}$, at a single time-point on day 19 (a time-point before the achievement of complete remission). Thus, taking into account differences among patients, it is difficult or nearly impossible to reproduce FC MRD diagnostics. This is especially true for centers/countries that are just starting out with FC MRD evaluation. Thus, we designed a study in both B precursor and T ALL patients that evaluated identical, pre-defined subpopulations/gates, regardless of the initial immunophenotype. We collected cell frequencies in each of these gates at individual time-points. The gates were made up of antigen combinations commonly used in FC RD studies. In total, we defined 29 gates/subpopulations in B cell precursor (BCP) acute lymphoblastic leukemia (ALL) and five subpopulations in T ALL. In this study, we included the samples with available RD using both FC and PCR, which enabled an exact assessment of both methods for MRD evaluation at individual time-points. The objective of the study was to develop a robust and reproducible FC RD approach for childhood ALL, even for centers/countries with limited experience in FC MRD analysis, and to precisely quantify the background present during therapy. This background was derived from cell frequencies in specimens whose MRD negativity was evident either because the patients suffered from ALL of the opposite lineage (T versus B) or because MRD was proven negative by PCR.

Patients and methods

Patients

Patients were recruited from the ALL IC BFM2002 protocol (started in XI/2002). The protocol was designed for countries with clinical experience with BFM based protocols but with limited experience and resources for MRD techniques. MRD was not used for patient stratification. This ran in parallel to the MRD-based (Ig/TCR RQ PCR) treatment protocol AIEOP–BFM ALL 2000. Laboratories from the Czech Republic, Israel, Croatia, Hungary and Hong Kong participated in the FC MRD study. Patients entered the study after their parents or guardians signed informed consent, and the study was approved by the institutional ethics committee. In total, 110 patients with newly diagnosed ALL entered the study (90 BCP ALL and 20 T ALL). The cohort reported here contained patients treated in 8 Czech centers, analyzed and acquired in a single FC laboratory. A summary of the patients' characteristics is shown in table 1. The diagnosis of ALL was established according conventional FAB and immunological criteria. All samples that met these criteria and were successfully analyzed both by FC and PCR were included in the study. In total, 612 samples were centrally evaluated (diagnosis: 110, day 8 bone marrow (BM): 103, day 8 peripheral blood (PB): 83, day 15 BM: 108, day 33 BM: 108, week 12 BM: 100). No patients were excluded as having an unsuitable immunophenotype for FC RD monitoring.
For background calculations, 49 specimens of B lineage patients were measured with T lineage combinations or vice versa (cross lineage controls).

Risk grouping, treatment and time-points

Patients were assigned to the standard risk group (SR) when they fulfilled the following criteria: age < 6 years, initial leukocytosis <20000/μL, good prednisone response (<1000 blasts/μL) at day 8 in peripheral blood, absence of BCR/ABL and MLL/AF4 fusion genes, and achieving a CR at day 33. Patients who fulfilled all of these SR criteria and had more than 25% blasts in BM by microscopy at day 15 were stratified into the intermediate risk group (IRG). Other patients with more than 25% blasts in BM by microscopy at day 15 were stratified into the high risk group (HRG). Other HRG criteria were BCR/ABL or MLL/AF4 fusion genes, poor

response to prednisone at day 8 (>1000 blasts/μL in peripheral blood), or >5% blasts at day 33 in BM. All remaining patients were assigned to the IRG.

All time-points shown here reflect remission induction therapy (day 8, day 15, day 33 and week 12). Remission induction therapy was scheduled over 9 weeks and included a 7-day steroid prephase with daily oral prednisone (60 mg/m^2 of body surface area daily, cumulative dose of prednisone at day 7 had to be greater than 210 mg/m^2) and a single dose of intrathecal methotrexate (age adjusted) on day 1, followed by prednisone (60 mg/m^2 daily) from day 8 to day 28, tapered thereafter over 9 days. It also included 8 doses of L-asparaginase (5000 U/m^2/day on days 12, 15, 18, 21, 24, 27, 30 and 33), daunorubicin (30 mg/m^2 on days 8 and 15, two other doses received IR and HR patients at day 22 and 29), vincristine (1.5 mg/m^2 on days 8, 15, 22, and 29) and two doses of intrathecal methotrexate (age-adjusted) on days 12 and 33 (2 additional doses on days 18 and 27 were administered in cases with leukemic CNS involvement or with traumatic lumbar puncture). From day 36 to 64, the regimen included: two doses of intrathecal methotrexate (age-adjusted) on days 45 and 59, cyclophosphamide (1 g/m^2 on days 36 and 64), cytarabine in 4 blocks (75 mg/m^2 daily on days 38–41, 45–48, 52–55, and 59–62) and 6-mercaptopurine (60 mg/m^2 daily from day 36 to day 62). Patients who had aplastic BM at day 33 underwent another BM puncture one week later (a sample with complete remission including regeneration in BM was analyzed as day 33). Patients who received a non-ALL IC therapy between day 33 and week 12 (4/6 BCR/ABL positive patients received imatinib mesylate according to the EsPhALL protocol: one patient received therapy according to the Interfant 2003 protocol after day 33) were excluded from the analyses that comprised week 12. Further therapy was not evaluated in this study.

Sample processing

Samples were processed within 24 hours after they were collected from patients. Sample preparation consisted of a 15-minute incubation with monoclonal antibodies (mAb) – sample-to-mAb volume ratios were used as recommended by the manufacturers. Red blood cells were then lyzed in a 15-minute incubation (ammonium chloride), followed by 5 minutes of centrifugation (500g), discarding of the supernatant, adding of PBS and immediate data acquisition. For intracellular staining, the Fix & Perm kit (An Der Grub Bioresearch, Austria) was used. All events were acquired and stored in listmode files, and no live gate strategy was used. At diagnosis, a minimum of 20,000 events per tube were acquired. During RD follow-up, the target minimum counts were 50,000 events (SYTO16 containing mAb combinations), 300,000 events (the most informative mAb combination) or 100,000 events (all other tubes).

Monoclonal antibodies

CD19 PE (clone SJ25C1), CD20 FITC (clone L27), CD45 PerCP and APC (clone 2D1) and CD99 FITC (clone TÜ12) were purchased from BD Biosciences (San Jose, CA, USA). CD10 FITC (clone ALB2), CD7 PE (clone 8H8.1), CD3 PC5/PC7 (clone UCHT1), CD5 PC5/PC7 (clone BL1a), CD19 PC5/PC7 (clone J4.119), CD34 APC (clone LIQ), CD58 FITC (clone AICDCD58) and CD66c PE (clone KOR-SA3544) were purchased from Immunotech (Marseille, France). CD10 PE (clone SS2/36) and TdT FITC (clone HT-6) were purchased from DAKO (Glostrup, Denmark). SYTO-16 (green fluorescent nucleic acid stain) was purchased from Invitrogen - Molecular Probes (Carlsbad, CA, USA).

Data analysis

Gating definitions and strategies are shown in figures 1 and 2. Uniform templates were designed in the software applications, Cellquest (BD, San Jose, CA, USA) and FlowJo (Tree Star, Oregon, USA). Gate positions were defined according to regenerating BM samples in patients with negative MRD by Ig- TCR RQ-PCR and/or using QSC beads (Quantum Simple Cellular, Bangs Labs, Fishers, IN, USA). In all B lineage and T lineage mAb combinations, the mAb reacting with a lineage-defining antigen (CD19 and CD7, respectively) was present. In all RD subsets, the target population was calculated as a fraction of nucleated cells (SYTO16^{pos}).

Reported subpopulations

The definition of subpopulations is shown in figures 1 and 2. All subpopulations were reported in all patients with ALL of the respective lineage, regardless of the detailed presenting immunophenotype. The true fraction of events within a defined region per total of lymphoid lineage cells (CD19^{pos} or CD7^{pos} cells, respectively) was multiplied by the fraction of CD19^{pos} or CD7^{pos} cells per total nucleated cells (SYTO16^{pos}). These levels of all reported subpopulations were stored in the database. To calculate the exact time point-specific background, we first selected a testing cohort, which consisted of a random half of the PCR^{neg} specimens at each time-point, and combined them with the cross lineage controls. For each reported value at all time-points, we calculated the 98th percentile. In order to encompass the random Poisson distribution of real data around the 98th percentile of the representative sub-cohort, we considered the three-fold multiplication of 98th percentile to be the background cut-off value. T lineage values were calculated using B lineage cross lineage controls only. All FC data reported as predefined subpopulations were compared with the subpopulation and time point-specific background cut-off

values; only when they were higher did we considered them to be positive. The highest FC^{pos} subpopulation above cut off was considered an FC RD (cut off values are shown in table 2.).

RQ PCR of Ig/TCR rearrangements

Follow-up BM or PB samples were processed by erythrocyte lysis and stored at -80°C. Genomic DNA was isolated by the QIAamp® DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany), and the DNA was stored at -20°C until further processing. Rearrangements of immunoglobulin heavy chain (*IGH*), immunoglobulin light chain kappa (*IGK*), T cell receptor delta (*TCRD*), T cell receptor gamma (*TCRG*) and T cell receptor beta (*TCRB*) were identified using single or multiplex PCR (11, 12). Clonality assessment, sequencing, patient-specific primer design and RQ-PCR with family-specific reverse primers and probes for *IGH*, *IGK*, *TCRD*, *TCRB* and *TCRG* were performed as described previously (13-15). The albumin gene was used to normalize DNA concentration and quality. The ESG-MRD-ALL (European Study Group on Minimal Residual Disease in ALL) criteria for RQ-PCR sensitivity and quantitative range were used (16). For statistics of continuous variables, numerical MRD values (representing the mean value of positive values within the triplicate) were always reported.

Statistics

FC sensitivity was defined as the percentage of samples positive by both methods among all PCR^{pos} samples. FC specificity was defined as the percentage of samples negative by both methods among PCR^{neg} samples. Spearman Rank correlation coefficient was used for correlations of RD between individual time-points and was calculated using STATISTICA software (StatSoft, Inc. (2006). STATISTICA (data analysis software system), version 7.1. www.statsoft.com., USA). A linear-by-linear association model (17) was used for analyses of correlations between FC RD subgroups at days 8 and 15 and PCR RD subgroups at day 33 and week 12. In order to fit linear by linear association, we used the R computing environment (www.r-project.org).

Results

Background identification for each subpopulation

Specimens with available PCR and FC RD analyses at day 15 were included in the study (n=104, B precursor ALL: xxx, T ALL: xxx). In total, xxx and xxx specimens (xxx cross-lineage control specimens, xxx PCR-proven negative) served as a basis for non-malignant background identification in B and T lineage ALL, respectively. The cutoff values representing the background of each reported subpopulation are listed in Table 2. Although it is frequently assumed that the non-malignant B precursor background is very low or absent at early time-points, our results do show non-zero background levels even at day 15, which must be taken into account in the data interpretation (similar situation appeared at day 33, data not shown).
Graphs showing cell frequencies of each individual FC subpopulation at day 15 as well as at days 8, 33 and week 12 are available (see supplemental information).

RD evaluation and comparison with RQ-PCR Ig-TCR

The FC RD value data represented the frequency of the most prominent subpopulation above its cutoff value. When comparing these FC RD data to the leukemic cell frequencies assessed by PCR, majority of PCR^{pos} specimens were positive also by FC (71 of 81 and 19 of 20 in B precursor and T ALL, respectively) at day 15. Starting at day 33, most of the FC data were below the cutoff values, and the percentage of PCR^{pos} specimens identified as FC^{pos} dropped profoundly (data not shown). Therefore, the cutoff-based, four-color FC focused at the cellular subpopulations deseribed here might serve as a basis for the design of RD investigation but it cannot be used as an RD evaluation at day 33 and week 12 of ALL therapy.

Prediction of molecular remission at day 33 and week 12 using FC stratification at day 15

Next, we asked whether day 15 FC RD could predict RD at day 33 and week 12 as evaluated by PCR. Using Spearman rank correlations, we compared FC RD values measured by FC at day 15 to PCR-assessed RD at day 33 and at week 12. In patients with B precursor ALL, this analysis indicated a significant correlation between day 33 or week 12 (PCR) and day 15 (FC) (R 0.59 and 0.31, $p<0.006$, respectively). This is in contrast to T ALL patients, whose data did not reach significance in any of these comparisons (R 0.04 and 0.22, $p > 0.3$, respectively). Therefore, we analyzed the levels of day 33 and week 12 RD (PCR) in categories defined by the FC RD level at day 15, exclusively in patients with B precursor ALL. As shown in Figure 4, day 15 FC RD categories (defined by intervals of FC RD in ascending order) correlated with day 33 RD by PCR ($p_{Fisher} < 10^{-7}$, Figure 4). A linear-by-linear association model (i.e., a model for contingency tables where both rows and columns are ordered and the scores of the rows and columns are given by theirs ranks) showed significant association between day 15 and day 33 ($p< 10^{-7}$, odds ratio is 2.321 (95% CI 1.603 – 3.360). The odds ratio describes the ratio of proportions of patients in columns and rows of any given 2x2 region with both day 33 categories (PCR) as well as day 15 categories (FC) ordered ascendingly. Similar but weaker correlations were

observed in B precursor ALL patients between FC RD in BM or peripheral blood at day 8 and RD (PCR) at later time-points (data not shown).

Contribution of individual values on day 15

We analyzed which of the recorded subpopulations contributed to the FC RD value. Among all specimens on day 15, only one of 34 recorded subpopulations ($CD45^{low}CD10^{low}CD66c^{pos}CD19^{pos}$) remained below the cutoff value in all cases. In addition, some values reached positivity but their levels were lower than the maximum positive value in all patients (i.e., lower than the overall FC RD value in 10 of 29 and 1 of 5 recorded values in B precursor and T ALL patients, respectively). Thus, we asked whether a reduced panel of mAb combinations (and a reduced number of recorded values) could provide acceptable information for day 15 FC RD. The RD information was considered acceptable if the reduced panel generated an RD value higher than 0.1 of the full panel RD value. Upon excluding the CD58/CD10/CD19/CD34 tube, the RD information was acceptable in 100% specimens (quantitatively, the reduced panel reduced the values to 0.64 to 1.0 of the full panel values; 5th percentile, 1.0). Further reduction to a set of just two mAb combinations (CD10/CD66c/CD19/CD45 and CD20/CD10/CD19/CD34) still generated acceptable results in 99% specimens, whereas selecting a set of any other two mAb combinations reduced the number of specimens to fewer than 90%. Excluding the SYTO16/CD19/CD45, CD10/CD66c/CD19/CD45 or CD20/CD10/CD19/CD34 mAb combination led to a reduction to 100%, 82% or 83% acceptable results, respectively. Excluding any of the two T-lineage mAb combinations reduced the frequency of acceptable results to fewer than 90% specimens. Similarly, reducing the B-lineage panel just to a single mAb combination reduced the frequency of acceptable results to fewer than 90% specimens in all cases (data not shown).

After reducing the B lineage panel to two mAb combinations (CD20/CD10/CD19/CD34 and CD10/CD66c/CD19/CD45), it was possible to further simplify the procedure by reducing the number of recorded subpopulations. In a reduced panel, we propose to record three $CD19^{pos}$ subpopulations with relatively high cutoff values ($CD34^{pos}$, $CD10^{pos}$ and $CD10^{neg}20^{neg}$), followed by daughter subsets of these $CD19^{pos}$ subpopulations ($CD10^{pos}34^{pos}$, $CD10^{neg}20^{neg}34^{pos}$, $CD10^{bright}$, $CD10^{pos}66c^{pos}$ and $CD10^{bright}66c^{pos}$ – the latter two also separately in $CD45^{neg}$, $CD45^{dim}$ and $CD45^{bright}$ fractions; in total, 14 recorded subsets). Obviously, all daughter subsets are less than or equal to their respective parent $CD19^{pos}$ subpopulations, which enables skipping some of the analyses. Such a reduced panel led to identical results in 71 of 83 positive specimens. Three of the 83 specimens would be considered negative, although very low level [10^{-4} to 10^{-3}] MRD was detected by the full panel; only one of these patients had an MRD above 10^{-4} at day 15, and the 5th percentile was 0.52 of the full panel values (25th percentile was 1.0). Similar reduction appears to be possible in T-ALL at day 15. If we report just $CD5^{pos}CD3^{neg}$, $CD99^{bright}CD5^{pos}$ and TdT^{pos} subpopulations of the $CD7^{pos}$ cells, the RD value remains identical in 18/19 positive specimens and is reduced by a factor of 0.92 in one case.

Discussion

Study design

The speed of FC, as well as its single-cell, quantitative nature, portrays FC as an ideal tool for RD and MRD measurement. The general lack of leukemia-specific antigens can be overcome by detecting LAIPs using combinations of mAbs labeled with different fluorochromes. A general and accepted definition of an LAIP is missing, however, and no consensus has been reached as to what constitutes an adequate negative control. LAIPs usually describe a subpopulation of cells of a given lineage at a particular differentiation stage with (a) aberrant molecular expression patterns, (b) asynchrony, and/or (c) profound over- or underexpression of molecules that are physiologically expressed at the given differentiation stage. Such molecular differences of leukemic cells may be highly specific when compared to BM of healthy subjects. Remission BM specimens, however, contain non-malignant cells in various phases of regeneration or otherwise affected by chemotherapy (18). Most strikingly, the B lineage regenerates, and B precursors are abundant at late phases of therapy, leading to potential problems with RD detection. Although this phenomenon is widely accepted, the possibility that subpopulations of the regenerating cells attain LAIP is often ignored. In addition, standardized approaches are necessary for RD detection in large and international protocols. So far, the interpretation of FC has been largely dependent on the expert experience of a cytometrist.

The presented approach largely disregards subjective evaluation of FC data. The distinction between leukemic cells and non-malignant background cells is quantitative. To our knowledge, this is the first study that sets the baseline as negative BM specimens from the same chemotherapy time-point. The presented approach may miss some clearly atypical cells, however, when they do not fit into the pre-defined regions. This appears to be the reason why the presented approach did not lead to a useful correlation at day 33 and week 12. Of note, even patient-tailored MRD investigation by 4-color FC may fail to generate clinically reliable data. The absolute number of relapsed patients among the FC MRD-negative subset (day 29) was the highest among all strata in a large published trial involving 2,113 patients by Children's Oncology Group Study (2). Thus, compared to a previous study (10) with recently updated results by Flohr et al. (19) (which was the basis for the AIEOP-BFM

ALL 2000 protocol), FC MRD at the end of induction treatment missed a much greater proportion of patients than Ig/TCR MRD. Recent data from Borowitz et al. (2) were less encouraging than several of the previous FC MRD reports. Here we show usefulness of day 15 FC RD; the fact that our approach failed at later time-points supports the recent caution (2), despite using a very different approach and a different chemotherapy protocol. Clearly, the use of FC at day 29 or later for MRD monitoring is still highly important. At day 29 or later, it reliably detects a high MRD burden, whereas its use at the level of 0.01% should rely on a multicolor approach and/or other methodological improvements.

MAb combinations - reasoning

The SYTO16/CD19 PE/CD45 PerCP and SYTO16/CD7 PE/CD45 PerCP combinations were used to quantify the proportion of B and T lineage cells, respectively, among nucleated cells. In addition, immature $CD45^{dim\ to\ neg}CD19^{pos}$ cells were recorded. The CD20 FITC/CD10 PE/CD19 PC7/CD34 APC evaluated B cell differentiation (20, 21). Changes in CD10 expression levels in the context of CD20 and CD34 are frequent in BCP ALL. The CD58 FITC/CD10 PE/CD19 PC7/CD34 APC combination was introduced to detect CD58 overexpression, commonly found in BCP ALL (22-24). The CD10 FITC/CD66c PE/CD19 PC7/CD45 APC evaluated CD66c, which is the most common aberrant myeloid antigen in childhood ALL and usually does not change between diagnosis and relapse (25). Possible temporary changes in its expression, however, would decrease its value in MRD. The CD99 FITC/CD7 PE/CD5 PC7/CD3 APC evaluated CD3 negativity and hyperexpression of CD99, both of which are common in T ALL (26, 27). The intra-TdT FITC/CD7 PE/CD3 PC7/intra CD3 APC evaluated the TdT expression in T cells, which is predominantly present in the thymus (28). Data interpretation and definition of individual subpopulations was done as described in figure 1 and 2.

Simplified RD testing

Recently Coustan-Smith et al. showed the prognostic relevance of $CD10^{pos}CD19^{pos}$ and $CD19^{pos}CD34^{pos}$ frequency at day 19 on the St. Jude protocol (29). The definitions of populations shown in this study are closest to $CD10^{bright}CD19^{pos}$ and $CD19^{pos}CD34^{pos}$. Here, we show a high background of these subpopulations at day 15 of the BFM-based ALL-IC BFM2002 protocol. Therefore, the published findings should not be generalized, and the possible presence of non-malignant $CD10^{pos}$ or $CD34^{pos}$ B cells should be considered in BM, even at early phases of chemotherapy. In agreement with the main conclusion of Coustan-Smith et al., negative findings with these simplified techniques are likely to specifically identify patients with low RD. Patients with higher numbers of $CD10^{pos}$ and/or $CD34^{pos}$ B lymphoblasts, however, may be free of RD, at least at day 15 of BFM-based protocols.

The relevance of non-malignant background

The strategy of using a wide panel of antibodies and reporting predefined subpopulations as described here was recently published in a childhood AML MRD study, where heterogeneity and phenotype instability in leukemic cells represented a problem in reporting MRD (30, 31). Although some data comparing FC MRD using patient specific LAIP to PCR MRD appear promising (32, 33), no reproducible gating strategy considering a time-point specific background has been published.

Although 4-color approaches are frequently used for RD detection, current equipment allows the use of an increasing number of molecules simultaneously. This may further improve the accuracy of distinguishing leukemic from non-malignant cells, even if no new antigens are introduced into the diagnostics. Knowledge of the non-malignant background, however, is vital for the correct interpretation of such RD data. The presented results show that the background level of immature cells of both the B and T lineage may exceed 0.1% even during the induction therapy.

The predictive value of early RD

As shown here, the evaluation of RD on day 15 using a cytometric strategy with predefined gating was predictive of MRD categories, especially at day 33. Day 15 cytometric RD detection is, thus, a notable alternative for PCR-based MRD at day 33, especially in settings where PCR-based MRD is unavailable. Although morphology in hypoplastic BM, which is typically present at day 8 and 15, can correctly identify patients with a high leukemic burden (BM infiltration over 25%), FC can specifically categorize patients using lower and meaningful thresholds (Figure 4). The fact that early FC RD did not correlate with later time-points in T lineage ALL might have been influenced by the lower number of patients in this ALL subset. A larger study for T lineage ALL should test whether RD in these patients requires a quantitatively different stratification. Both the instrument and the gating should be standardized for reliable RD monitoring, especially in a multi-center setting. The presented study shows background thresholds in precisely defined gates and the relevance of a time-point specific RD. The highest potential for the use of FC appears to be day 15 of a BFM-based protocol. These RD values may identify those patients at both high and low risk of MRD at the end of induction and at week 12 in BCP ALL.

Reduced panel for day 15 RD

A rather extensive panel was designed, and quite a high number of subpopulations were reported in this study. For practical reasons, limiting the number of analyzed subpopulations is vital, especially in B lineage ALL. In our cohort, a reduction in the number of subpopulations by one-half (14 subpopulations), omitting one mAb combination entirely and reducing the SYTO16/CD19/CD45 to SYTO16/CD19 provided comparable results to the full panel of 28 subpopulations with 4 mAb combinations. In T ALL, we noticed a decreased intensity of TdT expression in residual T lymphoblasts (data not shown). Not surprisingly, the two subsets of $TdT^{pos}CD7^{pos}$ subpopulations were already too infrequent and did not contribute to the overall RD values. Nevertheless, our data showed no predictive value of FC at day 15 in T ALL. Therefore, an alternative approach should be used for these patients. Perhaps day 15 could be replaced by another time-point in T ALL patients.

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Titles and legends to figures and tables

Table 1. Patients' characteristics

Hyperdiploid patients were negative for all fusion genes listed in this table. Standard, intermediate and high risk group (SRG, IRG, HRG) criteria are described in materials and methods.

Table 2. Cut-off values

Values represent the threshold for RD positivity (A. B lineage, B. T lineage). Numbers indicate the percentage of cells among nucleated cells above which all values are considered RD positive.

Table 3. FC sensitivity and specificity at different time-points

The number of $FC^{pos/neg}$ and $PCR^{pos/neg}$ (level 10^{-4}) patients at respective time-points and the corresponding sensitivity and specificity of FC RD.

Figure 1. Gating strategy in B lineage ALL

All reported gates for each mAb combination are shown in pseudocolor plots. Plots show residual leukemic cells (PCR RD $\geq 10^{-4}$) in regenerating bone marrow (week 12). In the CD10/CD66c/CD19/CD45 combination, subpopulations according to the levels of CD45 expression were also reported ($CD45^{++}$, $CD45^{dim}$, $CD45^{neg}$ defined by expression on $CD45^{dim}$ granulocytes).

Figure 2. Gating strategy in T ALL

All reported gates for each mAb combination are shown in pseudocolor plots. Plots show residual leukemic cells (PCR-RD > 10^{-1}) in a bone marrow (day 15) together with non-malignant T lymphocytes.

Figure 3. Correlation of RQ-PCR RD and FC RD at day 15

FC RD (calculated as the highest frequency of the subpopulation which exceeded the time point-specific background value) is compared to RQ-PCR RD calculated according to EAO principles. Only samples not used for background quantification are shown. The number of patients in each quadrant is shown.

Figure 4. Correlation between residual disease by PCR at day 33 and residual disease by FC at day 15 in BCP ALL

Patients were categorized based on PCR-detected RD at day 33 into: negative (white), below 10^{-4} (oblique hatching), below 10^{-3} (≥10^{-4} and < 10^{-3}, vertical hatching) and at or above 10^{-3} (black). The frequencies are shown in subsets of patients defined by FC RD at day 15.

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Figure 1.

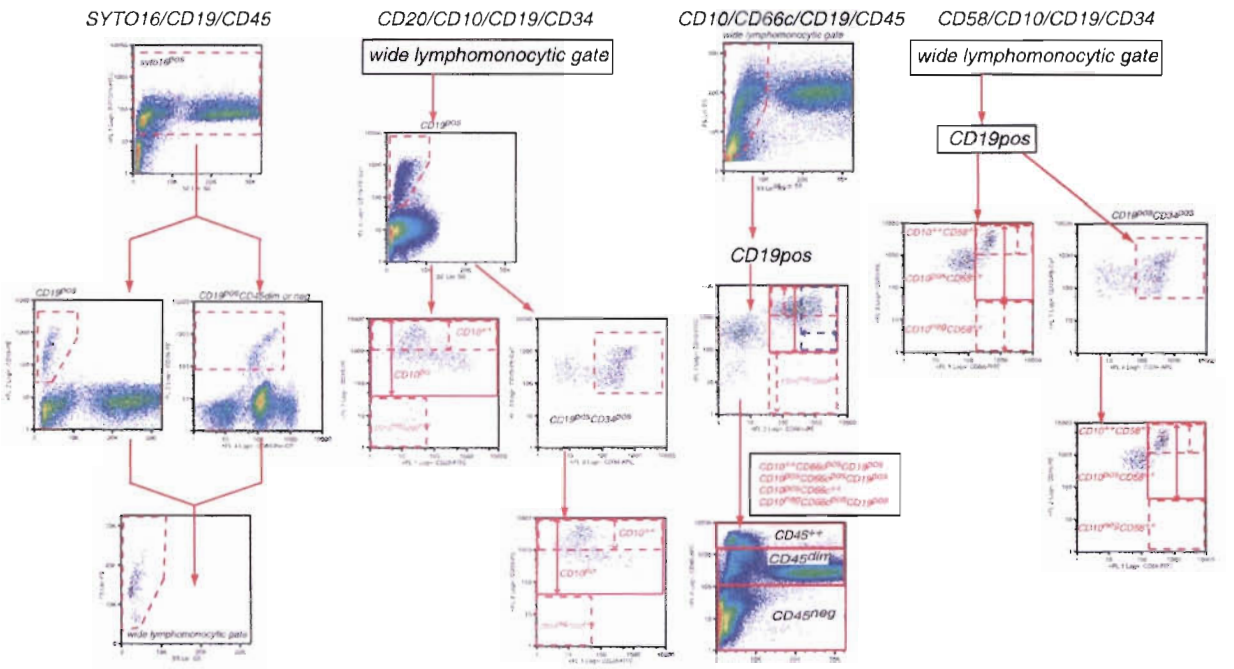

Figure 2.

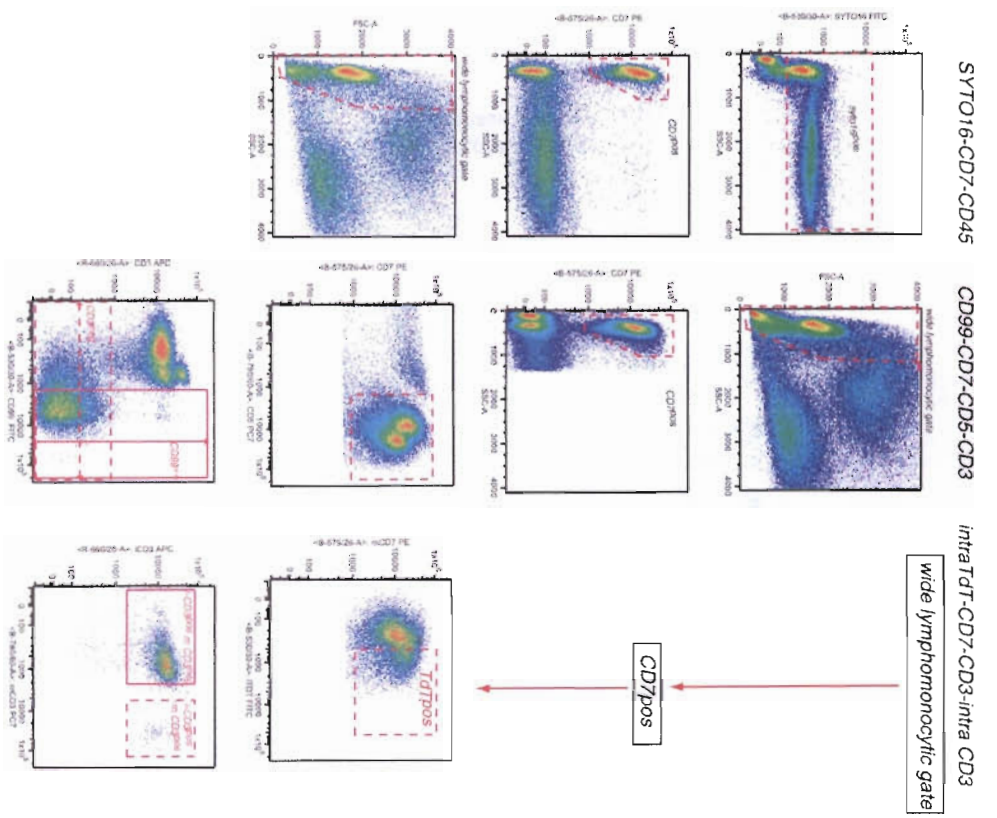

Figure 3.

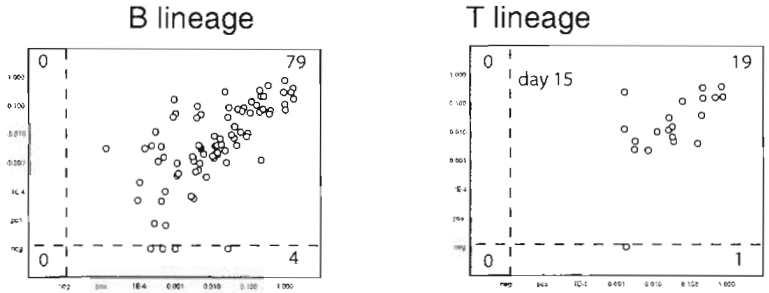

Figure 4.

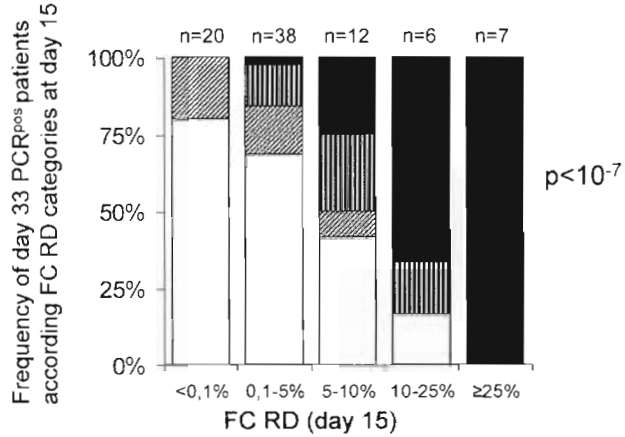

Table 1.

Characteristics at diagnosis (n)			
Male	67		
Female	43		
<6 years	68		
≥6 years	42		
BCP total	90	**T ALL**	20
BCP SRG	47	SRG	0
BCP IRG	33	IRG	12
BCP HRG	10	HRG	8
prae-B	17	mature T ALL	5
cALL	70	mature T ALL TCR alpha/beta^{pos}	2
AHL (cALL/My+)	1*	mature T ALL TCR gamma/delta^{pos}	2
pro-B	2	AHL (mature T ALL TCR gamma/delta^{pos}/My+)**	1
		intermediate T ALL	7
		prae-T	3
Prednisone good response	85	Prednisone good response	12
Prednisone poor response	5	Prednisone poor response	8
WBC at diagnosis ≥20000/uL	25	WBC at diagnosis ≥20000/uL	14
WBC at diagnosis <20000/uL	65	WBC at diagnosis <20000/uL	6
CR1 achieved at day 33	86	CR1 achieved at day 33	19
CR1 achieved later (till week 12)	3	CR1 achieved later (till week 12)	1
Death prior day 33	1	Death prior day 33	0
Genotype subgroups (BCP ALL)			
TEL/AML1	29		
BCR/ABL	7		
hyperdiploidy (DNA index ≥1.16<1.6)	28		
MLL/AF4	0		
others	26		

Table 2.

A

B lineage	day 15 cut off value
syto^{pos}CD45^{dim}CD19^{pos}	3.2
CD10^{bright}CD34^{pos}CD19^{pos}	0.0056
CD10^{pos}CD34^{pos}CD19^{pos}	0.11
CD10^{neg}CD20^{neg}CD34^{pos}CD19^{pos}	0.0072
CD34^{pos}CD19^{pos}	0.29
CD10^{bright}CD19^{pos}	0.024
CD10^{pos}CD19^{pos}	3.8
CD10^{neg}CD20^{neg}CD19^{pos}	0.58
CD10^{bright}CD58^{bright}CD34^{pos}CD19^{pos}	0.014
CD10^{pos}CD58^{bright}CD34^{pos}CD19^{pos}	0.13
CD10^{neg}CD58^{bright}CD34^{pos}CD19^{pos}	0.049
CD10^{bright}CD58^{bright}CD19^{pos}	0.03
CD10^{pos}CD58^{bright}CD19^{pos}	0.52
CD10^{neg}CD58^{bright}CD19^{pos}	0.95
CD10^{pos}CD66c^{pos} CD19^{pos}	0.2
CD10^{bright}CD66c^{pos} CD19^{pos}	0.017
CD10^{pos}CD66c^{bright} CD19^{pos}	0.028
CD45^{bright}CD10^{pos}CD66c^{pos} CD19^{pos}	0.052
CD45^{dim}CD10^{pos}CD66c^{pos} CD19^{pos}	0.12
CD45^{neg}CD10^{pos}CD66c^{pos} CD19^{pos}	0.083
CD45^{bright}CD10^{bright}CD66c^{pos} CD19^{pos}	0
CD45^{dim}CD10^{bright}CD66c^{pos} CD19^{pos}	0.01
CD45^{neg}CD10^{bright}CD66c^{pos} CD19^{pos}	0.012
CD45^{bright}CD10^{pos}CD66c^{bright} CD19^{pos}	0.0069
CD45^{dim}CD10^{pos}CD66c^{bright} CD19^{pos}	0.021
CD45^{neg}CD10^{pos}CD66c^{bright} CD19^{pos}	0.0043
CD45^{dim}CD10^{neg}CD66c^{pos} CD19^{pos}	0.12
CD45^{neg}CD10^{neg}CD66c^{pos} CD19^{pos}	0.26

B

T **lineage**	day 15 cut off value
CD5^{pos}CD99^{bright}CD7^{pos}	0.14
CD5^{pos}CD3^{neg}CD7^{pos}	0.18
iTdT^{pos} CD7^{pos}	0.36
iTdT^{pos}CD3^{pos}iCD3^{pos} CD7^{pos}	0.22
iTdT^{pos}CD3^{neg}iCD3^{pos} CD7^{pos}	0.014

6.4. Přiložené publikace bez impakt faktoru

Příloha 12

Imunofenotypizace dětských leukémií

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Imunofenotypizace dětských leukemií

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Souhrn
Imunofenotypizace je již dnes standardní metodou v diagnostice hemato-onkologických onemocnění. V současné době zažíváme rozvoj mnohobarevné průtokové cytometrie, jak ve výzkumu, tak i v klinické diagnostice. Práce shrnuje diagnostiku a sledování akutní lymfoblastické leukemie pomocí průtokové cytometrie.
Klíčová slova: imunofenotypizace, mnohobarevná průtoková cytometrie, akutní leukemie, expresní profilování, minimální reziduální nemoc

Summary
Kalina T., Mejstříková E., Vášková M., Hrušák O.: Immunophenotyping of childhood leukaemias

Immunophenotyping is a standard method used in diagnostics of hematological malignancies. Progress in number of multicolour cytometry as well as new targets identified by genomics bring new possibilities in research and diagnostics. These aspects are summarized in the article.
Key words: immunophenotyping, multicolour flow cytometry, acute leukaemia, expression profiling, minimal residual disease

Trans. Hemat. dnes, 12, 2006, No. 1, p. 20–25.

Úvod

Imunofenotypizace neboli stanovení povrchových znaků buněk pomocí průtokové cytometrie zažívá v poslední dekádě ohromný rozmach, jak po stránce dostupnosti, tak po stránce technické vyspělosti instrumentů a rozvoje technik a postupů uplatňujících se ve výzkumu i v diagnostice. To vede na jednu stranu k možnosti získání množství klinicky relevantních informací, ale zároveň je potřeba tyto informace zpracovat a klinicky správně interpretovat. Záměrem prezentovaného článku je poskytnout přehlednou informaci klinickým i laboratorním hemato-onkologům, která pomůže v uvažování během diagnostického a léčebného procesu. Výhodou průtokové cytometrie je její komplexní přístup a její schopnost dát relevantní odpověď i v situaci, kdy ošetřující lékař má více otázek než jasných odpovědí.

V hemato-onkologii je cytometrie centrální metodou, která řeší otázky diferenciálně diagnostické, typizuje leukemické buňky a má vliv na zařazení do léčebných protokolů. Zatím výzkumně se cytometrie používá ke sledování průběhu léčby (minimální reziduální nemoc) a k hledání nových znaků k predikci prognózy.

Leukemické buňky se vyvíjejí v kostní dřeni, v období plně rozvinuté choroby je nacházíme v periferní krvi nemocných, kudy recirkulují, podobně jako nezralé krevní buňky, ze kterých vycházejí. Schopnost zkoumat imunofenotyp jednotlivých buněk ve směsi buněk jakou je kostní dřeň i periferní krev představuje hlavní výhodu průtokové cytometrie.

V poslední době se do diagnostické průtokové cytometrie dostává možnost mnohobarevné analýzy. Současně přicházejí nové informace z genomiky a proteomiky, které mohou ovlivnit diagnostické možnosti imunofenotypizace.

Popis metody

Průtoková cytometrie používá jako zkoumaný materiál buněčnou suspenzi (např. kostní dřeň, periferní krev či likvor). Metoda vyžaduje čerstvý materiál (živé a neagregované buňky, obvykle do 24 hodin po odběru). K detekci jednotlivých povrchových nebo cytoplazmatických molekul používá monoklonální protilátky konjugované s fluorescenčními značkami. Suspenze buněk je po navázání fluorescenčně značených protilátek nasáta do průtokového cytometru, kde jsou buňky seřazeny do úzkého proudu kapaliny a vysokou rychlostí proudí komorou a jsou ozářeny laserovým paprskem. Laserové světlo excituje na buňkách navázané konjugáty protilátka-fluorescenční značka, které emitují světlo o vlnové délce určené typem fluorescenční značky. Současné průtokové cytometry umožňují zapojením jednoho až tří laserů excitovat 3 až 9 různých fluorescenčních značek zároveň. Pro každou buňku je zapsána kvantita každého fluorescenčního znaku a dva nefluorescenční parametry popisující velikost a granularitu buňky. V jednom vyšetření se obvykle použije simultánní kombinace znaků (v diagnostice leukemií cca 25 znaků, které jsou vyšetřeny v cca 15 oddělených alikvotech zkoumané suspenze), během sekund až minut se změří 20–200 tisíc buněk v každém alikvotu.

Ke grafické analýze těchto 5 až 11 parametrových dat se používá speciální software, kde se provádí výběr („gating") podskupin buněk na základě přítomnosti jednotlivých znaků (obr. 1). Komplexita změřených dat narůstá se zvyšováním počtu parametrů a vhodně zvolená strategie analýzy je velmi důležitá pro interpretaci dat. Interpretaci dat musí provádět vždy zkušený odborník-

-cytometrista. Při počtech nových diagnóz v dětském věku kolem 80 ročně je vhodná koncentrace vyšetření na jedno pracoviště, což je v ČR díky spolupráci všech dětských hematologických center zajištěno.

Cytometrie a akutní lymfoblastická leukemie

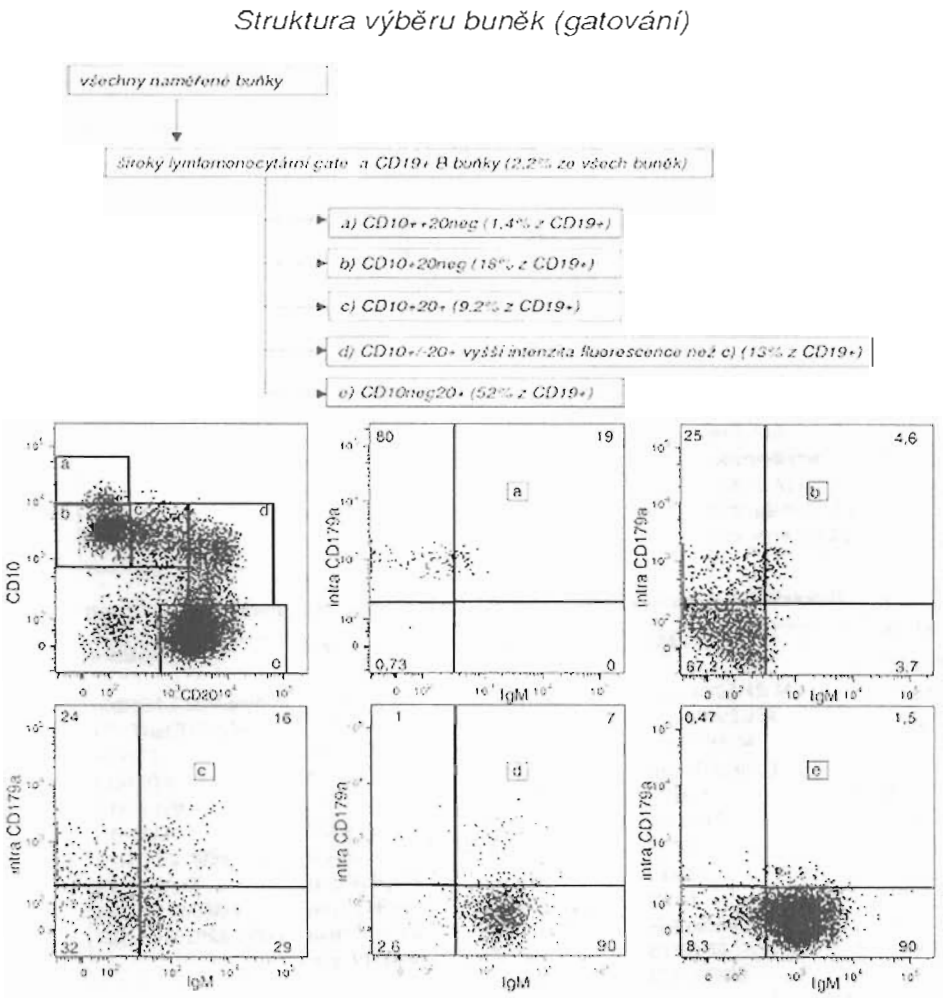
Predikce rizika

Přítomnost jednotlivých molekul na blastech ALL je intenzivně zkoumána z pohledu potenciálních prediktorů úspěšnosti terapie. Velká pozornost byla věnována aberantní expresi myeloidních znaků na buňkách ALL. Jednotlivé studie nalezly nepříznivý nebo žádný prognostický význam exprese těchto molekul při diagnóze. Tato diskrepance byla způsobena určitými nedostatky v designu studií. Různé myeloidní znaky byly často hodnoceny společně (např. CD13 a/nebo CD33) (1, 2). Ne vždy byla dostatečně dlouhá doba sledování (1) nebo se hodnotili pacienti s BCP-ALL a T- ALL společně. Vzhledem k léčebnému úspěchu léčiva založeného na monoklonální protilátce anti-CD33 a cytostatika calicheamicinu u myeloidní leukemie (3) je zajímavý nález korelace CD33 pozitivity u BCP-ALL se špatnou prognózou (4).

Imunofenotypizace při diagnóze

Cytometrie je díky rychlosti provedení vyšetření základním laboratorním vyšetřením vedoucím k diagnóze leukemie. Základní otázky, které diagnostické vyšetření odpovídá jsou:

- Jedná se o leukemii, o nemaligní proliferaci prekurzorů nebo o změnu procentuálního zastoupení subpopulací buněk např. při útlumu v kostní dřeni?
- Vychází leukemie z myeloidní nebo z lymfoidní řady?
- Jedná se o ALL z B-prekurzorů nebo z T-řady?
- Odpovědi na tyto diagnostické otázky mají význam pro potvrzení nebo vyloučení maligního onemocnění a pro zařazení pacienta do léčebného protokolu.



Obr. 1. Příklad použití mnohobarevné kombinace (CD34 FITC/CD10 PE/intra CD179a PC5/CD20 A405/IgM Dy647) znázorňující vývoj B řady v nemaligní KD. Vlevo nahoře jsou zobrazené CD19+ buňky ze širokého lymfomonocytárního gatu podle exprese CD10 a CD20 antigenu. Během vývoje B řady buňky postupně získávají antigen CD20 a ztrácí antigen CD10. Zralé B buňky, které jsou $CD20^{pos}$ a $CD10^{neg}$ nebo jen slabě + pak opouštějí KD. Na dalších obrázcích je pak rozvedena podle jednotlivých subpopulací exprese intracelulárního V preB receptoru (intra CD179a a povrchového IgM). Buňky postupně v průběhu svého vývoje přechodně exprimují VpreB receptor a postupně více buněk exprimuje na povrchu IgM. Osy zobrazují intenzitu fluorescence v grafu jmenovaných znaků.

B-prekurzorová akutní lymfoblastická leukemie (BCP-ALL)

Kostní dřeň je primárním místem vývoje prekurzorů krevních buněk. Sebeobnova a vývoj hematopoetických kmenových buněk, vývoj prekurzorů B lymfocytů a myeloidních buněk probíhá pouze v kostní dřeni, zatímco prekurzory T lymfocytů dozrávají v thymu. Imunofenotyp B-prekurzorových leukemií připomíná imunofenotyp nemaligních prekurzorů B řady. Klíčovým úkolem je tedy nalézt spolehlivé znaky, které odliší normální prekurzory od maligních buněk. Kombinace změn jako např. aberantní exprese, asynchronní exprese, hyperexprese, útlak normálních vývojových stádií či přítomnost prekurzorů v periferní krvi umožní stanovit správný diferenciálně diagnostický závěr.

Imunofenotypová klasifikace BCP-ALL podle návr-

hu skupiny EGIL (5) (tab. 1), kterou používáme v rámci BFM léčebných protokolů i v České republice rozlišuje tři – kategorie:

- proB ALL
- common ALL
- preB ALL

Tato klasifikace vychází z vývojového schématu B lymfopoézy. Ačkoliv imunofenotyp proB ALL je spojen s přestavbou MLL genu a horší prognózou, rozdíl v prognóze či biologických vlastnostech mezi common ALL a preB ALL nebyl prokázán. Zároveň se z pohledu ALL řeší i otázka hybridních leukemií, kdy jednotlivým znakům, je dáno hybridní skóre a pokud je skóre vyšší než 2, je leukemie klasifikována jako akutní hybridní leukemie (AHL – ALL/My+ nebo AML/Ly+).

V následujícím přehledu diskutujeme znaky používané v diagnostice ALL (tab. 2), více informací o jednotlivých CD znacích, pokud není uvedena reference, lze dále získat na webové stránce HLDA (Human Cell Differentiation Molecules Antigens Wokshop).

Liniově specifické znaky pro B-lymfocytární řadu

Pro zařazení vyžadujeme přítomnost alespoň dvou z následujících tří znaků:

CD19

Znak definující příslušnost k B řadě je především znak CD19, který se objevuje záhy po liniovém rozhodnutí společného lymfoidního prekurzoru směrem k B řadě, ve kterém hraje hlavní úlohu transkripční faktor Pax (Nutt, 1998). Tento znak zůstává na povrchu B buněk ve všech stádiích, ztrácí se až na zralé plazmatické buňce. Hraje nepostradatelnou roli jako koreceptor B-buněčného receptoru ve fyziologickém vývoji, aktivaci a diferenciaci B buněk.

CD79a

CD79a (Ig-alfa) je signální molekula, je rovněž B-liniově specifickým znakem, jeho cytoplazmatická exprese dokonce předchází znak CD19 a je první specifickou molekulou oddělující B-prekurzor od společného lymfoidního prekurzoru (7).

CD22

CD22 je specifický znak pro B lymfocytární řadu, v časných fázích vývoje je exprimován jen cytoplazmaticky, k povrchové expresi dochází v normálním vývoji v pozdních stadiích spolu s IgD a CD21 (8, 9). U leukemií z B řady se vyskytuje ve 3/4 případů (obvykle cALL a preB ALL). Molekula je příbuzná molekule CD33. Testuje se terapeutický účinek protilátky proti CD22 konjugované s cytostatikem calicheamicinem u různých CD22^{pos} malignit (10).

Tab. 1. Přehled klasifikace podle návrhu skupiny EGIL.

Kategorie	Kritéria	Podtřída	Kritéria
B prekurzorová	• 2 nebo 3 z následujících: CD19^{poz} (intra)CD79a^{poz} and CD22^{poz} • CD3^{neg} • intraCD3^{neg} • κ^{neg} a λ^{neg}	proB ALL	CD10^{neg} CD20^{neg}
		cALL	CD10^{poz} intraIgM^{neg}
		preB ALL	intraIgM^{poz}
zralá B	• 2 nebo 3 z následujících: CD19^{poz}, (intra)CD79a^{poz} and CD22^{poz} • CD3^{neg} • intraCD3^{neg} • κ^{poz} nebo λ^{poz}	*Bez další subklasifikace*	
T-ALL	• (intra)CD3^{poz} • CD7^{poz}	proT ALL	CD2^{neg} CD5^{neg} CD8^{neg}
		preT ALL	CD2^{poz} a/nebo CD5^{poz} a/nebo CD8^{poz}
		intermediární T- ALL	CD1a^{poz}
		zralá T-ALL	CD3^{poz} CD1a^{neg}
		TCRαβ^{poz} T-ALL	TCRαβ^{poz}
		TCRγδ^{poz} T-ALL	TCRγδ^{poz}

Tab. 2. Panely protilátek, které používáme při diagnostice akutní leukemie v naší laboratoři.

základní panel	T-ALL	BCP-ALL	AML
i-MPO/i-CD79acy/i-3	CD34/38/7	CD10/33/19	Syto-16/33/45/34
i-TdT/mCD7/m3/i-3	i-CD22	CD65/2/HLADR	CD19/56/33/34
i-CD22	CD1a (PE)	CD10/13/19	CD2/7/33/34
CD45/14	TcRγδ/TcRαβ	CD34/38/19	HLA DR/38/33
CD66c/19	CD7/NG2	CD79a	i-CD13
CD4/8/3	*CD64/13 (pokud*	CD22/24	CD33/13
CD20/10/19/34	*je AHL skóre2)*	CD19/NG2	CD41
CD99/7/5/3	Je-li TcRγδ^{poz}	*CD64(pokud*	CD42b
CD15/117/33/34	resp. TcRαβ^{poz},	*je AHL skóre 2)*	CD61
stanovení DNA cyklu	nastaví se Vδ	i-IgM/mCD19	glykoforin A (CD235a)
	resp.Vβ panel	κ/CD19	CD65/33
		λ/CD19	CD33/NG2
		mIgM	

Vysvětlivky:

m	povrchové značení
i	intracelulární značení
AHL skóre	viz text
Vδ panel	protilátky proti jednotlivým typům Vδ řetězců
Vβ panel	protilátky proti jednotlivým Vβ řetězců
Syto-16	fluorescenční barvička, značící buňky obsahující DNA, pomáhá k odlišení drti

Vývojová stadia B-prekurzorů

Další znaky popisují v normální kostní dřeni vývoj (CD10, CD34, CD20, cytoplazmatické TdT, povrchové i cytoplazmatické IgM).

CD10

Znak CD10, tzv. CALLA antigen, byl popsán jako typický znak pro „common

ALL" (11–14). B lymfocyty v periferní krvi tento znak nemají, na rozdíl od většiny B-prekurzorů v kostní dřeni. Leukemické blasty jej nesou ve více než 95 %, velmi často ve vysokých množstvích (tzv. hyperexprese CD10). V periferní krvi jsou B buňky většinou CD10 negativní, jen malá subpopulace je CD10 slabě pozitivní (tzv. transitional B buňky). CD10 hyperexprese v periferní krvi je tedy specifická pro leukemii (obr. 2). Není liniově specifický, vyskytuje se také u granulocytů (15) a ve stadiu kortikálního thymocytu i v rámci vývoje T řady. Část T-ALL je rovněž pozitivní, intenzita je ale zpravidla nižší než u BCP-ALL (16).

CD34

Znak časných progenitorů a kmenových buněk. Není liniově specifický.

Imunoglobulin M (IgM) těžký řetězec

V normální B lymfopoéze je po úspěšné VDJ rekombinaci nejprve exprimován těžký řetězec IgM cytoplazmaticky, poté je spolu s VpreB molekulou vystaven na povrch, což spouští signalizaci umožňující proliferaci prekurzoru s úspěšně přestavěným antigenním receptorem. Klasifikace leukemií používá detekci cytoplazmatického IgM jako marker pro preB ALL, povrchové IgM definuje zralou B-leukemii, která je nejméně obvyklá a liší se prognózou i léčbou.

CD20

se objevuje zhruba paralelně s dokončením přestavby těžkého řetězce a s jeho cytoplazmatickou expresí. U leukemií je často přítomen, někdy asynchronně s hyperexpresí CD10. Zpravidla je pozitivní jen na subpopulaci blastů, což limituje eventuální využití specifické anti-CD20 léčby u BCP-ALL. Zralá B-ALL je typicky vysoce pozitivní.

Terminální deoxy-nukleotidyl transferáza (TdT)

Cytoplazmatická TdT je typicky přítomna u leukemických buněk, v normální kostní dřeni se vyskytuje jen u malé subpopulace prekurzorů (především CD19+34+10+ až ++), lze využít pro odlišení maligní a nemaligní B lymfoproliferace.

CD58

Je znak nespecifický pro B řadu, ale jeho vysoká exprese spolu s vysokou expresí CD10 je charakteristická pro některé leukemie a lze proto využít pro sledování MRN (17).

Aberantní znaky u B-lymfocytární řady

Znaky omezené v normálním vývoji pro jiné řady, ale vyskytující se často na leukemických buňkách (18).

CD66c (KOR-SA3544 antigen)

Aktivační znak granulocytů, u nemaligních B prekurzorů se nevyskytuje, proto jej můžeme na B prekurzorech považovat za znak malignity (6).

CD33, CD13

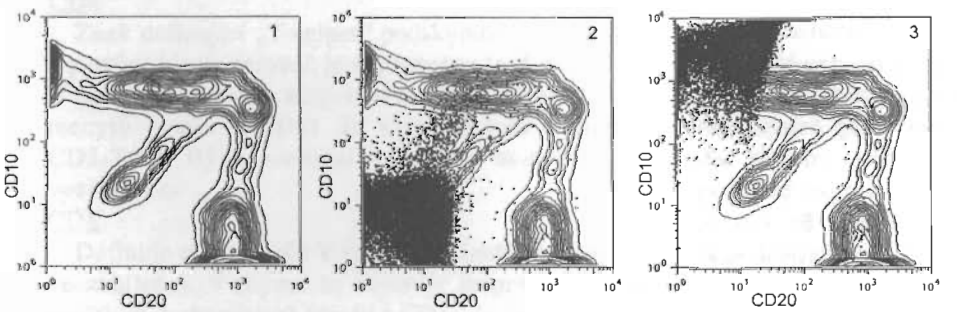
Znak monocytů a granulocytů, aberantně se vyskytuje na BCP-ALL.

CD15, CD65

Znak granulocytů, aberantně se vyskytuje na BCP-ALL.

NG2

Molekula chondroitin sulfátu, ve fyziologické hematopoéze tato molekula není exprimována. NG2 molekula se aberantně vyskytuje u leukemií (ALL i AML) s přestavbou genu MLL (obr. 3).



Obr. 2. Obrázek vlevo ukazuje normální vývoj B řady podle exprese antigenu CD20 a CD10, uprostřed na pozadí normální KD (kontury) je zobrazena CD10 negativní proB ALL a vpravo cALL, kde u většiny blastů je hyperexprese CD10. Všechny grafy jsou z gatu $CD19^{pos}$ buněk s odpovídajícími optickými vlastnostmi.

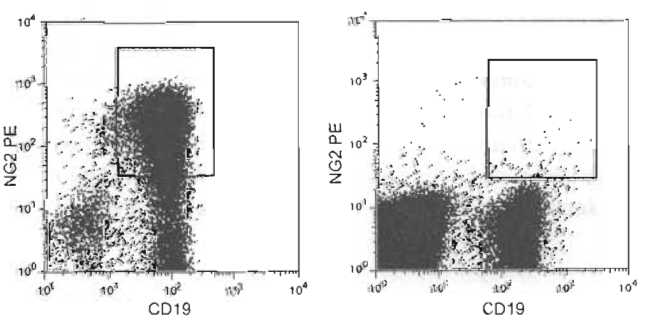
T-akutní lymfoblastická leukemie (T-ALL)

Primárním místem T lymfopoézy je thymus, leukemické buňky však cirkulují v periferní krvi a kostní dřeni. Přítomnost nezralých T lymfocytů v periferní krvi či kostní dřeni tedy ukazuje na diagnózu T-ALL. Typickou známkou malignity u T- ALL je koexprese CD7 a CD5 na CD3 negativních buňkách nebo koexprese CD4 a CD8 v periferní krvi nebo kostní dřeni.

Liniově specifické znaky pro T-lymfocytární řadu

CD7

CD7 je glykoprotein exprimovaný na zralých T lymfocytech, NK buňkách a thymocytech, ale i na některých nediferencovaných progenitorech. Je to senzitivní mar-



Obr. 3. Aberantní exprese NG2 na BCP ALL blastech u pacientky kojeneckou ALL s prokázaným fúzním genem MLL/AF4 (vlevo). Vpravo obrázek u téže pacientky 2 roky od diagnózy onemocnění v kompletní remisi a negativním fúzním genem MLL/AF4. Je zřetelné nenulové pozadí v regenerující KD, které limituje využití při detekci MRN.

ker T-ALL, ale není specifický, jelikož jej exprimují také některé typy myeloidních leukemií. Malá část nezralých progenitorů ve fyziologické KD je CD7+33+.

CD3

CD3 je součástí komplexu T-buněčného receptoru (TCR). CD3 je exprimován v cytoplazmě časnými thymocyty. Cytoplazmatická exprese CD3 je základním znakem, který zařazuje buňky k T řadě.

CD5 a CD2

Znaky CD2 a CD5 jsou exprimovány na thymocytech a zralých T lymfocytech. CD5 také definuje podskupinu B lymfocytů (tzv. T independentní B1 lymfocyty). CD2 nalézáme také na NK buňkách. Oba antigeny jsou exprimovány na více než 90 % T-ALL a používáme je jako podpůrné znaky. CD2 je aberantně exprimována u části AML, typicky u AML M3v (6).

Znaky definující vývojová stadia a funkční podskupiny

CD4

Znak definující „T-helper" podskupinu T lymfocytů v periferní krvi, zároveň je exprimován také monocyty. V thymu se objevuje nejprve u dvojitě pozitivních thymocytů (spolu s CD8). Je koreceptorem komplexu CD3-TCR. Bývá pozitivní u AML s monocytárním vyzráváním.

CD8

Definuje cytotoxické T lymfocyty (antigen specifické zabíječe). V thymu se objevuje nejprve u dvojitě pozitivních thymocytů (spolu s CD4). Je koreceptorem komplexu CD3-TCR.

TCRαß, TCRγδ

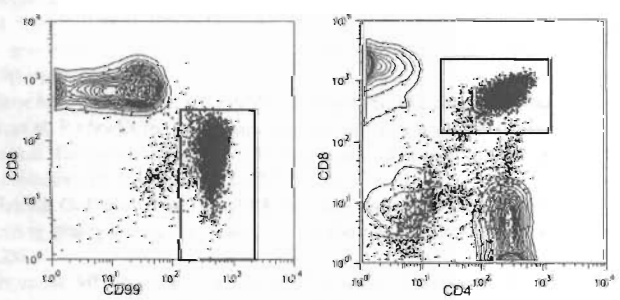
T-buněčný receptor, je základním nástrojem T buněk při specifickém rozeznávání antigenů.

CD1a

Je znak exprimovaný nezralými T lymfocyty v thymu. Definuje intermediární T-ALL (19).

CD99

CD99 je exprimováno na řadě hematopoetických buněk i progenitorů, v případě thymocytů a T-ALL je exprese kvantitativně zvýšená (20).



Obr. 4. Srovnání imunofenotypu nemaligních T lymfocytů (v konturách) a intermediární T-ALL (zvýrazněná populace obdélníkem). Na levém obrázku (kombinace CD99/7/5/3, gate $CD7^{poz}$ bez hyperexprese $CD5^{poz}$ pro kontury a $CD7^{++5}$ slabě poz $CD99^{++}$) typicky vysoká exprese CD99 a nízká až negativní povrchová CD3. Na pravém obrázku koexprese CD4 a CD8 z kombinace CD4/8/3.

Sledování průběhu léčby

Současný přístup k sestavování léčebných protokolů ALL používá strategii terapie šité na míru rizika selhání léčby. Jedním z parametrů je odpověď na léčbu. Již přes dvě dekády se hodnotí rychlost redukce počtu blastů při prednisonové předfázi. Nyní se zavádí v některých protokolech detekce minimální reziduální nemoci (MRN) v průběhu terapie jako kritérium pro přeřazení mezi rizikovými skupinami. K detekci MRN se používá metoda kvantitativní PCR a zatím pouze výzkumně také průtoková cytometrie. Ne docela vyřešeným problémem aplikace cytometrie ve sledování MRN je značná podobnost leukemických buněk a regenerujících B-lymfocytů, které v některých časových bodech terapie tvoří významné pozadí. Pro klinicky použitelné měření MRN je třeba detekovat leukemickou buňku na pozadí 1 000 až 10 000 buněk. Zlepšení specifity očekáváme od zavedení mnohobarevných protokolů (více než 4 fluorescenční znaky).

Nové prediktory

Studie expresního profilování (EP, expression profiling, gene chips, microarrays) přinášejí data o expresi desetitisíců genů. Jejich vyhodnocením je možné hledat skupiny (například ALL pacientů), které mají podobné expresní vzorce („expression patterns"), tzn. je pro ně charakteristická exprese některých genů, nebo hledat geny, které jsou typické pro jednotlivé skupiny (a vztahovat je k biologii, leukemogenezi a prognóze) (21). Odhady počtu genů, které jsou klíčové pro identifikaci určité skupiny pacientů (např. genotypové a rizikové), se liší. Někteří autoři předpokládají, že genů určujících např. genotypové poskupiny nemusí být více než 20 (např. Downing (22). Takový počet genů, či odpovídajících proteinů je potom možné studovat metodami molekulární biologie (RT-PCR) či průtokové cytometrie. Průtoková cytometrie má výhodu simultánního stanovení více molekul na jedné buňce a relativní nenáročnosti na kvalitu, množství vzorku a na provedení. V naší studii využíváme dat z EP k nalezení těchto klíčových molekul. Podařilo se nám nalézt dvě molekuly (CD44 a CD27) předpovídající přítomnost fúzního genu TEL/AML1 (23). Chen et al. (17) navrhli na základě dat z EP detekci molekuly CD58 při vyšetřování minimální residuální nemoci. Stále se také intenzivně pracuje na standardizaci EP pro diagnostické účely, ale jako racionálnější se jeví využití této metody pro výzkumné účely a pro nalezení nových diagnostických a prognostických znaků.

Závěr

Vhodné a metodicky dobře provedené použití průtokové cytometrie má významnou roli při diagnostice a diferenciální diagnostice leukemie. Imunofenotypizací odlišíme nemaligní prekurzory od leukemických buněk v aspirátu kostní dřeně či periferní krve. Na základě liniově specific-

kých znaků stanovíme typ leukemických buněk. Další znaky definují stupeň zralosti a můžou předpovědět i riziko selhání terapie. Mnohobarevná průtoková cytometrie je výzkumně používána k vyhledávání a stanovení prediktivní hodnoty dalších molekul a ke sledování MRN.

Seznam použitých zkratek

KD	kostní dřeň
BCP	B cell precursor
MRN	minimální reziduální nemoc
TCR	T cell receptor
ALL	akutní lymfoblastická leukemie
AML	akutní myeloidní leukemie
MLL	mixed lineage leukemia

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