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Exprese WT1 a jeho sestřihových variant v myeloidních leukémiích

Expression of WT1 and its isoforms in myeloid leukemia

Dizertační práce

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Poděkování

Děkuji své školitelce RNDr. Janě Moravcové, CSc. za převzetí vedení mého doktorandského studia od r. 2010, za její laskavý přístup, trpělivost a předávání praktických zkušeností pro práci v laboratoři i pro psaní odborných textů. Mé poděkování patří i Mgr. Kateřině Machové-Polákové, PhD., mé původní školitelce, za prvotní období práce na problematice mikroRNA. Cítím se velmi zavázána RNDr. Kateřině Kuželové, PhD., vedoucí oddělení buněčné biochemie ÚHKT, která mi umožnila po změně školitele na tomto oddělení dokončit dizertační práci a pomohla mi i kritickou revizí publikací a finálního textu této práce. Je mou milou povinností poděkovat i svým spolupracovníkům a spolupracovnicím z ÚHKT za konzultace a podporu. Děkuji také své rodině a příteli, kteří mě po celou dobu studia podporovali.

Abstrakt

Myeloidní leukémie představují maligní onemocnění charakterizovaná expanzí myeloidní krevní řady. Zatímco v případě chronické myeloidní leukémie (CML) je hlavní příčina onemocnění známa – t(9;22) a aktivita fuzního produktu BCR-ABL, s akutní myeloidní leukémií (AML) je spojena řada translokací a mutací. Cílem této práce je přispět ke zlepšení monitorování pacientů s myeloidními leukémiemi cestou detailního poznání exprese panleukemického markeru, genu Wilmsova tumoru (wt1). Prognostický význam celkové exprese wt1 je znám u pacientů s AML, nebyl ale jednoznačně potvrzen pro pacienty s CML. O expresi jednotlivých izoforem (celkem více než 36 proteinových produktů) existují velmi kusé informace v případě obou diagnóz i zdravé hematopoézy.

Většina této práce je soustředěna na CML, AML je věnována pouze omezená část. V rámci první části jsme prokázali vysoký prognostický význam celkové exprese mRNA wt1 pro pacienty s CML. Statistická hodnocení ukázala kritické hladiny wt1, které umožňují v porovnání s rutinně používaným markerem, hladinou transkriptu bcr-abl, upřesnit prognózu pacientů, kteří neodpovídají optimálně na léčbu. Dále jsme navrhli a optimalizovali reverzně transkriptázové PCR v reálném čase pro kvantifikaci vybraných variant wt1 (kombinace sestřihu exonu 5 a KTS sekvence, swt1 vs. wt1 plné délky). Zjistili jsme, že zatímco swt1 je exprimována ve velmi nízkých hladinách u pacientů s CML i AML a nelze ji proto považovat za kandidáta na prognostický marker, vysoké hladiny transkriptů variant -5/+KTS u pacientů s CML a +5/-KTS u pacientů s AML se jeví být novými, v porovnání s celkovou expresí wt1, specifičtějšími rizikovými markery. Význam sledování poklesu celkové hladiny mRNA wt1 jsme potvrdili také *in vitro* po ošetření primárních leukocytů léčivy pro sledování citlivosti k léčbě. Wt1 jako marker v tomto smyslu jsme aplikovali v rámci paralelní studie miRNA v CML, kde naše data spolu s literaturou naznačila existenci zpětnovazebné regulační smyčky mezi BCR-ABL a miR-451, mechanizmus potenciálně velmi významný pro udržení leukemického charakteru buněk.

Závěrem je možno shrnout, že celková hladina transkriptu wt1 může sloužit jako významný marker dalšího vývoje pacientů s CML, kteří neodpovídají dle současných kritérií optimálně na léčbu a u kterých rutinně používaný bcr-abl svůj význam často ztrácí. Vybrané varianty představují kandidátní rizikové markery specifické pro daný typ myeloidní leukémie. *In vitro* pomáhá hladina mRNA wt1 odhadnout účinnost působení léčiv a charakterizovat stav buněk.

Abstract

Myeloid leukemias include malignant diseases characterized by clonal expansion of the myeloid cell lineage. While in case of chronic myeloid leukemia (CML), the main cause of the disease has already been identified – t(9;22) and the aktivity of the fusion product of the translocation BCR-ABL, acute myeloid leukemia (AML) has been associated with plenty of different translocations and mutations. The aim of this work was to contribute to the improvement of monitoring of patients with myeloid leukemias via detailed study of the panleukemic marker Wilms tumor gene 1 (wt1) expression. Prognostic value of wt1 expression has been proved for AML patients, however, it has not yet been confirmed for CML patients. Expression of different wt1 variants (more then 36 protein products) is known very poorly in both, AML and CML as well as in normal hematopoiesis. Most of the study is focused on CML, only limited parts are dedicated to AML.

In the first part of the work, we clearly proved prognostic value of total wt1 mRNA expression for CML patients. Statistical evaluations revealed critical wt1 values which enable to specify prognosis of patients responding non-optimally to imatinib. Bcr-abl looses much of its prognostic value in these patients. Further, we have designed and optimized PCRs for selected wt1 variants (combination of exon 5 and KTS sequence splicing, swt1 vs. full lenght wt1). We found out, that swt1 was expressed at very low levels in CML and AML patients and it thus could not be considered a candidate marker. On the other hand, -5/+KTS and +5/-KTS seem to be novel candidates on prognostic markers for CML and AML patients, respectively. Expression of wt1 variants might serve as a more specific markers for given diagnosis as compared to total wt1 expression. We have also confirmed a correlation between wt1 mRNA expression and sensitivity to treatment *in vitro*. We applied those *in vitro* cultivations with BCR-ABL inhibitors in a paralel study of miRNA expression in CML and we identified negative-feed back regulatory relationship between miR-451 and BCR-ABL. This mechanism is potentially highly important for maintenance of the leukemic cell phenotype.

In conclusion, total wt1 mRNA expression can be useful as an additional molecular marker to bcr-abl to predict further disease course in CML patients who do not respond optimally to imatinib. We identified selected wt1 variants as novel candidate markers specific for different leukemia types. Wt1 mRNA expression measured *in vitro* after treating patients cells with kinase inhibitors helps in characterization of cell status and sensitivity to the drug.

Obsah

Poděkování 1
Abstrakt 2
Abstract 3
Úvod 6
1. Literární přehled 7
1.1. Myeloidní leukémie 7
1.1.1. Chronická myeloidní leukémie 7
1.1.1.1. Klinická charakteristika, časový proběh a epidemiologie CML 7
1.1.1.2. Biologie CML 8
1.1.1.2.1. Ph translokace, gen a protein BCR-ABL 8
1.1.1.2.2. Transformace CML buněk 9
1.1.1.2.3. Mechanizmy progrese CML 10
1.1.1.3. Současné léčebné přístupy k CML 11
1.1.1.3.1. Transplantace kmenových buněk 11
1.1.1.3.2. Léčba medikamentózní 11
1.1.1.3.3. Kurativní medikamentózní léčba CML? 13
1.1.1.3.4. Rezistence k léčbě 13
1.1.1.3.5. Odpovědi na léčbu a možnosti jejich predikce 14
1.1.2. Akutní myeloidní leukémie 16
1.1.2.1. Klinická charakteristika, časový průběh a epidemiologie AML 16
1.1.2.2. Biologie a klasifikace AML 16
1.1.2.3. Léčba AML 19
1.1.2.4. Monitorování pacientů s AML 19
1.2. Gen a protein Wilmsova tumoru (WT1) 20
1.2.1. Struktura genu wt1 a regulace jeho exprese 20
1.2.2. Struktura a funkce proteinu WT1, role jednotlivých izoforem 20
1.2.3. Význam WT1 v hematopoéze 22
1.2.4. Význam WT1 v onkologii a hematoonkologii 23
2. Cíle **25**
3. Výčet použitých metod **26**
4. Výsledky – komentář k publikacím **27**
4.1. Exprese mRNA wt1 v celkových leukocytech pacientů s myeloidními leukémiemi 27
4.1.1. Celková exprese mRNA wt1 u pacientů s CML (publikace č. 1) 27
4.1.2. Exprese sestřihových variant wt1 v myeloidních leukémiích 28

4.1.2.1. Exprese mRNA čtyř hlavních sestřihových variant wt1 v myeloidních leukémiích (publikace č. 2) 29
4.1.2.2. Exprese mRNA swt1 v myeloidních leukémiích (publikace č. 3) 30
4.2. Exprese mRNA wt1 po ošetření bunek inhibitory in vitro – aplikace wt1 (publikace č. 4, 5) 31
4.3. Exprese izoforem WT1 na proteinové úrovni 33
5. Publikace 34
Publikace č. 1 35
Publikace č. 2 58
Publikace č. 3 65
Publikace č. 4 68
Publikace č. 5 81
Publikace č. 6 85
6. Diskuse 106
7. Shrnutí 112
8. Seznam použité literatury 113
9. Seznam publikací 126
10. Konferenční příspěvky se vztahem k dizertační práci 127

Úvod

Ústav hematologie a krevní transfuze poskytuje léčebnou péči a provádí výzkum řady hemato-onkologických diagnóz. Pracovní skupina Dr. Moravcové se tu dlouhodobě zabývá studiem chronické myeloidní leukémie. V rámci grantových projektů byla v laboratoři Dr. Moravcové vyvinuta metodika pro monitorování exprese bcr-abl na úrovni mRNA, které je dnes prováděno rutinně. Monitorování hladiny transkriptu bcr-abl bylo původně zavedeno pro časnou detekci relapsu po transplantaci kostní dřeně, která byla v minulosti hlavní a prakticky jedinou léčebnou možností. Pozdě zjištěné relapsy byly pro pacienty fatální. V současné době je lékem první volby imatinib, selektivní inhibitor BCR-ABL tyrozinové kinázy, prototyp molekulárně cílené terapie. Ve fázi vývoje nebo dokonce v klinické praxi jsou inhibitory dalších generací. K progresi onemocnění dochází při léčbě kinázovými inhibitory velmi zřídka. K hematologickým relapsům dochází, v porovnání s relapsy po transplantaci kostní dřeně jsou však tyto relapsy pozvolnější. Zatímco transplantace směřuje k vymýcení BCR-ABL pozitivních buněk a jejich nahrazení zdravými buňkami, v případě kinázových inhibitorů jde o trvalé potlačování kinázové aktivity BCR-ABL. Takový typ léčby vyvíjí na buňky pacientů selekční tlak, v důsledku kterého může být selektován rezistentní klon. Spektrum mechanizmů rezistence k léčbě je široké, v řadě případů se dle současné literatury objevuje rezistence částečně až úplně nezávislá na aktivitě BCR-ABL. Grantové projekty skupiny Dr. Moravcové jsou proto soustředěny na hledání nových pomocných markerů vedle bcr-abl, které by pomohly určit prognózu pacienta a včas odhalit rozvíjející se rezistenci a tedy blížící se relaps i v případech, že se onemocnění stává více či méně nezávislým na BCR-ABL. Tyto markery by umožnily včasnou změnu léčby a přispěly k prodloužení bezpříznakového období nemoci a udržely vysokou kvalitu života pacientů. Individualizace léčby, k níž by nové markery přispěly, by umožnila aplikaci agresivních širokospektrých inhibitorů pouze u pacientů, kteří z takové léčby budou profitovat, a naopak by snížila zátěž pacientů, kteří mohou být dlouhodobě v remisi při užívání méně agresivních léčiv.

1. Literární přehled

1.1 Myeloidní leukémie

Myeloidní leukémie jsou maligní onemocnění, která se vyznačují expanzí myeloidní krevní řady. Zahrnují chronickou a akutní myeloidní leukémii. Rozdělení je především historického charakteru, konečná stádia chronické leukémie připomínají formu akutní.

1.1.1 Chronická myeloidní leukémie

1.1.1.1. Klinická charakteristika, časový průběh a epidemiologie CML

Chronická myeloidní leukémie (CML) je myeloproliferativní onemocnění, které vzniká v důsledku maligní transformace kmenové nebo primitivní progenitorové hematopoetické buňky. Bez cílené léčby probíhá CML ve třech stádiích: (1) chronická fáze (CP), fáze „preneoplastická“ charakterizovaná silnou expanzí myeloidní řady, buňky jsou většinou plně diferencované; (2) akcelerovaná fáze (AP) a (3) blastická krize (BC). AP je charakterizována alespoň jedním z následujících znaků: 10 až 30% blastů v periferní krvi nebo kostní dřeni, 20 a více % blastů a promyelocytů v periferní krvi nebo kostní dřeni, 20 a více % bazofilů v periferní krvi nebo kostní dřeni, trombocytopenie méně než $100x10^9$/l krve bez vztahu k léčbě, progresivní splenomegalie, přídatné chromozomální aberace. V BC má pacient více než 30% blastů v periferní krvi nebo kostní dřeni. AP a BC se souhrnně označují jako akutní fáze (připomínají akutní leukémii). U 60 % případů mají blasty myeloidní morfologii a exprimují myeloidní markery, v ostatních případech je fenotyp podobný akutní lymfoidní leukémii, vzácně se může jednat o megakaryoblasty a erytroblasty. V chronické fázi pacient dobře odpovídá na léčbu, akutní fáze, zvláště pak blastická krize jsou terapeuticky těžko zvladatelné. Do nedávné minulosti končila CML smrtí pacienta v blastické krizi. Při současné cílené léčbě (viz Léčba) je progrese onemocnění poměrně vzácný jev.

CML je onemocnění značně heterogenní, její agresivita a příznaky se liší u jednotlivých pacientů. K běžným příznakům patří jinak nevysvětlitelné horečky, ztráta váhy, zvýšená únava, bolesti kloubů a kostí atd.

Incidence CML se uvádí mezi 1 až 2 případy na sto tisíc lidí. V rámci všech leukémií tvoří CML asi 15% až 20%. Výskyt onemocnění roste s věkem, maxima dosahuje v šesté věkové dekádě (Meyer a spol. 2002, Adam a spol. 2001).

1.1.1.2. Biologie CML

1.1.1.2.1. Ph translokace, gen a protein BCR-ABL

Příčinou rozvoje CML je reciproká translokace mezi chromozómy 9 a 22 (Ph translokace), jejímž produktem je tzv. Philadelphský (Ph) chromozóm (zkrácený chromozóm 22), cytogenetický marker CML (Nowell a spol., 1960, Rowley, 1973). Ph chromozóm je detekován u 95% pacientů s CML, u 5% pacientů se vyskytuje komplexní translokace, která zahrnuje ještě další chromozómy (Adam, 2001). Jediným dokázaným vnějším faktorem pro vznik Ph translokace je ionizující záření, význam mohou mít také duplikony (76kb) v blízkosti genů bcr a abl (Salgio a spol. 2002) nebo přiblížení obou genů v jádře hematopoetických prekurzorů při přechodu z S do G2 a během G2 fáze buněčného cyklu (Neves et al. 1999).

Ve zlomových oblastech translokace se nacházejí dva geny – abl a bcr. Gen abl (9q,34, 225 kb) kóduje nereceptorovou tyrozinovou kinázu, lidský homolog v-abl onkogenu Abelsonova viru způsobujícího leukémii u myší. Jaderný ABL je významný proapoptotický protein, který hraje klíčovou roli v buněčné odpovědi na genotoxický stres, podílí se na spuštění apoptózy v buňkách vystavených ionizujícímu nebo gama záření (Agami a spol. 1999, Yuan a spol. 1999). Gen bcr kóduje Ser/Thr kinázu (160 kDa). Jednou z nejdůležitějších částí tohoto proteinu je oligomerizační doména s coiled-coil motivem, která je kódována prvním bcr exonem a zajišťuje oligomerizaci BCR jakož i BCR-ABL (viz dále) proteinu (McWhirter a spol., 1993).

Na Ph chromozómu vzniká Ph translokací fúzní gen bcr-abl, hlavní příčina rozvoje CML. Za počátečními exony genu bcr na 22q11 dochází k připojení téměř celého genu abl z 9q34. Zlomové oblasti jsou lokalizovány téměř výlučně v oblastech intronů. Zlom v genu abl nastává na jeho 5´ konci před 1b, za 1a, nebo nejčastěji mezi těmito dvěma exony (celkem více než 300 kb, Melo, 1996). Výsledný transkript bcr-abl je však vždy sestřižen tak, že sekvence abl v bcr-abl začíná exonem 2 (Morris a spol., 1991). Zlomové oblasti uvnitř genu bcr spadají při CML do jedné ze dvou oblastí, tzv. „break point cluster regions“: (1) Více než 90%pacientů s CML má zlom v oblasti zvané „major break point cluster region“ (M-bcr) mezi exony 12 až 16 (označováno také jako b1 až b5, celkem 5,8 kb). Alternativním sestřihem mohou vznikat fúzní transkripty se spojením e13a2 (b2a2) nebo e14a2 (b3a2). Obě mRNA jsou potom překládány do proteinu p210 BCR-ABL (Heisterkamp a spol., 1985), který je vedle CML spojován také s ALL, AML a vzácně dalšími onemocněními. (2) U pacientů s CML se zřídka vyskytuje také zlom v oblasti „minor break point cluster region“

(m-bcr) mezi alternativními exony e2´ a e2 (celkem 54,4 kb). Vzniklá e1a2 mRNA se překládá do proteinu p190BCR-ABL (Hermans a spol., 1987), někdy označovaného také jako p185. Vzácně se vyskytují případy CML s jinými spojeními bcr-abl, jako např. b2a3 (Moravcová a spol., 2005).

Přítomnost fúzního transkriptu bcr-abl byla prokázána i v krvi zdravých jedinců (Bose a spol., 1998). Předpokládá se, že v takových případech se buď imunitnímu systému podaří včas maligní klon vymýtit, nebo se jedná o buňku v terminálním stádiu diferenciace, která se již dále nedělí a nemůže z ní vzniknout maligní klon.

Pro úplnost je třeba zmínit, že Ph translokací vzniká také nový 9q+ chromozóm, na němž vzniká další fúzní gen abl-bcr. Přítomnost mRNA abl-bcr je nalézána přibližně u 70% pacientů s CML (Melo a spol., 1993).

1.1.1.2.2 Transformace CML buněk - působení proteinu BCR-ABL

BCR-ABL funguje v buňkách jako tyrozinová kináza, která se na rozdíl od ABL vyskytuje výhradně v cytoplazmě. V důsledku ztráty prvního exonu a fúze s BCR je BCR-ABL kináza konstitutivně aktivní, zcela neregulovaná. BCR-ABL vstupuje do regulace základních buněčných drah udržujících rovnováhu mezi buněčným přežitím a smrtí:

MAP-kinázová (mitogen-activated protein kinase) dráha

MAP-kinázová (Ras/Raf/MEK/ERK) dráha je centrální dráhou signální transdukce, která přenáší signál z různých povrchových receptorů buňky do buněčného jádra. Její stimulace vede k proliferaci buněk a inhibici apoptózy. Ras protein je membránová GTPáza, která se účastní v různých signálních drahách, mimo jiné právě v Raf/MEK/ERK. V řadě lidských maligních onemocnění se lze setkat s jejími mutacemi, v CML jsou však vzácné. BCR-ABL spolu s proteiny Grb2, Shc a Sos stabilizuje aktivní formu Ras proteinu (Skorski a spol., 1994). K deregulaci Ras dráhy v BCR-ABL+ buňkách přispívá zřejmě také nízká hladina produktu genu NF1, neurofibrominu, který funguje jako negativní regulátor Ras dráhy (Jongen-Lavrenic et al. 2005). Dále BCR-ABL aktivuje expresi Raf1 (Salomoni et al. 1998). Na úrovních níže od BCR-ABL je aktivován přes Rap-1 také B-Raf (Mizuchi et al. 2005).

JAK/STAT dráha

Signální dráha JAK/STAT přímo spojuje aktivaci cytokinových receptorů s genovou expresí. Skládá se ze tří rodin proteinů - JAK (Janus family of tyrosine kinases), STAT (signal transducers and activators of transcription) a CIS/SOCS (cytokine-induced SH2-containing proteins/supressors of cytokine signaling) rodiny, která tlumí aktivitu JAK/STAT

dráhy. Aktivovaná JAK kináza fosforyluje STAT na specifickém tyrozinu, následuje dimerizace STAT, zvýšení jeho stability a přesun do jádra, kde působí jako aktivátor transkripce. STAT faktory mohou být v CML buňkách aktivovány nezávisle na JAK kinázách proteinem BCR-ABL (Illaria a spol., 1996).

Fosfatidylinositol-3kinázová dráha

Fosfatidylinositolkinázy (PIK) představují rodinu proteinů katalyzujících přenos γ-fosfátu z ATP na fosfoinositidy. Aktivita PI3K je spojena především s cytoskeletární organizací, buněčným dělením a inhibicí apoptózy. PI3K se skládá z katalytické podjednotky p110 se serin/threoninovou kinázovou aktivitou a regulační p85 podjednotky, která stabilizuje a inhibuje p110. PI3K fosforyluje fosfoinositidy, které aktivují PDK fosforylující Akt (proteinkináza B, PKB). Pro proliferaci i ochranu BCR-ABL pozitivních buněk proti apoptóze je aktivita PI3 kinázové dráhy podmínkou (Shet et al. 2002). BCR-ABL nepřímo přes podjednotku p85 interaguje s PI3K. Ta je pak v komplexu s dalšími proteiny, jako je Gab2, aktivována (Sattler et al. 2002). Mimo to BCR-ABL inhibuje expresi SHIP1 fosfatázy, která je jednou z fosfatáz inhibujících aktivitu Akt kinázy (Sattler et al. 1999).

1.1.1.2.3 Mechanizmy progrese CML

Mechanizmus progrese CML do akutních fází není zcela jasný. Předpokládá se, že ke vzniku blastické krize stačí patrně samotné působení BCR-ABL proteinu mechanizmem alterované regulace genů závislým na dávce BCR-ABL (Calabretta a spol., 2004). Víme, že BCR-ABL prokazatelně navozuje genetickou nestabilitu buněk. Přestože je za normálních podmínek protein BCR-ABL výhradně cytoplazmatický, ukázalo se, že v leukemických buňkách vystavených genotoxickému stresu translokuje do jádra, kde narušuje opravu DNA (Dierov et al. 2004). Existuje také několik studií poukazujících na vztah mezi expresí BCR-ABL a aktivitou nebo expresí proteinů účastnících se oprav DNA, zvláště dvouřetězcových zlomů (Deutsch et al. 2001, Deutsch et al. 2003). Sekundární cytogenetické a molekulární změny jsou nalézány během progrese onemocnění směrem k blastické krizi u 60 až 80 % pacientů. V chronické fázi se tyto změny vyskytují pouze přibližně v 5% (Bacher et al. 2004). Chromozomální aberace zahrnují chromozómy 8, 17, 19 a 22. Mezi nejčastější patří duplikace Ph chromozómu a trizomie chromozómu 8 a naopak spíše výjimečně se objevuje ztráta 7. chromozómu (Bacher et al. 2004). Z molekulárních změn jsou u myeloidní blastické krize časté mutace v oblasti tumor supresorového genu p53 (Brusa et al. 2003). Přídatné změny jsou patrně současně příčinou i následkem probíhající progrese.

1.1.1.3 Současné léčebné přístupy k CML

Léčbu CML lze v zásadě rozdělit do dvou skupin - transplantaci a léčbu medikamentózní.

1.1.1.3.1 Transplantace kmenových buněk (TKB)

Pod tímto pojmem rozumíme přenos kmenových buněk zdravého dárce do oběhu příjemce, kde mají za úkol obnovit krvetvorbu příjemce, předtím chemoterapeuticky úplně nebo částečně zničenou. V klinické praxi jsou využívány tři typy transplantací - alogenní příbuzenská nebo nepříbuzenská, syngenní nebo autologní. Alogenní transplantace je stále jedinou metodou, která může vést k trvalému vyléčení. Riziko relapsu činí přibližně 20%. Přestože je TKB jedinou skutečně kurativní metodou, studie ukázaly, že s ohledem na riziko transplantační mortality a morbidity je výhodnější léčba medikamentózní (Hehlmann a spol., 2007). S výjimkou velmi mladých pacientů se tak dnes dává před transplantací přednost imatinibu (viz dále).

1.1.1.3.2 Léčba medikamentózní

Chemoterapie a interferon α (IFN)

V současné době se z chemoterapeutik v léčbě CML používá už pouze hydroxyurea, inhibitor ribonukleotidreduktázy, tedy syntézy DNA (Hehlmann et al. 1993), a to v okamžiku diagnózy ke snížení počtu bílých krvinek pacientů.

Monoterapie interferonem alfa (IFN) dokáže navodit hematologickou až cytogenetickou odpověď u pacientů s CML (Talpaz et al. 1986). Protinádorový účinek IFN je dán kombinací přímého antiproliferačního účinku a nepřímých účinků zprostředkovaných imunitním systémem. IFN působí stimulačně na NK-buňky a makrofágy a zároveň stimuluje expresi MHC gp I, nádorově specifických antigenů a adhezivních molekul nádorových buněk. Uvádí se také, že IFN má přímé cytostatické efekty na nádorové buňky (Jonasch a spol., 2001). Mezi nevýhody IFN patřily především vedlejší účinky. Randomizovaná studie IRIS srovnávající interferon alfa s imatinibem (viz dále; O´Brien a spol., 2003) ukázala na jasnou výhodu pokročilých léků – cílených kinázových inhibitorů. IFN se tak dnes používá pouze v případech, kdy nelze použít cílenou léčbu.

Molekulárně cílená terapie (MCT)

Principem MCT je specifická inhibice některé z molekul účastných na vzniku, resp. rozvoji CML. Většina pacientů je v současné době léčena právě tímto typem léků.

Imatinib mesylát (STI 571, obchodní názvy Glivec, v USA Gleevec, dále pouze imatinib) je selektivní inhibitor ABL, BCR-ABL, PDGFR, c-Kit a patrně také ARG. Imatinib kompetuje s ATP o vazebné místo v molekule kinázy (Buchdunger et al 2000, Okuda et al. 2001). Na buněčných liniích bylo ukázáno, že imatinib může navozovat apoptózu a/nebo diferenciaci leukemických buněk (např. Jacquel et al. 2003). Indukovaná autofágie může vést k zastavení buněčného cyklu a apoptóze, nebo může naopak rakovinným buňkám pomáhat recyklací proteinů a organel zničených léčbou (Ertmer et al. 2007). V léčbě CML byl imatinib poprvé použit v roce 1998, dnes je používán jako léčebný prostředek první volby. V porovnání s IFN imatinib navozuje signikantně více kompletních hematologických a cytogenetických odpovědí (O´Brien et al. 2003). Dosavadní studie ukazují, že kinázové inhibitory druhé generace mohou v první linii imatinib předčít, nicméně vzhledem ke dlouhé zkušenosti s imatinibem a jeho bezpečností dává většina lékařů stále přednost imatinibu.

Druhá generace MCT zahrnuje dasatinib a nilotinib:

Nilotinib (Tasigna, AMN107) je odvozen od imatinibu, je vysoce selektivní pro Abl1 a ve srovnání s imatinibem je 10-50x účinnější (Weisberg a spol., 2006, Rosti et al., 2009). Je účinný proti nemutovanému BCR-ABL a pouze některým mutantním formám. Na rozdíl od imatinibu se do buněk dostává difuzí a může být proto s výhodou podán v případě mnohočetné lékové rezistence.

Dasatinib (BMS-354825, Sprycel) je schopen vázat jak aktivní, tak i neaktivní formu kinázy BCR-ABL a inhibuje i kinázy Src rodiny (Tokarski et al. 2004). Ve srovnání s imatinibem je 300x účinnější. Dasatinib je účinný i proti mutantním formám BCR-ABL kinázy s výjimkou vysoce rezistentní formy s mutací T315I (Carter et al. 2005). Výsledky první fáze klinických testů ukázaly, že dasatinib navozuje hematologickou nebo až cytogenetickou remisi u pacientů, kteří netolerovali imatinib, nebo k němu byli rezistentní (Talpaz et al. 2006). Dasatinib je cílen na časnější progenitory než imatinib, nicméně nejprimitivnější CML buňky v klidovém stavu (G0 fázi) jsou rezistentní i k dasatinibu (Copland et al. 2006).

Ve fázi klinických studií je v současné době celá řada dalších cílených léků.

1.1.1.3.3 Kurativní medikamentózní léčba CML?

Nedořešenou otázkou současné léčby a důvodem, proč léčba kinázovými inhibitory není kurativní a vyžaduje doživotní užívání, je fakt, že tato léčba není účinná proti leukemickým kmenovým buňkám, zejména leukemickým kmenovým buňkám v G0 fázi. Jedním z důvodů je patrně nedávno prokázaná nezávislost přežití kmenových CML buněk na kinázové aktivitě BCR-ABL (Hamilton et al., 2012). Pokud bylo testováno vysazení léčby imatinibem v době kompletní cytogenetické remise, docházelo u pacientů k relapsům (např. Yhim a spol., 2012). Ukázalo se ale, že výskyt relapsů je nižší u pacientů, kteří byli předléčeni IFN. Nedávno byla publikována práce, která tento jev může vysvětlit – podání IFN aktivuje leukemické kmenové buňky v G0 fázi ke vstupu do buněčného cyklu (Essers a spol., 2010). Tyto buňky, o nichž se věří, že jsou příčinou relapsů po vysazení imatinibu, se tak stávají citlivými na následnou imatinibovou léčbu.

Dále se testují také některé další přístupy pro eradikaci leukemických kmenových buněk. Příkladem je použití inhibitoru GSK3 beta (glykogen synthase kinase 3 beta) v kombinaci s imatinibem (Reddiconto et al., 2012) nebo inhibice autofágie současně s TKI (Calabretta et al., 2012).

1.1.1.3.4 Rezistence k léčbě

Protože je dnes většina pacientů léčena imatinibem, je pozornost nejvíce zaměřena na mechanizmy rezistence právě k imatinibu. Lze rozlišit primární rezistenci, která se projeví nepřítomností účinku léčby již od jejího zahájení, nebo tzv. sekundární rezistenci, která se projeví ztrátou efektivity léčby po počáteční odpovědi (Hochhaus 2004). Podle mechanismu můžeme rozlišit rezistenci na BCR-ABL závislou a nezávislou. V praxi rozlišení umožňuje sledování fosforylace substrátů BCR-ABL jako např. CRKL (Hochhaus et al. 2002).

Donedávna se předpokládalo, že hlavním mechanizmem rezistence závislé na BCR-ABL jsou mutace v genu bcr-abl (Brandford et al. 2002). Pro vznik rezistence měly být významné především mutace v místech důležitých pro vazbu imatinibu, tj. především v kinázové doméně BCR-ABL, mutace v P smyčce, tj. v sekvenci, která se přímo účastní vazby ATP, mohou souviset s horší prognózou pacientů (Brandford et al. 2003). V nepřítomnosti imatinibu ale většina mutací nepřináší buňkám žádnou růstovou výhodu (Miething et al. 2006) a přítomnost mutované alely ne vždy znamená klinickou rezistenci k imatinibu (Khorashad et al. 2006). Dalším možným mechanizmem rezistence závislé na BCR-ABL je

overexprese BCR-ABL, k níž může docházet v důsledku duplikace Ph chromozómu (Oshikawa a spol., 2010) nebo genomické amplifikace bcr-abl (Virgili a spol., 2010).

Imatinib je do buněk transportován aktivně prostřednictvím hOCT1 proteinu (human organic cation transporter). Snížená exprese nebo aktivita tohoto proteinu by tak mohla být jednou z příčin rezistence k imatinibu, ať už primární nebo sekundární (Thomas et al. 2004). Existuje také mechanizmus mnohočetné lékové rezistence, kdy P-glykoprotein, produkt mdr1 genu, zajišťuje aktivní vylučování imatinibu z buňky (Mahon et al. 2003).

Mechanizmů rezistence nezávislé na BCR-ABL je patrně celá řada. Předpokládá se, že trvalá aktivace (exprese) některých molekul navozovaná BCR-ABL se může postupem času stát trvalou a na tomto původním stimulu nezávislou. Jako možná příčina progrese a/nebo rezistence k léčbě byla identifikována konstitutivně aktivní NfκB (Donato et al. 2003), nadměrná aktivace Src kináz, např. kinázy LYN spojená s overexpresí antiapoptotického proteinu BCL-2 (Donato et al. 2004, Dai et al. 2004), snížená exprese fosfatázy SHP-1 (Esposito et al., 2012), konstitutivní aktivace PI3K dráhy prostřednictvím mutace v PI3Kalfa (Quentmeier et al., 2012), aktivace Raf kinázy nezávislá na BCR-ABL i Ras (Hentschel et al., 2012), indukce cyklooxygenázy 2 (COX-2) (Kalle et al., 2012), aktivace ERK1/2 (Nambu et al., 2012). V některých případech může být rezistence důsledkem vývoje nemoci, během kterého se mohou objevit nové početní nebo strukturální cytogenetické aberace, které vedou k proliferaci leukemických buněk nezávisle na BCR-ABL.

1.1.1.3.5 Odpovědi na léčbu a možnosti jejich predikce

Odpověď na léčbu je hodnocena na úrovni klinické (celkový stav pacienta, stav sleziny, jater apod.), hematologické (krevní obraz a charakteristika jednotlivých krevních elementů), cytogenetické a molekulárně genetické. Cytogenetické metody využívají konvenční techniky, tzv. G-pruhování (barvení chromozómů podle Giemse) nebo metod FISH (fluroscence in situ hybridization). Citlivost těchto metod je zhruba 1 až 5%. Molekulárně genetické metody se zaměřují na sledování transkriptu BCR-ABL kvantitativní reverzně transkriptázovou PCR (Q-RT-PCR), v případě dosažení BCR-ABL negativity kvalitativní dvoustupňovou RT-PCR. Citlivost těchto metod (0,001 až 0,0001%) umožní zjistit jednu leukemickou buňku mezi 10^5 až 10^6 normálními leukocyty. Nárůst množství transkriptu BCR-ABL signalizuje špatnou odpověď na léčbu, pokles a nízká hladina BCR-ABL naopak dobrou odpověď na léčbu a dobrou prognózu. V indikovaných případech jsou vyšetřovány mutace v kinázové doméně BCR-ABL.

Na základě výsledků klinických, cytogenetických a molekulárně genetického vyšetření byly stanoveny jednotlivé odpovědi na léčbu imatinibem (Tab. 1- Baccarani a spol., 2009). U ztráty odpovědi na léčbu - relapsu onemocnění - rozlišujeme relaps hematologický, cytogenetický a molekulární. Hematologický relaps znamená zhoršení krevního obrazu nad normální hodnoty, nebo obecně nárůst hodnot. O cytogenetickém relapsu mluvíme, pokud je detekován Ph chromozóm po dosažení CCgR, nebo obecně dochází-li k nárůstu procentuálního počtu Ph+ metafází. Molekulární relaps je definován jako alespoň desetinásobný nárůst hladiny transkriptu BCR-ABL při CCgR.

Odpověď na léčbu	Definice
velká molekulární (MMR)	≤ 0,1% BCR-ABL (mezinárodní stupnice)
kompletní cytogenetická (CCgR)	0% Ph+
částečná cytogenetická (pCgR)	1-34% Ph+
malá cytogenetická (mCgR)	35-94% Ph+
kompletní hematologická (CHR)	leukocyty $<10*10^9$/L, trombocyty $<450*10^9$/L, nepřítonost nezralých buněk v periferní krvi, nepřítomnost splenomegalie
hematologická (HR)	jakékoliv zlepšení krevního obrazu

Tabulka 1. Definice odpovědí na léčbu (Baccarani a spol., 2009).

Na základě statistického hodnocení výsledků léčby imatinibem od jeho zavedení do praxe byly postupně stanoveny „dynamické odpovědi na léčbu“. Jde o stanovení prognózy pacientů na základě odpovědí dosažených ve stanovených časových bodech. Podle Evropské Leukemické Sítě (ELN) lze rozlišit odpověď optimální, suboptimální a selhání léčby (Baccarani a spol., 2009; Tab. 2). Podle doporučení ELN jsou pacienti v tzv. selhání léčby indikováni ke změně léčby, i když nerelabují (kompletní hematologická odpověď). Pacienti, kteří odpovídají suboptimálně, jsou do selhání léčby přeřazeni, pokud nedojde ke zlepšení.

Měsíce od nasazení imatinibu	Odpověď		
	optimální	suboptimální	selhání léčby
3	CHR	<CR	Bez CHR
6	≥pCgR	mCgR	žádná CgR
12	CCgR	pCgR	<pCgR
18	MMR	<MMR	<CCgR
kdykoliv	stabilní CCgR a MMR	ztráta MMR	ztráta HR
		mutace	ztráta CgR
		přídatné aberace	mutace

Tabulka 2. Dynamické odpovědi na léčbu imatinibem. (Baccarani a spol., 2009)

1.1.2 Akutní myeloidní leukémie (AML)

1.1.2.1 Klinická charakteristika a časový průběh, epidemiologie AML

Akutní myeloidní leukémie dospělých (AML) je klonální maligní onemocnění charakterizované akumulací leukemických blastů. AML je velmi heterogenní onemocnění s řadou subtypů.

Průměrná incidence výskytu AML v celé populaci je 3,4 případu na 100 000 obyvatel (1,2/100 000 v populaci mladší 30 let, 13,2/100 000 u lidí do 65 let a více než 20/100 000 obyvatel v populaci starší než 80 let). AML je převážně onemocnění starších lidí, medián věku při stanovení diagnózy AML je téměř 70 let.

AML nemá žádné specifické projevy (únava, horečky, noční pocení, bolesti kloubů aj.). Nedostatek červených krvinek se projevuje chudokrevností a anemickým syndromem. Nedostatek funkčních bílých krvinek znamená sníženou imunitu. Nedostatek krevních destiček se projeví zvýšenou krvácivostí (tvorba modřin, krvácení z nosu, nález krve ve stolici, nález krve v moči apod.).

Akutní myeloidní leukémie postupuje poměrně rychle a nemocného bez léčby usmrtí do několika týdnů až měsíců (Adam a spol., 2001).

1.1.2.2 Biologie a klasifikace AML

AML je v porovnání s CML ještě o poznání více heterogenní onemocnění. Neexistuje jednotná příčina. Je známo množství aberací, které mají dle statistických hodnocení vliv na

prognózu pacientů a jsou tak využity v klasifikaci jednotlivých subtypů AML (Grimwade a spol., 2009).

V praxi se paralelně používají dva klasifikační systémy. První představuje konsenzus francouzských, amerických a britských odborníků z roku 1976 (FAB, Bennett a spol., 1976; Tab. 3) a je založen na morfologickém a cytochemickém hodnocení blastů. WHO klasifikace zohledňuje kromě morfologie i klinická a imunofenotypizační kritéria a současně i nejnovější poznatky na poli genetiky (Vardiman a spol., 2002). Je značně nepřehledná, ale odráží rozdílnou leukemogenezi jednotlivých typů AML, což se odráží i v odlišném terapeutickém přístupu i prognóze (Tab. 4).

Na základě výsledků standardní karyotypizace leukemických buněk lze nemocné zařadit do 3 prognostických skupin: s příznivou, střední či špatnou prognózou, riziko je vztaženo k počtu komplexních remisí (complete remission - CR) a k celkovému přežití (overall survival - OS) (Tab. 5, Slovak a spol., 2000; Byrd a spol., 2002). Intermediární prognóza zahrnuje různé karyotypy včetně normálního – CN-AML (cytogenetically normal AML). V tomto případě je pozornost soustředěna na mutační status některých genů. Známé získané somatické mutace, které jsou klinicky relevantní, můžeme rozdělit do tří skupin: 1) mutace v npm1 (nukleofosmin), flt3 (FMS-like tyrosinové kináze 3), cebpa (CCAAT/enhancer vazebný protein alfa), které jsou dnes doporučovány k testování v době diagnózy; 2) mutace v genech idh (izocitrát dehydrogenázy), asxl1, ezh2, dnmt3alfa (DNA metyltransferáza 3 alfa), které pravděpodobně ovlivňují biologii AML prostřednictvím vlivu na epigenom, 3) ras, nf1 - skupina genů, které mohou indikovat efektivitu léčby.

„French-American-British“ (FAB) klasifikace
M0: minimálně diferencované leukémie
M1: myeloblastická leukémie bez vyzrávání
M2: myeloblastcká leukémie s vyzráváním
M3: hypergranulárni promyelocytární leukémie
M4: myelomonocytární leukémie
M4Eo: varianta: nárůst abnormáních dřeňových eosinofilů
M5: monocytární leukémie
M6: erythroleukémie (DiGuglielmova nemoc)
M7: megakaryoblastická leukémie

Tabulka 3. Francouzko-americko.britská klasifikace AML (Bennett a spol., 1976)

World Health Organization (WHO) klasifikace

I. AML s recurrentními genetickými abnormalitami

- AML s t(8;21)(q22;q22);*RUNX1/RUNX1T1*
- AML s abmormální eosinofilií v kostní dřeni [inv(16)(p13q22) or t(16;16)(p13;q22);*CBF*β/*MYH11*]
- Akutní promyelocytární leukémie [AML with t(15;17)(q22;q12) (*PML*/*RAR*α) and variants][b]
- AMLs 11q23 (*MLL*) abnormalitami

II. AML s multilineage dysplázií

- následující myelodysplastický syndrom, myelodysplastický syndrom, myeloproliferativní onemocnění
- bez předchozího myelodysplastického syndromu

III. AML a myelodysplastické syndromy, ve vztahu k léčbě

- Alkylačními látkami
- Inhibitory topoisomerase II
- jiné

IV. AML jinak nespecifikovaná

- AML minimálně diferencovaná
- AML bez vyzrávání
- AML s vyzráváním
- akutní myelomonocytární leukémie
- akutní monoblastická a monocytární leukémie
- akutní erythroidní leukémie
- akutní megakaryoblastická leukámie
- akutní basofilová leukémie
- akutní panmyelóza s myelofibrózou
- myeloidní sarcom

Tabulka 4. WHO klasifikace AML (Vardiman a spol., 2002)

Rizikové skupiny	abnormality	5ti leté přežití	výskyt relapsu
dobrá prognóza	t(8;21), t(15;17), inv(16)	70%	33%
intermediátní prognóza	normální, +8, +21, +22, del(7q), del(9q), abnormální 11q23, všechny jiné strukturní nebo početní změny	48%	50%
špatná prognóza	-5, -7, del(5q), abnormální 3q, komplexní cytogenetické změny	15%	78%

Tabulka 5. Klasifikace AML do prognostických skupin podle karyotypu (Slovak a spol., 2000; Byrd a spol., 2002)

1.1.2.3 Léčba AML

Léčba AML může být s kurativním cílem, paliativní nebo pouze suportivní (korekce anémie, trombocytopenie atd.). Při kurativním přístupu se terapie AML zahajuje indukční léčbou, jejímž cílem je navodit kompletní remisi (CR). Je-li jí dosaženo, následují terapie postremisní - konsolidační (mající konsolidovat CR), nebo udržovací (maintenance) terapie. Nedojde-li k navození CR, podává se záchranná (tzv. salvage) terapie.

1.1.2.4 Monitorování pacientů s AML

Stanovení genetických markerů AML je významné v době diagnózy pro stanovení prognózy a rozhodnutí o léčbě. Následně během chemoterapie a po transplantaci kmenových buněk se potom monitorováním těchto markerů sleduje výskyt minimální reziduální choroby a relapsu. Podle typu AML je na molekulární úrovni monitorována hladina fúzních transkriptů, početní nebo komplexní alterace jsou vyšetřovány pomocí interfázní FISH. V případě všech AML subtypů je pro monitorování minimální reziduální choroby významný především gen Wilmsova tumoru (wt1). Celková exprese mRNA wt1 má patrně pro většinu pacientů s AML v průběhu léčby vysoký prognostický význam (Andersson a spol., 2012; Polák a spol., 2012), i když toto pozorování ne všechny studie potvrzují (Yanada a spol., 2004).

1.2 Gen a protein Wilmsova tumoru (WT1)

1.2.1 Struktura genu WT1 a regulace jeho exprese

Gen Wilmsova tumoru 1 (wt1) leží na 11. lidském chromozómu v oblasti 11p3 a sestává z deseti exonů (Call a spol., 1990). Alternativní události, které dávají vznik velkému množství transkriptů a proteinových produktů WT1 jsou schématicky znázorněny na obr. 1. Sekvence genu WT1 zahrnuje dvě alternativně sestřihovaná místa – exon 5 a sekvenci KTS (lysin, treonin, serin). Varianty, které vznikají kombinací těchto dvou sestřihových míst jsou v literatuře označovány jako čtyři hlavní sestřihové varianty wt1 (Haber a spol., 1991). Alternativní první exon E1a dává vzniknout zkrácené variantě mRNA (swt1, Dallosso a spol., 2004, Hossain a spol., 2006). Tyto události se mohou dále kombinovat s editací RNA (záměna na konci exonu 6, Sharma a spol., 1994) a alternativními promotory (Bruening a spol.,1996; Scharnhorst a spol.,1999). Exprese další izoformy začínající na konci pátého intronu byla zatím popsána pouze u rakoviny prostaty, prsu a leukemických buněk (Dechsukhum a spol., 2000).

WT1 je významný pro vývoj urogenitálního traktu a přechod mesotelia v epitel. V dospělosti je exprese WT1 omezena na tkáně urogenitálního systému, mozku a hematopoetickou tkáň (shrnuto v Yang a spol., 2007). Na regulaci exprese se podílí patrně více transkripčních faktorů specifických pro konkrétní tkáně. V hematopoetických tkáních byla potvrzena regulace prostřednictvím GATA-1 a MYB (Wu a spol., 1995, Zhang a spol., 1997). V poslední době se v regulaci exprese WT1 ukazuje také role microRNA (miRNA), krátkých nekódujících RNA, které negativně regulují expresi cílových mRNA inhibicí translace nebo přímo štěpením cílových mRNA (Alemdehy a spol., 2012). U akutní myeloidní leukémie byla nedávno popsána regulační aktivita miR-132 a miR-212 ve vztahu k WT1 (Luesink a spol., 2009). Gao a spol. ukázali význam miR-15a a miR-16-1 v regulaci exprese WT1 (Gao a spol., 2011). O regulaci exprese jednotlivých izoforem je toho známo jen velmi málo. Sestřih sekvence KTS je patrně regulován (mimo jiné) intronickým enhancerem (Yang a spol., 2008).

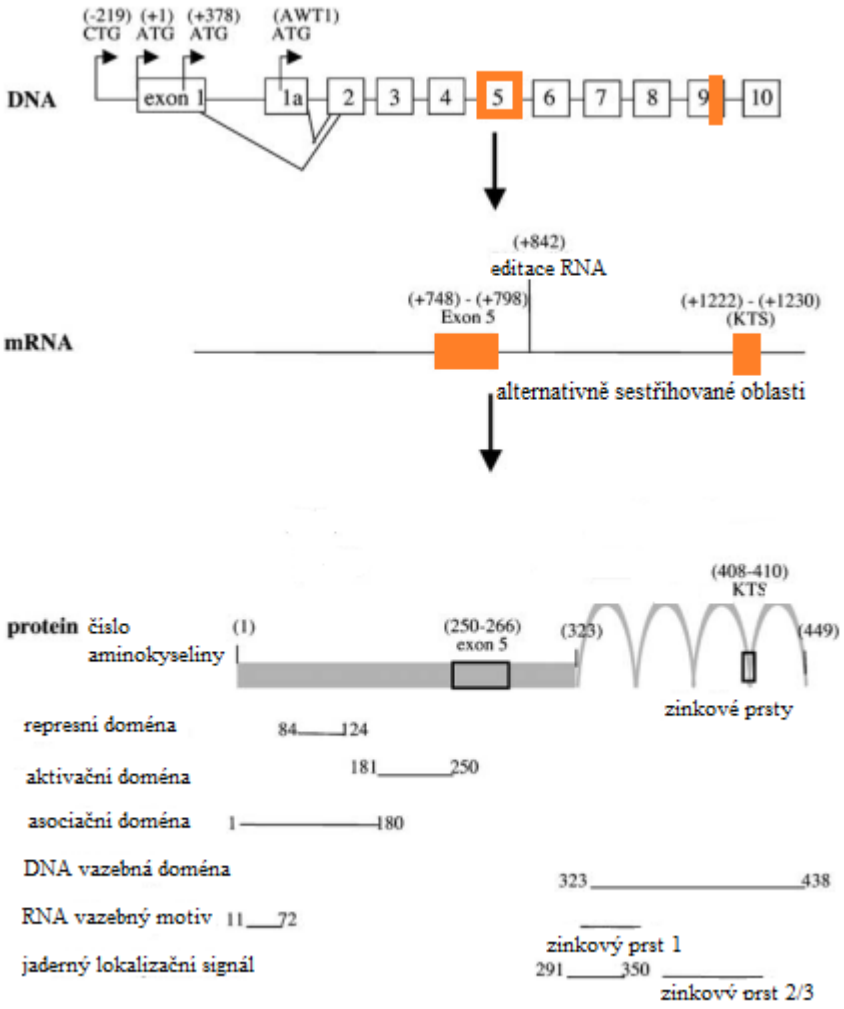
1.2.2 Struktura a funkce proteinu WT1, role jednotlivých izoforem

Struktura vybraných izoforem proteinu WT1 je znázorněna na obr. 1. S výše uvedenými změnami na úrovni transkripce se dále kombinují tři alternativní začátky translace. Hlavní AUG dává vznik proteinům o velikosti 52 (-5/-KTS) až 54 kDa (+5/+KTS) (Morris a spol,.

1991). Tomuto hlavnímu místu předchází CUG, ze kterého vznikají proteiny o velikosti 60 až 62 kDa (Bruening a spol., 1996). Naopak za hlavním sestřihovým místem ve směru translace je AUG, které dává vznik proteinům o velikosti 36 až 38 kDa (Scharnhorst a spol., 1999). Dohromady dávají všechny uvedené alternativní události vznik 36 izoformám WT1 proteinu. Přítomnost čtyř zinkových prstů typu Cys2His2 (DNA vazebná doména) na C-konci proteinu (exony 7 až 10) umožňuje WT1 vazbu na DNA a tedy funkci transkripčního regulátoru (Nurmemmedov a spol., 2006, Haber a spol., 1991). Díky přítomnosti transkripčně represní i aktivační domény může WT1 ovlivňovat expresi cílových genů pozitivně i negativně. U sWT1 dochází ke ztrátě transkripčně represní domény a v důsledku toho k aktivaci genů, které jsou WT1 plné délky reprimovány (Hossain a spol., 2006). Geny, jejichž expresi WT1 reguluje, lze zařadit mezi regulátory buněčného cyklu (p21, cyklin D), regulátory apoptózy (Bcl-2) nebo regulátory proliferace (Myb) (shrnuto v Yang a spol., 2007).

Čtyři hlavní izoformy WT1 se liší svými vlastnostmi a funkcí v buňce. Sekvence KTS ovlivňuje vzájemné postavení zinkových prstů (Laity a spol., 2000) – izoformy obsahující sekvenci KTS mají odlišný konsenzuální vazebný motiv na DNA (Hewitt a spol., 1996), preferují vazbu RNA (Nurmemmedov a spol., 2006, Nurmemmedov a spol., 2009), vykazují zrnité rozložení v buněčném jádře (Larsson a spol., 1995), asociují s některými komponentami sestřihového aparátu buňky a uvažuje se tak o jejich roli v sestřihu (Davies a spol., 1998, Englert a spol., 1995). Izoformy + i –KTS přechází mezi cytoplazmou a buněčným jádrem, asociují s ribonukleoproteinovými částicemi a aktivními polyzómy (Niksic a spol., 2004). Exon 5 kóduje protein-protein interakční doménu a sekvence jím kódovaná je tedy významná pro interakci s dalšími proteiny (Campbell a spol., 1994).

Působení WT1 dále ovlivňují také dva typy posttranslačních modifikací – fosforylace (Ser365, Ser393, Sakamoto a spol., 1997) a sumoylace na lysinech 17 a 173 (Smolen a spol., 2004). O konkrétním významu těchto modifikací není zatím mnoho známo. Sumo-1 je malá molekula příbuzná ubikvitinu, na rozdíl od něj však naopak stabilizuje cílový protein. Sumoylace neovlivňuje buněčnou lokalizaci WT1 (Smolen a spol., 2004). Sakamoto a spol., 1997 ukázali, že fosforylace může hrát roli v modulaci transkripčně regulační aktivity proteinu WT1 interferencí s jadernou translokací a zároveň inhibicí vazby k DNA. Fosforylací docházelo také k inhibici vazby na RNA. Fosforylaci může zprostředkovat např. cAMP dependentní protein kináza A.



Obr. 1. Struktura genových a proteinových produktů WT1
(upraveno podle Yang a spol., 2007)

1.2.3 Význam WT1 v hematopoéze

Role WT1 v hematopoéze není zatím zcela známá. Studie naznačují dílčí role: Exprese WT1 je patrně omezená především na primitivní CD34+ populaci buněk kostní dřeně, může být ale detekována i ve zralých krevních buňkách. Bylo ukázáno, že WT1 v progenitorových krevních buňkách hraje roli v jejich sebeobnovovacím potenciálu, přílišné zvýšení exprese ale vede k přechodu buněk do G0 fáze a podporuje myelo-monocytární diferenciaci (Svedberg a spol., 2001, Smith a spol., 1998, Loeb a spol., 2003). Jiné práce naopak uzavírají, že zvýšená exprese WT1 zastavuje diferenciaci (Svedberg H a spol., 1998, Gu a spol., 2005). WT1 je patrně důležitou komponentou regulace proliferace T lymfocytů závislé na oxidu dusnatém

(Marcet-Pacios M et al., 2007). Je patrné, že rozporuplné výsledky jsou způsobeny především nedostatečnou komplexitou studií, kterou si značné množství izoforem WT1 žádá.

1.2.4 Význam WT1 v onkologii a hematoonkologii

WT1 byl poprvé izolován jako gen zodpovědný za dětskou nefrologickou malignitu, Wilmsův tumor (Haber a spol., 1990). V tomto případě je WT1 inaktivován mutací a předpokládala se tak funkce tumor supresoru. Ukázalo se ale, že u řady malignit se WT1 chová naopak jako onkogen. WT1 je nadměrně exprimován u řady pevných nádorů (Oji a spol., 2002; Loeb a spol., 2001,Viel a spol., 1994; Rodech a spol., 1994; Campbell a spol., 1998; Amin a spol., 1995; Amini a spol., 2005; Oji a spol., 2004) a také u většiny leukémií (Miwa a spol., 1992). Více než 20 let výzkumu WT1 zatím nevysvětlilo mechanizmy, jakými funguje, pravděpodobně díky velkému množství genových produktů a modifikací WT1 vyžadujících při jeho studiu velmi komplexní přístup.

V případě leukémií existuje řada důkazů pro onkogenní působení WT1. Pokusy na buněčných liniích ukázaly, že WT1 je významný pro přežívání leukemických buněk (Yamagami T a spol., 1996, Ito K a spol., 2006). Zvýšená everexprese WT1 byla potvrzena na myším modelu jako druhý zásah v případě AML s fúzí aml1/eto (Nishida a spol., 2005).

Mutace wt1 jsou detekovány u velmi nízkého procenta pacientů s AML. Mutace jsou heterozygotní, obvykle se jedná o drobné inzerce, bodové mutace v oblasti zinkových prstů, nebo zkrácení proteinu. Vztah mezi přítomností mutované formy WT1 a horší prognózou pacientů s AML nebyl jednoznačně prokázán (Wang a spol., 2011).

V poslední době se již objevují také práce, které ukazují na odlišný význam jednotlivých izoforem. Overexprese variant wt1 obsahujících exon 5 chrání K562 buňky před buněčnou smrtí navozenou chemoterapeutiky (Ito K et al., Oncogene, 2006). Renshaw a spol. ukázali, že v důsledku léčby cytotoxickými látkami může docházet k narušení sestřihu exonu 5 (Renshaw a spol., 2004). Umlčení exprese izoforem obsahujících exon 5 na rozdíl od umlčení celkové exprese wt1 vede ke zvýšení citlivosti buněk k apoptóze navozené cytostatiky. Graidist a spol. ukázali, že izoformy +5/+KTS a +5/-KTS mohou fungovat jako antiapoptotické proteiny v buňkách rakoviny prsu (Graidista a spol., 2010).

Přestože mechanizmus fungování WT1 v leukemických buňkách ani zdravé hematopoéze není zatím vysvětlen, pro léčbu myeloidních leukémií jsou testovány vakcinace WT1 peptidem (Sugyiama et al., 2012; Oka a spol., 2010).

Především u dospělých AML pacientů a případně také u myelodysplastického syndromu (MDS) se používá celková exprese wt1 na úrovni mRNA jako marker pro monitorování minimální reziduální choroby (Andersson a spol., 2012; Polák a spol., 2013; Polák a spol., 2012; Gray a spol., 2012; Yamauchi a spol., 2012). Je však i několik prací, které takový význam pro WT1 nepotvrzují (Yanada a spol., 2004; Miglino a spol., 2011).

V případě CML pravděpodobně díky existenci vysoce specifického markeru bcr-abl je prací, které se věnují wt1 jako markeru podstatně méně. První nadějné výsledky ukazující na význam exprese wt1 pro pacienty s CML ukázalo několik málo prací (Kreutzer a spol., 2001; Cilloni a spol., 2003; Varma a spol., 2011). Zatímco práce Kreutzera a spol. byla soustředěna na pacienty po transplantaci kostní dřeně, práce Cilloni a spol. a Varma a spol. naznačily výzanm exprese wt1 také pro pacienty léčené imaitnibem.

2. Cíle

Cílem práce bylo přispět k efektivnějšímu monitorování pacientů s CML, a částečně také AML cestou detailního studia exprese wt1, které umožní jeho efektivnější a širší využití jako prognostického markeru a případně také jako terapeutického cíle.

Konkrétní cíle práce byly následující:

1. Ověřit možnost využití celkové exprese wt1 na úrovni mRNA jako markeru pro predikci dalšího vývoje onemocnění pro pacienty s CML
2. Vyvinout přístupy pro sledování exprese sestřihových variant wt1 na úrovni mRNA
3. Vyvinutými metodami stanovit expresní profil variant wt1 v době diagnózy pacientů s CML a AML a zjistit, zda profil koreluje s prognózou pacientů (retrospektivní studie)
4. Zjistit kinetiku exprese variant wt1 v průběhu léčby, ukázat zda kinetika exprese jednotlivých variant může mít význam pro včasnou predikci relapsu pacientů
5. Sledovat změny v expresi wt1 *in vitro* po kultivaci pacientských leukocytů s používanými léčivy a ověřit možnost stanovení účinnosti léčiv pomocí měření poklesu exprese mRNA wt1.

3. Výčet použitých metod

Izolace celkových leukocytů pacientů (osmotická lýze erytrocytů)

Příprava celkových lyzátů pro extrakci RNA, příprava proteinových lyzátů

Izolace celkové RNA, miRNA, plazmidových DNA (kyselá fenol-chloroformová extrakce, komerční kity)

Reverzně transkriptázová PCR v reálném čase (návrhy primerů a sond, in silico analýzy, *in vitro* optimalizace reakcí, PCR s interkalačními barvivy, PCR se sondami typu TaqMan)

Přímé sekvenování

Elektroforéza nukleových kyselin

PCR klonování (TOPO-TA clonning kit)

SDS elektroforéza (Laemmliho protokol, protokol Schager-Jagow, komerční systémy)

Western Blot („polosuchý“, „mokrý“ blot)

Vyhodnocování metody microarray

Kultivace buněčných linií a primárních leukocytů pacientů s kinázovými inhibitory

„Lab-on-chip“ přístupy pro detekci apoptózy (značení anexinem V, sledování míry degradace RNA)

Měření životaschopnosti buněk (barvení trypanovou modří, AlamarBlue assay)

4. Výsledky – komentář k publikacím

4.1 Exprese mRNA wt1 v celkových leukocytech pacientů s myeloidními leukémiemi

První část práce byla zaměřena na expresi wt1 na úrovni mRNA jako marker myeloidních leukémií. Exprese WT1 byla vyšetřována v celkových leukocytech periferní krve pacientů s CML (celková exprese wt1, exprese sestřihových variant wt1) a AML (exprese sestřihových variant wt1). Abychom zjistili, jaký význam může monitorování exprese wt1 pro pacienty mít, porovnávali jsme výsledky s prognostickými ukazateli používanými v rutinní klinické praxi.

4.1.1 Celková exprese mRNA wt1 u pacientů s CML

Publikace č. 1: Wt1: a helpful additional marker to BCR-ABL for patients with chronic myeloid leukemia. Tereza Lopotová, Sylvie Nádvorníková, Markéta Žáčková, Hana Klamová, Jana Moravcová; odesláno k recenzi

Přestože CML má svůj hlavní a nezastupitelný molekulární marker bcr-abl, existují pacienti, u nichž bcr-abl prognostický význam postrádá. Podle současných kritérií ELN pro odpovědi na léčbu imatinibem platí že, nedojde-li u suboptimálního odpovídače ke zlepšení odpovědi, přechází odpověď v selhání léčby. V takovém případě je doporučena změna léčby, i když pacient zůstává v kompletní hematologické odpovědi. Dle literatury (Mauro a spol., 2009, Rohoň a spol., 2011) i našich vlastních zkušeností především z pre-dasatinib a pre-nilotinib éry je však známo, že ne všichni pacienti s horší než optimální odpovědí hematologicky relabují. Skupina suboptimální odpovědi, resp. selhání léčby zahrnuje zároveň pacienty, kteří dosáhnou odpovědi později („pomalí odpovídači“) a pacienty s dosud neznámými obrannými mechanizmy proti CML, kteří přežívají dlouhodobě v kompletní hematologické odpovědi navzdory vysokým hladinám bcr-abl. Hladina transkriptu bcr-abl zůstává obvykle u suboptimálních odpovědí a selhání léčby stabilní a neumožňuje odhadnout spolehlivě blížící se hematologický relaps. Na základě literatury a našich vlastních předběžných dat (T. Lopotová, diplomová práce) jsme jako vhodný kandidátní pomocný marker pro upřesnění prognózy těchto pacientů zvažovali právě gen wt1.

V rámci současné studie byl soubor pacientů rozšířen na 32 a studie zaměřena přitom právě na suboptimální odpovídače a pacienty, kteří vykazují selhání léčby. Zjistili jsme, že celková exprese mRNA wt1 je u 60% pacientů schopna predikovat blížící se hematologický

relaps o 2,5 měsíce dříve v porovnání s bcr-abl. U většiny pacientů (téměř 70%) je nárůst exprese wt1 navíc výraznější a tedy jasněji interpretovatelný v porovnání s bcr-abl. Provedené analýzy dále ukázaly, že vedle kinetiky exprese má pro pacienty význam i konkrétní hladina. Statistické analýzy naznačily význam hladin wt1 v 6. a 12. měsíci od zahájení léčby imatinibem. Svou hladinou wt1 predikuje riziko hematologického relapsu pro dalších 35 měsíců léčby. ROC (reciever operating characteristic) křivkou stanovené kritické hladiny se navíc na základě zpětného hodnocení průběhu exprese Wt1 u jednotlivých pacientů v průběhu léčby zdají mít význam i déle než 35 měsíců od zahájení léčby. Relabující pacienty přitom od nerelabujících nebylo možné odlišit na základě hladin bcr-abl. Hladina transkritpu wt1 predikovala relaps stejně spolehlivě u pacientů, jimž byla detekována mutace v kinázové doméně BCR-ABL, jako u pacientů s divokým typem BCR-ABL.

Uzavřeli jsme, že kinetika exprese i konkrétní hladina wt1 jsou velmi vhodným markerem doplňujícím bcr-abl v monitorování pacientů s CML, především těch, kteří neodpovídají na imatinib optimálně. Naše výsledky podporují význam hladiny mRNA wt1 jako markeru, který právě pro svou obecnou povahu (overexprese napříč různými typy leukémií) odráží patrně aktivitu buněk pacientů obecně - nejen aktivitu původního leukemického klonu, ale i případných klonů rezistentních, jejichž závislost na BCR-ABL může být snížená.

4.1.2. Exprese sestřihových variant wt1 v myeloidních leukémiích

V první práci jsme ukázali vysoký prognostický význam celkové exprese wt1 pro pacienty s CML. Odpůrci využití wt1 v praxi poukazují na možný problém s případnou interpretací vyšetření s ohledem na nenulovou hladinu v periferní krvi s možnými drobnými výkyvy i u zdravých dárců. Hladina mRNA wt1 je marker pan-leukemický, kterému bývá vyčítána nízká specifita. Naše výsledky ukázaly, že celková hladina mRNA wt1 je u zdravých dárců natolik nízká, že patrně nebude dosahovat hodnot, jaké nacházíme u pacientů, kteří se blíží relapsu. Přesto jsme se chtěli pokusit dále zvýšit specifitu tohoto markeru. Bylo ukázáno, že exprese některých konkrétních izoforem, ale nikoliv celkové exprese wt1, může chránit buňky před apoptózou, navozenou cytotoxickými látkami (Renshaw a spol., 2004). Můžeme tedy předpokládat, že exprese některé konkrétní varianty nebo jejich vzájemný poměr mohou představovat specifičtější markery rozvíjející se rezistence (relapsu) v porovnání s celkovou hladinou wt1.

4.1.2.1 Exprese mRNA čtyř hlavních sestřihových variant wt1 v myeloidních leukémiích

Publikace č. 2: Expression of four major WT1 splicing variants in acute and chronic myeloid leukemia patients analyzed by newly developed four real-time RT PCRs. Tereza Lopotová, Jaroslav Polák, Jiří Schwarz, Hana Klamová and Jana Moravcová, Blood Cells Mol Dis 49(1):41-7 (2012), IF 2,351

Exprese čtyř hlavních sestřihových variant vznikajících kombinací sestřihu exonu 5 a KTS sekvence (+5/+KTS, +5/-KTS, -5/+KTS, -5/-KTS) byla dosud stanovována pouze semi-kvantitativními přístupy (oddělené stanovení variant s a bez exonu 5 nezávisle na přítomnosti či nepřítomnosti sekvence KTS a naopak). My jsme jako první vytvořili systém čtyř oddělených reverzně transkriptázových PCR v reálném čase pro stanovení exprese těchto čtyř hlavních sestřihových variant WT1 a aplikovali jej na vzorky pacientů s CML i AML (Lopotová a spol., ústní prezentace, Osaka, Japonsko, 2009; Lopotová a spol., poster, ASH, 2010; publikace II). Na rozdíl od dosud publikovaných přístupů naše reakce umožňují sledovat každou konkrétní variantu odděleně

Námi navržené reakce využívají primery, které hybridizují do oblastí sestřihových míst. V průběhu návrhu a optimalizace bylo třeba řešit především tyto problémy – dlouhý amplikon (cca 420 nt.) a velmi omezený prostor pro návrh, nepříznivá sekvence s adeninovými repeticemi a celkově malý rozdíl v + vs. –KTS variantách (KTS sekvence zahrnuje pouze 9 nukleotidů). Po otestování řady modifikací protokolu byla nakonec úspěšnou kombinací pro nejméně účinnou PCR vybraná LNA modifikace reverse primeru a nízká koncentrace DTT jako aditiva v reakční směsi. Funkčnost systému byla ověřena pomocí uměle připravených směsí plazmidových DNA imitujících různé poměry variant.

Pomocí nově vytvořené metodiky jsme zjistili, že profil AML a CML diagnózy se shoduje ve vysokém zastoupení variant pozitivních pro obě sestřihová místa, +5/+KTS. Zatímco u AML je druhou nejzastoupenější variantou +5/-KTS, u CML převládá varianta -5/+KTS. Abychom zjistili, zda varianty mohou mít význam pro odpověď na léčbu, provedli jsme u AML pacientů v okamžiku diagnózy porovnání profilů variant mezi různými FAB a WHO prognostickými skupinami. Zjistili jsme, že -5/+KTS je vysoce zastoupena u agresivních typů jako je AML M3. Porovnání skupin s dosažením vs. nedosažením odpovědi potom potvrdilo možnost fungování +5/-KTS jako nezávislého prognostického markeru. Vzorky několika CML pacientů z průběhu léčby umožnily nahlédnout do kinetiky exprese variant. Zjistili jsme, že v případě CML jako první a zároveň nejvýrazněji směrem do hematologického

relapsu narůstá varianta -5/+KTS. Naše výsledky tedy naznačily, že varianty -5/+KTS a +5/-KTS mohou fungovat jako rizikové markery CML a AML. Pro důkladné posouzení významu exprese těchto variant v porovnání s celkovou expresí mRNA wt1 je však třeba analyzovat větší soubor pacientů.

4.1.2.2. Exprese mRNA swt1 v myeloidních leukémiích

Publikace č. 3: N-terminally truncated WT1 variant (sWT1) is expressed at very low levels in acute myeloid leukemia and advanced phases of chronic myeloid leukemia. Tereza Lopotová, Sylvie Nádvorníková, Markéta Žáčková, Jaroslav Polák, Jiří Schwarz, Hana Klamová, Jana Moravcová, Leuk Res. 2012 Apr;36(4):e81-3. Epub 2012 Jan 9; IF 2,555

Druhou ze dvou studií navazujících na práci věnovanou celkové expresi mRNA wt1 je studie zaměřená na variantu sWT1. Tato varianta využívá oproti wt1 plné délky alternativní první exon, díky čemuž výsledný protein postrádá transkripčně represní doménu. Právě v tom viděli Hossain a spol. (2006) příčinu možného onkogenního působení této varianty.

Hossain a spol. prezentoval vysoké hladiny swt1 specificky u leukemických pacientů, neuvedl ale sekvence primerů, které použil. Vyvinuli jsme proto vlastní PCR, primery byly navržené do oblasti rozhraní exonů E1/E1a E2 podle referenčních sekvencí swt1 a wt1 plné délky databáze NCBI. Optimalizované reakce jsme aplikovali na soubor 12 CML a 34 AML diagnostických vzorků (Lopotová a spol., EHA 2010; publikace č. 3).

Exprese swt1 je dle našich analýz v celkových leukocytech periferní krve pacientů s CML i AML velmi nízká. Zatímco v případě AML pacientů jsme swt1 detekovali vždy, u pacientů s CML se swt1 objevovala až v blastické krizi. Zdá se tak, že její přítomnost souvisí s přítomností blastů ve vzorcích. Krátkou publikací jsme z části reagovali na práci Ishikawi a spol., kterým se rovněž nepodařilo data Hossaina a spol. potvrdit. Ishikawa a spol. (2011) analyzoval vedle vzorků pacientů s AML také vzorky kostní dřeně zdravých dárců. Stejně jako my pozoroval velmi nízké hladiny wt1 u pacientů s AML. V kostní dřeni zdravých dárců swt1 nedetekoval vůbec. Dohromady tak naše data spolu s prací Ishikawi a spol. naznačila, že se exprese swt1 je specifická pro leukemické blasty. V publikaci jsme zdůraznili také potřebu přesného popisu a standardizace použitých metodik tak, aby jednotlivé studie byly navzájem porovnatelné.

Varianta swt1 jistě není vhodným kandidátem na prognostický marker vzhledem k velmi nízké úrovni exprese, může být ale zajímavá pro další studium exprese a role této varianty v nezralých leukemických buňkách.

4.2. Exprese mRNA wt1 po ošetření buněk inhibitory *in vitro* – aplikace wt1

Publikace č. 4: Expression patterns of microRNAs associated with CML phases and their disease related targets. Kateřina Machová Poláková, Tereza Lopotová, Hana Klamová, Pavel Burda, Marek Trněný, Tomáš Stopka, Jana Moravcová, Mol Cancer. 2011 Apr 18;10:41

Publikace č. 5: MicroRNA-451 in chronic myeloid leukemia: miR-451-BCR-ABL regulatory loop? Tereza Lopotová, Markéta Záčková, Hana Klamová, Jana Moravcová, Leuk Res. 2011 Jul;35(7):974-7. Epub 2011 Apr 20; IF 2,555

Testování citlivosti pacientů k léčbě pomocí kultivací primárních leukocytů pacientů *in vitro* s používanými léčivy je v naší laboratoři zavedeno téměř do klinické praxe. Hodnotí se míra poklesu aktivity BCR-ABL nepřímo stanovením poklesu fosforylace proteinu Crkl, prominentního substrátu BCR-ABL kinázy, po ošetření buněk *in vitro* inhibitory. Pokles celkové exprese mRNA wt1 jako ukazatel citlivosti ke kinázovým inhibitorům resp. míry inhibice kinázové aktivity BCR-ABL byl již publikován skupinou Cilloni a spol., 2004, a dále v práci Otáhalové a spol., 2009.

Naším dílčím cílem bylo kultivační testy dále optimalizovat tak, abychom jimi mohli současně sledovat také závislost hladiny vybraných molekul na aktivitě kinázy BCR-ABL. Bylo třeba určit především dobu inkubace s inhibitorem tak, abychom mohli sledovat sekundární efekty v návaznosti na inhibici BCR-ABL přitom ale na ještě dostatečně životaschopných buňkách (Žáčková, Lopotová a spol., ASH abstrakt, 2011). Exprese wt1 by podle Svensona a spol., 2007, měla být v CML buňkách závislá mimo jiné právě na aktivitě BCR-ABL. Zatímco fosforylace Crkl klesá již po 2 hodinách inkubace buněk s 1 až 10 μM imatinibem, v případě hladiny mRNA wt1 dochází k průkaznému poklesu až po 24 hodinách. Po 72 hodinách je pak míra apoptózy natolik vysoká, že dochází k degradaci většiny mRNA (Žáčková, Lopotová a spol., ASH abstrakt, 2011). Optimalizované testy s inkubačním časem 48 hodin při koncentraci buněk 4 milióny/ml jsme využili v rámci paralelní studie miRNA v CML.

MiRNA jsou krátké nekódující RNA, které negativně regulují expresi cílových mRNA. Předpokládá se, že miRNA mohou regulovat přibližně jednu třetinu buněčných mRNA. V roce 2008, kdy jsme zahájili naši studii věnovanou miRNA v CML, bylo o expresi miRNA v CML známo velmi málo. V rámci prvotního screeningu exprese miRNA v CML jsme provedli microarray analýzu miRNA ve vzorcích pacientů s různou odpovědí na léčbu a zdravých dárců (publikace č. 4). Většina miRNA, jejichž expresi se podařilo detekovat, vykazovala zvýšenou nebo sníženou hladinu v blastické krizi oproti chronické fázi CML a zdravým dárcům, což mohlo mít souvislost s rozdílným diferenciačním stádiem testovaných buněk. Pouze 3 miRNA byly odlišně exprimované v průběhu CML a v porovnání se zdravými dárci – miR-451, miR-150 a miR-328. Exprese všech tří miRNA klesala se zhoršující se odpovědí na léčbu a fází CML. Zajímavé bylo, že k poklesu docházelo již v hematologickém relapsu, tedy ještě v rámci chronické fáze CML. Změny se tak zdály být v souvislosti s onemocněním, nikoliv diferenciačním stádiem buněk.

Otázkou, kterou jsme si položili, abychom vybrali miRNA potenciálně zajímavou pro podrobnější studie, bylo, zda je její exprese aberantní v důsledku aktivity BCR-ABL, nebo se může jednat o nezávislý faktor patogeneze CML. Pomocí výše zmíněných *in vitro* kultivací buněk leukemických linií, pacientů s CML a zdravých dárců jsme ukázali, že snížená exprese miR-451vzniká v důsledku působení BCR-ABL. Po ošetření buněk imatinibem docházelo k restaurování hladiny miR-451 spolu s poklesem fosforylace Crkl a mRNA exprese WT1. Iraci a spol. přitom v roce 2009 ukázali, že miR-451 má potenciál cílit přímo BCR-ABL. Dohromady tak naše data s literaturou dala vznik myšlence vzájemné regulační vazby mezi miR-451 a BCR-ABL. BCR-ABL svou aktivitou snižuje hladinu miR-451, jakožto svého vlastního negativního regulátoru. Naše výsledky týkající se miR-451 a námi navržený regulační mechanizmus, který může být velmi důležitý pro uchování maligního fenotypu CML buněk, jsme uvedli v krátké publikaci (publikace č. 5).

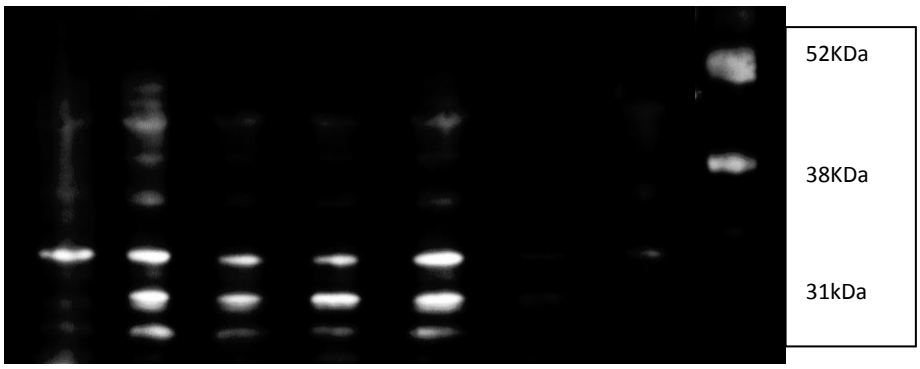
4.3 Exprese izoforem WT1 na proteinové úrovni

Studium exprese sestřihových variant wt1 na úrovni mRNA ukázalo, že přestože je wt1 považována za pan-leukemický marker, mechanizmus jejího působení se může lišit mezi jednotlivými diagnózami – u CML převažuje mRNA -5/+KTS, u AML +5/-KTS. Abychom mohli pochopit mechanizmus působení WT1, případně abychom mohli využít poznatky o expresi izoforem pro zvýšení efektivity studia terapeutických vakcín založených na WT1, bude dále nutné poznat hladinu efektorové molekuly, tedy proteinu.

V rámci projektu grantové agentury Univerzity Karlovy (GAUK č. 454511, 2011-2012) jsme tedy začali studium exprese WT1 také na proteinové úrovni. Toto je vedle vysokého počtu izoforem vznikajících alternativním sestřihem, využitím alternativního prvního exonu a alternativních začátků translace komplikováno dvěma posttranslačními modifikacemi – fosforylace (Ser 365 a Ser 393) a sumoylace (Lys 17 a Lys 173). V současné době neexistují specifické protilátky pro jednotlivé izoformy. Protilátky proti oběma fosforylovaným formám WT1 existují, jejich kvalita je ale dle našeho testování i hodnocení dalších uživatelů velmi nízká. V rámci komerčně dostupných jsme nakonec vybrali protilátku proti epitopu z C-konce proteinu, kde dosud nebyly popsány žádné alternativní události (ty jsou soustředěny ve středu proteinu nebo na jeho N-konci, viz Úvod). Taková protilátka by tedy měla detekovat všechny známé izoformy.

Metodami SDS-PAGE a Western Blot jsme zjistili, že po ošetření buněk linie K562, CML-T1 i JURL-MK1 ale i pacientů *in vitro* inhibitory BCR-ABL (imatinib, nilotinib, dasatinib) dochází k určitým změnám v profilu variant (obr. 2, př. linie CML-T1).

Abychom získali přesnější informaci o tom, které izoformy jsou jak zastoupeny, připravujeme v současné době kontrolní rekombinantní proteiny a optimalizujeme pro lepší rozlišení metody NEPHGE (nonequilibrium pH Gel Electrophoresis) a 2D-ELFO.



Obr. 2 Exprese WT1 po ošetření buněk kinázovými inhibitory. 1 a 10IM – 1 a 10uM koncentrace imatinibu v médiu; 10 a 100 DASA – 10 a 100 nM koncentrace dasatinibu v médiu; 0,2 a 2 nM koncentrace nilotinibu v médiu.

5. Publikace komentované v dizertační práci

Publikace č. 1

Odesláno k posouzení

Wilms´ tumor gene 1 – a helpful additional molecular marker to BCR-ABL in patients with chronic myeloid leukemia

Shortened running title: WT1 in chronic myeloid leukemia

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ABSTRACT

Chronic myeloid leukemia (CML) is a myeloproliferative disorder which arises from t(9;22) translocation. This translocation gives rise to BCR-ABL fusion gene. BCR-ABL kinase is the main cause of CML development and a target for therapy. Despite of impressive results with imatinib, selective BCR-ABL inhibitor, however, 20 to 30% of patients are resistant or intolerant. BCR-ABL transcript level and mutation status are the molecular markers of CML. As there are many different mechanisms of resistance more or less dependent on BCR-ABL, new markers additional to BCR-ABL would be useful for early relapse detection and prognosis. Here we report a complex retrospective study of Wilms´ tumor gene 1 (WT1) expression in CML patients treated with imatinib. Our data highlight the advantages of WT1 as general leukemic marker enabling to rapidly assess the effectiveness of treatment and risk of relapse.

INTRODUCTION

Chronic myeloid leukemia (CML) is a myeloproliferative disorder which arises from translocation of the *ABL* gene on chromosome 9 and the *BCR* gene on chromosome 22 to produce the Philadelphia (Ph) chromosome with the *BCR-ABL1* fusion gene. BCR-ABL codes for a constitutively active tyrosine kinase protein, which affects proliferation, differentiation, and apoptosis, leading to malignant transformation of cells. BCR-ABL represents the main driving force of CML development. Following the era of interferon-alpha (IFN-α) therapy, cytarabine, and hematopoietic stem cell transplantation as the standard treatment for CML patients, a break-through has come with the BCR-ABL-targeted therapy. The introduction of the tyrosine kinase inhibitor (TKI), imatinib mesylate (IM), has revolutionized the treatment of CML. Currently, imatinib is the first-line therapy for all newly diagnosed CML patients. Despite of impressive results of imatinib therapy, however, 20 to 30% of patients have primary refractory disease, intolerance or experience relapse after initial response. For successful therapy outcomes it is important to detect developing relapse early. For this purpose, patients are routinely examined for mRNA expression levels of the only molecular marker of CML BCR-ABL. Results from all haematological, cytogenetic and molecular examinations are used to stratify patients into three main groups with different risk of relapse and progression (EuropeanLeukemiaNet (ELN) criteria) [1]: optimal responders (OR) with low risk, suboptimal responders (SR) and therapy failures (TF) with increased risk.

BCR-ABL as the main cause of CML development has its irreplaceable role in CML monitoring. Its high prognostic value has been clearly proved for CML patients. Our experience, however, shows that there are also patients in whom BCR-ABL is unable to bring satisfactory information on prognosis. It is our own and others experience [2, 3] that suboptimal responders (ELN criteria) include both patients who are in high risk of relapse and "slow responders" group. CML mechanisms in such patients remain unknown, BCR-ABL levels remain usually stable and its monitoring looses much of its prognostic value in our own experience. If suboptimal responders do not achieve improvement of

response to therapy, they are considered therapy failures and they are recommended for therapy change although they do not relapse haematologically. There is increasing evidence that CML may become more or less independent of BCR-ABL and driven by other proteins and mechanisms over the time. That might explain why BCR-ABL on its own may stop being a reliable marker in "non-optimal responders" (SR, TF) to therapy (our own experience). There is an evident need for new CML biomarkers additionally to BCR-ABL (1) to early and reliably predict relapse, (2) to specify prognosis of suboptimal responses/therapy failures and indicate patients who require closer medical observation or (3) even redefine non-optimal responders according to actual risk of relapse (recently suggested also by Faber et al., 2012).

Wilms´ tumor gene 1 (WT1) encodes a zinc-finger transcriptional regulator important for cell growth and development. While in Wilms´ tumor, WT1 behaves as tumor suppressor gene, it is a proved oncogene in leukemias. [4] More than 36 WT1 isoforms probably enlarge the field of activities of the protein. While it was first suggested that WT1 expression might be restricted to leukemic blast cells, Inoue et al. showed that WT1 was also expressed in peripheral blood mononuclear cells from CML chronic phase. Total WT1 mRNA expression was found 10times higher in leukemias as compared to normal bone marrow cells. [5] Our own analyses have shown that certain WT1 variants might be restricted to blast cells only. [6] Total WT1 mRNA expression has already been used in clinical practice for monitoring patients with AML and MDS. [7, 8, 9] In 1994, Inoue et al. first suggested WT1 as new candidate on prognostic leukemic marker for monitoring minimal residual disease including CML. [5] Studies by Cilloni et al. [10] and more recent data by Varma et al. [11] indicated that WT1 might be useful also for CML patients on TKIs therapy.

Following promising data by Inoue et al., [5] Cilloni et al., [10] Varma et al. [11] we report herein a complex retrospective study of WT1 expression in CML patients treated with imatinib with the aim to test WT1 mRNA expression as a candidate additional CML biomarker which may improve early relapse prediction especially in suboptimal responders and therapy failures. Our data highlight the

advantages of WT1 as general leukemic marker enabling to rapidly assess the effectiveness of treatment and risk of relapse regardless of actual mechanisms underlying response or resistance to therapy.

MATERIAL AND METHODS

Patients, Cell Lines and Sample Processing

Totally, 39 patients with CML treated with imatinib (IM) in IHBT Prague between years 2001 – 2011 were included in this study. Thirty three patient samples of different responses to imatinib and CML phases (CCR, pCR, mCR, HR, Hr, AP/BC, definitions see below) were included in the primary evaluation of WT1 expression in CML (Figure 1), 396 samples of 32 patients were analysed during therapy (median: 10 samples per patient, range: 4-26, Figures 1-3). Median follow-up of patients was 41 months (range: 10-88 months). See Table I for detailed patients´ characteristics. All patients gave their written informed consent approved by the Ethics Comity of the Institute of Hematology and Blood Transfusion, Prague.

K562 cells used as positive controls were cultivated in RPMI medium under standard conditions and further processed in the same way as patients´ leukocytes. Total leukocytes were isolated by osmotic lysis and RNA was extracted by modified phenol-chlorophorm extraction. [12] cDNA was prepared using random hexamer primers and Superscript II reverse transcriptase (Invitrogen/Life Science, USA).

Response to Imatinib Criteria

The criteria for monitoring patients receiving TKIs are summarized in the European LeukemiaNet and National Comprehensive Cancer Network guidelines [1], briefly:

Haematological response (HR) is defined as the normalization of peripheral blood counts. Cytogenetic response is determined by bone marrow metaphase chromosome analysis (using at least

20 metaphases): complete (CCR; no Ph+ metaphases), partial (pCR; 1%-34% Ph+ metaphases), or minor (mCR; 35%-90% Ph+ metaphases). Major molecular response (MMR) is defined as a decrease of BCR-ABL level below 0.1% IS (international scale). Haematological relapse was defined as loss of complete HR (CHR).

Suboptimal response was defined as no CR at 3rd month, less than pCR at 6th month, pCR at 12th months, less than MMR at 18th month, and, in case of MMR loss, BCR-ABL mutations still sensitive to imatinib. Failure was defined as less than CHR at 3rd month, incomplete HR or no cytogenetic response at 6th month, less than pCR at 12th month, less than CCR at 18th month, and in case of CHR or CCR loss, clonal cytogenetic abnormalities, or BCR-ABL mutations.

Real-time PCR Analyses

All real-time PCR analyses were performed using Rotor Gene equipment (Qiagen) and B2M control gene. [12, 13] WT1 transcript level was quantified with primers and probe designed by Kreutzer et al. [14] Two delta delta CT method was used for WT1 data assessment; cDNA from K562 cells ten times diluted by cDNA from a mixture of leukocytes of healthy donors was applied as a calibrator. The results of BCR-ABL analyses were performed as previously described [12], data were taken from routine examinations.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 4.0 and MicroSoft Excell. Differences in WT1 levels between different therapy response groups were performed by Student t-test, ANOVA + Tukey test. The critical levels were estimated by ROC (receiver operator characteristic) curves. All the analyses were consulted with statistician.

RESULTS

WT1 Expression Levels in Different Responses to Imatinib

Patient samples of different responses to imatinib (n=33) were analysed for WT1 transcript level (Figure 1a). The results were compared with the analysis of BCR-ABL transcript levels in the same samples (Figure 1b). Samples of cytogenetic responses were included from around the 18th month of imatinib therapy (15-21), haematological remissions around the 4th month (2-5).

Patients with cytogenetic response exhibited low expression of WT1; expression in the CCR group was about the same as in normal individuals. WT1 levels were slightly increased in pCR and mCR groups (median 0.5 log) but the differences from CCR were not statistically significant ($p>0.05$). High WT1 levels with significant differences from cytogenetic responders were found in patients without cytogenetic response and then in disease progression ($p<0.05$). The difference in WT1 median level between mCR/pCR and BC was 3 logs (total expression range 5 logs). If we compare differences in BCR-ABL transcript levels with WT1 results, we found quite opposite situation. BCR-ABL, as a CML typical fusion transcript not present in normal individuals, exhibited great difference in cytogenetic responders; it was 3 logs in medians between CCR and mCR/pCR ($p<0.05$). Between mCR/pCR and BC, however, the difference was only 2 logs ($p<0.05$). It suggests that WT1 might be a candidate for marker of "un-optimal responders" but not for monitoring good responders to imatinib, where BCR-ABL is of advantage.

WT1 Expression Kinetics during the Course of CML treated with Imatinib

The kinetics of WT1 transcript level during therapy was retrospectively evaluated in 32 patients. We compared WT1 and BCR-ABL kinetics with regard to their ability to characterize disease status and to predict disease evolution, with focus on early (and clear) detection of haematological relapse.

Generally, WT1 as well as BCR-ABL levels are low and/or decreasing in good responders and high and/or increasing in poor responders with evolution of disease relapse or progression.

In patients with optimal response to imatinib, who achieved MMR before the 18 month since the therapy start, WT1 as well as BCR-ABL level exhibited sharp decrease (Figure 2a). WT1 achieved its

minimal level comparable with levels in healthy individuals and remained stable when the state of the disease was unchanged. BCR-ABL transcript level usually achieved negativity in these cases.

In relapsing patients (n=17; Figure 2b, c) the BCR-ABL expression was increasing or it showed stable levels. WT1 was increasing in all relapsing patients (n=17; Figure 2b, c). As compared to BCR-ABL, WT1 showed higher increase on average by 1 log in 65% (11/17) of patients. Total increase of WT1 was on average 2 logs as compared to only 1 log in case of BCR-ABL. More interestingly, WT1 was in nearly 60% (10/17) increased also earlier as compared to BCR-ABL. On average, WT1 increase preceded haematological relapse by 4.3 months while BCR-ABL by only 1.8 months in our patient cohort.

WT1 Expression in Patients with BCR-ABL Kinase Domain Mutation M244V

Several BCR-ABL mutations have been shown to exhibit patient to patient difference of clinical relevance. We tested whether WT1 might be of help in estimation of clinical relevance of detected mutation.

Four patients with the same mutation M244V were included in this study. While three out of those four patients relapsed haematologically within 10 to 30 months one patient remained in complete haematological remission for further 50 months. In relapsing patients, WT1 as well as BCR-ABL were increasing and thus signalized an on-going haematological relapse (Figure 2d). Decreasing levels of both WT1 and BCR-ABL (Figure 2e) differentiated patient who continued CCR from the others.

Mutations in the BCR-ABL kinase domain represent BCR-ABL-dependent mechanism of resistance and are associated with an increase in BCR-ABL transcript levels. WT1 might confirm the significance of risk of haematological relapse.

Predictive Value of WT1 Levels at selected Time Points of Imatinib Therapy for further Disease Course

Patients with SR or TF are in higher risk of haematological relapse than patients responding optimally (ELN criteria). However, these groups include patients who achieve response later ("slow responders"), patients who remain in long term complete haematological remission and patients who relapse haematologically. BCR-ABL transcript level is usually stabilised and loses thus much of its prognostic value. We wanted to find out whether WT1 levels would have a predictive value for further disease evolution in these non-optimal responders.

We tested 20 patients who did not achieved MMR but also did not develop haematological relapse within 18 months on imatinib (SR or TF according to ELN; see Table 1 for patients´ characteristics). We measured WT1 levels at the 6th and 12th month after beginning of imatinib therapy and evaluated haematological relapse incidence between 18th and 36th month. The patients were divided into 2 groups: a) patients with and b) patient without haematological relapse within 18-36 months. A group of 3 optimal responders was added for comparison. We found statistically significant differences in WT1 levels between all three groups ($P<0.0001$) (Figure 3a). When the same evaluation was performed for BCR-ABL transcript level, we did not find significant differences ($p=0.06$) (Figure 3b) and only optimal responders differed significantly from both remaining groups ($P<0.0001$).

ROC (receiver operating characteristic) curve analysis indicated that WT1 levels might be of predictive value and revealed the critical levels of WT1 expression in the 6th and 12th month: WT1 level > 0.12 at 6 months and WT1 level > 0.16 at 12 months were associated with significantly increased probability of haematological relapse.

Three CML Cases reporting predictive Value of WT1 Monitoring

The conclusion based on the above mentioned evaluations suggests that monitoring of BCR-ABL transcript levels complemented by monitoring of WT1 expression kinetics and particular levels may be of advantage. To illustrate how WT1 monitoring could be useful in patients with CML, we report finally three more interesting cases (Figure 4):

The first case (Fig. 4a) can be considered "slow cytogenetic responder" as defined by Tantiwiorat et al. Despite of achieving CCR more than a year later then it is recommended by ELN, patient remained in complete haematological remission for more than 85 months. Low WT1 levels indicated no immediate risk of Hr despite of BCR-ABL levels.

Figure 4b report results of a patient treated with imatinib in the pre-DASA and NILO era. The patient remained in complete haematological remission in the long term despite of 100% Ph positivity and extremely high BCR-ABL levels. Again, low WT1 expression indicated no immediate risk of haematological relapse. Further studies on defensive mechanisms against CML in such patients might be of high interest.

Patient in the Figure 4c exhibited primary resistance to imatinib and thus did not even achieve CHR. High WT1 levels together with clearly increasing trend since therapy initiation indicated aggressive and resistant form of CML. BCR-ABL levels were high and did not significantly differ from the other patients described in the previous paragraphs who did not relapse during the follow-up period (Figure 4a, b vs. Figure 4c).

DISCUSSION

CML is characterised by a typical fusion gene BCR-ABL which is used as a marker in monitoring the disease state in patients with CML. RT-PCR for monitoring of BCR-ABL transcript was introduced for early relapse detection in patients after transplantation as these relapses were abrupt and quickly progressed to untreatable blast crisis. In the era of targeted TKI therapy with high rate of molecular responses, BCR-ABL transcript monitoring is routinely used in all CML patients. The BCR-ABL transcript level well characterizes the state of the disease and the BCR-ABL kinetics has a prognostic value. Patients, who achieve decrease in BCR-ABL levels bellow 0.1% till the 18th month of imatinib therapy, are in low risk of relapse and progression. Recently, a prognostic value has been suggested for BCR-ABL expression already at the 3rd month of imatinib therapy. Hansftein et al. have showed that a BCR-ABL IS of 10% or more at the 3rd month of imatinib therapy is associated with a 5-year

overall survival of 87% suggesting the need for an early change of treatment. [15] On the other hand, BCR-ABL IS of 1% or less indicates a favorable 5-year overall survival of 97%. However, there are still cases where BCR-ABL quantification on its own is unable to bring satisfactory information.

Despite of BCR-ABL being the main causative factor in CML, the disease is very heterogenous. Other kinases cooperating with BCR-ABL might in some cases partially or even totally overtake the role of the driving force of the disease. In such cases, BCR-ABL on its own stops being reliable marker. In patients responding non-optimally to imatinib, BCR-ABL levels remain usually stable (1% to 100%). According to our own experience and others [2, 3], suboptimal responses as defined by ELN [1] include patients with truly inadequate response who are in a risk of relapse/progression, but also patients who could be called "slow responders". In our small patients´ cohort, we identified also patients remaining in complete haematological remission long term despite of Ph positivity and consistent BCR-ABL expression. CML mechanisms in such patients remain unknown. BCR-ABL monitoring losses much of its prognostic value and cannot predict relapse reliably on its own. Currently, no marker is available to distinguish sub-groups of suboptimal responses and therapy failures according to risk of haematological relapse and its immediacy. As we do not know all those side-players in CML pathogenesis a common marker reflecting the activity of the malignant cells might be useful. Based on literature data, Wilms´ tumor gene 1 seems to be suitable candidate. [5, 10, 11] WT1 gene sometimes even called "pan-leukemic" marker due to its overexpression in vast majority of leukaemias including CML. WT1 mRNA as compared to BCR-ABL may exhibit non-zero levels in leukocytes of healthy individuals. That was probably the reason why BCR-ABL showed higher sensitivity in relapse detection after transplantation. Herein we present a complex study of WT1 mRNA expression during the course of CML treated with imatinib.

We focused on the most problematic groups in terms of prediction of further disease course – suboptimal responses and therapy failures. Our data indicate that WT1 enables to improve risk of relapse assessment and prediction of response to therapy durability in those CML patients. As

compared to BCR-ABL, WT1 expression kinetics indicated on-going haematological relapse by higher and even earlier increase in 68.8 and 56% of patients, respectively. Both, expression kinetics and particular levels are probably of importance for further CML course prediction. While BCR-ABL did not allow to distinguish between suboptimal responders and therapy failures who relapsed haematologically vs. those who did not within the first 35 months of imatinib therapy, our statistical analyses revealed critical level for WT1 enabling to distinguish those patients at 12th month of imatinib therapy. Patients having WT1 levels over 0.116 in the 12th month on imatinib were found to be in high risk of haematological relapse for further 35 months. Levels below that critical level during all the course of therapy were associated with no immediate risk of relapse. As in the case of BCR-ABL, WT1 levels should be confirmed at least in three subsequent samples and taken into account together with the overall expression trend. Our data also suggest that WT1 might be helpful also in specification of clinical relevance of BCR-ABL kinase domain mutations. It seems that WT1 increase always means increased risk of relapse independently on the mechanism of resistance to therapy.

Recently, Tantiworawit et al. [17] and Hiwase et al. [18] discussed the phenomenon of so called sudden blast crisis. Sudden blast crisis develops in patients who achieved CCR and none of current markers or early response criteria can predict it. In our study, no patient with sudden blast crisis was included. However, based on our data showing excellent correlation of WT1 with CML status, we believe that WT1 is a highly suitable candidate marker which would warn and enable to detect progression also in these cases at time. This issue remains for further studies.

In conclusion, retrospective analyses of our CML patients revealed high suitability of WT1 monitoring for improvement of prediction of further disease course in CML patients. Our data indicate that both WT1 mRNA expression levels at selected time points of therapy and WT1 expression kinetics might help to better stratify "non-optimal responders" to imatinib according to actual risk of relapse and progression.

Total number of	32
Male	19
Female	13
Age at diagnosis	54

Patient no.	Sample used for Figure 1	OR/SR/TF (the 18th month) 18th	the best response on IM	Hr (months on IM)	total months on IM	therapy change	BCR-ABL KD mutation on IM
1	NA	OR	CMR	N	102	N	ND
2	NA	OR	CMR	N	78	N	ND
3	NA	OR	MMR	N	23	N	L273H
4	mCR	OR	CMR	N	100	N	ND
5	NA	TF	CCR	Y (27)	71	NILO	wt
6	CCR	TF	CCR	Y (17)	24	DASA	L378M
7	CCR	TF	CCR	Y (20)	28	SCT†	F370L, G250E
8	NA	TF	CCR	Y (29)	35	SCT	wt
9	mCR	TF	CCR	Y (58)	59	DASA	wt
10	pCR	TF	CCR (22)	Y (32)	33	DASA	F359V (21)
11	Hr	TF	CHR	Y (26)	26	NILO	wt
12	Hr	TF	CHR	Y (32)	60	DASA	M244V, H396R
13	BC/AP	TF	CHR	Y (16)	25	N†	wt
14	HR	TF	CHR	Y (18)	46	N†	M244V
15	HR	TF	CHR	Y (20)	67	DASA	M244V

16	Hr	TF	CHR	Y (24)	56	DASA†	F311I
17	Hr	TF	CHr	Y (22)	30	DASA, NILO	M351T
18	Hr	TF	CHR	Y (9)	50	SCT	F317L, M351T
19	NA	TF	CHR	Y (11)	14	N†	wt
20	HR; BC/AP	TF	CHR	Y (14)	19	SCT†	wt
21	Hr	TF	CHR	Y (20)	35	N†	wt
22	NA	TF	Prim rez	-	30	N†	H396R
23	NA	TF	CCR	N	119	N	wt/F317L/N358K
24	mCR	TF	CCR	N	44	DASA	E255K
25	pCR	TF	mCR	N	30	DASA	wt
26	HR	TF	pCR	N	78	N	wt
27	HR	TF	CHR	N	15	DASA	ND
28	HR	TF	CHR	N	34	N†	wt
29	CCR	TF	MMR	N	57	N	wt
30	pCR	TF	CCR	N	47	N	wt
31	CCR	SR	CCR	N	46	N	M244V
32	CCR	SR	CCR	N	60	N	wt
33	CCR	SR	MMR	N	63	N	wt
34	mCR	SR	MMR	N	102	N	wt
35	pCR	SR	CCR	N	18	DASA	wt
36	HR	SR	MMR	N	98	N	wt
37	HR	SL	CCR	Y (36)	58	DASA	Y253H

38	BC/AP	SL	pCR	NA*	10	N†	wt
39	BC/AP	SL	pCR	NA*	15	N†	T315I, G250E

Table I. Patients´ characteristics; BC – blast crisis, CCR – complete cytogenetic response, HR – haematological remission, Hr – haematological relapse, mCR – minor cytogenetic response, MMR – major molecular response, N – no, OR – optimal response, pCR – partial cytogenetic response, SR – suboptimal response, TF – therapy failure, wt – wild type, Y – yes, † - death; patients 33 to 39 were included in the evaluation on Figure 1 only due to low number of sequential analyses or short time on imatinib; *patients were administered imatinib in the accelerated phase of CML.

FIGURE LEGENDS

Figure 1. Comparison of WT1 (a) and BCR-ABL (b) expression in different responses to imatinib and CML phases; aligned dot plot, median with range; CCR – complete cytogenetic response, pCR – partial cytogenetic response, mCR – minor cytogenetic response, HR – haematological remission, Hr – haematological relapse, BC – blast crisis, N – healthy controls.

Figure 2. WT1 expression kinetics during the course of imatinib therapy; (a) patient no.1, optimal responder to imatinib (b) patient no.13, Hr 17th month on imatinib, high WT1 increase indicated hematological relapse completing warning given by stably high BCR-ABL levels, conclusive increase of WT1 preceded BCR-ABL by 2 month, total WT1 increase was 3 log as compared 1 log increase of BCR-ABL levels; (c) patient no.7, Hr 20 months after starting with imatinib, WT1 increase preceded BCR-ABL by 2 months; (d) patient no.31, M244V mutation in BCR-ABL kinase domain detected in the 10th month not followed by Hr – decreasing trend in both BCR-ABL and WT1; (e) patient no.15, M244V detected 2 months after starting with imatinib, increase in both, BCR-ABL and WT1 since imatinib therapy initiation.

Figure 3. Predictive value of WT1 expression for the first 35 months of imatinib therapy; (a) WT1 and (b) BCR-ABL levels in the 6th and the 12th month of IM therapy in SR/TF Hr (white box), SR/TF (grey box; Student t-test, $p<0.001$ and $p>0.05$, respectively). OR (black box) is shown as control. (c) ROC curve analysis indicated that the test might be useful in the 12th month of imatinib therapy (area under curve 0.7) for distinguishing SR/TF and SR/TF Hr. The 0.161 has been identified as the critical level.

Figure 4. Predictive value of combinatoral consideration of BCR-ABL kinetics and WT1 kinetics and levels; (a) patient no.23, "slow cytogenetic responder": CCR achieved in the 28th months after starting with imatinib, remaining in CCR for more then 85 months of further follow up; (b) patient no.26, patients achieving minorCG as the best response 60 months after starting with imatinib,

retaining complete hematological remission for further 18 months; (c) patient no.22, primary resistant patient, progression 10 months after starting with imatinib.

ACKNOWLEDGEMENT

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FIGURES

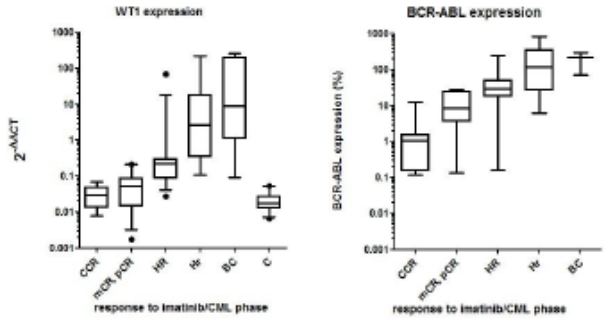


Figure 1.

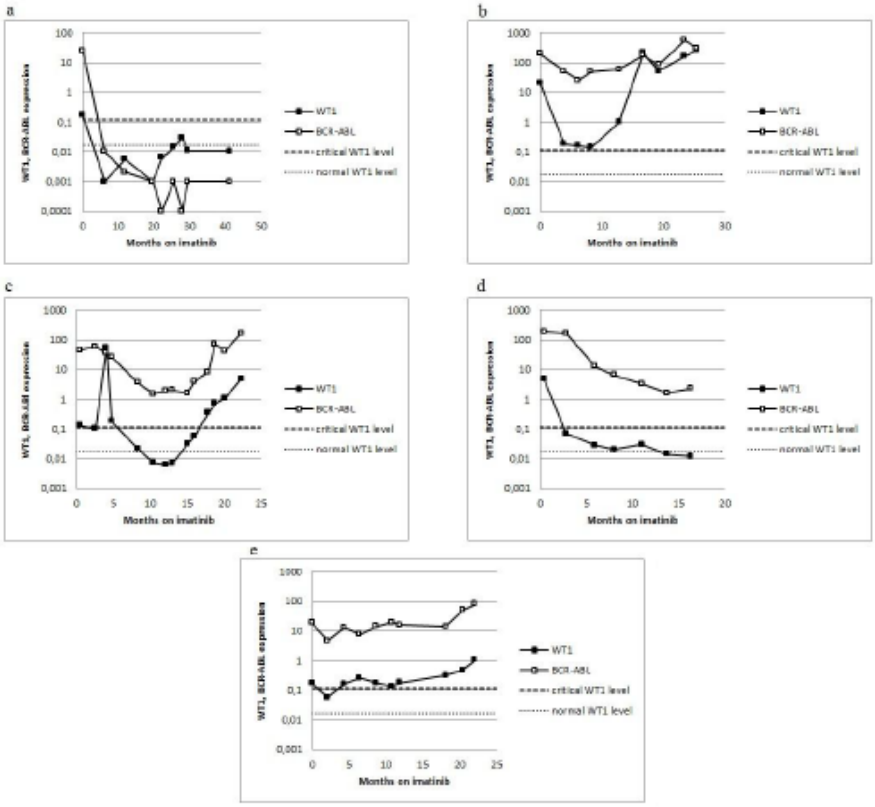
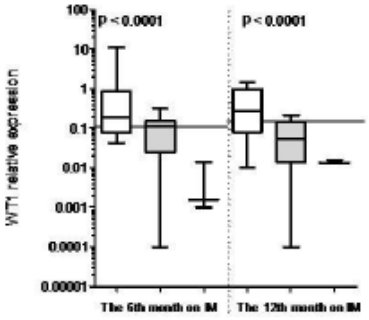


Figure 2.

a


p < 0.0001
p < 0.0001
WT1 relative expression
100
10
1
0.1
0.01
0.001
0.0001
0.00001
The 6th month on IM
The 12th month on IM

b

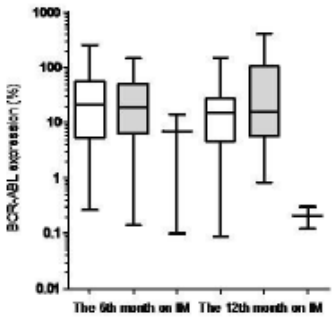

BCR-ABL expression [%]
1000
100
10
1
0.1
0.01
The 6th month on IM
The 12th month on IM

Figure 3.

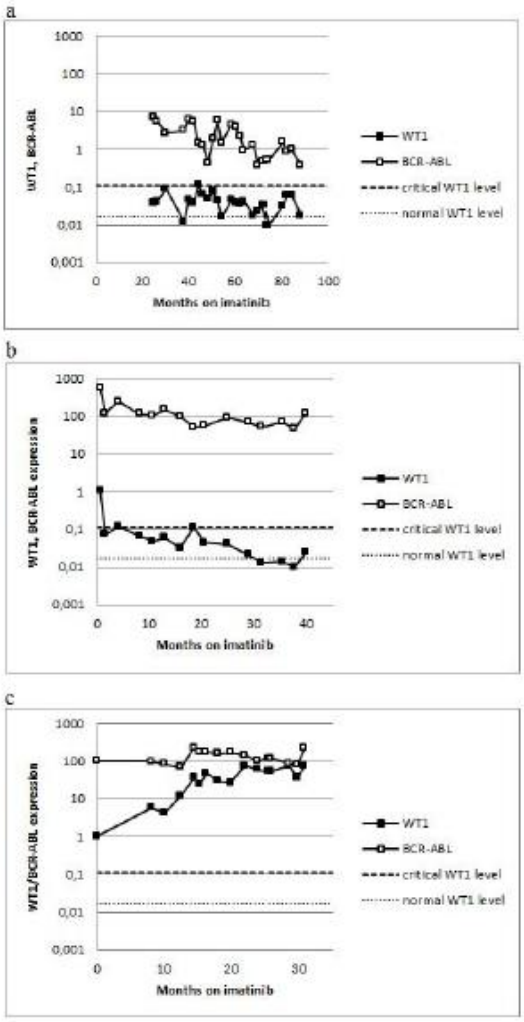

a
1000
100
10
1
0,1
0,01
0,001
WT1, BCR-ABL
0
20
40
60
80
100
Months on imatinib
WT1
BCR-ABL
critical WT1 level
normal WT1 level
b
1000
100
10
1
0,1
0,01
0,001
WT1, BCR ABL expression
0
10
20
30
40
Months on imatinib
WT1
BCR-ABL
critical WT1 level
normal WT1 level
c
1000
100
10
1
0,1
0,01
0,001
WT1/BCR-ABL expression
0
10
20
30
Months on imatinib
WT1
BCR-ABL
critical WT1 level
normal WT1 level

Figure 4.

23

Publikace č. 2

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Expression of four major WT1 splicing variants in acute and chronic myeloid leukemia patients analyzed by newly developed four real-time RT PCRs

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ABSTRACT

Although the mechanism of action of leukemic oncogene Wilms' tumor gene 1 (WT1) remains unclear, WT1 has already been used in monitoring of patients with acute myeloid leukemia (AML) and it is being tested for immunotherapy. More detailed understanding of the role of WT1 in leukemia may improve its utilization. At least 36 isoforms may be produced. Four major variants denoted as −5/−KTS, −5/+KTS, +5/−KTS and +5/+KTS are produced by combining splicing of exon 5 and KTS sequence. In this study, we report applicability of newly developed real-time RT PCRs enabling for the first time full quantification of the four major WT1 splicing variants. Following careful optimization and testing of quantification reliability of four assays, we analyzed 34 samples of patients with AML and 12 samples of patients with chronic myeloid leukemia (CML) at the time of diagnosis. Analyses of five more CML patients provided insight into WT1 variants expression kinetics. We found predominance of +5/+KTS in both diagnoses. Comparison of WT1 variant expression in AML and CML patients' groups differing in response to therapy suggested possible importance of particular WT1 variant levels as markers of further disease course.

Introduction

The Wilms' tumor gene (WT1) takes part in physiological developmental processes and also in tumorigenesis. While WT1 acts as a tumor suppressor gene in Wilms' tumor, it has oncogenic effects in most of leukemias [1,2]. Although the mechanism of WT1 action in leukemia has not yet been explained, total WT1 expression is already used to monitor minimal residual disease (MRD) in acute myeloid leukemia (AML) patients in our laboratory and in others [3–5], and WT1 peptides are being tested for vaccination [6]. Detailed understanding of the role of WT1 in leukemias may improve its utilization in diagnostics, prognostics and therapy.

WT1 encodes for a zinc finger transcriptional regulator which, depending on the cellular context, can either enhance or inhibit transcription of its target genes which include genes important for cellular growth and metabolism. Enormous amount of WT1 variants further extend the field of activity of WT1. At least 36 isoforms are produced by combining several transcriptional modifications, alternative sites for initiation of translation [7,8], alternative promoter [9], alternative first exon [10] and RNA editing [11]. Two splicing events including exon 5 and the KTS sequence in exon 9 are considered predominant. By combining these two events, four major WT1 splicing variants denoted as −5/−KTS, −5/+KTS, +5/−KTS and +5/+KTS are produced. Splicing of KTS removes three amino acids (lysine = K, threonin = T, serine = S) between zinc fingers 3 and 4 of the WT1 transcriptional regulator which alters the nucleic acid-binding properties of the protein [12–15]. Splicing of exon 5 removes a 17-amino-acid segment in the middle of protein–protein interaction domain which is involved in overcoming apoptosis [16,17]. Both splicing events may affect impact of cytotoxic drugs. Levels of individual variants thus might give additional information on patients' responsiveness to therapy and prognosis. Predominance of +5 variants at mRNA level has been previously found in AML patients' samples using a semi-quantitative approach [5,18].

In the present study, we demonstrate applicability of newly developed real-time RT PCRs for full quantification of four major WT1 splicing variants possibly exploitable to a number of diseases with altered WT1 expression. The method was originally presented on The Annual Meeting of American Society of Hematology 2009 [19] and subsequently further tuned and found imperfections rectified [20] until the form described in this article. Our data indicate possible importance of WT1 variants expression as markers for further disease course in patients with CML and AML.

Material and methods

Patients' samples and cell lines

Thirty four samples of patients with AML and 12 with chronic myeloid leukemia (CML) were analyzed at the time of diagnosis (i.e. untreated) in

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this study. AML patients were classified according to French–American–British (FAB) [22] and all AML subtypes were included. AML patients' characteristics are given in more detail in Table 1a. CML samples were characterized by Sokal and Hasford risk scores and by achieved responses to treatment defined by European LeukemiaNet (ELN) criteria [23]. Twelve CML patients' characteristics are given in more detail in Table 1b. Five more CML patients were analyzed during the course of imatinib therapy (characterization is given in Results, paragraph "CML patients") which provided some insight into kinetics of WT1 variants expression. All patients gave their written informed consent. The study was approved by the Ethics committee of the Institute. The cDNA of CML leukemic cell line K562 was used during development of real-time RT-PCRs.

Leukocytes were prepared from peripheral blood samples by red cell osmotic lysis within 12 hours after sample collection. K562 cells and patients' leukocytes were lysed in guanidium–thiocyanate buffer in the concentration of 10^7 leukocytes/ml.

RNA extraction and cDNA preparation

Total RNA was extracted by acidic phenol-chloroform extraction: cDNA synthesis was prepared using random hexamers and SuperScript II transcriptase (Invitrogen/Life Technologies, USA).

Plasmid standard preparation

Plasmid standards for *WT1* and *WT1* variants quantifications were prepared using PCR cloning with TOPO-TA cloning kit (Invitrogen/Life Technologies, USA) following manufacturers' instructions. The presence of proper inserts was checked by three times repeated sequencing of plasmid DNA in both forward and reverse directions using M13 primers (included in the cloning kit). Linearization was performed using NsiI restrictase (Sigma-Aldrich, USA), and plasmid DNA copy numbers were established. Linearized plasmid DNAs were diluted in sonicated Salmon Sperm DNA (Agilent Technologies, USA; 200 ng/μl in 1xTE buffer, pH 8).

Table 1a
AML patients' samples characteristics.

Total number of patients	34	Number of patients (%)
Sex	Male	17 (50)
	Female	17 (50)
Age at the time of diagnosis	18.4–74.1 (Median 41.2)	
FAB subtype	M0	1 (3.1)
	M1	8 (21.9)
	M2	8 (21.9)
	M3	4 (12.5)
	M4	5 (15.6)
	M5	5 (15.6)
	M6	1 (3.1)
	sec. AML	1 (3.1)
	AML-MLD	1 (3.1)
WHO subtype (cytogenetics based prognostic group)	Poor	4 (13.3)
	Intermediate	23 (66.6)
	Favorable	7 (20)
Response to induction	CR1	21 (61.8)
	CR2	7 (20.6)
	PR	1 (2.9)
	NR	5 (14.7)
Hematological relapse	Yes	8 (24.1)
	No	26 (75.9)

CR—complete remission, MLD—multilineage dysplasia, NR—no response to therapy, PR—partial remission.

Table 1b
CML patients' samples characteristics.

Total number of patients	12	Number of patients (%)
Sex	Male	4 (33)
	Female	8 (67)
Age at the time of diagnosis	22–61, 15 (Median 49.7)	
Sokal score	1	2 (16.7)
	2	6 (50)
	3	4 (33.3)
Hasford score	1	4 (33.3)
	2	5 (41.7)
	3	3 (25)
IFN pre-treatment		3 (25)
Imatinib as a first line therapy		9 (75)
Response to imatinib	Optimal	8 (67)
	Suboptimal/failure	4 (33)
Hematological relapse	Yes	1 (8)
	No	11(92)

Responses to imatinib were evaluated according to European LeukemiaNet criteria in the 18th month of imatinib therapy: Optimal response—achieving major molecular response (BCR-ABL below 0.1%), suboptimal response—less than major molecular response, therapy failure—less than complete cytogenetic response (0% of Ph positive cells).

Real-time PCR analyses

All PCRs were performed using Rotor-Gene equipment (Qiagen, USA).

Primers and probes for analyses of four major WT1 splicing variants (transcription variant A (−5/−KTS) [NM_000378.3], transcription variant B (+5/−KTS) [NM_024424.2], transcription variant C (−5/+KTS) [NM_024425.2], and transcription variant D (+5/+KTS) [NM_024426.3]) were designed using Primer 3 software V 0.4.021 (Rozen S. and Skaletsky H.J. 2000); sequences are listed in Table 2. The method was based on combination of four discriminating primers. Forward primers were positioned on exons 4/5 or 4/6 junction to distinguish +5 and −5 variants, respectively. Reverse primers hybridized to the region of spliced and non-spliced KTS sequence to distinguish −KTS and +KTS variants, respectively. All four primers were to be combined with one common TaqMan probe. Reaction mixes were as follows: (1) for −5/−KTS, +5/−KTS and +5/+KTS 1U Fast Start polymerase (Roche, Switzerland), 10× PCR buffer (Roche, Switzerland), 3.7 mM $MgCl_2$ (Roche, Switzerland), 200 nM dNTP (Promega), 200 nM primers and 100 nM probe were mixed into a final volume of 20 μl; (2) for −5/+KTS 5 mM DTT was added. Temperature profiles were the following: (1) for −5/−KTS 95 °C 10 min, followed by 45 cycles: 95 °C 10 s, 64 °C 45 s; (2) for −5/+KTS and +5/+KTS 95 °C 10 min, followed by 45 cycles: 95 °C 10 s, 60 °C 60 s; (3) +5/−KTS 95 °C 10 min followed by 45 cycles: 95 °C 10s, 60 °C 45 s. All samples were tested in duplicates. Expression of total WT1 was measured using primers and probe according to Kreutzer et al. [3]. Evaluation was performed using standard curves and the data were normalized to beta glucoronidase (GUS) expression levels [24,25]. Where denoted, the WT1 variants expression was expressed as a proportion of all four variants (%).

Table 2
Primers and probe sequences for expression analyses of the four major WT1 splicing variants.

Primer name (specificity)	Sequence
F1 (−5 specific forward)	**gagccaccttaaagggccaca**
F2 (+5 specific forward)	**ggacagaagggcagagcaacc**
R1 (−KTS specific reverse)	**gaagggcttttcacctgtatgag**
R2 (+KTS specific reverse)	**cttttcactTgTtttacctgt**
Probe	5′FAM **cgagagcgataaccacacaacgccc** 3′BHQ1

LNA modified nucleotides are underlined.

Statistical analyses

Statistical analyses including Spearmans' correlation test and two-tailed Mann–Whitney test were performed using GraphPad Prism version 4.1. (GraphPad Software). The level of statistical significance was set at a p-value 0.05.

Results

Optimization and verification of the assays for quantification of four major WT1 splicing variants

To optimize PCR parameters, we tested different annealing times, LNA modifications within the +KTS primer and various PCR additives. To check reaction parameters, we performed amplifications of serial dilutions of plasmid standards. When using the optimized protocol described in Materials and Methods, we found linearity of the measurement in a range from 10 to 1,000,000 copies. Representative examples of standard curves including reaction efficiency and sensitivity for each reaction are given in Fig. 1. Slight differences among reaction efficiencies (93%, 83%, 95% and 84%) aimed us to check quantification reliability of whole system. Primer positions moreover indicated low but non-negligible risk of cross-amplification. To check quantification reliability with respect to the risk of cross-amplification of the assays, we performed two tests.

First, we prepared mixtures of plasmid standards of known concentrations simulating 20 different ratios of four WT1 variants. All four variants were measured in all 20 mixtures, and obtained copy numbers and ratios were compared with known plasmids concentrations and ratios. Correlation coefficients $R^2 \geq 0.9$ confirmed quantification reliability in this artificial system.

Second, we compared amounts of total WT1 with sums of all four WT1 variants in real AML ($n=34$) and CML ($n=12$) patients' samples. PCR for total WT1 expression analyses amplifies a sequence lying between the two spice sites, i.e. amplicons of all four variants include this part (Fig. 1). The PCR for total-WT1 meets optimal real-time PCR parameters including 98% efficiency and <10 copies sensitivity. Correlation of the sum of all four variants normalized copy numbers with total WT1 normalized copies ($R=0.85$) confirmed quantification reliability of our method.

Application of the method

The above described method was subsequently used to retrospectively evaluate WT1 variants profile of AML and CML patients.

WT1 splicing variants expression profile of AML and CML diagnoses

The expression profile of the four major WT1 splicing variants for the investigated patients is shown in Fig. 2–a for AML and b for CML. In both AML and CML diagnoses, +5/+KTS variants represented nearly 50% of total WT1 expression. The AML and CML diagnostic samples differed in the proportion of +5/−KTS and −5/+KTS variants (Fig. 2a, b). While the +5/−KTS was more expressed in AML, higher expression of −5/+KTS was seen in CML. Differences in expressions of these two WT1 variants between AML and CML were statistically significant ($p<0.001$).

We found statistically significant differences among different AML FAB subtypes (Fig. 3a–i). While in the majority of AML FAB subtypes, the +5/−KTS and +5/+KTS were nearly equally expressed, levels of +5/−KTS were found much higher in AML M3, M6 and secondary AML as compared to remaining subtypes ($p<0.05$). The proportion of −KTS as compared to +KTS was increased in those patients. Total WT1 showed the highest levels in AML M2, sec. AML and AML M1 in our patients' cohort (Fig. 3d, g, i). Our data did not indicate any association of WT1 variants ratio with total WT1 levels.

Changes in WT1 variants expression in AML and CML patients associated with response to therapy

AML patients

We made a comparison of WT1 variants expression in diagnostic samples of patients who did vs. who did not achieve response to therapy in further follow-up. While there were no significant differences found among cytogenetics based AML risk groups, our data indicated certain difference in proportions of +5/−KTS and +5/+KTS from total WT1 in association with achieved response to therapy (Fig. 3j; $p>0.05$). Higher proportion of +5/−KTS and lower proportion of +5/+KTS were found in patients who did not achieve response to therapy. True assessment of statistical significance will however need larger patients' cohort.

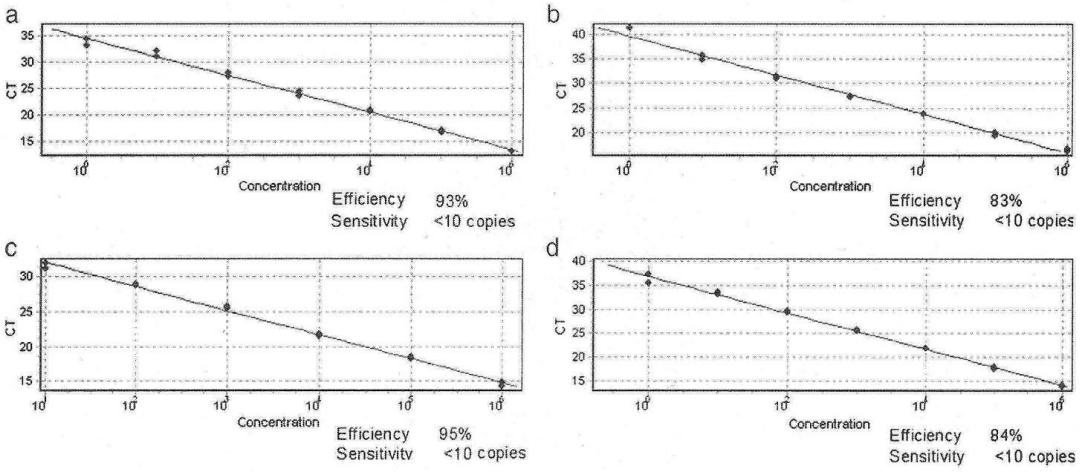


Fig. 1. Reaction parameters and calibration curves of real-time PCRs for expression analyses of four major WT1 splicing variants. Amplification of 10-dilutions of plasmid DNAs carrying PCR products of particular WT1 variants confirmed linearity of measurement from 10 to 1,000,000 copies. a, −5/−KTS; b, −5/+KTS; c, +5/−KTS; d, +5/+KTS.

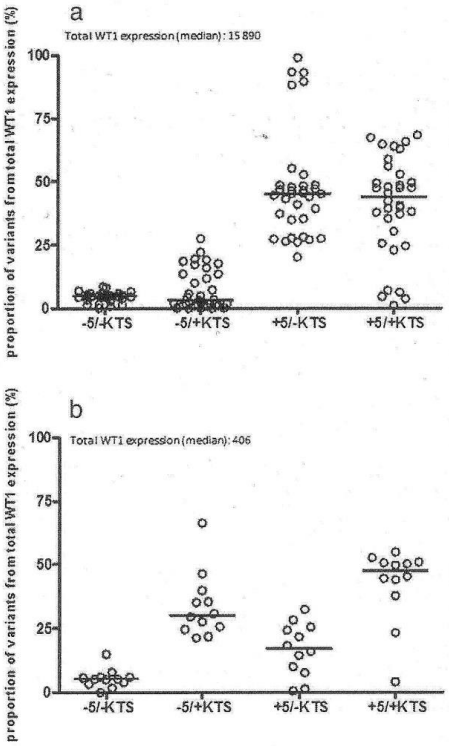


Fig. 2. The expression profile of four major WT1 splicing variants in diagnostic samples of AML and CML. WT1 splicing variants are expressed as proportion from total WT1 (%), median total WT1 expression is shown as copies per 100,000 copies of GUS; (a) AML patients' samples from the time of diagnosis, $n=34$; (b) CML patients' samples from the time of diagnosis, $n=12$. The $+5/-$KTS was found in higher proportion from total WT1 in AML, AND the $-5/+$KTS was found higher in CML (Mann–Whitney test, $p<0.001$).

Total WT1 levels were found in median only three times higher in patients' without remission as compared to those who achieved remission (Fig. 3j).

CML patients

Due to low number of patients in the CML diagnostic cohort ($n=12$), we could not evaluate differences in WT1 variants expression to assess any association with further disease course. Inclusion of another 5 patients at different times from imatinib therapy initiation into our CML patients' cohort provided a possibility to investigate WT1 variants expression kinetics (Fig. 4). In frame of this pilot study, we retrospectively examined 5 CML patients—3/5 hematologically relapsed, 2/5 remained in complete hematological remission. Total WT1 well correlated with the course of the disease in all cases: Total WT1 levels increased in all three patients with hematological relapse, preceding relapse by 3–5 months, but remained low in two patients remaining in complete hematological remission. The $-5/+$KTS was the first and most highly increased variant before and in relapse in all three relapsed patients. In two out of three hematologically relapsed patients, the $-5/+$KTS increase even preceded BCR-ABL and total WT1 increase (for example see Fig. 4a). Importantly, in two patients remaining in complete hematological remission, the $-5/+$KTS remained mostly undetectable (for example see Fig. 4b). Those figures, whatever low, suggest possible importance of $-5/+$KTS WT1 variant in early detection of CML relapse onset.

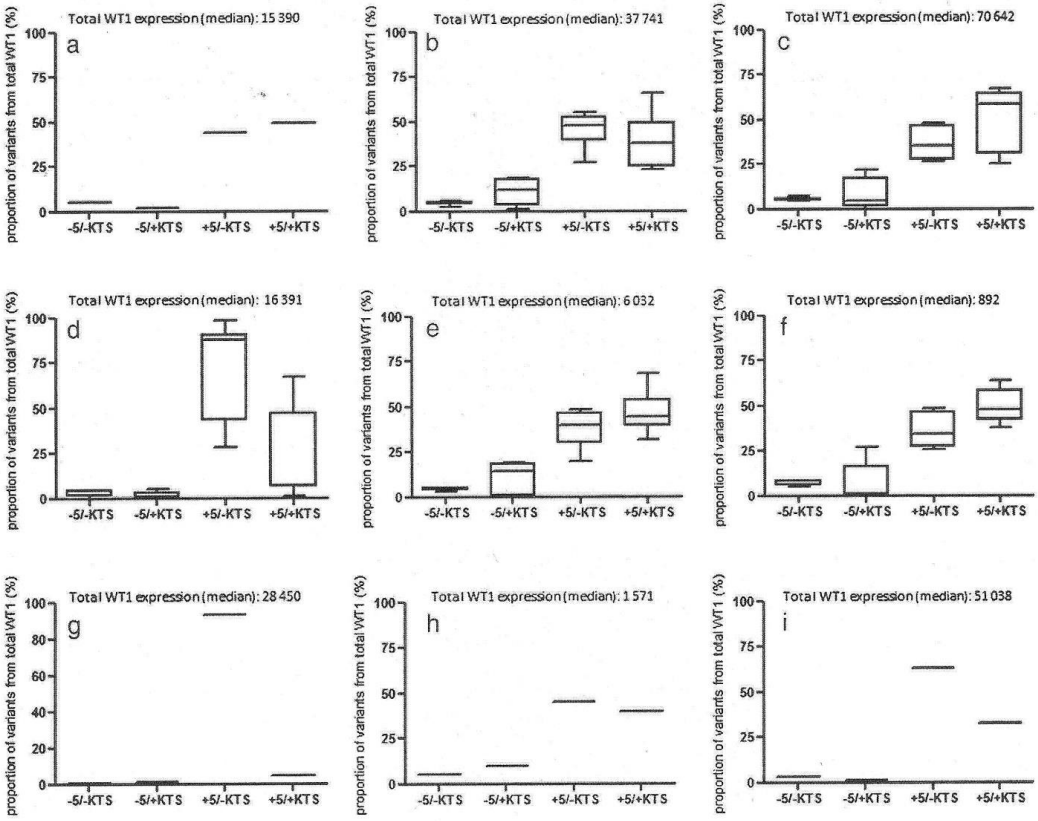
Discussion

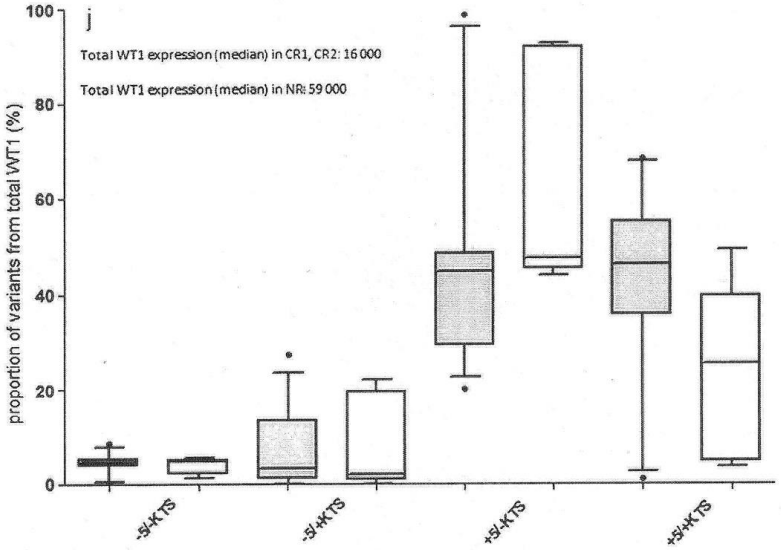
Although the exact role of WT1 in physiological and leukemic hematopoiesis has not yet been fully explained, prognostic value of total WT1 expression is well known and it has already been used in clinical practice in AML [3,4], MDS [21] and eventually also for CML patients. Splicing of *WT1* exon 5 has been previously associated with regulation of sensitivity to apoptosis [16,17]. We thus suppose that knowledge of expression levels of *WT1* splicing variants and their ratios might give additional and possibly more precise information on the disease state and prognosis, complementary to other markers including total *WT1* levels. Only two studies dealing with the analysis of expression of four major WT1 splicing variants ($-5/-$KTS, $-5/+$KTS, $+5/-$KTS and $+5/+$KTS) in leukemias have been reported, both of them using a semi-quantitative approach [5,18]. We have therefore developed fully quantitative assays for the four major WT1 splicing variants expression analyses which enabled us to assess quantitatively all four variants, each one separately. By means of it, we identified expression profile of four major WT1 variants in AML and CML patients' samples.

Our data showed predominance of $+5$ variants in diagnostic samples of AML patients which is in agreement with results previously published by Siehl et al., 2004 [18] and Gu et al., 2010 [5]. Further, we extended those observations also on CML patients and found higher incidence of $+5$ variants also in CML diagnostic samples. Moreover, as our assays enable us to obtain information on any particular WT1 variant expression, we showed that $+5/+$KTS is the most highly expressed in most of both CML and AML patients at the time of diagnosis. On the other hand, while the $+5/-$KTS was more expressed in AML, higher expression of $-5/+$KTS was seen in CML. In the majority of AML patients, the $+$ to $-$KTS ratio remained nearly 1. The $+$KTS variants were predominant in CML.

To see whether there might be any association between WT1 variants expression and responsiveness to therapy, we also compared WT1 variants expression among diagnostic samples of different AML subtypes and different responses to therapy in further follow-up. Analyses of five CML patients during the course of therapy provided insight into kinetics of WT1 variants expression in developing relapse vs. retaining hematological response. High expression of the $+5/-$KTS (increased to $+$KTS ratio) at the time of diagnosis seemed to be associated with aggressive (AML M3, sec. AML) and/or resistant AML (patients' without subsequent response to therapy). Total WT1 levels did not differ significantly in association with different sensitivity to therapy in our AML patients' cohort. WT1 variants thus might possibly be useful in specification of AML patients' prognosis. True assessment of statistical significance will however need larger patients' cohort. During CML relapse, the $-5/+$KTS became the predominant variant while it remained mostly undetectable in patient remaining in complete hematological remission. Careful evaluation of $-5/+$KTS variant expression during the course of CML in a larger patients' cohort may reveal its potential to serve as an early marker of relapse onset. This seems to be supported by our preliminary data from the five patients.

Fig. 3. Analyses of four major WT1 splicing variants expression in AML diagnostic samples; whiskers—5–95% percentile, dots—outliers. (a–i) Expression of WT1 variants in different AML FAB subtypes; a—M0, b—M1, c—M2, d—M3 (APL), e—M4, f—M5a, g—M6, h—AML-MLD, I—sec. AML; significantly higher proportions of $+5/-$KTS from total WT1 were found in AML M3 (APL), AML M6 and sec. AML as compared to other subtypes (Mann–Whitney test, $p<0.05$). (j) Expression of WT1 variants in diagnostic AML samples of patients who did (gray boxes) vs. who did not achieve (clear boxes) remission. Data indicated differences in $+5/-$KTS and $+5/+$KTS levels (Mann–Whitney test, $p>0.05$).


a
Total WT1 expression (median): 15 390
b
Total WT1 expression (median): 37 741
c
Total WT1 expression (median): 70 642
d
Total WT1 expression (median): 16 391
e
Total WT1 expression (median): 6 032
f
Total WT1 expression (median): 892
g
Total WT1 expression (median): 28 450
h
Total WT1 expression (median): 1 571
i
Total WT1 expression (median): 51 038
proportion of variants from total WT1 (%)
-5/-KTS
-5/+KTS
+5/-KTS
+5/+KTS


j
Total WT1 expression (median) in CR1, CR2: 16 000
Total WT1 expression (median) in NR: 59 000
proportion of variants from total WT1 (%)
-5/-KTS
-5/+KTS
+5/-KTS
+5/+KTS

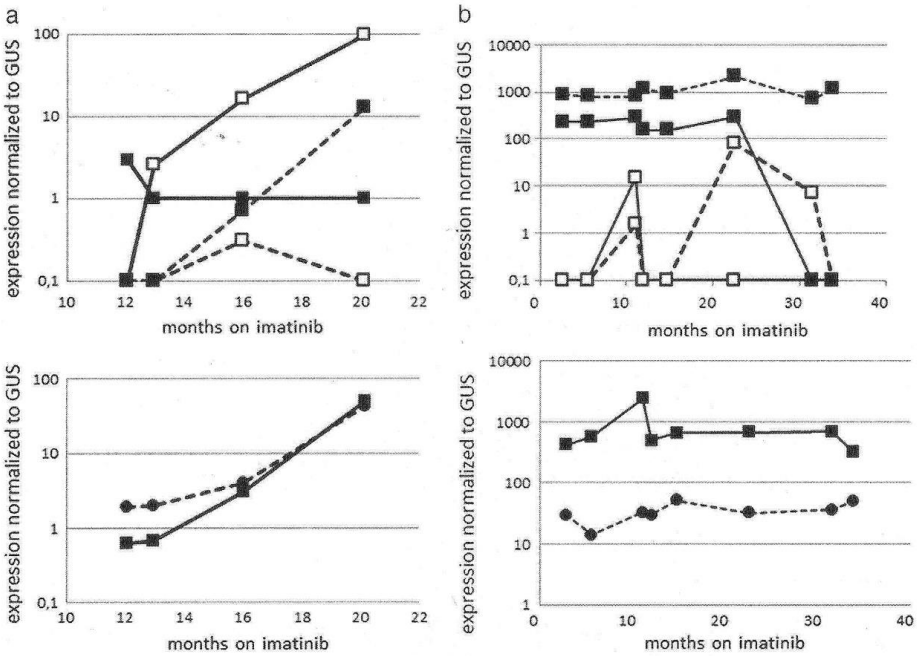


Fig. 4. Expression kinetics of four major WT1 splicing variants during the course of CML as compared to BCR-ABL and total WT1 expression. Upper graphs: expression of four major WT1 splicing variants (—□— −5/−KTS, —□— −5/+KTS, --■-- +5/−KTS, —■— +5/+KTS), lower graphs: expression of total WT1 (—■—) and BCR-ABL (--●--). (a) Forty-two-year old male with optimal response to imatinib in the 12th month of therapy (achieved CCR). He showed 1 log increase in *BCR-ABL* in the 18th month and hematologically relapsed in the 21th month. Interestingly, total *WT1* was increased 8.4-fold already 5 months before HR (lower graph). The most early (3 months even before total WT1) and the highest increase was seen in −5/+KTS (upper graph). (b) Sixty-two-year old man with failure in the 12th months of imatinib therapy (insufficient cytogenetic response). However, he did not develop relapse within further 30 months of imatinib therapy. BCR-ABL was high but relatively stable (lower graph). Also total WT1 (lower graph) and its variants showed no permanent increase (upper graph). Importantly, −5/+KTS stayed mostly undetectable.

Conclusions

In conclusion, our study demonstrates applicability of newly developed real-time RT PCRs for quantification of four major WT1 splicing variants possibly exploitable to a number of diseases with altered WT1 expression. Our analyses of CML and AML patients' samples suggest possible importance of WT1 variant levels as markers of further disease course in patients with myeloid leukemias. WT1 variants might specify the information given by total WT1 expression. Further analyses of a larger set of patients' samples are however necessary to confirm our results and to assess truly statistical significance of our observations.

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Letter to the Editor

N-terminally truncated WT1 variant (sWT1) is expressed at very low levels in acute myeloid leukemia and advanced phases of chronic myeloid leukemia

The Wilms' tumour gene 1 (WT1) encodes a multifunctional protein important for regulation of cell growth and survival. It plays a role in many physiological developmental processes and also in cancers including leukemia. WT1 is overexpressed in most of leukemias and therefore it is sometimes even called a "panleukemic marker". Total WT1 expression level is used in monitoring minimal residual disease in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) patients [1]. Besides this, WT1 is being currently tested for vaccination. Although the oncogenic behaviour of WT1 in leukemia has been proved, the mechanism has not yet been clearly explained. Detailed understanding of the role of WT1 in leukemia will improve the utilization of WT1 in diagnostics, prognostics and also in therapy. WT1 has an enormous number of variants due to alternative splicing, alternative initiation of translation etc. In 2004, a novel N-terminally truncated WT1 variant (sWT1) has been described by Dalloso et al. [2]. The sWT1 arises from alternative first exon E1a; it lacks the N-terminal transcriptional repression domain of full length WT1 (fWT1) and it activates expression of genes, which are repressed by fWT1. Hossain et al. [3] reported overexpression of sWT1 in leukemias and assumed that sWT1 might be the oncogenic WT1 variant based on in vitro experiments. Recently, we have read with high interest a study of Ishikawa et al. [4] which supplemented Hossain's and also our own data on sWT1 expression in myeloid leukemias.

In our study, we have tested sWT1 expression in chronic myeloid leukemia (CML) and AML patients. We designed discriminating forward primers hybridizing onto exon E1 or E1a and common probe and reverse primer hybridizing onto exon 2 to distinguish between fWT1 and sWT1: forward sWT1 5′-cctgcctactcctgggct-3′, forward fWT1 5′-cagcccgctattcgcaatc-3′, reverse 5′-tcatgcttgaatgagtggttgg-3′ and probe 5′-FAM cagcacggtcaccttcgacggga BHQ1-3′. We used cDNAs from K562, CML-T1 and JURL-MK1 cell lines as positive controls. All three cell lines expressed low levels of sWT1 (less than normalized 100 copies) and high levels of fWT1 (more than 10,000 normalized copies). The cDNAs from total leukocytes of four normal individuals were used as negative controls; WT1 expression was negligible. Plasmid standards prepared from K562 cell line were used for checking reaction sensitivity, preparing standard curves and performing quantification. Expression data were normalized to glucoronidase (GUS) gene expression [5]. We have analyzed samples of total leukocytes of peripheral blood (PB) in 48 AML and 18 CML patients. AML samples were collected at the time of diagnosis; different FAB subtypes were included in the study. Six of 18 CML patients' samples were collected in major molecular response (MMR, BCR-ABL levels ≤0.1%), 6 in hematological relapse (Hr, increase in leukocyte count over the physiological level of 10×10^9/L of peripheral blood) and 6 in accelerated phase (AP; 10–30% of blast cells in PB) or blast crisis (BC; ≥30% of blast cells in PB).

The sWT1 and fWT1 expression in different AML FAB subtypes are shown in Fig. 1A. Altogether, we detected sWT1 in 30 of 48 (63%) patients with AML. The highest rate of sWT1 positivity was found in M3 (10/12; 83%), M4 (6/9; 67%), M5b and M6 (100%). However, only a very low number of patients in M5b and M6 (2 and 1, respectively) was evaluated. The sWT1 levels were very low in all AML patients' samples except of M5b AML, where sWT1 expression was equal to fWT1 (Fig. 1A). In CML (Fig. 1B), sWT1 expression was detected exclusively in blast crisis or accelerated phase but its expression levels were very low similarly to the case of cell lines K562, CML-T1 and JURL-MK1 derived from CML blast crisis. The sWT1 was undetectable in CML chronic phase samples including hematological relapse, fWT1 was the only variant detected in CML chronic phase.

In the study of Hossain et al. [3], sWT1 mRNA expression was analyzed in 12 MDS, 26 AML, and 14 ALL patients; solely AML ($n = 237$) patients were analyzed in the study of Ishikawa et al. [4]. Our study included 48 AML and 18 CML patients. Therefore only the results of AML patients' analyses could be directly compared. Major differences were found in the rates of sWT1 positivity in these studies. It was 19% (45/237) in Ishikawa et al. [4], 63% (30/48) in our study and 85% (22/26) in Hossain et al.'s study [3].

Also sWT1 expression levels found in those studies differed. The sWT1 transcript level reported by Ishikawa et al. was much lower as compared to fWT1. By contrast, Hossain et al. found sWT1 rather highly expressed and notably, he found sWT1 as the single predominant isoform in 12/52 cases of adult leukemia. However, at the same time, Hossain mentioned that on the protein level, fWT1 was predominant. Our data correlate with those of Ishikawa et al. In further agreement with Ishikawa et al., we also found out that fWT1 level was higher in sWT1-epressing than in non-expressing AML patients. In our study, 27 out of 37 (72%) AML samples showing ≥1000 fWT1 normalized copies were sWT1 positive. On the other hand, sWT1 was detected only in 3 of 10 (30%) AML patients' samples showing ≤1000 fWT1 normalized copies. In CML, sWT1 was found only in AP/BC. In these CML advanced phases the fWT1 was higher than in CP. We found a statistically significant correlation of sWT1 with f WT1 ($R^2 = 0.9728$).

The differences among the results of these three studies might be the consequence of (1) different material used in these studies, as they were bone marrow samples in Ishikawa's and PB samples in Hossain's and our study, (2) to some extent different number of analyzed patients and of course (3) different PCR sensitivities or other parameters of methods used in individual laboratories.

The sWT1 expression was found in bone marrow [4] and leukocytes of peripheral blood of AML patients and in advanced phases of CML where a large number of immature cells is present but not in samples of CML CP. Therefore we can assume an association of sWT1 with immature cell character. As sWT1 was not detected in

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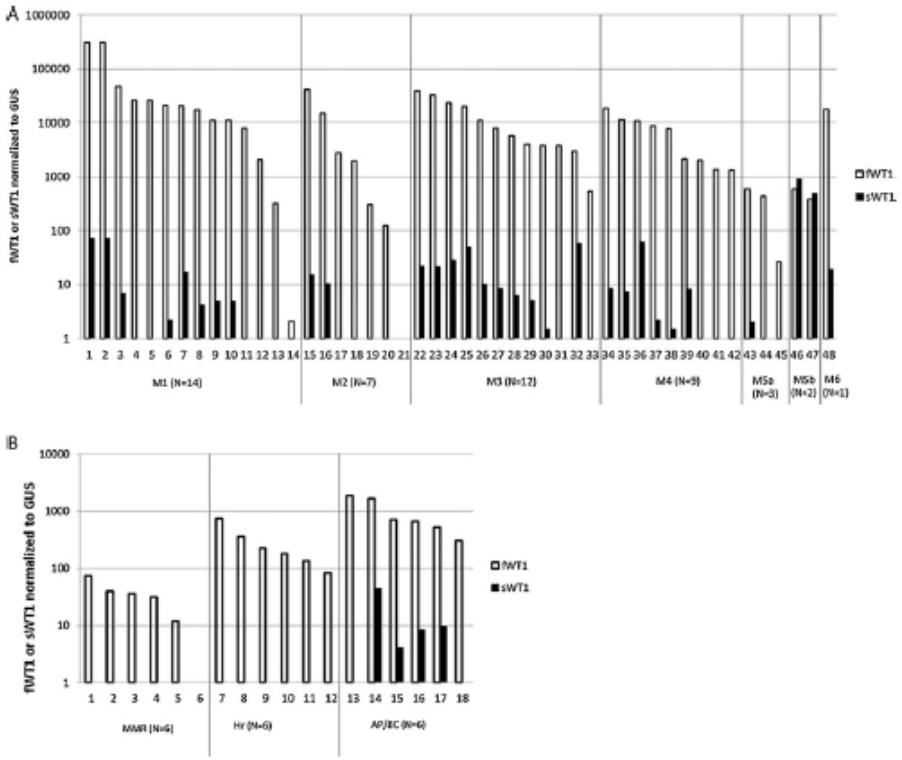

Fig. 1. (A) The sWT1 and fWT1 expression in diagnostic samples of different AML FAB subtypes [6] and (B) the sWT1 and fWT1 expression in different stages of CML; responses to therapy and CML phases were defined according to current EuropeanLeukemiaNet criteria [7]: MMR, major molecular response (BCR-ABL transcript level ≤ 0.1); Hr, hematological relapse (increase in leukocyte count over the physiological level of 10×10^9/L of peripheral blood); AP/BC, accelerated phase/blast crisis (10–30% in AP and more than 30% of blast cells in BC).

normal bone marrow [4] it seems probable that sWT1 is expressed only in immature cells of leukemic character.

In conclusion, our results demonstrate a low expression level of sWT1 variant in myeloid leukemias, which excluded sWT1 as a suitable marker for monitoring disease state. Current studies on CML and AML patients suggest that sWT1 is expressed preferentially in immature leukemic cells. Further studies are needed to clarify the role of WT1 and its variants in leukemias. The first step should be the standardisation of real-time RT-PCRs for WT1 variants to unify the results of different laboratories.

Conflict of interest

All authors have no conflict of interest.

Acknowledgements

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Contributions. TL contributed to the study design, data analyses, manuscript writing; MZ, SN and JP contributed to the data analyses, manuscript revision; JS and HK had taken the responsibility for collection of patient samples and clinical data; and JM is the consultant and supervisor throughout the study work, provided critical revision of the manuscript.

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Expression patterns of microRNAs associated with CML phases and their disease related targets

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Abstract

Background: MicroRNAs are important regulators of transcription in hematopoiesis. Their expression deregulations were described in association with pathogenesis of some hematological malignancies. This study provides integrated microRNA expression profiling at different phases of chronic myeloid leukemia (CML) with the aim to identify microRNAs associated with CML pathogenesis. The functions of *in silico* filtered targets are in this report annotated and discussed in relation to CML pathogenesis.

Results: Using microarrays we identified differential expression profiles of 49 miRNAs in CML patients at diagnosis, in hematological relapse, therapy failure, blast crisis and major molecular response. The expression deregulation of miR-150, miR-20a, miR-17, miR-19a, miR-103, miR-144, miR-155, miR-181a, miR-221 and miR-222 in CML was confirmed by real-time quantitative PCR. *In silico* analyses identified targeted genes of these miRNAs encoding proteins that are involved in cell cycle and growth regulation as well as several key signaling pathways such as of mitogen activated kinase-like protein (MAPK), epidermal growth factor receptor (EGFR, ERBB), transforming growth factor beta (TGFB1) and tumor protein p53 that are all related to CML. Decreased levels of miR-150 were detected in patients at diagnosis, in blast crisis and 67% of hematological relapses and showed significant negative correlation with miR-150 proved target *MYB* and with *BCR-ABL* transcript level.

Conclusions: This study uncovers microRNAs that are potentially involved in CML and the annotated functions of *in silico* filtered targets of selected miRNAs outline mechanisms whereby microRNAs may be involved in CML pathogenesis.

Introduction

Mammalian microRNAs (miRNA, miR) are short non-coding RNAs that regulate preferentially gene expression by inhibiting translation of specific target mRNAs. MiRNA-mRNA matching is based on imperfect sequence base-pairing with the required complementarity centered over positions 2 - 8 of mRNA's seed sequence [1]. Depending on specific target genes, miRNAs regulate many cellular functions such as developmental timing, signal transduction, apoptosis, cell proliferation and tumorigenesis [2-5]. Thus, gene expression and role of miRNAs are currently being largely studied in human malignancies and chemical compounds that regulate miRNA levels are potentially very important for developing new treatment strategies in chronic myeloid leukemia (CML). The first miRNA molecules that have been associated with human leukemia pathogenesis were found in chronic lymphocytic leukemia (CLL) [6]. MiR-15 and miR-16 are located in a genomic region that is frequently deleted in CLL, thus the expression of these two miRNAs is downregulated. Other works brought the evidence that many miRNAs are indeed found at chromosomal breakpoints and genomic regions associated with cancer [7,8].

In CML the following miRNAs were associated with the disease pathogenesis. For instance, the miR-203 was found to be epigenetically silenced in human leukemic Philadelphia chromosome-positive (Ph+) cell lines; this is in line with the observation that BCR-ABL and ABL kinases are miR-203 putative targets [9]. Derivative 9q+ chromosome deletions carrying miR-199b that occurred in some CML patients were associated with miR-199b

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decrease [10]. Venturini et al. [11] showed miR-17-92 cluster (onkomir-1) to be aberrantly expressed in CD34 + cells of CML patients. Agirre at al. [12] analyzed the expression of 157 miRNAs in mononuclear and CD34+ cells separated from bone marrow of 6 CML patients at diagnosis and found 11 miRNAs (e.g. miR-150, miR-151, miR-221, miR-127, miR-16) aberrantly expressed in CD34+ cells and 53 miRNAs differentially expressed in mononuclear cells (e.g. miR-150, miR-126, miR-221, miR-222, miR-21). Two recent works contributed to the knowledge about expression change in specific microRNAs associated with resistance to imatinib or responsiveness to imatinib after the treatment initiation in CML patients [13,14]. A group of 19 miRNAs (e.g. miR-191, miR-29a, miR-422b, miR-100, miR-326, miR-26a) were identified as possible predictors for clinical resistance to imatinib in patients with newly diagnosed CML [13]. A relatively rapid increase in the expression of miR-150 and miR-146a and decrease of miR-142-3p and miR-199b-5p in peripheral blood mononuclear cells (PBMCs) of patients newly diagnosed with CML was found two weeks after imatinib initiation [14].

In this study, we used an array platform to characterize differentially expressed miRNAs in peripheral blood total leukocytes of patients at different stages of CML including diagnosis, major molecular response, therapy failure, hematological relapse, accelerated phase and blast crisis with the aim to identify microRNAs associated with pathogenesis of CML. To the best of our knowledge, such integrated microRNA profiling during the course of CML has not yet been performed. Hierarchical clustering analysis based on expression profiles of 49 miRNAs clearly separated patients at diagnosis, hematological relapse and blast crisis from those in major molecular response and therapy failure. We used *in silico* analyses to better understand the targets of 17 selected miRNAs whose deregulation was confirmed by real-time quantitative PCR (RT-qPCR). Based on our previous results demonstrating that miR-150 downregulation is associated with CML [15], we further validated miR-150 expression in a larger number of patients (n = 70). As *MYB* represents functionally validated target of miR-150 [16], its gene expression analysis was performed on the same patient cohort. Our data provide significant inverse correlations between miR-150 and *MYB* expression and *BCR-ABL* transcript level and indicate that this relationship is potentially important for pathogenesis in CML.

Materials and methods

Patient samples

Twenty four patient samples of total leukocytes from peripheral blood (Table 1) were used to prepare pools representing different CML phases for microarray analysis: diagnosis (n = 5, Dg), major molecular response (n = 5, MMR), therapy failure (n = 5, TF), hematological relapse (n = 5, Hr), and blast crisis (n = 4, BC). Briefly, Dg, Hr and BC contain 100% of Ph+ cells. Therapy failure is defined here as complete hematological response with failure to achieve complete cytogenetic remission (CCgR). Hematological relapse is defined as increased number of WBC (range 14-28*10^9/L). MMR samples are characterized as *BCR-ABL* <0.1% (IS). BC samples contain blast cells in peripheral blood from 50% to 79%. Eleven healthy donors of age median 60 (range 45 - 78) and man/woman ratio 3/2 following CML incidence were used to create a control pool.

Seventy patient samples of total leukocytes from peripheral blood were used for miR-150 expression validation and *MYB* expression analyses (Table 2). Of these, 13 represented Dg, 16 = MMR, 14 = TF, 15 = Hr and 12 = AP (accelerated phase) together with BC (AP/BC). Therapy failure is defined here as non CCgR achievement; all patients achieved complete hematological remission and two patients major and minimal cytogenetic response, respectively. Hematological relapse is characterized by increased number of WBC and PLT (median 16*10^9/L and 448*10^9/L, respectively).

The percentage of *BCR-ABL* transcript level was observed from the routine monitoring using real-time qPCR that is standardized within the frames of international standardization [17]. Mutation analyses were performed by direct sequencing method [18].

All subjects donated their samples with informed consent approved by the Ethic Committee of the Institute of Hematology and Blood Transfusion, Prague.

Sample preparation

Cell pooling was applied for microarray analysis at the aim to reduce individual variability and to find common features of the disease stage. Pooling strategy was performed according to previously described recommendations [19,20]. Pools consisted of five patient samples; the blast crisis pool contained only four samples due to lack of appropriate material. Samples were selected for pooling according to their similar characteristics listed in Table 1. Each patient contributed to the pool by the same amount of leukocytes (10^7).

RNA extraction

Two different approaches were initially tested to extract total RNA containing small RNA molecules: acidic phenol-chloroform procedure and miRVana kit (Ambion®, Applied Biosystems, Foster City, CA, USA). The quality and quantity of RNA were evaluated using Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA) and spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), respectively. The miRVana kit

Table 1 Characteristics of patient samples in the pools

Pools	Patient number (gender)	IM therapy (months)	WBC count (*10^9/L PB)	Thrombocyte count (*10^9/L PB)	Blast count (% in PB)	BCR-ABL (%)	Ph+ cells (%)	Pretreatment	BCR-ABL mutations
Dg	1 (F)	0	71.4	749	0	60	100	HU	NA
	2 (F)	0	66.5	824	3	131	100	HU	NA
	3 (M)	0	22.3	509	1	144	100	HU	NA
	4 (M)	0	15.5	636	0	312	100	HU	NA
	5 (M)	0	198.5	550	1	139	100	HU, IFN	NA
MMR	6 (M)	14.8	5.7	244	0	0.001	0	HU	NA
	7 (F)	7.4	5.3	153	0	0.01	0	HU	NA
	8 (M)	9.2	6.1	181	0	0.001	0	HU	NA
	9 (M)	19.6	4.2	309	0	0.04	0	HU	NA
	10 (M)	13.1	4.8	230	0	0.02	0	HU	NA
TF	11 (F)	17.6	4.8	224	0	37	90	IFN	M351T
	12 (M)	14.3	4.2	142	0	15	100	HU	WT
	13 (F)	15.1	5.4	252	0	37	30	HU	WT
	14 (F)	13.5	2.4	100	0	23	60	HU, IFN	WT
	15 (M)	13.1	4.5	171	0	38	100	HU	WT
Hr	16 (M)	13.6	28.1	448	0	80	100	HU	M351T, F317L
	17 (F)	16.6	14.0	440	0	212	100	HU, IFN	F311I
	18 (M)	16.5	17.3	444	0	68	100	IFN, HU	M244V
	19 (F)	25.3	16.2	550	0	66	100	IFN	M351T
	20 (M)	22.4	26.1	245	0	164	100	IFN, HU	F317L
BC	21 (F)	55.4	65.6	147	62	1883	100	NA	M244V
	22 (M)	6.7	17.8	494	51	2188	100	NA	WT
	23 (M)	33.0	55.7	14	79	2500	100	NA	WT
	24 (M)	36.7	8.0	21	76	1000	100	NA	M351T, D276G

BC = blast crisis; Dg = Diagnosis; F = female; Hr = hematological relapse; HU = hydroxyurea; IF = interpheron alpha; IM = imatinib; M = male; MMR = major molecular response; PB = peripheral blood; TF = therapy failure; WBC = white blood cell, WT = wild type.

Table 2 Characteristics of patient samples in groups for miR-150 and *MYB* expression analysis

Disease stage	Number of patients	HU, IFN or combination pretreatment months from diagnosis median (range)	Months on imatinib median (range)	WBC x10^9/L PB median (range) PLT x10^9/L PB median (range)	Blasts in PB (%) median (range)	Ph+ cells (%) median (range)	BCR-ABL (%) median (range)	BCR-ABL KD mutation Number of patients
Dg	13	NA	NA	67 (22-457) 481 (130-824)	2 (1-5)	100	132 (61-312)	NA
AP/BC	12	24 (2-106)	24 (8-55)	34 (1.17-147) 81 (14-562)	18 (8-76)	100	510 (103-2500)	3
Hr	15	18 (1-66)	22 (10-54)	16 (6-28) 448 (71-1779)	10; 12	100 (32-100)	80 (23-827)	15
TF	14	14 (1-68)	18 (12-67)	Phy Phy	0	100 (40-100)	32 (11-91)	4
MMR	16	3 (1-11)	16 (7-24)	Phy Phy	0	0	0.02 (0.001-0.1)	NA

AP = accelerated phase; BC = blast crisis; Dg = Diagnosis; Hr = hematological relapse; MMR = major molecular response; PB = peripheral blood; Phy = physiological (WBC 4-10 x 10^9/L; PLT 150-400 x 10^9/L); PLT = platelets; TF = therapy failure; WBC = white blood cell.

and phenol-chloroform procedure gave comparable results with respect to RNA quality (RNA integrity numbers 7.8 - 9.1 and 8.5 - 9.0, respectively) and quantity (mean total RNA amounts 5 ug and 3 ug, respectively).

MiRVana kit however gave better 230/280 ratio and therefore was then selected for preparation of samples for microarrays. Acidic phenol-chloroform extraction was used for real-time qPCRs, which is a standardized method in our laboratory for *BCR-ABL* monitoring in international scale (IS).

Microarray analysis

PIQOR™ miRXplore arrays (Miltenyi Biotech GmbH, Cologne, Germany) were used for miRNA expression profiling and the whole procedure including miRXplore data analysis (control vs. sample) was performed within the genomic facility of the manufacturer. Total RNAs with controlled quality (RIN 8 - 9.2; A260/A280 1.86 - 2.01; A230/A260 1.95 - 2.1) and quantity (1.2 - 4.0 μg) were sent to Miltenyi Biotech laboratory on dry ice. RNA quality and quantity was checked after delivery with the comparable results. Microarray platform contained 872 probes for human miRNAs according to miRBase version 10.1 and an extensive system of controls. Raw data were derived from ImaGene® software (Biodiscovery, El Segundo, USA). Only spots with signal equal to or higher than 50% percentile of the background signal intensities were further analyzed.

The complete microarray data were deposited in Gene Expression Omnibus (GEO) database under the accession number GSE26260 (http://www.ncbi.nlm.nih.gov/geo/).

We applied MultiExperiment Viewer (MeV v4.0 release; http://www.tm4.org/mev) for k-means/medians and hierarchical clustering was performed using average linkage and average dot product metric.

Real-time qPCRs

Real-time qPCR was performed on RotorGene 6000 (Qiagen, San Francisco, CA, USA). The miRNA expression assay kits (Applied Biosystems) specific for selected miRNAs were used to perform reverse-transcriptions and RT-qPCRs. MiR-30c showed stable expression across all the patient and control samples analyzed (stable Ct/ngRNA) and was used as a housekeeping gene for normalization. Relative fold changes of gene expression were assessed using $2^{-\Delta\Delta CT}$ method. Mean of ΔCT values of 11 healthy donors was used as a calibrator. Results are presented as expression fold change of a patient to a healthy control.

TaqMan Gene Expression Assay (product number Hs00193527; Applied Biosystems) was used for *MYB* transcript quantification according to the manufacturer's recommendations. The *GUS* gene was used as the housekeeping gene with the primer set, probe, and protocol adopted from Beillard et al. [21].

Prediction of putative miRNA target genes

The TargetScan Human release 5.1. (http://www.targetscan.org) was used for prediction of miRNA targets. Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/) [22,23] was applied to annotate the biological functions of the predicted targets.

Statistical analyses

Analyses of *MYB* and miR-150 differential expression between different groups of samples were conducted using Kruskall Wallis's test and Dunn's multiple comparison test. Correlation analyses were calculated using the Spearman's rho correlation test. Statistical analyses and graphs were performed using GraphPad Prison version 4.03 (GraphPad Software, La Jolla, CA, USA).

Results

miRNA expression profiles in CML

Microarray analysis in CML resulted in the detection of 56 differentially expressed miRNAs (samples vs. control). Figure 1 shows three main gene clusters (clusters I, II, III) of altogether 49 miRNAs. A markedly (more than 1.5 fold; others are not indicated here) increased level over the control was found in BC pool for miR-19a, miR-19b, miR-221, miR-126, miR-106a, miR-17, miR-20a and miR-222 that belong to the cluster I. A distant gene cluster II grouped down-regulated miRNAs in BC; more than 1.5 fold change was detected for miR-24, miR-29b, miR-26b, miR-107, miR-103, miR-150, miR-451. The cluster III consisted of miRNAs with increased level in MMR; more than 1.5-fold change was found for miR-663, miR-638 and miR-720.

A separate cluster was created from MMR and TF pools and was distant from the cluster grouping Dg pool together with Hr and BC pools. MMR and TF pools are represented by the samples from patients during the imatinib treatment with optimal response and failure to achieve CCgR, respectively. The samples from MMR and TF pools were characterized by physiological blood count but different *BCR-ABL* transcript level and number of Ph+ metaphases (Table 1). The distant cluster of Hr, BC and Dg pools represented CML in progression and at diagnosis, respectively.

Validation of array data and sample pooling

Eight up-regulated (miR-19a, miR-19b, miR-221, miR-222, miR-126, miR-106a, miR-17 and miR-20a) and 3 down-regulated miRNAs (miR-103, miR-150 and miR-451) with more than 2.0-fold change in their expression (see Figure 1) over the control were selected for

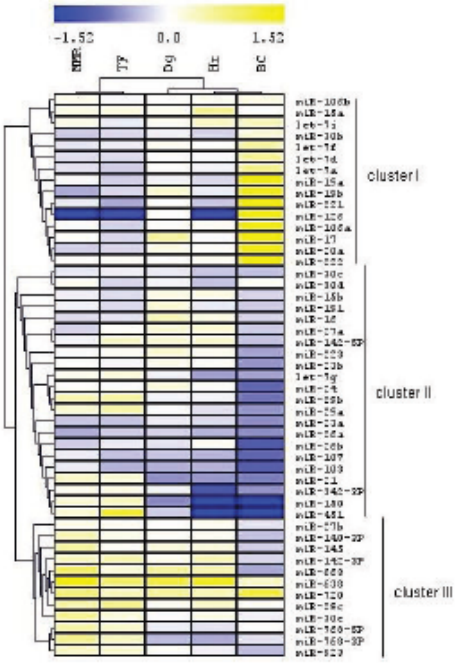

Figure 1 Hierarchical clustering analysis of expression data of 49 microRNAs from microarray analysis. Seven miRNAs are not displayed due to signal at the background level in at least one of the pools analyzed.

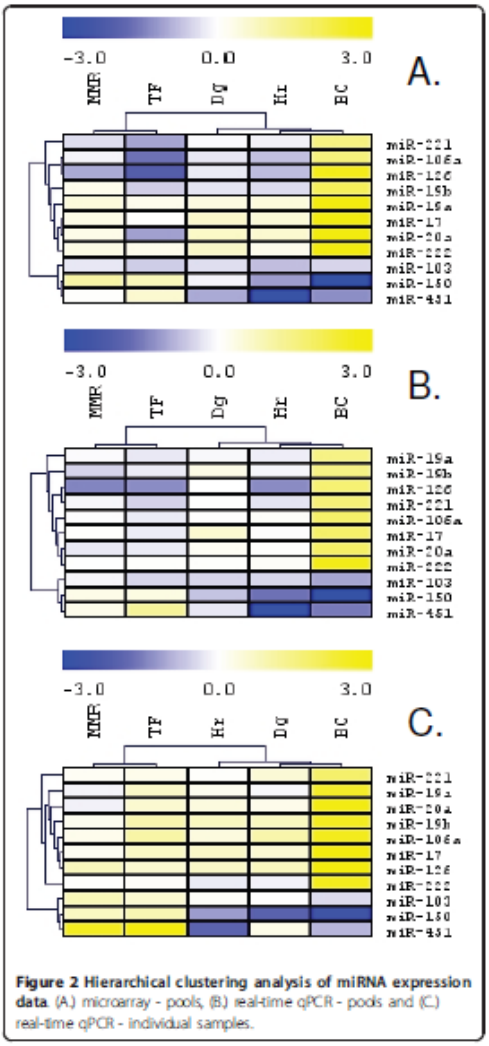

Figure 2 Hierarchical clustering analysis of miRNA expression data. (A.) microarray - pools, (B.) real-time qPCR - pools and (C.) real-time qPCR - individual samples.

array data validation and for evaluation of pooling precision by the RT-qPCR. MiRNAs expressions were measured in the pools and in individual samples of each pool. The heatmaps showed comparable results (Figure 2); on comparing array and RT-qPCR data of pools and averaged RT-qPCR data of individuals, hierarchical clustering formed similar gene and sample clusters. We noted also few discrepancies e.g. for miR-126 and miR-451. We evaluated RT-qPCR data of each sample using hierarchical clustering (Figure 3). Seven miRNAs (miR-181a, miR-181b, miR-92a, miR-146a, let7c, miR-144, miR-155) that were not displayed in Figure 1 and 2 due to low signal on the array analysis in at least one of the pools, were included into the RT-qPCR analysis because of strong change in their expressions in BC pool. Three prominent patient clusters and three prominent miRNA clusters were identified (Figure 3). Firstly, a gene cluster distant from the other two consisted of miR-103, miR-150, miR-451 and miR-144. These molecules showed a rather decreased level at Dg, in Hr and BC. The other two closely related clusters consisted of miR-19b, miR-19a, miR-17, miR-20a, miR-92a, miR-106a, miR-222, miR-126, miR-146a, miR-181a, miR-181b, let7c, miR-155 and miR-221 that were up-regulated in BC samples.

The BC samples formed one cluster that was mixed with two samples from hematological relapse (Hr 4, 5), one from diagnosis (Dg 3) and one therapy failure (TF 4). This cluster was related to the cluster that was formed by Hr samples (Hr 1-3), two Dg samples (Dg 1, 4) and one therapy failure (TF 3). The third cluster

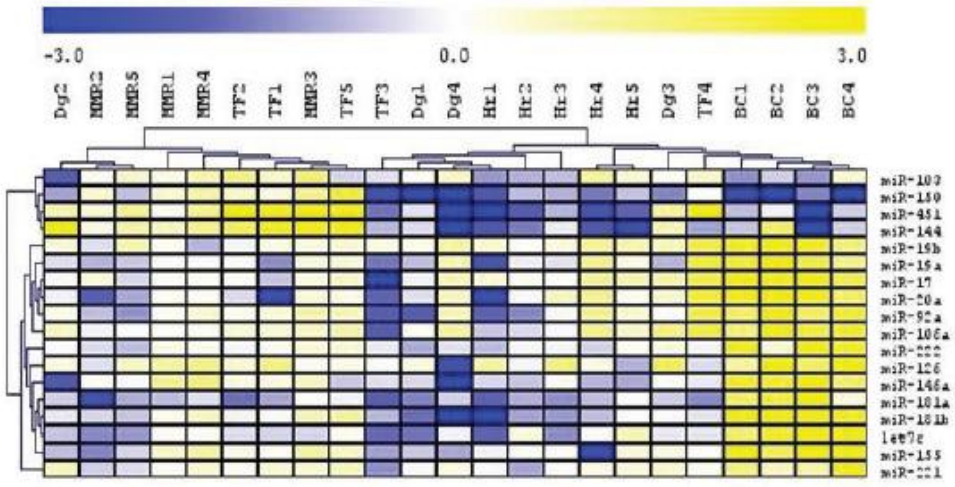

Figure 3 Hierarchical clustering analysis of detected levels of 17 selected miRNAs by real-time qPCRs.

distant from the other two consisted of all MMR samples, three TFs (TF 1, 2, 5) and was mixed with Dg 2 sample.

Target in silico analyses and functional annotation

The putative target genes were selected for 17 miRNAs with validated expression in different phases of CML (Figure 3). The predicted targets containing highly conserved sites were further studied according to the P_{CT} values [24] from the TargetScan release 5.1 except for miR-106a because of lack data in the database. Because the P_{CT} values are available only in the TargetScan we did not use another databases for miR-106a target prediction to preserve data consistency.

The targets were selected according to P_{CT} equal or higher than 0.1 and 0.5 of miRNAs with low and high number of targets in the database, respectively (see Additional file 1: Table S1). The P_{CT}, ranging between 0 and 1, corresponds to a Bayesian estimate of the probability that a site conserved to a particular branch length is conserved due to miRNA targeting [24]. The 30% of all of the targets with only conserved sites (Additional file 1: Table S1) are putative target genes of more than one of the selected miRNAs.

Functional annotation analysis of predicted targets (Table 3) revealed several biological processes ($P < 0.0001$). The encoded proteins are involved mainly in the regulation of transcription, intracellular signaling cascades, amino acid phosphorylation, regulation of RNA metabolic processes, regulation of apoptosis, regulation of cell proliferation and protein transport. Several proteins are implicated in hematopoietic or lymphoid organ development (n = 52; e.g. BMI1, WNT3A, MLL5, IL25, CDK6, MYH9, BCL2L11, CRKL, KIT, BCL2, RUNX1, TCF3, PIK3R1, NOTCH2, PKNOX1, SP1, SP3, TGFBR3), regulation of erythrocyte differentiation (ACVR2A, ACVR1B, ETS1, MAFB, SPI1, CDK6, FOXO3, INPP5D, ARNT) and regulation of myeloid cell differentiation (ZFP36, HMGB3, MAFB, KLF10, NDFIP1, SPI1, CDK6, FOXO3, PRDM16, PURB, ARNT, LIF, ACVR1B, ACVR2A, ID2, ETS1, GNAS, INPP5D, RUNX1).

Using KEGG database [25] we analyzed signaling pathways with significant hits ($P < 0.0001$) for predicted targets involved in endocytosis (hsa04144), pathways in cancer (hsa05200), mTOR signaling pathway (hsa04150), hedgehog signaling pathway (hsa04340), chronic myeloid leukemia (hsa05220), focal adhesion (hsa04510) and Wnt signaling (hsa04310) (Table 3).

Table 4 summarizes predicted targets associated with chronic myeloid leukemia. Most of them are involved in MAPK signaling (BCR, E2F2, E2F3, CBL, RAF1, CRK, CRKL, KRAS, SOS1, MAPK1). TGBR2, SMAD4 and ACVR1B play a role in transforming growth factor beta signaling pathway. Cyclin D1 (CCND1) and cyclin-dependent kinase 6 (CDK6) are important for the cell cycle and in the p53 pathway. Cell cycle is influenced by cyclin-dependent kinase inhibitor 1B (p27). ErbB

Table 3 Functional annotation of predicted targets

GO category	Count	% *	P-value
Biological process			
Regulation of transcription	407	22.0	4.28E-20
Intracellular signaling cascade	208	11.24	5.52E-12
Protein amino acid phosphorylation	127	6.86	2.03E-11
Regulation of RNA metabolic process	269	14.54	5.67E-10
Negative regulation of cellular biosynthetic process	107	5.78	8.68E-10
Phosphate metabolic process	159	8.59	7.5E-9
Regulation of small GTPase mediated signal transduction	58	3.14	1.41E-8
Regulation of Ras protein signal transduction	51	2.8	1.89E-8
Regulation of apoptosis	132	7.1	1.35E-7
Vesicle-mediated transport	101	5.5	2.2E-7
Negative regulation of signal transduction	50	2.7	2.77E-7
Protein transport	124	6.7	5.48E-7
Transmembrane receptor protein tyrosine kinase signaling pathway	49	2.65	1.04E-6
Regulation of cell migration	40	2.16	1.57E-6
Positive regulation of cell differentiation	49	2.65	2.03E-6
Intracellular transport	108	5.84	2.04E-6
Regulation of cell proliferation	124	6.7	2.99E-6
Hematopoietic or lymphoid organ development	52	2.8	7.41E-6
Regulation of protein kinase cascade	47	2.5	9.5E-5
Regulation of protein kinase activity	60	3.24	1.05E-4
Regulation of erythrocyte differentiation	9	0.5	2.41E-4
Regulation of myeloid cell differentiation	19	1.2	2.42E-4
Molecular function			
Transcription regulator activity	268	14.5	8.81E-19
GTPase regulator activity	82	4.4	1.32E-8
Protein kinase activity	110	5.95	2.33E-8
Cytoskeletal protein binding	90	4.86	1.06E-6
Transcription repressor activity	63	3.41	1.46E-6
Protein domain specific binding	65	3.51	1.67E-6
DNA binding	314	16.97	2.51E-6
Zinc ion binding	303	16.38	3.7E-5
Chromatin binding	34	1.84	4.03E-5
Cation binding	509	27.51	1.28E-4
SH3 domain binding	24	1.3	1.51E-4
Pathway			
Endocytosis	45	2.43	1.93E-8
Pathways in cancer	64	3.46	1.41E-7
mTOR signaling pathway	16	0.86	1.13E-4
Hedgehog signaling pathway	16	0.86	2.82E-4
Chronic myeloid leukemia	19	1.03	3.21E-4
Focal adhesion	37	2.0	3.39E-4
Wnt signaling pathway	29	1.57	8.77E-4

* from total 1850

Major Gene Ontology (GO) categories. The threshold of statistical significance for GO enrichment in the gene list was set up to $P \leq 10^{-4}$.

Table 4 Target annotation in pathways of chronic myeloid leukemia (hsa05220)

microRNAs	Targets*	Definition	P_{CT}	Pathway in CML
miR-20a	BCR	Breakpoint cluster region protein	0.56	
miR-222	E2F2	E2F transcription factor 2	0.32	
miR-17	E2F2		0.59	
miR-155	E2F2		0.78	
miR-17	E2F3	E2F transcription factor 3	0.54	
miR-150	CBL	E3 ubiquitin-protein ligase	0.45	
miR-222	CBL		0.33	
miR-155	CBL		0.67	
miR-19a	RAF1	RAF proto-oncogene serine/threonine-protein kinase	0.82	**MAPK signaling**
miR-126	CRK	Proto-oncogene C-crk	0.55	
miR-17	CRK		0.88	
miR-221	CRKL	Proto-oncogene C-crk	0.16	
miR-19a	KRAS	GTPase	0.92	
miR-155	KRAS		0.33	
miR-155	SOS1	Son of sevenless	0.53	
miR-181a	SOS1		0.78	
miR-19a	MAPK1	Extracellular signal-regulated	0.86	
miR-17	MAPK1	kinase 1/2 (ERK)	0.96	
miR-19a	TGFBR2	Transforming growth factor -beta	0.87	
miR-144	TGFBR2	receptor type-2	0.65	
miR-155	TGFBR2		0.28	**Transforming growth factor ß**
miR-144	SMAD4	Mothers against DPP homolog 4	0.58	
miR-17	ACVR1B	Transforming growth factor -beta receptor type-1	0.52	
miR-19	CCND1	Cyclin D1	0.86	
miR-17	CCND1		0.97	**p53 pathway**
miR-155	CCND1		0.55	
miR-103	CDK6	Cyclin-dependent kinase 6	0.73	
miR-222	CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27)	0.50	**Cell cycle**
miR-103	PIK3R1	Phosphoinositide-3-kinase,	0.73	
miR-221	PIK3R1	regulatory subunit	0.63	
miR-155	PIK3R1		0.28	**ErbB signaling**
miR-19a	PIK3R3	Phosphoinositide-3-kinase,	0.92	
miR181a	PIK3R3	regulatory subunit	0.81	
miR-17	RUNX1	Runt-related transcription factor 1	0.88	**Abnormality in**
miR-144	RUNX1	(AML1)	0.59	**growth inhibition**

* official gene symbol

signaling pathway encompasses PIK3R1 and PIK3R3. RUNX1 known as AML1 contributed to the abnormality in growth inhibition.

MiR-150 down-regulation and targeted MYB overexpression

Dramatic reduction of miR-150 in BC, at Dg and in Hr and its normal levels in patients under imatinib treatment (MMR and TF) (Figure 3) prompted us to determine this expression pattern on a larger cohort of patients (n = 70; Table 2). Significant down-regulation of the miRNA ($p < 0.05$) in comparison to healthy controls (n = 11) was confirmed for diagnosis and progressed phases of CML (Figure 4A.). MiR-150 level decreased more than 2-fold in 67% of hematological relapses (n = 10/15). There was no significant change in MMR and TF compared to controls. Among all patient samples analyzed, we found significant inverse correlation of miR-150 expression with *BCR-ABL* transcript level ($p = 0.01$; $r = -0.501$). To test whether miR-150 is regulated by BCR-ABL we have used a Ph+ cell line MOLM-7 and incubated it with two concentrations of

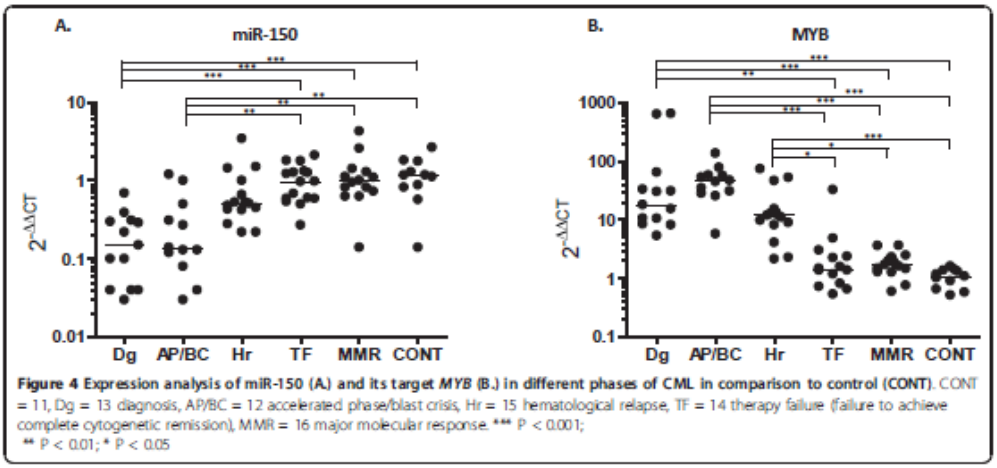

Figure 4 Expression analysis of miR-150 (A.) and its target *MYB* (B.) in different phases of CML in comparison to control (CONT). CONT = 11, Dg = 13 diagnosis, AP/BC = 12 accelerated phase/blast crisis, Hr = 15 hematological relapse, TF = 14 therapy failure (failure to achieve complete cytogenetic remission), MMR = 16 major molecular response. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

imatinib (1uM and 10 uM, Additional file 2: Figure S1A) for total 48 hours. We observed that following reduction of BCR-ABL tyrosine kinase activity (exemplified by decreased intensity of p-CRKL (Additional file 2: Figure S1B)) by imatinib the miR-150 levels were significantly upregulated. This paragraph provides link between levels of one particular microRNA, miR-150, identified by our microarray analysis and CML pathogenesis.

MYB is a confirmed target of miR-150 [16]. The expression pattern of *MYB* during the course of CML has not as yet been reported. We decided to analyze *MYB* transcript levels in the same cohort of patients. *MYB* was significantly increased at Dg, in AP/BC and Hr in comparison to controls ($p < 0.001$), to MMR ($p < 0.05$) and to TF ($p < 0.05$). Spearman's rho analysis displayed significant inverse correlation of *MYB* with miR-150 expression ($p = 0.01$; $r = -0.521$) and significant positive correlation between *MYB* expression and *BCR-ABL* transcript level ($p = 0.01$; $r = 0.771$).

Discussion

Specific microRNAs regulate hematopoietic cell differentiation and development [26]. The main interest is in whether there exists a link between levels of miRNAs and leukemia pathogenesis. The first work dealing with miRNA expression in CML demonstrated enhanced expression of the miR-17-92 cluster in CML CD34+ cells [11]. Other works that reported miRNA aberrant expression in CML appeared very recently. For example, it demonstrated that several miRNAs dysregulated in CML (miR-150, miR-146a, miR-142-3p, miR-199b-5p) were rapidly restored under imatinib treatment [14]. Several miRNAs (e.g. miR-191, miR-29a, miR-422b, miR-100, miR-326, miR-26a) are promising predictors of imatinib resistance in newly diagnosed CML patients [13].

This study investigates microRNA differential expression profiles that were initially analyzed at different stages of CML using microarrays. Pooling of patient samples was applied for microarray analysis to reduce individual variability and to find common features of the disease.

MiRNA array data showed similar expression pattern of 49 miRNAs in imatinib responders with MMR and patients with failure to achieve complete cytogenetic response. As expected, hierarchical clustering assembled the pools of samples at diagnosis, in hematological relapse and blast crisis, while MMR and TF pools formed a separate cluster (Figure 1). Total leukocytes from blast crisis peripheral blood that consisted of more than 50% blasts of each sample in the pool showed the highest number of strongly deregulated miRNAs.

We applied the functional annotation tool DAVID to look for the biological functions of predicted targets with only conserved sites and high P_{CT} values of the 17 miRNAs with real-time qPCR-confirmed up-regulation (miR-19a, miR-19b, miR-17, miR-20a, miR-92a, miR-221, miR-222, miR-126, miR-146a, miR-181a, miR-181b, let7c and miR-155) and down-regulation (miR-103, miR-150, miR-451 and miR-144) in blast crisis (Figure 3). Several targets were involved in the processes that were found to be important in CML; endocytosis [27], mTOR signaling pathway [28,29], hedgehog signaling [30,31], focal adhesion [32,33] and Wnt signaling [34,35]. We summarized 19 genes with the probability to be targeted by miR-20a, miR-17, miR-19a, miR-103,

miR-144, miR-150, miR-155, miR-181a, miR-221 and miR-222. The encoded proteins were annotated in pathways related to the CML (hsa05220). Out of these, 10 targets are involved in MAPK signaling (BCR, E2F2, E2F3, CBL, RAF1, CRK, CRKL, KRAS, SOS1, MAPK1). Interestingly, inhibition of MAPK signaling in Ph+ cell line K562 induced apoptosis [36]. Application of MAPK specific inhibitor U0126 showed synergistic effect with imatinib resulting in CD34+ progenitor reduction in CML [36].

Confirmed increase of miR-19a, miR-19b, miR-17, miR- 20a, miR-92a, miR-106a, miR-221, miR-222, miR-126, miR-146a, miR-181a, miR-181b, let7c and miR-155 was identified in samples of BC pool (Figure 3). This pattern was not found in Dg, Hr, TF or MMR pools. Overexpression of these miRs may be related to the immature character of blasts. Whether the increased level of these miRNAs may contribute to the CML pathogenesis or may simply reflect the stage of the disease is the matter of further investigation. Abnormal expression of onkomir miR-17-92 (miR-17; miR-19a; miR-19b; miR-20a; miR-92a) was described in CML CD34+ cells [11]. Agirre et al. [12] found up-regulated miR-221 and miR-222 in mononuclear cells of CML patients in comparison to healthy controls. MiR-155, miR-106a, miR-146a, miR-181 and miR-126 were reported as deregulated miRNAs in CML [13,14]. To our knowledge, let7c expression has so far not been described in CML. In this study, our *in silico* analyses revealed that miR-221 and miR-103 (P_{CT} 0.63 and 0.73, respectively) target PIK3R1. PIK3R3 is predicted to be regulated by miR-19a and miR-181a (P_{CT} 0.92 and 0.81, respectively). PI3K is annotated in ERBB, MAPK and mTOR signaling pathways. KRAS, which is involved in MAPK signaling, is a predicted target of miR-19a (P_{CT} 0.92). MAPK expression may be regulated by onkomirs miR-17 and miR-19a (P_{CT} 0.96 and 0.86, respectively). Interestingly, it was reported that RAS/MAPK signaling may contribute to the survival of BCR-ABL positive cells under imatinib selection pressure [37]. AKT1, a member of the antiapoptotic PI3K pathway, is involved in both, BCR-ABL mediated transformation as well as in response to the BCR-ABL kinase inhibitors. It was shown that the PI3K/AKT/mTOR signaling is activated in imatinib naive cells while under imatinib pressure it may enhance resistance to imatinib [38]. As shown in our real-time qPCR data (Figure 3), the rather decreased levels of miR-181a, miR-221 and miR-19a in some imatinib treated patients, and miR-103 down-regulation in a number of blast crisis, diagnosis and progressed CML may contribute to the increased level of PI3K and thus may be involved in the previously described PI3K/AKT/mTOR signaling activation and in the resistance development in some CML cases. Though no experimental therapy using miRNA modulation has as yet provided significant and curative approach, the knowledge of deregulation of miRNAs specific for CML may facilitate the development of such therapeutic strategies. Several candidate microRNAs (e.g. miR-181a, miR-221, miR-19a, miR-103) regulating expression in CML target important signaling pathways may represent promising candidate targets for CML therapy.

The real-time qPCR validated the down-regulation of miR-150, miR-451, miR-103 and miR-144 overall in individual samples of BC, Hr, Dg pools and in some samples of TF pool (Figure 3). These molecules may be related to the CML pathogenesis and may reflect transformation from chronic to accelerated phases. Agirre et al. found miR-150 downregulation in mononuclear cells and CD34+ cells separated from bone marrow in newly diagnosed CML patients (n = 6) in comparison to healthy donors (n = 6) [12]. MiR-150 was recently described to be downregulated in untreated CML patients [14]. Flamant et al. [14] suggest that miR-150 play a role in leukemic cells and potentially in the more primitive hematopoietic compartment in chronic phase CML patients. This is in line with the knowledge that miR-150 is important in the regulation of hematopoiesis. During normal erythroid differentiation its level is gradually decreased [39], however; it shows the highest expression in mature lymphocytes [40]. Others proved that miR-150 expression increases during B-lymphoid differentiation in contrast to myeloid differentiation. It seems likely that miR-150 regulates the development of other two different blood lineages; B lymphocytes and megakaryocytes [41,42]. Thus, miR-150 deregulation is found in hematological malignancies; miR-150 is decreased in polycytemia vera reticulocytes [43] and a marked decrease was recently also detected in MDS-del (5q) [44] while, in contrast, a twofold increase was found in CLL lymphocytes [45].

Based on our results [15] and recent results of others we expanded real-time qPCR assays of miR-150 on the larger cohort of CML patients. Decreased level of miR-150 was confirmed in patients at diagnosis, in the majority of patients with hematological relapse and in accelerated phase and blast crisis. Normal miR-150 level was observed in imatinib treated patients with major molecular response and failure to achieve CCgR. Our observations are consistent with the data of Flamant et al. [14] showing rapid increase of miR-150 expression after imatinib treatment initiation in patients with newly diagnosed CML. They further found that low miR-150 expression inversely correlated with white blood count and thus speculated that the level reflected the high leukocyte counts in newly diagnosed CML patients. We showed here a significant inverse correlation of miR-150 expression with *BCR-ABL* transcript level ($p = 0.01$; $r =$

-0.501). Non-treated newly diagnosed patients, patients with disease progression and resistant to imatinib showed a high level of *BCR-ABL* together with high leukocyte count and decreased amount of miR-150. Normal miR-150 level was detected in imatinib responders (MMR) and patients with failure to achieve CCgR (TF) with normal blood count and low *BCR-ABL* transcript level. As imatinib targets Ph+ cells, the normal level of miR-150 in imatinib treated patients in chronic phase with physiological blood count could be the result of the suppression of leukemic cells and the concomitant recovery of normal hematopoiesis under imatinib treatment. Our *in vitro* tests showed elevated expression of miR-150 and marked decrease of p-CRKL following imatinib *in vitro* treatment of Ph+ cell line MOLM-7. These findings suggest a potential functional relationship between miR-150 and BCR-ABL.

Gene expression of *MYB* in our study showed a significant inverse correlation with miR-150 transcript level (p = 0.01; r = -0,409). *MYB* is the proven target of miR-150 and encodes a transcriptional factor required for proliferation and survival of normal and leukemic blast cells. A recently published study on a mouse model of blast crisis reported that *c-MYB* is required for BCR-ABL dependent leukemogenesis [46]. Lidonnici et al. [46] speculated that miR-150 reduction might contribute to the *c-MYB* upregulation that is likely induced by BCR-ABL, and may be involved in BCR-ABL driven leukemogenesis in CML. Interestingly, we found a significant correlation between *MYB* expression and *BCR-ABL* transcript level (p = 0.01; r = 0,782) in CML patients, which is in line with the above described suggestion.

In summary, our data demonstrated that miR-150, miR-20a, miR-17, miR-19a, miR-103, miR-144, miR-155, miR-181a, miR-221 and miR-222 are deregulated in CML. Furthermore, *in silico* filtering identified targeted genes that are involved in cell cycle, growth inhibition, MAPK, ErBb, transforming growth factor beta and p53 signaling pathways that are reported in CML pathogenesis. MiR-150 expression showed significant negative correlation with its target *MYB* and with *BCR-ABL* transcript level. The results of this study outline the mechanisms whereby miRNAs may be implicated in CML pathogenesis. However, if they function in BCR-ABL dependent or independent manner has to be elucidated.

Additional material

Additional file 1: Table S1: Number of predicted targets of conserved miRNA families. Table summarizes number of the microRNA targets that were selected according to P_{CT} equal or higher than 0.1 and 0.5.

Additional file 2: Figure S1: MiR-150 exression is elevated and BCR-ABL activity is dropped after imatinib treatment in Ph+ MOML-7 cells. Data from *in vitro* test of miR-150 expression change after Ph+ cell line incubation with imatinib. (A) MiR-150 expression change after imatinib treatment in Ph+ MOML-7 cells. 3×10^6 MOLM-7 cells were incubated for 24 or 48hrs with or without (white bars; CTRL = control) imatinib. Two different concentrations of imatinib were tested (1 μM - gray bars and 10 μM - black). Cellular RNA was isolated by Trizol (Invitrogen), transcribed using High Capacity cDNA Reverse Transcription Kit (Roche Diagnostics). Real-time qPCR was performed using TaqMan protocol (Roche Diagnostics) and was run on the ABI 7900HT instrument. RNU44 was used as housekeeping gene. Data were evaluated by $2^{-\Delta\Omega}$ method. The viability of culture with imatinib decreased: 1 μM imatinib- from 94% after 24hrs to 21% after 48hrs; 10 μM imatinib- from 95% after 24hrs to 18% after 48hrs. (B.) The intensity change of p-CRKL after imatinib treatment in Ph+ MOLM-7 cells. The amount of p-CRKL (a client molecule of BCR-ABL tyrosine kinase) was measured by standard western blot analysis (p-CRKL (Tyr207) antibody; Cell Signaling Technology) after 24h culture cultivation with imatinib using both concentrations. Beta-actin (monoclonal antibody Anti-beta-Actin, Sigma) was used as the loading control and was measured by western blot analysis using alkaline phosphatase. The measurement of p-CRKL was not possible to perform in the culture after 48 incubation due to a marked viability decrease and thus to low amount of material.

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Authors' contributions

KMP - conception and design, *in silico* analyses, data evaluation and interpretation, manuscript drafting; TL - contribution to the manuscript drafting, real-time qPCR analyses and evaluation; HK - provision of patient samples, clinical data evaluation, critical revisions; PB - *in vitro* tests, contribution to data evaluation and interpretation, critical revision; MT - clinical data revision; critical revisions of the manuscript; TS - contribution to data evaluation and interpretation, critical revision; JM - critical revisions. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Brief communication

MicroRNA-451 in chronic myeloid leukemia: miR-451–BCR-ABL regulatory loop?

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ABSTRACT

Chronic myeloid leukemia (CML) is caused by constituve activity of BCR-ABL tyrosine kinase. Despite of high efficiency of imatinib, selective BCR-ABL inhibitor, about 30% of patients develop resistance. Novel markers and targets for therapy are thus necessary. MicroRNAs are small intereference RNAs whose role in physiological and malignant hematopoiesis has been shown. This study is focused on miR-451 in CML. Following our observation of miR-451 downregulation in CML, we further show its relation to BCR-ABL activity. Our data together with current literature indicate a more complex relationship of miR-451 and BCR-ABL in CML.

MicroRNAs are extremely short (21–23 nucleotides) single stranded RNA molecules which regulate gene expression via translational repression or mRNA cleavage. There is an increasing evidence in the literature that miRNAs belong among important regulators of both physiological hemopoiesis and abberant hemopoiesis of leukemias [1–3]. Abberant expression of miRNAs in chronic myeloid leukemia (CML) was reported previously [1–3], and in some of those miRNAs their relationship to BCR-ABL kinase was shown [1]. BCR-ABL tyrosin kinase constitutive activity was confirmed to be the main cause of CML and it has become a target for therapy. Imatinib (Glivec, imatinib mesylate, Novartis Oncology), selective BCR-ABL tyrosin kinase inhibitor is currently used as the first line therapy for all newly diagnosed CML patients. Non-negligible incidence of resistance to imatinib together with missing curative potential of tyrosine kinase inhibitors therapy call for novel biomarkers and molecules important in CML resistance, progression and pathogenesis in general.

In our previous study we tried to find possible novel biomarkers and/or players in CML pathogenesis among the miRNAs [4]. On the basis of literature data [1–3] and our own microarray data [4], we investigated miR-451 expression in CML patients by real-time PCR. We analyzed samples of total leukocytes in CML patients at the time of diagnosis (Dg, $n = 14$), in major molecular response (MMR; $n = 14$), in hematological relapse (Hr; $n = 17$) and in suboptimal response (SR/TF; $n = 7$). Response to therapy definitions and patients' samples characteristics are given in Table 1A. Total leukocytes of 11 healthy donors were used as controls. By this approach, we found miR-451 downregulated in most of the Dg and Hr in contrast to normal or slightly increased levels in MMR and SR/TF (Fig. 1).

The Ph positivity and BCR-ABL transcript levels correlated inversely with miR-451 expression at Dg, in Hr and MMR, but no such correlation was found in SR/TF (BCR-ABL as well as miR-451 levels were high in SR/TF). This discrepancy might be explained by results of our western blot analyses, which showed that there is a difference between Dg/Hr and SR/TF in BCR-ABL activity. While at Dg and Hr, BCR-ABL is not inhibited, BCR-ABL remains probably under partial control of imatinib in SR/TF (Fig. 1b). Our data thus indicated that the miR-451 downregulation might be related to BCR-ABL kinase activity.

To verify the hypothesized relation of miR-451 downregulation to BCR-ABL kinase activity we decreased BCR-ABL kinase activity by *in vitro* cultivations of cells with imatinib. We used leukocytes of imatinib-naïve CML patients, i.e., patients with the CML diagnosis but not yet treated with imatinib ($n = 6$, Table 1B); Ph+ (CML-T1, JURL-MK1) and Ph− cell lines (ML-2) and leukocytes of healthy donors as controls. Activity of BCR-ABL kinase was determined via phosphorylation of Crkl protein (western blots) [5]. Sensitivity of cells to imatinib was further checked by WT1 mRNA levels [6]. Based on literature data and our previous expe-

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Table 1
Characterization of patients samples.

(A) Disease stage	Number of patients' samples	Months on imatinib, median (range)	WBC × 10^9/l PB, median (range) PLT × 10^9/l PB median (range)	Blasts in PB (%) median (range)	Ph+ cells (%)	BCR-ABL (%), median (range)
Dg	14	0	71,38 (22,32–457) 502 (130–824)	0	100	132,1 (60,8–153,6)
MMR	14	18,93 (7,4–26,89)	Physiological Physiological	0	0	0.015 (0.0005–0.11)
SR/TF	7	21 (11–67)	Physiological Physiological	0	100	17,4 (11–69,83)
Hr	17	21,25 (10–53,85)	15,19 (5,83–28,13) 455,5 (71–2372)	0	100	73,7 (14,1–256,8)

(B) Group	Patient No	WBC × 10^9/l PB	PLT × 10^9/l PB	Blasts in PB (%)	Ph+ cells (%)	BCR-ABL (%)
B	1	81,17	606	2	100	179
	2	55,98	192	<1%	100	49
A	3	152	174	2	100	169
	4	188	206	1,5	100	41
	5	216	283	0,5	100	421
	6	86,57	683	2	100	139

(A) Characteristics of patients' samples of the course of CML Dg – diagnosis, samples prior to therapy; Hr – hematological relaps, increase in total leukocytes over the normal level of 10^9 10^9/L peripheral blood; SR/TF – suboptimal response/therapy failure, samples of complete hematological response of patients not achieving complete cytogenetic response within 12 or more month of imatinib therapy, MMR – major molecular response, decrease in BCR-ABL to less 0,1%; Scaling of responses to imatinib followed current criteria of European Leukemia Net. (B) Characteristic of patients used in cultivation analyses.

rience, 1 and 10 μM imatinib doses were applied on cells leading usually to partial and total inhibition of BCR-ABL activity, respectively [5]. Optimal time for *in vitro* cultivations was thoroughly tested [7]. It was necessary to find the time when changes of monitored parameters were not influenced by apoptotic/necrotic processes. We tested four different cultivation times: 2, 24, 48 and 72 h. Up to 48 h, number of apoptotic/necrotic cells (RNA degradation, Anexin V staining) was lower than 15% both in treated cells and non-treated controls. Changes of measured parameters at mRNA and protein levels were clearly detected in 48 h. At 72 h, high levels of RNA degradation and of apoptosis and necrosis (more than 60%, Anexin V staining) were found, which might distort results of our analyses. Therefore, 48 h were used in this study.

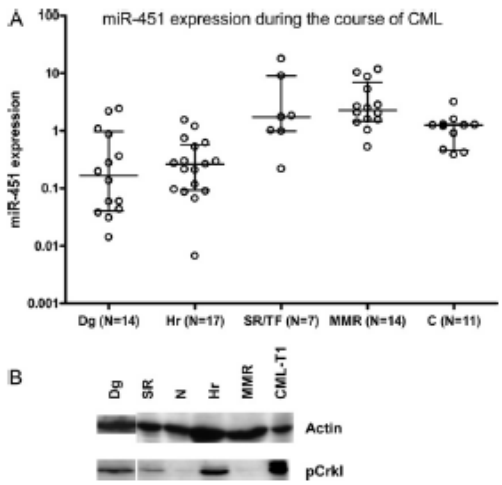

Fig. 1. MicroRNA-451 expression profile in the course of CML (A) MicroRNAs were quantified using comercially available assays (Life Technologies). miR-451 expression was evaluated with miR-30c as a control gene [4] and healthy control as a calibrator. (B) BCR-ABL activity during the course of CML was investigated by western blot analyses of pCrkl levels; results of representative samples of different responses to imatinib are shown.

We found BCR-ABL activity to be high in all Ph+ samples prior to cultivation and decreased or fully inhibited after imatinib treatment. No BCR-ABL activity was found in Ph– samples, which stayed unchanged after cultivation. Prior to cultivation, the miR-451 was downregulated in CML-T1 and JURL-MK1 cells in comparison to healthy leukocytes (0.00006 and 0.54, respectively). Interestingly enough, miR-451 was increased in both Ph+ cell lines after cultivation with imatinib (Fig. 2a, 2.8-fold in CML-T1 and 4.2-fold in JURL-MK1) suggesting relation of miR-451 downregulation to BCR-ABL activity in these cells. Control Ph– cell line ML-2 exhibited no changes in miR-451 levels after imatinib treatment which effectively excluded possible influence of imatinib via any other way then the BCR-ABL inhibition (Fig. 2a).

Concerning patient samples, samples of 4 out of 6 imatinib-naïve patients showed decreased miR-451 levels (group A) prior to cultivation (median of 0,04). Remaining 2 patients showed comparable levels of miR-451 to that of healthy leukocytes prior to cultivation (group B; median of 0.55). After *in vitro* cultivation with imatinib, miR-451 increased in samples from group A patients (Fig. 2c, 2.2 fold) and remained on almost the same level in group B (Fig. 2b). We detected no expression changes of miR-451 in healthy donor leukocytes after treating cells with imatinib (Fig. 2a). At present, we have no clear explanation of the differences in miR-451 expression between groups A and B. There were no significant differences in results of cytogenetic or BCR-ABL transcript and other analyses between patients of those two groups. Some other molecules may play a role, e.g. mediators between BCR-ABL and miR-451. To elucidate this, further studies are needed, preferentially on larger patient cohorts.

Nevertheless, the results of our *in vitro* experiments again confirmed our previous suggestion that expression changes of the miR-451 found in Ph+ cell lines and CML patients' samples are associated with BCR-ABL inhibition.

In conclusion, we showed that BCR-ABL activity plays certain role in downregulation of miR-451 in CML cells. On the other hand, Iraci et al. [8] showed previously that miR-451 had a potential to target BCR-ABL. Combination of those two findings suggests that there may exist a reciprocal regulatory loop between BCR-ABL and miR-451 (Fig. 3). BCR-ABL kinase activity

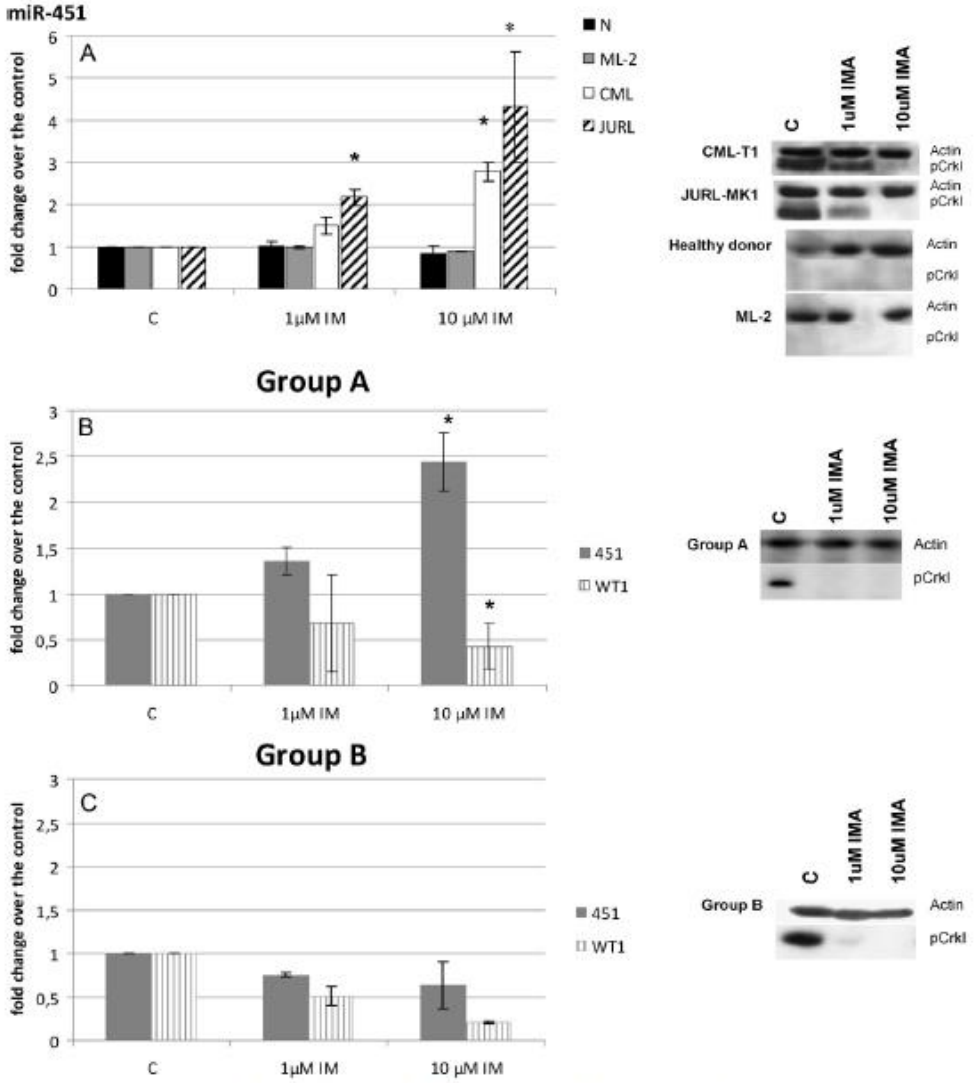

Fig. 2. Changes in miR-451 expression after 48 h cultivation with imatinib: Cells were incubated in RPMI, 10% FBS, penicillin, streptomycin and cultivated at 37 °C and 5% CO_2 atmosphere for 48 h with and without imatinib (1 and 10 μM). The miR-451 levels (A, C and E) are expressed as fold change over the un-treated control which is taken as the reference. (B, D and F) shows western blot analyses. (A and B) Ph negative cells (healthy donors leukocytes, ML-2 cell lines) and Ph positive cell lines (CML-T1, JURL-MK1). (Ph negative cells express negligible amount of WT1) (C and D) Patient Group A (E and F) Patient Group B.

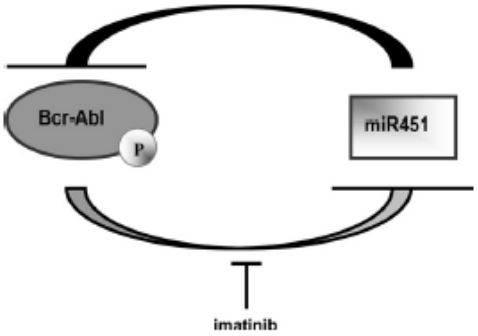

Fig. 3. Scheme of possible miR-451/BCR-ABL regulatory loop.

blocks expression of miR-451, its own negative regulator, increasing thus its own expression. This might be one of the ways by which leukemic state of the CML cells is preserved. Disruption of suggested regulatory loop could help to improve CML therapy.

Proving our hypothesis might help to understand the miR-451/BCR-ABL relationship, mechanism of CML pathogenesis and resistance to treatment in general.

Conflict of interest

All authors have no conflict of interest.

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Contributions. TL and MZ developed the study design, performed data analyses and wrote the manuscript, HK was responsible for collection of patient samples and clinical data, and JM served as consultant and supervisor throughout the study work and reviewed the manuscript.

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Publikace č. 6 - Přijata k publikaci

Chapter

MOLECULAR MONITORING OF CML: ROLE FOR NOVEL MARKERS ADDITIONAL TO BCR-ABL

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ABSTRACT

Tyrosine kinase inhibitors have revolutionized treatment of chronic myeloid leukemia (CML). However, despite of excellent results with imatinib, current first-line therapy, 20-35% of patients in chronic phase has primary refractory disease, intolerance or experience relapse after initial response. Early relapse and prognosis prediction is important for selection and timing of therapeutic interventions. BCR-ABL mRNA expression and BCR-ABL kinase domain mutation status are the molecular markers currently used in early prediction of relapse and in categorization of patients into three prognostic groups by ELN (Baccarani et al., 2009): optimal responders (OR), suboptimal responders (SR) and therapy failures (TF). All patients who do not respond to therapy optimally are considered to be in an increased risk of relapse according to current ELN criteria. However, it is our own and others experience that suboptimal responders include both patients who are at high risk of relapse and so called slow responders who achieve good response later. If suboptimal responders do not achieve any improvement, they are considered therapy failures and recommended for therapy change even in case they do not relapse. BCR-ABL transcript level remains usually stable and its monitoring is without prognostic value in these patients. There is increasing evidence that CML may become more or less independent on BCR-ABL and driven by other proteins and mechanisms over the time. That might explain why BCR-ABL stops being reliable marker in "non-optimal" responders (SR, TF). There is apparently need for novel CML biomarkers additional to BCR-ABL (1) to early and reliably predict relapse, (2) to specify prognosis of suboptimal response/therapy failure or (3) to even redefine not optimal

responders according to actual risk of relapse even in cases of limited contributions of BCR-ABL to the resistance. In principle, there are three possibilities to improve CML patient monitoring: (1) searching for unique mechanisms of resistance (2) selection of suitable marker among the general leukemic (cancer) biomarkers or (3) performing functional testing of sensitivity to drugs in vitro. In this chapter, the use of possible additional prognostic markers mostly referred in the current literature and ways and approaches to look for new ones will be discussed.

1. INTRODUCTION

Chronic myeloid leukemia (CML) is a myeloid neoplasm characterized by abnormal proliferation of myeloid lineage. It is the best characterized type of leukemia on the molecular level: the fusion protein BCR-ABL has been identified as the causative factor for CML development. Tyrosine kinase inhibitors targeting BCR-ABL have revolutionized treatment of chronic myeloid leukemia (CML). However, despite excellent results obtained with imatinib mesylate, a selective BCR-ABL inhibitor and current first-line therapy drug, 20-35% of patients in chronic phase have primary refractory disease, intolerance or experience relapse after initial response. Early relapse detection and prognosis is important for selection and timing of therapeutic interventions. BCR-ABL mRNA expression and BCR-ABL kinase domain mutation status are the molecular markers currently used for early prediction of relapse and in categorization of patients into three prognostic groups by EuropeanLeukemiaNet (ELN) [1]: optimal responders (OR), suboptimal responders (SR) and therapy failures (TF). Suboptimal responders and also therapy failures are heterogenous in terms of prognosis and include patients who relapse but also patients who remain in long term haematological remission. BCR-ABL remains usually stable and losses thus much of its prognostic value in "non-optimal" responders to imatinib. New markers for specification of prognosis and early relapse prediction are desirable as they may bring significant improvement in individualized treatment. In this chapter, possible use of additional prognostic markers suggested so far in the literature will be discussed, their pros and cons, novel approaches to search new markers, with respect to our own experience.

1.1. Current Status of CML Patients´ Monitoring and EuropeanLeukemiaNet (ELN) Criteria for Responses To imatinib

Table 1 summarizes current ELN criteria for different responses to imatinib and recommendations for therapeutic interventions [1]. According to those criteria, if suboptimal responders (SR) do not achieve any improvement, they are transferred into the group of therapy failures (TF) and recommended for therapy change even in case that they do not relapse. According to current ELN criteria, all patients who do not respond to imatinib optimally (SR, TF) are considered to be in an increased risk of relapse. However, our own and others experience [2, 3] shows that "non-optimal responders to imatinib" (SR, TF) as defined by ELN [1] include patients with truly inadequate response who are in a risk of relapse/progression, but also patients who could be called "slow responders" because they achieve response later. We have also identified a group of patients who remain in long term complete hematological remission despite of steadily high BCR-ABL levels (10 – 100%). [1]

Mechanisms of CML in non-optimal responders (SR, TF) are very poorly understood. In general, BCR-ABL transcript level remains usually stable and its monitoring has limited prognostic value in those patients. Taken together, no molecular marker is currently available to distinguish sub-groups of suboptimal responses and therapy failures according to the real risk of relapse and its urgency. Novel CML biomarkers additional to BCR-ABL are clearly needed (1) to enable early and reliable relapse prediction, (2) to specify prognosis of suboptimal response/therapy failure or (3) to redefine non-optimal responders according to the actual risk of relapse even in cases of limited contributions of BCR-ABL to the resistance.

There are apparently a number of mechanisms of resistance to tyrosine kinase inhibitors. The first and the best known mechanism of resistance to imatinib are mutations in BCR-ABL kinase domain which hamper imatinib binding (reviewed in [4]). Until very recently, BCR-ABL kinase domain mutations were considered the main cause of resistance to imatinib. With the exception of the highly resistant T315I mutation, only a weak correlation has been found between the *in vitro* sensitivity of mutated BCR-ABL forms to inhibitors and the *in vivo* responses. Many patients with resistance do not even have any BCR-ABL kinase domain mutations. [5] Those observations indicate that other mechanisms including the BCR-ABL independent ones take part in CML pathogenesis. As other proteins are necessary for the oncogenic effects of BCR-ABL (adaptor proteins, [6], [7]; other kinases cooperating with BCR-ABL, [8], [9]), sensitivity to drugs depends on the whole cell context. There is also increasing evidence that CML may become more or less independent from BCR-ABL and driven by other proteins which may overtake the role of the driving force of the disease during the time (Src family kinases, [10], PI3K, [11]).

Table 1. Response to imatinib definitions [1]

	Response to imatinib		
Month on imatinib	Optimal	Suboptimal	Therapy failure
3	CHR	<CHR	without HR
6	≥pCR	mCR	without CR
12	CCR	pCR	<pCR
18	MMR	<MMR	<CCR
any time	stable CCR and MMR	loss of MMR	loss of HR
		BCR-ABL mutation	loss of CR
		additional aberration	BCR-ABL mutation

CCR – complete cytogenetic response; HR – hematological response; mCR – minor cytogenetic response; MMR – major molecular response; pCR – partial cytogenetic response.

2. Potential Prognostic/Predictive Markers for CML Patients Additional to BCR-ABL

In principle, there are three possibilities to improve CML patient monitoring by adding supplemental markers to the traditionally used BCR-ABL: (1) searching for individual mechanisms of resistance (2) selection of a suitable marker among general leukemic (cancer) biomarkers or (3) performing functional testing of sensitivity to drugs *in vitro*. In the next

paragraphs, examples of potential new markers selected from current literature and based on our own experience will be described.

2.1. Causative Factors of Resistance to Imatinib

One of the ways to find new markers and to understand different mechanisms of resistance is the search for specific mechanisms of resistance using leukemic cell lines resistant to tyrosine kinase inhibitors (TKIs) developed and isolated in vitro under selective pressure of TKIs. A number of specific proteins which can cause resistance to imatinib have already been identified. Selected mechanisms and candidate markers most frequently encountered in the current literature will be listed and discussed below.

Protein Phosphorylation Status in CML

As described above, CML patients under treatment are routinely monitored for BCR-ABL mRNA levels, but not for protein levels neither for the kinase activity of BCR-ABL. Only few studies tested BCR-ABL activity for its predictive value for further therapy [12, 13]. As BCR-ABL protein is often degraded in patients´ cells during cell lysis, BCR-ABL activity is measured indirectly. The phosphorylation status of V-crk sarcoma virus CT10 oncogene homolog (avian)-like protein (Crkl) is the most widely used surrogate marker of BCR-ABL activity mainly in the *in vitro* testing of sensitivity to TKIs. Crkl is a prominent substrate of BCR-ABL/ABL kinase and its tyrosine phosphorylation occurs as a direct consequence of BCR-ABL activity [14-16]. White et al. [12] reported that a decrease in pCrkl levels in mononuclear cells at an early phase of imatinib treatment might be a significant predictor of the response to the therapy. Lucas then reported predictive value of pCrkl/Crkl ratio also in fresh peripheral blood samples of CML patients at diagnosis for response to therapy [13]. The pCrkl/Crkl levels can be considered a predictive marker of response to imatinib (TKIs), though the method for pCrkl/Crkl detection has to be standardized first and the results validated on larger patient cohort.

BCR-ABL can directly activate members of Src kinases family (Lyn, Fgr, Hck; [8]) and also affects negative feedback mechanisms regulating Src family kinases signalling [9]. While Src family kinases have been proved necessary for BCR-ABL-induced B-lymphoblastic leukemia, no such requirement was found for Src kinases in CML. [17] Yet, an important role of several Src kinases has been showed in CML resistance to imatinib and also to nilotinib. One of the first molecules to be associated with the term "BCR-ABL-independent resistance" was the Lyn kinase [10]. It is an important component of cytokine signal transduction in a variety of cell types and it has been shown to play a key role in the growth and apoptotic regulation of hematopoietic cells [18, 19]. Observations of Donato et al. have suggested a shift from BCR-ABL to Lyn kinase dependence induced through chronic imatinib exposure in imatinib-resistant K562 cells [10]. Further, Donato et al. identified increased expression of Lyn also in samples of patients resistant to imatinib. Those results have then been confirmed in a further study by Wu et al [20]. Pene-Dumitrescu et al. [21] showed that Hck kinase might play similar role in resistance to imatinib. The authors reported, that overexpression of Hck is sufficient to cause resistance to imatinib in wild-type BCR-ABL expressing cells. Evaluation of Src family kinases expression and activity may be important especially for patients with

suspected BCR-ABL independence who are candidates for therapy with dual Src/BCR-ABL inhibitors such as dasatinib.

Another pathway, BCR-ABL affects and interacts with, is the phosphatidylinositol-3-kinase (PI3K)/Akt. PI3K has been shown to be regulated by BCR-ABL by Skorski et al. more than 15 years ago [22]. Using cell lines, it has been shown that constitutive activity of PI3K/Akt pathway is associated with resistance to imatinib [11]. PI3K inhibitors were effective against imatinib-resistant K562 cells [23] and PI3K inhibition enhanced the antileukemic effect of imatinib on primary CML cells [24]. Importantly, Marley et al. reported that PI3K inhibition selectively decreases proliferation of CML but not of normal cells. Limited evidence in the literature suggests a role for specific PI3K isoforms which, however, remains to be further explored [25].

Protein phosphorylation is negatively regulated by protein phosphatases. SHP-1 and SHP-2 are two SH2-containing tyrosine phosphatases involved in the regulation of cell growth: SHP-1 has been described as a negative regulator of the Jak/Stat pathway; SHP-2 is a positive regulator of Ras/Raf signaling. SHP-1 is expressed at high levels in hematopoietic precursors and has been shown to be physically associated with BCR-ABL in CML cells [26, 27]. SHP-1 is probably able to block BCR-ABL–dependent transformation and to mediate PP2A-induced BCR-ABL proteasome degradation [28]. Esposito et al. [29] showed that low levels of SHP-1 are associated with a reduced degree of response or with a failure to IMA treatment.

Deregulation of activity (phosphorylation status) has been shown for many specific proteins in CML. Occasionally, those proteins may escape from the need of being activated by BCR-ABL kinase and become BCR-ABL independent driving force of the disease and resistance to imatinib (TKIs). With the increasing spectrum of TKIs with different targets, the need for a complex research of active protein profiles of CML patients is ever increasing.

Drug Transporters

Intracellular imatinib concentration is the critical parameter for effective BCR-ABL inhibition. Therefore, a set of studies was performed, focused on molecules implicated in the transport of imatinib into and out of the cells. Imatinib is transported into cells actively through the organic cation transporter, member 1 (Oct-1); efflux can be performed through ABCB1 [30]. Oct-1 is a member of solute carrier family of proteins, subfamily 22 which includes 18 members. Oct-1 activity has been shown to be of prognostic value at diagnosis when measured in mononuclear cell fraction but not in CD34+ cells [31]. Oct-1 activity in mononuclear cells seems to be related not only to major molecular response rates but also to the overall and event free survival [32]. A more simple examination would be to test Oct-1 expression on the mRNA level. However, data on the correlation of the Oct-1 mRNA expression with clinical outcomes remain still controversial [33-35]. Also, the association between ABCB1 (multidrug resistance protein 1, MDR1) expression or activity and response to therapy is not generally accepted [36, 37, 38, 39].

There are a number of other membrane transporters whose interaction with different TKIs was described. Intracellular uptake of second generation TKIs may also be independent of major drug transporters [40-43]. Mutual substitution of different transporters together with the possibility of passive diffusion decreases the importance of specific transporter proteins on their own as predictors of sensitivity to therapy.

Summary

Studies on cell lines selected *in vitro* for resistance to imatinib may reveal highly specific markers which might also be considered new potential targets for therapy. However, a lot of the mechanisms and markers found this way may have very limited incidence in patients. First, permanent cell lines are quite different from *ex vivo* patients´ cells. CML cell lines are derived from blast crisis cells in which other mechanisms take part as compared to chronic phase CML cells. Second, the number of studies identifying specific proteins as risk factors for CML resistance to imatinib is rapidly increasing and there are probably many ways how the cell can escape from the effects of the drug. Each of the suggested risk proteins has to be considered in the whole cell context. Rather than being used as individual specific markers, those proteins are candidates to be included in the list of risk proteins examinable simultaneously in an array-based approach.

2.2. General Cancer (Leukemic) Markers in CML Monitoring

The key regulators of cellular viability are highly conserved throughout various cell types and even organisms. Such molecules might serve as markers for cellular activity in different tumours. The Wilms´ tumor gene (WT1) and heat shock proteins (Hsps) represent molecules of such general importance in regulation of cell viability in many tumors including leukemia and CML in particular. WT1 and Hsps have already been suggested or even used as prognostic markers in clinical practice in other tumors.

Wilms´ Tumor Gene 1

Wilms´ tumor gene 1 (WT1) encodes a zinc-finger transcriptional regulator important for cell growth and development. While in Wilms´ tumor, WT1 behaves as a tumor suppressor gene, it is a proved oncogene in leukemias [44]. WT1 mRNA expression has already been used in clinical practice for monitoring patients with AML and MDS [45-47].

In 1994, Inoue et al. [48] first suggested WT1 as a new prognostic leukemic marker for monitoring CML minimal residual disease after allogenic stem cell transplantation. Studies by Cilloni et al., 2003 [49] and more recent data by Varma et al. 2012 [50] indicated that WT1 might be useful also for CML patients on TKIs therapy. Cilloni et al. [49] showed that WT1 expression had predictive value for response to imatinib. In advanced phases of CML, WT1 was suggested to be even superior to BCR-ABL for monitoring patients´ response to therapy [51]. In our lab, we have shown that WT1 expression may significantly improve early relapse prediction especially in suboptimal responders [52]. Our statistical analyses indicated that both WT1 mRNA expression levels at selected time points of the therapy and WT1 expression kinetics might help to better stratify "non-optimal responders" to imatinib according to the actual risk of relapse and progression. Schnittger et al., 2009 [53] showed another advantage of WT1 expression examination in CML patients. The authors found elevated WT1 expression in patients who developed Ph negative clonal evolution. Together, data of these studies highlight the advantages of WT1 as a general leukemic marker which enables rapid assessment of the effectiveness of the treatment and of the risk of relapse regardless of actual mechanisms underlying response or resistance to therapy.

The opponents of use of total WT1 mRNA expression as a marker for monitoring leukemic patients under treatment point to the non-zero and changing levels of WT1

expression in healthy peripheral blood. However, our own and other studies showed that WT1 levels were always very low in healthy individuals, well below the levels found in leukemic patients [52, 54].

The mechanism of WT1 function in both, normal and leukemic hematopoiesis remains to be elucidated. Effects of WT1 protein in cells are influenced by posttranslational modifications, spectra of interactive partners offered by the host cell and WT1 isoform ratio (reviewed in [55]). Up to now, more than 36 WT1 isoforms have been described. This widens the field of activities of the protein; besides its role of transcriptional regulator, WT1 probably takes part in regulation of splicing and translation [56, 57, 58, 59]. To classify WT1 isoforms, two splicing events are considered predominant: exon 5 and KTS sequence in exon 9. By combination, four major WT1 splicing variants are produced referred to as -5/-KTS, -5/+KTS, +5/-KTS, +5/+KTS. Renshaw et al. [60] showed an association of +5 isoforms with sensitivity to cytotoxic drugs. In general, +5 isoforms were associated with regulation of cell susceptibility to apoptosis [61]. Excess of +5 variants mRNA expression has already been shown in acute myeloid leukemia relapses [62]. Three studies so far have focused on all the four major WT1 isoform expression in myeloid acute leukemias [63-65]. Our lab also analysed CML patient´ samples and found excess of -5/+KTS in CML diagnosis and relapse [64]. Together, recent data on expression of the four major WT1 splicing variants in myeloid leukemia [62-65] indicate that particular variants may be applicable as new markers of higher specificity for particular diagnosis than the conventionally used total WT1 expression and warrant thus further more complex studies on WT1.

Heat Shock Proteins

Heat shock proteins belong among the most highly expressed cellular proteins across all species. Functions of Hsps include assisting in protein folding, stabilizing various proteins and aiding in protein degradation. Hsps are important regulators of the stabilization of various cellular oncogenes and their role has been shown in various tumors including leukemias [66-69]. There might also be increasing demand for Hsps during the course of CML relapse as increased levels of BCR-ABL and/or of other proteins need to be maintained to execute their pathological effects.

Heat Shock Protein 90 (Hsp90)

Besides other proteins, Hsp90 has been shown important in stabilization of Src and BCR-ABL kinases [70]. Therefore, we have analysed hsp90 protein expression in CML patients differentially responding to therapy [71]. We found out that Hsp 90 level in total leukocytes of peripheral blood is possible risk marker for CML patients, examinable at any time during the course of CML. Increased levels at diagnosis or at any later time point were associated with disease deterioration and relapse. In contrast to WT1, follow-up analyses of four patients during the course of imatinib therapy showed that Hsp90 probably did not change significantly during the course of the disease. Thus, increased Hsp90 expression level seems to indicate patient´s predisposition to less favorable outcome in general.

Heat shock protein 70 (Hsp70)

Hsp70 has been found to be abundantly expressed in malignant human tumors of various origins, whereas in normal cells, its expression is mainly stress-inducible [72-74]. Hsp70 has been demonstrated to enter the blood stream and travel throughout the body regulating various cellular processes, particularly stimulating the immune system and potentially

triggering anti-cancer immune response [75]. Guo et al. [76] found that Hsp70 inhibits apoptosis upstream and downstream of the mitochondria in blast cells from CML patients in blast crisis. In CML patients, levels of Hsp70 circulating in plasma are significantly higher than those of healthy donors and the increase has been reported to correlate with disease progression/resistance to imatinib therapy [77]. On the other hand, we have tested Hsp70 intracellular protein expression in whole white blood cells of CML patients in chronic phase and found no significant difference among patients groups with different responses to imatinib [71]. Thus, hsp70 protein levels in plasma may be more informative than its intracellular levels.

Summary

Our experience shows that general cancer markers may be very useful despite of or maybe thanks to their lowered specificity which is associated with their relative independence as to the mechanisms underlying resistance they warn against. General leukemic biomarkers may help to select CML patients who need closer surveillance. As showed on the example of Hsp90 or WT1, targets for therapy can also be found among the general leukemic markers. Hsp90 inhibitors have been shown effective for patients with T315I mutation which are highly resistant even to BCR-ABL inhibitors of second generation [78, 79]. WT1 targeted vaccination has been proved successful also in CML patients (reviewed in [80, 81]).

2.3. MicroRNAs (miRNAs)

MicroRNAs (miRNA) are short non-coding RNAs that regulate gene expression by inhibiting translation of specific target mRNAs. One miRNA can have thousands of targets and depending on particular target genes, miRNAs can regulate all cellular processes including apoptosis and proliferation. Many studies have shown various miRNAs to be aberrantly expressed in human malignancies including leukemia (reviewed in [82]). As compared to mRNAs, miRNAs are executive molecules similarly to proteins and can serve as a marker and a target for therapy at the same time. MiRNAs represent a specific group of possible prognostic markers for CML patients including molecules causing individual mechanisms of resistance and also molecules of more general role in CML pathogenesis.

The first miRNA reported in association with CML was the miR-203. BCR-ABL as well as WT1 is putative targets of miR-203 which has been found to be epigenetically silenced in human leukemic Philadelphia chromosome-positive (Ph+) cell lines [83]. The question remains open as to the expression of miR-203 in patient samples. Derivative 9q+ chromosome deletions occur in some CML patients and were associated with worse prognosis. This region includes location of miR-199b and a decrease of this miRNA can be the cause of unfavourable prognosis of patients with 9q+ deletions [84]. Both, miR-203 and miR-199b need to be tested for their expression during the course of CML to evaluate their potential to serve as molecular markers.

The analysis of miRNAs can reveal regulation mechanisms for proteins whose role in CML has already been established. Such miRNAs can then represent earlier biomarkers. In our microarray study, decreased levels of miR-451 in CML as compared to healthy donors were found [85, 86]. Sholl et al. [87] suggested miR-451 as possible predictor of the achievement of CCyR during imatinib therapy. Interestingly, miR-451 was found to be

potential negative regulator of BCR-ABL which makes miR-451 also a tool for therapy [88]. Further, we have found out that miR-451 levels were lowered due to BCR-ABL activity. Suggested regulatory loop between miR-451 and BCR-ABL might be of interest as a site for therapeutic intervention [86]. Two other miRNAs have recently been related to the regulation of WT1 expression. MiR-15a and miR-16-1 were shown to inhibit proliferation of leukemic cells via down-regulation of WT1 in leukemic cell lines [89]. Whether these miRNAs are aberrantly expressed in CML patients and whether these may serve as a marker superior to WT1 warrant further studies.

Only few studies focused directly on miRNAs expression in association with responsiveness to imatinib therapy. San José-Enériz et al. [90] identified 19 miRNAs that may predict clinical resistance to imatinib in patients with newly diagnosed CML. Oliver Bruhn et al. [91] identified distinct microRNA pattern comparing blood samples of responders and non-responders also prior to imatinib therapy. Predicted target genes were primarily transcription factors and oncogenes. Transporters and exocytotic pathways are in addition frequent targets of microRNAs deregulated after imatinib therapy.

Further studies are necessary to confirm the data and to validate and complete the list of miRNAs having possible prognostic value for CML patients. Smaller changes in miRNA as compared to mRNA expression handicap miRNAs in their application as prognostic markers, yet, due to advanced technologies of high accuracy and sensitivity (reverse transcriptase stem loop real-time PCR), miRNAs can be considered potential biomarkers.

2.4. Testing of Sensitivity to Imatinib (TKIs)

Imatinib Plasma Levels

Studies focused on imatinib pharmacokinetics revealed differences in imatinib plasma trough concentration in individual patients. Although in the majority of clinical studies, imatinib plasma levels were found to correlate with response rates [92, 93], other authors point out that importance of imatinib plasma levels in CML treatment outside the clinical trials is limited due to differences in CML mechanisms and patients´ compliance [94, 95]. The importance of imatinib plasma levels in clinical practice is thus still controversial.

In vitro testing of sensitivity to TKIs

Another approach to evaluate actual sensitivity to TKI and/or even predict therapy outcome is *in vitro* testing based on incubation of patients´ cells with TKIs *in vitro* and monitoring TKIs effects, mainly the inhibition of BCR-ABL activity. The activity of BCR-ABL is measured indirectly via the phosphorylation status of Crkl, the prominent target of BCR-ABL in CML cells (see above). As reported by Cilloni et al. [96] and confirmed by Otáhalová et al. [97], WT1 mRNA expression can also be used as the marker of sensitivity to drug and BCR-ABL inhibition. Predictive significance of *in vitro* testing has been discussed in many papers with controversial results so far. As described below, the results depend on cultivation conditions (TKIs concentrations, incubation time), cell type used (bone marrow cells, mononuclear cells, whole white blood cells, CD34+ cells), detection method for the phosphorylation status of Crkl (western blot, flow cytometry , fluorescence resonance energy transfer-based biosensor).

White and coworkers [98] tested *in vitro* imatinib sensitivity in samples of mononuclear cells collected at diagnosis and assessed prediction power of IC50 of imatinib (inhibitory

concentration 50%). They found IC50 values as a statistically significant predictor of patient outcome at the 12th month of imatinib therapy. While Holyoake´s lab [99] showed a predictive value of the decrease in pCrkl levels in CD34+ cells from leukapheresis or bone marrow, other authors failed to find any correlation in the CD34+ cell population [100, 101]. In our lab, we have tested sensitivity to TKIs *in vitro* in whole white blood cells. To reduce the amount of patients´ material required for the analysis, we selected only 2 concentrations of imatinib and harvested the cells after 48h [102]. We analysed samples of patients at diagnosis and also during treatment to assess the usefulness of this test. The results showed high predictability for response to therapy. Similar results to ours have been reported by Shiabata et. al. [103] who tested sensitivity in total peripheral blood cells incubated 24 hours with imatinib. Taken together, current data indicate a correlation of *in vitro* and *in vivo* sensitivity to TKIs when tested in whole peripheral white blood cells but not in the CD34+ progenitors. This seems to be in line with the recognition of general insensitivity of primitive progenitors to imatinib [104-106] mentioned already. *In vitro* sensitivity tests should be performed using the cells which are actually affected by imatinib. Whole peripheral white blood cells represent a highly heterogenous population but include all such cells. Besides the actual sensitivity/resistance of patients´ cells to therapy, these tests seem to have predictive value for further response to therapy (duration of response).

Mutations in the BCR-ABL kinase domain are the best described mechanism associated with secondary resistance but play only a limited, if any, role in primary resistance [107]. *In vitro* tests of sensitivity of different BCR-ABL mutants to TKIs were performed. The first studies were run on transfected cells, but it appears that the sensitivity of mutants to TKI also depends on the cell system used. Only highly resistant mutants as T315I were resistant in all tested systems [108-110]. *In vitro* 50% inhibitory concentration (IC50) values alone are not sufficient to guide the choice of a tyrosine kinase inhibitor, because of differences between sensitivity of cells transfected with BCR-ABL mutants and sensitivity of patient to treatment *in vivo* [111]. We believe that *in vitro* testing, using primary patients´ cells, gives the most relevant information as to the sensitivity to TKIs.

An obvious advantage of these *in vitro* tests of sensitivity to TKIs is that they resemble relatively closely the real effects the drug produces in patients´ cells *in vivo* and it can thus show sensitivity or resistance relatively independently of the mechanism of the disease. Mainly, such *in vitro* testing can be useful for patients exhibiting mutation in the BCR-ABL kinase domain to assess its clinical relevance. On the other hand, clear weak point of these tests *in vitro* is that these do not account for pharmacokinetics or immunological mechanism that may affect sensitivity of patient to the drug *in vivo*. Yet, those tests seem to be capable of bringing relevant information on the patients´ sensitivity to the drug in most cases.

Novel Approaches to Look for New Predictive/ Prognostic Markers

Current advances in molecular biology have brought novel high-throughput techniques which enable effective search for new markers, both on the mRNA and protein levels.

Microarray technology enables to study expression levels of thousands of genes in a single experiment. All [112] but one [113-116] microarray study of gene expression in CML

patients responding differentially to therapy reported a gene classifier predicting cytogenetic response at diagnosis. However, there was no overlap among the gene lists in those studies. Therefore, some authors turn attention on the analysis of less heterogenous samples, mostly on the primitive CD34+ cell population. McWeeney et al. [117] reported a set of 75 genes whose expression in CD34+ cell of bone marrow was of predictive value for achievement of major cytogenetic response. Importantly, those genes have already been associated with CML prognosis and disease stage.

Unlike mRNAs, the proteins represent the executive force and proteomic studies thus may identify not only new biomarkers but also targets for therapy. Mass spectrometric protein detection is a fundamental technique in clinical proteomics and the use of mass spectrometry based methods in myeloid leukemia research has recently been reviewed by Hjelle et al. [118]. A new possibility in complex cancer proteomic research is the use of protein arrays and microarrays, a rapidly developing approach applicable with the common laboratory equipment. In our lab, we have tested several types of protein arrays and found differences in active protein profiles of patients responding differentially to imatinib [119]. As in the case of mRNA expression arrays, array platform and list of proteins of prognostic value need to be validated prior to their introduction into clinical practice.

CONCLUSION

The number of kinase inhibitors is increasing rapidly and several other inhibitors besides imatinib are already tested for the first line therapy. Because of their widened specificity and because of multiple risk markers associated with resistance to TKIs which have been identified, it seems necessary to introduce the array-based approaches into clinical practice. Special attention will be focused on the markers predictive at the time of diagnosis helpful in distinguishing "high risk" patients who would benefit from aggressive therapy, from "low-risk" patients who could be spared from unnecessary high toxicity. Although TKIs brought a break-through in CML therapy as most patients on TKI therapy stay free of symptoms and progression in the long term, TKI therapy is probably not curative in most of them. The most primitive CML cells remain insensitive to BCR-ABL inhibitors. Research of the CD34+ population is highly important for finding new targets for therapy to develop drugs with curative potential for CML patients.

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6. Diskuze

Chronická myeloidní leukémie je nejlépe prostudovaným typem leukémie, léčba kinázovými inhibitory cílenými proti vlastní příčině onemocnění, kináze BCR-ABL, umožňuje dlouhodobé přežití pacientů v kompletní remisi. Existuje však přibližně 20 až 30% pacientů, kteří vykazují k imatinibu rezistenci, intoleranci, nebo u nich dochází k relapsu. Včasná detekce rozvíjející se rezistence je klíčová pro možnost efektivního terapeutického řešení.

Současné schéma ELN definuje jednotlivé odpovědi na léčbu a nabízí doporučení ke změně léčby v případě tzv. selhání léčby. Suboptimální odpovědi, nedojde-li ke zlepšení, jsou přeřazeni do skupiny selhání léčby a jsou tedy také doporučeni ke změně. Skupina Dr. Moravcové se monitorováním pacientů s CML během léčby imatinibem věnuje od jeho zavedení do praxe, tedy včetně období, kdy nebyly k dispozici kinázové inhibitory dalších generací a kdy tedy i pacienti, kteří na imatinib neodpovídali optimálně, setrvávali na téže léčbě. Naše zkušenosti jasně ukazují, že existuje nezanedbatelné procento pacientů, kteří přesto, že jsou definováni jako suboptimální odpovídači nebo selhání léčby, přežívají dlouhodobě na imatinibu v kompletní hematologické odpovědi. Tato naše pozorování jsou dokladována i několika současnými pracemi. Mauro a spol., 2009, vymezují novou skupinu v rámci „ne-optimálních odpovídačů“, tzv. pomalé odpovídače, kteří dosáhnou velké molekulární odpovědi později a dlouhodobě v ní zůstávají. Sami jsme dále identifikovali také skupinu pacientů, kteří přežívají dlouhodobě navzdory velmi vysokým hladinám bcr-abl. Rohoň a spol., 2011, přímo navrhli předefinování suboptimálních odpovědí. Dále existuje také několik prací, které popisují fenomén tzv. náhlé blastické krize, která se prudce rozvíjí u pacientů, kteří odpovídali do té doby uspokojivě (např. Tantiworawit a spol., 2012; Hiwase a spol., 2012). Je tedy jasné, že přesto, že CML je charakterizována jednoznačně přítomností BCR-ABL v okamžiku diagnózy, její prognóza je heterogenní a BCR-ABL jistě není jediným faktorem, který se v průběhu onemocnění v patogenezi CML uplatňuje. V literatuře se objevuje rostoucí množství prací, které identifikují proteiny, které mohou být příčinou rezistence k léčbě nezávislé na BCR-ABL. Především kinázy z rodiny Src byly ukázány jako proteiny schopné převzít roli BCR-ABL. Je proto třeba nalézt pomocné molekulární markery k bcr-abl, které umožní co nejpřesněji odhadnout prognózu, detekovat včas rozvíjející se rezistenci a přiblížit se tak více ideálu individualizované léčby, která je právě jen tak agresivní, jak bude pro daného pacienta výhodné.

Pro případy rezistence k imatinibu jsou dnes sice již k dispozici kinázové inhibitory druhé a dalších generací, střídání inhibitorů však není vždy možné bez rizika. Obecně s sebou změna jednoho cíleného léku na další nese potenciální riziko selekce multi-rezistentního klonu. Kinázové inhibitory s širší specificitou navíc otevírají otázku bezpečnosti při dlouhodobém užívání. V případě dasatinibu, duálního inhibitoru BCR-ABL a SRC kináz, se sice ukázal ve středně-dobém měřítku pozitivní vliv na imunitu pacientů (Rohoň a spol., 2011), při dlouhodobé inhibici molekul významných pro fungování zdravých krevních buněk však můžeme předpokládat i negativní efekty. Tento fakt jen dále dokládá, jak výhodné by bylo dokázat odlišit pacienty, kteří skutečně potřebují změnu léčby, od těch, kteří mohou dlouhodobě profitovat z imatinibu navzdory méně než optimální odpovědi podle současných kritérií.

Z praxe vidíme tři přístupy, jak vylepšit monitorování pacientů s CML: Můžeme hledat konkrétní mechanizmy pomocí rezistentních buněčných linií připravených dlouhodobým vystavením selekčnímu tlaku léčby, můžeme testovat citlivost primárních buněk *in vitro* (kultivace s inhibitory, měření imatinibu v plazmě/v buňkách pacientů), můžeme se pokusit najít marker obecnějšího charakteru mezi již známými obecnými nádorovými markery. Každý způsob má své výhody a nevýhody. Konkrétní mechanizmy identifikované pomocí rezistentních CML buněčných linií mohou přinést vysoce specifické markery a zároveň potenciální nové cíle léčby. Na druhé straně ale mechanizmy identifikované v umělém modelovém systému jako je buněčná linie, mohou být relativně vzácné u pacientů. V naší laboratoři se také snažíme hledat konkrétní mechanizmy, ale abychom se vyvarovali problémům plynoucím z práce s modelem, využíváme přímo vzorků pacientů. Zavedli jsme několik typů proteinových arrayí, pomocí kterých se snažíme najít další kandidátní molekuly účastnící se patogeneze CML nebo přímo rezistence k léčbě. První výsledky nás upozornily např. na roli phosphatidylinositol-3-kináz a nebo fosfatáz v CML (Žáčková, Lopotová, EHA, 2012). Kultivace s léčivy *in vitro* představují stav relativně blízký tomu, co se odehrává v krvi pacientů. Takové testy mohou dát patrně informaci o aktuální citlivosti k léčbě (v závislosti na způsobu hodnocení a podmínkách testu, viz dále), je ale otázka, zda testy mohou mít prognostický význam. Vzhledem k tomu, že možných mechanizmů, jakými buňky uniknou vlivu léčby, existuje patrně celá řada, výhodné mohou být markery obecnějšího charakteru. Tyto naše názory spolu s vlastními výsledky a příklady z literatury jsme shrnuli v kapitole v rámci knihy „Chronic Myeloid Leukemia: Signs/Symptoms, Classification and Treatment Options“ (Lopotová, Žáčková, 2012). V rámci této dizertační práce jsem se zaměřila na

obecný leukemický (nádorový) marker, gen wt1. Věříme, že nedostatečné poznání jeho exprese i funkce v myeloidních leukémiích (i zdravé hematopoéze) brzdí jeho plné využití v diagnostice, prognostice i přímo jako terapeutického cíle/nástroje.

Celková exprese mRNA wt1 nebyla dosud u pacientů s CML léčených kinázovými inhibitory komplexně studována. Dřívější práce zaměřené na potenciální význam tohoto markeru pro včasnou detekci relapsu po transplantaci kostní dřeně neukázaly wt1 jako vhodný marker pro pacienty s CML. V porovnání s bcr-abl se wt1 jevil nevýhodný jistě díky tomu, že zatímco bcr-abl není ve zdravé krvi přítomen a pouhá detekce tak znamená relaps, hladina wt1 stoupá z nenulových hodnot. V případě kinázových inhibitorů je situace v porovnání s transplantací odlišná. Nejedná se nutně o vymýcení maligního klonu, nýbrž o jeho trvalé potlačování. Naše studie potvrdila význam celkové exprese wt1 u pacientů s CML léčených imatinibem a jako první ukázala na možnost využití wt1 jako markeru pro upřesnění prognózy suboptimálních odpovídačů a pacientů s tzv. selháním léčby. Naše statistická hodnocení ukázala kritické hladiny wt1, které umožňují upřesnit prognózu těchto pacientů. Zdá se tedy, že pokud se nepodaří snížit hladinu wt1 pod určitou mez (dostatečně potlačit aktivitu buněk pacienta), je pravděpodobnost relapsu zvýšená. Kinetika exprese wt1 byla v porovnání s kinetikou exprese bcr-abl výraznější a predikovala tak jasněji blížící se relaps. Zatímco Na a spol., 2005 již dříve ukázali, že wt1 předčí bcr-abl v monitorování pacientů v akcelerované fázi, my jsme výhody WT1 prokázali také u pacientů v chronické fázi. Schnittger a spol., 2009, ukázali, že exprese wt1 korelovala s Ph negativní klonální evolucí u pacientů s CML, naše data ukázala nezávislost exprese wt1 na mechanismu rezistence alespoň pokud jde relaps s nebo bez mutace v kinázové doméně bcr-abl. Na základě našich výsledků i literárních dat věříme, že kombinace monitorování exprese wt1 spolu s bcr-abl může pomoci dále upřesnit prognózu pacientů s CML léčených imatinibem (kinázovými inhibitory).

WT1 je studován a testován pro terapeutické využití nejen jako marker, ale také jako antigen asociovaný s leukémií. Ve studiích testujících vakcíny založené na WT1 bylo ukázáno, že WT1 je na rozdíl od BCR-ABL značně imunogenní. Zároveň víme, že vybrané typy HLA chrání před CML (Posthuma a spol., 2000). Výjimky z pravidla „vysoká hladina wt1 – špatná prognóza, relaps, nízká hladina wt1 – dobrá prognóza“, mohou mít souvislost s parametry a stavem imunitního systému.

Jiná možnost vysvětlení „nekorelace“ celkové hladiny WT1 s vývojem nemoci může být také odlišné zastoupení jednotlivých izoforem. Současná literatura podporuje myšlenku, že onkogenní působení WT1 v leukémiích souvisí s konkrétní variantou WT1 nebo poměrem

variant. V práci Renshawa a spol., 2007 byl ukázán odlišný efekt současné inhibice všech variant od inhibice pouze určitých variant. Konkrétně varianty obsahující sekvenci kódovanou exonem 5 se zdají mít souvislost s ochranou buněk proti apoptóze navozené cytotoxickými látkami. Dříve publikovaný semi-kvantitativní přístup pro kvantifikaci čtyř hlavních sestřihových variant měl tu výhodu, že mohl být a logicky byl navržen tak, aby reakce měly optimální účinnost, citlivost i specifitu. My jsme jako vůbec první vyvinuli reverzně transkriptázové PCR reakce pro kvantifikaci čtyř hlavních sestřihových variant wt1 (Lopotová, ústní prezentace, Japonsko, 2009). Námi navržený přístup v porovnání s předchozím semi-kvantitativním přístupem umožňuje kvantifikovat a tedy monitorovat každou z variant odděleně. Účinnost a citlivost reakcí je sice nižší, nicméně rozsáhlá testování, která jsme provedli, jasně potvrdila kvantifikační schopnost našich reakcí.

V rámci studie čtyř hlavních sestřihových variant WT1 jsme vytipovali rizikové varianty pro CML i AML pacienty - varianty +5/-KTS a -5/+KTS se objevovaly ve zvýšených hladinách v souvislosti s relapsem AML a CML. Varianta +5/-KTS jako rizikový faktor AML byla potvrzena prací Kramarzové a spol., kteří velmi podobnou metodou analyzovali expresi čtyř hlavních sestřihových variant u dětských a dospělých AML a MDS. Přestože Kramarzová a spol. neshledali rozdíl signifikantní, pozorovali jisté zvýšení +5/-KTS varianty u pacientů s vyšším rizikem relapsu.

Pokud bychom přepokládali alespoň bazální korelaci mRNA izoforem s proteiny, můžeme na základě dat týkajících se exprese variant na úrovni mRNA a dosavadních informací o funkci jednotlivých variant hypotetizovat o mechanizmu fungování WT1 v leukémiích. Hossain a spol. původně předpokládali, že onkogenní izoforma bude právě sWT1 pro absenci transkripčně represní domény, která vede k tomu, že sWT1 aktivuje expresi genů, které běžně se vyskytující WT1 plné délky reprimuje. Nové výsledky obrací pozornost spíše ke čtyřem hlavním sestřihovým variantám. Nejzastoupenější u obou diagnóz byla varianta +5/+KTS. Víme, že +5 izoformy obecně mohou chránit buňky před apoptózou navozenou chemoterapeutiky, víme, že exon 5 kóduje doménu významnou pro interakci s partnery WT1. Můžeme tedy usuzovat na významnou roli právě interakčních partnerů WT1 v jeho onkogenním působení. Varianty +KTS upřednostňují vazbu RNA a asociují se sestřihovým aparátem buňky. Proto můžeme hypotetizovat také o tom, že onkogen WT1 může fungovat prostřednictvím své role regulátoru sestřihu spíše než transkripce. Z preliminárních dat k expresi WT1 na proteinové úrovni můžeme zatím uvažovat pouze o tom, že v rámci tří možných začátku translace je v CML nejvíce využíván třetí. Zda existuje rozdíl ve funkcích

proteinů vznikajících ze tří možných začátků translace, není zatím známo.

Zajímavé je dále také porovnání jednotlivých diagnóz, u nichž byl dosud poměr jednotlivých izoforem stanoven. V rámci naší práce to byli dospělí pacienti s AML nebo CML. V rámci práce Kramarzové a spol. byla analyzována dětská i dospělá AML a MDS. Zatímco v případě CML byla druhou nejzastoupenější variantou -5/+KTS, u AML to byla +5/-KTS. Kramarzová a spol. ukázala zároveň také profil variant ve vzorcích kostní dřeně zdravých dárců, kde byl podíl obou izoforem roven. Zdá se tedy, že význam pro leukemický charakter buněk může mít právě narušení poměru těchto dvou izoforem ať už jedním či opačným směrem. Odlišné profily izoforem u jednotlivých hemato-onkologických diagnóz navíc nabourávají zažitou představu o WT1 jako panleukemickém markeru, který funguje obdobným mechanizmem napříč těmito diagnózami.

Nejen naše data k expresi wt1 a jeho izoforem potvrdila, že využití obecného nádorového markeru může být v kombinaci se specifickým markerem BCR-ABL výhodné. Mimo rámec této dizertační práce jsme testovali jako potenciální prediktivní markery pro CML pacienty také heat shock proteiny (HSP), jejichž exprese je podobně jako v případě WT1 zvýšena u řady nádorů. HSP jsou významné pro správné složení, udržení stability a funkce buněčných proteinů včetně BCR-ABL (Wu a spol., 2008). V souvislosti s CML se mluví především o HSP90 a 70 (Chakraborty a spol., 2008, Peng a spol., 2007). HSP70 byl nalezen ve zvýšené hladině v plazmě pacientů s CML (Yeh a spol., 2009), zvýšená exprese v buňkách linie K562 byla spojena s rezistencí k imatinibu (Pocaly a spol., 2007). HSP90 tvoří komplex s BCR-ABL, který BCR-ABL stabilizuje a jehož narušení se zvažuje jako jeden z dalších možných terapeutických přístupů (Wu a spol., 2008). Naše pracovní hypotéza byla, že pokud se s časem v patogenezi CML, v její progresi nebo při rozvoji rezistence k léčbě uplatňuje vyšší množství proteinu BCR-ABL a/nebo více různých proteinů (kinázy rodiny SRC apod.), bude patrně narůstat i potřeba HSP proteinů. Zjistili jsme, že zatímco hladina HSP70 zůstává v buňkách pacientů s CML neměnná napříč různými odpověďmi na léčbu, v případě HSP90 jsme pozorovali korelaci se stavem onemocnění. Vyšší hladiny korelovaly s horším stavem a výskytem relapsu. Statistická hodnocení potom ukázala, že HSP90 má pro CML pacienty prognostický význam a to dokonce už v okamžiku diagnózy a dále kdykoliv během léčby. Na rozdíl od wt1 se hladiny HSP90 v průběhu onemocnění neměnily a tedy význam nemá kinetika exprese ale konkrétní hladina (Žáčková a spol., 2012). Inhibitory HSP90 se testují u CML pacientů pouze v pokročilých fázích a především se uvažují pro případ vysoce rezistentní mutace T315I. Naše studie naznačuje význam těchto proteinů také v chronické fázi CML.

Dále bude zajímavé zjistit, jaký vliv mají inhibitory HSP90 na expresi WT1. Naše předběžná data ukázala, že již po 48 hodinách inkubace buněk s novobiocinem dochází ke změnám v profilu izoforem a k celkovému poklesu hladiny proteinu WT1 současně s indukcí apoptózy a poklesem životaschopnosti buněk. Naše výsledky tak celkově podporují testování inhibitorů hsp jako cílených léků pro pacienty s CML.

Vzhledem k velkému počtu cílových molekul můžeme za potenciální obecné markery považovat i miRNA. Naše úvodní studie exprese miRNA u CML nás navedla k bližšímu zkoumání miR-451. Zjistili jsme pomocí kultivací buněčných linií, primárních buněk pacientů a zdravých dárců s inhibitory BCR-ABL *in vitro*, že snížená hladina této miRNA v CML vzniká v důsledku působení BCR-ABL. V naší publikaci jsme toto pozorování dali do souvislosti s předchozí prací Iraciho a spol., 2009, kteří ukázali, že miR-451 má potenciál inhibovat BCR-ABL, a navrhli jsme zpětnovazebnou regulační smyčku mezi těmito dvěma molekulami. Význam našeho pozorování zdůraznily dvě navazující práce, které je citují a dále rozvádí. Autoři Scholl et al., 2012 ukázali, že miR-451 je dokonce použitelná i přímo jako prognostický marker pro pacienty s CML. Liu a spol., 2012, ukázali, že miR-451/144 má vztah také k rezistenci k imatinibu a dále podpořili význam cílení těchto molekul v léčbě CML. Znovuobnovení zpětnovazebné regulace miR-451-BCR-ABL může být významné pro efektivní dlouhodobé potlačení BCR-ABL. Využití miR-451 jako terapeutického nástroje však jistě bude vyžadovat detailní poznání její role v CML i zdravé hematopoéze. Zatím víme, že miR-451 hraje roli v regulaci erytroidní diferenciace a její dodání leukemickým pacientům by tak mohlo mít vedle inhibice BCR-ABL pozitivní efekt také cestou obnovení nebo v případě CML spíše upevnění diferenciace progenitorových CML buněk.

Závěrem lze shrnout, že tato práce ukázala hladinu mRNA wt1 jako vhodný kandidátní marker pro lepší odhad prognózy pacientů s CML. Svůj význam má monitorování exprese ex vivo i *in vitro* po ošetření primárních buněk léčivy. Studium exprese sestřihových variant naznačilo možnost, jak zvýšit specificitu wt1 jako markeru a zároveň poskytlo cenný nový náhled do patogeneze myeloidních leukémií.

7. Shrnutí

V rámci této práce se podařilo prohloubit dosavadní znalosti o expresi wt1 v myeloidních leukémiích.

Vedle prokázání vysokého prognostického významu pro pacienty s CML, kde hlavní molekulární marker bcr-abl selhává, jsme dále vytvořili unikátní metodiku pro kvantifikaci šesti sestřihových variant wt1. Analýzy naznačily význam konkrétních variant jako rizikových markerů pro pacienty s AML i CML. Nově vytvořená metodika je navíc použitelná i pro další onemocnění, kde WT1 hraje roli.

Paralelní studie miRNA v CML přinesla ve spojení s aplikací měření exprese wt1 *in vitro* po ovlivnění buněk inhibitory BCR-ABL kinázy poznání zpětnovazebné regulační smyčky mezi BCR-ABL kinázou a miR-451. Pozorovaný vztah mezi BCR-ABL a miR-451 může být velmi zajímavý pro potenciální terapeutické využití miR-451.

Data získaná v rámci řešení této práce poskytla také motivaci a otázky pro další výzkum. Přestože je nyní wt1 chápán jako panleukemický marker a předpokládá se tak jednotný mechanizmus působení napříč hemato-onkologickými diagnózami, naše expresní data naznačila, že WT1 může fungovat odlišným mechanismem u různých hemato-onkologických diagnóz. Protože protein, nikoliv mRNA, je výkonnou molekulou, klíčové pro další pochopení role WT1 v patogenezi leukémií bude poznání exprese WT1 a jeho izoforem právě na proteinové úrovni. Řešení této problematiky je metodicky komplikované, nicméně bylo rovněž započato v rámci této práce.

Celkově naše práce ukazuje význam pomocných markerů k bcr-abl pro monitorování pacientů s CML léčených kinázovými inhibitory.

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9. Seznam publikací:

9.1 Publikace, které jsou podkladem dizertace

1. Lopotová T, Žáčková M, Nádvorníková S, Moravcová J: Wilms´ tumor gene 1 – a helpful additional molecular marker to BCR-ABL in patients with chronic myeloid leukemia. Odesláno do časopisu
2. Lopotová T, Polák J, Schwarz J, Klamová H, Moravcová J: Expression of four major WT1 splicing variants in acute and chronic myeloid leukemia patients analyzed by newly developed four real-time RT PCRs. Blood Cells Mol Dis. 2012 Jun 15;49(1):41-7. IF 2,716
3. Lopotová T, Nádvorníková S, Záčková M, Polák J, Schwarz J, Klamová H, Moravcová J.: N-terminally truncated WT1 variant (sWT1) is expressed at very low levels in acute myeloid leukemia and advanced phases of chronic myeloid leukemia. Leuk Res. 2012 Apr;36(4):e81-3. IF 2,555
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6. Kapitola v knize: Tereza Lopotová a Markéta Žáčková: Molecular Monitoring of CML: Role for Novel Markers Additional to BCR-ABL. kapitola knihy Chronic myeloid leukemia: Signs/Symptoms, Classification and Treatment Options; přijato k publikaci

9.2 Publikace bez vztahu k tématu dizertace

1. Machová Poláková K, Lopotová T, Klamová H, Moravcová J: High-resolution melt curve analysis: initial screening for mutations in BCR-ABL kinase domain. Leuk Res. 2008 Aug;32(8):1236-43. **IF 2,561**

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10. Konferenční příspěvky se vztahem k dizertační práci

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