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BCR/ABL preleukemic fusion gene in subpopulations of hematopoietic stem and progenitor cells from human UCB

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The BCR/ABL preleukemic fusion gene (PFG) is one of the most frequent fusion genes in acute lymphoblastic leukemia (ALL) and was also detected in hematopoietic cells from umbilical cord blood (UCB) of healthy newborns. Since hematopoietic stem/progenitor cells (HSPC) are considered to be a critical cellular target for origination of leukemia, we have studied the presence of BCR/ABL PFG in expanded subpopulations of HSPC and differentiated cells from UCB of those healthy newborns, who have previously been tested positive for BCR/ABL by screening of their UCB mononuclear cells using RT-qPCR and FISH methods. We isolated cells from human UCB samples positive for BCR/ABL and negative controls. The isolated cells were sorted into 5 hematopoietic and progenitor cell subpopulations. We analyzed BCR/ABL in sorted and expanded subpopulations of UCB using FISH and RT-qPCR. We found that the number of BCR/ABL positive cells was similar in each studied subpopulation and the same as in differentiated lymphocytes. Our data showed that there is no specific subpopulation of hematopoietic and progenitor stem cells with an increased leukemogenic potential due to the presence of higher copies of BCR/ABL.

Key words: BCR/ABL, preleukemic fusion gene, hematopoietic stem and progenitor cells, umbilical cord blood, children acute leukemia

Pediatric leukemia is a clonal disease arising from the transformation of a single pluripotent hematopoietic stem/progenitor cell (HSPC) [1–3]. The development of acute childhood leukemia is a multistep process driven by the accumulation of genetic abnormalities. First hit representing an initiating event is often a chromosomal translocation resulting in a preleukemic fusion gene (PFG) with a novel/modified activity, usually impairing differentiation of HSPC. PFG may arise predominantly *in utero* during fetal/embryonic development, producing a persistent, but clinically covert preleukemic clone [1, 3–5]. These clones were detected in umbilical cord blood (UCB) of newborns, usually at frequencies 100-fold higher then incidence of leukemia, suggesting relatively late origin and possible elimination of most preleukemic clones further in development [6]. While UCB HSPC have been used for transplantation in treatment of leukemia, the presence of an undiagnosed preleukemic clone in donor´s HSPC before transplantation may explain the development of donor cell leukemia (DCL), a rare complication that occurs after allogeneic HSPC transplantation [7] and causes very high mortality in leukemic recipients [8]. Therefore, the screening of UCB for preleukemic clones based on analysis of PFG may be of high importance for prevention of DCL. In addition, identification of specific HSPC subpopulation(s), in which the PFG is originated, can help to refine the target cells for therapy leading to a more specific and thus more effective therapy of primarily diagnosed and/or relapsed leukemia. These aspects are highly significant, especially for PFG-associated leukemias with a poor prognosis, including t(9;22)(q34;q11) reciprocal translocation resulting into the Philadelphia (Ph) chromosome and formation of the BCR/ABL fusion gene. Translocation t(9;22)(q34;q11) is the most frequent genetic aberration in adult acute lymphoblastic leukemia (ALL) and is found in 20–30% of patients overall; it increases with age, approaching 50% in patients older than 50 years [9, 10]. BCR/

ABL is one of the most frequent (3–5%) and prognostically important PFG associated with pediatric acute lymphoid leukemia (ALL).

BCR/ABL is formed by fusion of the break cluster region (BCR) gene with the Abelson tyrosine kinase (ABL1) gene [11]. Depending on the translocation breakpoint in BCR gene, different Bcr-Abl fusion protein isoforms are expressed, with all of them containing exons 2–11 of the ABL1 gene and different sections of BCR component [12]. The most common Bcr-Abl isoforms are p210 and p190, which is 501 amino acid residues shorter (~ 25%) and can be considered as internal deletion mutant of p210. The expression of p210 is the molecular hallmark of chronic myelogenous leukemia (CML) [12]. The Ph-chromosome is also present in 20–30% of adult B-cell ALL with ~ 1/4 and ~ 3/4 expressing p210 and p190, respectively [12].

BCR/ABL fusion gene results into constitutive expression of activated tyrosine kinase (TK). This gene is an important indicator for prognosis in ALL and is associated with poor overall survival and remission duration. The fusion results into an increased TK activity compared to ABL1 [13]. Since ABL1 regulates the evolution of T-lymphocytes and plays a pivotal role in the process of their cytoskeleton deformation [14], the screening for chimeric ABL1 genes could be considered in ALL-patients, especially in those with T-ALL.

Bcr-Abl is a constitutively active tyrosine kinase and its inhibitors (TKIs) are a paradigm for targeted cancer therapy [15]. P210 is the sole oncogenic driver, sufficient to establish and maintain CML. In contrast, Ph+ B-ALL is frequently associated with additional mutations [16].

Characterizing preleukemic HSPC and understanding the developmental origin and hierarchy in preleukemic HSPC may lead to identification of mechanisms for disease relapse and development of effective therapeutic strategies [17]. During recent years, the focus in treatment of leukemia relapse has shifted towards the development of novel immunotherapeutic strategies [18]. The recent development of novel T-cell-based immunotherapies, initially applied to the treatment of B-cell malignancies, represents an exciting new area of investigation in AML immune therapy [19]. Some of the most promising are drug-conjugated monoclonal antibodies, T-cell engaging antibody constructs, adoptive transfer with chimeric antigen receptor T cells (CAR T cells) and bifunctional antibodies such as bispecific T-cell engagers (BITE) [19, 20]. All these strategies require well characterized membrane markers of pathological cells to be targeted.

Overall, BCR/ABL is one of the most investigated PFG in healthy people; see for review [21]. The incidence of BCR/ABL p190 ranged from 0% up to 80% in studied subjects (newborns, children and adults). In this study, we expanded the rare populations of HSPC from UCB mononuclear cells (MNC) tested positive for BCR/ABL. The aim of this work is to study which cell subpopulation of hematopoietic system carries the PFG and is responsible for development of leukemia. This knowledge may provide a basis for developing immunotherapeutic strategies to eradicate the rare population of preleukemic stem cells.

Materials and methods

Sample preparation. The UCB cells isolated from healthy donors were obtained from Eurocord Slovakia s.r.o. – Slovak cord blood bank. Cells were thawed as previously described [22, 23] with addition of 100 μg/ml DNase I (Sigma-Aldrich, St. Louis, Missouri, United States) to the RPMI (Gibco, Thermo Fischer Scientific, Waltham, Massachusetts, United States) during thawing in order to prevent formation of clamps. After thawing, centrifugation and counting of cells using 0.4% trypan blue solution (Gibco, Thermo Fischer Scientific), cells were incubated in complete media (89% RPMI, 10% FBS, 1% ATB) for 30 min at 37 °C at 5% CO_2. Then cells were spun down for 15 min at 175×g, then the cell pellet was resuspended in 5 ml of complete media, and cell suspension was filtrated through 100 μm cell strainer (BD biosciences, San Jose, California, United States) to prevent formation of clogs during cell sorting. The final viability of cells in all experiments reached at least 95%.

Cell sorting. Sample pellets were resuspended in 400 μl of PBS containing 1% BSA (Sigma-Aldrich) with FC blocking (BD biosciences) in concentration 1:100 and incubated for 10 min at RT. For compensation, single-color stained controls and unstained control were prepared by adding 10 μl of cell solution to 30 μl of 1% BSA in PBS. The rest of cell solution was used for cell sorting. For immunophenotyping of different cell population following antibody conjugates were used: CD34-APC (Miltenyi Biotec, Bergisch-Gladbach, Germany), CD45-V450, CD38-PeCy7, CD45RA-FITC, CD90-PE and Lineage cocktail (CD56, CD8, CD3 and CD235a-PeCy5) (all BD Biosciences). The cells were then incubated for 60 min at 4 °C. Next, 500 μl of 1% BSA, PBS was added to each tube and samples were centrifuged for 10 min at 800×g. Sample was resuspended in 400 μl of 1% BSA, PBS and compensation controls in 200 μl. Finally, 2 μl and 1 μl of 7AAD were added to the sample and Lineage compensation tube, respectively.

Various subpopulations of HSPC were then sorted using BD FACSAria (BD Biosciences) into separate tubes with 200 μl of complete media according to the expression of different surface antigens:

HSPC: CD45+ CD45RA– CD34+
Progenitors: CD45+ CD45RA– CD34+ CD38+
HSC/MPP: CD45+ CD45RA– CD34+ CD38–
HSC: CD45+ CD45RA– CD34+ CD38– CD90+
MPP: CD45+ CD45RA– CD34+ CD38– CD90–

Purity of cell sorting was at least 95% in each one of the experiments.

Cell expansion. The sorted cell subpopulations were applied into wells on 24 well plates in 1.5 ml of complete expansion media (CEM). CEM for one well contained 1 ml of MEM alpha EAGLE with UltraGlutamine I, deoxyribo-

nucleoside, and ribonucleosides (αMEM) + 0.5 ml IMDM with HEPES w/L-Glutamine (both LONZA) with 10% of FBS (Fetal Bovine Serum, HYCLONE) with 1% of ATB/ATM (Antibiotic/Antimycotic Solution, PAA) containing stem cell factor (25 ng/ml), Flt-3L (25 ng/ml), and thrombopoietin (25 ng/ml) (all from R&D Systems). Mesenchymal stem cells (MSC) from umbilical cord in amount of $2{\times}10^4$/well/1 ml αMEM were added as "feeder" cells into the wells 48 h before set the expansion. Optimal concentration of starting cells for every CD34+ subpopulation was 1500 cells/well. The maximum level of expansion achieved was ~1,000× multiplication of starting culture. Cells were checked visually under the microscope in intervals of every 2–4 days and in day 8th of expansion were the cells harvested and transferred in fresh CEM into the wells with new MSC feeder. The optimal time for harvesting was defined before the reaching "plateau" phase during 10–12 days of expansion, when the multiplication is sufficient and the cells still contain the optimal amount of RNA.

Fusion gene and transcript detection. The RT-qPCR was performed as described previously [24]. However, the method of cDNA synthesis was dependent on the number of cells and thus RNA available for the screening. For standard reverse transcription (RT) reaction, 1 μg of total RNA was used. All "no template controls" (NTC) were negative (0/3) in all RT-qPCR assays. In case of expanded hematopoietic stem/progenitor cell subpopulations yielding often a limited number of cells and RNA, an amplification of cDNA has been introduced. Using this procedure based on Smart-seq2 [25] a controlled amplification of mRNA-derived cDNA (from 100- up to 140,000-fold) has been obtained.

FISH analysis. Dried slides were washed in KCl (0.55 g/100 ml; pH=6.8; 10 min; room temperature (RT)), placed in fixation solution (methanol 75% and 25% acetic acid; 10 min; RT) and dried. Next step was degreasing in xylene (15 min; RT) and dehydration in 96% ethanol (15 min; RT), repeat washing in KCl and fixation step. The slides were dried in a flow box. Next, ready to use or diluted DNA FISH probes were applied in volume of 3 μl. Slides were covered by cover slips, sealed by Fixogum (Marabu, Bietigheim-Bissingen, Germany) and applied into ThermoBlock at 75 °C (CytoCell Aquarius, Cambridge, United Kingdom) for 5 min. The samples were put into humid chamber in incubator at 37 °C overnight. The cover slips were removed and the slides were washed in 0.4× SSC (60 s; 72 °C) and immediately in 2× SSCT (30 s; RT). Next, the slides were dehydrated in ethanol row 50%, 70%, and 96% for 2 min in each concentration without drying. After this step the slides were dried in flow in dark. Afterwards, 3 μl DAPI in concentration 0.125 μg/ml containing antifade reagent (Cytocell, Cambridge, United Kingdom) was added. The slides were analyzed using fluorescent microscopy (Olympus BX51, Shinjuku, Japan) in spectrum DAPI, spectrum green and spectrum red. Depending on the quality of the samples 200–1,000 cells were analyzed manually.

Statistical analysis. Analysis of variance (ANOVA), Fisher exact test and LSD were applied using Statistica software (Dell software, Round Rock, Texas, United States). The results were considered significantly different at $p<0.05$.

Table 1. Real-time quantitative PCR analysis individually revealed BCR/ABL positivity in expanded UCB HSPC subpopulations.

proband/ subpopulation	HSPC	Progenitors	HSC/MPP	HSC	MPP
P327	0; 3; 5	10; 10; 0	0; 0; 3	0; 8; 13	2; 0; 3
P138	2; 4; 1	2; 1; 1	5; 2; 3	N/A	N/A
P292	16; 11; 18	31; 30; 41	8; 0; 63	N/A	N/A
P546	N/A	0; 0	0; 0; 3	5; 0; 0	5; 0; 0
P225	2; 0; 0	0; 0; 0	0; 0;	0; 0; 0	2; 0
P257	2; 0; 9	4; 11; 0	11; 15; 13	23; 11; 7	26; 7; 0
P428	9; 7; 3	4; 17; 5	3; 9; 7	1; 9; 13	13; 5
P431	N/A	14; 5; 7	4; 6; 4	A	40; 61; 28
P391	3; 5; 1	12; 6; 6	0; 0; 0	0; 0; 4	0; 5

Copy number of BCR/ABL (p190) per 100,000 cells in triplicates/duplicates are shown; probands originally tested as MNC-negative are designated in bold; subpopulations that failed to expand are shown as N/A; neat, 1:10 and 1:20 dilutions of amplified cDNA used as template in RT-qPCR, all three samples being positive, are designated by A.

Results

In previous study, UCB MNC from 500 newborns were screened by RT-qPCR for the presence of most prognostically important PFG [6]. From the same UCB cell bank, we have chosen six BCR/ABL positive and three BCR/ABL negative samples of frozen UCB MNC for sorting, subsequent cell expansion and identification of the subpopulation containing BCR/ABL fusion transcript and correspondent chromosomal translocation by RT-qPCR and FISH methods, respectively. UCB MNC were sorted into five subpopulations: HSPC: CD45+ CD45RA– CD34+; Progenitors: CD45+ CD45RA– CD34+ CD38+; HSC/MPP: CD45+ CD45RA– CD34+ CD38–; HSC: CD45+ CD45RA– CD34+ CD38– CD90+; and MPP: CD45+ CD45RA– CD34+ CD38– CD90–. The range number of expanded cells was from 40,000 to 6 million according to the number of expansion days and number of sorted cells entering in expansion, which ranged from 40 to 10,000 cells. The range of expansion rate was from 80 to 6,250 through all subpopulations. No significant correlation was found between time of expansion, number of cells at the start of expansion, and rate of expansion.

The results from RT-qPCR and FISH are shown in Table 1 and Table 2, respectively. The RT-qPCR analyses of MNC revealed the BCR/ABL positivity in HSPC of all nine probands, although in previous study MNC were tested as BCR/ABL negative in three of them using the same methodology. This positivity in some subpopulations of MNC, which have previously been tested negative, may be accounted for significant increase in the detection threshold due to cell sorting.

The RT-qPCR results show that individual UCB HSPC subpopulations have been tested positive for BCR/ABL, reaching different copy number of the BCR/ABL fusion transcript (from 1 to 76 copies per 100,000 cells). In general, all HSCPC subpopulations were found to be positive for BCR/ABL suggesting that this translocation may arise already in most primitive hematopoietic stem cells. The mean level of BCR/ABL positivity, estimated from RT-qPCR data including all probands and cell subpopulations, was ~7.05 transcripts per 100,000 cells. We did not observe any statistically significant differences between subpopulations of HSPC using ANOVA and Fisher LSD (p>0.05). Thus, our RT-qPCR results provide evidence that BCR/ABL PFG is present not only in a single UCB HSPC subpopulation, but more probably it spreads into all HSPC subpopulations.

Using FISH method we identified BCR/ABL chromosomal translocation only in one subpopulation of proband P428, namely in progenitors. The positivity was at the level of 1.5% in comparison with other subpopulations of this and other probands, in which the BCR/ABL was not found (Table 2). This positivity was unlikely false due to use of a "dual fusion" BCR/ABL DNA FISH probe, specially constructed to eliminate the false positivity that might be caused by a random overlap of two signals (Figure 1). Almost negative data on BCR/ABL chromosomal translocation obtained by FISH is in line with the RT-qPCR data that revealed extremely low incidence of BCR/ABL fusion falling below FISH detection threshold.

Table 2. FISH analysis revealed BCR/ABL fusion gene in UCB progenitors of one out from nine probands.

proband/ subpopulation	HSPC	Progenitors	HSC/MPP	HSC	MPP
P327	N/A	0/200	0/1000	0/1000	0/1000
P138	0/1000	0/1000	0/1000	N/A	N/A
P292	0/1000	0/1000	0/1000	N/A	N/A
P546	N/A	0/200	0/1000	0/1000	0/200
P225	0/200	N/A	N/A	0/500	N/A
P257	0/1000	0/1000	0/1000	0/1000	N/A
P428	0/200	3/200	0/500	0/1000	0/1000
P431	0/1000	0/1000	0/1000	0/1000	0/1000
P391	0/1000	0/1000	0/1000	0/1000	0/1000

Number of positive/analyzed cells is shown. The samples that failed to expand are shown as N/A.

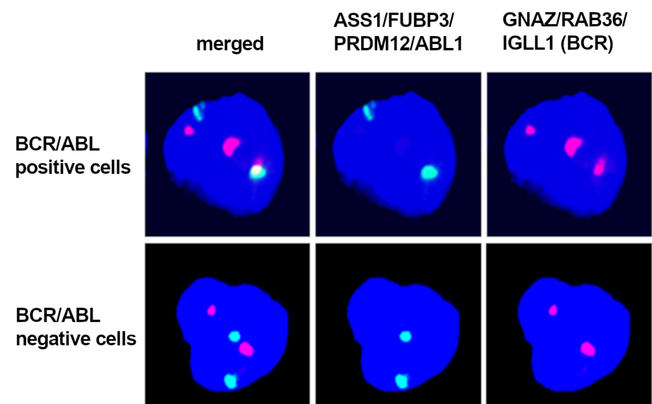

Figure 1. Representative image of BCR/ABL positive cell (upper row) and negative cells (bottom row). Nuclei of the progenitor cells are stained by DAPI in blue. Green and red spots represent the BCR and ABL region, correspondently. BCR/ABL negative cell displays two red spots and two green spots while BCR/ABL positive cell contains two green spots and three red spots (one intact, one co-localized with green spot showing BCR/ABL fusion, and one as a reciprocal product).

Discussion

For analyzing cell subpopulations, which may be responsible for leukemogenesis, we sorted HSPC subpopulations from UCB cells using appropriate membrane markers such as CD34, CD38, CD45, CD45RA, and Lin. In our experiments, UCB MNC tested previously as BCR/ABL positive or negative using standard RT-qPCR analysis in triplicates [6] were sorted and expanded. However, each cell subpopulation derived from the UCB MNC was individually tested BCR/ABL positive by RT-qPCR in these experiments. The sensitivity of our RT-qPCR has previously been analyzed and ratio of positive reactions to all reactions was defined as 0.4 when proportion of BCR/ABL is at least 1 copy/100,000 cells [24]. Thus, the positivity of HPSC populations from 3 tested negative MNC may be accounted for predominant formation of BCR/ABL in HPSC, which is not detectable in MNC due to low detection threshold. The positivity in subpopulations of previously tested negative MNC samples could also be explained by formation of BCR/ABL *trans*-spliced transcripts as a consequence of cell sorting and expansion process *in vitro* [26]. While chromosomal instability has been detected in *ex vivo* expanded HSC, this instability deals mostly with numerical chromosomal abnormalities with a negligible formation of chromosomal translocations [27]. Thus, it is unlikely that chromosomal translocation resulting in BCR/ABL would be formed during expansion.

Of note, in progenitors from proband P428, which were tested positive by FISH using dual fusion DNA probe with almost 0% false positivity [28], about 8 copies/100,000 cells were found by RT-qPCR analysis in triplicate. The same or even higher BCR/ABL copy number was established by RT-qPCR in some other subpopulations while no positivity was detected therein by FISH. However, relatively low sensitivity of FISH may underlie negative output of FISH analysis in these samples. Another explanation could be that RT-qPCR might detect aforementioned alternative *trans*-splicing product. Ability of FISH DNA probe to detect both BCR/ABL translocations, p190 and p210, while the RT-qPCR primers in our experiments were set for the p190 translocation, may also contribute to the obtained results.

CD34+ HSPC represented about 1% of UCB MNC. Proportion of specific HSPC increased in the sorted subpopulations as follows: CD34+ – by 100-fold, CD34+ CD38–, which represented 0.1% of MNC – by 1000, and CD34+ CD38– CD90+ represented 0.01–0.03% of MNC – by 10,000–3,333. If we assume the presence of specific HSPC subpopulations containing the BCR/ABL positive cells, the probability for identifying the positive cells would be significantly increased upon sorting. Given the BCR/ABL copy number observed by Kosik et al. [6] in BCR/ABL positive MNC samples selected for our experiments and possible preferential formation of BCR/ABL in a specific HSPC subpopulation, this specific sorted subpopulation would contain in average: CD34+ – ~1.315 copies per 1,000 cell; CD34+ CD38– – ~1.315 copies per 100 cells, and CD34+ CD38– CD90+ – ~1.315 copies per 10–30 cells. Thus, the sensitivity of FISH might be sufficient to detect PFG positive cells if hypothesis on specific localization of PFG in defined HSPC subpopulation is correct. In contrary to this hypothesis, our RT-qPCR results showed similar incidence of BCR/ABL+ cells in each subpopulation (mean value was ~7.05 transcripts per 100,000 cells) indicating a random distribution across all HSPC subpopulations. In addition, very similar BCR/ABL incidence was detected in BCR/ABL positive MNC chosen for this study. Thus, our data suggest that there is not specific HSPC subpopulation predisposed to formation of BCR/ABL. Finally, our data indicate that BCR/ABL fusion gene is formed predominantly in most primitive stem cells and then expand in the process of cell differentiation.

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