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DRUG RESISTANCE ASSOCIATED PROPERTIES OF BLASTS SUBPOPULATIONS WITH DIFFERENT CD34 EXPRESSION IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

T.V. Shman, U.U. Fedasenka, V.P. Savitski, A.M. Kustanovich, O.V. Aleinikova*
Belarusian Research Center for Pediatric Oncology and Hematology, Minsk, Belarus

***Aim*: The objective of this study was to investigate expression of drug resistance associated genes in CD34+ and CD34- leukemic subpopulations in childhood acute lymphoblastic leukemia (ALL). *Methods*: ALL samples with heterogeneous CD34 expression were separated into CD34-positive and CD34-negative subpopulations and mRNA levels of *MDR1*, *LRP* , *BCRP* and *BCL-2* genes were compared. *Results*: BCL-2 gene expression levels did not differ significantly between CD34+ vs CD34− subpopulations in most analyzed ALL cases. Oppositely, *MDR1* gene had >two-fold differences in expression levels between subpopulations in the majority of ALL cases. In T-lineage ALL CD34− fractions had increased level of *BCRP* and *LRP* genes in comparison with CD34+ ones whereas in most of B-lineage ALL expression of these genes did not differ. *Conclusion*: It was not found the unique pattern of resistance related genes expression in CD34+ *vs* CD34− subpopulations. However, in majority of studied pediatric ALL cases with CD34 heterogeneous expression one of subpopulations (positive or negative) could have an advantage for survival through elevated expression of drug resistance related genes.**

***Key Words*: acute lymphoblastic leukemia, CD34 heterogeneity, *BCL-2*, *MDR1*, *LRP*, *BCRP*.**

In spite of substantial improvement in treatment of childhood acute leukemia that has been achieved during the past decades, a relapse still proves to be a major obstacle on the way to a prolonged complete remission [1]. Patients diagnosed with acute lymphoblastic leukemia (ALL) and receiving treatment in accordance with current protocols still face an approximately 20% chance of a relapse [2–5].

Minimal residual disease (MRD) monitoring provides important information for early prediction of a recurrence of the illness, however immunophenotypic modulation (shifts in immunophenotypic markers of leukemic cells during treatment and at relapse) and presence of several immunophenotypic subpopulations provide additional difficulties for clinicians [6–7]. CD34 is a stage-specific antigen that is expressed on human hematopoietic stem and progenitor cells whose expression decreases with differentiation of the cell. CD34 marker is broadly used in ALL diagnostics and MRD monitoring [8–9]. However, it has been demonstrated significant reduction of CD34 expression during induction therapy in childhood ALL [10]. Those phenotypic changes could be a result of drug exposure or caused by a clonal selection during chemotherapy.

In this study we tried to elucidate if immunophenotypically heterogeneous subpopulations of leukemic cells differed in expression of drug resistance related genes in childhood ALL. Previously, some properties of $CD34^+$ vs $CD34^-$ leukemic subpopulations were investigated in acute myeloid leukemia (AML) samples. It was found that predominantly $CD34^+$ fractions were more resistant to apoptosis, had increased expression levels of antiapoptotic and drug resistance associated genes/proteins compared to a corresponding $CD34^-$ one [11–13]. Although other authors stated that during chemotherapy $CD34^+$ leukemic cells depleted more rapidly than $CD34^-$ cells [14]. Therefore, the absence of publications about "resistance associated" properties of $CD34^+/CD34^-$ leukemic fractions in ALL provides an interesting field for investigations in this area.

With regard to the facts mentioned above we studied bone marrow samples taken at diagnosis of childhood T- and B-lineage ALL. For an in-depth study, all samples with heterogeneous CD34 expression were separated into CD34-positive and negative subpopulations and mRNA levels of multidrug resistance genes, antiapoptotic gene *BCL-2* were compared.

MATERIALS AND METHODS

Patients. 17 children at the median age of 10 years (range, 1–18) with ALL were taken in this study. 11 patients were diagnosed as pro-B, common or pre-B ALL (B-cell precursor ALL, BCP-ALL), 6 patients were pro-/pre-T, cortical or mature T-ALL (T-lineage ALL, T-ALL). Heterogeneous CD34 expression and sufficient quantity of leukemic cells were the main criteria for choosing samples. ALL population was considered as heterogeneous when quantity of CD34 positive cells was between 10–90%.

This investigation was approved by the local Institutional Ethic Committee. Characteristics of patients are listed in Table 1.

Cell preparation. Leukemic cells of all used samples were isolated from bone marrow by gradient density centrifugation. Information about pattern of antigens expression was obtained from standard three-color immunophenotyping procedure.

Cell sorting. Leukemic cells were separated into $CD34^+$ and $CD34^-$ fractions after CD34 PE labeling (Becton Dickinson, San Jose, CA, USA). Additional

Received: June 27, 2011.
*Correspondence: Email – t_shman@yahoo.com
Fax: 375 (0) 17 265 42 22,

Abbreviations used: ALL – acute lymphoblastic leukemia; AML – acute myeloid leukemia; BCP-ALL – B-cell precursor acute lymphoblastic leukemia; MRD – minimal residual disease.

labeling and gating according to CD45 FITC fluorescence were used to exclude normal cells contamination. Dead cells were excluded using forward and side scatter gating. The purity of sorted subpopulations was always more than 98%. Gating strategy was performed as described previously [15]. Leukemic cells sorting were carried out using FACSVantage SE (BD).

Real-Time PCR. Total RNA from sorted cells was extracted using Gen Elute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St Louis, MO, USA). According to manufacturer's protocol after first step (cell lysing) samples were stored at -70 °C. When needed, samples were thawed and RNA was extracted. Quantity and quality of obtained total RNA were defined by spectrophotometry using Gene Quant RNA/DNA Calculator (GE Healthcare). Reaction of reverse transcription was carried out immediately after RNA extraction using Advantage RT-for-PCR Kit (BD) according to the manufacturer's protocol.

cDNA from one extraction from IM-9 cell line was used as a standard and a calibrator for analyzing *BCL-2*, *MDR1*, *LRP* and *BCRP* gene expression in leukemic bone marrow blasts where all these genes expressed at levels comparable with those in the cell line. Normal gene *GUS* was used as a loading control gene [16].

We evaluated expression levels of experimental genes using real-time PCR (iCycler, BioRad). For calculating relative expression levels standard curves method was used. Each standard curve was generated according to four points of 10-fold dilutions of cDNA obtained from IM-9 cell line. Amplifications were carried out in a total volume of 25 μl containing cDNA, Platinum Quantitative PCR SuperMix-UDG (Invitrogene, Carlsbad, CA, USA, final concentration of $MgCl_2$ was raised to 4mM), 300nM of forward and reverse primer, and 200nM of TaqMan probe. The following primers were used (5' to 3'): *BCL-2* forward primer: TTG GCC CCC GTT GCT T, reverse primer: CGG TTG TCG TAC CCC GTT CTC, TaqMan probe: FAM AGC GTG CGC CAT CCT TCC CAG BHQ1; *MDR1* forward primer: AGG AAG ACA TGA CCA GGT ATG C, reverse primer: CCA ACA TCG TGC ACA TCA AAC, TaqMan probe: FAM CCT GGC AGC TGG AAG ACA AAT ACA CAA BHQ1; *LRP* forward primer: CAG CTG GCC ATC GAG ATC A, reverse primer: TCC AGT CTC TGA GCC TCA TGC, TaqMan probe: FAM CAA CTC CCA GGA AGC GGC GGC BHQ1; *BCRP* forward primer: TGG CTG TCA TGG CTT CAG TA, reverse primer: GCC ACG TGA TTC TTC CAC AA, TaqMan probe: FAM AGC AGG GCA TCG AGC TCT CAC CCT G BHQ1.

Quantitative analysis of *TEL-AML1* fusion gene expression in $CD34^+$ and $CD34^-$ leukemic fractions was estimated with DNA-standards (Ipsogen, France) according to protocol of Gabert J. et al. [17] and normalized to *GUS* expression [16].

Statistical analysis. Student's test for paired samples was applied to determine significance of differences between $CD34^+$ and CD34- populations.

RESULTS

Bone marrow leukemic samples with heterogeneous expression of CD34 were separated into $CD34^+$ and $CD34^-$ fractions by fluorescent activated cell sorting. Characteristics of patients included in this investigation are listed in Table1. Median values of CD34 positive leukemic cells for all selected patients was 50 % (from 14 to 80 %).

Table 1. Patient Characteristics

Patient №	Age	ALL	CD34, %	Heterogeneous markers	Fusion gene
1	2	BCP	70	CD45, CD13	Negative
2	<1	BCP	48	CD15	MLL-AF1p
3	5	BCP	58	CD5, CD117	Negative
4	8	BCP	50	CD20, CD33	Negative
5	16	BCP	22	CD33, CD11b	Negative
6	2	BCP	33	CD45, CD10, CD19	TEL-AML1
7	2	BCP	34	CD45, CD20, CD15	TEL-AML1
8	6	BCP	59	DR, CD20, CD13, CD10, CD15	TEL-AML1
9	15	BCP, relapse	46	DR	Negative
10	3	BCP	70	CD20	TEL-AML1
11	15	BCP	46	DR, CD20,CD15	MLL-AF4
12	15	T, relapse	80	CD13, CD11b	Negative
13	15	T	14	CD8, CD13, TdT	SIL-TAL
14	13	T	58	CD1a	Negative
15	16	T	67	CD4	Negative
16	14	T	54	CD8, CD3, CD13, CD10, CD117, CD1a, TdT	Negative
17	10	T	47	CD4, CD3, CD117, CD33, CD11b, TdT,	Negative

Previously we described apoptosis and proliferations differences in small groups of childhood ALL and AML samples [15]. Here expression levels of *BCL-2, MDR1, LRP* and *BCRP* genes were analyzed in $CD34^+$/$CD34^-$ subpopulations of childhood ALL. Differences in expression level of genes between studied subpopulations in ⩾two-fold we considered as significant when differences <two-fold were accounted as insignificant.

In general, in BCP-ALL group we found no evident trend to higher expression levels of any studied genes in $CD34^+$ or $CD34^-$ fractions. In particular, *BCL-2* gene expression levels to be higher in $CD34^+$ cells vs $CD34^-$ cells only in 1 out of 11 samples (patient 1). Whereas in 2 out of 11 samples (patients 7, 8) expression levels of *BCL-2* were higher in $CD34^-$ cells, and in eight samples (73%) we found expression levels of this gene in $CD34^+$ and $CD34^-$ subpopulations to be not significantly different (Fig. 1, *a*).

Higher expression levels of *MDR1* gene in $CD34^+$ vs $CD34^-$ cells was registered in 3 out of 11 samples (patients 3, 4, 6). However, in 4 out of 11 samples we registered lower expression level of *MDR1* in $CD34^+$ fraction. In four samples (36%) expression levels of *MDR1* gene did not exceed two-fold difference (Fig. 1, B).

BCRP expression levels in $CD34^+$ cells was higher in 1 out of 11 samples (patient 1); in 2 out of 11 samples (patient 2, 3) its expression in $CD34^+$ subpopulation was lower; and eight samples (73%) we found no difference (figure 1C). For all BCP-ALL patients expression levels of *LRP* gene did not exceed two-fold difference between $CD34^+$ and $CD34^-$ subpopulations (figure 1D).

In T-ALL group differences in genes expression between $CD34^+$ and $CD34^-$ subpopulations are rep-

resented in Fig. 2. Antiapoptotic gene *BCL-2* in 1 out of 6 samples (patient 17) had >two-fold higher expression in CD34⁻ than CD34⁺ fraction while in other five samples we found negligible difference between fractions (figure 2a).

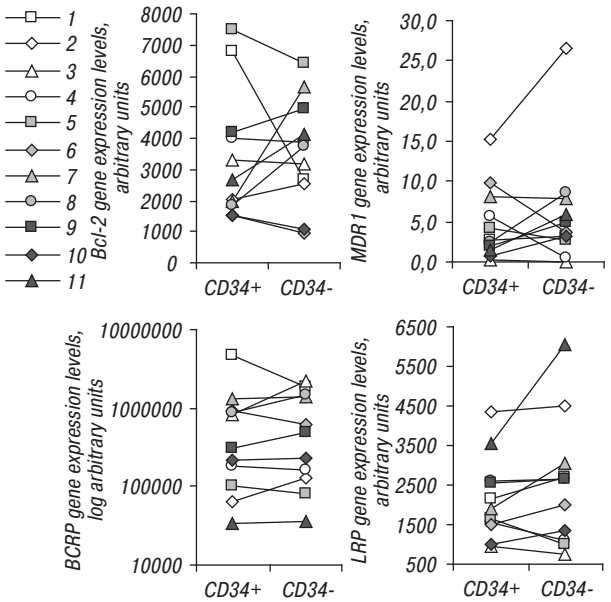

Fig. 1. Expression levels of *BCL-2* (*a*), *MDR1* (*b*), *BCRP* (*c*) and *LRP* (*d*) genes in CD34⁺ and CD34⁻ leukemic subpopulations in BCP-ALL

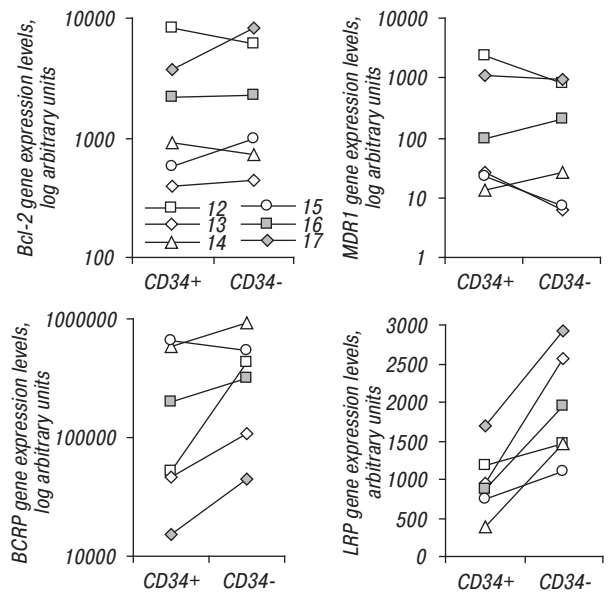

Fig. 2. Expression levels of *BCL-2* (*a*), *MDR1* (*b*), *BCRP* (*c*) and *LRP* (*d*) genes in CD34⁺ and CD34⁻ leukemic subpopulations in T-ALL

Expression levels of *MDR1* gene in samples from T-ALL group were significantly higher comparing to BCP-ALL group. For T-ALL samples median values of *MDR1* expression for CD34⁺ and CD34⁻ were 60.4 (range, 13.2–2384.0) and 117.2 (range, 6.3–967.2) arbitrary units respectively, while it were 2.7 (range, 0.16–15.3) and 3.6(range, 0.06–26.4) for CD34⁺ and CD34⁻ subpopulations for BCP-ALL cases.

In 3 out of 6 samples (patients 12, 13, 15) *MDR1* gene was expressed at higher levels in CD34⁺ cells than in CD34⁻ cells and in 2 samples (patients 14, 16) it was expressed at lower levels in CD34⁺ cells; one sample had no two-fold difference.

Interestingly, for all T-ALL cases *LRP* gene was expressed in higher level in CD34⁻ leukemic fractions than CD34⁺ ones. For patients 13, 14 and 16 differences in *LRP* expression levels between CD34⁻ *vs* CD34⁺ were 2.7, 3.9 and 2.3 fold respectively. *BCRP* gene levels were higher in CD34⁻ cells than CD34⁺ cells in 5 out of 6 samples, for three of them differences between CD34⁺ *vs* CD34⁻ cells were >two-fold.

Then we supposed that genetic alterations in leukemic cells could lead to a specific pattern of expression of investigated genes in CD34⁺ vs CD34⁻ leukemic fractions. *TEL-AML1* fusion gene was detected in four leukemic samples. It was found out that for all four samples CD34⁺ leukemic fractions had higher level of *TEL-AML1* expression, however parameters of genes expression were revealed in a variable extent (from no difference to > two fold difference) in CD34⁺/CD34⁻ leukemic subpopulations.

DISCUSSION

Currently it is evident that tumor tissues are composed of heterogeneous combinations of cells with different phenotypic characteristics, proliferation potentials, self-renewal ability, etc. [18]. In this work we made an attempt to investigate biologic properties of CD34⁻ and CD34⁺ leukemic cells with respect to a more "resistant phenotype" and a better chance of surviving during chemotherapy.

Apoptosis is an important mechanism that regulates survival of leukemic cells. It was described that an intact apoptosis signaling was important for efficient induction and maintenance of remission in childhood precursor B-cell ALL [19]; a relapse in childhood ALL was associated with decreased *BAX/BCL-2* ratio and loss of spontaneous caspase-3 processing *in vivo* [20]. *BCL-2* protein plays a critical role in inhibiting anticancer drug-induced apoptosis, constitutive overexpression of *BCL-2* is one of mechanisms of drug resistance in cancer [21]. Previously we described a decreased ability to apoptosis of CD34⁺ leukemic cells than CD34⁻ fraction [15]. Such difference in apoptosis levels between subpopulations can be a result of different expression of *BCL-2* gene. However, here our data did not reveal any considerable difference in *BCL-2* gene expression between CD34⁺ *vs* CD34⁻ fractions for BCP-ALL and for T-ALL.

Multidrug resistance phenotype is usually associated with efflux of anticancer agents from cells leading to a decreased drug accumulation. The role of *MDR1*, *BCRP*, *LRP* genes / proteins in drug resistance in leukemia has been studied for years, however, the prognostic role of these proteins in childhood ALL remains controversial [22–25].

Earlier, associations between expression of drug resistance associated genes/proteins, antiapoptotic *BCL-2* and CD34 expression were frequently described for AML patients [26–29]. However, these correlations were found rarely in ALL samples [23,

30–31]. Our results demonstrated that in the majority of B-lineage ALL cases $CD34^+/CD34^-$ leukemic fractions did not differ in expression of *BCRP* and *LRP* genes. On the other hand, for all analyzed T-lineage ALL samples CD34- leukemic fractions had increased level of *BCRP* (from 1.2 to 8.1 fold) and *LRP* (from 1.2 to 3.9 fold) genes in comparison with $CD34^+$. In the majority of patients with BCP- (7/11) and T-ALL (5/6) expression of *MDR1* gene had >two-fold difference between subpopulations, however increased *MDR1* gene expression was detected in $CD34^+$ as well as in $CD34^-$ subpopulations. However recently it was described that expression of CD34 was associated with elevated *MDR1* and *MRP1* mRNA expression levels, but not *LRP* in pediatric T-ALL [32].

Investigations of drug resistance properties of subpopulations became especially interesting in the light of new data that in childhood ALL blasts at all stages of immunophenotypic maturation have stem cell properties (ability to engraft and reconstitute leukemia in NOD/SCID mice) [33]. Normal hematopoietic and leukemic stem cells possess several characteristics that protect them from potential insults through the expression of ATP-associated transporters [34–35]. Therefore, we summarized all data comparing genes expression levels in $CD34^+$ vs $CD34^-$ leukemic fractions obtained from children with BCP-ALL and T-ALL. Conditionally we admit that if leukemic subpopulation ($CD34^+$ or $CD34^-$) had > two fold higher level at least of one of analyzed genes it means that this subpopulation possess "resistant" properties. Thus, we separated all analyzed cases into groups with "resistant" $CD34^+$ cells, "resistant" $CD34^-$ cells, a group of cases with both subpopulations showed signs of a "resistant" phenotype and a group that showed no difference between two subpopulations (Table 2).

Table 2. Comparison of "resistant" properties $CD34^+$ *vs* $CD34^-$ leukemic subpopulations

	BCP-ALL n=11	T-lineage ALL n=6
Only $CD34^+$ subpopulation had resistant properties	Patients 1, 4, 6	Patient 15
Only $CD34^-$ subpopulation had resistant properties	Patients 2, 7, 8, 9, 10, 11	Patients 14, 16, 17
$CD34^+$ and $CD34^-$ subpopulations had resistant properties	Patient 3	Patients 12, 13
No difference between $CD34^+$ and $CD34^-$ subpopulations	Patient 5	0

According to our assumption, it was found that both studied subpopulations showed indications of a resistant phenotype. Among of all analyzed cases only one sample (5.9%) revealed no difference in resistance associated features between cells with high and low CD34 expression. In 3 out of 17 cases (17.6%) both subpopulations displayed signs of a "resistant" phenotype. Surprisingly, only in 4 out of 17 ALL cases (23,5%) $CD34^+$ cells had more pronounced resistance properties. Finally, in 9 out of 17 cases (52,9 %) $CD34^-$ subpopulation had properties of a resistant phenotype.

In conclusion, it was not found the unique pattern of resistance related genes expression in $CD34^+$ *vs* $CD34^-$ subpopulations however, in majority of studied pediatric ALL cases with CD34 heterogeneous expression one of subpopulations (positive or negative) could have an advantage for survival through elevated expression of drug resistance related genes. Further investigation is required to elucidate reasons and patterns of such distinctions between subpopulations.

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