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Clinical impact of genomic analysis in children with B-acute lymphoblastic leukemia: A pilot study in Slovakia

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Acute lymphoblastic leukemia (ALL) belongs to a genetically heterogeneous disease associated with a wide range of chromosomal and molecular changes. Determining these changes at the time of diagnosis can help the therapeutic decision, and contributes to the prediction of patients' clinical outcomes. A part of B-ALL (B-other) lacks cytogenetic abnormalities with clinical relevance for prognosis. Our first goal was to retrospectively review genetic results of patients from 2013–2017 and identify number of B-other patients in Slovak population. The second goal was to implement single nucleotide polymorphism (SNP) array analysis to improve the diagnosis and risk stratification. In this study we reviewed 133 B-ALL patients. We found that nearly 40% of them (52 cases) belonged to the B-other ALL group. Eighteen B-other ALL patients were subjected to the analysis using SNP-array. Overall, we identified 126 cytogenomic changes and in 4 patients the SNP array revealed clinically relevant markers of adverse prognosis and high relapse risk. Integrating identified genetic changes into clinical practice can bring improvement of prognosis assessment for children with ALL in Slovakia.

Key words: children acute lymphoblastic leukemia, B-other, genetic markers, SNP-array

Acute lymphoblastic leukemia (ALL) is the most common malignant disease in childhood, accounting for 25% of all pediatric malignancies and about 80% of childhood leukemia. In the Slovak Republic approximately 30 new cases of ALL are diagnosed annually [1]. Over the past 60 years, remarkable progress has been made in the treatment of ALL patients. In the 1960s, less than 10% of children with ALL achieved a long-term survival. With current therapy, the chance for 5-year event-free survival and overall survival increased to almost 85% and 90%, respectively [2]. The dramatic improvement in the patient outcome was facilitated by stratification of treatment intensity based on the assessment of risk factors. These factors include clinical factors, patient response to induction therapy and cytogenetic abnormalities [1–3]. Patients with the greatest relapse risk receive the most aggressive treatment, and less intense regimens are given to patients at lower relapse risk. This approach was developed on the basis of the recognition that pediatric ALL is a heterogeneous disease comprised of different subtypes that differ significantly in response to chemotherapy [3].

The genetic profile of the leukemic blasts has a significant impact on prognosis. Genetic abnormalities can be primary or secondary events. Primary abnormalities are often structural (chromosomal translocations) or numerical abnormalities of chromosomes (gain or loss of multiple whole chromosomes), whereas secondary changes are usually copy number alterations (CNA, micro-deletions/duplications) or point mutations [4]. Primary genetic changes are present in all leukemic cells (clonal) and define the key features of leukemia. Conversely, secondary abnormalities are present only in a subclone of leukemic cells and lead to a complex branching of the subclone architecture [4, 5]. Since primary abnormalities define key features of leukemia, most screening algorithms for classifying patients into the risk groups and current treatment protocols recommend the detection of primary key chromosomal abnormalities [4].

In children with ALL, about 85% of cases develop from a precursor of B-cell lineage and the rest from a T-cell precursor. Approximately 75% of childhood B-ALL cases harbor primary chromosomal abnormalities detectable

by karyotyping, fluorescent *in situ* hybridization (FISH) or molecular techniques. Six major genetic subtypes, accounting for 75% B-ALL cases, are as follows: high hyperdiploidy (51–67 chromosomes per cell), *ETV6-RUNX1* (TEL-AML1) and *TCF3-PBX1* confer the most favorable prognosis, whereas hypodiploidy (less than 45 chromosomes per cell), *BCR-ABL1, KMT2A* (*MLL)* translocations are associated with poor prognosis [2–5]. In the remaining 25%, pediatric B-ALL patients lack abnormalities with clinical relevance for prognosis assessment. This group is called B-other. It is a heterogeneous group of patients with different response to therapy and high rate of relapse. Modern high-resolution methods have revolutionized the discovery of the ALL genome. Genome-wide SNP array method allows determination of both copy number aberrations and copy neutral loss of heterozygosity (CN-LOH) and therefore is instrumental in providing insights into often complex and unique genomic profiles of patients. They revealed many genetic changes related to the onset and prognosis of ALL, changes appropriate as new therapeutic targets. They also brought new insights into the complex interaction of genetic changes, that is, by integrating secondary abnormalities into existing classification, it is possible to reclassify all the genetic subgroups (including B-others) [5–8].

The aims of study were to: 1) retrospectively review clinical and cytogenetic results of patients diagnosed and monitored in a period from 2013 to 2017, 2) improve the genetic diagnosis and risk stratification of children with B-other ALL in Slovakia by implementing SNP-array.

Patents and methods

Patient cohort. We retrospectively reviewed clinical and biological results (flow cytometry, cytogenetic, FISH, PCR and MLPA) characteristics and treatment outcome for 152 consecutive ALL cases from the Department of Pediatric Hematology and Oncology of Comenius University between February 2013 and December 2017. Overall, 133 B-ALL children were included in this study and were statistically analyzed. We retrospectively identified a subgroup of 52 patients without a prognostic marker and in 18 of them we incorporated a SNP array analysis. All patients or their parents/guardians gave informed consent with enrolment in the study.

Immunophenotyping. Multiparameter flow cytometry was performed to determine a subtype of ALL blasts and measure of DNA content corresponding to ploidy (DNA index) in the blasts. DNA index (DNAI) of 1.0 is considered as normal (euploid), DNAI >1.16 defined hyperdiploidy and DNAI <0.8 denoted hypodiploidy.

Karyotype, fluorescent *in situ* hybridization (FISH), reverse transcription polymerase chain reaction (RT-PCR), multiplex ligation-dependent probe amplification (MLPA). Karyotyping was performed on at least 20 G-banded metaphase cells. Cases with less than 15 analyzable normal metaphase cells were considered inadequate. FISH analysis was performed using probe sets, including fusions *ETV6-RUNX1*, *BCR-ABL1*, *TCF3-PBX1*, *MLL* break apart. Karyotype and FISH results were interpreted according to the International System for Human Cytogenetic Nomenclature. FISH results were confirmed by RT-PCR analysis. The copy number states of commonly deleted genes (*IKZF1, CDKN2A/B, PAX5, ETV6, JAK2, BTG1, EBF1, RB1, PAR1* region) were assessed by multiplex ligation-dependent probe amplification (MLPA) on 113/133 samples using SALSA MLPA P335 by manufacturer's recommendations. MLPA data analysis was performed by Coffalyser.Net software.

SNP-array analysis. Twenty DNA samples from 18 patients (18 from diagnosis and 2 from relapse) for SNP-array analysis were extracted from frozen lymphocytes separated from the bone marrow. Isolation was carried out using a commercial isolation kit (Gentra Puregene kit, Qiagen) according to the manufacturer's recommended procedure. Genome-wide SNP array analysis was performed using the CytoScan HD microarray assay (Affymetrix/Thermo Fisher Scientific), which contains approximately 750 000 SNP-based oligonucleotide probes and 1.9 million non-polymorphic oligonucleotide probes, following manufacturer's instructions. Data were analyzed by the Affymetrix ChAS software version 3.1. All genomic positions were based on the hg19 human genome sequence build from February 2009. Results were compared to the Database of Genomic Variants containing known common copy number variation in healthy population. Copy number variations (CNV) or copy number alterations (CNA) that involved only intragenic regions or sequences not encoding protein coding genes were excluded from further analyses. The minimum number of probes was 50 for duplications and 25 for deletions. We considered a result clinically significant, when the gain/loss was within or included a clinically significant cancer-related gene, or the CNAs larger than 1 Mb outside the clinical oncology significant regions and loss of heterozygosity (CN-LOH) larger than 10 Mb.

Statistical analysis. The Overall survival (OS, %) rate was calculated from the date of diagnosis to the date of last follow-up or the date of death. Event-free survival (EFS, %) was calculated from the date of diagnosis to the date of the event. That could be therapy (non-response), relapse, secondary malignant neoplasm (SMN) or death from any cause. The Kaplan-Meier method was used to estimate survival rates. The Log-Rank test (Mantel-Cox test) was used to compare differences between groups defined by survival. Cumulative incidence curves for relapse (CIR) and deaths in complete remission were performed by the Gray test. Tests with ≤0.05 significance level indicated a statistically significant difference in the survival and cumulative incidence curves between compared groups. Analyses were carried out using XLSTAT 2014 and STATA/IC, version14.

Results

Clinical and genetic retrospective analysis. A total of 152 ALL cases were diagnosed between February 2013 and December 2017, of which 133 (87.5%) were classified as B-ALL and 19 (12.5%) as T-ALL. Based on immunophenotyping data, 9 (6.77%) cases showed early proB-ALL, 17 (12.78%) cases preB-ALL and 107 (80.45%) cases common ALL. DNAI was analyzed in 118 (88.72%) patients, of these 2 (1.69%) had a hypodiploid clone and 20 (16.95%) patients were hyperdiploid (Figure 1).

Cytogenetic results were available in 97 (72.93%) cases. In 42 (43.30%) analyzed cases karyotype was non-informative, due to reduced quality of cytogenetic preparations. The karyotype was informative in 43 (44.33%) cases and normal karyotype was found in 12 (12.37%) cases. Analyses by FISH and RT-PCR were performed in 133 (100%) of B-ALL. Recurring abnormalities with clinical relevance were observed in 81 (60.90%) patients. These abnormalities included hypodiploidy (n=3), hyperdiploidy (n=30), *ETV6-RUNX1* (n=39), *TCF3-PBX1* (n=4), *MLL* translocations (n=4), *BCR-ABL1* (n=1) (Figure 1). In 38/78 (48.72%) cases with non-informative or failed karyotype, FISH and RT-PCR identified recurrent translocation in patients. One of these methods revealed that 6/12 (50.00%) patients with normal karyotype harbored *ETV6-RUNX1* translocation and 1/12 (8.33%) had *MLL-AF10* fusion. No recurrent rearrangement was identified in 52/133 (39.10%) cases (Table 1).

MLPA was carried out in 112/133 (84.21%) of B-ALL cases. Out of 112 patients, 38 (33.93%), 33 (29.46%), 21 (18.58%), 13 (11.51%), and 7 (6.19%) carried no CNA, 1 CNA, 2-3 CNAs and >3 CNAs, respectively. This method revealed a total of 143 CNA: 106 (74.13%) deletions and 37 (25.87%) gains. The most frequently deleted genes were *ETV6* and *CDKN2A/B* occurring in 23.21% cases, further *PAX5* (14.29%), *IKZF1* (12.5%) and *JAK2* (8.93%). The highest number of deleted

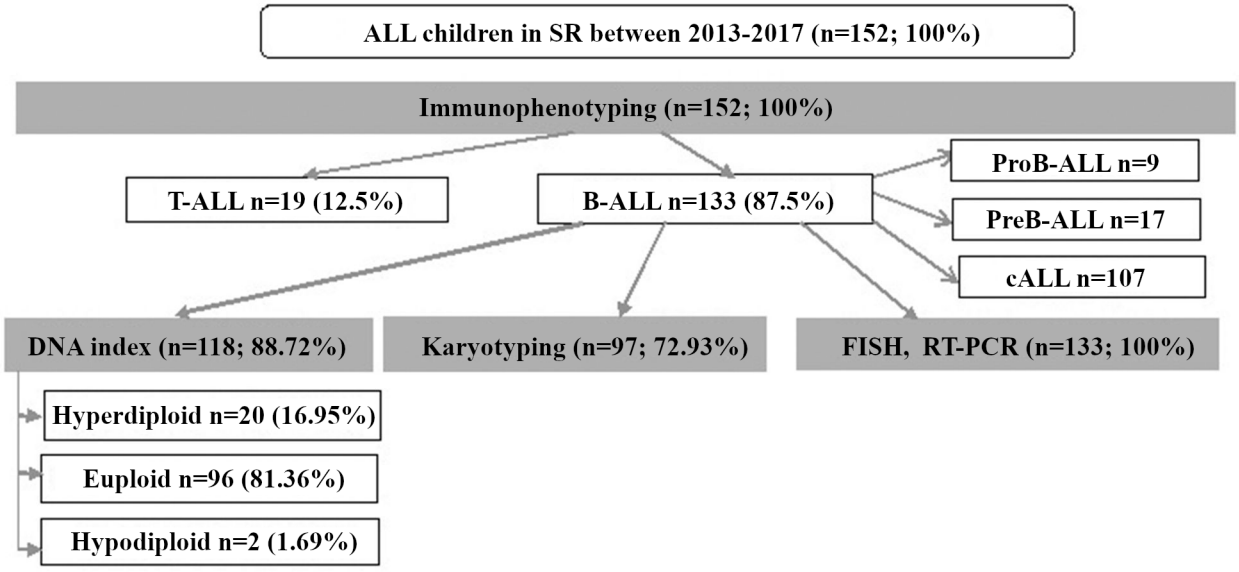

Figure 1. Review of immunophenotyping and standard genetic results of pediatric ALL patients from period 2013–2017 in Slovakia.

Table 1. Review of cytogenetic results by karyotype and FISH.

Recurrent abnormality	Whole cohort (n=133; 100%)	Informative karyotype (n=43/133; 23.33%)	Normal karyotype (n=12/133; 9.02%)	Non informative karyotype (n=42/133; 31.58%)	No karyotype (n=36/133; 27.07%)
Hypodiploid	**3 (2.26%)**	1 (2.33%)	0	1 (2.38%)	1 (2.78%)
Hyperdiploid	**30 (22.6%)**	21 (48.8%)	0	4 (9.52%)	5 (13.89%)
ETV6-RUNX1	**39 (29.3%)**	11 (25.6%)	6 (50%)	13 (30.95%)	9 (25%)
TCF3-PBX1	**4 (3.01%)**	1 (2.33%)	0	3 (7.14%)	0
MLL translocations	**4 (3.01%)**	2 (4.65%)	1 (8.33%)	0	1 (2.78%)
BCR-ABL1	**1 (0.75%)**	0	0	1 (2.38%)	0
B-other	**52 (39.10%)**	**7 (16.28%)**	**5 (41.67%)**	**20 (47.62%)**	**20 (55.56%)**

genes was observed together with ETV6-RUNX1 translocation (47/106; 44.34%) and with B-other (40/106; 37.74%) (Table 2).

Analyses of 133 B-ALL cases showed, that 73 (54.88%) patients had favorable cytogenetic features, 8 (6.02%) had unfavorable features (6.02%) and 52 (39.09%) patients were classified as B-other without a prognostically relevant cytogenetic marker. Due to the short monitoring period (4 year), the EFS and OS rates were similar for all 3 groups. Relapses occurred in the B-other group and in patients with favorable cytogenetic abnormalities. The 4-year CIR rate was 21.77% in the B-other and 17.75 % in the favorable cytogenetic group (Suppl Figure S1).

SNP array results. We retrospectively investigated 18/52 (34.62%) of B-other patients. The remaining cases could not be investigated due to the lack of DNA or poor DNA quality. Children included in this study were between 2 and 18 years old (median 7.2 years). The male/female ratio was 13:5. Based on therapeutic results the majority of analyzed patients (12/18, 66.6%) was classified into intermediate risk, 3/18 (16.6%) and 3/18 (16.6%) were classified into high and standard risk, respectively. For the whole group of 18 patients, the 5-year EFS and 3-year OS rates were 66.20% and 82.22%, respectively. The 5-year CIR of relapse was high, 39.83%.

All samples were successfully analyzed. In 17/18 (94.44%) of investigated cases CNAs were identified, only in one patient (D16) we did not identify any aberration. Our analyses revealed 126 CNAs, of which 49 (38.89%) were ≥10 Mb and 77 (61.11%) were ≤10 Mb. Overall, we identified 11 copy neutral loss of heterozygosity (CN-LOH) on chromosomes 2, 3, 4, 7, 11, 12, 13, 15, 16, 17 all of which were ≥10 Mb. The chromosomes most frequently affected by CNA were 3, 9, 11, 13, 21, and 22 (Figure 2A). Deletions were more common (67.7%) than gains (31.4%) (Figure 2B). The median number of CNAs per case was 6 (mean 5.48, range 1–11) (Figure 2C).

Clinically relevant aberrations with prognostic and therapeutic implications were found in 4/18 (22.22%) cases: masked hypodiploidy (1/4), intrachromosomal amplification of chromosome 21 (iAMP21) (1/4) and IKZF1^{PLUS} (2/4).

The first case (D1) had a non-informative karyotype and FISH analysis identified trisomy of chromosome 22 (45% interphase cells) and tetrasomy of chromosomes 11 and 21 (65% and 50% interphase cells). At diagnosis time, SNP array revealed copy neutral loss of heterozygosity (CN-LOH) of chromosomes 2, 3, 4, 7, 12, 13, 15, 16, 17 in addition to that tetrasomy of chromosomes 1, 6, 8, 11, 19, 21 and trisomy 5, 10, 14, 18, 20, 22 (Figure 3). This genomic profile was consistent with duplicated low hypodiploid clone bearing 37 chromosomes or masked hypodiploid. In this case SNP-array detected also 3 CNA including loss of *IKZF2* in 2q34.1 (156 kbp), *SSBP2* gene in 5q14.1 (215 kbp) and *RB1*gene in 13q14.2 (236 kbp). SNP-array performed at relapse showed CN-LOH of chromosomes 2, 3, 7, 8, 10, 12, 13, 15, 16, 17 (Figure 2). Deletions of *IKZF2*, *SSBP2* and *RB1* were also present at relapse.

The second case (D2) had a failed karyotype. FISH analysis showed amplification of the *RUNX1* signal ranging from 5–10 copies (67.5% interphase cells). SNP-array identified intrachromosomal segmental amplifications across the whole chromosome 21, a known recurrent abnormality in B-ALL often associated with inferior outcome. The degree of amplification was not even across the region (Figure 4). The highest level of amplification was a 9764 kbp region containing 5 genes: *TIAM1, RUNX1, DYRK1A, ERG*, *ETS2*. A deletion spanning 2971 kbp region including *MIR125B2* gene was observed at the proximal site of chromosome 21.

SNP array also revealed aberrations involving other chromosomes. Chromosome 7 showed an 18.9 Mb interstitial deletion from 7q31.31 to 7q34 involving genes *CREB3L2, TTC26, POT1, NRF1, ZC3HAV1, SPAM1, TRIM24, HIPK2* and focal loss in gene *IKZF1* in size 56 kbp. There was a loss of a 270 kbp region on chromosome 9p13.2 involving gene *PAX5.* Chromosome 12 showed loss of a 957 kbp long interstitial segment from 12p13.2 to 12p13.1 that included genes *CDKN1B, BCL2L14, ETV6*, and terminal copy neutral loss heterozygosity (CN-LOH) from 12q14.1 to 12q24.33 (71 Mb). All of these secondary aberrations had revealed at both diagnosis and relapse time. At relapse, additional

Table 2. Review of genetic results by MLPA.

Deleted genes	Whole cohort (n=112; 100%)	Hypodiploid (n=2/3; 66.67%)	*MLL*-translocations (n=4/4; 100%)	Hyperdiploid (n=21/30; 70%)	*ETV6- RUNX1* (n=36/39; 92.31%)	*TCF3-PBX1* (n=3/4; 75%)	B-other (46/52; 88.46%)
IKZF1	**14 (12.5%)**	0	0	2/21 (9.52%)	2/36 (5.56%)	0	10/46 (21.74%)
CDKN2A/B	**26 (23.21%)**	2/2 (100%)	1/4 (25%)	1/21 (4.76%)	10/36 (27.78%)	2/3 (66.66%)	10/46 (21.74%)
PAX5	**16 (14.29%)**	0	1/4 (25%)	1/21 (4.76%)	5/36 (13.89%)	2/3 (66.66 %)	7/46 (15.22%)
ETV6	**26 (23.21%)**	0	0	0	21/36 (58.33%)	0	5/46 (10.87%)
EBF1	**1 (0.89%)**	0	0	0	0	0	1/46 (2.17%)
JAK2ex23	**10 (8.93%)**	1/2 (50%)	0	1/21 (4.76%)	5/36 (13.89%)	2/3 (66.66%)	1/46 (2.17%)
BTG1	**3 (2.68%)**	0	0	0	2/36 (5.56%)	0	1/46 (2.17%)
RB1	**6 (5.36%)**	0	0	1/21 (4.76%)	1/36 (2.78%)	1/3 (33.33%)	3/46 (6.52%)
PAR1 region	**4 (3.57%)**	0	0	0	1/36 (2.78%)	0	2/46 (4.35%)
NEGAT MLPA	**38/112 (33.93%)**	0/2 (0%)	2/4 (50%)	5/21 (23.81%)	8/36 (22.22%)	1/3 (33.3%)	22/46 (47.83%)
Total n. of deletion	**106**	**3**	**2**	**6**	**47**	**7**	**40**

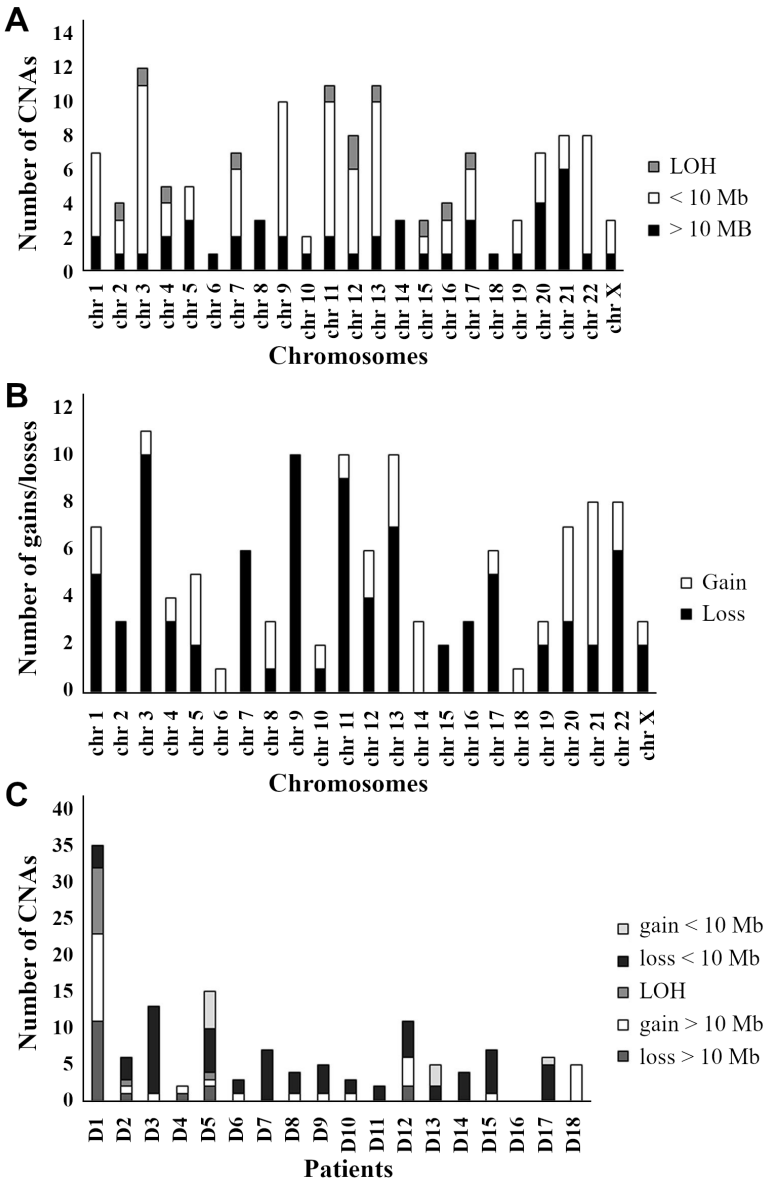

Figure 2. Number and distribution of CNAs. A) Distribution of all copy number alterations based on size. Smaller CNAs (<10 Mb) were more common than larger (>10 Mb). B) Distribution of losses and gains on chromosomes. C) Number and distribution of CNAs (gain, losses) and CN-LOH per patient in cohort at diagnosis.

segment deletion (1 172 kbp) developed on chromosome 12q24.11 within CN-LOH segment. This deletion included gene *SH2B3*.

Six cases (33.33%) had chromosomal abnormalities that did not represent the major recurring primary abnormalities. Among these cases, altogether 41 CNAs were identified with range of 2 to 14 CNAs per case, 7 CNAs in average. These included 13 chromosome gains (trisomy and tetrasomy of chromosome 21, trisomy of chromosome 5, partial trisomy of chromosome 14 and 20) and 5 segmental chromosome losses larger than 10 Mb (1p22.3-p21.3, 9p24.3-p13.2, 13q12.3-q34, 17p13.3-p11.2, 20q13.13-q13.33). Three of six cases had smaller deletion (<10 Mb) within 9p, 2/3 cases had heterozygous partial losses of *PAX5* and 1/3 case had heterozygous deletion of *CDKN2A/B*. In addition to the CNAs, one case had CN-LOH on chromosome 11 (18 Mb) (Suppl. Table S1).

Ten cases (55.55%) had a normal karyotype without gross chromosomal abnormalities identified. Nine cases had small (<10 Mb) CNAs. Total of 45 CNAs were revealed, ranging from 2 to 12 per case, 4 CNAs in average. Out of 45 CNAs, 41 (91.11%) were losses and 4 (8.88%) gains. Four (44.44%) of the nine cases had focal loss of gene *VPREB1*. The second

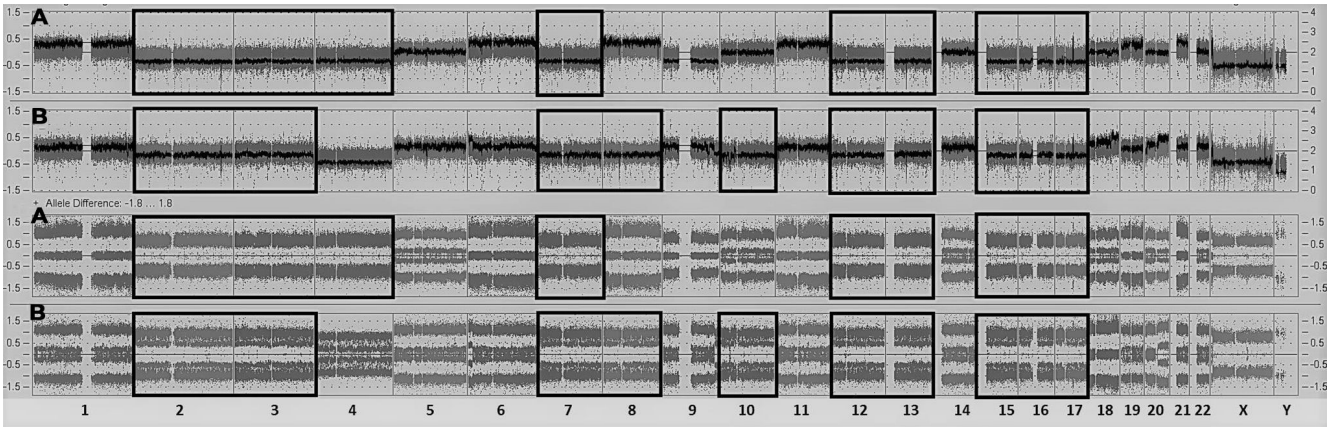

Figure 3. Masked low hypodiploidy in case D1. A) Sample at diagnosis, CN-LOH of chromosomes 2, 3, 4, 7, 12, 13, 15, 16, 17 (in black frames). B) Sample at relapse, CN-LOH of chromosomes 2, 3, 7, 8, 10, 12, 13, 15, 16, 17 (in black frames).

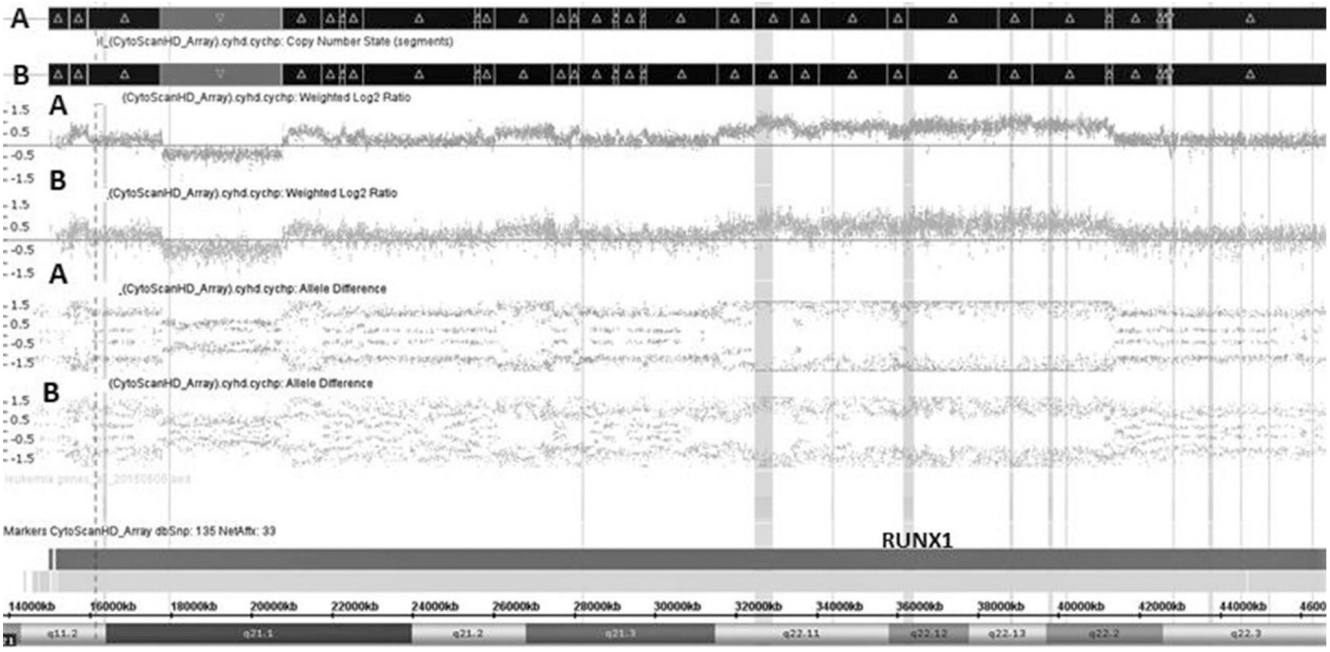

Figure 4. Intrachromosomal amplification of chromosome 21 at the time of diagnosis (A) and relapse (B) by SNP array.

most frequent loss included *IKZF1* (3/9; 33.33%), followed by loss of *CDKN2A/B* (2/9; 22.22%), *PAX5* (2/9; 22.22%) and deletion of PAR1 region (2/9; 22.22%) (Suppl Table S2). In this group of patients with normal karyotype, we identified 2 patients belonging to a recently described prognostic entity, IKZF1^{PLUS}. Patient D3 had a deletion of *IKZF1* with deletion of *PAX5* in the absence of *ERG* deletion. Patient D14 had also *IKZF1* deletion that co-occurred with deletion of *PAX5* and PAR1 region.

Discussion

Despite contemporary cure rates approaching 90%, ALL remains an important cause of morbidity and mortality in children. In the past decade, genomic investigations have revealed substantial new information about the genetic basis of leukemogenesis and treatment responsiveness [9]. Many of these findings have been implemented into clinical management of ALL. Genetic abnormalities detected by standard cytogenetic (karyotyping, FISH) and molecular (PCR) methods divide B-ALL into well-established genetic subtypes. This genetic classification is essential in prediction of prognosis and thus used for the risk stratification for therapy [10].

The first goal of our study was to review genetic results of patients from 2013–2017 and determine the effectiveness of routine genetic diagnostic methods. In B-ALL, approximately 25–30% of cases do not have any clinically relevant

abnormalities by routine cytogenetic analysis [3, 5, 11]. In this study, we found 60.9% of B-ALL patients with clinically relevant abnormalities by chromosome banding and/or by FISH analysis. The karyotype was informative only in 41.35% cases. Chromosome banding analysis has failed in 58.64% cases. In 27.07% it was not possible to perform cytogenetic analysis and in 31.58% the karyotype did not reveal chromosomal abnormalities. This high percentage of karyotype failure occurred because many cases did not have actively dividing cells or the metaphase cells were from poor material. Out of 133 B-ALL cases, about 9% of patients showed a normal karyotype. By FISH and RT-PCR, we found that 68.33% of cases with normal karyotype had either *ETV6-RUNX1* fusions or *MLL* translocation. The reason could be that some abnormalities are cryptic to metaphase analysis, because the limit of resolution is low (approximately 8–10 Mb). In addition, normal karyotype or informative karyotypes may still contain various submicroscopic changes. However, FISH method has higher resolution (100–300 kbp) and allows detection of cryptic changes, such as specific translocation, duplications or deletions. In our study, using FISH and RT-PCR methods, the detection rate for clinically relevant abnormalities increased from 32.33% to 60.9%. According to literature, this result is slightly lower, typically the detection rate is around 75% by using the abovementioned methods [3, 5, 11]. In our cohort nearly 40% of patients are B-other. This group of patients is heterogeneous with failed karyotypes, normal karyotypes and aberrations of unknown significance [12–15]. Out of 52 B-other, 76.92% had failed karyotype, 9.62% had normal karyotype and 13.46% had chromosomal aberration without clinical significance.

In contrast to gross chromosomal abnormalities, subchromosomal copy number alterations (CNAs) are usually cooperating aberrations that correlate with specific cytogenetic subtypes [8, 12, 16–18]. MLPA analysis allowed us to detect the 9 most commonly deleted genes involved in B-cell development, cell cycle control and hematopoiesis. Out of 112 analyzed patients by MLPA, 34% had no CNA alterations in any of these 9 genes, 29% of the patients harbored at least 1 CNA, whereas 18% and 11% of the cases showed 2 or 3 CNAs, respectively. The most commonly deleted genes were *ETV6* and *CDKN2A/B*, with deletions identified also in *PAX5*, *IKZF1* and *JAK2*. These results agree with the findings published by Singh *et al.* [13]. The highest number of CNAs observed were in the group of patients with *ETV6-RUNX1* fusion (45/106; 42.5%) and in B-other cases (40/106; 37.7%). Schwab *et al.* noted similar findings and suggested that CNAs are needed to induce leukemia onset [12]. The main disadvantages of this method is that it is not a suitable method to detect new prognostic markers and results may not be accurate if the sample from which the DNA was derived contained less than 50% blasts.

Our statistical data showed that the relapse rate (21.77%) was the highest in B-other group, followed by good risk cytogenetic group (17.75%). In contrast, patients with poor risk cytogenetics had the best outcomes. Nevertheless, longer follow-up is needed to determine the outcome of these 3 groups in our cohort more precisely, but these results indicate that the recurrent cytogenetic subgroups fail to demonstrate complete concordance with patients' outcome, and thus additional genetic markers are needed in risk stratification.

Due to the limitations of the previous methods used in diagnosis and prognosis of ALL in Slovakia, we implemented SNP-array method to improve diagnostic, prognostic and treatment strategies. In contrast to conventional diagnostic techniques, SNP-array is not dependent on the mitotically dividing cells and requires only a small amount of DNA. The main advantages of this method are high resolution, sensitivity, accuracy and capability of simultaneous detection of amplifications, deletions and copy neural losses of heterozygosity (CN-LOH) across the whole genome. CN-LOH affect regions containing tumor suppressor genes or oncogenes with mutations, microlesions, aberrantly methylated patterns that potentially confer positive selection of the leukemic clone and influence ALL development. It is defined as the loss of one allele at a given position caused by deletion, which was subsequently replaced by the same region from a homologous chromosome without the copy number changes. SNP-array is perfect method for the identification of large regions of homozygosity [19].

We analyzed only 18 (34.62%) B-other ALL patients using SNP-array, which is due to the retrospective nature of our pilot study. In this study group the rate of relapse was high, approximately 40%. Previous studies have shown that many of B-other cases harbor CNAs of prognostic relevance [14–18]. Altogether, we identified 126 CNA including 39% large aneuploidy changes and 61% subchromosomal changes. Deletions were more common (68%) than gains (32%). The median number of CNA per case was 6, which is consistent with published results by Mullighan *et al.* [18].

In 4/18 (22.2%) cases SNP-array revealed clinically relevant makers of poor prognosis. In the first case DNA index (1.4) and FISH results (trisomy of 22, tetrasomy of 11 and 21) suggest prognostically favorable hyperdiploidy. However, hyperdipoid cases are typically characterized by non-random gain of specific chromosomes, mostly trisomies of chromosomes X, 4, 6, 10, 14, 17, 18 and 21 (often may also be tetrasomic) [20]. In our case SNP-array revealed CN-LOH of chromosomes 2, 3, 4, 7, 12, 13, 15, 16, 17, normal diploid chromosome 9, gained X and Y chromosomes, trisomy of chromosomes 5, 10, 14, 18, 20, 22 and tetrasomy of chromosomes 1, 6, 8, 11, 19, 21. Besides aneuploid changes we identified loss of *IKZF2*, *SSBP2* and *RB1* genes. Hypodiploid clones can undergo endoreduplication of their entire chromosome complement, which leads to a secondary hyperdiploid clone. The hyperdiploid clone may be the predominant clone identified, hereby masking the presence of a hypodiploid karyotype [21, 22]. Masked hypodiploidy may be recognized based on gained chromosomes that are represented by heterozygous tetrasomies and other chromosomes

show CN-LOH. In contrast to hyperdiploidy, which harbor primarily heterozygous trisomies without CN-LOH [23]. Holmfeld *et al.* analyzed 8 masked low hypodiploid cases by SNP-array and demonstrated that masked low hypodiploidy displays characteristic patterns of chromosomal CN-LOH and gains, and showed that the majority of low hypodiploid ALL cases harbor TP53 alterations (91.2%), alterations of the lymphoid transcription factor IKZF2 (52.9%) and a high frequency (41.2%) of RB1 alterations [24]. Low hypodiploidy is found in less than 1% of B-ALL. Patients usually have poor prognosis with extremely poor 3-year EFS rate of 30% [21, 23, 24]. Case D1 after 2 years experienced hematological and CNS relapse and deceased shortly. Our SNP-array data indicate that this patient D1 had masked low hypodiploidy. The relapse clone was very similar to the original leukemia with additional changes including monosomy of chromosome 4 and CN-LOH on chromosome 10. *IKZF2* and *RB1* deletions were found at diagnosis and also at relapse. We did not identify additional affected genes.

In the second case (D2) we identified intrachromosomal amplification of chromosome 21 (iAMP21). This is a new genetic subgroup of the 2016 revised WHO classification accounting for about 2% of B-ALL cases and has been associated with poor outcome and high relapse rate when treated with standard therapy [22]. This finding may be also detected by FISH by using specific *RUNX1* probes. Five or more *RUNX1* signals per interphase cells define iAMP21, on SNP-array the structurally abnormal chromosome 21 with amplifications and deletions is typically observed [25]. Patients with iAMP21 display a unique spectrum of secondary genetic abnormalities, likely contributing to heterogeneous outcomes of these patients, which also may by revealed by SNP array [26–28]. Case D2 relapsed 3 years after the time of diagnosis and deceased shortly. We found following secondary abnormalities, which were identified also in relapsed sample: loss of chromosome 7q, deletion of *IKZF1*, *PAX5*, *ETV6* and CN-LOH on chromosome 12q. In relapse sample we identified an additional deletion of *SH2B3*. Baughn *et al.* suggested that aberrations including *SH2B3* gene are enriched in this subtype and may contribute to disease progression. It raises the possibility that these patients may be sensitive to tyrosine kinase inhibitors [28].

In 6 patients we found an atypical karyotype, where the most common deleted genes were: *CDKN2A/B*, *PAX5*, *VPREB1* and *RB1*. In patients with a normal karyotype, *IKZF1, CDKN2A/B, PAR1* region, *VPREB1* and *ETV6* are often deleted. The deletions of the *CDKN2A/B* and *VPREB1* gene occurred in patients with atypical and normal karyotype. Deletion of *IKZF1* was previously associated with poor prognosis. The frequency of IKZF1 deletions among B-other leukemia ranges from 15% to 20% [29]. Studies demonstrated that co-deletion of both *IKZF1* and *ERG* is associated with favorable outcome [30, 31]. Deletion of *VPREB1* was common in our study. Mangum *et al.* reported that high risk patient with deletion of *VPREB1* tend to have poorer survival [32]. In 2 patients with normal karyotype, we identified a new prognostic marker recently described: $IKZF1^{PLUS}$, defined as deletion of *IKZF1* with concurrent deletion of either *PAX5*, *CDKN2A/B* or the PAR1 region in the absence of *ERG* deletion. This subgroup accounts for about 6% of B-ALL cases and has very poor clinical outcome: 5-year EFS rates are low at around 50% and 5-year CIR 45%. The poor prognostic effect of $IKZF1^{PLUS}$ is restricted to those patients with MRD at least $10E^{-4}$ after induction treatment [33].

Our findings suggest, that integrating SNP-array among routine diagnostic methods provides more complete genetic characterization of leukemic clone and allows identification of clinically relevant primary and also secondary abnormalities that contribute to heterogeneity in patients' outcomes. In the near future, we plan to continue our study prospectively and expand our sample set. Identified genomic alterations we plan to use in clinical practice to improve diagnostic strategies and personalized treatment, which can improve prognosis of children with B-other ALL in Slovakia.

Supplementary information is available in the online version of the paper.

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