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Minimal residual disease detection using real-time quantitative PCR analysis of immunoglobulin and T-cell receptor gene rearrangements in the non-MRD-based ALL IC-BFM 2002 protocol for childhood ALL: Slovak experience.

A. KOLENOVA[1], I. HIKKEL[2], D. ILENCIKOVA[3], M. HIKKELOVA[2], D. SEJNOVA[1], E. KAISEROVA[1], A. CIZMAR[1], J. PUSKACOVA[1], E. BUBANSKA[4], I. ORAVKINOVA[5], M. GENCIK[2]

[1]Department of Pediatric Hematology and Oncology, Comenius University – Medical School and University Children's Hospital, Limbova 1, 833 40 Bratislava, Slovakia, e-mail: sasa.kolenova2@gmail.com [2]Laboratory of Medical Genetics, Medgene, Bratislava, Slovakia, [3]Department of Oncology and Genetics, National Institute of Oncology, Bratislava, Slovakia, [4]Department of Pediatric Hematology and Oncology, Children's Hospital, Banská Bystrica, Slovakia, [5]Department of Pediatric Hematology and Oncology, Children's Hospital, Košice, Slovakia

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Acute lymphoblastic leukemia is the most common form of cancer in children. The 10-year event-free survival ranged from 77 to 85% after having achieved complete remission rates of 93% or higher. The main cause of treatment failure is relapse arising from outgrowth of residual leukemic cells that are refractory to therapy. An intense effort has been made to develop methods to determine the degree of minimal residual leukemia cells present in patients considered to be in morphological remission. Because of the strong correlation between minimal residual disease (MRD) levels and risk of relapse, monitoring of MRD provides unique information regarding treatment response. The MRD monitoring based on real-time quantitative PCR detection of patient-specific immunoglobulin and T-cell receptor (Ig/TCR) gene rearrangements is currently considered to be the most reliable tool for MRD-based diagnosis in ALL. Because the significance of MRD monitoring has been strongly supported by several studies and because it has been implemented in the latest protocols, there has been a significant effort to develop MRD monitoring in the Slovak Republic since 2005. Between October 2006 and December 2009, 50 children with ALL who were treated at three Slovak centers were included in the RQ PCR MRD pilot project. A total of 40 patients with BCP-ALL (B cell precursor ALL) and 4 patients with T ALL were analyzed for Ig/TCR rearrangement. We identified 106 different rearrangements in the 44 ALL patients analyzed. Based on MRD stratification, we identified 26 patients who were stratified into the HRG (high risk group) (n = 3; 11.5%), IRG (intermediate risk group) (n = 14; 54%) and SRG) standard risk group) (n = 9; 34.5%). Morphology-based risk stratification allows the identification of most HRG patients identified also by MRD-based stratification, but fails to discriminate the IRG assigned to therapy reduction. Patients in the SRG and the IRG could profit from MRD-based risk assignment.

Key words: pediatric acute lymphoblastic leukemia, minimal residual disease, immunoglobulin and T cell receptor gene rearrangements -

Acute lymphoblastic leukemia (ALL) is the most common form of cancer in children, accounting for approximately 25% of all childhood cancers and about 80% of childhood leukemia. The treatment of childhood ALL is one of the true success stories of clinical oncology [1, 2]. In 2000, the treatment results of childhood ALL trials performed in the early 1990s by major study groups were uniformly presented. The 10-year event-free survival ranged from 77 to 85% after having achieved complete remission rates of 93% or higher [3, 4, 5, 6]. The main cause of treatment failure, which occurs in approximately 20% of patients, is relapse arising from outgrowth of residual leukemic cells that are refractory to therapy.

Over the last 2-3 decades, an intense effort has been made to develop methods to determine the degree of residual leukemia

Abbreviations: D33P – MRD positivity at day 33; D33N – MRD negativity at day 33; D78P – MRD positivity at day 78; D78N – MRD negativity at day 78; D15 – day 15; PGR – prednisone good responder; PPR – prednison poor responder; BM – bone marrow; IP – immunopheotype; WBC – white blood cell count (μl^{-1})

cells present in patients considered to be in morphological remission (i.e., to measure minimal residual disease). These efforts have resulted in assays that have a much greater sensitivity (100 times and more) than morphological assays [7, 8].

In pediatric ALL, the most useful minimal residual disease (MRD) detection methods are based on polymerase chain reaction (PCR) of antigen receptor genes and on flow cytometric detection of abnormal immunophenotypes.

Many studies have shown the prognostic importance of MRD as detected by flow cytometry [9, 10, 11]. Furthermore, studies of MRD by PCR have clearly demonstrated its prognostic importance [12, 13, 14]. Because of the strong correlation between MRD levels and risk of relapse, monitoring of MRD provides unique information regarding treatment response. Moreover, MRD has become a crucial component of contemporary treatment protocols in children with ALL [8]. The MRD monitoring performed in the AIEOP BFM 2000 study was based on real-time quantitative PCR detection of patient-specific immunoglobulin and T-cell receptor (Ig/TCR) gene rearrangements as markers of residual leukemic cells. This method is currently considered to be the most reliable tool for MRD-based diagnosis in ALL [15, 14].

Children with ALL in Slovakia are treated according to the ALL IC-BFM 2002 protocol, which was designed for countries in the I-BFM study group who, in 2002, were not able to apply routine MRD testing in clinical practice. Slovakia was one of the countries where risk-group stratification had not being performed with routine flow cytometry or PCR-based MRD. In the ALL IC-BFM 2002 study, the stratification into standard risk (SRG), intermediate risk (IRG) and high risk (HRG) groups was comprised of the response to a 7-day prednisone regimen and one treatment of intrathecal methotrexate, bone marrow (BM) morphology at days 15 and 33, the patient's age, white blood cell (WBC) count and the presence of BCR/ABL or MLL/AF4. A slow response to treatment detected as M3 BM at day 15 restratified patients to higher risk groups. The study asked the question whether it was possible to avoid MRD testing in some groups of patients. The answer to this question using data from the ALL IC BFM 2002 study was reported by [16].

However, as the results of several studies, morphology-based methods to assess treatment response are neither precise enough nor sensitive enough to measure the cytoreductive reliability [17]. Because the significance of MRD monitoring has been strongly supported by several studies and because it has been implemented in the latest protocols, there has been a significant effort to develop MRD monitoring in the Slovak Republic since 2005.

The aims of our study were to: 1) set up and monitor MRD among children with ALL using real-time quantitative PCR analysis of immunoglobulin and T-cell receptor gene rearrangements; 2) show that this time-consuming, logistically demanding and relatively expensive method can be applicable in Slovakia and that the results are within the European standard and 3) compare stratification into risk groups based on our non-MRD prognostic factors to the MRD-based stratification criteria.

In addition, we present a feasibility study of the standard strategy for the identification of Ig/TCR targets for MRD diagnostics in ALL pediatric patients. For this study, we identified the Ig/TCR gene rearrangement pattern using standard primer sets and protocols.

Materials and methods

Patients. Between October 2006 and December 2009, 50 children with ALL (age 1-18 years, median 4.1 years) who were treated at three Slovak centers [Bratislava (n = 27), Košice (n = 6) and Banská Bystrica (n = 17)] were included in this MRD pilot project. All children were treated according to the ALL IC-BFM 2002 protocol. The cohort of patients consisted of 16 girls and 34 boys. The MRD pilot project was approved by the local ethics committee, and all children or their parents gave informed consent to participate in the study.

Diagnostic tools. Diagnosis of ALL was based on the French-American-British classification and flow cytometric immunophenotyping using a standard set of monoclonal antibodies according to the European Group for Immunological Characterization of Leukemia (18). FISH and PCR screening for fusion genes TEL/AML1, BCR/ABL and MLL/AF4 were routinely performed on samples from each patient.

Treatment. The elements of this treatment protocol were previously published [16]. According to the protocol, the patients received a 7-day prednisone regimen with one treatment of intrathecal methotrexate, followed by an 8-week induction therapy consisting of eight agents.

For SR and IR, the consolidation phase was comprised of four courses of high-dose methotrexate (2 g/m^2) pre B-ALL is exact term for this B-ALL with IgM in cytoplasm (CD10-negat., membrane **K/λ** negat, CD19, CD20 and CD22-posit., cyt IgM-posit.), otherwise use terms B lymphoblastic AL and T lymphoblastic AL for pre B-ALL and 5 g/m^2 for T-ALL. The reinduction phase was randomized such that SRG patients received one treatment of protocol II or two treatments of protocol III. IRG patients were randomized to receive one treatment of protocol II or three treatments of protocol III.

HRG patients received three treatments of protocol III , six treatments of protocol III plus one treatment of protocol II or three treatments of protocol III and two treatments of protocol II. Maintenance therapy with a total treatment duration of 24 months consisted of daily mercaptopurine and a weekly treatment with methotrexate (Figure 1).

Response to treatment was evaluated according to a non-MRD-based protocol with cytomorphology. BM was classified as M1 (less than 5% blasts), M2 (5-24% blasts), or M3 (over 25% blasts) using standard morphological criteria. The bone marrow sampling for MRD monitoring was performed at the end of induction phase 1 (day 33) and preconsolidation at week 12 (day 78). Out of 50 children enrolled in the study, 26 patients were completely analyzed for MRD. Twelve patients

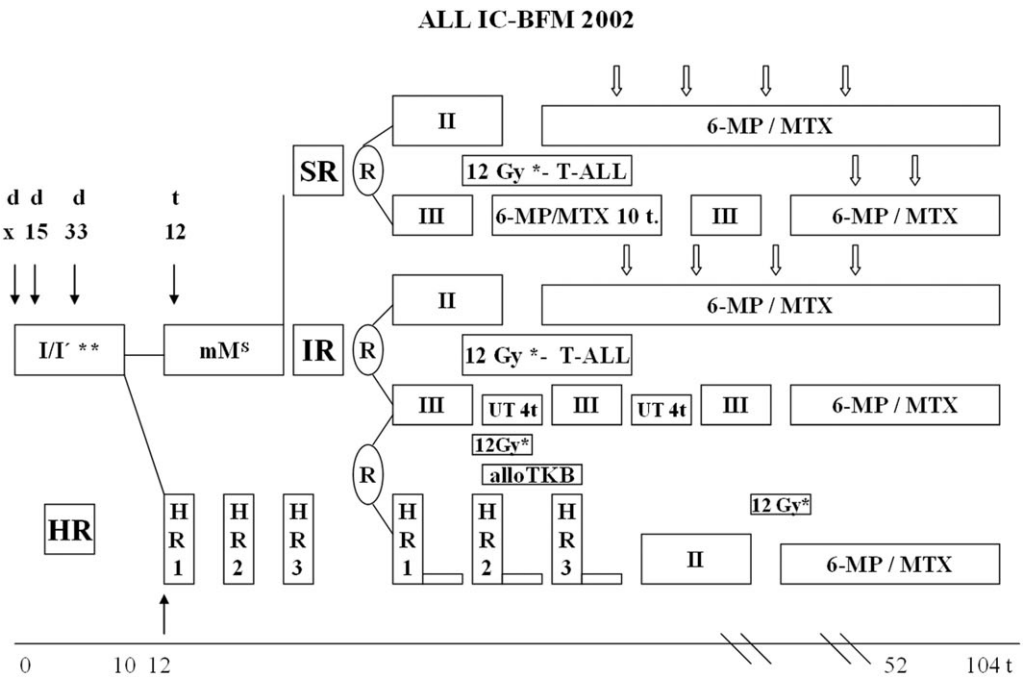

Figure 1: ALL IC BFM 2002 protocol scheme (non MRD based protocol)
(SR – standard risk, IR- intermediate risk, HR- high risk, 6-MP – merkaptopurin, MTX – metotrexat, d – day of bone marrow puncture), t- week)

were excluded because of poor quality DNA samples or missing diagnostic or follow-up DNA samples, and two patients were excluded due to early death.

Material and DNA isolation. Bone marrow samples were obtained before the commencement of treatment and at two follow-up time points (days 33 and 78). High molecular weight DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Samples with at least $1x10^7$ mononuclear cells at diagnosis and above $5x10^6$ mononuclear cells for the two follow-up samples were required for reliable MRD analysis.

Detection of Ig/TCR gene rearrangements. Detection of Ig/TCR rearrangements at the time of diagnosis was performed using PCR screening and examination of the obtained PCR products by heteroduplex analysis. Complete and incomplete *IGH* rearrangements were detected using six V_H and seven D_H primers in combination with one J_H consensus primer [7, 19]. At the time of diagnosis, genomic DNA samples were also screened for *IGK* deleting element rearrangements, *TCRG* rearrangements, complete and incomplete *TCRD* rearrangements and for the common V**δ**2-Jα29 rearrangement [20, 21]. Amplification was performed in a 20-μl reaction volume containing 50 ng of genomic DNA, 200 μM of dNTP, 10 pmol of the 5' and 3' oligonucleotide primers, 1.5 mM $MgCl_2$ and 1U Thermo-Start Taq DNA polymerase (Thermo Fisher Scientific, ABgene, Epsom, UK). The cycling conditions were as follows: initial denaturizing, 15 minutes at 95 °C, followed by 40 cycles of 45 seconds at 94 °C, 90 seconds at 60 °C and 2 minutes at 72 °C. After the last cycle, an additional extension step of 10 minutes at 72 °C was performed. The obtained PCR products were further examined using a standard heteroduplex analysis to discriminate between monoclonal and polyclonal PCR products derived from clonal leukemic cells and polyclonal reactive lymphocytes, respectively. Briefly, the PCR products were denaturized (5 minutes at 94 °C) and subsequently renaturated (1 hour at 4 °C) to allow duplex formation. The samples were immediately loaded onto 6% non-denaturizing polyacrylamide gel, run in 0.5 × TBE buffer, and stained with ethidium bromide.

Identification of rearrangements and design of allele-specific oligonucleotides. Monoclonal PCR products were processed with enzymatic treatment to remove unincorporated nucleotides and primers. The treated PCR products were sequenced in both directions using the PCR primers, a BigDye Terminator v1.1 Cycle Sequencing Kit and an ABI PRISM 3100-*Avant* Genetic Analyzer (Applied Biosystems, Foster City, USA). Enzymatic treatments were performed in 20 μl volume containing 10 μl PCR product, 2 U Exonuclease I (Fermentas GmbH, Opelstrasse, Germany) and 1.5 U Shrimp alkaline phosphatase (Fermentas, Opelstrasse, Germany) at 37 °C for 1 hour, followed by inactivation of enzymes at 80 °C for 20 minutes. Alternatively, the bands after heteroduplex analysis were excised from polyacrylamide gel, eluted and directly sequenced as described above. The identification of rearranged genes was performed by comparison of the obtained sequences with

germline sequences available in the databases IMGT (http://imgt.cines.fr; European Bioinformatics Institute, Montpellier, France) and VBASE (http://www.mrccpe.cam.ac.uk; Center for Protein Engineering, Cambridge, UK). Homology between the sequences was analyzed with the Geneious Pro 4.5.5 software (Biomatters Ltd., Auckland, New Zealand). The allele-specific oligonucleotide (ASO) primers were designed to be complementary to the junctional region of each target either manually or using the Primer Express software (Applied Biosystems, Foster City, USA).

MRD PCR analysis and data interpretations. The real-time quantitative PCR analysis of clonal rearrangements relied on the ASO primer approach. The forward or reverse (in the case of the V**δ**2-Jα29 rearrangement) ASO primer was combined with a consensus TaqMan probe and a consensus reverse or forward primer [19, 20; 21, 22]. The PCR reaction was performed using ABsolute QPCR ROX Mix (Thermo Fisher Scientific, ABgene, Epsom, UK) and a StepOne Real-Time PCR Instrument (Applied Biosystems, Foster City, USA) at standard annealing temperature (60°C). The experimental setup, data analysis and interpretation of MRD PCR results were performed in accordance with the guidelines of the European Study Group on MRD detection in ALL [21].

Statistical analyses. The Mann-Whitney or Kruskal-Wallis tests and Fisher's exact test were used to estimate significant differences between groups concerning MRD values or MRD positivity and negativity. All statistical analyses were performed using InStat version 3.06 (GraphPad Software, La Jolla, CA, USA).

Table 1 Clinical and biological characteristics of the patients at the time of diagnosis

	N	%
All patients	50	100
Age at diagnosis	1-18, m 4,1 y.	
1-6 years	33	66%
6-18 years	17	34%
Gender		
male	34	68%
female	16	32%
Center		
Bratislava	27	54%
Košice	6	12%
Banská Bystrica	17	34%
White blood cell count at diagnosis	1300-750000 m 10 400(ul/l)	
Less than 20 000/ul	30	60%
20 000- 100 000/ul	12	24%
more than 100 000 /ul	8	16%
Immunofenotyp		
CD 10 + BCP ALL	31	62%
CD 10 – BCP ALL	14	28%
T ALL	5	10%
Genetic features		
TEL/AML 1 pozit	9	18%
Hyperdiploidy	9	18%
Hypodiploidy	0	0%
MLL/AF 4	3	0,6%
Other	12	24%
Normálny karyotyp	17	34%

Results

From October 2006 to December 2009, 83 children were diagnosed with ALL in Slovakia. We obtained samples from 50 patients for the detection of clonal immunoglobulin and T-cell receptor gene rearrangements and for the monitoring of MRD.

Children included in this study were at between 1 and 18 years old (median 4.1 years). Of the 50 patients, 16 were girls and 34 were boys. Patients were treated at three centers according to the ALL IC BFM 2002 protocol [Bratislava (n = 27; 54%), Košice (n = 6; 12%), and Banská Bystrica (n = 17; 34%)] (Figure 1).

Of the 50 patients, 27 (60%) had an initial WBC count less than 20,000 μl^{-1}, 12 (24%) had between 20,000 μl^{-1} and 100,000 μl^{-1} and 8 children (16%) had more than 100,000 μl^{-1} WBCs at the time of diagnosis.

Immunophenotyping resulted in T-ALL in 5 patients (10%), CD 10 positive BCP-ALL in 31 (62%) and CD10 negative BCP-ALL in 14 patients (28%). Considering genetic features, 9 patients (18%) had TEL/AML 1, 9 (18%) had hyperdiploidy, 3 (6%) had an MLL rearrangement, 17 (34%) had normal karyotypes and 12 patients (24%) had other genetic features (Table 1).

A total of 40 patients with BCP-ALL (Figure 2) and 4 patients with T ALL (Figure 3) were analyzed for Ig/TCR rearrangement. Insufficient material for rearrangement analysis was in five cases of BCP-ALL and in one case of T-ALL. We identified 106 different rearrangements in the 44 ALL patients analyzed.

At least one Ig/TCR target was found in 92.5% of the analyzed BCP-ALL patients, and two or more rearrangements were found in 87.5% of the patients. The majority of recombination events were represented by an IGH rearrangement (in 95% of patients), with the majority being complete VH-JH joining. An incomplete DH-JH rearrangement was detected in four patients (10%). IGK-KDE rearrangements (V**κ**-KDE, intron-KDE) were detected in 62% of patients, TCRG rearrangements (V**γ**-J**γ**) were detected at a frequency of 55% and incomplete rearrangements in the TCRD/A region (V**δ**2-D**δ**3, D**δ**2-D**δ**3, V**δ**2-Jα29) was found in 68.5% of the BCP-ALL patients.

In four of the five analyzed patients with T ALL, at least one rearrangement was detected (Figure 3). The most frequent rearrangement in T ALL patients was represented by TCRG rearrangement (V**γ**-J**γ**) and was detected in two cases, the

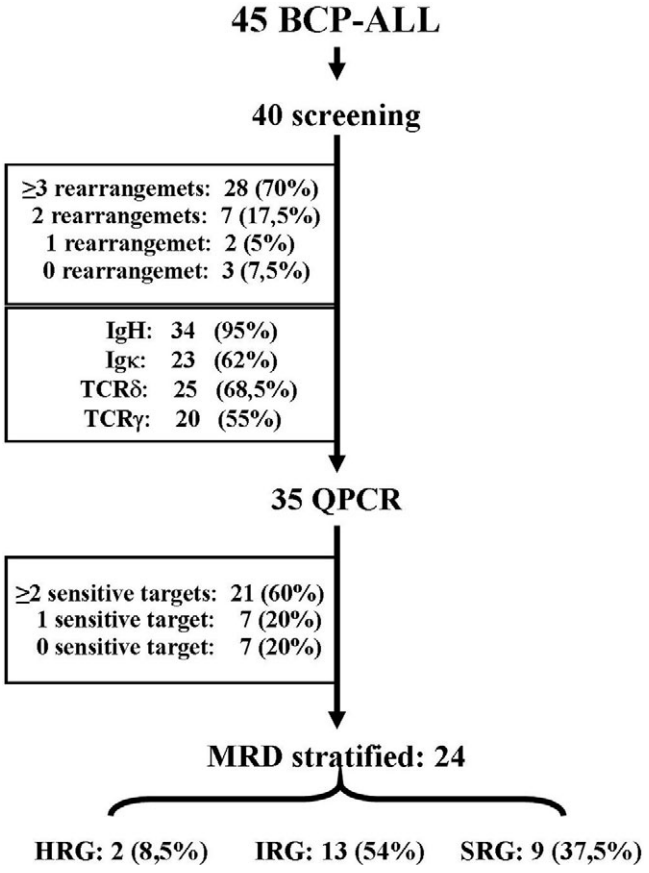

Figure 2: Frequency and sensitivities of Ig and TCR gene rearrangements as MRD PCR targets and MRD risk group distribution in precursor BCP-ALL patients.

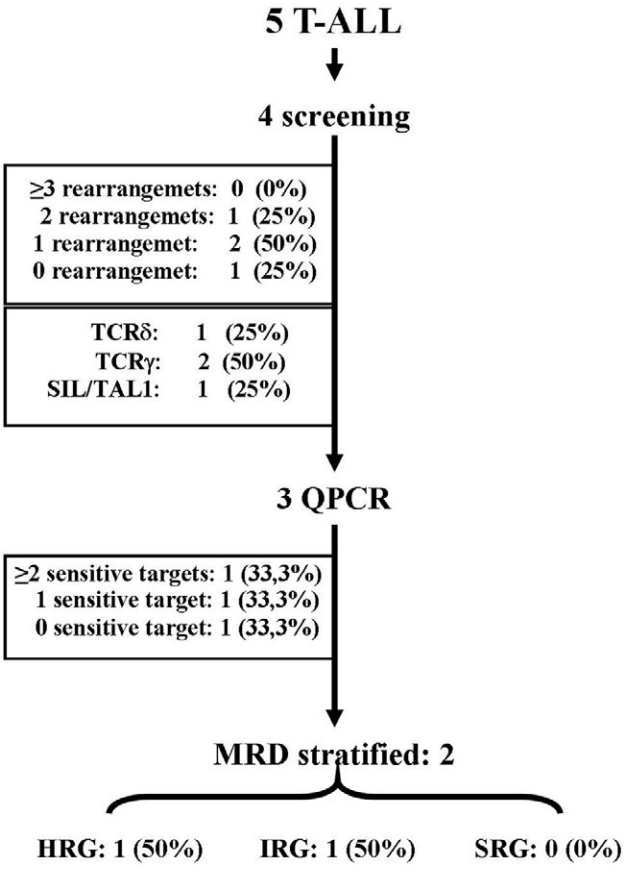

Figure :3: Frequency and sensitivities of Ig and TCR gene rearrangements as MRD PCR targets and MRD risk group distribution in T-ALL patients.

incomplete TCRD rearrangement D**δ2**-J**δ1** (V**δ**-J**δ1**, D**δ2**-J**δ1,** V**δ**2-D**δ**3, D**δ**2-D**δ**3 were tested) in one case as well as the SIL-TAL1 deletion in one case.

A total of 86 identified targets were analyzed for sensitivity: 4 targets in 3 of the T-ALL patients and 82 targets in 35 of the BCP-ALL patients (Figure 4). The percentage of sensitive targets was higher for IGH (71%) and TCRD (72%) rearrangements. The percentage of sensitive targets for IGK-KDE rearrangement was 66%. The lowest percentage of sensitive targets was identified as the TCRG rearrangement (50%). Only one T-ALL patient had two sensitive targets (detection limit ≤ 10^{-4}), and one other T-ALL patient had one sensitive target (Figure 3). In 21 (60%) of the 35 BCP-ALL patients, we identified at least two sensitive targets. One sensitive target was identified in 20% of these patients (Figure 2). In 21% of the 38 analyzed ALL patients, no targets with adequate sensitivity were identified.

Five of the 35 BCP ALL and one of three T-ALL patients with analyzed sensitivity of the identified targets had insufficient or no material from day 33 and/or day 78 for MRD analysis. Real-time quantitative PCR and MRD analyses were then performed with 30 BCP-ALL and two T-ALL patients. A total of 24 (80%) BCP-ALL and all two T ALL patients qualifying for MRD stratification according to the MRD-derived risk stratification in the AIEOP-BFM ALL 2000 study [14]. Six patients not qualifying for MRD stratification were represented by two of them with one sensitive target and MRD at day 78 < 10^{-3} and four with at least one target with a sensitivity of at least 10^{-3} and MRD at day 78 < 10^{-3}. Based on MRD stratification, we identified 26 patients who were stratified into the HRG (n = 3; 11.5%), IRG (n = 14; 54%) and SRG (n = 9; 34.5%). All 50 patients were also stratified based on non-MRD criteria, and the percentage of patients in the different risk groups was similar to that of the MRD-based stratification. Based on non-MRD criteria, we stratified 18 patients (36%) into SRG, 27 patients (54%) IRG and 5 patients (10%) into HRG. However, one patient assigned to the IRG based on non-MRD stratification was stratified into the HRG by MRD based stratification, and six patients assigned to the IRG were stratified into SRG. Nine patients assigned into the SRG based on non-MRD

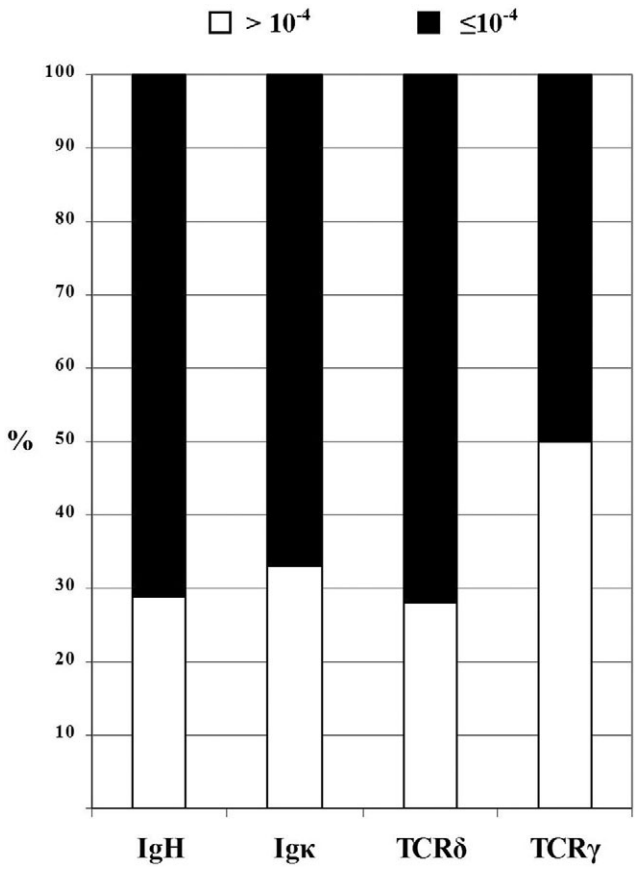

Figure 4: Sensitivities of immunoglobulin (Ig) and T-cell receptor (TCR) rearrangements used as MRD PCR targets.

Table 2. Treatment response (Prednison response on day 8, early bone marrow response on day 15, bone marrow response after induction, risk groups accordding non MRD ALL IC BFM 2002 criteria, Risk groups acording MRD criteria)

	N	%
	50	100
Prednison response		
PGR (less than 1000/ul blood blasts)	47	94%
PPR (more than 1000/ul blood blasts)	3	6%
Early bone marrow response day 15		
M1 (less than 5% blasts)	37	74%
M2 5-25% blasts	11	22%
M3 (more than 25% blasts)	2	4%
Response after induction day 33		
M1 (less than 5% blasts)	45	90%
M2 5-25% blasts	3	6%
M3 (more than 25% blasts)	1	2%
Risk groups accordding non MRD ALL IC BFM 2002 criteria		
SR	18	36%
IR	27	54%
HR	5	10%
Risk groups acording MRD criteria	26 p.	
SR	9	34,6%
IR	14	53,8%
HR	3	11,5%

stratification were stratified into the IRG using MRD based stratification.

Table 3 shows the distribution the MRD positivity and negativity at day 33 and day 78. We identified nine MRD-negative patients out of 17 IRG patients (53%), and only three MRD-negative out of 13 SRG patients (23%). Consequently,

Table 3.:Impact of clinical and biological factors on MRD status at day 33 and day 78.

	Risk group			Pred. response		Day 15, BM morphology		
	SRG	IRG	HRG	PGR	PPR	M1	M2	M3
D 33 P	10	8	2	20	1	15	5	1
D 33 N	3	9	0	11	0	9	2	0
	p=0,141	p=0,474	p=1,000	P= 1,000		P= 1,000	P= 1,000	P= 1,000
D 78 P	1	2	2	4	1	3	1	1
D 78 N	12	15	0	22	0	21	6	0
	P= 1,000	P=0,029	P=0,035	P=0,185		P= 1,000	P=0,250	P=0,160
	IP			Age		WBC		
	BCP-ALL	T-ALL		< 6	>=6	<20 000	>=20 000	
D 33 P	19	2		13	8	12	8	
D 33 N	11	0		6	5	7	5	
	P=0,534			P=0,721		P=1,000		
D 78 P	4	2		1	4	1	5	
D 78 N	26	0		18	9	18	8	
	P=0,292			P=0,132		P=0,029		

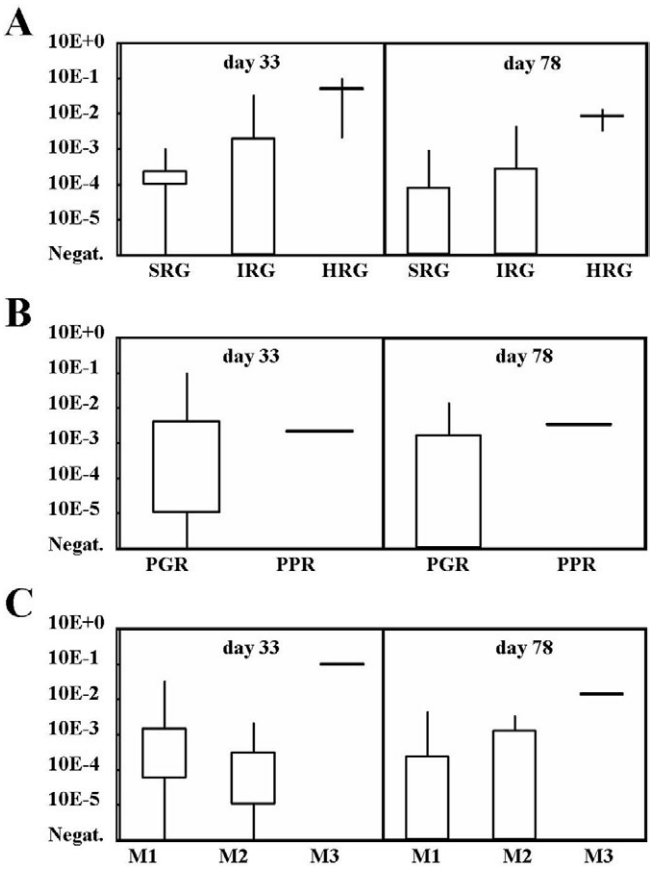

Figure 5: Relation of ALL IC-BFM 2OO2 risk-group stratification and early treatment response to MRD levels at day 33 and day 78 (columns represent the range between mean and median of the MRD levels). Quantitative MRD levels related to (A) IC-BFM 2002 risk-group stratification, (B) prednisone response at day 8 and (C) bone marrow morphology at day 15.

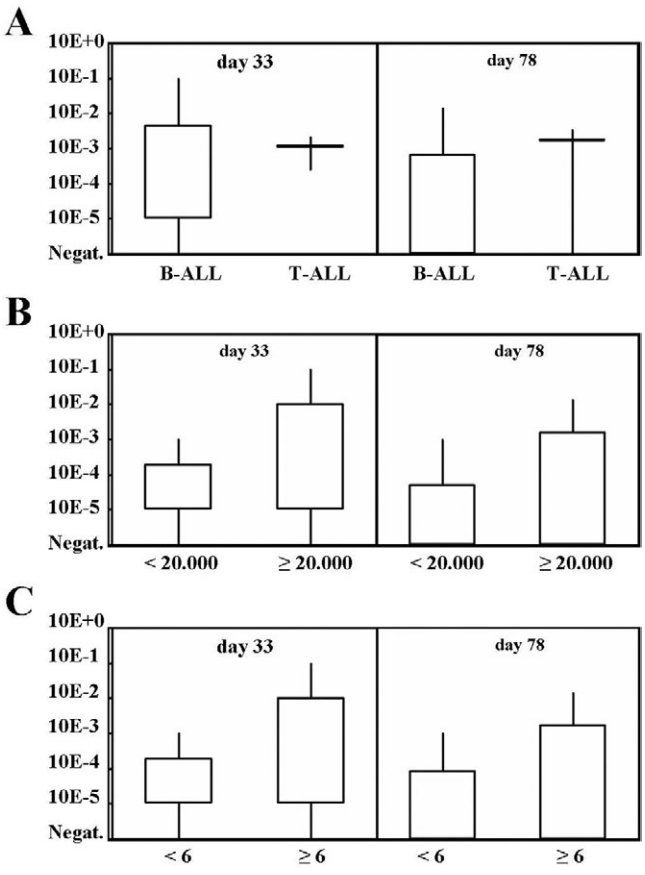

Figure 6: Relation of clinical features to MRD levels at day 33 and day 78 (columns represent the range between mean and median of the MRD levels). Quantitative MRD levels related to (A) immunophenotype, (B) white blood cell count (μl^{-1}) and (C) age at diagnosis.

the Fisher's exact test showed no significant difference in the response to treatment in terms of MRD negativity at day 33 ($p = 0.0575$) and at day 78 ($p = 1.0$) between patients assigned to the SRG and the IRG. In addition, the MRD level was not significantly correlated with non-MRD-based stratification into the SRG and the IRG at day 33($p = 0.146$) or at day 78 ($p = 0.659$) (Figure 5A). As opposed to employing MRD-based stratification, this disagreement appears to be the consequence of a significant discrepancy between classification into the SRG and the IRG based on non-MRD stratification. However, the MRD negativity and MRD value were significantly correlated with the non-MRD-based stratification into the HRG (Table 3, Figure 5A). This correlation was most clear on day 78. The mean MRD level at day 78 was 7.8×10^{-5} in SRG and 2.72×10^{-4} in IRG , compared to 8.7×10^{-3} in HRG ($p = 0.0034$).

Patients with T-ALL were less likely to achieve MRD negativity at day 33 and day 78 than those with BCP-ALL (Table 3). However, this difference was not statistically significant. One T-ALL patient had an MRD value higher than the mean MRD value of the BCP-ALL patients at day 33 and day 78. A second T-ALL patient who was analyzed for MRD had an MRD value around the median MRD value of the BCP-ALL patients at the both of the analyzed follow-up time points (Figure 6A).

Day 8 and day 15 BM morphology was assessed as part of the non-MRD-based risk group stratification (ALL IC-BFM 2002) in the Slovak Republic. As for the response to treatment, a good response to prednisone (PGR) was achieved in 47 patients (94%), and a poor response to prednisone (PPR) was observed in 3 patients (6%). An early response on day 15 with an M1 cytomorphology was achieved in 37 patients (72%), an M2 cytomorphology was achieved in 11 patients (22%) and an M3 cytomorphology in 2 patients (4%). Assessment of bone marrow after the induction of treatment on day 33 resulted in an M1 cytomorphology in 45 patients (90%), an M2 cytomorphology in 3 patients (6%) and an M3 cytomorphology in 1 patient (2%). One patient died before day 33. In the whole cohort of 32 patients who were analyzed for MRD at day 33 and day 78, we identified one patient with a PPR phenotype at

day 8 and one patient with M3 morphology at day 15. These two patients were stratified to the HRG by non-MRD as well as MRD-based risk stratification. They were all MRD-positive at day 33 and day 78. The patient with the PPR phenotype at on day 8 had an MRD value around the mean value of patients with the PGR phenotype (Figure 5B). The patients with an M3 morphology at day 15 had the highest MRD value of all patients in the cohort who were analyzed (32 patients) on day 33 and on day 8 (Figure 5C). Patients stratified in the HRG with both non-MRD and MRD-based risk stratification had an M2 or M3 cytomorphology at day 15. However, the patient stratified into the as IRG and HRG by the non-MRD and MRD-based classification had an M1 cytomorphology at day 15. Figure 8 shows a comparison between the day 8 and day 15 bone marrow morphology statuses with MRD levels at day 33 and day 78. There was no significant difference in MRD status between patients with M1 and M2 morphology at either of the time points. The same results were observed when MRD negativity and positivity was compared to bone marrow morphology. However, most of the patients with an M1 cytomorphology were MRD negative at day 33 as well as at day 78 (Table 3).

A total of 27 patients (60%) had an initial WBC count less than 20,000 μl^{-1}, 12 patients (24%) had between 20,000 and 100,000 μl^{-1} and 8 patients (16%) had more than 100,000 μl^{-1} at the time of diagnosis. Five patients from the six stratified into the HRG using both the non-MRD- and MRD-based criteria had a WBC count higher than 20,000 μl^{-1} at diagnosis. Using a cutoff of 20,000 μl^{-1}, we found a significant difference in these groups of patients at day 78 with respect to MRD positivity (Table 3). However, we found no significant difference in these groups with respect to MRD levels at any time point tested (Figure 6B).

Our cohort was comprised of children aged 1-18 years. Using an age cutoff of 6 years applied to the ALL IC-BFM 2002 stratification, we found no statistically significant differences in MRD positivity with respect to MRD levels between these two groups of patients (Table 3, Figure 6C). However, each patient stratified into the HRG was older than 6 years. Moreover, 18 of the 19 patients (95%) who were 1-5 years of age were MRD negative, and only 9 of 13 patients (69%) older than 6 years were MRD negative on day 78 (Table 3).

Discussion

The significance of minimal residual disease in childhood ALL has been shown in several retrospective studies since the late 1990s [9, 12, 13]. MRD detection is particularly useful for evaluation of early treatment response and consequently for improved front-line therapy stratification. MRD information is also significant for children undergoing allogeneic hematopoietic stem cell transplantation and those with relapsed ALL [23]. Currently, three highly specific and sensitive methodologies for MRD detection are available, namely multiparameter flow cytometric immunophenotyping, real-time quantitative PCR-based detection of fusion gene transcripts or breakpoints, and real-time quantitative PCR-based detection of clonal immunoglobulin and T-cell receptor gene rearrangements. The latest treatment protocols have implemented defined MRD monitoring into risk group stratification with the aim to intensify or reduce treatment. Currently, the most reliable method considered is a PCR analysis of clone-specific immunoglobulin and T-cell receptor gene rearrangements (14). Although this method requires good logistics, it is time-consuming and is relatively expensive. To keep up with another European countries and to have the possibility to use the most modern treatment protocols, a significant effort was used to implement this method in Slovakia as well.

Since January 2005, we have established this method in Slovakia to monitor MRD levels in our patients. We realize the fact that to guarantee the credibility of our results, regular inter-laboratory quality controls are required. To account for this, we underwent an international quality control led by colleagues from Prague. In this report, we present the outcome of 50 pediatric patients with ALL and the results of our MRD monitoring pilot study.

The incidence of pediatric ALL in Slovakia is about 30 new ALL cases per year [24]. Children with ALL are treated at three Slovakian centers (Bratislava, Košice and Banská Bystrica) according to an international non-MRD-based protocol from the ALL IC BFM 2002 study.

From October 2006 to December 2009, 83 children with ALL were diagnosed in Slovakia. Here we report samples from 50 patients, ages 1-18 years (median 4.1 years). Of these patients, 16 were girls and 34 were boys, 5 had T-ALL and 45 had BCP-ALL. There is room for improvement of the logistics with the aim to obtain samples from all patients. At least one Ig/TCR target was found in 92.5% of the analyzed BCP-ALL patients (37 patients), and two or more rearrangements were found in 87.5% of the patients. The majority of recombination was represented by an IGH rearrangement in 95% of patients. According to published data, at least two clonal rearrangements were detected in 86 and 91% of patients using the singleplex and singleplex-multiplex approaches, respectively [21].

Children were stratified using non-MRD criteria as SRG (36%), IRG (54%) or HRG (10%). Out of 50 children, 26 were stratified using MRD criteria as SRG (34.7%; two sensitive targets, MRD-negative at both time points), IRG (53.8%) or HRG (11.5%; MRD $\geq 10^{-3}$ at day 78). In comparison to the BFM study (14), 40% of patients were classified as SRG, 8% as HRG and 52% as IRG using the MRD based risk group stratification.

In our cohort, one patient assigned to the IRG based on non-MRD stratification was stratified into the MRD-HRG. Another six patients assigned to the IRG were stratified into the MRD-SRG, and nine patients assigned to the SRG based on MRD stratification were stratified into the MRD-IRG.

One of our patients had a PPR phenotype at day 8, and one patient had an M3 cytomorphology at day 15. These two patients were stratified to the HRG by both the non-MRD- and

MRD-based risk stratification, and these patients were MRD-positive at day 33 and day 78 respectively. The patient with the PPR phenotype at day 8 had an MRD value around the mean value of patients with the PGR phenotype. The patient with the M3 cytomorphology at day 15 had the highest MRD value of all values of the 26 analyzed patients at both time points, day 33 and 78 (Figure 8). The MRD negativity and MRD value were significantly correlated with non-MRD-based stratification into the HRG. These findings are consistent with those of Fronkova, Mejstrikova et al. [16], who found that morphology-based ALL IC risk stratification allows the identification of most HRG patients identified also by MRD-based stratification, but fails to discriminate the IRG assigned to therapy reduction. Patients in the SRG and the IRG could therefore profit from MRD-based risk assignment, which could inspire countries with non-MRD-based protocols to set up MRD techniques in structured cooperation among laboratories.

The 10-year update on the I-BFM-SG MRD study [14] demonstrated stable results [i.e., event-free survival]; SRG (93%), IRG (74%), and HRG (16%)]. PCR-based MRD discriminated prognosis better than previous AIEOP-BFM stratification criteria based on WBC count, age, early response to prednisone and genotype [including Ph(+) ALL]. The MRD response detected by sensitive real-time quantitative PCR at the two pre-defined time points was highly predictive for relapse in childhood BCP-ALL. This may reduce the relevance of conventional prognostic factors and improve adaptation of therapy [15].

It should be stressed that according to current standards for MRD diagnostics, the use of at least two clonal rearrangements for detecting a malignant clone is recommended to avoid false-negative results as a consequence of the loss of an MRD marker, which is due to secondary and ongoing Ig/TCR rearrangements and clonal evolution (described elsewhere) [25]. Monitoring of minimal residual disease in childhood and adult acute lymphoblastic leukemia is significantly correlated with clinical outcome. A particular emphasis is placed on inter-laboratory standardization, especially in view of the results obtained from the European collaborative BIOMED-1, BIOMED-2, the Europe against Cancer projects and recent developments by the European Study Group on MRD detection in ALL and Euro Flow Consortium. Standardized MRD techniques form the basis for stratification of patients into risk groups in new treatment protocols for childhood and adult ALL. Only the results of these studies can answer the question whether MRD-based treatment intervention is associated with an improved outcome.

In conclusion, assessment of minimal residual disease has acquired a prominent position in European treatment protocols for patients with acute lymphoblastic leukemia on the basis of its high prognostic value for predicting the outcome and the possibilities for implementing MRD diagnostics in treatment stratification. There is an increasing need for standardization of methodologies and harmonization of terminology. The current state of MRD diagnostics in ALL was summarized, and recommendations on the minimal technical requirements that should be fulfilled before implementation of MRD diagnostics into clinical trials were developed and were reviewed in [25].

In this study, first experiences are presented with monitoring of MRD in the Slovak Republic using real-time quantitative PCR analysis of immunoglobulin and T-cell receptor gene rearrangements in childhood ALL. Furthermore, we have opened the possibility of implementing MRD monitoring in clinical practice. We are currently able to provide MRD information for children undergoing allogeneic hematopoietic stem cell transplantation and those with relapsed ALL. Although the method is well established and it is undergoing inter-laboratory quality control assessments within international collaboration, we still need further improvement, especially with regard to the logistics. In 2009, we began an international collaboration with a laboratory in Vienna to implement monitoring of MRD by flow cytometry. In the near future, we hope to fulfill the international criteria and have the ability to use the most modern treatment protocol for ALL children in Slovakia.

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Bibliography:

[1] STANULLA, M. AND M. SCHRAPPE.:Treatment of childhood acute lymphoblastic leukemia. Semin Hematol 2009, 46: 52–63. doi:10.1053/j.seminhematol.2008.09.007

[2] Schrappe, M., NACHMAN.J. HUNGER, S., SCHMIEGELOW, K., CONTER, V. et al. :Educational symposium on long-term results of large prospective clinical trials for childhood acute lymphoblastic leukemia (1985-2000)'. Leukemia 2010, 24: 253–4. doi:10.1038/leu.2009.276

[3] MITCHELL, C., S. RICAHRDS "Long-term follow-up of the United Kingdom medicalresearch council protocols for childhood acute lymphoblastic leukaemia, 1980-2001." Leukemia 2010, 24: 406–18. doi:10.1038/leu.2009.256

[4] MORICKE, A., ZIMMERMAN, M., REITER, A., HENZE, G., SCHRAUDER, A. et al. : Long-term results of five consecutive trials in childhood acute lymphoblastic leukemia performed by the ALL-BFM study group from 1981 to 2000. Leukemia 2010, 24: 265–84. doi:10.1038/leu.2009.257

[5] SALZER, W. L., DEVIDAS, M., CARROLL, W. L., WINICK, N., PULLEN, J. et al.: Long-term results of the pediatric oncology group studies for childhood acute lymphoblastic leukemia 1984–2001: a report from the children's oncology group. Leukemia 2010, 24: 355–70. doi:10.1038/leu.2009.261

[6] PUI, C. H.:Toward a total cure for acute ly phoblastic leukemia. J Clin Oncol 2009, 27: 5121–3. doi:10.1200/JCO.2009.24.8518

[7] SZCZEPANSKI, T., WILLEMSE, M.J., VAN WERING, E. R., VAN WEERDEN, J. F., KAMPS, W. A. et al.:Precursor-B-ALL with D(H)-J(H) gene rearrangements have an immature

immunogenotype with a high frequency of oligoclonality and hyperdiploidy of chromosome 14. Leukemia 2001, 15: 1415–23. doi:10.1038/sj.leu.2402206

[8] CAMPANA, D.: Minimal residual disease in acute lymphoblastic leukemia. Semin Hematol 2009, 46: 100–6. doi:10.1053/j.seminhematol.2008.09.001

[9] COUSTAN-SMITH, E., BEHM, F. G., SANCHEZ, J., BOYETT, J. M., HANCOCK, M. L. et al. Immunological detection of minimal residual disease in children with acute lymphoblastic leukaemia. Lancet 1998, 351(9102): 550–4. doi:10.1016/S0140-6736(97)10295-1

[10] DWORZAK, M. N., FROSCHL, G., PRINTZ, D.MANN, G. POTSCHGER, U. et al.: Prognostic significance and modalities of flow cytometric minimal residual disease detection in childhood acute lymphoblastic leukemia. Blood 2002,99: 1952–8. doi:10.1182/blood.V99.6.1952

[11] BOROWITZ, M. J., DEVIDAS, M., HUNGER, S. P., BOWMAN, W. P., CARROLL, A. J.et al.: Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia and its relationship to other prognostic factors: a Children's Oncology Group study. Blood 111: 2008, 5477–85. doi:10.1182/blood-2008-01-132837

[12] CAVE, H., VAN DER WERFTEN BOSCH, J., SUCIN, S. GUIDAL, C. WATERKEYN, C. et al. : Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia. European Organization for Research and Treatment of Cancer--Childhood Leukemia Cooperative Group. N Engl J Med 1998, 339: 591–8. doi:10.1056/NEJM199808273390904

[13] VAN DONGEN, J. J., SERIU, T., PANZER-GRUNMYZER, E. R., BIONDI, A., PONGERS-WILLEMSE, M. J., et al.: Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. Lancet 1998, 52(9142): 1731–8. doi:10.1016/S0140-6736(98)04058-6

[14] FLOHR, T., SCHRAUDER, A., CAZZANIGA, G., PANZER-GRUNMAYER, R., VAN DER VELDEN, V. et al. : Minimal residual disease-directed risk stratification using real-time quantitative PCR analysis of immunoglobulin and T-cell receptor gene rearrangements in the international multicenter trial AIEOP-BFM ALL 2000 for childhood acute lymphoblastic leukemia. Leukemia 2008, 22: 771–82. doi:10.1038/leu.2008.5

[15] CONTER, V., BARTRAM, C. R., VALSECCHI, M. G.,SCHRAUDER, A., PANZER-GRUNMAYER, R.. et al.: Molecular response to treatment redefines all prognostic factors in children and adolescents with B-cell precursor acute lymphoblastic leukemia: results in 3184 patients of the AIEOP-BFM ALL 2000 study. Blood. 2010 Feb 12. [Epub ahead of print]

[16] FRONKOVA, E., MEJSTRIKOVA, E. AVIGAD, S., CHIK, K. W., CASTILLO, L. et al. :Minimal residual disease (MRD) analysis in the non-MRD-based ALL IC-BFM 2002 protocol for childhood ALL: is it possible to avoid MRD testing? Leukemia 2008, 22: 989–97. doi:10.1038/leu.2008.22

[17] PUI, C. H., ROBINSON, L. L, LOOK, A. T.: Acute lymphoblastic leukaemia. Lancet 2008, 371(9617): 1030–43. doi:10.1016/S0140-6736(08)60457-2

[18] BENE, M. C., CASTOLDI, G, KNAPP, W., LUDWIG, W. D. MATUTES, E. et al.: Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia 1995, 9: 1783–6.

[19] VERHAGEN, O. J., WILLEMSE, M. J., BREUNIS, W. B., WIJKHUIJS, A. J., JACOBS, D. C. et al. : Application of germline IGH probes in real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia. Leukemia 2000,14: 1426–35. doi:10.1038/sj.leu.2401801

[20] SZCZEPANSKI, T., VAN DER VELDEN, V. H., HOOGEVEEN, P. G., DE BIE, M., JACOBS, D. C. et al. "Vdelta2-Jalpha rearrangements are frequent in precursor-B-acute lymphoblastic leukemia but rare in normal lymphoid cells." Blood 103: 2004, 3798–804. doi:10.1182/blood-2003-08-2952

[21] VAN DER VELDEN, V. H., CAZZANIGA, G., SCHRAUDER, A., HANCOCK, J., BADER, P. et al. :Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. Leukemia 2007,21: 604–11.

[22] LANGERAK, A. W., WOLVERS-TETTERO I. L., VAN GASTEL-MOL, E. J.OUD, M. E., VAN DONGEN, J. J. et al.: Basic helix-loop-helix proteins E2A and HEB induce immature T-cell receptor rearrangements in nonlymphoid cells. Blood 2001, 98: 2456–65. doi:10.1182/blood.V98.8.2456

[23] BADER, P., H. KREYENBERG, HENZE, G. H. ECKERT, C. REISING, M. et al.: Prognostic value of minimal residual disease quantification before allogeneic stem-cell transplantation in relapsed childhood acute lymphoblastic leukemia: the ALL-REZ BFM Study Group. J Clin Oncol 27: 2009, 377–84. doi:10.1200/JCO.2008.17.6065

[24] KAISEROVÁ, E.: HEMATOLOGIC MALIGNANCIES. IN: ŠAŠINKA M, ŠAGÁT T, KOVÁCS L et al.:. Pediatria. Bratislava: Herba, spol. s.o.r., 2007: 726 (In Slovak)

[25] BRUGGEMANN, M., SCHRAUDER, A., RAFF, T.PFEIFER, H.DWORZAK, M et al. :Standardized MRD quantification in European ALL trials: Proceedings of the Second International Symposium on MRD assessment in Kiel, Germany, 18-20 September 2008. Leukemia 2009, 24: 521–35.