

# Implementation of the standard strategy for identification of Ig/TCR targets for minimal residual disease diagnostics in B-cell precursor ALL pediatric patients: Polish experience

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## Abstract

**Introduction:** Minimal residual disease (MRD), detected based on immunoglobulin and T-cell receptor (Ig/TCR) gene rearrangements as markers of residual leukemic cells, is currently the most reliable prognostic factor in acute lymphoblastic leukemia (ALL). A feasibility study is presented of the standard strategy for the identification of Ig/TCR targets for MRD diagnostics in Polish ALL patients by identifying Ig/TCR gene rearrangement pattern using standard primer sets and protocols.

**Materials and Methods:** The PCR-heteroduplex approach based on BIOMED-1 and BIOMED-2 protocols (recommended as the European standard) was used to detect *IGH*, *IGK*-Kde, *TCRD*, *TCRG*, and *TCRB* rearrangements in 58 Polish B-cell precursor ALL patients. Sequencing and homology analysis between the obtained and germline Ig/TCR sequences enabled identification of the rearrangements. The U-Gauss test was used for statistical analysis of the Ig/TCR rearrangement pattern in Polish patients compared with relevant data on other nationalities.

**Results:** The following pattern was identified: *IGH*: 83% (VH-JH: 74%, DH-JH: 9%), *IGK*-Kde: 41%, *TCRD*: 78% (incomplete *TCRD*: 55%, Vδ2-Dδ3: 45%, Dδ2-Dδ3: 21%, Vδ2-Jα: 35%), *TCRG*: 50%, and *TCRB*: 13%. Considerable convergence of the Ig/TCR pattern in Polish patients and those of other nationalities (mainly West Europeans) was demonstrated. Statistically relevant differences were only found between the incidence of DH-JH in Polish (9%) and Dutch patients (24%;  $p < 0.05$ ) and Polish and Italian patients (19%;  $p < 0.05$ ), VH-JH in Polish (74%) and Chilean patients (100%;  $p < 0.05$ ), and *TCRG* in Polish (50%) and Brazilian patients (69%;  $p < 0.05$ ).

**Conclusions:** The convergence of Ig/TCR patterns in Polish and European patients indicates that the strategy for Ig/TCR target identification based on standard primers and protocols might be directly used for the construction of Polish standards and recommendations for MRD diagnostics.

**Key words:** minimal residual disease, Ig/TCR gene rearrangements, acute lymphoblastic leukemia, BIOMED-1, BIOMED-2.

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## INTRODUCTION

The level of residual leukemic cells (minimal residual disease, MRD) is currently the most reliable prognostic factor in acute lymphoblastic leukemia (ALL).

Detection and quantitative assessment of MRD performed at fixed time-points during ALL treatment enables precise stratification of patients into risk groups and appropriate adjustment of treatment intensity. The clinical significance of MRD assessment in risk-adapted

management of ALL is already well documented [7, 26, 31].

Molecular diagnostics of MRD is currently most widely performed with the use of the real-time quantitative polymerase chain reaction approach (RQ-PCR) and rearranged immunoglobulin (Ig) and T-cell receptor (TCR) genes as patient-specific “fingerprint-like” markers of residual leukemic cells [25]. Protocols for MRD diagnostics (including the identification of Ig/TCR rearrangements and RQ-PCR MRD assessment) as well as guidelines for interpreting the quantitative data have been highly standardized through international collaboration, mainly within the confines of the BIOMED-1 Concerted Action, the BIOMED-2 Concerted Action, and the European Study Group on MRD Detection in ALL [14, 24, 30]. These standards are routinely applied, particularly in Western Europe, for the MRD-based stratification of ALL treatment. However, in some countries ALL therapeutic protocols are still being applied in which MRD diagnostics is not obligatory for risk-group assessment, such as the ALL-IC-BFM 2002 protocol. In these countries, like in Poland, an urgent need for the development of routine MRD diagnostics is indicated. The introduction of MRD assessment into clinical practice might be performed in a reasonably cost- and labor-effective manner through direct implementation of the existing European standards.

European collaboration implies that these standards were based on the rearrangements most commonly identified in patients from West European countries. However, according to some authors, the frequencies of Ig/TCR gene rearrangements might differ significantly in various populations [16–18]. Scrideli et al. [17] posed an hypothesis concerning socio-economic-based diversity of Ig and TCR gene rearrangement patterns (the frequency of rearrangements in individual loci and the utilization of Ig/TCR genes in the rearrangements), which might have implications for the choice of primers for MRD diagnostics. Therefore, for those aiming to implement MRD assessment in routine molecular diagnostics, it appears justified to assess the applicability of the chosen protocols and primer sets for efficient detection of MRD markers. Characterization of the rearrangement pattern, particularly the identification of distinctive features of the pattern in the target population, enables modification of the protocols to ensure successful selection of MRD markers. Since MRD diagnostics is currently being developed in Poland, we performed a feasibility study of the standard strategy for the identification of Ig/TCR MRD targets in Polish patients. We also evaluated the need for modifying European standards due to the specificity of the Polish pattern. To address these goals we performed an identification of the Ig/TCR gene rearrangement pattern using standard primer sets and protocols and compared the identified pattern with those reported for patients of other, mainly European, nationalities.

We previously reported our preliminary results on the identification of the antigen receptor gene

rearrangement pattern in a group of 23 Polish pediatric B-cell precursor ALL patients (BCP-ALL) [9]. In the present study we identified the rearrangement pattern in a larger cohort of 58 BCP-ALL children (including the 23 patients from our previous study), which enabled verification of our preliminary observations using statistical methods. We demonstrated convergence of the Ig/TCR rearrangement pattern identified in Polish patients with the results published for other European cohorts. This finding supports the idea of direct implementation of the standard strategy into MRD diagnostics in our country. Moreover, in the present study we assessed the efficacy of detecting Ig and TCR gene rearrangements using exclusively the singleplex PCR approach vs. a combined singleplex-multiplex approach, which might be applied for the design of a cost- and labor-effective MRD diagnostic approach. Our results might be useful for other countries undertaking the development of MRD diagnostics based on available European standards.

## MATERIALS AND METHODS

### *Patients*

We studied 58 consecutive patients with newly diagnosed BCP-ALL being treated according to the ALL IC BFM 2002 protocol, recruited (based on written informed consent and the availability of biological material of suitable amount and quality) between 2003–2006 in two hematological centers, Poznań and Bydgoszcz. The group was representative of the country in view of the considerable homogeneity of the Polish population. The cohort of patients consisted of 27 females and 31 males aged 1.1–16.7 years at the time of diagnosis (mean age: 6.9 years). Diagnosis of BCP-ALL was based on the French-American-British classification and flow cytometric immunophenotyping with the use of a standard set of monoclonal antibodies [15].

### *Material*

Bone marrow samples obtained before the commencement of treatment were subjected to Ficoll-Paque (Pharmacia Biotech, Sweden) or Gradisol L (Aqua-Medica, Poland) density gradient centrifugation for mononuclear cell (MNC) separation. Three methods were used for DNA extraction; the majority of DNA extractions were performed using QIAamp DNA Blood Mini Kit (Qiagen, Germany), some using the NaCl precipitation-based protocol (salting-out method), and a few MNC samples were processed by boiling in Qiagen PCR buffer (1 × containing 1.5 mM MgCl<sub>2</sub>, 95°C, 10 min).

### *Detection of Ig/TCR gene rearrangements by PCR amplification*

Detection of Ig/TCR rearrangements was performed with the use of the BIOMED-1 and BIOMED-2 proto-

cols and standard primers, including BIOMED-1 and BIOMED-2 primers. In all 58 patients a singleplex PCR approach using the BIOMED-1 primers was applied for amplification of the rearrangements *IGK*-Kde, incomplete *TCRD* (V $\delta$ 2-D $\delta$ 3, D $\delta$ 2-D $\delta$ 3), and *TCRG* [14]. Complete *IGH* rearrangements [33] and *TCRD/A* (V $\delta$ 2-J $\alpha$ 29) rearrangements were also detected with the singleplex approach using standard “non-BIOMED” primers [20]. Moreover, in 23 of these patients a multiplex approach was applied for detection of rearranged *TCRB* genes using BIOMED-2 primers [30]. Incomplete *IGH* [22] and V $\delta$ 2-J $\alpha$  [20] rearrangements were also detected in these 23 patients with the multiplex approach using standard “non-BIOMED” primers. Amplification was performed in a 50- $\mu$ l volume containing 50 ng of genomic DNA, 200  $\mu$ M of each dNTP, 7 and 14 pmol of the 5' and 3' oligonucleotide primers (Sigma/Oligo, Poland) in singleplex reactions or 10 pmol of the 5' and 3' primers (Sigma-Aldrich, USA) in the multiplex approach, 1.5 and 1.5–3 mM MgCl<sub>2</sub> (in the singleplex and multiplex approach, respectively), 0.5 U of TaqGold (Applied Biosystems, USA) or 0.65 U of HotStar-Taq (Qiagen, Germany) polymerase in singleplex reactions, and 1–2 U TaqGold (Applied Biosystems, USA) polymerase in the multiplex approach. In each PCR, negative and relevant positive controls were amplified. The cycling conditions were 95°C for 7 min, 35  $\times$  (94°C for 45 s, 60°C for 90 s, and 72°C for 2 min), and 72°C for 10 min. Standard agarose gel electrophoresis was used to select the PCR products containing the specific amplicons for further analysis.

#### *Heteroduplex analysis*

Heteroduplex analysis for discriminating between monoclonal and polyclonal PCR products derived from clonal leukemic cells and polyclonal reactive lymphocytes, respectively, was performed according to a standard protocol [11]. Briefly, the PCR products were denatured (94°C, 5 min) and subsequently renatured (4°C, 1 h) to allow duplex formation. The samples were immediately loaded onto 6% non-denaturing polyacrylamide gel, run in 0.5 $\times$ TBE buffer, and stained with ethidium bromide. Detection of homoduplexes (renaturation of identical DNA single strands) indicated monoclonality. Detection of heteroduplexes (renaturation of DNA strands of partially different sequence corresponding to the junction between the rearranged genes) was interpreted according to the migration pattern as a biclonal/biallelic rearrangement (mostly two homoduplex and two heteroduplex bands, alternatively co-migrating) or oligoclonality (more than two homoduplex and heteroduplex bands, a “PCR product ladder”). A smear of PCR products indicated polyclonality.

#### *Identification of rearrangements via sequence analysis*

Monoclonal PCR products were processed with the QIAquick PCR Purification Kit (Qiagen, Germany) or

were sequenced directly. Excision of the appropriate homoduplex or heteroduplex bands followed by DNA elution and precipitation was performed if more than one amplicon was detected in a single PCR (biclonal/biallelic rearrangement or multiplex PCR). Sequencing with the use of amplification primers [14, 20, 22, 30, 33] was carried out in both directions. 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.mrc-cpe.cam.ac.uk>; Center for Protein Engineering, Cambridge, UK). Homology between the sequences was analyzed with the use of DNAPLOT (W. Müller, H-H. Althaus, University of Cologne, Germany) and SeqMan II Lasergene v. 6 software (DNASTAR Inc., Madison, WI, USA).

## RESULTS

To determine the pattern of the rearrangements, i.e. the frequency of the rearrangements in individual Ig/TCR loci and the utilization of V (variable), D (diversity), and J (joining) genes, the rearrangements detected in the group studied were identified and characterized. Moreover, to address the efficacy of detection of Ig/TCR gene rearrangements with the use of the singleplex PCR vs. the combined singleplex and multiplex approach, relevant results obtained in the group of 58 patients were compared with those obtained for the subgroup of 23 patients, respectively.

#### *Detection and identification of Ig/TCR gene rearrangements*

In total, 211 clonal Ig/TCR gene rearrangements were detected (see Table 1 for a summary) comprising 60 *IGH* rearrangements (58 complete VH-(DH)-JH and 2 incomplete DH-JH), 33 *IGK* rearrangements (24 V $\kappa$ -Kde and 9 Intron-Kde), 48 incomplete *TCRD* rearrangements (34 V $\delta$ 2-D $\delta$ 3 and 14 D $\delta$ 2-D $\delta$ 3), 15 *TCRD/A* rearrangements (V $\delta$ 2-(D $\delta$ 3)-J $\alpha$ ), 51 *TCRG* rearrangements, and 4 *TCRB* rearrangements (3 complete V $\beta$ -(D $\beta$ )-J $\beta$  and 1 incomplete D $\beta$ -J $\beta$ ).

In the singleplex PCR approach, at least one clonal rearrangement was detected in 97% (56/58) of the BCP-ALL patients and at least two in 86% (50/58) of the patients. Insufficient quality of the DNA samples was excluded by amplification of the 100-, 200-, 400-, and 600-bp DNA fragments in multiplex PCR (BIOMED-2 Control Gene tube) in the case of the two patients in whom no PCR products were detected [30]. The number of rearrangements detected per patient ranged from 0 to 8, both cases occurring with a frequency of 3% (2/58), with 2 rearrangements as the most frequent number of rearrangements/patient (mode) occurring with a frequency of 24% (14/58). The mean number of

**Table 1.** Ig/TCR gene rearrangements found in the group of Polish B-cell precursor ALL patients

Patient	IGH	IGK-Kde	Incomplete TCRD	V $\delta$ 2-J $\alpha$	TCRG	TCRB	Total
1.1	VH3.66-DH1.26-JH3		V $\delta$ 2-D $\delta$ 3 V $\delta$ 2-D $\delta$ 3				3
3.1	VH4.39-JH6	V $\kappa$ 3d20-Kde		V $\delta$ 2-D $\delta$ 3-J $\alpha$ 29	V $\gamma$ 9-J $\gamma$ 1.3 V $\gamma$ 4-J $\gamma$ 1.3 V $\gamma$ 1-J $\gamma$ 1.3/2.3 <sup>a</sup>		6
5.1	VH3.21-DH2.8-JH6		D $\delta$ 2-D $\delta$ 3				2
7.1	VH3.9-DH5.5-JH4 DH-JH <sup>a</sup>						2
10.1	VH1.17-DH2.2-JH5		V $\delta$ 2-D $\delta$ 3 V $\delta$ 2-D $\delta$ 3 D $\delta$ 2-D $\delta$ 3		V $\gamma$ 4-J $\gamma$ 2.1 $\psi$ V $\gamma$ 11-J $\gamma$ 2.1		6
15.1		V $\kappa$ 1.16-Kde V $\kappa$ 2.30-Kde			V $\gamma$ 4-J $\gamma$ 2.3		3
18.1			V $\delta$ 2-D $\delta$ 3 D $\delta$ 2-D $\delta$ 3				2
19.1					V $\gamma$ 5-J $\gamma$ 2.3		1
20.1	VH2.26-JH6	V $\kappa$ 1.37-Kde		V $\delta$ 2-J $\alpha$ <sup>a</sup>	V $\gamma$ 8-J $\gamma$ 2.1 V $\gamma$ 9-J $\gamma$ 2.3		5
22.1	VH2.5-DH2.8-JH6		V $\delta$ 2-D $\delta$ 3		V $\gamma$ 3-J $\gamma$ 1.1 V $\gamma$ 1-J $\gamma$ 1.3/2.3 <sup>b</sup>		4
23.1	VH1.3-JH6						1
34.1	VH1.3-DH6.13-JH3 VH2.5-DH2.15-JH4	V $\kappa$ 2.24-Kde V $\kappa$ 2.30-Kde	V $\delta$ 2-D $\delta$ 3	V $\delta$ 2-J $\alpha$ 48	V $\gamma$ 3-J $\gamma$ 1.1 V $\gamma$ 4-J $\gamma$ 1.1		8
37.1	VH2.26-DH3.9-JH4 VH4.30-JH2	V $\kappa$ 2.30-Kde	D $\delta$ 2-D $\delta$ 3		V $\gamma$ 2-J $\gamma$ 2.3 V $\gamma$ 9-J $\gamma$ 2.3		6
40.1	VH3.13-DH2.2-JH3			V $\delta$ 2-D $\delta$ 3-J $\alpha$ 48			2
42.1	VH1.8-DH4.4-JH4 VH6.1-JH5			V $\delta$ 2-J $\alpha$ <sup>a</sup> V $\delta$ 2-J $\alpha$ <sup>a</sup>	$\psi$ V $\gamma$ 11-J $\gamma$ 2.3		5
43.1	VH3.13-DH3.3-JH4		V $\delta$ 2-D $\delta$ 3				2
44.1	VH2.5-DH2.8-JH6 VH4/6-JH <sup>a</sup> VH4/6-JH <sup>a</sup>		D $\delta$ 2-D $\delta$ 3		V $\gamma$ 3-J $\gamma$ 2.1 V $\gamma$ 5-J $\gamma$ 2.3 V $\gamma$ 1-J $\gamma$ 1.3/2.3 <sup>a</sup>		7
45.1		V $\kappa$ 2.30-Kde	V $\delta$ 2-D $\delta$ 3	V $\delta$ 2-D $\delta$ 3-J $\alpha$ 29	V $\gamma$ 3-J $\gamma$ 2.3 V $\gamma$ 9-J $\gamma$ 1.3	D $\beta$ 2-J $\beta$ 2.7	6
46.1	VH1.3-DH6.6-JH2		V $\delta$ 2-D $\delta$ 3 V $\delta$ 2-D $\delta$ 3 D $\delta$ 2-D $\delta$ 3		V $\gamma$ 5-J $\gamma$ 2.3		5
47.1	VH3.21-DH3.9-JH4	V $\kappa$ 1.33-Kde Intron-Kde	D $\delta$ 2-D $\delta$ 3 D $\delta$ 2-D $\delta$ 3	V $\delta$ 2-J $\alpha$ <sup>a</sup>	V $\gamma$ 2-J $\gamma$ 2.3		7
48.1	VH3.7-DH6.13-JH6 DH-JH <sup>a</sup>						2
49.1	VH3.49-DH3.22-JH4	V $\kappa$ 1.16-Kde	D $\delta$ 2-D $\delta$ 3	V $\delta$ 2-J $\alpha$ 29		V $\beta$ -(D $\beta$ )-J $\beta$ <sup>a</sup> V $\beta$ -(D $\beta$ )-J $\beta$ <sup>a</sup>	6
50.1	VH1.2-DH6.6-JH4 VH4.31-DH2.2-JH4		V $\delta$ 2-D $\delta$ 3		V $\gamma$ 9-J $\gamma$ 2.3	V $\beta$ -(D $\beta$ )-J $\beta$ <sup>a</sup>	5
51.1	VH1.69-DH1.14-JH4 VH4.59-DH4.17-JH4 VH3-JH <sup>a</sup> VH3-JH <sup>a</sup>	V $\kappa$ 1.8-Kde V $\kappa$ 3.15-Kde		V $\delta$ 2-D $\delta$ 3-J $\alpha$ 29 V $\delta$ 2-J $\alpha$ 29			8
52.1	VH3.64-DH5.24-JH4 VH6.1-DH6.19-JH4	Intron-Kde	V $\delta$ 2-D $\delta$ 3		V $\gamma$ 2-J $\gamma$ 1.1 V $\gamma$ 9-J $\gamma$ 2.3		6
54.1	VH4.61-JH6		D $\delta$ 2-D $\delta$ 3				2
57.1	VH3-JH <sup>a</sup> VH3-JH <sup>a</sup>				V $\gamma$ 4-J $\gamma$ 2.3		3
58.1		Intron-Kde	V $\delta$ 2-D $\delta$ 3				2
60.1	VH4/6-JH <sup>b</sup>						1
61.1	VH4.39-DH3.22-JH4		V $\delta$ 2-D $\delta$ 3				2
64.1	VH3.7-JH4		V $\delta$ 2-D $\delta$ 3				2
68.1		V $\kappa$ 2.30-Kde Intron-Kde	V $\delta$ 2-D $\delta$ 3 V $\delta$ 2-D $\delta$ 3		V $\gamma$ 5-J $\gamma$ 1.1		5

**Table 1.** Continued

Patient	IGH	IGK-Kde	Incomplete TCRD	V $\delta$ 2-J $\alpha$	TCRG	TCRB	Total
69.1	VH3.53-DH3.3-JH6		V $\delta$ 2-D $\delta$ 3	V $\delta$ 2-J $\alpha$ 29	V $\gamma$ 2-J $\gamma$ 1.1 V $\gamma$ 3-J $\gamma$ 1.3		5
70.1	VH3.21-JH4	V $\kappa$ 1.33-Kde Intron-Kde	V $\delta$ 2-D $\delta$ 3 V $\delta$ 2-D $\delta$ 3				5
72.1	VH3.23-JH4	V $\kappa$ 1.17-Kde V $\kappa$ 3.20-Kde		V $\delta$ 2-J $\alpha$ 29			4
73.1	VH4.61-DH2.2-JH6	Intron-Kde			$\psi$ V $\gamma$ 11-J $\gamma$ 2.3		3
74.1	VH3-JH <sup>b</sup>	Intron-Kde	D $\delta$ 2-D $\delta$ 3 V $\delta$ 2-D $\delta$ 3	V $\delta$ 2-J $\alpha$ 29	V $\gamma$ 9-J $\gamma$ 2.3		6
75.1							0
77.1	VH3.23-DH2.8-JH5 VH6.1-DH3.3-JH5	V $\kappa$ 1.27-Kde			V $\gamma$ 9-J $\gamma$ 1.3		4
78.1					V $\gamma$ 9-J $\gamma$ 1.1 $\psi$ V $\gamma$ 11-J $\gamma$ 2.3		2
79.1	VH3.30-DH4.4-JH4	V $\kappa$ 1.9-Kde V $\kappa$ 1-Kde <sup>a</sup>	V $\delta$ 2-D $\delta$ 3		V $\gamma$ 9-J $\gamma$ 2.3 V $\gamma$ 2-J $\gamma$ 2.3		6
80.1	VH1.8-DH2.8-JH4	V $\kappa$ 1.33-Kde V $\kappa$ 2.24-Kde					3
84.1					V $\gamma$ IV-J $\gamma$ 1.1/2.1 <sup>a</sup> V $\gamma$ IV-J $\gamma$ 1.1/2.1 <sup>a</sup> V $\gamma$ I-J $\gamma$ 1.3/2.3 <sup>a</sup> V $\gamma$ I-J $\gamma$ 1.3/2.3 <sup>a</sup>		4
85.1			V $\delta$ 2-D $\delta$ 3 D $\delta$ 2-D $\delta$ 3		V $\gamma$ 2-J $\gamma$ 2.3 V $\gamma$ I-J $\gamma$ 1.1/2.1 <sup>a</sup> V $\gamma$ I-J $\gamma$ 1.1/2.1 <sup>a</sup>		5
86.1	VH1.3-DH2.2-JH2						1
91.1			V $\delta$ 2-D $\delta$ 3				1
94.1		V $\kappa$ 4.1-Kde	V $\delta$ 2-D $\delta$ 3	V $\delta$ 2-J $\alpha$ 29			3
95.1	VH3.23-DH1.7-JH4						1
96.1		V $\kappa$ 2.30-Kde			V $\gamma$ 2-J $\gamma$ 2.3 V $\gamma$ 9-J $\gamma$ 2.3		3
97.1					V $\gamma$ I-J $\gamma$ 1.1/2.1 <sup>a</sup> V $\gamma$ I-J $\gamma$ 1.1/2.1 <sup>a</sup>		2
100.1	VH5.51-DH1.26-JH4 VH1.46-DH1.26-JH4	V $\kappa$ 2.30-Kde	V $\delta$ 2-D $\delta$ 3 V $\delta$ 2-D $\delta$ 3		V $\gamma$ 5-J $\gamma$ 2.3 V $\gamma$ I-J $\gamma$ 1.3/2.3 <sup>a</sup>		7
102.1	VH3-JH <sup>a</sup> VH3-JH <sup>a</sup>		V $\delta$ 2-D $\delta$ 3 V $\delta$ 2-D $\delta$ 3				4
103.1	VH2.26-DH6.13-JH6		D $\delta$ 2-D $\delta$ 3 D $\delta$ 2-D $\delta$ 3 V $\delta$ 2-D $\delta$ 3				4
104.1	VH6.1-JH4		V $\delta$ 2-D $\delta$ 3		V $\gamma$ 9-J $\gamma$ 2.3		3
106.1							0
108.1	VH2.26-DH6.13-JH4 VH3.13-DH3.10-JH4						2
110.1	VH3.23-JH6	Intron-Kde	V $\delta$ 2-D $\delta$ 3 V $\delta$ 2-D $\delta$ 3				4
113.1	VH3.43-DH3.3-JH4	Intron-Kde					2

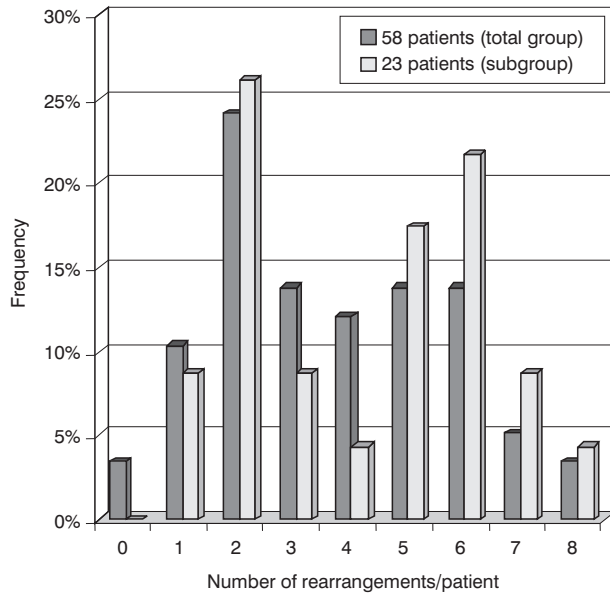
<sup>a</sup> Clonal rearrangements (based on heteroduplex analysis) not identified due to substantial proximity of migrating duplexes; it was impossible to cut the separate bands from the gel for further sequencing.

<sup>b</sup> Monoclonal rearrangements (based on heteroduplex analysis); V(D)J genes not unequivocally identified despite repeated sequencing.

rearrangements/patient was 3.6. In the case of the 23 patients (singleplex-multiplex approach), at least one clonal rearrangement was detected in 100% of the patients and at least two in 91% (21/23) of the patients. The number of rearrangements/patient ranged from 1 (2/23, 9%) to 8 (1/23, 4%), with a mode of rearrangements of 2 (6/23, 26%) and a mean of 4.2. The distribu-

tions of the number of rearrangements/patient detected in the groups of 58 and 23 patients are presented in Fig. 1.

In total, 179 rearrangements were identified, comprising 48 *IGH* rearrangements (37 VH-DH-JH and 11 VH-JH), 32 *IGK* rearrangements (23 V $\kappa$ -Kde and 9 Intron-Kde), 48 incomplete *TCRD* rearrangements (34



**Fig. 1.** Distribution of the number of rearrangements per patient detected in a total group of 58 patients (in a singleplex PCR approach) and in a subgroup of 23 patients (in a combined singleplex and multiplex PCR approach).

V $\delta$ 2-D $\delta$ 3 and 14 D $\delta$ 2-D $\delta$ 3), 11 *TCRD/A* rearrangements (6 V $\delta$ 2-J $\alpha$ 29, 3 V $\delta$ 2-D $\delta$ 3-J $\alpha$ 29, 1 V $\delta$ 2-J $\alpha$ 48, and 1 V $\delta$ 2-D $\delta$ 3-J $\alpha$ 48), 39 *TCRG* rearrangements, and 1 *TCRB* (D $\beta$ -J $\beta$ ) rearrangement. In Table 1 are listed the

Ig/TCR gene rearrangements found in the total group of patients.

#### Pattern of Ig/TCR gene rearrangements

The pattern of the frequencies of rearrangements detected in the individual Ig/TCR loci is presented in Table 2. The frequencies are depicted either for the total group of 58 patients (Ig/TCR loci screened in the singleplex approach) or for the subgroup of 23 patients (rearrangements detected in the multiplex approach, e.g. DH-JH, or in the combined singleplex-multiplex approach, e.g. total frequency for the *IGH* locus).

Moreover, the frequencies of variable, diversity, and joining genes in the rearrangements identified in the individual Ig/TCR loci were studied to determine the pattern of V(D)J utilization. The most relevant results follow. Rearrangements in the *IGH* locus most frequently involved the VH3, VH1, VH2, and VH4 family members in 40% (19/48), 21% (10/48), 15% (7/48), and 15% of the *IGH* rearrangements, respectively, DH2 (30%, 11/37), DH3 (24%, 9/37), and DH6 (19%, 7/37) family members, and JH4 (54%, 26/48), and JH6 (25%, 12/48) genes. Of the rearrangements in *IGK* locus, V $\kappa$ 1 (43%, 10/23) and V $\kappa$ 2 (39%, 9/23) family members were most frequently identified. In the *TCRD* locus, V $\delta$ 2-D $\delta$ 3 (71%, 34/48) rearrangements were predominantly identified over D $\delta$ 2-D $\delta$ 3 (29%, 14/48). In *TCRD/A* rearrangements, V $\delta$ 2 or V $\delta$ 2-D $\delta$ 3 genes were found to be rearranged with only two of the J $\alpha$  genes, J $\alpha$ 29 in

**Table 2.** The pattern of the frequency of the rearrangements detected in Ig/TCR loci in Polish B-cell precursor ALL patients

				Number of patients		Frequency of rearrangements
				subjected to PCR screening	with at least one rearrangement detected	
Ig	IGH	VH-(DH)-JH	58	43	74%	
		DH-JH	23	2	9%	
		total IGH	58 <sup>a</sup> , 23 <sup>b</sup>	43 <sup>a</sup> , 19 <sup>b</sup>	74% <sup>a</sup> , 83% <sup>b</sup>	
	IGK-Kde	V $\kappa$ -Kde	58	18	31%	
		Intron-Kde	58	9	16%	
		total IGK-Kde	58	24	41%	
Total Ig		58 <sup>a</sup> , 23 <sup>b</sup>	49 <sup>a</sup> , 21 <sup>b</sup>	84% <sup>a</sup> , 91% <sup>b</sup>		
TCR	TCRD	incomplete				
		V $\delta$ 2-D $\delta$ 3	58	26	45%	
		D $\delta$ 2-D $\delta$ 3	58	12	21%	
		total inc. TCRD	58	32	55%	
	V $\delta$ 2-J $\alpha$	V $\delta$ 2-(D $\delta$ 3)-J $\alpha$ 29	58	8	14%	
		V $\delta$ 2-(D $\delta$ 3)-J $\alpha$	23	5	22%	
		total V $\delta$ 2-J $\alpha$	58 <sup>a</sup> , 23 <sup>b</sup>	13 <sup>a</sup> , 8 <sup>b</sup>	22% <sup>a</sup> , 35% <sup>b</sup>	
		Total TCRD	58 <sup>a</sup> , 23 <sup>b</sup>	38 <sup>a</sup> , 18 <sup>b</sup>	66% <sup>a</sup> , 78% <sup>b</sup>	
	TCRG	V $\gamma$ -J $\gamma$	58	29	50%	
	TCRB	V $\beta$ -(D $\beta$ )-J $\beta$	23	2	9%	
D $\beta$ -J $\beta$		23	1	4%		
total TCRB		23	3	13%		
Total TCR		58 <sup>a</sup> , 23 <sup>b</sup>	47 <sup>a</sup> , 20 <sup>b</sup>	81% <sup>a</sup> , 87% <sup>b</sup>		
Total Ig/TCR		58 <sup>a</sup> , 23 <sup>b</sup>	56 <sup>a</sup> , 23 <sup>b</sup>	97% <sup>a</sup> , 100% <sup>b</sup>		

<sup>a, b</sup> Refer to groups of patients in whom particular rearrangements were screened for in either the singleplex or the combined singleplex-multiplex PCR approach.

**Table 3.** Comparison of Ig/TCR gene rearrangement patterns in Polish patients and other nationalities identified by the PCR-heteroduplex approach

Study	Country	Number of patients	Frequency of rearrangements in Ig/TCR loci [%]								
			VH-JH	DH-JH	IGH	IGK-Kde	incomplete TCRD	V $\delta$ 2-J $\alpha$	TCRD	TCRG	TCRB
Present study	Poland	58 <sup>a</sup> 23 <sup>b</sup>	<b>74<sup>a</sup></b>	<b>9<sup>b</sup></b>	83 <sup>b</sup>	41 <sup>a</sup>	55 <sup>a</sup> 45 <sup>a</sup> (V $\delta$ 2-D $\delta$ 3) 21 <sup>a</sup> (D $\delta$ 2-D $\delta$ 3)	35 <sup>b</sup>	78 <sup>b</sup>	<b>50<sup>a</sup></b>	13 <sup>b</sup>
van der Velden et al. [27]	The Netherlands	271	82	<b>24</b>	88	43	40 (V $\delta$ 2-D $\delta$ 3) 14 (D $\delta$ 2-D $\delta$ 3)	46	72	58	
Germano et al. [10]	Italy	53	77	<b>19</b>	83	42	62			62	
Meleshko et al. [13]	Belarus	67	78				63 51 (V $\delta$ 2-D $\delta$ 3) 36 (D $\delta$ 2-D $\delta$ 3)			45	
Barriga et al. [1]	Chile	32	<b>100</b>								
Scrideli et al. [17]	Brazil	79								<b>69</b>	
Szczepański et al. [20]	The Netherlands	339						41			
Brumpt et al. [4]	France	74 <sup>c</sup> 23 <sup>d</sup>			92 <sup>c</sup>		43 <sup>d</sup> (V $\delta$ 2-D $\delta$ 3)			66 <sup>c</sup>	
Cavé et al. [7]	France and Belgium	188					61			57	
van der Velden et al. [23]	The Netherlands	161									33

<sup>a, b, c, d</sup> Refer to groups of patients in whom particular rearrangements were screened for; frequencies depicted in bold mean that  $p < 0.05$  was obtained in the U-Gauss test.

82% (9/11) and J $\alpha$ 48 in 18% (2/11), of the rearrangements. In the *TCRG* locus the rearrangements most frequently involved V $\gamma$ I (59%, 23/39) and V $\gamma$ II (31%, 12/39) family member genes and J $\gamma$ 2.3 (59%, 23/39) gene.

#### Comparison of Ig/TCR rearrangement pattern in Polish patients and other nationalities

The frequencies of the rearrangements detected in individual Ig/TCR loci in the group of Polish patients were compared with the data obtained (using the PCR approach) in patients of other, mainly European, nationalities. The results of the analysis are presented in Table 3. The U-Gauss test was used to verify the statistical significance of the differences observed between the frequencies of the rearrangements in the studied group and those of other nationalities. The only statistically relevant differences ( $p$  values  $< 0.05$ ) were found between the incidence of DH-JH rearrangements in Polish (9%), Dutch (24%), and Italian patients (19%;  $p < 0.05$  in both tests), the frequency of VH-JH rearrangements in Polish (74%) and Chilean patients (100%;  $p < 0.05$ ), and the frequency of rearrangements in the *TCRG* locus in Polish (50%) and Brazilian patients (69%;  $p < 0.05$ ). In the remaining tests the  $p$  values were greater than 0.05.

## DISCUSSION

The current standards for MRD diagnostics in lymphoproliferative disorders are based on analyses per-

formed in patients from Western European countries. Thus the standard primers are aimed at the detection of the Ig and TCR gene rearrangements most frequently identified in these patients. However, there are data on inter-population diversity in the pattern of rearrangements and the existence of “population-specific” characteristics of the pattern [16, 17]. The impact of ethnic or socio-economic factors (e.g. earlier or greater exposure to bacterial and viral antigens) on the frequency of the rearrangements has been suggested [17]. Although this hypothesis still needs verification, it is beyond doubt that the determination of a specific Ig/TCR gene rearrangement pattern in a given population might have implications for the choice of primers for detecting the rearrangements. Therefore, this paper might be of particular interest for those aiming at introducing the European standards into molecular diagnostics of MRD.

Considerable convergence of the rearrangement pattern in Polish patients and those identified in European nationalities (mainly West Europeans) is demonstrated by comparison of the frequencies of the rearrangements in the Ig/TCR loci (Table 3). Except for the lower incidence of DH-JH rearrangements in the present study compared with those in Dutch [27] and Italian [10] patients (9% vs. 24% and 19%, respectively,  $p < 0.05$  in both tests), no statistically significant differences are observed between the rearrangement patterns in Polish and European patients. The lower frequency of DH-JH rearrangements detected in the group studied is rather unlikely to be Polish specific. It is most probably due to the fact that DH-JH rearrangements

**Table 4.** Frequency of detection of at least one and at least two clonal Ig/TCR gene rearrangements in Polish patients and in comparable studies of patients of other nationalities (based on the PCR-heteroduplex approach)

Study	Country	Number of patients	Rearrangements included in PCR screening								Frequency of rearrangements [%]		Mean number of rearrangements
			VH-JH	DH-JH	IGK-Kde	incomplete TCRD	Vδ2-Jα	complete TCRD	TCRG	TCRB	min 1	min 2	
Present study	Poland	58 <sup>a</sup>	+	-	+	+	+ <sup>a</sup>	-	+	-	97	86	3.6
		23 <sup>b</sup>	-	+	-	-	+ <sup>b</sup>	-	-	+	100	91	4.2
van der Velden et al. [27]	The Netherlands	271	+	+	+	+	+	-	+	-	>95	95	-
Germano et al. [10]	Italy	53	+	+	+	+	-	+	+	-	100	-	4
Szczepański et al. [21]	The Netherlands	89	+	+	+	+	-	-	+	-	98	-	4

- Relevant results were either not obtained or not reported in the cited papers.

<sup>a</sup> In the total group of 58 patients, Vδ2-Jα29 was the only Vδ2-Jα rearrangement screened for in the singleplex PCR approach.

<sup>b</sup> In the subgroup of 23 patients, Vδ2-Jα29 was screened for in the singleplex PCR approach; other Vδ2-Jα rearrangements were screened for in the multiplex-PCR approach.

Incomplete *TCRD* (Vδ2-Dδ3, Dδ2-Dδ3), complete *TCRD* (Vδ1-Jδ1, Vδ2-Jδ1, Dδ2-Jδ1).

are detected in BCP-ALL patients at a relatively low frequency combined with the small group of patients in whom these rearrangements were studied (a subgroup of 23 patients). We interpret the frequency of *TCRB* rearrangements, in the present study lower (though not significantly) than that reported for Dutch patients [23], similarly. In contrast, the statistically significant difference found between the frequencies of rearrangements in the *TCRG* locus in Polish and Brazilian patients might support the hypothesis by Scrideli et al. [17] of the socio-economic/ethnic-based diversity of rearrangements patterns. However, more studies with patients of different populations (from both “well-developed” and “low-resources” countries) need to be conducted to unequivocally determine the impact of these factors on the rearrangement patterns.

To provide more detailed insight into the pattern of Ig/TCR gene rearrangements in Polish patients, we also analyzed utilization of the VH, DH, JH, Vκ, Jα, Vγ, Jγ genes in the identified rearrangements and the profiles of Vκ-Kde vs. intron-Kde and Vδ2-Dδ3 vs. Dδ2-Dδ3 rearrangements. Comparison of our results with published data further confirms the convergence of the rearrangement patterns of Polish patients and other populations [2, 3, 5, 6, 8, 12, 13, 19, 20, 27–29, 32].

Moreover, to provide information potentially useful for the design of a cost-effective MRD diagnostic approach, we evaluated the efficiency of detecting Ig/TCR gene rearrangements based on the singleplex PCR approach and combined singleplex-multiplex approach. Our data show that use of exclusively singleplex PCRs (encompassing a broad gene rearrangement panel: complete *IGH*, *IGK*-Kde, Vδ2-Dδ3, Dδ2-Dδ3, Vδ2-Jα29, and *TCRG*) enables detection of at least one clonal rearrangement in 97% of BCP-ALL patients. Application of additional multiplex reactions (for incomplete *IGH*, Vδ2-Jα, and *TCRB* rearrangements)

results in the detection of at least one rearrangement in 100% of patients. The mean number of rearrangements detected per patient reaches 3.6 in the singleplex approach and 4.2 in the singleplex-multiplex approach. Our results are in concordance with those reported by other groups analyzing a comparably broad panel of rearrangements (Table 4).

However, it should be stressed that according to current standards for MRD diagnostics [24, 25], 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 due to secondary and ongoing Ig/TCR rearrangements and clonal evolution (described elsewhere) [21]. Our data show that at least two clonal rearrangements are detected in 86% of patients and in 91% of patients using the singleplex and singleplex-multiplex approaches, respectively. In a study by van der Velden et al. [27], at least two MRD markers were detected in 95% of patients in a combined singleplex-multiplex approach. This is more than the frequency detected in the present study, which might be because the cohort of Dutch patients substantially outnumbered that of the Polish patients (a subgroup of 23 patients for whom the combined singleplex-multiplex approach was used).

Detection of at least one clonal rearrangement in nearly 100% of patients and at least two rearrangements in 86% of patients (with the singleplex approach) contrasted with the efficiency obtained in the singleplex-multiplex approach (100 and 91%, respectively) indicates the need for MRD diagnostics based on a broad panel of rearrangements (detected preferably with both the singleplex and multiplex approach). However, if cost is a concern, it might be reasonable to perform singleplex PCRs (encompassing a broad gene rearrangement panel) as the basic PCR screening for all patients and, in cases of unsatisfactory results of Ig/TCR-MRD marker



selection, to broaden the PCR screening with multiplex PCRs.

In conclusion, the considerable convergence in the rearrangements patterns (frequencies of rearrangements and profiles of V(D)J gene utilization) demonstrated in Polish and European patients, including West European nationalities, indicates no Polish-specific characteristics of the rearrangement pattern. This enables us to conclude that in the case of Polish BCP-ALL patients, the strategy for detecting Ig/TCR gene rearrangements based on standard primers and protocols might be directly applied in the molecular diagnostics of MRD. Comparison of the efficiencies of detecting Ig/TCR rearrangements in a given population with singleplex and singleplex-multiplex PCR might be helpful in choosing a cost- and labor-effective Ig/TCR-PCR screening approach. It might be reasonable for other countries aiming to introduce European MRD-standards to perform similar feasibility studies of the standard primers and protocols in their particular populations of patients to identify or exclude population-specific characteristics of Ig/TCR rearrangement patterns and design an optimal diagnostic setting for MRD analysis.

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