

Comparative analysis of Ig and TCR gene rearrangements at diagnosis and at relapse of childhood precursor-B-ALL provides improved strategies for selection of stable PCR targets for monitoring of minimal residual disease

Tomasz Szczepański, Marja J. Willemsse, Bas Brinkhof, Elisabeth R. van Wering, Mirjam van der Burg, and Jacques J. M. van Dongen

Immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements are excellent patient-specific polymerase chain reaction (PCR) targets for detection of minimal residual disease (MRD) in acute lymphoblastic leukemia (ALL), but they might be unstable during the disease course. Therefore, we performed detailed molecular studies in 96 childhood precursor-B-ALL at diagnosis and at relapse ($n = 91$) or at presumably secondary acute myeloid leukemia ($n = 5$). Clonal Ig and TCR targets for MRD detection were identified in 94 patients, with 71% of these targets being preserved at relapse. The best stability was found for *IGK-Kde* rearrange-

ments (90%), followed by *TCRG* (75%), *IGH* (64%), and incomplete *TCRD* rearrangements (63%). Combined Southern blot and PCR data for *IGH*, *IGK-Kde*, and *TCRD* genes showed significant differences in stability at relapse between monoclonal and oligoclonal rearrangements: 89% versus 40%, respectively. In 38% of patients all MRD-PCR targets were preserved at relapse, and in 40% most of the targets ($\geq 50\%$) were preserved. In 22% of patients most targets (10 cases) or all targets (10 cases) were lost at relapse. The latter 10 cases included 4 patients with secondary acute myeloid leukemia with germline Ig/TCR genes. In 5 other

patients additional analyses proved the clonal relationship between both disease stages. Finally, in 1 patient all Ig/TCR gene rearrangements were completely different between diagnosis and relapse, which is suggestive of secondary ALL. Based on the presented data, we propose stepwise strategies for selection of stable PCR targets for MRD monitoring, which should enable successful detection of relapse in most (95%) of childhood precursor-B-ALL. (Blood. 2002;99:2315-2323)

© 2002 by The American Society of Hematology

Introduction

Several large prospective studies have clearly demonstrated the high prognostic value of minimal residual disease (MRD) monitoring in childhood acute lymphoblastic leukemia (ALL).¹⁻⁴ Based on the sensitive measurement of early response to cytotoxic treatment, it is currently possible to identify not only patients at high risk for relapse but also a group of low-risk patients with an excellent relapse-free survival of more than 95%.⁴ Hence, MRD information provides a new definition of remission in childhood ALL, which justifies incorporation of MRD data in current treatment protocols to refine risk assignment.⁵

Most MRD studies in pediatric precursor-B-ALL applied immunoglobulin (Ig) and/or T-cell receptor (TCR) gene rearrangements as polymerase chain reaction (PCR) targets for MRD detection. They can easily be identified in most patients at diagnosis with limited sets of PCR primers.^{6,7} Moreover, using these molecular targets, sensitivities of 10^{-4} to 10^{-6} (1 malignant cell within 10^4 to 10^6 normal cells) can be obtained routinely.⁷ With the advent of novel real-time quantitative (RQ) PCR techniques, Ig/TCR-based MRD techniques are now also quantitative.⁸⁻¹¹ However, it is also known that Ig and TCR gene rearrangements might change during the disease course, owing to secondary rearrangement processes mediated via ongoing activity of the V(D)J recombinase enzyme system (reviewed by Szczepański et

al¹²). This might lead to loss of the PCR target and consequently to false-negative MRD results. Such changes were most frequently described for Ig heavy chain genes (*IGH*) and to lesser extent for TCR genes and were found particularly in cases of precursor-B-ALL that already contained subclones at diagnosis.¹³⁻¹⁸

Although the presence of clonal evolution phenomena is widely acknowledged, its actual impact on the effectiveness of MRD monitoring has not been defined. So far, studies assessing the stability of Ig and TCR gene rearrangements at diagnosis and relapse of ALL either did not compare junctional region sequences or were limited to particular gene loci.¹³⁻¹⁸ Therefore, we studied the stability of the currently used Ig/TCR rearrangements (*IGH*, Ig κ light chain [*IGK*], TCR γ [*TCRG*], and TCR δ [*TCRD*] gene rearrangements) in a large series of 96 childhood precursor-B-ALL patients. This information is essential for reliable selection of MRD-PCR targets with minimal chance of false-negative results.

Patients, materials, and methods

Patients

Bone marrow or peripheral blood samples from 96 childhood precursor-B-ALL patients were obtained at initial diagnosis and at relapse (91 patients)

From the Department of Immunology, University Hospital Rotterdam/Erasmus University Rotterdam, The Netherlands; Department of Pediatric Hematology and Chemotherapy, Silesian Medical Academy, Zabrze, Poland; and Dutch Childhood Leukemia Study Group, The Hague, The Netherlands.

Submitted July 3, 2001; accepted November 20, 2001.

Supported by the Dutch Cancer Society/Koningin Wilhelmina Fonds (grants SNWLK 97-1567 and SNWLK 2000-2268).

Reprints: Jacques J. M. van Dongen, Dept of Immunology, Erasmus University Rotterdam, PO Box 1738, 3000 DR Rotterdam, The Netherlands; e-mail: vandongen@immu.fgg.eur.nl.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

© 2002 by The American Society of Hematology

or at presumably secondary acute myeloid leukemia (AML) (5 patients). The age distribution ranged from 1 month to 183 months. Eight children (8%) were infants (age < 1 year). The diagnosis of precursor-B-ALL was made according to French-American-British and standard immunophenotypic criteria.¹⁹⁻²¹ Immunologic marker analysis of the precursor-B-ALL revealed that 6 (6%) were pro-B-ALL, 59 (61%) were common ALL, and 31 (32%) were pre-B-ALL. Seven patients were studied at 2 subsequent leukemia relapses. Cell samples of 52 patients were obtained from the cell bank of the Dutch Childhood Leukemia Study Group.

Comparative immunophenotypic analysis revealed intralineage switches in 21% (18 of 86) of precursor-B-ALL patients with available detailed immunophenotypic data at relapse, which is slightly higher than reported previously.²²

The rationale, methodology, and pitfalls of the stepwise molecular comparison of the Ig/TCR gene rearrangements between diagnosis and relapse of precursor-B-ALL were previously exemplified in the case report of patient 5498, also included in these series.²³ A small subgroup of patients (n = 21) was studied before by comparative Southern blotting and PCR analysis of κ -deleting element (Kde) rearrangements at diagnosis and relapse.²⁴

Comparative Southern blot analysis

Mononuclear cells were separated from bone marrow or peripheral blood samples by Ficoll-Paque centrifugation (density 1.077 g/cm³; Pharmacia, Uppsala, Sweden). DNA was isolated from frozen mononuclear cells, digested with the *Bgl*III enzyme, and blotted to nylon membranes as described previously.²⁵ *IGH* and *IGK* gene configurations were analyzed with the *IGHJ6*, *IGKJ5*, *IGKC*, and *IGKDE* probes (DAKO, Carpinteria, CA).^{26,27} The configuration of the *TCRD* genes was analyzed with the *TCRDJ1* probe (DAKO).²⁸ The diagnosis and relapse samples of the 96 patients were always run in parallel lanes (Figure 1), except for 6 patients who were exclusively analyzed at diagnosis because of insufficient amounts of DNA from relapse samples. The Southern blot configuration of the Ig

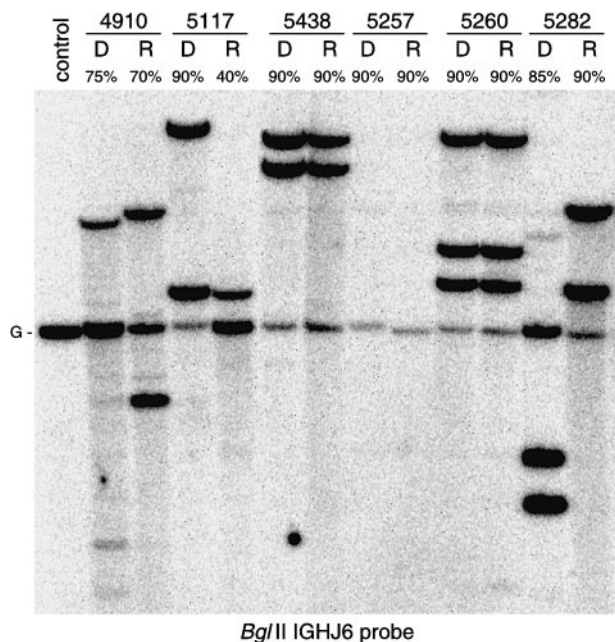


Figure 1. Comparative Southern blot analysis of *IGH* gene configuration in 6 precursor-B-ALL patients. Monoclonal patients 5438 and 5260 (patient with trisomy 14) show identical gene rearrangements at diagnosis and at relapse. In monoclonal patient 5117, one allele at relapse is deleted and another one is preserved. Patient 5257 demonstrates biallelic *IGH* deletion at both disease stages. Oligoclonal patient 4910 and monoclonal patient 5282 (the upper weak band in the diagnosis lane is derived from previous hybridization) show completely changed *IGH* gene rearrangement patterns. While sequence analysis has proven a clonal relationship between diagnosis and relapse in patient 4910, patient 5282 most likely represents a secondary ALL.

and/or TCR genes in 73 patients at diagnosis and in 30 patients at relapse has been reported previously.^{6,15}

PCR amplification and comparative heteroduplex analysis of PCR products

Independent of Southern blotting, PCR analysis could be performed on both diagnosis and relapse samples in 89 patients, essentially as described previously.^{7,29} Four patients with a secondary AML and 3 precursor-B-ALL patients with insufficient remaining DNA at diagnosis were not analyzed. In each 50 μ L PCR reaction, 50 ng DNA sample, 6.3 pM of the 5' and 3' oligonucleotide primers, and 0.5 U AmpliTaq Gold polymerase (PE Biosystems, Foster City, CA) were used. The sequences of the 25 oligonucleotide primer pairs used for amplification of *IGH* (5 VH family-specific framework 1 primers and 7 DH family-specific primers with consensus JH primer), *IGK*-Kde (4 V κ family-specific primers and intron recombination signal sequence [RSS] primer with Kde primer), *TCRG* (6 V γ -J γ primer combinations most frequently used in precursor-B-ALL), and *TCRD* gene rearrangements (V δ 2-D δ 3 and D δ 2-D δ 3 primer pairs) were previously published.^{7,30,31} PCR conditions were as follows: initial denaturation for 10 minutes at 94°C, followed by 40 cycles of 45 seconds at 92°C, 90 seconds at 60°C, and 2 minutes at 72°C using a Perkin-Elmer 480 thermal cycler (PE Biosystems). After the last cycle an additional extension step of 10 minutes at 72°C was performed. Appropriate positive and negative controls were included in all experiments.⁷

For heteroduplex analysis, the PCR products were denatured at 94°C for 5 minutes after the final cycle of amplification and subsequently cooled to 4°C for 60 minutes to induce duplex formation.³² Afterward the duplexes were immediately loaded on 6% nondenaturing polyacrylamide gels in 0.5 \times Tris-borate-EDTA buffer, run at room temperature, and visualized by ethidium bromide staining.³²

Relapse samples were at first analyzed with those primer combinations, which showed clonal PCR products at diagnosis. When the clonal PCR product was also found at relapse, its identity was subsequently compared with the PCR product found at diagnosis by means of mixed heteroduplex analysis, ie mixing of the diagnosis and relapse PCR products followed by heteroduplex analysis (Figure 2).²³ When clonal PCR products found at diagnosis were undetectable at relapse, the relapse sample was then analyzed with additional primer combinations for the involved gene loci.

Sequence analysis of Ig/TCR gene rearrangements

Clonal PCR products discordant between diagnosis and relapse of precursor-B-ALL as found by mixed heteroduplex analysis were directly sequenced. Sequencing was performed using the dye-terminator cycle sequencing kit with AmpliTaq DNA polymerase FS on an ABI 377 sequencer (PE Biosystems) as previously described.³¹ VH, DH, and JH segments were identified using DNAPLOT software (W. Müller, H.-H. Althaus, University of Cologne, Germany) by searching for homology with all known human germline VH, DH, and JH sequences obtained from the VBASE directory of human Ig genes (<http://www.mrc-cpe.cam.ac.uk/imt-doc/>).³³ V γ and J γ gene segments were identified by comparison to germline *TCRG* sequences as previously described.³⁴

Statistical analysis

Statistical analysis using the χ^2 test on a 2 \times 2 table was performed to compare the frequencies of particular Ig/TCR gene rearrangements between different precursor-B-ALL patient subgroups. Pearson correlation coefficient was calculated to test an association between variables. *P* values less than or equal to .05 were considered statistically significant.

Results

Southern blot configuration of Ig and TCR genes in relapsed ALL patients

The configuration of *IGH*, *IGK*, and *TCRD* genes was established with multiple Southern blot probes. This concerned all 96 patients

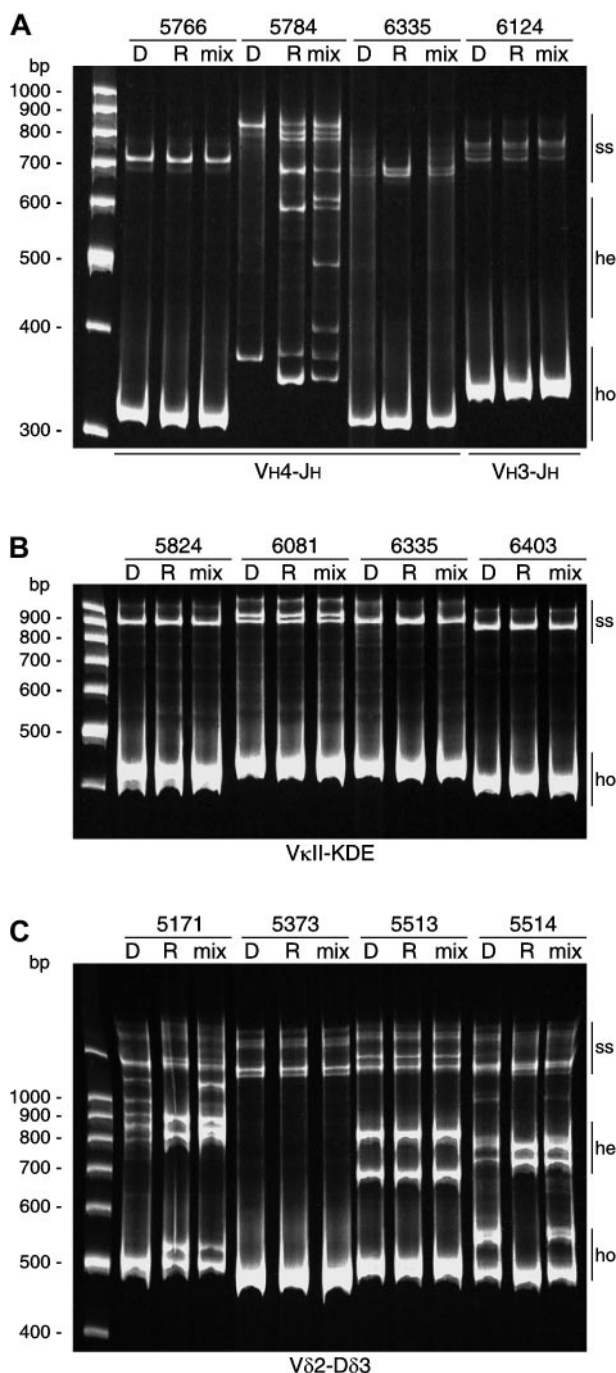


Figure 2. Examples of comparative heteroduplex PCR analysis. (A) Comparative heteroduplex analysis of *IGH* gene rearrangements. Monoallelic homoduplexes (ho) in patients 5766, 6335, and 6124 found at diagnosis and at relapse were of the same size. Mixing of the PCR products of these disease phases followed by heteroduplex PCR analysis demonstrated no heteroduplex (he) formation, proving that these gene rearrangements had identical junctional regions. In patient 5784, monoallelic homoduplexes found at diagnosis and at relapse slightly differed in size. Mixing of the VH4-JH PCR products followed by heteroduplex PCR analysis demonstrated clear heteroduplex formation, proving that these VH4-JH gene rearrangements had different junctional regions; (ss) indicates remaining single-strand fragments. (B) Comparative heteroduplex analysis of Kde rearrangements showed completely identical rearrangements at diagnosis and at relapse. (C) Comparative heteroduplex analysis of Vδ2-Dδ3 gene rearrangements. Patients 5373 and 5513 with monoallelic and biallelic rearrangements, respectively, had stable Vδ2-Dδ3 rearrangements. In contrast, Vδ2-Dδ3 joinings at diagnosis in patients 5171 and 5514 are oligoclonal, while in both cases 2 monoallelic Vδ2-Dδ3 rearrangements were found at relapse.

Table 1. Comparison of Ig and TCR gene rearrangement patterns based on Southern blotting

	Random precursor B-ALL at diagnosis*	Relapsed precursor-B-ALL at diagnosis†	Precursor-B-ALL at relapse
<i>IGH</i>			
Germline	0	0	0
R/G	1% (1/97)	2% (2/92)	5% (4/85)
R/R	88% (85/97)	88% (81/92)	85% (72/85)
R/D	8% (8/97)	7% (6/92)	7% (6/85)
D/G	2% (2/97)	1% (1/92)	1% (1/85)
D/D	1% (1/97)	1% (1/92)	2% (2/85)
Oligoclonal	36% (35/97)	42% (38/92)‡	28% (24/85)§
<i>IGK-Kde</i>			
Germline	50% (55/111)	51% (47/92)	49% (42/86)
R/G	28% (31/111)	21% (19/92)	23% (20/86)
R/R	23% (25/111)	28% (26/92)	28% (24/86)
Oligoclonal	Not determined	3% (3/92)	1% (1/86)
<i>TCRG</i>			
Germline	41% (79/192)	38% (35/91)	41% (37/91)
R/G or R/R	59% (113/192)	62% (56/91)	59% (54/91)
<i>TCRD</i>			
Germline	11% (22/202)	8% (7/91)	12% (10/85)
R/G	15% (30/202)	11% (10/91)	8% (7/85)
R/R	23% (47/202)	24% (22/91)	12% (10/85)
R/D	17% (35/202)	20% (18/91)	22% (19/85)
D/G	5% (11/202)	4% (4/91)	5% (4/85)
D/D	28% (57/202)	33% (30/91)	41% (35/85)
Oligoclonal	21% (13/62)	26% (24/91)	12% (10/85)

*The frequencies of particular Ig/TCR gene rearrangements in the random precursor-B-ALL group at diagnosis are derived from our previous studies.^{6,27,46}

†Including one patient with phenotypic switch to AML at relapse.

‡The frequency of *IGH* oligoclonality was significantly higher in infant ALL compared with noninfant ALL (75% vs 38%, $P < .05$).

§Not significant.

|| $P < .05$.

at diagnosis of precursor-B-ALL and 91 patients at subsequent relapse or secondary AML (Figure 1). This gave us the unique opportunity to address the question of whether there are any differences in Ig/TCR gene configuration between the patients who relapsed compared with the total childhood precursor-B-ALL group. This comparison is summarized in Table 1, which shows that the Ig/TCR gene rearrangement patterns at diagnosis and at relapse in patients included in this study are largely comparable to each other and to previously published data derived from large series of random childhood precursor-B-ALL cases at initial diagnosis.^{6,27} Only 2 immunogenotypic features were more prevalent in ALL at relapse. The *TCRD* gene configuration at relapse was characterized by significantly less frequent Vδ2-Dδ3 and Dδ2-Dδ3 rearrangements and more frequent *TCRD* deletions ($P < .05$), which reflects ongoing deletional rearrangements. Secondly, the frequency of *IGH* and *TCRD* oligoclonality at relapse was slightly less frequent, but this difference was only significant for *TCRD* ($P < .05$).

The configuration of Ig/TCR genes compared between 2 subsequent relapses in the 7 patients analyzed showed evidence for clonal evolution in only 2 cases (concerning 1 or 2 gene rearrangements), while in 5 of the 7 cases we observed some changes in gene rearrangement patterns between diagnosis and first relapse.

Southern blot analysis in 4 of 5 patients with a presumed secondary AML demonstrated the complete absence of clonal Ig/TCR gene rearrangements (Figure 3), which is in line with the AML diagnosis. However, in one patient (3991) 3 clonal Southern blot bands were preserved in the AML clone, and the sequence identity was confirmed by comparative PCR analysis for 2 VH-JH

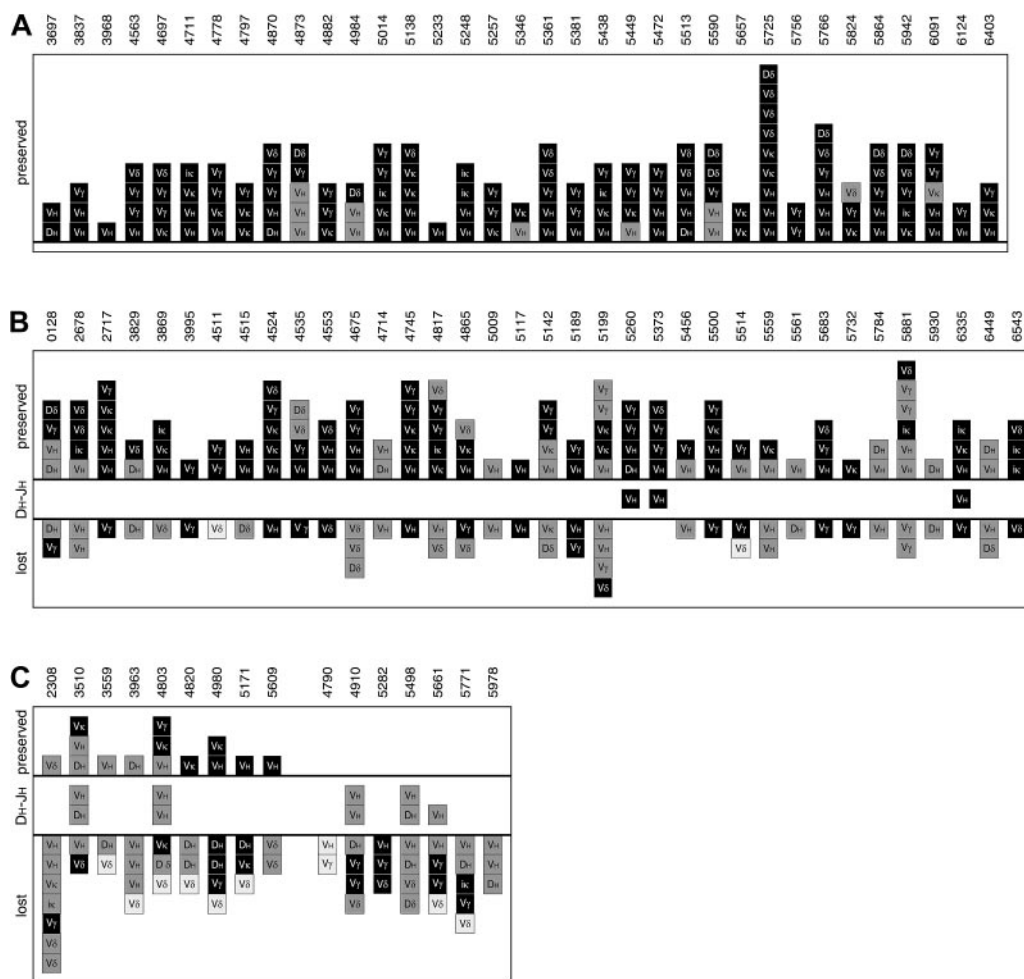


Figure 4. Ig/TCR gene rearrangement stability patterns in 87 precursor-B-ALL patients subjected to comparative heteroduplex PCR analysis at diagnosis and at relapse. Black squares represent monoclonal rearrangements; gray squares, biconal/oligoclonal rearrangements; white squares, oligoclonal/polyclonal rearrangements detectable as weak Southern blot bands. All squares above the upper line represent stable MRD-PCR targets, while all squares below the lower line represent Ig/TCR gene rearrangements lost owing to clonal evolution. VH squares between the 2 lines have common DH-JH stems shared by different *IGH* rearrangements at diagnosis and at relapse. (A) Thirty-five patients with all PCR-MRD targets identified at diagnosis preserved at relapse; (B) 36 cases with some targets (ranging from one target to half of the targets) lost during disease course; (C) 16 patients with most or all clonal markers absent at relapse. Patient 4790 was not included in our evaluation of MRD-PCR target stability because the detected *IGH* and *TCRG* gene rearrangements at diagnosis were oligoclonal. VH indicates VH-JH gene rearrangements; DH, DH-JH gene rearrangements; V_k, V_k-K_de gene rearrangements; ik, intronRSS-K_de gene rearrangements; V_γ, V_γ-J_γ gene rearrangements; V_δ, V_δ2-D_δ3 gene rearrangements; D_δ, D_δ2-D_δ3 gene rearrangements.

relationship between diagnosis and relapse was confirmed by the identification of a common DH-JH stem shared by respective VH-JH gene rearrangements. In another 2 cases additional analyses^{35,36} showed at both disease stages identical DNA sequences of V_k-J_k (5771) and chimeric *MLL-AF4* fusion genes (5978), respectively (data not shown). Finally, in one patient (5282) there was no evidence for a common origin of diagnosis and relapse clones (Figures 1 and 4C). Because all original monoclonal rearrangements were lost in this case at relapse, we believe that this is in fact a secondary ALL.

Stability of MRD targets is not related to age, blood cell counts, or remission duration

Stability of MRD-PCR targets did not significantly correlate with age or white blood cell count at diagnosis or with remission duration, ie, time span between diagnosis and relapse.

IGH gene rearrangements

Clonal *IGH* gene rearrangements were detected by PCR in 75 of the 90 studied childhood precursor-B-ALL patients. In 66 cases

(88%) at least one *IGH* MRD-PCR target was preserved at relapse. In 12 additional patients PCR analysis did not result in identification of clonal *IGH* rearrangements, while Southern blot data suggested the preservation of at least one target in all 12 patients (fully identical *IGH* configuration in 9 cases). In 3 patients deletions of JH region were identified by Southern blotting both at diagnosis and at relapse of ALL. The Southern blot results of these 15 patients were not used for calculating the stability of the *IGH* PCR targets.

The stability of the *IGH* PCR targets was markedly different between oligoclonal and monoclonal patients, ie, at least one MRD-PCR target was preserved in 76% and 98% of patients, respectively (Table 2). This significant difference was even more pronounced at the MRD-PCR target level, with 63 (85%) of 74 monoclonal *IGH* gene rearrangements being stable compared with only 36 (44%) of 85 oligoclonal rearrangements. Taking into account the type of *IGH* gene rearrangement, complete VH-JH recombinations were characterized by a higher stability compared with incomplete DH-JH rearrangements, with 69% versus 43% of targets preserved, respectively.

IGK deletional rearrangements

A total of 60 *IGK* deletional rearrangements were detected in 40 childhood precursor-B-ALL patients at diagnosis: 44 V κ -Kde, 15 intron-Kde, and 1 rarely occurring V κ -intron RSS recombination. At least one of the rearrangements was preserved in 37 cases (93%). In fact, *IGK*-Kde recombinations represented the most stable MRD-PCR targets, with 90% of all targets preserved. Most (55 of 60) Kde rearrangements were monoclonal and highly stable (52 targets preserved; 95%), while only 2 of 5 oligoclonal *IGK*-Kde targets were found at relapse. No significant difference in stability was found between V κ -Kde and intron-Kde rearrangements.

TCRG gene rearrangements

A total of 87 *TCRG* gene rearrangements were detected in 53 precursor-B-ALL patients at diagnosis, and in 44 cases (83%) at least one MRD-PCR target was preserved at relapse. Because accurate oligoclonality detection in *TCRG* locus is rather complex, even by Southern blotting,³⁷ we did not evaluate whether there were any differences in MRD-PCR target stability between monoclonal and oligoclonal patients.

TCRD gene rearrangements

A total of 60 clonal *TCRD* gene rearrangements (43 V δ 2-D δ 3, 16 D δ 2-D δ 3, and 1 V δ 3-J δ 1) were identified by PCR in 39 precursor-B-ALL patients. At least one of the clonal rearrangements was preserved in 27 (69%) of 39 cases. Once again we observed striking differences in stability between monoclonal and oligoclonal patients, ie, at least one target was preserved in 88% and 36% of patients, respectively (Table 2). Only 26% of oligoclonal targets were preserved compared with 86% of monoclonal targets. Moreover, in 10 patients Southern blot data suggested the presence of a clonally rearranged band corresponding to V δ 2-D δ 3 rearrangements, while heteroduplex PCR analysis of these rearrangements showed oligoclonality or even polyclonality.⁶

Patterns of clonal evolution in precursor-B-ALL patients with unstable targets

Based on combined Southern blot, PCR, and sequence analysis, it was possible to follow the patterns of clonal evolution leading to disappearance of rearrangements that were originally present at diagnosis.

Clonal evolution in the *IGH* locus

Owing to clonal evolution phenomena, 62 *IGH* targets in 36 patients were lost. We could determine the exact patterns of clonal evolution in 8 patients with monoclonal *IGH* gene rearrangements and in 26 patients with an oligoclonal rearrangement pattern.

In 7 patients (2 monoclonal and 5 oligoclonal) Southern blot rearrangement patterns between diagnosis and relapse were identical, while PCR analyses showed disappearance of a single rearrangement. This might be explained by the disappearance of minor subclones undetectable by Southern blotting. In 3 patients with monoclonal *IGH* configuration, one of the rearrangements was changed both in Southern blotting and PCR, while sequence comparison showed VH replacement with a preserved VH-N-DH-N-JH junction. In another 2 monoclonal patients we observed clonal "regression" of one of the rearrangements to germline. Finally, in a single patient (5282) both monoclonal *IGH* rearrange-

ments were replaced by 2 new unrelated rearrangements; this patient was suspected of having developed a secondary ALL (Figure 4C).

In 11 oligoclonal patients, the *IGH* configuration at relapse was monoclonal, which is suggestive of clonal selection during the treatment. In another 10 oligoclonal patients, *IGH* was also oligoclonal at relapse, with several rearrangements lost and with emergence of new (sub)clones. Sequence comparison was fully completed in 12 oligoclonal patients with changes at relapse, indicating ongoing VH to DH-JH joinings with preservation of common DH-JH stems in 5 patients and possibly secondary rearrangements in 5 other cases. In the remaining 2 oligoclonal patients, the *IGH* sequences were unrelated and suggestive of the development of diagnosis and relapse clones from a common clonal progenitor via independent secondary rearrangements.

Clonal evolution in the *TCRG* locus

Clonal evolution of *TCRG* gene rearrangements was observed in 19 patients, leading to loss of 22 MRD-PCR targets. In 9 patients this concerned "regression" of clonal rearrangements most probably to germline configuration. In 5 patients the new rearrangements at relapse contained upstream V γ and downstream J γ segments as compared with the V γ -J γ rearrangements at diagnosis, which is suggestive of ongoing recombination with V γ -J γ replacement. In the remaining 5 patients the sequence comparison of V γ -J γ rearrangements at diagnosis and at relapse excluded secondary rearrangements and indicated the emergence of a clone related to the initial (pre)leukemic clone but different from the predominant clone at diagnosis.

Clonal evolution in the *TCRD* locus

Clonal evolution in the *TCRD* locus resulted in the loss of 22 MRD-PCR targets in 17 patients (5 monoclonal and 12 oligoclonal cases). In 6 patients (including 4 patients with monoclonal V δ 2-D δ 3 rearrangements) ongoing deletions were observed. In contrast, in 8 patients (including 1 patient with monoclonal V δ 2-D δ 3) the rearrangement pattern "regressed" to germline configuration. Finally, in the remaining 3 cases new rearrangements were found at relapse, including 1 V δ 2-D δ 3 joining and 2 unidentified rearrangements to the D δ 3-J δ 1 region. Interestingly, 5 of 10 cases with oligoclonal/polyclonal V δ 2-D δ 3 rearrangements at diagnosis had a monoclonal *TCRD* configuration at relapse with a single V δ 2-D δ 3 joining. In the remaining 5 patients, ongoing *TCRD* deletion was assumed in 2 cases, 2 patients demonstrated "regression" to germline configuration, and only a single patient preserved the oligo/polyclonal V δ 2-D δ 3 rearrangement pattern at relapse.

Discussion

Our comparative Southern blot, PCR, and sequencing analyses of childhood precursor-B-ALL at diagnosis and relapse have provided detailed insight in the stability and changes of Ig and TCR gene rearrangements during the disease course. This information is essential for reliable application of Ig/TCR gene rearrangements as MRD-PCR targets in childhood ALL. However, one should be cautious with extrapolating these data to adolescent or adult precursor-B-ALL patients, because the immunogenotype of adult precursor-B-ALL has more immature features.²⁹

The Ig/TCR gene rearrangement patterns at diagnosis in relapsed patients appeared to be comparable to those in a random series of newly diagnosed pediatric precursor-B-ALL, implying that the various Ig/TCR gene characteristics at diagnosis have no prognostic value. This is in contrast to the previously reported strong predictive value of the V δ 2-D δ 3 gene rearrangement or the overall clonal diversity.^{38,39} The overall Ig/TCR gene configuration patterns at relapse were largely comparable to those at diagnosis but were characterized by less oligoclonality and more frequent *TCRD* gene deletions. These 2 differences fit with the concept of ongoing clonal selection and continuing rearrangements.

The detailed molecular analyses proved the clonal relationship between diagnosis and relapse in 88 of 89 childhood precursor-B-ALL with identified MRD-PCR markers at diagnosis. In only one patient (5282) were the Ig/TCR gene rearrangement patterns at diagnosis and relapse completely different with unrelated junctional region sequences (Figures 1 and 4C). Consequently, the presumed ALL relapse in this child (3.5 years after diagnosis) might in fact represent a secondary leukemia. This single patient confirms previous observations that ALL rarely occurs as second malignancy after previous cytotoxic treatment.⁴⁰ This is in contrast to secondary AML, which affects approximately 4% of children treated for ALL with cytotoxic regimens containing topoisomerase II inhibitors.⁴¹ We indeed proved the presence of secondary AML with germline Ig/TCR genes in 4 (4%) of the 96 patients (Figure 3). However, in a fifth patient we demonstrated the clonal relationship between the precursor-B-ALL at diagnosis and the presumed secondary AML, which in fact represented a phenotypic switch.

The comparison of Ig/TCR gene rearrangement patterns between diagnosis and relapse showed marked heterogeneity in the occurrence of clonal evolution phenomena. In 40% of patients all PCR-identified clonal Ig/TCR rearrangements were present at relapse (Figure 4A), and in another 42% of cases at least half of the identified gene rearrangements remained stable at relapse (Figure 4B). Extreme clonal evolution with differential outgrowth of subclones characterized the remaining 18% of cases (Figure 4C), in whom most or even all clonal Ig/TCR gene rearrangements identified at diagnosis were lost during disease course. Interestingly, in contrast to the frequent occurrence of clonal evolution between diagnosis and relapse, we did not observe major clonal instability of Ig/TCR genes between 2 consecutive relapses (7 cases), which is in line with previous observations.¹⁴

Previous studies suggested that the risk of changes in Ig/TCR rearrangement patterns increases with time.^{15,42} We did not find a significant correlation between remission duration and target stability in this extensive study. This is in line with the report that clonal selection processes can already occur in early treatment phases and the reports on clonal identity between diagnosis and very late relapse of precursor-B-ALL.⁴³⁻⁴⁵

In this extensive molecular study, we wished to identify the factors associated with the occurrence of clonal evolution and therefore the increased risk of false-negative MRD-PCR results. It is entirely clear from our study that discrimination between monoclonality versus oligoclonality at diagnosis is the most powerful predictor of clonal evolution during the ALL disease course. All other variables, such as age, white blood cell count, and immunophenotype at diagnosis, failed to identify patients prone to clonal evolution of their Ig/TCR gene rearrangements. Monoclonal MRD-PCR targets were characterized by high stability, with 89% of all targets detectable at relapse. In contrast, only 40% of the oligoclonal MRD-PCR targets were preserved at relapse. Therefore, it is probably important to discriminate between monoclonal

and oligoclonal Ig/TCR rearrangements, which requires a combined Southern blot and PCR approach. Southern blotting is particularly informative for detection of oligoclonality in *IGH* and *IGK* gene rearrangements, whereas heteroduplex PCR analysis in combination with Southern blotting is informative for detection of oligoclonal *TCRD* gene rearrangements. Southern blotting needs more DNA and is more labor intensive and time consuming than PCR techniques. However, with a single *Bgl*III restriction enzyme digestion it is possible to detect oligoclonality in *IGH*, *IGK*, and *TCRD* loci.²⁶⁻²⁸ Judging clonality solely from the number of PCR products per gene would result in marked underestimation of oligoclonality; eg, at least one third of the oligoclonal *IGH* targets (29 of 85, including 15 lost MRD-PCR markers) would have been classified as monoclonal.

The herein presented detailed comparison of Ig/TCR gene rearrangement patterns provides important information for appropriate selection of PCR targets for MRD monitoring. It is already accepted that preferably 2 MRD-PCR targets should be used per patient. Furthermore, our data show that monoclonal targets should be chosen as first option. As previously suggested, monoclonal Kde rearrangements were characterized by the best stability (95%), owing to their end-stage character.^{24,30} In addition, approximately 85% of monoclonal *IGH* and *TCRD* gene rearrangements remained stable at relapse (Table 2). In monoclonal VH-JH rearrangements, it is particularly attractive to position the patient-specific primers/probes at the VH-DH part of the junctional region, which is a preferred strategy in current RQ-PCR based strategies.⁹⁻¹¹ Identification of preferably 2 monoclonal MRD-PCR targets (*IGH*, *IGK*, and/or *TCRD*) was possible in 67 (77%) of 87 patients. When applying these monoclonal targets, the detection of relapse would have been possible in 65 patients, but false-negative results would have been obtained in 2 patients: one with presumably secondary

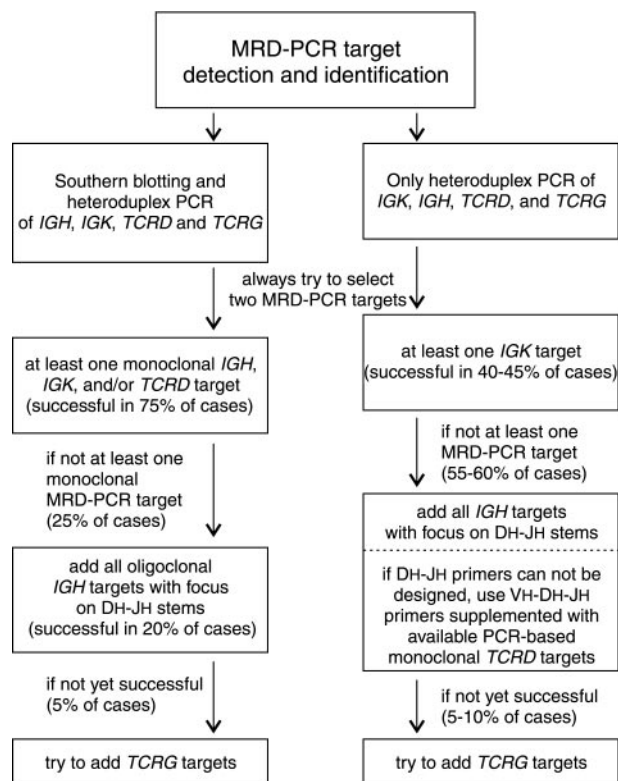


Figure 5. Flow diagram. Flow diagram for the stepwise selection of MRD-PCR targets, dependent on the choice of techniques for detection and identification of *IGH*, *IGK*, *TCRD*, and *TCRG* gene rearrangements.

ALL and an infant case characterized by extensive clonal Ig/TCR evolution. The second choice for target selection should concern oligoclonal *IGH* gene rearrangements. Although these rearrangements are particularly prone to ongoing and secondary recombination processes, they are the sole MRD-PCR targets in approximately 10% of childhood precursor-B-ALL patients.⁴⁶ In the case of oligoclonal *IGH* targets, the patient-specific primers/probes should preferably be positioned at the D-N-J junctions. Moreover, all identified clonal DH-JH stems should be followed because restriction to 2 targets would increase the risk of false-negative MRD results. In our series this approach could have been used in an additional 17 (20%) of 87 patients with available MRD-PCR targets and should have resulted in detection of relapse in 15 cases (false negativity in patients 2308 and 5978 with secondary *IGH* gene rearrangements). Finally, successful MRD detection in the remaining 3 patients could have been accomplished by usage of *TCRG* gene rearrangements, which were sole MRD-PCR targets in these patients. One could argue for the preferred usage of *TCRG* gene rearrangements instead of oligoclonal *IGH* targets. However, in at least 2 of our patients (4910, 5661) usage of common DH-JH stems would have been superior to V γ -J γ targets (Figure 4C). More importantly, *TCRG* gene rearrangements are generally less sensitive markers in RQ-PCR analyses, owing to their limited combinatorial diversity and the abundance of polyclonal V γ -J γ joinings in normal T-cells in postinduction follow-up samples.⁴⁷ Finally, our study clearly shows that oligoclonal V δ 2-D δ 3 and D δ 2-D δ 3 rearrangements should not be used as MRD-PCR targets, because most of them (about 75%) would be modified through clonal evolution processes. The Southern blot and PCR-based strategy for MRD-PCR target selection (priority order: 2 monoclonal *IGH*, *IGK*, and/or *TCRD* targets, followed by DH-JH stems of oligoclonal *IGH* rearrangements, followed by *TCRG* targets with full exclusion of oligoclonal *IGK* and *TCRD* targets) would enable successful detection of relapse in 83 (95%) of 87 patients with the currently available MRD-PCR targets (Figure 5).

The above-presented strategy is based on combined Southern blot and PCR analyses for discrimination between monoclonal and oligoclonal Ig/TCR gene configuration. However, many MRD-PCR laboratories do not routinely perform Southern blotting, implying that they will underestimate the occurrence of oligoclonality in *IGH* (44% of patients in this series), *TCRD* (36%), and *IGK* (8%) genes. In an exclusively PCR-based strategy for MRD target selection, Kde rearrangements should be chosen as first option.

When applying all available Kde targets, the detection of relapse would have been possible in 37 patients in our series (43%), but false-negative results would have been obtained in 2 patients. The second choice for target selection should concern *IGH* gene rearrangements. Using all identified *IGH* gene rearrangements with patient-specific oligonucleotides positioned at the DH-JH stems should have resulted in detection of relapse in 43 cases (49%), with false-negative results in 2 patients. If the design of DH-JH oligonucleotides is not successful, one might decide to design VH-DH-JH oligonucleotides supplemented with the usage of PCR-based monoclonal *TCRD* targets. Finally, successful MRD detection in the remaining 3 patients could have been accomplished by usage of *TCRG* gene rearrangements (Figure 5). Similarly to the combined Southern blot/PCR-based approach, the exclusively PCR-based approach would enable successful detection of relapse in 95% of patients. Thus, the lack of Southern blot information for discrimination between monoclonal and oligoclonal PCR targets might be compensated by monitoring of a higher number of *IGH* and *TCRD* targets (Figure 5).

Both described strategies for selection of MRD-PCR targets have their advantages and limitations, which should be carefully weighed in the context of the facilities and experience of each MRD-PCR laboratory. Nevertheless, each strategy would enable successful detection of relapse in 95% of patients. If one assumes that the actual relapse rate in childhood precursor-B-ALL is 25% to 30%, the current Ig/TCR-based MRD-PCR methodology should be "effective" in 97% to 98% of cases with identifiable MRD-PCR targets at diagnosis.

Acknowledgments

We are grateful to Prof dr R. Benner and Prof dr D. Sońta-Jakimczyk for their continuous support, Mrs F. J. Weerden for assistance in statistical analyses, Mrs W. M. Comans-Bitter and Mr T. van Os for preparation of the figures, and Mrs J. A. Boon for her secretarial support. We thank the Dutch Childhood Leukemia Study Group for kindly providing precursor-B-ALL cell samples. Board members of the Dutch Childhood Leukemia Study Group are P. J. van Dijken, K. Hählen, W. A. Kamps, E. Th. Korthof, F. A. E. Nabben, A. Postma, J. A. Rammeloo, G. A. M. de Vaan, A. J. P. Veerman, and R. S. Weening.

References

- Jacqy C, Delepaut B, Van Daele S, et al. A prospective study of minimal residual disease in childhood B-lineage acute lymphoblastic leukemia: MRD level at the end of induction is a strong predictive factor of relapse. *Br J Haematol*. 1997; 98:140-146.
- Cave H, van der Werff ten Bosch J, Suci S, et al. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia. *N Engl J Med*. 1998;339:591-598.
- Coustan-Smith E, Behm FG, Sanchez J, et al. Immunological detection of minimal residual disease in children with acute lymphoblastic leukemia. *Lancet*. 1998;351:550-554.
- Van Dongen JJM, Seriu T, Panzer-Grumayer ER, et al. Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. *Lancet*. 1998;352:1731-1738.
- Pui CH, Campana D. New definition of remission in childhood acute lymphoblastic leukemia. *Leukemia*. 2000;14:783-785.
- Szczepański T, Beishuizen A, Pongers-Willemsse MJ, et al. Cross-lineage T-cell receptor gene rearrangements occur in more than ninety percent of childhood precursor-B-acute lymphoblastic leukemias: alternative PCR targets for detection of minimal residual disease. *Leukemia*. 1999;13:196-205.
- Pongers-Willemsse MJ, Seriu T, Stolz F, et al. Primers and protocols for standardized detection of minimal residual disease in acute lymphoblastic leukemia using immunoglobulin and T cell receptor gene rearrangements and TAL1 deletions as PCR targets: report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. *Leukemia*. 1999;13:110-118.
- Pongers-Willemsse MJ, Verhagen OJHM, Tibbe GJM, et al. Real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia using junctional regions specific TaqMan probes. *Leukemia*. 1998;12:2006-2014.
- Verhagen OJHM, Willemsse MJ, Breunis WB, 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;14:1426-1435.
- Brüggemann M, Droese J, Bolz I, et al. Improved assessment of minimal residual disease in B cell malignancies using fluorogenic consensus probes for real-time quantitative PCR. *Leukemia*. 2000;14:1419-1425.
- Donovan JW, Ladetto M, Zou G, et al. Immunoglobulin heavy-chain consensus probes for real-time PCR quantification of residual disease in acute lymphoblastic leukemia. *Blood*. 2000;95:2651-2658.
- Szczepański T, Pongers-Willemsse MJ, Langerak AW, van Dongen JJM. Unusual immunoglobulin and T-cell receptor gene rearrangement patterns in acute lymphoblastic leukemias. *Curr Top Microbiol Immunol*. 1999;246:205-215.
- Steward CG, Goulden NJ, Katz F, et al. A polymerase chain reaction study of the stability of Ig

- heavy-chain and T-cell receptor delta gene rearrangements between presentation and relapse of childhood B-lineage acute lymphoblastic leukemia. *Blood*. 1994;83:1355-1362.
14. Steenbergen EJ, Verhagen OJ, van Leeuwen EF, van den Berg H, von dem Borne AE, van der Schoot CE. Frequent ongoing T-cell receptor rearrangements in childhood B-precursor acute lymphoblastic leukemia: implications for monitoring minimal residual disease. *Blood*. 1995;86:692-702.
 15. Beishuizen A, Verhoeven MA, van Wering ER, Hählen K, Hooijkaas H, van Dongen JJM. Analysis of Ig and T-cell receptor genes in 40 childhood acute lymphoblastic leukemias at diagnosis and subsequent relapse: implications for the detection of minimal residual disease by polymerase chain reaction analysis. *Blood*. 1994;83:2238-2247.
 16. Taylor JJ, Rowe D, Kylefjord H, et al. Characterization of non-concordance in the T-cell receptor γ chain genes at presentation and clinical relapse in acute lymphoblastic leukemia. *Leukemia*. 1994;8:60-66.
 17. Baruchel A, Cayuela JM, MacIntyre E, Berger R, Sigaux F. Assessment of clonal evolution at Ig/TCR loci in acute lymphoblastic leukaemia by single-strand conformation polymorphism studies and highly sensitive PCR derived methods: implication for a general strategy of minimal residual disease detection. *Br J Haematol*. 1995;90:85-93.
 18. Rosenquist R, Thunberg U, Li AH, et al. Clonal evolution as judged by immunoglobulin heavy chain gene rearrangements in relapsing precursor-B acute lymphoblastic leukemia. *Eur J Haematol*. 1999;63:171-179.
 19. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol*. 1976;33:451-458.
 20. Van Dongen JJ, Adriaansen HJ, Hooijkaas H. Immunophenotyping of leukaemias and non-Hodgkin's lymphomas. Immunological markers and their CD codes. *Neth J Med*. 1988;33:298-314.
 21. Groeneveld K, te Marvelde JG, van den Beemd MW, Hooijkaas H, van Dongen JJM. Flow cytometric detection of intracellular antigens for immunophenotyping of normal and malignant leukocytes. *Leukemia*. 1996;10:1383-1389.
 22. Van Wering ER, Beishuizen A, Roeffen ET, et al. Immunophenotypic changes between diagnosis and relapse in childhood acute lymphoblastic leukemia. *Leukemia*. 1995;9:1523-1533.
 23. Szczepański T, Willemsse MJ, Kamps WA, van Wering ER, Langerak AW, van Dongen JJM. Molecular discrimination between relapsed and secondary acute lymphoblastic leukemia—proposal for an easy strategy. *Med Pediatr Oncol*. 2001;36:352-358.
 24. Van der Velden VHJ, Willemsse MJ, van der Schoot CE, van Wering ER, van Dongen JJM. Immunoglobulin κ deleting element rearrangements in precursor-B acute lymphoblastic leukemia are stable targets for detection of minimal residual disease by real-time quantitative PCR. *Leukemia*. In press.
 25. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes. Part I: basic and technical aspects. *Clin Chim Acta*. 1991;198:1-91.
 26. Beishuizen A, Verhoeven MA, Mol EJ, Breit TM, Wolvers-Tettero ILM, van Dongen JJM. Detection of immunoglobulin heavy-chain gene rearrangements by Southern blot analysis: recommendations for optimal results. *Leukemia*. 1993;7:2045-2053.
 27. Beishuizen A, Verhoeven MA, Mol EJ, van Dongen JJM. Detection of immunoglobulin κ light-chain gene rearrangement patterns by Southern blot analysis. *Leukemia*. 1994;8:2228-2236.
 28. Breit TM, Wolvers-Tettero ILM, Beishuizen A, Verhoeven M-AJ, van Wering ER, van Dongen JJM. Southern blot patterns, frequencies and junctional diversity of T-cell receptor δ gene rearrangements in acute lymphoblastic leukemia. *Blood*. 1993;82:3063-3074.
 29. Szczepański T, Langerak AW, Wolvers-Tettero ILM, et al. Immunoglobulin and T cell receptor gene rearrangement patterns in acute lymphoblastic leukemia are less mature in adults than in children: implications for selection of PCR targets for detection of minimal residual disease. *Leukemia*. 1998;12:1081-1088.
 30. Beishuizen A, de Bruijn MAC, Pongers-Willemsse MJ, et al. Heterogeneity in junctional regions of immunoglobulin κ deleting element rearrangements in B cell leukemias: a new molecular target for detection of minimal residual disease. *Leukemia*. 1997;11:2200-2207.
 31. Szczepański T, Pongers-Willemsse MJ, Langerak AW, et al. Ig heavy chain gene rearrangements in T-cell acute lymphoblastic leukemia exhibit predominant DH6-19 and DH7-27 gene usage, can result in complete V-D-J rearrangements, and are rare in T-cell receptor $\alpha\beta$ lineage. *Blood*. 1999;93:4079-4085.
 32. Langerak AW, Szczepański T, van der Burg M, Wolvers-Tettero ILM, van Dongen JJM. Heteroduplex PCR analysis of rearranged T cell receptor genes for clonality assessment in suspect T cell proliferations. *Leukemia*. 1997;11:2192-2199.
 33. Cook GP, Tomlinson IM. The human immunoglobulin VH repertoire. *Immunol Today*. 1995;16:237-242.
 34. Breit TM, Van Dongen JJ. Unravelling human T-cell receptor junctional region sequences. *Thymus*. 1994;22:177-199.
 35. Van der Burg M, Tumkaya T, Boerma M, de Bruijn-Versteeg S, Langerak AW, van Dongen JJM. Ordered recombination of immunoglobulin light chain genes occurs at the IGK locus but seems less strict at the IGL locus. *Blood*. 2001;97:1001-1008.
 36. Reichel M, Gillert E, Breitenlohner I, et al. Rapid isolation of chromosomal breakpoints from patients with t(4;11) acute lymphoblastic leukemia: implications for basic and clinical research. *Leukemia*. 2001;15:286-288.
 37. Szczepański T, Langerak AW, Willemsse MJ, Wolvers-Tettero ILM, van Wering ER, van Dongen JJM. T cell receptor γ (TCRG) gene rearrangements in T cell acute lymphoblastic leukemia reflect "end-stage" recombinations: implications for minimal residual disease monitoring. *Leukemia*. 2000;14:1208-1214.
 38. Diaz MA, Garcia-Sanchez F, Vicario JL, Ramirez M, Balas A, Madero L. Clinical relevance of T-cell receptor delta gene rearrangements in childhood B-precursor cell acute lymphoblastic leukaemia. *Br J Haematol*. 1997;99:308-313.
 39. Green E, McConville CM, Powell JE, et al. Clonal diversity of Ig and T-cell-receptor gene rearrangements identifies a subset of childhood B-precursor acute lymphoblastic leukemia with increased risk of relapse. *Blood*. 1998;92:952-958.
 40. Hunger SP, Sklar J, Link MP. Acute lymphoblastic leukemia occurring as a second malignant neoplasm in childhood: report of three cases and review of the literature. *J Clin Oncol*. 1992;10:156-163.
 41. Pui CH, Ribeiro RC, Hancock ML, et al. Acute myeloid leukemia in children treated with epipodophyllotoxins for acute lymphoblastic leukemia. *N Engl J Med*. 1991;325:1682-1687.
 42. Wasserman R, Yamada M, Ito Y, et al. VH gene rearrangement events can modify the immunoglobulin heavy chain during progression of B-lineage acute lymphoblastic leukemia. *Blood*. 1992;79:223-228.
 43. Vora A, Frost L, Goodeve A, et al. Late relapsing childhood lymphoblastic leukemia. *Blood*. 1998;92:2334-2337.
 44. LoNigro L, Cazzaniga G, DiCataldo A, et al. Clonal stability in children with acute lymphoblastic leukemia (ALL) who relapsed five or more years after diagnosis. *Leukemia*. 1999;13:190-195.
 45. De Haas V, Verhagen OJ, von dem Borne AE, Kroes W, van den Berg H, van der Schoot CE. Quantification of minimal residual disease in children with oligoclonal B-precursor acute lymphoblastic leukemia indicates that the clones that grow out during relapse already have the slowest rate of reduction during induction therapy. *Leukemia*. 2001;15:134-140.
 46. Szczepański T, Willemsse MJ, van Wering ER, Weerden JF, Kamps WA, van Dongen JJM. Precursor-B-ALL with DH-JH gene rearrangements have an immature immunogenotype with a high frequency of oligoclonality and hyperdiploidy of chromosome 14. *Leukemia*. 2001;15:1415-1423.
 47. Van Wering ER, van der Linden-Schrevel BEM, van der Velden VHJ, Szczepański T, van Dongen JJM. T lymphocytes in bone marrow samples of children with acute lymphoblastic leukemia during and after chemotherapy might hamper PCR-based minimal residual disease studies. *Leukemia*. 2001;15:1031-1033.