DETECTION OF MINIMAL RESIDUAL DISEASE IN ACUTE LYMPHOBLASTIC LEUKEMIA

Opsporen van kleine aantallen tumorcellen bij patiënten met acute lymfatische leukemie

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Opsporen van kleine aantallen tumorcellen bij patiënten met acute lymfatische leukemie

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In te Domine, speravi:
non confundar in aeternum.

(Te Deum Laudamus)

DETECTION OF MINIMAL RESIDUAL DISEASE IN ACUTE LYMPHOBLASTIC LEUKEMIA

Opsporen van kleine aantallen tumorcellen bij patiënten met acute lymfatische leukemie

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1

CHAPTER 1.1

IMMUNOBIOLOGY OF ACUTE LYMPHOBLASTIC LEUKEMIA*

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IMMUNOPHENOTYPE OF ACUTE LYMPHOBLASTIC LEUKEMIA

The cells in acute lymphoblastic leukemias (ALL) are generally regarded as malignant counterparts of immature lymphoid cells.[1-5] Immunophenotyping enables the determination of the differentiation stage of normal and abnormal populations and subpopulations. Therefore, it allows identification and classification of ALL.[6,7] Using immunophenotyping it is possible to discriminate at least seven different ALL subtypes.[2,4,7,8] As has been drawn in Figure 1, these concern four types of B-lineage ALL and at least three types of T-ALL.

Immunophenotypic characteristics of precursor-B-ALL

The immunophenotypes of B-lineage ALL are summarized in Table 1. All four forms of precursor-B-ALL (pro-B-ALL, common ALL, pre-B-ALL and transitional pre-B-ALL) are positive for terminal deoxynucleotidyl transferase (TdT). The B-lineage ALL types are positive for HLA-DR, CD19, CyCD79, and for CD22. CD79 is completely B-cell specific.[9,10] Except for a small part of the pre-B-ALL, expression of the CD79-antigen in precursor-B-ALL is restricted to the cytoplasm.[9,10] CD10, pre-B CyIgμ, and SmIgμ are important markers for discrimination between the four subtypes of B-lineage ALL (see Table 1 and Figure 1). An example of flow cytometric immunophenotyping of a common ALL is given in Figure 2. For the diagnosis of pre-B-ALL, expression of pre-B CyIgμ is a prerequisite: at least 10% to 20% of the ALL cells should express this marker.[7,11,12] In about 5% of pre-B-ALL, faint expression of the Igμ-CD79 complex without normal Ig light chains is seen on the surface membrane (pre-B complex).[13] These ALL have been defined as transitional pre-B-ALL.[13]

The distribution of the immunophenotypic subgroups in ALL is age-related.

**Adapted from Chapter 6 'Immunobiology of Leukemia' in Henderson E.S., Lister T.A. and Greaves M.F. (eds.) Leukemia, 7th edition, Philadelphia, WB Saunders Company, 2002; 85-129*

Figure 3 summarizes the ALL subgroups in childhood.[14] In infants, pro-B-ALL predominates, whereas in young children (one to six years old), high frequencies of CD10^{+} precursor-B-ALL (i.e., common ALL and pre-B-ALL) are found. Figure 3 illustrates that the relatively high incidence of ALL during early childhood mainly concerns CD10^{+} precursor-B-ALL. It has been proposed that altered patterns of infection due to an improved socioeconomic situation and hygiene may partly explain this high incidence in the developed countries.[15]

Figure 1.
Hypothetical scheme of lymphoid differentiation. The expression of relevant immunologic markers is indicated for each differentiation stage; markers in parentheses are not always expressed. The bars represent the various types of leukemias and non-Hodgkin lymphomas (NHL) and indicate where these malignancies can be located according to their maturation arrest. Abbreviations used: AITL = angioimmunoblastic T-cell lymphoma, ALCL = anaplastic large cell lymphoma (of T-cell type), ALL = acute lymphoblastic leukemia, ATLL = adult T-cell leukemia lymphoma, AUL = acute undifferentiated leukemia, CLL = chronic lymphocytic leukemia, CTCL = cutaneous T-cell lymphoma, CTLL = cutaneous T-cell leukemia lymphoma (mycosis fungoides/Sézary syndrome), DLBCL = diffuse large B-cell lymphoma, ETTL - enteropathy-type T-cell lymphoma, HCL = hairy cell leukemia, HTLV = human T-cell leukemia virus, LGL = large granular lymphocyte, MALT = mucosa associated tissue lymphoma, NHL = non-Hodgkin's lymphoma, PLL = prolymphocytic leukemia (see reference[4]).

Table 1. Immunophenotypic characteristics of precursor-B-ALL.

Markers	pro-B-ALL	common ALL	pre-B-ALL	transitional-pre-B-ALL[a]
TdT	++	++	++	++
CD10	—	++	++	++
CD19	++	++	++	++
CD20	—	+	+	+
CD22	++	++	++	++
CyCD79	++	++	++	++
Pre-B CyIgμ	—	—	++	++
SmVpre-B/λ5	—	—	—	++
SmIg-CD79	—	—	—	++
CD34	+	+	+	+
HLA-DR	++	++	++	++

a. approximately 5% of pre-B-ALL patients have a 'transitional pre-B-ALL', defined by both pre-B CyIgμ and surface membrane expression of the pre-B-cell complex (pre-B SmIgμ-CD79) without expression of mature κ or λ Ig light chains.[13]

—, <10% of the leukemias is positive; +, 25-75% of the leukemias is positive; ++, >75% of the leukemias is positive.

Immunophenotypic characteristics of T-ALL

Virtually all T-ALL are positive for TdT, CD2, CD7, and CyCD3.[2,4,16] Markers, which can be used for additional characterization, concern CD1, surface membrane CD3 (SmCD3), CD4, CD5, and CD8.[4,7,8,17] In fact, using only CD1 and SmCD3 at least three types of T-ALL can be recognized, i.e. immature T-ALL ($CD1^-$/$SmCD3^-$), common thymocytic (cortical) T-ALL ($CD1^+$/$SmCD3^{-\text{ or }+}$), and mature T-ALL ($CD1^-$

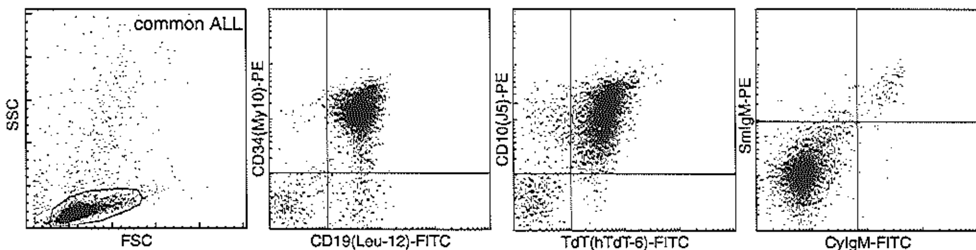


Figure 2.
Flow cytometric immunophenotyping of mononuclear blood cells from a precursor-B-ALL patient at diagnosis. The CD19/CD34, CD10/TdT and CyIgμ/SmIgμ double immunofluorescence stainings were analyzed within the indicated FSC/SSC gated cell population. The precursor-B-ALL cells expressed CD19, CD10, CD34 on the cell surface and TdT intracellularly but they did not produce Igμ protein. Such immunophenotype is typical for common ALL.

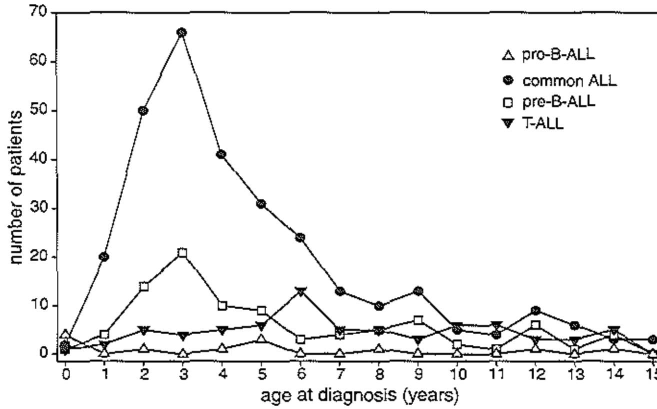


Figure 3.
Age-related distribution of immunophenotypic subgroups in childhood ALL in the Netherlands (1986-1991), according to the Dutch Childhood Leukemia Study Group. (Adapted from. Coebergh JWW et al.).[14]

Table 2. Immunophenotypic characteristics of T-ALL.

	immature T-ALL		common thymocytic T-ALL		mature T-ALL
Markers	prothymocytic (pre-T-ALL)	immature thymocytic	$SmCD3^-$	$SmCD3^-$	
TdT	++	++	++	++	++
CD1	—	—	++	++	—
CD2	+	++	++	++	++
CyCD3	++	++	++	++	++
SmCD3	—	—	—	++	++
$CD4^-/CD8^-$	++	+	—	—	—
$CD4^+/CD8^-$	—	±	±	±	+
$CD4^-/CD8^+$	—	±	±	±	±
$CD4^+/CD8^+$	—	—	+	+	±
CD5	—	++	++	++	++
CD7	++	++	++	++	++
TCRαβ	—	—	—	60%-70%	
TCRγδ	—	—	—	30%-40%	
HLA-DR	+	—	—	—	—

-, <10% of the leukemias is positive; ±, 10-25% of the leukemias is positive; +, 25-75% of the leukemias is positive; ++, >75% of the leukemias is positive.

/SmCD3$^+$) (Table 2; see Figure 1).[4,7] The immature T-ALLs either concern the rarely occurring prothymocytic T-ALL (pro-T-ALL) or the immature thymocytic T-ALL (pre-T-ALL).[4,18] The common thymocytic T-ALL can be divided further in SmCD3$^-$ and SmCD3$^+$ types (Table 2). The distinction between SmCD3$^-$ T-ALL and SmCD3$^+$ T-ALL is used widely; SmCD3 expression is found in approximately 35% of all T-ALL.[19] The CD3 antigen on the cell surface membrane is associated with TCR molecules. Approximately 65% of T-ALL is SmCD3$^-$, approximately 20% is TCR$\alpha\beta^+$, and approximately 15% is TCR$\gamma\delta^+$.[19] SmCD3$^+$ T-ALL could be further characterized with the monoclonal antibodies specific to particular Vβ, Vγ, or Vδ gene segments.[20,21] Immunophenotypic classification of T-ALL gives significant prognostic information. The most immature T-ALL (CD1$^-$/SmCD3$^-$) is associated with unfavorable outcome, while CD1$^+$ (and/or CD4$^+$/CD8$^+$) cortical T-ALL is a distinct subgroup with an excellent prognosis under intensive high-risk treatment.[8,22-24] Within SmCD3$^+$ T-ALL, event-free survival is significantly better in TCR$\gamma\delta^+$ T-ALL when compared to TCR$\alpha\beta^+$ T-ALL.[25]

Burkitt's Leukemia/Lymphoma (B-ALL)

French-American-British classification of ALL includes morphological type L3, which corresponds immunophenotypically to B-ALL. Only rare cases of ALL-L3 have immunophenotype of T-ALL.[26] B-ALL is characterized by bright surface expression of IgM and negativity for TdT and CD34. Many cases are also CD10 positive. B-ALL comprises 2% to 3% of pediatric and adult ALL.[27] Based on its similar morphology, the presence of characteristic chromosomal aberrations involving *C-MYC* gene and clinical behavior, B-ALL is currently perceived as leukemic form of Burkitt's lymphoma.

Cross-lineage marker expression in ALL

The expression of myeloid markers in ALL cells has been defined as cross-lineage marker expression.[28,29] This mainly concerns expression of CD13, CD14, CD15, CD33 and/or CD65 antigens in up to one third of precursor-B-ALL and T-ALL patients.[28-30] Such observations have been interpreted as lineage infidelity or lineage promiscuity. Terms such as hybrid acute leukemia, mixed lineage leukemia, biphenotypic acute leukemia, and biclonal acute leukemia have been used to describe acute leukemia with cross-lineage marker expression.[31] It has been suggested that these leukemias exhibit aberrant gene expression, but coexpression of lymphoid and myeloid genes may also occur in normal immature cells.[31] There are only a few lineage-specific antigens, i.e. CD79 and Ig molecules in B-cells, CD3 and TCR molecules in T-cells and MPO in myeloid cells.[9,10,17,32]

Applications of Immunophenotyping of ALL

Immunophenotyping is useful for discrimination among ALL, acute myeloid leukemia, and acute undifferentiated leukemia, which is the most important step for initial selection of the effective treatment strategy.[2,4] Furthermore, immunophenotypic subclassification of ALL is clinically relevant, particularly the distinction between precursor-B-ALL and T-ALL. T-lineage immunophenotype of ALL is more frequently associated with several high-risk features when compared with precursor-B-ALL. Inclusion of T-ALL patients into more intensive treatment protocols or even establishment of T-ALL phenotype-specific treatment regimens has been associated with significantly improved long-term survival.[33]

Detailed immunophenotyping can also be used for identification of ALL subgroups with poor prognosis, such as pro-B ALL and immature ($CD1^-/CD3^-$) T-ALL.[8,22] Myeloid marker expression in adult ALL was traditionally associated with poor prognosis.[8,28] In childhood ALL this correlation was not clear, because of conflicting data that were attributed to differences in treatment protocols.[8,28,29] With the current intensive treatment protocols, myeloid antigen expression lacks prognostic significance both in adult and pediatric ALL.[30,34-36]

Several studies indicate that particular chromosome aberrations are associated with specific immunophenotypes. The t(4;11) is seen in pro-B-ALL with cross-lineage expression of the myeloid markers CD15 and CD65 and ectopic expression of the NG2 antigen , i.e., human homologue of the rat chondroitin sulfate proteoglycan.[37-39] Hyperdiploidy especially occurs in $CD10^+$ precursor-B-ALL[40], and t(1;19) seems to be restricted to $CD10^+/CD19^+/CD34^-$ precursor-B-ALL, especially pre-B-ALL.[41,42] The t(12;21) occurring in more than 25% of pediatric precursor-B-ALL is characteristically CD66c negative, CD9 negative or partly positive, CD20 negative or partly positive, and frequently positive for myeloid antigens CD13, CD33, or both.[43-45] The most frequent aberration in adult ALL, i.e., t(9;22), is significantly linked with CD25 and CD66c positivity.[46,47]

Finally, immunologic marker analysis can be used in ALL patients to study the effectiveness of treatment by monitoring bone marrow and blood samples for the occurrence of 'minimal residual disease' (see Chapter 1.2).

IMMUNOGENOTYPIC CHARACTERISTICS OF ALL

Lymphopoiesis results in the continuous production of mature B lymphocytes and T lymphocytes, which together form the antigen-specific immune system.[48-50] Both types of lymphocytes express unique receptors on their cell membrane that allow the specific recognition of antigens.[49,50] The antigen-specific receptors are different on each lymphocyte, but each single lymphocyte or lymphocyte clone expresses thousands (approximately 10^5) of receptors with the same antigen speci-

ficity. Surface membrane-bound Ig molecules (SmIg) represent the antigen-specific receptors of B-lymphocytes, whereas TCR molecules exhibit this function in T-lymphocytes.[50,51]

Rearrangement and expression of Ig and TCR genes

The immune system encounters millions of different antigens and antigenic epitopes. The ability to recognize specifically these antigens is based on the enormous diversity (at least 10^9) of antigen-specific receptors. If the entire repertoire of Ig and TCR molecules were to be encoded by separate genes, they would occupy a large part of the human genome. Instead, a limited number of gene segments is able to code for the receptor diversity owing to the fact that combinations of gene segments are made, which are different in each lymphocyte or lymphocyte clone.[48,49,52]

Immunoglobulin molecules and their encoding genes

Ig molecules consist of two Ig heavy (IgH) chains and two Ig light chains, held together by disulfide bonds. The Ig class or subclass is determined by the isotype of the involved IgH chain and is independent of the Ig light chain. Each B-lymphocyte or B-lymphocyte clone expresses only one type of Ig light chain (Igκ or Igλ), whereas multiple IgH chains can be expressed.[50] The majority of B-lymphocytes in peripheral blood express IgM and IgD on the cell membrane, whereas a minority expresses IgG or IgA. In lymph nodes and other lymphoid tissues, higher frequencies of IgG- and/or IgA-bearing lymphocytes are present. SmIg molecules are closely associated with a disulfide-linked heterodimer, which consists of the CD79a protein chain (pre-

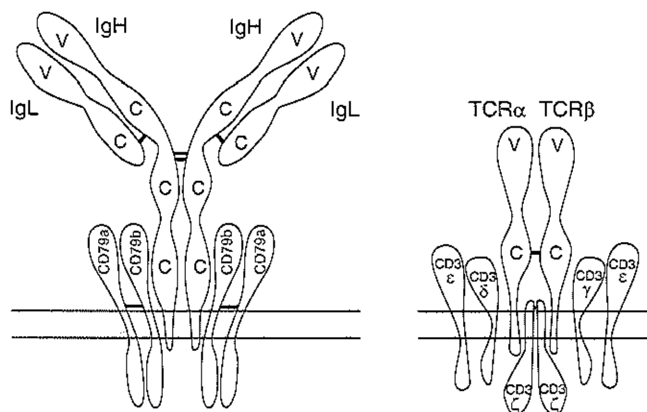
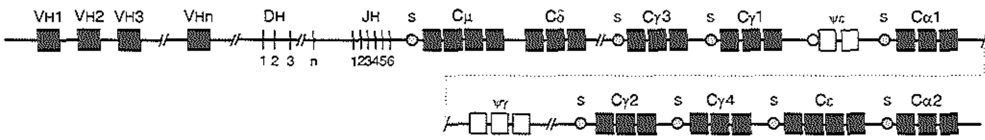
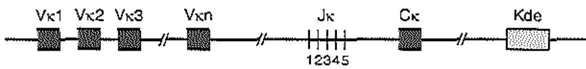


Figure 4.
Schematic diagram of SmIg and TCR molecules on the surface membrane of a B lymphocyte and T lymphocyte, respectively. SmIg molecules are closely associated with disulfide-linked CD79 protein chains, whereas TCR molecules are associated with CD3 protein chains. V and C: variable and constant domains of Ig or TCR chains.





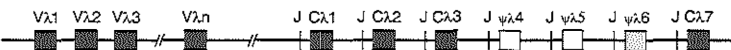


Figure 5.
Schematic diagram of the human Ig gene complexes. The *IGH* gene complex consists of at least 40 functional VH gene segments, 27 DH gene segments, 6 functional JH gene segments, and several CH gene segments, which together encode the various IgH class and subclass constant domains. Most CH gene segments are preceded by a switch gene(s), which plays a role in IgH (sub)class switch. The *IGK* gene complex consists of approximately 35 functional Vκ gene segments, five Jκ gene segments, and a single Cκ gene segment. The Kde (kappa-deleting element) plays a role in the deletion of the Jκ-Cκ or Cκ gene regions in B-cells, which rearrange their *IGL* genes. The *IGL* gene complex consists of approximately 30 Vλ gene segments and four functional Cλ genes, all of which are preceded by a Jλ gene segment. Pseudogenes (ψ) are indicated as open symbols.

viously mb1 or Igα) and the CD79b chain (previously B29 or Igβ). In the SmIg-CD79 complex, the CD79 protein chains mediate the transmembrane signal transduction (Figure 4).[51]

The Ig protein chains are composed of one variable domain, which is involved in antigen recognition, and one constant domain in case of Ig light chains or three to four constant domains in case of IgH chains (Figure 4). Each domain of an Ig chain is encoded by a separate exon (Figure 5). The variable domain of an IgH chain is encoded by an exon, which consists of a combination of a V (variable), a D (diversity) and a J (joining) gene segment. A combination of a V and a J gene segment encodes the variable domain of an Ig light chain.[19,50] During B-cell differentiation, combinations of the available V, D, and J gene segments are made through a process of gene rearrangement.[52,53] The constant domains of the Ig chains are encoded by C (constant) gene segments, depending on the isotype of the Ig chain (Figure 5).[54-57]

T-cell receptor molecules and their encoding genes

TCR molecules consist of two chains, which are generally disulfide-linked. Two types of TCR have been recognized: the 'classical' TCR, which consists of a TCRα

and a TCRβ chain (TCRαβ) and the 'alternative' TCR, which is composed of a TCRγ and a TCRδ chain (TCRγδ).[19,49] The majority of mature T-lymphocytes (85 to 98%) in peripheral blood and most lymphoid tissues express TCRαβ; a minority (2% to 15%) expresses TCRγδ.[19] Both types of TCR molecules are closely associated with the CD3 protein chains, which together form the TCR-CD3 complex (Figure 4). After antigen recognition by the TCR molecule, transmembrane signal transduction is mediated via the CD3 protein chains.[51]

Each TCR chain consists of two domains: a variable domain and a constant domain. Analogous to the Ig chains, the variable domain of a TCR chain is encoded by a combination of the available V and J gene segments in case of TCRα and TCRγ chains, or by a combination of the available V, D, and J gene segments in case of TCRβ and TCRδ chains.[19,49] The constant domains of the TCR chains are encoded by C gene segments: one C gene segment for the constant domain of the TCRα chain and one for the TCRδ chain, and two C gene segments are available for the constant domains of the TCRβ and TCRγ chains (Figure 6).[57-60]

Gene rearrangement: V-(D-)J joining

During early B- and T-cell differentiation, the germline V, (D,) and J gene seg-

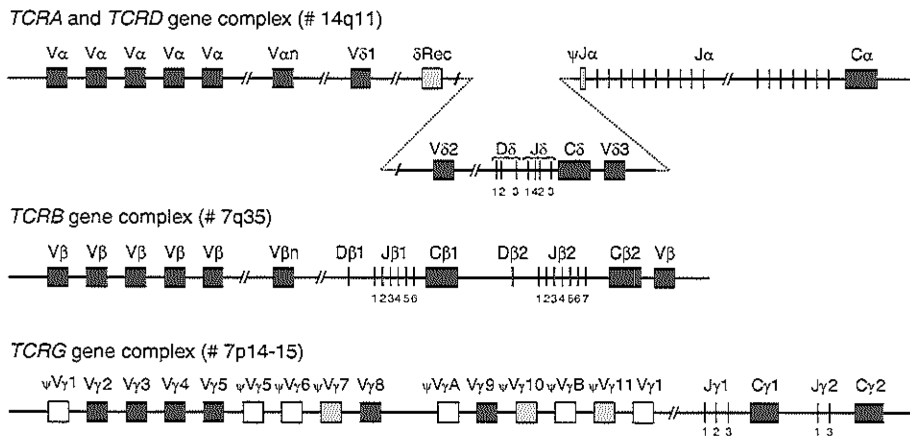


Figure 6.

Schematic diagram of the four human TCR genes. The *TCRA* gene complex consists of >50 Vα gene segments, a remarkably long stretch of 61 functional Jα gene segments, and one Cα gene segment. The major part of the *TCRD* gene complex is located between the Vα and Jα gene segments and consists of eight Vδ, three Dδ, and four Jδ gene segments and one Cδ gene segment. The δREC and ψJα gene segments play a role in *TCRD* gene deletions, which precede *TCRA* gene rearrangements in developing T-cells. The *TCRB* gene complex consists of 65 Vβ gene segments and two Cβ gene segments, both of which are preceded by one Dβ and six or seven Jβ gene segments. The *TCRG* gene complex consists of a restricted number of Vγ gene segments (six functional Vγ gene segments and nine pseudogene segments) and two Cγ gene segments, each preceded by two Jγ1 or three Jγ2 gene segments. Pseudogenes (ψ) are indicated with open symbols.

ments of the Ig and TCR gene complexes rearrange, and each lymphocyte thereby obtains a specific combination of V-(D-)J segments.[48,49,52] An example of an *IGH* gene rearrangement is illustrated in Figure 7: one of the J gene segments is joined to one of the D gene segments, and subsequently a V to D-J joining occurs, thereby deleting all intervening sequences. After the process of rearrangement, the gene is transcribed into a precursor mRNA, which is transformed into mature mRNA by splicing and eliminating all noncoding intervening sequences. Comparable rearrangement and transcription processes occur in the other Ig and TCR gene complexes.

The gene rearrangement processes are mediated by a recombinase enzyme system, which probably contains several components, including the protein products of the so-called recombinase activating genes (*RAG-1* and *RAG-2*).[61,62] The recombinase complex recognizes specific recombination signal sequences (RSS), which are well conserved during evolution and consist of a palindromic heptamer and nonamer sequence, separated by spacer regions of 12 or 23 base pairs.[63] Complete RSS, starting with the heptamer, border the 3′ side of V gene segments, both sides of D gene segments and the 5′ side of J gene segments.[63] A gene rearrangement first involves a back-to-back fusion of the heptamer-nonamer RSS. This is followed by deletion of these RSS and all intervening sequences in the form of a circular excision product and by joining of the two gene segments (Figure 8).[63]

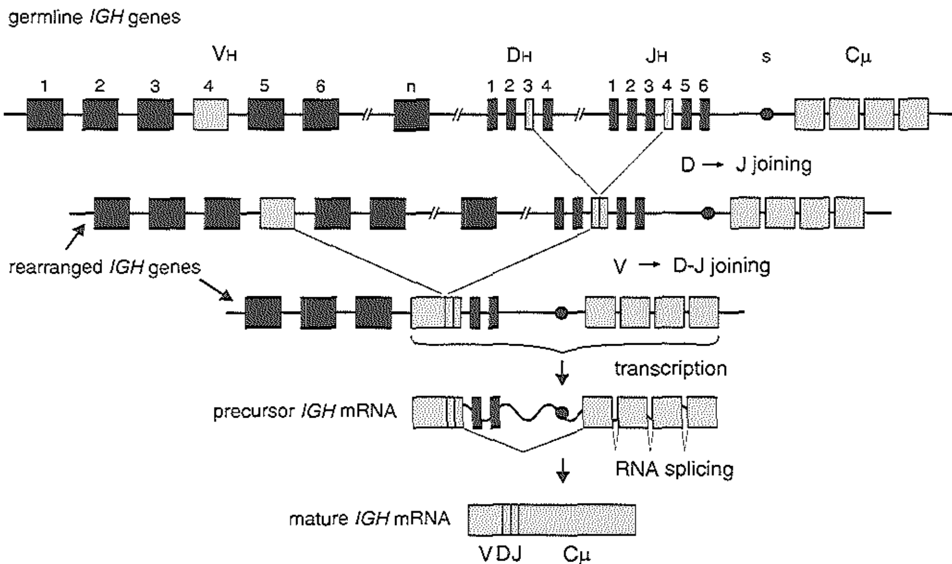


Figure 7.
Schematic diagram of human *IGH* gene rearrangement. In this example DH3 is first joined to JH4, followed by VH4 to DH3-JH4 joining, thereby deleting all intervening sequences. The rearranged gene complex can be transcribed into precursor mRNA, which will be transformed into mature mRNA by splicing out all noncoding intervening sequences.

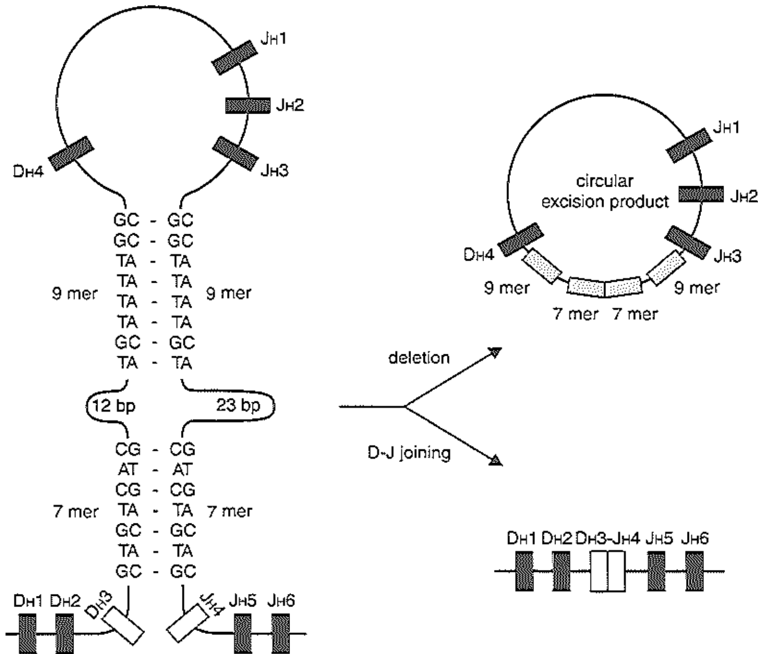


Figure 8.
Schematic diagram of the role of heptamer-nonamer recombination signal sequences (RSS) during gene rearrangement. The downstream RSS of DH3 and upstream RSS of JH4 fuse back to back, followed by DH3-JH4 joining and deletion of the intervening sequences in the form of a circular excision product. The presented RSS are not the exact RSS of the DH3 and JH4 gene segments, but represent the consensus heptamer-nonamer RSS, which are well conserved in Ig and TCR genes.

Secondary gene rearrangements

Ig and TCR gene rearrangements are complex processes in which the joining of the gene segments is imprecise.[49] Because of the triplet reading frame of DNA sequences, approximately two out of three joinings will be out-of-frame; that is, an mRNA is produced that cannot be transcribed into a complete protein.[49] The high frequency of out-of-frame rearrangements and the generation of stop codons at the joining sites may explain why most B-cells have rearranged both *IGH* alleles and why most T-cells have biallelic rearrangements of their *TCRB* and *TCRG* genes.[52,64]

In addition to the biallelic rearrangements, secondary gene rearrangements appear to occur that are assumed to rescue precursor B- and T-cells with nonproductive Ig or TCR genes. Three types of secondary rearrangements can occur, dependent on the involved Ig or TCR gene complex and the type of preexisting rearrangement (Table 3). DH-JH replacements in B-cells replace preexisting DH-JH complexes by joining an upstream DH gene segment to a downstream JH gene seg-

Table 3. Occurrence of secondary rearrangements in Ig and TCR genes.

	Ig genes			TCR genes			
	IGH	*IGK*	*IGL*	*TCRA*	*TCRB*	*TCRG*	*TCRD*
D-J replacement	+	-	-	-	(+)	-	(+)
V-J replacement	-	+	(+)	+	-	+	-
V replacement	+	-	-	-	+	(+)	-

+, replacement reported to occur; (+), replacement can potentially occur, but not (yet) reported;
-, replacement not likely to occur.

ment (Figure 9).[65] D-J replacements can also potentially occur in *TCRB* and *TCRD* genes. In a comparable way, V-J replacements replace a preexisting V-J complex in *TCRA*, *TCRG*, and Ig light chain genes.[66] Both types of replacements can occur repeatedly on the same Ig or TCR allele as long as germline V, (D,) and J gene segments are available (Table 3).

The third type of secondary rearrangement concerns V replacement in complete V(D)J complexes.[67,68] The V gene segment of the V(D)J complex is replaced by a new upstream V gene segment (Figure 9). This process is mediated via an internal heptamer RSS in the 3′ part of the V gene segments in *IGH*, *TCRB*, and *TCRG* genes, but this heptamer RSS is not present in V gene segments of Ig light chain, *TCRA*, and *TCRD* genes.[67-69] So far, V replacements have especially been observed in *IGH* genes and have also been reported to occur in *TCRB* genes (Table 3).

Secondary rearrangements have been found to replace not only preexisting nonproductive rearrangements but also productive rearrangements, such as productive Vα-Jα rearrangements. This suggests that secondary rearrangements not only rescue precursor-B- and T-cells from nonproductive rearrangements but are also involved in selection processes, such as thymic selection of the T-cell repertoire.[66]

Repertoire of Ig and TCR molecules

The enormous diversity of antigen-specific receptors of lymphocytes is mediated by the described rearrangements of the gene segments that code for the variable domains of Ig and TCR molecules. The extent of the potential primary repertoire of antigen-specific receptors is based on the combinatorial diversity (i.e. the number of possible V-D-J combinations) and the junctional diversity (i.e. diversity due to imprecise joining of the V, D, and J gene segments).[48,49]

The so-called combinatorial diversity can be calculated from the possible combi-

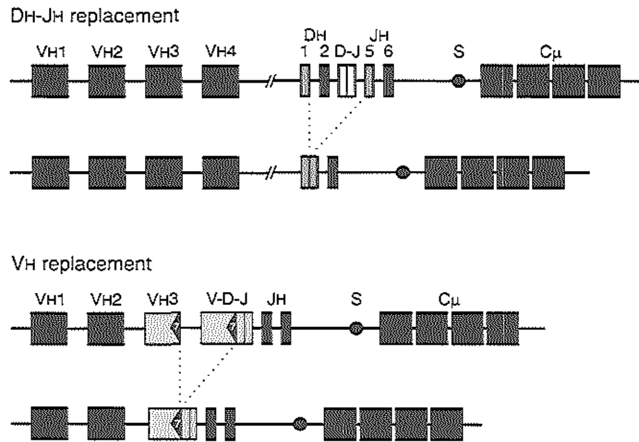


Figure 9.
Examples of secondary gene rearrangements in *IGH* genes.
(A) DH-JH replacement: an upstream DH gene segment rearranges to a downstream JH gene segment, thereby replacing the preexisting DH-JH rearrangement.
(B) VH replacement: the VH gene segment in a complete V-D-JH rearrangement is replaced by an upstream VH gene segment via a rearrangement process, which is mediated via heptamer RSS (indicated as: 7) within the VH gene segments.

nations of the available V, D, and J gene segments per gene complex and the pairing of two different protein chains per antigen-receptor molecule, i.e. IgH with Igκ or Igλ, TCRα with TCRβ, and TCRγ with TCRδ.[48,49] For example, the *IGH* gene complex contains at least 40 functional VH gene segments, 25 functional DH gene segments, and 6 functional JH gene segments, resulting in at least 6000 possible VH-DH-JH combinations.[70-72] Together with the V-J combinations of the *IGK* and *IGL* genes, a potential combinatorial diversity of more than 2×10^6 can be obtained. A comparable diversity can be obtained for the TCRαβ molecules (Table 4). However, the combinatorial diversity of TCRγδ molecules is less extensive due to the limited number of V,(D,) and J gene segments in the encoding gene complexes. Nevertheless, because of multiple Dδ gene usage, a combinatorial repertoire of potentially more than 5000 TCRγδ molecules can be produced.

The calculations in Table 4 are based on the assumption that the available functional V, D, and J gene segments are used randomly. This is not always the case. For instance, fetal B-cells use a restricted set of VH gene segments, related to JH proximity.[73,74] TCRαβ$^+$ cells tend to use Jβ2 gene segments more frequently than Jβ1 gene segments.[75] Peripheral TCRγδ$^+$ T-lymphocytes exhibit preferential usage of Vγ9-Jγ1.2 and Vδ2-Jδ1 gene segments.[76,77] However, it might well be that the whole potential combinatorial repertoire is present but that particular receptor specificities dominate due to clonal selection and expansion.[78]

Table 4. Estimation of the potential primary repertoire of human Ig and TCR molecules.[a]

	Ig molecules			TCRαβ molecules		TCRγδ molecules	
	IgH	Igκ	Igλ	TCRα	TCRβ	TCRγ	TCRδ
Number of functional gene segments:							
V gene segments	40-46	34-37	30-33	45	44-47	6	6
D gene segments	25[b]	-	-	-	2[b]	-	3[b]
J gene segments	6	5	4	50	13	5	4
Combinatorial diversity		$>2 \times 10^6$		$>2 \times 10^6$		>5000	
Junctional diversity	++	±	±	+	++	++	++++
ESTIMATION OF TOTAL REPERTOIRE		$>10^{12}$		$>10^{12}$		$>10^{12}$	

a. Based on the international IMGT (ImMunoGeneTics) Database.[57]
b. In *TCRD* gene rearrangements, multiple D segments might be used; this implies that the number of junctions can vary from one to four. In *IGH* and *TCRB* gene rearrangements, only one D gene segment is generally used.

The junctional diversity of Ig and TCR genes is based on deletion of germline nucleotides by trimming the ends of the rearranging gene segments as well as by insertion of nucleotides between the joined gene segments. Insertion of nucleotides (N region insertion) at the junction sites is mediated by TdT, which is able to add nucleotides to 3´ ends of DNA breakpoints without need for a template.[79,80] The junctional regions of Ig and TCR genes encode for the so-called third complementarily determining region (CDR3), which contributes considerably to the antigen recognition site of the variable protein domains. Therefore N-region insertion drastically increases the diversity of antigen receptors, especially when multiple junction sites occur in a junctional region, such as in *IGH*, *TCRB*, and especially *TCRD* genes (Table 4). The random insertion and deletion of nucleotides at the junction sites of V, (D,) and J gene segments make the junctional regions into 'fingerprint-like' sequences, which are most probably different in each lymphocyte (Figure 10).

Absence or decreased TdT activity during Ig or TCR gene rearrangements leads to the absence of N-region insertion, as is found in fetal thymocytes and B-cell precursors in fetal liver.[81-83] Also rearranged Ig light chain genes in mature B-cells virtually lack N-region insertion,[48,49,84] suggesting that the Ig light chain genes rearrange in the absence of TdT activity. This is in contrast to the junctional regions

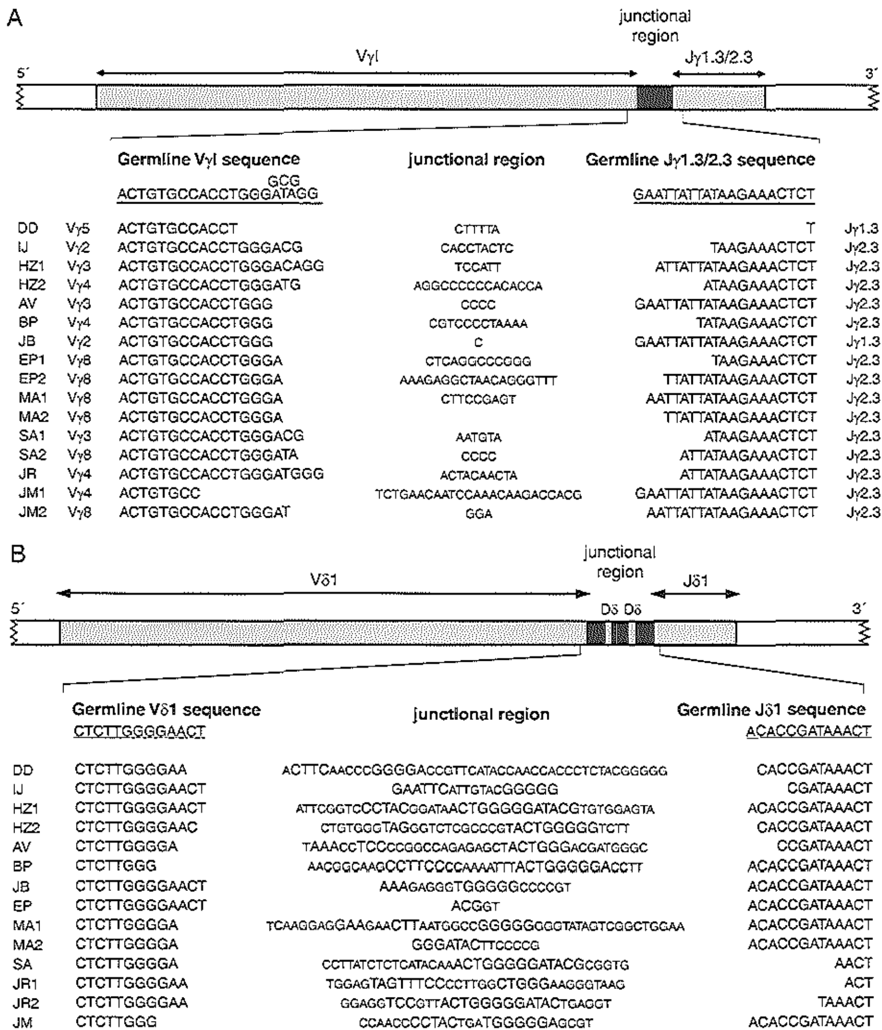


Figure 10.

(A) Schematic diagram of the Vγl gene segment joined to the Jγ1.3/2.3 gene segment via a junctional region. PCR-mediated amplification of the joined *TCRG* gene segments and subsequent sequencing of the junctional region in the obtained PCR products can be performed. The presented junctional region sequences are derived from T-ALL patients and illustrate the deletion of nucleotides from the germline sequences as well as the size and composition of the junctional regions.

(B) Schematic diagram of the Vδ1 gene segment joined to the Jδ1 gene segment via a junctional region. *TCRD* junctional regions may contain one, two, or three Dδ gene segments. The presented *TCRD* junctional region sequences are derived from the same T-ALL patients as in panel A and illustrate that deletion and insertion of nucleotides are more extensive than in the case of *TCRG* junctions. Dδ gene segments and inserted nucleotides are indicated by capital letters and small capital letters, respectively.

of postnatally rearranged TCR genes, all of which contain N regions. This discrepancy in N-region diversity between Ig and TCR genes is in line with the fact that SmIg$^+$/TdT$^+$ immature B-cells are extremely rare, whereas TCR$^+$/TdT$^+$ T-cells occur in a great amount in the thymus.[19] Apparently TdT activity decreases early during B-cell differentiation (after *IGH* gene rearrangement), whereas TdT is expressed throughout thymic T-cell differentiation.

Rearrangement and expression of Ig and TCR genes during lymphoid differentiation

Ig and TCR gene rearrangements start early during lymphoid differentiation and occur in a hierarchical order. One of the earliest events during normal B-cell differentiation are incomplete DH-JH rearrangements, which are already found in CD34$^+$/CD19$^-$/CD10$^+$ precursor cells.[85,86] Most of the more mature CD34$^+$/CD19$^+$/CD10$^+$ B-lineage precursors contain at least one DH-JH rearranged allele and frequently also complete VH-(DH-)JH rearrangements.[86,87]. *IGH* gene rearrangements are followed by recombination in the *IGK* locus. If the latter rearrangements are nonfunctional, the *IGL* genes will rearrange.[48,52] Generally, *IGL* gene rearrangements occur after or coincide with *IGK* gene deletions.[88,89] All *IGK* gene deletions are mediated via rearrangement of the so-called kappa deleting element (Kde), which is located downstream of the Cκ gene segment (Figure 5).[90,91] Kde rearranges to a heptamer RSS in the Jκ-Cκ intron, thereby deleting the Cκ gene segment, or to a Vκ gene segment, thereby deleting a large part of the *IGK* gene, including the Jκ and Cκ gene segments.[90,92] Functional rearrangement of *IGH* and Ig light chain genes results in SmIg$^+$ B-lymphocytes.

During T-cell differentiation, *TCRD* genes rearrange followed by *TCRG* gene rearrangements, which might result in TCRγδ$^+$ T-lymphocytes if the rearrangements are functional. TCRαβ$^+$ T-lymphocytes most probably develop via a separate differentiation lineage with *TCRB* gene rearrangements prior to *TCRA* gene rearrangements.[52] *TCRA* rearrangements are preceded by deletion of the *TCRD* genes because the major part of the *TCRD* gene complex is located between the Vα and Jα gene segments[59,93-95] and is flanked by the so-called δREC and ψJα gene segments (Figure 6).[96,97] *TCRD* gene deletion is mediated via rearrangement of the δREC and ψJα gene segments.[82,96,97] These rearrangement and deletion processes in the *TCRA/D* locus probably play a crucial role in the divergence of the TCRγδ and TCRαβ differentiation pathways.[19] It is not yet clear in which differentiation stage this divergence occurs, but it is remarkable that virtually all TCRαβ$^+$ T-lymphocytes have rearranged *TCRG* genes and that most TCRγδ$^+$ T-lymphocytes have rearranged *TCRB* genes (Figure 11).[21,52]

The SmIg-CD79 complex (Figure 4) is expressed on B-cells as soon as functional *IGH* and Ig light chain gene rearrangements are completed. However in the pre-B-cell differentiation stage, the result of functional *IGH* gene rearrangement can

be seen, i.e. the weak cytoplasmic expression of Igμ heavy chain (pre-B-CyIgμ).[11,98] In a part of the pre-B-cells also, a weak membrane expression of the so-called pre-B-cell complex (pre-B-SmIgμ-CD79) can be found.[13,51] This pre-B-cell complex consists of CD79 and Igμ protein chains and a pseudo Ig light chain, which is derived from nonrearranging Igλ-like gene segments.[99] The precise function of this transiently expressed pre-B-cell complex is still unclear, but it is assumed that it plays a role in the regulation of early B-cell development.[51]

Surface membrane expression of TCR-CD3 complexes depends on the functional rearrangement of *TCRD* and *TCRG* genes or *TCRB* and *TCRA* genes.

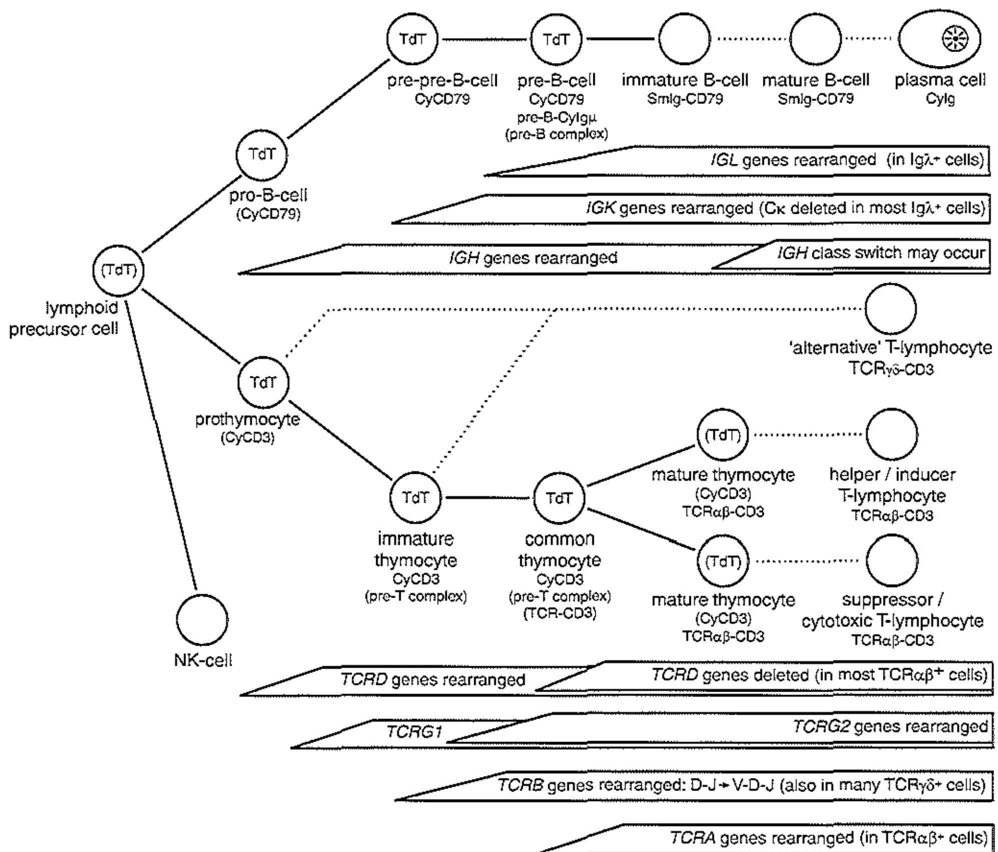


Figure 11.

Hypothetical diagram of Ig and TCR gene rearrangements during lymphoid differentiation. The ordered rearrangement of Ig and TCR genes is indicated with bars. The expression of nuclear TdT, cytoplasmic CD3 (CyCD3), cytoplasmic CD79 (CyCD79), pre-B-Ig molecules (pre-B-CyIgμ and pre-B SmIgμ-CD79), pre-T complex, and the mature SmIg-CD79 and TCR-CD3 antigen receptor complexes are indicated per differentiation stage.

Analogous to the pre-B-cell complex, the so-called pre-T-cell complex is weakly expressed on the surface membrane of immature thymocytes of the TCRαβ differentiation lineage.[100] In addition to CD3 and TCRβ protein chains, this pre-T-cell complex contains a second TCR chain.[100]

DETECTION OF CLONAL IG AND TCR GENE REARRANGEMENTS

Rearrangements in Ig and TCR genes result in relocation and joining of gene segments with simultaneous deletion of the intervening gene segments. In principle, these rearrangements are assumed to be identical in all cells of a particular leukemia, because leukemias are clonal cell proliferations. The deletion-relocation-joining processes can be studied by Southern blotting and PCR-based techniques. Southern blotting allows detection of deletion and relocation of gene segments based on changes in distances between cleavage sites of restriction enzymes in the DNA. PCR analysis of Ig and TCR gene rearrangements is based on the (selective) amplification of junctional regions of rearranged Ig or TCR gene segments. Such amplification is possible only when the Ig or TCR gene segments are juxtaposed through rearrangement, as the distance between these gene segments in germline configuration is far too large for PCR amplification. It should be noted that clonality studies by Southern blotting take advantage of the combinatorial diversity, i.e., the relocation of gene segments, whereas clonality studies by PCR-based methods employ mainly the junctional diversity of Ig and TCR gene rearrangements.

Southern blotting

In Southern blot studies the DNA samples are digested with restriction enzymes.[101] The obtained DNA fragments (restriction fragments) are size-separated by agar electrophoresis, transferred (blotted) onto a nitrocellulose or nylon membrane, and immobilized.[101] This membrane is incubated with a radiolabeled DNA probe that hybridizes to complementary sequences of Ig and TCR genes.[52] Unbound probe is washed away, and the location of the probe and thereby the size of the recognized restriction fragments can be detected by autoradiography or by phosphorimaging. If appropriate combinations of restriction enzymes and DNA probes are used, the detected restriction fragments of rearranged Ig or TCR genes will differ from those of germline genes.[52,94,102,103]

Figure 12 illustrates various aspects of Southern blot analysis of *IGH* genes: the germline restriction map of the JH-Cμ region with an appropriate JH probe (IGHJ6), the separation of restriction fragments in an agarose gel and the autoradiographic results of hybridization with the radiolabeled IGHJ6 probe.[52,102]

Optimally designed Ig/TCR probes recognize sequences just downstream of the rearranging gene segments and should be used in combination with at least two dif-

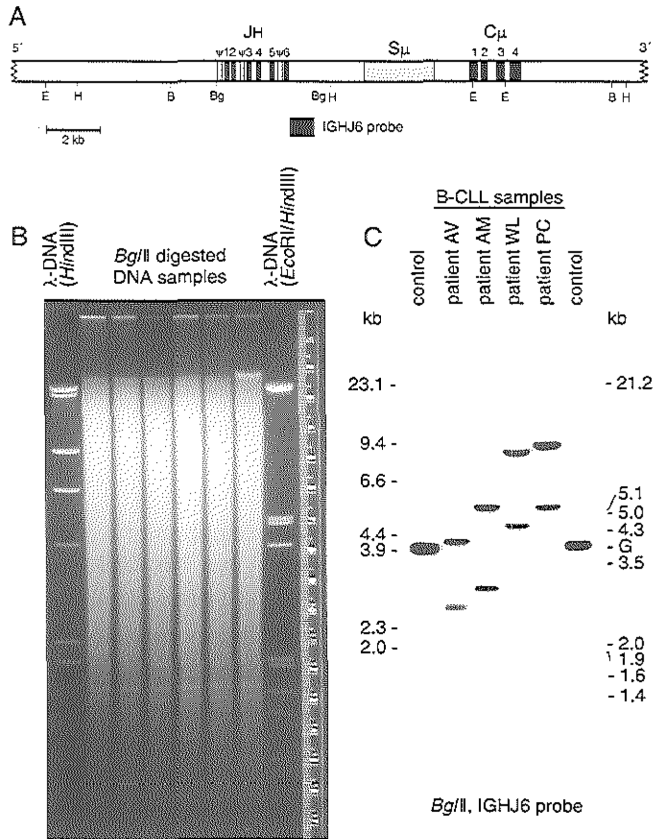


Figure 12.
Southern blot analysis of *IGH* genes.
(A) Restriction map of JH-Cμ region. The position of the relevant *Eco*RI (E), *Hin*dIII (H), *Bam*HI (B), and *Bgl*II (Bg) restriction sites are indicated. Also the location of the switch region (Sμ) is indicated. The solid bar represents the JH probe (IGHJ6).
(B) Ethidium bromide-stained agarose gel with size-separated *Bgl*II restriction fragments of control DNA and four different B-cell chronic lymphocytic leukemia (B-CLL) DNA samples. The two outer lanes contain size markers (*left*: *Hin*dIII digested λ DNA; *right*: *Eco*RI/*Hin*dIII digested λ DNA). The DNA fragments were blotted to a nylon filter.
(C) X-ray film after exposure to the nylon filter, which was hybridized to the ^{32}P-radiolabeled IGHJ6 probe. The size of the germline band (G) and the position of the size markers are indicated. The two control lanes contain the 3.9 kb germline band, whereas each of the four B-CLL lanes show two clonally rearranged bands, due to biallelic *IGH* gene rearrangements.

ferent restriction enzyme digests, which result in germline restriction fragments that are not affected by polymorphisms and which are smaller than or the size of 10 kb. The latter is important for prevention of comigration of germline and/or rearranged bands.[52,103-107]

Because Ig and TCR gene rearrangements in ALL are identical, the (identical) restriction fragments give rise to a clearly visible rearranged band, which is different from the germline band.[52] Furthermore, two rearranged bands of comparable density will be visible if the clonal cell population has rearranged both Ig or TCR alleles (Figure 12). In contrast to clonal cell populations, reactive (polyclonal) lymphoid cell proliferations contain many different Ig and TCR gene rearrangements that are detectable as a characteristic background pattern or smear of multiple faint rearranged bands. Thus, Southern blot analysis of Ig and TCR genes allows discrimination between clonal rearrangements and polyclonal rearrangements.[52,64] Leukemic cell populations can be detected with a sensitivity of approximately 5%, whereas the detection limit is 10% to 15% if a clonal cell population has to be identified within a background of polyclonal, reactive cells.

PCR analysis

The PCR technique allows the selective amplification of a particular DNA region while it is still incorporated in the total genomic DNA.[108,109] Knowledge of the precise nucleotide sequences, which flank the target DNA region, is a prerequisite for the PCR technique. Based on this information, two synthetic oligonucleotides are prepared that can hybridize to the flanking sequences of opposite strands (primer annealing), after the DNA has been denatured to single-stranded DNA.[108,109] The two oligonucleotides serve as primers for the *Taq* polymerase-mediated DNA synthesis (primer extension), which proceeds across the target DNA region using this region as template (Figure 13). The PCR process involves temperature-regulated cycles of DNA denaturation (94°C), primer annealing (55-65°C), and primer extension (72°C). Because the PCR product of one primer can serve as a template for the other primer in subsequent cycles, each successive PCR cycle essentially doubles the number of PCR products (Figure 13). Continuation of the PCR procedure for 20-30 cycles theoretically results in 2^{20}-2^{30} times amplification of the target DNA region. In principle, the PCR technique can amplify target DNA sequences up to 10 kb, but preferably the PCR target should not be longer than 2 kb in routinely performed PCR analyses.[109,110] The PCR primers for amplification of junctional regions are designed at opposite sides of the junctional region, generally within a distance of less than 500 bp.

Because most PCR studies on Ig and TCR gene rearrangements in leukemias are performed at the DNA level, the primers are complementary to exon and/or intron sequences of V, D, and/or J gene segments, depending on the type and completeness of the rearrangement.[111-113] Also, mRNA of complete V-(D-)J-C transcripts can

be used as target for the PCR technique after the mRNA has been transcribed into complementary (c)DNA by use of reverse transcriptase (RT-PCR). Such studies generally use V exon primers in combination with a single C exon primer.[114-116] It is obvious that the choice of primers depends on the type of Ig or TCR gene and the type of rearranged gene segments. It may be possible to design general or consensus primers, which recognize virtually all V or J gene segments of a particular Ig or TCR gene complex; family-specific primers, which recognize families of V or J gene segments; or even member-specific primers, which recognize individual V or J gene segments.[111-113,117]

It should be noted that the Ig and TCR genes not only contain functional V, (D), and J gene segments but also nonfunctional (pseudo) gene segments. Such segments can be involved in gene rearrangements, provided they are flanked by a proper RSS. Table 5 summarizes the estimated number of gene segments and families of the three Ig genes and four TCR genes.

Complete *IGH*, *TCRG*, and *TCRD* gene rearrangements can be analyzed relatively easily with the PCR technique, because the *IGH* gene complex contains only seven VH families and six functional JH gene segments and because the *TCRG* and

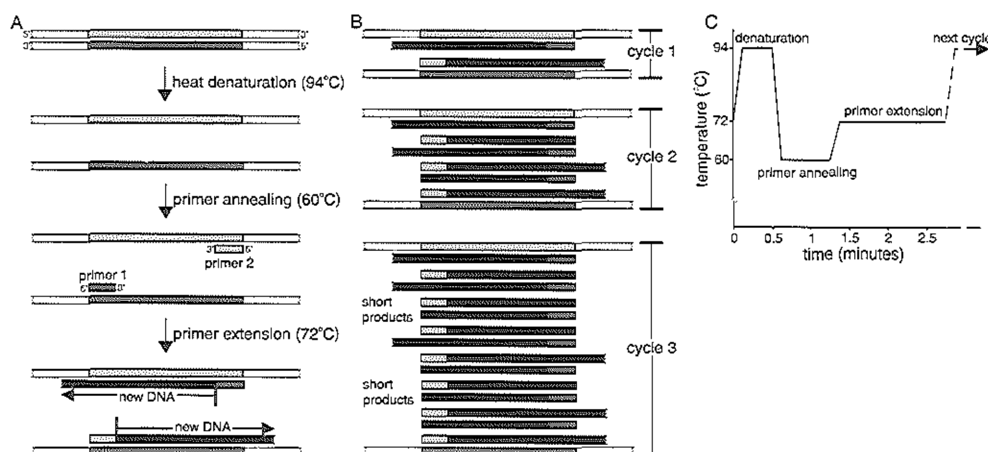


Figure 13.

Schematic diagram of PCR.

(A) Temperature-regulated PCR cycle in which double-stranded DNA is heat denatured at 94°C, followed by primer annealing at 60°C, and primer extension by *Taq* polymerase at 72°C. In the first PCR cycle, synthesis of the new DNA proceeds across the target DNA, resulting in long PCR products.

(B) In subsequent cycles, the PCR products of the previous cycle(s) can serve as template, resulting in short PCR products. Each PCR cycle essentially doubles the number of PCR products.

(C) Temperature cycle for denaturation, primer annealing, and primer extension. Each cycle takes 5 to 10 minutes, depending on the size of the PCR product (i.e., the duration of primer extension).

Table 5. Estimated number of human V, D, and J gene segments that can be potentially involved in Ig or TCR gene rearrangements.[a]

Gene segment	*IGH*	*IGK*	*IGL*	*TCRA*	*TCRB*	*TCRG*	*TCRD*
V (family)	~70 (7)	~60 (7)	~40 (11)	~60 (32)	~65 (30)	9 (4)	7[b]
D (family)	25 (7)	-	-	-	2	-	3
J (family)	6	5	5[c]	61[c]	13	5 (3)	4

a. The estimated numbers include non-functional (pseudo) gene segments with functional RSS, which allow rearrangement; orphan genes are excluded. The calculations are based on the international IMGT (ImMunoGeneTics) Database[57] and complete genomic sequences of *IGH*, *TCRB*, and *IGL*.[56,60,71]
b. These numbers include the non-functional δREC gene segment (*TCRD* locus) and the ψJα gene segment (*TCRA* locus).
c. Two of the seven Jλ gene segments (Jλ4 and Jλ5) have never been observed in *IGL* gene rearrangements, probably because of their inefficient RSS (Tümkaya et al., unpublished results).

TCRD genes contain a limited number of V and J gene segments (Table 4 and Figure 6).[54,94,118,119] This implies that only a restricted number of oligonucleotide primers is needed.[120,121] In principle, PCR analysis of *IGK*, *IGL*, *TCRA* and *TCRB* gene rearrangements is also possible, but it requires more primers, especially for the many different V and J gene segments in *TCRA* and *TCRB* gene complexes (Figure 6). RT-PCR analysis of *TCRA* and *TCRB* V-(D-)J-C transcripts still requires many different Vα or Vβ primers, although these can be used in combination with a single Cα or Cβ primer.[114-116]

One disadvantage of Ig or TCR gene analysis by PCR-based techniques, as compared with Southern blot analysis, is that the detectability of (clonal) rearrangements is limited by the choice of primers, raising the possibility of false-negative results. A more essential drawback is the risk of false-positive results owing to the fact that not only clonally rearranged Ig and TCR genes but also Ig and TCR rearrangements from normal, polyclonal cells are amplified.

Analysis of PCR-amplified Ig and TCR gene rearrangement products

PCR-mediated detection of clonal Ig and TCR gene rearrangements is relatively easy if the percentage of leukemic cells is high (e.g., greater than 90%). In such cell samples, the background of Ig and TCR gene rearrangements in normal polyclonal cells generally does not hamper the PCR studies of leukemic cells. This is illustrated in Figure 14, which shows that PCR products of comparable clonal *TCRD* rearrangements in different T-ALLs are clearly different from each other, based on differences in the size of their junctional regions (compare with Figure 10).[112] If many normal polyclonal B- or T-cells are present in the cell sample, many polyclonal PCR products will also be present. Discrimination between clonal (leukemia-derived) PCR

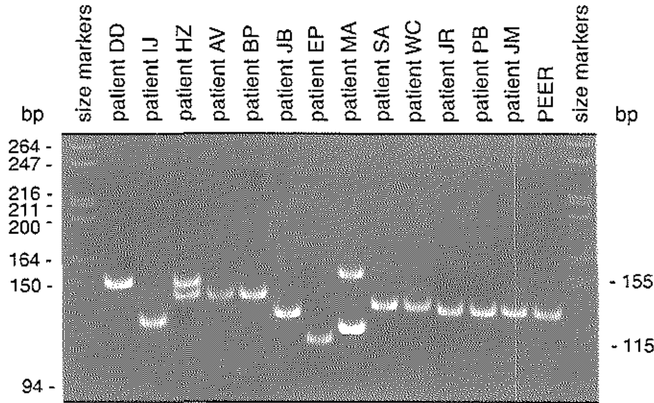


Figure 14.
Vδ1-Jδ1 PCR products in cases of T-ALL with monoallelic or biallelic Vδ1-Jδ1 rearrangements. The PCR products were separated in a polyacrylamide gel, resulting in optimal size separation. The positions of the size markers are indicated in the left margin. The differences in size of the PCR products (115-155 bp) are due to differences in size of the junctional regions (see Figure 10B).

products and polyclonal (normal) PCR products with standard gel electrophoresis is hampered by the fact that the clonal PCR products have to be identified as a dominant band within a background of multiple weaker bands of slightly different sizes, representing polyclonal PCR products.[113] Because junctional regions are 'fingerprint-like' sequences that differ between lymphocytes or lymphocyte clones, it is assumed that they represent specific markers for each individual leukemia.[111,117] Thus, strategies should be followed that employ the junctional regions of amplified rearranged Ig and TCR genes as PCR targets for discrimination between polyclonal and clonal cell populations. Methods that have been successfully applied to solve this background problem include direct sequencing of PCR products,[122] single-strand conformation polymorphism analysis,[123] denaturing gradient gel electrophoresis,[124] heteroduplex analysis (Figure 15),[125] temperature gradient gel electrophoresis,[126] high resolution radioactive fingerprinting,[113] and fluorescent gene scanning analysis.[127]

Ig and TCR gene rearrangements in B-lineage ALL

Based on their immunophenotypic characteristics, precursor-B-ALL are generally regarded as clonal malignant counterparts of normal precursor-B-cells. In line with this assumption, our studies indicate that more than 95% of precursor-B-ALL have *IGH* gene rearrangements and that most of them contain *IGK* gene rearrangements (30%) or deletions (50%); even 20% of precursor-B-ALL cases have *IGL* gene rearrangements (Table 6).[64,103,128] Deletions in the *IGK* genes are predominantly

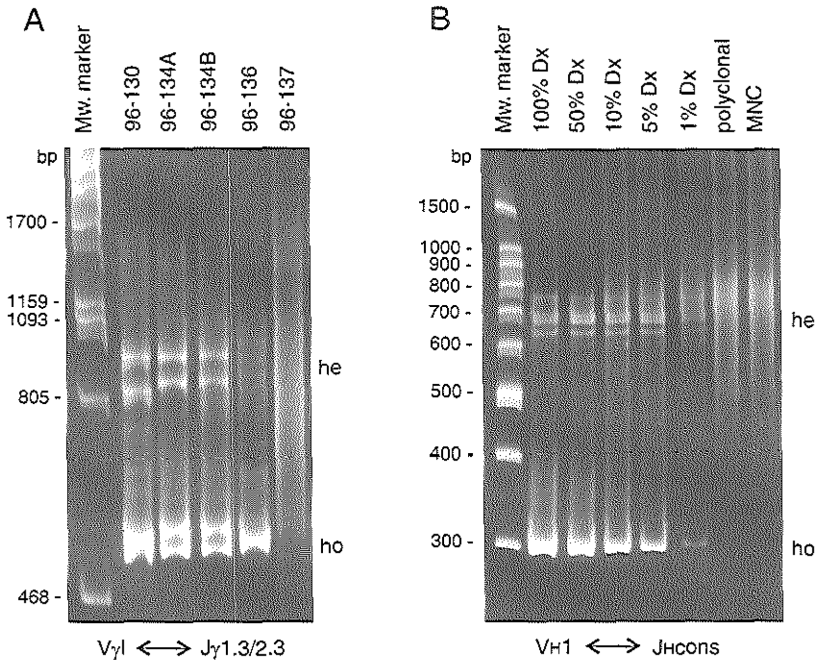


Figure 15.
(A) Heteroduplex analysis of Vγl-Jγ1.3/2.3 PCR products from several T-ALL and precursor-B-ALL patients in a non-denaturing 6% polyacrylamide gel. Following denaturation and renaturation of PCR products, the presence of homoduplexes in samples 96-130, 96-134A, 96-134B, and 96-136 proves the monoclonal character of these samples. The smear of heteroduplexes that is seen in 96-137 is indicative of a polyclonal cell population. The additional bands that are seen in samples 96-130, 96-134A, and 96-134B represent clonal heteroduplexes, resulting from renaturation of single-stranded fragments derived from biallelic rearrangements. The two homoduplexes from these biallelic rearrangements are easily seen in 96-134A and 96-134B (bone marrow and peripheral blood samples, respectively, from the same patient), but not in 96-130 owing to comigration.
(B) Heteroduplex PCR analysis of *IGH* in precursor-B-ALL patient. Malignant lymphoblasts at diagnosis contained a monoclonal VH1-JH gene rearrangement. Dilution of the diagnosis bone marrow DNA into polyclonal MNC DNA derived from healthy blood donors showed a sensitivity threshold of approximately 1%. he = heteroduplexes; ho = homoduplexes; Mw marker = 100-bp molecular weight marker.

mediated via the Kde sequence, which implies that *IGK* gene deletions can be identified as Kde rearrangements, which occur on one allele or both alleles in 20% and 30% of precursor-B-ALL cases, respectively.[103] Cross-lineage TCR gene rearrangements occur in high frequency in precursor-B-ALL cases: *TCRB*, *TCRG*, and *TCRD* gene rearrangements and/or deletions are found in 35%, 60%, and 90% of cases, respectively.[129,130]

Several studies have shown that newly diagnosed precursor-B-ALL are frequently oligoclonal because they contain multiple *IGH* gene rearrangements (30% to

40% of cases) and even multiple *IGK* gene rearrangements (5% to 10% of cases).[128,131] These multiple Ig gene rearrangements can result from both continuing rearrangement processes (e.g., continuing VH to DH-JH joining) and from secondary rearrangements (e.g., DH-JH replacements, VH-JH replacements, and Vκ-Jκ replacements), which result in one or more subclones.[131-135]

Comparative studies between cell samples taken at diagnosis and at relapse revealed that the ongoing rearrangements and secondary rearrangements can cause changes in the Ig and TCR gene configuration at the time of relapse.[134,136-139] Such changes are particularly prevalent in cases of precursor-B-ALL that already contain subclones at diagnosis.[137]

TCR and Ig gene rearrangements in T-ALL

The immunophenotype of T-ALL is fully comparable to those of cortical thymocytes. Subclassification of T-ALL into CD3$^-$, TCRγδ$^+$, and TCRαβ$^+$ subgroups also reveals major differences in TCR gene rearrangement patterns.[19,64] Although the frequency of TCR gene rearrangements in the total group of T-ALL is very high, approximately 10% of CD3$^-$ T-ALL still have all TCR genes in germline configuration;[19,64] this mainly concerns immature CD1$^-$/CD3$^-$ T-ALL of the prothymocytic/pre-T-ALL subgroup (see Table 2). The *TCRD* genes in CD3$^-$ T-ALL are rearranged in most cases (approximately 80%) and contain biallelic deletions in approximately 10% of cases.[19,94] As expected, all TCRγδ$^+$ T-ALL have *TCRG* and *TCRD* gene rearrangements and the majority (approximately 95%) also contain *TCRB* gene rearrangements.[21,94,112] All TCRαβ$^+$ T-ALL contain *TCRB* and *TCRG* gene rearrangements and have at least one deleted *TCRD* allele (= *TCRA* rearrangement); the second *TCRD* allele is also deleted in two-third of cases.[64,94] Cross-line-

Table 6. Frequencies of detectable Ig and TCR gene rearrangements and deletions in ALL.

	IGH		*IGK*		*IGL*	*TCRB*	*TCRG*	*TCRD*	
	R	D	R	D	R	R	R	R	D
Precursor-B-ALL	96%	3%	30%[a]	50%[a]	20%	35%	60%	55%	35%
T-ALL									
CD3$^-$	20%	0%	0%		0%	85%	90%	80%	10%
TCRγδ$^+$	50%	0%	0%		0%	95%	100%	100%	0%
TCRαβ$^+$	<5%	0%	0%		0%	100%	100%	35%	65%

a. A total of ~60% of precursor-B-ALL have IGK gene rearrangements and/or deletions.[103]
R, at least one allele rearranged; D, both alleles deleted.

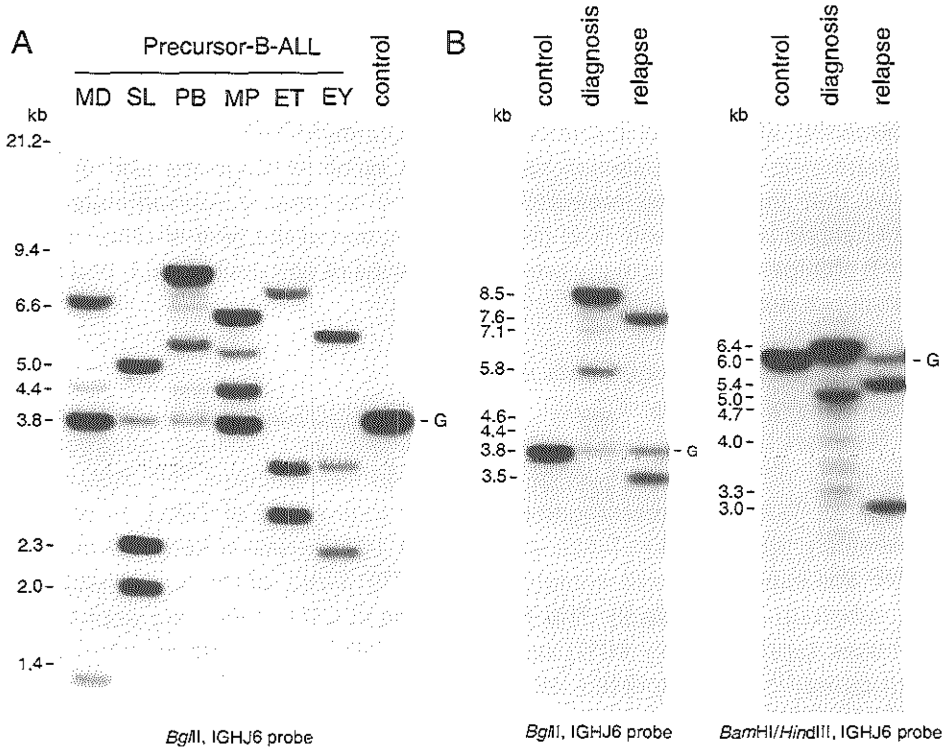


Figure 16.
Southern blot analysis of *IGH* genes in precursor-B-ALL.
(A) Analysis at diagnosis with the restriction enzyme *Bgl*II and the IGHJ6 probe (see Figure 12). Multiple rearranged bands are present in each precursor-B-ALL lane. In patient SL, this is due to trisomy 14, but in all other cases subclone formation (biclonality or oligoclonality) is the cause.
(B) Comparative analysis of DNA samples of patient peripheral blood at diagnosis and relapse with *Bgl*II and *Bam*HI/*Hin*dIII digests and the IGHJ6 probe. Multiple rearranged bands of different density are present in both digests at diagnosis. However, at relapse only two rearranged bands are present, which probably have the same size as two faint rearranged bands at diagnosis.

age Ig gene rearrangements occur at low frequency in T-ALL (approximately 20%) and involve virtually only *IGH* genes (Table 6).[64,140]

Comparative studies at diagnosis and at relapse revealed that continuing and secondary TCR rearrangements could also occur in 40% to 50% of T-ALL patients.[137-139] Nevertheless, TCR oligoclonality is rarely seen at diagnosis in T-ALL cases,[64,137] except for a few $CD3^-$ cases that showed a faint band of δREC-ψJα rearrangements normally involved in *TCRD* gene deletions.[82] Detailed studies of these T-ALL cases revealed that the detected δREC-ψJα rearrangements were fully polyclonal but were derived from otherwise monoclonal T-ALL.[141]

Analysis of ALL at diagnosis and subsequent relapse

Analysis of Ig and/or TCR gene rearrangement patterns at diagnosis and subsequent relapse can prove whether the recurrence of an ALL represents a relapse or a secondary malignancy. Southern blot analysis has been considered the gold standard for the comparison of the Ig/TCR gene rearrangement patterns between diagnosis and recurrence of a lymphoid malignancy (Figure 16).[137] Currently, comparative junctional region analysis can be easily performed by mixing the PCR products of both disease stages and evaluating them by heteroduplex method. In case of relapsed leukemia, mixing will result in a single homoduplex band upon heteroduplex analysis, whereas in case of a secondary malignancy two distinct homoduplexes as well as two heteroduplexes will be found (Figure 17). These heteroduplexes result from cross-renaturation of single-strand molecules of the two different malignant cell populations. Finally, when only nonidentical clonal PCR products at diagnosis and at relapse are detected with mixed heteroduplex PCR analyses, direct sequencing of

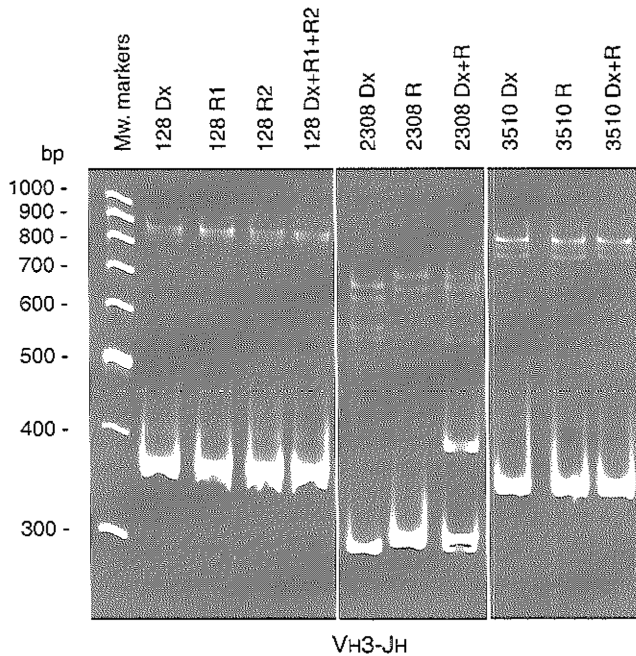


Figure 17.

Comparative heteroduplex analysis of VH3-JH PCR products in three precursor-B-ALL patients at diagnosis and at leukemia relapse. In two patients (0128 and 3510), identical clonal VH3-JH PCR products were found at both leukemia phases (identical size of homoduplexes and no heteroduplex formation after mixing and subsequent denaturation/renaturation). In patient 2308 monoclonal homoduplexes found at diagnosis and at relapse slightly differed in size. Mixing of the VH3-JH PCR products followed by heteroduplex PCR analysis demonstrated clear heteroduplex formation, proving that these VH3-JH gene rearrangements had different junctional regions.

clonal PCR products can be performed to exclude clonal evolution due to ongoing rearrangements; e.g. different V_H to D_H-J_H rearrangements but with an identical pre-existing D_H-J_H rearrangement.

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CHAPTER 1.2

MINIMAL RESIDUAL DISEASE IN LEUKEMIA PATIENTS*

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ABSTRACT

Because of developments in diagnosis of hemopoietic malignant diseases during the past two decades, routine and reliable identification of very low numbers of malignant cells, known as minimal residual disease (MRD), is now possible. Several large-scale studies have shown that monitoring of MRD in hemopoietic malignant disease predicts clinical outcome. In acute lymphoblastic leukemia, MRD detection is useful for evaluating early response to treatment and consequently for improving stratification, including treatment reduction. In acute promyelocytic leukemia and chronic myeloid leukemia, MRD information at specific time points enables effective early treatment intervention. MRD monitoring is also possible in other leukemia subtypes, but in these disorders the clinical value of MRD detection is not yet known.

INTRODUCTION

Modern treatment protocols lead to complete remission in a high proportion of patients with acute leukemia, but many of these patients relapse. Clearly, not all clonogenic malignant cells are killed, although the patients are classified as being in complete remission according to clinical and morphological criteria (Figure 1). For hemopoietic malignant disease to be detected with morphological techniques, malignant cells must make up at least 1–5% of the total cells. Consequently, morphology

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provides limited information about the effectiveness of treatment because only patients with a very poor prognosis (not achieving remission) can be identified. In patients who achieve remission, morphology cannot discriminate between patients with a high or low risk of relapse. Therefore, more sensitive techniques are needed to detect small numbers of malignant cells during and after treatment – detection of minimal residual disease (MRD).

Reliable techniques to detect MRD should have the following features:

- sensitivity of at least 10^{-3} (one malignant cell within 10^3 normal cells), but sensitivities of 10^{-4} to 10^{-6} are preferred;
- ability to discriminate between malignant and normal cells, without false-positive results;
- stable leukemia-specific markers – it should not give false-negative results because the MRD targets are lost during the course of disease;
- reproducibility between laboratories - this is essential for multicenter treat ment protocols;
- easy standardization and rapid collection of results;
- a method of quantifying MRD.

MRD detection techniques can be used for more accurate stratification of therapy in hemopoietic malignant disease. This stratification includes treatment intensification in patients at high risk of relapse and reduction in low-risk patients, who are being overtreated.

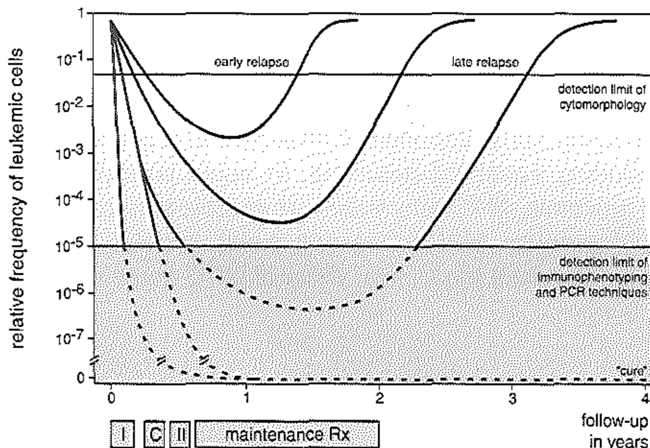

Figure 1.
Diagram of the frequencies of leukemic cells in peripheral blood or bone marrow of patients with acute leukemia, during and after chemotherapy, and during development of relapse. The detection limit of morphologic techniques, as well as the detection limit of flow cytometric immunophenotyping and PCR techniques are indicated. I, induction treatment; C, consolidation treatment; II, re-induction treatment.

TECHNIQUES AND TARGETS FOR MRD MONITORING

During the past 15 years, several methods of MRD detection have been developed and evaluated. These include conventional cytogenetics, cell-culture systems, fluorescent *in situ* hybridization, Southern blotting, immunophenotyping, and polymerase chain reaction (PCR) techniques. Most of these techniques are not suitable for clinical MRD detection because of limited sensitivity, specificity, or applicability. However, current multiparameter flow-cytometric immunophenotyping and PCR-based approaches for MRD monitoring can reach sensitivities of 10^{-3} to 10^{-6} and are quantitative, sufficiently specific, and broadly applicable. Flow-cytometric MRD detection is based on the occurrence of leukemia-associated immunophenotypes, such as aberrant, unusual, or ectopic antigen expression. PCR techniques can be used to detect tumor-specific sequences, such as junctional regions of rearranged immunoglobulin and T-cell receptor (TCR) genes, or breakpoint fusion regions of chromosome aberrations.

Principles of MRD monitoring by flow-cytometric immunophenotyping

Acute and chronic leukemias can be regarded as malignant counterparts of cells in immature and more mature stages of hemopoiesis, respectively (Figure 2). Consequently, the presence of normal hemopoietic cells limits the detection of leukemic cells.[1,2] Nevertheless, immunophenotypic MRD detection is possible in many hemopoietic malignant disorders, because the cells express aberrant or unusual antigens, or have clonal patterns of immunoglobulin or TCR protein expres-

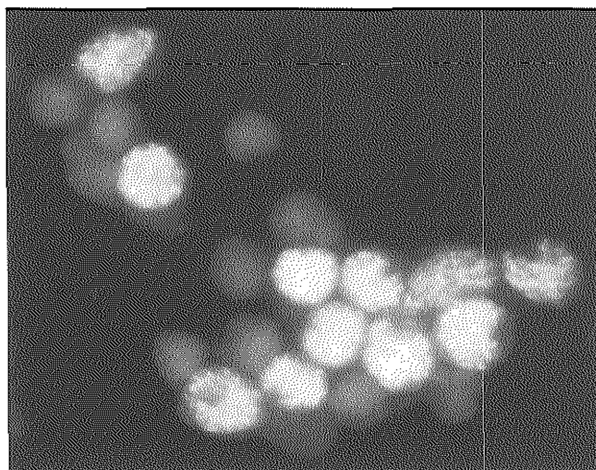

Figure 2.
Immunofluorescence staining of terminal deoxynucleotidyl transferase (TdT) in the nucleus of ALL cells, representing a typical marker of immature lymphoid blast cells.

sion.

Aberrant or unusual immunophenotypes are the result of cross-lineage antigen expression, maturational asynchronous expression of antigens, antigen overexpression, absence of antigen expression, and/or ectopic antigen expression (Figure 3).[1,2] Such leukemia-associated immunophenotypes can be identified in the majority of acute leukemias, but they are rare in mature hemopoietic malignant diseases. Asynchronous antigen expression occurs when two or more antigens not present at the same time during normal differentiation are coexpressed. In cross-lineage antigen expression, typical myeloid antigens are expressed on lymphoid cells or *vice versa* and B-lineage antigens on T-lineage cells or *vice versa*.[1,2] Ectopic antigen expression refers to the presence of particular antigens on cells outside their normal sites of production or homing areas, or to the expression of antigens that are normally expressed only on non-hemopoietic cells. Examples are the combined expression of terminal deoxynucleotidyl transferase (TdT) and a T-cell marker outside the thymus on T-lineage acute lymphoblastic leukemia (ALL) blasts, and the expression

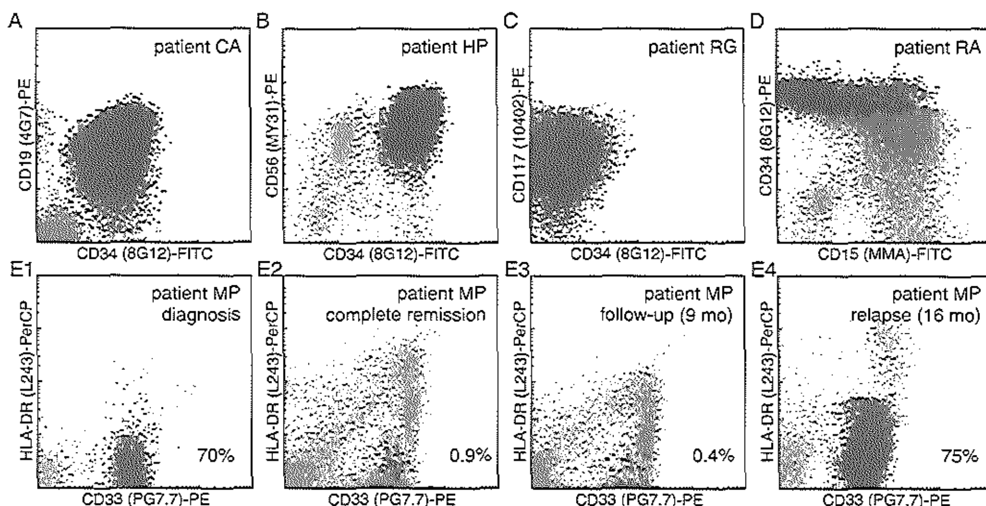

Figure 3.
(Top panel) Flow cytometric MRD monitoring showing examples of aberrant or unusual antigen expression patterns in four AML patients at diagnosis. (A) Cross-lineage expression of the B-lineage marker CD19 on CD34-positive myeloid blast cells. (B) Asynchronous expression of the more mature marker CD56 on CD34-positive myeloid blast cells. (C) Lack of CD34 expression on CD117 weakly-positive non-monocytic blast cells. (D) Overexpression of CD34 in a subset of AML blasts.
(Lower panel) Flow cytometric MRD monitoring in one AML patient. (E_1) 70% of CD33-positive AML blasts at diagnosis showed aberrant lack of HLA-DR expression as well as lack of CD34, CD15, CD14, and CD11b expression (detected with a cocktail of antibodies used with a third fluorochrome). Two follow-up samples show the persistence of moderate MRD levels (0.9% and 0.4%) at the time of first complete remission (E_2) and nine months after diagnosis (E_3), respectively. At relapse (16 months after diagnosis) 75% of AML blasts showed the aberrant immunophenotype again (E_4).

of the NG2 antigen in a subset of ALL and acute myeloid leukemias (AML) with *MLL* gene rearrangements, respectively. Since ALL blasts frequently display complex aberrant immunophenotypic features, they fall into so-called 'empty spaces' outside the multiparameter flow-cytometric dot plot templates of normal lymphoid cells in normal bone marrow and peripheral blood.[3,4]

Clonal immunoglobulin molecules are expressed by most chronic B-cell leukemias and B-cell lymphomas, and can be identified by single immunoglobulin light chain expression, i.e. immunoglobulin κ or immunoglobulin λ (Table 1). Clonal TCR$\alpha\beta$ molecule expression is detectable with antibodies against the variable (V) domains of TCRβ chains, which together cover 65–75% of TCR$\alpha\beta$-positive cells, and consequently allow detection of monotypic Vβ domain expression in most TCR$\alpha\beta$-positive T-cell malignant disorders.[5-7] Antibodies against the variable domains of TCRγ and TCRδ chains might contribute to clonality assessment of suspect TCR$\gamma\delta$-positive T-cell proliferations, but their sensitivity is limited because of the limited number of γ and δ variable domains.[8]

One pitfall of immunophenotypic MRD detection is that patterns of antigen expression can change during the course of disease. This is a particular characteristic of acute leukemias and may affect up to two-thirds of patients.[9,10] Frequently, leukocyte antigens are involved, which are not essential for MRD detection. In fact, at least one leukemia-associated antigen combination is retained by leukemic cells at relapse in more than 80% of patients.[10,11] Nevertheless, preferably two leukemia-associated antigen combinations per patient should be used for immunophenotypic MRD monitoring, in order to prevent false-negative results.

Immunoglobulin and TCR gene rearrangements as patient-specific 'fingerprints'

During early differentiation of B and T-cells, the germline variable (V), diversity (D) and joining (J) gene segments of the immunoglobulin and TCR gene complexes rearrange. Each lymphocyte gets a specific combination of V-(D-)J segments that codes for the variable domains of immunoglobulin or TCR molecules. The random insertion and deletion of nucleotides at the junction sites of V, (D,) and J gene segments make the junctional regions of immunoglobulin and TCR genes into fingerprint-like sequences, which probably differ in each lymphocyte and thus also in each malignant lymphoid disease. Therefore, junctional regions can be used as leukemia-specific targets for PCR analysis of MRD. Such targets can be identified at initial diagnosis in > 95% of patients with lymphoid malignant disease and in about 10% of patients with AML (Table 1) by using various PCR primer sets (Figure 4). The PCR products need to be analyzed for their clonal origin, e.g. by heteroduplex analysis or by gene scanning, to confirm that they are derived from the malignant cells and not from contaminating normal cells with similar immunoglobulin or TCR gene rearrangements.[12] Subsequently, the precise nucleotide sequence of the junctional

Table 1. Application of MRD techniques in hemopoietic malignancies.[a]

Disease category	Flow cytometric immunophenotyping (sensitivity)		PCR or RT-PCR analysis (sensitivity)	
	Aberrant immunophenotypes (10^{-3}-10^{-4})	Igκ/Igλ distribution or TCR-V analysis (10^{-2}-10^{-3})[b]	Junctional regions of Ig/TCR genes (10^{-3}-10^{-6})	Chromosome aberrations (10^{-4}-10^{-6})[c]
Precursor-B-ALL				
- children	80-90%	NA	~95%	40-50%
- adults	70-80%	NA	~90%	35-45%
T-ALL				
- children	>95%	30-35%[d]	>95%	10-25%
- adults	>95%	?	~90%	5-10%
Chronic B-cell leukemias	rare (<5%)	>95%	>95%	rare (<5%)
Chronic T-cell leukemias	rare (5-10%)	60-65%[e]	~95%	rare (<5%)
B-cell lymphomas	rare (<5%)	>95%	70-80%[f]	25-30%
T-cell lymphomas	20-25%[g]	50-60%[e]	~95%	10-15%
AML	70-90%	NA	~10%	10-30%[h]
CML	NA	NA	NA	>95%

a. The percentages indicate the applicability of the MRD techniques per category of hemopoietic malignancies; J.J.M. van Dongen, unpublished results. NA = not applicable.
b. Sensitivity can be improved to $\leq 10^{-3}$ in triple labelings with specific markers like BCL2, cytoplasmic CD3, or ALK proteins.
c. Only concern chromosome aberrations with fusion genes because of their detectability at the DNA level or RNA level, such as t(14;18) with the *BCL2-IGH* fusion in some B-cell lymphomas, and t(2;5) with the *NPM-ALK* fusion gene in some T-cell lymphomas.
d. Based on the expression of TCRαβ molecules (20% of cases) or TCRγδ molecules (~10% of cases); this information is not available for adult ALL.
e. Antibodies to TCR-V recognize 65% to 70% of TCRαβ molecules and most TCRγδ molecules.
f. Somatic mutations hamper primer annealing in some patients with B-CLL or B-cell lymphomas.
g. Mainly based on T-ALL-like immunophenotype in T-lymphoblastic lymphoma and NPM-ALK expression in ~50% of large cell anaplastic lymphomas of T-cell lineage.
h. Frequency of PCR-detectable chromosome aberrations (with fusion genes) is age dependent: ~30% in childhood AML; ~20% in adult AML (≤60 years); ~10% in elderly AML (>60 years); see also Table 2.

Abbreviations: B-ALL, B-cell lineage acute lymphoblastic leukaemia.T-ALL, T-cell lineage acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CML, chronic myeloid leukaemia; Ig, immunoglobulin; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; TCR, T-cell receptor.

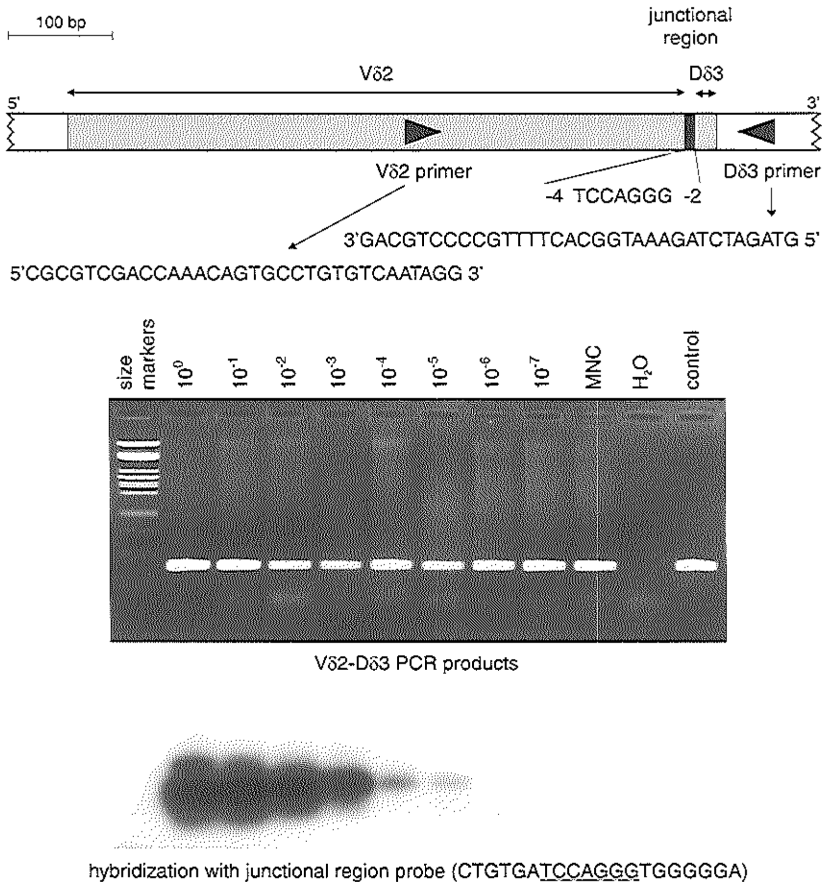

Figure 4.
PCR target for MRD detection in a patient with precursor-B-ALL with a Vδ2-Dδ3 rearrangement. The specificity of the junctional region is based on the deletion of six nucleotides and the random insertion of seven nucleotides (top panel). This sequence information was used to design a patient-specific junctional region probe. DNA from the ALL cells was diluted into DNA from normal blood mononuclear cells (MNC) and analyzed by PCR with Vδ2 and Dδ3 primers. PCR products were size-separated on an agarose gel (middle panel), blotted onto a nylon membrane, and hybridized with the junctional region probe (inserted nucleotides are underlined) (lower panel). In all dilution steps and in the mononuclear cells, Vδ2-Dδ3 PCR products were found, but only the first five dilution steps showed leukemia-derived PCR products, i.e. a sensitivity of 10^{-5} was reached.

regions should be determined. This sequence information allows the design of junctional region-specific oligonucleotides. These oligonucleotides can be used to detect malignant cells among normal lymphoid cells during follow-up of patients in two different ways. One uses the oligonucleotides as patient-specific junctional region probes in hybridization experiments to detect PCR products derived from the malignant cells (Figure 4). Alternatively, the junctional-region-specific oligonucleotide can

be used as a primer to specifically amplify the rearrangements of the malignant clone.[12]

During B-cell differentiation, immunoglobulin gene rearrangements can undergo further rearrangement, or might be subjected to somatic hypermutation processes. These events may modify PCR targets for MRD during the disease course and potentially generate false-negative results – this should be taken into account in the design of PCR strategies to detect MRD in ALL as well as for follicular B-cell malignant diseases.

Breakpoint fusion regions of chromosome aberrations as leukemia-specific markers

An advantage of using chromosome aberrations as tumor-specific PCR targets for MRD detection is their stability during the disease course. However, many hemopoietic malignant diseases do not have specific chromosome aberrations that can be detected by PCR (Table 1).

Chromosome aberrations can be used as targets only if the PCR primers are chosen at opposite sides of the breakpoint fusion region, preferably within a distance of < 2 kb. However, only in a few hemopoietic malignant diseases the breakpoints cluster in a small area, enabling PCR-mediated amplification of breakpoint fusion sequences at the DNA level. This is the case in t(14;18) in follicular-cell lymphoma, where most breakpoints are clustered in a few small regions of the *BCL2* gene, which are next to one of the JH gene segments of the immunoglobulin heavy-chain locus. Other examples include T-ALL-associated aberrations involving the *TAL1* gene (Table 2). The nucleotide sequences of breakpoint fusion regions differ between patients; therefore, these regions are unique patient-specific MRD-PCR targets, which can be reliably identified by use of patient-specific oligonucleotide probes.

In most translocations, the breakpoints of different patients are spread over one or more introns, which makes identification of breakpoint fusion regions at the DNA level laborious and time-consuming. However, several malignant diseases with chromosome aberrations have tumor-specific fusion genes, which are transcribed into fusion-gene mRNA molecules that are similar in individual patients despite distinct breakpoints at the DNA level (Figure 5).[13] The most frequently occurring fusion genes in acute leukemias are summarized in Table 2.[13,14] Other examples include *BCR-ABL* transcripts in the case of chronic myeloid leukemia (CML) and *NPM-ALK* transcripts in anaplastic large-cell lymphoma with t(2;5). Depending on the type of chromosome aberration, sensitivities of 10^{-3} to 10^{-6} can be reached with PCR.[13] However, because of this high sensitivity, cross-contamination of reverse transcriptase PCR (RT-PCR) products between patients' samples is a major pitfall in PCR-mediated MRD detection studies. Such cross-contamination is difficult to recognize, since leukemia-specific fusion-gene RT-PCR products are not patient-specific.

Table 2. Chromosome aberrations with fusion genes as PCR targets for MRD detection in acute leukemias.[a]

Disease category Aberration	Target (mRNA or DNA level)	Frequency of applicability[b]	
		children	adults
Precursor-B-ALL			
t(1;19)(q23;p13)	*E2A-PBX1* (mRNA)	5-8%	3-4%
t(4;11)(q21;q23)	*MLL-AF4* (mRNA)	3-5%[c]	3-4%
t(9;22)(q34;q11)	*BCR-ABL* (mRNA)	5-8%	25-40%
11q23 aberrations	*MLL* fusion genes (mRNA)	5-6%	<5%
t(12;21)(p13;q22)	*TEL-AML1* (mRNA)	25-30%	1-3%
TOTAL:		40-50%	35-45%
T-ALL			
TAL1 deletion	*SIL-TAL1* (DNA or mRNA)	10-25%	5-10%
AML			
t(8;21)(q22;q22)	*AML1-ETO* (mRNA)	10-15%	2-5%
t(15;17)(q23;q21)[e]	*PML-RARA* (mRNA)	8-10%	1-3%
inv(16)(p13q22)	*CBFB-MYH11* (mRNA)	5-8%	2-5%
11q23 aberrations	*MLL* fusion genes (mRNA)	15-20%	3-7%
TOTAL:		35-40%	10-20%[d]

a. Detection limit of PCR analysis of chromosome aberrations is 10^{-3} to 10^{-5}.
b. Represent frequencies within the precursor-B-ALL, T-ALL, and AML groups.
c. In infant ALL, the frequency of t(4;11) can be as high as 70%.
d. Mainly concern adult AML patients <60 years old. In AML patients >60 years the total frequency of PCR-detectable fusion genes is <10%.
e. In southern European regions the frequency of t(15;17) with *PML-RARA* is essentially higher than in northern European regions.
B-ALL, B-cell lineage acute lymphoblastic leukaemia; T-ALL, T-cell lineage acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; PCR, polymerase chain reaction; MRD, minimal residual disease.

Quantification of MRD by use of PCR analysis

Whereas immunophenotyping gives direct quantitative information, MRD quantification by PCR analysis is a complex process. First, the isolated DNA or RNA must be of good quality and have potential to be amplified. Furthermore, minor variations in RT efficiency, primer annealing, and primer extension may lead to major variations at the end of PCR, i.e. after 30–35 PCR cycles. The difficulty of quantifying MRD in follow-up samples can be partly overcome by comparison with serial dilutions of leukemic-cell DNA or RNA from diagnosis into DNA or RNA of normal mononuclear

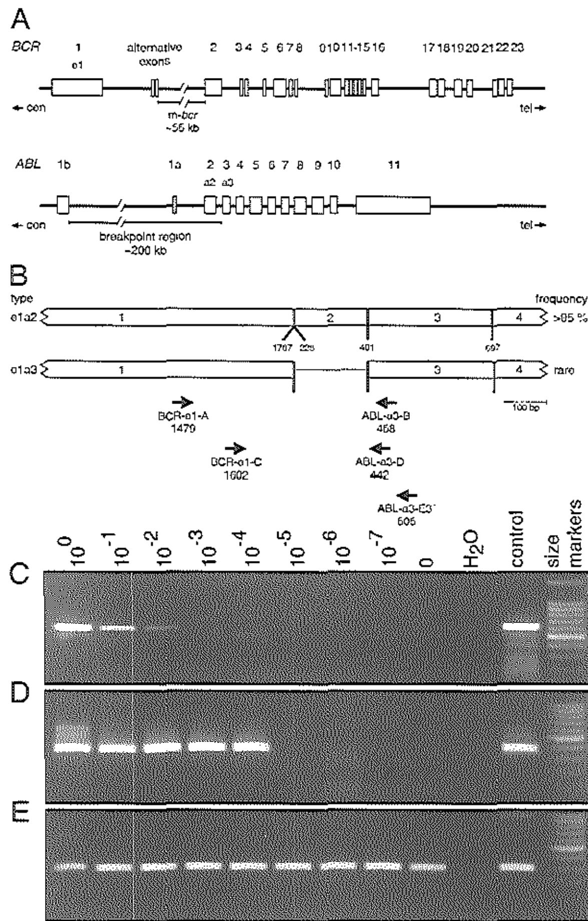

Figure 5.
RT-PCR analysis of *BCR-ABL* fusion gene transcripts for MRD detection. (A) Schematic diagram of the exon/intron structure of the *BCR* and *ABL* genes involved in t(9;22)(q34;q11). The centromeric (cen) and telomeric (tel) orientation, exon numbering, and relevant breakpoint regions are indicated. The old nomenclature for *BCR* exon 1 and *ABL* exons 2 and 3 is also shown. (B) Schematic diagrams of the *BCR-ABL* p190 type fusion gene transcripts. The numbers under the fusion gene transcript refer to the first (5') nucleotide of the involved exon, except when the last (3') nucleotide of the upstream gene is indicated. The arrows indicate the relative position of the primers, the numbers refer to the 5' nucleotide position of each primer. The outer primers A and B (BCR-e1-A and ABL-a3-B) are used for first round amplification and the internal primers C and D (BCR-e1-C and ABL-a3-D) are used for the nested RT-PCR reaction. Primer E is the so-called 'shifted' primer used exclusively to confirm the positive results obtained with A↔B primers. See reference [13] for detail of development of primers (C) Agarose gel electrophoresis of first round amplification of serially diluted leukemic cells derived from a patient with precursor-B-ALL. In the first round, RT-PCR products can be detected down to 10^{-1} to 10^{-2} dilution mixtures. (D) Agarose gel electrophoresis of a nested RT-PCR reaction of the same serially diluted samples. (E) Agarose gel electrophoresis of a control RT-PCR amplification using primers for the constitutively expressed *ABL* gene.

cells. This dilution series is run in parallel with the follow-up samples to estimate the tumor load in each follow-up sample semiquantitatively by comparison of the hybridization signals.[12] A more precise, but laborious, quantification method is based on limiting dilution of MRD-positive follow-up samples.[15] To make this assay reliable, replicate experiments need to be done to find out the amount of MRD. Another less tedious strategy for quantification is 'competitive PCR', which uses an internal standard that is coamplified with the target. This technique compares the PCR signal of the leukemia-specific target with that of known concentrations of an internal standard (the competitor).[16]

Recently, a new technology has become available, 'real-time quantitative PCR' (RQ-PCR). In contrast to PCR endpoint quantification techniques, RQ-PCR permits accurate quantification during the exponential phase of PCR amplification. This method has a very large dynamic detection range over five orders of magnitude, thereby eliminating the need for serial dilutions of follow-up samples. Also, the quantitative data are quickly available since post-PCR processing is not necessary. Therefore, RQ-PCR is suitable for the quantitative detection of MRD, by use of junctional regions of immunoglobulin and *TCR* gene rearrangements, or breakpoint fusion regions of chromosome aberrations as PCR targets (Figure 6).[17,18]

CLINICAL VALUE OF MRD MONITORING IN HEMOPOIETIC MALIGNANT DISEASES

MRD monitoring in ALL for assessment of early treatment response

Several studies, mainly in children and based on immunoglobulin/TCR gene rearrangements as PCR targets for monitoring MRD, have shown that the most important application of MRD monitoring in ALL is to assess initial responses to cytotoxic therapy.[19-22] Low amounts or absence of MRD in bone marrow after induction therapy seem to predict good outcome, and the risk of relapse is proportional to the level of MRD.[19-22] Multivariate analyses showed that the level of MRD after induction therapy is the most powerful prognostic factor, independent of other clinically relevant risk factors, such as age, blast count, immunophenotype, presence of chromosome aberrations at diagnosis, and response to prednisone.[20-22] The results from the large prospective MRD study of the International BFM Study Group showed that information on the kinetics of the decrease in tumor load after induction treatment and before consolidation treatment, is more useful than analyzing MRD at one time point (Figure 7).[22] It makes distinctions between patients at low risk, with no MRD at both time points (5-year relapse rate of 2%); patients at high risk, with intermediate (10^{-3}) or high ($\geq 10^{-2}$) MRD at both time points (5-year relapse rate of 80%); and patients at intermediate risk (5-year relapse rate of 22%). The low-risk group is of substantial size (about 45%), similar to the frequency of survivors of childhood ALL

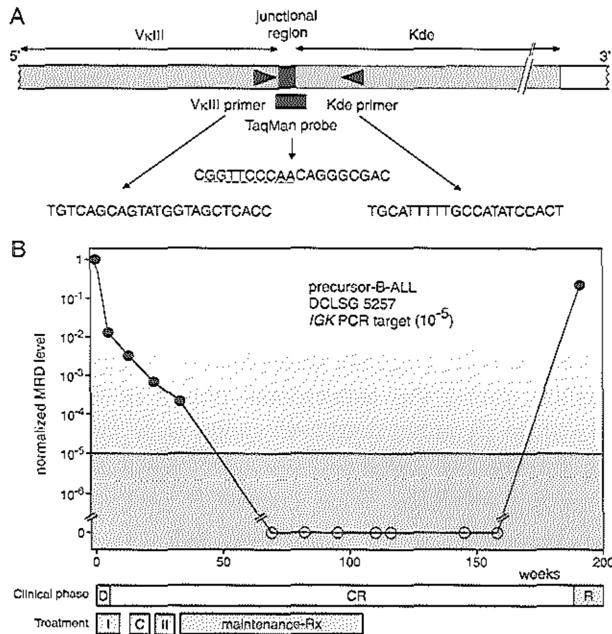

Figure 6.
(A) Schematic representation of VκIII-Kde rearrangement as MRD-PCR target in a patient with precursor-B-ALL treated with the International BFM Study Group treatment protocol. The junctional region sequence of this rearrangement had a deletion of 18 germline nucleotides and a random insertion of nine nucleotides. Sequences and positions of the germline VκIII and Kde primers are indicated as well as the patient-specific TaqMan probe (with junctional region nucleotides underlined), which was used for RQ-PCR analysis (see ref.[17] for details). **(B)** MRD kinetics during the follow-up of the patient measured by RQ-PCR using the VκIII-Kde gene rearrangement. Based on high MRD levels during the first two time points this patient was classified into MRD-based high-risk group (see Figure 7). Although the patient achieved an MRD-negative status during treatment, he relapsed after cessation of maintenance treatment. D, diagnosis; I, induction treatment; C, consolidation treatment; II, re-induction treatment; CR, complete remission; R, relapse.

in the 1970s, before treatment intensification was introduced. This group might particularly profit from treatment reduction. On the other hand, the high-risk group is larger than any previously identified high-risk group in childhood ALL (about 15%) and has an unusually high 5-year relapse rate of 80%. This group might benefit from further intensification of treatment, including bone-marrow transplantation during first remission or new treatment approaches.

Results from MRD studies based on flow-cytometric immunophenotyping were similar. In a large series of studies in pediatric patients with ALL, Coustan-Smith and colleagues[23] showed that patients with high amounts of MRD ($\geq 10^{-2}$) at the end of induction phase (6 weeks) or moderate to high levels of MRD ($\geq 10^{-3}$) at week 14 of continuation therapy, had particularly poor outcome. Moreover, detectable MRD lev-

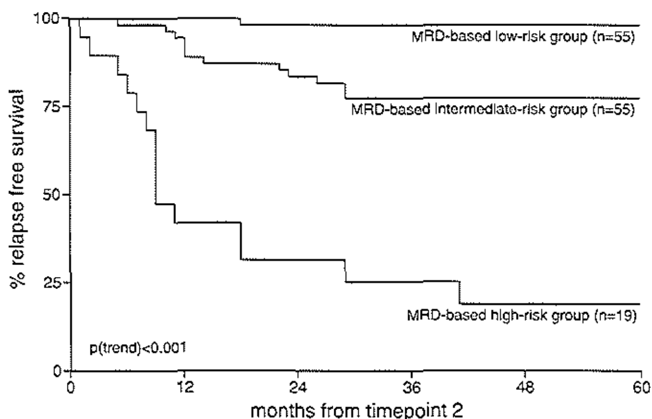

Figure 7.
Relapse-free survival of the three MRD-based risk groups of children treated for ALL according to protocols of the International BFM Study Group. The three risk groups were defined by combined MRD information at the end of induction treatment (time point 1) and before consolidation treatment (time point 2).[22] Patients in the low-risk group have no MRD at both time points (43% of patients), patients in the high-risk group have MRD degrees of $\geq 10^{-3}$ at both time points (15% of patients), and the remaining patients form the MRD-based intermediate-risk group (43% of patients).

els ($\geq 10^{-4}$) at each consecutive time point were associated with a higher relapse rate. Thus, flow cytometric MRD monitoring also provides independent prognostic information in children with ALL. Ciudad and colleagues[24] showed that flow cytometric detection of MRD was associated with relapse not just in childhood ALL, but also in adult ALL .

Continuous MRD monitoring in ALL throughout chemotherapy has shown that steady decrease of MRD levels is associated with a favorable prognosis, whereas persistently high or steadily increasing MRD levels generally lead to clinical relapse.[20-24] This information is of limited clinical value when compared with assessment of early treatment response, but might be advantageous for high-risk patients to evaluate the effectiveness of bone marrow transplantation or alternative treatment approaches. For example, no detectable MRD before bone marrow transplantation seems to be a prerequisite for a successful outcome in childhood ALL patients.[25]

MRD detection in CML for post-bone marrow transplantation monitoring

In virtually all (>95%) CML patients, MRD monitoring can be performed by RT-PCR detection of *BCR-ABL* fusion transcripts (Table 1 and Figure 5). The most relevant clinical application of MRD in CML is the assessment of treatment response after bone marrow transplantation, because virtually all patients on chemotherapy, interferon-α therapy, and the novel STI-571 therapy, remain RT-PCR positive.[16,26,27] After bone marrow transplantation, the vast majority of patients are PCR positive dur-

ing the first 6-9 months, and *in vitro* experiments have shown that some *BCR-ABL*-positive cells keep their clonogenic potential. Negative PCR results within 1 year of bone marrow transplantation indicate that disease has been cured, while patients with positive PCR results more than 1 year after bone marrow transplantation have a significantly greater risk of relapse.[16] The group of high-risk patients could be identified with serial quantitative PCR analyses, which generally show increasing MRD levels several months before hematological or cytogenetic relapse. Patients who stay in remission generally have decreasing or persistently low MRD levels, but some patients test positive for *BCR-ABL* mRNA up to 10 years after allogeneic bone marrow transplantation.[28] Quantitative MRD studies in CML have enabled the definition of molecular relapse after allogeneic bone marrow transplantation. It is equivalent to rising or persistently high MRD levels (*BCR-ABL/ABL* ratio of > 0.02%) in two consecutive specimens more than 4 months after bone marrow transplantation.[29] Quantitative MRD analysis has also been used for monitoring the response to immunotherapy, i.e. donor lymphocyte infusions for patients relapsing after allogeneic bone marrow transplantation.[30] Preliminary data indicate that such immunotherapy is more effective when given at the cytogenetic or molecular relapse phase, when the burden of disease is low. In responders, this early treatment can lead to sustained negative PCR results.[30]

MRD detection in APL as example of continuous monitoring for treatment titration

Current acute promyelocytic leukemia (APL) treatment protocols combine all-*trans*-retinoic acid (ATRA) with consolidation chemotherapy. Most patients achieve clinical remission, but 20–30% relapse. MRD studies in APL are based on RT-PCR monitoring of *PML-RARA* fusion mRNA associated with t(15;17), which is present in about 90% of APL patients. The results from several retrospective and prospective RT-PCR studies in APL patients, showed variable MRD levels during chemotherapy. To get clinically relevant information, continuous prospective MRD monitoring is required during the first 6–12 months after consolidation treatment. Reappearance of detectable MRD in this time characterizes patients at increased risk of relapse and generally precedes hematological relapse at a median time of 2–3 months. This information helped to define molecular relapse in APL, which is manifested by conversion from negative to positive RT-PCR results in two successive bone marrow samplings during follow-up.[31] Patients treated at the time of molecular relapse have much better 2-year event-free survival rates compared with patients treated at the time of hematological relapse (92% versus 44%).[32]

Relevance of MRD detection in non-APL subtypes of AML

PCR targets for MRD investigation in AML subtypes other than APL are only available in less than 20% of patients (Table 2). Therefore, the only MRD technique

available for the vast majority of AML patients is flow cytometric immunophenotyping (Table 1). Multiparameter flow cytometry studies indicate that persistence and/or increase of cells with leukemia-associated immunophenotypes precede hematological relapse (Figure 3). This suggests that continuous MRD monitoring in AML could be beneficial. However, three studies showed a strong prognostic association between MRD levels at the end of induction treatment and the risk of AML relapse. This is consistent with the estimation of early treatment response.[33-35] San Miguel and colleagues[34] showed that the level of MRD detected both after induction and intensification therapy, is associated with the risk of relapse, as well as with multidrug resistance at diagnosis. In a more recent study involving 126 patients with AML, early flow cytometric MRD analysis after induction therapy was used to identify different risk groups. Such information can contribute to post-induction treatment stratification.[36] The three-year cumulative frequency of relapse was 84% for high-risk patients (MRD $> 10^{-2}$), 45% for the intermediate-risk group, and 14% for the low-risk group (MRD $< 10^{-3}$). High MRD levels were also indicative of a poor prognosis when APL and other AMLs were analyzed separately. Adverse cytogenetic subtypes, necessity for two or more chemotherapy cycles to induce complete remission, and high leukocyte counts at diagnosis, were all associated with high MRD levels. Moreover, multivariate analysis showed that the MRD level was the most powerful independent prognostic factor, followed by cytogenetics, and the number of cycles required to achieve complete remission.[36]

The clinical value of the RT-PCR-based MRD studies in AML patients with either t(8;21) or inv(16) is not certain. These chromosome aberrations are present in a small subset of AML patients (10-20%) with a fairly good prognosis. Also, several RT-PCR studies suggest that *AML1-ETO* or *CBFB-MYH11* fusion transcripts are detectable in the bone marrow and peripheral blood of patients in long-term remission, while other research shows that the transcripts disappear in individuals in complete remission.[37-40] Preliminary results from several semi-quantitative RT-PCR and/or RQ-PCR studies indicate that a gradual reduction of fusion mRNA levels or a change from positive to negative PCR results during the course of disease, is associated with durable clinical remission. In contrast, persistently high MRD levels during treatment are associated with hematological relapse.[41]

MRD detection in chronic B-lineage malignant diseases

MRD monitoring is possible in virtually all mature lymphoid malignant diseases through PCR analysis of immunoglobulin/TCR gene rearrangements and, to lesser extent, by immunophenotyping (Table 1). The detection of MRD might be particularly valuable in more aggressive disorders, which can be cured with intensive treatment approaches including bone marrow transplantation.[42,43]

MRD data in B-CLL are preliminary, mostly retrospective, and lack obvious clinical value. It is clear that MRD kinetics in B-CLL differs depending on the treatment

protocol.[42] Nevertheless, patients with high-risk B-CLL might profit from more precise treatment monitoring.

MRD detection might be particularly important in high and intermediate-grade lymphomas, where circulating lymphoma cells are frequently found in bone marrow and peripheral blood. The vast majority of clinical studies have concentrated on follicular lymphomas with t(14;18) using the *BCL2-IGH* fusion gene as a DNA target for MRD-PCR analysis. Some studies showed that no detectable MRD during cytotoxic treatment is associated with longer relapse-free survival.[44] In contrast, others showed that there are circulating t(14;18)-positive cells in patients in long-term clinical remission after therapy for localized follicular lymphoma or even in healthy individuals.[45,46] These data imply that positive results in sensitive *BCL2-IGH* PCR studies should be interpreted with caution. Further investigations and standardization between laboratories are needed to establish quantitative criteria for molecular remission in follicular lymphoma, so that MRD information may be used in clinical decision making.

Patients with mantle-cell lymphoma continuously tested positive for MRD in bone marrow and/or peripheral blood during chemotherapy when *IGH* gene rearrangements and *BCL1-IGH* fusion genes are used as DNA targets. In the majority of patients, MRD levels varied between 10^{-2} and 10^{-3}, indicating significant resistance to conventional chemotherapy regimens.[47]

CONCLUSIONS

Reliable quantitative PCR and immunophenotyping techniques are available for MRD detection in patients with hemopoietic malignant diseases. Each MRD technique has its advantages and disadvantages, which have to be weighed up carefully before making an appropriate choice for each disease category. False-positive and false-negative results should be prevented and the MRD detection techniques should be sufficiently sensitive. These requirements can generally be met with PCR analysis of chromosome aberrations, if adequate precautionary measures are taken to prevent cross-contamination of PCR products. PCR analysis of junctional regions of immunoglobulin/TCR gene rearrangements can be used in all categories of lymphoid malignant disease, and are highly sensitive. However, the ongoing and secondary rearrangements in ALL as well as continuing somatic hypermutation in a subset of mature B-lineage malignant diseases, poses a risk for false-negative results. Flow cytometric immunophenotyping is less sensitive than PCR-based MRD techniques. However, this sensitivity can still be satisfactory for obtaining clinically relevant information in some disease categories. Particularly in AML, immunophenotyping is the only MRD detection technique available for the majority of patients.

Even when highly sensitive MRD detection techniques give negative results,

there may still be malignant cells present. Each MRD test only screens 10^5 to 10^6 cells, which is a tiny fraction of the total number of hemopoietic cells in a human body. In addition, the distribution of low numbers of malignant lymphoid cells throughout the body may not be homogeneous, so the cell sample may not be representative.

Finally, the clinical impact of MRD detection in different hemopoietic diseases is not the same. In ALL, the main application of MRD information is the evaluation of early treatment response, with precise measurement of tumor-load reduction during remission induction therapy. In contrast, in CML and APL, MRD levels have to be monitored over a period of time before the information is clinically useful – it can then be used for attuning treatment. By analyzing MRD in CML and APL, patients at high risk of relapse can be identified at an early stage, when the tumor load is low (molecular relapse) and when restarting treatment is more effective. This might also apply in other subtypes of AML and mature lymphoid malignant diseases. However, further studies are required to fully define the disease-specific 'MRD windows' (time-span and minimal sensitivity) for obtaining clinically relevant MRD information in AML, chronic lymphocytic leukemias, and malignant lymphomas. Furthermore, MRD detection techniques can be applied for several other specific aims, like detection of minimal central nervous system involvement in ALL, early diagnosis of leukemia and lymphoma in patients with unexplained cytopenias, improved staging of lymphomas, and the detection of malignant cells in autologous stem-cell grafts.

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2

CHAPTER 2.1

JUNCTIONAL REGIONS OF REARRANGED IMMUNOGLOBULIN AND T-CELL RECEPTOR GENES AS LEUKEMIA-SPECIFIC 'FINGERPRINTS'

The variable domains of the immunoglobulin (Ig) and T-cell receptor (TCR) molecules are involved in antigen recognition and constitute the enormous antigen-specific receptor diversity of the immune system. Each variable domain is encoded by two or more gene segments, which are joined to each other via gene rearrangement processes during early lymphoid differentiation.[1,2] The combinations of variable (V), diversity (D), and joining (J) gene segments and the combination of two protein chains with different variable domains per antigen-receptor molecule determines the so-called combinatorial diversity. The repertoire of Ig and TCR molecules is further extended by the so-called junctional diversity, which is based on deletion of germline nucleotides by trimming the ends of the rearranging gene segments and by random insertion of nucleotides between the joined gene segments.[1,2]

Consequently, the junctional regions of rearranged Ig and TCR genes are unique "*fingerprint-like*" sequences, which are assumed to be different in each lymphoid cell and thus also in each lymphoid malignancy.[3,4] Therefore, junctional regions can be used as tumor-specific targets for PCR-based detection of minimal residual disease (MRD).[5-7] For this application, the various clonal Ig and/or TCR gene rearrangements have to be identified in each lymphoid malignancy at diagnosis by use of selected PCR primer sets and distinguished from polyclonal rearrangements derived from normal lymphocytes. For that purpose, we applied a modified heteroduplex PCR technique, which generated results largely comparable to Southern blotting, which is considered as gold standard for Ig/TCR clonality assessment (see Chapters 2.2, 2.4 and 2.8).

This chapter presents PCR-based strategies for detection and identification of lineage-specific and cross-lineage Ig/TCR gene rearrangements as molecular targets for MRD monitoring in acute lymphoblastic leukemia (ALL). Firstly, we developed a PCR approach for the detection of clonal incomplete D_H-J_H gene rearrangements (see Chapters 2.3 and 2.7), since previous PCR studies in precursor-B-ALL employing V_H-J_H primer sets showed generally lower frequencies of *IGH* gene rearrangements as compared to Southern blot analyses.[8-10] Because *IGH* gene rearrangements in precursor-B-ALL are frequently oligoclonal and thus potentially unstable during the disease course,[11] we evaluated the occurence of cross-lineage TCR gene rearrangement patterns (see Chapter 2.4). We also developed a multiplex PCR approach for identification and characterization of cross-lineage Vδ2-Jα gene rearrangements in precursor-B-ALL patients (see Chapter 2.5).

In T-ALL, we focused on the comprehensive characterization of TCR gamma gene rearrangements as principal MRD targets (see Chapter 2.6) in addition to previously characterized TCR delta gene rearrangements.[12] Immunobiology and MRD applicability of cross-lineage *IGH* gene rearrangements in T-ALL were the subjects of another study (see Chapter 2.7).

We also thoroughly characterized Ig and TCR gene rearrangements in adult ALL patients and compared them to rearrangement patterns in childhood ALL patients (see Chapter 2.8). Particularly in precursor-B-ALL, we and others showed that the gene rearrangement patterns seem to be age-related.[13] Finally, information on Ig and TCR gene rearrangement patterns in ALL provides insight in immunobiological aspects of normal lymphopoiesis as well as in oncogenetic processes in early lymphoid precursors (reviewed in Chapter 2.9).

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CHAPTER 2.2

HETERODUPLEX PCR ANALYSIS OF REARRANGED T-CELL RECEPTOR GENES FOR CLONALITY ASSESSMENT IN SUSPECT T-CELL PROLIFERATIONS*

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ABSTRACT

Molecular analysis of T-cell receptor (TCR) genes is frequently used to prove or exclude clonality and thereby support the diagnosis of suspect T-cell proliferations. PCR techniques are more and more being used for molecular clonality studies. The main disadvantage of the PCR-based detection of clonal TCR gene rearrangements, is the risk of false-positive results due to 'background' amplification of similar rearrangements in polyclonal reactive T lymphocytes. Therefore, PCR-based clonality assessment should include analyses that discern between PCR products derived from monoclonal and polyclonal cell populations. One such method is heteroduplex analysis, in which homo- and heteroduplexes resulting from denaturation (at 94°C) and renaturation (at lower temperatures) of PCR products, are separated in non-denaturing polyacrylamide gels based on their conformation. After denaturation/renaturation, PCR products of clonally rearranged TCR genes give rise to homoduplexes, whereas in case of polyclonal cells heteroduplexes with heterogeneous junctions are formed.

We studied heteroduplex PCR analysis of TCR gene rearrangements with respect to the time and temperature of renaturation and the size of the PCR products. Variation in time did not have much influence, but higher renaturation temperatures (>30°C) clearly showed better duplex formation. Nevertheless, distinction between monoclonal and polyclonal samples was found to be more reliable at a renaturation temperature of 4°C, using relatively short PCR products. To determine the sensitivity of heteroduplex analysis with renaturation at 4°C, (c)DNA of T-cell malignancies with proven clonal rearrangements was serially diluted in (c)DNA of

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polyclonal mononuclear peripheral blood cells and amplified using V and C primers (*TCRB* genes) or V and J primers (*TCRG* and *TCRD* genes). Clonal *TCRB* and *TCRD* gene rearrangements could be detected with a sensitivity of at least 5%, whereas the sensitivity for *TCRG* genes was somewhat lower (10-15%). The latter could be improved by use of Vγ member primers instead of Vγ family primers.

We conclude from our results that heteroduplex PCR analysis of TCR gene rearrangements is a simple, rapid and cheap alternative to Southern blot analysis for detection of clonally rearranged TCR genes.

INTRODUCTION

Acute lymphoblastic leukemias can easily be diagnosed by routine cytomorphologic examination and/or by immunophenotyping. The latter is based on homogenous expression of particular atypical leukemia-specific marker combinations, including the expression of terminal deoxynucleotidyl transferase (TdT).[1-3] Immunophenotypic diagnosis of the more mature leukemias and lymphomas is generally more complex. In case of mature B-cell proliferations, single immunoglobulin (Ig) light chain (i.e. Igκ or Igλ) expression as determined by immunophenotyping, is indicative of the presence of a clonal B-cell population.[4,5] However, clonality of suspect mature (TdT-negative) T-cell proliferations is difficult to establish by immunophenotyping, because of the absence of tumor-specific markers. As lymphoid leukemias and lymphomas can be considered as malignant counterparts of normal T-cells in their various differentiation stages,[6,7] most T-cell malignancies contain rearranged T-cell receptor (TCR) genes. Similar to other neoplasms, T-cell malignancies are derived from a single malignantly transformed cell, implying that the TCR gene rearrangements within all the malignant cells are identical. Molecular analysis of TCR genes is therefore generally performed to prove or exclude clonality.[8-12] So far, this mainly concerned Southern blot analysis, which is a reliable method, if proper probe/restriction enzyme combinations are used.[10,11] Because Southern blot analysis of TCR genes is time-consuming and labor-intensive, PCR techniques are frequently being used as alternatives.

Although PCR techniques can be sensitive and fast, there are two major drawbacks in diagnostic clonality studies of TCR genes. Firstly, the possibility of identifying clonal rearrangements by PCR is limited by the choice of primers. Incomplete V-D or D-J rearrangements (in case of *TCRB* and *TCRD* genes) will remain undetected if only V and J primers are used. Furthermore, rearrangements involving particular V gene segments (for the *TCRB* genes) might remain undetected when only consensus primers are used. The second more important drawback is the risk of false-positive results due to 'background' amplification of similar rearrangements in polyclonal, reactive T lymphocytes. The latter implies that PCR analysis is not sufficient

for clonality assessment, unless it is followed by analyses that discern between PCR products derived from polyclonal and monoclonal T-cell populations. Methods that have been applied to solve this PCR 'background problem' include: direct sequencing of the PCR products,[13,14] single-strand conformation polymorphism (SSCP)-based analysis,[15,16] denaturing gradient gel electrophoresis (DGGE),[17-20] heteroduplex analysis,[21-23] temperature gradient gel electrophoresis (TGGE),[24] and gene scanning analysis.[25-28] From these techniques, heteroduplex analysis is probably the simplest, fastest, and cheapest method for analysis of PCR products of rearranged TCR genes.

In heteroduplex analysis, PCR products are denatured at high temperature and subsequently renatured to induce homo- or heteroduplex formation. Originally the heteroduplex technique was designed for mutation detection in genetic diseases. However, it has been shown in several reports that it can also be applied for analysis of PCR products from TCR gene rearrangements.[21-23] Usage of heteroduplex analysis enables discrimination between PCR products derived from monoclonal and polyclonal lymphoid cell populations, based on the presence of homoduplexes (PCR products with identical junctional regions) or a smear of heteroduplexes (derived from PCR products with heterogeneous junctional regions), respectively (Figure 1). In this report heteroduplex analysis of rearranged TCR genes is studied with respect to time and temperature of renaturation and the size of the PCR products in order to further optimize the applicability of this technique and to determine its sensitivity for clonality assessment in suspect T-cell proliferations.

MATERIALS AND METHODS

Cell samples

Cell samples were obtained from several TCR$\gamma\delta^+$ T-cell acute lymphoblastic leukemias (T-ALL) and TCR$\alpha\beta^+$ T-cell large granular lymphocyte (T-LGL) proliferations, containing >90% of malignant cells at initial diagnosis. Clonality of the T-cells was proven with Southern blot analysis of TCR genes.[10] Mononuclear cells (MNC) were isolated from peripheral blood (PB) or bone marrow (BM) by Ficoll-Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. The cell samples were frozen and stored in liquid nitrogen.

DNA isolation, RNA isolation, and reverse transcriptase (RT) reaction

DNA was isolated from frozen MNC as described previously.[10] Total RNA was isolated essentially according to the method of Chomczynski.[29] cDNA was prepared from mRNA as described before,[30]using oligo(dT) and AMV reverse transcriptase.

(RT-)PCR amplification

PCR was essentially performed as described previously.[31] In each 100 µl PCR reaction 0.1-1 µg DNA sample, 12.5 pmol of 5′ and 3′ oligonucleotide primers, and 1 U *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA) were used. The oligonucleotides used for amplification of *TCRG* and *TCRD* gene rearrangements were published before.[12] For *TCRB* RT-PCR amplification cDNA (5 µl out of 20 µl RT reaction volume) was amplified in 100 µl reaction mixtures, containing 12.5 pmol of 5' and 3' primers and

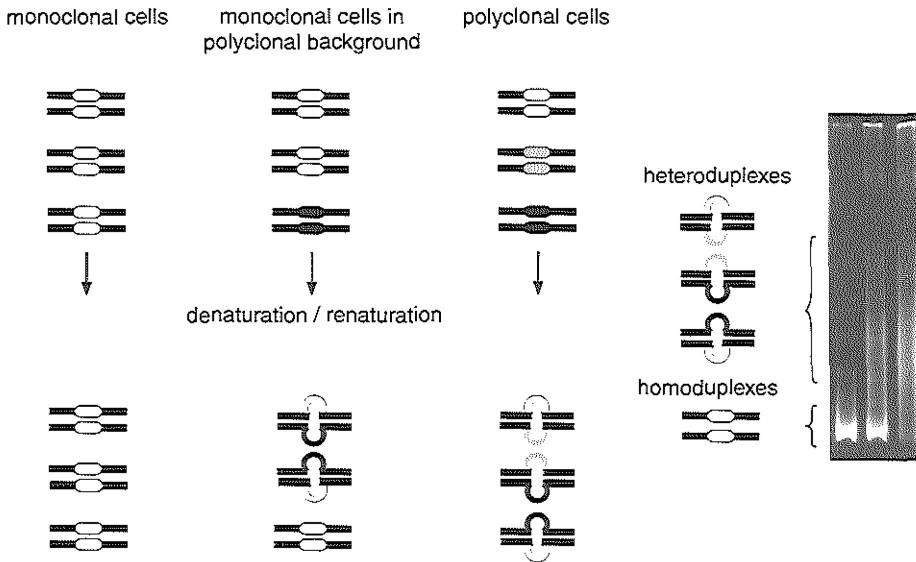

Figure 1.
Schematic diagram of the heteroduplex analysis technique. In order to discern between PCR products derived from monoclonal and polyclonal lymphoid cell populations, the junctional region heterogeneity of PCR products of rearranged Ig or TCR genes can be studied by heteroduplex analysis. In heteroduplex analysis PCR products are heat-denatured and subsequently rapidly cooled to induce duplex (homo- or heteroduplex) formation. In cell samples which consist of clonal lymphoid cells, the PCR products of rearranged Ig or TCR genes give rise to homoduplexes after denaturation and renaturation, whereas in samples which contain polyclonal lymphoid cell populations the single-strand PCR fragments will mainly form heteroduplexes upon renaturation (left part). In case of admixture of monoclonal cells in a polyclonal background, both hetero- and homoduplexes are formed. Because of differences in conformation, homo- and heteroduplexes can be separated from each other by electrophoresis in non-denaturing polyacrylamide gels. Homoduplexes with perfectly matching junctional regions migrate more rapidly through the gel than heteroduplex molecules with less perfectly matching junctional regions. The latter form a background smear of slower migrating fragments (right part).

1 U *Taq* polymerase. An RT-PCR procedure with a single Cβ primer in combination with 30 Vβ family-specific framework 3 (FR3) primers was set up, in order to avoid differences in primer annealing to the members of the 25 well-defined Vβ families when using a consensus Vβ primer.[32] The Vβ family-specific primers as well as the Cβ primer used for this *TCRB* RT-PCR were adapted from Gorski et al.[33] All oligonucleotides were synthesized on an ABI 392 DNA synthesizer (Applied Biosystems, Foster City, CA, USA) using the solid-phase phosphotriester method. PCR conditions were 1 min at 94°C, 1 min at 55° to 65°C, and 2 min at 72°C for 30 to 40 cycles using a Perkin-Elmer thermal cycler (Perkin-Elmer Cetus). After the last cycle an additional step of 7 min at 72°C was performed for final extension. RT-PCR conditions were 1 min at 94°C, 1 min at 65°C, and 2 min at 72°C for 35 cycles, also followed by a final extension step (7 min, 72°C).

Heteroduplex analysis

For heteroduplex analysis, the PCR or RT-PCR products were denatured at 94°C for 5 min, after the final cycle of amplification, and subsequently cooled (to a lower temperature) to induce duplex formation. This renaturation step was performed at different temperatures (range 4-40°C) for different time periods

(range 15-60 min). After duplex formation the hetero- and/or homoduplexes were immediately loaded on 6% non-denaturing polyacrylamide gels in 0.5 x Tris-Boric acid-EDTA (TBE) buffer, run at room temperature, and visualized by ethidium bromide staining. *Pst*I-digested lambda DNA was used as a size marker.

RESULTS

Influence of renaturation time and temperature on duplex formation

To find the optimal conditions for renaturation in heteroduplex analysis, the influence of both time and temperature of renaturation was studied for PCR products of rearranged TCR genes. For this purpose we used DNA from a TCR$\gamma\delta^+$ T-ALL patient with biallelic Vγ8-Jγ2.3 rearrangements. As both *TCRG* gene rearrangements in this patient involve the same gene segments, PCR amplification with VγI and Jγ1.3/2.3 primers[12] resulted in almost identical PCR products of around 450 bp. The two products only differed in their junctional regions. The heterogeneity was characterized by a 13 bp-size difference and a difference in nucleotide composition. Upon denaturation and renaturation of these PCR products, four double-strand fragments were formed. These included two homoduplexes, representing the two PCR amplified rearranged alleles, and two distinct heteroduplex molecules resulting from renaturation of single-strand fragments of the PCR products from the two different alleles (Figure 2).

Following PCR amplification with VγI and Jγ1.3/2.3 specific primers, heteroduplex analysis was performed at variable renaturation temperatures to compare the degree of duplex formation on polyacrylamide gels. When renaturation was performed for one hour at relatively high temperatures (30°C or 40°C) the two homoduplexes and the two slower migrating heteroduplexes were clearly detectable (Figure 2). After renaturation at room temperature (22°C), duplex formation was not complete, as slow-migrating single-strand products were also visible. At renaturation temperatures of 10°C or 4°C, single-strand fragments were even more clearly present in parallel with a lower intensity of the duplex band (Figure 2). This is probably due to the slower renaturation process at these lower temperatures. Analysis of biallelic Vγ8-Jγ2.3 rearrangements (11 nucleotides size difference) from a second TCR$\gamma\delta^+$ T-ALL patient revealed a similar pattern of duplex bands and single-strand products. Analysis of biallelic Vδ1-Jδ1 PCR products (around 450 bp, with a size difference of 33 nucleotides) in the latter patient sample also showed a comparable effect of temperature on duplex formation. Interestingly, in all cases the single-strand fragments showed different migration patterns at the different renaturation temperatures, suggesting that the conformation of single-strand DNA products is also temperature-dependent.

To test the effect of renaturation time on duplex formation, several different *TCRG* and *TCRD* gene rearrangements were amplified and subsequently denatured and renatured for different time periods (15-60 min.). However, in contrast to the dif-

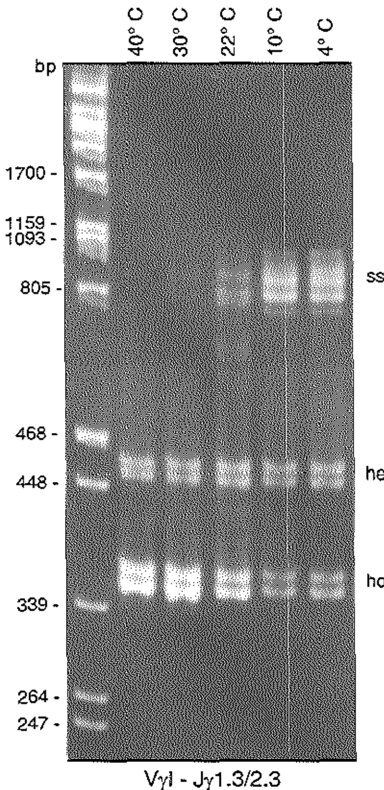

Figure 2.
Analysis of the influence of the renaturation temperature on duplex formation of (biallelic) *TCRG* PCR products from T-ALL DNA. After amplification of the clonal *TCRG* rearrangements with Vγl and Jγ1.3/2.3 primers, PCR products were denatured for 5 min at 94°C and renatured for 1 h at the indicated renaturation temperatures. Electrophoresis was performed in 6% non-denaturing polyacrylamide gels, using *Pst*I-digested lambda DNA as size marker. As a result two homo- and two heteroduplexes can be identified. At higher temperatures (>30°C) the renaturation process is (almost) complete, whereas upon renaturation at lower temperatures single-strand fragments can still be identified as more retarded fragments. ss, single-strand fragments; he, heteroduplexes (of clonal origin); ho, homoduplexes.

ferences observed upon variation in renaturation temperature, differences in time of renaturation (at a given temperature) did not have much effect on duplex formation (data not shown). Irrespective of the type of PCR product and the renaturation temperatures, maximum levels of homo- and heteroduplexes were generally observed after 30 to 45 min of renaturation. Duplex formation was only slightly lower after 15 min of reannealing. This illustrates that the degree of duplex formation is largely dependent on renaturation temperature rather than duration of the renaturation process. To guarantee optimal renaturation, we used renaturation times of one hour in all further experiments.

Influence of renaturation temperature and size of the PCR product on clonality assessment

Although the above-mentioned results clearly indicate that duplex formation is more optimal at higher renaturation temperatures, we wanted to know whether the discussed denaturation/renaturation strategy can effectively be applied to discern between monoclonal and polyclonal cell populations. For this we took 100%, 20%, and 0% dilutions of T-ALL DNA in polyclonal MNC DNA, PCR amplified the Dδ2-Jδ1

rearrangement, and subsequently performed heteroduplex analysis with renaturation at either 40°C or 4°C for one hour. After renaturation the samples were kept on ice until loading of the gel, using ice-cold loading buffer. The polyacrylamide gels were run at room temperature.

The Dδ2-Jδ1 rearrangement was amplified using a Jδ1 primer in combination with either one of two distinct Dδ2 primers. The proximal primer, Dδ2, is located relatively close to the Dδ2 gene segment, resulting in a PCR product of around 240 nucleotides, whereas the distal primer (Dδ2up) lies far more upstream of the Dδ2 gene segment (and even upstream of the Dδ1 gene segment), giving rise to a much larger PCR product of around 800 bp. These two different Dδ2 primers were employed to see whether the size of the PCR product might be an important determinant for reliable clonality assessment as well. Amplification with the Dδ2up and Jδ1 primers, followed by denaturation and renaturation at 40°C, resulted in clear homoduplex bands of the expected size in the 100% and 20% leukemic samples, but a band of comparable size was also observed in the healthy control MNC sample (Figure 3A). When renaturation was performed at 4°C, the intensity of the homoduplex bands in the 100% and 20% samples was slightly lower (Figure 3A), as was expected from the above described temperature experiments. Most importantly however, no homoduplexes were found in the control sample at this low renaturation temperature. The problem of detecting a 'false-positive' homoduplex band in the non-leukemic control sample could also largely be resolved when the more proximal Dδ2 primer was used for Dδ2-Jδ1 amplification (Figure 3B). Nevertheless, using this smaller PCR product, a faint homoduplex band could still be detected within a background smear in the polyclonal control sample. The 'false-positive' homoduplex band again entirely disappeared when renaturation was performed at 4°C (Figure 3B). Similar results were obtained with Vβ-Cβ RT-PCR products of even smaller size (around 200 bp), where renaturation at 40°C resulted in faint homoduplex bands that disappeared when reannealing was performed at 4°C (data not shown). These experiments showed that larger PCR products gave rise to stronger false-positive homoduplex-like bands at 40°C renaturation, whereas renaturation at 4°C never gave false-positive results.

Sensitivity of heteroduplex analysis for clonality detection

The above data suggested that a reliable distinction between monoclonal and polyclonal samples by heteroduplex PCR analysis requires renaturation at a low temperature (4°C). However, the initial experiments clearly indicated that duplex formation is less optimal at lower temperatures, suggesting that the sensitivity of the heteroduplex technique would be lowered by performing renaturation at 4°C. We therefore performed dilution experiments for several targets to study the sensitivity of detection of clonal homoduplexes upon renaturation at 4°C.

Firstly, sensitivity of clonality detection was determined for several frequently

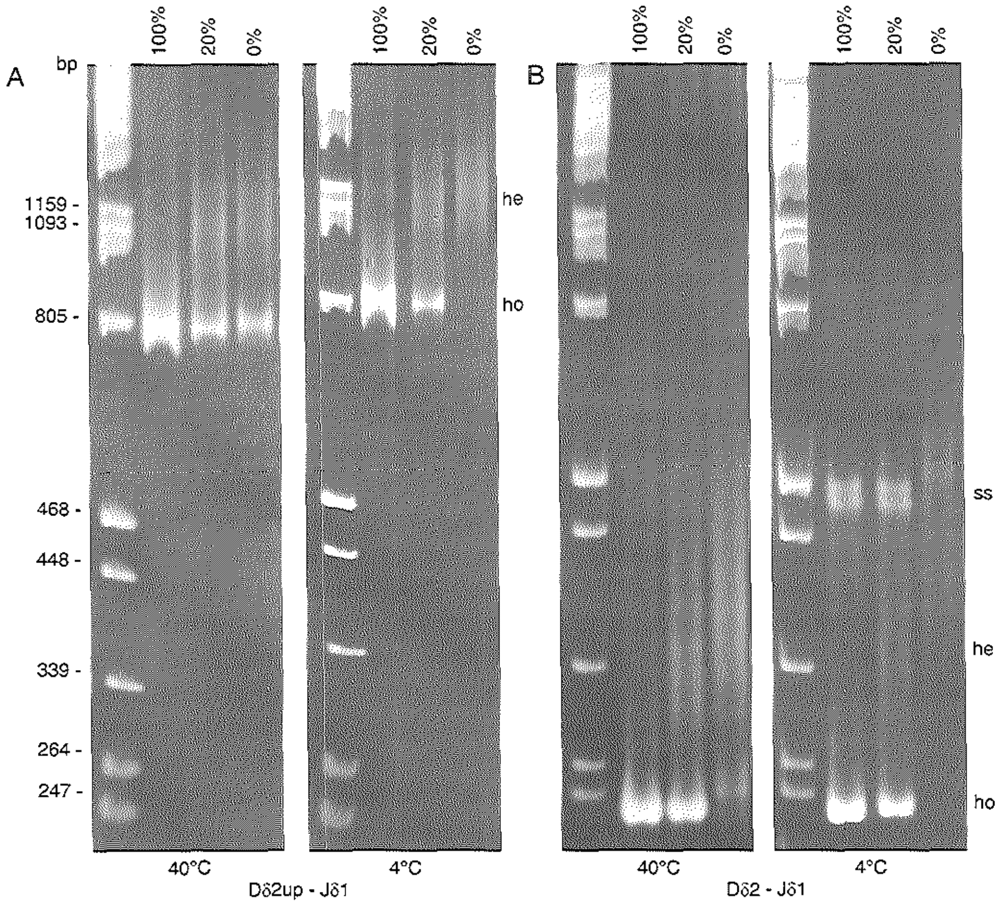

Figure 3.
Clonality assessment via heteroduplex analysis of TCR junctional regions. T-ALL DNA was serially diluted (100%, 20%, and 0%) in polyclonal MNC DNA. The clonal *TCRD* rearranged gene was subsequently PCR amplified using either Dδ2up and Jδ1 primers (A) or Dδ2 and Jδ1 primers (B). The resulting PCR products were subjected to heteroduplex analysis; denaturation (5 min, 94°C) was followed by renaturation for 1 h at 40°C or 4°C. Samples were run in 6% non-denaturing polyacrylamide gels. *Pst*I-digested lambda DNA was used as size marker. In case of the large PCR products (A) a homoduplex-like band was visible in the polyclonal control sample after renaturation at 40°C, but not at 4°C. Although less clearly, this band was also visible upon renaturation of smaller PCR products (B) at 40°, but again disappeared upon renaturation at 4°C. The single-strand fragments as visible in panel (B) (4°C) are not visible in panel (A) due to the slow mobility of the larger single-strand fragments. ss, single-strand fragments; he, (smear of) heteroduplexes; ho, homoduplexes.

occurring *TCRG* and *TCRD* gene rearrangements. Heteroduplex analysis of Vδ1-Jδ1 and Dδ2-Jδ1 amplified products, using serial dilutions of several TCRγδ^{+} T-ALL, yielded clear homoduplex bands in samples containing only 5% tumor cells (Figure

4A and 4B). Occasionally faint homoduplex bands were even observed in 1% dilutions. Homoduplexes were never found when using polyclonal DNA. Vγll(Vγ9)-Jγ1.3/2.3 homoduplexes could also be detected with a sensitivity of around 5% (Figure 5B). However, clonality detection of Vγl-Jγ1.3/2.3 rearrangements by heteroduplex analysis turned out to be more difficult, resulting in a sensitivity of around 10% (Figure 5A). This limited sensitivity can probably be explained by the fact that all rearrangements involving members of the Vγl family are amplified when using a Vγl family-specific oligonucleotide primer. We therefore determined the detection limit using Vγl member-specific primers to amplify *TCRG* rearrangements. This resulted in improved detection of homoduplexes with a sensitivity of at least 5%, which is at least two-fold better than in the case of the consensus Vγl family primer

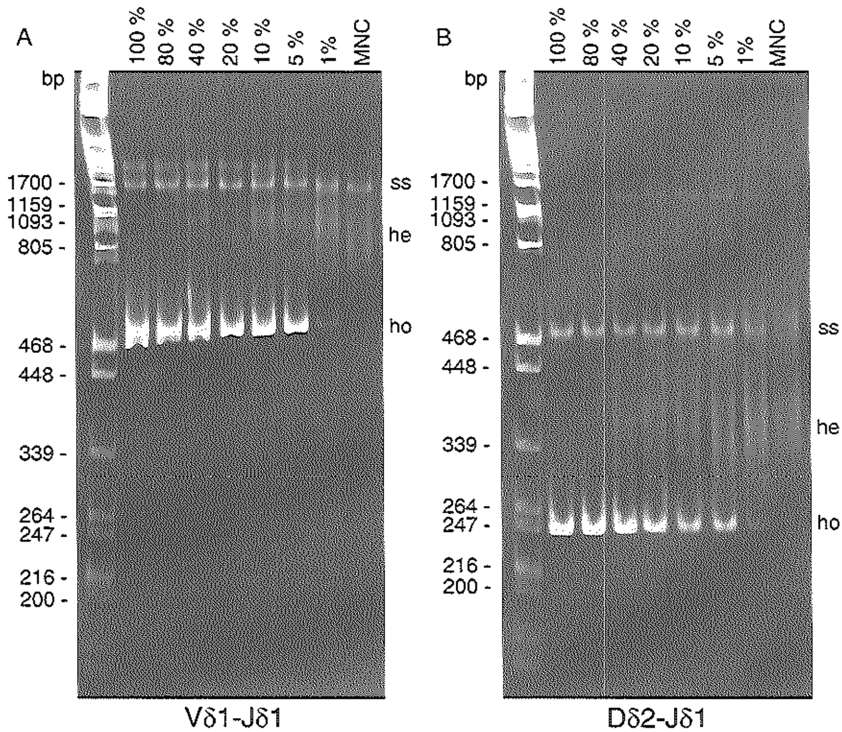

Figure 4.
Sensitivity of heteroduplex analysis for clonality detection of *TCRD* rearranged gene products. T-ALL DNA was serially diluted in polyclonal MNC DNA. The clonal Vδ1-Jδ1 and Dδ2-Jδ1 rearrangements in this DNA sample were amplified with specific primer sets, i.e. either Vδ1 (A) or Dδ2 (B) primers in combination with a Jδ1 primer. The resulting PCR products were subjected to heteroduplex analysis (5 min, 94°C; 1 h 4°C) prior to electrophoresis in 6% non-denaturing polyacrylamide gels. In both panels clonal homoduplexes could be detected with a sensitivity of 1-5%. ss, single-strand fragments; he, (smear of) heteroduplexes; ho, homoduplexes.

(Figure 5A).

PCR analysis of *TCRG* and *TCRD* genes can easily be performed at the DNA level because of the relatively limited number of V, (D), and J gene segments. As the high number of Vβ and Jβ gene segments would require many different primer combinations, analysis of *TCRB* genes is often performed via RT-PCR with Vβ family-specific primers and a single Cβ primer. Using this RT-PCR procedure, *TCRB* analysis was performed on several T-LGL proliferations as well as TCRαβ^{+} T-ALL. Subsequently, cDNA from clonal Vβ2, Vβ7, and Vβ23 positive lymphoproliferations was serially diluted in polyclonal MNC cDNA and the PCR products were subjected to heteroduplex analysis with renaturation at 4°C. Homoduplexes were clearly visible in the 5% dilutions, whereas in the 1% dilutions a faint homoduplex could still be detected (Figure 6). In the polyclonal cDNA lane only a smear of heteroduplexes was seen.

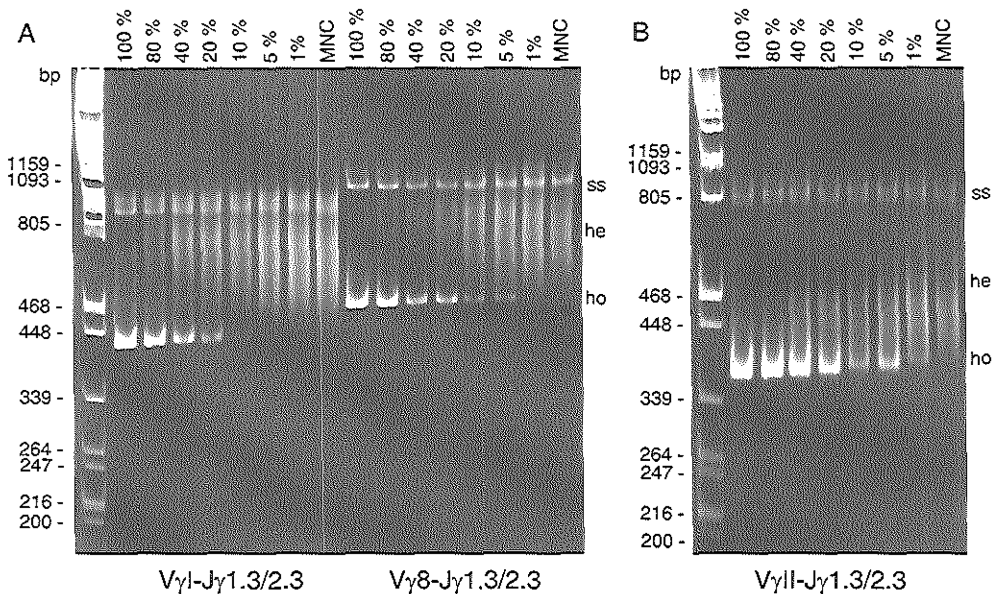

Figure 5.

Sensitivity of heteroduplex analysis for clonality detection of *TCRG* rearranged gene products. T-ALL DNA was serially diluted in polyclonal MNC DNA. The clonal Vγ8-Jγ/2.3 and Vγ9-Jγ2.3 rearrangements in this DNA sample were amplified with a family-specific V primer (VγI) or a member-specific V primer (Vγ8) in combination with a Jγ1.3/2.3 primer (A) and a VγII primer in combination with a Jγ1.3/2.3 primer (B). The resulting PCR products were subjected to heteroduplex analysis (5 min, 94°C; 1 h 4°C) before electrophoresis in 6% non-denaturing polyacrylamide gels. Clonal VγII-Jγ2.3 homoduplexes could be detected with a sensitivity of 1-5%. Sensitivity of detection of clonal VγI-Jγ1.3/2.3 duplexes was similar, provided that member-specific Vγ primers were used. ss, single-strand fragments; he, (smear of) heteroduplexes; ho, homoduplexes.

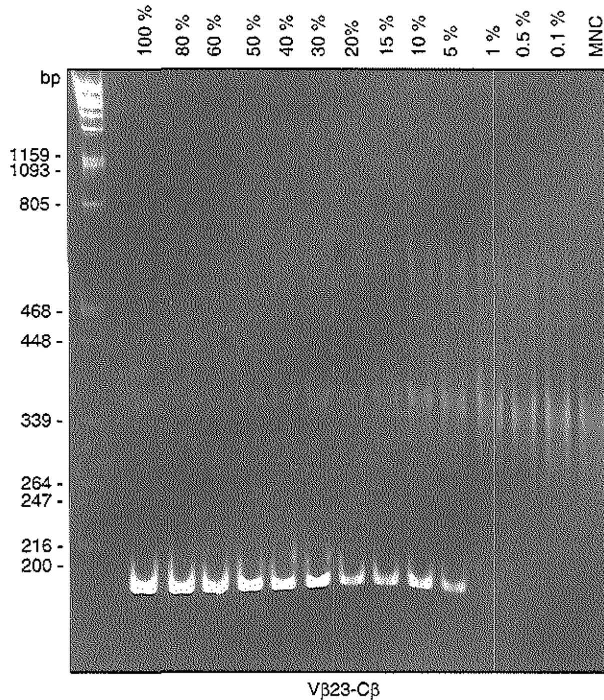

Figure 6.
Sensitivity of heteroduplex analysis for clonality detection of *TCRB* rearranged gene products. T-LGL cDNA was serially diluted in polyclonal MNC cDNA. After reverse transcription, the clonal Vβ-Jβ rearrangement in this sample was amplified with specific primers, i.e. a family-specific V primer (Vβ23) in combination with a Cβ primer. The resulting PCR products were subjected to heteroduplex analysis (5 min, 94°C; 1 h 4°C) before electrophoresis in 6% non-denaturing polyacrylamide gels. Clonal homoduplexes could be detected with a sensitivity of 1-5%. ss, single-strand fragments; he, (smear of) heteroduplexes; ho, homoduplexes.

DISCUSSION

Southern blot analysis has long been the only reliable method for clonality assessment in suspect lymphoproliferations, but over the last years several PCR-based methods have been devised as an alternative. One of the main difficulties of PCR-based clonality detection is discrimination between monoclonal and polyclonal PCR products. This problem can be solved by further analyzing the PCR products in various ways, ranging from high resolution polyacrylamide gel electrophoresis (fingerprinting) and gene scanning[25-28] to DGGE[17-20] and heteroduplex analysis.[21-23]

In heteroduplex analysis hetero- and homoduplexes, resulting from denaturation and renaturation of PCR products, are separated in non-denaturing polyacrylamide

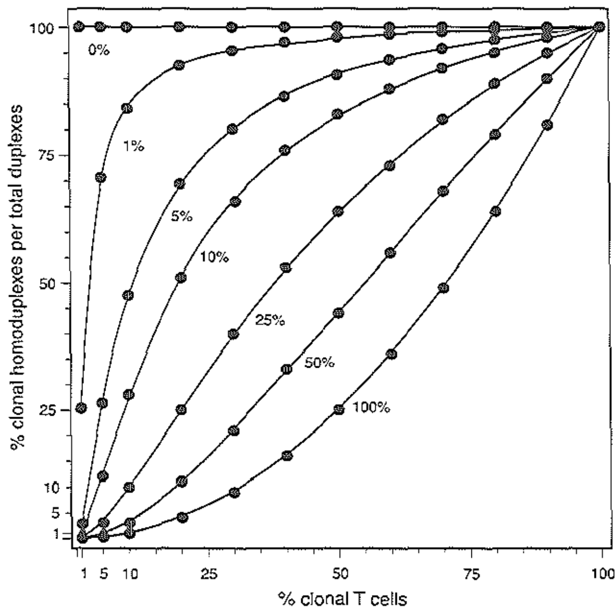

Figure 7.
Calculated frequencies of clonal homoduplexes within the total pool of hetero- and homoduplexes, assuming that duplex formation is a fully random process as it seems to be at 4°C. The seven curves illustrate that the frequency of normal polyclonal T-cells within the non-tumor cell fraction (0%, 1%, 5%, 10%, 25%, 50%, and 100%) drastically influences clonal homoduplex formation, since the frequency of clonal homoduplexes progressively decreases with the increase in the polyclonal T-cell frequency. The percentage of clonal homoduplexes being formed by renaturation of single strand fragments, was calculated according to the formula H = M/(M+(100-M).P/100) x M/(M+(100-M).P/100) x 100%, in which H is the percentage of homoduplexes, M is the percentage of monoclonal T-cells, and P is the percentage of polyclonal T-cells in the non-tumor fraction.

gels based on their conformation. The presence of clear homoduplex bands or a smear of heteroduplexes enables discrimination between monoclonality and polyclonality, respectively (Figure 1). Therefore, heteroduplex analysis seems to be a simple, rapid and cheap technique of PCR product analysis, which can be introduced easily in most diagnostic laboratories, as no radioactive substrates are involved and no expensive equipment other than ordinary laboratory tools is required.

The number of clonal homoduplexes that is formed upon renaturation of denatured PCR products is related to the percentage of clonal T-cells in the analyzed sample (Figure 7). However, based on the assumption that random pairing occurs between single-strand fragments of clonal and polyclonal T lymphocytes, the presence of polyclonal T-cells in the same cell sample will lead to the formation of heteroduplexes. The more polyclonal T-cells are present in the sample, the less homoduplexes (derived from monoclonal T-cells) are formed (Figure 7). Therefore, the sensitivity of heteroduplex PCR analysis for identification of clonal T-cell populations

between polyclonal T-cells is dependent on the detectability of (renatured) homoduplexes amongst heteroduplexes. To optimize the heteroduplex technique for clonality assessment in suspect T-cell proliferations further, we studied several assay parameters, like the time and temperature of renaturation and the size of the analyzed PCR products.

The temperature at which the denatured fragments renature rather than the time used for renaturation appeared to be an important determinant in duplex formation. Duplex formation is more optimal at higher renaturation temperatures (40°C) but clonality analysis appears to be more reliable at lower temperatures (4°C), especially in case of small suspect cell populations. This is caused by the presence of a faint, somewhat fuzzy homoduplex band in polyclonal control samples at 40°C, which is absent when renaturation is performed at 4°C. We assume that the higher movement of molecules at higher temperatures (e.g. 40°C) supports the non-random formation of 'non-clonal' homoduplexes due to preferential pairing of 'matching' polyclonal single-strand molecules. As a result many non-clonal homoduplexes with Gaussian size distribution are present at this high temperature, causing the fuzzy homoduplex band in polyclonal control samples (Figs. 3A and 3B). This problem of 'false-positive' homoduplex bands is more prominent in case of larger PCR products (>600 bp) than in case of smaller (<250 bp) PCR products, in which the contribution of the junctional region is relatively high. It is our assumption that for large PCR products, in which the contribution of the junctional region nucleotides to the entire size of the PCR product is relatively small, the resolution of the PAGE system was not sufficient to discern between homoduplexes of identical size derived from clonal cells and differently-sized non-clonal homoduplexes derived from polyclonal cells. Furthermore, large heteroduplex molecules with a few non-matching nucleotides will differ only slightly from homoduplexes in their mobility rate, since differences in electrophoretic mobility between heteroduplexes and homoduplexes progressively decrease when the PCR products become larger.

Heteroduplex analysis of TCR genes is thus a reliable technique for clonality assessment in suspect T-cell proliferations, provided that the appropriate conditions are used, i.e. PCR products of preferably 150-250 bp and renaturation at 4°C. Analysis of serial dilutions of tumor DNA in polyclonal control DNA, revealed sensitivities of 1-5% for PCR products of *TCRD* gene rearrangements and also for RT-PCR products of *TCRB* transcripts. In case of $V\gamma II(V\gamma 9)$-$J\gamma 1.3/2.3$ rearrangements a sensitivity of 5% was achieved as well, but $V\gamma I$-$J\gamma 1.3/2.3$ rearrangements showed sensitivities of only around 10%. This reduced sensitivity is probably caused by the high background of these rearrangements, as they frequently occur in normal $TCR\alpha\beta^+$ T-cells (Figure 7). Use of $V\gamma$ member-specific primers instead of a $V\gamma I$ family-specific primer can help to improve the sensitivity to at least 5% (Figure 5A). The less optimal duplex formation at 4°C could partly be compensated by prolonged renaturation (24 or even 48 hours), without formation of false homoduplex bands in

polyclonal control samples (data not shown). Increasing the renaturation time extensively may thus help to improve the sensitivity of heteroduplex analysis, which seems especially important for *TCRG* gene rearrangements. A detection limit of 1-5% for most TCR gene rearrangements is similar to or even better than Southern blot analysis. Moreover, the sensitivities that we reach under conditions that guarantee reliable clonality assessment, are largely comparable to those mentioned by other authors who used serial dilutions in polyclonal MNC DNA and performed high resolution non-denaturing PAGE[24] or DGGE.[17-20]

Sensitivities of around 5% are clinically relevant for initial diagnosis, but they are certainly not sufficient for detection of minimal residual disease (MRD) in ALL patients which requires sensitivities of 10^{-4} to 10^{-5}.[35] It has been suggested that sensitivities of 10^{-2} to 10^{-3} as determined by PCR might be predictive for slow remission upon chemotherapy,[36] but such sensitivities cannot easily be reached via heteroduplex analysis of PCR products, unless most of the polyclonal T-cells are depleted before DNA or RNA extraction. Nevertheless, heteroduplex PCR analysis of TCR gene rearrangements is sufficiently sensitive for monitoring patients with chronic T-cell leukemias as well as patients with oligoclonal T-cell proliferations in order to predict the possible outgrowth of a dominant cell population.

Heteroduplex analysis of rearranged TCR gene products can be a valuable technique in several other applications as well. Proof or exclusion of the common clonal origin of two distinct lymphoproliferations in one patient can be obtained upon analysis of homo- and heteroduplexes after mixing PCR products of the two samples. Another application involves direct sequencing of homoduplex bands of PCR amplified rearranged TCR genes which obviates the need for cloning in order to sequence their junctional regions for MRD studies. Furthermore, heteroduplex analysis can also be employed for studying the diversity of the TCR repertoire in immune responses during infections or in autoimmune disorders, as has already been reported for the *TCRB* and *TCRG* genes.[21,22] However, this application suffers from a few technical limitations. Firstly, the heteroduplex method is more qualitative than quantitative; secondly, scarcity of material or low frequencies of T lymphocytes in the studied cell sample may give rise to a kind of pseudo-oligoclonality or even pseudo-monoclonality, which does not reflect the actual heterogeneity.

We conclude from our data that the described PCR procedure of TCR gene amplification followed by denaturation of the resulting PCR products and renaturation at 4°C serves to assess clonality in suspect T-cell proliferations. This is especially the case when relatively short PCR products (150-250 bp) are analyzed. Heteroduplex analysis of PCR products is a simple, rapid, and cheap alternative to Southern blot analysis for TCR gene clonality assessment. Moreover, heteroduplex analysis may even be superior to other methods like fingerprinting, DGGE, and gene scanning, since no expensive equipment is needed, no radioactivity is involved, and different conditions do not seem to be required for all different TCR gene primer

combinations. Our current results suggest that this procedure is not only useful for TCR gene analysis, but might also be applied for clonality assessment in suspect B-cell proliferations via analysis of *IGH*, *IGK*, and *IGL* genes. However, one should be aware that PCR studies of rearranged Ig genes in mature (post)follicular B-cells might be hampered by the occurrence of somatic mutations.

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CHAPTER 2.3

PRECURSOR-B-ALL WITH DH-JH GENE REARRANGEMENTS HAVE AN IMMATURE IMMUNOGENOTYPE WITH A HIGH FREQUENCY OF OLIGOCLONALITY AND HYPERDIPLOIDY OF CHROMOSOME 14*

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ABSTRACT

The *IGH* gene configuration was investigated in 97 childhood precursor-B-ALL patients at initial diagnosis. Rearrangements were found by Southern blotting in all but three patients (97%) and in 30 cases (31%) we observed oligoclonal *IGH* gene rearrangements. Heteroduplex PCR analysis revealed at least one clonal PCR product in all Southern blot-positive cases. In 89 patients (92%) complete V(D)J rearrangements were found, while incomplete DH-JH rearrangements occurred in only 21 patients (22%). In 5% of cases the DH-JH rearrangements were the sole *IGH* gene rearrangements Sequence analysis of the 31 identified incomplete rearrangements revealed preferential usage of segments from the DH2, DH3 and DH7 families (78%). While DH2 and DH3 gene rearrangements occur frequently in normal B-cells and B-cell-precursors, the relatively frequent usage of DH7-27 (19%) in precursor-B-ALL patients is suggestive of leukemic transformation during prenatal lymphopoiesis. Among JH gene segments in the incomplete DH-JH rearrangements, the JH6 segment was significantly overrepresented (61%). This observation together with the predominant usage of the most upstream DH genes indicates that many of the identified clonal DH-JH gene rearrangements in precursor-B-ALL probably represent secondary recombinations, having deleted pre-existing DH-JH joinings.

The patients with incomplete DH-JH gene rearrangements were frequently char-

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acterized by hyperdiploid karyotype with additional copies of chromosome 14 and/or by *IGH* oligoclonality. The presence of incomplete DH-JH joinings was also significantly associated with a less mature immunogenotype: overrepresentation of VH6-1 gene segment usage, absence of biallelic *TCRD* deletions, and low frequency of *TCRG* gene rearrangements. This immature immunogenotype of precursor-B-ALL with incomplete *IGH* gene rearrangements was not associated with more aggressive disease.

INTRODUCTION

DH-JH joinings are assumed to be the first steps in the recombination process of the immunoglobulin heavy chain (*IGH*) locus and represent one of the earliest events in B-cell development (reviewed in Ref. 1). DH-JH joinings hallmark the differentiation of common lymphoid precursors into the B-cell lineage and are already found in $CD34^+/CD19^-/CD10^+$ precursor cells.[2,3] Most of the more mature $CD34^+/CD19^+/CD10^+$ B-lineage precursors contain at least one DH-JH rearranged allele and frequently also complete VH-(DH-)JH rearrangements.[3,4]

Based on their immunophenotype, precursor-B acute lymphoblastic leukemias (ALL) are generally regarded as clonal malignant counterparts of normal $CD34^+/CD19^+/CD10^+$ B-lineage precursors in the bone marrow. In line with this assumption, Southern blot studies have shown that >95% of precursor-B-ALL have *IGH* gene rearrangements, that most of them contain immunoglobulin (Ig) kappa light chain gene rearrangements (60%), and that even 20% of precursor-B-ALL have Ig lambda light chain gene rearrangements.[5-8] Newly diagnosed childhood precursor-B-ALL are frequently oligoclonal and contain multiple *IGH* gene rearrangements (30% to 40% of cases).[6,9] These multiple *IGH* gene rearrangements are caused by continuing rearrangement processes (e.g. continuing VH to DH-JH joining) as well as secondary rearrangements (e.g. DH-JH replacements, VH replacements), which result in one or more subclones.[9-13]

PCR studies in precursor-B-ALL employing VH-JH primer sets showed generally lower frequencies of *IGH* gene rearrangements as compared to Southern blot analyses.[14-18] This was assumed to be caused in large by the presence of incomplete DH-JH rearrangements. Two limited studies indeed revealed clonal DH-JH rearrangements in a total of 14 out of 19 precursor-B-ALL patients (74%) with biallelic rearrangements in Southern blotting but no clonal VH-(DH-)JH rearrangements by PCR.[16-19] However, no systematic investigations have been published on the frequency and types of clonal DH-JH rearrangements in a random group of precursor-B-ALL patients. Most published data on DH-JH rearrangements in precursor-B-ALL have in fact been obtained indirectly from the sequences of clonal VH-(DH-)JH joinings.[15-20] This limited information about DH-JH rearrangements is partly caused by

the fact that the complete sequence of the DH region was published only recently.[21,22]

In this study we aimed at a thorough analysis of DH-JH rearrangements in childhood precursor-B-ALL patients using combined Southern blot and heteroduplex PCR analyses. We first determined the precise frequency of clonal DH-JH rearrangements in a non-selected series of 97 children with precursor-B-ALL. Secondly, we correlated the presence of clonal incomplete DH-JH joinings with several immunobiological and clinical features of these patients. Finally, we critically assessed the potential application of clonal DH-JH rearrangements as PCR targets for monitoring of minimal residual disease (MRD).

MATERIALS AND METHODS

Patients

Peripheral blood (PB) or bone marrow (BM) samples from 97 precursor-B-ALL patients were obtained at initial diagnosis. The age distribution ranged from 3 months to 190 months. Four children (4%) were infants (age <1 year). The diagnosis of precursor-B-ALL was made according to FAB and standard immunophenotypic criteria.[23-25] Immunological marker analysis of the precursor-B-ALL revealed that four (4%) were pro-B-ALL, 64 (66%) were common ALL, and 29 (30%) were pre-B-ALL. The status of chromosome 14 was based on routine cytogenetics as available in the Dutch Childhood Leukemia Study Group (DCLSG) database. Most patients (91 out of 97) were treated according to the DCLSG ALL-8 treatment protocols.[26]

Southern blot analysis

Mononuclear cells (MNC) were separated from PB or BM samples by Ficoll-Paque centrifugation (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden). DNA was isolated from MNC, digested, and blotted to nylon membranes as described previously.[27] *IGH* gene rearrangements were studied by use of the ^{32}P labeled IGHJ6 probe (DAKO, Carpinteria, CA, USA) in *Bgl*II and *Bam*HI/*Hin*dIII digests.[28] In 88 patients the configuration of the T-cell receptor (TCR) genes was established as previously reported.[29]

PCR amplification and heteroduplex analysis of PCR products

PCR was essentially performed as described previously.[30,31] In each 50µl PCR reaction 50 ng DNA sample, 6.3 pmol of the 5′ and 3′ oligonucleotide primers, and 0.5 U AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA) were used. The sequences of the 12 oligonucleotides used for amplification of complete VH-JH and incomplete DH-JH gene rearrangements (Figure 1) were published before.[32,33] In addition 91 patients were tested with a special VH6-JH primer combination to identify patients with VH6-1 gene rearrangements.[32] PCR conditions were pre-activation of the enzyme for 10 min at 94°C, followed by 35 cycles of 45 s at 92°C, 90 s at 60°C, and 2 min at 72°C using a Perkin-Elmer 480 thermal cycler (Applied Biosystems). After the last cycle an additional extension step of 10 min at 72°C was performed. Appropriate positive and negative controls were included in all experiments.[31]

In order to distinguish between polyclonal and monoclonal rearrangements, heteroduplex analysis of the obtained PCR products was performed as described previously.[34] In short, the PCR products were denatured at 94°C for 5 min to obtain single-stranded PCR products. Subsequently the single-stranded products were cooled to 4°C for 60 min to induce random renaturation (duplex formation).[34] In case of monoclonal gene rearrangements homoduplexes are formed (identical junctional regions), whereas heteroduplexes are found in case of polyclonal gene rearrangements (heterogeneous junctional regions). The duplexes obtained were immediately loaded on 6% non-denaturing polyacrylamide gels in 0.5 x Tris-Borate-EDTA (TBE) buffer, run at room temperature, and visualized by ethidium bromide staining in order

to discriminate between the presence of rapidly migrating homoduplex bands or slowly migrating heteroduplexes smears; the remaining single-strand molecules migrate very slowly (Figure 2).

Sequence analysis of *IGH* gene rearrangements

Sequence analysis concerned both VH-JH and DH-JH gene rearrangements in all patients in whom at least one clonal incomplete DH-JH rearrangement was found by heteroduplex analysis. When heteroduplex PCR analysis revealed more than two clonal bands i.e. either two homoduplexes, or an additional upper band resulting from extension to downstream JH segments, or a DH7-27-JH1 germline band accompanying a DH7-27-JH rearrangement, homoduplexes were excised from the polyacrylamide gel and eluted as described before.[33] The eluted PCR products were either directly sequenced or subjected to second step PCR with the same primer pair to increase the amount of template for sequence analysis. Sequencing was performed using the dye-terminator cycle sequencing kit with AmpliTaq DNA polymerase FS on an ABI 377 sequencer (Applied Biosystems) as described before.[33] 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/).[35]

Statistical analysis

Statistical analyses using the χ^2 test or Fisher's exact test on a 2 x 2 table were performed for comparison of patient and disease characteristics (age, sex, white blood count at diagnosis, immunophenotype, DNA ploidy, the presence of cross-lineage TCR gene rearrangements, steroid response) between precursor-B-ALL patients with DH-JH gene rearrangements (DH-JH-positive group) vs. patients without DH-JH gene rearrangements (DH-JH-negative group). A value of $p < 0.05$ was regarded to be statistically significant.

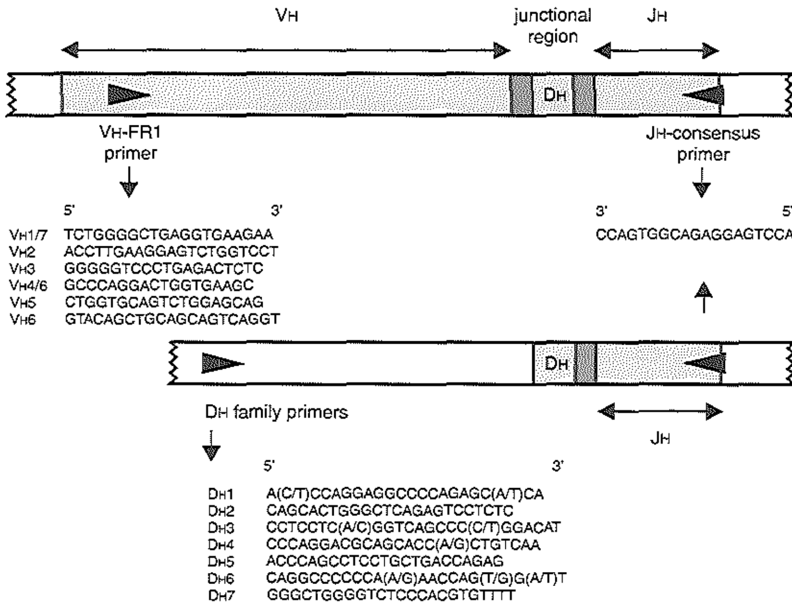

Figure 1.
Schematic diagrams of VH-DH-JH and DH-JH junctional regions with primers for PCR analysis. The sequence, approximate position and orientation (5' → 3') of the VH family specific framework 1(VH-FR1) and DH family specific primers as well as of the JH-consensus primer are indicated.[32,33]

Event-free survival (EFS) was defined as the time from diagnosis to induction failure, relapse, death in remission, or the occurrence of a second tumor. For patients event-free alive at the latest follow-up time point (censored observations) EFS was calculated till this latest follow-up. Patients who did not achieve remission, were included in the analysis and considered as treatment failures with EFS of 0 days. Survival curves and standard errors were calculated according to the Kaplan-Meier method.

RESULTS

Southern blot configuration of *IGH* genes

Clonal *IGH* gene rearrangements were demonstrated by Southern blot analysis in 94 out of 97 precursor-B-ALL patients (97%). In the other three patients monoallelic (two cases) or biallelic (one patient) *IGH* deletions were assumed based on the percentages of leukemic blasts and the relative density of rearranged and germline bands. Biallelic rearrangements were found in 85 out of 97 patients (88%), in eight patients the second *IGH* allele was deleted (8%), and only in a single patient the second allele was in germline configuration. In 13 patients three and in two patients four *IGH* gene rearrangements with Southern blot bands of identical density were found suggesting trisomy and tetrasomy 14, respectively. In 30 out of 97 precursor-B-ALL patients (31%) the presence of rearranged bands of different densities or additional weak bands suggested oligoclonality.

Complete VH-(DH-)JH and incomplete DH-JH rearrangements

Detailed heteroduplex PCR analysis of the *IGH* locus in the 97 precursor-B-ALL patients was based on 12 primer combinations (Figure 1) theoretically covering the majority of complete VH-(DH-)JH joinings and all incomplete DH-JH rearrangements. With this approach at least one clonal PCR product was demonstrated in all 94 patients with Southern blot detectable *IGH* gene rearrangements. However, in only 43 patients (46%) the number of clonal PCR products exactly matched the number of Southern blot bands. In 45 patients (48%) the number of clonal homoduplexes was lower as compared to the number of rearrangements in Southern blot analysis. This concerned 29 patients with monoclonal *IGH* rearrangements and 16 patients with oligoclonal *IGH* gene rearrangements. In contrast, in six patients (6%) the number of clonal homoduplexes exceeded the number of rearrangements in Southern blot analysis. Five of these six patients were considered to have monoclonal *IGH* gene rearrangements based on Southern blotting, which indicates that the additional clonal PCR products most probably represented minor subclones, not detectable by Southern blotting.

Clonal complete VH-(DH-)JH rearrangements were found in 89 patients (92%) as exemplified in Figure 2, while incomplete DH-JH rearrangements were demonstrated in only 21 patients (22%) (exemplified in Figure 3 for the most frequently observed

DH-JH couplings). In only five patients (5%) the incomplete DH-JH joinings were the sole *IGH* rearrangements. Hence, 16 patients had both complete and incomplete *IGH* gene rearrangements.

Usage of DH gene segments in incomplete DH-JH gene rearrangements

In 21 patients with clonal DH-JH rearrangements we identified a total of 31 DH-JH joinings, including five of the DH-DH-JH type. The frequencies of the different DH family members found among the 36 identified DH sequences are summarized in Table 1. Usage of gene segments from the DH2 and DH3 families was most prominent (36% and 33%, respectively), with the DH2-2 and DH3-9 gene segments being used most frequently (each comprising 19% of all identified DH sequences) (Table 1 and Figure 3). The single member of the DH7 family (DH7-27) is located most proximal to the JH cluster and was also found in seven DH-JH joinings (19%). Three rearrangements (8%) contained DH segments of the DH1 family, whereas a single rearrangement utilized the DH6-13 gene segment (Table 1). Taken together, 26 of the 36 rearranged DH segments (72%) belonged to the more upstream part of the DH region (Table 1).

The sizes of the DH-JH junctional regions ranged from 0 to maximally 43

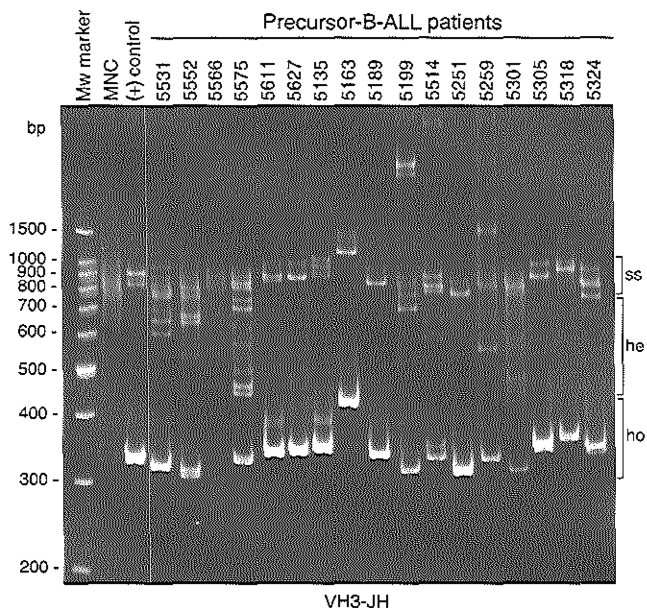

Figure 2.
Heteroduplex PCR analysis in 17 precursor-B-ALL patients to distinguish between polyclonal and monoclonal *IGH* gene rearrangements. Using the VH3-JH primer combination clonal homoduplexes were found in the majority (16/17) of the presented precursor-B-ALL patients. ho, position of homoduplexes; he, heteroduplexes; ss, single strand PCR products.

Table 1. Usage of different DH families and gene segments in incomplete DH-JH gene rearrangements in childhood precursor-B-ALL

New family name[a]	Old family name[a]	Number and frequency of rearrangements	Segments used
DH1	DM	3 (8%)	DH1-1: 2 DH1-7: 1
DH2	DLR	13 (36%)	DH2-2: 7 DH2-8: 3 DH2-15: 2 DH2-21: 1
DH3	DXP	12 (33%)	DH3-3: 3 DH3-9: 7 DH3-10: 2
DH4	DA	0	-
DH5	DK	0	-
DH6	DN	1 (3%)	DH6-13: 1
DH7	DQ52	7 (19%)	DH7-27: 7

a. The new nomenclature is derived from Corbett et al.[21], whereas the old nomenclature is according to Ichihara et al.[49]

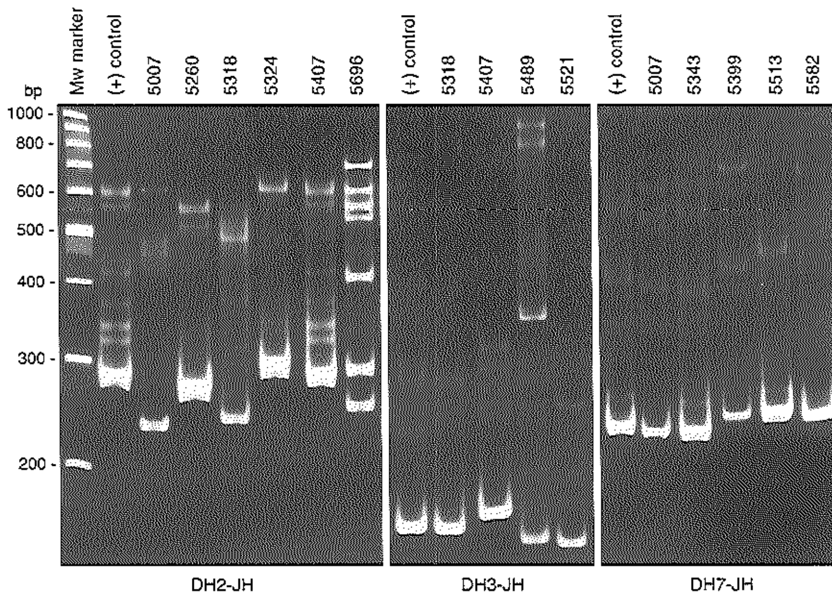

Figure 3.
Heteroduplex PCR-based detection of incomplete DH-JH gene rearrangements in several precursor-B-ALL patients. As illustrated, the usage of gene segments from the DH2, DH3, and DH7 families was most prominent.

nucleotides with an average of 8.1 nucleotides. Seven out of 31 DH-JH junctions (23%) in six patients did not show any randomly inserted N-region nucleotides.

Usage of JH gene segments in incomplete DH-JH gene rearrangements

The frequencies of different JH gene segments in the 31 DH-JH gene rearrangements are summarized in Table 2. Remarkably, the JH6 gene segment was found in more than 60% of the joinings, followed by JH4 and JH5, each occurring in 16% of cases. The JH3 gene segment was found in two DH-JH gene rearrangements (7%), while the two most upstream JH1 and JH2 genes were not utilized in the 31 analyzed ncomplete DH-JH joinings.

Sequences of complete VH-(DH)-JH gene rearrangements in precursor-B-ALL patients with incomplete DH-JH gene rearrangements

A total of 27 VH-(DH)-JH sequences were identified in the 16 precursor-B-ALL patients showing both complete VH-JH and incomplete DH-JH gene rearrangements. This series of 16 patients included 10 oligoclonal patients. Remarkably, the most downstream VH6-1 gene segment was found most frequently, i.e. in 10 sequences (37%), while 12 different VH gene segments were used in the remaining 17 sequences. Because of the unexpectedly high frequency of the VH6-1 gene segment in the DH-JH-positive group, we also performed a VH6-JH specific PCR analysis (see ref. 32) on 70 patients of the DH-JH-negative group and found that the VH6-1 gene segment was utilized in only 11% of *IGH* rearrangements in the DH-JH-negative group, which is significantly less than in the DH-JH-positive group ($p < 0.01$).

Table 2. Usage of JH gene segments in complete and incomplete *IGH* gene rearrangements in human BM precursor-B-cells, PB B-lymphocytes, precursor-B-ALL, and in cross-lineage *IGH* gene rearrangements in T-ALL.

JH segment	Human BM precursor-B-cells VH-DH-JH[a] (n=63)	Human PB B lymphocytes VH-DH-JH[b] (n=111)	Precursor-B-ALL VH-DH-JH[c] (n=68)	Precursor-B-ALL DH-JH (n=31)	T-ALL VH-DH-JH and DH-JH[d] (n=39)
JH1	0%	1%	2%	0%	13%
JH2	4%	0%	2%	0%	15%
JH3	14%	9%	11%	7% (2)	10%
JH4	32%	53%	32%	16% (5)	33%
JH5	17%	15%	22%	16% (5)	8%
JH6	33%	22%	32%	**61%** (19)	21%

a. Raaphorst et al.[43]
b. Yamada et al.[37]
c. Steenbergen et al.[15]
d. Szczepański et al.[33]

Sequence analysis helped to establish the relationship between VH-(DH)-JH and DH-JH gene rearrangements in the DH-JH-positive group. In four of the ten oligoclonal patients and also in three monoclonal patients a common D-J stem was identified in VH-(DH)-JH and DH-JH joinings indicating ongoing VH to DH-JH joining (see examples in Table 3). In another four of the ten oligoclonal patients the VH-(DH)-JH and DH-JH sequences were not related, which might be caused by secondary rearrangements, which replaced pre-existing rearrangements. In the last two patients, the oligoclonality was based on the presence of two rearranged bands of different density on Southern blot analysis; in one of the two patients the identified sequences were not related and in the other patient the type of relation could not be established because one of the *IGH* rearrangements remained unidentified.

IGH gene configuration and chromosome 14 status in precursor-B-ALL patients with incomplete DH-JH gene rearrangements as compared to DH-JH-negative patients

Based on Southern blot analysis, 10 of the 21 precursor-B-ALL patients with incomplete DH-JH rearrangements were classified as oligoclonal (48%), which is markedly higher than in the DH-JH-negative group (19 of 76 patients, 25%). In addition, five DH-JH-positive patients (24%) were assumed to have trisomy 14 and one patient probably had tetrasomy 14, based on the presence of three or four rearranged bands of equal density on Southern blot analysis. Although these frequencies are higher than in the DH-JH-negative group, the differences did not reach statistical significance. However, patients with monoclonal biallelic *IGH* gene rearrangements were significantly underrepresented in the DH-JH-positive group (24% vs. 59% in the DH-JH-negative group, $p < 0.01$).

Cytogenetic analysis revealed the presence of additional copies of chromosome 14 in 23 out of 83 patients (28%) with conclusive cytogenetic data (Table 4). Trisomy 14 and tetrasomy 14 were demonstrated in nine and one of 18 DH-JH-positive patients with conclusive cytogenetic data (56%) as compared to 12 and one out of 65 DH-JH-negative cases (20%, $p < 0.01$). The presence of additional copies of chromosome 14 was correctly anticipated by Southern blot data in seven of the 23 patients. Nine patients showed monoclonal biallelic *IGH* rearrangements, which might indicate that the additional copies of chromosome 14 in these cases characterized minor subclones. In the remaining seven patients the Southern blot based *IGH* configuration was oligoclonal. Interestingly, two of these oligoclonal cases were classified as biclonal based on the presence of two rearranged bands of different densities in Southern blotting. In both patients two clonal VH-(DH)-JH rearrangements were demonstrated by heteroduplex PCR analysis. This suggests that trisomy 14 in these patients resulted from duplication of a chromosome 14 with an existing rearranged *IGH* gene. In another two oligoclonal patients and in at least three monoclonal cases with trisomy 14, the sequence analysis revealed identical DH-JH stems

Table 3. Examples of junctional region sequences of *IGH* gene rearrangements in patients with incomplete DH-JH recombinations and oligoclonal *IGH* gene configuration due to ongoing VH to DH-JH joinings.

VH gene	N-region	VH/DH gene	N-region	DH gene	N-region	JH gene
Patient 5498						
		DH2-2 (-1)	AAG	(-7) DH6-13 (0)	CGGGGATG	(-6) JH6b
VH6-1 (-6)	GAGGGAGGG	(-14) DH2-2 (-1)	AAG	(-7) DH6-13 (0)	CGGGGATG	(-6) JH6b
		DH3-3 (-5)	CCTAATCCCTCTTATAC	(-7) DH1-7 (0)	CCACGAGA	(-6) JH6b
VH5-51 (-2)	GG	(-5) DH3-3 (-5)	CCTAATCCCTCTTATAC	(-7) DH1-7 (0)	CCACGAGA	(-6) JH6b
Patient 5565						
				DH3-10 (-6)	GG	(-10) JH6b
VH6-1 (-1)	CGGG			(-6) DH3-10 (-6)	GG	(-10) JH6b
VH1-3 (0)	AGGGAC			(-6) DH3-10 (-6)	GG	(-10) JH6b
VH6-1 (-1)	GG			(0) DH3-16 (-4)	CTCCCTCAGACGCTCCAAA	(-6) JH4b
Patient 5696						
				DH2-2 (-3)	TTGAAGTGACGGTATTAC- GACCTCGCCTAGGCTTCCCCGT	(-4) JH6b
				DH2-2 (-7)	TGCCCCTCCGAGGTTCTCTTT- GACCTCGCCTAGGCTTCCCCGT	(-4) JH6b
VH1-3 (0)		TGTAC		(-4) DH2-2 (-7)	TGCCCCTCCGAGGTTCTCTTT- GACCTCGCCTAGGCTTCCCCGT	(-4) JH6b
				DH2-2 (-4)	CGACGCTTG	(-10) JH6c
VH3-11 (-1)	CTCCCATCAGAGTA			(-13) DH2-2 (-4)	CGACGCTTG	(-10) JH6c

in at least two of the rearrangements. Apparently, in these patients the chromosome duplication developed after the incomplete DH-JH recombination, but before ongoing VH to DH-JH joining. Finally, in two oligoclonal cases the sequences of *IGH* rearrangements were completely unrelated, which is indicative of duplication of chromosome 14 as a very early event preceding recombination in the *IGH* locus.

TCR gene configuration in precursor-B-ALL patients with incomplete DH-JH gene rearrangements as compared to DH-JH-negative patients

Cross-lineage TCR gene rearrangements were found in 66 of 69 (96%) DH-JH-negative patients and in 17 of 19 (90%) of DH-JH-positive patients. However, detailed TCR gene rearrangements patterns significantly differed between the two groups. TCR gamma (*TCRG*) gene rearrangements were observed in only two DH-JH-positive patients (11%) as compared to 49 DH-JH-negative patients (71%, $p < 0.01$). Although the recombination in TCR alpha/delta locus occurred with similar frequencies in both groups (approximately 90% of patients), it rarely concerned TCR delta (*TCRD*) deletions in the DH-JH-positive group: monoallelic deletions in two patients (11%) and no biallelic deletions. This was in striking contrast ($p < 0.01$) to the DH-JH-negative group, where monoallelic and biallelic deletions were found in 19 (28%) and 21 patients (30%), respectively. Finally, TCR beta (*TCRB*) gene rearrangements were slightly less prevalent in the DH-JH-positive group (4/19 patients, 21% vs. 26/69 DH-JH-negative patients, 38%; not significant) and exclusively concerned monoallelic incomplete Dβ-Jβ2 rearrangements. In striking contrast, *TCRB* gene rearrangements were biallelic in 50% (13/26) of DH-JH-negative patients, while complete Vβ-Jβ2 joinings were found in 69% (18/26) of patients.

Clinical and laboratory characteristics of DH-JH-positive patients

Clinical and laboratory characteristics of DH-JH-positive vs. DH-JH-negative patients are summarized in Table 4. The two patient groups showed similar age and sex distribution. The only significant clinical difference concerned the frequency of infant ALL, which comprised 14% (three cases) of DH-JH-positive precursor-B-ALL as compared to 1% (one case) of DH-JH-negative patients ($p < 0.05$). However, owing to the low frequency of infant ALL this difference should be interpreted with caution. Both DH-JH-positive and DH-JH-negative groups showed similar distribution of precursor-B immunophenotypes. Strikingly, cytogenetic and DNA index analyses demonstrated a statistically significant overrepresentation of patients with hyperdiploidy >50 chromosomes and increased DNA index (≥1.16) in the DH-JH-positive group (40-50% as compared to 20% in DH-JH-negative patients; $p < 0.01$). The stratification into the three treatment risk groups was comparable between the DH-JH-positive and the DH-JH-negative patients. Both groups showed similar early treatment response to prednisone. Finally, the presence of incomplete DH-JH rearrangements influenced neither the event-free survival nor the overall survival (Figure 4).

Table 4. Clinical and laboratory characteristics of DH-JH positive and DH-JH negative subgroups of precursor-B-ALL.

Parameters	DH-JH positive group (n = 21)	DH-JH negative group (n = 76)
Age		
< 1 year	3 (14%)[b]	1 (1%)[b]
1 – 5 years	13 (62%)	42 (55%)
5 – 10 years	1 (5%)	23 (30%)
> 10 years	4 (19%)	10 (14%)
Gender		
Boys	12 (57%)	42 (55%)
Girls	9 (43%)	34 (45%)
Treatment group[a]		
SRG	6 (29%)	21 (30%)
MRG	12 (57%)	43 (61%)
HRG	3 (14%)	6 (9%)
Steroid response		
good	20 (95%)	72 (95%)
poor	1 (5%)	2 (3%)
not determined	0	2 (3%)
Immunophenotype		
pro-B ALL	2 (10%)	2 (3%)
common ALL	14 (67%)	50 (65%)
pre-B ALL	5 (24%)	24 (32%)
Cytogenetics		
diploid	1 (5%)	14 (18%)
hypodiploid (< 46 chr.)	0	4 (5%)
hyperdiploid (47-50 chr.)	2 (10%)	6 (8%)
hyperdiploid (> 50 chr.)	11 (52%)[b]	15 (20%)[b]
pseudodiploid	4 (19%)	19 (25%)
not determined	3 (14%)	18 (23%)
DNA index		
< 1.00	0	0
1.00	7 (33%)	51 (67%)
>1.00 and < 1.16	4 (19%)	5 (7%)
≥1.16 and ≤ 1.60	9 (43%)[b]	12 (16%)[b]
>1.60	0	1 (1%)
not determined	1 (5%)	7 (9%)
Chromosome 14 status		
monosomy	0	1 (1%)
normal	8 (38%)[b]	51 (67%)[b]
trisomy	9 (43%)[b]	12 (16%)[b]
tetrasomy	1 (5%)	1 (1%)
not determined	3 (14%)	11 (14%)
6-year relapse-free survival (No. of relapses)[a]	61% ± 11% (8)	64% ± 6% (25)

a. These data exclusively concern 91 patients treated with DCLSG ALL-8 treatment protocol

b. Parameters statistically significantly different between the DH-JH positive and the DH-JH negative subgroups of precursor-B-ALL ($p < 0.05$).

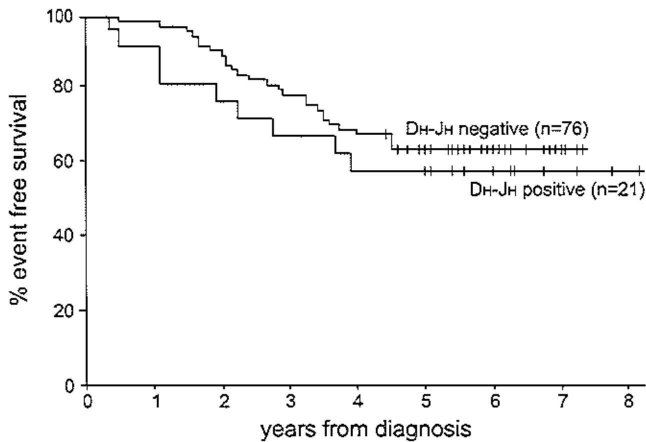

Figure 4.
Event-free survival of the DH-JH positive vs. DH-JH negative patients treated with DCLSG ALL-8 chemotherapy protocols.

DISCUSSION

The frequency and molecular characteristics of incomplete DH-JH gene rearrangements were investigated in precursor-B-ALL patients. Using seven DH family specific primer combinations, covering theoretically all incomplete recombinations, we identified clonal DH-JH gene rearrangements in 21 of 97 patients (22%). DH-JH joinings were the sole clonal *IGH* rearrangements in only 5% of patients. At first sight, this might suggest that the final oncogenic event in precursor-B-ALL rarely concerns early-B progenitors, characterized by the exclusive presence of incomplete *IGH* gene rearrangements. However, the continuing rearrangement processes in ALL can easily change the original Ig gene configuration present at the time of the oncogenic transformation.[36] This probably also explains the relatively low frequency of incomplete DH-JH gene rearrangements on the second *IGH* allele (18% of cases with biallelic *IGH* rearrangements).

We observed non-random DH gene segment usage in incomplete DH-JH gene rearrangements with predominance of three families, i. e. DH2, DH3, and DH7, comprising 36%, 33% and 19%, respectively (Table 1). Gene segments of the DH2 and DH3 families are known to be preferentially utilized in complete VH-DH-JH joinings in normal and malignant B-cells and B-cell precursors.[15,20,37,38] In contrast, the segments of the DH5 and DH6 families, accounting for 10-15% of identified DH genes in the third *IGH* complementarity-determining regions (HCDR3) in B-lineage cells, were virtually absent in incomplete DH-JH gene rearrangements in precursor-B-ALL.

Another striking finding was the frequent usage of the DH7-27gene (19%), which is located immediately upstream to JH gene complex. This single member of the DH7 family is rarely (approximately 1%) identified in HCDR3 of normal and malignant B-cells and B-cell precursors,[15,20,37,38] while DH7-27gene rearrangements are predominantly (approximately 40%) found in fetal B-lineage cells.[39,40] This finding together with the frequent lack of N-nucleotide insertions in DH-JH junctions supports the hypothesis that a significant proportion of childhood precursor-B-ALL originates *in utero*.[41,42]

Concerning the JH gene usage, we observed a striking predominance of the most downstream JH6 segment, which was found in 61% of clonal DH-JH gene rearrangements. This is twice as much as reported for HCDR3 in normal and malignant B-lineage-cells (Table 2).[15,37,43] This observation together with the finding of predominant usage of DH genes from the more upstream part of the DH region (78%) suggests that most DH-JH gene rearrangements in precursor-B-ALL actually represent secondary recombinations deleting pre-existing DH-JH joinings.

The patients with incomplete DH-JH gene rearrangements were frequently characterized by hyperdiploid karyotype (52%) with additional copies of chromosome 14 (56%) and/or by *IGH* oligoclonality (52%). Combined cytogenetic, Southern blot and PCR data revealed a complex relationship between the hyperdiploidy 14, *IGH* gene configuration, and subclone formation. In our series, additional copies of chromosome 14 were found in 28% of precursor-B-ALL patients, which is in line with previously published cytogenetic data.[44] Sequence analysis showed that this type of chromosomal aberration might occur before *IGH* gene rearrangements, but more frequently parallels ongoing VH to DH-JH joining or might even affect cells with an end-stage (stable) *IGH* configuration. Although it is difficult to explain how the presence of DH-JH rearrangements is related to trisomy 14, it suggests that having extra copies of chromosome 14 freezes the ALL cells in a more immature immunogenotypic stage. In this context it was striking to observe that hyperdiploidy of chromosome 14 was inversely associated with *TCRD* gene deletions, which are regarded as a more mature immunogenotypic feature. The association between DH-JH gene rearrangements and *IGH* oligoclonality is obviously explained by the presence of ongoing and secondary recombination events. The presence of incomplete DH-JH joinings was also significantly associated with a less mature immunogenotype as reflected by overrepresentation of the most downstream VH6-1 gene segment, the virtual absence of monoallelic *TCRD* deletions, the full absence of biallelic *TCRD* deletions, low frequency of *TCRG* gene rearrangements, the absence of complete Vβ-Jβ rearrangements, and the absence of biallelic *TCRB* rearrangements. However, these distinct immature immunogenotypic features of precursor-B-ALL with clonal incomplete *IGH* gene rearrangements were not associated with more aggressive disease (Figure 4). The frequency of high-risk features at diagnosis, early treatment response, as well as long-term event-free survival, did not significantly differ between

DH-JH-negative and DH-JH-positive patients (Table 4). Thus, the presence of clonal incomplete *IGH* gene rearrangements has no direct clinical implications, although associated with immunobiological features of immature B-cell differentiation stages.

In this study, we demonstrated that clonal VH-(DH-)JH gene rearrangements can be easily identified in the vast majority of precursor-B-ALL patients and can serve as PCR targets for MRD monitoring (Figure 2). However, in almost half of the cases the number of clonal PCR products was lower than the number of rearrangements in Southern blot analysis, including 29 monoclonal cases with an undetectable second allele. Our PCR analyses correctly assigned 105 alleles in 67 patients with monoclonal biallelic *IGH* rearrangements, which gives an overall sensitivity of 78%. Although chromosome translocations involving the *IGH* gene might occur, the recombinations undetectable by PCR most probably represent unidentified VH-(DH-)JH gene rearrangements. Particularly the newly discovered VH pseudogenes might be involved in these rearrangements since many of them contain proper recombination signal sequences, while they frequently lack framework one sequences, which are recognized by the currently used VH family-specific primers.[22]

Since the types of preferential DH-JH gene rearrangements have now been identified, it would be relatively easy to screen precursor-B-ALL patients for the presence of incomplete *IGH* gene rearrangements and apply them as PCR targets for MRD monitoring, particularly in patients without detectable clonal complete VH-(DH-)JH joinings. Three primer combinations (DH2, DH3, and DH7 in combination with a JH consensus primer) can identify 89% of incomplete DH-JH rearrangements in precursor-B-ALL (Figure 3) and result in clonal PCR products in all patients without VH-JH rearrangements. However, it should be noted that incomplete DH-JH gene rearrangements are associated with *IGH* oligoclonality in at least 52% of patients. Moreover, in one third of patients with clonal DH-JH gene rearrangements we found evidence for continuing VH to DH-JH recombination already at diagnosis (Table 3). *IGH*-based MRD detection in such patients is still possible and could rely on so-called common DH-JH stems, which are preserved in different VH-(DH-)JH joinings. Such an approach is feasible with the currently used real-time quantitative PCR approaches for MRD detection based on TaqMan technology.[45-48] In some cases this strategy might be hampered by the lack of N-nucleotide insertions, which concerns 20-30% of *IGH* gene rearrangements in precursor-B-ALL.[15] DH-JH stems in rearrangements involving the most downstream JH6 gene segment (i.e. 60% of all incomplete joinings) could be perceived as "end-stage" rearrangements, which probably are stable throughout the disease course. This is not the case with the DH-JH rearrangements involving upstream JH gene segments, which might be subjected to DH-JH replacements. We found evidence for such secondary (replacement) rearrangement patterns in 20% of patients at diagnosis. If they occur during the disease course, they might lead to false-negative MRD-results. In conclusion, incomplete DH-JH gene rearrangements can be perceived as a supplementary MRD-PCR target, particular-

ly useful for patients, who do not have other leukemia-specific targets, such as complete VH-(DH-)JH joinings, Ig kappa deleting element (Kde) rearrangements, and cross-lineage *TCRG* and *TCRD* gene rearrangements or in whom the other MRD-PCR targets are not suitable because of insufficient sensitivity.

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CHAPTER 2.4

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*

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ABSTRACT

A large series of 202 childhood precursor-B-cell acute lymphoblastic leukemia (ALL) patients was analyzed by Southern blotting (SB) for cross-lineage rearrangements and/or deletions in the T-cell receptor *TCRB*, *TCRG*, and *TCRD* loci. In 93% (187/201) of the precursor-B-ALL patients one or more genes were rearranged and/or deleted. *TCRB* gene rearrangements were found in 35% (69/196), *TCRG* gene rearrangements in 59% (113/192), *TCRD* gene rearrangements in 55% (112/202), and isolated monoallelic or biallelic deletions of *TCRD* loci in 34% (68/202) of the cases.

TCRB gene rearrangements involved exclusively the Jβ2 locus with complete V(D)Jβ2 joinings in 53% of gene rearrangements and incomplete Dβ-Jβ2 gene rearrangements in 33%. *TCRG* gene rearrangements frequently occurred on both alleles (65% of cases) and in approximately 70% concerned rearrangements to Jγ1 gene segments. Most rearranged *TCRD* alleles (80%) represented incomplete Vδ2-Dδ3 or Dδ2-Dδ3 gene rearrangements, while the remaining *TCRD* gene rearrangements remained unidentified.

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Subsequently, we evaluated, whether heteroduplex PCR analysis of rearranged *TCRG* and *TCRD* genes can be used for reliable identification of PCR targets for detection of minimal residual disease (MRD). The concordance between SB and heteroduplex PCR analysis for detection of the various types of clonal *TCRG* and *TCRD* gene rearrangements ranged between 78% and 87%. The discrepancies could be assigned to the presence of 'atypical' *TCRD* gene rearrangements or translocations only detecable by SB, but also to efficient PCR-based detection of rearrangements derived from small subclones, which are difficult to detect with SB. Indications for oligoclonality were observed in 38% and 30% of patients with *TCRG* and *TCRD* gene rearrangements, respectively, which is comparable to the frequency of oligoclonality in *IGH* locus.

Based on the combined data it was possible to reduce the broad panel of six *TCRD* and 12 *TCRG* primer combinations for MRD studies to two *TCRD* combinations (Vδ2-Dδ3 and Dδ2-Dδ3) and six *TCRG* combinations (VγI, VγII, VγIV family-specific primers with Jγ1.1/2.1 and Jγ1.3/2.3 primers) resulting in the detection of 80% and 97% of all *TCRD* and *TCRG* gene rearrangements, respectively. Finally, the heteroduplex PCR data indicate that MRD monitoring with *TCRG* and/or *TCRD* targets is possible in approximately 80% of childhood precursor-B-ALL patients; ~55% of patients even have two *TCRG* and/or *TCRD* targets.

INTRODUCTION

The enormous diversity of immunoglobulin (Ig) and T-cell receptor (TCR) molecules is generated during B and T-cell differentiation by a series of ordered rearrangements of variable (V), diversity (D), and joining (J) gene segments.[1,2] Initially, Ig and TCR gene rearrangements were regarded as B-lineage and T-lineage specific markers, respectively. However, the finding of cross-lineage expression of 'lineage-specific' immunological markers and cross-lineage Ig and TCR gene rearrangements has disputed this view.[2,3]

Cross-lineage rearrangements of Ig heavy-chain (*IGH*) genes have been observed in 10-15% of T acute lymphoblastic leukemia (T-ALL) and in ~5% of mature T-cell leukemias and lymphomas, but cross-lineage rearrangements of Ig light chain genes in malignant T-cell proliferations are rare (<1%).[4-5] On the other hand, TCR beta (*TCRB*), TCR gamma (*TCRG*), and TCR delta (*TCRD*) gene rearrangements and/or deletions have been found in ~30%, ~50%, and ~80% of precursor-B-ALL, respectively.[5-13] However, only a few limited studies have described the configuration of all three TCR genes in precursor-B-ALL.[5,7,10,11] The detection of rearrangements in the TCR alpha (*TCRA*) locus by Southern blotting (SB) and PCR is difficult due to the long stretch of Jα gene segments (about 85 kb) (Figure 1), but the occur-

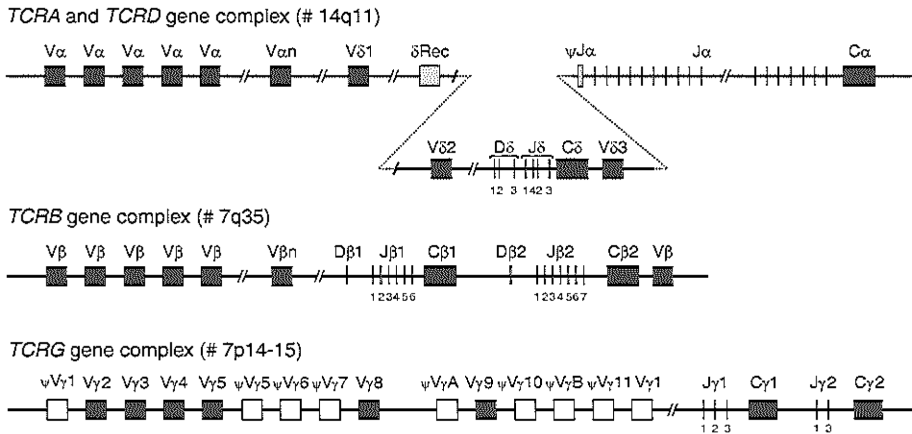


Figure 1.
Schematic diagram of the four human TCR genes. The *TCRA* gene complex consists of >50 V gene segments, a remarkably long stretch of 61 functional J gene segments, and one C gene segment. The major part of the *TCRD* gene complex is located between the Vα and Jα gene segments and consists of eight V, three D, and four J gene segments and one C gene segment. The δREC and ψJα gene segments play a role in *TCRD* gene deletions, which precede *TCRA* gene rearrangements in developing T-cells. The *TCRB* gene complex consists of 65 V gene segments and two C gene segments, both of which are preceded by one D and six or seven J gene segments. The *TCRG* gene complex consists of a restricted number of V gene segments (six functional V gene segments and nine pseudogene segments) and two C gene segments, each preceded by two Jγ1 or three Jγ2 gene segments. Pseudo genes (ψ) are indicated with open symbols.

rence of *TCRA* gene rearrangements in precursor-B-ALL can be deduced from *TCRD* gene deletions. Therefore, whenever *TCRD* gene deletions are detected, rearrangements in the *TCRA* locus can be anticipated.

Junctional regions of rearranged Ig and TCR genes in lymphoid malignancies can be regarded as clonal 'tumor-specific' markers, because they arise during the recombination of different V, (D), and J gene segments by deletion and random insertion of nucleotides at the junctions of the gene segments.[1] Hence, each junctional region of rearranged Ig and TCR genes is different in each leukemia and lymphoma. Therefore, the polymerase chain reaction (PCR) has been used for amplification of the 'tumor-specific' junctional regions of cross-lineage rearranged *TCRG* and *TCRD* genes to detect minimal residual disease (MRD) in precursor-B-ALL.[14-17]

The aims of our study were firstly to determine the precise frequency of cross-lineage TCR gene rearrangements in a large series of childhood precursor-B-ALL and to evaluate the occurrence of preferential TCR gene rearrangements and the occurrence of oligoclonality at the TCR gene level. Secondly, based on SB data we estimated the number of precursor-B-ALL patients with identifiable cross-lineage TCR gene rearrangements, which can be used for PCR-mediated MRD detection. Thirdly, we wished to evaluate whether heteroduplex analysis of PCR products can

be used for reliable identification of rearranged *TCRG* and *TCRD* genes as molecular targets for MRD monitoring, because heteroduplex PCR analysis was shown to be a rapid, cheap, non-radioactive, and easy alternative approach for detection of clonal Ig and TCR gene rearrangements with a sensitivity of 1-5%.[18-21] The configuration of the *TCRB*, *TCRG*, and/or *TCRD* genes was analyzed by SB in 202 well-characterized cases of precursor-B-ALL and the results were compared with previously published childhood precursor-B-ALL cases. In addition, 62 precursor-B-ALL patients were subjected to detailed heteroduplex PCR analysis of the *TCRG* and *TCRD* gene loci.

MATERIALS AND METHODS

Cell samples

Bone marrow (BM) or peripheral blood (PB) samples from 202 children with precursor-B-ALL were obtained at initial diagnosis. The age distribution was from 2 months until 16 years and 6 months (mean 5 years and 8 months). Twenty-five children were younger than 2 years and eight of them were infants (age <1 year). The diagnosis of ALL was made according to the FAB classification[22] and was always confirmed by the laboratory of the Dutch Childhood Leukemia Study Group.

Mononuclear cells (MNC) were isolated from PB or BM samples by Ficoll-Paque centrifugation (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden). The freshly obtained MNC samples were subjected to a detailed immunophenotyping according to standard protocols.[23-25] Remaining MNC were stored in liquid nitrogen. A leukemia was considered to be a precursor-B-ALL if the malignant cells were positive for terminal deoxynucleotidyl transferase (TdT), CD19 and HLA-DR (pro-B-ALL), for TdT, CD10, CD19 and HLA-DR (common ALL), or for TdT, CD10, CD19, HLA-DR and cytoplasmic Ig heavy-chain μ (CyIgμ) (pre-B-ALL). Immunological marker analysis of the precursor-B-ALL revealed that eight were pro-B-ALL, 135 were common ALL, and 59 were pre-B-ALL.

Southern blot analysis

DNA was isolated from fresh or frozen MNC fractions as described previously.[2] Fifteen micrograms of DNA were digested with the appropriate restriction enzymes (Pharmacia), size-separated in 0.7% agarose gels and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as described.[2] Incomplete and complete *TCRB* gene rearrangements were detected with TCRBD1U, TCRBD1, TCRBJ1, TCRBD2U, TCRBD2, TCRBJ2, and TCRBC probes (DAKO Corporation, Carpinteria, CA, USA) in *Eco*RI and *Hind*III digests.[47] *TCRG* gene rearrangements were analyzed with the Jγ1.2 probe in *Bgl*II digests and the Jγ1.3 and Jγ2.1 probes in *Eco*RI digests.[2,26,27] The configuration of the *TCRD* genes was analyzed with the TCRDJ1 probe in *Bgl*II, *Eco*RI, and *Hind*III digests and in 102 cases additionally with TCRDV1, TCRDV2, TCRDV3, TCRDRE, TCRDD1, TCRDD3, TCRDJ2, TCRDJ3, TCRDC, and TCRAPJ probes (DAKO Corporation).[13]

PCR amplification

Sixty-two precursor-B-ALL patients were subjected to detailed PCR study of *TCRG* and *TCRD* gene loci. PCR was essentially performed as described previously.[13,28,29] In each 100 μl PCR reaction 0.1 μg DNA sample, 12.5 pmol of the 5′ and 3′ oligonucleotide primers, and 1 U *Taq* polymerase (PE Biosystems, Foster City, CA, USA) were used. The sequences of the majority of oligonucleotides used for amplification of *TCRG* and *TCRD* gene rearrangements were published before.[13,29] Some primers were newly designed during the BIOMED-1 Concerted Action '*Investigation of minimal residual disease in acute leukemia: international standardization and clinical evaluation*'.[30] All primers were synthesized on an ABI 392 DNA synthesizer (PE Biosystems) using the solid-phase phosphotriester method. PCR conditions

Table 1. Frequencies of cross-lineage TCR gene rearrangements in childhood precursor-B-ALL.[a]

Precursor-B-ALL	TCR gene rearrangements and/or deletions				
	TCR G/G	*TCRB* R/G or R/R	*TCRG* R/G or R/R	*TCRD*	
				R/G, R/R or D/R	D/G or D/D
Pro-B-ALL	25% (2/8)	0% (0/8)	13% (1/8)	38% (3/8)	25% (2/8)
Common ALL	2% (3/134)	40% (51/129)	63% (79/125)	61% (83/135)	32% (43/135)
Pre-B-ALL	15% (9/59)	31% (18/59)	56% (33/59)	44% (26/59)	39% (23/59)
Total	7% (14/201)	35% (69/196)	59% (113/192)	55% (112/202)	34% (68/202)

a. Number of patients with germline or rearranged cross-lineage TCR genes per total group of precursor-B-ALL patients in parentheses.

G, allele in germline configuration; R, rearranged allele; D, deletion of the Cδ gene segment.

were: initial denaturation step of 3 min at 92°C followed by 45 s at 92°C, 90 s at 60°C, and 2 min at 72°C for 35 cycles using a PE Biosystems thermal cycler. After the last cycle an additional extension step of 10 min at 72°C was performed. Appropriate positive and negative controls were included.[30]

Heteroduplex analysis of PCR products

For heteroduplex analysis, the PCR products were denatured at 94°C for 5 min after the final cycle of amplification and subsequently cooled to 4°C for 60 min to induce duplex formation.[19] Afterwards the duplexes were immediately loaded on 6% non-denaturing polyacrylamide gels in 0.5 x Tris-Boric acid-EDTA (TBE) buffer, run at room temperature, and visualized by ethidium bromide staining.[19] *Pst*I-digested lambda DNA or a 100-bp DNA ladder (Promega Corporation, Madison, WI, USA) were used as size markers.

Both SB and heteroduplex analysis of PCR products were performed in parallel in a double-blind manner. Concordance between the two methods was calculated assuming that the following gene configurations concerned the same alleles: rearranged band in SB = presence of clonal homoduplex after PCR; germline band and/or deletion in SB = no clonal PCR product.

RESULTS

SB analysis of *TCRB* gene rearrangements

DNA samples from 196 precursor-B-ALL at diagnosis were examined for the

Table 2. Allelic frequencies of *TCRB* gene rearrangements in 196 childhood precursor-B-ALL patients as detected by SB analysis.

TCRB gene configuration (No. of alleles)		Pro-B-ALL (n=16)	Common ALL (n=260)[a]	Pre-B-ALL (n=118)	Total group of precursor-B-ALL (n=394)[a]
Germline alleles		16 (100%)	184 (71%)	94 (80%)	294 (75%)
Rearranged alleles		0	76 (29%)	24 (20%)	100 (25%)
Number of rearrangements[b]		0	79[b]	27[b]	106[b]
TCRB1 region: Vβ- Dβ1, Dβ1- Jβ1, and/or Vβ- Jβ1		0	0	0	0
TCRB2 region:	Vβ- Dβ2	0	3 (4%)	2 (7%)	5 (5%)
	Dβ1- Dβ2	0	2 (3%)	0	2 (2%)
	Vβ- Jβ2	0	43 (54%)	14 (52%)	57 (53%)
	Dβ1- Jβ2	0	12 (15%)	5 (19%)	17 (16%)
	Dβ2- Jβ2	0	16 (20%)	2 (7%)	18 (17%)
	Unidentified to β2	0	3 (4%)	4 (15%)	7 (7%)

a. The number of indicated alleles includes two precursor-B-ALL patients showing three monoclonal rearrangements, most probably as a result of trisomy 7.
b. The number of rearrangements is higher than the number of rearranged alleles, because of the incidental occurrence of subclones in these patients.

presence of rearranged *TCRB* genes by SB (Figure 2A). In all eight pro-B-ALL no *TCRB* gene rearrangements were detected. In common ALL and pre-B-ALL, *TCRB* gene rearrangements were detected in 40% (51/129) and 31% (18/59), respectively (Table 1). The allelic frequencies of *TCRB* gene rearrangements are summarized in Table 2. Germline configuration was found in 75% (294/394) of the *TCRB* alleles. No rearrangements were detected with the TCRBD1 and Jβ1 probes; only rearrangements in the *TCRB*2 locus were observed. The majority concerned complete V(D)Jβ2 rearrangements (53%) or incomplete Dβ-Jβ2 rearrangements (33%) (Table 2). In six patients (3%), SB analysis revealed weak bands, most probably derived from subclones.

SB analysis of *TCRG* gene rearrangements

Diagnosis DNA samples from 192 precursor-B-ALL patients were analyzed by SB for the presence of rearranged *TCRG* genes (Figure 2B). We detected *TCRG* gene rearrangements in 59% (113/192) of precursor-B-ALL; this finding concerned monoallelic rearrangements in 35% (39/113) and biallelic rearrangements in 65% (74/113). The distribution of the *TCRG* gene rearrangements in the three precursor-B-ALL subgroups was 13% (1/8) in pro-B-ALL, 63% (79/125) in common ALL, and

Table 3. Allelic frequencies of *TCRG* gene rearrangements in childhood precursor-B-ALL.[a]

TCRG gene configuration	Total group (n=192) analyzed by SB		Subgroup of 62 patients			
			Analyzed by PCR		Analyzed by combined SB/PCR[b]	
Germline alleles	196	(51%)	-		68	(55%)
Deleted alleles	1	(< 1%)	-		0	
Rearranged alleles	187	(49%)	84		56	(45%)
Number of rearrangements	187	84	56			
Involving Jγ1.1/2.1	56	(30%)	17	(20%)	12	(21%)
VγI-Jγ1.1/2.1	37	(20%)	15	(18%)	10	(18%)
VγII-Jγ1.1/2.1	12	(6%)	1	(1%)	1	(2%)
VγIII-Jγ1.1/2.1	0		0	0		
VγIV-Jγ1.1/2.1	5	(3%)	1	(1%)	1	(2%)
not identified to Jγ1.1/2.1	2	(1%)	0	0		
Involving Jγ1.2	1	(< 1%)	1	(1%)	1	(2%)
Involving Jγ1.3/2.3	130	(70%)	66	(79%)	43	(77%)
VγI-Jγ1.3/2.3	93	(50%)	47	(56%)	31	(55%)
VγII-Jγ1.3/2.3	29	(16%)	15	(18%)	9	(16%)
VγIII-Jγ1.3/2.3	1	(< 1%)	0	0		
VγIV-Jγ1.3/2.3	6	(3%)	4	(5%)	3	(5%)
not identified to Jγ1.3/2.3	1	(< 1%)	0	0		

a. Since frequencies of particular TCRG gene rearrangements are slightly different in the group studied by heteroduplex PCR analysis from the total group analyzed by SB, both groups are described separately.
b. Excluding weak gene rearrangements, probably derived from subclones.

56% (33/59) in pre-B-ALL (Table 1). The allelic frequencies of *TCRG* gene rearrangements are summarized in Table 3. *TCRG* genes in germline configuration were found in 51% of alleles. Rearrangements to Jγ1.3 or Jγ2.3 gene segments predominated, comprising 70% of recombinations, while Jγ1.1 or Jγ2.1 gene rearrangements were found in the vast majority of the remaining cases. Whenever SB allowed a reliable discrimination between Jγ1 and Jγ2 gene rearrangements (Figure 1) (172 of the 187 rearranged alleles), we found that approximately 70% of the *TCRG* gene rearrangements occurred to Jγ1 gene segments.

V gene segments of the VγI family were used most frequently (70%), followed by VγII (Vγ9) (22%) and VγIV (Vγ11) (6%). In up to 10% of all patients (19/192), i.e. 17% (19/113) of patients with *TCRG* gene rearrangements, either multiple bands of heterogeneous densities or weak bands in addition to germline bands were found suggesting oligoclonality in the *TCRG* locus (Figure 3).

Table 4. Allelic frequencies of *TCRD* gene rearrangements or deletions in childhood precursor-B-ALL as detected by SB analysis.

TCRD gene configuration (no. of alleles)	Pro-B-ALL (n=16)	Common ALL (n=273)[a]	Pre-B-ALL (n=119)[a]	Total group of precursor-B-ALL (n=408)[a]
Germline configuration	8 (50%)	47 (17%)	31 (26%)	86 (21%)
TCRD gene rearrangements	3 (19%)	121 (44%)	38 (32%)	162 (40%)
TCRD gene deletion[b]	5 (31%)	105 (38%)	50 (42%)	160 (39%)
Number of rearrangements[c]	3	131	47	181
Vδ2-Dδ3	3 (100%)	92 (70%)	27 (57%)	122 (67%)
Dδ2-Dδ3	0	15 (11%)	8 (17%)	23 (13%)
other[d]	0	24 (18%)	12 (26%)	36 (20%)

a. The number of indicated alleles includes four precursor-B-ALL patients showing three monoclonal rearrangements, most probably as a result of trisomy 14.
b. Based on rearrangements of Vδ2 or Dδ2 gene segments in the absence of the Cδ gene region, we assume that in 35% (36/102) of these *TCRD* gene deletions a Vδ2 or Dδ2 gene segment rearranged to a Jα gene segment. A Vα-Jα rearrangement was assumed in 60% (61/102) based on the fact that no hybridization signal was obtained when all *TCRD* and γJα probes were used for hybridization.
c. The number of rearrangements is higher than the number of rearranged alleles, because of the occurrence of subclones.
d. Rearranged *TCRD* bands in the presence of the Cδ gene segment are probably caused either by rearrangements of Vα gene segments to the Dδ3-Jδ1 locus or by translocations or other chromosomal aberrations in the *TCRD* locus.

SB analysis of *TCRD* gene rearrangements

DNA samples from 202 precursor-B-ALL were analyzed for the presence of *TCRD* gene rearrangements and/or deletions (Figure 2C). In the total group of precursor-B-ALL patients monoallelic or biallelic *TCRD* gene rearrangements were found in 55% (112/202) and monoallelic or biallelic *TCRD* gene deletions in the absence of *TCRD* rearrangements were found in 34% (68/202), equally distributed over the three different precursor-B-ALL subgroups (Table 1). The allelic frequencies of germline *TCRD* genes (21%), *TCRD* gene rearrangements (40%), and deletions (39%) are summarized in Table 4. In 67% of rearranged alleles a Vδ2-Dδ3 rearrangement and in 13% a Dδ2-Dδ3 rearrangement was found. The remaining *TCRD* gene rearrangements detected by use of the Jδ1 probe could not be identified precisely. They are probably caused either by rearrangements of a Vα gene segment to the Dδ3-Jδ1 region or by translocations or other chromosomal aberrations in the *TCRD* locus.

In addition, 102 patients were subjected to detailed SB *TCRD* analysis with multiple Vδ, Dδ, Jδ, probes as well as Cδ, δREC, and ψJα probes. Rearrangements of Vδ or Dδ gene segments in the absence of the Cδ gene region allowed us to conclude that in 36% (36/102) of the *TCRD* gene deletions a Vδ2 or Dδ2 gene segment most

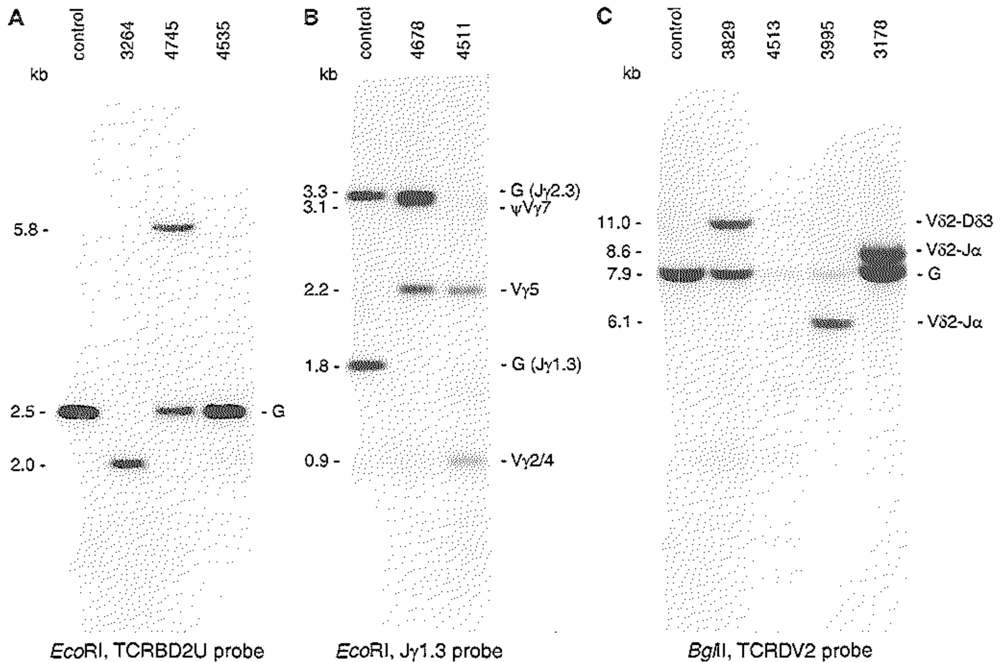
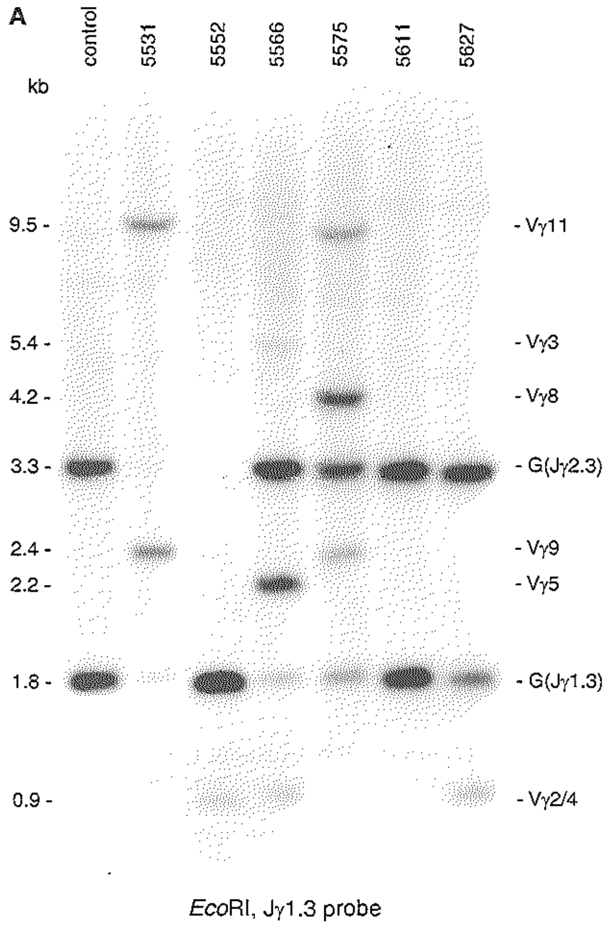


Figure 2.
SB analysis of TCR genes in several precursor-B-ALL patients. Control DNA and DNA from precursor-B-ALL patients were digested with the appropriate restriction enzymes, size-separated and blotted onto nylon membrane filters, which were hybridized with ^{32}P-labeled probes. (**A**) *TCRB* gene analyses using *Eco*RI with the TCRBD2U probe. (**B**) *TCRG* gene analyses using *Eco*RI with the Jγ1.3 probe. (**C**) *TCRD* gene analyses using *Bgl*II with the TCRDV2 probe. The germline bands (G) and several TCR gene rearrangements are indicated.

likely rearranged to a Jα gene segment (Figure 2C). Furthermore, we assume that in the majority of *TCRD* gene deletions (60%) a Vα-Jα gene rearrangement occurred, because upon hybridization with all *TCRD* and ψJα probes no hybridization signal was observed. In four precursor-B-ALL patients (five alleles) a unique hybridization pattern was found in which Vδ (except for the Vδ3 probe), δREC, and Dδ1 probes resulted in germline hybridization signals, whereas all Jδ, Cδ, and the ψJα probes showed no hybridization signal, indicating deletion of these DNA gene segments. Therefore, it cannot be excluded that potential targets for rearrangements are present within the Dδ2-Dδ3 region or that chromosome aberrations in the *TCRD/A* locus are present. In four patients three *TCRD* gene rearrangements of identical intensity were found suggesting trisomy 14 (Table 4 and Figure 4). In up to 7% (15/202) of precursor-B-ALL patients, i.e. 13% (15/112) of cases with *TCRD* gene rearrangements, the presence of additional weak SB bands suggested oligoclonality.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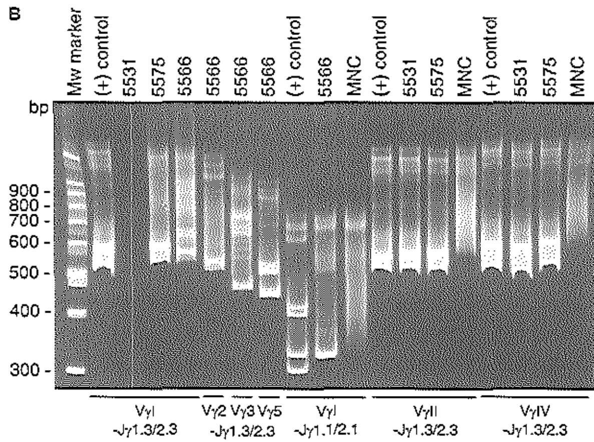
- Vγ11
- Vγ3
- Vγ8
- G(Jγ2.3)
- Vγ9
- Vγ5
- G(Jγ1.3)
- Vγ2/4
EcoRI, Jγ1.3 probe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Vγ2 Vγ3 Vγ5 -Jγ1.3/2.3
VγI -Jγ1.1/2.1
VγII -Jγ1.3/2.3
VγIV -Jγ1.3/2.3

Figure 3.
Clonality assessment via SB analysis and heteroduplex PCR analysis of *TCRG* genes.
(**A**) DNA from precursor-B-ALL patients was digested with *Eco*RI. The filter was hybridized with the ^{32}P-labeled Jγ1.3 probe. In two patients (5566 and 5575) multiple rearranged bands of different densities were found reflecting *TCRG* oligoclonality. (**B**) Heteroduplex PCR analysis with four family specific V primers in combination with Jγ1.1/2.1, Jγ1.2, and Jγ1.3/2.3 primers. Five clonal PCR products were found in patient 5566 sample, and three in patient 5575. There was no obvious difference in the intensity of homoduplexes between true 'allelic' rearrangements (patient 5531) and recombinations derived from subclones (patient 5575)

Comparison of SB and heteroduplex PCR analysis of *TCRG* and *TCRD*

DNA-based PCR amplification was simultaneously performed with SB analysis in a double-blind manner for *TCRG* and *TCRD* genes in 62 cases. Heteroduplex PCR analysis of *TCRG* with 12 primer pair combinations (four Vγ family-specific primers with Jγ1.1/2.1, Jγ1.2, and Jγ1.3/2.3 primers) revealed all except one allelic gene rearrangements detected by the SB technique. In seven cases with *TCRG* gene rearrangements estimated as monoallelic based on SB, two homoduplexes were found, indicating the presence of two rearrangements of the same gene segments. Furthermore, with the same primer combinations we were able to amplify 10 minor TCR gene rearrangements, demonstrated by SB analysis as weak bands (Figure 3). They were visualized as 12 homoduplexes of intensities equal to products derived from monoallelic gene rearrangements. Finally, nine rearrangements were detected exclusively by heteroduplex PCR analysis (six rearrangements to Jγ1.1/2.1, two VγII-Jγ1.3/2.3, and one VγIV-Jγ1.3/2.3 recombination). Overall, combined SB and PCR data indicate the presence of minor subclones of *TCRG* gene rearrangements in up to 26% (16/62) of childhood precursor-B-ALL patients, i.e. 38% (16/42) of the patients with *TCRG* gene rearrangements.

Using six primer pair combinations for heteroduplex PCR analysis of *TCRD* (Vδ1-Jδ1, Vδ2-Jδ1, Vδ3-Jδ1, Vδ2-Dδ3, Dδ2-Dδ3, Dδ2-Jδ1) we have not found any clonal PCR products of rearrangements to the Jδ1 segment. Moreover, samples shown by SB to contain clonal Vδ2-Dδ3 gene rearrangements revealed monoclonal homoduplexes in all but four cases (Figure 4). In those four patients heteroduplex PCR analysis showed an oligoclonal pattern of the Vδ2-Dδ3 joining. All samples found by SB to contain clonal Dδ2-Dδ3 recombinations were also monoclonal in heteroduplex PCR analysis (Figure 4). This also concerned weak SB bands, probably derived from subclones. In cases, when 'atypical' rearrangements to Dδ3/Jδ1 locus were detected with SB (the sizes of clonal bands could not be assigned to a particular Vδ-Jδ joining) we did not find any clonal PCR products with the applied primer sets. Based on combined SB and PCR data there was an evidence for oligoclonality in *TCRD* gene locus in up to 21% of precursor-B-ALL patients (13/62), i.e. 30% (13/43) of the patients with *TCRD* gene rearrangements. The overall concordance between SB and heteroduplex PCR analysis for detection of clonal *TCRG* and *TCRD* gene rearrangements ranged from 78 to 87%.

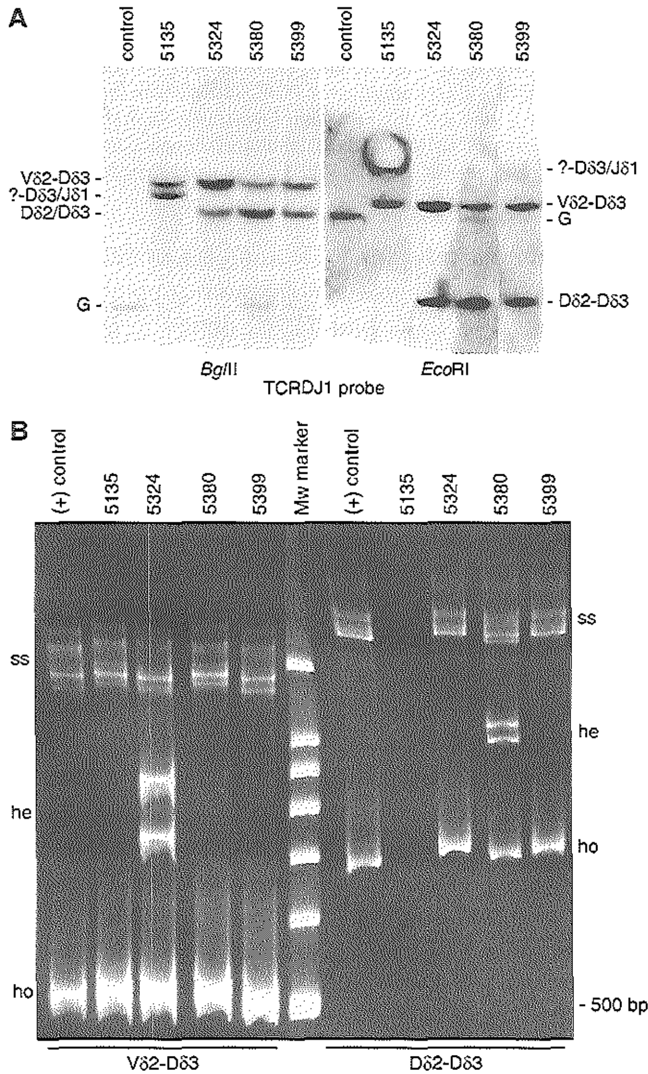

Figure 4.
Clonality assessment via SB analysis and heteroduplex PCR analysis of *TCRD* genes.
(**A**) DNA from precursor-B-ALL patients was digested with *Bgl*II and *Eco*RI. The filter was hybridized with the ^{32}P-labeled TCRDJ1 probe. Based on SB band intensity and sizes 'triallelic' *TCRD* rearrangement was assumed in patients 5324 (two times Vδ2-Dδ3, and single Dδ2-Dδ3) and 5380 (two times Dδ2-Dδ3, and single Vδ2-Dδ3), and biallelic in patients 5135 (Vδ2-Dδ3 and ?-Dδ3/Jδ1) and 5399 (Vδ2-Dδ3 and Dδ2-Dδ3), respectively. (**B**) Heteroduplex PCR analysis with Vδ2 and Dδ2 primers in combination with a Dδ3 primer showed fully concordant results with SB analysis. ss, single-strand fragments; he, heteroduplexes; ho, homoduplexes; Mw marker, 100-bp molecular weight marker.

Combinations of cross-lineage TCR gene rearrangements based on SB analysis

The different combinations of cross-lineage TCR gene rearrangements in our total group of precursor-B-ALL are summarized in Table 5. Twenty-seven percent of all precursor-B-ALL patients had simultaneously rearranged and/or deleted *TCRB*, *TCRG*, and *TCRD* genes. These combined rearrangements were not found in eight pro-B-ALL patients, whereas in common ALL and pre-B-ALL 30% and 25% of cases had all three TCR genes rearranged or deleted.

Twenty-six percent of all precursor-B-ALL cases showed *TCRD* gene rearrangements and/or deletions, with germline *TCRB* and *TCRG* genes. In contrast, *TCRB* and/or *TCRG* gene rearrangements without *TCRD* gene rearrangements and/or deletions were observed in only 4% of cases. Similarly, 32% of all precursor-B-ALL cases had a rearranged *TCRG* gene with a germline *TCRB* gene, while only 8% showed *TCRB* gene rearrangements without *TCRG* gene rearrangements. These data indicate that *TCRD* gene rearrangements and/or deletions occur most frequently and that virtually all *TCRB* and *TCRG* rearrangements coincide with *TCRD* gene rearrangements and/or deletions.

Table 5. Combination of TCR gene rearrangements as detected by SB analysis in childhood precursor-B-ALL from our data and data reviewed from the literature.

	Cross-lineage TCR gene rearrangements and/or deletions							
References	G	only β	only γ	only δ	only β+ γ	only β+ δ	only γ+ δ	β+ γ+ δ
Hara[7] (n=29)	10% (3)	0% (0)	0% (0)	34% (10)	0% (0)	3% (1)	24% (7)	28% (8)
Dyer[5] (n=12)	0% (0)	0% (0)	0% (0)	25% (3)	0% (0)	8% (1)	17% (2)	50% (6)
Felix[10] (n=52)	15% (8)	4% (2)	0% (0)	25% (13)	0% (0)	6% (3)	29% (15)	21% (11)
Fey[11] (n=7)	0% (0)	0% (0)	0% (0)	43% (3)	0% (0)	14% (1)	29% (2)	14% (1)
This study (n=192)	7% (14)	2% (3)	2% (4)	26% (50)	0%((0)	6% (12)	30% (57)	27% (52)
Total group of precursor-B-ALL (n=292)	9%(25)	2% (5)	1% (4)	27% (79)	0% (0)	6% (18)	28% (83)	27% (78)

G, germline configuration; β, *TCRB* gene rearrangement; γ, *TCRG* gene rearrangement; δ, *TCRD* gene rearrangement and/or deletion

DISCUSSION

We investigated a large group of 202 precursor-B-ALL patients for the configuration of their cross-lineage TCR gene rearrangements. Rearrangements and/or deletions of the *TCRB*, *TCRG* and *TCRD* genes were found in 35%, 59% and 89%, respectively. Most *TCRB* gene rearrangements (53%) were monoallelic complete V(D)Jβ2 rearrangements, *TCRG* genes most frequently (~70%) contained rearrangements to the Jγ1 gene segments, and 80% of *TCRD* gene rearrangements represented incomplete Vδ2-Dδ3 or Dδ2-Dδ3 rearrangements, which is in agreement with previously published data.[5-12] Our earlier literature review on cross-lineage TCR gene configuration in childhood precursor-B-ALL,[6] suggested that *TCRB* gene rearrangements occur in 33% of precursor-B-ALL, *TCRG* gene rearrangements in 44%, and *TCRD* gene rearrangements and deletions in 57% and 26%, respectively. These percentages of TCR gene rearrangements and/or deletions are all lower than in the current study of childhood precursor-B-ALL (Table 1). This difference is probably due to the extended panels of TCR DNA probes and restriction enzymes used for this study.[2,13,27] Ig and TCR gene rearrangements can be missed when only one DNA probe is used in combination with a single restriction enzyme, especially when this results in large germline restriction fragments. Furthermore, especially the use of upstream and downstream Dβ probes has given more detailed insight in the configuration of the *TCRB* locus. We found a significantly higher frequency of incomplete *TCRB* gene rearrangements (42% of all *TCRB* gene rearrangements) than reported previously (Table 2).[5,7,10,11] This is most probably owing to the usage of the four different Dβ probes, which allow identification of incomplete V-Dβ, D-Dβ, and D-Jβ gene rearrangements.

Four other research groups analyzed and compared all three cross-lineage TCR genes in a total number of 100 cases.[5,7,10,11] Combining these published data and the data presented here show that *TCRD* gene rearrangements and/or deletions occurred in the majority of cases (~90%) and that 27% of childhood precursor-B-ALL had *TCRD* gene rearrangements and/or deletions with germline *TCRG* and *TCRB* genes (Table 5). In contrast, *TCRG* and/or *TCRB* gene rearrangements without *TCRD* gene rearrangements and/or deletions were found in only 3% (9/292) of cases (Table 5). Similarly, 29% (87/292) of precursor-B-ALL cases had rearranged *TCRG* genes with germline *TCRB* genes, while only 8% (23/292) showed *TCRB* gene rearrangements without *TCRG* gene rearrangements (Table 5). Apparently in precursor-B-ALL, similarly to the hierarchy during early T-cell development, rearrangements in the *TCRD* locus occur first, followed by *TCRG* gene rearrangements, and subsequently by *TCRB* gene rearrangements.

The frequencies of cross-lineage TCR gene rearrangements seem to be related to the maturation stages of B-cells. The frequency of TCR gene rearrangements is lower in immature precursor-B-ALL (pro-B-ALL) as compared to $CD10^+$ precursor-

B-ALL (common ALL and pre-B ALL) (Table 1) and if present, rearrangements exclusively concern a single locus in pro-B-ALL. When comparing our common ALL and pre-B-ALL subgroups, it is striking that in the 'mature' ($CyIg\mu^+$) pre-B-ALL group a higher frequency of patients have all TCR genes in germline configuration (15% in pre-B-ALL *vs* 2% in common ALL; Table 1). Moreover, cross-lineage TCR gene rearrangements are rare (<5%) in mature B-cell malignancies, such as chronic lymphocytic leukemias (CLL) or non-Hodgkin's lymphomas (NHL)[6,31,32]

The occurrence of cross-lineage TCR gene rearrangements in precursor-B-ALL can be explained in several ways. Based on the fact that cross-lineage TCR gene rearrangements seem to be rare in normal precursor-B-cells, TCR gene rearrangements may only occur in early precursor cells and the occurrence of TCR gene rearrangements in precursor-B-cells may stop their further differentiation and maturation.[6] As a consequence, similarly to cortical thymocytes in the thymus, a large fraction of proliferating, immature BM precursor-B-cells without functional *IGH* rearrangement and/or with cross-lineage gene rearrangements undergo apoptosis unless they become neoplastic.[3,33,34] However, this would imply that oncogenic transformation preferentially affects precursor-B-cells with cross-lineage TCR gene rearrangements. An alternative explanation could be that cross-lineage TCR gene rearrangements in precursor-B-ALL result from the continuing activity of the V(D)J recombinase system after the malignant transformation and maturation arrest.[3] We favor the latter hypothesis, since this would explain the low frequency of cross-lineage TCR gene rearrangements in normal precursor-B-cells and in mature B-cell malignancies. From our data it is also apparent that common ALL is a seemingly distinct subentity with a highly active recombination machinery and accessible Ig and TCR genes. In very immature B-cells (pro-B-ALL) and more mature B-cells (pre-B-ALL, B-CLL, and B-NHL) the V(D)J recombinase is less active and the mature B-cell malignancies have already passed the lineage commitment point so that TCR genes are not accessible any more and rearrangements are no longer possible.

Owing to their enormous diversity, junctional regions of rearranged Ig and TCR genes represent ideal PCR targets for the detection of MRD.[14,17,35] In precursor-B-ALL, this especially concerns junctional regions of *IGH*, *IGK*, *TCRG* and *TCRD* genes.[15] For this purpose the precise configuration of the gene rearrangements (i.e. the V(D), and J gene segments) has to be identified at diagnosis. We determined the frequency of precursor-B-ALL, which have an identifiable type of *TCRG* (59%) and/or *TCRD* (49%) gene rearrangement and found that in at least 79% (159/202) of precursor-B-ALL one or more rearranged *TCRG* and/or *TCRD* genes can be used as targets for PCR-mediated MRD detection. In 55% of patients even two *TCRG* and/or *TCRD* targets were present.

Comparative SB and heteroduplex PCR analysis of *TCRG* and *TCRD* in 62 patients showed concordance of approximately 80% between the two techniques. One explanation for the discrepancies is the efficient PCR detection of rearrange-

ments derived from small subclones, which are difficult do detect by SB. Secondly, 15 rearrangements to the Dδ3/Jδ1 region as detected by SB could not be assigned to a particular Vδ-Jδ joining based on the sizes of clonal bands. In those cases no clonal PCR products were found with the *TCRD* applied primer sets. These rearrangements probably reflect Vα-Jδ1 rearrangements or translocations into the *TCRD* locus. Furthermore, four precursor-B-ALL patients with seemingly 'clonal Vδ2-Dδ3 bands' on SB analysis revealed an oligoclonal pattern by heteroduplex PCR analysis.

Based on combined SB/PCR data and on reports from the literature the frequency of cross-lineage TCR oligoclonality in precursor-B-ALL can be estimated to be ~20%. This seems to be significantly lower as compared to *IGH*, where multiple rearranged *IGH* gene bands, generally differing in density, were found in 30-40% of patients.[36-41] However, when focusing on the group of patients with *TCRG* and *TCRD* rearrangements, the frequency of oligoclonality is comparable to *IGH* gene. Taking into account *IGH* oligoclonality and the relative instability of *IGH* rearrangements,[36-38,42,43] TCR gene rearrangements seem to be at least equally suitable MRD-PCR targets. The stability of the leukemia-specific TCR gene junctional regions is reasonably high as found in studies of ALL patients at diagnosis and relapse, which showed that in 80-90% of leukemias at least one rearranged TCR allele remained stable.[37,44,45]

Heteroduplex PCR analysis appeared to be a valuable method for the identification of *TCRG* and *TCRD* MRD-PCR targets. This method is more rapid and less expensive than SB analysis and requires only small amounts of DNA. Heteroduplex PCR data indicate that MRD monitoring is possible using *TCRG* and *TCRD* targets in approximately 80% of childhood precursor-B-ALL patients. Nevertheless, reliable quantitative molecular techniques such as TaqMan technology are needed to distinguish between rearrangements from major and minor subclones.[46]

Our study indicates that cross-lineage TCR gene rearrangements and/or deletions in childhood precursor-B-ALL occur at high frequency, in a hierarchical order, and are incomplete in a large number of cases (*TCRB* and *TCRD* genes). In at least 79% of precursor-B-ALL one or more of the *TCRG* and *TCRD* gene rearrangements can be identified and can be used as targets for PCR-mediated MRD detection. Based on the combined data it is also possible to reduce the broad panel of six *TCRD* and 12 *TCRG* primer combinations to two *TCRD* combinations (Vδ2-Dδ3 and Dδ2-Dδ3) and six *TCRG* combinations (VγI, VγII, VγIV family specific primers with Jγ1.1/2.1 and Jγ1.3/2.3 primers) resulting in the detection of 80% and 97% of all *TCRD* and *TCRG* gene rearrangements, respectively.

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CHAPTER 2.5

THE MAJORITY OF Vδ2-Jα GENE REARRANGEMENTS IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA CONCERN Vδ2-Jα29 JOININGS COMPRISING SENSITIVE RQ-PCR TARGETS FOR MINIMAL RESIDUAL DISEASE MONITORING*

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ABSTRACT

This study aimed at identification and detailed immunobiological characterization of Vδ2-Jα gene rearrangements in precursor-B-ALL. We developed a multiplex heteroduplex PCR assay wit 61 Jα primers and analyzed 339 childhood precursor-B-ALL, of which 153 cases were also studied by Southern blotting.

A total of 158 clonal Vδ2-Jα gene rearrangements were identified in 141 of the 339 patients (41%). The combined PCR/Southern blot studies in the subgroup of 153 patients showed that among 70 cases containing Vδ2-Jα rearrangements, 39 (56%) showed monoclonal and 31 (44%) showed oligoclonal rearrangement patterns. Based on sequence analysis, preferential usage of the Jα29 gene segment was found in 85 of the 158 Vδ2-Jα sequences (54%). The remaining 73 sequences used 26 other Jα segments, which included two additional clusters, one involving the most upstream Jα segments, i.e. Jα48 to Jα61 (23%), and the second cluster located around the Jα9 gene segment (8%). The stability of Vδ2-Jα joinings analyzed at relapse in 42 patients was excellent for monoclonal rearrangements (88% stable) as compared to oligoclonal Vδ2-Jα rearrangements (only 40% stable). Finally, real-time quantitative (RQ)-PCR experiments revealed that Vδ2-Jα rearrangements can be used as patient-specific targets for detection of minimal residual disease (MRD) with reproducible sensitivities of at least 10^{-4} in most cases. Using RQ-PCR, we demon-

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strated that Vδ2-Jα rearrangements were detectable in normal bone marrow and peripheral blood samples, albeit at very low levels (generally below 10^{-3}). In conclusion, *monoclonal* Vδ2-Jα gene rearrangements might serve as principal MRD-PCR targets in ~25% of precursor-B-ALL.

INTRODUCTION

Current diagnostics in hematopoietic malignancies frequently employs polymerase chain reaction (PCR)-based techniques for detection of low levels of malignant cells, known as minimal residual disease (MRD).[1] In childhood acute lymphoblastic leukemia (ALL), MRD information has high prognostic value, as was shown by several large multi-center prospective studies.[2-6] MRD studies can precisely assess the early response to induction treatment and thereby contribute to an improved definition of remission in ALL. Consequently, MRD detection is currently being incorporated into stratification of treatment protocols.[7,8]

In childhood ALL, detection of MRD most frequently relies on patient-specific junctional regions of immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements, mainly concerning Ig heavy chain (*IGH*) gene, Ig kappa deleting element (*IGK*-Kde), TCR gamma (*TCRG*) gene and TCR delta (*TCRD*) gene.[8-11] These Ig/TCR gene rearrangements can be identified at initial diagnosis in approximately 95% of ALL patients, comprising at least two sufficiently sensitive targets ($\leq 10^{-4}$) for real-time quantitative (RQ)-PCR based MRD detection in approximately 80% of patients.[8] Thus, in 20-25% of ALL cases, the Ig/TCR MRD-PCR targets need still to be optimized. For instance, *TCRG* gene rearrangements are potentially less sensitive markers owing to the limited number of different Vγ and Jγ gene segments and the frequent occurrence of *TCRG* gene rearrangements in polyclonal T-cells in bone marrow (BM) follow-up samples.[12] In fact, in not more than half of the *TCRG* gene rearrangements in precursor-B-ALL patients, a maximal sensitivity of 10^{-4} could be reached in RQ-PCR experiments.[13] Moreover, in approximately 15% of precursor-B-ALL patients, oligoclonal *IGH* gene rearrangements are the only available MRD targets.[9] In such patients all oligoclonal targets should be monitored to prevent false negative results, which is costly and time consuming.[14] In addition, substantial expansions of normal precursor-B-cells with polyclonal *IGH* gene rearrangements in regenerating BM after cessation of maintenance therapy might affect the sensitivity of MRD detection using Ig gene rearrangements as PCR targets.[15] Therefore, particularly precursor-B-ALL patients might profit from the introduction of new MRD-PCR targets.

Vδ2-Jα gene rearrangements might be attractive new MRD-PCR targets, but have not yet been evaluated for this purpose. Several earlier studies indicated that *TCRD* gene deletions with rearrangements in the Jα locus occur frequently in pre-

cursor-B-ALL (Figure 1).[16-21] Our detailed Southern blot study indicated that at least 40% of *TCRD* alleles in precursor-B-ALL are deleted, which might be largely due to Vδ2-Jα gene rearrangements.[21] Therefore, we developed a multiplex PCR strategy for easy identification and characterization of clonal Vδ2-Jα gene rearrangements in a large series (n = 339) of precursor-B-ALL patients. In a subset of patients, we assessed the stability of these rearrangements at relapse of ALL. We also investigated the presence of the most frequent Vδ2-Jα rearrangements in normal lymphoid

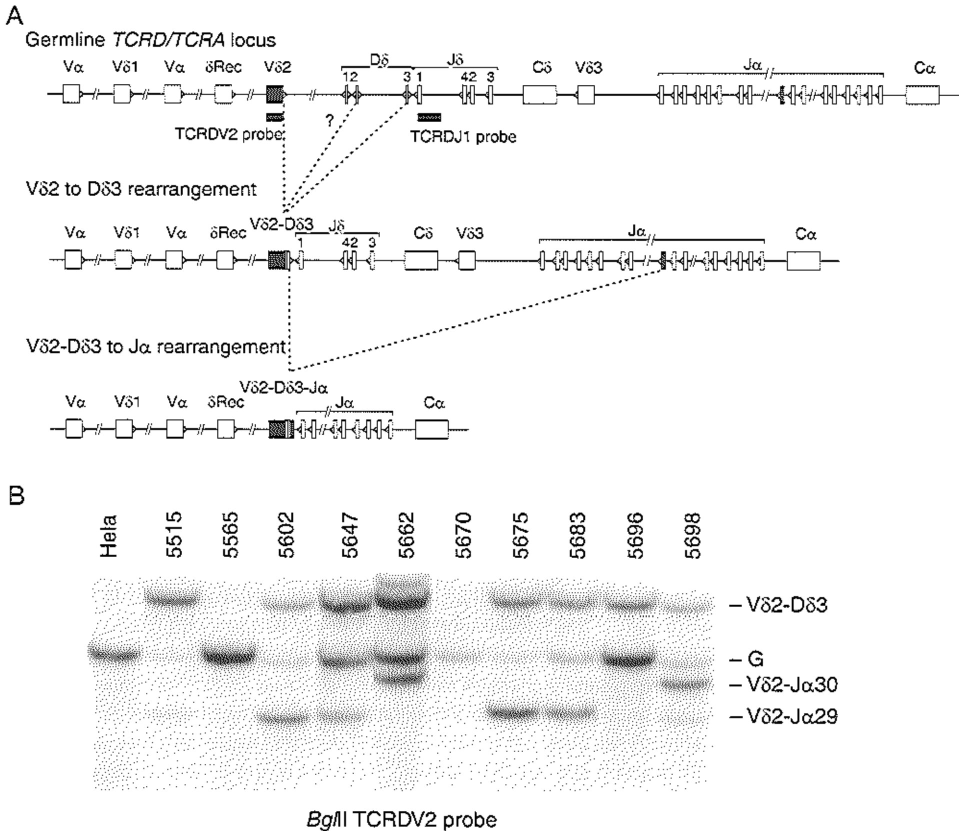

Figure 1.
(A) Consecutive rearrangements in the *TCRD/A* locus involving the Vδ2 gene segment, which are characteristic for precursor-B-ALL. The main pathway concerns consecutive Vδ2-Dδ3 → Vδ2-Jα recombinations. Dδ2-Dδ3 and Dδ2-Jα gene rearrangements can also occur albeit at much lower frequencies. Solid boxes below the gene segments represent the probes used for Southern blot hybridization.
(B) Southern blot analysis with TCRDV2 probe in 10 precursor-B-ALL patients. Vδ2-Dδ3 and/or Vδ2-Jα29 gene rearrangements in patients 5602, 5675, 5683, and 5696 are monoclonal. The presence of several rearranged bands of different densities in patients 5515, 5647, 5662, and 5698 is consistent with oligoclonality. Both Vδ2 alleles in patient 5670 are deleted, while patient 5565 has both Vδ2 alleles in germline configuration.

tissues. Finally, we evaluated the sensitivity of Vδ2-Jα rearrangement as RQ-PCR targets for MRD monitoring.

PATIENTS, MATERIALS AND METHODS

Patients

BM or peripheral blood (PB) samples from 339 children with precursor-B-ALL were obtained at initial diagnosis. The age distribution ranged from 1.5 months until 15.9 years. The diagnosis of precursor-B-ALL was made according to FAB and standard immunophenotypic criteria.[22,23] Immunological marker analysis of the precursor-B-ALL revealed that 12 were pro-B-ALL, 226 were common ALL, and 101 were pre-B-ALL.

Southern blot analysis

Mononuclear cells (MNC) were isolated from BM or PB samples by Ficoll-Paque centrifugation (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden). DNA was isolated from fresh or frozen MNC fractions as described previously.[24,25] Fifteen micrograms of DNA were digested with the appropriate restriction enzymes (Pharmacia), size-separated in 0.7% agarose gels and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as described.[24] The configuration of the *TCRD* genes was analyzed with the TCRDJ1 and TCRDV2 probes (DAKO Corporation, Carpinteria, CA, USA) in *Bgl*II, *Eco*RI, or *Hin*dIII digests.[26]

Primer design and heteroduplex PCR analysis

Vδ2 and Dδ2 primers have been developed by the BIOMED-2 Concerted Action BMH4-CT98-3936 "PCR-based clonality studies for early diagnosis of lymphoproliferative disorders" (Van Dongen et al. submitted for publication). Based on the available nucleotide sequence of the human 3' terminal end of the *TCRA/D* locus (EMBL accession no. M94081[27]), 61 Jα primers were designed compatible to the Vδ2 primer, using OLIGO 6.0 software (Dr. W. Rychlik; Molecular Biology Insights, Inc., Cascade, CO, USA) and applying previously described guidelines (Table 1).[11] Oligonucleotide Jα primers of 17 to 23 bp were positioned 111 to 169 bp downstream of the involved recombination signal sequence (RSS). Secondary structures such as primer dimers and hairpins were avoided, and the melting temperature (Tm) was 68°C ± 3°C. The 61 Jα primers were subsequently checked for absence of "cross"-dimer formation, using the OLIGO 6.0 software. This allowed as to design seven Vδ2-Jα multiplex PCR tubes (Table 2).

The multiplex Vδ2-Jα PCR analyses were performed in all 339 patients, essentially as described previously.[28,29] In each 50 μl PCR reaction 100 ng DNA sample, 10 pmol of the 5′ and 3′ oligonucleotide primers, and 1 U AmpliTaq Gold polymerase (PE Biosystems, Foster City, CA) were used. PCR conditions were: initial denaturation for 10 min at 94°C, followed by 35 cycles of 45 sec at 92°C, 90 sec at 60°C, and 2 min at 72°C using a Perkin-Elmer 480 thermal cycler (PE Biosystems). After the last cycle an additional extension step of 10 min at 72°C was performed. Appropriate positive and negative controls were included in all experiments.[11]

Additionally, the presence of clonal Vδ2-Dδ3 and Dδ2-Dδ3 gene rearrangements was tested using our classical monoplex approach.[11] Multiplex Dδ2-Jα PCR was performed in 11 patients, pre-selected based on Southern blot and PCR information (i.e., germline Vδ2 allele with deleted Dδ3/Jδ1 area and the absence of clonal Vδ2-Jα rearrangements).

For heteroduplex analysis, the PCR products were denatured at 94°C for 5 min after the final cycle of amplification and subsequently cooled to 4°C for 60 min to induce duplex formation.[30] Afterwards the duplexes were immediately loaded on 6% non-denaturing polyacrylamide gels in 0.5 x Tris-borate-EDTA

(TBE) buffer, run at room temperature, and visualized by ethidium bromide staining (Figure 1).[30] A 100-bp DNA ladder (Promega Corporation, Madison, WI, USA) was used as size marker.

Comparative heteroduplex analysis of PCR products

Comparative heteroduplex analysis of Vδ2-Jα PCR products at diagnosis and relapse concerned 42 relapsed precursor-B-ALL, which contained Vδ2-Jα rearrangements at diagnosis. The relapse samples of these patients were at first analyzed in monoplex PCR 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, i.e. mixing of the diagnosis and relapse PCR products followed by heteroduplex analysis (Figure 2).[14,31] When clonal PCR products found at diagnosis were undetectable at relapse, the relapse sample was analyzed with all 7 Vδ2-Jα multiplex tubes.

Sequence analysis of Vδ2-Jα gene rearrangements

Direct sequencing of Vδ2-Jα rearrangements was performed with the Vδ2 primer using the dye-terminator cycle sequencing kit with AmpliTaq DNA polymerase FS on an ABI 377 sequencer (PE Biosystems) as previously described.[10] When heteroduplex PCR analysis revealed more than two clonal bands i.e. two homoduplexes, or an additional upper band resulting from extension to downstream Jα segments, homoduplexes and/or heteroduplexes were excised from the polyacrylamide gel, eluted and directly sequenced as described before.[32] Also clonal PCR products discordant between diagnosis and relapse of precursor-B-ALL as found by mixed heteroduplex analysis, were subsequently sequenced and the sequences were evaluated for the presence of common Vδ2-Dδ3 stems. Jα gene segments were identified by comparison to germline sequences as previously described.[33] For alignments of Dδ2 and Dδ3 segments in Vδ2-Jα junctional regions, it was required to have at least 4 and 5 consecutive matching nucleotides, respectively.[33]

RQ-PCR-based detection of clonal Vδ2-Jα gene rearrangements

RQ-PCR-based detection of clonal Vδ2-Jα gene rearrangements relied on allele specific oligonucleotide (ASO) primer approach as described previously.[13,34] The germline TaqMan probe (5'-AGACCCTTCATCTCTCTCTGATGGTGCAAGTA-3') and forward primer (5'-TGCAAAGAACCTGGCTGTACTTAA-3') were designed in the Vδ2 gene segment. The ASO primers were positioned at the junctional regions, preferably covering the Dδ3-Jα, and sometimes also the Vd2-Dd3 junction (Table 3). A standard annealing temperature of 60°C was used. To determine the efficiency of amplification and sensitivity of the PCR target, diagnostic DNA was diluted in 10-fold steps into control MNC DNA, from 10^{-1} down to 10^{-6}. The serial dilutions of diagnostic DNA were subjected to RQ-PCR analysis together with negative controls (H_2O and control MNC DNA). Serial dilutions of diagnostic samples were analyzed in triplicate. To correct for the quantity and quality (amplifiability) of DNA, RQ-PCR analysis of the albumin gene was used.[35]

Non-specific amplification was defined as any amplification observed in control MNC DNA. The reproducible sensitivity of a primers/probe combination was defined as the maximal 10-fold dilution step with a maximal difference in cycle threshold (C_T) value of 1.5 between the duplicate of the involved dilution samples and with a maximal C_T value of 40 cycles.[13,36] The standard curve within this reproducible range should have a correlation coefficient of at least 0.95 for precise quantification. Furthermore, the C_T values of the reproducible sensitivity had to be at least three cycles lower than the C_T values of control MNC DNA (tested in two- to six-fold). In case non-specific amplification was observed, the maximal sensitivity was defined as the maximal 10-fold dilution of the diagnostic sample giving a reproducible C_T value and with the highest C_T at least 1 cycle lower than the lowest C_T of non-specific amplification. If non-specific amplification was not observed, the maximal sensitivity was defined as the maximal 10-fold dilution of the diagnostic sample giving specific but non-reproducible amplification.[13,36]

Table 1. **Primers developed for PCR and sequence analysis of Vδ2-Jα and Dδ2-Jα gene rearrangements.**

Primer code	Size of primer (bp)	Position in bp[a]	Sequence (5' → 3')
Jα1	18	153	GGGACCCAGAAATCAGAA
Jα2	18	166	CTACAGCAAGCCTCACCA
Jα3	20	160	GAGCCCACAGAGGAAAATAC
Jα4	21	151	CCCCAAGTTTGCTATAGATCA
Jα5	23	129	TTCATCATCTAAGAAAGCAGAGT
Jα6	23	160	GATGGAATAGATCACAACACAAA
Jα7	18	131	AGAGGCTCTCCAGCACAG
Jα8	23	113	ATATATGCCCAATATTGAGGATA
Jα9	23	169	TTTAACTGGCAGACAAAACTATG
Jα10	22	123	TTCTTCCACTTATTGTCACCAG
Jα11	21	139	ATGAGGATAACACGCAATACA
Jα12	22	151	TCCAGCTCATTTTGCATTATAC
Jα13	22	120	TTACGGTCTGAGAGAAGACAAC
Jα14	22	120	GCAGTAAGTTTAGTGGGTCTCA
Jα15	20	133	ATTTGGTCACCTGTGCAATA
Jα16	20	150	AACATTTGGCAGTCCACTTA
Jα17	21	144	GACATTAATTTGGGCCAATAC
Jα18	21	124	CTCCCCTTTTAATTTCTCCAC
Jα19	21	162	CTCCCATCAGAAAGCAATTAC
Jα20	23	169	TGGGAAAGCTCTTAGAATTTAGT
Jα21	20	153	GACCCAAAATGCAAAATAAA
Jα22	21	146	CTCAGGCCCATTAAGTTACAT
Jα23	21	163	TCCCCCTCTCTAAACATTCTT
Jα24	20	167	GGCTTCCTTTCAGATGTGTT
Jα25	23	115	AGTTTTCCTCTTGGAGATAATCA
Jα26	18	141	GACTCCTGGCCTCAAGAC
Jα27	21	162	ATTAATAAAGAGCCCAACCAG
Jα28	19	157	GCAAAGAAAACACCACCTG
Jα29	21	146	GGCAAAAGCATTCTAGGTACA
Jα30	18	153	GCCACCCACATGTCTTAG
Jα31	23	125	ATTAAATCTCCACTAACTTCACG
Jα32	19	129	TGCTGTCGCTTCCTACTTG
Jα33	21	157	GCAGGCTGACTTGTTCTTAAA
Jα34	20	146	CAACAAGGAGCAAAACTTCA
Jα35	19	159	ACTGGAAAATGGGTGTGTG
Jα36	20	125	GTGTCTGGGATGTGAGAACT
Jα37	23	148	TTTGGTTAGAAGTTGAGACAGAG
Jα38	22	107	AGGAGGCAGTTTCTGAGATATT
Jα39	18	127	GCTCAGTGCTACGGCTTC
Jα40	20	120	CCTCAAACATGAACACCAAC
Jα41	18	120	AACAGGTCCCATTGGATT
Jα42	20	124	TTGCCCAGAGTGACAAAGTA
Jα43	18	124	GAAACTGCCCAGAACAGC

Continued Table 1

Primer code	Size of primer (bp)	Position in bp[a]	Sequence (5' → 3')
Jα44	21	127	TCTGCAGTATCCCCTGTTTTA
Jα45	21	148	ATGAGCCAAGGTTTAGAAATG
Jα46	19	144	CCAAAGGAGGACAACTCAA
Jα47	18	128	TCCCAGCCAGAAAAAGTT
Jα48	19	114	TCCCCAGAATCTTATGCAG
Jα49	19	111	GCAGTTTAAAGGGTTTGCT
Jα50	17	154	CAACCACGATGCCATCT
Jα51	19	143	GGATTTATGCTGCCACTGA
Jα52	17	118	GGGGAAGGGAGCAAAAG
Jα53	18	130	ACCTGACACTGGGGTGAC
Jα54	21	130	GAGGGGCAAGTAATTAAATCA
Jα55	21	152	AGTATACGTCCCTCAAGGAGA
Jα56	21	142	AGGAGATTCGGTTATCTTTCA
Jα57	21	143	GGACCTGGGTTATAAAACAGA
Jα58	19	163	GACTTGAATGTGGCAGAGA
Jα59	20	149	ATCAAATCCTCAGGGAGAAG
Jα60	19	120	CTGATTGCCAGGTGTTAGG
Jα61	23	135	GTTTGTTAAGGCACATTAGAATC

a. The position of the 5' end of the primer is indicated downstream relative to the RSS of the involved Jα gene segments.

RQ-PCR-based detection of polyclonal Vδ2-Jα gene rearrangements in normal cell samples

Normal tissue samples tested for the presence of Vδ2-Jα gene rearrangements included normal PB, E-rosette$^+$ PB-cells (T-cells), E-rosette$^-$ PB-cells (B-cells, NK-cells and monocytes), normal BM, sorted BM B-cells and B-cell precursors, tonsils, lymph nodes, thymuses, and post-chemotherapy regenerating BM samples, which are known to contain high frequencies of normal precursor-B-cells.[15,37] Whenever possible, at least two different samples were tested per category, each sample in triplicate. To analyze the presence of Vδ2-Jα gene rearrangements in normal tissue samples, the above-described germline

Table 2. Composition of the multiplex tubes for detection of Vδ2-Jα gene rearrangements.

Tube code	Tube composition
Vδ2-Jα Tube 1	Vδ2 + Jα1, Jα6, Jα7, Jα22, Jα26, Jα40, Jα54, Jα57
Vδ2-Jα Tube 2	Vδ2 + Jα2, Jα3, Jα5, Jα8, Jα11, Jα12, Jα13, Jα19
Vδ2-Jα Tube 3	Vδ2 + Jα4, Jα10, Jα14, Jα20, Jα23, Jα29, Jα42, Jα48
Vδ2-Jα Tube 4	Vδ2 + Jα9, Jα16, Jα35, Jα37, Jα38, Jα41, Jα49, Jα51, Jα56, Jα60
Vδ2-Jα Tube 5	Vδ2 + Jα15, Jα18, Jα28, Jα34, Jα36, Jα43, Jα44, Jα45, Jα46
Vδ2-Jα Tube 6	Vδ2 + Jα17, Jα21, Jα24, Jα25, Jα27, Jα31, Jα32, Jα33, Jα39
Vδ2-Jα Tube 7	Vδ2 + Jα30, Jα47, Jα50, Jα52, Jα53, Jα55, Jα58, Jα59, Jα61

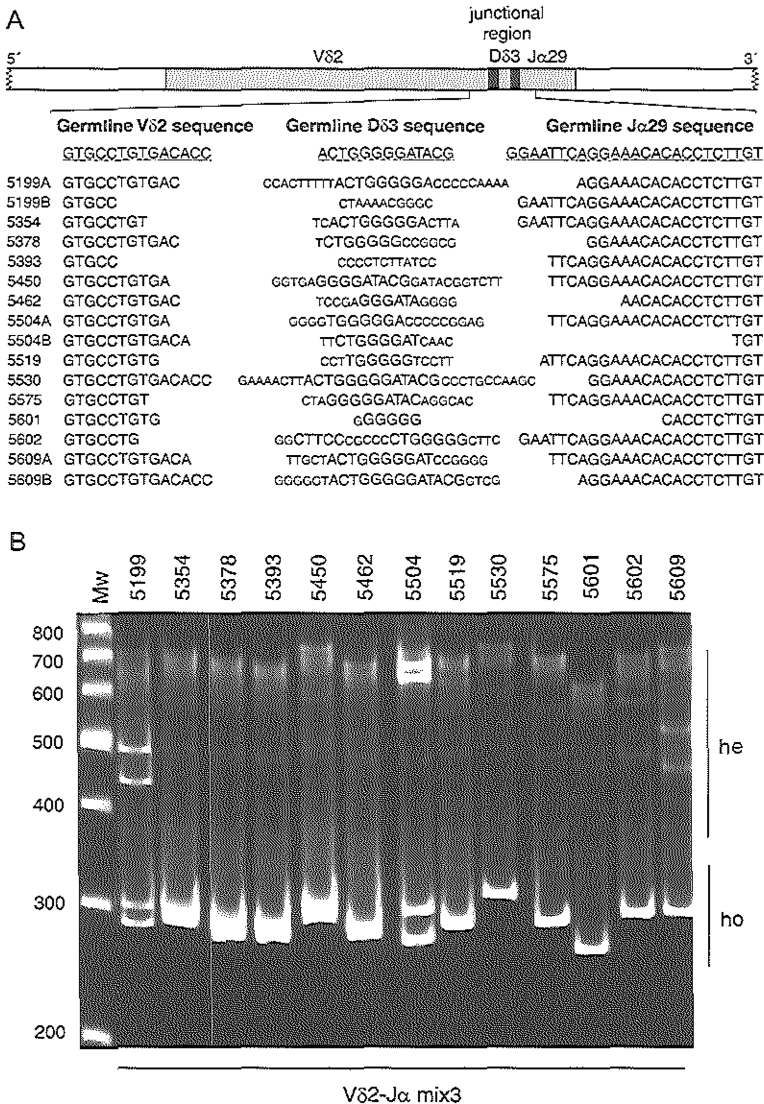

Figure 2.

(A) Schematic diagram of the Vδ2 gene segment joined to the Jα29 gene segment via a junctional region. The presented Vδ2-Jα29 junctional region sequences are derived from precursor-B-ALL patients and illustrate the deletion of nucleotides from the germline sequences as well as the size and composition of the junctional regions. Dδ gene segments and inserted nucleotides are indicated by capital letters and small capital letters, respectively.

(B) Multiplex heteroduplex PCR analysis with Vδ2 in combination with a Jα primers (mix 3) showed clonal Vδ2-Jα homoduplexes (ho) in all patients tested. Sequence analysis (see part A) showed that all these rearrangements involved the Jα29 gene segment. The presence of heteroduplexes (he) in patients 5199, 5504, and 5609 indicated the presence of double Vδ2-Jα29 rearrangements.

TaqMan probe and forward primer, positioned in the Vδ2 gene, were used together with a reverse Jα primer. Based on the frequencies of particular Vδ2-Jα gene rearrangements in precursor-B-ALL (see below), Jα9, Jα29, Jα58, and Jα61 primers were tested (Table 1). To determine the efficiency of amplification and sensitivity of the PCR target, diagnostic DNA from the precursor-B-ALL containing particular Vδ2-Jα gene rearrangement was diluted in 10-fold steps (10^{-1} down to 10^{-6}) into DNA from the cell line CEM, known to have deleted both *TCRD* alleles. To correct for the quantity and quality (amplifiability) of DNA, RQ-PCR analysis of the albumin gene was used.[35]

RESULTS

Clonal *TCRD* gene rearrangements in precursor-B-ALL

Southern blot analysis with two *TCRD* probes (TCRDJ1 and TCRDV2 probes) was performed in 153 patients. Based on the combined Southern blot and PCR results, clonal Vδ2 gene rearrangements were found in 77% (117/153) of precursor-B-ALL patients. Clonal Vδ2-Dδ3 rearrangements were detected in 39% (60/153) of patients. In an additional 7% (11/153) of cases Southern blot indicated the presence of a clonal Vδ2-Dδ3 recombination, which turned out to be oligo/polyclonal by PCR analysis.[29,38] Vδ2-Jα rearrangements were found in 46% (70/153) of patients and combined Southern blot/PCR data showed that 56% (39/70) of these Vδ2-Jα joinings were monoclonal. The vast majority (79%) of monoclonal Vδ2-Jα rearrangements were monoallelic. A significant proportion (44%; 31/70) of Vδ2-Jα joinings were oligonoclonal. Oligoclonality was assumed either when the Southern blot revealed presence of rearranged bands of different densities (13 patients; Figure 1B) or when the number of clonal Vδ2-Jα and Vδ2-Dδ3 homoduplexes exceeded the number of Vδ2 rearrangements in Southern blot analysis (18 patients).

Clonal Dδ2-Dδ3 rearrangements were detected in 10% (15/153) of patients. Monoclonal Dδ2-Jα gene rearrangements were found in only 3 of the 11 patients with a germline Vδ2 allele but a deleted Dδ3/Jδ1 region. This indicates that Dδ2-Jα rearrangements are rare (~2%) in childhood precursor-B-ALL.

Spectrum of Vδ2-Jα gene rearrangements in precursor-B-ALL

In the total group of 339 patients studied with our multiplex PCR strategy, a total of 158 clonal Vδ2-Jα gene rearrangements were detected in 141 cases (42%). The sequence analysis of clonal Vδ2-Jα PCR products revealed that 26 different Jα segments were used (Figure 3). Surprisingly, the Jα29 gene segment was present in 54% (85/158) of all clonal Vδ2-Jα joinings (Figures 2 and 3). Together with Jα30 and Jα31 genes they formed a first cluster comprising 59% of Vδ2-Jα gene rearrangements. A second cluster frequently involved in Vδ2-Jα recombination concerned the Jα segments most proximal to the *TCRD* locus. Altogether, 10 of the most upstream Jα genes were found in 23% (36/158) of Vδ2-Jα joinings, with Jα48, Jα54, Jα58, and

Table 3. RQ-PCR analysis of Vδ2-Jα gene rearrangements for sensitive detection of MRD in precursor-B-ALL patients.

Patient	Vδ2-Jα	Junctional region[a] rearrangement	ASO sequence 5'→3'[b]	T(°C)[c]	Reproducible sensitivity[d]	Maximal sensitivity	Non-specific amplification[e]	C_T 10^{-1} dilution
5161	Vδ2-Jα29	2/24(Dδ3)/7	TTCCTTGTCCCCAGTACCCC	60	10^{-3}	10^{-4}	5/6	28.3
5172	Vδ2-Jα29	3/15/3	CCTGAATAAGGGTCGGGGT	60	10^{-5}	10^{-5}	-	25.0
5236	Vδ2-Jα29	1/30(Dδ3)/5	AGCCATCTTTAGGAATTCTCGATC	60	10^{-4}	10^{-4}	-	27.5
5462	Vδ2-Jα29	3/15(Dδ3)/11	GTTCCCCTATCCCTCGGAGTC	60	10^{-4}	10^{-5}	1/6	25.9
6338	Vδ2-Jα29	3/16(Dδ3)/8	AAGAGGTGTGTTTCCCCCGTAT	60	10^{-2}	10^{-2}	6/6	30.5
6395	Vδ2-Jα54	3/21(Dδ2, Dδ3)/0	GTTTATAGTTGCTACCTCCACTATTCTCCTAT	60	10^{-4}	10^{-5}	-	23.9
6403	Vδ2-Jα29	9/22(Dδ3)/3	GGTGTGTTTCCTGAATCCTCGT	60	10^{-4}	10^{-5}	1/6	23.5
6436	Vδ2-Jα29	4/14/12	TGTGTCCCTAACGGTCGAAT	60	10^{-4}	10^{-5}	1/6	23.5
6439	Vδ2-Jα48	3/25(Dδ3)/15	AAAGGTTAATTTCTCATCGGGAGTAT	60	10^{-4}	10^{-5}	-	25.2
6464	Vδ2-Jα56	3/13/21	GTCAGCCCCTGGACAGTAAGTC	60	10^{-5}	10^{-5}	-	25.5
6527	Vδ2-Jα29	0/20/18	GTCCCGTAAGAAACCCTCTCG	60	10^{-4}	10^{-5}	-	26.7
6571	Vδ2-Jα29	1/11(Dδ3)/6	TGTTTCCTGGGAGTATCCTCGT	60	10^{-3}	10^{-3}	-	30.7
6615	Vδ2-Jα29	5/13(Dδ3)/12	AAAGACAAGAGGTGTGTCGGT	60	10^{-4}	10^{-5}	-	23.4
6673	Vδ2-Jα29	0/28(Dδ3)/0	TGTTTCCTGAATTCCGAGGAT	60	10^{-4}	10^{-5}	-	23.5
6687	Vδ2-Jα58	3/18(Dδ3)/3	TGGTTTCTTCCCACTATCCCTGA	60	10^{-3}	10^{-4}	1/6	29.2

a. Sequences of the junctional region were aligned with the 3' end of the Vδ2 germline sequence and the 5' end of the Jα germline sequences. The number of 5'-deleted, inserted, and 3'-deleted nucleotides as well as the presence of Dδ2 and Dδ3 segments are indicated.

b. The nucleotides of the ASO primer that overlap with the Dδ3 segment are underlined.

c. Temperature of annealing and extension during the RQ-PCR reaction.

d. The reproducible sensitivity of a primers/probe combination was defined as the dilution step with a maximal difference in C_T value of 1.5 between the duplicate dilution samples and with a maximal C_T value of 40 cycles.

e. Nonspecificity refers to amplification of normal MNC DNA. The number of positive wells per total number of wells is shown.

Jα61 genes used most frequently (Figure 3). The third and most downstream cluster was located around the Jα9 segment and comprised 8% (12/158) of Vδ2-Jα gene rearrangements. In line with these results, the three identified Dδ2-Jα gene rearrangements contained the Jα9, Jα29, and Jα58 gene segments, respectively.

Characteristics of clonal Vδ2-Jα gene rearrangements in precursor-B-ALL

The vast majority of Vδ2-Jα gene rearrangements (78%, 122 of 156 fully sequenced clonal PCR products) contained a part of the Dδ3 gene segment (9 nucleotides on average, range 5-13 nucleotides). In striking contrast, remnants of the Dδ2 gene segment were found in only 7% (11/156) of the Vδ2-Jα sequences. Overall sizes of the Vδ2-Jα junctional regions were extensive with 18.8 nucleotides on average (range 0-47). The sizes of the Vδ2-Dδ3 and Dδ3-Jα junctions were comparable: 5.9 nucleotides on average (range 0-26). The 3' deletions of the Vδ2 gene segment (mean 4.4, range 0-43 nucleotides) were comparable to the 5' deletions in the Jα gene segments (mean 5.9, range 0-33 nucleotides).

Stability of Vδ2-Jα gene rearrangements in monoclonal and oligoclonal precursor-B-ALL patients at relapse

A total of 55 clonal Vδ2-Jα gene rearrangements in 42 precursor-B-ALL patients were subjected to comparative heteroduplex PCR analysis at diagnosis and relapse:

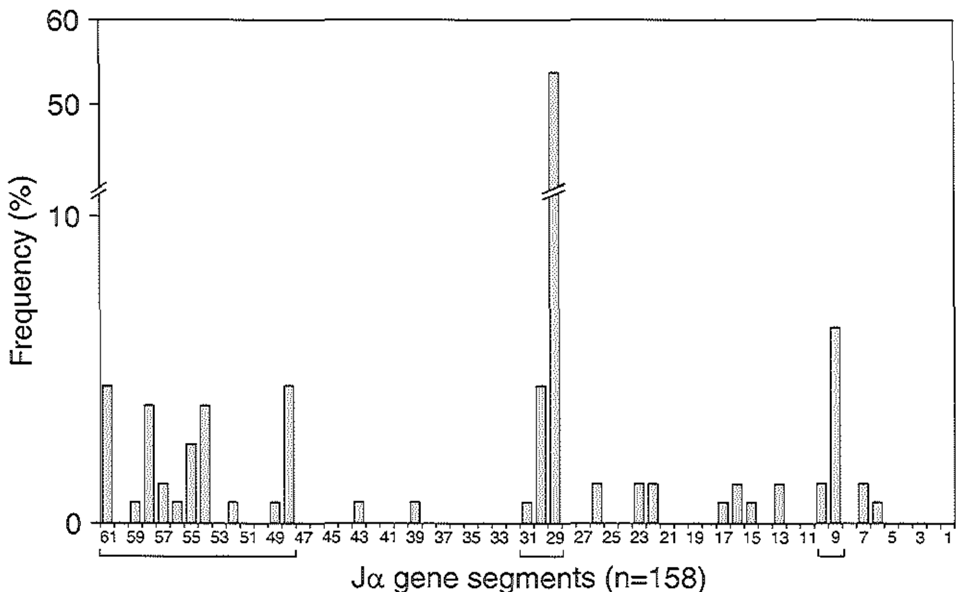

Figure 3.
Bar diagram summarizing the usage of particular Jα segments in Vδ2-Jα gene rearrangements in precursor-B-ALL.

33 of the 55 rearrangements were found to be stable (60%). In 27 patients (64%) at least one Vδ2-Jα rearrangement was preserved at relapse. The stability of the Vδ2-Jα gene rearrangements was markedly different between monoclonal and oligoclonal patients; i.e. at least one rearrangement was preserved in 86% (18/21) and 43% (9/21) of patients, respectively. The significant difference between monoclonal and oligoclonal rearrangements was even more pronounced at the allelic level, with 21 of 24 monoclonal Vδ2-Jα gene rearrangements being stable (88%) as compared to only 12 of 31 oligoclonal rearrangements (39%).

Owing to clonal evolution phenomena, 22 Vδ2-Jα rearrangements were lost in 18 patients. In 13 patients, this concerned either "regression" of (sub)clonal rearrangements to germline configuration or disappearance (deletion) of the Vδ2-Jα joinings, probably owing to secondary Vα-Jα recombinations. In five patients, new Vδ2-Jα gene rearrangements were detected at relapse. In one of these five patients, the Vδ2-Jα23 sequence at diagnosis and the Vδ2-Jα29 sequence at relapse shared a common Vδ2-Dδ3 stem confirming their origin from a common (pre)leukemic progenitor cell with a Vδ2-Dδ3 rearrangement. In the remaining four cases, the junctional regions of the Vδ2-Jα gene rearrangements at diagnosis and at relapse were completely different suggesting that the presumed leukemic progenitor probably had germline *TCRD* genes.

Vδ2-Jα gene rearrangements as MRD-PCR targets in precursor-B-ALL patients

Vδ2-Jα gene rearrangements were tested as MRD-PCR targets in TaqMan-based RQ-PCR assays employing a germline Vδ2 forward primer and a germline Vδ2 TaqMan probe together with patient-specific reverse primers located in the Vδ2-Jα junctional regions (Table 3). In 11 of 15 patients (73%), a reproducible sensitivity of 10^{-4} was achieved at the routine annealing temperature of 60°C, i.e. no optimization was necessary. In all except two patients the maximal sensitivity was at least 10^{-4}. Repeated background amplification in normal MNC was found in only two cases (Table 3).

Vδ2-Jα gene rearrangements in normal lymphoid tissues

Using RQ-PCR assays with a germline Vδ2 forward primer and TaqMan probe and one of four reverse germline Jα primers (Jα61, Jα58, Jα29, and Jα9), according to the most frequent Vδ2-Jα gene rearrangements in precursor-B-ALL, we demonstrated that such preferential Jα usage is not characteristic for normal lymphoid tissues. Relatively high levels of Vδ2-Jα58 and Vδ2-Jα61 gene rearrangements (10^{-3} to 10^{-2}) were only found in thymus samples (Table 4). Ten-fold lower levels (10^{-4} to 10^{-3}) were repeatedly detected in PB, particularly in a fraction of E-rosette selected T-cells. Lower frequencies of Vδ2-Jα58 and Vδ2-Jα61 gene rearrangements were detected in normal BM, lymph nodes, and tonsils (generally $\leq 10^{-4}$). Vδ2-Jα29 gene rearrangements were consistently found in the thymus sam-

Table 4. Vδ2-Jα rearrangements in normal lymphoid tissues as compared with precursor-B-ALL.

	Vδ2-Jα61	Vδ2-Jα58	Vδ2-Jα29	Vδ2-Jα9
Precursor-B-ALL (% of Vδ2-Jα positive patients)	4.4%	3.8%	53.8%	6.3%
$CD19^+/CD10^{++}/CD20^-$ sorted normal BM B-cell precursors (n = 1)[a]	–	–	–	–
$CD19^+/CD10^+/CD20^{\pm}$ sorted normal BM B-cell precursors (n = 1)[a]	–	–	±	–
$CD19^+/CD10^-/CD20^+$ sorted normal BM B-cells (n = 1)[a]	–	–	–	–
Regenerating BM (n = 3)[b]	±	–	–	–
Normal BM (n = 3)	±/+	±	–	–
Normal PB (n = 2)	+	±	–	–
E-rosette+ PB MNC (n = 1)	+	±/+	–	–
E-rosette- PB MNC (n = 1)	–	–	–	–
Tonsil (n = 2)	±	±	±	–
Lymph node (n = 3)	±	±/+	±	–
Thymus (n = 3)	++	+/++	±	–

a. Normal BM was stained with CD10, CD20, and CD19 monoclonal antibodies and the indicated cell populations were sorted using a Becton Dickinson Diva flow cytometer.

b. Regenerating BM after cessation of chemotherapy is known to contain high frequencies of precursor-B-cells (5-30%)[15,37]

Symbols: ++, Vδ2-Jα levels > 10^{-3}; +, Vδ2-Jα levels between 10^{-4} and 10^{-3}; ±, Vδ2-Jα levels between 10^{-5} and 10^{-4}; -, Vδ2-Jα levels ≤ 10^{-5} or undetectable.

ples at very low levels of ~10^{-5}. Incidental positivity at similarly low levels was also found in PB, BM, and tonsils. In contrast, Vδ2-Jα9 gene rearrangements were virtually undetectable in all tested normal lymphoid tissues, including the thymus (Table 4). Overall, these RQ-PCR data indicate that Vδ2-Jα gene rearrangements in normal lymphoid tissues are restricted to T-cell compartment and the prevalence of particular Jα segments in Vδ2-Jα gene rearrangements is mainly determined by the proximity to the *TCRD* locus.

DISCUSSION

The results of our study indicate that the Vδ2 gene segment is a 'hot spot' for V(D)J recombination in precursor-B-ALL. This single gene segment is involved in

various gene rearrangements in 75 to 80% of patients. In 39% of cases, Vδ2-Dδ3 joinings were found, which are known to be the most prevalent *TCRD* gene rearrangements in precursor-B-ALL.[21,26,39] In 42% of patients our combined Southern blot and PCR analyses confirmed the presence of Vδ2 rearrangements to various Jα segments. The junctional regions of most (78%) Vδ2-Jα gene rearrangements contained the Dδ3 segment, which indicates that recombination to Jα was preceded by a Vδ2-Dδ3 rearrangement (Figure 1). The Dδ2 segment is another gene, which is reported to be involved in *TCRD/A* gene rearrangements in precursor-B-ALL.[19,26] However, Dδ2-Dδ3 gene rearrangements were found in only approximately 10% of precursor-B-ALL patients, which is in line with literature data.[19,21,26] Parts of the Dδ2 gene segments were found in 7% of Vδ2-Jα junctional regions, which is in striking contrast to the frequent detection of Dδ3 segments. Clonal Dδ2-Jα gene rearrangements occur even more seldom, since we were able to show clonal Dδ2-Jα PCR products in only 3 patients (2%), who were pre-selected based on Southern blot data. Thus Vδ2, Dδ2, Dδ3, and several Jα genes are preferentially involved in recombinations in the TCRD/A locus in precursor-B-ALL, with the main pathway being Vδ2-Dδ3 → Vδ2-Jα29 (Figure 1). The next step might concern secondary Vα-Jα rearrangements, deleting the whole TCRD locus as well as pre-existing Vδ2-Jα joinings.[19,21] An explanation for the limited number of gene segments involved in the Vδ2 gene rearrangements is differential accessibility of gene segments within the *TCRD* locus in precursor-B-ALL. Some *TCRD* regions, particularly Vδ1 and all Jδ genes, seem to be fully closed for the persistent activity of the V(D)J recombinase in precursor-B-ALL, because rearrangements involving these gene segments were reported only anecdotally.[40] On the other hand, there might be additional factors related to the oncogenic process such as over-expressed transcription regulators, which might influence selection of particular gene segments, i.e. Vδ2, Dδ3, or Jα29.

The spectrum of Vδ2-Jα gene rearrangements in precursor-B-ALL is clearly not random. The single gene segment Jα29 was found in 54% of all Vδ2-Jα joinings. Such non-random usage of Jα gene segments was previously suggested by Southern blot data but was never confirmed at the PCR and sequence level.[17,18] The remaining Vδ2-Jα sequences contained 25 different Jα segments, most of them belonging to two additional clusters. The first cluster involved gene segments located most proximally to the TCRD locus, with Jα61, Jα58, Jα54, and Jα48 gene segments used most frequently. Altogether, 23% of Vδ2-Jα gene rearrangements involved Jα gene segments in this proximal cluster. The second cluster was located around the Jα9 gene segment and accounted for 8% of Vδ2-Jα joinings. The preferential usage of Jα29 might be related to the fact that the RSS of Jα29 is fully identical to the consensus RSS. However, no preferential usage was found for the other Jα gene segments with a full consensus RSS, i.e. Jα15 and Jα34. Apparently, a combination of several factors determines the preferential usage of several Jα gene

segments, such as: (1), proximity to the *TCRD* locus (e.g. for Jα61, Jα58, Jα54, and Jα48)[41-43]; (2), leukemia-associated differential accessibility, potentially related to specific transcription factors; and (3), presence of consensus RSS.

Vδ2-Jα gene rearrangements can also occur at low levels in normal lymphoid tissues (Table 4). They are relatively frequent in the thymus, where they represent one of the *TCRD* deletion pathways for commitment to the TCRαβ lineage.[41-43] Similarly to our previously published data,[43] the majority of Vδ2-Jα gene rearrangements in the thymus involved the most proximal Jα genes (in our study represented by Jα58 and Jα61) and the frequency of such recombinations ranged from 10^{-3} to 10^{-2}. The same type of rearrangements were also detectable in other lymphoid tissues including PB-MNC, BM, lymph nodes, and tonsils but at generally more than 10-fold lower levels as compared to the thymus (i.e. $< 10^{-3}$). Such difference in frequency of Vδ2-Jα gene rearrangements between the thymus and PB was suggested before based on qualitative PCR data.[43] In striking contrast, Vδ2-Jα29 and Vδ2-Jα9 joinings were virtually undetectable in normal lymphoid tissues (Table 4). This suggests that the preferential usage of the Jα9 and Jα29 clusters in the Vδ2-Jα rearrangements of precursor-B-ALL (Figure 3) is a leukemia-specific characteristic.

Our multiplex PCR strategy can easily identify clonal Vδ2-Jα gene rearrangements, which can be applied as PCR targets for MRD monitoring. In fact, based on the limited number of Jα segments involved in the Vδ2-Jα gene rearrangements in precursor-B-ALL, the multiplex strategy can be further simplified. The junctional regions of Vδ2-Jα joinings are extensive, containing approximately 19 nucleotides on average. Therefore, it should be relatively easy to design optimal patient-specific oligonucleotides reaching sensitivities of at least 10^{-4}, which is required for recognition of the MRD-based low-risk patients.[3,6] Our preliminary results in 15 patients showed that in 11 of these cases Vδ2-Jα gene rearrangements were sufficiently sensitive and specific MRD-PCR targets, without need for any assay optimization. This is in striking contrast to *TCRG* gene rearrangements, which fail as MRD-PCR targets in approximately 60% of precursor-B-ALL due to their limited specificity and/or sensitivity.[13] Another advantage of Vδ2-Jα gene rearrangements as MRD-PCR targets is the extremely low background of polyclonal Vδ2-Jα joinings in normal BM and PB, irrespective of the treatment phase.

The major disadvantage of Vδ2-Jα gene rearrangements is their potential instability owing to different clonal evolution phenomena with secondary rearrangements causing oligoclonality. Approximately 45% of Vδ2-Jα joinings in precursor-B-ALL are oligoclonal. Comparative studies on *IGH*, *IGK*-Kde, and *TCRD* gene rearrangements at diagnosis and relapse reported a much lower stability for the oligoclonal rearrangements as compared to monoclonal gene rearrangements.[14,36,44] This is also the case for Vδ2-Jα gene rearrangements, where 87% of monoclonal rearrangements were preserved as compared to only 40% of the oligoclonal rearrangements. Oligoclonal Vδ2-Jα gene rearrangements usually reflect ongoing

Vδ2-Dδ3 to Jα joinings. In fact, small Vδ2-Jα subclones with a common Vδ2-Dδ3 stem were reported in the vast majority of precursor-B-ALL with clonal Vδ2-Dδ3 gene rearrangements.[20] Monoclonal Vδ2-Jα gene rearrangements as confirmed by combined Southern blot and PCR strategy, are excellent MRD-PCR targets that are equally valuable to monoclonal *IGH* and *TCRD* gene rearrangements. The usage of oligoclonal Vδ2-Jα gene rearrangements as MRD-PCR targets is not recommended owing to their low stability. When the applied MRD-PCR strategy does not include Southern blotting, Vδ2-Jα gene rearrangements can only be used as supplementary MRD-PCR targets in exceptional cases.

In conclusion, Vδ2-Jα gene rearrangements are frequent cross-lineage recombinations in *TCRD/A* locus of precursor-B-ALL, which is in striking contrast to their infrequent occurrence in normal B-cells and B-cell precursors. The spectrum of Vδ2-Jα gene rearrangements in precursor-B-ALL is not random with preferential usage of Jα29 gene segment. The extensive junctional regions, the low background in normal BM and PB, and the good stability (87%) of monoclonal rearrangements are the features that favor the usage of monoclonal Vδ2-Jα gene rearrangements as principal MRD-PCR targets in approximately 25% of precursor-B-ALL.

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CHAPTER 2.6

T-CELL RECEPTOR GAMMA (*TCRG*) GENE REARRANGEMENTS IN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA REFLECT 'END-STAGE' RECOMBINATIONS: IMPLICATIONS FOR MINIMAL RESIDUAL DISEASE MONITORING*

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ABSTRACT

The T-cell receptor gamma (*TCRG*) gene configuration was established in a large series of 126 T-cell acute lymphoblastic leukemia (T-ALL) patients using combined Southern blotting (SB) and heteroduplex PCR analyses. The vast majority of T-ALL (96%) displayed clonal *TCRG* gene rearrangements, with biallelic recombination in 91% of patients. A small immature subgroup of $CD3^-$ T-ALL (n = 5) had both *TCRG* genes in germline configuration, three of them having also germline *TCRD* genes. In five patients (4%) combined SB and PCR results indicated oligoclonality. In five rearrangements detected by SB, the Vγ gene segment could not be identified suggesting illegitimate recombination. Altogether, 83% of *TCRG* gene rearrangements involved either the most upstream Vγ2 gene (including four cases with interstitial deletion of 170 bp in Vγ2) and/or the most downstream Jγ2.3 segment, which can be perceived as 'end-stage' recombinations.

Comparative analysis of the *TCRG* gene configuration in the major immunophenotypic subgroups indicated that $TCR\gamma\delta^+$ T-ALL display a less mature immunogenotype as compared to $TCR\alpha\beta^+$ and most $CD3^-$ cases. This was reflected by a significantly increased usage of the more downstream Vγ genes and the upstream Jγ1 segments. Comparison between adult and pediatric T-ALL patients did not show any

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obvious differences in *TCRG* gene configuration.

The high frequency, easy detectability, rare oligoclonality, and frequent 'end-stage' recombinations make *TCRG* gene rearrangements principal targets for PCR-based detection of minimal residual disease (MRD) in T-ALL. We propose a simple heteroduplex PCR strategy, applying five primer combinations, which results in the detection of approximately 95% of all clonal *TCRG* gene rearrangements in T-ALL. This approach enables identification of at least one *TCRG* target for MRD monitoring in 95% of patients, and even two targets in 84% of T-ALL.

INTRODUCTION

Nowadays, T-cell receptor gamma (*TCRG*) gene rearrangements are frequently utilized as clonality markers and as PCR targets for the detection of minimal residual disease (MRD) in lymphoproliferative disorders. The latter application is particularly relevant in acute lymphoblastic leukemia (ALL), since *TCRG* gene rearrangements occur in the vast majority of T-ALL and in approximately 60% of precursor-B-ALL patients.[1,2] Several retrospective studies and recently also two large prospective MRD studies successfully employed *TCRG* gene rearrangements as leukemia-specific PCR targets.[3-7]

The frequencies and patterns of *TCRG* gene rearrangements in T-ALL were addressed by several Southern blot (SB)-based studies.[8-12] Biallelic rearrangements were found in the vast majority of patients and preferential usage of the Jγ2 gene complex was strongly suggested.[1,12] There was some indication on differential Vγ gene utilization in $CD3^+$ versus $CD3^-$ negative T-ALL subsets, but such observations were generally based on limited numbers of patients.[11] Currently, PCR-based methodologies are more frequently applied for the detection of clonal *TCRG* gene rearrangements. However, the vast majority of PCR-based studies on *TCRG* genes in T-ALL, were based either on small or restricted patient groups or on limited primer combinations.[13-17] Therefore, we performed extensive heteroduplex PCR analysis of *TCRG* gene rearrangements in a large group of 126 T-ALL patients. The majority of patient samples were also studied in parallel by SB as a reference technique. Based on the combined SB and heteroduplex PCR results, we were able to determine the presence of oligoclonality and illegitimate recombinations as well as to compare *TCRG* gene rearrangement patterns between childhood and adult T-ALL patients, and to compare between different immunophenotypic subgroups of T-ALL.

MATERIALS AND METHODS

Patient description

Peripheral blood (PB) or bone marrow (BM) samples from 126 T-ALL patients (90 children and 36 adults) were obtained at initial diagnosis.[16,18] Mononuclear cells (MNC) were isolated from PB or BM samples by Ficoll-Paque centrifugation (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden) and subjected to detailed immunophenotyping according to standard protocols.[19,20] To analyze sufficient numbers of CD3$^+$ T-ALL (especially TCR$\gamma\delta^+$ T-ALL) T-ALL cell samples were selected based on their CD3/TCR immunophenotype resulting in 64 CD3$^-$ T-ALL (51% of the total series), 33 TCR$\alpha\beta^+$ T-ALL (26%), and 29 TCR$\gamma\delta^+$ T-ALL (23%). In an entirely random series of T-ALL this immunophenotype distribution would approximate 70%, 20%, and 10%, respectively.[21]

Southern blot analysis

SB analysis was performed in 117 of the 126 patients. DNA was isolated from frozen MNC, digested, and blotted to nylon membranes as described previously.[22] The *TCRG* gene configuration was studied using the TCRGJ13 probe (DAKO Corporation, Carpinteria, CA, USA) in *Eco*RI digests together with either the TCRGJ21 probe (DAKO) in *Pst*I digests or a combination of the Jγ2.1 probe in *Eco*RI digests and the Jγ1.2 probe in *Bgl*II digests.[12,23] The information on the SB configuration of *TCRG* genes was reported before for 70 T-ALL patients.[12,16,17]

PCR amplification and heteroduplex analysis of PCR products

All patient samples were subjected to detailed PCR analysis, which was essentially performed as described previously.[16,24] In each 50µl PCR reaction 50 ng DNA sample, 6.3 pmol of the 5′ and 3′ oligonucleotide primers, and 0.5 U AmpliTaq Gold polymerase (PE Biosystems, Foster City, CA, USA) were used. The sequences of the oligonucleotides used for amplification of *TCRG* gene rearrangements (four Vγ family-specific primers, six VγI member-specific primers, and three Jγ primers) were published before.[24] PCR conditions were: initial denaturation for 10 min at 94°C, followed by 35 cycles of 45 s at 94°C, 90 s at 60°C, and 2 min at 72°C using a Perkin-Elmer 480 thermal cycler (PE Biosystems). After the last cycle an additional extension step of 10 min at 72°C was performed. Appropriate positive and negative controls were included in all experiments.[24] The results of PCR analysis of *TCRG* gene rearrangements in 22 adult T-ALL patients were reported before.[16] All other 104 T-ALL patients were newly analyzed for this study.

For heteroduplex analysis, the PCR products were denatured at 94°C for 5 min after the final cycle of amplification and subsequently cooled to 4°C for 60 min to induce duplex formation.[25] Afterwards the duplexes were immediately loaded on 6% non-denaturing polyacrylamide gels in 0.5 x Tris-borate-EDTA (TBE) buffer, run at room temperature, and visualized by ethidium bromide staining.[25] A 100-bp DNA ladder (Promega Corporation, Madison, WI, USA) was used as size marker.

Sequence analysis of *TCRG* gene rearrangements

When assignment for Vγ and/or Jγ gene segments was not possible in particular rearrangements based on combined SB and PCR information, clonal PCR products as found by heteroduplex analysis were sequenced directly. 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.[26] Vγ and Jγ gene segments were identified by comparison to germline *TCRG* sequences as described before.[27]

Statistical analysis

Statistical analysis using the χ^2 test on a 2 x 2 table was performed to compare the frequencies of particular *TCRG* gene rearrangements between childhood and adult T-ALL patients, and between different immunophenotypic subgroups of T-ALL. A value of $p < 0.05$ was regarded to be statistically significant.

RESULTS

Spectrum of *TCRG* gene rearrangements in T-ALL

Based on the combined SB and heteroduplex PCR analyses, we were able to determine the *TCRG* gene configuration in a large group of 126 T-ALL patients in detail (Table 1). In the vast majority of cases (115 patients; 91%) both *TCRG* alleles were rearranged (Figure 1). In one of these patients three rearranged bands of comparable density were found by SB, strongly suggesting trisomy 7. In five patients (4%) both *TCRG* alleles were in germline configuration, suggesting an immunogenotypically very immature T-ALL. Monoallelic rearrangements were found in six patients (5%), with the second allele in germline configuration in three cases and deleted in the other three cases. In only five patients (4%) did combined SB and PCR data indicate the presence of minor subclones as identified by additional *TCRG* gene

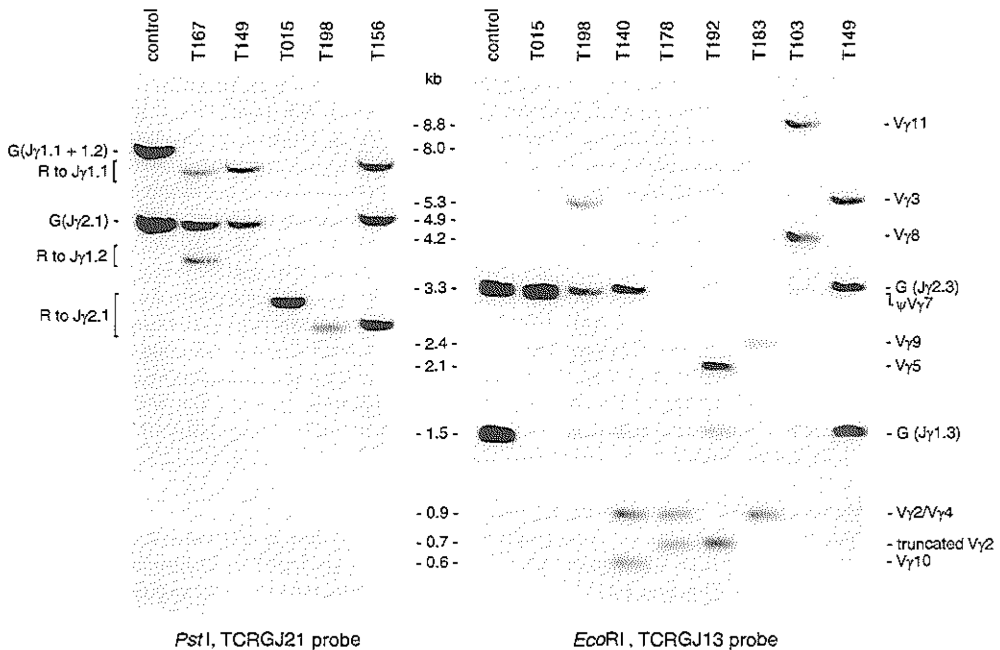

Figure 1.
Southern blot analysis of the *TCRG* gene configuration in ten of the studied T-ALL patients. Hybridization of *Pst*I digests with the TCRGJ21 probe and *Eco*RI digests with the TCRGJ13 probe revealed biallelic rearrangements in all ten cases. In two patients (T178 and T192) SB indicated the presence of a truncated Vγ2 segment, which was subsequently confirmed by heteroduplex PCR analysis. In patient T015 the rearrangement of the ψVγ7 gene to Jγ2.3 segment is virtually indistinguishable from the germline Jγ2.3 band derived from the other allele.

Table 1. ***TCRG* gene rearrangement patterns in childhood versus adult T-ALL patients.**

		Childhood T-ALL (90 patients; 180 alleles)	Adult T-ALL (36 patients; 73 alleles)[a]	Total T-ALL (126 patients; 253 alleles)[a]
Germline alleles		6 (3%)	7 (10%)	13 (5%)
Deleted alleles		2 (1%)	1 (1%)	3 (1%)
Rearranged alleles		172 (96%)	65 (89%)	237 (94%)
VγI	Vγ2	44 (26%)	18 (28%)	62 (26%)
	Vγ3	16 (9%)	4 (6%)	20 (8%)
	Vγ4	24 (14%)	10 (16%)	34 (14%)
	Vγ5	9 (5%)	3 (5%)	12 (5%)
	ψVγ7	4 (2%)	1 (2%)	5 (2%)
	Vγ8	30 (17%)	12 (19%)	42 (18%)
VγII	Vγ9	22 (13%)	4 (6%)	26 (11%)
VγIII	ψVγ10	12 (7%)	6 (9%)	18 (8%)
VγIV	ψVγ11	7 (4%)	6 (9%)	13 (5%)
Not identified		4 (2%)	1 (2%)	5 (2%)
Jγ1	Jγ1.1	7 (4%)	7 (11%)	14 (6%)
	Jγ1.2	1 (1%)	1 (2%)	2 (1%)
	Jγ1.3	14 (8%)	6 (9%)	20 (8%)
Jγ2	Jγ2.1	19 (11%)	3 (5%)	22 (9%)
	Jγ2.3	131 (76%)	48 (75%)	179 (76%)

a. In a single adult T-ALL patient three rearranged bands of comparable density were found on SB analysis, suggesting trisomy 7.

rearrangements, demonstrating that oligoclonality in *TCRG* genes is rare in T-ALL.

Analysis of Vγ gene segment usage revealed the utilization of the most upstream Vγ2 segment (Figure 2) in 62 (26%) rearrangements. Interestingly, in four patients the Vγ2-Jγ2.3 rearrangement contained an interstitial deletion of approximately 170 bp (Figure 1). Two other members of the VγI family were also frequently used, i.e. the Vγ4 and the Vγ8 gene segments on 34 (14%) and 42 (18%) alleles, respectively. Altogether, the VγI family segments were involved in 175 (73%) rearrangements. The single members of the other three families were identified in 57 joinings, with the Vγ9 (VγII) gene used most frequently (26 rearrangements; 11%). In five rearrangements detected by SB the Vγ segment could not be identified, which suggests that in these cases an illegitimate recombination might have occurred, e.g. a chromosomal translocation involving the *TCRG* locus.

Analysis of Jγ gene utilization showed a preferential usage of the most downstream Jγ2.3 segment (Figure 2), which was identified in 179 (76%) rearrangements. The other gene segment of the second Jγ cluster, i.e. the Jγ2.1 gene, was identified in 22 rearrangements (9%). Recombination to the first Jγ cluster was found on 36

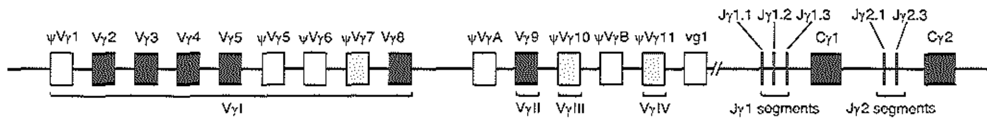

Figure 2.
Organization of the human *TCRG* locus on chromosome 7p14-p15. The *TCRG* locus consists of two constant region gene segments (Cγ) preceded by two or three joining gene segments (Jγ) and at least 14 variable gene segments (Vγ) located upstream of the two Jγ-Cγ regions. Six Vγ gene segments are functional (solid blocks), three pseudogenes can rearrange to Jγ gene segments (shaded blocks), whereas the other pseudogenes (open blocks) have no functional recombination signal sequences. Nine upstream Vγ gene segments belong to family I, whereas families II, III and IV each consist of a single gene segment assigned as Vγ9, Vγ10 and Vγ11, respectively. Five joining segments have been identified: 3 Jγ1 segments i.e. Jγ1.1, Jγ1.2 and Jγ1.3 upstream of Cγ1 and 2 Jγ2 segments i.e. Jγ2.1 and Jγ2.3 upstream of Cγ2.

alleles (15%) with Jγ1.1 and Jγ1.3 segments used most frequently on 14 (6%) and 20 (8%) alleles, respectively. Rearrangements involving the Jγ1.2 gene segment were identified in only two patients; both joinings concerned the Vγ9 gene coupling. One of these Vγ9-Jγ1.2 rearrangements contained a canonical junctional region based on the absence of N-nucleotide insertion and with a deletion of three overlapping homologous nucleotides, which is preferentially expressed by normal peripheral blood $TCR\gamma\delta^+$ cells.[28] In contrast, the junctional region of the other Vγ9-Jγ1.2 rearrangement contained 11 inserted N-nucleotides.

TCRG gene rearrangements in adult versus pediatric patients

TCRG gene rearrangement patterns were compared between adult and pediatric patients (Table 1). This analysis revealed that the *TCRG* configuration was largely comparable between these two patient groups. Although in adult patients the *TCRG* alleles were more frequently in germline configuration (11% vs. 4% in pediatric ALL), frequently involved the Jγ1 segments (22% vs. 13%), and used the Vγ9 gene segment less frequently (6% vs. 13%), these findings did not reach statistical significance.

TCRG gene rearrangements in different immunophenotypic subsets

Analysis of *TCRG* gene rearrangements in the three major immunophenotypic T-ALL groups revealed largely comparable configuration patterns (Table 2). Nevertheless, several discrete differences were observed. Firstly, within the $CD3^-$ group there was a small subset with *TCRG* genes in germline configuration (5 patients), whereas all $CD3^+$ leukemias displayed at least a monoallelic rearrangement. Secondly, $TCR\gamma\delta^+$ T-ALL displayed several distinct characteristics. This immunophenotypic subset was characterized by preferential usage of the Vγ9 (VγII) gene, which was found in 22% of rearrangements, as compared to 8% in the non-$TCR\gamma\delta^+$ T-ALL group ($p < 0.01$). This was still significant when $TCR\gamma\delta^+$ T-ALL was

independently compared to TCRαβ^{+} and CD3^{-} T-ALL ($p < 0.05$). Altogether, the three most downstream Vγ members were slightly overrepresented in TCRγδ^{+} T-ALL (36% versus 20% in non-TCRγδ^{+} T-ALL; $p < 0.05$). Moreover, the Jγ segments of the upstream Jγ1 cluster, particularly the Jγ1.3 gene, were more frequently used in TCRγδ^{+} T-ALL (28%), when compared to non-TCRγδ T-ALL (11%; $p < 0.01$).

DISCUSSION

We studied the *TCRG* gene configuration in a large series of 126 T-ALL patients. Based on the combined SB and PCR results, we were able to establish several char-

Table 2. ***TCRG*** **gene rearrangement patterns in major immunophenotypic subsets of T-ALL**

		CD3- T-ALL (64 patients; 129 alleles)			TCRγδ^{+} T-ALL (29 patients; 58 alleles)			TCRαβ^{+} T-ALL (33 patients; 66 alleles)		
Germline alleles		10	(8%)		3	(5%)		0		
Deleted alleles		2	(2%)		1	(2%)		0		
Rearranged alleles		117	(91%)		54	(93%)		66	(100%)	
VγI	Vγ2	37	(32%)	77%	10	(19%)	62%	15	(22%)	77%
	Vγ3	9	(8%)		6	(11%)		5	(8%)	
	Vγ4	15	(13%)		8	(15%)		11	(17%)	
	Vγ5	5	(4%)		3	(6%)		4	(6%)	
	ψVγ7	2	(2%)		0			3	(4%)	
	Vγ8	23	(20%)		6	(11%)		13	(20%)	
VγII	Vγ9	10	(9%)	20%	12	(22%)	36%[b]	4	(6%)	21%
VγIII	ψVγ10	7	(6%)		4	(7%)		7	(11%)	
VγIV	ψVγ11	6	(5%)		4	(7%)		3	(4%)	
Not identified		3	(3%)		1	(2%)		1	(2%)	
Jγ1	Jγ1.1	3	(3%)	10%	6	(11%)	28%[b]	5	(8%)	16%
	Jγ1.2	2	(2%)		0			0		
	Jγ1.3	6	(5%)		9	(17%)		5	(8%)	
Jγ2	Jγ2.1	9	(8%)	90%	3	(6%)	72%	10	(15%)	84%
	Jγ2.3	97	(83%)		36	(67%)		46	(70%)	

a. In a single adult CD3^{-} T-ALL patient three rearranged bands of comparable density were found on SB analysis, suggesting trisomy 7.

b. Gene segments significantly overrepresented in *TCRG* gene rearrangements of TCRγδ^{+} T-ALL as compared to non-TCRγδ T-ALL.

acteristics of *TCRG* genes in T-ALL, which were also suggested in previous molecular studies.[8-10] Firstly, we found *TCRG* gene rearrangements in 96% of patients, involving both alleles in most cases. Nevertheless, we could identify a small subset of five $CD3^-$ T-ALL with both *TCRG* genes in germline configuration. They most probably are immunogenotypically very immature leukemias, because three of these patients also had germline T-cell receptor delta (*TCRD*) genes. Secondly, we confirmed the earlier observation of preferential usage of Jγ segments from the downstream Jγ2 cluster (85% of rearrangements), particularly of the Jγ2.3 gene segment (76%).[12,29] In addition, we showed preferential usage of the Vγ2 segment, which was present in more than a quarter of all joinings. Together, rearrangements concerning this most upstream Vγ2 gene segment and/or the most downstream Jγ2.3 gene were found on 83% of rearranged alleles and can be perceived as 'end-stage' recombination events. Moreover, four of the Vγ2-Jγ2.3 joinings contained an interstitial deletion of approximately 170 bp in the Vγ2 gene segment, most probably resulting from rearrangement to a recombination signal sequence within the Vγ2 gene.[30]

We could also demonstrate that *TCRG* oligoclonality is a rare phenomenon in T-ALL, occurring in less than 5% of patients. The *TCRG* gene rearrangement patterns in T-ALL are strikingly different from the cross-lineage *TCRG* gene rearrangements in precursor-B-ALL. The latter involve Jγ1 segments in approximately 70% of alleles and show oligoclonality in 38% of precursor-B-ALL patients with *TCRG* gene rearrangements.[2] This on one hand might reflect differences in accessibility of the *TCRG* locus to V(D)J recombinase activity between T-ALL and precursor-B-ALL. On the other hand, lineage promiscuity of *TCRG* rearrangements in precursor-B-ALL and lack of lineage-specific enhancement might favor the usage of more proximal gene segments. The rare occurrence of *TCRG* oligoclonality in T-ALL might indicate that the malignant transformation occurred in thymocytes with completed *TCRG* gene rearrangement processes. In contrast, the more frequent *TCRG* gene oligoclonality in precursor-B-ALL is one of the indicators that such cross-lineage recombinations are post-oncogenic events.[2,31]

Comparative immunogenotypic analysis of pediatric and adult T-ALL subgroups showed largely similar *TCRG* gene rearrangement patterns. This confirms our previous observation and is in striking contrast to precursor-B-ALL, where immunoglobulin and TCR gene rearrangement patterns are clearly less mature in adults than in children.[16]

Comparison of *TCRG* gene configurations between the three major immunophenotypic T-ALL subgroups revealed limited but significant differences. The $TCR\gamma\delta^+$ subgroup was distinct from non-TCRγδ cases with respect to a more frequent usage of the more downstream Vγ segments (with a striking preference for Vγ9) and the more upstream Jγ1 segments (particularly Jγ1.3). This might reflect the rapid cessation of *TCRG* gene accessibility to V(D)J recombinase activity as soon as a func-

tional *TCRG* gene rearrangement has been achieved. This is in line with a previous hypothesis that $TCR\gamma\delta^+$ T-ALL should be regarded as cells that branched off T-cell development at an early stage of completion of *TCRD* and *TCRG* gene rearrangement processes, while the recombinase activity is still retained.[17,21,31] Retained V(D)J activity is for instance reflected by the frequent occurrence of complete Vβ-Jβ and cross-lineage *IGH* gene rearrangements, detectable in 60% and 50% of $TCR\gamma\delta^+$ T-ALL patients, respectively.[17,32] We could not confirm the previous observation of Hara et al.[11] suggesting that in $CD3^-$ T-ALL Vγ genes more proximal to Jγ are more frequently rearranged, whereas $CD3^+$ T-ALL show a high frequency of rearrangements involving Vγ genes more distal from Jγ. We believe that this might be a bias owing to the relatively small group of T-ALL (24 cases) studied.

Our study also aimed at establishment of an optimal PCR strategy for identification of clonal *TCRG* gene rearrangements as molecular targets for MRD monitoring in T-ALL patients. Using five principal Vγ-Jγ primer combinations (Table 3), we found 94% of clonal *TCRG* gene rearrangements. These five primer combinations detected at least one rearrangement in all but one T-ALL patient with rearranged *TCRG* genes. Therefore, we routinely apply this set of primers on new diagnostic T-ALL samples. With three supplementary Vγ-Jγ primer combinations (Table 3), an additional 3% of clonal rearrangements were detected. This set may be reserved for T-ALL cases negative with the first set of primers, while SB analysis can be limited to rare cases with questionable PCR results.

Occurrence of *TCRG* gene rearrangements in virtually all patients, easy detectability of these rearrangements, and rare oligoclonality make *TCRG* genes principal molecular targets for MRD monitoring in T-ALL, particularly when *TCRD*

Table 3. PCR primer combinations for optimal detection of *TCRG* gene rearrangements as molecular targets for MRD monitoring in T-ALL.

PCR primer combination	Relative frequency of *TCRG* gene rearrangements
I. Principal combinations	
VγI-Jγ1.3/2.3	60%
VγI-Jγ1.1/2.1	13%
VγII-Jγ1.3/2.3	10%
VγIII-Jγ1.3/2.3	7%
VγIV-Jγ1.3/2.3	4%
II. Supplementary combinations	
VγII-Jγ1.2	1%
VγIII-Jγ1.1/2.1	1%
VγIV-Jγ1.1/2.1	1%

genes are deleted. The only potential disadvantage is a decreased sensitivity of patient-specific PCR owing to competition with *TCRG* gene rearrangements in normal T-cells. Nevertheless, in the extensive study of the BIOMED-1 Concerted Action, 86% of *TCRG* targets reached a sensitivity of at least 10^{-4}, which is a prerequisite for reliable identification of MRD-based risk groups.[7,24] Moreover, the 'end-stage' character of most *TCRG* gene rearrangements in T-ALL probably results in stability during the disease course. This was also suggested by several studies comparing *TCRG* gene configuration at diagnosis and at relapse of T-ALL.[7,33-37] Interestingly, a single study by Taylor et al.[37] described a so-called clonal regression in two T-ALL patients with biallelic *TCRG* gene rearrangements at diagnosis and both alleles in germline configuration at relapse. This observation was not confirmed by other studies, which showed that clonal evolution of *TCRG* genes is a rare event in T-ALL, and if clonal evolution occurred, this mainly concerned 'non-end-stage' rearrangements resulting in ongoing recombination of more upstream Vγ and more downstream Jγ segments.[7,33,34,36]

In conclusion, we could determine the *TCRG* gene configuration in a large group of T-ALL patients, showing biallelic gene rearrangements in the vast majority of cases. Analysis of different immunophenotypic subsets showed that $TCR\gamma\delta^+$ T-ALL are characterized by a less mature *TCRG* gene configuration when compared to $CD3^-$ and $TCR\alpha\beta^+$ cases. Since the majority of *TCRG* gene rearrangements in T-ALL represent 'end-stage' recombinations, they presumably are relatively stable molecular MRD targets. Based on our study, we propose a rapid and easy heteroduplex PCR strategy enabling identification of approximately 95% of clonal *TCRG* gene rearrangements with only five primer combinations, resulting in at least one MRD-PCR target in 95% of T-ALL patients and two PCR targets in 84% of cases.

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CHAPTER 2.7

IG HEAVY CHAIN GENE REARRANGEMENTS IN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA EXHIBIT PREDOMINANT DH6-19 AND DH 7-27 GENE USAGE, CAN RESULT IN COMPLETE V-D-J REARRANGEMENTS, AND ARE RARE IN T-CELL RECEPTOR αβ LINEAGE*

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ABSTRACT

Rearranged *IGH* genes were detected by Southern blotting in 22% of 118 cases of T-cell acute lymphoblastic leukemia (ALL) and involved monoallelic and biallelic rearrangements in 69% (18/26) and 31% (8/26) of these cases, respectively. *IGH* gene rearrangements were found in 19% (13/69) of $CD3^-$ T-ALL and in 50% of $TCR\gamma\delta^+$ T-ALL (12/24), whereas only a single $TCR\alpha\beta^+$ T-ALL (1/25) displayed a monoallelic *IGH* gene rearrangement. The association with the T-cell receptor (TCR) phenotype was further supported by the striking relationship between *IGH* and TCR delta (*TCRD*) gene rearrangements, i.e., 32% of T-ALL (23/72) with monoallelic or biallelic *TCRD* gene rearrangements had *IGH* gene rearrangements, whereas only 1 of 26 T-ALL with biallelic *TCRD* gene deletions contained a monoallelic *IGH* gene rearrangement.

Heteroduplex polymerase chain reaction (PCR) analysis with VH and DH family-specific primers in combination with a JH consensus primer revealed a total of 39 clonal products, representing 7 (18%) VH-(DH-)JH joinings and 32 (82%) DH-JH rearrangements. Whereas the usage of VH gene segments was seemingly random, preferential usage of DH6-19 (45%) and DH7-27 (21%) gene segments was

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observed. Although the JH4 and JH6 gene segments were used most frequently (33% and 21%, respectively), a significant proportion of joinings (28%) used the most upstream JH1 and JH2 gene segments, which are rarely used in precursor-B-ALL and normal B-cells (1% to 4%). In conclusion, the high frequency of incomplete DH-JH rearrangements, the frequent usage of the more downstream DH6-19 and DH7-27 gene segments, and the most upstream JH1 and JH2 gene segments suggests a predominance of immature *IGH* rearrangements in immature (non-TCR$\alpha\beta^+$) T-ALL as a result of continuing V(D)J recombinase activity. More mature $\alpha\beta$-lineage T-ALL with biallelic *TCRD* gene deletions apparently have switched off their recombination machinery and are less prone to cross-lineage *IGH* gene rearrangements. The combined results indicate that *IGH* gene rearrangements in T-ALL are post-oncogenic processes, which are absent in T-ALL with deleted *TCRD* genes and completed TCR alpha (*TCRA*) gene rearrangements.

INTRODUCTION

When rearranged immunoglobulin (Ig) and T-cell receptor (TCR) genes were found to be useful clonal leukemia-specific markers, they were initially considered to be lineage-specific.[1,2] However, the recognition of "abnormal" TCR gene rearrangements in precursor-B-acute lymphoblastic leukemia (ALL) and in some mature B-cell malignancies together with the finding of rearranged Ig heavy chain (*IGH*) genes in some T-ALL raised the question of lineage infidelity or promiscuity.[2,3] TCR gene rearrangements in precursor-B-ALL were observed in 60% to 80% of patients, indicating that these recombinations should rather be regarded as an ubiquitously occurring (cross-lineage) phenomenon in this type of B-cell malignancy.[4-7] In a recent study on 202 precursor-B-ALL patients, we even found cross-lineage TCR gene rearrangements in 93% of the patients.[8] The occurrence of cross-lineage rearranged TCR genes is related to the maturation stage, being significantly lower in immature $CD10^-$ precursor-B-ALL as compared with $CD10^+$ precursor-B-ALL.[8,9] Analogous to the hierarchical order during early T-cell development, rearrangements and/or deletions in the TCR delta (*TCRD*) locus occur most frequently (89% of patients), followed by TCR gamma (*TCRG*) (59%) and TCR beta (*TCRB*) (35%) gene rearrangements.[4-6,8] Furthermore, most *TCRB* gene rearrangements are restricted to the Jβ2 locus, the majority of *TCRG* gene rearrangements involve the Jγ1 gene segments, and a striking predominance of incomplete Vδ2-Dδ3 and Dδ2-Dδ3 rearrangements in the *TCRD* locus is observed.[4-11]

In contrast to the high frequency of cross-lineage TCR gene recombinations in precursor-B-ALL, *IGH* gene rearrangements in T-ALL are relatively rare. In a meta-analysis of previously reported small patient groups, the prevalence of rearranged *IGH* genes was estimated at 10% to 15% of lymphoblastic T-cell malignancies.[11] All

early studies were exclusively based on Southern blotting (SB) and at that time, the configuration of *IGH* diversity (D) regions was not completely known. Therefore, it was not possible to reliably discern between incomplete DH-JH and complete VH-(DH-)JH gene rearrangements and to identify cross-lineage *IGH* gene rearrangement patterns, which are characteristic for T-ALL.[11,12] The only consistent finding was, that a significant proportion of *IGH* rearrangements in human leukemic T-cells involved the DH7-27 (DQ52) gene segment, which is located immediately upstream of the JH region (Figure 1).[13,14]

To study cross-lineage *IGH* gene rearrangements in T-ALL in detail, we analyzed a large group of 118 T-ALL patients by Southern blotting to determine the precise incidence of rearranged *IGH* genes. Heteroduplex polymerase chain reaction (PCR) analysis and subsequent sequencing were applied to identify the rearranged *IGH* gene segments as well as the junctional regions of the rearrangements. In addition to VH family-specific primers and a JH consensus primer, we designed a new set of DH family-specific primers to detect and identify all complete VH-JH and incomplete DH-JH gene rearrangements.

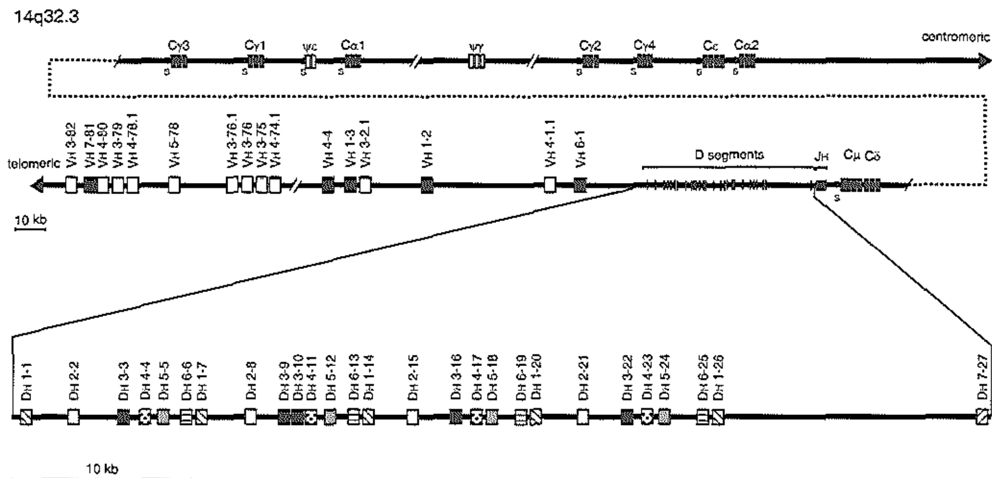

Figure 1.
Schematic representation of the human *IGH* locus on chromosome 14q32.3. The *IGH* gene complex consists of numerous (>120) V gene segments, 27 D gene segments, 6 functional J gene segments, and C gene segments for the constant domains of the various IgH classes and subclasses, most of which are preceded by switch sequences (s).[23,28,52,53] Pseudogenes are represented as open bars. The 27 DH gene segments are grouped in seven families based on sequence homology. Members of the same DH family are depicted with the same shading pattern.

MATERIALS AND METHODS

Cell samples

Peripheral blood (PB) or bone marrow (BM) samples from 118 T-ALL patients (84 children and 34 adults) were obtained at initial diagnosis.[7,15] Mononuclear cells (MNC) were isolated from PB or BM samples by Ficoll-Paque centrifugation (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden) and subjected to detailed immunophenotyping according to standard protocols.[16,17] To analyze sufficient numbers of CD3$^+$ T-ALL (especially TCR$\gamma\delta^+$T-ALL) we selected T-ALL cell samples based on their CD3/TCR immunophenotype, resulting in 69 CD3$^-$T-ALL (58% of the total series), 25 TCR$\alpha\beta^+$T-ALL (21%), and 24 TCR$\gamma\delta^+$T-ALL (20%). In an entirely random series of T-ALL this immunophenotype distribution would approximately be 70%, 20%, and 10%, respectively.[18] Although in two CD3$^+$T-ALL cases no information about TCR protein expression was available, they were included into the TCR$\gamma\delta^+$T-ALL group based on the Southern blot finding of biallelic *TCRD* rearrangements in both cases.

Southern blot analysis

DNA was isolated from fresh or frozen MNC fractions as described previously.[2] Fifteen micrograms of DNA was digested with the appropriate restriction enzymes (Pharmacia), size-separated in 0.7% agarose gels, and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as described.[2,19] *IGH* gene configuration was analyzed in the 118 T-ALL patients with the IGHJ6 probe (DAKO Corporation, Carpinteria, CA) in *Bgl*II, *Bam*HI/*Hin*dIII, *Eco*RI and/or *Hin*dIII digests.[20] The configuration of the *TCRD* genes was analyzed in 101 out of 118 patients with the TCRDV2, TCRDD2, TCRDJ1, TCRDJ2, TCRDJ3, TCRDRE, and TCRAPJ probes (DAKO Corporation) in *Bgl*II, *Eco*RI, and *Hin*dIII digests.[15]

Primer design and heteroduplex PCR analysis

PCR was essentially performed as described previously.[7,19] In each 50µl PCR reaction 50 ng DNA sample, 6.3 pmol 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 oligonucleotides used for amplification of complete VH-JH gene rearrangements (6 *IGH* framework-1 VH-family specific primers, and 1 JH consensus primer) were published before.[21,22] Based on recently published data of germline DNA D-region sequences of the human *IGH* locus[23] (EMBL accession no. X97051; for the detailed organization of the *IGH* D-region see Figure 1), 7 family-specific DH primers were designed using OLIGO 6.0 software (Dr. W. Rychlik; Molecular Biology Insights, Inc., Plymouth, MN) applying previously described guidelines (Table 1).[24] Oligonucleotide primers of 22 to 24 bp were positioned at least 50 bp upstream of the involved recombination signal sequence (RSS). Secondary structures such as primer dimers and hairpins were avoided, and the melting temperature (Tm) was 68°C ± 3°C. All primers were synthesized on an ABI 392 DNA synthesizer (PE Biosystems) using the solid-phase phosphotriester method.

PCR conditions were: initial denaturation for 3 minutes at 92°C, followed by 35 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.[24]

Heteroduplex analysis of PCR products included denaturation at 94°C for 5 minutes after the final cycle of amplification and subsequent renaturation at 4°C for 60 minutes to induce duplex formation.[25] Afterwards the duplexes were immediately loaded on 6% nondenaturing polyacrylamide gels in 0.5 x Tris-Boric acid-EDTA (TBE) buffer, run at room temperature, and visualized by ethidium bromide staining.[25] A 100-bp DNA ladder (Promega Corporation, Madison, WI) was used as size marker.

Sequence analysis of *IGH* gene rearrangements

PCR products found to be clonal by heteroduplex analysis were directly sequenced except for cases in which heteroduplex PCR analysis showed more than two clonal bands, i.e., either two homoduplexes, or an additional upper band resulting from extension to downstream JH segments, or a DH7-27-JH1 germline band accompanying a DH7-27-JH rearrangement. In such cases homoduplexes were excised from the polyacrylamide gel and eluted as described before.[26,27] The eluted PCR products were either

Table 1. Forward primers developed for PCR and sequence analysis of incomplete DH-JH gene rearrangements.

Primer code	Size of primer (bp)	Position in bp[a]	Sequence (5' → 3')
DH1	22	-79	A(C/T)CCAGGAGGCCCCAGAGC(A/T)CA
DH2	24	-187	CAGCACTGGGCTCAGAGTCCTCTC
DH3	24	-109	CCTCCTC(A/C)GGTCAGCCC(C/T)GGACAT
DH4	24	-129	CCCAGGACGCAGCACC(A/G)CTGTCAA
DH5	23	-245	ACCCAGCCTCCTGCTGACCAGAG
DH6	22	-144	CAGGCCCCCCA(A/G)AACCAG(T/G)G(A/T)T
DH7	24	-181	GGGCTGGGGTCTCCCACGTGTTTT

a.The position of the 5' end of the primer is indicated upstream (–) relative to the 3' RSS of the involved DH gene segment.

directly sequenced or subjected to second step PCR with the same primer pair to increase the amount of template for sequence analysis. Sequencing was performed using the dye-terminator cycle sequencing kit with AmpliTaq DNA polymerase FS on an ABI 377 sequencer (PE Biosystems). Briefly, 50-200 ng of PCR product and 3.2 pmol primer were used in a 15 µl reaction volume. The cycling protocol was 96°C for 30 seconds followed by 60°C for 4 minutes for a total of 25 cycles. Each PCR product was sequenced in two directions.

Interpretation of sequence data

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/)[28]. For alignments of D segments in VH-DH-JH or DH-DH-JH rearrangements, it was required to have at least 10 consecutive matching nucleotides.[23] Palindromic (P-region) nucleotides (maximally 2) generated during the joining process were recognized as being palindromic to the juxtaposed nucleotides of an untrimmed rearranged gene segment.[29] Extensive N-regions (nucleotides that cannot be assigned to V, D, J gene segments or P-regions) were analyzed in more detail by comparing them to the most recent update of GenBank using the BLAST sequence similarity-searching tool (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/ BLAST/).[30]

RESULTS

Southern blot analysis of *IGH* gene rearrangements in T-ALL

IGH gene rearrangements were found in 22% (26/118) of T-ALL patients, and were equally distributed between different age groups, i.e., 23% (19/84) of children and 21% (7/34) of adults. In the majority of cases this concerned monoallelic rearrangements (69% [18/26]), and in 31% (8/26) biallelic rearrangements were observed. In 2 of these patients, SB analysis revealed weak bands, most probably derived from subclones. Cross-lineage *IGH* gene rearrangements were found in 19% (13/69) of $CD3^-$ T-ALL and in 50% of $TCR\gamma\delta^+$ T-ALL (12/24), whereas only a single $TCR\alpha\beta^+$ T-ALL (4% [1/25]) displayed a rearranged *IGH* gene on one allele.

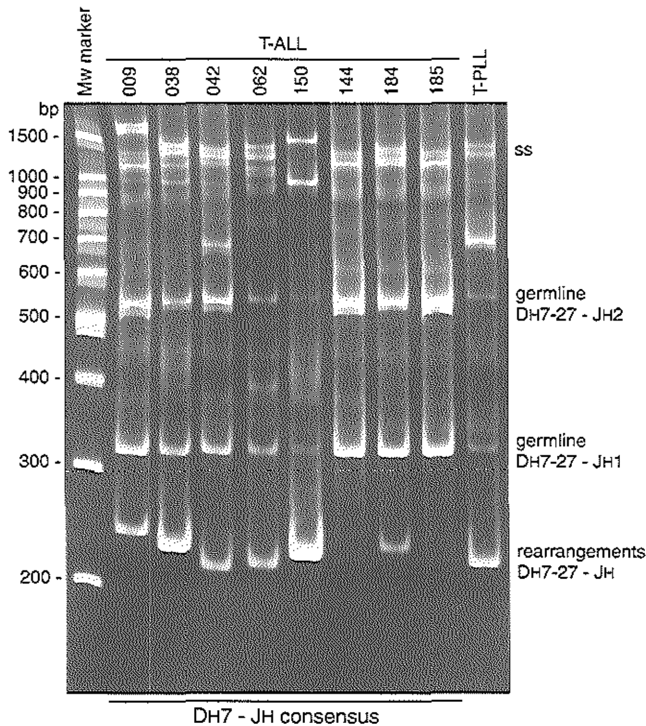

Figure 2.
Heteroduplex PCR analysis of DH7-27-JH cross-lineage rearrangements in T-ALL. Subsequent to agarose gel electrophoresis samples containing PCR products were subjected to heteroduplex PCR analysis, separated in a 6% polyacrylamide gel, and visualized by ethidium bromide staining. Based on the size of clonal PCR products DH7-27-JH rearrangements (~250 bp) were identified in T-ALL patients T009, T038, T042, T062, T150, and T184 as well as in a T-cell prolymphocytic leukemia (T-PLL). In addition to homoduplexes resulting from DH7-27-JH rearrangement, the germline DH7-27-JH1 and DH7-27-JH2 homoduplexes were consistently present, except for cases with biallelic *IGH* rearrangements and a very high tumour load (i.e., patient T150). To obtain a clonal sequence of DH7-27-JH rearrangements, homoduplexes of the correct size were excised from the polyacrylamide gel, eluted, and sequenced. ss = single strand DNA.

IGH gene rearrangements coincide with *TCRD* gene rearrangements

Because CD3⁻ T-ALL theoretically represent precursor stages of both TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ T-ALL, we analyzed the configuration of the *TCRD* genes in 57 $CD3^-$ T-ALL cases and used it as an additional marker for further subdivision of this group.[15,16] The *TCRD* gene configuration of each allele can potentially pass three consecutive stages: germline, rearrangement, and deletion.[15-18] Except for 1 case, all *IGH* gene rearrangements were found in patients with at least one *TCRD* gene rearrangement; in this single $CD3^-$ T-ALL a monoallelic *IGH* gene rearrangement

Table 2. Usage of different DH families in (in)complete cross-lineage *IGH* gene rearrangements in T-ALL.

New family name[a]	Old family name[a] of rearrangements	No. (frequency)	Segments used	
DH1	DM	7 (18%)	DH1-7:	2
			DH1-20:	2
			DH1-26:	3
DH2	DLR	1 (3%)	DH2-2:	1
DH3	DXP	1 (3%)	DH3-22:	1
DH4	DA	2 (5%)	DH4-4:	1
			DH4-23:	1
DH5	DK	1 (3%)	DH5-18:	1
DH6	DN	18 (47%)	DH6-6:	1
			DH6-19:	17
DH7	DQ52	8 (21%)	DH7-27:	8

a. The new nomenclature is derived from Corbett et al.[23] whereas the old nomenclature is according to Ichihara et al.[51]

was observed in combination with deletion of both *TCRD* alleles.

Based on the above-described results, we analyzed the association of cross-lineage *IGH* gene rearrangements and *TCRD* gene configuration for 101 of the 118 T-ALLs. Rearrangements in the *IGH* locus appeared to be almost exclusively associated with *TCRD* gene rearrangements. They were evenly distributed between cases with 1 rearranged and 1 germline *TCRD* allele (1 of 3 cases [33%]), cases with biallelic *TCRD* rearrangements (15 of 47 cases [32%]) and cases with 1 rearranged and 1 deleted *TCRD* allele (7 of 22 cases [32%]). Remarkably, only 1 of 26 cases with biallelically deleted *TCRD* genes (4%) displayed a cross-lineage *IGH* gene rearrangement.

Taking these data together, cross-lineage *IGH* gene rearrangements occurred in only 5% (2/38) of $\alpha\beta$-lineage T-ALL, i.e., either TCR$\alpha\beta^+$ T-ALL (n = 25) or CD3$^-$ T-ALL with biallelic *TCRD* deletion (n = 13).

Complete VH-(DH-)JH and incomplete DH-JH rearrangements

Detailed PCR analysis of the *IGH* locus in the 26 patients with Southern blot documented *IGH* gene rearrangements was based on 13 primer combinations covering the vast majority of complete VH-(DH-)JH joinings and potentially all incomplete DH-JH rearrangements. Heteroduplex PCR analysis revealed a total of 39 clonal homoduplexes, reflecting seven (18%) complete VH-(DH)-JH joinings and 32 (82%) incomplete DH-JH rearrangements. Complete VH-(DH)-JH rearrangements were found in 4 CD3$^-$ T-ALL and 2 TCR$\gamma\delta^+$ T-ALL patients, with 1 TCR$\gamma\delta^+$ T-ALL showing biallelic

VH-(DH-)JH joinings. Heteroduplex PCR analysis for incomplete DH7-27-JH rearrangements is shown in Figure 2.

Sequence analysis confirmed monoclonality in 7 complete VH-(DH-)JH rearrangements, which involved 7 different gene segments from 4 families: VH1-3, VH1-69, VH3-13, VH3-23, VH3-33, VH4-4, and VH6-1. None of the rearrangements was potentially functional. Six sequences were out-of-frame joinings, whereas the single rearrangement with an in-frame VH-JH contained a stop codon in the junctional region.

DH6-19 and DH7-27 are preferentially used in *IGH* gene rearrangements in T-ALL

Sequence analysis of the junctional regions of complete VH-(DH-)JH recombinations allowed identification of a D segment in 5 of 7 joinings. Moreover, 1 incomplete rearrangement was of the DH-DH-JH type. The frequencies of different DH family members found among the 38 identified DH sequences in the complete and incomplete cross-lineage *IGH* gene rearrangements are summarized in Table 2. Usage of the DH6 family was most prominent (47%), with the DH6-19 gene segment being preferentially used (45% of all identified DH sequences; Table 3). The second most frequently used DH segment was DH7-27 (21%). Seven rearrangements (18%) contained DH segments of the DH1 family, whereas 5 other rearrangements used various segments of the remaining 4 DH families (Table 2). Taken together, only 5 of the 38 rearranged DH segments belonged to the most upstream part of the DH region, whereas all other 33 DH gene segments (87%) belonged to the most downstream part of the DH region (Table 2). No relationship between age and DH gene segment usage was observed.

The sizes of the DH-JH junctional regions ranged from 0 to maximally 32 nucleotides, with an average of 7.6 nucleotides. Three of 37 DH-JH junctions (8%) did not have any randomly inserted N-region nucleotides. P-nucleotides, indicating the absence of deletion, were present in 7 DH-JH joinings (19%).

Table 3. Junctional region sequences of oligoclonal *IGH* gene rearrangements in a T-ALL patient (T061) illustrating the ongoing recombination process.

No	Upstream VH segment	N- and P-nucleotides[b]		DH6-19		N- and P-nucleotides[a]		JH gene segments
1.	–	–		GGGTATAGCAGTGGCTGGT	(-2)	TTTTCTTCTA	(-6)	JH6c
2.	VH1-03 (0)	TAGT	(-2)	GTATAGCAGTGGCTGGT	(-2)	TTTTCTTCTA	(-6)	JH6c
3.	VH3-33 (-1)	TAAGGGTGTGATG-TGTTTTGTGGA	(-11)	TGGCTGGTAC	(0)	GAGGCTGGCAGGGGGA	(-14)	JH6b

a. Underlined nucleotides represent P-nucleotides.

Table 4. Usage of different JH gene segments in incomplete and complete cross-lineage *IGH* gene rearrangements in T-ALL as compared with precursor-B-ALL, human BM precursor-B-cells, and PB B lymphocytes.[a]

JH segment	T-ALL (n=39)	Precursor-B-ALL	Human BM precursor-B-cells	Human PB B lymphocytes
JH1	13% (5)	2%	0%	1%
JH2	15% (6)	2%	4%	0%
JH3	10% (4)	11%	14%	9%
JH4	33% (13)	32%	32%	53%
JH5	8% (3)	22%	17%	15%
JH6	21% (8)	32%	33%	22%

a. The B-lineage data were obtained in comparable PCR-based studies by Steenbergen et al.[37] for precursor-B-ALL, by Raaphorst et al.[36] for human BM precursor-B-cells and by Yamada et al.[35] for human blood B lymphocytes.

Usage of JH gene segments in cross-lineage *IGH* gene rearrangements

The frequencies of different JH gene segments in *IGH* gene rearrangements in T-ALL are summarized in Table 4. The JH4 gene segment was found most frequently in approximately one third of joinings, followed by JH6 in 20% of cases. The remaining 4 JH segments were almost equally used, each comprising 10% to 15% of the rearrangements.

Oligoclonality in cross-lineage *IGH* gene rearrangements in T-ALL

SB analysis and heteroduplex PCR analysis showed fully concordant results in 20 of 26 cases with cross-lineage *IGH* gene rearrangements in T-ALL. In 1 case with a single rearranged band on SB, we were not able to amplify the clonal rearrangement with the applied primer sets. In the remaining 5 cases the number of clonal PCR-detected homoduplexes was higher than the number of rearranged bands in SB, which may suggest the presence of minor subclones undetectable by SB. In 1 of these seemingly oligoclonal cases the identified incomplete and complete rearrangements (1 and 2 in Table 3) shared the same DH-JH fragment suggesting ongoing VH to DH-JH joining. In 4 other patients, the detected rearrangements were not related in their used gene segments. This may reflect secondary DH-JH joining, with concomitant deletion of a pre-existing DH-JH rearrangement.

Based on the combined SB/PCR results, we found evidence for *IGH* oligoclonality at diagnosis in 27% (7/26) of T-ALL patients with this type of cross-lineage recombination. This includes the above-mentioned 5 cases with the higher number of PCR-detected homoduplexes than the number of rearranged bands in SB and 2 additional PCR-positive cases with weak bands on SB analysis, apparently derived from subclones.[20]

DISCUSSION

We investigated a large group of 118 T-ALL patients for the presence of cross-lineage *IGH* gene rearrangements. Based on SB analysis we identified such rearrangements in 22% of T-ALL, which is slightly higher than previously reported.[11,12] The vast majority (82%) of *IGH* gene rearrangements in T-ALL concerned incomplete DH-JH joinings. However, complete VH-(DH-)JH recombinations were also documented in our group of T-ALL patients. The usage of VH gene segments was seemingly random and not limited to the ones most proximal to the JH cluster. Nevertheless, 6 of 7 involved VH gene segments were derived from the proximal 3' portion of the *IGH* locus, a pattern that is seen in first trimester fetal VH-(DH)-JH rearrangements.[31] None of the seven VH-(DH-)JH rearrangements was potentially functional. To our knowledge this is the first extensive evidence for the occurrence of clonal complete *IGH* gene rearrangements in T-ALL. They were previously reported in a single case of T-lymphoblastic non-Hodgkin's lymphoma, which is the lymphomatous counterpart of T-ALL.[32]

In both the VH-(DH-)JH and DH-JH rearrangements we found a strikingly preferential usage of the more downstream DH gene segments (87% of identified DH sequences), especially DH6-19 (45%) and DH7-27 (21%). The DH6-19 gene segment is one of the gene segments that were recently discovered thanks to complete sequencing of the DH region.[23] Retrospective analysis of nearly 900 *IGH* junctional regions of B-lineage cells showed DH6-19 involvement in approximately 5% of the rearrangements, which is significantly higher than would be expected on a random basis.[23] The same holds true for 8 other DH segments, but 6 of them were not found in our T-ALL patients. The preferential usage of DH6-19 could not be explained by a more optimal RSS at the 3' end in comparison to other DH segments, either. Further studies are needed to define whether this gene segment is also preferentially rearranged in precursor-B-cells or whether this finding only relates to the cross-lineage phenomenon in T-ALL. In contrast to DH6-19, earlier reports indicated that the DH7-27 gene segment is involved in a significant proportion of *IGH* gene rearrangements in T-ALL.[13,14] Interestingly, we also found this gene segment in a cross-lineage *IGH* gene rearrangement of a TCR$\alpha\beta^+$ T-prolymphocytic leukemia (Figure 2). The DH7-27 gene segment is also preferentially used by fetal B-cells but is rarely observed in adult BM and PB.[33-36] In the analysis of nearly 900 *IGH* junctional regions of B-lineage cells, DH7-27 was found in only 0.5% of the rearrangements.[23] Since DH7-27 consists of only 11 nucleotides, it is more difficult to identify this gene segment in a junctional region after moderate trimming during V(D)J recombination, if stringent assignment criteria are used.

The analysis of JH segment usage revealed a more frequent use of JH4 and JH6 gene segments, which is also the case in normal and leukemic B-lineage cells (Table 4).[35-37] However, in our T-ALL group a significant proportion of joinings involved the

most upstream JH1 and JH2 gene segments (28%), which are rarely used by B-lymphocytes (~1%) and B-cell precursors (3% to 4%).[35-37] In conclusion, the high frequency of incomplete DH-JH rearrangements together with the frequent usage of the more downstream DH gene segments and the most upstream JH1 and JH2 gene segments suggest a predominance of the most immature *IGH* rearrangements in T-ALL. This particular DH-JH rearrangement pattern appears to be nonrandom and is not comparable with any known stage of B-cell ontogeny or B-cell differentiation.[31]

Since the types of preferential *IGH* gene rearrangements have now been identified, it would be relatively easy to screen T-ALL patients for the presence of cross-lineage *IGH* gene rearrangements and apply them as PCR targets for monitoring of minimal residual disease (MRD) in T-ALL. Three primer combinations (DH1, DH6, and DH7 in combination with a JH consensus primer) can identify 85% of incomplete DH-JH rearrangements in T-ALL. However, we observed oligoclonality in the *IGH* locus in 27% of T-ALL patients with rearranged *IGH* genes. In 1 case, we found evidence for continuing VH to DH-JH recombination, whereas VH replacements have been described previously during disease progression of a T-lymphoblastic lymphoma.[32] Secondary rearrangements via continuing VH to DH-JH joining, VH gene replacements, and de novo *IGH* gene rearrangements have been reported for precursor-B-ALL.[38-40] These processes may lead to the emergence of clones with secondary *IGH* rearrangements. In a previous study, we compared the *IGH* gene rearrangement patterns between diagnosis and relapse in 40 ALL patients and found that at least one major *IGH* rearrangement was stable in most cases.[41] Therefore, cross-lineage *IGH* gene rearrangements might be useful as supplementary MRD target in addition to leukemia-specific *TCRG* and *TCRD* gene rearrangements, and *TAL1* gene deletions.[24,42]

Cross-lineage *IGH* gene rearrangements occurred most frequently in TCR$\gamma\delta^+$ T-ALL (50% of patients) and in 20% of CD3$^-$ T-ALL, but we found them in a single case of TCR$\alpha\beta^+$ T-ALL (4%). Moreover, there was a striking association between the presence of rearranged *TCRD* genes and the occurrence of *IGH* recombination. A similar association was previously found for cross-lineage gene rearrangements in acute myeloid leukemia, suggesting that *TCRD* and *IGH* genes are concomitantly accessible for V(D)J recombinase in early hematopoietic precursors.[43] Furthermore, in the genotypically most mature T-ALL subgroup with biallelic *TCRD* gene deletions, an *IGH* gene rearrangement was observed in only 1 of 26 cases. Because *IGH* gene rearrangements are also rare in mature T-cell malignancies (~5%),[11] this suggests that the *IGH* locus may be accessible to V(D)J recombinase activity only in cells at earlier stages of T-cell differentiation. In this context, TCR$\gamma\delta^+$ T-ALL should be regarded as cells that branched off T-cell development at an early stage of completion of *TCRD* and *TCRG* gene rearrangement processes, when the recombinase activity is still retained.[16]

Incomplete DH-JH rearrangements are one of the earliest events during normal B-cell development and are already found in $CD34^{+}/CD19^{-}/CD10^{+}$ precursor cells.[44-45] Most of the more mature $CD34^{+}/CD19^{+}/CD10^{+}$ B-lineage precursors contain at least 1 DH-JH rearranged allele and frequently also complete VH-(DH-)JH rearrangements.[45-46] One could therefore speculate that cross-lineage *IGH* gene rearrangements in T-ALL might reflect malignant transformation of a thymocyte derived from a $CD34^{+}/CD19^{-}/CD10^{+}$ precursor cell with rearranged *IGH* genes. This idea may be supported by a murine model, in which *IGH* rearrangements were only found at an intermediate stage of thymocyte development.[47] Nevertheless, this phenomenon was not observed in normal human thymocytes.[44] Furthermore, the absence of D-J-Cμ transcripts in fetal human thymocytes depleted of $CD34^{+}$- and/or $CD19^{+}$-bearing cells suggest that DH-JH rearrangements in humans may be restricted to normal B-lineage differentiation.[44,48] It has also been suggested, that *IGH* gene rearrangements in T-cell-precursors may be an aberrant event directing cells into apoptotic pathway, unless they become immortalized by malignant transformation.[3] An alternative explanation could be that cross-lineage *IGH* gene rearrangements in T-ALL are postoncogenic events resulting from the ongoing activity of the common B- and T-cell V(D)J recombinase system on accessible gene loci.[3,49,50] We favor the last hypothesis, because this would explain the virtual absence of *IGH* gene rearrangements in normal thymocytes and mature T-cell malignancies on the one hand and the presence of *IGH* oligoclonality and secondary VH-(DH-)JH rearrangements in T-ALL on the other hand.[41] The virtual absence of *IGH* gene rearrangements in αβ-lineage T-ALL with biallelic *TCRD* gene deletions suggests that the recombination system is not active in this more mature type of T-ALL. Apparently, the recombinational activity is switched off as soon as the rearrangement and deletion processes in the *TCRA/TCRD* locus are completed.

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CHAPTER 2.8

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*

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ABSTRACT

In order to gain insight into immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements in adult acute lymphoblastic leukemia (ALL), we studied 48 adult patients: 26 with precursor-B-ALL and 22 with T-ALL. Southern blotting (SB) with multiple DNA probes for the *IGH, IGK, TCRB, TCRG, TCRD* and *TAL1* loci revealed rearrangement patterns largely comparable to pediatric ALL, but several differences were found for precursor-B-ALL patients. Firstly, adult patients showed a lower level of oligoclonality in the *IGH* gene locus (five out of 26 patients; 19%) despite a comparable incidence of *IGH* gene rearrangements (24 out of 26 patients; 92%). Secondly, all detected *IGK* gene deletions (n = 12) concerned rearrangements of the kappa deleting element (Kde) to Vκ gene segments, which represent two thirds of the Kde rearrangements in pediatric precursor-B-ALL and only half of the Kde rearrangements in mature B-cell leukemias. Thirdly, a striking predominance of

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immature Dδ2-Dδ3 cross-lineage recombinations was observed (seven out of 16 *TCRD* rearrangements; 44%), whereas more mature Vδ2-Dδ3 gene rearrangements occurred less frequently (six out of 16 *TCRD* rearrangements; 38% versus >70% in pediatric precursor-B-ALL). Together these data suggest that the Ig/TCR genotype of precursor-B-ALL is more immature and more stable in adults than in children.

We also evaluated whether heteroduplex analysis of polymerase chain reaction (PCR) products of rearranged Ig and TCR genes can be used for identification of molecular targets for minimal residual disease (MRD) detection. Using five of the major gene targets (*IGH, IGK, TCRG, TCRD* and *TAL*1 deletion), we compared the SB data and heteroduplex PCR results. High concordance between the two methods ranging from 96 to 100% was found for *IGK*, *TCRG*, and *TAL*1 genes. The concordance was lower for *IGH* (70%) and *TCRD* genes (90%), which may be explained by incomplete or 'atypical' rearrangements or by translocations detectable only by SB. Finally, the heteroduplex PCR data indicate, that MRD monitoring is possible in almost 90% of adult precursor-B-ALL and >95% of adult T-ALL patients.

INTRODUCTION

Recent advances in molecular biological and cytogenetic analysis of acute lymphoblastic leukemia (ALL) have revealed great heterogeneity within the disease[1,2] Based on the biological features of the leukemic clone, it is possible to predict the clinical aggressiveness of the malignant process and to select a treatment strategy in several subtypes.

In contrast to pediatric ALL with long-term event-free survival in more than 70% of patients, adult ALL is not associated with such a favorable prognosis.[3,4] Some factors responsible for this difference have been characterized, namely advanced age and hyperleukocytosis at presentation, higher frequency of more immature B or T lineage phenotypes, lack of *TEL* gene rearrangements, and the relatively high frequency of the Ph^1 translocation t(9;22), that is found in 30% of adult precursor-B-ALL patients.[3,5] So far, information about the immunogenotype of adult ALL is sparse and usually is derived from studies involving both adult and childhood patients.[2,6,7] This information is determined by analyzing the configuration of immunoglobulin (Ig) and T-cell receptor (TCR) genes, generally by means of Southern blotting (SB).[8]

SB of Ig and TCR gene rearrangements is a very reliable technique for clonality assessment in suspect lymphoproliferations, with a sensitivity of 1-5%.[8] It is considered to be the gold standard for this type of diagnostics.[8-10] However, SB is time-consuming, laborious and relatively expensive, limiting its applicability to specialized molecular laboratories. During the last decade polymerase chain reaction (PCR) techniques have proven to be extremely useful in molecular diagnostics. PCR analy-

sis is also successfully applied for clonality assessment based on the analysis of Ig and TCR gene recombinations.[11-14] However, due to the sensitivity of PCR methods, gene rearrangements in normal lymphocytes are also amplified, generally resulting in PCR products detectable in agarose or polyacrylamide gels. In order to distinguish between monoclonality and polyclonality, additional analysis of PCR products is necessary by means of denaturing gradient gel electrophoresis, fingerprinting technique, gene scanning analysis, or direct sequencing; these techniques require special equipment and/or radioactivity facilities.[15-19] Heteroduplex analysis of PCR-amplified junctional regions of Ig and TCR genes was found to be a cheap, non-radioactive, and easy alternative approach leading to reliable detection of clonal lymphoid cell populations in a polyclonal background.[20-22]

In order to determine the configuration of the Ig and TCR genes in adult ALL, we performed SB analysis on a series of 48 cases with an extensive set of DNA probes. Using the SB results as a reference, heteroduplex PCR analysis was evaluated for clonality assessment in this series of adult ALL. This PCR approach is especially important for rapid and easy identification of Ig and TCR gene rearrangements as PCR targets for sensitive monitoring of minimal residual disease (MRD).[23-25]

MATERIALS AND METHODS

Cell samples

Cell samples were obtained from 48 newly identified adult ALL patients enrolled in the HOVON-18 study in four centers (University Hospital Rotterdam, Free University Hospital Amsterdam, University Hospital Leuven and University Hospital Utrecht). This concerned 15 female patients and 33 male patients with a mean age of 33 years (range: 15-61 years). The precursor-B or T lineage of the ALL samples was determined by flow cytometric immunophenotyping, based on standard criteria, using a wide panel of monoclonal antibodies.[26,27] Mononuclear cells (MNC) were isolated from peripheral blood (PB) or bone marrow (BM) by Ficoll-Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. Some of the cell samples were frozen and stored in liquid nitrogen.

Southern blot analysis

DNA was isolated from fresh or frozen MNC fractions as described previously.[8] Fifteen micrograms of DNA were digested with the appropriate restriction enzymes (Pharmacia), size-separated in 0.7% agarose gels and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as described.[8] Ig heavy chain (*IGH*) and Ig kappa light chain (*IGK*) gene configurations were analyzed with the IGHJ6, IGKJ5, IGKC and IGKDE probes (DAKO Corporation, Carpinteria, CA, USA) in *Bgl*II and *Bam*HI/*Hin*dIII digests.[28,29] Incomplete and complete TCR beta (*TCRB*) gene rearrangements were detected with TCRBD1U, TCRBD1, TCRBJ1, TCRBD2U, TCRBD2, TCRBJ2 and TCRBC probes (Langerak et al, manuscript in preparation, DAKO Corporation) in *Eco*RI and *Hin*dIII digests. TCR gamma (*TCRG*) gene rearrangements were analyzed with the Jγ1.2 probe in *Bgl*II digests and the Jγ1.3 and Jγ2.1 probes in *Eco*RI digests.[8,30,31] The configuration of the TCR delta (*TCRD*) genes was analyzed with the TCRDV2, TCRDD2, TCRDJ1, TCRDJ2, TCRDJ3, TCRDRE, and TCRAPJ probes (DAKO Corporation) in *Bgl*II, *Eco*RI, and *Hin*dIII digests.[32] The *TAL1* gene was screened by means of the SILDB probe in *Bgl*II, *Hin*dIII, and *Eco*RI digests.[33]

PCR amplification

PCR was essentially performed as described previously.[34] In each 100μl PCR reaction 0.1 μg DNA sample, 12.5 pmol of the 5′ and 3′ oligonucleotide primers, and 1 U *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA) were used. The sequences of the majority of oligonucleotides used for amplification of *IGH*, *IGK* (kappa deleting element; Kde), *TCRG*, *TCRD*, and *TAL1* gene rearrangements were published before.[33,35-37] Some of the primers were newly designed during the BIOMED-1 Concerted Action '*Investigation of minimal residual disease in acute leukemia: international standardization and clinical evaluation*' (Pongers-Willemse et al., manuscript in preparation). All primers were synthesized on an ABI 392 DNA synthesizer (Applied Biosystems, Foster City, CA, USA) using the solid-phase phosphotriester method. PCR conditions were 1 min at 94°C, 1 min at 55° to 60°C, and 2 min at 72°C for 30 to 35 cycles using a Perkin-Elmer thermal cycler (Perkin-Elmer Cetus). After the last cycle an additional extension step of 7 min at 72°C was performed. Appropriate positive and negative controls were included.

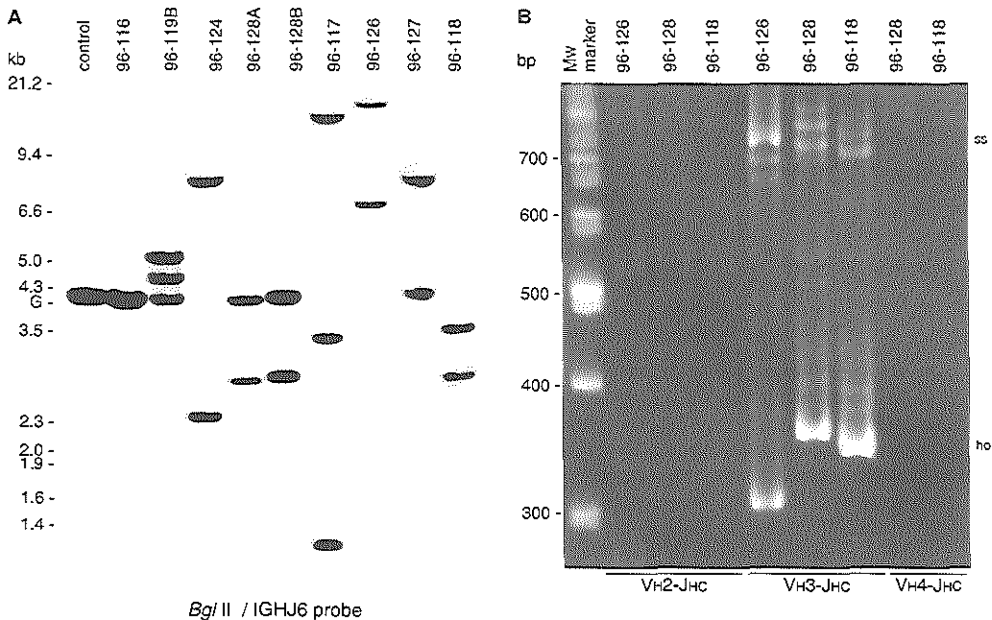

Figure 1.
(A) SB analysis of the *IGH* genes in several adult precursor-B-ALL and one T-ALL. Control DNA and DNA from ALL samples were digested with *Bgl*II, size separated, and blotted onto nylon membrane filters, which were hybridized with the ^{32}P-labeled IGHJ6 probe. The sizes (in kb) of the molecular weight marker are indicated. In all presented precursor-B-ALL cases at least one *IGH* gene rearrangement was detected, while the single T-ALL case (96-116) revealed germline configuration. In one precursor-B-ALL patient (96-117) three rearranged bands of comparable density were demonstrated, suggesting trisomy 14. **(B)** Heteroduplex PCR analysis for three patients showing the results with VH family primers for the three most commonly used VH families and a JH consensus primer. Samples showing positivity on an agarose gel were subsequently subjected to heteroduplex PCR analysis. In patients 96-118 and 96-126 one clonal VH3-JH homoduplex was found whereas SB showed biallelic rearrangements. In patient 96-128 the monoclonal VH3-JH homoduplex corresponded to the monoallelic rearrangement as detected by SB.

Heteroduplex analysis of PCR products

For heteroduplex analysis, the PCR products were denatured at 94°C for 5 min after the final cycle of amplification and subsequently cooled to 4°C for 60 min. to induce duplex formation.[22] After duplex formation the hetero- and/or homoduplexes were immediately loaded on 6% non-denaturing polyacrylamide gels in 0.5 x Tris-Boric acid-EDTA (TBE) buffer, run at room temperature, and visualized by ethidium bromide staining. *Pst*I-digested lambda DNA was used as a size marker. Both SB and heteroduplex analysis of PCR products were performed in parallel in a double-blind manner. Concordance between the two methods was calculated assuming that the following gene configurations concerned the same alleles: rearranged band in SB = presence of clonal homoduplex in PCR; germline band and/or deletion in SB = no clonal PCR product.

RESULTS

Patients

A total of 48 adult ALL patients enrolled in the HOVON-18 study were analyzed for Ig and TCR gene rearrangements as well as for the presence of *TAL1* gene deletion. This concerned 26 precursor-B-ALL and 22 T-ALL.

SB analysis of *IGH* gene rearrangements

DNA samples from 47 adult ALL patients were examined for the presence of *IGH* gene rearrangements (Figure 1). In the total group of 26 precursor-B-ALL patients, *IGH* gene recombination was detected in 24 cases (92%)(Table 1), while the remaining two cases revealed a germline configuration. In four precursor-B-ALL patients (15%) more than two rearrangements were found. Obvious differences in density of two or more rearranged bands, suggesting the presence of subclones, were observed in five precursor-B-ALL patients (19%). In one patient three rearranged

Table 1. Frequencies of Ig, TCR, and *TAL1* gene rearrangements in adult ALL as detected by SB analysis.

Type of gene rearrangement	Precursor-B-ALL (n = 26)[a]	T-ALL (n = 22)
IGH	24 (92%)	5 (24%)[b]
IGK (Jκ)	4 (15%)	0[b]
IGK (Kde)	9 (35%)	0[b]
TCRB	7 (27%)	18 (82%)
TCRG	15 (58%)	19 (86%)
TCRD	12 (46%)	15 (68%)
TAL1 deletion	0	2 (9%)

a. Excluding patients with weak gene rearrangements only, probably derived from subclones.
b. One T-ALL patient was not analyzed by SB with *IGH* and *IGK* probes, because of an insufficient amount of DNA.

bands of comparable density were demonstrated (Figure 1), suggesting trisomy 14 but cytogenetic analysis was inconclusive. Additionally, *IGH* gene rearrangements on one or even both alleles were found in five of the 21 tested T-ALL patients (24% of cases) (Table 1).

SB analysis of *IGK* gene rearrangements

46% (12 out of 26) of precursor-B-ALL patients contained rearranged *IGK* genes. Four patients (15%) demonstrated Vκ to Jκ rearrangements (Table 1). The deletional Kde rearrangements (n = 12) exclusively involved Vκ genes and were found in nine patients (35%) (Table 1). No deletional rearrangements of Kde to the recombination signal sequence in the Jκ-Cκ intron (intron RSS) were found.[29] *IGK* gene rearrangements were not detected in any of the 21 T-ALL patients studied (Table 1).

SB analysis of *TCRB* gene rearrangements

Using our well-defined TCRBD1U, TCRBD1, TCRBJ1, TCRBD2U, TCRBD2, TCRBJ2, and TCRBC probes, it was possible to distinguish between complete Vβ-Jβ and incomplete Dβ-Jβ rearrangements in the β1 and β2 regions.

Table 2. Allelic frequencies of particular *TCRB* gene rearrangements in adult ALL as detected by SB analysis.

TCRB gene configuration	Precursor-B-ALL (n = 26 patients, 52 alleles)	T-ALL (n = 22 patients, 44 alleles)
Germline	42 (81%)	11 (25%)
Rearranged	10 (19%)	33 (75%)
Number of rearrangements[a]	14[a]	41[a]
*TCRB*1 region:		
Vβ-Dβ1	0	0
Dβ1-Jβ1	0	4 (10%)
Vβ-Jβ1	0	6 (15%)
Unidentified to β1	0	1 (2%)
*TCRB*2 region:		
Vβ-Dβ2	0	0
Dβ1-Dβ2	0	0
Vβ-Jβ2	5 (36%)	19 (46%)
Dβ1-Jβ2	1 (7%)	2 (5%)
Dβ2-Jβ2	1 (7%)	6 (15%)
Unidentified to β2	7 (50%)	3 (7%)

a. The number of rearrangements is higher than the number of rearranged alleles, because of the incidental occurrence of rearrangements in the β1 region as well as the β2 region of the same allele and/or because of subclones.

Eighteen T-ALL patients (82%) had rearranged *TCRB* genes (Table 1). Allelic frequencies of the different types of *TCRB* recombinations are summarized in Table 2. Predominance of *TCRB2* rearrangements was characteristic for T-ALL ('*TCRB2/TCRB1* ratio' of 2.7:1). More than half (61%) of the detected *TCRB* rearrangements in T-ALL concerned complete Vβ-Jβ recombinations (Table 2).

Cross-lineage *TCRB* gene rearrangements were found in seven (27%) precursor-B-ALL (Table 1). These were all restricted to the *TCRB2* locus; no rearrangements were detected with the *TCRB1* region probes (Table 2).

SB analysis of *TCRG* gene rearrangements

Eighty six percent (19 out of 22 cases) of T-ALL patients were found to have rearranged *TCRG* genes (Table 1), predominantly on both alleles (77%). In the vast majority V gene segments of the VγI family were found to be rearranged to Jγ1.3 or Jγ2.3 gene segments (Figure 2 and Table 3). Only two T-ALL patients were found to have rearrangements to Jγ1.1 or Jγ2.1 gene segments, while in a single case a clon-

Table 3. Allelic frequencies of particular *TCRG* gene rearrangements in adult ALL as detected by SB analysis.

TCRG gene configuration	Precursor-B-ALL (n = 26 patients, 52 alleles)	T-ALL (n = 22 patients, 45 alleles)[a]
Germline	28 (54%)	7 (16%)
Deletion	2 (4%)	1 (2%)
Rearrangements	22 (42%)	37 (82%)
Number of rearrangements	23[b]	37
Involving Jγ1.1/2.1	3 (13%)	2 (5%)
Involving Jγ1.2	0	1 (3%)[c]
Involving Jγ1.3/2.3	20 (87%)	34 (92%)
VγI: Vγ2/4-Jγ1.3/2.3	5 (22%)	14 (38%)
Vγ3-Jγ1.3/2.3	3 (13%)	3 (8%)
Vγ5-Jγ1.3/2.3	2 (9%)	1 (3%)
Vγ7-Jγ1.3/2.3	1 (4%)	0
Vγ8-Jγ1.3/2.3	0	8 (22%)
VγII-Jγ1.3/2.3	7 (30%)	2 (5%)
VγIII-Jγ1.3/2.3	0	5 (14%)
VγIV-Jγ1.3/2.3	2 (9%)	0
Unidentified to Jγ1.3/2.3	0	1 (3%)

a. The number of indicated alleles is 45 instead of 44, because one T-ALL patient showed three mono clonal rearrangements, most probably as a result of trisomy 7.

b. Including one weak rearrangement, probably derived from a subclone.

c. One T-ALL patient showed a monoclonal Vγ9-Jγ1.2 rearrangement, typical for peripheral blood T-cells, but rarely found in T-ALL cells.

al Vγ9-Jγ1.2 rearrangement was discovered. In one patient three rearranged bands of comparable density were demonstrated, suggesting trisomy 7. Allelic frequencies of *TCRG* gene rearrangements are shown in detail in Table 3.

Cross-lineage *TCRG* recombinations were discovered in 58% of precursor-B-ALL (Table 1), and in two patients this was associated with *TCRG* gene deletion on one allele. Similarly to T-ALL, rearrangements to Jγ1.3 or Jγ2.3 gene segments predominated, being demonstrated in 15 patients on 19 alleles, while Jγ1.1 or Jγ2.1 rearrangements were found on only three alleles (Table 3).

SB analysis of *TCRD* gene rearrangements

TCRD gene rearrangements and/or deletions were observed in 21 out of 22 T-ALL patients studied. In 15 patients (68%) a rearranged *TCRD* gene was found on at least one allele (Figure 3 and Table 1). *TCRD* deletion of at least one allele occurred in 12 patients. Only one T-ALL patient had both *TCRD* alleles in germline configuration. This patient also had germline *TCRB* and *TCRG* genes. Precise frequencies of different *TCRD* gene rearrangements are summarized in Table 4.

In 12 out of 26 patients (46%) with precursor-B-ALL *TCRD* rearrangements were found (Table 1). In 13 patients (50%) *TCRD* genes were deleted on one or both alleles. All SB identifiable *TCRD* rearrangements were incomplete Vδ2-Dδ3 or Dδ2-Dδ3 recombinations (Table 4). In three precursor-B-ALL patients (12%) weak rearranged

Table 4. Allelic frequencies of particular *TCRD* gene rearrangements in adult ALL as detected by SB analysis.

TCRD gene configuration	Precursor-B-ALL (n = 26 patients, 52 alleles)	T-ALL (n = 22 patients, 44 alleles)
Germline	21 (40%)	3 (7%)
Deletion	18 (35%)	17 (39%)
Rearrangement	13 (25%)	24 (54%)
Number of rearrangements	16[a]	24
Vδ2-Dδ3	6 (38%)	0
Dδ2-Dδ3	7 (44%)	1 (4%)
Vδ1-Jδ1	0	9 (38%)
Dδ2-Jδ1	0	4 (17%)
Vδ2-Jδ1	0	1 (4%)
Vδ3-Jδ1	0	1 (4%)
Vδ6-Jδ2	0	1 (4%)
Unidentified to Jδ1	3 (19%)	7 (29%)

a. Including weak rearrangements (one Vδ2-Dδ3 and two unidentified rearrangements) most probably derived from subclones.

bands were observed suggesting the presence of subclones in these cases (Figure 4). Thirty-one percent of precursor-B-ALL patients were found to have the *TCRD* genes in germline configuration on both alleles.

SB analysis of *TAL1* gene rearrangements

All ALL patients were tested for the presence of *TAL1* rearrangements using the SILDB probe. A typical type 1 *TAL1* deletion, resulting from fusion of sildb and tal1db breakpoints was discovered in two T-ALL patients (Table 1). Both *TCRD* alleles were deleted in these two cases. No other *TAL1* gene rearrangements were found.

Comparison of SB and heteroduplex PCR analysis of Ig, TCR, and *TAL1* genes

DNA-based PCR amplification was simultaneously performed in a double blind manner for several of the Ig and TCR genes studied by SB analysis. Clonal V_H-J_H rearrangements were found by heteroduplex PCR analysis in 17 out of 25 analyzed precursor-B-ALL patients and in a single T-ALL patient (Figure 1). In contrast to 49 rearranged alleles found by classical SB, heteroduplex PCR analysis revealed 27 monoclonal products assumed to be derived from those alleles (Table 5). Using $V\kappa$

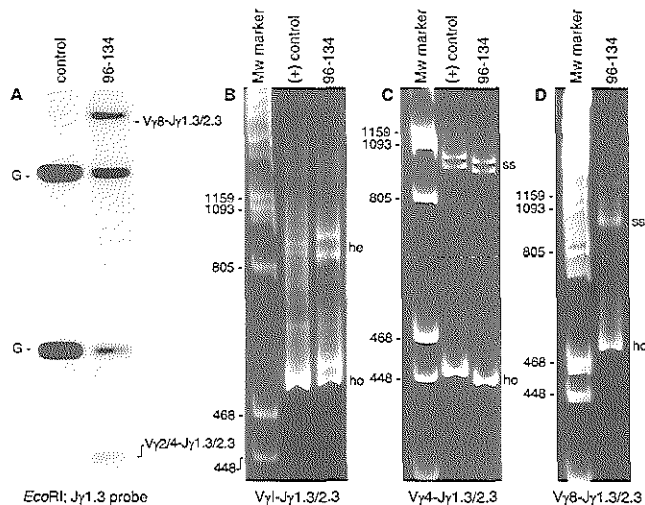

Figure 2.
Clonality assessment via SB analysis and heteroduplex PCR analysis of *TCRG* genes.
(A) DNA from a T-ALL patient was digested with *Eco*RI. The filter was hybridized with the ^{32}P-labeled Jγ1.3 probe and two rearrangements were found: Vγ2/4 to Jγ1.3 or Jγ2.3 and Vγ8 to Jγ1.3 or Jγ2.3. **(B)** Heteroduplex PCR analysis with a family-specific V primer (VγI) in combination with a Jγ1.3/2.3 primer showed two clonal VγI-Jγ1.3/2.3 homoduplexes as well as two heteroduplexes of clonal origin. **(C and D)** Further analysis with member-specific primers revealed two rearrangements: Vγ4-Jγ1.3/2.3 and Vγ8-Jγ1.3/2.3, identical to those detected by SB. ss, single-strand fragments; he, heteroduplexes; ho, homoduplexes.

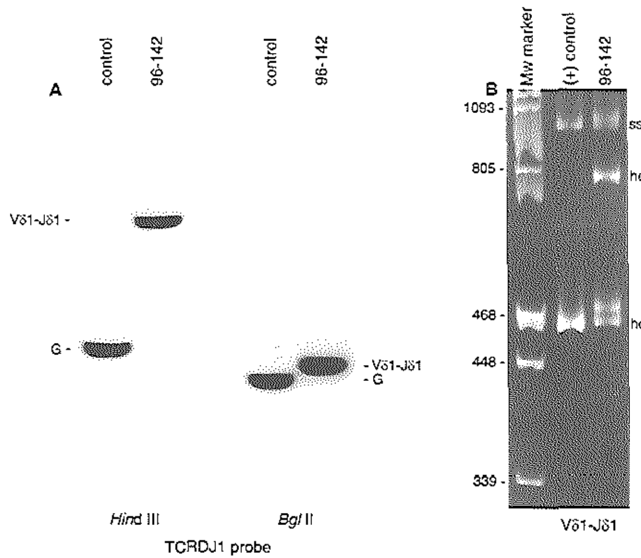

Figure 3.
Clonality assessment via SB analysis and heteroduplex PCR analysis of *TCRD* genes.
(A) DNA from a T-ALL patient was digested with *Bgl*II and *Hin*dIII. The filter was hybridized with the ^{32}P-labeled TCRDJ1 probe and biallelic Vδ1-Jδ1 rearrangements were found. **(B)** Heteroduplex PCR analysis with a Vδ1 primer in combination with a Jδ1 primer showed two clonal Vδ1-Jδ1 homoduplexes as well as (comigrating) heteroduplexes of clonal origin. ss, single-strand fragments; he, heteroduplexes; ho, homoduplexes.

family-specific primers and a Kde primer it was possible to amplify Vκ-Kde rearrangements in the eight precursor-B-ALL patients, which were also detected by means of SB. In contrast to 12 rearranged alleles found by SB, heteroduplex PCR analysis resulted in clonal products in 10 out of these 12 alleles (Table 5). PCR analysis of the *TCRG* genes revealed all except one rearrangement detected by the SB technique as well as three additional recombinations, all of them involving Jγ1.1 or Jγ2.1 gene segments (Table 5). Except for the presumed Vδ3-Jδ1, Vδ6-Jδ2 and ten unidentified rearrangements to Jδ1, all DNA samples that were found to contain clonal *TCRD* rearrangements with SB also revealed clonal PCR products (Figure 3 and Table 5). However, in contrast to the SB results, two precursor-B-ALL patients were found to have an oligoclonal pattern of Vδ2-Dδ3 rearrangements by heteroduplex PCR analysis (as exemplified in Figure 4). In concordance with the SB results, *TAL1* deletion PCR products were observed in both T-ALL patients (Table 5).

Table 5. Comparison of results obtained by SB analysis and heteroduplex PCR analysis.[a]

Gene (segment)	Rearrangements detected by SB analysis	Rearrangements detected by heteroduplex PCR analysis	Concordance between the two techniques
IGH	49 (57)	27[b]	71% (65%)
IGK (Kde)	12	10	98%
TCRG	58 (60)	62	94% (96%)
TCRD	37 (40)	28[c]	91% (90%)
TAL1	2	2	100%

a. Concordance between SB analysis and heteroduplex PCR analysis was calculated assuming that the following gene configurations concerned the same alleles: rearranged band in SB = presence of clonal (homoduplex) PCR product; germline band and/or deletion in SB = no clonal PCR product. The numbers and percentages in brackets express concordance between the two methods when weak rearranged SB bands (most probably derived from subclones) were included.

b. Including one precursor-B-ALL patient with a single PCR product of low intensity.

c. Including two precursor-B-ALL patients with oligoclonal Vδ2-Dδ3 rearrangements in heteroduplex PCR analysis.

DISCUSSION

The samples of leukemic cells from 48 adult ALL patients were analyzed for the configuration of Ig and TCR genes. Based on SB results *IGH* gene recombinations were detected in 92% of precursor-B-ALL patients, most of them (~80%) having two rearranged alleles. This is comparable to childhood ALL.[38,39] Germline *IGH* genes were found in two patients, probably reflecting malignant transformation of a very early B-cell precursor. Multiple rearranged bands were found in only 19% of cases, which is essentially lower than the 30-45% observed in childhood ALL.[39,40] Similarly to childhood precursor-B-ALL, *IGK* gene rearrangements were found in 46% of adult patients.[29] However, Kde rearrangements to intron RSS were not found in any of our adult ALL cases, although they comprise 31% of Kde rearrangements in childhood precursor-B-ALL and 54% of Kde rearrangements in chronic B-cell leukemias.[29,37] These differences in Ig gene rearrangement patterns might reflect biological differences between adult and childhood ALL, with adult precursor-B-ALL showing tendency towards a more immature and stable genotype.

Analysis of TCR gene rearrangements in adult ALL revealed configurations comparable to those previously discovered for childhood ALL patients.[25,41,42] Nevertheless, several discrepancies were found considering cross-lineage *TCRD* gene rearrangements in precursor-B-ALL. First of all, complete germline TCR gene patterns were found in 19% of adult precursor-B-ALL patients, which was mainly

caused by a lower incidence of *TCRD* deletions (35%) as compared to childhood ALL (45%).[32] In addition, incomplete Dδ2-Dδ3 rearrangements occurred most frequently, comprising approximately 45% of the *TCRD* recombinations, followed by the more mature Vδ2-Dδ3 rearrangements (38%). The latter rearrangement was found to be dominant in childhood precursor-B-ALL, comprising >70% of the rearrangements, while Dδ2-Dδ3 occurred in only 10% of the *TCRD* gene rearrangements.[32,43,44] Furthermore, heteroduplex PCR analysis demonstrated that three out of six Vδ2-Dδ3 rearrangements were derived from minor subclones. This phenomenon was previously reported in some cases of pediatric ALL.[44,45] Taken together, these data suggest that adult ALL has a more immature genotype as compared to pediatric ALL.

Southern blotting is considered to be the gold standard for clonality diagnostics. However, this technique is laborious, time consuming, relatively expensive, and requires large amounts of high quality DNA. This generally limits the applicability of the method to more specialized laboratories. PCR techniques offer good alternatives, as they are more rapid, less expensive, and require only small amounts of DNA. However, due to the enormous sensitivity, false-positive results might be obtained, if monoclonal PCR products cannot be distinguished from the background signals resulting from similar gene rearrangements in normal polyclonal lymphocytes. Heteroduplex analysis of PCR products was shown to be an effective procedure for such discrimination between monoclonal and polyclonal rearrangements.[22] Comparison of SB analysis and heteroduplex PCR analysis of *IGK* (Kde), *TCRG*, *TCRD*, and *TAL1* gene rearrangements showed a high concordance (>90%) between the two techniques. In four cases (two Vκ-Kde, one presumed Vδ3-Jδ1, and one presumed Vδ6-Jδ2 recombination) no clonal PCR products were obtained repeatedly. Unusual gene recombinations resulting in restriction fragments of comparable size on SB and/or extensive deletions of nucleotides in junctional regions might explain these negative PCR results. Ten rearrangements to Jδ1 as detected by SB could not be assigned to a particular Vδ-Jδ joining based on the sizes of clonal bands.[32] In those cases no clonal products were found with the applied *TCRD* primer sets. These rearrangements might therefore reflect Vα-Jδ1 rearrangements or translocations into the *TCRD* locus. In contrast, three additional rearrangements to Jγ1.1/2.1 were detected with the heteroduplex PCR technique as compared to SB analysis. These additional positive PCR results may be the consequence of the low sensitivity of the currently used Jγ2.1 SB probe. Furthermore, in two precursor-B-ALL patients with 'clonal Vδ2-Dδ3 bands' on SB analysis, heteroduplex PCR analysis revealed an oligoclonal pattern. Homoduplexes representing VH-JH rearrangements were found on only 27 alleles in contrast to 49 clonal bands detected by SB with the IGHJ6 probe. The overall concordance between SB and heteroduplex PCR analysis for *IGH* reached only 71%. Rearrangements not identified by PCR may include incomplete DH-JH recombinations or translocations to the JH region.[46] The overall

results indicate that heteroduplex PCR analysis is an effective, rapid, and reliable approach for Ig and TCR gene rearrangement studies in ALL. However, particular rearrangements like biallelic *TCRD* deletions or atypical rearrangements (e.g. Vα-Jδ1, incomplete DH-JH rearrangements or translocations) can only be detected by conventional SB analysis, unless many additional sets of PCR primers are used. An additional advantage of heteroduplex PCR analysis is that excised homoduplex bands can be successfully used for direct cycle sequencing, as was demonstrated by Beishuizen et al.[37] for Vκ-Kde rearrangements.

Based on clonal Ig and TCR gene rearrangements identified at diagnosis, it is possible to monitor MRD by use of PCR analysis in most adult ALL patients (Table 6). Heteroduplex PCR analysis revealed at least one patient-specific MRD-PCR target in 95% of T-ALL and almost 90% of precursor-B-ALL. Monitoring with two MRD-PCR targets would be possible for > 75% of precursor-B-ALL cases and 86% of T-ALL cases.

In conclusion, our data indicate a largely comparable pattern of Ig and TCR gene rearrangements between adult ALL and pediatric patients. However, the lower fre-

Table 6. Frequencies of Ig and TCR gene rearrangements in adult ALL, which can be used for PCR-based MRD detection.[a]

Ig and TCR gene rearrangements	Precursor-B-ALL	T-ALL
IGH:		
VH-JH	68%[b]	6%[b]
IGK:		
Vκ-Kde	31%	0%
intron-Kde	0%	0%
TCRG:		
Vγ-Jγ	62%	91%
TCRD:		
Vδ2-Dδ3 or Dδ2-Dδ3	35%	5 %
Dδ2-Jδ1 or Vδ-Dδ-Jδ1	0%	41%
total *TCRD*	35%	46%
TAL1 deletion	0%	9%
at least one PCR target	88%	95%
at least two PCR targets	77%	86%
at least three PCR targets	58%	50%

a. Weak rearranged bands as detected by SB and oligoclonal rearrangements as detected by heroduplex PCR analysis were excluded. This exclusion mainly concerned *TCRD* gene rearrangements (Vδ2-Dδ3 rearrangements) in precursor-B-ALL.

b. One precursor-B-ALL patient and six T-ALL patients were not analyzed by VH-JH PCR because of an insufficient amount of DNA.

quency of oligoclonal *IGH* gene rearrangements, the absence of intron RSS-Kde rearrangements, the higher frequency of germline *TCRD* genes and incomplete Dδ2-Dδ3 rearrangements, and the lower frequency of *TCRD* deletions were characteristic for adult precursor-B-ALL patients as compared to childhood precursor-B-ALL, suggesting a more immature genotype in adults. Furthermore, our results show that the genotype of malignant cells can be studied reliably by means of both SB and heteroduplex PCR analysis, because these techniques give highly comparable results in ALL. Finally, identification of clonally rearranged Ig and/or TCR genes as molecular targets for PCR-based MRD monitoring is possible in almost 90% of precursor-B-ALL and 95% of T-ALL in adults.

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CHAPTER 2.9

UNUSUAL IMMUNOGLOBULIN AND T-CELL RECEPTOR GENE REARRANGEMENT PATTERNS IN ACUTE LYMPHOBLASTIC LEUKEMIAS*

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ABSTRACT

Immunoglobulin (Ig) and T-cell receptor (TCR) genes are rearranged in virtually all acute lymphoblastic leukemia (ALL) cases. However, the recombination patterns display several unusual features as compared to normal lymphoid counterparts. Cross-lineage gene rearrangements occur in more than 90% of precursor-B-ALL and in ~20% of T-ALL, whereas they are rare in normal lymphocytes. Approximately 25-30% of the Ig and TCR gene rearrangements at diagnosis are oligoclonal, and can undergo continuing or secondary recombination events during the disease course. Based on our extensive molecular studies we hypothesize that the unusual Ig and TCR gene rearrangements in ALL occur as an early postoncogenic event resulting from the continuing V(D)J recombinase activity on accessible gene loci. This hypothesis is on the one hand supported by the virtual absence of cross-lineage gene rearrangements in normal lymphocytes and mature lymphoid malignancies and on the other hand by the presence of oligoclonality and secondary Ig and TCR gene rearrangements in ALL.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a malignancy of immature lymphoid cells. Leukemic lymphoblasts are considered as the malignant counterparts of normal bone marrow B-cell precursors and thymic T-cell precursors that have undergone

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malignant transformation resulting in precursor-B-ALL and T-ALL, respectively. The oncogenic events block further differentiation, freezing the cell in a particular stage of the development, and subsequently enable uncontrolled proliferation. In virtually all ALL cases leukemic cells contain rearranged immunoglobulin (Ig) and/or T-cell receptor (TCR) genes.[1,2] This is probably related to the fact that Ig and TCR gene rearrangements take place very early during lymphoid differentiation i.e. before surface expression of the pan-B-cell marker CD19 in B-cell progenitors and in $CD34^+/CD1a^-$ thymocytes, respectively.[3,4] In exceptional ALL cases, most probably derived from very immature progenitor cells, the Ig and TCR gene loci are preserved in germline configuration.

In contrast to normal lymphoid development, Ig and TCR gene rearrangements in ALL have several unusual characteristics. Owing to the observation of cross-lineage TCR gene rearrangements in precursor-B-ALL and in some mature B-cell malignancies together with the finding of rearranged Ig heavy chain (*IGH*) genes in some T-ALL, Ig and TCR gene rearrangements cannot be regarded as specific markers for B-lineage and T-lineage, respectively.[5] In contrast, cross-lineage gene rearrangements are not observed in normal human thymocytes, whereas their frequency in normal B-cells is very low (below 0.5%).[3,6] Other unusual characteristics of Ig and TCR gene rearrangements in ALL as compared to their normal counterparts are the overrepresentation of particular rearrangement types, e.g. the restriction to certain gene segments, the usage of pseudogenes, the presence of 'end-stage' rearrangements, and the occurrence of asynchronous combinations of gene rearrangements.[1,2,7]

Since a leukemic clone originates from a single malignantly transformed lymphoid cell, all lymphoblasts in principle have identically rearranged Ig and TCR genes i.e. monoclonal gene rearrangements. However, in a substantial proportion of ALL there is evidence of oligoclonality. Subclones frequently show junctional region sequences related to the major clone, reflecting a continuing rearrangement process. Ongoing recombinations may also result in changes of leukemia-specific rearrangements at relapse of the disease.[8-11]

The occurrence of unusual gene rearrangements in ALL can be explained in several ways. It could be that cross-lineage gene rearrangements occur in early lymphoid progenitors leading to a stop in further differentiation and maturation. As a consequence, a large fraction of such proliferating, immature lymphoid precursor cells without functional lineage-specific rearrangements and/or with cross-lineage gene rearrangements would normally undergo apoptosis unless they become neoplastic. An alternative explanation could be that unusual and cross-lineage gene rearrangements in ALL represent postoncogenic events resulting from the ongoing activity of the common B and T-cell V(D)J recombinase system on gene loci, which are accessible at the stage of maturational arrest and initial proliferation. Secondary and ongoing rearrangements would then take place during the proliferative phase as well.

Here we present the results of our extensive studies on Ig and TCR gene rearrangements patterns in ALL, which in our opinion favor the hypothesis of unusual gene recombination in ALL as a predominantly postoncogenic event.

RESULTS AND DISCUSSION

Lineage-specific Gene Rearrangements in ALL

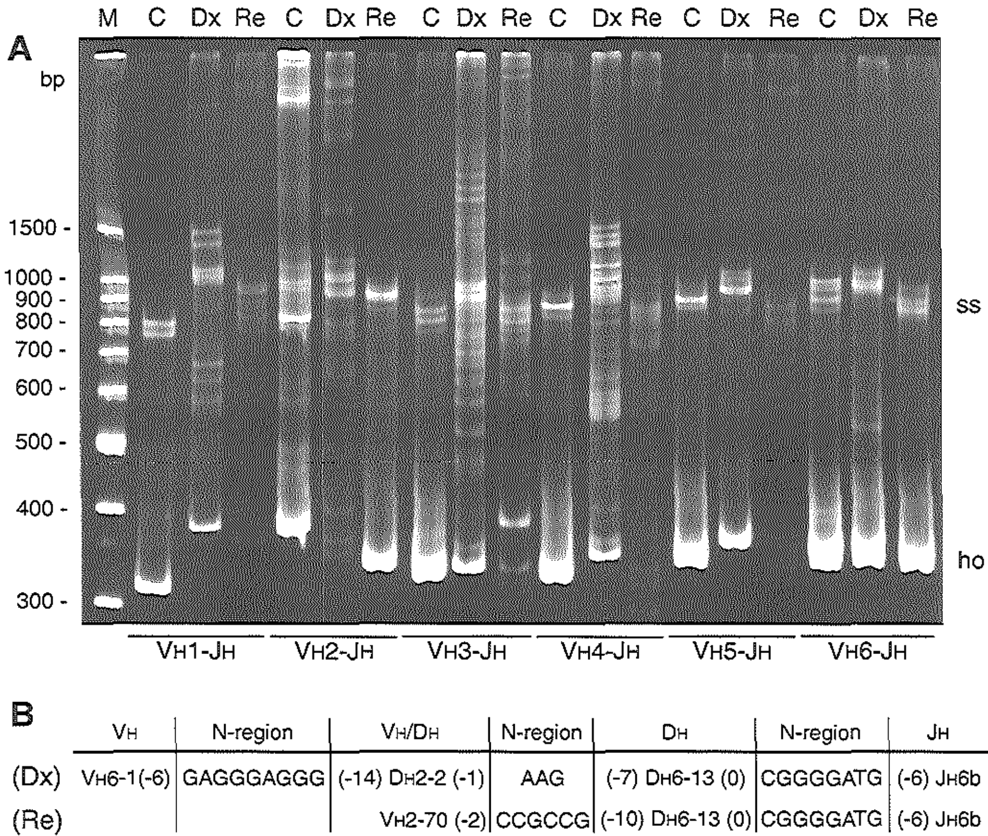
During the last years we performed detailed analysis of Ig and TCR gene rearrangements in 340 ALL patients at diagnosis (Table 1).[1,12-16] In virtually all precursor-B-ALL cases (>95%) Southern blot analysis revealed clonal *IGH* gene rearrangements. In a significant proportion of these patients (~40%) obvious differences in density of two or more rearranged bands were found, reflecting oligoclonality at the *IGH* locus.[12] Since PCR analysis is more sensitive than Southern blotting, one can expect that *IGH* oligoclonality might in fact even concern a larger fraction of precursor-B-ALL cases. Figure 1 illustrates a case with multiple clonal *IGH* gene rearrangements at diagnosis as detected by heteroduplex PCR analysis.[17] Furthermore, PCR studies have already shown that the vast majority of *IGH* gene rearrangements were not functional, and that D_H-D_H-J_H fusions could be identified in ~20% of patients.[18,19]

Ig light chain gene rearrangements were found in 60% of cases (Table 1). Rearrangements of the Ig kappa light chain (*IGK*) gene occur more frequently than in the Ig lambda light chain (*IGL*) gene locus and often concern deletional rearrangements to the kappa deleting element (Kde).[20] In contrast to normal B-cells we observed an unusually high frequency of *IGK* deletional rearrangements compared to *IGK* and *IGL* gene rearrangement frequencies. Oligoclonality of *IGK* gene rearrangements was observed in <10% of precursor-B-ALL patients.[12] Interestingly,

Table 1. Relative frequencies of Ig and TCR gene rearrangements in ALL.[a]

	IGH		*IGK*		*IGL*	*TCRB*	*TCRG*	*TCRD*	
	R	D	R	D				R	D
Precursor-B-ALL (children)	95%	3%	30%	30%	20%	30%	60%	55%	35%
T-ALL	20%	0%	0%	0%	0%	90%	95%	70%	25%
Total ALL	85%	2%	25%	25%	15%	45%	65%	60%	35%

a. Gene configurations as determined by interpretation of Southern blot results: R, rearrangement of at least one allele; D, deletion on both alleles or one allele deleted and the other in germline configuration.



	VH	N-region	VH/DH	N-region	DH	N-region	JH
(Dx)	VH6-1(-6)	GAGGGAGGG	(-14) DH2-2 (-1)	AAG	(-7) DH6-13 (0)	CGGGGATG	(-6) JH6b
(Re)			VH2-70 (-2)	CCGCCG	(-10) DH6-13 (0)	CGGGGATG	(-6) JH6b

Figure 1.
(A) Heteroduplex PCR analysis of bone marrow DNA from a precursor-B-ALL patient at diagnosis (Dx) and at relapse (Re) using six VH family-specific primers together with a JH consensus primer. The results illustrate oligoclonality both at diagnosis (five *IGH* gene rearrangements) and at relapse (three *IGH* gene rearrangements). None of the rearrangements was identical between diagnosis and relapse. ss, single-strand fragments; ho, homoduplexes; C, positive control; M, 100 bp molecular weight marker.
(B) Fluorescent sequencing of clonal PCR products at diagnosis and relapse showed that two different rearrangements shared an identical DH6-13-JH6b junctional region, thereby proving the clonal relationship between the diagnosis and relapse cell samples.

a significant proportion (~20%) of *IGL* gene rearrangements in precursor-B-ALL involves the non-functional J-Cλ6 gene region (Tümkaya et al., submitted for publication).

Analysis of a small group of adult precursor-B-ALL patients revealed several intriguing differences as compared to childhood patients.[21] Firstly, we observed a lower level of *IGH* oligoclonality (~20% versus ~40% in children) despite a compa-

rable incidence of *IGH* gene rearrangements. Secondly, all detected *IGK* gene deletions concerned rearrangements of the Kde to Vκ gene segments, which represent two thirds of the Kde rearrangements in pediatric precursor-B-ALL and only half of the Kde rearrangements in mature B-cell leukemias.[20,21]

TCR gene rearrangements occur in more than 95% of T-ALL cases (Table 1). The TCR delta (*TCRD*) locus first undergoes rearrangement in normal thymocyte development and likewise is the most frequently rearranged gene in T-ALL (~95%). In contrast to frequent subclone formation in Ig genes in precursor-B-ALL, this phenomenon is rarely observed for TCR gene rearrangements in T-ALL (Langerak et al., unpublished data).

Interestingly, in several T-ALL patients an atypical TCR gamma (*TCRG*) gene rearrangement was demonstrated showing an interstitial deletion of ~170 bp of the downstream part of the Vγ2 gene segment.[22]

Cross-lineage TCR Gene Rearrangements in Precursor-B-ALL

Our analysis of a large group of 202 pediatric precursor-B-ALL patients revealed TCR beta (*TCRB*), *TCRG*, and *TCRD* gene rearrangements in 35%, 59%, and 89% of cases, respectively (Table 1).[15] In total, in 93% of the precursor-B-ALL cases one or more TCR genes were rearranged and/or deleted, indicating that cross-lineage TCR gene rearrangements are ubiquitously present in malignant B-lineage lymphoblasts. However, the spectrum of cross-lineage TCR gene rearrangements in precursor-B-ALL is very limited (Table 2). *TCRB* gene rearrangements are restricted to the Jβ2 region. This is in contrast to normal T-cells and T-ALL, which employ both Jβ1 and Jβ2 gene regions. *TCRG* gene rearrangements in precursor-B-ALL most

Table 2. Unusual TCR gene rearrangements in precursor-B-ALL as compared to normal T-cells and T-ALL.

	Precursor-B-ALL	Normal T-lymphocytes/T-ALL	
TCRB genes	exclusively to Jβ2: Vβ-Jβ2: 53% Dβ-Jβ2: 35%	both to Jβ1 and Jβ2	
TCRG genes	Vγ-Jγ1.3: 40% Vγ-Jγ2.3: 28% Vγ-Jγ1.1: 27%	$TCR\alpha\beta^+$ T-cells: $TCR\gamma\delta^+$ T-cells : T-ALL blasts:	mainly to Jγ1.3/2.3 mainly VγII-Jγ1.2 mainly to Jγ1.3/2.3
TCRD genes	Vδ2-Dδ3: 40-70% Dδ2-Dδ3: 10-40%	$TCR\alpha\beta^+$ T-cells/T-ALL: $TCR\gamma\delta^+$ T-cells: $TCR\gamma\delta^+$ T-ALL:	mainly biallelic deletion mainly Vδ2-Jδ1 mainly Vδ1-Jδ1

frequently (~70%) concern rearrangements to Jγ1 region gene segments. Curiously, 80% of *TCRD* gene rearrangements represent incomplete Vδ2-Dδ3 or Dδ2-Dδ3 joinings, whereas complete rearrangements to the Jδ1 gene segment, characteristic for normal T-cells and T-ALL blasts (particularly TCRγδ$^+$ [23]) have never been reported in precursor-B-ALL. Apparently, only certain parts of the *TCRB* and *TCRD* genes are accessible for continuing activity of the V(D)J recombinase in precursor-B-ALL. Using a transgenic mouse model, Lauzurica et al.[24] showed that V-D to J recombination in human *TCRD* genes is controlled by the *TCRD* enhancer, whereas rearrangements to the Dδ3 gene segment are controlled by a separate mechanism. It seems highly probable that in precursor-B-ALL *TCRD* gene rearrangements are restricted to those that are independent of the *TCRD* enhancer. Furthermore, Vδ2 and Dδ2 gene segments in precursor-B-ALL can further undergo rearrangements to Jα gene segments and the majority of *TCRD* gene deletions reflect secondary Vα-Jα rearrangements.[6,15]

The analysis of combinations of cross-lineage TCR gene rearrangements in precursor-B-ALL patients showed that *TCRD* gene rearrangements and/or deletions occurred in the majority of cases (~90%) and that 27% of childhood precursor-B-ALL had *TCRD* gene rearrangements and/or deletions with germline *TCRG* and *TCRB* genes.[15] This implies that the hierarchy of cross-lineage TCR gene events is similar to early T-cell development, where rearrangements in the *TCRD* locus occur first, followed by *TCRG*, and subsequently by *TCRB* gene rearrangements. Nevertheless, in a few cases we found rearranged *TCRB* genes with *TCRD* and *TCRG* loci in germline configuration. Furthermore, the frequencies of cross-lineage TCR gene rearrangements seem to be related to the maturation stages of malignant B-cells. The frequency of TCR gene rearrangements is lower in immature precursor-B-ALL (pro-B-ALL) as compared to CD10$^+$ precursor-B-ALL (common ALL and pre-B-ALL). When comparing common ALL and pre-B-ALL subgroups, it is striking that in the 'mature' (CyIgμ$^+$) pre-B-ALL group a higher frequency of patients have their TCR genes in germline configuration (15% versus 2% in common ALL). TCR gene rearrangements are still present in the most mature precursor-B-ALL subgroup with simultaneous cytoplasmic and membrane Igμ expression (transitional-pre-B-ALL). However, in mature B-cell malignancies cross-lineage TCR gene rearrangements are rare (<5%).[1,25]

Based on combined Southern blotting and heteroduplex PCR analysis we showed oligoclonality in 38% and 30% of precursor-B-ALL patients with *TCRG* and *TCRD* gene rearrangements, respectively, which is comparable to the frequency of oligoclonality found in the *IGH* locus.[15]

In adult precursor-B-ALL a striking predominance of immature Dδ2-Dδ3 cross-lineage recombinations was observed (44%), whereas the more mature Vδ2-Dδ3 gene rearrangements occurred less frequently (38%). This is in contrast to the high frequency (>70%) of the more mature Vδ2-Dδ3 gene rearrangements in childhood

precursor-B-ALL. Together with the characteristic *IGH* and *IGK* gene rearrangement patterns this suggests that the Ig and TCR genotype of precursor-B-ALL in adults is less mature than in children.[21]

Cross-Lineage *IGH* Gene Rearrangements in T-ALL

In contrast to the frequent cross-lineage TCR gene rearrangements in precursor-B-ALL, *IGH* gene rearrangements in T-ALL occur in ~20% of patients (19% of $CD3^-$ T-ALL, 50% of $TCR\gamma\delta^+$ T-ALL, and only 4% of $TCR\alpha\beta^+$). Using heteroduplex PCR analysis we found a high frequency (~80%) of incomplete DH-JH rearrangements as well as preferential usage of DH6-19 and the most downstream DH7-27 gene segment together with the most upstream JH1 and JH2 gene segments. We have not found VH gene restriction in the complete VH-JH recombinations, which comprised 18% of cross-lineage *IGH* gene rearrangements in T-ALL patients. Furthermore, oligoclonality in the *IGH* locus was found in 27% of T-ALL patients with rearranged *IGH* genes (Szczepański et al., submitted for publication). Cross-lineage *IGH* gene rearrangements are rare in mature T-cell malignancies and Ig light chain gene rearrangements have only been reported anecdotally in malignant T lymphoblasts.[1]

Secondary and Ongoing Gene Rearrangements in ALL

Another striking feature of Ig and TCR gene rearrangements in ALL is the occurrence of secondary recombination events. Secondary rearrangements via continuing VH to DH-JH joining, VH gene replacements, and 'de novo' *IGH* gene rearrangements have frequently been reported for precursor-B-ALL (Figure 1).[26,27] Southern blot comparison between *IGH* configuration at diagnosis and at relapse provided evidence for clonal changes in 40% of precursor-B-ALL cases.[8] A few limited PCR studies estimated the frequency of *IGH* clonal evolution at ~30% of cases.[9,28]

Recently, we found evidence for continuing VH to DH-JH recombination in T-ALL, while VH replacement has been described previously during disease progression of a T-lymphoblastic lymphoma.[29]

Based on Southern blot analysis, differences in *TCRB*, *TCRG*, and *TCRD* gene rearrangements between diagnosis and relapse were found in 30%, 20%, and 10% of T-ALL, respectively.[8] Limited PCR studies estimated the frequency of *TCRG* and *TCRD* clonal evolution at 10-40% of T-ALL cases.[10,11] Interestingly, we demonstrated in several T-ALL cases ongoing, polyclonal δREC-ψJα gene rearrangements within otherwise monoclonal leukemic populations.[30]

Cross-lineage *TCRG* and *TCRD* gene rearrangement patterns in precursor-B-ALL were found discordant between diagnosis and relapse in 30-50% of cases by Southern blotting. However, in a few cases seemingly clonal Vδ2-Dδ3 rearrangements appeared to be oligoclonal upon PCR analysis.[15,21] Moreover, ongoing Vδ2-Dδ3 to Jα recombination was demonstrated in the vast majority of Vδ2-Dδ3-positive precursor-B-ALL.[6] Finally, in a few precursor-B-ALL cases with clonal *TCRG*

rearrangements at diagnosis the disease recurred with *TCRG* genes in germline configuration (clonal regression).[8,10]

CONCLUSIONS

ALL blasts display a variety of characteristic gene rearrangements. The majority of lineage-specific rearrangements probably reflect the gene configuration status of the lymphoid precursor cell that was transformed at a particular stage of differentiation. Cross-lineage gene rearrangements that are found in ALL could potentially be acquired before malignant transformation as well. However, then one would expect them to occur in normal lymphocytes at much higher frequencies. Since the majority of cross-lineage gene rearrangements are monoclonal and not oligoclonal, they should have occurred early after malignant transformation. The rare occurrence of cross-lineage gene rearrangements in TCR$\alpha\beta^+$ lineage T-ALL as well as in mature B-cell and T-cell malignancies can be explained by the absence of recombinase activity in these more mature types of lymphoid malignancies. Oligoclonality at diagnosis, clonal regression, ongoing and secondary recombination are unequivocally later postoncogenic events. Taken together, we hypothesize that the unusual gene rearrangements in ALL are caused by post-transformation continuing activity of the V(D)J recombinase enzyme system on accessible gene loci.

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3

CHAPTER 3.1

CLONAL EVOLUTION OF IMMUNOGLOBULIN AND T-CELL RECEPTOR GENE REARRANGEMENTS BETWEEN DIAGNOSIS AND RELAPSE OF ACUTE LYMPHOBLASTIC LEUKEMIA

The initial enthusiasm after establishing PCR strategies for detection of minimal residual disease (MRD) in acute lymphoblastic leukemia (ALL) based on immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements was challenged by the description of clonal evolution phenomena. Ig and TCR gene rearrangements in precursor-B-ALL and T-ALL might be prone to subclone formation due to continuing rearrangements or secondary rearrangements mediated via the continuously active recombinase enzyme system in these immature lymphoid malignancies (reviewed in Chapter 2.9). Especially the *IGH* gene rearrangements are known to change during the disease course, resulting in different rearrangement patterns between diagnosis and relapse. In fact, at diagnosis multiple *IGH* gene rearrangements are already found in 30 to 40% of precursor-B-ALL, indicating the presence of biclonality or oligoclonality.[1] Such oligoclonality usually reflects continuing or secondary *IGH* gene rearrangements, representing V_H to D_H-J_H rearrangements or V_H replacements, respectively.[2,3] The problem of oligoclonality at diagnosis is the uncertainty which clone is going to emerge at relapse and should be monitored with MRD-PCR analysis. Changes in *IGH* gene rearrangement patterns at relapse were shown to occur at high frequency in childhood precursor-B-ALL, particularly when subclone formation is already present at diagnosis.[4,5]

TCR gene oligoclonality is rarely seen at diagnosis in T-ALL (see Chapter 2.6).[4,6] In contrast, combined Southern blot and PCR data show that the frequency of oligoclonality in cross-lineage TCR gene rearrangements of precursor-B-ALL is approximately 20%, which is slightly less than in the *IGH* gene (see Chapter 2.4). Initially, subclone formation at diagnosis was thought to be less frequent for the *TCRD* gene complex, as suggested by Southern blotting.[7] However, PCR heteroduplex analysis and sequencing have shown that Vδ2-Dδ3 and Dδ2-Dδ3 rearrangements in newly diagnosed precursor-B-ALL are oligoclonal in 30 to 40% of cases (see Chapters 2.4 and 2.8).[8] Again, frequent TCR oligoclonality in precursor-B-ALL might be associated with further clonal evolution and loss of particular rearrangements at relapse.[4,5,9] For instance, oligoclonal Vδ2-Dδ3 rearrangements are prone to continuing rearrangements, particularly to Jα gene segments with concomitant deletion of the Cδ exons.[8,10]

False-negative results due to clonal evolution are a major drawback of using Ig/TCR gene rearrangements as PCR targets for MRD detection. In order to minimize false-negative results, detailed studies have been performed on the stability of

different Ig and TCR gene rearrangements between diagnosis and relapse in large series of childhood precursor-B-ALL and T-ALL patients (see Chapters 3.2 and 3.3). Based on the comparative information, it is possible to define optimal strategies for MRD-PCR target selection. Furthermore, using a stepwise molecular approach, it is possible to identify rare cases of true secondary ALL, which can be distinguished from presumably late ALL relapses (Chapter 3.4). Finally, based on preserved identical Ig/TCR gene rearrangements, molecular analyses can identify cases among presumably secondary acute myeloid leukemias, which actually represent unusual phenotypic shifts of initial ALL (Chapters 3.2 and 3.5).

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CHAPTER 3.2

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*

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ABSTRACT

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 rearrangements (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 (≥ 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 four patients with secondary acute myeloid leukemia with germline Ig/TCR genes. In five

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other patients additional analyses proved the clonal relationship between both disease stages. Finally, in one 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.

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} (one 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 al.[12]). 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 kappa light chain (*IGK*), TCR gamma (*TCRG*) and TCR delta (*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) 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] Immunological marker analysis of the precursor-B-ALL revealed that six (6%) were pro-B-ALL, 59 (61%) were common ALL, and 31 (32%) were pre-B-ALL. Seven patients were studied at two 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]

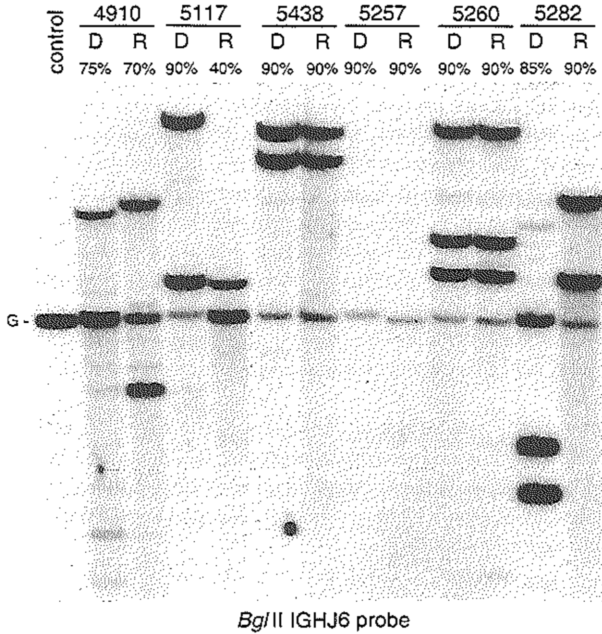
Comparative Southern blot analysis

Mononuclear cells were separated from bone marrow or peripheral blood samples by Ficoll-Paque centrifugation (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden). DNA was isolated from frozen mononuclear cells, digested with the *Bgl*II 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 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 three precursor-B-ALL patients with insufficient remaining DNA at diagnosis were not analyzed. In each 50μl 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 V_H family-specific framework 1 primers, and 7 D_H family-specific primers with consensus J_H primer), *IGK*-Kde (4 V_κ family-specific primers and intron recombination signal sequence [RSS] primer with Kde primer), *TCRG* (6 Vγ-Jγ primer combinations most frequently used in precursor-B-ALL), and *TCRD* gene rearrangements (Vδ2-Dδ3 and Dδ2-Dδ3 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]

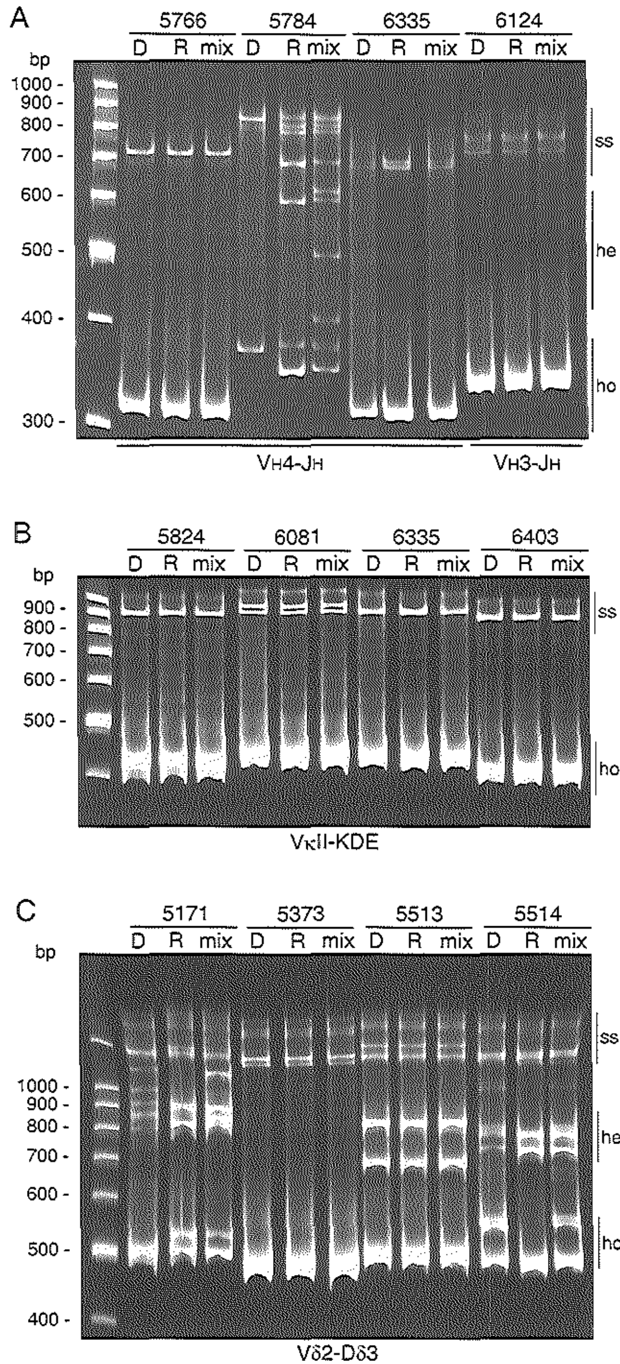
For heteroduplex analysis, the PCR products were denatured at 94°C for 5 minutes after the final cycle of amplification and subsequently cooled to 4°C for 60 minutes to induce duplex formation.[32]



BglII IGHJ6 probe

Figure 1.
Comparative Southern blot analysis of *IGH* gene configuration in six 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.

Figure 2.
Examples of comparative heteroduplex PCR analysis. **(A)** Comparative heteroduplex analysis of *IGH* gene rearrangements. Monoclonal 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, monoclonal 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 two monoclonal Vδ2-Dδ3 rearrangements were found at relapse.


A
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Vδ2-Dδ3

Afterwards the duplexes were immediately loaded on 6% nondenaturing polyacrylamide gels in 0.5 x 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, i.e. 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] V_H, D_H, and J_H segments were identified using DNAPLOT software (W. Müller, H-H. Althaus, University of Cologne, Germany) by searching for homology with all known human germline V_H, D_H, and J_H sequences obtained from the VBASE directory of human Ig genes (http://www.mrc-cpe.cam.ac.uk/imt-doc/).[33] $V\gamma$ and $J\gamma$ 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 x 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 0.05 were considered to be 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 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 two immunogenotypic features were more prevalent in ALL at relapse. The *TCRD* gene configuration at relapse was characterized by significantly less frequent $V\delta2$-$D\delta3$ and $D\delta2$-$D\delta3$ rearrangements and more frequent *TCRD* deletions ($p<0.05$), 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* ($p<0.05$).

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

	Random precursor-B-ALL at diagnosis[a]	Relapsed precursor-B-ALL at diagnosis[b]	Precursor-B-ALL at relapse
IGH			
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)[c]	28% (24/85)*
IGK-Kde			
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)
TCRG			
Germline	41% (79/192)	38% (35/91)	41% (37/91)
R/G or R/R	59% (113/192)	62% (56/91)	59% (54/91)
TCRD			
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)**

* not significant
** $p < 0.05$
a. 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]
b. Including one patient with phenotypic switch to AML at relapse
c. The frequency of *IGH* oligoclonality was significantly higher in infant ALL compared with noninfantALL (75% versus 38%, $p < 0.05$)

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

Southern blot analysis in four out of five patients with a presumed secondary AML demonstrated the complete absence of clonal Ig/TCR gene rearrangements

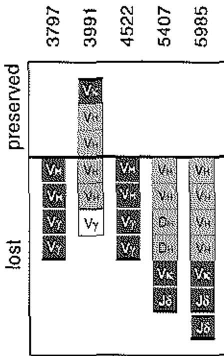

Figure 3.
Ig/TCR gene rearrangement stability in five precursor-B-ALL patients subjected to comparative heteroduplex PCR analysis at diagnosis and at presumably secondary AML. All Ig/TCR gene rearrangements identified at diagnosis in patients 3797, 4522, 5407, and 5985 were lost, which is in line with secondary AML. In contrast several *IGH* and *IGK* gene rearrangements in patient 3991 were preserved, suggesting a phenotypic shift from ALL to AML. Black squares represent monoclonal rearrangements; gray squares, biclonal/oligoclonal rearrangements; white squares, oligoclonal/polyclonal rearrangements. All squares above the line represent stable MRD-PCR targets, while all squares below the line represent Ig/TCR gene rearrangements lost owing to clonal evolution. VH indicates VH-JH gene rearrangements; DH, DH-JH gene rearrangements; Vκ, Vκ-Jκ gene rearrangements; Vγ, Vγ-Jγ gene rearrangements; Jδ, rearrangements to Jδ1-Dδ3 region detected by Southern blot analysis.

(Figure 3), which is in line with the AML diagnosis. However, in one patient (3991) three clonal Southern blot bands were preserved in the AML clone, and the sequence identity was confirmed by comparative PCR analysis for two VH-JH gene rearrangements. Apparently the original ALL clone underwent a phenotypic switch to AML. This patient was included in our further comparative diagnosis-relapse analyses despite the phenotypic shift, while the other four patients were excluded.

PCR detectability of Ig and TCR gene rearrangements in relapsed precursor-B-ALL patients

A total of 362 clonal PCR products of different Ig/TCR gene rearrangements were identified at diagnosis in 87 (98%) of 89 patients, with an average of four targets per patient. In one patient no clonal gene rearrangements were detected by PCR, while Southern blotting showed a single weak rearranged *IGH* band, identical between the diagnosis and relapse sample. The second patient had an infant ALL and was fully oligoclonal at diagnosis, which precluded identification of clonal Ig/TCR markers for PCR-based MRD monitoring. Generally, *IGH* oligoclonality at diagnosis was more prevalent in infant ALL patients (6 of 8 cases), compared with the non-infant precursor-B-ALL cases (32 of 84 cases), $p<0.05$.

Stability of particular MRD-PCR targets in monoclonal and oligoclonal precursor-B-ALL patients at relapse

A total of 256 (71%) of 362 clonal Ig/TCR gene rearrangements identified with heteroduplex PCR analysis at diagnosis in 87 patients were preserved at relapse. This concerned 99 (64%) of 155 *IGH*, 54 (90%) of 60 *IGK*-Kde, 65 (75%) of 87 *TCRG*, and 38 (63%) of 60 *TCRD* gene rearrangements (Table 2). In three addition-

Table 2. Stability of MRD-PCR targets in patients with monoclonal and oligoclonal Ig and TCR gene configuration

	STABILITY OF MRD-PCR TARGETS			STABILITY IN PATIENTS (all / ≥1)		
	monoclonal	oligoclonal	total	monoclonal	oligoclonal	total
IGH						
VH-JH	88% (n = 67)	47% (n = 60)	69% (n = 127)	81% / 98%	37% / 73%	63% / 88%
DH-JH	57% (n = 7)	38% (n = 21)	43% (n = 28)	67% /67%	27% / 53%	33% / 57%
all	85% (n = 74)	44% (n = 81)	64% (n = 155)	76% / 98% (n=42)	24% / 76% (n=33)	52% / 88% (n=75)
IGK-Kde						
Vκ-Kde	95% (n = 39)	40% (n = 5)	91% (n = 44)	94% / 97%	33% / 66%	88% / 94%
Intron-Kde	86% (n = 15)	0%	87% (n = 15)	92% / 92%	0%	85% / 85%
all* (n = 55)	95% (n = 5)	40% (n = 60)	90% (n = 37)	95% / 95% (n = 3)	33% / 66% (n = 40)	88% / 93%
tCRG						
Vγ-Jγ	ND	ND	75% (n = 87)	ND	ND	64% / 83% (n = 53)
TCRD						
Dδ2-Dδ3	100% (n = 9)	14% (n = 7)	63% (n = 16)	100%	14% / 14%	60% / 60%
Vδ2-Dδ3	81% (n = 27)	31% (n = 16)	63% (n = 43)	75% / 80%	20% / 50%	59% / 71%
all **	86% (n = 37)	26% (n = 23)	63% (n = 60)	80% / 88% (n =25)	14% / 36% (n =14)	56% / 69% (n =39)

* including one Vκ-intronRSS rearrangement
** including one Vδ3-Jδ1 rearrangement
ND not done

al patients, Southern blot analysis provided conclusive information about stability of gene rearrangement patterns.[6,15]

In 36 patients (including patient 4616 studied exclusively by Southern blotting) all MRD-PCR targets identified at diagnosis were preserved at relapse (Figure 4A). In 38 cases (including patients 2665 and 4501 studied exclusively by Southern blotting) at least half of the targets remained stable during the disease course (Figure 4B). In another ten patients (including patient 3991 with AML at relapse; Figure 3) most

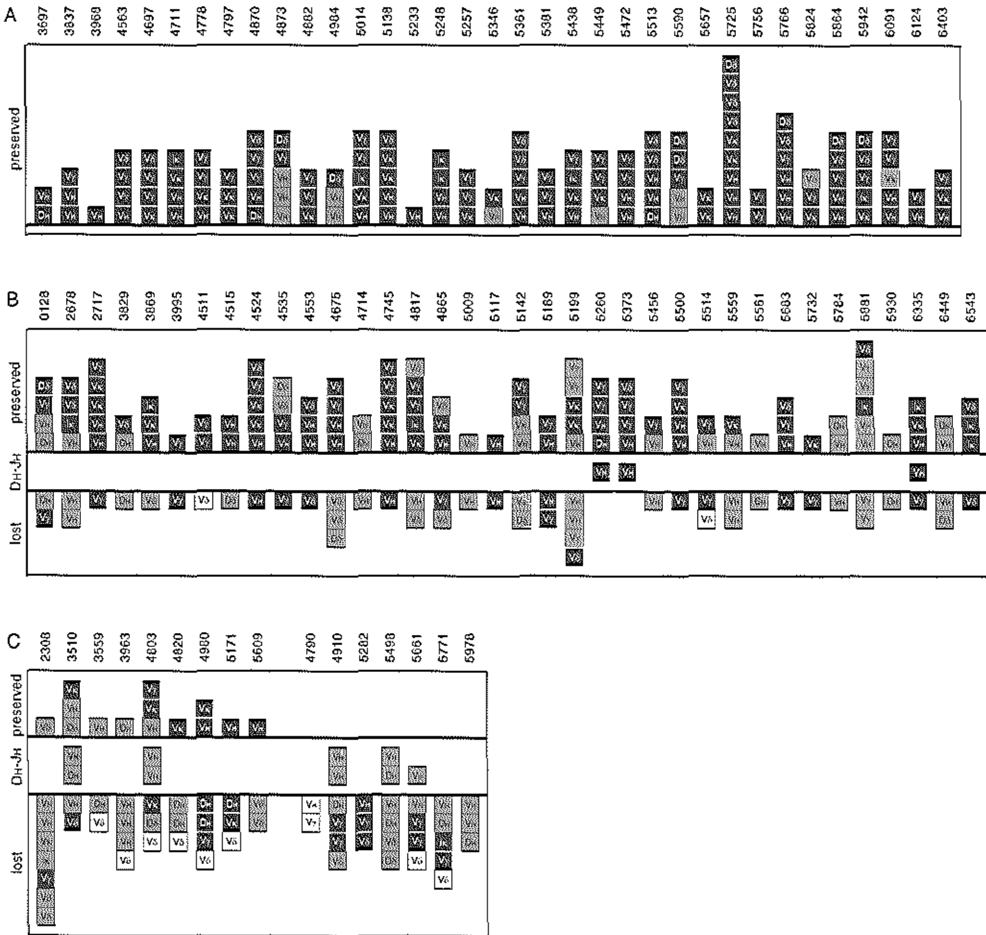

Figure 4.
Ig/TCR gene rearrangement stability patterns in 87 precursor-B-ALL patients subjected to comparative heteroduplex PCR analysis at diagnosis and at relapse. Black squares represent monoclonal rearrangements; gray squares, biclonal/oligoclonal rearrangements; white squares, oligoclonal/polyclonal 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 two lines have common DH-JH stems shared by different *IGH* rearrangements at diagnosis and at relapse. **(A)** 35 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; Vκ, Vκ-Kde gene rearrangements; iκ, intronRSS-Kde gene rearrangements; Vγ, Vγ-Jγ gene rearrangements; Vδ, Vδ2-Dδ3 gene rearrangements; Dδ, Dδ2-Dδ3 gene rearrangements.

MRD-PCR targets were absent at relapse but at least one rearrangement was common for both diagnosis and relapse samples (Figure 4C). Consequently, at least one MRD-PCR target was preserved at relapse in 84 (93%) of 90 patients with available clonal MRD-PCR targets at diagnosis. In the remaining six patients all clonal markers found at diagnosis seemed to be lost. However, in three of these six cases the clonal 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 two cases additional analyses[35,36] showed at both disease stages identical DNA sequences of Vκ-Jκ (case 5771) and chimeric *MLL-AF4* fusion genes (case 5978), respectively (data not shown). Finally, in one patient (case 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, i.e. 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 three 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; i.e. 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% vs. 43% of targets preserved, respectively.

IGK deletional rearrangements

A total of 60 *IGK* deletional rearrangements were detected in 40 childhood pre-

cursor-B-ALL patients at diagnosis: 44 Vκ-Kde, 15 intron-Kde and one rarely occurring Vκ-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 two of five oligoclonal *IGK*-Kde targets were found at relapse. No significant difference in stability was found between Vκ-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 didn't 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 Vδ2-Dδ3, 16 Dδ2-Dδ3, and 1 Vδ3-Jδ1) 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; i.e. 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 Vδ2-Dδ3 rearrangements, while heteroduplex PCR analysis of these rearrangements showed oligoclonality or even polyclonality.

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, which were originally present at diagnosis.

Clonal evolution in *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 seven patients (two monoclonal and five 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 three 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 two monoclonal patients we observed clonal "regression" of one of the rearrangements to germline. Finally in a single patient (5282) both monoclonal *IGH* rearrangements were replaced by two 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 five patients and possibly secondary rearrangements in five other cases. In the remaining two 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 *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 five patients the new rearrangements at relapse contained upstream Vγ and downstream Jγ segments compared with the Vγ-Jγ rearrangements at diagnosis, which is suggestive of ongoing recombination with Vγ-Jγ replacement. In the remaining five patients the sequence comparison of Vγ-Jγ 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 *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 four patients with monoclonal Vδ2-Dδ3 rearrangements) ongoing deletions were observed. In contrast, in 8 patients (including one patient with monoclonal Vδ2-Dδ3) the rearrangement pattern "regressed" to germline configuration. Finally, in the remaining 3 cases new rearrangements were found at relapse, including one Vδ2-Dδ3 joining and two unidentified rearrangements to the Dδ3-Jδ1 region. Interestingly, 5 out of 10 cases with oligo/polyclonal Vδ2-Dδ3 rearrangements at diagnosis had a monoclonal *TCRD* configuration at relapse with a single Vδ2-Dδ3 joining. In the

remaining five patients, ongoing *TCRD* deletion was assumed in two cases, two patients demonstrated "regression" to germline configuration, and only a single patient preserved the oligo/polyclonal Vδ2-Dδ3 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 Vδ2-Dδ3 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 two 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 four (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 iden-

tified 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 two 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*II 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; e.g. 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 two 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 two 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 two patients: one with presumably secondary 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 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 two targets would increase the risk of false-negative MRD results. In our series this approach could have been used in 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 three 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 two of our patients (4910, 5661) usage of common DH-JH stems would have been superior to Vγ-Jγ 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 Vγ-Jγ joinings in normal T-cells in postinduction follow-up samples.[47] Finally, our study clearly shows that oligoclonal Vδ2-Dδ3 and Dδ2-Dδ3 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: two 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 cho-

sen 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 two 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 two 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 three 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 compensat-

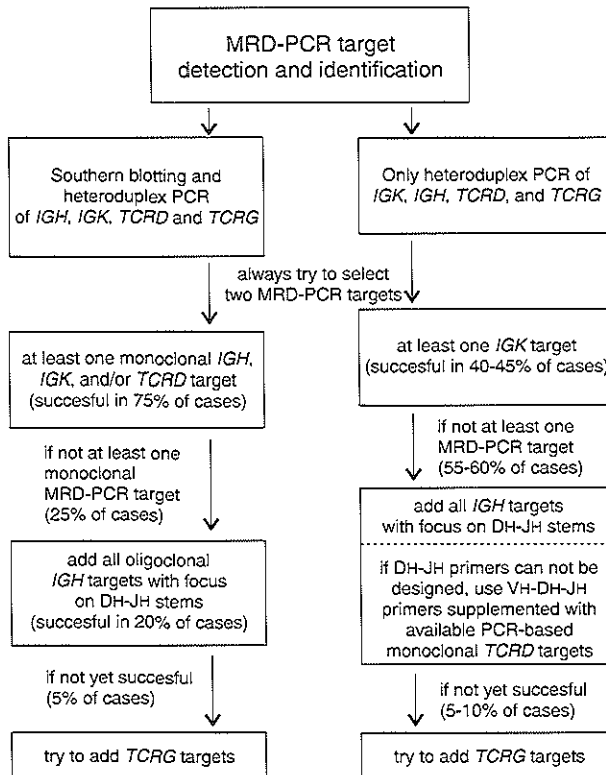

Figure 5.
Flow diagram for the stepwise selection of MRD-PCR targets, dependent on the choice of techniques for detection and identification of *IGH*, *IGK*, *TCRD*, and *TCRG* gene rearrangements.

ed 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.

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CHAPTER 3.3

COMPARATIVE ANALYSIS OF T-CELL RECEPTOR GENE REARRANGEMENTS BETWEEN DIAGNOSIS AND RELAPSE OF CHILDHOOD T-ALL SHOWS HIGH STABILITY OF CLONAL MARKERS FOR MRD MONITORING AND REVEALS OCCURRENCE OF SECONDARY T-ALL*

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ABSTRACT

Twenty-eight children with T-ALL were subjected to detailed analyses of T-cell receptor (TCR) and *TAL1* genes at diagnosis and relapse using combined Southern blotting and PCR.

A total of 120 clonal TCR and *TAL1* gene rearrangements were identified at diagnosis; in each patient at least two PCR targets for minimal residual disease (MRD) detection were found. In 16 patients (62%) all rearrangements and in 9 patients (35%) most (≥60%) rearrangements identified at diagnosis were preserved at relapse. In one T-ALL patient three clonal rearrangements were lost and only one rearrangement was preserved at relapse. Finally, two patients displayed completely different TCR gene rearrangement sequences between diagnosis and relapse and the rearrangement patterns were highly suggestive of secondary ALL. Strikingly, both patients experienced very late T-ALL recurrences, 6 and 10 years from diagnosis, respectively. This is in striking contrast to the remaining 26 patients, who relapsed within 37 months from diagnosis.

Ninety-five (86%) of 111 clonal rearrangements identified at diagnosis in true relapsed T-ALL were preserved at relapse. The best stability was found for the *TCRD* gene rearrangements and *SIL-TAL1* fusion genes (both 100%). Vγ-Jγ gene rearrangements identified in all 26 patients were generally stable targets (42/49,

*Submitted

86%). Finally, the stability of *TCRB* gene rearrangements approximated 80% (35/44). Based on the stability of TCR gene rearrangements and their sensitivity as MRD-PCR targets, we propose a stepwise strategy for PCR target selection (*TCRD* + *SIL-TAL1* → *TCRB* → *TCRG*), which would enable successful detection of MRD in all (100%) T-ALL patients.

INTRODUCTION

Detection of low levels of malignant cells, known as minimal residual disease (MRD), is becoming a routine tool in diagnostics and management of various hematopoietic malignancies.[1] MRD information is particularly valuable for childhood ALL patients as shown by several large multi-center prospective studies.[2-6] MRD information on early response to induction treatment contributes to an improved definition of remission in ALL, and therefore is currently being incorporated into stratification of treatment protocols.[7,8] MRD monitoring is particularly attractive in T-ALL patients, because it can be performed by analysis of peripheral blood without the need for repetitive traumatic bone marrow punctures.[9]

In childhood ALL detection of MRD most frequently relies on patient-specific junctional regions of immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements (reviewed in [8]). These leukemia-specific 'fingerprints' can be identified in virtually all ALL patients and they enable routine MRD detection with sensitivities of 10^{-4} to 10^{-5} (i.e. one malignant cell in the background of 10^4 to 10^5 normal cells). Clonal Ig and TCR gene rearrangements are easily identified with molecular biology techniques including Southern blot, polymerase chain reaction (PCR) and sequencing methods, and the results are reproducible between laboratories as proven by international standardization.[10] With the advent of real-time quantitative PCR (RQ-PCR) analysis of Ig and TCR gene junctional regions, precise quantification of MRD levels is routinely achievable.[11-15]

One of the most important pitfalls of MRD detection using Ig/TCR gene rearrangements is clonal evolution caused by the persistent activity of the V(D)J recombinase machinery in leukemic blasts (reviewed in [16]). Ongoing or secondary rearrangements might lead to the loss of MRD-PCR targets and consequently to false negative results. Such clonal selection processes might even occur early during induction treatment hampering reliable stratification into MRD-based risk groups.[17] Our extensive study in precursor-B-ALL patients showed differences in Ig/TCR gene rearrangement patterns between diagnosis and relapse in 62% of patients.[18] However, based on the stability of the individual Ig and TCR gene rearrangements, we proposed a stepwise strategy for selection of PCR targets enabling successful detection of MRD in the vast majority (95%) of precursor-B-ALL patients.

Although the presence of clonal evolution phenomena has also been reported in T-ALL, the studies comparing the TCR gene rearrangement patterns at diagnosis and relapse in T-ALL either did not compare junctional region sequences or were limited to TCR gamma (*TCRG*) and/or TCR delta (*TCRD*) gene loci.[19-22] Therefore we studied the stability of the TCR gene rearrangements currently used for MRD monitoring in T-ALL, i.e. TCR beta (*TCRB*), *TCRG*, *TCRD* gene rearrangements and *SIL-TAL1* gene fusion in a series of 28 T-ALL patients. This information forms the basis for reliable selection of MRD-PCR targets in T-ALL patients with minimal chance of false-negative MRD results.

PATIENTS, MATERIALS, AND METHODS

Patients

Bone marrow, peripheral blood samples or lymph node biopsy from 28 T-ALL patients were obtained at initial diagnosis and at relapse. The age distribution ranged from 2 years until 15.9 years. The diagnosis of T-ALL was made according to FAB and standard immunophenotypic criteria.[23,24] Immunophenotyping of the T-ALL revealed that 18 (64%) were $CD3^-$ T-ALL, 8 (29%) were $TCR\alpha\beta^+$ T-ALL, and 2 (7%) were $TCR\gamma\delta^+$ T-ALL. Three patients were studied at two subsequent leukemia relapses.

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 described in detail previously.[25]

Comparative Southern blot analysis

Mononuclear cells (MNC) were separated from bone marrow or peripheral blood samples by Ficoll-Paque centrifugation (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden). DNA was isolated from frozen MNC, digested with the appropriate restriction enzymes, and blotted to nylon membranes as described previously.[26] *TCRB* gene rearrangements were detected with TCRBJ1, TCRBJ2, and TCRBC probes (DAKO Corporation, Carpinteria, CA, USA) in *Eco*RI and *Hin*dIII digests.[27] The configuration of the *TCRD* genes was analyzed with the TCRDJ1 probe (DAKO) in *Eco*RI and *Hin*dIII digests.[28] The *TCRG* gene configuration was studied using the TCRGJ13 probe (DAKO) in *Eco*RI digests together with either the TCRGJ21 probe (DAKO) in *Pst*I digests or a combination of the Jγ2.1 probe in *Eco*RI digests and the Jγ1.2 probe in *Bgl*II digests.[29,30] The diagnosis and relapse samples of the 28 patients were always run in parallel lanes. The Southern blot configuration of the TCR genes in 14 patients at diagnosis and in 10 patients at relapse has been reported previously.[19,27,28,31]

PCR amplification and comparative heteroduplex analysis of PCR products

PCR analysis was performed on both diagnosis and relapse samples in all patients as described previously.[10,32] The sequences of the oligonucleotides used for amplification of *TCRG* (four Vγ family-specific forward primers, six VγI member-specific forward primers, and three reverse Jγ primers), *TCRD* (Vδ1-Jδ1, Vδ2-Jδ1, Vδ3-Jδ1, Dδ2-Jδ1, Vδ2-Dδ3 and Dδ2-Dδ3 primer pairs) and *SIL-TAL1* gene rearrangements were published before.[10] In each 50 μl PCR reaction 50 ng DNA sample, 6.3 pmol of the forward and reverse primers, and 0.5 U AmpliTaq Gold polymerase (PE Biosystems, Foster City, CA, USA) were used. PCR conditions were: initial denaturation for 10 min at 94°C, followed by 40 cycles of 45 sec at 92°C, 90 sec at 60°C, and 2 min at 72°C using a Perkin-Elmer 480 thermal cycler (PE Biosystems). After the last cycle an additional extension step of 10 min at 72°C was performed. Appropriate positive and negative controls were included in all experiments.[10]

Identification of clonal *TCRB* gene rearrangements was based on multiplex strategy using 23 Vβ, 2 Dβ, and 13 Jβ primers as developed by BIOMED-2 Concerted Action "PCR-based clonality studies for early diagnosis of lymphoproliferative disorders" {Van Dongen et al. submitted for publication}.

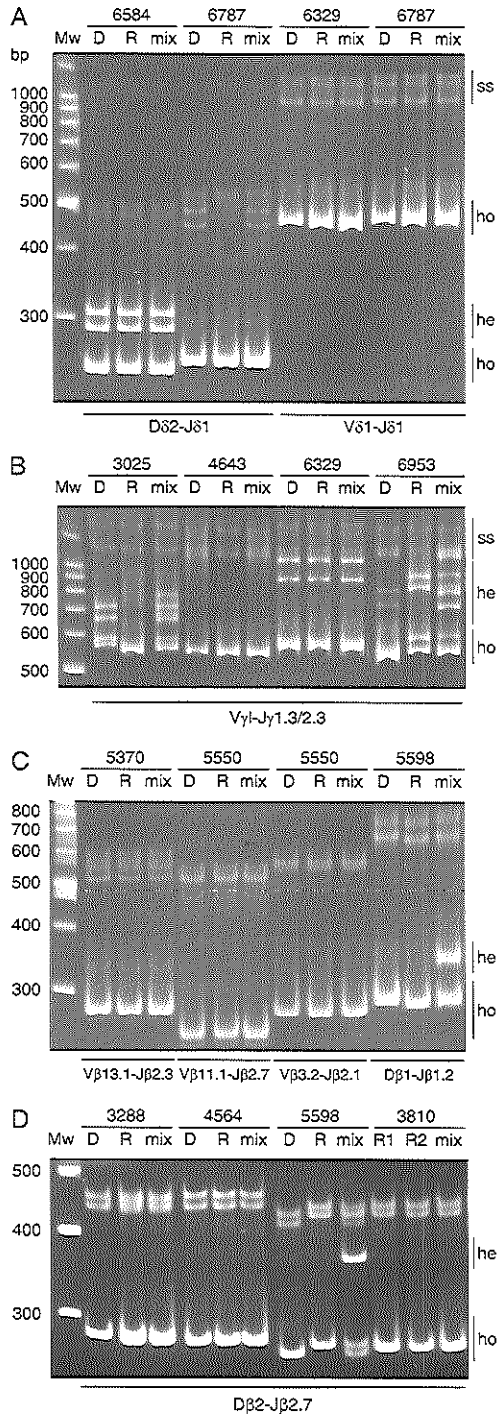
For heteroduplex analysis, the PCR products were denatured at 94°C for 5 min after the final cycle of amplification and subsequently cooled to 4°C for 60 min to induce duplex formation.[33] Afterwards the duplexes were immediately loaded on 6% non-denaturing polyacrylamide gels in 0.5 x Tris-borate-EDTA (TBE) buffer, run at room temperature, and visualized by ethidium bromide staining.[33] For identification of gene segments involved in clonal *TCRB* gene rearrangements, homoduplexes of appropriate size were excised from the polyacrylamide gel and eluted as described previously.[34,35] The eluted PCR products were directly sequenced either with Dβ or multiplex Vβ primers {Van Dongen et al. submitted for publication}.

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, i.e. mixing of the diagnosis and relapse PCR products followed by heteroduplex analysis (Figure 1).[18,25] 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 described before.[35] Vβ, Dβ, and Jβ segments were identified using DNAPLOT software (W. Müller, H-H. Althaus, University of Cologne, Germany) by searching for homology with all known human germline Vβ, Dβ, and Jβ sequences obtained from the IMGT directory of human TCR genes (http://imgt.cnusc.fr:8104).[36] Vγ, Vδ, Dδ, Jγ, and Jδ gene segments were identified by comparison to germline *TCRG* and *TCRD* sequences as described before.[37]

Figure 1.
Examples of comparative heteroduplex PCR analysis. **(A)** Comparative heteroduplex PCR analysis of *TCRD* gene rearrangements. Monoclonal homoduplexes (ho) in patients 6329, 6584, and 6787 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. **(B)** Comparative heteroduplex PCR analysis of *TCRG* gene rearrangements showed identical rearrangements at diagnosis and relapse in patients 4643 and 6329. In patients 3025 and 6953 monoclonal homoduplexes found at diagnosis and at relapse slightly differed in size. Mixing of the Vγ-Jγ PCR products followed by heteroduplex PCR analysis demonstrated clear heteroduplex formation, proving that these Vγ-Jγ gene rearrangements had different junctional regions. **(C)** Comparative heteroduplex PCR analysis of *TCRB* gene rearrangements. Patients 5370, and 5550 with mono and biallelic rearrangements, respectively, had stable Vβ-Jβ rearrangements. In contrast, in patient 5598 with presumed secondary T-ALL, monoclonal Dβ1-Jβ1.2 homoduplexes found at diagnosis and at relapse had different junctional regions confirmed by heteroduplex formation after mixing of the PCR products. **(D)** Comparative heteroduplex PCR analysis of Dβ2-Jβ2.7 gene rearrangements. Patients 3288 and 4564 with monoallelic rearrangements had stable Dβ2-Jβ2.7 rearrangements between diagnosis and relapse. In patient 5598 with presumed secondary T-ALL, Dβ2-Jβ2.7 joinings at diagnosis and relapse were different as shown by mixed heteroduplex analysis. Patient 3810 was negative for Dβ2-Jβ2.7 rearrangement at diagnosis but had identical Dβ2-Jβ2.7 rearrangements in two subsequent relapse samples.



A
6584
6787
6329
6787
Mw D R mix D R mix D R mix D R mix
bp
1000
900
800
700
600
500
400
300
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ho
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Dδ2-Jδ1
Vδ1-Jδ1
B
3025
4643
6329
6953
Mw D R mix D R mix D R mix D R mix
1000
900
800
700
600
500
ss
he
ho
Vγl-Jγ1.3/2.3
C
5370
5550
5550
5598
Mw D R mix D R mix D R mix D R mix
800
700
600
500
400
300
he
ho
Vβ13.1-Jβ2.3
Vβ11.1-Jβ2.7
Vβ3.2-Jβ2.1
Dβ1-Jβ1.2
D
3288
4564
5598
3810
Mw D R mix D R mix D R mix R1 R2 mix
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Dβ2-Jβ2.7

Statistical analysis

Statistical analysis using the χ^2 test on a 2 x 2 table was performed to compare the frequencies of clonal evolution between different immunophenotypic subgroups of T-ALL. A value of $p < 0.05$ was regarded to be statistically significant.

RESULTS

PCR detectability of TCR gene rearrangements in relapsed T-ALL patients

A total of 120 clonal PCR products of different TCR gene rearrangements and *SIL-TAL1* fusion gene were identified at diagnosis in the 28 T-ALL patients with an average of 4.3 per patient and a range from two to six rearrangements per patient. Consequently, in each T-ALL patient at least two MRD-PCR targets were available.

TCR gene rearrangement patterns in two patients suspected of a secondary T-ALL

Within the studied T-ALL group, two patients (3025 and 5598) displayed completely different TCR gene rearrangement sequences between diagnosis and presumed relapse (3 and 6 rearrangements lost, respectively) and the rearrangement patterns were highly suggestive of a secondary ALL (Table 1). Moreover, the emergence of a new chromosome aberration, i.e. t(10;14), at relapse in patient 5598 indicated the development of a secondary malignancy. Both patients experienced very

Table 1. TCR gene rearrangement patterns in the two T-ALL patients suspected of secondary T-ALL.

	Patient 3025		Patient 5598	
	Diagnosis	Relapse	Diagnosis	Relapse
TCRB	R - Vβ7.9 - Jβ2.5 G	R - Dβ2 - Jβ2.7 R - not identified to Jβ2 R - Vβ6.3 - Jβ1.2 R - not identified to Jβ1	R - Vβ2.2 - Jβ2.7 R - Dβ1 - Jβ1.2 R - Dβ2 - Jβ2.7	R - Vβ20.1 - Jβ1.2 R - Vβ6.1 - Jβ1.1 RN - Dβ1 – Jβ1.2 RN - Dβ2 - Jβ2.7
TCRG	R - Vγ2 - Jγ2.3 R - Vγ4 - Jγ2.3	R - Vγ7 - Jγ2.3 R - Vγ9 - Jγ2.3	R - Vγ10 - Jγ2.3 R - Vγ11 - Jγ2.3	R - Vγ2 - Jγ2.3 R - Vγ4 - Jγ2.3
TCRD	D D	R - Vδ1 - Jδ1 R - Dδ2 - Dδ3	R - Vδ1 - Jδ1 D	R - ? t(10;14) G

G, allele in germline configuration; R, rearranged allel; D, deletion of the Cδ gene segment; RN, rearrangement using the same gene segments but with different junctional region as compared to rearrangement at diagnosis

late relapses at 6 and 10 years from initial diagnosis, respectively. This is in striking contrast to the remaining 26 patients, who relapsed within 37 months from diagnosis (mean: 14 months). Therefore, we concluded that these two patients developed a secondary T-ALL, and consequently they were excluded from further calculations on the stability of the rearrangements.

Stability of gene rearrangements in T-ALL patients at relapse

A total of 95 (86%) of 111 clonal TCR and *SIL-TAL1* gene rearrangements identified with heteroduplex PCR analysis at diagnosis in 26 T-ALL patients were preserved at relapse (Figure 1). This concerned 17 of 17 (100%) *TCRD*, 42 of 49 (86%) *TCRG*, 35 of 44 (80%) *TCRB* gene rearrangements and a single *SIL-TAL1* fusion gene (Table 2).

In 16 patients all TCR gene rearrangements identified at diagnosis were preserved at relapse (Figure 2A). In 9 cases some targets (ranging from one to 40%) were lost during the disease course (Figure 2B). Finally, in one patient (6953) only one *TCRB* rearrangement was common for both the diagnosis and relapse samples, whereas the other three TCR gene rearrangements were absent at relapse. Consequently, at least one rearrangement was preserved at relapse in all 26 T-ALL patients.

Clonal evolution was observed in seven of 16 patients with $CD3^-$ T-ALL (44%), two of eight patients with $TCR\alpha\beta^+$ T-ALL (25%) and one of two $TCR\gamma\delta^+$ T-ALL (50%). $CD3^-$ group can be further subdivided based on *TCRD* gene configuration. Clonal evolution was more frequent in T-ALL with biallelic *TCRD* deletions (two of three cases; 67%) as compared with five of 13 T-ALL with at least one *TCRD* gene rearrangement (38%), but this difference did not reach statistical significance.

It should be noted that in the eight $TCR\alpha\beta^+$ T-ALL at least one *TCRB* rearrangement (in six patients all *TCRB* rearrangements) remained stable and that also in the two $TCR\gamma\delta^+$ T-ALL no changes were observed in the *TCRG* or *TCRD* gene rearrangements. Apparently, at least the expressed TCR alleles were not subjected to continuing rearrangements.

Stability and patterns of clonal evolution of particular gene rearrangements in T-ALL patients

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

***TCRD* gene rearrangements**

A total of 17 clonal *TCRD* gene rearrangements (10 Vδ1-Jδ1, 4 Dδ2-Jδ1, 1 Vδ2-Jδ1, 1 Vδ3-Jδ1 and 1 Vδ2-Dδ3) were identified by PCR in 13 T-ALL patients. All clonal rearrangements were preserved at relapse. In four additional patients PCR analy-

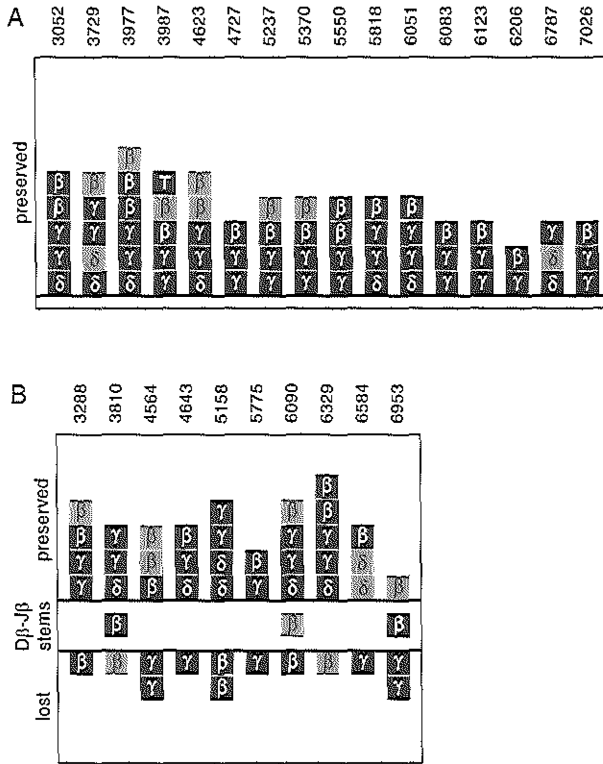

Figure 2.

TCR gene rearrangement stability patterns in 26 T-ALL patients subjected to comparative heteroduplex PCR analysis at diagnosis and relapse. Black squares represent complete rearrangements, while gray squares reflect incomplete rearrangements. All squares above the (upper) line represent stable MRD-PCR targets, while all squares below the (lower) line represent TCR gene rearrangements lost owing to clonal evolution. β squares between the two lines have common Dβ-Jβ stems shared by different *TCRB* rearrangements at diagnosis and at relapse. **(A)** 16 patients with all rearrangements identified at diagnosis preserved at relapse, **(B)** 9 cases with some TCR gene rearrangements (ranging from one to 40%) lost during the disease course and one patient (6953) with the majority of clonal markers absent at relapse.

Abbreviations: β, *TCRB* gene rearrangements; δ, *TCRD* gene rearrangements; γ, Vγ-Jγ gene rearrangements; T, *SIL-TAL1* fusion gene.

sis did not result in identification of clonal *TCRD* rearrangements, while Southern blot data showed rearrangements to the Dδ3/Jδ1 region, which could not be assigned to a particular Vδ-Jδ joining based on the size of the clonal bands. Identically rearranged bands, most probably representing Vα-Jδ1 rearrangements or translocations into the *TCRD* locus, were also present in these four patients at relapse.

Table 2. Stability of MRD-PCR targets in 26 T-ALL patients.

	STABILITY OF MRD-PCR TARGETS		STABILITY IN PATIENTS			
			All targets preserved		At least one target preserved	
TCRB						
Vβ-Jβ	79%	(23/29)	74%	(17/23)	78%	(18/23)
Dβ-Jβ	80%	(12/15)	75%	(9/12)	83%	(10/12)
all *TCRB*	80%	(35/44)	76%	(19/25)	92%	(23/25)
TCRG						
Vγ-Jγ	86%	(42/49)	81%	(21/26)	89%	(23/26)
TCRD						
Vδ-Jδ or Vδ-Dδ	100%	(13/13)	100%	(12/12)	100%	(12/12)
Dδ2-Jδ1 or Dδ2-Dδ3	100%	(4/4)	100%	(3/3)	100%	(3/3)
all *TCRD*	100%	(17/17)	100%	(13/13)	100%	(13/13)
SIL-TAL1	100%	(1/1)	100%	(1/1)	100%	(1/1)

Clonal evolution in *TCRG* locus

A total of 49 *TCRG* gene rearrangements were detected in 26 T-ALL patients at diagnosis and in 23 cases (89%) at least one *TCRG* gene rearrangement was preserved at relapse. Clonal evolution of *TCRG* gene rearrangements was observed in 5 patients leading to loss of 7 MRD-PCR targets. In two patients (5775, 6584) this concerned "regression" of clonal rearrangements most probably to germline configuration. In one patient (4643) Southern blot data indicated the deletion of a second allele. In one patient (4564) the new rearrangements at relapse contained upstream Vγ and downstream Jγ segments as compared to the Vγ-Jγ rearrangements at diagnosis, which is suggestive of ongoing recombination with Vγ-Jγ replacement. Finally, in the fifth patient (6953) the sequence comparison of Vγ-Jγ 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 *TCRB* locus

A total of 44 *TCRB* gene rearrangements were detected in 25 T-ALL patients at diagnosis and in 23 cases (92%) at least one MRD-PCR target was preserved at relapse. Clonal evolution of *TCRB* gene rearrangements was observed in 6 patients leading to loss of 9 MRD-PCR targets. In two patients (3288 and 6329) combined Southern blot and PCR information showed that subclones found at diagnosis disappeared at relapse. In one patient (6090), the configuration at relapse reflected

ongoing Vβ to Dβ2-Jβ2 joining, deleting the Vβ-Jβ1 rearrangement present at diagnosis (Figure 3). In two patients (3810 and 6953), the sequences of the Vβ-Jβ rearrangements contained common Dβ-Jβ stems, which confirms their origin from a common (pre)leukemic progenitor with such Dβ-Jβ rearrangement (Figure 3). Finally, in one TCRγδ^{+} T-ALL patient (5158), the sequences of the *TCRB* gene rearrangements at diagnosis and at relapse were completely different but the *TCRG* and *TCRD* rearrangements were identical suggesting that the presumed leukemic progenitor probably had germline *TCRB* genes.

An additional example of clonal evolution in the *TCRB* genes was found at relapse of patient 5598. Two of the oligoclonal rearrangements at relapse (Vβ20.1-Jβ1.2 and Dβ1-Jβ1.2) contained identical Dβ1-Jβ1.2 junctions, which is consistent with ongoing Vβ to Dβ-Jβ joining.

DISCUSSION

When compared to precursor-B-ALL, T-ALL are generally characterized by more high-risk clinical features at presentation and by a more aggressive clinical course.[38] This is also clearly visible in the MRD kinetics during the first year of treatment. The frequency of MRD-positive patients and the MRD levels are generally higher in T-ALL than in precursor-B-ALL, reflecting the more frequent occurrence of resistant disease in T-ALL.[6] This more aggressive disease kinetics is also associated with shorter progression free survival in T-ALL patients.[6,39] In fact, the vast majority of T-ALL relapses occur within the first 2 to 3 years from diagnosis.[39,40]

Surprisingly, two patients in our study with very late T-ALL relapses (6 and 10 years from initial diagnosis, respectively) showed fully changed TCR gene configurations, very much suggestive of a secondary leukemia (Table 1). This was in striking contrast to the other 26 patients, which showed moderate-to-high stability of identified MRD-PCR targets. The only alternative explanation for so different TCR gene rearrangement patterns would be the emergence of clones at diagnosis and relapse from a common progenitor with all TCR genes in germline configuration. The secondary character of one of the two T-ALL was further supported by the finding of a novel genetic aberration at relapse.

Secondary ALL are rare, comprising only 5-10% of secondary leukemias and secondary T-ALL was previously described only anecdotally and never in association with a primary T-ALL.[41-45] In fact, our hypothesis that a proportion of very late T-ALL recurrences represent secondary T-ALLs has never been put forward previously. In contrast, Lo Nigro and colleagues[22] studied *TCRD* and *TCRG* gene configurations in two T-ALL patients relapsing 72 and 77 months from diagnosis and found in both patients at least one identical clonal TCR gene rearrangement at diagnosis and at relapse. Therefore, it would be of great importance to study more patients with

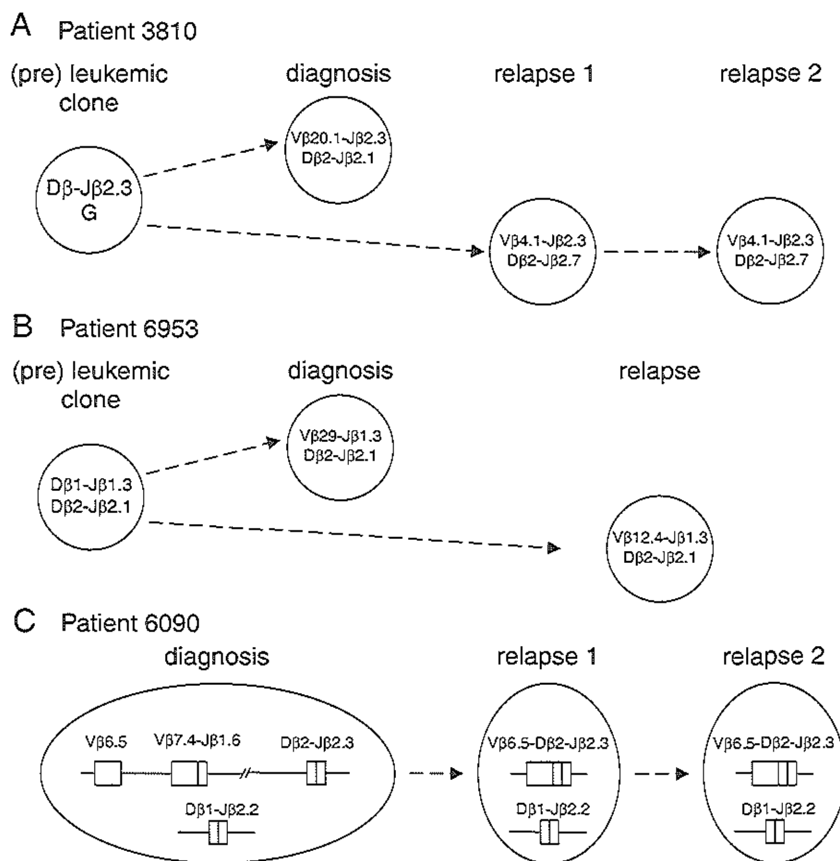

Figure 3.

Examples of clonal evolution in *TCRB* locus. **(A)** On one *TCRB* allele in patient 3810, the Vβ20.1-Jβ2.3 rearrangement at diagnosis and the Vβ4.1-Jβ2.3 rearrangement at relapse had identical Dβ-Jβ2.3 junctions. The Dβ2-Jβ2.1 rearrangement at diagnosis and the Dβ2-Jβ2.7 rearrangement at both relapses were not related. This indicates the origin of clones at diagnosis and at relapses from a common (pre)leukemic clone with a Dβ-Jβ2.3 rearrangement on one allele and a second *TCRB* allele in germline configuration. **(B)** In patient 6953, the Vβ29-Jβ1.3 rearrangement at diagnosis and the Vβ12.4-Jβ1.3 rearrangement at relapse had identical Dβ1-Jβ1.3 junctions. On the same allele, the Dβ2-Jβ2.1 rearrangement identified at diagnosis was preserved at relapse. This indicates the origin of clones at diagnosis and at relapses form a common (pre)leukemic clone with double Dβ1-Jβ1.3 and Dβ2-Jβ2.1 rearrangements on one allele. The second allele at diagnosis was in germline configuration. **(C)** Example of complex secondary rearrangement and ongoing Vβ to Jβ joining in patient 6090. At diagnosis, two rearrangements were found on one allele: Vβ7.4-Jβ1.6 and Dβ2-Jβ2.3. Ongoing rearrangement of the Vβ6.5 segment, located immediately upstream to the Vβ7.4 segment, to Dβ2 resulted in a new Vβ6.5-Jβ2.3 rearrangement with a preserved Dβ2-Jβ2.3 stem at relapse. The configuration on the second *TCRB* allele (Dβ1-Jβ2.2 rearrangement) was identical at diagnosis and relapse.

very late T-ALL recurrences to address the question of secondary T-ALL in this group. In fact, such patients might have an inherited predisposition for T-ALL development.

Our comparative Southern blot, PCR, and sequencing analyses of T-ALL at diagnosis and relapse have provided detailed insight in the stability and changes of TCR and *SIL-TAL1* gene rearrangements during the disease course. This information is essential for reliable application of such clonal rearrangements as PCR targets in MRD studies in T-ALL. Our previous study in a large group of precursor-B-ALL patients demonstrated extreme clonal evolution in approximately 20% of patients, with all MRD-PCR targets lost in 7% of patients.[18] In contrast, the loss of virtually all MRD-PCR targets was observed in only one T-ALL patient, in whom one of the four clonal TCR gene rearrangements was still present at relapse. Also the proportion of patients with all MRD-PCR targets preserved at relapse was markedly higher in T-ALL (62%) compared with precursor-B-ALL (40%).

DNA breakpoints of chromosome aberrations are ideal targets for MRD detection, since they are linked to the oncogenic process and are therefore stable throughout the disease course.[46] However, such aberrations, namely *TAL1* deletion with *SIL-TAL1* gene fusion, can be identified in only 10-25% of T-ALL patients.[47,48] Only one T-ALL case in our study group had a *TAL1* deletion with an identical breakpoint sequence at diagnosis and at relapse.

Analyzing the stability of particular MRD-PCR targets, *TCRD* gene rearrangements were uniformly preserved at relapse in all T-ALL patients. Recombination of the *TCRD* locus is one of the presumably earliest events in T-cell development in the thymus. Oncogenic transformation in T-ALL, i.e. malignant proliferation of thymocytes, usually (in 95% of patients) occurs when *TCRD* gene rearrangement is completed on at least one allele.[28] The *TCRD* gene rearrangements might be potentially lost during the disease course via ongoing recombination in the *TCRA* locus, thereby deleting the *TCRD* genes.[49] Such clonal evolution at relapse has indeed been described in two patients, associated in one case with a phenotypic shift from $CD3^-$ T-ALL to $TCR\alpha\beta^+$ T-ALL.[21,22] Nevertheless, in concordance with our study, the vast majority of previously described T-ALL patients analyzed at diagnosis and relapse showed a fully stable *TCRD* configuration.[19,21,22]

In contrast to *TCRD* gene rearrangements, which are detectable in only half of the T-ALL patients, *TCRG* gene rearrangements are present in more than 95% of T-ALL patients[31] and accordingly could be identified in all patients in our study. In 81% of patients, the *TCRG* gene configuration was fully identical between diagnosis and relapse, and in 89% of patients at least one *TCRG* rearrangement was preserved. Such high stability might be anticipated from the finding that most of the *TCRG* gene recombinations are end-stage rearrangements involving either the most upstream $V\gamma2$ or the most downstream $J\gamma2.3$ gene segments.[31] Nevertheless, in a subgroup of patients we and others[20] observed clonal "regressions" to a less mature configura-

tion, which in fact represented the outgrowth of different subclone at relapse. In these cases (patients 5775, 6584 and 6953), the oncogenic event most probably occurred before the *TCRG* gene rearrangement. Another potential disadvantage of using *TCRG* junctions as MRD-PCR targets might be their limited sensitivity owing to the abundant background of polyclonal *TCRG* gene rearrangements in normal T-cells.[15,50] The currently used RQ-PCR techniques aim at sensitivities of $\leq 10^{-4}$, but in approximately one-third of *TCRG* gene rearrangements in T-ALL such sensitivity cannot easily be reached.[15]

The development of multiplex approaches for detection of *TCRB* gene rearrangements, resulted in an additional PCR target for MRD detection (Van Dongen et al. submitted for publication). Clonal *TCRB* gene rearrangements were identified in all except one patient in our series. Although their general stability (80%) was inferior to *TCRG and TCRD* gene rearrangements, at least one *TCRB* PCR target was preserved at relapse in 92% of patients. Particularly, complete Vβ-Jβ rearrangements are attractive MRD PCR targets because of their extensive junctional regions, which guarantee good sensitivities in RQ-PCR analysis in virtually all patients (Brüggemann et al, submitted for publication). However, we observed several examples of clonal evolution phenomena owing to continuing rearrangements as well as resulting from the selection of drug-resistant subclones with partly related or unrelated *TCRB* genes (Figure 3). In patients with extreme clonal evolution, the *TCRB* gene rearrangements might represent post-oncogenic events (see Figure 3), which might be related to the observation that *TCRB* gene rearrangements occur relatively late during T-cell differentiation.

Based on the comparative analysis of the TCR gene configuration at diagnosis and relapse of T-ALL, we propose a stepwise strategy for MRD-PCR target selection. In precursor-B-ALL, the molecular approach for MRD-PCR target identification significantly profits from Southern blot information, which distinguishes a subgroup of precursor-B-ALL patients with oligoclonal and generally unstable Ig/TCR gene rearrangements.[18] In contrast, the strategy developed for T-ALL can be exclusively based on PCR data, since TCR oligoclonality is rare in T-ALL patients. In our study at least one MRD-PCR target was preserved in all patients, but we recommend that two MRD-PCR targets should be used per patient. *TCRD* and *SIL-TAL1* gene rearrangements are highly stable targets and should be selected as the first choice (Figure 4). *TCRB* gene rearrangements are slightly more stable at the patient level than *TCRG* gene rearrangements (see Table 2) and therefore should be selected as the second choice. Ideally, patient-specific oligonucleotides should be positioned on Dβ-Jβ junctions to prevent false negative results owing to ongoing Vβ to Dβ-Jβ rearrangements. Finally, *TCRG* gene rearrangements have a fair stability but are less sensitive RQ-PCR targets and should therefore be selected as third-choice MRD-PCR targets. In our study group, such stepwise strategy (*TCRD* + *SIL-TAL1* → *TCRB* → *TCRG*) would enable successful detection of MRD in all T-ALL patients.

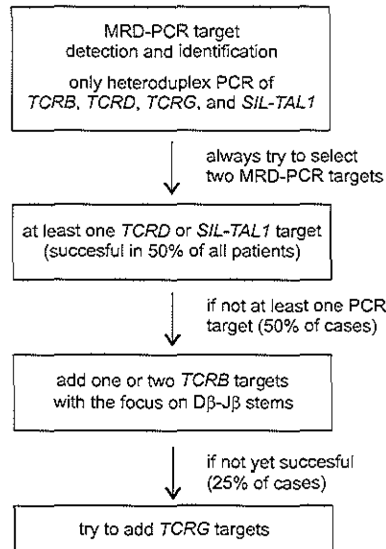

Figure 4.
Flow diagram for the stepwise selection of PCR targets for MRD detection in T-ALL.

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CHAPTER 3.4

MOLECULAR DISCRIMINATION BETWEEN RELAPSED AND SECONDARY ACUTE LYMPHOBLASTIC LEUKEMIA: PROPOSAL FOR AN EASY STRATEGY*

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ABSTRACT

Background

Discrimination between late relapse of acute lymphoblastic leukemia (ALL) and secondary ALL might be clinically important, because the former might still respond favorably to chemotherapy and/or bone marrow transplantation, whereas secondary ALL is associated with poor prognosis.

Procedure

We present a pre-B-ALL patient in whom disease recurred two years after completion of treatment. Differences in cytomorphology and immunophenotyping raised a suspicion of secondary ALL. We performed detailed molecular studies of immunoglobulin and T-cell receptor genes for discrimination between relapsed and secondary ALL.

Results

Southern blot analysis showed an oligoclonal immunoglobulin heavy chain (*IGH*) gene configuration at diagnosis and a monoclonal configuration at relapse. The size of one of the rearranged bands at relapse was identical to one of the faint rearranged

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bands at diagnosis. However, heteroduplex PCR analysis demonstrated that none of the clonal *IGH* gene rearrangements at diagnosis and at relapse was fully identical. Sequencing of several clonal PCR products revealed an identical DH6-13↔JH6b junction shared by two different rearrangements at diagnosis and one rearrangement at relapse, thereby proving the clonal relationship between diagnosis and late relapse in this patient.

Conclusions

We propose a stepwise molecular approach for discrimination between relapsed and secondary ALL based on the rapid and cheap heteroduplex PCR technique, including mixing of clonal (homoduplex) PCR products identified at diagnosis and at relapse. Direct sequencing and comparative sequence analysis of *IGH* gene rearrangements at diagnosis and at relapse should be regarded as an ultimate standard, but can be limited to the rare cases, in which no identical clonal PCR products at diagnosis and at relapse were detected with the mixed heteroduplex PCR analyses.

INTRODUCTION

Despite major progress in the treatment of childhood acute lymphoblastic leukemia (ALL), approximately 25-30% of patients relapse after successful remission induction.[1,2] In a proportion of cases there are obvious cytomorphological, immunophenotypic and/or cytogenetic differences between diagnosis and relapse of ALL.[3-5] Detailed Southern blot (SB) analysis of clonal immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements, showed one or more changes in the rearrangement patterns between diagnosis and relapse of ALL in approximately 70% of patients.[6] However, in 75% to 90% of ALLs at least one major Ig heavy chain (*IGH*), TCR gamma (*TCRG*), or TCR delta (*TCRD*) gene rearrangement remained present at relapse.[6] Subsequent PCR-based studies of clonal Ig and TCR junctional regions demonstrated that the vast majority of changes between diagnosis and relapse resulted from ongoing rearrangements.[7-10] In more than 90% of studied ALL cases, the junctional V-(D)-J sequences of at least one rearrangement were identical at diagnosis and at relapse.[8-10] Thus, in 5-10% of cases no clonal relationship could be assessed between the leukemic clone at diagnosis and during recurrence of the disease, suggesting that in fact a secondary ALL might have emerged.

Secondary acute leukemias comprise a substantial proportion of secondary malignancies as a side effect of intensive chemotherapy. In more than 90% of cases this concerns treatment-related acute myeloid leukemia (AML) characterized by an aggressive clinical course, the presence of *MLL* gene rearrangements, and an unfavorable outcome.[11] Prior usage of topoisomerase II inhibitor and/or alkylating agents

is significantly associated with the development of treatment-related AML.[11,12] Secondary ALL occurs less frequently, comprising only 5-10% of secondary leukemias. In several secondary ALL the presence of the t(4;11) translocation was demonstrated and the outcome was found to be generally unfavorable.[13] Discrimination between late relapse of ALL, usually still favorably responding to treatment, and secondary ALL might thus be of clinical importance.

Here, we present an ALL patient in whom disease recurred two years after treatment cessation. Based on differences in cytomorphology and immunophenotyping, secondary ALL was strongly suspected. However with a stepwise molecular diagnostic procedure for discrimination between relapsed and secondary ALL, we were able to show the clonal relationship between the leukemic cells at diagnosis and at relapse.

MATERIALS AND METHODS

Case report

A 10-year-old child was admitted to the Beatrix Children's Hospital in Groningen with the suspicion of acute leukemia. Cytomorphology of FAB-L1 and immunophenotype ($CD19^+/CD22^+/CD10^+/TdT^+/$ $/HLA\text{-}DR^+/CD34^+$ with 36% of blasts expressing CyIgμ) were compatible with pre-B-ALL. Based on the estimated leukemic cell mass at diagnosis, and the absence of the t(9;22) translocation and *MLL* gene rearrangements, the patient was stratified into the standard-risk treatment group of the Dutch Childhood Leukemia Study Group (DCLSG) ALL-8 protocol, which has a BFM (Berlin-Frankfurt-Münster) backbone.[14] Complete clinical remission was successfully induced and the patient received full standard-risk chemotherapy. Two years after treatment cessation, the child was readmitted to the hospital and relapse of ALL was assumed. However, the morphology of lymphoblasts differed from diagnosis (FAB-L2) and all cells co-expressed the CD5 antigen, whereas the expression of CD34 was lost. Virtually all TdT^+ cells (>98%) were now expressing CyIgμ. Both the morphological and the immunological changes raised a suspicion of secondary ALL.

Southern blot analysis

Mononuclear cells (MNC) were isolated from bone marrow (BM) samples by Ficoll-Paque centrifugation (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden). DNA was isolated from fresh or frozen MNC fractions as described previously.[15] Fifteen micrograms of DNA was digested with the appropriate restriction enzymes (Pharmacia), size-separated in 0.7% agarose gels and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as described.[16] *IGH* and Ig kappa light chain (*IGK*) gene configurations were analyzed with the IGHJ6, IGKJ5, IGKC and IGKDE probes (DAKO Corporation, Carpinteria, CA) in *Bgl*II and *Bam*HI/*Hin*dIII digests, whereas the Ig lambda light chain (*IGL*) gene configuration was analyzed with the IGLC3 probe in *Eco*RI/*Hin*dIII digest.[17-19] The configuration of the *TCRD* genes was analyzed with the TCRDJ1 probe in *Bgl*II, *Eco*RI, and *Hin*dIII digests (DAKO).[20] Incomplete and complete *TCRB* gene rearrangements were detected with the TCRBD1U, TCRBD1, TCRBJ1, TCRBD2U, TCRBD2, TCRBJ2, and TCRBC probes (DAKO) in *Eco*RI and *Hin*dIII digests.[21] *TCRG* gene rearrangements were analyzed with the Jγ1.2 probe in *Bgl*II digests and the TCRGJ13 and Jγ2.1 probes in *Eco*RI digests.[22,23]

PCR amplification and heteroduplex analysis of PCR products

PCR was essentially performed as described previously.[24,25] In each 50µl PCR reaction 50 ng DNA sample, 6.3 pmol 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 oligonucleotides used for amplification of complete VH-JH and incomplete DH-JH gene rearrangements were published before.[26,27] PCR conditions were: initial denaturation for 10 min at 94°C, followed by 35 cycles of 45 sec at 92°C, 90 sec at 60°C, and 2 min at 72°C using a Perkin-Elmer 480 thermal cycler (PE Biosystems). After the last cycle, an additional extension step of 10 min at 72°C was performed. Appropriate positive and negative controls were included in all experiments.[25]

For heteroduplex analysis, the PCR products were denatured at 94°C for 5 min after the final cycle of amplification and subsequently cooled to 4°C for 60 min to induce duplex formation.[28] Afterwards, the duplexes were immediately loaded on 6% non-denaturing polyacrylamide gels in 0.5 x Tris-borate-EDTA (TBE) buffer, run at room temperature, and visualized by ethidium bromide staining.[28] A 100-bp DNA ladder (Promega Corporation, Madison, WI) was used as size marker. Usage of heteroduplex analysis enables discrimination between PCR products derived from monoclonal and polyclonal lymphoid cell populations, based on the presence of homoduplexes (PCR products with identical junctional regions) or a smear of heteroduplexes (derived from PCR products with heterogeneous junctional regions), respectively.

Sequence analysis of *IGH* gene rearrangements

Clonal PCR products as found by 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 described before.[27] 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/).[29]

RESULTS

Southern blot (SB) analysis

SB analysis at diagnosis revealed an oligoclonal pattern in the *IGH* gene locus with two major rearrangements and four to five minor rearrangements (Figure 1), whereas the *IGK* and *IGL* genes were in germline configuration. SB analysis of the *TCRD* locus revealed a strong germline band as well as two weak subclonal rearranged bands (Vδ2-Dδ3 and Dδ2-Dδ3 rearrangements). The *TCRG* and *TCRB* genes were in germline configuration.

At relapse, SB analysis of *IGH* revealed a monoclonal pattern with two strong non-germline bands of equal intensity, corresponding to biallelic rearrangements (Figure 1). The size of one of the rearranged bands was identical to one of the minor rearrangements at diagnosis. The *TCRD* genes were now in germline configuration.

Heteroduplex PCR analysis

Detailed heteroduplex PCR analysis of the *IGH* locus was performed with thir-

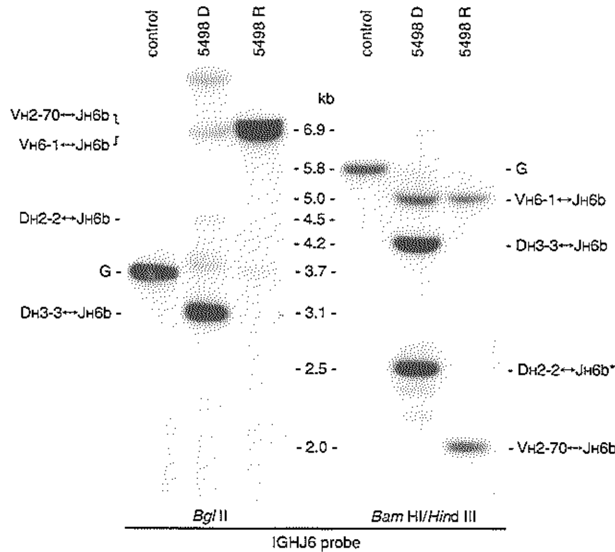

Figure 1.
Southern blot analysis of the *IGH* gene in patient 5498. Control DNA and patient's DNA isolated from diagnosis (D) and relapse (R) BM samples were digested with *Bgl*II and *Bam*HI/*Hind*III restriction enzymes, size separated, and blotted onto nylon membrane filters, which were hybridized with the ^{32}P-labeled IGHJ6 probe.[17] At diagnosis multiple bands of different densities were found, reflecting *IGH* oligoclonality. In contrast, the relapse sample displayed a monoclonal pattern with biallelic *IGH* gene rearrangements. Based on the germline sequence and the restriction map of the *IGH* locus,[30] rearrangements found by direct sequencing were assigned to corresponding bands on the Southern blot. The asterisk indicates the comigration of a faint rearranged band corresponding to the incomplete DH2-2↔DH6-13↔JH6b rearrangement with a major non-identified rearranged band in the *Bam*HI/*Hind*III digest.

teen primer combinations (six *IGH* framework-1 VH family-specific primers and seven family-specific DH primers in combination with one JH consensus primer). At diagnosis, monoclonal homoduplexes were found with the following primer combinations: VH5-JH, VH6-JH, DH2-JH, and DH3-JH, whereas the VH1-JH, VH3-JH and VH4-JH PCR products were seemingly oligoclonal (Figure 2A). At relapse, monoclonal homoduplexes were found with both the VH2-JH and VH6-JH primer combinations. The VH6-JH monoclonal products at diagnosis and at relapse slightly differed in size. When mixed together and subjected to denaturation and renaturation they formed a heteroduplex band, confirming that the VH6-JH PCR products at diagnosis and relapse were not identical (Figure 2B).

Sequence analysis

Four monoclonal PCR products found at diagnosis and two monoclonal PCR products found at relapse were sequenced resulting in identification of the involved

Table 1. Junctional region sequences of different *IGH* gene rearrangements found in patient 5498 at diagnosis and at relapse of ALL.[a]

Disease stage	VH gene segment	N-REGION	VH/DH gene segment	N-REGION	DH gene segment	N-REGION	JH gene segment	Reading frame
Diagnosis			DH2-2 (-1)	AAG	(-7) DH6-13 (0)	CGGGGATG	(-6) JH6b	Not applicable
	VH6-1 (-6)	GAGGGAGGG	(-14) DH2-2 (-1)	AAG	(-7) DH6-13 (0)	CGGGGATG	(-6) JH6b	(+)
			DH3-3 (-5)	CCTAATCCCTCTTATAC	(-7) DH1-7 (0)	CCACGAGA	(-6) JH6b	Not applicable
	VH5-51 (-2)	GG	(-5) DH3-3 (-5)	CCTAATCCCTCTTATAC	(-7) DH1-7 (0)	CCACGAGA	(-6) JH6b	(+)
Relapse			VH2-70 (-2)	CCGCCG	(-10) DH6-13 (0)	CGGGGATG	(-6) JH6b	(-)
			VH6-1 (0)	ACGG	(-1) DH2-8 (-6)	CCACCCCC	(-7) JH6b	(+)

[a] Numbers in parentheses indicate the extent of nucleotide deletion by trimming of the 5' and 3' part of the involved gene segments.

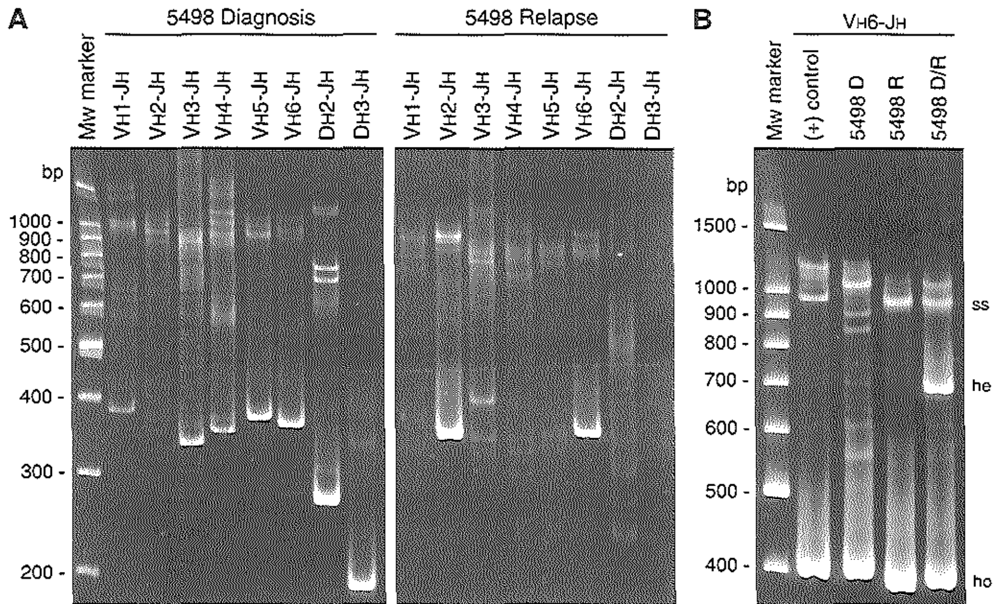

Figure 2.

(A) Heteroduplex PCR analysis of *IGH* gene rearrangements at diagnosis and at relapse of the ALL clone in patient 5498. Several clonal PCR products were found at diagnosis, confirming *IGH* oligoclonality, whereas the relapse sample revealed two major VH-JH gene rearrangements.

(B) Heteroduplex PCR analysis of VH6-JH PCR products. Monoclonal homoduplexes (ho) found at diagnosis and at relapse slightly differed in size. Mixing of the VH6-JH PCR products followed by heteroduplex PCR analysis demonstrated clear heteroduplex (he) formation, proving that these VH6-JH gene rearrangements had different junctional regions. (ss) = single strands.

gene segments and the junctional regions (Table 1). The four sequences at diagnosis were found to be derived from two different incomplete rearrangement stems: DH2-2↔DH6-13↔JH6b and DH3-3↔DH1-7↔JH6b, illustrating ongoing VH to D-J joining. The junctional region of the DH6-13↔JH6b stem was also preserved in the VH2-70↔DH6-13↔JH6b rearrangement at relapse. The sequence of the VH6-1↔DH2-8↔JH6b gene rearrangement found at relapse did not have any relationship with the sequences found at diagnosis, also not with the VH6-1↔DH2-2↔DH6-13↔JH6b rearrangement at diagnosis (Table 1).

Functionality of *IGH* gene rearrangements and its correlation with immunophenotype

Based on the sequenced clonal *IGH* gene rearrangements at diagnosis and at relapse and the availability of the complete sequence of the human *IGH* locus,[30] the sizes of restriction fragments containing the respective rearrangements were calcu-

lated (Figure 1). At diagnosis the incomplete DH3-3↔DH1-7↔JH6b rearrangement corresponded to a major rearranged band, whereas the DH2-2↔DH6-13↔JH6b and VH6-1↔DH2-2↔DH6-13↔JH6b rearrangements represented faint bands.

Based on the SB rearranged band density, the in-frame VH6-1↔DH2-2↔DH6-13↔JH6b rearrangement was largely contributing to CyIgμ expression found in 36% of leukemic cells at diagnosis, whereas the functional (in-frame) VH5-51↔DH3-3↔DH1-7↔JH6b rearrangement (expected size of 1.3 kb in *Bgl*II and 8.0 kb in *Bam*HI/*Hin*dIII) was not visible in the SB analyses, implying that this rearrangement was derived from a minor subclone (<5%). The two rearranged bands at relapse had sizes corresponding with the out-of-frame VH2-70↔DH6-13↔JH6b and the in-frame VH6-1↔DH2-8↔JH6b rearrangements. The latter was consistent with the finding that virtually all blasts at relapse were expressing CyIgμ. Curiously, the functional VH6-1↔JH6b rearrangements at diagnosis and at relapse were completely different (Table 1).

DISCUSSION

SB analysis is considered to be the gold standard for detection of clonal Ig and TCR gene rearrangements.[16] This technique has also been used in a large comparative study of leukemia-specific molecular markers between diagnosis and recurrence of ALL.[6] The finding of identically rearranged bands at diagnosis and at relapse, however, only reflects usage of identical gene segments, but does not necessarily imply identical junctional regions. For reliable proof of clonal identity, the identically sized rearranged bands should represent major gene rearrangements and preferably this should be found for multiple Ig and TCR gene loci. This is particularly important for patients with oligoclonal Ig and TCR gene rearrangements. When the only indication for clonal relationship is based on the identity of a single band, comparative analysis of junctional regions is mandatory, like in our patient with a subclone at diagnosis, which seemed to result in a major rearrangement at relapse. Such comparative junctional region analyses can be performed with the current PCR-based methods.

Using multiple primer combinations followed by heteroduplex PCR analysis, we were able to detect several clonal *IGH* gene rearrangements at diagnosis and two at relapse, but only the VH6-JH primer combination resulted in clonal PCR products both at diagnosis and at relapse. However, the VH6-JH homoduplex PCR products slightly differed in size, whereas denaturation and renaturation of the mixed PCR products resulted in obvious heteroduplex formation (Figure 2B), confirming a difference in the junctional regions of the VH6↔JH gene rearrangements. Indeed in both rearrangements the same VH6-1 and JH6b gene segments were found, but the junctional regions were completely different (Table 1). These two different VH6-1↔JH6b

gene rearrangements gave rise to identical bands in the diagnosis and relapse samples on SB analysis. Only after sequencing of several clonal PCR products, an identical DH6-13↔JH6b junction stem shared by two rearrangements at diagnosis and one at relapse was observed (Figure 3), finally confirming the clonal relationship between the diagnosis and late relapse of ALL, and excluding secondary malignancy. Thus comparative sequence analysis of different *IGH* gene rearrangements at diagnosis and at relapse should be regarded as an ultimate standard for the discrimination between the relapse of ALL and a secondary leukemia.

Coincidentally, the two distinct VH6-1↔JH6b gene rearrangements both encoded for functional CyIgμ chains. The fact that the VH6-1↔JH6b rearrangement was only present as a minor rearrangement at diagnosis (Figure 1), explains that the CyIgμ expression was found in only one-third of the leukemic blasts at diagnosis. The functional VH6-1↔JH6b gene rearrangement at relapse characterized the total monoclonal population, which indeed uniformly expressed CyIgμ. As explained above, the VH6-1↔JH6b gene rearrangements at diagnosis and at relapse were derived from different chromosomes. This means that although both disease stages were classified as pre-B-ALL, the expressed CyIgμ protein chains were different. The finding of a pre-B-ALL phenotype at diagnosis and at relapse is therefore pure coincidence and illustrates that the absence of an immunophenotypic shift does not necessarily mean that the leukemic cells at diagnosis and at relapse are identical.

Clonal evolution in the *IGH* gene locus in our patient clearly demonstrates the pitfalls of using *IGH* gene rearrangements as PCR targets for minimal residual disease (MRD) monitoring. Frequent *IGH* oligoclonality at diagnosis (at least 40% of precursor-B-ALL cases) as well as ongoing and secondary gene rearrangements may hamper MRD detection resulting in false-negativity.[7,31-33] Therefore, the initial large-scale prospective MRD studies in childhood precursor-B-ALL included more stable although less frequently occurring TCR and *IGK* deletional element gene rearrangements.[34,35] Nevertheless, in 10-15% of precursor-B-ALL cases, including our patient, *IGH* gene rearrangements would be the only available PCR targets for MRD monitoring.[36,37] Based on the common DH6-13↔JH6b junction stem in our patient, we performed real-time quantitative PCR (RQ-PCR) analysis.[38-40] This RQ-PCR

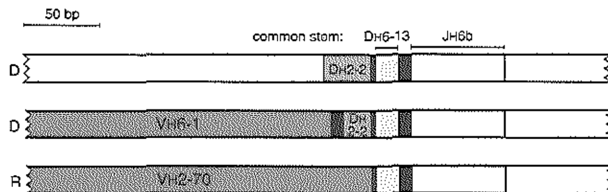

Figure 3.
Schematic representation of different *IGH* gene rearrangements at diagnosis (D) and at relapse (R) with the common DH6-13↔JH6b stem.

analysis revealed that the common junction stem, which was preserved at relapse could in principal have been used as RQ-PCR target for MRD monitoring (data not shown). This information is particularly important for patients with oligoclonal *IGH* gene rearrangements. Our findings also confirm the need to use at least two independent molecular MRD targets,[34] since in our patient the second common DH1-7↔JH6b junction stem found at diagnosis was not present at relapse.

Using a stepwise molecular approach, we were able to prove the clonal relationship between diagnosis and late relapse of ALL, thereby excluding the possibility of secondary leukemia. Such discrimination is relevant for tailoring the treatment strategy and intensity. We also demonstrated the usefulness of mixed heteroduplex PCR analysis for rapid discrimination between identical or different clonal PCR products at diagnosis and at relapse. This technique was already proven to be a rapid and cheap alternative for the more laborious and time-consuming SB analysis to identify clonal Ig and TCR gene rearrangements.[28,36] Direct sequencing can be limited to those cases, in which non-identical clonal PCR products at diagnosis and at relapse are detected with mixed heteroduplex PCR analyses.

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CHAPTER 3.5

ACUTE LYMPHOBLASTIC LEUKEMIA FOLLOWED BY A CLONALLY-UNRELATED EBV-POSITIVE NON-HODGKIN'S LYMPHOMA AND A CLONALLY-RELATED MYELOMONOCYTIC LEUKEMIA CUTIS*

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ABSTRACT

Background

Malignant hematopoietic proliferations might severely hamper the course of acute lymphoblastic leukemia (ALL) in patients with an otherwise good prognosis. It is important to distinguish whether such neoplastic proliferations represent an ALL relapse or secondary treatment-related malignancies.

Procedure

We present a precursor-B-ALL patient in whom maintenance treatment of ALL was complicated by an isolated ALL relapse in the brain, nodular lymphoproliferations in liver, and an isolated myelo-monocytic leukemia cutis. All these hemato-oncologic malignancies occurred in the background of an iatrogenic immunodeficiency

Results and conclusions

Using a stepwise molecular approach, we were able to demonstrate that the liver infiltrates were Epstein-Barr virus (EBV)-positive, contained monoclonal mature B-cells with immunoglobulin heavy chain gene (*IGH*) gene rearrangements unrelated to the primary ALL, and thus represented a true secondary Non-Hodgkin's lymphoma (NHL). In contrast, the skin infiltrates consisted of myelo-monocytic cells with

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*Submitted

clonal *IGH* and T-cell receptor gamma gene rearrangements, identical to the precursor-B-ALL blasts at diagnosis. Thus, the disease course of the precursor-B-ALL patient was complicated by two different isolated extramedullary relapses and a secondary EBV$^+$ B-NHL.

INTRODUCTION

Among acute hemato-oncologic complications of childhood acute lymphoblastic leukemia (ALL) relapse of the disease is the most frequent problem. Despite significant progress in treatment of ALL in last decades 25-30% of ALL patients relapse and most children with an early relapse die of disease progression.[1,2] Another serious malignant side-effect of ALL treatment is acute myelogenous leukemia (AML) affecting approximately 4% of ALL patients.[3] Most of these AML are secondary malignancies with translocations involving *MLL* gene on chromosome 11q23, particularly developed after treatment with topoisomerase II inhibitors, such as epipodophyllotoxins or alkylating agents.[3] Nevertheless, detailed molecular analyses of immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements revealed that a subset of presumed secondary AML represent in fact a phenotypic shift of the same leukemic clone.[4,5]

Epstein-Barr virus (EBV) related lymphoproliferative disorders and EBV-related non-Hodgkin's lymphomas (NHL) are rare acute complications of ALL treatment.[6-12] These disorders usually are associated with profound and prolonged immunosuppression such as in patients with severe combined immunodeficiency, recipients of organ or bone marrow transplants, and AIDS patients.[13,14]

Here we present a child with precursor-B-ALL who suffered from several consecutive acute hemato-oncologic complications, namely isolated central nervous system involvement, multiple liver tumors, and skin infiltrations. The application of various molecular techniques could discriminate between ALL relapse and treatment-related secondary malignancies.

MATERIALS AND METHODS

Case report

An eleven-year-old, previously healthy girl (patient 4687) was admitted to the University Hospital in Nijmegen with a suspicion of acute leukemia. On physical examination she presented with generalized lymphadenopathy and prominent hepatosplenomegaly. More than 90% of bone marrow nucleated cells and peripheral blood leukocytes (WBC of 220 x 10^9/l) were lymphoblasts of FAB-L1 morphology and common-ALL immunophenotype (CD19$^+$/CD10$^+$/TdT$^+$/CyIgμ^-). Cytogenetic analysis of leukemic blasts revealed a normal 46XX karyotype. The treatment was started according to the Dutch Childhood Leukemia Study Group (DCLSG) ALL-7 protocol.[15,16] The response to steroid treatment was good and

cytomorphological remission was successfully induced already after two weeks. Further induction as well as consolidation treatment was well tolerated.

During maintenance treatment she developed several periods of absolute neutropenia and hypogammaglobulinemia complicated by protracted infections. A year after initial diagnosis the child presented with headache, hesitant speech, and wordfinding difficulties, subsequently combined with periods of visual hallucinations, confusion and vomiting without nausea. Neurological examination revealed a fronto-temporal dysfunction and a slight left-sided hearing disturbance. Ophtalmological and otolaryngological examinations were normal. Lumbar puncture provided clear cerebrospinal fluid (CSF) with a normal cell count and slightly elevated protein concentration. Cytomorphology and immunophenotyping of CSF cells revealed only normal lymphocytes and macrophages. Since the neurological symptoms persisted despite empiric broad-spectrum anti-bacterial/anti-mycotic treatment and computed tomography revealed multiple brain lesions a small brain cortex biopsy was performed. Histomorphology showed patchy necrosis and perivascular cuffs of infiltrating leukemic lymphoblasts. This was consistent with the isolated central nervous system ALL relapse. Taking into account the persistent iatrogenic immune deficiency the child was not exposed to an intensive systemic re-induction therapy. Instead cranial irradiation was administered to a total dose of 30 Gy combined with monthly intravenous L-asparaginase and intrathecal methotrexate injections in addition to the normal maintenance therapy. The neurological symptoms gradually resolved and follow-up by computed tomography scan demonstrated disappearance of the cerebral lesions.

After subsequent two months, the child presented with the bouts of fever, while abdominal ultrasonography showed multiple liver lesions with a diameter of 2 – 5 cm. The open liver biopsy procedure showed multiple round gray-yellowish tumors in both liver lobes. A good representative biopsy was obtained. The bacteriologic and mycological cultures were negative. Histopathologic investigation showed several areas of liver necrosis, surrounded by polymorphous lymphoid infiltration. Immunophenotyping revealed a mixed population of both T and B-cells, and the staining pattern for immunoglobulin light chains was polyclonal. The overall picture was very much suggestive of polymorphic B-cell lymphoma, which is typically associated with Epstein-Barr virus (re-)infection in the background of immunodeficiency. Since the follow-up ultrasonography showed a steady increase of liver lesions, the whole liver was irradiated to a total dose of 21 Gy. Radiotherapy was well tolerated by the child and follow-up ultrasound investigation showed complete resolution of the lesions.

Three months later the girl presented with multiple slightly elevated red-bluish skin lesions on the arms. The nodules were neither pruritic nor tender. The sections of the skin biopsy showed small nodular proliferations of cells with monocytic/histiocytic morphology infiltrating the dermis and subcutaneous fat, extending to periadnexal regions. Immunohistochemistry showed that the infiltrating cells were strongly positive for CD14 and CD68, and negative for B-cell, T-cell, and epithelial markers. The histomorphological picture was strongly suggestive of an aleukemic leukemia cutis of myelomonocytic subtype (AML-M4).

Very shortly after the appearance of the skin lesions, the patient developed severe bronchopneumonia, which progressed to overt cardio-respiratory insufficiency and child's death. The autopsy showed extensive necrotizing pneumonia with the presence of Aspergillus fumigatus and Pneumocystis carinii. Aspergillus infection was widely disseminated into heart, brain and large blood vessels. Remarkably, bone marrow, central nervous system and liver did not show any macroscopic or microscopic evidence for the persistence of malignant cells.

Southern blot analysis of Ig and TCR gene rearrangements

Mononuclear cells (MNC) were isolated from bone marrow (BM) sample at diagnosis by Ficoll-Paque centrifugation (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden). DNA was isolated from frozen MNC and from snap-frozen tissue samples (liver and skin biopsies) after homogenization with a tissue grinder (Tamson, Zoetermeer, The Netherlands) as described previously.[17] Unfortunately, there was no material available for molecular analysis of the central nervous system infiltrate. Fifteen µg of DNA was digested

with the appropriate restriction enzymes (Pharmacia), size-separated in 0.7% agarose gels and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as described.[17] For comparative analysis the diagnosis and biopsy-derived DNA samples were run in parallel lanes (Figure 1). Ig heavy chain (*IGH*) and Ig kappa light chain (*IGK*) gene configurations were analyzed with the IGHJ6, IGKJ5, IGKC and IGKDE probes (DAKO Corporation, Carpinteria, CA) in *Bgl*II, *Xba*I and *Bam*HI/*Hin*dIII digests, whereas the Ig lambda light chain (*IGL*) gene configuration was analyzed with the IGLC3 probe in *Eco*RI/*Hin*dIII digest.[18-20] The configuration of the TCR delta (*TCRD*) genes was analyzed with the Vδ2, TCRDJ1, Jδ2, TCRDC4 probes in *Bgl*II, *Eco*RI, and *Hin*dIII digests (DAKO).[21] TCR beta (*TCRB*) gene rearrangements were detected with the TCRBJ1, TCRBJ2, and TCRBC probes (DAKO) in *Eco*RI and *Hin*dIII digests.[22] TCR gamma (*TCRG*) gene rearrangements were analyzed with the Jγ1.2 probe in *Bgl*II digests and the TCRGJ13 and Jγ2.1 probes in *Eco*RI digests.[23,24]

Southern blot based detection of EBV genome

The presence of clonal EBV DNA was analyzed with a ^{32}P labeled *Xho*I probe. This probe is a 1.9 kb fragment, which recognizes unique EBV-DNA sequences, adjacent to the repeat sequence at each terminus.[25,26] The DNA isolated from BM at diagnosis and from the liver and skin biopsies was analyzed with this probe in *Bam*HI/*Hin*dIII digests.

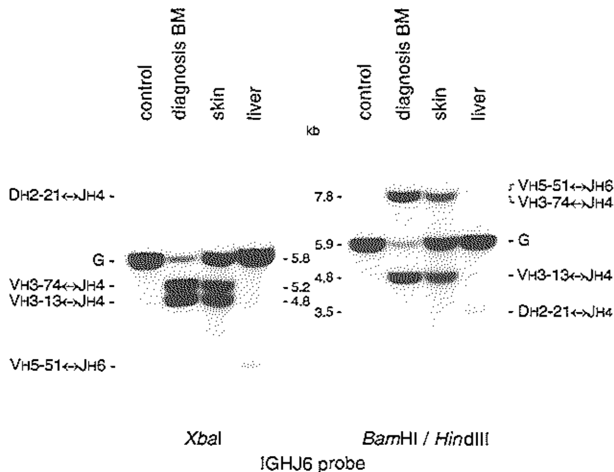

Figure 1.

Southern blot analysis of the *IGH* genes in patient 4687. Control DNA and patient's DNA isolated from BM at diagnosis as well as from liver and skin biopsies were digested with *Xba*I and *Bam*HI/*Hin*dIII restriction enzymes, size separated, and blotted onto nylon membrane filters, which were hybridized with the ^{32}P-labeled IGHJ6 probe.[18] At diagnosis as well as in the skin biopsy two bands of identical size were found, reflecting identical, monoclonal *IGH* gene rearrangements. In contrast, the liver biopsy displayed a germline band with two faint *IGH* gene rearrangements of different sizes as compared to BM at diagnosis and the skin infiltrate. This fits with a presence of a minor clonal population in an otherwise polyclonal EBV-related lymphoproliferation. Based on the germline sequence and the restriction map of the *IGH* locus,[34] rearrangements found by direct sequencing could be assigned to the corresponding bands in the Southern blot.

PCR amplification and heteroduplex analysis of PCR products

PCR was essentially performed as described previously.[27,28] In each 50μl PCR reaction 50 ng DNA sample, 6.3 pmol 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 oligonucleotides used for amplification of complete VH-JH and incomplete DH-JH as well as Vγ-Jγ gene rearrangements were published before.[28,29] PCR conditions were: initial denaturation for 10 min at 94°C, followed by 35 cycles of 45 sec at 92°C, 90 sec at 60°C, and 2 min at 72°C using a Perkin-Elmer 480 thermal cycler (PE Biosystems). After the last cycle an additional extension step of 10 min at 72°C was performed. Appropriate positive and negative controls were included in all experiments.[28]

For heteroduplex analysis, the PCR products were denatured at 94°C for 5 min after the final cycle of amplification and subsequently cooled to 4°C for 60 min to induce duplex formation.[30] Afterwards the duplexes were immediately loaded on 6% non-denaturing polyacrylamide gels in 0.5 x Tris-borate-EDTA (TBE) buffer, run at room temperature, and visualized by ethidium bromide staining.[30] A 100-bp DNA ladder (Promega Corporation, Madison, USA) was used as size marker. Usage of heteroduplex analysis enables discrimination between PCR products derived from monoclonal and polyclonal lymphoid cell populations, based on the presence of homoduplexes (PCR products with identical junctional regions) or a smear of heteroduplexes (derived from PCR products with heterogeneous junctional regions), respectively.

When a particular primer combination detected clonal PCR products in two different DNA samples, their identity was subsequently compared by means of mixed heteroduplex analysis, i.e. mixing of the PCR products followed by heteroduplex analysis (Figure 2).[31]

Sequence analysis of *IGH* gene rearrangements

Clonal PCR products as found by 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 described before.[32] 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] Based on the germline sequence and the restriction map of the *IGH* locus,[34] rearrangements found by direct sequencing were assigned to corresponding bands on the Southern blot as described previously.[31] Vγ and Jγ gene segments were identified by comparison to germline *TCRG* sequences as described before.[35]

RESULTS

Southern blot analysis

Southern blot analysis at diagnosis of ALL demonstrated a monoclonal pattern in the *IGH* gene locus with two major rearrangements (Figure 1), whereas the *IGK* and *IGL* genes were in germline configuration. Southern blot analysis of the *TCRD* locus revealed a strong germline band and a rearranged band of similar density; the size of this rearranged band did not correspond to any of the well-established *TCRD* gene rearrangements.[21] The *TCRG* locus showed two rearranged bands with the Jγ2.1 probe most probably reflecting biallelic rearrangements. The *TCRB* genes were in germline configuration.

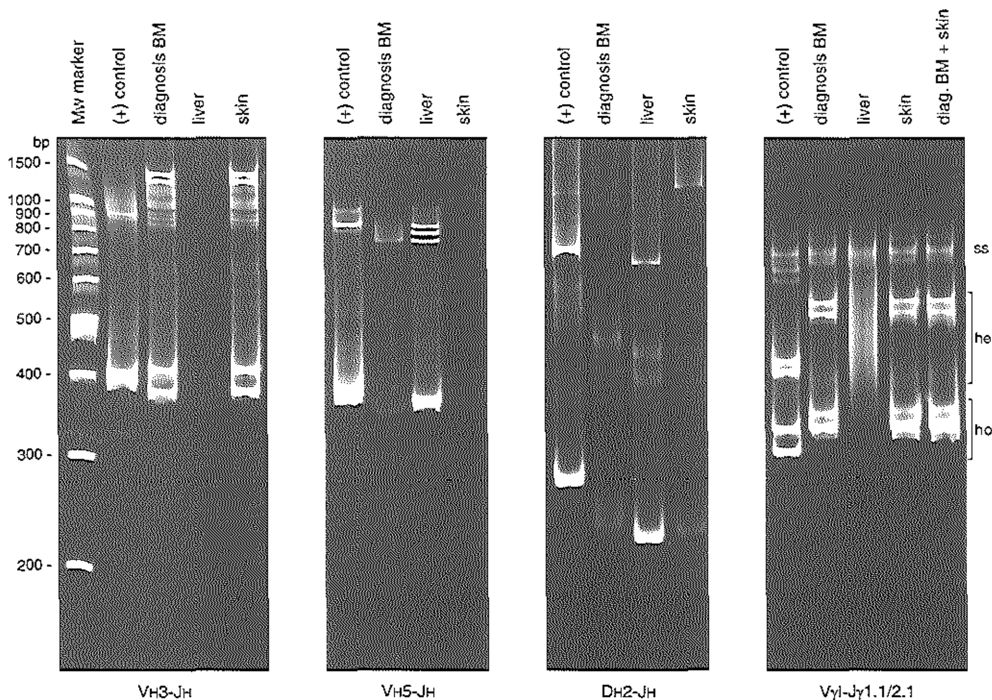

Figure 2.
Heteroduplex PCR analysis of *IGH* and *TCRG* gene rearrangements in BM at diagnosis of ALL as well as in liver and skin biopsies from patient 4687. In diagnosis BM and in the monocytic skin infiltrate identical clonal PCR products of two VH3-JH rearrangements and two Vγl-Jγ1.1/2.1 were found. Mixing of these PCR products followed by heteroduplex PCR analysis (as exemplified for Vγl-Jγ1.1/2.1 rearrangements) demonstrated no additional heteroduplex formation, thereby proving that these gene rearrangements had identical junctional regions. In contrast, PCR analysis of *IGH* rearrangements in liver proliferation showed clonal VH5-JH and DH2-JH joinings, not present in the ALL clone. No clonal *TCRG* gene rearrangements were demonstrated in the liver biopsy. Abbreviations: ss, single strands; he, heteroduplexes; ho, homoduplexes.

In the liver biopsy two rearranged *IGH* gene bands and one rearranged *IGK* gene band were found, which differed completely from the pattern at diagnosis, while their density suggested a minor clonal component among otherwise polyclonal cells (Figure 1). All TCR genes were in germline configuration.

Curiously, the configuration of the Ig and TCR genes in the skin monocytic infiltrate was identical to the pattern identified at diagnosis, except for the presence of a monoallelic Jκ rearrangement; the size of this *IGK* band was different from the one identified in the liver biopsy.

DNA samples from the diagnosis BM as well as from the liver and skin biopsies were examined for the presence of EBV genome. In the diagnosis and the skin biop-

sy cell samples no EBV genome was found, whereas the liver cell sample showed a clonal EBV-DNA band.

Heterodupiex PCR analysis

Detailed heteroduplex PCR analysis of the *IGH* locus was performed with twelve primer combinations (five *IGH* framework-1 V_H-family specific primers and seven family-specific D_H primers in combination with one J_H consensus primer). In diagnosis BM as well as in the skin biopsy, two monoclonal homoduplexes were found with the V_H3-J_H primer combination (Figure 2). When samples from both disease stages were mixed together and subjected to denaturation and renaturation they did not form any additional heteroduplex band, confirming that the V_H3-J_H PCR products at diagnosis and at skin relapse were identical (Figure 2B). Similarly, identical biallelic Vγl-Jγ1.1/2.1 gene rearrangements were characteristic both for ALL at diagnosis and for the skin infiltrate.

In contrast, heteroduplex analysis of PCR products derived from the liver biopsy did not disclose any of the clonal PCR products found at diagnosis, while it showed monoclonal homoduplexes with the V_H5-J_H and D_H2-J_H primer combinations.

Sequence analysis

Two monoclonal *IGH* PCR products found at diagnosis and two monoclonal PCR products found in liver biopsy sample were sequenced resulting in identification of the involved gene segments and the junctional regions. In the two *IGH* sequences at diagnosis (V_H3-13↔D_H2-2↔D_H6-13↔J_H4b and V_H3-74↔D_H6-19↔J_H4b) the junctional regions were completely unrelated to those found in liver biopsy (V_H5-51↔J_H6b and D_H2-21↔J_H4b). The sequences of the V_H3 gene segments in the V_H-D_H-J_H rearrangements at diagnosis were 100% concordant with the germline, but the in-frame sequence of the V_H5-51↔J_H6b gene rearrangement derived from the liver tumor contained 6% somatic mutations.

Based on the sequenced clonal *IGH* gene rearrangements at diagnosis and at relapse and the availability of the complete sequence of the human *IGH* locus,[34] the predicted sizes of restriction fragments containing the respective rearrangements were calculated (Figure 1), which confirmed the full concordance of Southern blot and PCR results.

The sequence analysis of *TCRG* gene rearrangements at diagnosis revealed clonal Vγ3-Jγ1.1 and Vγ8-Jγ1.1 gene rearrangements.

DISCUSSION

We present an unusually complicated course of ALL in a child with initially high tumor mass but with an excellent response to the induction treatment and rapid

achievement of complete remission. Therefore, the early isolated cerebral relapse was highly unexpected, particularly because repetitive normal findings in cerebrospinal fluid. One might speculate that the iatrogenic immunodeficiency during the maintenance treatment hampered the clearance of residual ALL cells from the central nervous system. Shortly before the child died from a heavily disseminated Aspergillus infection and Pneumocystis carinii pneumonia, she presented with an aleukemic leukemia cutis of AML-M4 type. Using detailed molecular studies,[31] we clearly proved that this was not a secondary malignancy, since we demonstrated identical *IGH* and *TCRG* gene rearrangements in BM at diagnosis of ALL and in the skin infiltrate. It is not clear whether this unusual skin relapse is induced by the immunodeficiency status.

EBV-related lymphoproliferative disorders are usually associated with congenital or acquired immunodeficiencies. Seven well-documented cases of such an acute hemato-oncologic complication were described in patients receiving ALL treatment.[6-11] They were uniformly associated with (re)-activation of EBV infection, usually in the background of profound treatment-related immunodeficiency. The EBV-positive lymphoproliferation in our patient contained a monoclonal B-cell component in an otherwise polymorphous tumor. The sequences of the clonal *IGH* gene rearrangements in the liver infiltrates were completely different from those found in the ALL clone. Moreover, the high frequency of somatic mutations in the V_H gene sequences were compatible with a mature B-cell lymphoproliferation in contrast to the unmutated *IGH* genes in the precursor-B-ALL.[36] Consequently, the EBV-related polymorphic Non-Hodgkin's lymphoma represented a true secondary malignancy, unrelated to the preceding precursor-B-ALL.

In conclusion, the iatrogenic immunodeficiency status in a girl with ALL resulted in an unusually complex and fatal disease course with an early isolated central nervous system relapse, an EBV-related B-cell lymphoma in the liver, an isolated leukemia relapse in the skin with AML-M4 immunophenotype, and life-threatening disseminated infections.

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4

CHAPTER 4.1

SPECIFIC APPLICATIONS OF PCR-BASED DETECTION OF MINIMAL RESIDUAL DISEASE IN ACUTE LYMPHOBLASTIC LEUKEMIA

Several minimal residual disease (MRD) studies used immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements as PCR targets, and demonstrated that evaluation of early treatment response has high predictive value for clinical outcome in childhood acute lymphoblastic leukemia (ALL).[1-3] Therefore, MRD monitoring is currently being applied for treatment stratification in several front-line ALL treatment protocols. MRD information can also be relevant for specific ALL subsets, which are characterized by common features, like age, immunophenotype, or the presence of particular chromosomal aberrations. One such specific patient group that might profit from MRD-based treatment modification is infant ALL. Chapter 4.2 exemplifies how currently available MRD techniques can provide insight into treatment effectiveness in infant ALL.

In addition to the "classical" application of MRD-PCR techniques for evaluation of the kinetics of ALL cell disappearance, PCR studies can be applied for many other purposes (Table 1). For instance, Ig/TCR gene rearrangements can be used as clonality markers for confirmation or exclusion of the common origin of two phenotypically different malignancies (see Chapters 3.5 and 4.3).

Early diagnosis of ALL in patients with smoldering leukemia

Another specific application of MRD-PCR techniques is the early diagnosis of ALL in cases of 'smoldering' leukemia, i.e. in patients with initially hypoplastic bone marrow with less than 25% of lymphoblasts, who subsequently developed overt ALL. A few case reports demonstrated the presence of identical clonal cell populations during the hypoplastic phase, the subsequent recovery phase, and the overt leukemia phase using patient-specific Ig/TCR gene rearrangements as PCR targets.[4,5] In contrast, monoclonal gene rearrangements could not be detected in patients with idiopathic hypoplastic anemia.[5]

Assessment of central nervous system involvement in ALL

MRD-PCR techniques can also be used for detection of central nervous system (CNS) involvement in ALL patients. One possibility is to screen for TdT^+ or $CD34^+$ cells, which normally are not found in cerebrospinal fluid (CSF); consequently, their presence in CSF provides evidence for meningeal leukemic infiltration.[6] Monitoring of MRD in CSF samples of more than one hundred patients with a TdT^+ malignancy during a 5-year follow-up period showed the development of overt CNS involve-

Table 1. Application of MRD-PCR technology using Ig/TCR gene rearrangements as targets.

Classical applications of PCR-based MRD detection in ALL
- MRD based risk-group classification of ALL patients according to the kinetics of leukemia cell decrease (front-line treatment and relapse treatment);
- evaluation of added value of individual treatment blocks, particularly in high-risk patients;
- evaluation of treatment effectiveness in specific ALL subtypes, such as infant ALL and T-ALL;
- evaluation of the pre-transplant MRD levels and assessment of the effectiveness of post-transplant immunotherapy;

Specific applications of PCR-based MRD detection in ALL
- assessment of common origin of two phenotypically different malignancies;
- early diagnosis of ALL in patients with smoldering leukemia, i.e. patients initially presenting with aplastic bone marrow with low percentages of lymphoblasts;
- assessment of CNS involvement at diagnosis and monitoring of CSF during follow-up for prediction of CNS leukemia;
- detection of bone marrow involvement during 'isolated' extramedullary relapse of ALL
- evaluation of testicular biopsies, e.g. at the end of treatment or evaluation of the contralateral testis in case of testicular relapse;
- evaluation of autologous stem cell transplants, before and after purging;
- evaluation of cord blood and later blood samples for the presence of transplacentally-migrated maternal ALL cells;
- evaluation of Guthrie cards and blood samples of monozygotic twins in case of ALL diagnosis (early diagnosis of ALL in the second twin and proving of the clonal relationship between the ALL cells of both twins).

ment in 70% of the patients with repeated finding of TdT^+ cells in CSF, despite normal cell morphology.[6] On the other hand, patients with CSF pleocytosis or "suspicious" morphology but TdT negativity (probably indicative of activated lymphocytes) had no evidence of subsequent CNS disease.[6,7] Also patient-specific *TCRD* rearrangements have been used as a MRD-PCR target for detection of CNS involvement in childhood precursor-B-ALL.[8] Identical rearrangements were found both in bone marrow and CSF in 43% of the analyzed patients, which confirms the clinical assumption that asymptomatic CNS involvement occurs much more frequently than diagnosed on the basis of classical cytomorphological criteria.[8] Moreover, preliminary data indicate that the molecular finding of MRD in CSF during ALL treatment as assessed via patient-specific Ig/TCR gene rearrangements is inevitably associated with subsequent CNS relapse.[9]

Detection of bone marrow involvement during 'isolated' extramedullary relapse of ALL

MRD techniques can be also employed for detection of bone marrow involvement during 'isolated' extramedullary relapse of ALL (e.g. in CNS or testis). Such sub-microscopic bone marrow involvement is confirmed in most of ALL patients with isolated extramedullary relapse.[10-12] This is in concordance with the clinical obser-

vation that full systemic reinduction therapy is required in these patients to prevent hematological relapse. Nevertheless, some ALL patients with isolated CNS relapse did not have detectable MRD levels in BM.[10,13]

Evaluation of testicular biopsies for the presence of leukemic infiltration

MRD positivity of histologically normal end-of-treatment testicular biopsies was shown to be followed by overt testicular relapse.[14] Moreover, PCR-based MRD assays at the time of a unilateral testicular relapse allowed reliable exclusion of occult leukemic blasts in the histologically normal contralateral testis.[14]Nevertheless, in some patients testicular relapse did occur despite MRD negativity in testicular biopsies.[14]

Evaluation of autologous stem cell transplants, before and after purging

Another attractive application of MRD detection is the evaluation of autologous stem cell grafts for contamination with leukemic blasts. Autologous purged stem cell transplantation following intensified chemotherapy is currently being evaluated as new treatment modality in high-risk ALL patients in second complete remission, who do not have a matched related donor. PCR studies showed that MRD-positivity of the autologous bone marrow graft before purging was the most predictive factor of treatment failure in ALL, regardless of a seemingly successful purging procedure (MRD-negative graft).[15] In fact, the remission duration after autologous stem cell transplantation significantly correlated with MRD levels before the purging procedure. On the other hand, infusion of MRD-negative purged grafts in patients with MRD-negative pre-transplantation bone marrow was associated with durable clinical remission.[16]

Evaluation of cord blood and later blood samples for the presence of transplacental ALL cells

Finally, we used PCR-based MRD studies for assessing the disappearance of transplacentally-migrated maternal ALL blasts from the blood of a newborn child (see Chapter 4.4). Transplacental migration of leukemic cells has also been observed in monozygotic twins with monochorionic placenta, developing ALL at different ages (reviewed by MF Greaves[17]). The finding of identical DNA sequences of Ig/TCR gene rearrangements and identical translocation breakpoints has proven the prenatal origin of ALL in one of the twins with subsequent transplacental dissemination to the second twin. Using MRD-PCR techniques, it is also possible to detect or exclude the presence of ALL clone in the unaffected monozygotic twin of the ALL patient.[18] Extensive MRD-PCR analyses of Guthrie cards of childhood ALL patients showed the presence of clonotypic ALL sequences already at birth in the vast majority of cases, confirming *in utero* origin of childhood ALL.[17-19]

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CHAPTER 4.2

INTENSIFIED THERAPY FOR INFANTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA*

Results from the Dana-Farber Cancer Institute Consortium

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We read with great interest the report by Silverman et al.[1] summarizing treatment outcome in 23 infants treated with Dana-Farber Cancer Institute Consortium protocols. Intensified multidrug therapy resulted in significantly improved long term, event free survival in 54% ± 11% of infants. This included at least three patients with *MLL* gene rearrangements, which are known to be associated with multidrug-resistant disease.

The authors describe two infants in whom the blasts at the time of recurrence differed phenotypically from those at diagnosis. They considered the possibility of secondary leukemia, but also speculated about recurrence of the leukemia with a phenotypic shift. In our opinion, the latter explanation appears most probable. In acute lymphoblastic leukemia (ALL) occurring in infants, particularly in cases with *MLL* gene rearrangements, leukemogenesis affects early progenitor cells. In such patients cross-lineage expression of myeloid antigens such as CD13, CD15, CD33, and CD65 frequently is observed,[2] in a minority of cases even biphenotypic acute leukemia has been diagnosed based on simultaneous expression of lineage specific antigens.[3] Using clone specific markers such as clonal immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements, it is possible to distinguish between recurrence and secondary, therapy-related leukemia. We previously described a pre-B-ALL patient who developed acute myeloid leukemia 17 months after diagnosis, suggesting the development of secondary leukemia. However, the Ig and TCR gene rearrangement pattern was identical between diagnosis and recurrence, implying cytomorphologic and immunophenotypic evolution of the same clone.[4]

In contrast to previous reports,[3] Silverman et al.[1] clearly showed that infant ALL in principle can be cured. However, 50% of patients still recur. This implies that

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analysis of specimens at diagnosis is not sufficient for predicting treatment response and that more insight is needed into *in vivo* effectiveness of treatment during the follow-up. This is possible with the currently available standardized techniques for the detection of minimal residual disease (MRD).[5] To exemplify this strategy we show the monitoring of MRD in a 10-month-old infant with common-ALL using a patient specific oligonucleotide probe to the junctional region of an *IGK* gene rearrangement (Figure 1).[6] Despite cytomorphologic remission at the end of induction therapy, we still could detect low levels of malignant cells. The recurrence 14 month after diagnosis was predicted 3 months earlier with molecular MRD analysis. We believe that such prospective MRD monitoring can be used for the assessment of treatment response and can be applied toward individualization of therapy to improve the outcome of infant leukemia further.

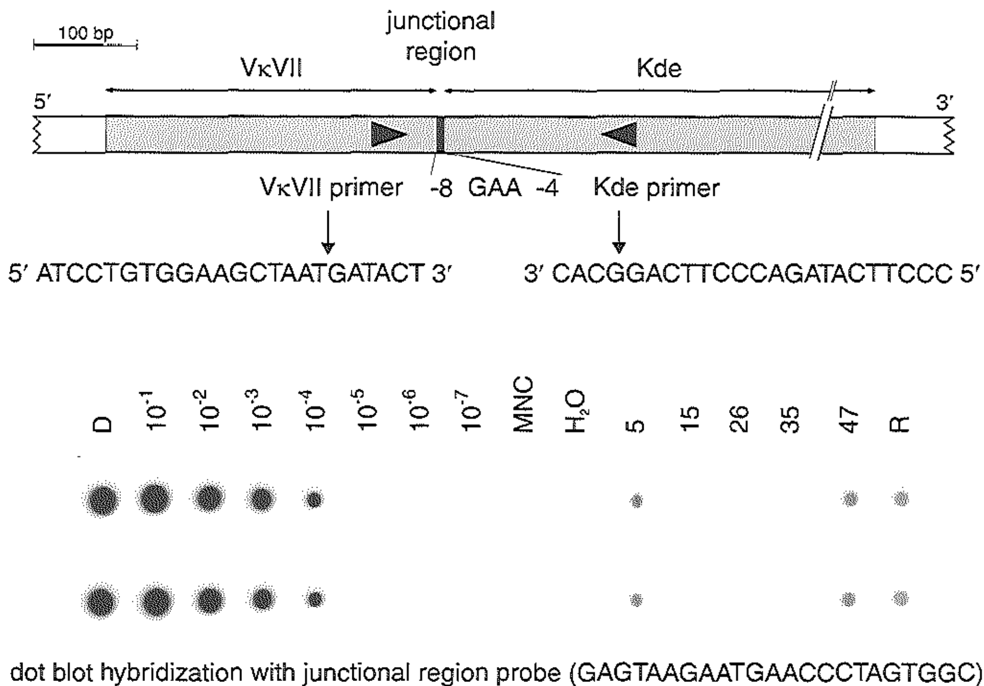

Figure 1.
VkVII and Kde primers were used for polymerase chain reaction (PCR) amplification of bone marrow DNA samples at diagnosis (D) as well as during follow-up. The PCR products were spotted onto a nylon membrane, which was hybridized with the ^{32}P-labeled junctional region probe. Tenfold dilution series of diagnosis DNA revealed a sensitivity of 10^{-4} (one acute lymphoblastic leukemia cell between 10^{4} normal cells). During follow-up the bone marrow became negative after Week 15, but at Week 47 PCR positivity was found again (i.e. 3 months before clinical relapse [R]). bp: base pairs; MNC: DNA from normal mononuclear cells.

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CHAPTER 4.3

TWO CONSECUTIVE IMMUNOPHENOTYPIC SWITCHES IN A CHILD WITH IMMUNOGENOTYPICALLY STABLE ACUTE LEUKEMIA*

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ABSTRACT

A 12-year-old girl presented with a $CD33^+$ precursor B-acute lymphoblastic leukemia (ALL) and seemed to respond well to ALL treatment. However, 2 weeks after diagnosis her leukocyte count rose rapidly with a predominance of myeloid blasts with M5b morphology and $CD19^+$ myeloid immunophenotype. Acute myeloid leukemia (AML) treatment was started and remission was achieved after one course of chemotherapy; the AML treatment was continued for 6 months. Two months after cessation of chemotherapy, the patient developed a bone marrow relapse, this time with an undifferentiated blast morphology and a precursor B immunophenotype.

Molecular analysis of the immunoglobulin and T-cell receptor genes showed several clonal gene rearrangements at diagnosis: two *IGH*, two *IGK* and two *TCRD* gene rearrangements. All rearrangements were also detected during the AML phase of the disease, suggesting a phenotypic shift of the same leukemia. At relapse, 8 months later, all rearrangements were preserved except for one *TCRD* (Vδ2-Dδ3) rearrangement.

The first phenotypic shift in the genotypically stable leukemia was remarkably fast. The most probable explanation for our observations is an oncogenic event in an undifferentiated hematopoetic progenitor clone, with a highly versatile phenotype.

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INTRODUCTION

In contrast to the vast majority of normal lymphoblasts, a significant proportion of acute lymphoblastic leukemias (ALLs) co-express myeloid antigens, such as CD13, CD14, CD15, CD33, CD65 and/or CD66c at diagnosis.[1-3] Also, several acute myeloid leukemias (AMLs) co-express lymphoid antigens such as CD2, CD4, CD7 and CD19.[4,5] Rarely, acute leukemias co-express several antigens from two differentiation lineages; if so, the diagnosis of acute biphenotypic leukemia is assumed.[6]

Interlineage switch of acute leukemia at relapse usually concerns a switch from ALL to AML and has been reported to occur in 5-7% of cases.[7,8] Such phenotype switches generally occur late in the course of the disease and are most frequently assumed to represent therapy-induced secondary malignancies. However, in rare cases analysis of immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements have suggested that both malignancies were derived from the same stem cell clone.[9] In contrast, an early lineage switch during induction chemotherapy is an extremely rare event. Such an early phenotypic switch might be the result of aberrant differentiation of the malignant clonogenic cells or selection of a minor subfraction from a mixed population as the result of selective killing by chemotherapy.

We present the clinical course and the immunophenotypic and immunogenotypic investigations in a case of childhood ALL showing an early phenotypic switch and a second later switch.

PATIENT AND METHODS

Clinical course

A 12-year-old girl presented with a history of tiredness and easy bruising. Her hemoglobin was 6.77 g/dl, platelets were 45 x 10^9/l and the leukocyte count was 15 x 10^9/l (with 18% blasts). Her bone marrow (BM) contained 96% lymphoblasts with a precursor-B-ALL phenotype and cross-lineage expression of CD33 and dimCD13 (Table 1)(Figure 1A). Her cerebrospinal fluid was free of blast cells. Cytogenetic analysis of BM did not show any abnormalities. She was started on the non-high-risk ALL-9 protocol of the Dutch Childhood Leukemia Study Group (DCLSG).

Her initial chemotherapy consisted of dexamethasone, vincristin and intrathecal triple therapy. She responded well to this treatment in the first week and was discharged from the hospital on day 7. Two weeks after diagnosis, a rapidly rising leukocyte count (62 x 10^9/l) was found with the re-emergence of 84% blast cells in her blood, but this time with a different morphology (Figure 1B). BM cytomorphology revealed 65% blast cells with a phenotypic switch to AML (Table 1). Cytogenetic analysis again showed a normal karyotype. Her treatment was accordingly changed to the Medical Research Council (MRC) AML 12/DCLSG ANLL 97 protocol and after one induction course she attained remission. She received a total of two ADE courses (cytosine-arabinoside/etoposide/daunorubicin), one MACE (cytosine-arabinoside/etoposide/amsacrine) and one MidAc course (high-dose cytosine-arabinoside/mitoxantrone). Treatment was electively stopped after 6 months.

The patient developed a BM relapse 8 months after the initial diagnosis. This time, the morphology

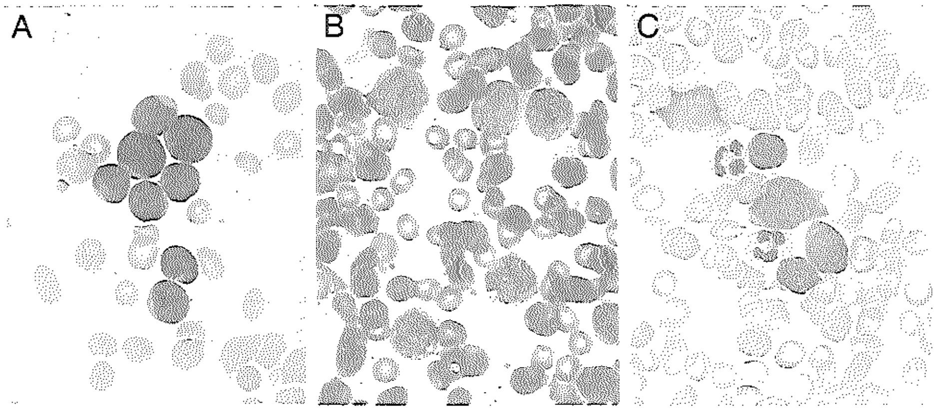

Figure 1.
Morphology of blast cells at initial diagnosis **(A)**, 2 weeks later **(B)**, and at relapse **(C)**. (Magnification x 1000).

of the blast cells was undifferentiated (Figure 1C), while the immunophenotype was of precursor-B-lineage (common ALL) (Table 1). Cytogenetic investigation revealed a 46, XX, del (9) (p21) [9] / 46, XX [23]; i.e. a mosaicism of normal cells and cells with a deletion of the short arm of chromosome 9. This deletion is seen in ALL-L1 and ALL-L2.

The patient was started on the DCLSG ALL-relapse protocol. Reinduction was with vincristin, dexamethasone, L-asparaginase, VM-26 and cytosine-arabinoside. After 6 weeks, her BM was again in remission. Six weeks after the newly attained remission, she was transplanted with CD34 selected (CliniMACS, Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) blood stem cells from her father (one HLA-B locus mismatch). Conditioning was with etoposide (cumulative dose 700 mg/m^2), cyclophosphamide (cumulative dose 120 mg/kg) and total body irradiation (2 x 6 Gy). Anti-thymocyte globulin was used as serotherapy. She engrafted in 21 days (neutrophil count > 0.5 x 10^9/l) and chimerism studies showed full donor chimerism. Cyclosporin A, used as graft-versus-host disease (GvHD) prophylaxis, was rapidly tapered. There was no sign of GvHD. She died in complete remission from liver failure of unknown cause 10 months after her allogeneic transplant.

Immunophenotypic analysis

Immunophenotypic studies at diagnosis and relapse were performed on lysed whole BM or blood samples by triple staining with directly conjugated monoclonal antibodies, as described previously.[10,11] The specificity and source of the monoclonal antibodies used in this study are summarized in Table 1.

Measurements were performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). For data acquisition, CELLQUEST software was used with the PAINT-A-GATE PRO program for data analysis (Becton Dickinson). The percentage of positivity for the various monoclonal antibodies was determined after gating of the blast populations as defined by their forward scatter and sideward scatter characteristics.

Southern blot analysis of Ig and TCR gene rearrangements

DNA was isolated from frozen mononuclear cell fractions as described previously.[12] Fifteen micrograms of DNA was digested with the appropriate restriction enzymes, size-separated in 0.7% agarose

gels and transferred to Nytran-13N nylon membranes as described.[13] Ig heavy chain (*IGH*) and Ig kappa light chain (*IGK*) gene configurations were analyzed after hybridization with the IGHJ6, IGKJ5, IGKC, and IGKDE probes in *Bgl*II digests.[14,15] The configuration of the TCR delta (*TCRD*) genes was analyzed with the TCRDJ1 and TCRVD2 probes in *Bgl*II and *Eco*RI digests.[16]

Table 1. Morphological, cytochemical, immunophenotypic and immunogenotypic characteristics at different disease phases.

	Diagnosis	AML (day 14)	Relapse (day 245)
Cytomorphology (FAB)	ALL-L1	AML-M5b	AUL
Blast percentage in bone marrow	96	65	30
Cytochemistry			
Periodic-acid -Schiff (PAS)	negative	negative	negative
Peroxidase	negative	negative	negative
Alpha naphtyl-acetate esterase	weak positive, positive	spots positive	diffuse
Alpha naphtyl-acetate esterase + Na F	not done	inhibited	not done
Immunophenotype (% positivity within the gated blast cell population)			
TdT(HTdT6)[a]	91	0	81
CD34 (HPCA-2)[b]	93	98	85
HLA-DR (anti-HLA-DR)[b]	95	98	91
CD10 (J5)[c]	88	0	81
CD19 (Leu-12)[b]	93	91	83
CyCD79a (HM47)[c]	97	0	78
CD22 (Leu-14)[b]	96	0	82
CD13 (My-7)[c]	92 (dim)	87 (dim)	92 (dim)
CD14 (RMO52)[c]	not done	96	0
CD15 (Leu-M1)[b]	0	19	0
CD33 (D3HL60.251)[c]	86	99	79
CD65 (VIM2)[d]	0	24	0
MPO (MPO-7)[e]	0	5	0
Immunogenotype			
IGH gene	R/R	R/R	R/R
IGK gene	Vκl-Jκ/Vκl-Jκ (+2 x intronRSS-Kde)	Vκl-Jκ/Vκl-Jκ (+2 x intronRSS-Kde)	Vκl-Jκ/Vκl-Jκ (+2 x intronRSS-Kde)
TCRD gene	Vδ3-Jδ1/Vδ2-Dδ3	Vδ3-Jδ1/Vδ2-Dδ3	Vδ3-Jδ1/D

a. Supertechs, Bethesda, MD, USA
b. Becton Dickinson, San Jose, CA, USA
c. Coulter/Immunotech, Westbrook, ME, USA
d. Caltag, San Francisco, CA, USA
e. DAKO, Glostrup, Denmark

R, rearranged allele; D, deleted allele

Polymerase chain reaction (PCR) heteroduplex analysis of Ig and TCR gene rearrangements

PCR was essentially performed as described previously.[17] In each 50μl PCR reaction, a 50 ng DNA sample, 6.3 pmol of the 5′ and 3′ oligonucleotide primers, and 0.5 U of AmpliTaq Gold polymerase (PE Biosystems, Foster City, CA, USA) were used. The sequences of the oligonucleotides used for amplification of *IGK* and *TCRD* gene rearrangements have been published.[18] For heteroduplex analysis, the PCR products were denatured at 94°C for 5 min after the final cycle of amplification and subsequently cooled to 4°C for 60 min to induce duplex formation.[19]

RESULTS

Cytomorphological and immunophenotypic characterization

Cytomorphology and immunophenotyping at diagnosis showed an ALL with a CD10-positive precursor-B-ALL phenotype with co-expression of two myeloid markers (CD33 and CD13^{dim}). Two weeks later, an acute leukemia of monocytic cell type could be seen with α-naphthyl-acetate-esterase positivity inhibited by sodium fluoride; this was supported by positivity for CD13 (dim), CD14 and CD33, but negativity for TdT and the B-cell markers CD10, CD22 and CD79a; only CD19 was expressed. At relapse, the morphology was undifferentiated and the original CD10-positive precursor-B-ALL phenotype had reappeared again (Table 1). Morphologically, the size of the blast cells (twice the size of an erythrocyte) in combination with a low nucleus/cytoplasm ratio, several large nucleoli and the absence of granulation and negative peroxidase reactivity, were suggestive of an undifferentiated phenotype.

Immunogenetic characterization of the disease phases

Southern blot analysis at diagnosis revealed monoclonal *IGH* gene rearrangements on both alleles. Also, in the *IGK* locus two monoclonal rearrangements were observed that both concerned Vκ-Jκ recombinations coupled to intron RSS to Kappa deleting element (Kde) recombinations on the same allele (Figure 2A). Southern blot analysis of the *TCRD* locus revealed a strong rearranged band corresponding to a Vδ3-Jδ1 recombination as well as a weak subclonal Vδ2-Dδ3 rearrangement.

At the AML phase and at relapse, Southern blot analysis of *IGH* and *IGK* revealed exactly the same rearrangements (Figure 2A). The *TCRD* gene rearrangements of the AML phase were identical to initial diagnosis, but at relapse the subclonal Vδ2-Dδ3 rearrangement was lost while the Vδ3-Jδ1 rearrangement was preserved.

Based on the Southern blot information, PCR heteroduplex analysis was performed with Vδ3-Jδ1, Vδ2-Dδ3, Vκ-Jκ and intron RSS-Kde primer combinations. At all three disease phases, monoclonal Vδ3-Jδ1 homoduplexes of equal size were found. When mixed together and subjected to denaturation and renaturation, they

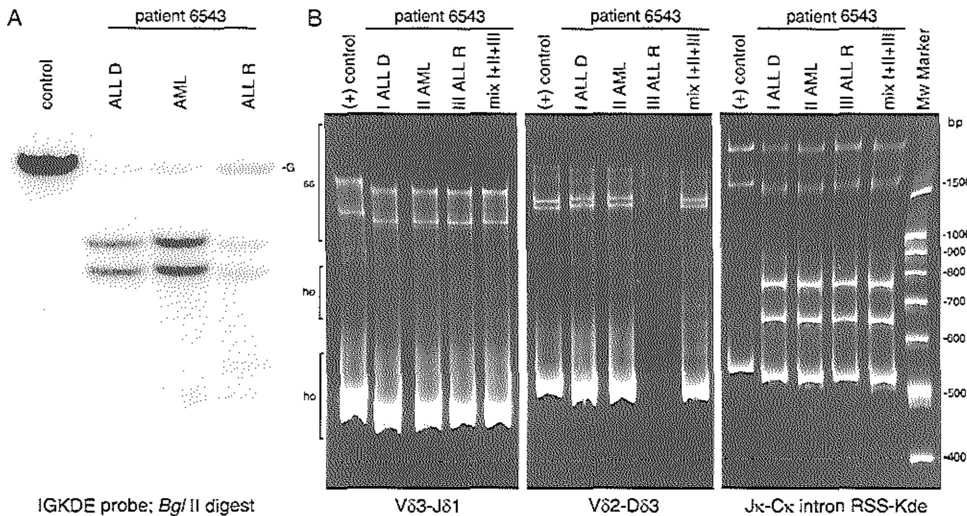

Figure 2.

(A) Southern blot analysis of the *IGK* gene. Control DNA and patient DNA at diagnosis (ALL D), AML phase (AML) and relapse (ALL R) were digested with the *Bgl*II restriction enzyme, size separated and blotted onto nylon membrane filters, which were hybridized with the ^{32}P-labelled IGKDE probe. In all three disease phases, an identical monoclonal pattern with biallelic *IGK* gene rearrangements was identified.

(B) Heteroduplex PCR analysis of Vδ3-Jδ1, Vδ2-Dδ3 and intron RSS-Kde PCR products. Monoclonal homoduplexes (ho) found at three disease phases 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. ss, remaining single strand fragments.

did not form any additional (heteroduplex) bands, confirming that the PCR products at diagnosis, at the AML phase and at relapse were identical (Figure 2B). Similarly, identical Vδ2-Dδ3 homoduplexes were found at initial diagnosis and at AML phase, while repeated PCR analysis did not reveal clonal Vδ2-Dδ3 PCR products at the ALL/acute undifferentiated leukemia (AUL) relapse. Finally, two VκI-Jκ and two intron RSS-Kde homoduplexes were found by PCR analysis, representing biallelic rearrangements that were identical at all three disease phases (Figure 2B).

DISCUSSION

We describe a patient with three different phases of acute leukemia. A remarkably early switch was observed from a precursor–B-ALL, initially responding to standard ALL therapy, to AML5b 2 weeks after diagnosis. This switch was apparent both

morphologically and immunophenotypically. After completion of AML treatment, a relapse occurred. This third phase of the disease was morphologically AUL but had immunophenotypic characteristics of precursor-B-ALL.

Biallelic *IGH* and *IGK* gene rearrangements were found at diagnosis. Also, two *TCRD* gene rearrangements were detected, a Vδ3-Jδ1 and a Vδ2-Dδ3 rearrangement, the latter being derived from a subclone. The finding of a complete Vδ3-Jδ1 gene rearrangement was unusual for precursor-B-ALL, because complete *TCRD* gene rearrangements are characteristic of T-lineage ALL and are rarely observed in precursor-B-ALL.[16,20] Ig/TCR gene rearrangements, including complete *TCRD* rearrangements, can be found in AML, albeit with low frequency (5-15%) and usually concerning single loci.[21,22] Multiple Ig gene rearrangements particularly involving the *IGK* gene are almost exclusively observed in precursor-B-ALL. All identified Ig and TCR gene rearrangements were stable throughout the disease course, except for the subclonal Vδ2-Dδ3 rearrangement, which was lost at relapse. These immunogenetic results prove the clonal relationship between the three disease phases in this leukemia patient.

Sensitivity to chemotherapy was remarkably good at all three stages of the disease. There was no apparent development of drug resistance and, even at the time of relapse, conventional ALL-type chemotherapy was sufficient to obtain remission again.

Two patients with a similar early ALL to AML switch have been described previously but without any proof for clonal relationship.[23,24] There may be several mechanisms to explain the described phenomenon of phenotypic switch in a leukemic clone. Clearly, a 2-week period is too short for therapy-induced secondary leukemia. However, chemotherapy might have selected a therapy-insensitive myeloid subclone, which represented a minority in the larger population of leukemic cells with a different (lymphoid) phenotype. Such a shift in lineage predominance rather than a true phenotypic change has been described previously in two infants with congenital leukemia.[25,26] Both patients had biphenotypic leukemias at diagnosis, with lymphoid and myeloid blasts sharing the same *MLL* gene abnormality and *IGH* gene rearrangement in the two disease phases. This is in contrast to our case, in which there was no cytomorphological and immunophenotypic evidence for the presence of phenotypically biclonal leukemia at diagnosis. An alternative explanation for the early phenotype switch would be reprogramming of a malignant pluripotent stem cell, capable of both lymphoid and myeloid differentiation.

The dynamics of the phenotype switch in our patient - especially apparent in the first episode - is puzzling. Within days, chemotherapy was capable of killing the lymphoid leukemic cells, while the leukemic stem cell could change its program into a different (myeloid) direction, resistant to the chemotherapy used in that phase. The second shift, with the same immunogenotype, may have been the initial phenotype or alternatively a third phenotype.

We conclude that this case illustrates the rare potential of leukemic clones to adapt to chemotherapy by changing phenotype. This phenotype switch did not, however, lead to clinically apparent multidrug resistance. Cases like our patient may provide more insight into adaptive mechanisms in the behavior of leukemias.

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CHAPTER 4.4

CLEARANCE OF MATERNAL LEUKEMIC CELLS IN A NEONATE*

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ABSTRACT

A 36-week pregnant woman was diagnosed with acute lymphoblastic leukemia. Delivery was initiated prematurely, and a healthy child was born. Cord blood and peripheral blood samples from the neonate (obtained at 6 weeks, 3 months and 6 months) were analyzed for the presence of minimal residual disease by polymerase chain reaction analysis of a leukemia-specific *IGH* gene rearrangement and the *E2A-PBX1* fusion gene transcript. In the cord blood sample, a tumor load of $\approx 4 \times 10^{-4}$ was found, whereas all later blood samples were negative. Our data indicate that the maternal leukemic cells did not engraft in the neonate.

INTRODUCTION

It has become widely accepted that the placenta is not an absolute barrier, but that single cells can pass from mother to child and vice versa.[1-3] Recently, Catlin *et al*[4] reported transplacental transmission of a maternal natural killer cell lymphoma and subsequent engraftment of these cells in the fetus, with fatal consequences for the infant. In addition, direct evidence has been obtained for intraplacental metastasis of (pre)leukemic cells between monozygotic twins who developed concordant acute lymphoblastic leukemia (ALL) several years after birth.[5] The clonal origin of the twin leukemias was proved by the presence of exactly the same clonotypic fusion

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sequence of a chromosome aberration and an identically rearranged *IGH* allele.

Reports of patients with ALL during pregnancy are rare. In most cases reported so far, chemotherapy was given during pregnancy and a healthy child was born.[6,7] We report the presence of maternal ALL cells in cord blood (CB) of a neonate, born to a mother who was diagnosed with ALL during late pregnancy.

MATERIALS AND METHODS

Case report

A previously healthy pregnant woman at 36 weeks' gestation presented with symptoms of abdominal pain, extensive night sweating and nose bleeding. She was referred to the St. Antonius Hospital (Nieuwegein, The Netherlands) and diagnosed with ALL of pre-B-ALL immunophenotype (TdT^+, $CD10^+$, $CD19^+$, $CD22^+$, cytoplasmic $CD79^+$, $HLA\text{-}DR^+$, and cytoplasmic $Ig\mu^+$) with 90% blast cells in her peripheral blood (PB). Before the start of the cytotoxic treatment, delivery was initiated prematurely, and a healthy female child (2.72 kg) was born. No abnormalities were found upon physical examination of the newborn, and her PB was normal by routine biochemical and cytomorphological analyses. The child developed normally, was released from the hospital 3 weeks after birth and has continued to do well for 30 months.

A CB sample was taken, and PB samples were obtained from the newborn at 6 weeks, 3 months and 6 months after birth. Mononuclear cells (MNCs) were obtained by Ficoll density centrifugation and used for molecular investigations.

Molecular analysis of blood samples from the newborn using Ig/TCR gene rearrangements as targets

To detect clonal immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements in the maternal ALL cells, DNA was extracted from MNCs obtained at diagnosis. Polymerase chain reaction (PCR) analysis was performed using 26 different primer combinations to detect rearrangements of the *IGH, IGK, TCRD,* and *TCRG* gene loci.[8] PCR products were denaturated and renatured (60 min at 4°C) for heteroduplex analysis to confirm the clonality of the detected rearrangements. Monoclonal rearrangements were subjected to direct sequence analysis of the junctional regions.[8]

The tumor load in the CB and PB samples from the child were analyzed by dot blotting followed by hybridization with a patient-specific junctional region probe[8,9] and by real-time quantitative PCR (RQ-PCR) analysis as described previously.[10] Tenfold serial dilutions of ALL DNA into DNA from normal MNCs (10^{-1} – 10^{-6}) were made to define the sensitivity of the two PCR techniques and for assessment of the tumor load.

Molecular analysis of blood samples from the newborn using fusion gene transcripts as PCR target

RNA was extracted from maternal MNCs at diagnosis, and cDNA was prepared. Reverse transcription (RT)-PCR was performed to detect the most common chromosome aberrations in ALL.[11] RNA samples from CB and PB MNCs from the child were analyzed by a nested RT-PCR approach using *E2A* and *PBX1* primers as described previously.[11] To check for the integrity of the RNA samples, an *ABL* RT-PCR was performed. In addition, RQ-PCR analysis was performed for the *E2A-PBX1* fusion transcript. The forward primer (5'-GAC TCC TAC AGT GCT TCC CTG TTT AT-3') and TaqMan probe (AGC CCA GGA GGA

GGA ACC CAC AGA) were positioned in exon 13 of the *E2A* gene and the reverse primer (5'-CGC TAA CAG CAT GTT GTC CAG-3') was positioned in exon 2 of the *PBX1* gene. Tenfold serial dilutions of ALL RNA into HL-60 RNA (10^{-1} – 10^{-6}) were made to define the sensitivity of the two PCR techniques and for assessment of the tumor load.

RESULTS

PCR heteroduplex analysis and subsequent sequencing revealed two clonal *IGH* gene rearrangements, of which a VH3-11-JH6c rearrangement was selected for this study. A junctional region-specific probe was developed for PCR dot-blot analysis reaching a sensitivity of 10^{-5} (Figure 1). Analysis of the CB sample showed the presence of maternal leukemic cells, with an estimated tumor load of 10^{-3}. The PB samples taken at 6 weeks and 3 months were negative (Figure 1). RQ-PCR analysis of the VH3-11-JH6c rearrangement resulted in a sensitivity of 10^{-4}. The calculated tumor load of the CB sample was 5 x 10^{-4}, whereas the later neonatal PB samples were negative.

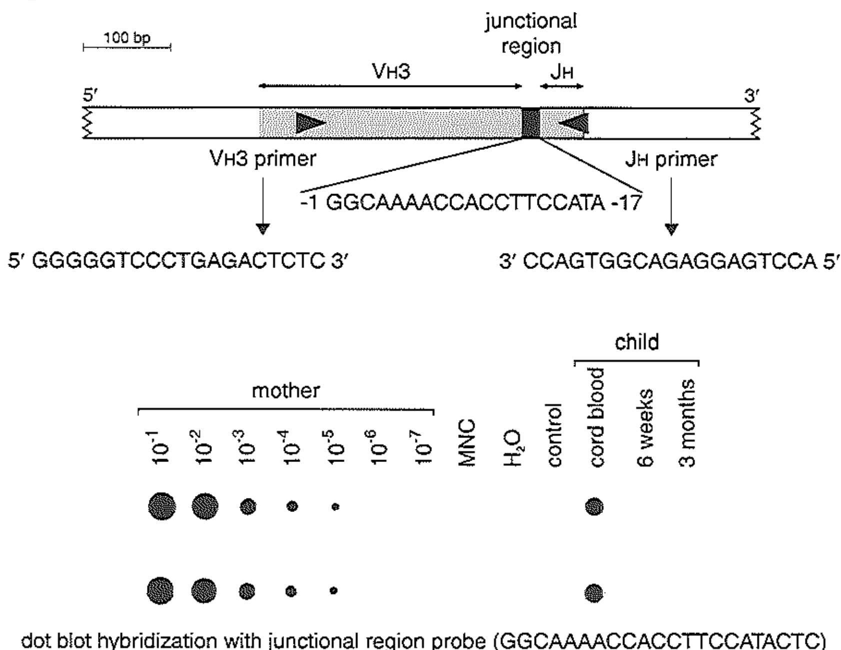

Figure 1.
Dot-blot hybridization after PCR amplification of the VH3-11-JH6c target. The PCR products obtained were hybridized with the patient-specific probe as indicated. This technique reached a sensitivity of 10^{-5}. The CB MNCs were clearly positive, with an estimated tumor load of 10^{-3} based on comparison of the hybridization signals with those of the dilution series. The samples obtained from the child at 6 weeks and 3 months of age were negative.

RT-PCR analysis of the diagnosis sample identified the *E2A-PBX1* fusion transcript, characteristic for t(1;19) and in line with the pre-B-ALL immunophenotype. Via nested RT-PCR analysis, *E2A-PBX1* fusion transcripts were found in the CB sample, whereas all neonatal PB samples were negative. RQ-PCR analysis of the *E2A-PBX1* fusion gene transcript resulted in a sensitivity of 5×10^{-5}. The relative tumor load of the CB sample was 3×10^{-4}, whereas all PB samples were negative.

DISCUSSION

This is the first report on the presence of maternal ALL cells in the CB sample of a neonate. The occurrence of maternal leukemic cells in the CB sample and in the child's PB samples was determined by analysis of a clonal *IGH* rearrangement and *E2A-PBX1* fusion gene transcripts via classical PCR approaches [8,9,11] as well as via the recently developed RQ-PCR technique.[10] Fully concordant results were obtained with the four different minimal residual disease (MRD) PCR techniques, and accurate quantification of the leukemic load ($\approx 4 \times 10^{-4}$) was achieved by the RQ-PCR analyses.

Formally, we cannot exclude the possibility that the CB sample has been contaminated with a minor amount of maternal PB during sampling, but we took all necessary precautions to obtain uncontaminated CB. However, it is fair to assume that the maternal leukemic cells passed the placental barrier, like normal maternal leucocytes[1-3] and natural killer cell lymphoma cells.[4] Consequently, we conclude that these maternal leukemic cells did not engraft in the fetus: within 6 weeks after birth, the leukemic cells became undetectable and were probably eliminated by the immune system.

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5

CHAPTER 5.1

MOLECULAR MONITORING OF RESIDUAL DISEASE USING ANTIGEN RECEPTOR GENES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA*

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ABSTRACT

Immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements are assumed to be unique '*fingerprint-like*' sequences for each acute lymphoblastic leukemia (ALL). Various clonal Ig/TCR gene rearrangements can be identified at diagnosis in virtually all childhood ALL patients, representing molecular targets for detection of minimal residual disease (MRD) during follow-up analysis. The usage of at least two MRD-PCR targets per patient generally ensures high sensitivity (1:10^4 normal cells) and prevents false-negative results owing to ongoing or secondary rearrangements.

MRD monitoring in childhood ALL employing Ig/TCR gene rearrangements as PCR targets has significant prognostic value. This is particularly powerful for evaluation of early treatment response and consequently can be used for improved therapy stratification. Prolonged continuous MRD monitoring might be important for patients at intermediate or high risk of relapse. MRD monitoring in second complete remission identifies patients with excellent drug sensitivity and predicts outcome after stem cell transplantation.

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INTRODUCTION

Modern treatment protocols induce complete remission in virtually all (>95%) children with acute lymphoblastic leukemia (ALL), but approximately 20-30% of these patients relapse.[1] Apparently, chemotherapy regimens are not capable of killing all clonogenic malignant cells in these patients although they reached complete remission according to clinical and morphological criteria. The detection limit of morphological techniques in ALL is not lower than 1-5% of malignant lymphoblasts between normal bone marrow (BM) cells. Therefore, it was postulated that more sensitive techniques would discern cases at high risk of relapse from patients with excellent prognosis. In childhood ALL detection of malignant cells at low frequencies during and after treatment, i.e. detection of minimal residual disease (MRD), most frequently relies on patient-specific immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements as molecular markers for investigation.

JUNCTIONAL REGIONS OF IG/TCR GENE REARRANGEMENTS AS PCR TARGETS FOR MRD DETECTION

During early B- and T-cell differentiation the germline variable (V), diversity (D), and joining (J) gene segments of the Ig and TCR gene complexes rearrange, and each lymphocyte thereby obtains a particular combination of V-(D-)J segments.[2-4] Moreover, deletion of germline nucleotides by trimming the ends of the rearranging gene segments as well as insertion of nucleotides between the joined gene segments by terminal deoxynucleotidyl transferase (TdT) creates an enormous junctional diversity (Figure 1). Therefore, the junctional regions of rearranged Ig and TCR genes are unique '*fingerprint-like*' sequences that are assumed to be different in each lymphoid precursor cell. Consequently, the junctional region can be considered as a '*DNA-fingerprint*' of each particular ALL because ALL cells are clonal lymphoid precursor proliferations (Figure 1).[4,5] Several early studies have shown that junctional regions indeed can be used as tumor-specific targets for MRD monitoring.[6-8] For this purpose, the various Ig and/or TCR gene rearrangements have to be identified in each leukemia patient at initial diagnosis.

Identification of junctional regions of Ig and TCR gene rearrangements as MRD targets

To identify leukemia-specific junctional region sequences precisely, clonal Ig and TCR gene rearrangements should first be amplified by PCR analysis, followed by sequencing of the PCR product obtained. PCR amplification is possible only when the Ig or TCR gene segments are juxtaposed through rearrangement, as the dis-

A

	Germline Vκ3-20 sequence	junctional region	Germline Kde sequence
	TGTCAGCAGTATGGTAGCTCACCTCC		TGGAGCCCTAGTGGCAGCCCAGGGCGACTCC
5142	TGTCAGCAGT	CCCGAAGGGA	AGGGCGACTCCT
5257	TGTCAGCAGTATGGTAGCTCACCTCC	GGTTCCCAA	CAGGGCGACTCCT
5346	TGTCAGCAGTATGGTAGCTCACCT	ACCGAGAGA	AGCCCTAGTGGCAGCCCAGGGCGACTCCT
5500	TGTCAGCAGTATGGTAGCTCACCTCC	TCCCC	CCCTAGTGGCAGCCCAGGGCGACTCCT
6229	TGTCAGCAGTATGGTAGCTCACC	CATCCCCGAGG	GCCCTAGTGGCAGCCCAGGGCGACTCCT
6364	TGTCAGCAGTATGGTAGCTCACCTCC	CC	GCCCTAGTGGCAGCCCAGGGCGACTCCT
6429	TGTCAGCAGTATGGTAGCTCACC	CTC	GGCAGCCCAGGGCGACTCCT
7092	TGTCAGCAGTATGGTAGCTCACC	GGG	CCTAGTGGCAGCCCAGGGCGACTCCT
7102A	TGTCAGCAGTATGGTAGCTCACCTCC		GAGCCCTAGTGGCAGCCCAGGGCGACTCCT
7102B	TGTCAGCAGTATGGTAGCTCACCTCC	AA	AGCCCAGGGCGACTCCT

B

	Germline VH6-1 sequence	junctional region	Germline JH sequences	
	CTGTGCAAGAGA		ATTACTACTACTACTACGGTATGGACGTCTGG	JH6
			ACAACTGGTTCGACTCCTGG	JH5
			ACTACTTTGACTACTGG	JH4
			ATGCTTTTGATATCTGG	JH3
3963	CTGTGCAA	TAT	CTACTTTGACTACTGG	JH4
5161A	CTGTGCAAGAG	GACGAAACAGTAACTGC	CTACTACTACTACGGTATGGACGTCTGG	JH6
5161B	CTGTGCAAGAG	CCTCTCTCCACTGGGATGGGGGG	CTACTGG	JH4
5161C	(-31)	CG	CCTGG	JH5
5260	CTGTGCA	CGGTAGTACCAGCTGCCCATACGATTTTTGGAGTGGTTACCCCGACTGGG	ACTACGGTATGGACGTCTGG	JH6
5357	CTGTGCAAGAG	GACTTTGG	ATGCTTTTGATATCTGG	JH3
5561	CTGTGCAAGAG	CAGCAGCTCGGCCCC	CTTTGACTACTGG	JH4
5565A	CTGTGCAAGAG	GGGTATTATGATTACGTTTGGGGGAGTTATCGTTACTCCCTCAGACGCTCAAA	TTTGACTACTGG	JH4
5565B	CTGTGCAAGAG	CGGGCTATGGTTCAGGGAGTTATGG	CTACTACGGTATGGACGTCTGG	JH6
5582	CTGTGCAAGAG	GGTGGGAGCTACTA	GACTACTGG	JH4
5584	CTGTGCAAGAGA	TCCGGGCAGCTCGTTT	TGCTTTTGATATCTGG	JH3
5602	CTGTGCAAGAGA	GATAGTATAGCAGCTCGT	ACAACTGGTTCGACTCCTGG	JH5
5670	CTGTGCAAG	GGTAGCTAAAC	CTTTGACTACTGG	JH4

Figure 1.

(A) Schematic diagram of the Vκ3-20 gene segment joined to the Kde gene segment via a junctional region. PCR-mediated amplification of the joined *IGK* gene segments and subsequent sequencing of the junctional region in the PCR products obtained can be performed. The junctional region sequences presented are derived from precursor-B-ALL patients and illustrate the deletion of nucleotides from the germline sequences as well as the size and composition of the junctional regions.

(B) Schematic diagram of the VH6-1 gene segment joined to one of the JH gene segments via a junctional region. *IGH* junctional regions may contain one, or rarely, two DH gene segments. The *IGH* junctional region sequences presented illustrate that deletion and insertion of nucleotides are more extensive than in the case of *IGK*-Kde junctions. DH gene segments and inserted nucleotides are indicated by capital letters and small capital letters, respectively.

tance between these gene segments in germline configuration is far too large for PCR amplification. Because most PCR studies on Ig and TCR gene rearrangements in ALL are performed at the DNA level, the primers are complementary to exon and/or intron sequences of V, D and/or J gene segments, depending on the type and completeness of the rearrangement.[5,6,8] Ig heavy chain (*IGH*), TCR gamma (*TCRG*), and TCR delta (*TCRD*) gene rearrangements can be analyzed relatively easily with the PCR technique because the *IGH* gene complex contains only seven VH families, seven DH families, and six functional JH gene segments (recognized by five VH family-specific and seven DH family-specific primers in combination with one consensus JH primer) and because the *TCRG* and *TCRD* genes contain a limited number of V, (D), and J gene segments.[5,9-11] This implies that only a restricted number of oligonucleotide primers are needed.[11,12] Also PCR analysis of Ig kappa (*IGK*), Ig lambda (*IGL*), TCR alfa (*TCRA*) and TCR beta (*TCRB*) gene rearrangements is possible, but this requires more primers, especially for the many different V and J gene segments in *TCRA* and *TCRB* gene complexes.

The detection of (clonal) Ig/TCR gene rearrangements by PCR analysis is limited by the choice of primers, raising the possibility of false-negative results. A more essential drawback is the risk of false-positive results owing to the fact that not only clonally rearranged Ig and TCR genes are amplified but also Ig and TCR rearrangements from normal, polyclonal cells, which have rearranged the same gene segments. Discrimination between clonal (leukemia-derived) and polyclonal (reactive and normal lymphoid cell-derived) PCR products is virtually impossible by means of standard agarose gel electrophoresis. Therefore, PCR-amplified rearranged gene products need additional analysis for distinction between polyclonal and monoclonal PCR products. Methods that have been successfully applied for this purpose include direct sequencing of PCR products,[13] single strand conformation polymorphism analysis,[14] denaturing gradient gel electrophoresis,[15] heteroduplex analysis,[16,17] temperature gradient gel electrophoresis,[18] high-resolution radioactive fingerprinting[19] and fluorescent gene scanning analysis.[20] Of these, heteroduplex analysis is probably the simplest, fastest and most cost-effective. In the heteroduplex analysis, the double-strand PCR products are denatured at 94°C and subsequently renatured at 4°C to induce homoduplex formation (duplexes with identical, clonal junctions) or heteroduplex formation (duplexes with different junctional regions).[17] Homoduplexes and heteroduplexes are subsequently separated from each other by polyacrylamide gel electrophoresis, which clearly discriminates between the presence of rapidly migrating homoduplex bands or slowly migrating heteroduplex smears.[17]

Ig and TCR gene rearrangements in precursor-B-ALL

Based on their immunophenotypic characteristics, precursor-B-ALL are generally regarded as clonal malignant counterparts of normal precursor-B-cells. In line with this assumption, our studies indicate that >95% of childhood precursor-B-ALL have

IGH gene rearrangements and that most of them contain *IGK* gene rearrangements (30%) or deletions (50%); even 20% of precursor-B-ALL cases have *IGL* gene rearrangements (Table 1).[21-24] The vast majority of *IGH* gene rearrangements represent complete VH-(DH)-JH joining, while incomplete DH-JH rearrangements could be identified in 22% of patients, being the sole *IGH* gene rearrangements in only 5% of patients.[11] Deletions in the *IGK* genes are predominantly mediated via the *IGK* deleting element (Kde) sequence, which implies that *IGK* gene deletions can be identified as Kde rearrangements. Kde rearranges either to a heptamer recombination signal sequence (RSS) in the Jκ-Cκ intron, thereby deleting the Cκ gene segment, or to a Vκ gene segment, thereby deleting a large part of the *IGK* gene, including the Jκ and Cκ gene segments, or very rarely to one of the RSS flanking Jκ gene segments.[25,26] *IGK*-Kde rearrangements occur on one allele or both alleles in 20% and 30% of precursor-B-ALL cases, respectively.[22]

Cross-lineage TCR gene rearrangements occur at high frequency in childhood precursor-B-ALL: *TCRB*, *TCRG* and *TCRD* gene rearrangements and/or deletions are found in 35%, 60% and 90% of cases, respectively.[27,28] However, the spectrum of cross-lineage TCR gene rearrangements in precursor-B-ALL is very limited. *TCRB* gene rearrangements are restricted to the Jβ2 region. This is in contrast to normal

Table 1. Frequencies of identifiable Ig and TCR gene rearrangements as MRD-PCR targets in childhood ALL.[a]

Gene	Rearrangement type	Precursor-B-ALL	T-ALL
IGH	VH-JH	93%	5%
	DH-JH	20%	23%
	total *IGH*	98%	23%
IGK	Vκ-Kde	45%	0%
	intron RSS-Kde	25%	0%
	total *IGK*-Kde	50%	0%
TCRG	Vγ-Jγ	55%	95%
TCRD	Vδ-Jδ1 or Dδ-Jδ1	<1%	50%
	Vδ2-Dδ3 or Dδ2-Dδ3	40%	5%
	Total *TCRD*	40%	55%
	At least one PCR target	>95%[b]	>95%[b]
	At least two PCR targets	~90%[b]	~90%[b]
	At least three PCR targets	~65%	~50%

a. The indicated frequencies refer solely to the presence of PCR-detectable rearrangements.

b. When a high sensitivity of $\leq 10^{-4}$ is included as an extra criterion, the frequency of at least one sensitive PCR target drops to 85-90% and the frequency of at least two sensitive PCR targets drops to approximately 80%.

T-cells and T-ALL, which employ both Jβ1 and Jβ2 gene regions. *TCRG* gene rearrangements in childhood precursor-B-ALL most frequently (~70%) concern rearrangements to Jγ1 region gene segments. Curiously, 80% of *TCRD* gene rearrangements represent incomplete Vδ2-Dδ3 or Dδ2-Dδ3 joinings, whereas complete rearrangements to the Jδ1 gene segment, characteristic for normal T-cells and T-ALL blasts (particularly $TCR\gamma\delta^+$)[29] have been reported in precursor-B-ALL only anecdotally.[30] Furthermore, Vδ2-Dδ3 rearrangements are prone to continuing rearrangements, particularly to Jα gene segments with concomitant deletion of the Cδ exons and subsequent Vα-Jα recombination.[31,32] In fact, 40% of *TCRD* gene deletions in childhood precursor-B-ALL result from a Vδ2-Jα recombination, while the remaining 60% of Cδ deletions are caused most probably by Vα-Jα rearrangements.[28] Interestingly, the occurrence of cross-lineage TCR gene rearrangements seems to be age-dependent.[33,34] For example, the incidence of incomplete Vδ2-Dδ3 gene rearrangements significantly decreases with patient age, while *TCRG* gene rearrangements are rarely found in patients below 2 years of age.[33,34]

Junctional regions of *IGH*, *IGK* (especially *IGK*-Kde), *TCRG*, and *TCRD* gene rearrangements are convenient MRD-PCR targets because they can be identified wit only a limited number of PCR primer sets.[11,12] Moreover, the vast majority (>95%) of precursor-B-ALL patients can be monitored by application of junctional regions of *IGH*, *IGK*, *TCRG* and/or *TCRD* gene rearrangements (Table 1).[28,35]

TCR and Ig gene rearrangements in T-ALL

The immunophenotype of T-ALL is comparable to those of cortical thymocytes. Subclassification of T-ALL into $CD3^-$, $TCR\gamma\delta^+$, and $TCR\alpha\beta^+$ subgroups reveals major differences in TCR gene rearrangement patterns.[21,36] Although the frequency of TCR gene rearrangements in the total group of T-ALL is very high, approximately 10% of $CD3^-$ T-ALL still have all TCR genes in germline configuration;[21,36] this concerns mainly immature $CD1^-/CD3^-$ T-ALL of the prothymocytic/pre-T-ALL subgroup. The *TCRD* genes in $CD3^-$ T-ALL are rearranged in most cases (approximately 80%) and contain biallelic deletions in approximately 10% of cases.[9,36] As expected, all $TCR\gamma\delta^+$ T-ALL have *TCRG* and *TCRD* gene rearrangements and the vast majority (approximately 95%) also contain *TCRB* gene rearrangements.[9,29] All $TCR\alpha\beta^+$ T-ALL contain *TCRB* and *TCRG* gene rearrangements and have at least one deleted *TCRD* allele (*TCRA* rearrangement); the second *TCRD* allele is also deleted in two third of cases.[9,21] Analysis of the *TCRG* gene configuration in T-ALL showed that $TCR\gamma\delta^+$ T-ALL display a less mature *TCRG* immunogenotype as compared to $TCR\alpha\beta^+$ and most $CD3^-$ cases.[37] This is reflected by significantly more frequent usage of the more downstream Vγ genes and the upstream Jγ1 segments in $TCR\gamma\delta^+$ T-ALL.[29,37] Despite the described immunobiological differences between the T-ALL subsets, in virtually all childhood T-ALL (>95%) *TCRG* and/or *TCRD* junctional regions are potentially suitable targets for MRD monitoring (Table 1).[9,37]

Cross-lineage Ig gene rearrangements occur at relatively low frequency in T-ALL (approximately 20%) and involve virtually only *IGH* genes (Table 1).[21] Interestingly, cross-lineage *IGH* gene rearrangements occur more frequently in $CD3^-$ T-ALL (approximately 20%) and $TCR\gamma\delta^+$ T-ALL (approximately 50%) than in $TCR\alpha\beta^+$ T-ALL (<5%).[38]

Heteroduplex PCR analysis showed a high frequency (approximately 80%) of incomplete DH-JH rearrangements as well as preferential usage of DH6-19 and the most downstream DH7-27 gene segment together with the most upstream JH1 and JH2 gene segments. Complete VH-JH recombinations comprised only 18% of cross-lineage *IGH* gene rearrangements in T-ALL patients.[36]

Applicability of the junctional regions of Ig/TCR gene rearrangements for MRD analysis

The homoduplex PCR products can be used for direct sequencing of the junctional regions of the clonal Ig/TCR gene rearrangements. This sequence information allows the design of junctional region-specific oligonucleotides. These oligonucleotides can be applied for detection of malignant cells among normal lymphoid cells in two different ways. One possibility is the use of the oligonucleotides as patient-specific junctional region probes in hybridization experiments to detect PCR products derived from the malignant cells. The other possibility is to use the junctional region-specific oligonucleotide as primer in a PCR to amplify the rearrangements of the malignant clone specifically.

Sensitivity of MRD monitoring by PCR analysis of junctional regions

Theoretically the detection limit of the PCR technique is approximately 10^{-6} if a DNA segment is used as PCR target.[5] This detection limit can indeed be reached, but generally varies between 10^{-4} and 10^{-6}.[5,39,40] The sensitivity of MRD-PCR analysis of junctional regions is dependent on the type of rearrangement and on the 'background' of normal lymphoid cells with comparable Ig or TCR gene rearrangements. Junctional regions of complete V-D-J rearrangements are extensive, whereas junctional regions of V-J rearrangements are generally three to four times smaller. Normal cells can contain the same rearranged gene segments as the leukemic cells. For instance, Vδ1-Jδ1 rearrangements frequently occur in T-ALL, but also in a small fraction (0.1-2%) of normal peripheral blood (PB) T-cells; VγI-Jγ1.3 and VγI-Jγ2.3 joinings comprise 50-60% *TCRG* gene rearrangements in ALL, but are also found in a large fraction (70-90%) of normal PB T-cells. This might significantly influence sensitivity levels, particularly taking into account the abundance of polyclonal Vγ-Jγ joinings in normal T-cells in post-induction follow-up samples.[41] Therefore, MRD-PCR analysis of long Vδ1-Jδ1 junctional regions in PB samples is generally more sensitive (10^{-4} to 10^{-6}) than MRD-PCR analysis of short Vγ-Jγ junctional regions (10^{-3} to 10^{-5}).[12] Similarly, substantial expansions of normal precursor-B-

cells with polyclonal *IGH* gene rearrangements in regenerating BM after cessation of maintenance therapy may affect sensitivity of MRD detection using Ig gene rearrangements as PCR targets.[42,43]

Ig/TCR gene rearrangements can be identified at diagnosis in more than 95% of patients, and at least two targets per patient are available for 90% of childhood ALL.[40] However, as explained above, not all Ig/TCR targets can reach a sensitivity of $\leq 10^{-4}$. Preliminary results of first 500 childhood ALL patients treated with current BFM (Berlin-Frankfurt-Münster) Study Group treatment protocol indicate that it is generally possible to identify two sensitive MRD targets ($\leq 10^{-4}$) in approximately 80% of patients (T. Flohr et al., unpublished results).

Quantification of MRD levels detected by PCR analysis of junctional regions

MRD quantification by PCR analysis of Ig/TCR gene rearrangements is a complex process, which is essential for reliable disease monitoring. First, the quality and amplifiability of isolated DNA should be ensured. Second, minor variations in primer annealing and primer extension may lead to major variations after 30-35 PCR cycles.

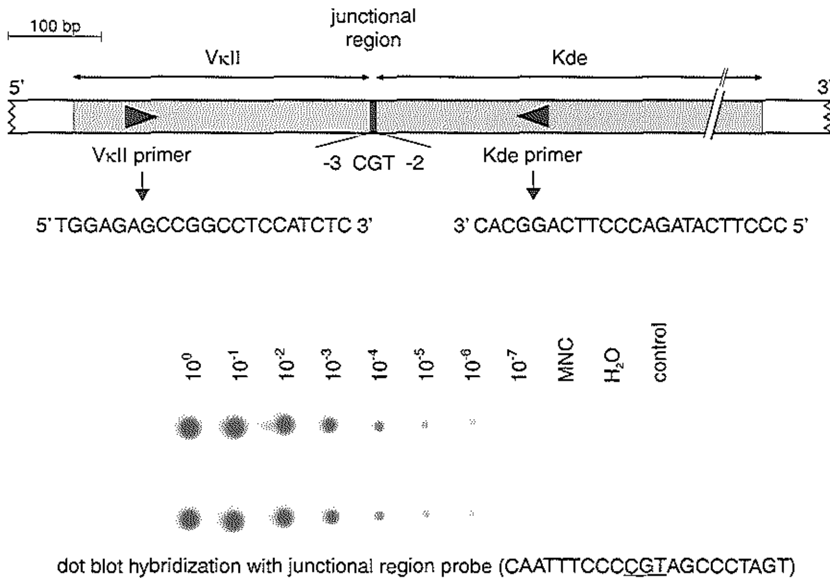
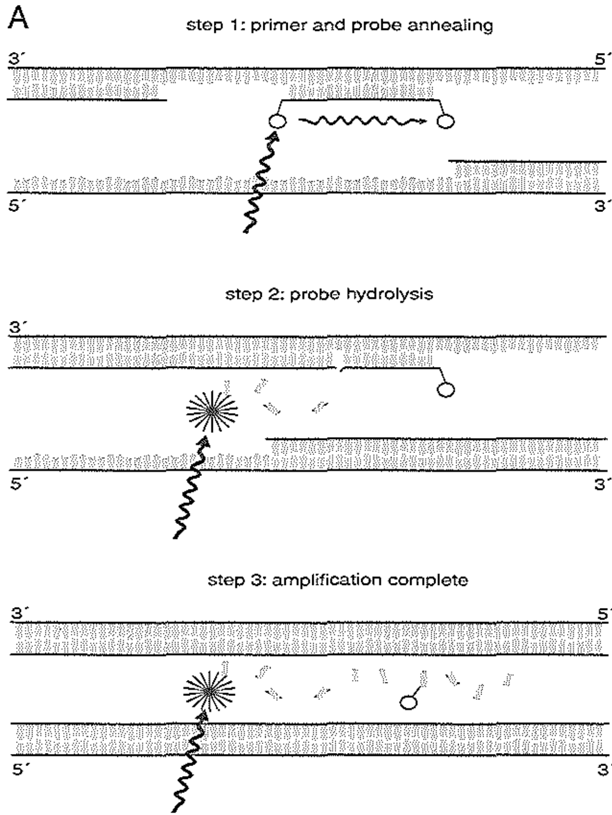


Figure 2.
A VκII-Kde rearrangement of a precursor-B-ALL as sensitive MRD-PCR target. The patient-specific junctional region probe was designed according to the junctional region sequence with deletion of five nucleotides and insertion of only three nucleotides. Tenfold dilutions were made from DNA of the leukemic cells into DNA from mononuclear cells. PCR analysis with VκII and Kde primers was performed on the dilution samples. The PCR products were blotted in duplicate on a nylon membrane and hybridized with the junctional region probe. This specific MRD-PCR target reached a sensitivity of 10^{-6} without background signals, despite the relatively short junctional region.

The disadvantages of PCR end-point quantification might (partly) be overcome by using serial dilutions of DNA isolated from the leukemic cell sample at diagnosis into DNA of normal mononuclear cells (Figure 2).[12] The same dilution series of diagnosis DNA is generally used to determine the tumor load in a follow-up sample in a semi-quantitative manner by comparison of the hybridization signals. This approach gives an indication of the tumor burden in the follow-up sample. A more precise but also more laborious quantification method is based on limiting dilution of MRD positive remission samples.[44,45] To make this assay reliable, it is necessary to perform replicate experiments to determine the level of MRD positivity. Another less tedious strategy for quantitative PCR uses an internal standard that is co-amplified with the target of interest. Quantification by competitive PCR is performed by comparing the PCR signal of the specific target DNA with that of known concentrations of an internal standard, the competitor.[46]

Recently, a novel technology has become available, the 'real-time' quantitative PCR (RQ-PCR).[47,48] In contrast to the above-described PCR end-point quantification techniques, RQ-PCR permits accurate quantification during exponential PCR amplification. The first available RQ-PCR technique was based on TaqMan technology (Figure 3). This assay exploits the 5'→3' nuclease activity of the *Taq* polymerase to detect and quantify specific PCR products as the reaction proceeds.[49] Upon amplification, an internal target-specific TaqMan probe (hydrolysis probe) conjugated with a reporter and a quencher dye is degraded, resulting in emission of a fluorescent signal by the reporter dye that accumulates during the consecutive PCR cycles. Because of the real-time detection, the method has a very large dynamic detection range over five orders of magnitude, thereby eliminating the need for performing serial dilutions of follow-up samples (Figure 3). Quantitative data can be obtained in a short period of time because post-PCR processing is not necessary. Several groups have shown that RQ-PCR via the TaqMan technology can be used for the quantitative detection of MRD in childhood ALL using junctional regions of Ig/TCR gene rearrangements as leukemia-specific PCR targets.[50-52] Another type of RQ-PCR technology exploits hybridization probes via fluorescence resonance energy transfer (FRET) technology, which is usually performed using rapid-cycle RQ-PCR (LightCycler) technology. This method requires two hybridization probes complementary to neighboring sequences, one labeled with a fluorescent dye at the 3' end and the other carrying a fluorochrome at the 5' end. One dye is a donor fluorochrome, whereas the other fluorochrome (acceptor) emits light if it is positioned close to the donor dye. Fluorescence is measured during each annealing step, when both probes hybridize to adjacent target sequences on the same strand.[47,53] Also in this hybridization probe-based RQ-PCR analysis, the fluorescent signal is exponentially increasing during the consecutive cycles, in line with the amount of PCR product formed. A third possibility for RQ-PCR is detection of SYBR green I (DNA-intercalating dye) fluorescence during PCR employing patient-specific primers (Figure


A
step 1: primer and probe annealing
3´
5´
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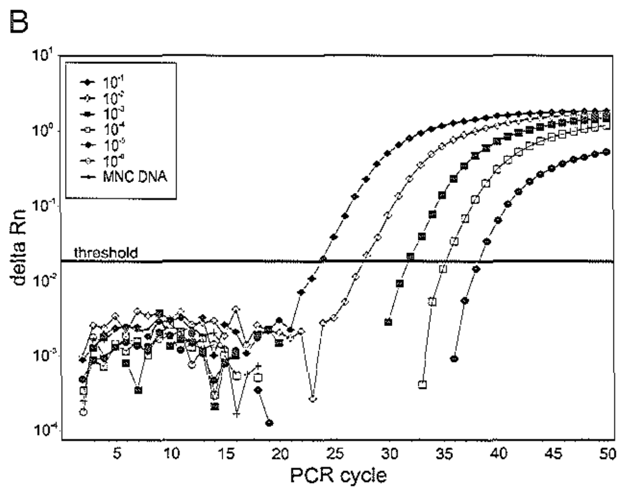
step 2: probe hydrolysis
3´
5´
3´
step 3: amplification complete
3´
5´
5´
3´


B
10¹
10⁰
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delta Rn
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MNC DNA
5
10
15
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25
30
35
40
45
50
PCR cycle

4).[47,54] The SYBR Green I dye binds to the minor groove of double-stranded DNA, which greatly enhances its fluorescence. During the consecutive PCR cycles, the amount of double-stranded PCR product will increase, and therefore more SYBR Green I dye can bind to DNA and emit its fluorescence. Maximal SYBR Green I dye binding will occur at the end of each elongation phase. This approach is most cost-effective and is sufficiently sensitive for MRD detection.[54] Several studies are in progress to assess whether SYBR green I-based detection of PCR products ensures satisfactory specificity; the first results look very promising (T. Flohr et al., unpublished results).

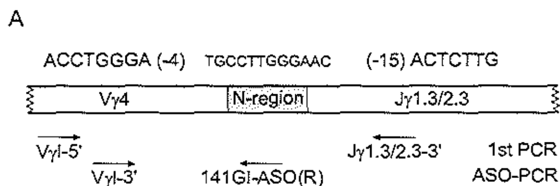
Oligoclonality and stability of Ig and TCR gene rearrangements

Ig and TCR gene rearrangements in precursor-B-ALL and T-ALL might be prone to subclone formation owing to continuing rearrangements or secondary rearrangements mediated via the active recombinase enzyme system in these immature lymphoid malignancies (reviewed in ref. 55). Several studies have shown that newly diagnosed childhood precursor-B-ALL are frequently oligoclonal because they contain multiple *IGH* gene rearrangements (30% to 40% of cases) and even multiple *IGK* gene rearrangements (5% to 10% of cases).[23,56] These multiple Ig gene rearrangements can result from both continuing rearrangement processes (e.g., continuing VH to DH-JH joining) as well as secondary rearrangements (e.g., DH-JH replacements, VH-JH replacements, and Vκ-Jκ replacements), which result in one or more subclones.[56-58] *IGH* gene rearrangement patterns might even differ between BM and PB in the same patient.[59] Furthermore, oligoclonality in the *IGH* locus is also found in 27% of T-ALL patients with rearranged *IGH* genes.[36]

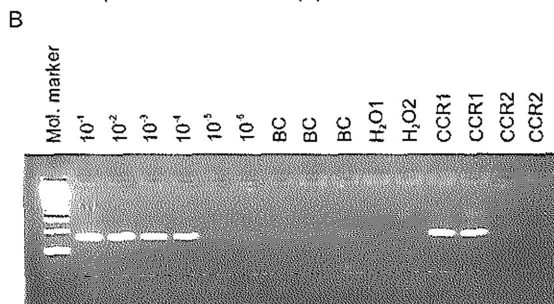
The problem of oligoclonality at diagnosis is the uncertainty as to which clone is going to emerge at relapse and which should therefore be monitored with MRD-PCR techniques. Secondary and ongoing *IGH* gene rearrangements might also occur in the time period between diagnosis and relapse, resulting in loss of leukemia-specific MRD targets.[56,60] These continuing or secondary *IGH* gene rearrangements in ALL frequently represent VH to DH-JH rearrangements or VH replacements, respectively.[38,57] During these two types of rearrangements the DH-JH junctional region remains unaffected, leading to the concept of designing the primers around the rel-

Figure 3.
Real-time quantitative PCR analysis by use of the TaqMan technique. **(A)** The TaqMan probe contains a reporter dye (R) and a quencher dye (Q), which prevents emission of the reporter dye as long as the reporter dye and the quencher dye are closely linked. During the extension phase of each PCR cycle, the annealed TaqMan probe is hydrolyzed by the 5'→3' exonuclease activity of the *Taq* polymerase, thereby separating the reporter dye from the quencher dye. This results in a fluorescent signal (delta R_n), which further increases during each subsequent PCR cycle.
(B) Example of an RQ-PCR analysis using the TaqMan approach. Tenfold dilutions of a diagnostic sample in normal mononuclear cell DNA were analyzed using an *IGH* rearrangement and a junctional region-specific primer.

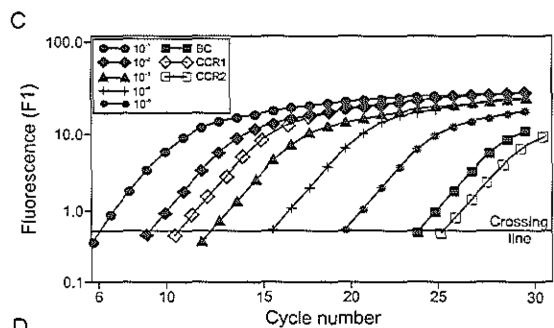
A



B



C



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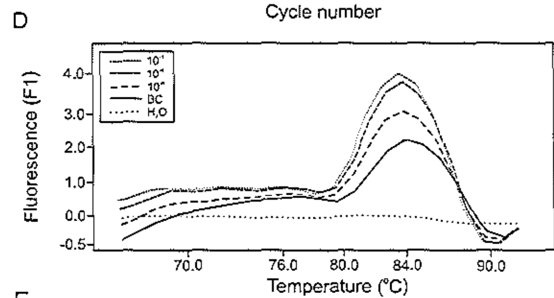

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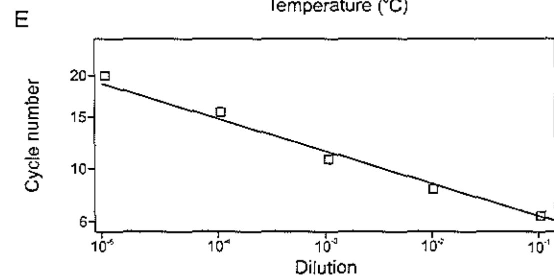

atively stable DH-JH region in order to prevent false-negative PCR results.[57]

TCR gene oligoclonality is rarely seen at diagnosis in T-ALL.[21,37,60] However, combined Southern blot and PCR data show that the frequency of oligoclonality in the cross-lineage TCR gene rearrangements of precursor-B-ALL is approximately 20%, which is slightly less than in the *IGH* gene.[28] Initially, subclone formation at diagnosis was thought to be less frequent for the *TCRD* gene complex, as suggested by Southern blotting.[9] However, heteroduplex PCR analysis and sequencing have shown that Vδ2-Dδ3 and Dδ2-Dδ3 rearrangements in newly diagnosed precursor-B-ALL are oligoclonal in 30-40% of cases.[28,33,61] Vδ2-Dδ3 rearrangements are also prone to continuing rearrangements, particularly to Jα gene segments with concomitant deletion of the Cδ exons.[32,61]

Monoclonal MRD-PCR targets in childhood precursor-B-ALL are characterized by high stability, with approximately 90% of all targets detectable at relapse. In contrast, only 40% of the oligoclonal MRD-PCR targets are preserved at relapse (T. Szczepański et al., unpublished observations). Therefore, it is probably important to discriminate between monoclonal and oligoclonal Ig/TCR rearrangements; this requires a combined Southern blot and PCR approach. Southern blotting is particularly informative for detection of oligoclonality in *IGH* and *IGK* gene rearrangements,[21,23] whereas heteroduplex PCR analysis in combination with Southern blotting is informative for detection of oligoclonal *TCRD* gene rearrangements.[28,61]

False-negative results due to clonal evolution are a major drawback of using Ig/TCR gene rearrangements as PCR targets for MRD detection. Changes in *IGH* gene rearrangement patterns at relapse occur at high frequency in childhood precursor-B-ALL, particularly when subclone formation is already present at diagnosis.[60] Changes in *TCRG* and *TCRD* gene rearrangements at relapse are found in both precursor-B-ALL and T-ALL, but generally concern only one allele.[58,60,62] At present, the *IGK*-Kde gene rearrangements are considered to be the most stable MRD-PCR targets, probably because they are rarely oligoclonal and represent end-stage rearrangements, which do not allow continuing or secondary rearrangements (Van der Velden et al., unpublished observations). The risk of changes in rearrangement patterns was suggested to increase with time (i.e. with remission duration).[60] However, clonal selection processes can occur already during early treatment phases,[63] whereas clonal identity was frequently reported between diagnosis and very late relapse of precursor-B-ALL.[64] Moreover, in relapse patients further clonal evo-

Figure 4.
RQ-PCR based MRD analysis using the Light Cycler technology and SYBR green I detection format. **(A)** Schematic representation of the allele-specific oligonucleotide (ASO) PCR strategy. For *TCRG* gene rearrangement, first-round PCR was performed using the VγI family-specific primer and the Jγ1.3/2.3 consensus primer. An inner VγI member-specific primer (Vγ4-3') and an ASO were used for ASO-PCR. **(B)** ASO-PCR results after 25 PCR cycles at 62°C, including five initial touch-down cycles with a decrease in temperature of 1°C per cycle from 67°C to 62°C on a block thermocycler and gel electrophoresis. **(C)** Amplification profile. **(D)** Melting curve analysis. **(E)** Calibration graph (see reference 54 for details).

lution is generally not observed at second or third relapse.[32,60] Despite the high frequency of immunogenotypic changes in childhood ALL at relapse, at least one rearranged *IGH*, *TCRG* and/or *TCRD* allele remains stable in 75-90% of precursor-B-ALL and in 90% of T-ALL.[58,60,62,65] More importantly, in most ALL patients at least two suitable PCR-targets are available (Table 1). Therefore, it is now generally accepted that MRD-PCR studies should preferably employ at least two Ig/TCR targets per patient. Such approach should result in a major reduction of false-negative MRD results.

CLINICAL VALUE OF MRD IN CHILDHOOD ALL

Assessment of early treatment response

The initial retrospective and small prospective studies with relatively short follow-up indicated that the detection of MRD in ALL in childhood has potent clinical value, although the results of these clinical studies were not fully concordant.[8,46,66-79] This was attributed to differences in MRD methods and particularly differences in sensitivities of MRD monitoring as well as to differences in intensity of cytotoxic treatment protocols. Several recent large prospective studies confirmed the clinical value of MRD monitoring, justifying incorporation of the MRD information to refine risk assignment in current childhood ALL treatment protocols.[65,80-84]

The most significant application of MRD monitoring in childhood ALL is the estimation of the initial response to chemotherapy. Traditionally, good clinical response with blast count in PB less than 1000/μl after a week of single agent steroid therapy or absence of circulating blasts after 7 days of multi-agent induction chemotherapy were found to be important prognostic factors.[85] As a logical continuation of these clinical findings, low levels or absence of MRD after completion of induction therapy seems to predict good outcome, as found by PCR-based MRD studies.[65,68,72,77,78,80,82] Meta-analysis of published MRD studies showed that approximately 50% of children with ALL are MRD positive at the end of induction treatment and approximately 45% of these MRD-positive patients will ultimately relapse.[86] The risk of relapse is proportional to the detected MRD levels.[65,72,77,80] The level of MRD-PCR positivity after induction therapy is the most powerful prognostic factor. Multivariate analyses showed that this prognostic value is independent of other clinically relevant risk factors, including age, blast count at diagnosis, immunophenotype at diagnosis, presence of chromosome aberrations, response to prednisone, and classical clinical risk group assignment, provided that accurate MRD quantification on adequate BM samples is performed.[65,80,82]

The meaning of MRD positivity differs in cytogenetic subgroup of ALL. Philadelphia-positive (Ph^+) ALL with t(9;22) is characterized by high drug-resistance and a very poor prognosis.[87] This leukemia subtype is also associated with an

increased percentage of MRD positive patients and increased MRD levels at the end of induction treatment.[88] Chemotherapy can lower the degree of MRD by only 2 to 3 logs in most Ph^+ ALL patients, which is not enough for prolonged hematological remission. Nevertheless, in some patients with favorable prognostic features (e.g., low initial leukocyte count or good prednisone response) the disease can be controlled with intensive chemotherapy.[87] MRD monitoring might identify this small drug-sensitive subgroup of Ph^+ patients. However, due to the poor prognosis of ALL with t(9;22) and the very low frequency of complete cure in this patient group, this hypothesis should be confirmed by large multi-center studies. The same holds true for other cytogenetic ALL subgroups with increased risk of relapse – as, for instance, precursor-B-ALL with t(4;11). It is possible to identify a small subgroup of t(4;11)-positive patients with rapid achievement of molecular remission after intensive chemotherapy and/or allogeneic BM transplantation (BMT) and persistent PCR-negativity in long-term complete remission.[89] Therefore, prospective MRD monitoring can be used for assessment of treatment response and can be applied for individualization of therapy in order further to improve the outcome of high-risk t(4;11)-positive leukemia, including infant ALL patients.[90] MRD studies in patients with the prognostically favorable *TEL-AML1* aberration suggest that the MRD levels at the end of induction therapy are generally below the threshold associated with bad outcome.[91] Nevertheless, using quantitative MRD analysis of early treatment response, it is possible to identify the subgroup of *TEL-AML1* positive patients at high risk of leukemia relapse.[91]

The results from the large prospective MRD study of the International BFM Study Group (I-BFM-SG) indicated that analysis of MRD at a single time point is not sufficient for recognition of patients with poor prognosis as well as patients with good prognosis.[65] In contrast, combined information on the kinetics of tumor load decrease at the end of induction treatment and before consolidation treatment appeared to be highly informative. This combined MRD information distinguishes patients at low-risk with MRD negativity at both time points (5-year relapse rate of 2%); patients at high risk with an intermediate (10^{-3}) or high ($\geq 10^{-2}$) degree of MRD at both time points (5-year relapse rate of 80%); and the remaining patients at intermediate risk (5-year relapse rate of 22%) (Figure 5).[65] The MRD-based low-risk patients make up a group of a substantial size (approximately 45%), comparable to the frequency of survivors of childhood ALL before treatment intensification was introduced.[92] Within the MRD-based low-risk group, half of the patients have already low ($\leq 10^{-4}$) or undetectable MRD levels after two weeks of treatment.[83] This group might particularly profit from treatment reduction. On the other hand, the group of patients at MRD-based high risk is larger than any previously identified high-risk group (approximately 15%) and has an unprecedented high 5-year relapse rate of 80%. This group might benefit from further intensification of treatment protocols including BMT during first remission or novel treatment modalities - for instance the

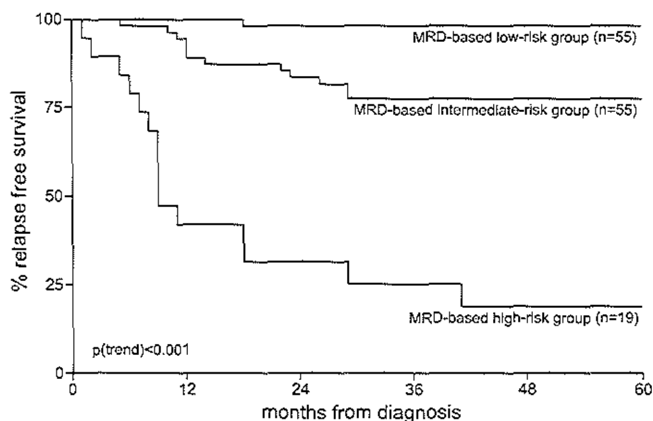

Figure 5.
Relapse-free survival of the three MRD-based risk groups of children treated for ALL according to protocols of the International BFM Study Group. The three risk groups were defined by combined MRD information at the end of induction treatment (time point 1) and before consolidation treatment (time point 2).[65] Patients in the low-risk group have MRD negativity at both time points (43% of patients), patients in the high-risk group have MRD degrees of $\geq 10^{-3}$ at both time points (15% of patients), and the remaining patients form the MRD-based intermediate-risk group (43% of patients).

inclusion of antibodies conjugated with immunotoxins or tyrosine kinase inhibitors. It is important to bear in mind that optimal time-points and sensitivities for determination of early treatment response ('MRD window') should be determined as per ALL treatment protocol. Modifications in chemotherapy regimens and the corresponding BM sampling time points might result in the loss of clinical significance of MRD information if the MRD window is not simultaneously adjusted.[93]

The predictive value of MRD monitoring is particularly clear after first relapse.[73,94] This can be perceived as assessment of early treatment response after second induction treatment. The BFM group demonstrated the high prognostic value of MRD detection at day 42 of the ALL-REZ treatment protocol. Patients with MRD levels below 10^{-3} had a probability of relapse-free survival of 88%, whereas MRD levels $\geq 10^{-3}$ were uniformly predictive of dismal outcome (probability of relapse-free survival of 0%).[94]

The MRD data from a single study suggest slower kinetics of leukemia clearance in T-ALL as compared to precursor-B-ALL patients.[76] Virtually all (16 of 17) patients were found to be MRD-positive at the end of induction treatment, while a later time point at the beginning of maintenance treatment carried the most significant prognostic information. All but one (seven of eight) MRD-positive patients at this *late* time point subsequently relapsed, while all eight MRD-negative patients remained in continuous complete remission.[76] This is in contrast to the MRD study of the I-BFM-SG,

which showed that PCR-based measurement of *early* treatment response at two consecutive time points after induction treatment and before consolidation treatment results in highly prognostic MRD information: ~25% low-risk patients with 0% relapses, ~50% intermediate-risk patients with 25% relapses, and ~25% high-risk patients with 100% relapses (M. J. Willemse et al., unpublished observations).

The value of continuous MRD monitoring in childhood ALL

Continuous MRD monitoring in childhood ALL has shown that a steady decrease of MRD levels to negative PCR results during treatment is associated with favorable prognosis,[71] whereas persistence of high MRD levels or a steady increase of MRD levels generally leads to clinical relapse.[65-67,69,78] Therefore, persisting MRD levels during treatment can be regarded as the best indicator of resistance to treatment. PCR-based MRD monitoring was shown to be able to select the group of 'poor-responders' with early relapse during maintenance treatment.[46,65,80,82] Sequential sampling generally shows positive MRD-PCR results prior to clinical relapse. However, the time span between the re-appearance of MRD positivity and overt hematological relapse might be too short for earlier implementation of re-induction treatment.[69] Real false-negative results might be obtained due to continuing or secondary Ig or TCR gene rearrangements (clonal evolution). Such false-negative results can generally be prevented by using two Ig/TCR targets per patient (see above).[65]

Low levels of MRD after therapy might be associated with late development of relapse, but absence of MRD at the end of treatment is not sufficient to assure that the patient is cured.[65,70,78] Despite the high sensitivity of most MRD techniques, it should be noted that MRD negativity does not exclude the presence of leukemic cells in the patient because each MRD test only screens a minor fraction of all BM and PB leukocytes. Curiously, one report claims that multiple PCR analyses (testing a higher number of cells) gave evidence for residual leukemia at very low levels in ~90% (15/17) of patients remaining in long-term clinical remission.[75] In 7 out of these 15 patients this PCR result was confirmed in a blast colony assay. So far these data have not been confirmed by other investigators, even by using extremely sensitive 10-fold PCR analyses (sensitivities: 10^{-6} to 10^{-7}).[65] In contrast, large prospective studies showed 0 to 10% of patients being positive at the end of treatment, with the majority of these MRD positive patients relapsing later on.[65-78]

The value of MRD monitoring in childhood ALL patients undergoing BMT

MRD monitoring is particularly informative for ALL patients undergoing BMT. High levels of MRD positivity (10^{-2} to 10^{-3}) before allogeneic BMT are invariably associated with relapse after transplantation, while 2-year event-free survival in patients with low-level MRD positivity (10^{-3} to 10^{-5}) approximated 35%.[95] This is particularly true for patients receiving T-cell-depleted grafts, while a strong graft-ver-

sus-leukemia effect associated with non-depleted grafts might even overcome high levels of MRD positivity in selected cases.[96] In contrast, MRD-negativity before allogeneic transplantation significantly correlated with better outcome and a 2-year event-free survival of 73%.[95] Therefore, patients with a high MRD burden prior to BMT might be offered alternative treatment (e.g. further cytoreduction before BMT, intensified conditioning, and/or early post-BMT immunotherapy) in order to improve their generally poor outcome.[95,97]

MRD-PCR positivity in ALL patients after BMT is suggestive of impending relapse.[98] This is true both for high-risk patients transplanted in first remission and patients subjected to BMT in second remission after leukemia relapse. MRD was shown to occur in post-BMT samples in 88% of patients who subsequently relapsed, while only 22% of patients in long-term complete remission showed MRD at any time after BMT, mostly at low levels.[98] The patients with persistent MRD positivity after BMT may be candidates for early treatment with immunotherapy, including donor lymphocyte infusions to increase graft-versus leukemia effects.[98]

Potential usage of peripheral blood for MRD monitoring in childhood ALL

One of the serious limitations of continuous MRD monitoring is the need of multiple traumatic BM aspirations. Replacement of BM sampling by PB sampling has been a topic of debate in MRD studies for the last 15 years. The data from a single PCR study showed 11.7 difference between BM and PB during induction treatment of precursor-B-ALL patients.[99] This implies that MRD techniques need to be at least tenfold more sensitive (i.e. $\leq 10^{-5}$), when PB samples are monitored.[65] The difference between BM and PB might be lower in Ph^+ precursor-B-ALL, most probably because of a generally higher degree of MRD as compared to other ALL subtypes. Undoubtedly, the differences in MRD levels between BM and PB are additionally influenced by the degree of dilution of BM aspirates with PB. This is suggested by the finding of 4.1-fold greater mean MRD level in the trephine BM biopsies as compared to the BM aspirates.[100] Nevertheless, more information is needed to decide whether or not, and to what extent, the more traumatic BM sampling can be replaced by PB sampling.

SUMMARY

Antigen receptor gene rearrangements are reliable patient-specific targets for PCR-based MRD monitoring in childhood ALL. Such Ig/TCR rearrangements can be identified at diagnosis in more than 95% of patients through relatively easy and cost-effective approaches such as heteroduplex PCR analysis or fluorescent gene scanning. This should result in MRD-PCR targets, which are at least two orders of magnitude more sensitive than conventional cytomorphological techniques. Usage of at

least two sensitive targets per patient (available for approximately 80% of ALL) is strongly advised to prevent false-negative results owing to target loss via secondary or ongoing recombination throughout the course of the disease. With currently available RQ-PCR methods, MRD monitoring via Ig/TCR gene rearrangements is quantitative and highly reproducible between laboratories.

Several studies have demonstrated that MRD monitoring in childhood ALL applying Ig/TCR gene rearrangements as PCR targets significantly predicts clinical outcome. This is particularly useful for evaluation of early treatment response, which distinguishes high-risk patients with poor prognosis from low-risk patients with excellent prognosis. Similarly, high MRD positivity before stem cell transplantation is strongly prognostic of relapse after transplantation. Prolonged continuous MRD monitoring might be important for patients at high risk of relapse and for patients at intermediate risk of relapse, i.e. patients with a relatively slow early response to treatment.

As detection of MRD has proven to be of clinical importance, it is currently being incorporated into stratification of childhood ALL treatment protocols. Further studies should prove whether MRD-based individualization of therapy improves treatment outcome in high-risk ALL patients and prevents unnecessary treatment-related toxicity in low-risk ALL patients.

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PRACTICE POINTS

- junctional regions of Ig and TCR gene rearrangements are `fingerprint-like' sequences which are unique for each acute lymphoblastic leukemia. They can therefore be used as PCR targets for sensitive detection of low frequencies of leukemic cells, i.e. minimal residual disease (MRD)
- it is possible to identify clonal immunoglobulin and T-cell receptor gene rearrangements in virtually all childhood precursor-B-ALL and T-ALL patients
- using immunoglobulin and T-cell receptor gene rearrangements, it is possible to monitor quantitatively minimal residual disease with high specificity and high sensitivity, at least 100-fold more sensitive than with routine morphology

- MRD monitoring in childhood ALL employing Ig/TCR gene rearrangements as PCR targets has significant predictive value superior to that of other clinically relevant prognostic factors
- evaluation of early treatment response via MRD monitoring results in an unprecedentedly accurate MRD-based risk group classification which can be used for treatment stratification in childhood ALL
- prolonged continuous MRD monitoring might be important for patients at intermediate risk or high risk of relapse for assessment of long-term response to more aggressive treatment
- MRD monitoring in second complete remission identifies patients with excellent drug-sensitivity and predicts outcome after stem cell transplantation

RESEARCH AGENDA

- for a subgroup of childhood ALL patients there is still the requirement to identify new sensitive MRD-PCR targets. This might be accomplished by further characterization of Ig/TCR gene rearrangement patterns with subsequent identification of new PCR targets
- further standardization of RQ-PCR techniques should provide rapid and quantitative MRD information, which is essential for clinical decision-making.
- ongoing prospective multi-center studies should answer the question: does MRD-based intensification of ALL treatment improve the outcome in MRD-based high-risk ALL patients?
- future treatment protocols should show whether less intensive chemotherapy in MRD-based low-risk ALL patients (40 ± 45% of childhood ALL) decreases treatment-related toxicity and does not result in an increase in the rate of relapse

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CHAPTER 5.2

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Several studies have proven that minimal residual disease (MRD) monitoring in acute lymphoblastic leukemia (ALL) patients has significant prognostic value, which can be used for improved therapy stratification. At present such clinically relevant MRD information can be obtained with three different techniques (reviewed in ref. [1-3]):

- flow cytometric immunophenotyping using aberrant or "leukemia-associated" phenotypes;
- PCR techniques using chromosome aberrations that result in fusion gene transcripts or aberrant expression of transcripts;
- PCR techniques using patient-specific junctional regions of rearranged immunoglobulin (Ig) and T-cell receptor (TCR) genes.

The characteristics of the three currently used MRD techniques in ALL are summarized in Table 1. Each of the three MRD techniques has specific advantages and disadvantages, which should be weighed against each other when plannings are made for large-scale clinical MRD studies. Particularly, the required sensitivity and the applicability will play an important role, because these two characteristics determine which patients can actually be monitored.

Flow cytometric MRD detection

The immunophenotypic "targets" in flow cytometric MRD detection mainly concern aberrant or "leukemia-associated" immunophenotypes, which are rare (or sometimes absent) in normal bone marrow (BM) and peripheral blood (PB) (reviewed in ref. [2,4]). Current triple and quadruple labelings allow detection of such leukemia-associated phenotypes in the majority (60-98%) of precursor-B-ALL and in virtually all T-ALL.[5-8] Immunophenotypic shifts during the disease course do occur, although their reported frequency is variable, mainly because different definitions are used for phenotypic shifts.[2,9] Nevertheless, most groups agree that preferably two different leukemia-associated phenotypes should be monitored per patient to prevent false-negative results.[2,4]

The sensitivity of flow cytometric MRD detection remains unclear. Flow cytometric evaluation of 10^6 cells is technically not a problem, but detection of 50 to 100 malignant precursor-B-cells between 10,000 to 50,000 normal precursor-B-cells in BM is not easy, particularly in regenerating BM during or after therapy.[10] However, this is essential to reach sensitivities of 10^{-4}. Some experienced centers claim that a detection limit of 10^{-4} can be reached routinely in virtually all ALL patients, but other centers agree that the detection limit varies between 10^{-3} and 10^{-4} for most

Table 1. Characteristics of the techniques currently employed for MRD detection in ALL.

	Flow cytometric immunophenotyping	PCR analysis of chromosome aberrations (mainly detection of fusion gene transcripts)	PCR analysis of Ig/TCR genes (junctional region specific approach)
Sensitivity	10^{-3}–10^{-4}	10^{-4}–10^{-6}	10^{-4}–10^{-5}
Applicability			
- Precursor-B-ALL	60-98%	40-45%*	90-95%
- T-ALL	90-95%	15-35%**	90-95%
Advantages	- applicable for most patients - relatively cheap - rapid: 1-2 days	- relatively easy and cheap - sensitive and leukemia-specific - stable target during disease course - rapid: 2-3 days - suitable for monitoring of uniform patient groups (e.g., Ph^+ ALL)	- applicable for virtually all patients if *IGH*, *IGK*-Kde, *TCRG*, and *TCRD* gene rearrangements are used as targets - sensitive and patient-specific - rapid during follow-up: 2-3 days (if junctional region is identified and if RQ-PCR is used)
Disadvantages	- limited sensitivity - need for preferably two aberrant immunophenotypes per patient, because of chance of immunophenotypic shifts	- useful in only a minority of patients - cross-contamination of PCR products leading to false-positive results (even at diagnosis)	- time-consuming at diagnosis: identification of the junctional regions and sensitivity testing - relatively expensive - need for preferably two PCR targets per patient, because of chance of clonal evolution

* In childhood ALL this particularly concerns t(12;21)(*TEL-AML1*) and in adult ALL particularly t(9;22)(*BCR-ABL*)

** This mainly concerns del(1)(p32 p32) with *SIL-TAL1* fusion and t(5;14) with aberrant *HOX11L2* expression, together occurring in 25-35% of childhood T-ALL and in 15-20% of adult ALL.[11,12]

precursor-B-ALL, while in virtually all T-ALL a detection limit of 10^{-4} can indeed be reached, because of their specific thymocytic phenotype.

PCR analysis of chromosome aberrations

Structural chromosome aberrations are ideal leukemia-specific PCR targets, which remain stable during the disease course and can reach excellent sensitivities of 10^{-4} to 10^{-6}. In ALL, these PCR targets mainly concern fusion gene transcripts (e.g. *TEL-AML1*, *BCR-ABL*, and *SIL-TAL1*) or aberrantly expressed specific transcripts (e.g., *HOX11L2* and *WT1*), which can be detected via reverse transcriptase (RT) PCR analysis.[3,11-13]

Two main disadvantages limit the application of chromosome aberrations as MRD-PCR targets: applicability in only a minority of ALL patients and the chance of false-positive results via cross-contamination of PCR products.

PCR analysis of Ig and TCR gene rearrangements

The junctional regions of rearranged Ig and TCR genes are fingerprint-like sequences, which differ in length and composition per lymphocyte or lymphocyte clone and consequently also per each lymphoid malignancy, such as ALL.[14] These patient-specific MRD-PCR targets can be detected in the vast majority of precursor-B-ALL and T-ALL (Table 2) and generally reach sensitivities of 10^{-4} to 10^{-5}.[1,15-17]

These high sensitivities require the precise identification of the junctional region sequences of Ig and TCR genes in each ALL, because these sequences are need-

Table 2. Frequencies and stability of MRD-PCR targets in childhood precursor-B-ALL.

PCR target	Frequency* at diagnosis	Monoclonality at diagnosis*	Stability at relapse**		
			monoclonal	oligoclonal	total
IGH	95%	60-70%	85%	44%	64%
- VH-JH	95%		88%	47%	69%
- DH-JH	20%		57%	38%	43%
IGK-Kde	50%	90%	95%	40%	90%
- Vκ-Kde	45%		95%	40%	91%
- intron-Kde	25%		86%	0	87%
TCRG (Vγ-Jγ)	55%	60-65%	NT	NT	75%
TCRD	40%	60%	86%	26%	63%
-Vδ2-Dδ3	35%		81%	31%	63%
-Dδ2-Dδ3	7%		100%	14%	63%

* see ref.[15-17]
** see ref.[20]
NT not tested

ed to design patient-specific oligonucleotides. Subsequently, sensitivity testing has to be performed via serial dilution of DNA obtained at diagnosis in order to assess whether the required sensitivity can indeed be reached.

If only sensitivities of 10^{-2} to 10^{-3} are required, it is possible to skip the sequencing of the junctional regions and focus on differences in length of the junctional regions, which can be evaluated by PCR product length assessment via fluorescent GeneScanning. An internal competitor containing the same type of rearrangement can be used for quantification of the Ig/TCR gene target.[18]

The main disadvantage of using Ig/TCR gene rearrangements as MRD-PCR targets in ALL is the occurrence of continuing rearrangements during the disease course as has been identified by comparing the Ig/TCR gene rearrangement patterns at diagnosis and relapse.[19,20] Such changes in rearrangement patterns will lead to false-negative PCR results.

In precursor-B-ALL, changes at relapse were particularly observed in patients with more than one leukemic subclone (oligoclonality) at diagnosis.[20] The occurrence of subclones differs per type of Ig/TCR gene rearrangement and consequently also the stability differs per type of rearrangement (Table 2). For example, *IGK* gene rearrangements involving the so-called kappa deleting element (Kde) are more stable than *IGH*, *TCRG*, and *TCRD* gene rearrangements.[20]

In T-ALL, the changes in TCR gene rearrangement patterns at relapse are more limited, probably related to the fact that T-ALL rarely contains oligoclonal TCR gene rearrangement patterns.[16,19] Nevertheless, it is now generally accepted that preferably two Ig/TCR gene targets should be used for reliable and sensitive MRD detection in ALL patients.

Real-time quantitative PCR techniques

PCR-based MRD methodologies are increasingly achievable thanks to the development of real-time quantitative (RQ-) PCR techniques (reviewed in ref. [21]).

Fusion gene transcripts from chromosome aberrations or aberrantly expressed transcripts are excellent RQ-PCR targets for the detection of MRD in ALL.[22,23] Copy numbers of the relevant transcript in BM or PB follow-up samples can be calculated via a dilution curve of known amounts of plasmids containing the fusion gene sequences. A standardized approach has been developed for the most frequent fusion gene transcripts in ALL via the Europe Against Cancer program.[24]

Several studies have proven that RQ-PCR can be effectively used for quantitative detection of MRD using junctional regions of Ig and TCR gene rearrangements as PCR targets. Uniform approaches with a junctional region-specific primer in combination with a germline TaqMan probe and primer have been developed for the classical Ig/TCR targets: *IGH*, *IGK*-Kde, *TCRG* and *TCRD*.[25-28] Introduction of new MRD-PCR targets like *TCRB* gene rearrangements is currently a subject of an European BIOMED-2 Concerted Action.[29]

PROGNOSTIC VALUE OF MRD DETECTION IN ALL

Early retrospective and small prospective studies with relatively short follow-up indicated that the detection of MRD in childhood ALL predicts treatment outcome, although the results of these clinical studies were not fully concordant (reviewed in ref. [1]). This was attributed to differences in sensitivities of MRD monitoring as well as to differences in intensity of cytotoxic treatment protocols. Several recent large-scale prospective studies confirmed the clinical value of MRD monitoring, justifying incorporation of MRD information in current childhood ALL treatment protocols to refine risk assignment.[5,8,30-33]

Significance of MRD detection during front-line treatment of childhood ALL

The most significant application of MRD monitoring for front-line treatment of ALL is the evaluation of the initial response to cytotoxic therapy. Low levels or absence of MRD in BM after completion of induction therapy appears to predict good outcome.[5,8,30-34] Depending on the treatment protocol and the end-of-induction sampling time point, MRD-negativity is associated with overall relapse rates of only 2-10% (Figure 1 and Table 3)[5,8,30-33] Moreover, sensitive MRD detection *during* the induction phase seems capable of identifying 20% of childhood ALL patients with a very rapid leukemia clearance and long-term relapse-free survival.[35] On the other

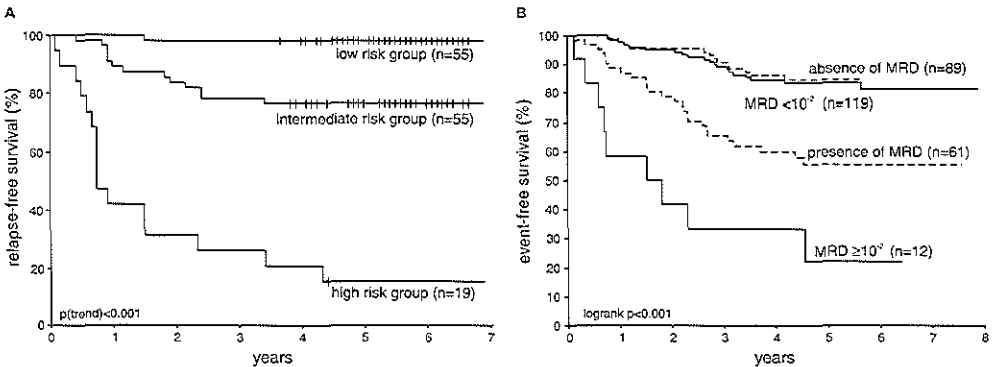

Figure 1.
(A) Relapse-free survival of the three MRD-based risk groups of children treated for ALL according to protocols of the International BFM Study Group. The three risk groups were defined by combined MRD information at the end of induction treatment and before consolidation treatment (see[30] for details). **(B)** Relapse-free survival according to the qualitative (presence or absence) and quantitative detection of MRD after the completion of induction therapy in EORTC trial 58881 (with courtesy to Dr. H. Cavé and Prof. E. Vilmer).[32,36]

Table 3. Comparison between large prospective MRD studies in pediatric ALL.*

Study group	I-BFM-SG	BFM-Austria	NOPHO	EORTC	St-Jude
Treatment protocol	ALL-BFM 90/ AEIOP ALL91 /DCLSG – ALL8	ALL-BFM 95	NOPHO ALL MRD 95	EORTC protocol 58881	Total Therapy Study XIIIA and XIIIB
Induction intensity before MRD sampling	Standard (PRED + Mtx i.th. + VCR + DAUNO + L-ASP)	Standard (PRED + Mtx i.th. VCR + DAUNO + L-ASP)	Standard (PRED + Mtx i.th. VCR +DOXO + L-ASP)	Standard (PRED + Mtx i.th. VCR +DOXO + L-ASP)	Intensive (Mtx + PRED + VCR + Dauno + L-ASP + VP-16 + Ara-C + triple i.th.)
Number of patients	169	98	100	151	165
Relapse rate	27% at 5 years	12% at 3.5 years	15% at 4 years	21% at 3 years	16.3% ± 3.5% at 4 years
Technique	Ig/TCR PCR	Flow cytometry	Ig/TCR PCR	Ig/TCR PCR	Flow cytometry
Standardization	multi-center	single-center	single-center	multi-center	single-center
MRD time point at the end of induction treatment	5 weeks	5 weeks	4 weeks	5 weeks	6 weeks

MRD status at the end of induction treatment	I-BFM-SG Distribution % (No.)	I-BFM-SG Relapse rate % (No.)	BFM-Austria Distribution % (No.)	BFM-Austria Relapse rate % (No.)	NOPHO Distribution % (No.)	NOPHO Relapse rate % (No.)	EORTC Distribution % (No.)	EORTC Relapse rate % (No.)	St-Jude Distribution % (No.)	St-Jude Relapse rate % (No.)
MRD-negative	42% (71)	3% (2)	60% (59)	7% (4)	53%	2%	66% (88)	8% (7)	75% (123)	7% (9)
MRD ≤ 10^{-4}	20% (33)	24% (8)	5% (5)	20% (1)	(53)	(1)	23% (30)	17% (5)	12% (19)	~ 30%
MRD ≈ 10^{-3}	22% (38)	39% (15)	32% (31)	6% (5)	32% (32)	28% (9)	11%	73%	8% (14)	
MRD ≥ 10^{-2}	16% (27)	74% (20)	3% (3)	100% (3)	15% (15)	33% (5)	(15)	(11)	5% (9)	72% ± 21%

Continued Table 3.

Risk group classification	Combined two time points		Combined two time points		Single time point		Single time point		Combined two time points	
	Distribution	Relapse rate	Distribution	Relapse rate	Distribution	Relapse rate	Distribution	Relapse rate	Distribution	Relapse rate
MRD-based LRG	43% (55)	2% (1)	90%	3%	40% (40)	0%	89%	10%	79% (123)	7% (9)
MRD-based IRG	43% (55)	24% (13)	(94)	(3)	60%	25%	(118)	(12)	9% (14)	7% (1)
MRD-based HRG	15% (19)	84% (16)	10% (10)	100% (10)	(60)	(15)	11% (15)	73% (11)	12% (18)	56% (10)

* see ref.[5,8,30-32]

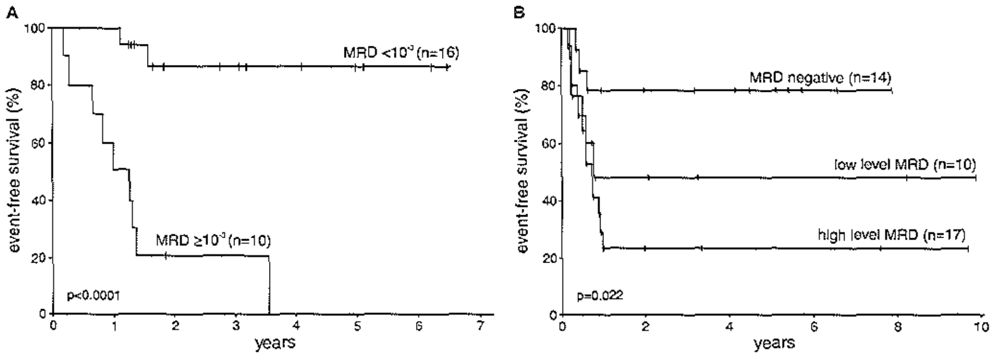

Figure 2.
(A) Probability of event-free survival according to the MRD levels after the second block of the ALL-REZ BFM treatment protocols (with courtesy to Dr. C. Eckert and Prof. G. Henze).[38] (B) Probability of event-free survival after allogeneic BMT according to the MRD levels in pre-BMT BM samples (with courtesy to Dr. P. Bader).[42]

hand, several studies proved that high MRD levels at the end of induction treatment are associated with high relapse rates of 70–100% (Figure 1 and Table 3).[5,8,30,32,36] Statistical analyses have shown that the MRD status after induction therapy is the most significant prognostic factor, independent of other clinically relevant risk factors, such as age, blast count, immunophenotype, presence of chromosome aberrations at diagnosis, and response to prednisone.[30,32,37]

Clinical value of MRD during treatment of relapsed ALL

The predictive value of MRD monitoring is particularly clear after ALL relapse, as shown by the BFM group.[38] This can be perceived as assessment of early treatment response after second induction treatment. Low MRD levels ($<10^{-3}$) were associated with a probability of relapse-free survival of 86%, whereas MRD levels $\geq 10^{-3}$ were uniformly predictive of dismal outcome (probability of relapse-free survival of 0%; see Figure 2).[38] Apparently, MRD information also has a high predictive value in relapsed ALL. However, the patient numbers are still small and the results need further confirmation.[38]

Clinical value of MRD detection before bone marrow transplantation in childhood ALL

MRD monitoring was shown to be very informative for ALL patients undergoing bone marrow transplantation (BMT).[39-43] In patients receiving T-cell depleted grafts, high levels of MRD-PCR positivity (10^{-2} to 10^{-3}) before allogeneic BMT were invariably associated with relapse after transplantation.[39,42,43] The 2-year event-free survival in patients with low level of MRD positivity (10^{-3} to 10^{-5}) was 35 to 50%, irre-

spective of graft manipulation.[39,42,43] It has been suggested that significant graft-versus-host disease associated with non-depleted grafts might overcome MRD positivity, even high MRD levels.[42,43] In contrast, MRD-negativity before allogeneic transplantation significantly correlated with better outcome and 2-year event-free survival higher than 70% (Figure 2).[39-43] Therefore, patients with a high MRD burden prior to BMT might be offered alternative treatment (e.g. further cytoreduction before BMT, intensified conditioning, and/or early post-BMT immunotherapy) in order to improve their generally poor outcome.[39,41]

Clinical value of MRD detection in adult ALL

The preliminary results of MRD studies in adult ALL showed molecular response to chemotherapy similar to childhood ALL, but with higher frequencies of persistent MRD positivity in adults.[44,45] Not only the frequencies of MRD positivity, but also the MRD levels in adult patients were significantly higher than in comparably treated children, which is consistent with a higher *in-vivo* drug-resistance of adult ALL.[44,45] A single prospective MRD study in t(9;22) negative adult precursor-B-ALL patients demonstrated the strong predictive value of MRD information at all investigated time points within two years of treatment, particularly 3-5 months after remission induction and beyond.[46]

Recognition of MRD-based risk groups

The results of the large prospective MRD studies in childhood ALL indicate that MRD analysis at a single time point gives highly significant prognostic information, but a single time point is not sufficiently precise for defining MRD-based low-risk *and* high-risk groups.[5,8,30-32] Depending on the MRD study, the end-of-induction MRD status either identifies only patients at low risk of relapse[30,31] or more frequently identifies exclusively high-risk patients (Table 3).[32,37] In contrast, information on the kinetics of tumor load decrease, e.g., at the end of induction treatment and before consolidation treatment, appeared to be highly reliable for the recognition of all clinically relevant risk groups.[30,33] As shown by the International BFM Study Group (I-BFM-SG), this combined MRD information distinguished patients at low-risk with MRD negativity at both time points (5-year relapse rate of 2%); patients at high risk with intermediate (10^{-3}) or high ($\geq 10^{-2}$) MRD levels at both time points (5-year relapse rate of 84%); and the remaining patients at intermediate risk (5-year relapse rate of 24%) (Figure 1).[30] The group of MRD-based high-risk patients was larger than any previously identified high-risk group (approximately 15%) and had an unprecedentedly high 5-year relapse rate of 84%. On the other hand, the MRD-based low-risk patients made up a group of a substantial size (approximately 45%), comparable to the frequency of survivors of childhood ALL in the 1970s before treatment intensification was introduced.[47] Such a precise MRD-based risk stratification has not yet been demonstrated for adult ALL.

CAN WE COMPARE THE MRD RESULTS OF DIFFERENT CLINICAL ALL TRIALS?

The prognostic value of MRD detection during the early treatment phases of childhood and adult ALL has been established. In fact, every published study has confirmed the high prognostic value of MRD data obtained at the end of induction treatment.[5,8,30-32] Nevertheless, the reported large-scale MRD studies show remarkable differences in the meaning of MRD level information at the end of induction (Table 3). Also the MRD-based risk groups are defined differently, resulting in different distributions of the patients over the risk groups and different relapse rates (Table 3).

The major differences in risk group definition and corresponding relapse rates might be related to the type of treatment protocol, the timing of the follow-up samples, or the applied MRD technique (e.g., sensitivity and/or stability of the applied targets). Consequently, it will be impossible to extrapolate data from one clinical treatment protocol to another. This implies that for each treatment protocol the "MRD window" has to be defined precisely: sampling time points versus required sensitivity. In practice, this means that MRD-based treatment intervention should always be designed according to earlier-obtained MRD results from the same treatment protocol.

HOW TO TRANSLATE MRD INFORMATION INTO NEW CLINICAL TREATMENT PROTOCOLS?

When MRD information from existing treatment protocols is translated into new clinical treatment protocols, several MRD-related aspects will influence the implementation of the new protocol:

- MRD-based stratification can only be introduced in the protocol *after* the actual MRD measurements, i.e., 6 to 13 weeks after start of treatment.
- The treatment blocks *before* the MRD sampling time points cannot be changed, because this would directly change the prognostic value of the MRD results.
- Preferably at least two early MRD sampling time points should be used, because this results in a more accurate definition of MRD-based risk groups.

MRD-based high-risk and low-risk patients

Protocol Committees will choose for specific MRD-based aims in new protocols, depending on the results of the preceding clinical MRD studies (Table 3). *MRD-based high-risk patients* (with relapse rates of 75 to 100%) might profit from further treatment intensification including stem cell transplantation or new treatment modalities. For example, this was a logical choice for the ongoing MRD-based BFM-AIEOP

and EORTC studies (Figure 1).

MRD-based low-risk patients (with relapse rates 0-2%) represent a group of substantial size in childhood ALL and might profit from treatment de-intensification, e.g., reduction of re-intensification treatment, as aimed for by the current BFM-AIEOP protocol (M. Schrappe, personal communication). It should be emphasized that truly low-risk patients can only be identified if the applied MRD technique is sufficiently sensitive ($\leq 10^{-4}$).

MRD-based intermediate-risk patients

The group of *MRD-based intermediate-risk patients* (relapse rate of 20 to 30%) comprises ALL patients, who are MRD-positive at the end of induction treatment and are MRD-negative or positive at low levels (i.e. $\leq 10^{-4}$) before consolidation treatment.[30,33] This group can have a substantial size and would need further treatment intensification, but this would imply that ~75% of patients in this group are overtreated. The I-BFM-SG has shown that MRD information at later time points (e.g. at one year) has added value for the MRD-based intermediate-risk patients: MRD-positive patients (generally with low MRD levels) have a high chance of relapse (65%), whereas MRD-negative patients have a low chance of relapse (~10%).[30]

The vast majority of relapses (~90%) in the MRD-based intermediate-risk group occur after one year of treatment (see Figure 1A).[30,33] Consequently, the MRD information at one year can potentially be used for treatment modification in MRD-positive intermediate-risk patients only.

MRD-based stratification of relapsed ALL patients

As suggested by the results of BFM study group,[38] there is a subset of relapsed ALL patients with a good molecular response to re-induction chemotherapy. For such patients, BMT might not be required, which should be proven in a prospective treatment trial.

MRD-based stratification of ALL patients undergoing BMT

Based on available data,[39-42] MRD information should play an important role in the clinical BMT setting. It is generally accepted that BMT should be performed within the first half a year of remission, but patients with high MRD levels ($\geq 10^{-3}$) at this time point are at very high risk of relapse post BMT. Future prospective trials should answer the question, whether further pre-transplant tumor load reduction can be achieved with intensified chemotherapy or post-transplant immunotherapy and whether this would improve post-BMT survival.

CHOICE OF MRD TECHNIQUES

The three different MRD techniques differ in their sensitivity and applicability. Consequently, the choice of MRD techniques is dependent on the requirements of the clinical MRD study and vice-versa the applied MRD technique determines the possibilities for MRD-based treatment intervention (see Table 3).

If only high-risk patients need to be identified, GeneScanning of Ig/TCR gene rearrangements and flow cytometric MRD detection are well-suited (cheap and rapid) methods. If the clinical MRD study is focusing on the recognition of high-risk *and* low-risk patients within a specific subgroup of ALL, a sensitive MRD technique can be selected for that specific subgroup. For example, flow cytometry in case of a clinical MRD study in T-ALL or RT-PCR analysis of *BCR-ABL* fusion gene transcripts in case of a clinical MRD study focusing on Ph^+ ALL.

Usage of different MRD techniques for different patients within the same treatment protocol should be avoided, unless full proof is provided that the obtained MRD results are *fully identical*, as has been demonstrated for RQ-PCR analysis of patient-specific Ig/TCR gene rearrangements and *TEL-AML1* transcripts.[48] Small single-center studies showed that results of MRD detection by flow cytometry and quantitative PCR of patient-specific Ig/TCR gene rearrangements are largely comparable.[49,50] However, differences have been found in follow-up samples with low MRD levels (around 10^{-4}), which are essential for recognition of low-risk patients.[49,50] These discrepancies are probably caused by differences in cell sample processing, sensitivity, and expression of MRD results. So, harmonization of these topics is essential before MRD data obtained with different techniques become interchangeable.

Thus far, RQ-PCR detection of patient-specific Ig/TCR gene rearrangements is the most broadly applicable (> 90%) and sufficiently sensitive ($\leq 10^{-4}$) MRD technique in ALL, which has proven to be realizable in multi-center clinical MRD studies.[30,33,38] This probably explains why most European MRD studies in ALL use RQ-PCR analysis of Ig/TCR genes.

CONCLUSIONS

Sensitive and quantitative MRD detection has proven to be clinically relevant in childhood ALL patients. Particularly the evaluation of early treatment response has high prognostic value, because this allows identification of low-risk and high-risk patients, which may profit from treatment reduction or treatment intensification, respectively. Also the pre-BMT MRD status has predictive value for post-BMT relapse-free survival. Comparable results are currently being obtained in adult ALL

MRD studies, albeit that the relative sizes of the MRD-based risk groups differ from those in childhood ALL.

Although the prognostic value of MRD has been established, the reported MRD studies show remarkable differences in MRD-based risk group definition and the corresponding relapse-free survival rates. This might be related to the type of treatment protocol, the timing of the follow-up samples, or the applied MRD technique (particularly its sensitivity). Consequently, the precise prognostic value of MRD information has to be assessed carefully for each treatment protocol *before* MRD-based intervention can be implemented. Furthermore, standardization of MRD techniques and quality control of MRD results are essential for multi-center clinical MRD studies that aim for MRD-based treatment intervention.

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ABBREVIATIONS

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
ASO	allele-specific oligonucleotide
AUL	acute undifferentiated leukemia
BFM-SG	Berlin-Frankfurt-Münster Study Group
BM	bone marrow
BMT	bone marrow transplantation
CB	cord blood
CD	cluster of differentiation
CDR	complementarity-determining region
C gene segment	constant gene segment
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CNS	central nervous system
CSF	cerebro-spinal fluid
Cy	cytoplasmic
DCLSG	Dutch Childhood Leukemia Study Group
D gene segment	diversity gene segment
DGGE	denaturing gradient gel electrophoresis
EBV	Epstein-Barr virus
EFS	event-free survival
FR	framework
FRET	fluorescence resonance energy transfer
Ig	immunoglobulin
IGH	immunoglobulin heavy chain gene
IGK	immunoglobulin kappa light chain gene
IGL	immunoglobulin lambda light chain gene
J gene segment	joining gene segment
Kde	immunoglobulin kappa deleting element
LGL	large granular lymphocytes
MNC	mononuclear cells
MRD	minimal residual disease
NHL	non-Hodgkin's lymphoma
PAGE	polyacrylamide gel electrophoresis
PB	peripheral blood
PCR	polymerase chain reaction
precursor-B-ALL	precursor-B-cell acute lymphoblastic leukemia
RAG	recombination-activating gene

RQ-PCR	real-time quantitative polymerase chain reaction
RSS	recombination signal sequence
RT	reverse transcriptase
SB	Southern blotting
SCT	stem cell transplantation
Sm	surface membrane
SSCP	single-strand conformation polymorphism
T-ALL	T-cell acute lymphoblastic leukemia
TCR	T-cell receptor
TCRA	T-cell receptor alpha gene
TCRB	T-cell receptor beta gene
TCRD	T-cell receptor delta gene
TCRG	T-cell receptor gamma gene
TdT	terminal deoxynucleotidyl transferase
TGGE	temperature gradient gel electrophoresis
V gene segment	variable gene segment

SUMMARY

Acute lymphoblastic leukemia (ALL) represents the most frequent malignancy in childhood. Last decades brought enormous progress in ALL treatment and in the understanding of ALL biology (see Chapter 1.1), but still 20 to 30% of children suffer from relapse and many of them will ultimately die of disease progression. The currently used cytomorphological (microscopic) techniques can only detect 1 to 5% of malignant cells, which is not sufficiently sensitive for identification of patients who are prone to relapse and who might be rescued by treatment intensification. During the past 15 years several approaches have been developed for detection of much lower numbers of malignant cells, i.e. for detection of minimal residual disease (MRD) in various hematopoietic malignancies (see Chapter 1.2). Monitoring of MRD with sensitivities of 10^{-4} to 10^{-6} (i.e. one malignant cell within the background of 10^{4} to 10^{6} normal cells) has significantly higher prognostic value than conventional cytomorphological techniques and other clinical parameters at diagnosis and is therefore currently implemented into clinical practice in several hematopoietic malignancies, including ALL.

In childhood ALL, detection of MRD most frequently relies on patient-specific immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements as molecular markers for PCR studies. The junctional regions of rearranged Ig and TCR genes are unique "*fingerprint-like*" sequences, which are assumed to be different in each lymphoid cell and thus also in each lymphoid malignancy. They can be easily identified and characterized for instance by using heteroduplex PCR analysis (see Chapter 2.2) and direct sequencing.

This thesis aimed at detailed evaluation of Ig and TCR gene rearrangements in ALL with regard to the following aspects:

- characterization of Ig/TCR gene rearrangements patterns in precursor-B-ALL and T-ALL;
- immunobiological differences between malignant and normal lymphoid cells;
- stability of clonal Ig/TCR gene rearrangements at relapse of ALL;
- applicability of Ig/TCR gene rearrangements as PCR targets for detection of MRD.

Virtually all precursor-B-ALL (96%) have rearranged Ig heavy chain (*IGH*) genes. In most cases (80-90%) this concerns complete VH-DH-JH rearrangements on at least one allele. Incomplete DH-JH rearrangements could be identified in 22% of patients, being the sole *IGH* gene rearrangements in only 5% of patients (see Chapter 2.3). Most precursor-B-ALL contain Ig kappa (*IGK*) light chain gene rearrangements (30%) or deletions (50%); 20% of precursor-B-ALL cases even have Ig lambda (*IGL*) gene rearrangements. Deletions in the *IGK* genes are predominantly mediated via the *IGK* deleting element (Kde) sequence. Such Kde rearrangements occur in 50% of precursor-B-ALL cases.

Cross-lineage TCR gene rearrangements occur at high frequency in childhood precursor-B-ALL: TCR beta (*TCRB*), TCR gamma (*TCRG*), and TCR delta (*TCRD*) gene rearrangements and/or deletions were found in 35%, 60%, and 90% of cases, respectively (see Chapter 2.4). Moreover, in approximately 40% of childhood precursor-B-ALL patients Vδ2-Jα gene rearrangements could be identified, which might serve as principal MRD-PCR targets in approximately 25% of precursor-B-ALL (see Chapter 2.5). Interestingly, the occurrence of cross-lineage TCR gene rearrangements seems to be age-dependent. For example, the incidence of incomplete *TCRD* gene rearrangements significantly decreases with the age of the patient, while *TCRG* gene rearrangements are rarely found in patients below 2 years of age. Alltogether, analysis of Ig and TCR gene rearrangement patterns suggests that the Ig and TCR genotype of adult precursor-B-ALL is less mature than in childhood precursor-B-ALL (see Chapter 2.8).

Analysis of the *TCRG* gene configuration in T-ALL showed that TCRγδ$^+$ T-ALL display a less mature *TCRG* immunogenotype as compared to TCRαβ$^+$ and most CD3$^-$ cases. This is reflected by significantly higher usage of the more downstream Vγ genes and the more upstream Jγ1 segments in TCRγδ$^+$ T-ALL (see Chapter 2.6). Despite immunobiological differences between the T-ALL subsets, in virtually all childhood T-ALL (>95%) *TCRG* and/or *TCRD* junctional regions are potentially suitable targets for MRD monitoring. Cross-lineage Ig gene rearrangements occur at relatively low frequency in T-ALL (approximately 20%) and almost exclusively involve *IGH* genes. Interestingly, cross-lineage *IGH* gene rearrangements occur more frequently in CD3$^-$ T-ALL (approximately 20%) and TCRγδ$^+$ T-ALL (approximately 50%) than in TCRαβ$^+$ T-ALL (<5%). Heteroduplex PCR analysis showed a high frequency (approximately 80%) of incomplete DH-JH rearrangements as well as preferential usage of DH6-19 and the most downstream DH7-27 gene segment together with the most upstream JH1 and JH2 gene segments (see Chapter 2.7).

Ig and TCR gene rearrangements in precursor-B-ALL and T-ALL might be prone to continuing rearrangements or secondary rearrangements mediated via the active recombinase enzyme system, resulting in subclone formation in these immature lymphoid malignancies (reviewed in Chapter 2.9). This might lead to loss of Ig/TCR gene rearrangements during the disease course and thereby to false-negative MRD results. Chapter 3 contains several reports on detailed comparative analysis of Ig and TCR gene rearrangements at diagnosis and relapse of precursor-B-ALL and T-ALL. Despite the high frequency of immunogenotypic changes in childhood ALL at relapse, at least one rearranged *IGH*, *IGK*-Kde, *TCRG* and/or *TCRD* allele remained stable in > 90% of precursor-B-ALL and in > 95% of T-ALL. Based on these data, we proposed strategies for MRD-PCR target selection in order to minimize the risk of false-negative results (Chapter 3).

In childhood ALL, MRD monitoring is particularly powerful for evaluation of early response during the first months of treatment and consequently allows identification

of low-risk and high-risk patients, who may profit from treatment reduction or treatment intensification, respectively (see Chapters 5.1 and 5.2). MRD monitoring at later time points might be important for patients at intermediate or high risk of relapse. MRD information is therefore highly valuable in ALL subgroups with increased risk of relapse such as infant ALL (see Chapter 4.2) or t(9;22)-positive ALL. MRD monitoring after relapse can identify patients with a good therapy response, who probably do not need bone marrow transplantation (BMT) but who might be cured with cytotoxic treatment. Also the MRD status before BMT has predictive value for post-BMT relapse-free survival. Moreover, techniques for MRD detection can be further applied for several specific purposes, e.g., detection of central nervous system involvement, assessment of the common clonal origin of two phenotypically different malignancies, early detection of smoldering leukemia in patients with aplastic bone marrow, detection of residual leukemic cells in autologous stem cell grafts, etc. (see Chapters 4.1, 4.3 and 4.4).

In conclusion, Ig and/or TCR gene rearrangements can be identified at diagnosis in more than 95% of patients, comprising at least two MRD targets per patient in 90% of childhood ALL. Moreover, when knowledge about clonal evolution is employed in the process of MRD-PCR target selection, MRD monitoring should enable successful detection of relapse in the majority of patients. Nevertheless, two sensitive ($\leq 10^{-4}$) Ig/TCR targets, required for recognition of low risk patients, are only available in approximately 80% of patients. Thus, future studies should focus on improvement of currently available MRD-PCR targets and introduction of new sensitive MRD markers, such as *TCRB* gene rearrangements in precursor-B-ALL and T-ALL and DNA breakpoint fusion regions of *MLL* gene rearrangements in infant ALL.

The results of our studies contribute to the immunobiological knowledge of Ig and TCR gene rearrangements in ALL, which is essential for performing reliable PCR-based MRD studies in ALL patients. Careful assessment of the prognostic value of MRD information in each treatment protocol will enable MRD-based treatment intervention with treatment intensification and treatment reduction (see Chapter 5.2). The ultimate goal is to improve overall outcome in childhood ALL with concomitant increase of quality of life and reduction of long-term sequelae.

SAMENVATTING

Acute lymfatische leukemie (ALL) is de meest voorkomende maligniteit op de kinderleeftijd. De afgelopen jaren is een enorme vooruitgang geboekt in de behandeling van ALL en in het begrip rondom de biologie van ALL (zie Hoofdstuk 1). Desondanks krijgt 20 à 30% van de kinderen met ALL een recidief, van wie uiteindelijk een groot deel zal overlijden aan de ziekte. Met de huidige cytomorfologische (microscopische) technieken kan slechts 1 tot 5% maligne cellen worden gedetecteerd, wat niet voldoende gevoelig is voor het herkennen van patiënten die een grote kans hebben op het krijgen van een recidief en die mogelijk zouden kunnen worden genezen met een intensievere behandeling. De afgelopen 15 jaar zijn diverse technieken ontwikkeld voor de detectie van zeer kleine aantallen maligne cellen bij patiënten met een hematopoïetische maligniteit, d.w.z. de detectie van "minimale restziekte" ofwel "*minimal residual disease*" (MRD) (zie Hoofdstuk 1.2). Het vervolgen van MRD met gevoeligheden tussen 10^{-4} en 10^{-6} (d.w.z. één maligne cel tussen 10^{4}-10^{6} normale cellen) heeft een zeer grote prognostische waarde vergeleken met conventionele cytomorfologische technieken en andere klinische parameters bij diagnose. Daarom wordt MRD diagnostiek op dit moment geïmplementeerd in therapieprotocollen voor verschillende typen hematopoïetische maligniteiten, waaronder ALL.

Detectie van MRD bij kinderen met ALL is meestal gebaseerd op patiënt-specifieke immunoglobuline (Ig) en T-celreceptor (TCR) genherschikkingen, die worden gebruikt als moleculaire markers voor PCR studies. De "junctional regions" van herschikte Ig en TCR genen zijn unieke sequenties ("DNA vingerafdruk"), waarvan wordt verondersteld dat ze verschillend zijn in elke lymfatische cel en dus ook in elke lymfatische maligniteit. Deze junctional regions kunnen op eenvoudige wijze worden geïdentificeerd en gekarakteriseerd door bijvoorbeeld heteroduplex PCR analyse (zie Hoofdstuk 2.2) en direct sequencen.

Het onderzoek in dit proefschrift was gericht op gedetailleerde analyse van Ig en TCR genherschikkingen bij ALL met speciale aandacht voor:

- karakterisering van de Ig/TCR genherschikkingspatronen bij precursor-B-ALL en T-ALL;
- immunobiologische verschillen tussen maligne en normale lymfatische cellen;
- stabiliteit van Ig/TCR genherschikkingen bij recidieven van ALL;
- toepasbaarheid van Ig/TCR genherschikkingen als PCR targets voor MRD diagnostiek.

Vrijwel alle precursor-B-ALL patiënten (96%) hebben herschikte Ig zware keten (*IGH*) genen in hun leukemiecellen. In de meeste gevallen (80-90%) betreft dit complete VH-DH-JH herschikkingen op tenminste één allel. Incomplete DH-JH herschikkingen konden worden geïdentificeerd in 22% van de leukemieën en waren de enige *IGH* genherschikkingen bij 5% van de leukemieën (zie Hoofdstuk 2.3). De

meeste precursor-B-ALL bevatten Ig kappa (*IGK*) lichte keten genherschikkingen (30%) of deleties (50%); 20% van de precursor-B-ALL hebben zelfs Ig lambda (*IGL*) genherschikkingen. Deleties in de *IGK* genen worden voornamelijk gemedieerd via het *IGK* deletie element (Kde). Dergelijke *IGK*-Kde herschikkingen komen voor bij 50% van de precursor-B-ALL.

"Cross-lineage" TCR genherschikkingen komen zeer frequent voor in kinder precursor-B-ALL: TCR bèta (*TCRB*), TCR gamma (*TCRG*) en TCR delta (*TCRD*) genherschikkingen en/of deleties werden gevonden bij respectievelijk 35%, 60% en 90% van de patiënten (zie Hoofdstuk 2.4). Tevens worden bij ongeveer 40% van de precursor-B-ALL op de kinderleeftijd Vδ2-Jα genherschikkingen geïdentificeerd, die bij ongeveer 25% van de precursor-B-ALL het meest belangrijke MRD-PCR target vormen (zie Hoofdstuk 2.5). Het voorkomen van cross-lineage TCR genherschikkingen lijkt bovendien gerelateerd te zijn aan de leeftijd. De incidentie van incomplete *TCRD* genherschikkingen daalt bijvoorbeeld significant met de leeftijd van de patiënt, terwijl *TCRG* genherschikkingen nauwelijks worden gevonden bij voorloper-B-ALL patiënten jonger dan 2 jaar. Analyse van Ig en TCR genherschikkingspatronen suggereert in alle opzichten dat het Ig en TCR genotype van precursor-B-ALL bij volwassenen minder rijp is dan bij kinder precursor-B-ALL (zie Hoofdstuk 2.8).

Analyse van de *TCRG* genconfiguratie bij T-ALL liet zien dat $TCR\gamma\delta^+$ T-ALL een minder rijp *TCRG* immunogenotype vertonen vergeleken met $TCR\alpha\beta^+$ T-ALL en de meeste $CD3^-$ T-ALL. Dit blijkt uit een significant groter gebruik van de meer downstream gelegen Vγ genen en de meer upstream gelegen Jγ1 segmenten in $TCR\gamma\delta^+$ T-ALL (zie Hoofdstuk 2.6). Ondanks de immunobiologische verschillen tussen de drie T-ALL subgroepen, zijn *TCRG* en/of *TCRD* junctional regions bij vrijwel alle kinder T-ALL patiënten (>95%) bruikbare targets voor MRD monitoring. Cross-lineage Ig genherschikkingen komen in relatief lage frequentie voor bij T-ALL (ongeveer 20%) en betreffen met name *IGH* genen. Interessant is daarbij dat cross-lineage *IGH* genherschikkingen frequenter voorkomen bij $CD3^-$ T-ALL (ongeveer 20%) en $TCR\gamma\delta^+$ T-ALL (ongeveer 50%) dan bij $TCR\alpha\beta^+$ T-ALL (<5%). Heteroduplex PCR analyse toonde een hoge frequentie (ongeveer 80%) van incomplete D_H-J_H herschikkingen aan en tevens een preferentieel gebruik van D_H6-19 en het meest downstream gelegen D_H7-27 gensegment samen met de meest upstream gelegen J_H1 en J_H2 gensegmenten (zie Hoofdstuk 2.7).

Ig en TCR genherschikkingen bij precursor-B-ALL en T-ALL kunnen gevoelig zijn voor doorgaande herschikkingen of secundaire herschikkingen, die gemedieerd worden via het actieve recombinase enzymsysteem en resulteren in het ontstaan van subklonen (samengevat in Hoofdstuk 2.9). Dit kan leiden tot een verlies van Ig/TCR genherschikkingen gedurende het ziekteproces en op deze wijze resulteren in valsnegatieve MRD resultaten. Hoofdstuk 3 bevat verscheidene studies over vergelijkende analyses van Ig en TCR genherschikkingen bij diagnose en recidief van precursor-B-ALL en T-ALL patiënten. Ondanks de hoge frequentie van immunogeno-

typische veranderingen in kinder ALL bij recidief blijft tenminste één herschikt *IGH*, *IGK*-Kde, *TCRG* en/of *TCRD* allel stabiel in >90% van de precursor-B-ALL en >95% van T-ALL. Op basis van deze data hebben we strategieën voorgesteld voor de selectie van MRD-PCR targets om de kans op vals-negative resultaten te minimaliseren (Hoofdstuk 3).

Bij ALL op de kinderleeftijd is MRD monitoring vooral belangrijk voor de evaluatie van de vroege therapierespons tijdens de eerste maanden van de behandeling, waarbij het mogelijk is om laag-risico en hoog-risico groepen te definiëren, die profijt kunnen hebben van respectievelijk een reductie of intensivering van de behandeling (zie Hoofstukken 5.1 en 5.2). MRD monitoring op latere tijdstippen kan belangrijk zijn voor patiënten met een intermediair of hoog risico op het krijgen van een recidief. MRD onderzoek blijkt bijzonder waardevol te zijn bij hoog-risico ALL subgroepen, zoals ALL bij zuigelingen (zie Hoofdstuk 4.2) en t(9;22)-positieve ALL. MRD monitoring bij kinderen met een recidief kan patiënten herkennen met een hoge gevoeligheid voor medicijnen, die waarschijnlijk geen beenmergtransplantatie (BMT) nodig hebben, maar kunnen worden genezen met een cytotoxische behandeling. Daarnaast heeft de MRD status vóór BMT voorspellende waarde voor de recidief-vrije overleving na BMT. Tenslotte kunnen MRD technieken worden toegepast voor verschillende specifieke doeleinden, zoals aantonen van uitbreiding naar het centrale zenuwstelsel, vaststellen of uitsluiten van de gemeenschappelijke klonale afkomst van twee fenotypisch verschillende maligniteiten, vroege detectie van sluimerende leukemie bij patiënten met aplastisch beenmerg, detectie van resterende leukemiecellen in autologe stamceltransplantaten, etc. (zie Hoofdstukken 4.1, 4.3 en 4.4).

Concluderend kan worden gesteld dat Ig en/of TCR genherschikkingen bij diagnose kunnen worden geïdentificeerd bij meer dan 95% van de patiënten, waarbij in 90% van de kinder ALL tenminste twee MRD targets per patiënt worden gevonden. Indien bovendien kennis over klonale evolutie wordt gebruikt tijdens het proces van MRD-PCR target selectie, zal MRD monitoring de succesvolle detectie van recidivering mogelijk maken in de meerderheid van de patiënten. Desondanks zijn twee gevoelige ($\leq 10^{-4}$) Ig/TCR targets, die noodzakelijk zijn voor de herkenning van laag-risico patiënten, slechts beschikbaar in ongeveer 80% van de patiënten. Daarom zouden vervolgstudies gericht moeten zijn op het verbeteren van de huidig beschikbare MRD-PCR targets en de introductie van nieuwe gevoelige MRD-PCR targets, zoals *TCRB* genherschikkingen in precursor-B-ALL en T-ALL en DNA breukpunt fusiegen gebieden van *MLL* genherschikkingen in ALL bij zuigelingen.

De resultaten van onze studies dragen bij aan de immunobiologische kennis van Ig en TCR genherschikkingen in ALL, die essentieel is voor het uitvoeren van betrouwbare PCR-gebaseerde MRD studies bij ALL patiënten. Zorgvuldige beoordeling van de prognostische waarde van MRD informatie voor ieder behandelingsprotocol zal MRD-gebaseerde behandelingsinterventie mogelijk maken met

zowel intensivering als reductie van behandeling (zie Hoofdstuk 5.2). Het uiteindelijke doel is om de kans op genezing van kinderen met ALL te verhogen, parallel aan een verbetering van de kwaliteit van leven en een reductie van de lange termijn effecten.

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Seven years ago, in September 1995, I started my scientific adventure at the Department of Immunology of the Erasmus University in Rotterdam. At that time, being a freshly graduated physician without any scientific experience, I did not expect that this would result in a Ph. D. thesis even in my wildest dreams. What happened afterwards, since these early days until the day of the promotion, was only possible thanks to the numerous people to whom I would like to dedicate this book.

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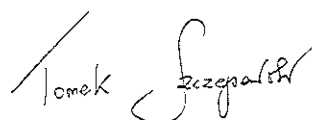

CURRICULUM VITAE

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Education:

1984-1988	High school: I[st] Secondary School in Zabrze
1988-1994	Medical Studies at the Silesian Academy of Medicine in Katowice
April 1991-June 1991	Clinical Training: Department of Pediatrics, Gothenburg's University, Göteborg (Sweden) Supervisor: Prof. Dr. I. Kjellmer Subject: *General Pediatrics for Medical Students*
August-September 1993	Research Training: Department of Immunology, Gothenburg's University, Göteborg (Sweden) Supervisor: Prof. Dr. L. Å. Hanson Subject: *Clinical Immunology*
June 1994	M.D. exam (cum laude) at the Silesian Academy of Medicine in Katowice
July-August 1994	Clinical and Research fellowship: Academic Medical Centrum /University of Amsterdam and Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam (The Netherlands) Supervisors: Dr. H. Behrendt and Dr. C.E. van der Schoot Subject: *Diagnostics of Pediatric Leukemia*
October 1994 - September 1995	Clinical fellowships: Departments of Internal Medicine, Pediatrics, Pediatric Surgery, Gynecology, and Obstetrics; 1st Clinical Hospital of the Silesian Academy of Medicine in Zabrze, Poland Supervisors: Prof. Dr. E. Rogala, Prof. Dr. D. Sońta-Jakimczyk, Prof. Dr. J. Dzielicki, and Prof. Dr. J. Dudkiewicz.
June 1995	Cambridge Certificate in Advanced English

October 1995 - July 1996	Research fellowship: Department of Immunology, Erasmus University Rotterdam, Rotterdam, The Netherlands (Ambassadorial Scholarship of Rotary International) Supervisors: Prof. Dr. J.J.M. van Dongen and Dr. A.W. Langerak. Subject: *Molecular analysis of immunoglobulin and T-cell receptor genes in lymphoid malignancies*
August 1996 - up to now	Clinical fellowship: Department of Pediatric Hematology and Chemotherapy, Silesian Academy of Medicine in Zabrze, Poland Supervisor: Prof. Dr. D. Sońta-Jakimczyk.
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April 2001	1st degree specialization certificate in pediatrics (Zabrze, Poland).
October 1997 - up to now	Ph. D. Research: Department of Immunology, Erasmus University, Rotterdam, The Netherlands Supervisor: Prof. Dr. J.J.M. van Dongen Subject: *Detection of minimal residual disease in acute lymphoblastic leukemia*

LIST OF PUBLICATIONS

International Publications

1. Szczepański T, Sońta-Jakimczyk D, Janik-Moszant A, Olejnik I. Generalized lymphadenopathy as initial presentation of toxocariasis in a 7-year-old boy. *Pediatr Inf Dis J* 1996; 15: 717-718.
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