RAPID COMMUNICATION

Ig Heavy Chain Gene Rearrangements in T-Cell Acute Lymphoblastic Leukemia Exhibit Predominant DH6-19 and DH7-27 Gene Usage, Can Result in Complete V-D-J Rearrangements, and Are Rare in T-Cell Receptor αβ Lineage

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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 TCRγδ$^+$ T-ALL (12/24), whereas only a single TCRαβ$^+$ 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, ie, 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 showed 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 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-TCRαβ$^+$) T-ALL as a result of continuing V(D)J recombinase activity. More mature αβ-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.

WHEN REARRANGED 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 (Fig 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 rear-

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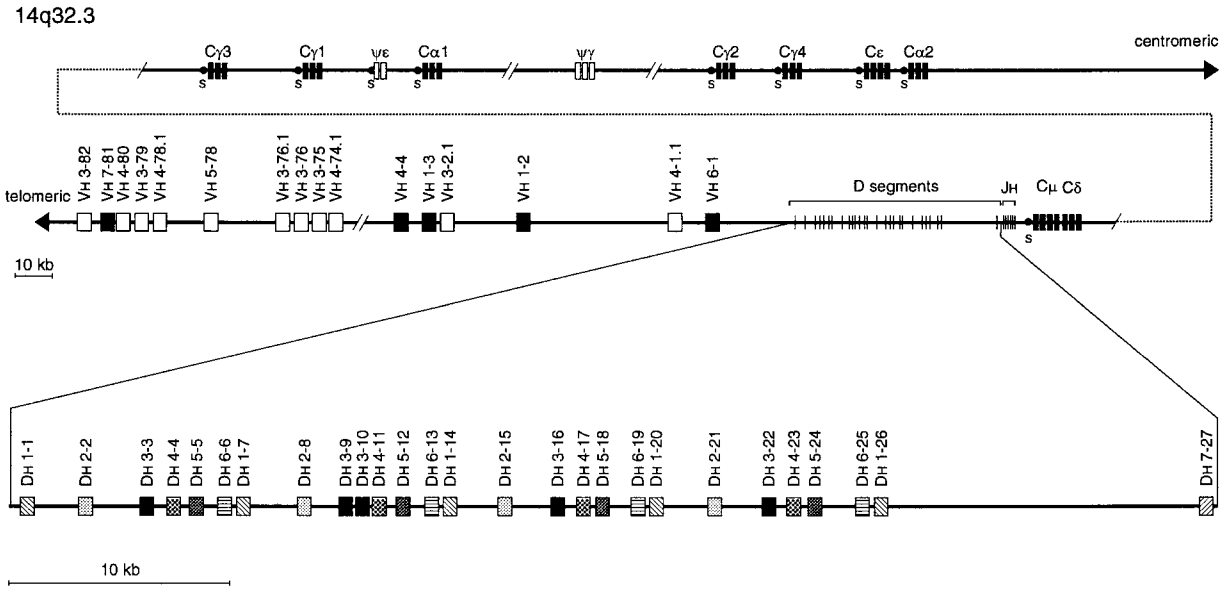

Fig 1. Schematic representation of the human *IGH* locus on chromosome 14q32.3. The *IGH* gene complex consists of numerous (>120) V gene segments, 27 D gene segments, 6 functional J gene segments, and C gene segments for the constant domains of the various IgH classes and subclasses, most of which are preceded by switch sequences (s).[23,28,52,53] Pseudogenes are represented as open bars. The 27 DH gene segments are grouped in 7 families based on sequence homology. Members of the same DH family are depicted with the same shading pattern.

ranged *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.

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/mL; 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 2 $CD3^+$ 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 Corp, 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 of 118 patients with the TCRDV2, TCRDD2, TCRDJ1, TCRDJ2, TCRDJ3, TCRDRE, and TCRAPJ probes (DAKO Corp) 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 50 μL 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 Fig 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

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*	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

*The position of the 5′ end of the primer is indicated upstream (−) relative to the 3′ RSS of the involved DH gene segment.

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× Tris-boric acid-EDTA (TBE) buffer, run at room temperature, and visualized by ethidium bromide staining.[25] A 100-bp DNA ladder (Promega Corp, 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, ie, 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 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 to 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, 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 necessary 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 the different age groups, ie, 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 showed 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 TCRγδ$^+$ T-ALL (12/24), whereas only a single TCRαβ$^+$ T-ALL (4% [1/25]) displayed a rearranged *IGH* gene on one allele.

IGH gene rearrangements coincide with TCRD gene rearrangements. Because CD3$^-$ T-ALL theoretically represent precursor stages of both TCRαβ$^+$ and TCRγδ$^+$ 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.[15,18] 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 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 αβ-lineage T-ALL, ie, either TCRαβ$^+$ 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 showed a total of 39 clonal homoduplexes, reflecting 7 (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 TCRγδ$^+$ T-ALL patients, with 1 TCRγδ$^+$ T-ALL showing biallelic VH-(DH-)JH joinings. Heteroduplex PCR analysis for incomplete DH7-27-JH rearrangements is shown in Fig 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

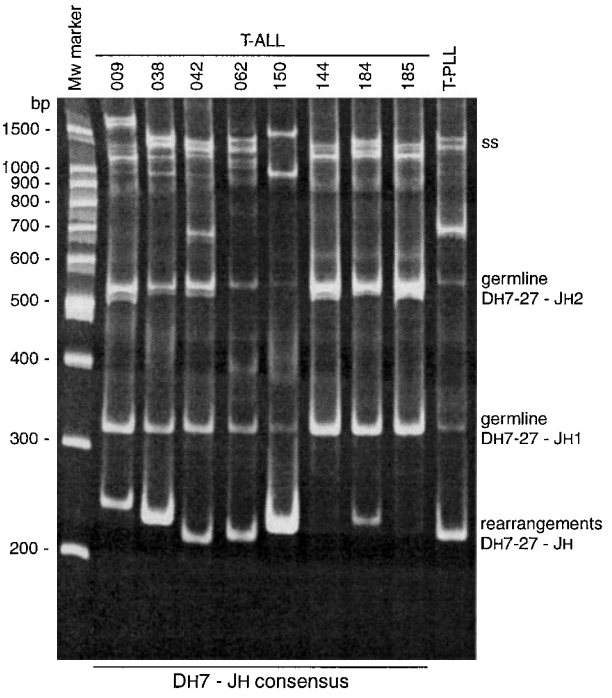

Fig 2. Heteroduplex PCR analysis of DH7-27-JH cross-lineage rearrangements in T-ALL. Subsequent to agarose gel electrophoresis, samples containing PCR products were subjected to heteroduplex PCR analysis, separated in a 6% polyacrylamide gel, and visualized by ethidium bromide staining. Based on the 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 tumor load (ie, 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.

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 2. Usage of Different DH Families in (In)complete Cross-Lineage *IGH* Gene Rearrangements in T-ALL

New Family Name*	Old Family Name*	No. (frequency) of Rearrangements	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

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

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

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*		DH6-19		N- and P-Nucleotides*		JH Gene Segments
1.	—	—		GGGTATAGCAGTGGCTGGT	(−2)	TTTTCTTCTA	(−6)	JH6c
2.	VH1-03 (0)	TAGT	(−2)	GTATAGCAGTGGCTGGT	(−2)	TTTTCTTCTA	(−6)	JH6c
3.	VH3-33 (−1)	TAAGGGTGTGATGTGTTTTGTGGA	(−11)	TGGCTGGTAC	(0)	GAGGCTGGCAGGGGGA	(−14)	JH6b

*Underlined nucleotides represent P-nucleotides.

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 (Fig 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] Because 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 showed 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]

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

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%

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.

Because 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.[18]

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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REFERENCES

1. Tonegawa S: Somatic generation of antibody diversity. Nature 302:575, 1983

2. Van Dongen JJM, Wolvers-Tettero ILM: Analysis of immunoglobulin and T cell receptor genes. Part I: Basic and technical aspects. Clin Chim Acta 198:1, 1991

3. Greaves MF, Chan LC, Furley AJ, Watt SM, Molgaard HV: Lineage promiscuity in hemopoietic differentiation and leukemia. Blood 67:1, 1986

4. Dyer MJ: T-cell receptor delta/alpha rearrangements in lymphoid neoplasms. Blood 74:1073, 1989

5. Felix CA, Poplack DG: Characterization of acute lymphoblastic leukemia of childhood by immunoglobulin and T-cell receptor gene patterns. Leukemia 5:1015, 1991

6. Hara J, Benedict SH, Champagne E, Takihara Y, Mak TW, Minden M, Gelfand EW: T cell receptor delta gene rearrangements in acute lymphoblastic leukemia. J Clin Invest 82:1974, 1988

7. Szczepański T, Langerak AW, Wolvers-Tettero ILM, Ossenkoppele GJ, Verhoef G, Stul M, Petersen EJ, de Bruijn MAC, van't Veer MB, van Dongen JJM: Immunoglobulin and T cell receptor gene rearrangement patterns in acute lymphoblastic leukemia are less mature in adults than in children: Implications for selection of PCR targets for detection of minimal residual disease. Leukemia 12:1081, 1998

8. Szczepański T, Beishuizen A, Pongers-Willemse MJ, Hählen K, van Wering ER, Wijkhuijs JM, Tibbe GJM, De Bruijn MAC, van Dongen JJM: Cross-lineage T-cell receptor gene rearrangements occur in more than ninety percent of childhood precursor-B-acute lymphoblastic leukemias: Alternative PCR targets for detection of minimal residual disease. Leukemia 13:196, 1999

9. Nuss R, Kitchingman GR, Cross A, Zipf TF, Antoun GR, Bernstein I, Behm F, Pullen DJ, Crist W, Mirro J Jr, Goorha R: T cell receptor gene rearrangements in B-precursor acute lymphoblastic leukemia correlate with age and the stage of B cell differentiation. Leukemia 2:722, 1988

10. Biondi A, Francia di Celle P, Rossi V, Casorati G, Matullo G, Giudici G, Foa R, Migone N: High prevalence of T-cell receptor V delta 2-(D)-D delta 3 or D delta 1/2-D delta 3 rearrangements in B-precursor acute lymphoblastic leukemias. Blood 75:1834, 1990

11. Van Dongen JJM, Wolvers-Tettero ILM: Analysis of immunoglobulin and T cell receptor genes. Part II: Possibilities and limitations in the diagnosis and management of lymphoproliferative diseases and related disorders. Clin Chim Acta 198:93, 1991

12. Kitchingman GR, Rovigatti U, Mauer AM, Melvin S, Murphy SB, Stass S: Rearrangement of immunoglobulin heavy chain genes in T cell acute lymphoblastic leukemia. Blood 65:725, 1985

13. Mizutani S, Ford AM, Wiedemann LM, Chan LC, Furley AJ, Greaves MF, Molgaard HV: Rearrangement of immunoglobulin heavy chain genes in human T leukaemic cells shows preferential utilization of the D segment (DQ52) nearest to the J region. EMBO J 5:3467, 1986

14. Ozaki M, Asada M, Tamura C, Zhong WK, Nakamura K, Fujimoto J, Miyashita T, Kobayashi N, Mizutani S: Molecular analysis of 5′ J region of immunoglobulin heavy chain gene in human acute leukemias. Leukemia 4:415, 1990

15. Breit TM, Wolvers-Tettero ILM, Beishuizen A, Verhoeven M-AJ, van Wering ER, van Dongen JJM: Southern blot patterns, frequencies and junctional diversity of T-cell receptor δ gene rearrangements in acute lymphoblastic leukemia. Blood 82:3063, 1993

16. Van Dongen JJ, Adriaansen HJ, Hooijkaas H: Immunophenotyping of leukaemias and non-Hodgkin's lymphomas. Immunological markers and their CD codes. Neth J Med 33:298, 1988

17. Groeneveld K, te Marvelde JG, van den Beemd MW, Hooijkaas H, van Dongen JJM: Flow cytometric detection of intracellular antigens for immunophenotyping of normal and malignant leukocytes. Leukemia 10:1383, 1996

18. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J: Development of human T lymphocytes and their thymus-dependency. Thymus 16:207, 1990

19. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual (ed 2). Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1989

20. Beishuizen A, Verhoeven MA, Mol EJ, Breit TM, Wolvers-Tettero ILM, van Dongen JJM: Detection of immunoglobulin heavy-chain gene rearrangements by Southern blot analysis: Recommendations for optimal results. Leukemia 7:2045, 1993

21. Aubin J, Davi F, Nguyen-Salomon F, Leboeuf D, Debert C, Taher M, Valensi F, Canioni D, Brousse N, Varet B, Flandrin G,

Macintyre EA: Description of a novel FR1 IgH PCR strategy and its comparison with three other strategies for the detection of clonality in B cell malignancies. Leukemia 9:471, 1995

22. Küppers R, Zhao M, Rajewsky K, Hansmann ML: Detection of clonal B cell populations in paraffin-embedded tissues by polymerase chain reaction. Am J Pathol 143:230, 1993

23. Corbett SJ, Tomlinson IM, Sonnhammer ELL, Buck D, Winter G: Sequence of the human immunoglobulin diversity (D) segment locus: A systematic analysis provides no evidence for the use of DIR segments, inverted D segments, ''minor'' D segments or D-D recombination. J Mol Biol 270:587, 1997

24. Pongers-Willemse MJ, Seriu T, Stolz F, d'Aniello E, Gameiro P, Pisa P, Gonzalez M, Bartram CR, Panzer-Grumayer ER, Biondi A, San Miguel JF, van Dongen JJM: Primers and protocols for standardized MRD detection in ALL using immunoglobulin and T cell receptor gene rearrangements and *TAL1* deletions as PCR targets. Report of the BIOMED-1 Concerted Action: Investigation of minimal residual disease in acute leukemia. Leukemia 13:110, 1999

25. Langerak AW, Szczepański T, van der Burg M, Wolvers-Tettero ILM, van Dongen JJM: Heteroduplex PCR analysis of rearranged T cell receptor genes for clonality assessment in suspect T cell proliferations. Leukemia 11:2192, 1997

26. Beishuizen A, de Bruijn MAC, Pongers-Willemse MJ, Verhoeven M-AJ, van Wering ER, Hählen K, Breit TM, de Bruin-Versteeg S, Hooijkaas H, van Dongen JJM: Heterogeneity in junctional regions of immunoglobulin kappa deleting element rearrangements in B cell leukemias: A new molecular target for detection of minimal residual disease. Leukemia 11:2200, 1997

27. Ghali DW, Panzer S, Fischer S, Argyriou-Tirita A, Haas OA, Kovar H, Gadner H, Panzer-Grumayer ER: Heterogeneity of the T-cell receptor delta gene indicating subclone formation in acute precursor B-cell leukemias. Blood 85:2795, 1995

28. Cook GP, Tomlinson IM: The human immunoglobulin VH repertoire. Immunol Today 16:237, 1995

29. Lafaille JJ, DeCloux A, Bonneville M, Takagaki Y, Tonegawa S: Junctional sequences of T cell receptor gamma delta genes: Implications for gamma delta T cell lineages and for a novel intermediate of V-(D)-J joining. Cell 59:859, 1989

30. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 25:3389, 1997

31. Schroeder HW Jr, Mortari F, Shiokawa S, Kirkham PM, Elgavish RA, Bertrand FE 3rd: Developmental regulation of the human antibody repertoire. Ann NY Acad Sci 764:242, 1995

32. Rosenquist R, Lindh J, Roos G, Holmberg D: Immunoglobulin VH gene replacements in a T-cell lymphoblastic lymphoma. Mol Immunol 34:305, 1997

33. Schroeder HW Jr, Wang JY: Preferential utilization of conserved immunoglobulin heavy chain variable gene segments during human fetal life. Proc Natl Acad Sci USA 87:6146, 1990

34. LeBien TW, Elstrom RL, Moseley M, Kersey JH, Griesinger F: Analysis of immunoglobulin and T-cell receptor gene rearrangements in human fetal bone marrow B lineage cells. Blood 76:1196, 1990

35. Yamada M, Wasserman R, Reichard BA, Shane S, Caton AJ, Rovera G: Preferential utilization of specific immunoglobulin heavy chain diversity and joining segments in adult human peripheral blood B lymphocytes. J Exp Med 173:395, 1991

36. Raaphorst FM, Raman CS, Tami J, Fischbach M, Sanz I: Human Ig heavy chain CDR3 regions in adult bone marrow pre-B cells display an adult phenotype of diversity: Evidence for structural selection of DH amino acid sequences. Int Immunol 9:1503, 1997

37. Steenbergen EJ, Verhagen OJ, van Leeuwen EF, Behrendt H, Merle PA, Wester MR, von dem Borne AE, van der Schoot CE: B precursor acute lymphoblastic leukemia third complementarity-determining regions predominantly represent an unbiased recombination repertoire: Leukemic transformation frequently occurs in fetal life. Eur J Immunol 24:900, 1994

38. Kitchingman GR: Immunoglobulin heavy chain gene VH-D junctional diversity at diagnosis in patients with acute lymphoblastic leukemia. Blood 81:775, 1993

39. Choi Y, Greenberg SJ, Du TL, Ward PM, Overturf PM, Brecher ML, Ballow M: Clonal evolution in B-lineage acute lymphoblastic leukemia by contemporaneous VH-VH gene replacements and VH-DJH gene rearrangements. Blood 87:2506, 1996

40. Steenbergen EJ, Verhagen OJ, van den Berg H, van Leeuwen EF, Behrendt H, Slater RR, von dem Borne AE, van der Schoot CE: Rearrangement status of the malignant cell determines type of secondary IgH rearrangement (V-replacement or V to DJ joining) in childhood B precursor acute lymphoblastic leukemia. Leukemia 11:1258, 1997

41. Beishuizen A, Verhoeven MA, van Wering ER, Hählen K, Hooijkaas H, van Dongen JJM: Analysis of Ig and T-cell receptor genes in 40 childhood acute lymphoblastic leukemias at diagnosis and subsequent relapse: Implications for the detection of minimal residual disease by polymerase chain reaction analysis. Blood 83:2238, 1994

42. Van Dongen JJM, Seriu T, Panzer-Grumayer ER, Biondi A, Pongers-Willemse MJ, Corral L, Stolz F, Schrappe M, Masera G, Kamps WA, Gadner H, van Wering ER, Ludwig W-D, Basso G, de Bruijn MAC, Cazzaniga G, Hettinger K, van der Does-van den Berg A, Hop WCJ, Riehm H, Bartram CR: Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. Lancet 352: 1731, 1998

43. Adriaansen HJ, Soeting PW, Wolvers-Tettero ILM, van Dongen JJM: Immunoglobulin and T-cell receptor gene rearrangements in acute non-lymphocytic leukemias. Analysis of 54 cases and a review of the literature. Leukemia 5:744, 1991

44. Bertrand FE III, Billips LG, Burrows PD, Gartland GL, Kubagawa H, Schroeder HW Jr: Ig D(H) gene segment transcription and rearrangement before surface expression of the pan-B-cell marker CD19 in normal human bone marrow. Blood 90:736, 1997

45. Davi F, Faili A, Gritti C, Blanc C, Laurent C, Sutton L, Schmitt C, Merle-Beral H: Early onset of immunoglobulin heavy chain gene rearrangements in normal human bone marrow $CD34^+$ cells. Blood 90:4014, 1997

46. Ghia P, ten Boekel E, Sanz E, de la Hera A, Rolink A, Melchers F: Ordering of human bone marrow B lymphocyte precursors by single-cell polymerase chain reaction analyses of the rearrangement status of the immunoglobulin H and L chain gene loci. J Exp Med 184:2217, 1996

47. Born W, White J, Kappler J, Marrack P: Rearrangement of IgH genes in normal thymocyte development. J Immunol 140:3228, 1988

48. Bertrand FE III, Billips LG, Gartland GL, Kubagawa H, Schroeder HW, Jr: The J chain gene is transcribed during B and T lymphopoiesis in humans. J Immunol 156:4240, 1996

49. Yancopoulos GD, Blackwell TK, Suh H, Hood L, Alt FW: Introduced T cell receptor variable region gene segments recombine in pre-B cells: Evidence that B and T cells use a common recombinase. Cell 44:251, 1986

50. Breit TM, Verschuren MCM, Wolvers-Tettero ILM, van Gastel-Mol EJ, Hählen K, van Dongen JJM: Human T cell leukemias with continuous V(D)J recombinase activity for TCR-delta gene deletion. J Immunol 159:4341, 1997

51. Ichihara Y, Matsuoka H, Kurosawa Y: Organization of human immunoglobulin heavy chain diversity gene loci. EMBO J 7:4141, 1988

52. Matsuda F, Ishii K, Bourvagnet P, Kuma K, Hayashida H, Miyata T, Honjo T: The complete nucleotide sequence of the human immunoglobulin heavy chain variable region locus. J Exp Med 188:2151, 1998

53. Lefranc MP, Giudicelli V, Ginestoux C, Bodmer J, Müller W, Bontrop R, Lemaitre M, Malik A, Barbi V, Chaume D: IMGT, the international ImMunoGeneTics database. Nucleic Acids Res 27:209, 1999