

# V $\delta$ 2-J $\alpha$ rearrangements are frequent in precursor-B–acute lymphoblastic leukemia but rare in normal lymphoid cells

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The frequently occurring T-cell receptor delta (*TCRD*) deletions in precursor-B–acute lymphoblastic leukemia (precursor-B–ALL) are assumed to be mainly caused by V $\delta$ 2-J $\alpha$  rearrangements. We designed a multiplex polymerase chain reaction (PCR) assay with 61 J $\alpha$  primers and identified clonal V $\delta$ 2-J $\alpha$  rearrangements in 141 of 339 (41%) childhood and 8 of 22 (36%) adult precursor-B–ALL. A significant proportion (44%) of V $\delta$ 2-J $\alpha$  rearrangements in childhood precursor-B–ALL were oligoclonal. Sequence analysis

showed preferential usage of the J $\alpha$ 29 gene segment in 54% of rearrangements. The remaining V $\delta$ 2-J $\alpha$  rearrangements used 26 other J $\alpha$  segments, which included 2 additional clusters, one involving the most upstream J $\alpha$  segments (ie, J $\alpha$ 48 to J $\alpha$ 61; 23%) and the second cluster located around the J $\alpha$ 9 gene segment (7%). Real-time quantitative PCR studies of normal lymphoid cells showed that V $\delta$ 2 rearrangements to upstream J $\alpha$  segments occurred at low levels in the thymus ( $10^{-2}$  to  $10^{-3}$ ) and were rare (gener-

ally below  $10^{-3}$ ) in B-cell precursors and mature T cells. V $\delta$ 2-J $\alpha$ 29 rearrangements were virtually absent in normal lymphoid cells. The monoclonal V $\delta$ 2-J $\alpha$  rearrangements in precursor-B–ALL may serve as patient-specific targets for detection of minimal residual disease, because they show high sensitivity ( $10^{-4}$  or less in most cases) and good stability (88% of rearrangements preserved at relapse). (Blood. 2004;103:3798-3804)

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## Introduction

Rearrangements of T-cell receptor (TCR) delta (*TCRD*) genes represent one of the earliest events in normal T-cell development.<sup>1-3</sup> However, recombinations in *TCRD* genes are not fully restricted to the T-cell lineage. The presence of cross-lineage *TCRD* gene rearrangements is a frequent phenomenon both in childhood and adult precursor-B–acute lymphoblastic leukemia (precursor-B–ALL).<sup>4-6</sup> Nevertheless, the spectrum of *TCRD* gene rearrangements in precursor-B–ALL is very limited, with 80% of detected rearrangements representing incomplete V $\delta$ 2-D $\delta$ 3 or D $\delta$ 2-D $\delta$ 3 joinings.<sup>5,7,8</sup> Similarly, only D $\delta$ 2-D $\delta$ 3 and V $\delta$ 2-D $\delta$ 3 joinings can be found in normal B-cell precursors or even in mature B cells.<sup>9,10</sup> Moreover, exactly the same types of incomplete *TCRD* gene rearrangements can be induced in nonlymphoid tissues transfected in vitro with basic helix-loop-helix transcription factors.<sup>11</sup> Interestingly, V $\delta$ 2-D $\delta$ 3 rearrangements in precursor-B–ALL are prone to continuing rearrangements, particularly to J $\alpha$  gene segments with concomitant deletion of the C $\delta$  exons and subsequent V $\alpha$ -J $\alpha$  recombination (Figure 1A).<sup>4,5,9,12-14</sup> 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 V $\delta$ 2-J $\alpha$  rearrangements.<sup>5</sup> Limited, mainly qualitative data indicate that V $\delta$ 2-J $\alpha$  rearrangements are infrequent in normal lymphoid tissues.<sup>15,16</sup> Other immunobiologic characteristics of V $\delta$ 2-J $\alpha$  rearrangements in normal and malignant lymphoid cells are largely unknown.

We developed a multiplex polymerase chain reaction (PCR) strategy for easy identification and characterization of clonal V $\delta$ 2-J $\alpha$  gene rearrangements in a large series (n = 361) of precursor-B–ALL. Subsequently, we investigated the presence of the most frequent V $\delta$ 2-J $\alpha$  rearrangements in various types of normal lymphoid tissues. Finally, we evaluated the sensitivity and stability of V $\delta$ 2-J $\alpha$  rearrangements as real-time quantitative (RQ)-PCR targets for detection of minimal residual disease (MRD).<sup>17</sup>

## Patients, materials, and methods

### Patients

Bone marrow (BM) or peripheral blood (PB) samples from 339 children with precursor-B–ALL were obtained at initial diagnosis (age range, 1.5 months to 15.9 years). Immunologic marker analysis revealed 12 pro-B-ALL, 226 common ALL, and 101 pre-B–ALL.<sup>18</sup>

In addition, diagnosis samples from 22 adult precursor-B–ALL were analyzed. The clinical, immunophenotypic, and immunogenotypic characteristics of these adult patients were reported previously.<sup>6</sup>

Patient samples were obtained after informed consent according to the guidelines of the Medical Ethics Committee of the Erasmus MC, Rotterdam.

### Southern blot analysis

Mononuclear cells (MNCs) were isolated from BM or PB samples by Ficoll-Paque centrifugation (density, 1.077 g/cm<sup>3</sup>; Pharmacia, Uppsala,

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Sweden). DNA was isolated from fresh or frozen MNC fractions as described previously.<sup>19,20</sup> 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 & Schuell, Dassel, Germany) as described.<sup>19</sup> The configuration of the *TCRD* genes was analyzed with the TCRDJ1 and TCRDV2 probes (DAKO, Carpinteria, CA) in *Bgl*II, *Eco*RI, or *Hind*III digests.<sup>8</sup> Southern blot analysis was successfully performed in 208 precursor-B-ALL.

### Primer design and heteroduplex PCR analysis

V $\delta$ 2 and D $\delta$ 2 primers have been developed by the BIOMED-2 Concerted Action BMH4-CT98-3936 "PCR-based clonality studies for early diagnosis of lymphoproliferative disorders."<sup>21</sup> Based on the available nucleotide sequence of the human 3' terminal end of the *TCRA/D* locus (European Molecular Biology Laboratory [EMBL] accession no. M94081),<sup>22</sup> 61 J $\alpha$  primers compatible with the V $\delta$ 2 primer were designed using OLIGO 6.0 software (developed by Dr W. Rychlik; Molecular Biology Insights, Cascade, CO) and applying previously described guidelines.<sup>23</sup> The sequences of the primers and the composition of 7 V $\delta$ 2-J $\alpha$  multiplex PCR tubes are available upon request.

The multiplex V $\delta$ 2-J $\alpha$  PCR analyses were performed in all 339 patients, essentially as described previously.<sup>6,23</sup> In each 50  $\mu$ L PCR reaction, 100 ng DNA sample, 10 pmol of the 5' and 3' oligonucleotide primers, and 1 unit AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) were used. PCR conditions were initial denaturation for 10 minutes at 94°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 (Applied 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.<sup>23</sup> Heteroduplex analysis of PCR products was performed as described previously.<sup>24</sup>

The presence of clonal V $\delta$ 2-D $\delta$ 3 and D $\delta$ 2-D $\delta$ 3 gene rearrangements was tested using our classical monoplex approach.<sup>23</sup> Multiplex D $\delta$ 2-J $\alpha$  PCR was performed in 11 patients, preselected based on Southern blot and PCR information (ie, germline V $\delta$ 2 allele with deleted D $\delta$ 3/J $\delta$ 1 area and absence of clonal V $\delta$ 2-J $\alpha$  rearrangements).

### Comparative heteroduplex analysis of PCR products

Comparative heteroduplex analysis of V $\delta$ 2-J $\alpha$  PCR products at diagnosis and relapse concerned 43 of 91 relapsed precursor-B-ALL patients,<sup>25</sup> selected for the presence of V $\delta$ 2-J $\alpha$  rearrangements at diagnosis. The relapse samples were first analyzed in a monoplex PCR with those primer combinations that 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 mixed heteroduplex analysis—that is, mixing of the diagnosis and relapse PCR products followed by heteroduplex analysis.<sup>25,26</sup> When clonal PCR products found at diagnosis were undetectable at relapse, the relapse sample was analyzed with all 7 V $\delta$ 2-J $\alpha$  multiplex tubes.

### Sequence analysis of V $\delta$ 2-J $\alpha$ rearrangements

Direct sequencing of V $\delta$ 2-J $\alpha$  rearrangements was performed with the V $\delta$ 2 primer using the dye-terminator cycle sequencing kit with AmpliTaq DNA polymerase FS on an ABI 377 sequencer (Applied Biosystems) as previously described.<sup>27</sup> When heteroduplex PCR analysis revealed more than 2 clonal bands (ie, 2 homoduplexes or an additional upper band resulting from extension to a downstream J $\alpha$  segment), the bands were excised from the polyacrylamide gel, eluted, and directly sequenced as described before.<sup>28</sup> Recognition of D $\delta$ 2 and D $\delta$ 3 segments in V $\delta$ 2-J $\alpha$  junctional regions required at least 4 and 5 consecutive matching nucleotides, respectively.<sup>29</sup>

### RQ-PCR detection of V $\delta$ 2-J $\alpha$ rearrangements in normal tissue samples

Normal tissue samples tested for the presence of V $\delta$ 2-J $\alpha$  rearrangements included normal PB, E-rosette-positive PB cells (T cells), E-rosette-

negative PB cells (B cells, natural killer [NK] cells, and monocytes), normal BM, sorted BM B cells and B-cell precursors, tonsils, lymph nodes, thymuses, and postchemotherapy regenerating BM samples, which are known to contain high frequencies of normal precursor-B-cells.<sup>30,31</sup> Whenever possible, at least 2 different samples were tested per category, each sample in triplicate. To analyze the presence of V $\delta$ 2-J $\alpha$  gene rearrangements in normal tissue samples, the germline V $\delta$ 2 TaqMan probe (5'-AGACCCTTCATCTCTCTCTGATGGTGCAAGTA-3') and the germline V $\delta$ 2 forward primer (5'-TGCAAAGAACCCTGGCTGTACTTAA-3') were used together with a germline reverse J $\alpha$  primer. Based on the frequencies of particular V $\delta$ 2-J $\alpha$  gene rearrangements in precursor-B-ALL ("Results"), J $\alpha$ 9, J $\alpha$ 29, J $\alpha$ 58, and J $\alpha$ 61 primers were selected for analysis in normal lymphoid cells. To determine the efficiency of amplification and sensitivity of the RQ-PCR, diagnostic DNA from precursor-B-ALL containing the same V $\delta$ 2-J $\alpha$  gene rearrangements was 10-fold serially diluted (10<sup>-1</sup> down to 10<sup>-6</sup>) into DNA from the cell line CEM, known to have 2 deleted *TCRD* alleles. To correct for the quantity and quality (amplifiability) of DNA, RQ-PCR analysis of the albumin gene was used.<sup>32</sup> Bovine serum albumin was added to each RQ-PCR reaction to prevent inhibition.<sup>33</sup>

### RQ-PCR detection of patient-specific V $\delta$ 2-J $\alpha$ rearrangements

RQ-PCR-based detection of clonal V $\delta$ 2-J $\alpha$  rearrangements relied on the allele-specific oligonucleotide (ASO) primer approach as described previously.<sup>34-36</sup> The above-described germline V $\delta$ 2 TaqMan probe and V $\delta$ 2 forward primer were combined with the ASO primers positioned at the junctional regions, preferably covering the D $\delta$ 3-J $\alpha$ , and sometimes also the V $\delta$ 2-D $\delta$ 3 junction. A standard annealing temperature of 60°C was used. The serial dilutions (10<sup>-1</sup> down to 10<sup>-6</sup>) of diagnostic DNA into control MNC DNA were subjected to RQ-PCR analysis together with negative controls (H<sub>2</sub>O and control MNC DNA). Each dilution step was analyzed in triplicate. RQ-PCR data were analyzed as described previously.<sup>37</sup>

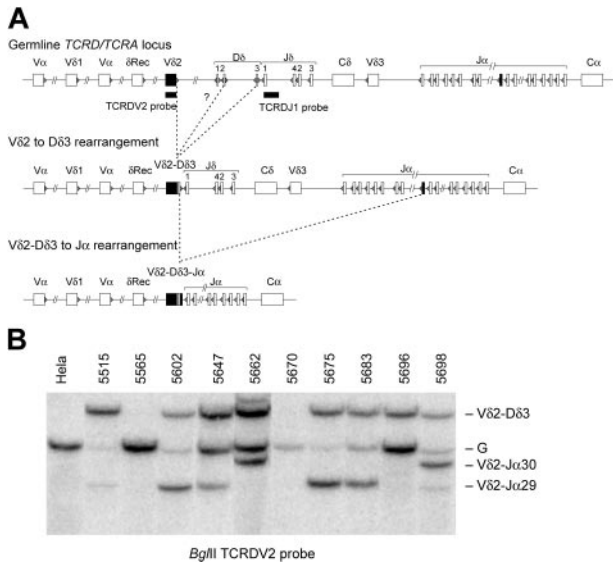
## Results

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

Based on the combined Southern blot and PCR heteroduplex results, clonal V $\delta$ 2 rearrangements were found in 69% (144 of 208) of childhood precursor-B-ALL cases (Figure 1B). Clonal V $\delta$ 2-D $\delta$ 3 rearrangements were detected in 40% (83 of 208) of leukemias. In an additional 7% (14 of 208) of cases, Southern blot indicated the presence of a clonal V $\delta$ 2-D $\delta$ 3 recombination that turned out to be oligoclonal/polyclonal by PCR analysis.<sup>6,38</sup> V $\delta$ 2-J $\alpha$  rearrangements were found by PCR in 46% (95 of 208) of cases. Consequently, 27% (57 of 208) of precursor-B-ALL had both V $\delta$ 2-D $\delta$ 3 and V $\delta$ 2-J $\alpha$  rearrangements (Figure 1).

In 56% (53 of 95) of V $\delta$ 2-J $\alpha$ -positive precursor-B-ALL, V $\delta$ 2-J $\alpha$  joinings were monoclonal (in 83% monoallelic), but in a significant proportion (42 of 95; 44%) the V $\delta$ 2-J $\alpha$  rearrangements were oligoclonal. Oligoclonality was assumed either when the Southern blot revealed presence of rearranged bands of different densities (22 cases) or when the number of PCR-detected clonal V $\delta$ 2-J $\alpha$  and V $\delta$ 2-D $\delta$ 3 recombinations exceeded the number of V $\delta$ 2 rearrangements detected by Southern blot analysis (20 cases).

Clonal D $\delta$ 2-D $\delta$ 3 rearrangements were detected in 10% (20 of 208) of precursor-B-ALL cases. Monoclonal D $\delta$ 2-J $\alpha$  rearrangements were found in only 3 of the 11 leukemias with a germline V $\delta$ 2 allele but a deleted D $\delta$ 3/J $\delta$ 1 region.



**Figure 1. TCRD/A gene rearrangements in precursor-B-ALL.** (A) Consecutive rearrangements in the TCRD/A locus involving the Vδ2 gene segment that are characteristic for precursor-B-ALL. The main pathway concerns consecutive Vδ2-Dδ3 → Vδ2-Dδ3-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 samples. Vδ2-Dδ3 and/or Vδ2-Jα29 gene rearrangements in cases 5602, 5675, 5683, and 5696 are monoclonal. The presence of several rearranged bands of different densities in cases 5515, 5647, 5662, and 5698 is consistent with oligoclonality. Both Vδ2 alleles in case 5670 are deleted, while case 5565 has both Vδ2 alleles in germline (G) configuration.

**Spectrum of Vδ2-Jα rearrangements in childhood precursor-B-ALL**

In the group of 339 childhood precursor-B-ALL cases studied with our multiplex PCR strategy, a total of 172 clonal Vδ2-Jα rearrangements were detected in 141 cases (42%). The frequency of Vδ2-Jα joinings was slightly lower in pro-B-ALL (3 of 12; 25%) as compared with common ALL (95 of 226; 42%) and pre-B-ALL (43 of 101; 43%), but this was not statistically significant.

Sequence analysis of the 172 clonal Vδ2-Jα PCR products revealed that 27 different Jα segments were used (Figure 2A). Surprisingly, the Jα29 gene segment was present in 54% (93 of 172) of all clonal Vδ2-Jα joinings (Figure 2). Together with Jα30 and Jα31 segments, the Jα29 segment 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 segments were found in 23% (40 of 172) of Vδ2-Jα joinings, with Jα48, Jα54, Jα58, and Jα61 segments used most frequently (Figure 2). The third and most downstream cluster was located around the Jα9 segment and comprised 7% (12 of 172) of Vδ2-Jα rearrangements. In line with these results, the 3 identified Dδ2-Jα rearrangements contained the Jα9, Jα29, and Jα58 gene segments, respectively.

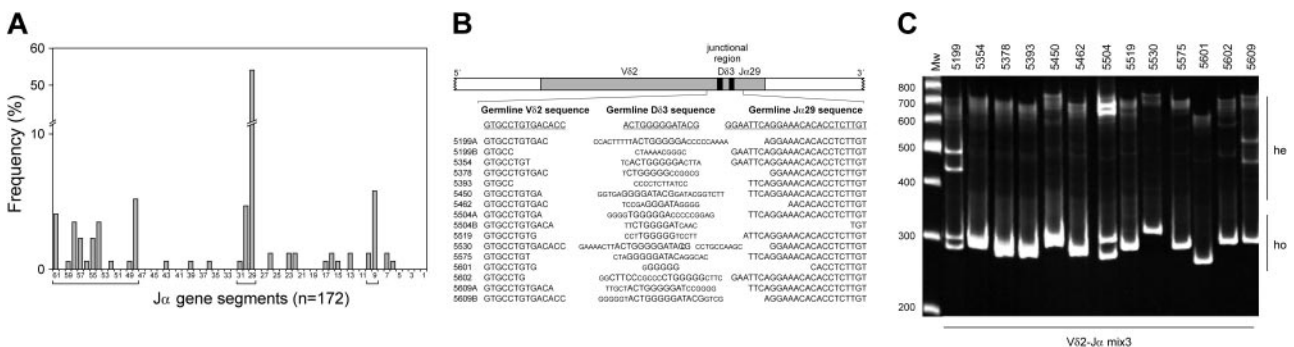
Most of the Vδ2-Jα rearrangements (79%; 132 of 167 fully sequenced clonal PCR products) contained a part of the Dδ3 gene segment. In striking contrast, remnants of the Dδ2 gene segment were found in only 8% (13 of 167) of the Vδ2-Jα sequences. Overall sizes of the Vδ2-Jα junctional regions were extensive, with 18.6 nucleotides on average.

**Vδ2-Jα rearrangements in adult precursor-B-ALL**

Heteroduplex PCR analysis showed a total of 9 clonal Vδ2-Jα rearrangements in 8 of 22 (36%) adult precursor-B-ALL cases. Interestingly, 7 of 9 Vδ2-Jα junctions (78%) contained the Jα29 gene segment. The remaining 2 Vδ2-Jα rearrangements contained Jα48 and Jα54, respectively. A Dδ3 gene segment was identified in 6 Vδ2-Jα junctions. Based on combined Southern blot and PCR assessment, monoclonality in the TCRD/A locus was assumed in all except 1 precursor-B-ALL case with a subclonal Vδ2-Jα rearrangement.

**Vδ2-Jα rearrangements in normal lymphoid tissues**

Using RQ-PCR assays with the germline Vδ2 forward primer, the germline Vδ2 TaqMan probe, and 1 of 4 reverse germline Jα primers (Jα61, Jα58, Jα29, and Jα9), according to the most frequent Vδ2-Jα 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 rearrangements ( $10^{-3}$  to  $10^{-2}$ ) were only found in thymus samples (Table 1 and Figure 3). 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 (generally  $10^{-4}$  or less) of Vδ2-Jα58 and Vδ2-Jα61 rearrangements were detected in normal BM, lymph nodes, and tonsils. Vδ2-Jα29 rearrangements



**Figure 2. Spectrum of Vδ2-Jα rearrangements in childhood precursor-B-ALL.** (A) Bar diagram summarizing the usage of Jα segments in Vδ2-Jα rearrangements in precursor-B-ALL. (B) 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 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 uppercase and small uppercase letters, respectively. (C) Multiplex heteroduplex PCR analysis with the Vδ2 primer in combination with 8 Jα primers (mix 3) showed clonal Vδ2-Jα homoduplexes (ho) in all samples tested. Sequence analysis (B) showed that all these rearrangements involved the Jα29 gene segment. The presence of heteroduplexes (he) in cases 5199, 5504, and 5609 indicated the presence of double Vδ2-Jα29 rearrangements.

**Table 1. V $\delta$ 2-J $\alpha$  rearrangements in normal lymphoid tissues as compared with precursor-B-ALL**

	V $\delta$ 2-J $\alpha$ 61	V $\delta$ 2-J $\alpha$ 58	V $\delta$ 2-J $\alpha$ 29	V $\delta$ 2-J $\alpha$ 9
Precursor-B-ALL, % of V $\delta$ 2-J $\alpha$ -positive cases	4.4	3.8	53.8	6.3
CD19 <sup>+</sup> /CD10 <sup>+</sup> /CD20 <sup>-</sup> sorted normal BM B-cell precursors; n = 1*†	–	–	–	–
CD19 <sup>+</sup> /CD10 <sup>+</sup> /CD20 <sup>+/–</sup> sorted normal BM B-cell precursors; n = 1†	–	–	±	–
CD19 <sup>+</sup> /CD10 <sup>-</sup> /CD20 <sup>+</sup> sorted normal BM B cells; n = 1†	–	–	–	–
Regenerating BM; n = 3‡	±	–	–	–
Normal BM; n = 3	±/+	±	–	–
Normal PB; n = 2	+	±	–	–
E-rosette-positive PB MNCs; n = 1	+	±/+	–	–
E-rosette-negative PB MNCs; n = 1	–	–	–	–
Tonsil; n = 2	±	±	±	–
Lymph node; n = 3	±	±/+	±	–
Thymus; n = 3	++	+/++	±	–

V $\delta$ 2-J $\alpha$  rearrangements were quantified by RQ-PCR, and the obtained values were grouped into the following categories: ++, V $\delta$ 2-J $\alpha$  levels more than 10<sup>-3</sup>; +, V $\delta$ 2-J $\alpha$  levels between 10<sup>-4</sup> and 10<sup>-3</sup>; ±, V $\delta$ 2-J $\alpha$  levels between 10<sup>-5</sup> and 10<sup>-4</sup>; –, V $\delta$ 2-J $\alpha$  levels 10<sup>-5</sup> or less or undetectable.

\*Numbers correspond to number of samples tested.

†Normal BM was stained with CD10, CD20, and CD19 monoclonal antibodies, and the indicated cell populations were sorted using a Diva flow cytometer (BD Biosciences, San Jose, CA).

‡Regenerating BM after cessation of chemotherapy is known to contain high frequencies of precursor-B cells (5% to 30%).<sup>30,31</sup>

were found in thymus, lymph node, and tonsil samples at very low levels (10<sup>-5</sup> to 10<sup>-4</sup>) but were virtually absent in BM and PB. V $\delta$ 2-J $\alpha$ 9 rearrangements were virtually undetectable in all tested normal lymphoid samples, including the thymus (Table 1).

#### Stability of V $\delta$ 2-J $\alpha$ rearrangements in 43 precursor-B-ALL at relapse

Forty-three relapsed precursor-B-ALL cases with a total of 56 clonal V $\delta$ 2-J $\alpha$  rearrangements at diagnosis were also evaluated at relapse. Altogether, 34 of the 56 (61%) clonal V $\delta$ 2-J $\alpha$  rearrangements found at diagnosis were stable at relapse. In 28 precursor-B-ALL (65%), at least 1 V $\delta$ 2-J $\alpha$  rearrangement was preserved at relapse. The stability was markedly different between monoclonal and oligoclonal V $\delta$ 2-J $\alpha$  gene rearrangements, with 21 of 24 monoclonal V $\delta$ 2-J $\alpha$  joinings being stable (88%) as compared with only 13 of 32 oligoclonal rearrangements (41%).

Owing to clonal evolution phenomena, 22 V $\delta$ 2-J $\alpha$  rearrangements were lost in 18 precursor-B-ALL. In 13 cases, this concerned either “regression” of (sub)clonal rearrangements to germline configuration or disappearance (deletion) of the V $\delta$ 2-J $\alpha$  joinings, probably owing to secondary V $\alpha$ -J $\alpha$  recombinations. In 5 precursor-B-ALL, new V $\delta$ 2-J $\alpha$  rearrangements were detected at relapse. In 1 of these 5 cases, the V $\delta$ 2-J $\alpha$ 23 sequence at diagnosis and the V $\delta$ 2-J $\alpha$ 29 sequence at relapse shared a common V $\delta$ 2-D $\delta$ 3 stem, confirming their origin from a common (pre)leukemic progenitor cell with a V $\delta$ 2-D $\delta$ 3 rearrangement. In the remaining 4 cases, the V $\delta$ 2-D $\delta$ 3 junctional regions and the J $\alpha$  segments of the V $\delta$ 2-J $\alpha$  rearrangements at diagnosis and at relapse were completely different, suggesting that the common leukemic progenitor probably had germline *TCRD* genes. It is tempting to speculate that some of the new V $\delta$ 2-J $\alpha$  rearrangements at relapse might have been already present at diagnosis at low frequency as has been described in literature for other immunoglobulin (Ig)/TCR gene rearrangements.<sup>39-41</sup>

#### V $\delta$ 2-J $\alpha$ rearrangements as MRD-PCR targets in precursor-B-ALL

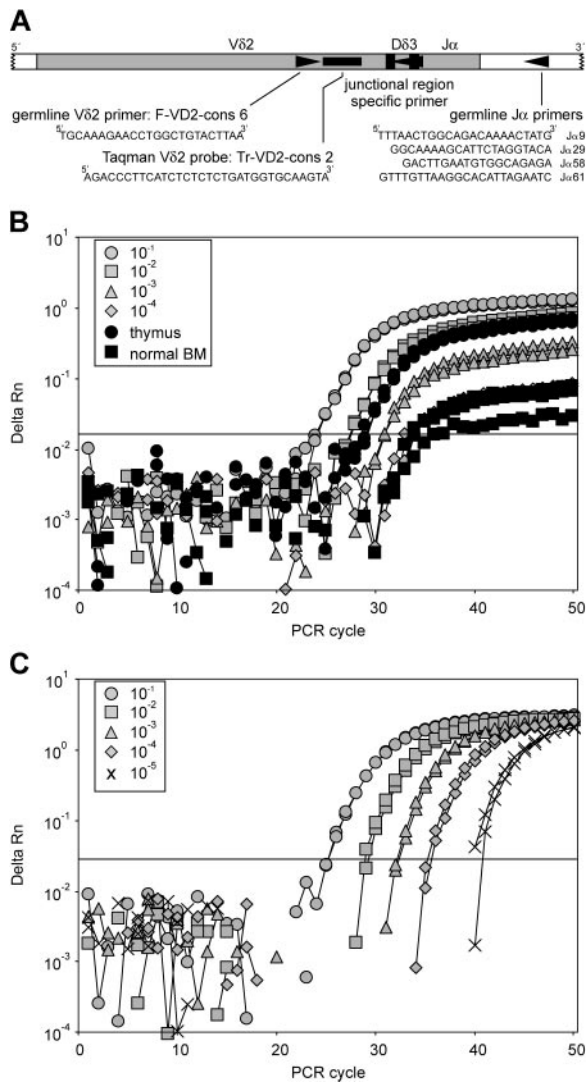
V $\delta$ 2-J $\alpha$  rearrangements were tested as MRD-PCR targets in TaqMan-based RQ-PCR assays employing the germline V $\delta$ 2 forward primer and the germline V $\delta$ 2 TaqMan probe together with ASO reverse primers. In 21 of 32 cases (66%), a quantitative range

of 10<sup>-4</sup> was achieved at the routine annealing temperature of 60°C (ie, requiring no optimization). In 27 of 32 cases (84%) the sensitivity was at least 10<sup>-4</sup>. Very low levels of background amplification in normal MNCs was found in only 7 cases (22%). If observed, limited sensitivity of the MRD-PCR assay was mainly due to the presence of the V $\delta$ 2-J $\alpha$  rearrangement in a subclone only, as indicated by the relatively high threshold cycle (C<sub>T</sub>) value of the 10<sup>-1</sup> dilution.

## Discussion

Our study indicates that the V $\delta$ 2 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 approximately 70% of precursor-B-ALL. By combined Southern blot and PCR analyses, V $\delta$ 2-D $\delta$ 3 joinings were found in 40%, whereas V $\delta$ 2-J $\alpha$  rearrangements were found in 42% of precursor-B-ALL. In 27% of cases, V $\delta$ 2-D $\delta$ 3 and V $\delta$ 2-J $\alpha$  joinings occurred simultaneously. The junctional regions of most (79%) V $\delta$ 2-J $\alpha$  rearrangements contained the D $\delta$ 3 segment, which indicates that recombination to J $\alpha$  was preceded by a V $\delta$ 2-D $\delta$ 3 rearrangement (Figure 1). D $\delta$ 2-D $\delta$ 3 rearrangements were found in only 10% of precursor-B-ALL, and the D $\delta$ 2 segment was found in only 8% of V $\delta$ 2-J $\alpha$  junctional regions. Clonal D $\delta$ 2-J $\alpha$  rearrangements occur even more seldom, because we were able to detect clonal D $\delta$ 2-J $\alpha$  PCR products in only 3 precursor-B-ALL (less than 2%). Thus, V $\delta$ 2, D $\delta$ 3, and several J $\alpha$  genes are preferentially involved in recombinations in the *TCRD/A* locus in precursor-B-ALL, with the main pathway being V $\delta$ 2-D $\delta$ 3 → V $\delta$ 2-D $\delta$ 3-J $\alpha$  (Figure 1). The next step might concern secondary V $\alpha$ -J $\alpha$  rearrangements, deleting the whole *TCRD* locus as well as preexisting V $\delta$ 2-J $\alpha$  joinings.<sup>5,14</sup> The limited number of *TCRD/A* gene segments involved in these rearrangements may be explained by differential accessibility of gene segments within the *TCRD* locus in precursor-B-ALL. Some *TCRD* regions, particularly V $\delta$ 1 and all J $\delta$  gene segments, 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.<sup>42-44</sup>

The spectrum of J $\alpha$  segment usage in V $\delta$ 2-J $\alpha$  rearrangements in precursor-B-ALL was not random. The single J $\alpha$ 29 segment was



**Figure 3. RQ-PCR analysis of Vδ2-Jα rearrangements.** (A) Schematic representation of RQ-PCR analysis of Vδ2-(Dδ3)-Jα rearrangements. The positions and sequences of the germline Vδ2 TaqMan probe, germline Vδ2 forward primer, and 4 germline Jα primers are indicated. (B) Real-time amplification plots of the serial dilutions of a precursor-B-ALL DNA containing clonal Vδ2-Jα61 gene rearrangement into DNA of the cell line CEM, known to have 2 deleted *TCRD* alleles. RQ-PCR analysis was performed using the germline Vδ2 TaqMan probe, the Vδ2 forward primer, and the Jα61 primer. Relatively high levels of Vδ2-Jα61 rearrangements were found in thymus ( $6 \times 10^{-3}$ ). Such rearrangements were also detectable in normal BM, albeit at low levels (less than  $10^{-4}$ ). (C) Real-time amplification plots of the serial diagnosis DNA dilutions into MNC DNA in precursor-B-ALL. RQ-PCR analysis by use of the TaqMan technique was performed using a Vδ2-Jα56 rearrangement with the junctional region-specific primer approach. The reproducible sensitivity in this case reached  $10^{-5}$ .

found in 54% of all Vδ2-Jα joinings. Such nonrandom usage of Jα segments was previously suggested by Southern blot data but was never confirmed at the PCR and sequence level.<sup>4,13</sup> The remaining Vδ2-Jα sequences contained 26 different Jα segments, most of them belonging to 2 additional clusters. The preferential usage of Jα29 might be related to the fact that the recombination signal sequence (RSS) of Jα29 is fully identical to the consensus RSS. However, no preferential usage was found for the other 2 Jα gene segments with a full consensus RSS (ie, 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 (eg, for Jα61, Jα58, Jα54, and Jα48); (2) leukemia-associated differential accessibility, potentially related to specific

(yet unknown) transcription factors; and (3) presence of a consensus RSS.

Vδ2-Jα rearrangements can occur at low levels in normal lymphoid tissues (Table 1). They are relatively frequent in the thymus, where they might represent an infrequent *TCRD* deletion pathway for commitment to the TCRαβ lineage.<sup>15,16,45</sup> Most of the Vδ2-Jα gene rearrangements in the thymus involved the most proximal Jα genes (in our study represented by Jα58 and Jα61),<sup>15,16</sup> and the frequency of such recombinations ranged from  $10^{-3}$  to  $10^{-2}$ . The same spectrum of rearrangements was detectable at more than 10-fold lower levels (ie, less than  $10^{-3}$ ) in other lymphoid tissues, including PB MNCs, BM, lymph nodes, and tonsils. In striking contrast, Vδ2-Jα29 and Vδ2-Jα9 joinings were virtually undetectable in normal lymphoid cells (Table 1). This suggests that the preferential usage of the Jα9 and Jα29 clusters is a leukemia-specific characteristic in precursor-B-ALL.

Our multiplex PCR strategy can easily identify clonal Vδ2-Jα rearrangements that can be applied as PCR targets for MRD monitoring.<sup>46</sup> In fact, based on the limited spectrum of Vδ2-Jα rearrangements in precursor-B-ALL, the assay for Vδ2-Jα detection can be further simplified. Using 2 tubes, one with Vδ2 and Jα29 primers and the second with Vδ2 and 12 Jα primers (Jα9, Jα30, Jα48, Jα49, Jα52, Jα54, Jα55, Jα56, Jα57, Jα58, Jα59, Jα61), we could reliably detect 87% of Vδ2-Jα rearrangements (data not shown). Because the junctional regions of Vδ2-Jα joinings are extensive, it is relatively easy to design optimal patient-specific oligonucleotides reaching sensitivities of at least  $10^{-4}$ , which is required for reliable recognition of the MRD-based risk groups.<sup>47,48</sup> Another advantage of Vδ2-Jα 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.

Many Vδ2-Jα joinings (about 45%) were oligoclonal, comparably to Ig heavy chain gene rearrangements (30% to 40%) and *TCRD* gene rearrangements (about 25%) in precursor-B-ALL.<sup>49,50</sup> Monoclonal Vδ2-Jα gene rearrangements are excellent MRD-PCR targets with good stability (88% of monoclonal rearrangements preserved at relapse) and high sensitivity of at least  $10^{-4}$  in virtually all cases. The usage of oligoclonal Vδ2-Jα rearrangements as MRD-PCR targets is not recommended owing to their low stability at relapse (41%). When the applied MRD-PCR strategy does not include Southern blotting for detection of oligoclonality, one might decide to use our germline Vδ2-Jα RQ-PCR as used for characterization of polyclonal Vδ2-Jα gene rearrangements in normal tissues (Figure 3). Based on the obtained high  $C_T$  values (compared with monoclonal Vδ2-Jα controls), it is possible to identify subclonal Vδ2-Jα joinings when they contribute to less than 10% of the tumor load (data not shown).

In conclusion, Vδ2-Jα rearrangements are frequent cross-lineage recombinations in 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 rearrangements in precursor-B-ALL is not random with preferential usage of the Jα29 gene segment. The extensive junctional regions, the low background in normal BM and PB, and the high stability (88%) of monoclonal rearrangements are the features that favor the usage of monoclonal Vδ2-Jα rearrangements as principal MRD-PCR targets in approximately 25% of precursor-B-ALL.

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