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PILOT STUDY

Clonal stability in children with acute lymphoblastic leukemia (ALL) who relapsed five or more years after diagnosis

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Although most relapses of childhood acute lymphoblastic leukemia (ALL) occur 24–36 months after first CR has been achieved, few patients relapse 5 or more years after CR achievement. The assessment of clonality has proved to be useful in determining whether even those very late events represent the reoccurrence of the original clone or alternatively a secondary leukemia. To gain further information on clonal stability in such late relapse, we performed detailed comparative Southern blotting and PCR analyses of TcRδ and TcRγ gene rearrangements in five ALL at presentation and subsequent relapse which occurred more than 5 years after diagnosis. At least one stable rearranged allele of the TcRδ and TcRγ loci was traced in all cases at presentation and clinical relapse despite a wide heterogeneity of the pattern of rearrangements. Our study extends to a larger series of patients previous findings which have sought to analyze the phenomenon of clonal evolution in children relapsed after more than 5 years of CCR. With respect to the potential pitfalls in monitoring minimal residual disease in childhood ALL for the presence of clonal evolution, our results highlight the combination of two target genes (such as TcRγ and TcRδ) as a tool to reduce false negative MRD results.
Keywords: late relapse; childhood ALL; clonality

Introduction

Children diagnosed with acute lymphoblastic leukemia (ALL) have a 70–75% long-term, relapse-free survival rate with current therapy.[1] In the remaining cases, unpredictable relapses occur mainly during treatment or during the first year after its suspension.[2,3] Only few patients relapse 5 or more years after complete remission (CR) has been achieved; thus, 5-year event-free survival is commonly considered a valuable endpoint for clinical evaluation of treatment protocols.[4]

In the last decade, the assessment of Immunoglobulin (Ig) and T cell receptor (TcR) gene rearrangements as a marker of clonality in human lymphoid neoplasms, have provided insight into the mechanism(s) of relapsing children with ALL.[5,6] Data have been obtained by comparative Southern blot (SB) and polymerase chain reaction (PCR) of Ig and TcR gene rearrangement patterns at diagnosis and subsequent relapse.[7–10] Although different processes of clonal evolution between diagnosis and relapse have been described,[11,12] there is cumulative evidence that relapse in most cases indicates the persistence of the original leukemic clone that resists cytotoxic treatment.[7,13,14] However, in most cases only relapses occurring early during treatment or shortly after its interruption have been so far evaluated. Since adequate samples from long-term survivors may be difficult to obtain, only few studies have assessed clonality at presentation and recurrence in late-relapsing children with ALL.[13,15] However, since the PCR and SB are clone-specific rather than leukemia-specific, clonal evolution, clonal selection, emergence of independent new clones and secondary leukemias can all hamper detection of MRD.[16]

In order to gain further information on clonal stability in late relapses occurring in children with ALL, we performed detailed comparative Southern blotting and PCR analyses of TcRδ and TcRγ gene rearrangements in five ALL at presentation and subsequent relapse which occurred more than 5 years after diagnosis.

Materials and methods

Patients

After informal consent, bone marrow (BM) samples were obtained from five children with ALL (three precursor B-ALLs and two T-ALLs) at initial diagnosis and at first relapse occurring at least 60 months after presentation of childhood ALL. Diagnosis of ALL was made according to standard cytomorphology, cytochemistry and immunological marker analyses.[17]

Southern blot analysis

High molecular weight DNA was isolated from mononuclear cells with standard phenol–chloroform extraction procedure. Fifteen micrograms of DNA was digested with *Eco*RI and *Hin*dIII restriction enzymes (Boehringer Mannheim, Mannheim, Germany), size-fractionated in 0.8% agarose gels and transferred on to nylon membranes (Genescreen Plus, New England Nuclear, Boston, MA, USA). Hybridization and washing were performed as previously described.[18] The configuration of the TcRγ genes was analyzed by use of the PH 60 probe.[19] TcRδ gene rearrangements were detected with the TcRDJ1 probe (kindly provided by Dr JJM van Dongen, Rotterdam, The Netherlands). Probes were labeled with the Megaprime Kit (Amersham, Life Science, Buckinghamshire, UK) with ^{32}P-α-dCTP.

PCR amplification and sequencing of PCR products

PCR was essentially performed as follow: a 100 μl reaction mixture contained 500 ng high molecular weight DNA, 200 mmol/l dNTP, 10 mmol/l Tris-HCl pH 8, 30 mmol/l $MgCl_2$, 50 mmol/l KCl, 1 U Taq polymerase (Amplitaq; Perkin Elmer Cetus, Norwalk, CT, USA), 12 pmol of each

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oligonucleotide and 0.001% gelatin (w/v). All samples were investigated for both TcRδ-PCR and TcRγ-PCR. For TcRδ we used primers that have been previously published;[20] for TcRγ, according to the Southern blotting results, we used primers specific for each gene member of the Vγ family and primers for Jγ1.3/2.3.[20] The reaction mixture was first incubated at 94°C for 3 min to denature double-stranded DNA and then cooled at 55°C for 2 min. Primer extension was assessed to proceed for 3 min. Following this initial-round, denaturing, annealing and extension steps were performed at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, respectively, for 35 cycles in an automatic PCR processor (DNA Thermal Cycler; Perkin Elmer Cetus).

Direct dideoxynucleotide sequencing of double-stranded PCR products was performed using the pMOS Blue T-Vector cloning system (Amersham, Life Science) and the Sequenase 2.0 Kit (Amersham, Life Science) with internal sequencing primers (see Table 2). Sequencing products were run on a 8% denaturing polyacrylamide sequence gel, dried, and exposed for 12–72 h at –70°C.

Results

The main clinical and laboratory features of the five ALL patients included in the study are shown in Table 1. Length of first continued complete remission (CCR) ranged between 60 and 77 months. No significant changes were observed in immunophenotype between diagnosis and relapse (data not shown). By contrast, with the exception of case 2, a significant heterogeneity in TcRγ and TcRδ gene rearrangement patterns was found between presentation and recurrence of disease, as summarized in Table 2.

Patient 1

The same TcRδ rearrangement (Vδ2Dδ3) was observed at diagnosis and relapse (Figure 1). However, when the Vδ2Dδ3 rearrangements were amplified by PCR and the amplification products used for direct sequence, a distinct junctional sequence was identified between DNA from presentation and relapse marrow (Table 2). Southern blot analyses of TcRγ gene from the presentation bone marrow of this case showed germline bands, an equally intense rearranged band for Vγ7 and a second weakly hybridizing band for Vγ9. At relapse, the intense rearranged band (Vγ7) was lost and a new, strongly hybridizing band (Vγ2/4) appeared. The band at the position of the weakly hybridizing Vγ9 rearranged band, shown at diagnosis, increased its intensity. PCR amplification and sequence analyses confirmed the nature of Vγ7 and Vγ4 for the two non-concordant rearrangements at diagnosis and relapse, respectively (data not shown). Sequence analysis of the junctional V–J region of the rearranged Vγ9 demonstrated an identical junctional region at presentation and relapse (Table 2).

Patient 3

The analysis of TcRδ gene displayed the same pattern at diagnosis and at relapse. Southern blot analysis of DNA from presentation marrow after digestion with *Eco*RI and *Hin*dIII and the TcRγ probe hybridization showed two germline and one rearranged band (Vγ2/4) (Figure 1). At relapse both the germline band and the Vγ2/4 rearranged band persisted, while a new rearranged band (Vγ5) also appeared. Sequence analysis of the corresponding TcRγ rearranged segment confirmed the identity of the same Vγ4 rearrangement at diagnosis and at relapse.

Patient 4

Clonal variation at the TcRδ locus was documented as a Vδ1–Jδ1 rearrangement at diagnosis whereas a Vδ3–Jδ1 was observed at recurrence (Figures 1 and 2). Of interest, when PCR amplification and sequence analyses were performed at presentation and relapse, the presence of the same Vδ1–Jδ1 rearrangement (detectable only at diagnosis by Southern blot) was confirmed even at the time of relapse (Table 2). Southern blot analysis of TcRγ gene from the presentation bone marrow of this case showed a Vγ2/4 rearranged band, whereas at relapse a second equally intense rearranged band

Table 1 Clinical and laboratory features of the analyzed patients

Patient	*Sex/ Age*	*Disease stage (months from diagnosis)*	*WBC $\times 10^9$/l*	*Immunophenotype*	*Leukemic cells (%)*	*Protocol treatment*[a]	*Site of relapse*	*Clinical outcome*[b]
1	M/9	D	36.1	common ALL	98	8602		2nd Rel. (+ 11 mo). Dead for progression (+ 12 mo)
		R (69)	9.8	common ALL	95	9102-REC	BM	
2	M/3	D	126.0	common ALL	98	8503B		Dead in 2nd CR for hesit failure (+ 6 mo)
		R (60)	14.0	common ALL	85	9103	BM	
3	M/5	D	5.4	common ALL	95	8802		PBSC-BMT (+ 4 mo). Dead for multi-organ failure (+ 32 mo)
		R (72)	1.2	common ALL	90	9103	BM + CNS	
4	M/2	D	135.0	T-ALL	90	8503A		Syngeneic BMT (+ 4 mo). CCR (+ 5 y)
		R (72)	27.0	T-ALL	70	VCR + Epidoxorubicin	BM + CNS + Kidney	
5	M/5	D	173.0	T-ALL	95	8503A		Allo BMT (+ 6 mo). Dead for multi-organ failure (+3 mo)
		R (77)	141.8	T-ALL	80	REC89	BM	

[a]Duration of front-line protocols was 24 months.
[b]Time points refer to the date of first relapse.

Table 2 Rearrangement patterns by Southern blotting, PCR analysis and sequencing of the 'N' region

Patient	*Status*	*Southern blotting*		*PCR*		*TcR gamma sequence*	*TcR delta sequence*
		TcR gamma	*TcR delta*	*TcR gamma*	*TcR delta*		
1	D	Vγ7–Vγ9	Vδ2Dδ3	Vγ9	Vδ2Dδ3	Vγ9 tgtgcctAGAGGtataagaaa	Vδ2 cctgtgacgCCactggggg
	R	Vγ2/4–Vγ9	Vδ2Dδ3	Vγ2, Vγ4 Vγ9	Vδ2 Dδ3	Vγ4 tattacCCCTGGGACGGacaa Vγ9 tgtgcctAGAGGtataagaaa	Vδ2 cctgtTCGTactggggg
2	D	Vγ9	Vδ2Dδ3	ND	Vδ2Dδ3	ND	Vδ2 tgtgaccccGAGGtggggg
	R	Vγ9	Vδ2Dδ3	ND	Vδ2Dδ3	ND	Vδ2 tgtgaccccGAGGtggggg
3	D	Vγ2/4	D/G	Vγ2, Vγ4	/	Vγ4 tgtgccaccCtataagaaa	/
	R	Vγ2/4–Vγ5	D/G	Vγ2, Vγ4 Vγ5	/	Vγ4 tgtgccaccCtataagaaa Vγ5 ggggtcCTGGGgaattatt	/
4	D	Vγ2/4	Vδ1Jδ1	Vγ2, Vγ4	Vδ1Jδ1	Vγ4 gggaGCAGGTGGCGTCGatt	Vδ1 aacCcctactgggggataTCGGaa
	R	Vγ2/4–Vγ11	Vδ3Jδ1	Vγ2, Vγ4 Vγ11	Vδ3Jδ1 Vδ1Jδ1	Vγ2 acggTCACGGGAtataa ND	Vδ3 cctttaTCGGTTtaaactc Vδ1 aacCcctactgggggataTCGGaa
5	D	Vγ2/4–Vγ8	Vδ1Jδ1 Vδ3Jδ1	Vγ2, Vγ4 Vγ8	Vδ1Jδ1 Vδ3Jδ1	Vγ2 acgggCGAGTTTTTGgaaa Vγ8 gggataAAAGGTTGTCGTC GGGgaaa	Vδ1 ggggaacAATGCATGCACAGCTcctt ccACgggggatacgCAGGGTAGacc Vδ3 cctttCAAGGTACTGAgaaaAAGccttccC GGAAGactgggggatacgGAAGACTcacc
	R	Vγ2/4–Vγ8	Vδ1Jδ1	Vγ2, Vγ4 Vγ8	Vδ1Jδ1	Vγ2 acgggCGAGTTTTTGgaaa Vγ8 gggataAAAGGTTGTCGTC GGGgaaa	Vδ1 ggggaacAATGCATGCACAGCTcctt ccACgggggatacgCAGGGTAGacc

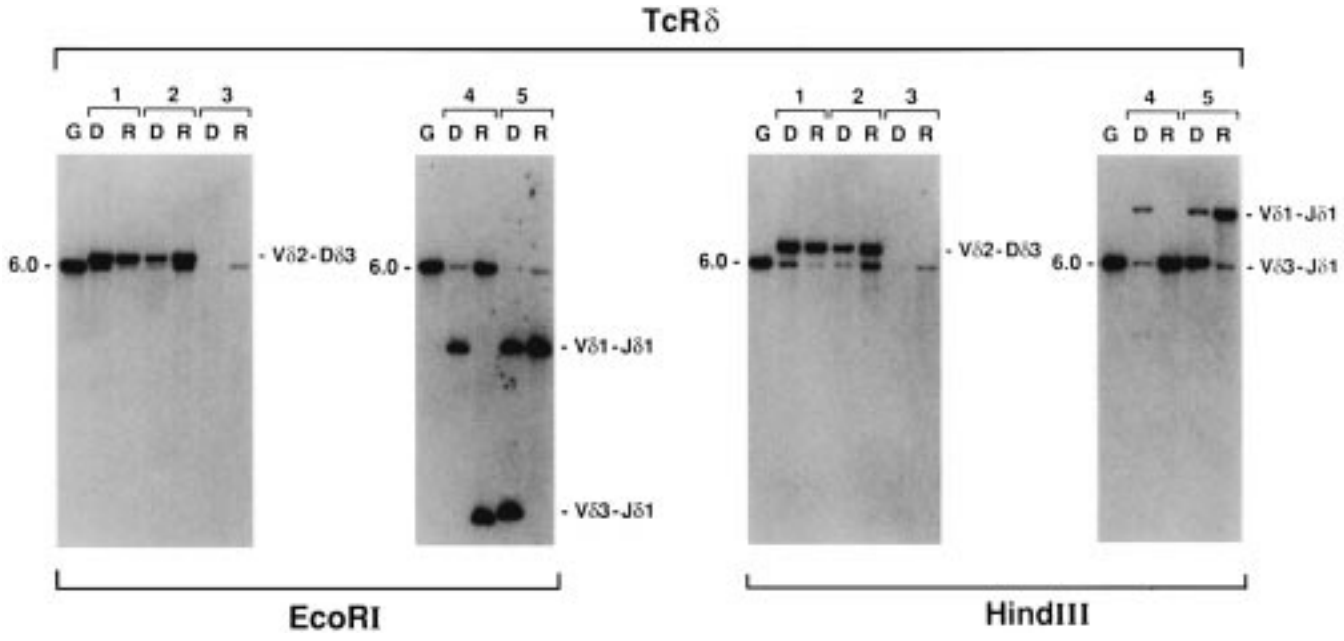

Figure 1 Southern blot analysis with the TcRDJ1 probe of *Eco*R1 and *Hin*dIII cut DNA from bone marrow mononuclear cells of the five patients at diagnosis (D) and first relapse (R). G, germline pattern from placental DNA of a healthy donor. The samples are coded according to Table 1. Rearrangement patterns for Vδ2–Dδ3, Vδ1–Jδ1 and Vδ3–Jδ1 are indicated on the right of each panel. The size (kb) of the germline fragment is indicated on the left of each panel.

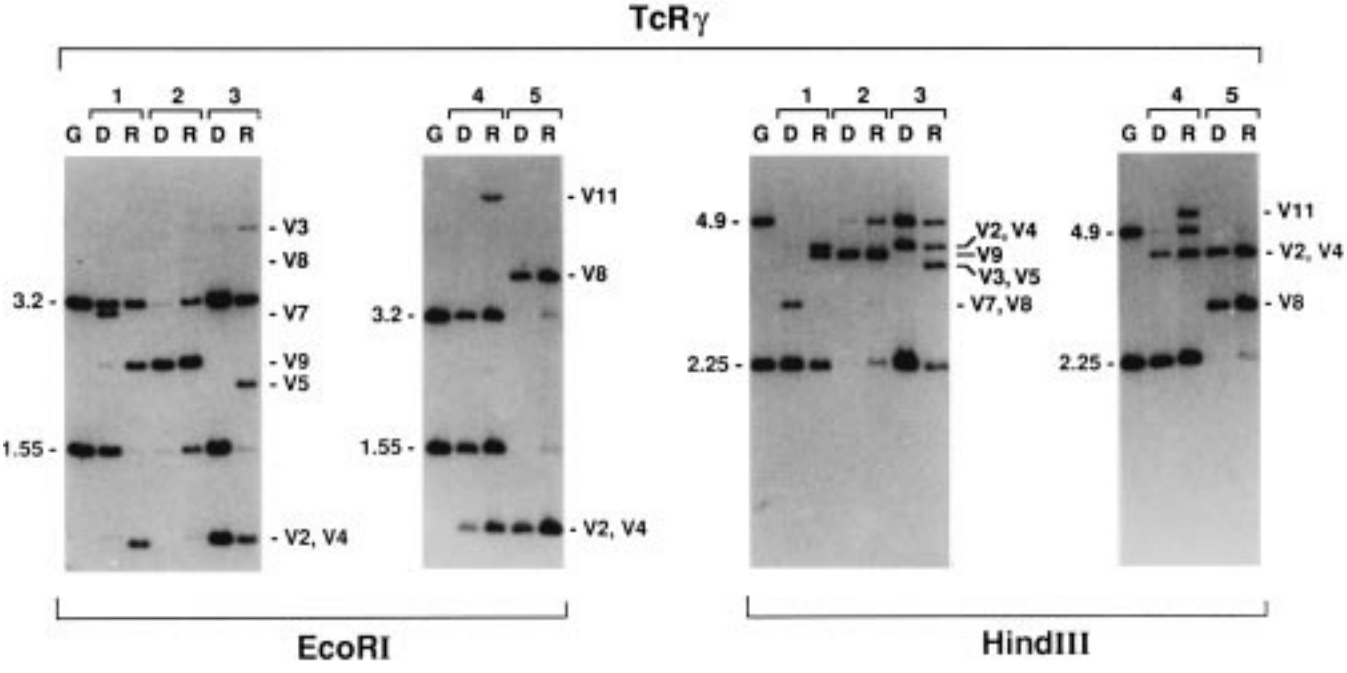

Figure 2 Southern blot analysis with the PH60 probe of *Eco*RI and *Hin*dIII cut DNA from bone marrow mononuclear cells of the five patients at diagnosis (D) and first relapse (R). G, germline pattern from placental DNA of a healthy donor. The samples are coded according to Table 1. Rearrangement patterns for Vγ2/4, Vγ3, Vγ5, Vγ7, Vγ8 and Vγ9 Vγ family members are indicated on the right of each panel. The sizes (kb) of the germline fragments are indicated on the left of each panel.

(corresponding to a Vγ11 rearrangement) became apparent. PCR amplification and sequence analyses showed a Vγ4–Jγ1 rearrangement from the presentation marrow, whereas a Vγ2–Jγ1 was observed from the relapse marrow.

Patient 5

Rearranged bands at similar positions (corresponding to a Vδ1–Jδ1 rearrangement) were detected with the TcRδ probe in both the presentation and relapse marrows, the former having an additional rearranged band corresponding to a Vδ3–Jδ1 rearrangement. PCR amplifications and sequence analyses showed the same junctional region of the Vδ1–Jδ1 rearranged allele at diagnosis and at relapse. No clonal variation was observed at the TcRγ locus, a similar biallelic rearranged pattern (Vγ2/4 and Vγ8) being detected in both the presentation and relapse marrow by Southern blot (Figures 1 and 2) and further confirmed by PCR and sequence analyses.

Discussion

Even with modern front-line therapy protocols for childhood ALL, relapse remains the most important obstacle to overcome.[1–3] Although most events occur 24–36 months after first CR has been achieved,[2] few patients relapse 5 or more years after CR achievement. Such very late relapse is generally considered a different biological and clinical entity, because of the long-term duration of first CR. In that context, the assessment of clonality has proved to be useful in determining whether even those very late events represent the reoccurrence of the original clone or alternatively a secondary leukemia.[7,10,13,14] Our study extends to a larger series of patients previous findings which have sought to analyze the phenomenon of clonal evolution in children relapsed after more than 5 years of CCR.[13,15]

At least one stable rearranged allele of the TcRδ and TcRγ loci was traced in all cases at presentation and clinical relapse despite a wide heterogeneity of the pattern of rearrangements. Differences at diagnosis and relapse with respect to TcRγ locus occurred in these cases. In two cases (Nos 1 and 4) further rearrangements of Vγ genes, which in germline configuration are located upstream of the Vγ genes rearranged at the presentation, occurred at relapse. By contrast, the occurrence of new clonal rearrangements can be observed at relapse in case Nos 3 and 4.

The existence of a significant heterogeneity of the TcRδ gene leading to bi/oligoclonality in precursor B-ALLs, as reported by Ghali *et al*[16] might explain the pattern observed in case No. 1. Discordant junctional sequences of the Vδ2–Dδ3 rearrangement were found at presentation and relapse, suggesting either a different response to chemotherapy of the subclone present at diagnosis or a different proportional composition. No experiments have been done to explore further the latter hypothesis. Clonal variation of VDJ rearrangements at the TcRδ locus was documented in case Nos 4 and 5. Of interest, PCR analysis revealed the persistence in the relapse sample of the same Vδ1–Jδ1 rearrangement observed at diagnosis, although undetectable by Southern blot.

The existence of at least one concordant rearranged allele favors the hypothesis that the new rearrangement observed at relapse probably originates from the presentation clone. A certain degree of clonal stability demonstrated in all cases of relapsing children after a long-term CCR (>5 years), is further supported by the few reports indicating that even relapses occurring more than 10 years after diagnosis are due to true re-emergence of the original leukemic clone.[13,15,21] It is intriguing to interpret a very long period of clinical remission with the persistence of minimal residual disease, being detectable only by using very sensitive PCR techniques.[22] What mechanism(s) can suppress the malignant clone for so long a time and which events can lead to its re-emergence, are still unanswered questions.

With respect to the potential pitfalls in monitoring minimal residual disease in childhood ALL for the presence of clonal evolution, our results highlight the combination of two target genes (such as TcRγ and TcRδ) as a tool to reduce false negative MRD results. Those findings further extend (even for late-relapse events) those recently reported in the largest series of children ALL prospectively analyzed for the prognostic impact of MRD detection.[23]

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