Assessment of clonal stability of minimal residual disease targets between 1st and 2nd relapse of childhood precursor B-cell acute lymphoblastic leukemia

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Background and Objectives. Immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements are excellent patient-specific targets for polymerase chain reaction (PCR)-based detection of minimal residual disease (MRD) in acute lymphoblastic leukemia (ALL). Nevertheless, instability of these targets during the course of the disease has important implications for PCR-based MRD monitoring and may lead to false negative results.

Design and Methods. Several studies have shown that Ig and TCR targets are reasonably stable in ALL between diagnosis and first relapse, but up to now, there are no data on the stability of these targets between first and second relapse. We, therefore, performed a PCR-study on bone marrow samples from 49 children with precursor B-ALL at first and second relapse. Homo-heteroduplex PCR analyses were used for identification of clonal *IGH*, *IGK*-Kde, *TCRG* and *TCRD* gene rearrangements. Clonal targets were studied by sequencing and/or comparative homo-heteroduplex analysis.

Results. In 52% (25/48) of the patients, all PCR targets identified at first relapse were preserved at second relapse; in 92% (44/48) of the patients at least one target and in 73% (35/48) at least two targets remained stable. Best stability was found for *IGH* and *TCRG* gene rearrangements.

Interpretation and Conclusions. Based on these first data about clonal stability of Ig and TCR targets between first and second relapse of childhood precursor B-ALL, we developed a stepwise strategy for appropriate selection of stable PCR targets for MRD monitoring. This strategy was applicable in 84% of the relapsed patients and resulted in at least one stable MRD-PCR target per patient in 98% of these children.

Key words: Ig and TCR gene rearrangements, childhood relapsed ALL, clonal evolution, minimal residual disease.

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Acute lymphoblastic leukemia (ALL) is the most common form of cancer in children. Over the last three decades dramatic improvements in the treatment of this disease have turned this once uniformly fatal condition into one that is potentially curable with a long-term survival rate of about 75%.[1,2] However, despite this progress, about 20-30% of children will eventually suffer a relapse.[3] For these children, the overall cure rate is only 30% at present.[4,5]

Several large prospective studies have demonstrated the high prognostic value of quantification of the leukemia burden (minimal residual disease - MRD) after induction treatment in newly diagnosed ALL.[6-8] This sensitive evaluation of early response to cytotoxic treatment enables identification not only of patients at high risk of relapse but also of those low-risk patients with a relapse-free survival of more than 95%.[7] Recent data suggest that also in children with relapsed ALL, monitoring of MRD during the first weeks of therapy has a high predictive value.[8] Consequently, MRD information provides new and promising opportunities of offering risk-assigned treatment in childhood ALL.

Three different techniques - allowing detection of leukemic cells with a sensitivity of 10^3-10^6 are mainly used: flow-cytometric immunophenotyping (using aberrant or *leukemia-associated* phenotypes), polymerase chain reaction (PCR) analysis of breakpoint fusion regions of chromosome aberrations, and detection of clone-specific immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements by PCR amplification.[7,9,10] This last technique is the most widely used for MRD studies in patients with ALL. The antigen receptor genes are made up of several discontinuous germline segments that undergo rearrangement processes during early lymphoid development. Each gene rearrangement is unique and therefore a specific target for the particular leukemic cell clone of an individual patient.[9]

Nevertheless, it is already known that these Ig and TCR targets might change or even be lost during the course of the disease (clonal evolution), in particular, because of continuous rearrangement processes within the leukemic cell population as well as the selection or expansion of subclone populations without *Ig/TCR* gene rearrangements or with different ones.[7,11-18] This will lead to false-negative MRD-results and potentially dramatic consequences for the young patients.

Although the presence of clonal evolution phenomena between newly diagnosed ALL and first relapse is well known and widely acknowledged,[7,11-18] so far only a few

quite heterogeneous studies have occasionally monitored clonal stability between first and second relapse (total number of patients: 30; focal point: *IgH* and *TCRD*) in addition to their main investigations of clonal stability between newly diagnosed ALL and first relapse.[17-22]

We, therefore, studied the stability of the most frequently used *Ig* and *TCR* gene rearrangements (*IgH*, *IGK*-Kde, *TCRG*, and *TCRD* gene rearrangements) in a series of 49 children with precursor B-ALL patients between first and second relapse. This information is essential for reliable selection of PCR targets in order to avoid false-negative MRD results in ALL-relapse trials.

Design and Methods

Patients' material

Bone marrow samples were obtained from 49 children with precursor B-ALL at first and at second relapse (48 patients) or at the finding of a presumably secondary acute myeloid leukemia (AML) (one patient). In four patients additional bone marrow smears were used (patients #4, 16, 35, and 38). The samples were collected from the centers enrolled in the ALL-REZ BFM (Berlin-Frankfurt-Münster) relapse trials and date from 1988 to 2001. The age distribution of the patients ranged from 2 years to 18 years at the time of first relapse. The diagnosis of precursor B-ALL was made according to French-American-British and standard immunophenotypic criteria.[23-25] Immunologic marker analysis at first relapse revealed that 6 (12%) were pro-B ALL, 32 (65%) were common ALL, and 11 (22%) were pre-B ALL. Comparative immunophenotypic analysis between first and second relapse revealed intralineage switches in 20% (9/44) of precursor B-ALL patients with available detailed immunophenotypic data at second relapse: four pre-B ALL converted into common ALL (patients #1, 9, and 44) or pro-B ALL (patient #13); three common ALL turned into pre-B ALL (patients #22 and 49) or AML (patient #6); one pro-B ALL turned into common ALL (patient #43), and one pro-B ALL turned into pre-B ALL (patient #8).

Mononuclear cells were isolated by Ficoll density gradient centrifugation (Seromed; Biochrom KG, Berlin, Germany; density 1.077 g/mL). DNA was isolated using a QIAamp Kit (Qiagen; Hilden, Germany).[26] DNA concentration was determined by UV spectrophotometry.[27] Integrity and quantitative analysis of DNA were confirmed by PCR amplification of the β-globin gene by LightCycler real-time quantitative (RQ) PCR technology.[28,29]

Representativeness of the material

As mentioned above, the samples from the patients were collected from the centers enrolled in the ALL-REZ BFM relapse trials and were, therefore, selected on the basis of availability. We compared our study group with a reference group in order to evaluate the extent of selection and representativeness. We compared the following characteristics: sex, time of first relapse (very early, early and late), site of first relapse (bone marrow, isolated or combined), stratification group of the protocol (intermediate [S2], high risk [S3, S4]) at first relapse, fusion gene *TEL-AML1* at first relapse, peripheral blasts at first relapse diagnosis, stem cell transplantation during second remission, site of second relapse.

Time of first relapse, stratification group at first relapse and stem cell transplantation were significantly different distributed. Our study group is composed of patients who had significantly more late first relapses (p=0.021), belonged significantly more to the stratification group S2 (p=0.007) and underwent significantly fewer stem cell transplantations (p=0.027).

The reasons for these significant differences are as follows: most of the patients who suffer a very early or an early first relapse (with bone marrow involved) belong to the stratification groups S3 and S4 (both high risk): stem cell transplantation is indicated for these patients. When these patients suffer a second relapse they are treated palliatively because they have a very bad prognosis. Understandably, clinicians and the parents are no longer interested in any molecular assessments and, therefore, we did not receive bone marrow or blood from these patients.

Identification of clonal targets

To detect and identify the Ig/TCR targets we used primer sets for seven *IGH*,[30] five *IGK*-Kde,[31] twelve *TCRD*,[32] and six *TCRG* (*Peter A, et al. in preparation*) *PCR*-products. Based on further sequence analyses of these 30 PCR-products we discriminated 102 different gene rearrangements (36 *IGH*, 4 Vκ-Kde (κ deletion element) and one RSS *intron*-Kde (recombination signal sequence), 55 *TCRG*, and 6 *TCRD*).

The PCR products were analyzed for clonality by homo-heteroduplex analyses.[33-35] PCR products were denatured for 5 min at 94°C. After denaturation, the samples were rapidly cooled to 50°C and incubated for at least 60 min at this temperature.[36] Size separation of the generated homoduplexes and heteroduplexes was performed in a 12% non-denaturing polyacrylamide gel. Heteroduplex bands were excised, eluted, and further amplified by PCR and sequenced. PCR products which showed a homoduplex band in the polyacrylamide gel were directly sequenced.

Additional comparative homo-heteroduplex analyses were carried out in a few samples. We, therefore, repeated the same procedure as for

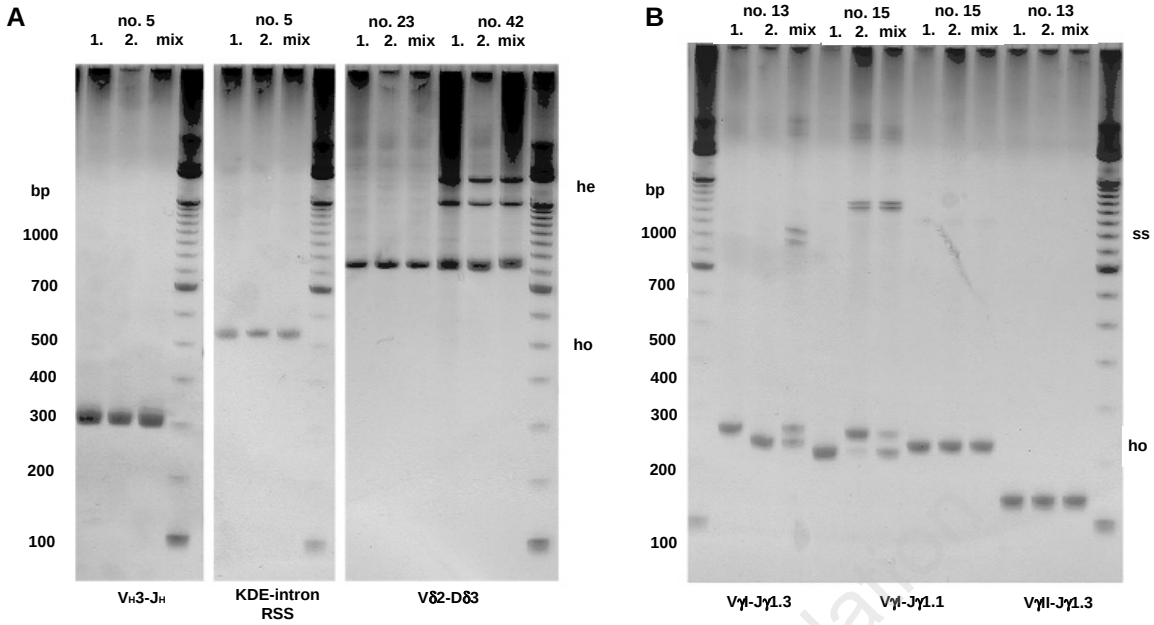

Figure 1. Examples of comparative homo-heteroduplex analysis. (A) Comparative homo-heteroduplex analysis of *IgH, IgK*-Kde-intron RSS (recombination signal sequence), and *TCRD* gene rearrangements. Monoclonal homoduplexes (ho) in patients# 5 and# 23 found at first and second relapse were of the same size. Mixing of the first and second relapse PCR products (mix) demonstrated no heteroduplex (he) formation, proving that these gene rearrangements had identical junctional regions. Patient# 42 showed a stable biallelic Vδ2-Dδ3 gene rearrangement. Mixing of the first and second relapse PCR products (mix) demonstrated the same homo- (ho) and heteroduplex (he) formations, proving that these Vδ2-Dδ3 gene rearrangements had identical junctional regions. (B) Comparative homo-heteroduplex analysis of *TCRG* gene rearrangements. In patient# 13 and# 15 monoclonal Vγ I-Jγ1.3 homoduplexes (ho) found at first and second relapse differed in size. Mixing of the first and second relapse PCR products followed by homo-heteroduplex PCR analysis demonstrated clear heteroduplex (he) formation, proving that these VgI-Jg1.3 gene rearrangements had different junctional regions. (ss) = remaining single strand fragments. Patient# 13 as well as# 15 yielded identical monoclonal homoduplexes (ho) at first and second relapse (Vγ I-Jγ1.1 and Vγ II-Jγ1.3, respectively). These results were confirmed by sequencing.

homo-heteroduplex analysis but compared on one gel the homo-heteroduplex banding of the PCR products of the first and the second relapse samples with a mixture of both (Figure 1).[22,33,34] Sequences of the clone-specific gene rearrangements were obtained using Big Dye Terminator Cycle Sequencing on a ABI Prism 377 Automated Sequencer (both Applied Biosystems; Foster City, CA, USA). For those patients in whom questions arose about the gain or loss of targets between first and second relapse while targets of other *Ig/TCR* gene loci remained fully stable, or about the total absence of targets at second relapse, we tried to collect sufficient quantities of DNA to perform additional Southern blot analyses. Unfortunately, only for three patients (patients #2, 6, and 30), could we obtain sufficient quantities. For these patients, *IGH*, *IGK*, and *TCRD* gene configurations were analyzed using ^{32}P-labeled *IgHJ*6, *TCRDJ*1, and IgKDE probes (DAKO, Carpinteria, CA, USA) in Bg/II digest.[31,34,35,37]

Statistical analysis

Statistical analysis using a χ^2 test on a 2×2 table was performed, first to compare the frequencies of clonal evolution at *Ig/TCR* gene loci when one or more than one rearrangement per gene locus was detectable, and secondly, to compare the frequencies of clonal evolution at the *Ig/TCR* gene loci and the duration of remission. Pearson's 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

Frequency and stability of Ig and TCR gene rearrangements between 1st and 2nd relapse in precursor B-ALL patients

One hundred and forty-nine clonal *Ig/TCR* gene rearrangements were identified at first relapse in 48 of 49 patients (98%), with an average of 3.1 MRD-PCR targets per patient. In one patient (#17) no clonal gene rearrangement was detectable, either at first or at second relapse. One hundred and nine of these 149 gene rearrangements (73%) identified at first relapse were preserved at second relapse. The best stability was found for *IGH* and *TCRG* gene rearrangements (42/56: 75%; 26/34: 76%) followed by *TCRD* (17/24: 71%) and IGK-Kde (24/35:

Table 1. Incidence, stability and changes in *TCRD*, *TCRG*, *IgK-Kde*, and *IgH* gene rearrangement patterns of 49 precursor B-ALL patients between first and second relapse.

	TCRD		*TCRG*		*IGK*		*IGH*	
	%	n	%	n	%	n	%	n
Incidence of rearranged Ig/TCR gene loci (mean: number of rearrangements per gene locus and patient)	37	18/49 (1.3)	53	26/49 (1.3)	51	25/49 (1.4)	71*	35/49 (1.6)
Patients with two or more gene rearrangements per gene locus	33	6/18	31	8/26	40	10/25	49	17/35
Patients in whom all gene rearrangements remained stable	67	12/18	69	18/26	68	17/25	71	25/35
Patients in whom at least one gene rearrangement remained stable	72	13/18	85	22/26	72	18/25	89	31/35
Total number of stable gene rearrangements	71	17/24	76	26/34	69	24/35	75	42/56

**Difference statistically significant ($p<0.05$).*

69%; Table 1), but none of these results achieved statistical significance ($p>0.05$). Gain and loss of Ig/TCR gene rearrangements between first and second relapse appeared with almost equal frequencies (Table 2).

Stability: difference between Ig/TCR gene loci with only one and more than one rearrangement (multiple rearranged)

Only for the *IgH* gene locus did we find a significant correlation between the presence of more than one rearrangement per gene locus at first relapse and the increase in clonal evolution ($p<0.05$). Multiple rearranged *IgK*-Kde, *TCRD*, and *TCRG* gene loci also yielded a higher frequency of clonal evolution than did gene loci with only one rearrangement, but this did not achieve statistical significance ($p>0.05$; Table 3). Nevertheless, in 35 of all 41 multiple rearranged *Ig/TCR* gene loci (85%) at least one target per gene loci remained fully stable (Table 3). Beyond this, at multiple rearranged *TCRD* and *TCRG* gene loci, the stability of at least one target was even higher than for gene loci with only one rearrangement detected at first relapse (Table 3).

Patterns of clonal evolution: focusing on each particular gene locus

Table 4 summarizes the patterns of clonal evolution at each particular *Ig/TCR* gene locus. Gene loci which remained without any changes between first and second relapse are not shown (Table 2). In comparison with the *IGK*-Kde, *TCRD*, and *TCRG* gene loci, the *IGH* locus showed a greater stability of at least one target despite a high number of changes (lines 1-3). When changes occurred at the *IGK*-Kde gene locus they were characterized by the loss of all targets at second relapse (lines 5 and 6), and at the *TCRG* gene locus by the gain of new targets (lines 4 and 6).

Patterns of clonal evolution in patients

Despite a high frequency of clonal evolution (36/48; 75%) – also involving patients in whom no *Ig/TCR* gene rearrangements were detectable at first relapse, but in whom one or more gene rearrangements occurred at second relapse (Tables 2 and 4) - in 92% of the patients (44/48) at least one marker and in 73% (35/48) two or more markers remained stable between first and second relapse. The number, type or sequence of gene rearrangements had changed from first to second relapse at the *IgH*, *IgK-Kde*, *TCRG*, and *TCRD* gene locus in 49% (20/41), 41% (11/27), 45% (15/33), and 48% (10/21) of the patients, respectively (Table 4). ALL rearrangements in *IgH*, *IgK-Kde*, *TCRG*, and *TCRD* gene loci were preserved in 71% (25/35), 68% (17/25), 69% (18/26), and in 67% (12/18) of the patients, respectively, between first and second relapse (Table 1). At least one clonal marker per rearranged *IgH*, *IgK*-Kde, *TCRG*, and *TCRD* gene locus remained stable in 89% (31/35), 72% (18/25), 85% (22/26), and 72% (13/18), respectively (Table 1).

All Ig/TCR targets preserved at second relapse

In 25 patients (52%), all Ig/TCR gene rearrangements identified at first relapse were preserved at second relapse (Table 2). In 13 patients additional Ig/TCR gene rearrangements occurred at second relapse. This might have been caused by continuing rearrangement processes within the leukemic cell population or the outgrowth of subclone populations with first detectable Ig/TCR gene rearrangements at second relapse. Patient #31 developed a third relapse two and a half months after the diag-

Table 2. *Ig/TCR* gene rearrangement stability patterns in 49 precursor B-ALL patients between first and second relapse.

Patient no.	*Age at first relapse*	*Phenotype of ALL 1st/2nd relapse*	*TCRD*	*TCRG*	*IGK-Kde*	*IGH*	*Loss / total no. of rearrangements at 1st relapse*	*Gain of at rearrangements 2nd relapse*	*Time of CCR between 1st/2nd relapse (months)*
1ph	7	pre-B/cALL	–/–	R/id	R/id	–/–	0/2	–	23
2	13	pro-B/pro-B	R/–	–/–	–/–	–/–	1/1	–	14
3	5	cALL/cALL	–/–	–/–	R_1R_2/-	–/–	2/2	–	19
4	4	cALL/cALL	R/id	–/R	–/–	–/R	0/1	2	10
5ex	18	cALL/cALL	–/–	R_1R_2/id	R_1/id+R_2R_3	R/id	0/4	1	17
6ph	7	cALL/AML	–/–	–/–	–/–	R/–	1/1	–	21
7	13	cALL/cALL	–/–	R_1R_2 /R_1id	R_1R_2 /–	R_1R_2/R_1id	4/6	–	40
8ph	10	pro-B/pre-B	–/–	R_1R_2/id	–/–	R_1R_2/R_3	2/4	1	17
9ph	9	pre-B/cALL	–/–	R/id	–/–	R/id	0/2	–	14
10	18	cALL/cALL	–/R	–/–	–/–	$R_1R_2R_3$/id	0/3	1	40
11ex	2	pre-B/pre-B	–/–	R/id	R/id	R_1R_2/id+R_3	0/4	1	3
12	16	pre-B/pre-B	–/–	R/id	–/–	R_1R_2/id	0/3	–	10
13ph	8	pre-B/pro-B	R1R2/–	R_1R_2/ R_1id+R_3	R_1R_2/id	R_1R_2/R1id	4/8	1	44
14	7	pre-B/pre-B	–/–	–/–	–/–	R/id	0/1	–	2
15	13	cALL/cALL	–/–	R_1R_2/ R_1id+R_3	–/–	R/id	1/3	1	15
16	6	cALL/cALL	–/–	–/–	–/–	R/–	1/1	–	10
17	7	pre-B/n.a.	–/–	–/–	–/–	–/–	0/0	–	4
18	15	cALL/cALL	–/–	–/R	–/–	R/id	0/1	1	17
19	9	cALL/n.a.	–/–	R/id	–/ R_1R_2	R/id	0/2	2	7
20	6	cALL/cALL	–/–	R_1R_2/ R_1id+R_3	R_1R_2/id	–/–	1/4	1	14
21	9	cALL/cALL	–/R	–/ R1R_2	R_1R_2/id	–/R_1R_2	0/2	5	14
22ph	9	cALL/pre-B	R/id	–/R	R/id	R/id	0/3	1	26
23ex	3	cALL/cALL	R/id	R_1R_2/id	R_1R_2/ R_1/id	R_1id+R_2R_3	1/6	2	14
24	8	cALL/cALL	R/id	R/–	R_1/R_2	R_1R_2/id	2/5	1	16
25	11	pre-B/pre-B	R/id	R/id	–/–	R/id	0/3	–	24
26	5	cALL/cALL	R/id	–/–	–/–	$R_1R_2R_3$/id	0/4	–	17
27	11	cALL/cALL	R_1R_2/id	–/–	–/–	–/–	0/2	–	17
28	11	cALL/cALL	–/–	–/R	R_1R_2/id	–/R	0/2	2	19
29	11	pro-B/pro-B	R/–	–/–	R/id	R_1/id+R_2	1/3	1	38
30	9	pro-B/pro-B	–/–	R_1/R_2	R/–	$R_1R_2R_3$/ R_1id+R4	4/5	2	5
31	12	cALL/cALL	–/–	R/id	R/id	–/R	0/2	1	8
32	10	cALL/cALL	R_1/R_2	–/R	R/id	R/id	1/3	2	15
33ex	10	cALL/cALL	R_1R_2/ R_1id	R/–	R/id	$R_1R_2R_3$/ R_1id	4/7	–	16
34	12	cALL/cALL	–/–	R_1/R_2	–/–	R/id	1/2	1	23
35	9	cALL/cALL	R_1R_2/id	–/–	–/–	–/–	0/2	–	16
36	5	cALL/n.a.	–/–	R/id	R_1R_2/id	–/R_1R_2	0/3	2	23
37ex	9	cALL/cALL	–/–	–/R	R_1R_2/ id	–/R	0/2	2	18
38	6	cALL/cALL	–/–	R/id	R/id	R_1R_2/id	0/4	–	3
39	3	pre-B/n.a.	R_1R_2/id	–/–	–/–	R_1R_2/id	0/4	–	15
40	8	cALL/cALL	–/–	R/id	R/id	R_1R_2/id	0/4	–	13
41	7	pre-B/pre-B	R/–	R/id	R/id	R/id	1/4	–	57
42	8	cALL/cALL	R_1R_2/id+R_3R_4	–/–	R_1R_2/–	R_1R_2/ id	2/6	2	33
43ph	7	pro-B/cALL	–/–	–/–	–/–	R_1/id+R_2	0/1	1	48
44ph	13	pre-B/cALL	–/–	–/–	–/–	R_1R_2/R_1id	1/2	–	17
45	14	pro-B/pro-B	R/id	R_1R_2/id	–/–	R/id	0/4	–	42
46	12	cALL/cALL	R/id	–/–	R/–	R/id	1/3	–	89
47	18	cALL/n.a.	–/–	R/id	–/–	R_1R_2/–	2/3	–	50
48	11	cALL/cALL	–/–	R/id	R_1/R_2	–/–	1/2	1	35
49ph	7	cALL/pre-B	–/R	R/id	–/R	R_1R_2/R_1id	1/3	2	58

id: idem; rel.: relapse; n.a.: not available; R_{1-4}: clonal gene rearrangement ($_{1-4}$: numbered from 1 to 4); -: no gene rearrangement; no.ph: shift of immunophenotype; no.ex: additional extramedullary site; CCR: continuous clinical remission.

Table 3. Association between detection of more than one clonal PCR product per *Ig/TCR* gene locus and clonal evolution.

	TCRD		*TCRG*		*IGK*		*IGH*	
	%	n	%	n	%	n	%	n
Stability, when only one gene rearrangements per gene locus was detected	67	8/12	78	14/18	73	11/15	89	16/18
Stability, when two or more gene rearrangement per gene locus were detected	67	4/6	50	4/8	60	6/10	53	9/17
Stability of at least one gene rearrangement per gene locus within the latter group	83	5/6	100	8/8	70	7/10	88	15/17

Table 4. Comparison of patterns of clonal evolution between 1st and 2nd relapse in precursor B-ALL patients focusing on *Ig/TCR* gene loci in which clonal evolution occurred.

	TCRD	*TCRG*	*IGK*	*IGH*	
Patients who showed clonal evolution	10	15	11	20	
Patients with loss of rearrangements, while one or more remained stable	1	1	1	5	
Patients with gain of rearrangements at 2nd relapse, while the first relapse rearrangements remained stable		1	–	1	4
Patients with loss and gain of rearrangements, while the first relapse rearrangements remained stable	–	3	–	1	
Patients with gain of rearrangements at 2nd relapse, while at first relapse no rearrangements were detectable	3	7	2	6	
Patients with total loss of all gene rearrangements	4	2	5	4	
Patients with total loss and gain of rearrangements, while none were preserved at 2nd relapse	1	2	2	1	

nosis of second relapse. The newly detected *IgH* gene rearrangement at second relapse remained fully stable between this patient's second and third relapse. We assumed related leukemias between first and second relapse in all 25 patients.

At least half of the targets preserved at second relapse

In 15 patients (31%) at least half of the *Ig/TCR* gene rearrangements identified at first relapse were preserved at second relapse (mean: 2.5 of 3.9 gene rearrangements per patient). Thirteen of these 15 patients (87%) showed gain of 1 or 2 *Ig/TCR* gene rearrangements at second relapse. In these 15 patients, we again assumed related leukemias between first and second relapse.

Most targets lost at 2nd relapse with retention of at least one Ig/TCR target

In four patients (8%; patients #7, 30, 33, 46) most gene rearrangements were absent at second relapse but at least one rearrangement was common to both first and second relapse (Table 2). Approximately one-third of the gene rearrangements identified at first relapse were preserved at second relapse (mean: 1.8 of 5.3 rearrangements per patient). Three patients showed gene rearrangements of three different gene loci (mean: 4.7 rearrangements per patient), and in one patient (#33) rearrangements of all *Ig/TCR* gene loci were seen (7 gene rearrangements). Some possible explanations for this are continuing rearrangement processes within the leukemic cell population, and selection or expansion of subclone populations without particular *Ig/TCR* gene rearrangements.

Complete loss of Ig/TCR targets at second relapse

In four patients (8%; patients #2, 3, 6, and 16) all *Ig* and/or *TCR* gene rearrangements were lost at second relapse (Table 2). In all four of these patients, only one gene locus was rearranged at first relapse (1 or 2 rearrangements per patient). Additional Southern blot analysis of first and second relapse samples (patients #2 and #6) was not able to contribute to clarifying whether the leukemias were related or not. We therefore assumed the selection or expansion of subclones without detectable *Ig/TCR* gene rearrangements. In patient #6 an intralineage shift from pre-B-ALL to AML occurred (Table 2). We could not further describe the relation between the first and second relapse leukemia in this patient.

Clonal evolution and duration of second continuous complete remission

The median remission duration between the first and second relapse was 17 months (mean 22 months) and ranged between 2 and 89 months. According to ALL-REZ BFM criteria,[4,5] twenty-five children relapsed very early (< 18 months after diagnosis and < 6 months after the end of therapy), seven early (≥ 18 months after diagnosis and < 6 months after the end of therapy), and 17 late (≥ 6 months after the end of therapy). Among those patients with clonal evolution (36/48), late (15/36) and early (6/36) relapses occurred more frequently than among patients without clonal evolution (2/12; 1/12), but this did not achieve statistical significance ($p>0.05$). In contrast, among the patients with no immunogenotypic changes (n=12), very early relapses occurred significantly more frequently (9/12) than in those with changes (15/36; $p<0.05$).

Discussion

Stability-ranking

Regarding stability of each individual gene rearrangement as well as the necessity for at least one stable rearrangement per gene locus in order to prevent false-negative *MRD* results, the *TCRG* and *IgH* gene loci appear more reliable than *TCRD* and *IgK*-Kde gene loci (Tables 1 and 2). However, none of these stability results reached statistical significance ($p>0.05$).

The stability of *IgK*-Kde gene rearrangements between first and second relapse appears rather low in our study, since recently published studies on clonal stability between newly diagnosed ALL and first relapse reported a high stability of 72% to 92%.[7,13,16] *IgK*-Kde rearrangements are 'end-stage' rearrangements which cannot undergo a further rearrangement process. Therefore, these targets are likely to remain stable during the course of the disease.[16,38–40] Nevertheless, the unexpected loss of all *IgK*-Kde targets in 7 of 8 patients in whom immunogenotypic changes occurred at second relapse (Table 1) could be explained by successful eradication of the leukemic clone with the *IgK*-Kde rearrangements and expansion of a less chemotherapeutic sensitive subclone without *IgK*-Kde rearrangements. For instance, patient #30 showed one VκI-Kde rearrangement at first relapse which was not detectable at second relapse. Southern blot analysis of first and second relapse samples of patient #30 confirmed these findings and demonstrated both alleles of the second relapse sample in germline configuration.

Two targets or two gene loci?

In Southern blot-supported clonality studies between newly diagnosed ALL and first relapse it is already accepted that two, preferentially monoclonal MRD-PCR targets should be used per patient (i.e. two targets either detected from one or two *Ig/TCR* gene loci).[22] Southern blot analysis can distinguish between MRD targets of monoclonal and oligoclonal leukemic cell populations. Szczepanski *et al.* described this discrimination as the most powerful predictor of clonal evolution in Ig/TCR gene rearrangements between newly diagnosed ALL and first relapse.[22] Nevertheless, because of the large amounts of DNA needed and the more labor-intensive and time-consuming procedures, increasingly more MRD-PCR laboratories are not routinely performing additional Southern blotting. PCR-based analysis cannot discriminate between monoclonality and oligoclonality. However, the advantage of Southern blot analysis can be compensated by monitoring strategies which follow two rules: i) the use of all PCR-detected MRD targets of two *Ig/TCR* gene loci and ii) the priority order: *IGH>TCRG>TCRD>IGK-Kde* (our experiences with the patients in this study suggests that the selection of two *Ig/TCR* gene loci will be based mainly on the availability of detected gene rearrangements).

Selection-strategy

Based on these first data about clonal stability of *Ig/TCR* gene rearrangements between first and second relapse we designed a strategy to provide first guidelines for appropriate selection of PCR targets for *MRD* monitoring in children with relapsed precursor B-ALL (Figure 2). First of all, we suggest the use of MRD-PCR targets of two different *Ig/TCR* gene loci per patient (Figure 2: *first choice*) - provided that all detected rearrangements at these two gene loci are used as MRD targets. If more than two rearranged *Ig/TCR* gene loci are available, *IGH* and *TCRG* gene rearrangements should be preferred (priority order: *IgH>TCRG >TCRD>IgK-Kde*). This strategy would be applicable to 67% (33/49) of the patients in the current study, with a mean of 3.1 targets per patient and would enable successful detection of second relapse or, rather, prevent false-negative MRD-results in 100% of the patients (33/33).

In order to increase the applicability of MRD detection in children with ALL relapse, patients with only one rearranged *Ig/TCR* gene locus at first relapse but two or more rearrangements at this gene locus are also acceptable for MRD-PCR monitoring (*second choice*). In this series, another 8 patients could be included (8+33/49; 84%). Combining the *first* and *second choice* strategies, successful detection of second relapse would be possible in 98% (40/41) of the patients. The remaining 8 patients (16%) in this series would not be suitable for MRD-PCR monitoring. Seven patients showed one gene rearrangement at first relapse – this was preserved in only four at second relapse. One patient showed no detectable gene rearrangements, either at first or at second relapse.

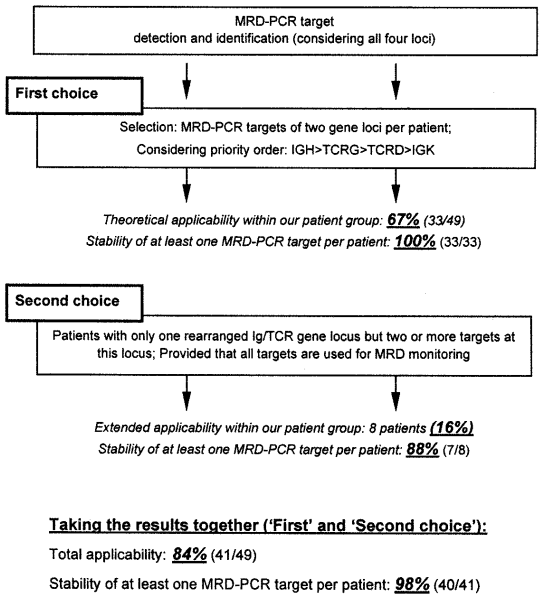

Figure 2. Flow diagram for the selection of MRD-PCR targets in relapsed childhood precursor B-ALL. The selection depends on the availability of two *Ig/TCR* gene loci (*first* and *second choice*) and the preference of *IgH* and *TCRG* gene loci. When *Ig/TCR* gene loci with more than one target have been selected, it is essential to use all targets of this particular gene locus for MRD-monitoring.

Table 5. Comparison of the stability of *Ig/TCR* targets in precursor B-ALL patients with or without immunophenotypic changes between 1st and 2nd relapse (n=44; in five patients, detailed information about the second relapse immunophenotype was not available).

	Patients with immunophenotypic-shift (n=9)		*Patients without immunophenotypic-shift (n=35)*	
	n	*(%)*	*n*	*(%)*
Patients in whom at least one target remained stable	8/9	(89)	32/35	(91)
Patients in whom at least two targets remained stable	6/9	(67)	26/35	(74)
Patients without any changes at all four gene loci	2/9	(22)	8/35	(23)
Applicability	7/9	(78)	30/35	(86)
Expected successful MRD detection following the strategy in this study	7/7	(100)	29/30	(97)

Remission duration

Some previous studies of clonal evolution between newly diagnosed ALL and first relapse have observed a correlation between the occurrence of clonal evolution and increasing duration of remission.[11,13,41] The authors suggested that the instability of targets increases as a function of time and therefore concluded that the incidence of clonal evolution between first and second relapse should be lower than that between newly diagnosed ALL and first relapse because of a shorter mean remission duration (mean interval second remission: 17 months; first remission: 30 months).[17,41] In our study we observed an almost similar stability of clonal targets between first and second relapse (mean interval: 21 months) as that published for the stability between newly diagnosed ALL and first relapse.[7,11,14,15,17,18,42,43]

Comparative immunophenotypic analysis

In this study we could not find any correlation between the appearance of immunophenotypic changes within the leukemic cell population from first to second relapse and the increase or decrease of clonal evolution (loss or gain of *Ig/TCR* targets). Table 5 compares the stability of *Ig/TCR* targets in patients with and without immunophenotypic changes. The phenomenon that clonal evolution at *Ig/TCR* gene loci does not correlate with the occurrences of immunophenotypic changes had been previously described in children between newly diagnosed ALL and first relapse.[44-46]

In view of the expected reliability of *Ig/TCR* targets in MRD-monitored studies following our selection strategy we could not find differences (Tables 3 and 5).

Representativeness of the group of patients

It is very difficult to assess a representative group in the context of clonality studies between first and second relapse, because it would be ethically discussible to request all samples (see *Design and Methods*). Nevertheless, we think that the selection in this study group is acceptable, since our study does not aim to make prognostic statements. We hope to confirm our data in a prospective study which is underway.

Conclusions

This study shows that *IgH*, *IgK-Kde*, *TCRD*, and *TCRG* gene rearrangements are reasonably stable targets for MRD monitoring in children with precursor B-ALL relapse. We produced a strategy for appropriate selection of PCR targets for MRD monitoring based on the recommendation of using all detected *Ig/TCR* gene rearrangements of two different gene loci (Figure 2). This strategy includes a priority order for the selection of *Ig/TCR* gene loci (*IgH>TCRG>TCRD>IgK-Kde*), even if the selection of two *Ig/TCR* gene loci will be based predominantly on the availability and the attainable sensitivity of detected gene rearrangements (e.g. *TCRG* targets are less sensitive in RQ-PCR analyses).[47] Based on these first data about stability of MRD-PCR targets in childhood precursor B-ALL between first and sec-

ond relapse, it is rather difficult to make any final recommendations. We are, therefore, extending these findings by a prospective study, presently underway. For precursor B-ALL patients who fall into the *second choice* strategy, we would recommend the use of additional MRD-PCR targets such as *TCRB* gene rearrangements. In the remaining patients (8/49; 16%), who would have to be excluded from MRD-PCR monitoring, we suggest the use of additional MRD methods, if available, such as flow cytometry or real-time quantitative reverse transcriptase-PCR of fusion gene transcripts in the hope of extending MRD detection to all children suffering from relapsed ALL.[48,49]

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Pre-publication Report & Outcomes of Peer Review

Contributions

AG, CE, GH, HGE and KS: responsible for the conception and design of the study; AG, CE, CH, TT, TS and VvV performed the experiments. AG, CE, CH and TT did the DNA-isolations, PCR-analysis, homo-heteroduplex-analysis and the sequencing. AG analysed and compared the sequences. VvdV and TS did the Southern blot analyses. AG, CE and TT managed the database of the study. AG, HGE and GH performed and discussed the statistical analysis. AG, CE, TT, TS, VvdV and CH discussed the data. AG, CE, CH, TT and HGE drafted the paper. TS, VHJ vdV, GH and KS revised critically for important intelectual content the paper. AG, CE, CH, TT, HGE, TS, VHJ vdV, GH and KS approved the final version to be published.

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Disclosures

Conflict of interest: none.

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Manuscript processing

This manuscript was peer-reviewed by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received October 25, 2002; accepted May 26, 2003.

In the following paragraphs, Professor Cazzola summarizes the peer-review process and its outcomes.

What is already known on this topic

Based on investigations between newly diagnosed acute lymphoblastic leukemia (ALL) and first relapse, it has been suggested that the instability of clonal markers increases as a function of time.

What this study adds

This study shows that a few immunoglobulin and T-cell receptor gene rearrangements are reasonably stable targets for MRD monitoring in children with precursor B-ALL relapse.