Minimal residual disease analysis in children with t(12;21)-positive acute lymphoblastic leukemia: comparison of Ig/TCR rearrangements and the genomic fusion gene

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Quantification of minimal residual disease (MRD) based on clonotypic immunoglobulin/T-cell receptor (Ig/TCR) gene rearrangements is widely used as an independent prognostic parameter in childhood acute lymphoblastic leukemia (ALL). In this study we compared MRD by quantification of Ig/TCR targets and genomic *ETV6-RUNX1* specific sequences. In ten of twelve patients with t(12;21)+ ALL we observed concordance with rapid blast reduction in nine, and high-level persistence in one case. The two remaining patients showed low-level persistence of the genomic breakpoint specific sequence. These patients have remained in complete remission for 38 and 41 months, so far, indicating that a small *ETV6-RUNX1*-positive clone is not detrimental to the short-term prognosis of affected children.

Key words: t(12;21) ALL, *ETV6-RUNX1* fusion gene, minimal residual disease, MRD, *TEL-AML1*.

Haematologica 2006; 91:683-686

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Minimal residual disease (MRD) has evolved as an independent prognostic parameter in childhood acute lymphoblastic leukemia (ALL) and is currently used in clinical trials for treatment stratification.[1,2] Development in technologies has made it possible to quantify the residual tumor load accurately. Immunoglobulin/T-cell receptor (Ig/TCR) gene rearrangements are the most commonly used DNA targets for the molecular monitoring of treatment response because of their high prevalence in lymphoid leukemia.[3] Although they provide a fingerprint for the leukemia clone these rearrangements are not causally related to the leukemia development and can undergo clonal selection and/or evolution processes leading to potential loss of the marker. In contrast, genomic fusion sites generated by chromosomal translocations are not only patient-specific but also appear to be identical in the translocation target cell and the evolving progeny. The *ETV6-RUNX1* fusion, also known as *TEL-AML1*, which results from the t(12;21)(p13;q22) chromosomal translocation, is an early or even initiating event in leukemia development.[4] It is, however, insufficient to produce a clinically overt leukemia. Secondary events, as proposed for the deletion of the non-rearranged *ETV6* gene, are necessary for the manifestation of the disease.[5] Differences in the boundaries of these *ETV6* deletions as well as changes of the Ig/TCR rearrangement between initial and relapsed leukemias support the hypothesis that at least some of the relapses of this leukemia subtype derive from the persistent *ETV6-RUNX1* pre-leukemic master clone by independent secondary events.[6-9] The detection of diverse Ig/TCR rearrangements at relapse of *ETV6-RUNX1* positive ALL provided the unique opportunity of tracking the different subclones, i.e. the dominant clone at initial diagnosis and the relapse clone, by their unique clonotypic rearrangements in two children with a late recurrence of leukemia.[7] We demonstrated that the dominant clone from initial diagnosis responded rapidly to chemotherapy while the relapsed clone that was present as a small subclone at initial manifestation reacted more slowly. Consequently, we wondered whether a fusion gene positive clone, which differs in its Ig/TCR rearrangements from the dominant clone at diagnosis, would be detectable in children with *ETV6-RUNX1* positive ALL. We thus evaluated response to treatment in 12 children using real-time quantitative polymerase chain reaction (RQ-PCR) of the genomic *ETV6-RUNX1* specific sequence as well as of clonotypic Ig/TCR rearrangements.

Design and Methods

Twelve children with t(12;21)-positive ALL (median age at diagnosis 3.0 years; range 1.5-8.3) were selected according to the availability of complete sample sets (DNA from bone marrow aspirations obtained at diagnosis and follow-up time points), the presence of at least two clonotypic Ig/TCR rearrangements in a leukemia appearing to be monoclonal (i.e. not more than two distinct rearrangements per each Ig/TCR locus)

Table 1. Clinical characteristics and molecular response of children with *ETV6-RUNX1* positive ALL.

Pt. ID	*Age at Diagnosis (yrs)*	*1st Remission Duration (mo)*	*Molecular Positivity (wks)§ Ig/TCR targets*	*Fusion gene*
1	2.9	36	2	2
2	1.9	35	2	2
3	5.3	45	2	2
4	3.1	32	2	2
5	3.7	61	0	0
6	8.3	29	5	8$
7	2.3	58	0	5$
8	3.1	29	5	0$
9	2.7	94	0	5$
10	**1.5**	**41**	**2**	**12***
11	2.8	55&	24	24
12	**5.7**	**38**	**2**	**104***

*§:molecular response in weeks (after the start of treatment) up to which the respective marker was detectable; samples were analyzed at weeks 2, 5, 8, 12, 24, 52 and 104; 0, indicates that in these patients no follow-up DNA from week 2 was available and the respective marker was negative at the next time point, i.e. week 5; °: the child underwent stem cell transplantation for high risk leukemia 8 months after initial diagnosis; $: discordant amount of target sequences measured at a single time point at the detection limit (10^{-4} or higher) of the other marker(s); *: discordant PCR levels on more than one occasion and at more than the 1 log level.*

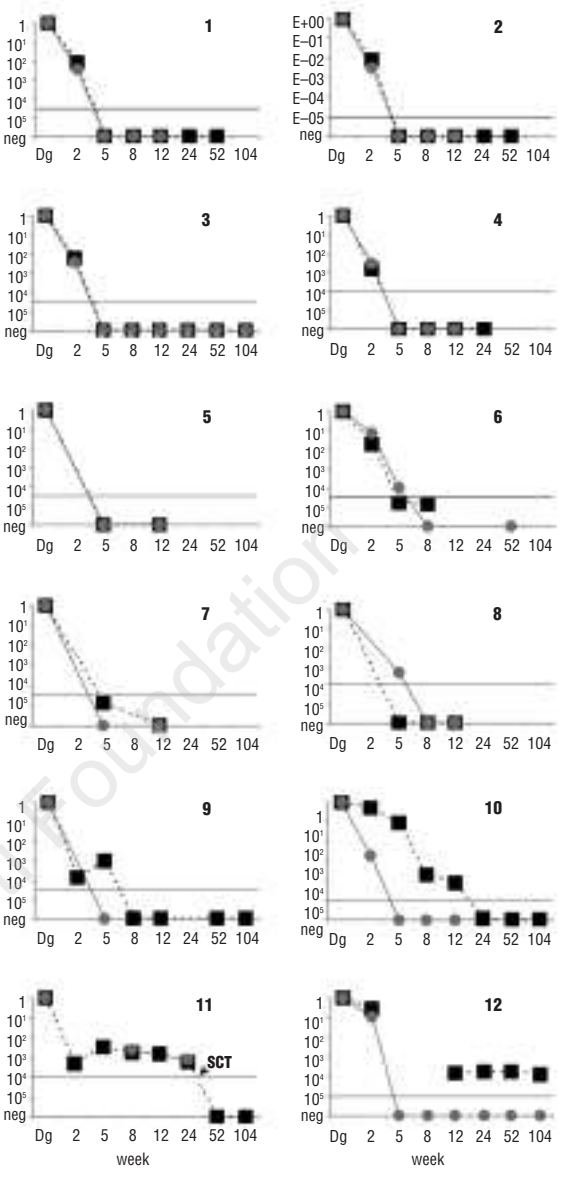

Figure 1. Kinetics of *ETV6-RUNX1* fusion gene and Ig/TCR rearrangements carrying clones during chemotherapy. Graphs show the clearance of *ETV6-RUNX1* fusion gene positive clones (black squares) as well as Ig/TCR positive populations (gray circles) in children with t(12;21) positive ALL during chemotherapy. On the y-axis, logarithmic levels indicate the amount of the respective gene in the bone marrow at defined time points during treatment, depicted on the x-axis. The gray line represents the detection limit of the target gene(s). In case of divergent results between fusion gene and Ig/TCR targets the gray line indicates the sensitivity of the negative target.

and a genomic *ETV6-RUNX1* specific sequence that allowed the design of specific probe sets with appropriate sensitivity (quantitative range of at least 10^{-4}) for RQ-PCR. All children were treated according to the ALL-BFM 2000 protocol.[10] The leukemias were characterized as part of the routine diagnostic procedures and in accordance with the required standards this included fluorescent *in situ* hybridization (FISH) analysis for the *ETV6* and *RUNX1* genes as described previously.[7] Informed consent for inclusion in this study was obtained from the parents or guardians of the children and the ethical committee of the collaborating institutions approved the study. The clinical characteristics of the study population are shown in Table 1.

DNA extraction and screening for clonotypic Ig/TCR rearrangements were performed according to standardized methods.[11] The amount of DNA used for standard curve and test samples was 500ng DNA per well. At least two clonotypic rearrangements were selected for the characterization of the leukemia clone according to established criteria used for MRD analysis. Quantification of rearrangements and interpretation of the data was done according to the guidelines established by the I-BFM MRD and ESG-MRD Study Groups.[12] Genomic *ETV6-RUNX1*-specific sequences were amplified by nested multiplex long-range PCR. Four primer sets, each with one *ETV6* sense, and four *RUNX1* antisense primers were used with the Expand Long Template PCR System (Roche) according to the manufacturer's instructions. Primer sequences have already been published.[7] Breakpoint-spanning PCR products were directly sequenced and specific primer/probe sets were designed for each patient (Primer Express® software, Applied Biosystems). RQ-PCR (ABI PRISM 7700 Sequence Detection System) was performed separately with two different aliquots of DNA, each in triplicate. The genomic *ETV6-RUNX1* specific sequences of all cases were submitted to GenBank (Acc No. AJ888040, DQ100455, AJ888041, AJ888038, DQ100456, AJ888037, DQ100457, AJ888036, DQ100458, AJ888033, DQ100459, AJ888035).

Results and Discussion

The simultaneous detection and quantification of at least two leukemia clone-specific Ig/TCR rearrangements and the patient-specific genomic *ETV6-RUNX1* specific sequence was prospectively assessed in 41 follow-up bone marrow samples from 12 children by DNA-based RQ-PCR. This molecular approach differs from that described in previously published studies,[13] as DNA markers allow quantification of absolute cell numbers as opposed to relative values obtained from fusion transcript-based reverse transcriptase PCR. In two of the 12 children (no. 10 and 12) in this study we observed varying, but clear-cut discrepancies with regard to the size of the clones as well as their persistence over time. The divergent results of the genomic breakpoint-specific positivity from the Ig/TCR targets were represented by a high, up to 2 log, difference in amplification in early follow-up samples, as well as by a low level detection at several time points up to two years of treatment. The remaining ten children had concordant results. Minor discrepancies observed in four of them (no. 6-9) were the consequence of different sensitivities of the Ig/TCR and the genomic breakpoint specific PCR, or were visible at only one early time point during treatment (Figure 1 and Table 1). As expected, the majority of children responded rapidly to the treatment, as they were already MRD-negative at week 5 of treatment. In nine of them, MRD negativity was determined concordantly using both target types while in two patients a breakpoint-positive clone was not detectable by Ig/TCR rearrangements.

In the remaining patient (no. 11) high-level MRD was concordantly detected and only dropped after stem cell transplantation, which was considered when the child was found to be at high-risk for relapse based on MRD analysis (equal or more than 10^{-3} at week 12 of treatment) (Table 1 and Figure 1).

The distribution of molecular clearance at week 5 by Ig/TCR targets in this study is similar to the overall results in 79 consecutively diagnosed children with *ETV6-RUNX1*-positive ALL treated according to the BFM-ALL 2000 protocol with a leukemia clearance of about 60-70% at week 5. Low level positivity (less than 10^{-4}) for Ig/TCR targets beyond week 5 of treatment - as was observed in this study with genomic breakpoint specific sequences in two cases - occurred in about 5% of the patients in the larger series and lasted up to week 12 of treatment, but not thereafter, probably representing a biologically similar *slow-responding* clone as detected by the patient-specific genomic breakpoint specific sequence in patients no. 10 and 12 from this study (*ERP-G, unpublished observation*). Persisting high levels of MRD, as detected in one case (no. 11) in this study, is known to be a rare event but exact data are not available. Of note, these low-level positivities do not affect the current risk group assignment within the BFM treatment protocol[14] and do not seem to affect the short-term outcome since none of the patients had an early (up to 30 months after diagnosis) recurrence. The persistence of a small, slowly responding clone further supports our assumption that this clone differs biologically from the fully malignant leukemia clone. The true nature of these cells does, however, remain elusive since these rare cells cannot be sufficiently enriched from limited volumes of partially regenerating bone marrow aspirates to allow for extensive characterization.

Our observation of a persisting *ETV6-RUNX1*-positive clone that lacks the clonotypic Ig/TCR rearrangements from diagnosis is not due to different sensitivities of the two types of targets since both reached equally high sensitivities (Figure 1). Based on our data[7,9,15] we envisage, as an explanation for the observed phenomenon, that the leukemia progenitor cell that is transformed by the fusion gene has already started to rearrange *Ig/TCR* genes. In the resulting pre-leukemic clone, rearrangements become heterogeneous due to the high potential for further recombination[15,16] and the cell that becomes fully leukemic may not share all its rearrangements with the remaining cells of the *ETV6-RUNX1* clone. Thus, a fusion gene positive clone may persist that has Ig/TCR rearrangements other than the dominant clone from initial diagnosis. This scenario is supported by recent data from comparisons of diagnostic and relapse samples indicating that clonal selection and evolution processes lead to diverse rearrangements at relapse, yet complete changes are rare.[17] We cannot, however, exclude the possibility that the fusion gene occurs in a progenitor cell before the start of somatic recombination, as suggested previously.[18]

We conclude that low-level positivity by genomic breakpoint specific amplification during treatment does not currently indicate a change in short-term prognosis in patients treated with BFM-based protocols. Its prognostic relevance for late relapses, whether detected by Ig/TCR rearrangements or the genomic *ETV6-RUNX1* specific sequence, needs to be demonstrated on a larger number of patients after an extended observation period, given the particular biological features of this leukemia subtype laeding to late relapses.[1]

MM, GM and ER P-G were responsible for the design and conception of the study, analysis and interpretation of data and for writing the manuscript. UM, ML, CG, TF, SV, TL and OAH performed the experiments and contributed to drafting the manuscript. MS and HG provided patients' samples and clinical data. All authors approved the final version of the manuscript. Susanna Fischer is a further coauthor. The authors acknowledge the participating centers of the ALL BFM 2000 study, Martina Peham for initial experiments and Marion Zavadil for proofreading the manuscript. This collaboration was performed using the network of the I-BFM Biology Group. The authors declare that they have no potential conflicts of interest.

This work was supported in part by GENAU-CHILD, the Österreichische Kinderkrebsforschung and private donations to the CCRI. MM, CG and TL were supported by the Wilhelm Sander Foundation, TF and SV by the KPOH.

Manuscript received November 18, 2005. Accepted March 6, 2006.

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