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ORIGINAL ARTICLE

Acute lymphoblastic leukemia with t(4;11) in children 1 year and older: The 'big sister' of the infant disease?

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The t(4;11)-positive acute lymphoblastic leukemia (ALL) is a rare disease in children above the age of 1 year. We studied the clinical and biological characteristics in 32 consecutively diagnosed childhood cases (median age 10.0 years, range 1.0–17.1 years). Immunophenotyping revealed a pro-B and a pre-B stage in 24 and eight cases, respectively. *IGH* genes were rearranged in 84% of leukemias with a predominance of incomplete DJ_H joints. Whereas *IGK*-Kde and *TCRD* rearrangements were rare, *TCRG* rearrangements were present in 50% of cases and involved mainly Vγ11 or Vγ9 together with a Jγ1.3./2.3 gene segment, an unusual combination among t(4;11)-negative B-cell precursor ALL. Oligoclonality was found in about 30% as assessed by heterogeneous *IGH* and *TCRG* rearrangements. Our data are in line with transformation of a precursor cell at an early stage of B-cell development but retaining the potential to differentiate to the pre-B cell stage *in vivo*. Although a distinct difference between infant and older childhood cases with t(4;11) became evident, no age-related biological features were found within the childhood age group. In contrast to infants with t(4;11)-positive ALL, childhood cases had a relatively low cumulative incidence of relapse of 25% at 3.5 years with BFM-based high-risk protocols.
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Introduction

The t(4;11)(q21;q23) chromosomal translocation, resulting in the *MLL-AF4* fusion gene, is the most prevalent of the numerous *MLL* fusion genes and is mainly associated with pro-B acute lymphoblastic leukemia (ALL) in infants (less than 1 year at diagnosis).[1,2] In this age group the t(4;11) comprises about 50% of ALL, with a predominance in the first 6 months and a clear decline with age to reach a 10% incidence by the end of the first year.[3,4] The general incidence of t(4;11) in childhood ALL up to the age of 15 years, excluding infancy, is approximately 2%.[5,6] Although the prognosis was shown to be similarly poor for infant and childhood *MLL-AF4*-positive ALL a decade ago,[7] recent studies suggested a better outcome in children 1 year of age or older.[8–10] The reason for this age-related difference is still a matter of debate, as is a potentially biased distribution of the leukemia-specific *MLL* breakpoints of patients as a function of age.[11]

Immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements are regarded as a molecular fingerprint of each individual lymphocyte and its progeny and are therefore widely used as clonal markers in lymphoid malignancies and as polymerase chain reaction (PCR) targets for the detection of minimal residual disease.[12,13] In B precursor cells, the recombination of *IGH* genes follows a hierarchical order: first, a D_H segment is joined to a J_H segment on both alleles, before one V_H segment is rearranged to one of the DJ_H joints. If this rearrangement is not potentially productive, the other incomplete DJ_H rearrangement undergoes further recombination. If successful, an Igμ protein is expressed in the cytoplasm. On the other hand, if this attempt – including additional rescue mechanisms – fails to produce a functional Igμ protein, the cell will undergo apoptotic cell death unless it carries a mutation that interferes with the execution of that decision.[14,15] This order of recombination seems to be maintained in B-cell precursor (BCP) ALL[16]. The frequency and types of rearrangements have been related to the genotype of BCP leukemias in the past and they also appear to be influenced by the target cell of the chromosomal translocation and the effects of the resulting fusion gene (*TEL-AML1, E2A-PBX1*).[17,18] In addition, the age of the affected children may add further restrictions to the repertoire of rearrangements.[18,19] Although an extensive analysis of Ig/TCR rearrangements was recently performed in infant ALLs including those with the *MLL-AF4* fusion gene,[3] no such data are available on the small subgroup of older children with this particular fusion gene carrying ALL.

We thus assessed the frequency and type of clonotypic Ig/TCR rearrangements of *MLL-AF4*-positive ALLs from 32 children 1 year of age or older as well as their short-term prognosis. We describe here a distinct pattern of Ig/TCR rearrangements that differs from the infant cases with regard to its maturity, rather resembling those of the adult patients. Further, the short-term relapse free survival was fairly good with 25% cumulative incidence of relapses after a median observation time of 3.5 years with BFM-based treatment protocols.

Patients and methods

Patients and leukemias

We collected 32 t(4;11)-positive ALL from children above the age of one and adolescents up to the age of 18 years, who were consecutively assigned to one of the current BFM-based

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treatment protocols in Austria ($n=5$), Germany ($n=7$), Italy ($n=17$) and The Netherlands ($n=3$).[20] All materials were obtained after informed consent of the parents and with approval of the Ethics Committee of the Children's Cancer Research Institute, the St Anna Children's Hospital and the collaborating institutions.

Morphological diagnosis of ALL and immunophenotyping was performed according to standard criteria in the reference laboratories of the collaborating countries.[21] The presence of a t(4;11)(q21;q23) translocation and/or the *MLL-AF4* fusion gene was demonstrated by one or more of the following methods: metaphase cytogenetic analysis, PCR with reverse transcription (RT-PCR)[22] and fluorescence *in situ* hybridization (FISH)[23] as part of the diagnostic work-up.

Detection and analysis of clonotypic Ig/TCR rearrangements

For the immunogenotyping of leukemias (i.e. the summary of all Ig/TCR rearrangements per leukemia), the incomplete and complete *IGH* rearrangements, *IGK*-Kde, *TCRD* and *TCRG* rearrangements were analysed. All laboratory procedures for the identification and characterization of rearrangements starting from cell separation, DNA extraction, PCR-based screening for the detection of the indicated Ig/TCR rearrangements, as well as the sequencing and interpretation of the data were standardized between the four laboratories and were performed as described earlier (Van der Velden *et al*., in press).[12,17] In the majority of cases, all obtained PCR products were sequenced independent of their abundance. However, some very faint PCR products were not sequenced and thus not included in our analysis.

Definition of clonality and oligoclonality

Assuming that a cell carries two alleles of each Ig and TCR gene, a leukemia was considered monoclonal if a maximum of two unrelated rearrangements per Ig/TCR gene were detected. In any other case, be it the presence of partially related rearrangements or more than two rearrangements, the leukemia was defined as oligoclonal.

Statistical analysis

To compare the frequency of Ig/TCR rearrangements or specific types of rearrangements between different age groups and/or BCP ALL with different genotypes, the two-sided Fisher exact test was used. The cumulative incidence of relapses was estimated by the method of Kaplan and Meier. Only children who survived the first 5 weeks of treatment were included in the analysis.

Results and discussion

Immunophenotype

Thirty-two children with a median age of 10.0 years (range 1.0–17.1) at diagnosis of a t(4;11)+ ALL were included in this study. Interestingly, the age distribution of the childhood cases above 1 year did not show a further decrease with age, which would have been expected from the infant cases where a decrease from almost 50% in the first 2 months to 10% at the end of the first year has been described.[3] Indeed, the 32 cases were evenly distributed over the entire age period. For further potential age-related association we assigned the patients to one of the two age groups, aged up to 9 years ($n=14$, 44%) and 9 years and older ($n=18$, 56%), based on the prognostic influence of age in children with BCP ALL[8]. Immunophenotyping revealed a pro-B and a CD10-negative pre-B (cytoplasmic Igμ positivity in >20% of leukemic cells) type in 24 (75%) and 8 (25%) leukemias, respectively, which was not influenced by age. This is in contrast to infant cases with t(4;11), which have been reported to lack Igμ positivity.[3] Thus, it appears that the pre/leukemic clone in children 1 year and older retained, in general, the potential to rearrange functionally and express its *IGH* genes. Since this differentiation to the pre-B immunophenotype was equally spread over the childhood age, it cannot solely be attributed to a longer latency period, provided the leukemia initiating event indeed occurred *in utero*. This aspect of leukemia development, however, has, so far, not been addressed in the childhood *MLL-AF4* cases.

In the total group of childhood BCP ALL, independent of the genetic subtype, the pre-B phenotype occurs in about 20–35% of cases,[17,24] potentially reflecting the overall success rate of normal precursor B cells to productively rearrange their *IGH* genes, as has been suggested in the past.[25] The t(1;19)+ALL is an exception to the above consideration since for this subgroup convincing evidence was provided that the transforming event takes place at the pre-B stage.[26]

Frequency of Ig/TCR rearrangements

All but two cases from this study had PCR-detectable Ig and/or TCR rearrangements with a median number of two rearrangements (range 1–7) per leukemia. In the leukemias that lacked Ig/TCR rearrangements a potential failure for specific amplification was excluded by satisfying control gene amplification and repeated negative results of the screening PCRs. It may therefore be assumed that these leukemias had rearrangements that were not covered by the primer sets used or that in these children the translocation had occurred in a cell before the start of V(D)J recombination. Twenty-seven of the 32 leukemias (84%) had the *IGH* locus rearranged with a total number of 56 rearranged alleles (Table 1). Among these, the incomplete DJ_H rearrangements dominated over the complete VDJ_H rearrangements (31 and 25, respectively). Interestingly, only six leukemias appeared to have a monoallelic rearrangement (DJ_H, $n=1$; VDJ_H, $n=5$) whereas the others had at least two rearrangements. Of the 21 leukemias with two (or more) *IGH* rearrangements five had

Table 1 Frequency of rearranged Ig/TCR loci in *MLL-AF4*-positive ALLs in different age groups

Antigen receptor gene locus	*Childhood cases (n=32) (%)*[a]	*Infant cases (n=51) (%)*[b]	*Adult cases (n=12) (%)*[c]
D_H-J_H	66*	90	NA
V_H-DJ_H	66*	41	NA
Mono/biallelic *IGH*	19/66	NA	NA
Total *IGH*	84	92	79
IGK-Kde	13	6	NA
TCRD	19	24	38
TCRG	50*	16	50
Mono/biallelic *TCRG*	56/44	NA	57/43

Abbreviation: NA, not analysed.
[a]This study.
[b]Jansen *et al*.[3]
[c]Brumpt *et al*.[18]
*Indicates a significant difference between childhood and infant cases (two-sided Fisher exact test, $P<0.05$), but not adult cases (where applicable).

exclusively incomplete DJ_H rearrangements but the vast majority of cases had already a combination of at least one DJ_H with a VDJ_H rearrangement ($n=15$). A single case had two complete VDJ_H rearrangements. Again, there was no significant difference with regard to the two age groups in our cohort of patients when considering the frequency of incomplete and complete *IGH* rearrangements.

We further compared the types of *IGH* rearrangements (DJ_H and VDJ_H) with published data and demonstrated a tendency towards a more mature (i.e. VDJ_H) pattern of *IGH* rearrangements as compared to infant cases,[3] whereas the frequency of *IGH* rearranged leukemias is similar in all age groups including adults[18]. Of note, the data from this study were exclusively obtained by PCR analysis, as were the data from adults, but infant cases were studied by both PCR and Southern blotting.

Further characteristics of these *IGH* rearrangements with regard to the usage of V_H, D_H and J_H segments revealed a comparable distribution as found in other BCP ALL in children (including TEL-AML1+ ALLs as well as the control group comprising hyperdiploid and 'others').[17]

The incidence of *IGK*-Kde rearrangements was only 13% (Table 1), with three of the four positive cases having a pre-B immunophenotype. The number of pre-B ALLs, however, is too small to analyse them separately with regard to Ig/TCR rearrangement status.

Cross-lineage *TCRD* (Dδ2-Dδ3/Vδ2-Dδ3) rearrangements occurred in six leukemias, one Dδ2-Dδ3 together with a Vδ2-Dδ3 and five Vδ2-Dδ3. In contrast to this low frequency of *TCRD* rearrangements, the *TCRG* locus was rearranged in 50% of cases – similar to the adults but unlike the infant cases with only 16% of cases (Table 1). The frequency of *TCRG* rearranged cases among the children in this study was not associated with age (50 and 53% in $<$9- and $\geqslant$9-year olds, respectively).

TCRG Vγ and Jγ segment usage

As shown in Table 2, usage of the Vγ11 (VγIV) segment prevailed, followed by Vγ9 (VγII) and, less frequent, by a member of the VγI family. Two-thirds of rearrangements involved the Jγ1.3/2.3 gene segments, whereas one-third involved Jγ1.1/2.1gene segments, a distribution that was independent of the Vγ segment usage. This skewed pattern of *TCRG* rearrangements was comparable to the one reported from a small number of adult *MLL-AF4* cases (Table 2) and thus seems uniquely associated with the *MLL-AF4* fusion gene in non-infant cases. This is in contrast to childhood BCP ALLs with other genotypes: the *TEL-AML1*-positive ALL had a high frequency of mature 'end-stage' *TCRG* rearrangements and also *TEL-AML1*-negative BCP (hyperdiploid and others) show usage of mainly upstream VγI gene segments.

Table 2 Characteristics of rearranged *TCRG* alleles in ALL as a function of *MLL-AF4* status and age

Gene segment usage	*MLL-AF4+ALL This study (29 alleles) %*	*MLL-AF4+adult cases[a] (10 alleles) %*	*BCP ALL TEL-AML1+ (260 alleles) %[b]*	*BCP-ALL hyperdiploid and 'others' (145 alleles)[b]*
VγI	28*	10	63	70
VγII	24	30	36	27
VγIII	0	20	0	0
VγIV	48*	40	1	3
Jγ1.1/2.1	34[c]	20	11	38
Jγ1.3/2.3	66[c]	80	89	62

[a]Brumpt *et al*.[18]
[b]Huebner *et al*.[17]
[c]Statistical difference between childhood *MLL-AF4*-positive leukemias and *TEL-AML1*-positive cases (two-sided Fisher exact test, $P<0.001$).
*Indicates a significant difference between the childhood *MLL-AF4*-positive and *MLL-AF4*-negative childhood ALL cases (two-sided Fisher exact test, $P<0.001$), but not the adult cases.

Next, we assessed whether the recombination of Ig and TCR genes followed the classical hierarchical order of V(D)J recombination and whether this would be related to the different maturation stages as defined by the presence of exclusively incomplete DJ_H rearrangements and those that are characterized by the appearance of a complete VDJ_H. Four of the six cases (67%) with exclusive DJ_H rearrangements and seven of the 21 cases (33%) with at least one complete VDJ_H rearrangement had no other Ig or TCR genes rearranged. Thus, leukemias with a more immature *IGH* rearrangement seem to have less additional rearrangements. As expected, cross-lineage *TCRD* and *TCRG* rearrangements coincided frequently.

Oligoclonality

The frequency of oligoclonality in this study cannot be directly compared with other reports, which present the overall frequency as a combined information of PCR plus Southern-blot analysis and, as a consequence, have much higher percentages of clonal variation. The data from this study, however, are comparable to our recent investigation on the large subgroup of *TEL-AML1*+ leukemias and their respective control group that comprised hyperdiploid as well as cytogenetically not homogenously characterized BCP ALLs (17), since they were analysed by the same PCR approach. The *MLL-AF4*+ subgroup clearly shows a higher incidence of clonal heterogeneity (31%) than the *TEL-AML1* and hyperdiploid subgroups (*TEL-AML1*+ ALL 20% and *TEL-AML1*-negative cases – including about 50% of hyperdiploid leukemias with three chromosomes 14–34%). Interestingly, clonal variations in the *IGH* locus were present in 22% and did not overlap with those of the *TCRG* locus with 9%. In the *IGH* locus, oligoclonality was almost exclusively derived from multiple unrelated rearrangements and only one case had two clones characterized by a partial sequence homology. In this leukemia, a V_H segment was joined to a pre-existing incomplete DJ_H rearrangement. These findings together with multiple unrelated *IGH* rearrangements in our study are in line with the overall immature immunogenotype in t(4;11)-positive ALL[27] as well as with the proposed undifferentiated target cell of this translocation.

Ig/TCR rearrangements in relation to age

Since age-related patterns of Ig/TCR rearrangements within the childhood BCP ALL have been reported previously[18,19], we also evaluated the possibility that such association was present in our cohort. We could, however, not detect such a correlation between age and any of the rearranged Ig/TCR loci, suggesting that the reported age-related changes are mainly because of the different frequencies of particular genetic subgroups (e.g. *TEL-AML1* or hyperdiploid).

Clinical outcome

Apart from immunogenotyping, we assessed the relapse rate in our patient group. So far, six of the 26 children with a minimal observation time of one year (median, 3.5 years; range, 1–7.3 years) have relapsed (median remission duration, 0.8 years; range, 0.5–1.4 years) after initial diagnosis. Thus, the estimated

3.5-year cumulative incidence of relapse according to Kaplan and Meier is 25% (±9). There were four patients with a treatment-related death (early death, $n=3$; one transplant-related death 1 year after diagnosis) resulting in a cumulative incidence of treatment related fatalities of 16% (±7). Hence, the 3-year event free survival is 59% (±10). These data are in line with the reported early relapse incidence in the majority of t(4;11)-positive cases[8] and further support a superior relapse free survival of children older than 1 year of age when treated according to BFM-based protocols, which classifies them as high risk and thereby makes them eligible for stem cell transplantation. The overall prognostic significance of this preliminary observation as well as of the treatment-related mortalities, however, needs to be re-evaluated after a prolonged observation time, preferably in a larger cohort.

Collectively, our immunogenotype data in t(4;11)-positive ALL from children 1 year or older at diagnosis demonstrated a more mature pattern of *IGH* rearrangements as compared to infant cases. This maturation is also reflected by the ability to create a productive *IGH* rearrangement and consequently express the Igμ protein in the cytoplasm. However, the 25% of cases with a pre-B immunophenotype still did not express CD10, in line with the reported lack of CD10 expression in *MLL-AF4*-positive ALL, a hallmark of this translocation.[1,28] In line with our results the potential of leukemic cells to differentiate *in vivo* was independent of the children's age (1–18 years), but clearly differed from the infant cases that were of the pro-B immunophenotype. The higher number of *TCRG* rearrangements, as reported here, shows, in a similar way, an age-group related distribution, since it increased from infancy to childhood by more than threefold and retained a similar high frequency in adult cases. The tendency of infant ALL to undergo *TCRG* rearrangements also depends on the genotype of the leukemia since 17% of infant leukemias with *MLL*-rearrangement, but 31% without *MLL* rearrangement have *TCRG* genes rearranged.[3]

Up to now, it remains unclear which mechanisms contribute to the biological differences of t(4;11)-positive ALL in infants as opposed to older children. However, several pieces of evidence have emerged lately suggesting that the target cell, the microenvironment or the exposure to genotoxic chemicals, either one of these three factors alone or, more likely, a combination thereof, may modulate the disease. One possibility is that the target cell as well as the microenvironment differs between infants and older children. The liver serves as the primary site of hematopoiesis in the fetus and *MLL* rearrangements can be induced by topoisomerase II inhibitors in hematopoietic stem cells from this organ.[29,30] Another possibility is that the duration of transplacental injury to hematopoietic stems cells during pregnancy, either continuous or intermittent, might render the cell vulnerable not only to the initiating *MLL* gene fusion but also to the rapid acquisition of secondary events resulting in the very short latency of infant leukemias.[30,31] This may also suggest that the t(4;11) ALL after infancy might arise later in an ontogenetically younger cell from a different microenvironment (e.g. the bone marrow) in combination with a shorter exposure to specific toxins. There is only circumstantial evidence for a different target cell/environment of the leukemia in the older children that supports the differentiation of the leukemic cells up to the pre-B stage as observed in 25% of the patients aged 1 year or older. In support of a changed microenvironment are studies of the leukemic t(4;11) cell line BLIN3, derived from a 3-month-old infant, which indeed differentiated in culture on bone marrow feeder layers to the pre-B stage.[32] This might imply that even infant leukemias are able to differentiate under certain conditions that are not generally present in the fetus. So far, these explanations remain, however, speculative and only further research will eventually elucidate the mechanisms leading to the infant and childhood disease, respectively.

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