Frequent and sex-biased deletion of SLX4IP by illegitimate V(D)J-mediated recombination in childhood acute lymphoblastic leukemia

Barbara Meissner1,2, Thies Bartram1, Cornelia Eckert3, Jan Trka4, Renate Panzer-Grümayer5, Ivana Hermanova4, Eva Ellinghaus6, Andre Franke6, Anja Möricle1, André Schrauder1, Andrea Teigler-Schlegel7, Petra Dörge1, Arend von Stackelberg3, Giuseppe Basso8, Claus R. Bartram9, Renate Kirschner-Schwabe3, Beat Bornhäuser10, Jean-Pierre Bourquin10, Giovanni Cazzaniga11, Julia Hauer12, Andishe Attarbaschi15, Shai Izraeli13, Marketa Zaliova2,4, Gunnar Cario1, Martin Zimmermann2, Smadar Avigad14, Magdalena Sokalska-Duhme15, Markus Metzler16, Martin Schrappe1, Rolf Koehler9, Geertruy te Kronnie8 and Martin Stanulla2,

1Department of Pediatrics, University Hospital Schleswig-Holstein, Kiel, Germany 2Pediatric Hematology and Oncology, Hannover Medical School, Hannover, Germany 3Pediatric Hematology and Oncology, Charité University Hospital, Berlin, Germany 4Childhood Leukaemia Investigation Prague (CLIP), Department of Pediatric Hematology/Oncology, 2nd Faculty of Medicine, Charles University Prague, Prague, Czech Republic 5Department of Pediatrics, St. Anna Children’s Hospital and Children’s Cancer Research Institute, Medical University Vienna, Vienna, Austria 6Institute for Clinical Molecular Biology, Christian-Albrechts-University, Kiel, Germany 7Oncogenetic Laboratory, Pediatric Hematology and Oncology, University of Giessen, Giessen, Germany 8Department of Women’s and Children’s Health, University of Padua, Padua, Italy 9Department of Human Genetics, University of Heidelberg, Heidelberg, Germany 10University Children’s Hospital, Zurich, Switzerland 11M. Tettamanti Research Center, Children’s Hospital, University of Milan-Bicocca, Monza, Italy 12Department of Pediatric Oncology, Hematology and Immunology, Heinrich-Heine-University, Düsseldorf, Germany 13Sheba Medical Center Tel-Hashomer, Ramat Gan, Israel 14Pediatric Hematology Oncology, Schneider Children’s Medical Center of Israel, Petach Tikva, Israel 15Pediatric Hematology and Oncology, Olga Children’s Hospital, Stuttgart, Germany 16Pediatric Hematology and Oncology, University Children’s Hospital Erlangen, Erlangen, Germany

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Acute lymphoblastic leukemia (ALL) accounts for ~25% of pediatric malignancies. Of interest, the incidence of ALL is observed ~20% higher in males relative to females. The mechanism behind the phenomenon of sex-specific differences is presently not understood. Employing genome-wide genetic aberration screening in 19 ALL samples, one of the most recurrent lesions identified was monoallelic deletion of the 5′ region of SLX4IP. We characterized this deletion by conventional molecular genetic techniques and analyzed its interrelationships with biological and clinical characteristics using specimens and data from 993 pediatric patients enrolled into trial AIEOP-BFM ALL 2000. Deletion of SLX4IP was detected in ~30% of patients. Breakpoints within SLX4IP were defined to recurrent positions and revealed junctions with typical characteristics of illegitimate V(D)J-mediated recombination. In initial and validation analyses, SLX4IP deletions were significantly associated with male gender and ETV6/RUNX1-rearranged ALL (both overall P < 0.0001). For mechanistic validation, a second recurrent deletion affecting TAL1 and caused by the same molecular mechanism was analyzed in 1149 T-cell ALL patients. Validating a differential role by sex of illegitimate V(D)J-mediated recombination at

*To whom correspondence should be addressed at: Department of Pediatric Hematology and Oncology, Hannover Medical School, Carl-Neuberg-Strasse 1, D-30625, Germany. Tel: +49 5115327978; Fax: +49 5115329120; Email: stanulla.martin@mh-hannover.de

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the TAL1 locus, 128 out of 1149 T-cell ALL samples bore a deletion and males were significantly more often affected (P = 0.002). The repeatedly detected association of SLX4IP deletion with male sex and the extension of the sex bias to deletion of the TAL1 locus suggest that differential illegitimate V(D)J-mediated recombination events at specific loci may contribute to the consistent observation of higher incidence rates of childhood ALL in boys compared with girls.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy (1–3). Despite intensive research efforts, the causes of childhood ALL remain largely unknown. Sex bias—characterized by a 20% higher incidence in males relative to females—is consistently observed in childhood ALL (2–4). In addition, male sex is associated with worse treatment response and outcome (5,6). Therefore, a better understanding of this phenomenon could directly enhance our understanding of disease etiology and improve therapeutic approaches to ALL.

From a genetic perspective, ALL is characterized by recurrent numeric and/or structural somatic aberrations (7). Hyperdiploidy or the cryptic chromosomal translocation t(12;21)—leading to an ETV6/RUNX1 gene fusion—are detectable in 20–25% of cases each, making them the most common genetic subtypes of childhood ALL (7,8). Important for pathomechanistic insights, the molecular features associated with the breakpoints of structural genetic aberrations in ALL are multifold and include, for example, an open chromatin context during gene transcription, Alu and other repeat sequences, or illegitimate V(D)J-mediated recombination (7).

V(D)J recombination is a physiological process by which segments (variable (V), diversity (D), joining (J)) of immunoglobulin (Ig) or T-cell receptor (TCR) genes are rearranged and lead to great diversity of the Ig/TCR repertoire. This process is mediated by lymphocyte-specific endonucleases (RAG1, RAG2) which cut the regional V(D)J genes at flanking recombination signal sequences (RSS) consisting of specific highly conserved heptamer and nonamer sequences with an unconserved spacer (12 or 23 nucleotides) in-between (9,10). Subsequently, the coding segments are joined using the classical non-homologous end-joining (NHEJ) pathway. Paradoxically, RAG proteins can recognize a variety of slightly modified RSS sequences increasing the chance of erroneously targeting RSS-like sequences (“cryptic RSS”) elsewhere in the genome. Several chromosomal translocations between Ig/TCR loci and proto-oncogenes as well as deletions of non-antigen receptor loci in lymphoid malignancies are thought to be generated by such illegitimate V(D)J-mediated recombination events (11,12). Recently, interstitial deletion of the B-cell translocation gene 1 (BTG1) in 9% of precursor B-cell ALL has been attributed to this mechanism (13). Also, subtypes of CDKN2A and IKZF1 deletions as well as all TAL1 deletions have been associated with off-target action of the RAG complex (12,14–16).

In the present study, we describe a highly frequent deletion in SLX4IP—a gene encoding a currently uncharacterized DNA repair-related protein—by illegitimate V(D)J-mediated recombination (17). SLX4IP deletion has previously been reported in childhood ALL, but at a far lower frequency (18,19). We correlate this finding to demographic and clinical characteristics in a large cohort of children with ALL and describe its specific association with ETV6/RUNXI-rearranged ALL and male sex.

RESULTS

In our initial screen for recurrent genetic aberrations in childhood ALL employing comparative genomic hybridization (CGH) analysis, 5 out of 19 samples—making it one of the most common observations—harbored a monoallelic deletion encompassing the first two exons of the SLX4IP gene with tight breakpoint clustering to a defined position (HG18, chromosome 20: ~10 363 654–10 404 199 bp; Fig. 1A). Sequencing analysis in the five deletion-positive samples revealed patient-specific breakpoints within only a few nucleotides of each other—confirming site specificity of the recombination event—and demonstrated near perfect matches to 5’ and 3’ heptamer sequences (consensus 5’-CACAGTG, non-templated ‘N’ nucleotides and ‘nibbling away’ of nucleotides at the junction points as hallmarks of illegitimate V(D)J-mediated recombination (Fig. 1B–D)(11,12,14,16,20). No convincing AT-rich nonamer sequences (consensus 5’-ACAAAAACC) were found at 12 or 23 nucleotides distance of the heptamers. As all five patients displaying the SLX4IP deletion at initial diagnosis relapsed, we next analyzed ALL samples obtained at disease recurrence for maintenance of aberrations during leukemic evolution and detected the same patient-specific deletion breakpoints in all samples (Fig. 1D).

As the above analyses were performed on selected patients, we next aimed at a reliable assessment of the SLX4IP deletion frequency in a cohort of 512 patients enrolled into AIEOP-BFM ALL 2000 (Cohort 1, Table 1, Supplementary Material, Fig. S1) (5,6). Employing a PCR assay, the deletion could be detected in 164 children (32.0%). When analyzing the association of SLX4IP deletion with clinical characteristics, the most significant positive interrelationships were observed for male sex (P < 0.001) and ETV6/RUNXI-rearranged ALL (P < 0.001) (Table 1). In an analysis stratified by ETV6/RUNX1 translocation status, the male sex bias stayed significant in translocation positive (P < 0.001) as well as negative patients (P < 0.001) (Tables 2 and 3). No association of SLX4IP deletion with treatment outcome was detected (Supplementary Material, Fig. S2).

To validate the observed interrelationships, we next screened an independent cohort of 232 patients with ETV6/RUNXI-rearranged ALL from AIEOP-BFM ALL 2000 (Cohort 2) and identified 145 (62.5%) deletion-positive samples. In these analyses, both the association of SLX4IP deletion with male sex (P = 0.049) and its high incidence in ETV6/RUNXI-rearranged ALL could be confirmed (Table 3, Supplementary Material, Table S1). In a second validation step, we screened 249 leukemic ETV6/RUNXI-negative samples from AIEOP-BFM ALL 2000 patients enrolled in the years 2001 and 2002 (Cohort 3) and, again, found a significant positive association of SLX4IP...
deletion status with male sex (P = 0.029) (Table 3, Supplementary Material, Table S2).

To consolidate our findings on SLX4IP deletion characteristics at the nucleotide level which were based on five samples only, we continued by sequencing 38 additional leukemic patient DNA samples and detected site-specific SLX4IP breakpoint junctions with typical characteristics of illegitimate V(D)J-mediated recombination in all specimens (Supplementary Material, Table S3). This suggests that—similar to T-cell ALL with its high frequency of illegitimate V(D)J-mediated recombination events—precursor B-cell ALL bears site-specific recombination events with non-dispersed breakpoints in association with cryptic RSS at a previously underestimated high frequency (>25%). Of importance, ~50% of samples exhibited signs of oligoclonality, indicating that different SLX4IP-deleted subclones existed in parallel.

Recently, it was demonstrated that subclones in ALL have variegated genetics associated with a dynamic clonal architecture in the lead-up to a diagnosis and in relapse (21). To characterize the behavior of the SLX4IP deletion during leukemic

![Figure 1. Deletion of the first two exons of SLX4IP on chromosome 20 in five ETV6/RUNX1-negative ALL samples. (A) Custom made NimbleGen fine-tiling CGH-array illustrating a uniform deletion at diagnosis. (B) PCR product of the same samples showing the SLX4IP deletion at diagnosis and relapse. No PCR products regarding SLX4IP deletion are discovered in remission (germline). Patient samples are depicted in the following order: KY-3: lines 1, 6 and 11; KY-2: lines 2, 7 and 12; KY-5: lines 3, 8 and 13; KY-1: lines 4, 9 and 14; KY-4: lines 5, 10 and 15. bp = 100 base pair DNA ladder. L = negative control. HPRT exons 7 and 8 were used as positive control. (C) The SLX4IP deletion junction sequence is displayed and remains unchanged from diagnosis to relapse. Dotted lines illustrate breakpoints at the 5′ and 3′ end of the deletion and N-nucleotides are allocated in-between. (D) The SLX4IP deletion junction sequence at diagnosis and relapse is shown in alignment with wild-type sequence. Signs of illegitimate V(D)J-mediated recombination are displayed. The pseudo-heptamer as part of the pseudo-RSS is depicted as black box. The potential pseudo-nonamer sequence is underlined, separated either by a 12-RSS or 23-RSS.
evolution, we extended our above described preliminary analyses of initial diagnosis and relapse samples to a total of 22 pairs (Fig. 1; Supplementary Material, Table S4). The deletion was confirmed at relapse in 21 paired samples by PCR. Sequence analysis of the respective breakpoints showed the same monoclonal sequences in nine patients indicating stability of $SLX4IP$ deletions during disease progression. The remaining samples either developed heterogeneity of $SLX4IP$ breakpoints at relapse subsequent to monoclonal sequences ($n = 1$) or already showed an oligoclonal pattern at initial diagnosis ($n = 11$) (Supplementary Material, Fig. S3). Backtracking of $SLX4IP$ deletions to birth in seven $ETV6/RUNX1$-positive patients using material derived from Guthrie cards did not yield any positive results suggesting that deletion of $SLX4IP$ is a secondary event occurring after birth (Supplementary Material, Fig. S4). Although backtracking of $SLX4IP$ deletions may have been hampered by the sensitivity of the detection method, a secondary nature of the deletions is supported by the observation that in monozygotic twins with $ETV6/RUNX1$-positive ALL and identical translocation breakpoints, the $SLX4IP$ deletion was detectable in one diagnostic leukemic sample only while the second twin was negative (Supplementary Material, Fig. S5). None of 134 available matching bone marrow remission samples from Cohort 1 exhibited the $SLX4IP$ deletion as well as none of 145 peripheral blood samples derived from healthy blood donors.

To gain information on functional consequences of $SLX4IP$ deletion, we next compared its expression in 60 diagnostic ALL specimens carrying the deletion with 60 deletion-negative samples (Supplementary Material, Table S5). We did not find significant differences by $SLX4IP$ deletion status ($P = 0.793$), but $ETV6/RUNX1$-positive samples demonstrated higher $SLX4IP$ expression compared with $ETV6/RUNX1$ negatives (Fig. 2, $P < 0.001$). Next, we investigated a potential effect of $SLX4IP$ deletion on the expression of neighboring genes ($MKKS$ and $JAG1$, Supplementary Material, Table S5 and S6) (22,23). While no differential expression of the $MKKS$ gene was seen ($P = 0.826$), patients carrying a $SLX4IP$ deletion demonstrated significantly higher expression of $JAG1$ ($P < 0.001$). Also here, we performed stratified analysis by $ETV6/RUNX1$ status and observed significantly higher expression of $JAG1$ in $SLX4IP$-deleted samples in the $ETV6/RUNX1$-negative subgroup ($P = 0.011$), whereas positive patients demonstrated uniformly high $JAG1$ expression with no discernable differences according to $SLX4IP$ deletion status ($P = 0.999$) (Fig. 2).
### Table 1. Clinical characteristics of 512 patients with ALL from trial AIEOP-BFM ALL 2000 (Cohort 1) by SLX4IP deletion status

<table>
<thead>
<tr>
<th></th>
<th>Patients positive for SLX4IP deletion (n = 164)</th>
<th>Patients negative for SLX4IP deletion (n = 348)</th>
<th>P-valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>124 (76.5)</td>
<td>181 (52.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female</td>
<td>40 (24.4)</td>
<td>167 (48.0)</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>1–&lt;5</td>
<td>79 (48.2)</td>
<td>161 (46.3)</td>
<td></td>
</tr>
<tr>
<td>5–&lt;10</td>
<td>49 (29.9)</td>
<td>90 (25.9)</td>
<td></td>
</tr>
<tr>
<td>10–&lt;15</td>
<td>23 (14.0)</td>
<td>73 (21.0)</td>
<td></td>
</tr>
<tr>
<td>≥15</td>
<td>13 (7.9)</td>
<td>24 (6.9)</td>
<td>0.286</td>
</tr>
<tr>
<td>Initial WBC (×10⁹/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10 000</td>
<td>80 (48.8)</td>
<td>156 (44.8)</td>
<td></td>
</tr>
<tr>
<td>10 000–&lt;50 000</td>
<td>56 (34.1)</td>
<td>111 (31.9)</td>
<td></td>
</tr>
<tr>
<td>50 000–100 000</td>
<td>16 (9.8)</td>
<td>46 (13.2)</td>
<td></td>
</tr>
<tr>
<td>DNA indexb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1.16</td>
<td>117 (71.3)</td>
<td>208 (59.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥1.16</td>
<td>8 (4.9)</td>
<td>64 (18.4)</td>
<td></td>
</tr>
<tr>
<td>ETV6/RUNXI positive</td>
<td>71 (43.3)</td>
<td>39 (11.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Unknown</td>
<td>39 (23.8)</td>
<td>76 (21.8)</td>
<td></td>
</tr>
<tr>
<td>BCR/ABL positive</td>
<td>5 (3.0)</td>
<td>8 (2.3)</td>
<td>0.463</td>
</tr>
<tr>
<td>Negative</td>
<td>82 (50.0)</td>
<td>283 (81.3)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>11 (6.7)</td>
<td>26 (7.5)</td>
<td></td>
</tr>
<tr>
<td>MLL/AF4 positive</td>
<td>158 (96.4)</td>
<td>332 (95.4)</td>
<td>0.765</td>
</tr>
<tr>
<td>Negative</td>
<td>1 (0.6)</td>
<td>8 (2.3)</td>
<td></td>
</tr>
<tr>
<td>Prednisone responsec</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good</td>
<td>151 (92.1)</td>
<td>294 (84.5)</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>12 (7.3)</td>
<td>52 (14.9)</td>
<td>0.015</td>
</tr>
<tr>
<td>Risk group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>61 (37.2)</td>
<td>112 (32.2)</td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>83 (50.6)</td>
<td>169 (48.6)</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>20 (12.2)</td>
<td>67 (19.3)</td>
<td>0.123</td>
</tr>
</tbody>
</table>

aWBC, white blood cell count at diagnosis.

bRatio of DNA content of leukemic G0/G1 cells to normal diploid lymphocytes.

cGood: < 1000 leukemic blood blasts/μl on treatment Day 8; poor: ≥ 1000 μl⁻¹.

To find out whether an increased male susceptibility to illegitimate V(D)J-mediated recombination was restricted to the SLX4IP locus or could be extended to other loci, we finally analyzed illegitimate V(D)J-mediated deletions of TAL1 in a cohort 1149 T-cell ALL patients from trials AIEOP-BFM ALL 2000 and ALL IC-BFM 2002 (Cohort 4). We were able to identify 128 (11.1%) TAL1-deleted patients with 110 (85.9%) out of them being male. Similar to SLX4IP, this distribution resulted in a significant positive association of TAL1 deletion status with male sex (P = 0.002) (Table 3). Thus, an increased male susceptibility to the event of illegitimate V(D)J-mediated recombination could also be observed at the TAL1 locus. In additional analyses employing multiplex ligation-dependent probe amplification (MPLA) data on IKZF1 and BTG1 deletions which were previously described as being V(D)J-mediated we could not detect a sex-biased distribution of deletions (Supplementary Material, Table S7) (13,24,25).

### DISCUSSION

Here, we describe a site-specific deletion within the 5′ region of the SLX4IP locus in ~30% of childhood ALL in general and >60% of ETV6/RUNXI-rearranged ALL, making the deletion of SLX4IP one of the most common aberrations in childhood ALL described so far. As SLX4IP deletions were previously only described at far lower frequencies in array-based genome-wide analyses of representative large cohorts (18,19), our observation of SLX4IP deletion at a much higher percentage by PCR analysis implies that the subclonal architecture of ALL in association with methodological sensitivity thresholds introduces bias in frequency estimates of genetic aberrations by conventional SNP array or CGH analyses. Most likely, a larger proportion of SLX4IP deletions detected in our study occurred at later stages of leukemic evolution secondary to ETV6/RUNXI rearrangements and were, therefore, only present in a fraction of leukemic cells. These subclonal levels would have been missed by array techniques requiring presence of a lesion in a majority of analyzed cells for reliable detection. The particular sensitivity of the SLX4IP locus to secondary recombination events is further exemplified by the fact that breakpoint sequencing revealed oligoclonality in nearly half of the SLX4IP deletion-positive samples analyzed at initial diagnosis of ALL.

The described deletion in the 5′ region of SLX4IP differs from other recurrently detectable genetic aberrations of comparable frequencies in childhood ALL in the way that it occurs in a site-specific fashion and consistently demonstrates features of a single causal mechanism—illegal V(D)J-mediated recombination. The repeatedly detected association of SLX4IP deletion with male sex in our study and the extension of the sex bias to deletion of the TAL1 locus imply that differential susceptibility by sex to illegitimate V(D)J-mediated recombination at specific loci may contribute to the consistent observation of higher incidence rates of childhood ALL in boys compared with girls. We were not able to extend our findings to two additional loci recurrently displaying features of illegitimate V(D)J-mediated recombination at their breakpoints—IKZF1 and BTG1 (13,14). This suggests that our thoroughly validated observation of sex-specific acquisition of SLX4IP and TAL1 deletions by illegitimate V(D)J-mediated recombination cannot be generalized to all loci demonstrating susceptibility to illegitimate V(D)J-mediated recombination. The mechanism underlying these locus-specific findings remains to be clarified. Potential explanations could include locus-specific differences, variation in sex-specific susceptibility to illegitimate V(D)J-mediated recombination for biological subgroups of ALL, or SLX4IP and TAL1 deletions occurring with equal frequencies in males and females, but underlying sex-specific differences in selection processes during leukemia development.

Sex differences exist in a variety of diseases including, besides others, autoimmune disorders and infectious diseases, and are...
largely believed to represent differential effects of sex-specific hormone action even in prepubertal children (26,27). For the hematopoietic system, it was demonstrated that 17β-estradiol treatment can modify the differentiation, proliferation and survival of early B-cell precursors with a direct influence on Ig gene rearrangements (28). How these observations could mechanistically relate to sex-specific differences in illegitimate V(D)J-mediated recombination in childhood ALL is currently only subject to speculation, but it may well be that differences in endogenous sex hormone exposure could play a modifying role here. Although sex hormone levels between boys and girls already differ during infancy, it is of interest that sex bias in childhood ALL becomes most obvious in children during puberty when hormonal differences become more profound (27). That nuclear receptors themselves are implicated in the mechanism of genomic aberrations was recently demonstrated by an intriguing observation demonstrating that binding of the androgen receptor to intronic regions introduces inter- and intrachromosomal interactions, leading to double-strand breaks and ligation by the NHEJ pathway (29). Future mechanistic studies taking into account this available information may shed some light on the complex interplay of factors with potential involvement in sex-biased genetic aberrations in childhood ALL.

If the high frequency of the deletion indicates a pathomechanistic role for SLX4IP in ALL leukemogenesis and confers a selective advantage to the affected leukemic clone or simply reflects an increased locus-specific susceptibility to recombination events without ‘leukemogenic’ consequences remains to be evaluated in further detail. Nevertheless, we found that SLX4IP expression did not differ between leukemic samples carrying the deletion and those not, indicating that loss at SLX4IP does not lead to haploinsufficiency at the transcriptional level. In contrast, we could demonstrate higher expression of the androgen receptor to intronic regions introduces inter- and intrachromosomal interactions, leading to double-strand breaks and ligation by the NHEJ pathway (29). Future mechanistic studies taking into account this available information may shed some light on the complex interplay of factors with potential involvement in sex-biased genetic aberrations in childhood ALL.

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Table 3. Gender distribution by \(SLX4IP\) or \(TAL1\) deletion status in four childhood ALL cohorts from trials AIEOP-BFM ALL 2000 and ALL IC-BFM 2002

<table>
<thead>
<tr>
<th>Gender</th>
<th>SLX4IP deletion-positive ((n = 164))</th>
<th>SLX4IP deletion-negative ((n = 348))</th>
<th>SLX4IP deletion-positive ((n = 71))</th>
<th>SLX4IP deletion-negative ((n = 39))</th>
<th>SLX4IP deletion-positive ((n = 25))</th>
<th>SLX4IP deletion-negative ((n = 224))</th>
<th>SLX4IP deletion-positive ((n = 283))</th>
<th>SLX4IP deletion-negative ((n = 39))</th>
</tr>
</thead>
<tbody>
<tr>
<td>m(^c)</td>
<td>124 (75.6)</td>
<td>167 (48.0)</td>
<td>181 (52.0)</td>
<td>27 (69.2)</td>
<td>119 (53.1)</td>
<td>105 (46.9)</td>
<td>154 (54.4)</td>
<td>271 (26.5)</td>
</tr>
<tr>
<td>f(^c)</td>
<td>40 (24.4)</td>
<td>167 (48.0)</td>
<td>181 (52.0)</td>
<td>27 (69.2)</td>
<td>119 (53.1)</td>
<td>105 (46.9)</td>
<td>154 (54.4)</td>
<td>271 (26.5)</td>
</tr>
</tbody>
</table>

\(^a\)Analysis in Cohort 1 included stratification regarding \(ETV6/RUNXI\) translocation and DNA index.

\(^b\)\(x^2\)- or Fisher’s exact test.

\(^c\)m, male; f, female.

\(^d\)Cohort 2 comprised only \(ETV6/RUNXI\) translocation-positive patients.

\(^e\)Cohort 3 comprised only \(ETV6/RUNXI\) translocation-negative patients.

\(^f\)Cohort 4 consisted of 1149 children with T-cell ALL who have been investigated for \(TAL1\) deletions and were recruited from the German ALL-BFM 2000 and additional national trial groups from Austria, Italy, Czech Republic and Israel.
was suggested to be responsible for the increase of JAG1 transcription in ETV6/RUNX1-positive ALL (23). However, although we detected differential JAG1 expression by SLX4IP deletion status in the overall patient group and in the ETV6/RUNX1-negative subgroup, this effect could not be observed in ETV6/RUNX1-positive patients. This observation contradicts a selective advantage of a leukemic cell acquiring a SLX4IP deletion by dysregulation of JAG1 and supports a model where higher regional transcriptional activity at SLX4IP and the neighboring JAG1 is common to ETV6/RUNX1-rearranged and a subgroup of alike other ALLs which predisposes to SLX4IP deletion through an open chromatin context.

Thus, SLX4IP deletion may act as a surrogate of a local transcriptional profile common to ETV6/RUNX1-positive ALL and a group of ETV6/RUNX1-like ALL with a similarly good prognosis.

In conclusion, the here reported increased susceptibility of males to illegitimate V(D)J-mediated recombination at SLX4IP and TAL1 may serve as an important etiological hint to the currently only poorly understood sex bias in childhood ALL and, at the same time, may serve the hypothesis of sex-specific differences in recombination frequencies being associated with higher male incidences in a majority of hematological neoplasms and their partly less favorable treatment outcome.
MATERIALS AND METHODS

Study individuals

Patients from Austria, Germany, Italy and Switzerland were enrolled in multicenter trial AIEOP-BFM ALL 2000 on treatment of childhood ALL. Diagnosis, characterization and treatment of ALL were performed as previously described (see also Supplementary Material) (5,6,30–35).

German patients of AIEOP-BFM ALL 2000 were included for SLX4IP deletion screening and sequencing, TAL1 deletion analysis as well as gene expression analysis, Austrian and Italian patients were only included in TAL1 deletion analysis. Patients from Czech Republic and Israel were treated in multicenter trial ALL IC-BFM 2002. Czech patients were included in backtracking experiments and subjected to TAL1 deletion analysis, Israeli patients only employed for TAL1 analysis. German patients with recurrent ALL were enrolled in relapse trials ALL-Rez BFM 95/96 and ALL-Rez BFM 2002 and included in SLX4IP sequencing analyses. Outcome analysis was only performed for German patients treated on AIEOP-BFM ALL 2000. All trials are described in detail elsewhere and were approved by the respective institutional review boards (Hannover Medical School, Hannover; 2nd Faculty of Medicine, Charles University Prague; Charité University Medicine, Berlin) (5,6,36–38). Informed consent for the use of specimen for research was obtained from all study individuals, parents or legal guardians.

Samples, cell and DNA isolation

Bone marrow samples were obtained at initial diagnosis, treatment Days 15, 33, 52, 78 and at relapse. Mononuclear cells were isolated by Ficoll-Paque gradient centrifugation...
(Pharmacia, Freiburg, Germany) from bone marrow samples followed by extraction of high-molecular-weight DNA according to standardized protocols using either the Puregene DNA isolation system (Gentra Systems, Minneapolis, MN, USA) or Qiagen DNA Blood Kits (Qiagen, Hilden, Germany). Quality and quantity of genomic DNA was determined by spectrophotometry. DNA yielded from bone marrow aspirates collected in remission served for germline \textit{SLX4IP} deletion screening. An anonymized cohort of blood donors served as healthy control group.

**Comparative genomic hybridization analysis**

Nineteen diagnostic DNA samples (blast content >80\%) from German AIEOP-BFM ALL 2000 patients (Supplementary Material, Table S8) who later relapsed and matching remission bone marrows were hybridized to high-resolution custom-made fine-tiling CGH arrays covering 62 loci comprising 27 210 197 bp (Supplementary Material, Table S9). Locci selection was based on own preliminary experiments employing 100K Affymetrix arrays (Affymetrix, Santa Clara, CA, USA) and publications reporting genomic aberrations in ALL (18).

UCSC Human genome build March 2006 (HG18) was used as a reference. The designed oligonucleotide array comprised 385 000 probes (probe length 50–75mer) and was manufactured, hybridized and scanned by imaGenes GmbH (NimbleGen service: ROCHE NimbleGen, Reykjavik, Iceland). Repeat regions were excluded and oligonucleotide spacing was <71 bp on average (Supplementary Material, Table S9). Data were analyzed using SignalMap Version 1.9.0.03 (NimbleGen Systems, Inc., Madison, WI, USA).

**SLX4IP and TAL1 deletion screening and genomic sequencing, MLPA**

\textit{SLX4IP} deletion breakpoints were amplified by genomic PCR using primers (5′ → 3′) forward: gataattcacccggctatttttatt and reverse: atgccccggctactccac (39). Product length was \( \sim 705 \) bp when \textit{SLX4IP} deletion was present; sequencing primers (5′ → 3′) were forward: tggctcgactgtctetat and reverse: cctgtatagtactccaac. For \textit{TAL1} deletion analysis, diagnostic leukemic DNA samples were screened using the BIOMED-1 primer set (40).

Amplification of \textit{SLX4IP} deletion breakpoints was performed by genomic PCR (forward primer (5′ → 3′): gataattcacccggctactccac and reverse primer (5′ → 3′): atgccccggctactccac (39)). Fifty nanograms of leukemic blast DNA or human control DNA were amplified using the Qiagen Mutation Detect PCR Kit (Qiagen). As a positive control, a fragment encompassing \textit{HPRT} exons 7 and 8 was amplified using the following primers: forward (5′ → 3′): gataattttttacacgtctggtgc and reverse (5′ → 3′): tagctctgtggaattttgtgc (product length 423 bp). PCR conditions were: 95°C for 5 min followed by 35 cycles of 95°C for 30 s and 61°C for 1 min and 72°C for 3 min. PCR products were analyzed on a multicapillary electrophoresis system (QIAxcel, Qiagen). Before direct sequencing, PCR products were run on a 1.0% agarose gel and purified using the Qiagen gel extraction Kit (Qiagen). Product length was \( \sim 705 \) bp when \textit{SLX4IP} deletion was present. The nucleotide sequences of both strands of the PCR products were directly determined using an automated fluorescent sequencer (ABI Prism 310 Genetic Analyzer, Perkin Elmer Corporation, Wellesley, MA, USA). Primer sequences used for sequencing were the following: forward (5′ → 3′): tgtcaaatagttattttttt and reverse (5′ → 3′): cctgtatatagtactccaac. When distinct breakpoints with non-ambigous sequencing results continued behind the breakpoint junction in forward and reverse sequencing reactions were observed, monoclonality of \textit{SLX4IP} deletion was assumed (Fig. 1C). In the case of non-ambiguous sequencing results before the breakpoint junction (in forward and reverse reactions) with sudden indistinct sequencing signature or with one major-sequence as well as another upcoming minor sequence signature behind the typical breakpoint junction, oligoclinality was hypothesized (Supplementary Material, Tables S3 and S4 and Fig. S3). In these cases, true breakpoints might deviate by 1–2 nucleotides from those depicted in Supplementary Material, Tables S5 and S6, due to ambiguous breakpoint junction assignment.

Primer design in the BIOMED-1 approach for the \textit{TAL1} deletion was restricted to the breakpoint in the \textit{SIL} gene and the two most frequent breakpoints in the \textit{TAL1} gene, type 1 and type 2 (taldb1 and taldb2), covering at least 95\% of all known \textit{TAL1} deletions. PCR products obtained were further examined by heteroduplex or gene scanning analyses to discriminate between amplifications derived from monoclonal or polyclonal lymphoblastic cell populations and junctional regions of clonal PCR products were sequenced.

MLPA was performed as described previously (25).

**Deletion backtracking in Guthrie cards and \textit{ETV6/RUNXI} genomic breakpoint cloning**

For backtracking of \textit{SLX4IP} deletions to the time of birth, genomic DNA from Guthrie cards was isolated using the InstaGene Dry Blood Kit (Bio-Rad, Hercules, CA, USA). Three separate sections (1/8) of one Guthrie card were used in PCR reactions. For \textit{SLX4IP} deletion screening, specific primers were designed. The amplified products were analyzed by automated chip-based microcapillary electrophoresis on an Agilent 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA). Assay specificity was determined using serial dilutions of individual diagnostic DNA in healthy donor DNA. Guthrie card PCRs were performed in a separate room in a separate PCR box. For \textit{SLX4IP} deletion screening in Guthrie cards specific primers were designed: forward (5′ → 3′): agaaacacacacagaaag and reverse (5′ → 3′): gggtggttagaacacagt. PCR conditions were: initial denaturation at 95°C for 10 min; 35 cycles at 94°C for 30 s, 58.5°C for 30 s, and 72°C for 3 s, final extension 72°C for 7 min. \textit{ETV6/RUNXI} genomic breakpoints in monozygotic twins were cloned as described previously (41).

**RNA isolation, reverse transcription and gene expression analysis by real-time quantitative PCR**

Total RNA was isolated with Triazol reagent (Invitrogen, Paisley, UK) and subsequently passed over a Qiagen RNeasy column (Qiagen) for removal of small fragments. Total RNA was quantified and validated for integrity using the Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA). Real-time quantitative polymerase chain reaction (RQ-PCR) analysis was performed after
random hexamer priming and MuLV reverse transcription (Fermentas, Hanover, MD, USA) to generate cDNA. PCR was carried out on an Applied Biosystems Model 7900 HT Sequence Detector (Darmstadt, Germany) using the QuantiTect SYBR Green PCR kit (Qiagen) as described in the manufacturer’s instructions. The expression level of the SDHA and ABL1 were used to normalize for differences in input cDNA. QuantiTect Primer Assays were used to measure mRNA abundances of the SLX4IP, MKKS and JAG1 genes (Qiagen). Melting curve analyses were performed to verify the amplification specificity. Each sample was tested in duplicate. The expression ratio was calculated as 2^n, where n was the threshold cycle (C_{T}) value difference normalized by the CT difference of a calibrator sample.

For SLX4IP and MKKS expression analysis 120 German patients from trial AIEOP-BFM ALL 2000 with known SLX4IP deletion status and RNA availability were selected. Selection criteria were as following: half of the 120 patient samples were either SLX4IP deletion-negative (n = 60) or positive (n = 60). Within each of the two subgroups, half of the patients were ETV6/RUNX1 translocation-negative (n = 30) or positive (n = 30). Exclusion criteria comprised: BCR/ABL1 or MLL rearrangement, T-ALL and hyperdiploidy (Supplementary Material, Table S5). For JAG1 expression analysis, 319 German patients from trial AIEOP-BFM ALL 2000 with known SLX4IP deletion status and RNA availability were selected (Supplementary Material, Table S6).

**Statistical analysis**

Differences in the distribution of categorical variables among patient subsets were analyzed using Fisher’s exact or χ²-test. Comparisons of gene expression levels between groups were performed by Mann–Whitney U-test. Event-free survival was defined as the time from diagnosis to the date of last follow-up in complete remission or to the first event. Events were resistance to therapy (non-response), relapse, secondary neoplasm or death from any cause. Failure to achieve remission due to early death or non-response was considered as event at time zero. Patients lost to follow-up were censored at the time of their withdrawal. The non-response was considered as event at time zero. Patients lost from any cause. Failure to achieve remission due to early death or therapy (non-response), relapse, secondary neoplasm or death (Supplementary Material, Table S5).

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**REFERENCES**


