Novel cryptic chromosomal rearrangements in childhood acute lymphoblastic leukemia detected by multiple color fluorescent in situ hybridization

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Background and Objectives. It is often difficult to obtain good karyotypes of cells from children with acute lymphoblastic leukemia (ALL) because of poor morphology and spreading. Detailed karyotyping can be further hampered by the presence of multiple rearrangements. Our objective was to search for cryptic rearrangements in childhood ALL.

Design and Methods. A series of eight cases of childhood ALL with at least two structural defects were selected and studied by multiple color fluorescent in situ hybridization (M-FISH).

Results. Four previously not reported translocations were detected: a t(14;20)(q32;q11.2) in a 3-year old girl with T-ALL, a cryptic t(7;11)(q35;q24) in association with an at(1;14)(p32;q32) in a patient with T-ALL and two translocations possibly involving the same 6q26 region on the distal end of the long arm of chromosome 6. Further FISH analysis on the t(7;11) indicated rearrangement of the TCRB locus at 7q35 suggesting that this t(7;11) leads to overexpression of an as yet unidentified gene at 11q24. This observation also triggered further screening for TCRB rearrangements in T-ALL. FISH analysis of the t(14;20) with an IGH locus-specific probe provided evidence for an unusual rearrangement of the IGH gene, in the variable gene segment region. Finally, we also observed cryptic insertions of AF4 and ETV6 in combination with complex rearrangements, leading to MLL/AF4 and ETV6/RUNX1 gene fusions.

Interpretation and Conclusions. This study underscores the importance and power of M-FISH analysis in unraveling complex karyotypes and identifying cryptic chromosomal rearrangements. It also sheds some light on the implication of cryptic TCRB rearrangements in T-ALL.

Key words: childhood ALL, M-FISH, complex karyotypes, TCRB

Acute lymphoblastic leukemia (ALL) represents about 85% of childhood leukemias. The current cure rate is nearly 80%, reflecting the remarkable progress in identifying and treating resistant subtypes of the disease.1,2 The classification of ALL into therapeutically relevant risk categories relies on both clinical parameters, including age, leukocyte count, immunophenotype, central nervous system (CNS) involvement, as well as on the blast cell karyotypes.1 Conventional cytogenetic analysis of ALL is often hampered by the difficulty in obtaining good quality chromosomes for analysis. Improvements in cytogenetic techniques and complementation with interphase and multiple color fluorescence in situ hybridization (M-FISH) have increased the success rate in detecting recurrent chromosome changes in ALL. A large number of chromosomal rearrangements have now been described for which the genetic alterations and effect on prognosis are well known.2,3 These include ETV6/RUNX1 fusion and hyperdiploidy, associated with a favorable outcome, and hypodiploidy, BCR/ABL1, and MLL rearrangements associated with a poor prognosis in ALL. Recurrent genetic changes in T-cell ALL have also been identified, but their effect on outcome is less pronounced.4,5 Translocations observed among T-lineage ALL preferentially involve the TCRAD locus at 14q11 and the TCRB locus at 7q35, and are present in 20-25% of these patients.3 Despite the significant progress which has been made in identifying the molecular defects occurring in childhood ALL, no known recurrent chromosomal changes are found in a considerable number of (near)-diploid ALL. Recent reports describing the finding of cryptic translocations, e.g. the t(5;14)(q35;q32) in 22% of children with T-ALL6 and the recurrent t(7;12)
(q36;p13) and t(5;11)(q35;p15.5) in childhood acute myeloid leukemia, encouraged us to perform further M-FISH analysis on a series of eight selected cases of childhood ALL and indeed resulted in the detection of new cryptic rearrangements.

**Design and Methods**

**Patients**

A total of eight cases of 66 childhood ALL diagnosed between 1998 and 2001 at the Ghent University Hospital were selected based on the presence of complex karyotypes and the availability of sufficiently well-spread metaphases (Table 1). All but two patients are still alive and in remission. Patient 3 and patient 4 died 30 months and 11 months post-diagnosis, respectively.

**Cytogenetic and molecular cytogenetic analysis**

Metaphase chromosome spreads from diagnostic bone marrow samples were prepared and G-banded according to standard procedures. Karyotypes were described according to the ISCN guidelines.

The 24Xcyte probe kit was purchased from Meta-Systems (Altussehm, Germany) and contains combinatorially labeled chromosome paints obtained from degenerated oligonucleotide primer polymerase chain reaction (DOP-PCR) amplified microdissected chromosomes. Fluorescein isothiocyanate (FITC), SpectrumOrange™, TexasRed® and diethylcoumarine (DEAC) were used for direct detection, whereas biotin was indirectly visualized with Streptavidin Cy5®. The M-FISH procedure was performed as previously described. All six fluorochromes including the DAPI counterstain were sequentially captured previously described.

The M-FISH ISIS software (Meta-Systems) for image capturing and modification of false colors was used. At least five metaphase cells from each patient were analyzed by M-FISH.

Subsequent to M-FISH analysis, some results were confirmed or further characterized with whole chromosome plasmid libraries (kindly provided by Dr. Collins, Livermore, CA, USA) or region-specific probes. Probe-labeling and FISH was performed according to Van Roy et al. For multiple color FISH, a combination of three or four different fluorochromes was used following the procedure described. Routine FISH for the detection of BCR/ABL1 fusion, ETV6/RUNX1 fusion, and MLL rearrangements, respectively, was performed using commercial probes: LSI® ABL/BCR ES probe, LSI® ETV6(TEL)/RUNX1 (AML1) ES probe, and LSI® MLL dual color probe (Vysis, Downers Grove, IL, USA). Additional region-specific probes used in this study were: BAC clones RP11-362J18, RP11-64A1, RP11-397E7, RP11-168E22, RP11-71J3, and RP11-476C8 covering the AF4 locus; BAC clones RP11-556113, RP11-282G13, RP11-1160C9, RP11-1220K2, and RP11-17D6 covering the TCRB locus and RP11-242H9 and RP11-447G18 for the TCRA locus; ten selected BACs in the 11q24 region; the subtelomeric 21q probe GS-63-H24, and commercial probes LSI® IGH/CCND1, LSI® IGH dual color and LSI® D20S108 (Vysis).

**Reverse transcription polymerase chain reaction (RT-PCR) analysis**

Total RNA was extracted from bone marrow using TriZol (Gibco-BRL, Gaithersburg, MD, USA) or the RNeasy® Mini kit (Qiagen, Hilden, Germany) according to the manufacturers’ protocols. Routine screening of acute leukemias at diagnosis, for the presence of 28 different translocations or chromosomal rearrangements was performed with a multiplex PCR (mD® HemaVision®, DNA Technology A/S, Aarhus, Denmark), according to the manufacturer’s instructions. Screening of HOX11/L2 overexpression in T-ALL, was performed with a standard PCR using primers HOX11L2-F and HOX11L2-R as described previously by Bernard et al.

**Results**

**Cytogenetic, FISH and PCR analysis**

Details of the cytogenetic findings are summarized in Table 1. High hyperdiploid karyotypes (> 50 chromosomes) were observed in three patients, whereas the remaining patients had pseudodiploid karyotypes. Structural abnormalities included multiple marker chromosomes and unidentified additional material, five deletions, two duplications, a novel balanced translocation t(6;12)(q27;q13), and a recurrent t(1;14) (p32; q11).

FISH was routinely performed for detection of ETV6/RUNX1 and BCR/ABL1 gene fusions and MLL rearrangement. MLL rearrangement was observed in patient #4 whereas patient #5 was positive for the ETV6/RUNX1 gene fusion. Multiplex RT-PCR at diagnosis identified an MLL/AF4 fusion in patient #4, and confirmed the presence of ETV6/RUNX1 in patient #5. Both fusion transcripts were confirmed by single specific RT-PCR analysis.

Following M-FISH, revision of the G-banded karyotypes was possible in all the selected cases (Table 1). FISH allowed confirmation and/or correction of observed numerical changes, identification of the chromosomal origin of marker chromosomes and small translocated segments (adds), elucidation of the nature of complex chromosome rearrangements and...
identification of novel/cryptic rearrangements.

In total, four novel reciprocal translocations were detected: t(14;20)(q32;q11), t(6;12)(q26;q13), t(6;10)(q26;q24), and t(7;11)(q35;q24).

In patient #1, M-FISH confirmed the presence of translocated 20q material on the derivative chromosome 14 and a partial deletion of the long arm of chromosome 6. Dual color FISH with the LSI® IGH dual color probe showed a split of the IGH probe covering the variable gene segments. Subsequent FISH analysis using the LSI® IGH/CCND1 (Vysis) and the LSI®D20S108 (Vysis) probes showed the presence of one signal for IGH on chromosome 20 (Figure 1A), thus providing evidence for the reciprocal nature of this translocation t(14;20)(q32;q11.2).

In patient #2, in addition to the recurrent t(1;14)(p32;q11), two novel cryptic translocations, t(7;11)(q34;q24) and t(6;10)(q26;q24), were found. The possible involvement of the TCRB gene at 7q34 was investigated using BAC clones covering the TCRB locus in combination with a chromosome 11 specific library. The combined BAC clones covering the TCRB locus were found to be split: one signal was retained on the der(7), whereas the other signal was translocated to the derivative chromosome 11 (Figure 1B). The LSI® MLL dual color probe was retained on the der(11). Further mapping with BAC clones allowed identification of a breakpoint spanning BAC clone (RP11-784K23) (not shown). Putative target genes in the breakpoint flanking region on 11q24 are TRIM29 and POU2F3, among others. Results of the ongoing expression studies will soon be reported.

### Table 1. Diagnosis, the initial karyotype descriptions based on banding and revised karyotypes for eight childhood ALL and the positive results of standard FISH analysis for BCR/ABL1, ETV6/RUNX1 and MLL abnormalities at diagnosis.

<table>
<thead>
<tr>
<th>Nr</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>FISH</th>
<th>PCR</th>
<th>Karyotype</th>
<th>Revised karyotype</th>
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<tr>
<td>1</td>
<td>3 y</td>
<td>F</td>
<td>Mature T-ALL</td>
<td>Split IGH locus</td>
<td>46,XX,del(6)(q16q24),add(14)(q32),del(20)(q11.2q13.1)</td>
<td>46,XX,del(6)(q16q24),t(14;20)(q32;q11)</td>
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<tr>
<td>2</td>
<td>4 y</td>
<td>M</td>
<td>Intermediate T-ALL</td>
<td>Split TCRB and B locus</td>
<td>46,XY,t(1;14)(q32;q11),add(9)(p22)</td>
<td>46,XY,t(1;14)(q32;q11),t(6;10)(q26;q24),ins(9)(q22?),t(7;11)(q35;q24)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>14 y</td>
<td>F</td>
<td>Common ALL</td>
<td>46,XX,del(1)(q12q43),del(9)(q33?),13,del(13)(q32),del(13)(q26q24),+3mar(9),46,XX(6)</td>
<td>46,XX,del(1)(q12q43),del(9)(q33?),der(13)(13pter→13q32::13q34::13qter)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>7 w</td>
<td>F</td>
<td>Pro B/early B-ALL</td>
<td>MLL/AF4+</td>
<td>46,XX,t(1;17)(q10p10),t(11;?)</td>
<td>46,XX,der(1)(1pter→1p13::17q21::17qter)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>7 y</td>
<td>F</td>
<td>Common ALL</td>
<td>MLL/AF4+</td>
<td>47,XX,5.add(8)(q24),der(11)(q21),add(12)(p13),add(15)(p12),+21,mar</td>
<td>47,XX,der(5)(5pter?5q13::21q22::21qter)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>13 y</td>
<td>M</td>
<td>Early T-ALL</td>
<td>HOX11L2+</td>
<td>56,XY,+4,+6,+8,+10,add(11)(p13),+16,19,+C,+6,mar</td>
<td>56,XY,+4,+6,+8,+10,der(11)(p11)</td>
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<tr>
<td>7</td>
<td>6 y</td>
<td>M</td>
<td>Common ALL</td>
<td>57,XX,+2B,+4C,+D,+E,+21,+21</td>
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<tr>
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<td>M</td>
<td>Common ALL</td>
<td>56,XY,dup(1)(q12q43),+4,+6,+8, +10,14,+16,+18,+21,+C, +x,+14,+16,+17,+18,+21</td>
<td>56,XY,dup(1)(q12q43),+4,+6,+8,+10, +12,14,+15,+16,+17,+18,+21</td>
<td></td>
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Figure 1. Partial G-banding (A1) and M-FISH identified a t(14;20)(q32;q11) in patient n. 1 (A2). FISH using the LSI® IGH dual color probe revealed a split of the probe spanning the IGH variable gene segment (green) (arrowhead) (A3). Dual color FISH revealed a split of the IGH spanning probe (green), and fusion of one signal with the LSI® D20S108 (red) located at 20q12 (arrowhead). (A4) Partial G-banding (B1) and M-FISH analysis in patient #2 revealed a recurrent t(1;14)(p32;q11), and 2 cryptic translocations (B2). Dual color FISH with chromosome 11 library (red) and TCRB locus spanning probes (green) showing a split of the probe spanning TCRB and the probe signal moved to the der(11) (B3). Karyotype (C1) and M-FISH analysis in patient #3 showed the presence of a complex chromosome 13, 14, 15, and 16, a dup(1)(q12q43) and a balanced translocation t(6;12)(q26;q13) (C2). G-banding (D1) and M-FISH analysis (D2) and FISH using locus-specific probes to unravel a complex rearrangement of chromosomes 1, 11, and 17 leading to the MLL/AF4 fusion in patient #4. Triple color FISH using the LSI® MLL dual color (distal probe red, proximal probe green), and AF4 spanning probes (yellow) revealed a fusion signal (arrowhead) (D3). Triple color FISH using LSI® MLL dual color probe and a chromosome 11 specific library (yellow) showed the proximal MLL probe (green) on the der(11) (arrowhead) and the distal MLL probe (red) on the der(11) (D4). Partial G-banding (E1) and M-FISH (E2), showing a complex chromosome 5, 8, 12, 21 rearrangement in patient #5. Triple color FISH using LSI® ETV6(TEL)/RUNX1(AML1) ES probe (5' ETV6 in green, RUNX1 spanning probe in red) and GS-63-H24 probe located at 21qter (yellow), showed that the ETV6/RUNX1 fusion is inserted in the derivative chromosome 21 (E3). G-banding (E4) and M-FISH image of a complex chromosome change involving chromosomes X, 15 and 18 in patient #6 (E5).
A novel reciprocal translocation t(14;20)(q32;p11), t(7;11)(q35;q24), t(6;10) (q26;q24), and t(6;12)(q26;q13). A cryptic translocation t(7;11)(q35;q24) was detected in a patient with T-ALL in association with a recurrent t(1;14)(p32;q11), a second cryptic translocation t(6;10) (q25;q24), and an ins(9;10)(p22;?) . FISH analysis showed rearrangement of a BAC clone spanning the TCRB locus and led us to assume that this translocation, in analogy to other translocations involving TCR and IGH loci, has caused juxtaposition of a proto-oncogene to the TCRB locus resulting in aberrant expression of this gene. This translocation is reminiscent of the recently detected cryptic t(5;14)(q35;q32) in T-ALL, which has now been shown to be present in as many as 22% of T-ALL patients. HOX11L2, an orphan homeobox gene located close to the chromosome 5 breakpoints was found to be transcriptionally activated as a result of this translocation. The t(7;11) also involves distal chromosome arm ends and cannot be detected upon banding analysis only, and thus can be considered to be truly cryptic. The 11q24 breakpoint was mapped up to the BAC level. Possible candidate genes which might be over-expressed due to juxtaposition to enhancers of the TCRB locus include TRIM29, POU2F3 and ARHGEF12. Further studies are ongoing in order to identify the target gene. The simultaneous presence of two distinct translocations, resulting one each from TCRα and TCRβ rearrangements, is a new observation. The order of occurrence is undetermined and the exact contribution of each translocation to the T-cell malignant phenotype remains to be determined. Nevertheless, this observation clearly illustrates the importance of several genetic defects in T-cell oncogenesis.

The finding of a cryptic translocation involving the TCRB locus in this study prompted us to perform further screening for cryptic rearrangements in T-ALL and led to the discovery of a new recurrent inversion of chromosome 7, inv(7)(p15q34) causing upregulation of HOXA10 and HOXA11 expression in a subset of mature T-ALL (T3-4 according to EGIL recommendations) and a peculiar immunophenotype with CD2+, CD4+, and CD8+ blasts. Details of this study were published elsewhere. Moreover, evidence for other possible new T-cell oncogenes involved in TCRB rearrangements was found and overall a high involvement of TCRB chromosomal rearrangement in T-ALL was observed. Therefore, the present study illustrates the power of M-FISH in detecting new recurrent chromosomal rearrangements in selected cases. A novel reciprocal translocation t(14;20)(q32;q11) was detected by banding analysis and FISH with IGH spanning probes, and confirmed by M-FISH in a patient with T-ALL. The involvement of the IGH locus in T-ALL is unusual. However, the breakpoint within the IGH gene was located in the variable gene segments, distal to the typically involved J or switch.
region segments. Mapping of the 20q11.2 breakpoint was precluded due to limited patient material.

Two reciprocal translocations, one of which was cryptic in nature, appeared to involve the same chromosome band, 6q26. Further mapping is required to determine whether the same locus is involved in both cases. If proven, this gene/locus may, as observed for other genes located at the distal ends of chromosome arms (e.g. MLL and ETV6), lead to formation of hidden translocations, as exemplified by one of the two 6q26 translocations observed here. The fact that these two cases are of different cell lineage (T-ALL and c-ALL, respectively) does not preclude the involvement of the same oncogene as illustrated by the occurrence of MLL gene fusion in T- and B-lineage acute lymphoblastic and myeloid leukemias.

In addition to the above-described reciprocal translocations, we also identified insertions of AF4 and ETV6 in combination with complex chromosomal rearrangements, leading to formation of MLL/AF4 and ETV6/RUNX1.

The classical translocation t(4;11)(q21;q23) and resulting MLL/AF4 fusion is strongly associated with infant leukemia and topoisoerase inhibitor-induced secondary leukemias, and has a poor prognosis. Despite the demonstration of an MLL/AF4 fusion by FISH with locus-specific probes and RT-PCR, neither G-banding nor M-FISH could show involvement of chromosome 4 in patient #4. A complex rearrangement involving chromosomes 1, 11, and 17 was apparent from this analysis. Further analysis showed that the MLL/AF4 fusion on the derivative 11 resulted from an insertion of 5' AF4 sequences into the MLL gene on the der(11) thus indicating further complexity of the translocation. A cryptic MLL/AF4 fusion, as a result of insertion of the 5' MLL sequences in chromosome 4q21, was recently demonstrated, although in that case a normal karyotype was observed by G-banding. The current observation clearly demonstrates the need for systematic screening of MLL/AF4 (either with FISH or RT-PCR) in ALL.

A similar insertion event was observed in an ETV6/RUNX1 positive ALL patient with complex karyotypic changes i.e. the 5' part of ETV6 was inserted into the RUNX1 gene on a derivative chromosome 21, the latter being implicated in a complex translocation involving chromosomes 5, 8, and 12. A similar observation of insertion of RUNX1 into ETV6 on 12p13 was reported in a case of ALL with a normal karyotype. Cryptic insertions of 5' RUNX1 have been observed in complex karyotypes. The latter reports and the present observation further document the occurrence of cryptic insertions as an alternative mechanism for formation of fusion genes in leukemic cells.

To date, only a limited number of studies have used 24-color FISH to identify chromosomal abnormalities in childhood ALL. These studies evaluated the efficiency of spectral karyotyping and M-FISH in the detection of chromosome aberrations previously diagnosed using chromosome banding and/or RT-PCR, and attempted to identify complex or novel rearrangements. Three novel, cryptic rearrangements were observed in a study of 20 patients whose leukemic blast cells were normal by conventional cytogenetics. Other novel translocations have been found in patients with multiple chromosome rearrangements. Using the IPM-FISH technology, a novel multi-color approach that obtains stronger hybridization signals at the telomeric ends of the chromosome, Helias et al. observed the novel recurring t(5;14)(q35;q32) in T-ALL. The finding of a possible recurrent cryptic t(7;11)(q35;q24) involving the TCRB locus in our study offers new avenues for further research to characterize the involvement of candidate genes on 11q24 and to determine its frequency of occurrence. This study thus demonstrates the great potential of M-FISH in detecting previously unidentified translocations.

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