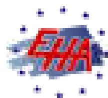

Novel cryptic chromosomal rearrangements in childhood acute lymphoblastic leukemia detected by multiple color fluorescent *in situ* hybridization

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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 a t(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*

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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-ALL[6] and the recurrent t(7;12)

(q36;p13)[7,8] and t(5;11)(q35;p15.5)[9] 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.[10]

The *24Xcyte* probe kit was purchased from MetaSystems (Altlussheim, 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.[11] All six fluorochromes including the DAPI counterstain were sequentially captured with a Zeiss axioplan epifluorescence microscope (Carl Zeiss, Zaventem, Brussels) using single-band-pass filters. The M-FISH ISIS software (MetaSystems) 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)[12] or region-specific probes. Probe-labeling and FISH was performed according to Van Roy *et al.*[27] For multiple color FISH, a combination of three or four different fluorochromes was used following the procedure described.[13] 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, RP11-711J3, and RP11-476C8 covering the *AF4* locus; BAC clones RP11-556I13, RP11-282G13, RP11-1160C9, RP11-1220K2, and RP11-17D6 covering the *TCRB* locus and RP11-242H9 and RP11-447G18 for the *TCRAD* 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 (*m*Dx® HemaVision®, DNA Technology A/S, Aarhus, Denmark), according to the manufacturer's instructions. Screening of *HOX11L2* overexpression in T-ALL, was performed with a standard PCR using primers HOX11L2-F and HOX11L2-R as described previously by Bernard *et al.*[6]

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 karyotyopes. 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

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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. In

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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.

Nr	*Age*	*Sex*	*Diagnosis*	*FISH*	*PCR*	*Karyotype*	*Revised karyotype*
1	3 y	F	Mature T-ALL	Split *IGH* locus		46,XX,del(6)(q16q24),add(14)(q32), del(20)(q11.2q13.1)	46,XX,del(6)(q16q24), t(14;20)(q32;q11), del(20)(q11.2q13.1)
2	4 y	M	Intermediate T-ALL	Split *TCRA* and *B* locus	*HOX11L2 – TAL+*	46,XY,t(1;14)(p32;q11),add(9)(p22)	46,XY,t(1;14)(p32;q11),t(6;10)(q26;q24), ins(9;10)(p22;?),t(7;11)(q35;q24)
3	14 y	F	Common ALL			46,XX,dup(1)(q12q43),t(6;12)(q27;q13), del(9)(q33?),-13,del(13)(q13q32), -14,-15,add(16)(q24), +3mar(9)/46,XX(6)	46,XX,dup(1)(q12q43),t(6;12)(q26;q13), del(9)(q33?),der(13)(13pter→13q32 ::15p?q?::16p?q?),der(13)(13pter→13 q::14p?q?),der(14)(14pter→14q ::13p?q?),der(15)ins(14;15q),der(16) (16pter→16q11.2::14::13q12→13qter)
4	7 w	F	Pro B/early B-ALL	*MLL/AF4+*	*MLL/AF4+*	46,XX,t(1;17)(q10;p10),t(11;?)(q23;?)	46,XX,der(1)(1qter→1p13::17p?→17p? ::11q23→11qter),der(11)(11pter→11q 23::17),der(17)(1pter→1p13::17p11.2 →17qter)
5	7 y	F	Common ALL	*ETV6/RUNX1+*	*ETV6/ RUNX1+*	47,XX,-5,add(8)(q24),del(11)(q21), add(12)(p13),add(15)(p12),+21,+mar	47,XX,der(5)(5pter?5q13::21q22→21 qter),der(8)(8pter→8q24::5q?),der(12) (12qter→12p13::5q?::8q24→8qter), der(15)(Xp?q?::15p12→15qter),+21(6)/ 47,XX,der(5)(5pter?5q13::21q22?21 qter),der(8)(8pter?8q24::5q?),der(11) (11pter?11q23::Xp?q?),der(12) (12qter?12p13::5q?::8q24?8qter),+21(6)
6	13 y	M	Early T-ALL		*HOX11L2 –*	56,XY,+4,+6,+8,+10,add(11)(p13), +16,+19,+C,+G,+mar	56,XY,+4,+6,+8,+8,+10,+der(11)t(1;11) (p?;p15),+16,+der(18)(18pter?18 q21::X::15),+19,+22
7	6 y	M	Common ALL			57,XXY,+2B,+4C,+D,+E,+21,+21	57,XXY,+4,+4,+6,+8,+9,+10,+14,+18,+21,+21
8	6 y	M	Common ALL			56,XY,dup(1)(q12q43),+4,+6,+8, +10,+14,+16,+18,+21,+E,+C	56,XXY,dup(1)(q12q43),+4,+6,+8,+10, x+14,+16,+17,+18,+21

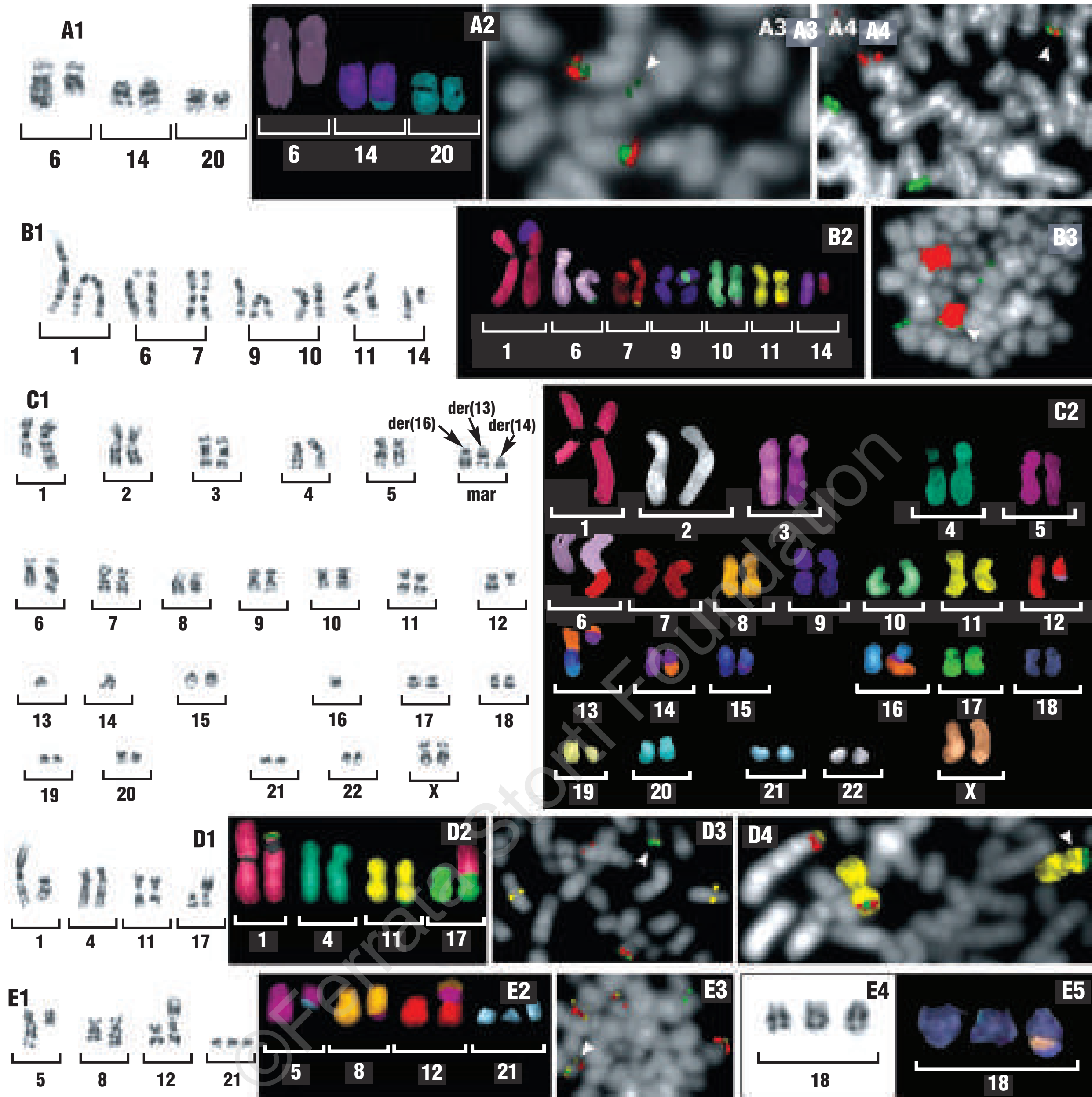

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Figure 1. Partial G-banding (A1) and M-FISH identified a t(14;20)(q32;q11) in patient n. 1 (A2). FISH using the LSI® IGH dual color probe revealed a split of the probe spanning the *IGH* variable gene segment (green) (arrowhead)(A3). Dual color FISH 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), 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(1) (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 21qtel (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).

patient #3, we identified a reciprocal t(6;12)(q26;q13) with an apparently similar 6q26 breakpoint as the cryptic t(6;10)(q26;q24) in patient #8. Additional complex rearrangements involving chromosomes 13, 14, 15, and 16 were observed (Figure 1C).

Routine RT-PCR revealed the presence of an *MLL/AF4* fusion gene in patient #4. However, G-banding analysis and M-FISH showed the presence of a complex rearrangement involving chromosomes 1, 11, and 17, but provided no evidence for the involvement of chromosome 4 (Figure 1D). Triple color FISH with the clones spanning *AF4* in combination with the LSI® MLL dual color probe (Vysis), revealed a fusion signal on the der(11). This was further evidenced by triple color FISH using the LSI® MLL dual color probe (Vysis) and a chromosome 11 specific library. The *3'MLL* probe moved to the der(1) (Figure 1D). Therefore, we can conclude that the *MLL/AF4* fusion results from a complex chromosomal mechanism. We assume that an insertion of 3'*AF4* into 11q23 coincided with complex translocations involving chromosomes 1, 11, and 17.

Patient #5 was positive for *ETV6/RUNX1* by PCR and FISH analysis. Triple color FISH with the LSI® ETV6 (TEL)/RUNX1(AML1) ES probe (Vysis) and a chromosome 21 specific library, showed the *ETV6/RUNX1* fusion signal on the der(21) (*not shown*). Results from triple color FISH using the LSI® ETV6(TEL)/RUNX1(AML1) ES (Vysis) and probe GS-63-H24 located at 21qter (Figure 1E), demonstrated that the *ETV6/RUNX1* fusion on the der(21) most probably resulted from an insertion of 5'ETV6 into the *RUNX1* gene on 21q22.

Chromosomal material of unidentified origin

In patient n. 6, M-FISH showed the additional material on the der(11) to be derived from chromosome 1, whereas the marker chromosome was shown to result from a complex rearrangement involving chromosomes X, 15, and 18 (Figure 1F).

In patient #5, the chromosome changes observed by banding analysis were revised as der(5) (5pter→5q13::21q22→21qter),der(8)(8pter→8q24::5q→),der(12)(12qter→12p13::5q→::8q24→8qter),der(15)(Xp→q→::15p12→15qter),der(11)(11pter→11q23::Xp→q→) (Figure 1E).

Discussion

Encouraged by the discovery of new and/or hidden translocations in previous multicolor FISH studies in ALL,[14-16] we decided to investigate eight cases of childhood ALL, selected for the presence of only partially characterized, well-spread metaphases, with combined cytogenetic and M-FISH analysis. Four novel balanced translocations were identified in three patients: 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.[6] 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 *TCRA* and *TCRB* 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.[17] 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*

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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 topoisomerase inhibitor-induced secondary leukemias, and has a poor prognosis.[18] 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 *3′ 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.[19] 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.[20] Cryptic insertions of *5' RUNX1* have been observed in complex karyotypes.[21] 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.[14-16, 22-26] 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.[15] Other novel translocations have been found in patients with multiple chromosome rearrangements.[14-16] 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.*[28] 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.

Conception and design: BP, BC, ADP, YB, FS; analysis and interpretation of data: BP, BC, FS, HVL, NY, JP, BV; drafting the article or revising it critically for important intellectual content: BP, BC, ADP, FS; final approval of the version to be published: all authors. The authors declare that they have no potential conflict of interest.

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