Incidence and Characterization of MLL Gene (11q23) Rearrangements in Acute Myeloid Leukemia M1 and M5

By Hélène Poirel, Katrina Rack, Eric Delabesse, Isabelle Radford-Weiss, Xavier Troussard, Caroline Debert, Daniel Lebœuf, Christian Bastard, Françoise Picard, Agnès Veil-Buzyn, Georges Flandrin, Olivier Bernard, and Elizabeth Macintyre

To determine the incidence of MLL rearrangement in acute myeloid leukemia (AML) French-American-British (FAB) type M1 and to evaluate optimal screening strategies for the characterization of such abnormalities, we analyzed specimens from 41 patients with AML by Southern blotting with two MLL genomic probes and compared the capacities of reverse transcription-polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH) to identify the types of rearrangement found in AML M1 with those observed in AML M5. MLL rearrangement was found in 6 of 29 (20%) AML M1 and 6 of 10 AML M5 cases. RT-PCR characterization of 11 cases showed four MLL self-fusions, four MLL-AFG, two MLL-AF6, including a novel AF9 breakpoint, and one uncharacterized t(11;19). Only 5 of 10 MLL-rearranged cases tested demonstrated karyotypic 11q23 abnormalities. FISH analysis of nine cases with an MLL-specific yeast artificial chromosome (YAC) confirmed the cytogenetic abnormalities in two cases, clarified them in one, and did not detect six cases, including three MLL self-fusions, one case with a probable MLL-rearranged subclone not represented karyotypically, and two MLL-AFG. A whole chromosome 11 paint detected one of these MLL-AF6, and an AF6 cosmid demonstrated that the other was probably due to insertion of a submicroscopic portion of chromosome 6, including part of AF6, into an apparently normal chromosome 11. We conclude that MLL rearrangements are common in adult AML M1, that MLL self-fusion and MLL-AFG are the most frequent types of abnormalities, and that RT-PCR is preferable to 1q23 FISH analysis for their characterization.

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Fig 1. (A) Physical map of the MLL gene. Selected BamHI (B), HindIII (H), and EcoRI (E) restriction sites are shown, as are the positions of the different probes used for Southern blot analysis. The zone involved in self-fusion is underscored, with the dashed line corresponding to the region including the 5' breakpoints. The overlined regions represent localization of the different self-fusion 5' breakpoints detected by Southern blot. Numbered boxes indicate exons. (B) Southern blot analysis. Hybridization of the same BamHI DNA digests with 6 and 9 MLL probes and hybridization of EcoRI DNA digests with the 11 probe. (C) RT-PCR analysis. Left: The four patients with MLL self-fusion exhibit the same fusion transcript with the exon 6-sense and exon 3-antisense primers. PC, positive control; NC, an HL60 myeloid cell line negative control; numbers refer to patient UPNs. Middle: MLL exon 6-AF6 (UPNs 336, 313, and 562) and MLL exon 7-AF6 (UPN 510 and, as a minor alternative transcript, UPN 313) fusion transcripts detected with the MLL-Ex5S-ext and AFGAS-ext primers. Right: MLL-Ex5S-ext and AFSAS3 generated, in addition to the previously described AF9 “A” (PC, positive ALL control) and “B” (UPN 313) fusion transcripts, a novel AF9 “C” transcript in patient UPN 427.

11q23 abnormalities. In childhood ALL, 11q23 abnormalities not involving MLL have favorable clinical features, thus rendering comparison of prognostic evaluation based on cytogenetic and molecular data difficult. These discrepancies suggest that molecular detection of MLL rearrangement in AML other than AML M4/M5 and characterization of the type of abnormality will be important for adequate evaluation of prognostic impact.

After detection by Southern blotting, MLL abnormalities can be characterized by FISH or by reverse transcription-polymerase chain reaction (RT-PCR) amplification of the various fusion transcripts described to date. It is not yet clear which combination of techniques will best complement classical karyotype analysis in large-scale screening programs. On initial screening by Southern blotting, we identified MLL rearrangements, not only in monocytic AML, but also in AML M1. This subtype of AML is heterogeneous and is often associated with an apparently normal karyotype. We, therefore, undertook to determine the incidence of MLL rearrangements in AML M1 and to compare the type of abnormalities with those in AML M5 by additional Southern, RT-PCR, and FISH analyses. We demonstrate that MLL rearrangement occurs in approximately 20% of AML M1, that MLL rearrangements in M1 and M5 are predominantly due to MLL self-fusion and MLL-AF6, and that RT-PCR screening of rearranged cases is preferable to FISH analysis for identification of the type of rearrangement.

MATERIALS AND METHODS

Patient Morphologic, Immunophenotypic, and Cytogenetic Analysis

Forty-one patients with diagnoses of AML and treated in three different centers between 1989 and 1994 were studied. The patients with AML M5 and neonatal AML were recruited from a single
peripheral blood smears stained by May-Grunwald-Giemsa, ac-
tically. Morphologic analysis was performed

techiques. Cases were considered positive if greater than 20% of

RT-PCR Analysis

Southern Blot Analysis and DNA Probes

High-molecular-weight DNA was extracted from cryopreserved
cells, digested with BamHI, electrophoresed on 0.7% agarose, trans-
ferred to nylon membranes (Hybond N+; Amersham, Buckingham-
shire, UK), and hybridized with a random primed 32P-labeled probe. Membranes were washed at a final stringency of 0.1
saline sodium
citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) at 65°C before
autoradiography. Rearranged cases were also digested with HindIII
and, in selected cases, with EcoRI. 15 and 19 are genomic probes

Table 1. Sequences of Primers Used for RT-PCR Analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense*</td>
<td></td>
</tr>
<tr>
<td>MLL-Ex5S-ext</td>
<td>5'-GAGGATCCTCCTCCAAAAAGAAAAAG-3'</td>
</tr>
<tr>
<td>MLL-Ex5S-int</td>
<td>5'-GCCTGATCCAAAGGACCCACGT-3'</td>
</tr>
<tr>
<td>MLL-Ex5S</td>
<td>5'-GCAAAAGAAAAATGCGCTCCCGG-3'</td>
</tr>
<tr>
<td>HRX-Ex6</td>
<td>5'-gcaaatTCAGACAGCAGCAAGAACAG-3'</td>
</tr>
<tr>
<td>MLL-Ex7</td>
<td>5'-gcaaatTCAGACACTCTCCTCCAATGG-3'</td>
</tr>
<tr>
<td>Anti-sense</td>
<td></td>
</tr>
<tr>
<td>HRX-Ex3B</td>
<td>5'-AGAAgtCCTTTCTTCAATTA-3'</td>
</tr>
<tr>
<td>AF6-AS-ext</td>
<td>5'-CTCGGATCTAGATCCTTACATG-3'</td>
</tr>
<tr>
<td>AF6-AS-int</td>
<td>5'-TACCTGGAAGAGAGACATTGCG-3'</td>
</tr>
<tr>
<td>AF9-AS1</td>
<td>5'-CAGAGTCTATTGCTGATTCCTCCAAC-3'</td>
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<tr>
<td>AF9-AS3</td>
<td>5'-TCACGATCTGTCGAGAATGTGT-3'</td>
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<td>ENL-AS2</td>
<td>5'-AGCTGATCCCAGACTCCTACCTTG-3'</td>
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<td>ENL-AS3</td>
<td>5'-GACGAAGAGTGCTCCTCCTCGGACT-3'</td>
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<tr>
<td>ELL-AS-ext</td>
<td>5'-CAGACTCGGTCAGGAAAGG-3'</td>
</tr>
<tr>
<td>ELL-AS-int</td>
<td>5'-GCGGATGTGAGACTGATGAGA-3'</td>
</tr>
</tbody>
</table>

Lowercase bases represent mutations introduced for cloning purposes.
Abbreviation: nt, nucleotide.

* MLL nucleotide nomenclature from Gu et al.5

Southern Blot Analysis and DNA Probes

High-molecular-weight DNA was extracted from cryopreserved
cells, digested with BamHI, electrophoresed on 0.7% agarose, trans-
ferred to nylon membranes (Hybond N++; Amersham, Buckingham-
shire, UK), and hybridized with a random primed 32P-labeled probe. Membranes were washed at a final stringency of 0.1
saline sodium
citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) at 65°C before
autoradiography. Rearranged cases were also digested with HindIII
and, in selected cases, with EcoRI. 15 and 19 are genomic probes

RT-PCR Analysis

RNA was extracted, and cDNA was synthesized from 2 μg RNA, as
previously described.39 For PCR amplification, 4 μL (0.4 μg)
cDNA, PCR buffer, 2.5 mmol/L MgCl2, 0.2 mmol/L deoxynucleo-
tide triphosphate (dNTP), 5% dimethyl sulfoxide (DMSO), 0.4 μmol/
L oligonucleotide primers, and 2 U Taq polymerase in a total volume
of 50 μL were amplified for 35 cycles in a DNA thermal cycler
(Biometra, Göttingen, Germany) with a stepwise program consisting
of 92°C for 30 seconds, 60°C for minute, and 72°C for 1 minute (2
minutes for AF9-AS3). This was preceded by a denaturation step
of 92°C for 3 minutes before the first cycle. The final extension
phase was increased to 10 minutes. Single-stage PCR was performed
using the MLL-Ex5-prim and either AF6-AS-ext, AF9-AS1, AF9-AS3, ENL-AS2, ENL-AS3, ELL-AS-ext, or ELL-AS-int (Table
1). Confirmation of the identity of positive bands was undertaken
by hybridization with an internal MLL probe or by a second amplifi-
cation with a different MLL primer. Only bands that corresponded
with the expected size with MLL primers specific for at least two
exons were considered positive. Detection of the Ex2-Ex6 MLL
duplication was performed with HRX-Ex6 and HRX-Ex3B primers,
as previously described.33 PCR products were size-fractionated by
electrophoresis in a 2% agarose gel and visualized with ethidium
bromide. Quality of RNA was assessed by concurrent amplification
of PBGD transcripts from the same cDNA using exon 1 and exon
7 oligonucleotide primers. HL60 RNA and no-RNA negative con-
trols were reverse-transcribed and amplified in parallel with all pa-
tient samples. Standard precautions were undertaken to prevent prod-
tuct carry-over. Exclusion of reagent/RNA contamination by PCR
product as a cause of positive results in RT-PCR–positive, cytoge-
etic-negative cases was assessed by the failure to detect a PCR
product after amplification, after reverse transcription in the absence
of reverse transcriptase.

Sequence Analysis

For sequencing, the PCR product was purified on a SpinX column
according to the manufacturer’s instructions (Costar, Cambridge,
MA) and cloned in pMosBlue (Amersham, Buckinghamshire, UK). The 1.2-kb fragment from UPN 427 was BanHl-digested, and a
970-bp fragment was subcloned into pBlueScript KS(+) (Stratagene,
La Jolla, CA). PCR products were sequenced using the Prism Dye
Primer kit with T7 and T3 primers and analyzed on an Applied
 Biosystems 373A automated analyzer (Perkin Elmer/ABI, Roissy,
France).

FISH Analysis

In situ hybridization was performed as previously described.39
The 13H14 yeast artificial chromosome (YAC),33 hybridizing to
Table 2. Clinical, Morphologic, Cytogenetic, RT-PCR, and Southern Data on the 12 AML Cases With MLL Rearrangement

<table>
<thead>
<tr>
<th>Case</th>
<th>Specimen</th>
<th>FAB</th>
<th>Sex/Age</th>
<th>Karyotype [no. of mitoses analyzed]</th>
<th>Southern</th>
<th>RT-PCR</th>
<th>FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>427</td>
<td>PB</td>
<td>M1</td>
<td>F/6 mos</td>
<td>46,XX,t(9;11)(p21;q23)[24]</td>
<td>R/G</td>
<td>R/G</td>
<td>G</td>
</tr>
<tr>
<td>510</td>
<td>PB</td>
<td>M1</td>
<td>M/68 yrs</td>
<td>46,XY<a href="q23">11</a>[9]</td>
<td>R/G</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>303</td>
<td>PB</td>
<td>M1</td>
<td>F/45 yrs</td>
<td>46,XX[22]</td>
<td>R/G</td>
<td>G/R</td>
<td>G</td>
</tr>
<tr>
<td>313</td>
<td>BM</td>
<td>M1</td>
<td>M/52 yrs</td>
<td>ND</td>
<td>R/G</td>
<td>G/G</td>
<td>G</td>
</tr>
<tr>
<td>506</td>
<td>BM</td>
<td>M1</td>
<td>F/82 yrs</td>
<td>ND</td>
<td>R/G</td>
<td>G/G</td>
<td>G</td>
</tr>
<tr>
<td>532</td>
<td>BM</td>
<td>M1</td>
<td>F/25 yrs</td>
<td>46,XX[18]</td>
<td>R/G</td>
<td>G/R</td>
<td>G</td>
</tr>
<tr>
<td>18</td>
<td>PB</td>
<td>M5B</td>
<td>F/1.5 mos</td>
<td>46,XXt(11;19)(q23;p13)[23]</td>
<td>R/G</td>
<td>G/G</td>
<td>G</td>
</tr>
<tr>
<td>507</td>
<td>PB</td>
<td>M5A</td>
<td>M/6 yrs</td>
<td>47,XYt(9;11)[p21;q23],+8[10]/48,del(9)[5]</td>
<td>R/G</td>
<td>G/G</td>
<td>ND</td>
</tr>
<tr>
<td>336</td>
<td>BM</td>
<td>M5B</td>
<td>M/18 yrs</td>
<td>46,XY[25]</td>
<td>R/G</td>
<td>G/G</td>
<td>G</td>
</tr>
<tr>
<td>306</td>
<td>BM</td>
<td>M5B</td>
<td>F/44 yrs</td>
<td>46,XX[18]</td>
<td>R/G</td>
<td>G/R</td>
<td>G</td>
</tr>
<tr>
<td>236+</td>
<td>PB</td>
<td>M5A</td>
<td>M/22 yrs</td>
<td>55,XYt(11)(q10),+3,+5,+6,+8,+18,+19,+20,+22[30]/55,del(11;3)[q23;p24][2]</td>
<td>R/G</td>
<td>R/G</td>
<td>ND</td>
</tr>
<tr>
<td>582</td>
<td>BM</td>
<td>M5B</td>
<td>F/36 yrs</td>
<td>46,XXt(6;11)[q27;123][17]</td>
<td>R/G</td>
<td>G/G</td>
<td>G</td>
</tr>
</tbody>
</table>

Abbreviations: PB, peripheral blood; BM, bone marrow; ND, not done; R, rearranged; G, germline; N, normal.
* Type of fusion transcript; data in italics refer to the exons involved, with exon, for example, indicating the presence of both exon 6 and exon 7 transcripts.
† Analyzed at relapse.
‡ Detected by Southern blot analysis.

RESULTS

Patient Characteristics

The 41 patients analyzed included 29 patients with AML-M1, 10 with AML-M5, and two with neonatal AML with trisomy 21, one of which demonstrated trisomy 21 as a constitutional finding and the other as an acquired abnormality limited to the myeloid cells (the fibroblast karyotype was normal). Excluding the latter two patients, median age was 56 years (range, 1.5 months to 82 years); 22 were males, and 17 were females. The AML M5 cases included three patients aged less than 18 years. All but one AML M1 case were aged over 18 years (range, 20 to 82 yrs.) Karyotype analysis was abnormal in 14 of 28 cases tested. Chromosome 11q23 abnormalities were observed in five cases (2 of 21 AML M1 cases, and three of seven M5 cases). Clinical details, morphologic classification, and karyotype of the cases demonstrating 11q23 abnormalities and/or MLL rearrangement are listed in Table 2.

Detection of MLL Rearrangement by Southern Blot Analysis

MLL rearrangement was observed in 12 samples after hybridization of BamH1-digested DNA with the 15 probe (Fig 1B) and was confirmed in HindIII digests for 11 samples with sufficient DNA (Table 2). After rehybridization with the I9 probe (Fig 1A), only 4 of the 12 samples demonstrated an abnormal BamH1 fragment (Fig 1B), all of which differed in size from the I5 rearranged fragment, indicating that the putative chromosome 11 breakpoint lay between the two probes. The germline pattern observed in the eight other samples can be attributed to either deletion of the telomeric part of the MLL locus or MLL self-fusion not encompassing the I9 probe. To discriminate between these two possibilities, additional hybridizations were performed with I1, specific

ch...
for MLL intron 1. Three samples (UPNs 303, 306, and 532) demonstrated an abnormal fragment in both EcoRI (Fig 1B) and BamHI digests, compatible with MLL self-fusion. Hybridization of 11 on HindIII digests from these cases revealed only germline bands, thus localizing the duplication start point between the BamHI site and the most 3′ HindIII site in intron 1 (Fig 1A).

Therefore, of the 41 patients tested, 12 were shown to have a rearranged MLL locus. These included 6 of 29 (20%) AML M1 cases, (5 of 28 adult M1 cases) and 6 of 10 M5 cases, including two of four M5A and three of six M5B cases, although it has been suggested that 11q23 abnormalities are more frequent in M5A. Neither case of neonatal AML demonstrated rearrangement. Median age of MLL-rearranged patient at diagnosis was 48 years, not significantly different from that of MLL germinal M1 cases (58 years). The unexpectedly high incidence in AML M1 was not due to misclassification of early M4/M5 cases, because morphologic re-evaluation confirmed their M1 nature and did not demonstrate any particular distinguishing features. All five cases tested were α-naphthyl butyrate esterase (NBE)-negative. Immunologic comparison, however, demonstrated more frequent expression of the CD11c (MLL rearranged, five of six cases positive; MLL-unrearranged, 6 of 16 positive) and CD4 (four of six cases v 6 of 16) monocytic-associated markers in MLL rearranged M1 cases, suggesting that these leukemias more commonly demonstrate early immunologic monocytic differentiation than their MLL-unrearranged M1 counterparts. However, expression of the more mature CD14 and CD36 monocytic markers was not seen (data not shown). It has been suggested that MLL-rearranged AML cases frequently express lymphoid markers, including CD7, CD19, and CD22. This was not confirmed in the MLL-rearranged M1 and M5 cases analyzed here; CD7 expression was observed in only 1 of 11 patients tested, and CD10, CD19, and CD22, in none (11, 10, and 10 cases tested, respectively). This discrepancy may be due to the fact that the cases identified by Cuneo et al were predominantly AML M4, which were not analyzed in the present series.

**RT-PCR Characterization of Cases With MLL Rearrangement**

Of the 10 MLL-rearranged patients who underwent cytogenetic analysis, only five were shown to have an 11q23 cytogenetic abnormality (Table 2). None of the germline MLL patients showed apparent 11q23 abnormalities. Because Southern blot analysis with MLL probes does not identify the MLL partner, except in MLL self-fusions rearranged with the 11 probe, further analyses were undertaken by RT-PCR (MLL-AF6, MLL-AF9, MLL-ENL, and MLL-ELL and MLL self-fusion) and FISH in all MLL-rearranged cases with appropriate material available. UPN 236, with complex karyotypic abnormalities, lacked material for RT-PCR.

**Detection of MLL self-fusion by RT-PCR.** MLL self-fusion was investigated in UPNs 303, 313, 508, 532, 507, 336, and 306, all of which were rearranged with the 15 probe and germline with the 19 probe. RT-PCRs were performed using an exon 3 antisense primer and an exon 6 sense primer (Fig 1A), thus allowing amplification only from a duplicated MLL transcript. As shown in Fig 1C, a discreet 292-bp fragment was observed in the lanes corresponding to UPNs 303, 306, 508, and 532, consistent with partial duplication of MLL exon 2 to exon 6, as previously described. UPN 508, who demonstrated only a germline BamHI and EcoRI pattern with the 11 probe, was interpreted as harboring an MLL self-fusion with a duplication start point lying 5′ to the intron 1 EcoRI site (Fig 1A). All three MLL self-fusion cases tested karyotypically were normal, without trisomy 11 (Table 2). The possibility that MLL self-fusion could be somatically inherited was investigated in UPN 306. Single-stage RT-PCR performed on RNA extracted from apparently normal bone marrow obtained 4 months after diagnosis was negative (data not shown).

**MLL-AF6.** UPNs 510, 313, 562, 507, 336, and 306 were investigated by MLL-AF6 RT-PCR. A specific fragment was observed in four of six cases (UPNs 313, 336, 510, and 562; Fig 1C). The size of the amplified fragments observed with two independent pairs of primers in UPNs 313, 336, and 562 was compatible with the previously described MLL-AF6 fusion transcript. A larger, 667-bp fragment was found in UPN 510 and was shown to contain a fusion of MLL exon 7 to the same AF6 exon (data not shown). An apparently identical-sized minor band was also observed in UPN 313, probably representing an alternative transcript (Fig 1C). RT-PCR amplification, therefore, detected MLL-AF6 in both patients with a karyotypic t(6;11): one with a normal karyotype and one in whom chromosome analysis had not been performed.

**MLL-AF9.** RT-PCR detected, as expected, a specific fragment in lanes corresponding to UPNs 427 and 507 (Fig 1C), both of which demonstrated a t(9;11), but not in cDNAs from UPNs 510, 313, and 336. UPN 507 was positive with the MLL-Ex5S-extAF9-AS3 primer pair but negative with the MLL-Ex5S-extAF9-AS1 primer pair, compatible with an MLL exon 6 fusion to the AF9/LTG9 type B fusion previously described. UPN 427 demonstrated an approximately 2-kb fragment after amplification with MLL-Ex5S-extAF9-AS3 (Fig 1C) and two distinct bands of approximately 1.1 and 1.2 kb after amplification with MLL-Ex5S and AF9-AS1 (data not shown). Sequence analysis of the larger band demonstrated a novel AF9 fusion site, fused to MLL exon 8 (Fig 2). This AF9 fusion (type C) was situated 704 bp upstream of the most frequently described type A fusion. The smaller, 1.1-kb band is likely to correspond to an MLL-Ex7-AF9 type C alternative fusion transcript.

**MLL-ENL/LTG19 and MLL-ENL/MEN.** The one remaining, uncharacterized, MLL-rearranged patient (UPN 18) demonstrated a t(11;19)(q23;p13), confirmed by FISH (see below). RT-PCR amplification with MLL-Ex6S and ENL-AS2 (specific for the ENL gene at 19p13.3) primers did not generate any specific bands from this patient, although PBGD controls showed amplifiable cDNA and parallel amplification of a t(11;19) ALL demonstrating an MLL exon 7β-ENL “A” fusion transcript showed that the primers used were capable of amplifying this type of transcript (data not shown). Further amplifications using various MLL exon 5 and 7 primers and the ENL-AS2 and AS3 primers previously described were also negative. RT-PCR amplifications with
MLL exons 5, 6, and 7 sense and two ELL antisense primers (specific for the ELL/MEN gene at 19p13.1; Table 1)15,16 were also negative (data not shown). It is, therefore, likely that the breakpoint in chromosome 19 is distinct from those previously described.

Detection of Chromosome 11q23 Abnormalities by FISH

FISH hybridization was performed with the 13HI4 YAC encompassing the MLL locus (in 10 cases) and a chromosome 11 paint (in four cases). In three patients, analysis with the MLL YAC showed three signals on metaphase cells and on interphase nuclei (Table 2), confirming the presence of a reciprocal 11q23 rearrangement. These included one t(9;11), one t(11;19), and one initial del(11)(q23) [UPN 510]. Re-evaluation of the latter karyotype in light of the RT-PCR and FISH findings showed that it was, in fact, a t(6;11), known to be difficult to detect by banding analysis.9,18 FISH analysis of the t(11;19), which could not be amplified with either MLL-ENL or MLL-ELL primers, did not allow improved resolution of the 19p13 breakpoint. No signal was seen with the MLL YAC on the der(6) in metaphases from UPN 562, known to possess a t(6;11). A strong signal on chromosome 6 was, however, obvious when FISH was performed with a chromosome 11 paint. Since this case demonstrated no rearrangement with 19, it is likely that the translocation was associated with deletion of the distal part of MLL.

Metaphases from four patients with MLL rearrangement but with a normal karyotype were analyzed by FISH. As expected, in mitoses from the three cases with MLL duplication (UPNs 306, 303, and 532), the MLL YAC showed only two signals, one on each chromosome 11. MLL duplication has been demonstrated frequently, but not exclusively, in association with trisomy 11.23,30 None of the current three duplicated cases demonstrated trisomy 11. MLL YAC analysis of 200 to 400 interphase nuclei from all three demonstrated only two signals in over 90% of cells, thus excluding the possibility that a trisomy 11 was present in a nonmitotic population.

The one remaining MLL-rearranged patient with a normal karyotype (UPN 336) did not demonstrate any apparent abnormality by FISH with both the MLL YAC and the chromosome 11 paint. In this case, characterized by an MLL-AF6 transcript by RT-PCR, failure of the YAC to detect the translocation may be explained by deletion of the telomeric, 3' part of MLL, as evidenced by the absence of Southern rearrangement with 19. Failure to detect any translocation with the chromosome 11 paint suggested either an unbalanced translocation or failure of the paint to detect the 11qter, although it did detect the t(6;11) from UPN 562, also characterized by an MLL 3' deletion. Analysis with an AF6 cosmid demonstrated three signals — on two chromosomes 6 and one chromosome 11 — compatible with hybridization to one normal chromosome 6 and duplication or splitting of the AF6 locus between the second chromosome 6 and the chromosome 11. Dual hybridization with the MLL YAC and the AF6 cosmid demonstrated colocalization on one apparently normal chromosome 11 (Fig 3). These data are compatible with an interstitial insertion of a submicroscopic portion of chromosome 6, including part of the AF6 gene, into the MLL locus, with an associated deletion of part of 11q, including the 3' portion of MLL. Further characterization of this case is underway.

The MLL YAC and the chromosome 11 paint also did not detect any abnormality in UPN 236, characterized by a
complex karyotype without any apparent chromosome 11 abnormalities. This result is more unexpected than in the previous cases, as the I9 Southern probe was rearranged. Although it was not possible to test for MLL self-fusion by RT-PCR in this case, the I9 rearrangement and the absence of 11 rearrangement after an EcoR1 digest makes this an unlikely explanation for the absence of FISH abnormalities. Southern analysis, however, showed MLL rearrangement in a minority population, compatible with the fact that this case presented with only 30% blasts in the blood and bone marrow. The complex karyotypic abnormalities detected, however, preclude analysis of normal residual cells as an explanation for the negative FISH results and suggest that the MLL rearrangement detected by Southern blot occurred in a different subclone to those analyzed karyotypically. In keeping with this, at least two karyotypically abnormal clones were identified (Table 2).

**DISCUSSION**

In this study, we evaluated optimal screening strategies for the detection and characterization of MLL abnormalities in AML by studying a series of predominantly adult FAB M1 AML by Southern blotting and subsequent RT-PCR and FISH. Southern blot screening, without prior cytogenetic selection, demonstrated MLL rearrangement in 6 of 29 (20%) AML M1 and 6 of 10 M5 cases. As MLL rearrangement is already known to be frequent in AML M5, this small series
of M5 cases was included to enable us to compare the types of MLL abnormalities in AML M1 and monocytic AMLs.

The high incidence of MLL rearrangement in M1 was unexpected. Sporadic reports of 11q23 abnormalities in AML M1,4 some of which have been shown to involve MLL,7,38 exist. The incidence of MLL rearrangement in non-monoblastic AML has not been widely studied but has recently been suggested to be low.21 The data presented here suggest that this is not the case for AML M1. The discrepancy between published cytogenetic data and the molecular results presented here may be related to the fact that three of six MLL rearrangements detected in M1 cases were self-fusions (see below).

MLL rearrangement was seen in the single case of infant AML M1 studied, but was also found in 18% of adult patients. The median age of patients with MLL-rearranged M1 was not significantly different from that observed in the MLL-negative M1 group, suggesting that the predominance of MLL rearrangements in younger AML patients21 is restricted to monocytic cases. Morphologic reanalysis excluded misclassification, although immunophenotyping suggested that MLL-rearranged M1 cases demonstrate early monocytic differentiation more frequently than their MLL-germline counterparts.

RT-PCR analysis of the MLL-rearranged cases demonstrated several noteworthy findings. All four MLL self-fusions involved duplications of exons 2-6, although alternative duplications involving exons 2-8 have been described in AML.22,23 All occurred in adult patients, as have all seven previously published cases, many of which occurred in AML M1 and M2.23,39 In five of these seven cases, MLL self-fusion was associated with trisomy 11. The data presented here confirm that this abnormality also occurs frequently in the absence of such abnormalities.

All four MLL-AF6 cases demonstrated the same AF6 fusion transcript as the two previously described cases,12 but with variable MLL exon usage, as previously described for several other fusion transcripts.38,40 The high incidence of MLL-AF6 and MLL self-fusions, and their relative homogeneity by RT-PCR, will render them useful markers for patient follow-up.

Analysis of MLL-AF9 fusion transcripts identified one alternative 3′ MLL-AF9 fusion, previously described in ALL,41 and labeled AF9′B′.38 Southern and RT-PCR analyses have suggested clustering of AML breakpoints in the 5′ AF9′A′ region (Fig 2) and ALL breakpoints in the 3′ AF9′B′ region.37,38 Identification of a type 'A′ transcript in ALL43 and the present demonstration of an AF9′B′ breakpoint in AML M5 shows that this is not absolute. A novel, third MLL-AF9 fusion was identified in the second case. Sequence analysis showed that the breakpoint (AF9′C′; Fig 2) occurred 704 nucleotides 5′ to the AF9′A′ breakpoint. Of the seven (t9;11) AML cases analyzed by Yamamoto et al.,24 two were found to be negative by RT-PCR. It is possible that these cases correspond to the AF9′C′ identified here, although our case was identified using the same primers.38

RT-PCR did not detect an MLL-ENL or MLL-ELL fusion in the (t11;19) case. Despite the lack of an available positive control for ELL, the failure to detect a specific PCR product suggests that further breakpoints may exist in either gene, or even that the 19p13 breakpoint may involve a distinct gene.

MLL rearrangement was detected in approximately 20% (5 of 23) of AML cases without apparent 11q23 abnormalities, including four cases with a normal karyotype and one with complex abnormalities. RT-PCR in these cases demonstrated three MLL self-fusions and one MLL-AF6, whereas FISH analysis with an MLL YAC was negative in the two MLL self-fusions tested, as expected, but also in the MLL-AF6. Further analysis with an AF6 cosmid showed that this was due to insertion of a submicroscopic portion of chromosome 6, including part of AF6, into an apparently normal chromosome 11. This type of cytogenetic abnormality is well recognized in t(9;22)-negative chronic myeloid leukemia (CML), with interstitial insertion of a small part of chromosome 9 including the 3′ part of ABL next to BCR on an apparently normal chromosome 2241 and has also been described in other AMLs, such as PML-RARA-positive, t(15;17)-negative AML M3.42 However, to our knowledge, it has not been described in rearrangements involving MLL.

Comparison of the contribution of RT-PCR versus FISH analysis to the characterization of MLL rearrangements, therefore, showed that RT-PCR for the three major types of MLL abnormalities identified 10 of 11 cases, whereas FISH with a combination of an MLL-specific probe and a chromosome 11 paint detected four of nine cases. RT-PCR failed to detect one (t11;19), and FISH with the MLL YAC did not detect three MLL self-fusions: one karyotypic (t6;11), one hidden MLL-AF6, and one case with a probable MLL subclone distinct from those analyzed karyotypically.

The majority of molecular studies identifying MLL partner genes are based on preselection of cytogenetically abnormal cases. In the present study, their relative frequencies are independent of karyotypic findings and are, thus, likely to reflect those present in AML M1 and M5. We identified four MLL-AF6, four MLL self-fusions, two MLL-AF9, one uncharacterized (t11;19), and one uncharacterized MLL rearrangement. These relative frequencies are different from those identified in the study by Yamamoto et al.,24 in which patients with 11q23 abnormalities were analyzed for MLL-AF4, MLL-AF9, and MLL-ENL. Of the eight AML cases (two M4, five M5, and one M1), seven demonstrated t(9;11), five of which were shown to be MLL-AF9, and one t(11;19) M5 showed an MLL-ENL.38 From our results, it is apparent that the two most common abnormalities, present in at least two thirds of cases, are either difficult or impossible to detect by classical karyotype analysis. The number of cases characterized here is too low to determine whether the types of MLL abnormalities are different in AML M1 and M5. It is, however, worth noting that three of four of the MLL self-fusions occurred in AML M1.

AML M1, M4, and M5 represent 50% of AML cases.43 The prior demonstration that approximately 20% of adult AML M4 and M5 cases have MLL rearrangements,20,21 taken together with the data presented here, make this the most common specific genetic abnormality in AML and suggests that routine screening in a prospective study is justified. Furthermore, chromosome 11q23 abnormalities are also rec-
ognized in AML M2, M6, and M7. It is, therefore, probably justifiable to undertake initial screening in all de novo AML cases other than the M3 subtype, increasingly treated on separate protocols.

Given the diversity of MLL abnormalities, screening strategies need to be chosen with care. Southern hybridization with an MLL probe after a single BamHI digestion detects the vast majority of MLL rearrangements, although rearrangements not detected on BamHI digests have been described. Several groups screen for MLL rearrangement using a cDNA probe including exons 5-11. This probe has the advantage of simultaneously detecting MLL rearrangement and 3' deletion, but does not allow mapping of breakpoints on EcoRI digests, which is possible with the IS and IG genomic probes. The information provided by these two strategies is, however, otherwise similar. An interesting alternative to Southern analysis is initial multiplex screening by RT-PCR, although this strategy will not detect rare or currently uncharacterized abnormalities. Initial MLL FISH analysis should theoretically detect rearrangement regardless of the partner, but frequent 3' telomeric deletions, MLL self-fusions, and, now, the demonstration of interstitial insertions, limit the use of this approach for screening purposes in AML. Furthermore, these techniques are dependent on the availability of metaphases that are representative of different tumor subclones. Based on the data presented here, we would recommend initial screening of a single BamHI digest and hybridization with one cDNA or two genomic MLL probes, with subsequent single or multiplex RT-PCR characterization. FISH analysis is useful in doubtful or unidentified cases, or where confirmation of certain RT-PCR results is required. Characterization of MLL rearrangements by these strategies will determine whether the different subtypes have different biologic characteristics or prognostic significance.

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Incidence and characterization of MLL gene (11q23) rearrangements in acute myeloid leukemia M1 and M5


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