Rearrangements of the MLL Gene in Therapy-Related Acute Myeloid Leukemia in Patients Previously Treated With Agents Targeting DNA-Topoisomerase II


Chromosome band 11q23 is frequently involved in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) de novo, as well as in myelodysplastic syndromes (MDS) and lymphoma. Five percent to 15% of patients treated with chemotherapy for a primary neoplasm develop therapy-related AML (t-AML), that may show rearrangements, usually translocations involving band 11q23 or, less often, 21q22. These leukemias develop after a relatively short latent period and often follow the use of drugs that inhibit the activity of DNA-topoisomerase II (topo II). We previously identified a gene, MLL (myeloid-lymphoid leukemia or mixed-lineage leukemia), at 11q23 that is involved in the de novo leukemias. We have studied 17 patients with t-MDS/t-AML, 12 of whom had cytogenetically detectable 11q23 rearrangements. Ten of the 12 t-AML patients had received topo II inhibitors and 9 of these, all with balanced translocations of 11q23, had MLL rearrangements on Southern blot analysis. None of the patients who had not received topo II inhibitors showed an MLL rearrangement. Of the 5 patients lacking 11q23 rearrangements, some of whom had monoblastic features, none had an MLL rearrangement, although 4 had received topo II inhibitors. Our study indicates that the MLL gene rearrangements are similar both in AML that develops de novo and in t-AML. The association of exposure to topo II-reactive chemotherapy with 11q23 rearrangements involving the MLL gene in t-AML suggests that topo II may play a role in the aberrant recombination events that occur in this region both in AML de novo and in t-AML.

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Chromosomal abnormality involving 11q23 were included. These samples were obtained from 1985 to 1992. Similar material was provided by the University Hospital, Copenhagen, Denmark, including Ficoll-Isopaque-isolated mononuclear cells or granulocytes from the same patients. We also analyzed 5 patients who lacked cytogenetic 11q23 rearrangements, but who developed t-AML or t-MDS (with monoblastic features) after treatment with topo II-reactive drugs or after other types of chemotherapy. Cytogenetic analysis was performed using trypsin-Giemsa banding.

**DNA analysis.** DNA was extracted from leukemia cells using standard techniques. Ten micrograms of each sample was restricted with BamHI or HindIII, separated by electrophoresis on 1% agarose gels, and transferred to Genescreen (New England Nuclear Research Products, Boston, MA) nylon membranes. The MLL0.7B probe was cloned from a cDNA library as described elsewhere. The probes corresponding to the extreme 5' and 3' ends of the 8.3-kb BamHI fragment were made by polymerase chain reaction (PCR) amplification of MLL0.7B sequences. Probes were 32P-labeled by the random priming method (Pharmacia, Piscataway, NJ). Hybridization to Southern blots was performed with 50% formamide at 42°C. Blots were washed to a final stringency of 1x or 0.1 x SSC, 1% sodium dodecyl sulfate (SDS), at 65°C, before autoradiography.

**RESULTS**

DNA from 17 patients with t-AML or t-MDS was analyzed for rearrangements of the MLL gene. Clinical information for all patients is presented in Table 1. Included are 12 samples (nos. 1 through 12) that showed cytogenetically detectable 11q23 rearrangements (Table 2). Ten of these 12 patients had received chemotherapy that included topo II inhibitors. On Southern blot analysis, the 0.74-kb BamHI MLL cDNA fragment, MLL0.7B, detected DNA rearrangements in 9 of these 10 patients. All 9 showed balanced translocations of 11q23, cytogenetically. The other patient (no. 12) who showed no rearrangement with the probe had an unbalanced translocation (Tables 2A and 3, Fig 2, and data not shown). Two patients who had unbalanced rearrangements of 11q23 (nos. 10 and 11 with interstitial deletions q13-14q23) had received no topo II inhibitors. No DNA rearrangements were detected in these patients (Tables 2B and 3). A third subset of 5 patients (nos. 13 through 17) presented with t-AML or t-MDS but had no cytogenetically detectable 11q23 rearrangement. Four of the 5 (nos. 13 through 17) presented with t-AML or t-MDS but had no cytogenetically detectable 11q23 rearrangement. Four of the 5 (nos. 13 through 15 and 17) had received topo II inhibitors, whereas 1 (no. 16) had received only other types of chemotherapy. Three (nos. 14, 16, and 17) presented with M4 t-AML. No sample from this group showed any rearrangements with the MLL0.7B probe. Some of the patient samples were flow-sorted into granulocytes and mononuclear cells. In the two cases in which rearrangements were noted in these samples (nos. 7 and 9), both types of cells exhibited the rearrangement.

We reported previously that the MLL0.7B probe detected two rearranged bands, originating from both derivative chromosomes, in approximately 70% of the samples of leukemia de novo, as well as an 8.3-kb BamHI germline fragment. Probes made from each end of the MLL0.7B cDNA...
fragment, when hybridized separately, detected only one or the other of the two rearranged bands, indicating that each probe hybridized to the MLL gene on either the derivative 11 or the derivative chromosome formed with the other translocation partner. In samples in which only one rearranged band was detected, exon sequences telomeric to the breakpoint were shown to be deleted, whereas the centromeric exons were always retained. We have analyzed that showed rearrangements with the MLL0.7B probe, 1 (no. 9) exhibited a deletion of exon sequences telomeric to the breakpoint (data not shown). Moreover, we have detected rearrangements in 9 of 10 t-AML patients with 11q23 translocations, whereas a single patient (no. 12) who had an unbalanced 11q23 aberration and who had not received top0 II inhibitors, did not have rearrangements of this gene. We wanted to determine whether any of this group might have a cryptic MLL rearrangement. However, no MLL rearrangement was observed in any of these patients (Tables 1B and 2B).

**DISCUSSION**

We recently identified the MLL gene, which is rearranged in both common and rare 11q23 translocations in acute leukemia de novo. We have now detected molecular rearrangements in the same 8.3-kb region of the MLL gene in 9 of 17 patients who developed t-AML (Table 2A, Fig 2; and data not shown). Moreover, we have detected MLL rearrangements in 9 of 10 t-AML patients with 11q23 translocations, who had received chemotherapy that included topo II-targeting agents (Table 3). All 9 patients had balanced translocations, whereas a single patient (no. 12) who had an unbalanced 11q23 translocation cytogenetically had a germline BamHI fragment with the MLL0.7B probe. We studied this patient by fluorescence in situ hybridization (FISH) with a series of DNA probes from 11q23. We observed that the breakpoint is proximal to the entire MLL locus; thus, the rearrangement in this sample involves another gene or genes, not simply another region of MLL.

### Table 1. Clinical Patient Information

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex/Age (yr)</th>
<th>Primary Tumor</th>
<th>Treatment for Primary Tumor</th>
<th>Mo to Secondary Disease*</th>
<th>Diagnosis of Secondary Disease</th>
<th>Survival (mo)</th>
</tr>
</thead>
</table>
| A. Patients with MLL rearrangements
| 1 | M/64 | Large-cell lymphoma | CHOP | 9 | 1 |
| 2 | F/51 | Breast | Dex/Ctx/Tam; RT | 18 | 10 |
| 3 | M/29 | Germ cell | Cisp/Bleo/Vbl; Cisp/ActD | 31 | 12 |
| 4 | F/48 | Ovarian | Ctx/Dox 5FU; Cisp/Hex | 24 | 6 |
| 5 | F/69 | Lung | Etop/Cisp | 35 | 1 |
| 6 | F/33 | Breast | RT; 4-epidox/Cisp | 18 | 45+ |
| 7 | F/52 | Lung | Etop/CTX/CCNU/VCR; RT | 15 | 1 |
| 8 | F/25 | Rhabdomyosarcoma | ActD/Vcr/Ctx; RT; Ifos/Etop | 31 | 0 |
| 9 | M/25 | Non-Hodgkin’s lymphoma | CHOP; RT | 24 | 5 |

B. Patients with no MLL rearrangements

| 10 | F/48 | Polycythemia vera | 5FU; HU | Unknown | t-AML | 1 |
| 11 | M/44 | Hodgkin’s lymphoma | RT; MOPP | 53 (none) | t-MDS | 98 |
| 12 | M/43 | Hodgkin’s lymphoma | ABVD/MOPP | 40 (40) | t-MDS | 9 |
| 13 | M/53 | Non-Hodgkin’s lymphoma | Ctx/Vcr/Pred; CHOP/Bleo/CCNU; RT; BCNU/Vbl | 72 (60) | t-MDS | 7 |
| 14 | F/5329 | Breast | Ctx/Cisp | 56 (33) | t-AML (M4) | 0 |
| 15 | F/32 | Germ cell | Etop/Cisp/Bleo; RT | 68 (68) | t-AML | 30+ |
| 16 | M/69 | Rectal | BCNU/Vcr/OTIC/5FU | 104 (none) | t-AML (M4) | 1 |
| 17 | F/48 | Non-Hodgkin’s lymphoma | CHOP | 52 (52) | t-AML (M4) | 9 |

Topo II inhibitors are in bold.

Abbreviations: Ctx, cyclophosphamide; Dox, doxorubicin; Vcr, vincristine; CHOP, Ctx, Dox, Vcr, prednisone; Tam, tamoxifen; RT, radiotherapy; Cisp, cisplatin; Bleo, bleomycin; Vbl, vinblastine; Etop, etoposide; 5FU, 5-fluorouracil; Hex, hexamethylmelamine; 4-epidox, 4-epidoxorubicin; CCNU, lomustine; ActD, actinomycin D; Ifos, ifosamide; HU, hydroxyurea; MOPP, nitrogen mustard, vincristine, procarbazine, prednisone; ABVD, Dox, Bleo, Vbl, dacarbazine; BCNU, carmustine; Mtx, methotrexate; DTIC, dacarbazine.

* Total months from initial treatment; the number of months from first exposure to use of topo II inhibitors in parentheses.
Samples from 3 patients were flow sorted into mononuclear cells and granulocytes. In the 2 samples (patients no. 7 and 9, with t-AML, and M4 and M5b, respectively) that showed MLL rearrangements, the same rearranged band was present in both cell types. Patient no. 11 (with MDS) showed no rearrangement in either cell type. Our data suggest that the recombination event involving the MLL gene occurs in a monocytic-granulocytic (or earlier) precursor cell and that the rearrangement does not inhibit the differentiation of granulocytic precursors present in the mononuclear fraction of leukemia cells. The involvement of MLL in both myeloid and lymphoblastic leukemias as well as in mixed-lineage and biphenotypic leukemias suggests that some of the recombination events involving this gene could occur even earlier in hematopoietic differentiation, perhaps in a pluripotent stem cell.

In our previous study of MLL rearrangements in AML/ALL de novo, we observed that, in about 30% of the samples, one or more exons 3' (telomeric) of the breakpoint cluster region are deleted, whereas the 5' (centromeric) exons are always retained. The preservation of the 5' exons as well as the consistent conservation of the der (11) chromosome in variant translocations (involving a third chromosome) suggests that the der (11) contains the critical junction for the development of these leukemias. We have studied 2 t-AML samples that showed deletions of at least some of the centromeric exons of the 8.3-kb breakpoint region. This result may indicate that there is a difference in t-AML/tMDS and acute leukemias that develop de novo with respect to the critical regions of the MLL protein. However, our observations may simply show that exons important for the development of both types of leukemias are further centromeric to the breakpoint cluster region.

Although the MLL gene is rearranged in the majority of 1q23 translocations, a human lymphoma cell line, RC-K8, with a t(11;14)(q23;q32), has been shown to involve another gene, RCK, that is 110 to 400 kb telomeric to the MLL locus. Another study showed that a patient with a null-cell acute lymphoblastic leukemia with an (11;14)(q23;q11) translocation had a breakpoint on 11 that is distal to both MLL and RCK, involving an as yet unidentified gene. A second gene, PLZF, was identified at the junction of a t(11;17)(q23;q21) translocation in a patient with acute promyelocytic leukemia. The rearrangement resulted in a fusion of this gene with the retinoic acid receptor \( \alpha \) gene on chromosome 17. Thus, there are a number of other genes involved in the 11q23 rearrangements that are observed in human leukemia cells.

The leukemogenic nature of chemotherapeutic drugs has been recognized for a number of years. The earliest reported cases of t-AML were attributed to prior treatment with alkylating agents. These patients usually present with MDS after a median latent period of approximately 5 years. Cytogenetic features include a loss of chromosomes 5 and/ or 7 or the long arm of these chromosomes. The MDS progresses to overt AML that is relatively resistant to chemotherapy; the prognosis is poor and the survival time is short. Ratain et al first reported the association of high doses of the epipodophyllotoxin, etoposide, for treatment of a lung cancer, with the subsequent development of t-AML.

Table 3. Association of Molecular MLL Rearrangements and Prior Therapy With Top0 II Inhibitors

<table>
<thead>
<tr>
<th>MLL Rearrangement</th>
<th>Prior Treatment With Topo II Inhibitors</th>
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<tbody>
<tr>
<td>+</td>
<td>9 (nos. 1 through 9)</td>
</tr>
<tr>
<td>-</td>
<td>2 (nos. 10 and 11)</td>
</tr>
</tbody>
</table>

Numbers of patients in each category are listed first, with patient numbers in parentheses. All patients listed had cytogenetic 11q23 rearrangements \( (P = .01, \) Fisher’s exact test).
Fig 2. Southern blot analysis of t-AML and t-MDS patient DNA. Samples were digested with BamHI and Southern blot was hybridized to the MLL0.7B probe. Lanes are marked above with patient numbers from Table 1. Patients no. 3, 7, and 9 show one rearranged band in addition to the germline 8.3-kb BamHI fragment. Patients no. 4 and 6 show two rearranged bands. None of the other samples exhibited rearrangements. (Data for other patients in study not shown.) Both lanes of patient no. 9 material show apparent hybridization to a high molecular weight band. This was not seen in prior or subsequent hybridizations and is a nonspecific signal at the boundary of the compression zone. C, control DNA; mo, mononuclear cells; gr, granulocytes.

*MLL* rearrangements had balanced translocations and developed overt t-AML after a relatively short latency period. Those patients who showed no *MLL* involvement had unbalanced rearrangements, rare translocations, or normal karyotypes and developed t-MDS or t-AML after a much longer latent period (Tables 1 and 2, and Fig 3).

Although the desired effect of treatment with topo II inhibitors is to interfere with the essential functions of topo II
in dividing neoplastic cells, the actual biologic activities of these drugs vary in vivo. Both the epipodophyllotoxins and the anthracyclines appear to stabilize the DNA-enzyme complex after double-strand cleavage, resulting in the accumulation of DNA double-strand breaks. However, the anthracyclines, such as adriamycin, act by intercalating into the DNA, whereas the epipodophyllotoxins do not intercalate. In addition, in at least one cell line (HL60), the activities of these two types of drugs differ. Treatment with VM-26 results in degradation of DNA in S-phase, whereas doxorubicin causes arrest in S and G2 with no such degradation of the DNA. In another study, the epipodophyllotoxins have been implicated in the direct or indirect induction of apoptosis in addition to causing DNA double-strand breaks. Another group of drugs, known as the dioxipiprazones, has recently been associated with t-AML. In one study, the most frequently detected chromosome rearrangements were the t(15;17)(q22;q12) and the t(8;21)(q22;q22). These drugs appear to inhibit the catalytic activity of topo II by competing with DNA for direct binding to the enzyme; thus, they would inhibit, rather than induce, double-strand breaks.

The association of the use of topo II inhibitors with the development of t-AML may provide insights into the mechanism of aberrant recombination at 11q23 (and 21q22) in both t-AML and t-AML de novo. VP-16 and VM-26 have been observed to induce nonrandom chromosome aberrations, rearrangements of specific genes, and reciprocal exchanges between nonhomologous chromosomes in vitro. It has been proposed that, in the presence of these drugs, topo II molecules become trapped and covalently integrated in the DNA, thus becoming more abundant and available to mediate aberrant recombination. Both prokaryotic and eukaryotic purified topo II proteins have been shown to mediate recombination between nonhomologous DNA molecules in vitro. Finally, topo II has been implicated in aberrant recombination events as a component of chromatin structure, specifically as a major scaffold attachment region (SAR) protein. SARs have been localized to translocation breakpoint junctions, some of which have been shown to bind topo II specifically and to act as “hot spots” for double-strand cleavage.

The genomic MLL breakpoint cluster region (8.3-kb BamHI fragment) has recently been sequenced. Its overall sequence composition is extremely AT-rich and includes several Alu repeats. More importantly, the fragment contains several in vitro and in vivo consensus binding sites for topo II. At this time, we speculate that the region may contain a number of active topo II binding sites or may be especially prone to cleavage, acting as a “hot spot” for double-strand breakage, which, under the appropriate conditions, leads to aberrant recombination with a number of other chromosomal regions. Certain selected recombinations in hematopoietic cells would provide a proliferative advantage that ultimately results in leukemia. Evidence for translocation breakpoints occurring at or near topo II sites has recently been reported for the t(15;17).

In summary, rearrangements of the MLL gene are observed in t-AML, especially after treatment for a primary tumor with topo II-reactive drugs. The breakpoint cluster region within the MLL gene is the same as that observed in AML de novo. We are currently investigating the chromatin structure of the breakpoint region and testing for evidence of the ability of topo II to bind and to cut in this region. These studies should lead to an understanding of the possible role of topo II in the frequent illegitimate recombination events that occur both in AML de novo and in t-AML.

ACKNOWLEDGMENT
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