RAPID COMMUNICATION

HRX Involvement in De Novo and Secondary Leukemias With Diverse Chromosome 11q23 Abnormalities

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Chromosome band 11q23 is a site of recurrent translocations and interstitial deletions in human leukemias. Recent studies have shown that the 11q23 gene HRX is fused to heterologous genes from chromosomes 4 or 19 after t(4;11)(q21;q23) and t(11;19)(q23;p13) translocations to create fusion genes encoding proteins with structural features of chimeric transcription factors. In this report, we show structural alterations of HRX by conventional Southern blot analyses in 26 of 27 de novo leukemias with cytogenetically diverse 11q23 abnormalities. The sole case that lacked HRX rearrangements was a t(11;17)-acute myeloid leukemia with French-American-British M3-like morphology. We also analyzed 10 secondary leukemias that arose after therapy with topoisomerase II inhibitors and found HRX rearrangements in 7 of 7 with 11q23 translocations, and in 2 of 2 with unsuccessful karyotypes. In total, we observed HRX rearrangements in 35 leukemias involving at least nine distinct donor loci (1q32, 4q21, 6q27, 7p15, 9p21-24, 15q15, 16p13, and two 19p13 sites). All breakpoints localized to an 8-kb region that encompassed exons 5-11 of HRX, suggesting that fusion proteins containing similar portions of HRX may be consistently created in leukemias with 11q23 abnormalities. We conclude that alteration of HRX is a recurrent pathogenetic event in leukemias with 11q23 aberrations involving many potential partners in a variety of settings including acute myeloid leukemia, acute lymphoblastic leukemia, chronic myelogenous leukemia in blast crisis, and topoisomerase II inhibitor-induced secondary leukemias of both the myeloid and lymphoid lineages.

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alkylating agents and composed of patients with AML/ MDS with trilineage dysplasias, is associated with visible abnormalities of chromosomes 5 and/or 7.\textsuperscript{36,37}

In this report, we assessed the prevalence of HRX involvement by a wide variety of I1q23 aberrations in leukemias. HRX DNA rearrangements were detected in 25 of 26 de novo leukemias with I1q23 translocations and in 1 case with an I1q23 deletion. HRX rearrangements were also observed in 9 of 10 secondary leukemias that arose after therapy with topo II inhibitors, consistent with an analogous role in a subset of therapy-induced malignancies. In total, at least nine distinct cytogenetic loci were juxtaposed to HRX. These results suggest that HRX fusion proteins play a pleiotropic role in the pathogenesis of human leukemias in diverse clinical settings.

MATERIALS AND METHODS

Patient materials. Leukemic cells used in these studies were collected from patients enrolled in treatment protocols of the Pediatric Oncology Group (POG), the Southwest Oncology Group (SWOG), or institutional protocols of the Children's Hospital at Stanford or the University of New Mexico, which had been approved by the respective Institutional Review Boards. All patients and/or their parents/guardians gave informed consent for treatment and for collection of material for biologic studies.

Eight of 10 patients with secondary leukemia (patients no. 1 through 8) were enrolled in one of a series of POG protocols for the treatment of T-cell ALL (T-ALL) or stage III lymphoblastic lymphoma (NHL-III) and were treated with intensive rotational polychemotherapy that included significant doses of the epipodophyllotoxin teniposide as well as the topo II inhibitor doxorubicin combined with cyclophosphamide. As of December 2, 1992, 16 patients enrolled in these studies had developed a secondary leukemia of non-T-cell phenotype. Material for molecular analysis was available in 8 cases. The incidence of secondary leukemias and further details on these patients and their therapy are described in Table 2 and elsewhere.\textsuperscript{38}

Patient no. 9 was an 11-year-old male originally treated at the Children's Hospital at Stanford in 1981 for stage III abdominal Burkitt's lymphoma with a regimen that included high doses of cyclophosphamide (CTX) administered in combination with doxorubicin (DOX) as well as teniposide (VM26) (9 cycles of CTX at 1,000 mg/m\textsuperscript{2} and DOX at 50 mg/m\textsuperscript{2} and 6 cycles of VM26 at 100 mg/m\textsuperscript{2} over an 18-month period). Forty-six months after the initial diagnosis (27 months after completion of therapy), he developed a secondary AML that was classified as FAB subtype M5. Patient no. 10 was a 32-year-old woman who presented in 1989 with a diagnosis of diffuse noncleaved large-cell lymphoma (B cell); therapy at Brigham and Women's Hospital and the National Institutes of Health (NIH) included high doses of CTX and DOX. Thirty-eight months after initial diagnosis, she developed a secondary AML, classified as FAB M5.

The secondary leukemias were clearly myeloid in nine cases. Case no. 4 was classified as a secondary ALL, as the blast cells expressed the B-lineage cell-surface antigens CD10, CD19, CD22, CD24, and HLA DR and were negative for T-cell- (CD1, CD2, CD3, CD4, CD5, CD7, and CD8) and myeloid- (CD13 and CD33) associated markers. In all cases, the diagnosis and subclassification of leukemias was based on standard morphologic, cytochemical, and immunologic studies.

Cyto genetic studies. For the patients enrolled in POG studies, material was shipped by overnight carrier to the POG reference cytogenetic laboratory in Birmingham, AL and analyzed as described previously.\textsuperscript{39} SWOG patient samples were analyzed at approved SWOG reference laboratories and all submitted karyotypes were reviewed by the SWOG Cytogenetics Working Group. For patients on the POG T-ALL/NHL studies, repeat immunophenotyping and karyotyping were performed at the time of any suspected recurrence of leukemia. For some patients, karyotyping of the secondary malignancy was performed in local clinical cytogenetics laboratories.

Gene rearrangement analyses. High molecular weight DNA was digested to completion with restriction endonucleases (see below), separated by electrophoresis in 0.8% agarose gel, and immobilized on nylon membranes. Hybridization and washing of the blots was performed under stringent conditions, as described previously.\textsuperscript{40} For these studies, a 0.86-kb BamHI fragment of the HRX cDNA (referred to as B9) was used as a hybridization probe on BamHI-digested genomic DNA.\textsuperscript{10,11} For analyses of T-cell receptor \(\beta\) (TCR\(\beta\)) gene rearrangements, a cocktail of the \(J_\beta_1 + J_\beta_2\) probes\textsuperscript{41} was used to analyze \(\beta\)lI-digested genomic DNA. Some of the leukemias included in Table 1 have been reported in earlier studies.\textsuperscript{10,13}

RESULTS

A collection of 27 de novo leukemias with cytogenetically identified 11q23 abnormalities (26 translocations and 1 deletion) was analyzed for HRX gene rearrangements. The probe (B9) consisted of a fragment of HRX cDNA previously shown to span the fusion sites of chimeric transcripts resulting from the t(11;19) and t(4;11) translocations.\textsuperscript{10,13} This probe hybridized to HRX exons 5-11 contained on an 8-kb germline BamHI fragment as illustrated in Fig. 1. As shown in Table 1, the single leukemia with an 11q23 deletion and 25 of 26 with translocations involving at least eight different donor loci had rearrangements detected with this probe. In most cases, two rearranged bands were observed,

| Table 1. HRX Gene Rearrangements in De Novo Leukemias With 11q23 Cytogenetic Abnormalities |
|-----------------------------------|----------------|----------------|
| Cyto genetic Abnormality | Leukemia Type | No. of HRX Rearranged/ No. of Cases Analyzed |
| t(11;11)(q32;q23) | CML, bc | 1/1 |
| t(4;11)(q21;q23) | ALL | 5/5 |
| t(6;11)(q27;q23) | T-ALL | 1/1 |
| t(6;11)(q27;q23) | AML | 1/1 |
| t(7;11)(q15;q23) | AML-M7 | 1/1 |
| t(9;11)(q21-p24;q23) | AML | 5/5 |
| t(11;11)(p23;q10) | ALL | 1/1 |
| t(11;11)(p23;q21) | AML | 0/1 |
| t(11;19)(q23;q13) | ALL | 6/6 |
| t(11;19)(q23,p13) | AML | 4/4 |
| del (11)(q23) | ALL | 1/1 |

* One case was cytogenetically characterized as a t(9;11)(p13;q13).

As HRX rearrangements were clearly present in this leukemia, it is likely that interstitial deletions accompanied the translocation. It is uncertain whether this case involves the same chromosome 9 locus as do other t(9;11)s.

19p13 breakpoints were not distinguishable cytogenetically; however, earlier studies\textsuperscript{42,43} show different 19p13 breakpoints in myeloid versus lymphoid leukemias (also supported by preliminary molecular studies: Tkachuk et al\textsuperscript{45} and unpublished observations of D.C.T. and M.L.C.)
indicating that the translocations were reciprocal and that the breakpoints clustered within the 8-kb genomic region subtended by the B9 probe.

We then analyzed a series of 10 secondary leukemias (9 AML and 1 ALL) that developed after therapy that included significant doses of topo II inhibitors (predominantly the epipodophyllotoxin teniposide in 8 cases and the combination of DOX/CTX in 2 cases). Eight cases (nos. 1 through 8) were derived from a series of 16 patients who developed secondary leukemia after treatment on one of several POG T-ALL/NHL protocols. Pertinent clinical features of these patients and results of gene rearrangement studies are summarized in Table 2. Nine of the 10 secondary leukemias had HRX rearrangements detected with the B9 probe, including 1 case of secondary ALL that carried a t(11;16)(q23;p13) and 8 AMLs. Of these 10 patients, 7 had cytogenetic 11q23 abnormalities (which were not detected in the original malignant clone) at the time of diagnosis of secondary leukemia and each had HRX rearrangements. Two secondary AMLs had an unsuccessful karyotype, yet molecular analysis showed HRX rearrangements in both. The one therapy-induced leukemia in which HRX was germline was an AML that carried a t(3;13)(q26;q12) without any cytogenetically evident 11q23 abnormalities.

Paired samples of primary T-ALL and secondary AML/ALL were analyzed for both HRX and TCRβ gene rearrangements in five of these patients (nos. 1 through 5 in Table 2 and 1 through 4 in Fig 2). HRX was in germline configuration in each of the primary T-ALLs and was rearranged in four of five secondary leukemias. In contrast, the TCRβ loci were rearranged in each of the primary T-ALLs and were germline in the secondary leukemias. Taken together, these results show that the initial and secondary leukemias were phenotypically and genetically unrelated.

**DISCUSSION**

Chromosomal translocations involving exchanges of band 11q23 with more than a dozen different donor loci and interstitial deletions are frequently observed in de novo leukemias. The translocations are especially common in infants, being present in 50% of those less than 1 year of age and fully 75% of those diagnosed before the age of 6 months. The AMLs typically display monocytic features and are usually subclassified as FAB subtype M4 or M5. The ALLs are usually of very early B lineage, are frequently CD10 (CALLA) negative, and often coexpress myeloid-associated antigens. Typically, these leukemias respond poorly to therapy and are associated with an increased risk of relapse when compared with similar leukemias that lack 11q23 abnormalities. In addition, 11q23 abnormalities are frequent among leukemias with “lineage infidelity” that display different immunophenotypes at relapse compared with initial diagnosis.
Due to the frequency and variety of translocations involving chromosome band 11q23, this area has been the subject of intensive investigation by several laboratories. These investigations have culminated in the identification of a human homolog of the Drosophila trithorax gene called HRX (also termed MLL, ALL-1, and HTRX13), which is structurally interrupted by various 11q23 translocations, two of which have been molecularly characterized to date.10,15 Although these translocations are usually balanced, cytogenetic evidence suggests that the derivative 11 chromosome is conserved in complex rearrangements and likely codes for the leukemogenic product.46 Cloning of t(11;19) and t(4;11) fusion cDNAs has shown that in each case a similar amino-terminal portion of HRX, which includes protein motifs implicated in minor groove DNA binding, is fused with portions of serine/proline-rich proteins, creating chimeras with structural features suggestive of chimeric transcription factors.10,15

In this study, we provide molecular evidence for consistent interruption of HRX in leukemia-associated 11q23 translocations involving exchanges with many potential partners in a variety of clinical settings including AML, ALL, blast crisis of chronic myelogenous leukemia (CML-bc), and topo II inhibitor-induced secondary leukemias of both myeloid and lymphoid lineages. We found HRX to be juxtaposed to at least nine distinct cytogenetic loci—i.e., 1q32, 4q21, 6q27, 7p15, 9p21-24, 15q15, 16q13, and two separate 19q13 sites. HRX was also rearranged in the one leukemia with an 11q23 interstitial deletion, the molecular consequences of which are not yet defined. The sole case in our series with an 11q23 translocation that did not involve HRX was an AML with FAB M3-like morphology that carried a t(11;17)(q23;q21). Chen et al17 have recently reported a similar case that was molecularly characterized and shown to fuse the novel 11q23 gene PLZF to RARα. The t(11;17) reported in our series also involves these genes, as a PLZF-RARα fusion cDNA was amplified by reverse-transcription polymerase chain reaction (RT-PCR) (A. Scott, J. Licht, and C.L.W., unpublished data). Thus, it appears that the overwhelming majority of 11q23 translocas-
sions observed in leukemia will interrupt HRX, whereas at least one additional 11q23 gene, PLZF, will be involved in a small minority of cases. As our series was limited to leukemias, we cannot comment on the frequency of HRX involvement in other tumors with 11q23 cytogenetic abnormalities (such as lymphomas).

A striking observation in our series was the restricted distribution of 11q23 breakpoints in the HRX gene, which is quite large and spans 90 kb of 11q23. All HRX breakpoints in this study mapped within 8 kb between exons 5 and 11 because they were detected with the B9 probe. By analogy to the characterized t(11;19) and t(4;11) fusion transcripts, our results indicate that essentially all HRX translocations will disrupt the HRX protein within a 250 amino acid region fusing a similar amino-terminal portion of HRX to a variety of different recipient proteins. The basis for this restricted breakpoint distribution is not clear, but may relate to genomic features predisposing to recombination such as the density of Alu repeat elements or the presence of cryptic heptamer and nonamer sequences in this region of HRX. It is also likely that the biologic properties of resultant HRX chimeras confer a selective advantage and promote expansion of clones with such translocations.

The fusion of HRX to at least nine different recipient loci has no precedent among other translocations and raises the issue of the specific contribution of each partner in determining the clinical and biologic features of 11q23 leukemias. The multitude of partners (most of which are currently uncharacterized) may indicate that the non-HRX sequences are interchangeable with respect to their leukemogenic properties. Although the t(4;11) and t(11;19) fusion partners FEL and ENL contain no recognizable protein motifs, each is unusually rich in proline and serine residues, features previously associated with proteins that function as transcriptional activators. In light of this finding, we hypothesize that the various HRX translocation partners might each contribute domains with similar functional properties (such as transactivation), but that they may not necessarily be structurally related to one another. The amino terminal portion of HRX, which contains motifs implicated in minor groove DNA binding at AT-rich sites, likely plays a primary and critical role in conferring the common clinical properties shared by leukemias with 11q23 translocations. As some 11q23 translocations occur essentially only in ALL [eg, the t(4;11)] and others only in AML [eg, the t(9;11)], it is possible that some fusion partners may influence leukemic lineage, although these associations could also reflect the susceptibility of various partner genes to DNA recombination in different lineages.

Over the past several years, 11q23 translocations have also been linked to a very distinct subtype of secondary leukemias (predominantly AMLs) recognized among patients treated with topo II inhibitors, particularly the epipodophyllotoxins. These leukemias generally present without a preceeding myelodysplastic phase and are largely of the M4 or M5 subtype. The largest series of such leukemias has been described by Pui et al from St Jude Children's Research Hospital (SJCRH). Among patients in these series, 80% (16 of 20) with an abnormal karyotype had 11q23 translocations. Similarly, eight of nine successfully karyotyped patients in the series of secondary AMLs reported by Winick et al had 11q23 abnormalities. The actuarial risk of secondary leukemia is substantial, reaching approximately 12% at 6 years in SJCRH patients who received epipodophyllotoxins in weekly or twice-weekly doses for prolonged periods. Other investigators have reported similar secondary AMLs and occasional ALLs after chemotherapy for various primary malignancies in both adults and children. The unifying factor among these cases is prior exposure to topo II inhibitors, particularly the epipodophyllotoxins.

In this study, we observed HRX gene rearrangements in 9 of 10 cases of topo II inhibitor-associated secondary leukemias, including 7 of 7 with visible 11q23 abnormalities and 2 of 2 with an unsuccessful karyotype. Cytogenetic and gene rearrangement data indicate that HRX abnormalities were not present in the original malignancies, suggesting they were a direct result of prior therapy with epipodophyllotoxins or other topo II inhibitors. Thus, it is likely that most, but not all, patients with this class of secondary leukemia have translocations that create HRX fusion proteins. In a small subset of topo II inhibitor-induced secondary leukemias, translocations involving other chromosomal sites such as 21q22 (the site of the AML1 gene) have been observed. The one case in our series without HRX rearrangements was a case of t(3;13)-AML. One of the secondary leukemias in our series with HRX rearrangements was an ALL (which was cytogenetically, phenotypically, and genotypically distinct from the primary T-ALL) carrying a t(11;16)(q23;p13). Others have reported occasional secondary t(4;11)-ALLs in both adults and children after therapy of solid tumors with topo II inhibitors. These studies suggest that HRX fusion proteins are implicated in the pathogenesis of 80% to 90% of topo II inhibitor-associated leukemias of either myeloid or lymphoid derivation.

The mechanism by which topo II inhibitors might induce such translocations is unclear. It is postulated that these agents stabilize a complex between the enzyme and DNA and inhibit repair, thereby promoting DNA breaks that might then allow illegitimate crossovers. The same structural features that favor HRX translocations in de novo leukemias may also promote recombination with other loci once DNA breaks are induced. Identification of the critical region of HRX affected by these rearrangements opens the possibility for in vitro experiments to look at DNA crossovers at this site; such a system might facilitate a search for nonleukemogenic epipodophyllotoxin analogs. In the near future, it is likely that RNA-PCR tests will be available to amplify HRX fusion mRNAs. Our results, in combination with prior cytogenetic studies, indicate that the vast majority of topo II inhibitor-associated secondary leukemias will express chimeric HRX mRNAs. Given the high risk seen in subgroups of patients treated with frequent administration of such agents, one could consider the possibility of PCR monitoring for presumptomatic detection of secondary leukemias. Further biologic studies should clarify the role of HRX fusion proteins in transformation and shed additional
light on the unusual multilineage characteristics of these leukemias.

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