RAPID COMMUNICATION

The t(9;14)(p13; q32) Chromosomal Translocation Associated With Lymphoplasmacytoid Lymphoma Involves the PAX-5 Gene

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The t(9;14)(p13; q32) translocation is associated with approximately 50% of lymphoplasmacytoid lymphoma (LPL), a subtype of B-cell non-Hodgkin’s lymphoma (NHL). We cloned the chromosomal breakpoint of der (14) from an LPL case (1052) and showed that it involved a junction between 9p13 and the switch region of the Ig heavy chain locus (IgH) on 14q32. Using a YAC contig spanning 1.5 megabase (Mb), we determined that the 9p13 breakpoint in one case (1052) mapped within a 270-kb restriction fragment containing two previously reported 9p breakpoints associated with a heavy chain disease case (MAL) and Ki-1 positive diffuse large cell lymphoma (DLCL) cell line (KIS-1). The same fragment also contained the PAX-5 gene which encodes a B-cell specific transcription factor involved in the control of B-cell proliferation and differentiation. The breakpoints of KIS-1 and 1052 were mapped within the 5' noncoding region of PAX-5, while the 9p13 breakpoint of MAL mapped 230 to 270 kb upstream to PAX-5. In all three cases, the translocation caused the juxtaposition of the PAX-5 gene to the IgH locus in the opposite direction of transcription. When compared with six other DLCL cell lines lacking t(8;14)(p13; q32), the KIS-1 cell line showed an 11-fold overexpression of PAX-5 mRNA and a significantly reduced expression of the p53 gene, which is normally regulated by PAX-5. Moreover, metaphase and interphase fluorescence in situ hybridization (FISH) analysis using a YAC clone spanning 1 Mb including the PAX-5 as a probe identified chromosomal translocations in 5 of 7 cases carrying 9p13 translocations. These findings suggest that the PAX-5 gene is the target of the t(9;14) in LPL whereby its expression may be deregulated by juxtaposition to IgH regulatory elements, thus contributing to lymphomagenesis.

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Nonrandom Chromosomal translocations are typically associated with lymphoid neoplasms where they involve a number of protooncogene loci. In B-cell non-Hodgkin's lymphoma (NHL), translocations deregulate these proto-oncogenes by juxtaposition with regulatory elements of genes expressed normally in the cells, most frequently immunoglobulin genes (Ig). Different chromosomal translocations are associated with specific subtypes of lymphoma, and substantial experimental evidence, including studies in transgenic mice, indicates that most of these oncogenes contribute specifically to lymphomagenesis. For instance, t(8;14)(q24; q32), t(8;22)(q24; q11), and t(2;8)(p12; q24) lead to the deregulated expression of the MYC proto-oncogene by juxtaposition to one of the Ig loci in 100% of Burkitt's lymphoma. Similarly, rearrangement and deregulation of the antiapoptotic gene, BCL-2, caused by t(14;18) (q32; q21) has been shown in 70% to 90% of follicular lymphomas. In approximately 50% of mantle cell lymphomas, the t(11;14)(q13; q32) results in the overexpression of BCL-1(Cyclin D1/PRAD1), which regulates cell-cycle progression, by juxtaposition with the IgH gene. The BCL-6 gene, encoding a POZ-zinc-finger transcription factor, is altered by rearrangements or mutations involving its 5' noncoding region in the majority of diffuse large cell lymphomas (DLCL) and less frequently in follicular lymphomas.

The t(9;14)(p13; q32) is associated with 50% of lymphoplasmacytoid lymphoma (LPL)/immunocytoma (Revised European-American Classification of Lymphoid Neoplasms [REAL]; small lymphocytic lymphoma with plasmacytoid differentiation according to the Working Formulation). This rare type of lymphoma is characterized by the infiltration of B cells displaying a lymphoplasmacytoid phenotype, the presence of cytoplasmic Ig and serum paraproteins, and an indolent clinical course followed by transformation into large cell lymphoma. LPL are associated with translocations involving chromosome 9p13, which are "promiscuous" in that various chromosome loci including 1q43q2, 1q25, 3q27, 7q11, 12q13, 12q21, 19p13, and 9q13 can act as reciprocal translocation partners in different cases.

Two distinct breakpoint regions linking 9p11 and 9p13 sequences to the Ig heavy-chain locus (IgH) on 14q32 have already been cloned from a case (MAL) of a-heavy chain disease and a Ki-1 positive DLCL cell line (KIS-1), respectively. However, the gene on 9p13 involved in these translocations has not been identified.

In further pursuit of the target gene involved in 9p13 translocation, we analyzed the breakpoint of a typical LPL case and compared its genomic loci with two previously identified cases. This approach led us to identify that the translocation breakpoints at 9p13 of all three cases are located within a 270-kb region containing the PAX-5 (paired homeobox-5) gene. A YAC probe spanning this region could also identify chromosomal rearrangements in five out of seven (71%) cases with 9p13 translocations by fluorescence in situ hybridization (FISH) analysis. This structural
analysis together with the finding of overexpression of the PAX-5 mRNA in the KIS-1 cell line suggest that the t(9;14)(p13;q32) translocation deregulates the expression of the PAX-5 gene by juxtaposition to the Ig regulatory elements.

MATERIALS AND METHODS

Tumor biopsies and cell lines. The biopsy (case 1052, 79-year-old woman) sample analyzed was derived from a retroperitoneal lymph node diagnosed as LPL. Cytogenetic analysis showed that 16 of 16 metaphases had 46, XX, t(9;14)(p13;q32). Detailed clinical characteristics were previously reported.17 The KIS-1 cell line derived from a Ki-1 positive DLCL with t(9;14)(p13;q32) was previously described.17 The RC-K9 and LY1, 2, 3, 17, 18 cell lines were kindly provided by Dr I. Kubonishi (Kouchi Medical College, Kouchi, Japan) and Dr H.A. Messner (Ontario Cancer Institute, Toronto, Canada), respectively.18,19

Southern blot analysis. Southern blot analysis was performed as described previously.18 A 1.3-kb EcoRI-EcoRI Cµ probe and a 6.6-kb BamHI-HindIII JH probe were kindly provided by S.J. Korsmeyer (Washington University, St Louis, MO).

Cloning of chromosomal breakpoints. High-molecular-weight DNA from case 1052 was partially digested with Sau3AI and size-fractionated by using a low-melting point agarose gel. DNA fragments ranging from 15 to 23 kb were gel-purified, ligated into the λ dash II vector (Stratagene, La Jolla, CA) and in vitro packaged. Recombinant clones, 5 × 106 were screened by plaque hybridization using the Cµ and JH probes. A human placental DNA library purchased from Stratagene was used for the isolation of 9p13 germline sequences.

Polymerase chain reaction (PCR) primers, probe preparation, and YAC library screening. STS primers (sense 5′-GGAAT- GCCCTCCTACTG-3′; antisense 5′-GACAAGCCATGTCT- AAG-3′) were designed from the pI313 sequence in the vicinity of MAL case's breakpoint.23 The amplified 153-bp PCR product was also used as a probe (MAL). PCR primers (sense 5′-CACAGTGCC- AACCTTCAG-3′; antisense 5′-TGAGAGACCTGGCTCTAG-3′) derived from the pI313 sequence surrounding the 1052 breakpoint were prepared based on the sequence of the 1.5-kb HindIII fragment, which was also used as a probe (1052-1.5H) (Fig 1B). These PCR primers were used to screen a human CEPH YAC DNA pools (Research Genetics, Huntsville, AL). Extraction of YAC DNA was performed according to standard techniques.24 PAX-5 gene probes were prepared by reverse transcription (RT)-PCR using primer sets to amplify nucleotides 46 to 675 (PAX-5 5′; sense 5′-GTCCATTCC- GAAATCGTAACCGTTCGTACGAGAATCGCT-3′; antisense 5′-GACTCTGAATACATCCCTG-3′) and 1,721 to 2,370 (PAX-5 3′; sense 5′-CATCCAGGCGACATCT- TAG-3′; antisense 5′-CAGAAGTTCTGGCTGTAG-3′) of the PAX-5 cDNA sequence.15 The pBS8.6BB clone containing pIUS was prepared by the guanidium thiocyanate method and poly(A) RNA was selected using Oligotex coated with poly(T) (Qiagen, Chatsworth, CA). Northern blot analyses were performed as described previously.24 1 × 10⁶ plaques from a cDNA library constructed from the BJAB cell line were screened with the PAX-5 5′ cDNA probe.

DNA sequencing. DNA sequencing was performed by the di-deoxy chain termination method using an automated sequencing system, ABI 473A (Applied Biosystems, Foster City, CA).

RESULTS

Molecular cloning of the t(9;14) breakpoint region in an LPL case. Southern blot analysis of BamHI-digested DNA from case 1052 using the Cµ probe showed two rearranged bands (Fig 1A). One of these bands (8.0 kb) did not co-migrate with the JH band in the BamHI digest, suggesting the existence of a chromosomal breakpoint between the JH and Cµ loci of the IgH gene. To clone this fragment, a genomic library was constructed and screened with both Cµ and JH probes. As a result, we isolated a clone (A4) containing the IgH Sµ region juxtaposed to an unknown fragment (Fig 1B). FISH analysis showed that clone A4 hybridized to both 14q32 and 9p13 chromosomal loci on normal metaphase spreads (data not shown), indicating that it represented the breakpoint region of the der (14) chromosome. Localization to chromosome 9 was also confirmed by hybridization to a somatic cell hybrid panel with the 1052-1.5H probe (data not shown).

Three NHL-associated pI313 breakpoints map within a 270-kb genomic region containing the PAX-5 locus. Restriction mapping of clone A4 showed that the sequences involved in the breakpoints of case 1052 and those previously reported in MAL and KIS-1 were independent from each other. To investigate the genomic distance between these three breakpoints, we isolated YAC clones spanning this region. For this purpose, we designed STS primers in the
vicinity of each breakpoint, and screened a CEPH YAC library. Overlapping YAC clones spanning 1.5 Mb were isolated and mapped to the 9p13 locus by FISH analysis (Fig 2). FISH data not shown). A representative YAC contig (Fig 2) was constructed based on the overlapping hybridization pattern given by YAC end probes. Using mapping by PFGE, we found that the three breakpoints were located within 270 kb which also contained the PAX-5 gene, recently assigned to the 9p13 locus. In addition, two of the breakpoints (case 1052 and KIS-1) mapped within the same 150-kb Nru I fragment (Fig 3), while the breakpoint of case MAL mapped 230 to 270 kb upstream to the PAX-5 locus.

Relationship between 9p13 breakpoints and the PAX-5 gene. To precisely determine the genomic distance between the breakpoints and PAX-5 gene sequences, we isolated the 5' region of this gene by screening a cDNA library derived from the BJAB cell line. Two kinds of PAX-5 cDNA clones were isolated, which differed in their 5' termini. One of the cDNA species was identical to that previously reported for PAX-5. The other cDNA species, represented by phage ABJ-11, contained a different 5' cDNA sequence (Fig 4A). Nucleotide sequencing showed that this clone contained a putative alternative in-frame translation initiation site (ATG), partly corresponding to Kozak's consensus sequence (Fig 4B). The N-terminal sequences of the protein putatively encoded by this cDNA species differ from those previously reported for PAX-5, although the main functional domains of the protein remain conserved. Consistent with these two cDNA species, Northern analysis showed two alternatively spliced transcripts of 10 kb and 10.5 kb, using a PAX-5 probe derived from the 3' end (Fig 4C). On the other hand, the 10-kb and 10.5-kb transcripts were specifically detected by the pKIS and 1.7B genomic probes, respectively, suggesting that the 5' region of PAX-5 contains two alternatively used first exons (1a and 1b in Fig 4D). Most B-cell lines (eg, RC-K8 and KIS-1) express both the 10-kb and the 10.5-kb transcripts, which use the first (ATG1) and second translation initiation site (ATG2), respectively. Only the 10-kb transcript is detectable in the RAMOS cell line by Northern analysis, although the 10.5-kb mRNA species was also identified by RT-PCR analysis (data not shown).

The breakpoints of KIS-1 and case 1052 mapped 2.5 kb upstream to the ATG1 site and just 5' to the ATG2 site within exon 1b (Fig 4B and D). In both cases, the PAX-5 gene was juxtaposed to the IgH locus to the opposite direction to transcription as a result of the (t9;14) translocation. In case 1052, t(9;14) removed the enhancer \( \mu \) (\( \mu \)) element and the ATG1 site of PAX-5, while in the case of the KIS-1 cell line, the \( \mu \) sequences and both PAX-5 exon 1s remain on der (14).
The PAX-5 gene is overexpressed in a cell line carrying t(9;14). To determine whether the juxtaposition to IgH had any effect on PAX-5 gene expression, we compared PAX-5 RNA levels in KIS-I and other DLCL cell lines sharing the same histological subtype, but lacking t(9;14)(p13;q32) (Fig 5). Densitometric analysis of the hybridization bands showed an 11-fold overexpression in the KIS-I cell line when compared with the mean value of the other six cell lines.

The p53 gene, which is negatively regulated by PAX-5, is downregulated in an NHL cell line containing t(9;14) and overexpressing PAX-5. The expression of the p53 gene is physiologically modulated by PAX-5. Because the KIS-1 cell line overexpresses PAX-5, we examined whether this was associated with p53 downregulation by analyzing p53 RNA levels in KIS-1 cells versus 6 control NHL lines. The results showed that p53 RNA expression is significantly repressed in KIS-1 cells (Fig 5).

Frequent detection of 9p13 breakpoint in LPL by FISH analysis. To investigate the frequency of the PAX-5 gene involvement in B-cell NHL, we analyzed 20 LPL cases, including 2 cases with t(9;14) and a panel of 50 lymphoma cases representative of the main NHL subtypes by Southern blot analyses using the MAL, pKIS, and 1.7B probes. However, we did not detect any rearrangement (data not shown), suggesting that none of the probes used was representative of a rearrangement cluster. We then decided to use the CEPH788C3 YAC DNA as a probe for metaphase and/or interphase FISH analysis to examine a larger genomic region spanning the PAX-5 gene. Seven B-cell NHL cases, including 3 LPL and 2 DLCL cases with t(9;14)(p13;q32) and 2 DLCL cases with other translocations involving 9p13 (Table 1) were selected for this analysis. The results show that 3 LPL and a DLCL case carrying t(9;14), together with 1 DLCL case with a t(3;9)(q27;p13) show rearrangements of 9p13 within the genomic region represented by the YAC clone (Table 1, Fig 6).

DISCUSSION
The t(9;14)(p13;q32) translocation is a recurrent chromosomal abnormality associated with a significant fraction of LPL cases and less frequently with other NHL subtypes. Our results identify a chromosomal subregion at 9p13 which is involved in this translocation in most cases of LPL and provides evidence in a few cases that the chromosomal breakpoints directly involve the PAX-5 gene. These results have implications for the consequences of t(9;14)(p13;q32) on PAX-5 expression, for the role of PAX-5 in lymphomagenesis, and for the possible diagnostic use of 9p13 breakpoints in NHL diagnosis.

Consequences of t(9;14)(p13;q32) on PAX-5 gene expression. Our results show that 9p13 chromosomal breakpoints are located within a 270-kb genomic region which contains the PAX-5 gene in three cases. In addition, the fact that the 9p13 breakpoints were detected by the 1-Mb YAC containing the PAX-5 gene in 6 of 8 NHL cases by FISH analysis indicates that the breakpoints may be in relative proximity to the PAX-5 gene in most LPL and DLCL cases. In analogy to the chromosomal translocations involving the IgH locus and the BCL-1, BCL-2, or c-MYC loci in other types of NHL, it is conceivable that at these distances IgH gene regulatory elements such as IgH transcriptional enhancers and locus control regions may deregulate PAX-5 expression. This model is directly supported by the findings in case KIS-1 and case 1052, where the IgH locus...
is directly juxtaposed to the PAX-5 gene in the opposite direction of transcription, and by the striking overexpression of the PAX-5 mRNA in the KIS-1 cell line. In addition, the observation that the 9p13 locus seems to be disrupted also in reciprocal translocations involving partner chromosomes other than 14q32 (see case 1615 in Table I) suggests that multiple chromosomal regions may contribute to PAX-5 deregulation perhaps by juxtaposing different regulatory elements, a mechanism analogous to that observed for chromosomal translocations involving the BCL-6 gene in DLCL.8

Role of PAX-5 in lymphomagenesis. The PAX gene family include a number of transcription factors which share homologous DNA binding and dimerization domains, designated as paired domains, and are characterized by a highly conserved α-helical structure at the N terminus.32,33 Through transcriptional regulation of downstream genes, PAX genes control embryonal development and organogenesis. In addition, various PAX genes have been implicated in oncogenesis.34 The PAX-3 and PAX-7 genes have been shown to fuse to fork head domain gene (FKHR) by t(2; 13)(q35;q14) and t(1; 13)(p36;q 14) translocations in alveolar rhabdomyosarcoma.35,36 The PAX-2 and PAX-5 genes have been found highly expressed in Wilms’ tumor,37 and in brain tumors such as medulloblastoma and glioblastoma,38 respectively, although the mechanism responsible for their altered expression has not been identified.
Fig 5. PAX-5 mRNA is overexpressed in KIS-1 cell line carrying t(9;14). A 10-μg aliquot of total RNA was loaded onto each lane, blotted, and hybridized with PAX-5 3' and p53 cDNA probes, together with GAPDH probe to control for the amount of RNA. All the cell lines except for HELA, which acted as the negative control for PAX-5 expression, are of B-cell lineage. Histological subtype of the original patients' sample are indicated on the top. BL, Burkitt's lymphoma; DLCL, diffuse large cell lymphoma.

The PAX-5 gene was cloned as a human homolog of the sea urchin TSAP (tissue-specific activator protein), and turned out to be the gene encoding 50-kD BSAP (B-cell lineage specific activator protein). PAX-5 is expressed in fetal brain and liver during the embryonic period and, after birth, is exclusively expressed in B lymphocytes and testis. Several observations suggest that PAX-5 plays a significant role in the control of B-cell proliferation and differentiation. Its loss of function results in a maturation arrest at the pro-B cell stage and its overexpression results in proliferation of splenic B cells. On the other hand, treatment with antisense oligonucleotides strikingly reduces lipopolysaccharide-induced proliferation of splenic B cells and spontaneous proliferation of B-lymphoma cell lines. Furthermore, PAX-5 gene expression is upregulated by the product of the E2A proto-oncogene, whereas PAX-5 itself downregulates the expression of p53. Taken together, these observations suggest that PAX-5 is crucial in the control of proliferation of mature B cells and that its deregulated expression may contribute to abnormal proliferation and thus to lymphomagenesis.

In this study we have also identified alternative transcripts and translation initiation sites that had not been previously reported for the PAX-5 gene. Interestingly, the amino acid sequence (MEIHCKHDPFASMH) encoded by the novel exon Ib identified in this study is 92% homologous to that (MDMHCKADPFSAMH) encoded by exon 1 of the PAX-2 gene, suggesting that this domain may have functional significance. Alternatively spliced mRNAs encoding different open reading frames are found in the same (class III) PAX family genes including PAX-2 and PAX-8, and in the PAX-8 gene such alternative forms are developmentally regulated and generate isoforms with different transactivation properties. These observations suggest that the two PAX-5 mRNAs (10.0 kb and 10.5 kb) which are expressed in most B-cell lines may encode functionally distinct proteins, whose role in normal B-cell development and possibly lymphomagenesis requires further investigation.

Table 1. Rearrangement of YAC(CEPH788C3) Signal in Tumors With 9p13 Chromosomal Translocations

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Histology</th>
<th>9p13 Aberration</th>
<th>YAC Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1052</td>
<td>LPL</td>
<td>t(9;14)(p13;q32)</td>
<td>Rearranged</td>
</tr>
<tr>
<td>1113</td>
<td>LPL</td>
<td>t(9;14)(p13;q32)</td>
<td>Rearranged</td>
</tr>
<tr>
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<td>1800</td>
<td>DLCL</td>
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<td>NT</td>
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<tr>
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<td>DLCL</td>
<td>t(9;14)(p13;q32)</td>
<td>Rearranged</td>
</tr>
<tr>
<td>1615</td>
<td>DLCL</td>
<td>t(3;9)(q27;p13)</td>
<td>NT</td>
</tr>
<tr>
<td>1068</td>
<td>DLCL</td>
<td>t(7;9)(q32;p13)</td>
<td>NT</td>
</tr>
</tbody>
</table>

Abbreviation: NT, not tested.

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Fig 6. Rearrangement of nonchimeric YAC(CEPH 788C2) in lymphomas with 9p13 chromosomal translocations. (1 through 4) Case 1113. (1 and 2) A partial metaphase of a tumor cell showing hybridization signals at the breakpoints of der (9) and der (14) chromosomes (arrowheads). (1) and the corresponding DAPI image showing a G-banding like staining of chromosomes (2). (3 and 4) Interphase nuclei from the same case showing two signals in a normal nucleus (3) and three signals in a tumor nucleus (4). (5 and 6) Interphase nuclei from case 1052 showing two signals in a normal nucleus (5) and three signals in a tumor nucleus (6). Positive signals in interphase FISH analysis are indicated by arrows.

or substitute conventional cytogenetic analysis for detecting these chromosomal abnormalities. In addition, this approach may facilitate the detection of cytogenetically undetectable 9p13 rearrangements. Finally, 9p13 rearrangements, which are common in LPL, but occasionally present in DLCL, may represent useful clonal markers to determine whether some LPL cases can progress into DLCL.

ACKNOWLEDGMENT

We are grateful to Dr N.Z. Parsa for organizing the tumor bank, and to R.S. Hauptschein for advice on YAC cloning. The exon 1b sequence of the PAX-5 gene reported in this paper has been deposited in the GenBank data base (accession no. U62539).

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