RAPID COMMUNICATION

p53 Mutations Are Associated With Histologic Transformation of Follicular Lymphoma

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The majority of low-grade non-Hodgkin's lymphomas (NHL) undergo clinical progression toward intermediate- and high-grade lymphomas. This progression is often associated with histologic transformation from follicular to diffuse-type NHL. The pathogenetic mechanisms underlying this evolution are presently unknown. In this study, we have analyzed the role in NHL progression of relevant genetic lesions affecting proto-oncogenes and tumor suppressor genes. Sequential biopsies from 21 patients with clinical progression with (5 cases) or without (16 cases) evidence of histologic transformation were analyzed for karyotypic changes, c-myc rearrangements and deletions affecting 6q27 by Southern blot analysis, and p53 mutations by single-strand conformation polymorphism (SSCP) analysis coupled with direct sequencing of polymerase chain reaction-amplified products. No novel cytogenetic aberration was detected in association with progression, and all samples analyzed displayed a normal c-myc gene. Mutations of the p53 gene were detected in 4 of 5 cases displaying histologic transformation from follicular to diffuse-type NHL and in none of the 16 cases displaying clinical progression in the absence of histologic transformation. In 1 of these positive cases, the same mutation was also present in the pretransformation biopsy, correlating with the presence of diffuse-type areas within a predominant follicular pattern. In 1 of these cases, a deletion of 6q27 was also detected in the posttransformation biopsy along with a p53 mutation. These findings indicate that p53 mutations are associated with and may be responsible for histologic transformation of follicular lymphoma.

MATERIALS AND METHODS

Tumor biopsies. Between January 1984 and December 1991, 781 consecutively ascertained specimens of histologically confirmed NHL seen at the Memorial Sloan Kettering Cancer Center were subjected to genetic analysis. Biopsy material was divided for histopathologic, cytogentic, and immunophenotypic/immunogenotypic analyses, which were performed as previously described. Karyotypes were defined and described according to ISCN (1991). Of the 781 specimens, 480 showed clonal chromosomal abnormalities. In 47 cases, multiple biopsies were obtained from the same patient in the setting of clinical progression of lymphoma. Of these, 21 paired samples of DNA were available that showed clonal rearrangement of the Ig gene in both specimens. Cases were classified according to the International Working Formulation.

Southern blot analysis. High molecular weight DNA was ob-

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Submitted July 7, 1993; accepted August 10, 1993.

Supported in part by National Institutes of Health Grants No. CA-44029 (to R.D.F.) and CA-34775 and CA-08748 (to R.S.K.C.). G.G. has been supported by a fellowship for AIDS research from Istituto Superiore di Sanita', and F.L.C. by AIL, Rome, Italy. K.O. has been supported by a Clinical Oncology Cancer Development award of the American Cancer Society.

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0006-4971/93/8208-0039$3.00/0
tained by proteinase K digestion, phenol/chloroform extraction, and precipitation by ethanol. After digestion with appropriate restriction enzymes, 5 to 10 μg of DNA was size-fractionated by electrophoresis on 0.8% agarose gel, denatured, and transferred to Dur- 
aloase filters (Stratagene, La Jolla, CA) according to standard procedures. Filters were hybridized as reported elsewhere with specific probes labeled with 32P by random priming, washed in 0.2% SSC/0.5% sodium dodecyl sulfate (SDS) for 2 hours at 60°C and exposed for autoradiography at −80°C for 24 to 48 hours using intensifyingscreens.

DNA probes. In gene rearrangement analysis was performed on HindIII and EcoRI digests by using a 32P probe kindly provided by Dr S. Korsmeyer. The configuration of the c-myc locus was analyzed by hybridizing EcoRI- and HindIII-digested DNA to the MCI41RC probe, representative of the third exon of the c-myc gene.21,22 The presence of 6q deletions was studied by loss of constitutive heterozygosity (LOH) analysis using the highly polymorphic cosm id clone CEB4/D6S133 (a gift of Dr G. Vergnaud) on Promethion digest.

Oligonucleotide primers. All the oligonucleotides used for polymerase chain reaction (PCR) amplification were synthesized using an Applied Biosystem synthesizer (Foster City, CA). The oligonucleotides used to amplify the sequences of p53 exons 5 through 9 have been previously reported.

Single-strand conformation polymorphism (SSCP) analysis. SSCP analysis was performed according to an adapted version of a previously published method. Briefly, PCR was performed with 100 ng of genomic DNA, 10 pmol of each primer, 2.5 μmol/L dNTPs, 1 μCi of [α-32P] dCTP, 10 mmol/L Tris-HCl (pH 8.8), 50 mmol/L KCl, 1 mmol/L MgCl2, 0.1% gelatin, 0.5 U Taq polymerase (Cetus, Emeryville, CA), in a final volume of 10 μL. Thirty cycles of denaturation (94°C), annealing (63°C for reactions amplifying exons 5, 6, 7, and 9; and 58°C for exon 8), and extension (72°C) were performed on an automated heat-block (Perkin-Elmer/Cetus). The reaction mixture (2 μL) was diluted 1:25 in 0.1% SDS, 10 mmol/L EDTA and further mixed 1:1 with a sequencing stop solution containing 20 mmol/L NaOH. Samples were heated at 95°C for 5 minutes, chilled on ice, and immediately loaded (3 μL) onto a 6% acrylamide/TBE gel containing 10% glycerol. Gels were run at 8 W for 12 to 15 hours at room temperature, fixed in 10% acetic acid, air dried, and exposed at −80°C for 6 to 72 hours using intensifying screens.

Direct sequencing of PCR products. PCR was performed with 500 ng of genomic DNA, 20 pmol of each primer, 200 pmol/L dNTPs, 10 mmol/L Tris-HCl (pH 8.8), 50 mmol/L KCl, 1 mmol/L MgCl2, 0.01% gelatin, 2.5 U Taq polymerase. The number and conditions of amplification cycles were as described above. Sequencing reactions were performed as previously reported. Briefly, 10 pmol of one of the two primers used for DNA amplification was labeled with [γ-32P] ATP (New England Nuclear, Boston, MA; specific activity, 3,000 Ci/mmol) by means of T4 polynucleotide kinase (Biolabs, Beverly, MA) and purified through a Bio-gel P4 fine column (Bio-Rad, Richmond, CA). Approximately 0.25 pmol of each fragment, previously isolated after electrophoresis in low-melting point agarose (BRL, Gaithersburg, MD), was denatured and annealed in the presence of 2 pmol of labeled primer. Sequencing reactions were performed with reagents supplied in a T7 polymerase sequencing kit (US Biochemicals, Cleveland, OH), following the manufacturer’s recommendations. Both strands were sequenced for each DNA segment analyzed.

RESULTS

Relevant clinical, histologic, and genetic features of the 21 patients selected for this study are summarized in Table 1. The criteria for the inclusion of the sequential biopsies were (1) evidence of clinical progression (defined by enlargement of peripheral or visceral adenopathy, involvement of new sites, or stage progression), with or without associated histologic transformation (change in the infiltration pattern from predominantly follicular to diffuse type); and (2) evidence of identical IgH gene rearrangements documenting the identical clonal derivation of the two biopsies (Fig 1).

Sixteen of the 21 cases selected were representative of clinical progression in the absence of histologic transformation, whereas 5 cases displayed clinical progression associated with typical histologic transformation. The initial biopsy was taken before treatment in 10 cases and after treatment in 11 cases. The median time between the sequential biopsies was 12 months. Most of the cases exhibited progression posttreatment (18) or off-treatment (2 cases); in 1 case, progression occurred while on treatment (Table 1).

Cytogenetic analysis. In 14 cases, including the 5 cases displaying histologic progression, a t(14;18) was detected. In all cases (9) in which cytogenetic analysis could be performed on both samples the t(14;18) translocation persisted in the postprogression phase. Although there were additional cytogenetic aberrations acquired in the second biopsies, none of these aberrations were observed in more than a single sample pair.

c-myc oncogene rearrangements. Translocations involving chromosome 8 can lead to c-myc deregulation by different molecular mechanisms in distinct tumor types.27,28 In the most frequent t(8;14) translocation (80% of cases), typically associated with sporadic Burkitt’s lymphoma as well as a subset of diffuse large-cell NHL, c-myc is activated by truncations within its first exon, first intron, or flanking sequences. These truncations can be detected by Southern blot analysis using restriction enzymes cutting outside c-myc sequences (eg, EcoRI and HindIII).29 This assay showed a normal c-myc allele in all the biopsies tested (not shown), confirming previous reports indicating that c-myc oncogene activation is infrequently associated with NHL progression and transformation.14

Deletions in chromosome band 6q27. Previous cytogenetic studies have proposed that 6q deletions may be associated with tumor progression in some cases.8-10 Our recent molecular analysis has shown that these deletions represent a relatively frequent event in B-NHL that can be detected at the molecular level in cases displaying no microscopically visible deletions.20 By using a highly polymorphic probe (CEB4/D6S133; heterozygosity >95%)23 mapping within the region of minimal deletion, the presence of deletions can be rapidly detected as loss of heterozygosity in Southern blot hybridization assays. This probe detected heterozygosity and thus was informative in 20 of 21 cases tested. However, loss of heterozygosity was detected in association with histologic transformation in only 1 case (RC1141, Fig 2). This deletion was not detected at the cytogenetic level.

p53 mutations. p53 inactivation in human tumors is most frequently due to point mutations in the coding sequence of exons 5 through 9 in one allele with or without loss of the other allele.31,32 To define the occurrence of p53
lesions in association with NHL progression, a two-step strategy was used, as previously reported. P53 exons 5 through 9 were analyzed in genomic DNA of the 21 paired NHL samples by the PCR-SSCP technique (Fig 3A). Fragments displaying an altered electrophoretic mobility by SSCP analysis were then reamplified in a separate reaction and analyzed by PCR-direct sequencing to confirm and characterize the nature of the mutation (representative examples are shown in Fig 3B). Our previous control studies had shown that, under our experimental conditions, the SSCP method is sensitive at the level of at least 1% and is specific, as evaluated by the 100% concordance between the results obtained by direct sequencing versus SSCP analysis of p53 exons 5 through 9.

Using this approach, p53 mutations were found in 4 of 5 cases of histologic transformation, whereas all the cases dismissed in association with NHL progression, a two-step strategy was used, as previously reported.26 p53 exons 5 through 9 were analyzed in genomic DNA of the 21 paired NHL samples by the PCR-SSCP technique (Fig 3A). Fragments displaying an altered electrophoretic mobility by SSCP analysis were then reamplified in a separate reaction and analyzed by PCR-direct sequencing to confirm and characterize the nature of the mutation (representative examples are shown in Fig 3B). Our previous control studies had shown that, under our experimental conditions, the SSCP method is sensitive at the level of at least 1% and is specific, as evaluated by the 100% concordance between the results obtained by direct sequencing versus SSCP analysis of p53 exons 5 through 9.

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playing clinical progression in the absence of histologic transformation displayed normal p53 genes (Table 1). In 3 of the 4 cases displaying mutated p53 genes, the mutation was present only in the posttransformation biopsy, suggesting that this lesion was associated with the transformation event. In the remaining case (RC1055/1130), the mutation was present in both biopsies. Interestingly, the first biopsy of this case was classified as having areas of diffuse growth mixed within a predominantly follicular pattern, suggesting that the p53 mutation may be associated with the developing diffuse pattern. One of the cases carrying a p53 mutation in the posttransformation sample (RC1141) also carried a 6q deletion (Fig 2 and Table 1).

The characteristics of the observed p53 mutations in transformed follicular NHL are described in Table 2. In all 4 cases, the mutations were analogous for type and position to the ones observed in other types of tumors such as brain and colon cancers.

The identification of the molecular mechanisms that are associated with the clinical progression of NHL represents a critical issue with implications for the pathogenesis as well as for diagnosis and management of these malignancies. The present report confirms previous studies showing that neither frequent cytogenetic abnormalities nor c-myc activation are consistently associated with tumor progression. On the other hand, it provides the first evidence of a genetic alteration, p53 mutation, which is associated with tumor progression in cases displaying histologic transformation.

With respect to the role of recurrent chromosomal abnormalities, deletions of 6q27, 13q32, and 17p, and trisomy of chromosomes 2, 3, 7, and 12 have been reported in association with tumor progression in several studies, although...
none of these lesions accounted for a significant fraction of the cases analyzed. Considering only the subset of cases displaying histologic transformation, loss/deletion of 6q and trisomy 7 were observed as secondary aberrations in 20% to 40% of t(8;14)-carrying FLs, although these observations were not confirmed in another study. Our results did not confirm the acquisition of specific cytogenetic aberrations in a panel of 16 cases of clinical progression.

### Table 2. Type of p53 Mutations in NHL Histologic Progression

<table>
<thead>
<tr>
<th>Biopsy No.</th>
<th>Histology</th>
<th>p53 Mutation</th>
<th>p53 Exon</th>
<th>Codon</th>
<th>Mutation</th>
<th>AA Substitution</th>
</tr>
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<tbody>
<tr>
<td>RC96</td>
<td>MX-F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Arg → Hys</td>
</tr>
<tr>
<td>RC201</td>
<td>IMB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RC266</td>
<td>SCC-F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RC1141</td>
<td>MX-D</td>
<td>+</td>
<td>5</td>
<td>175</td>
<td>CGC → CAC</td>
<td>Arg → Hys</td>
</tr>
<tr>
<td>RC1039</td>
<td>MX-F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RC1188</td>
<td>LC-D</td>
<td>+</td>
<td>5</td>
<td>151</td>
<td>CCC → ACC</td>
<td>Pro → Thr</td>
</tr>
<tr>
<td>RC1055</td>
<td>LC-F(D)</td>
<td>+</td>
<td>6</td>
<td>190</td>
<td>CCT → CTT</td>
<td>Pro → Leu</td>
</tr>
<tr>
<td>RC1130</td>
<td>LC-D</td>
<td>+</td>
<td>6</td>
<td>190</td>
<td>CCT → CTT</td>
<td>Pro → Leu</td>
</tr>
<tr>
<td>RC358</td>
<td>MX-F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RC363</td>
<td>MX-F(D)</td>
<td>+</td>
<td>8</td>
<td>282</td>
<td>CGG → TGG</td>
<td>Arg → Trp</td>
</tr>
</tbody>
</table>
without histologic transformation. With regard to histologic transformation, our limited panel of cases does confirm that 6q deletions can occasionally be observed.

Previous observations of two cases of NHL carrying rearranged bcl-2 and c-myc genes were taken to suggest that c-myc oncogene activation was associated with histologic transformation. In the present study, we analyzed the region of c-myc most frequently associated with c-myc activation. In the present study, we analyzed the region of c-myc most frequently associated with c-myc activation. This conjecture was supported by in vitro evidence of a synergistic effect of c-myc and bcl-2 in causing cell transformation and, most notably, by the observation that, in mice transgenic for bcl-2, tumor progression was frequently associated with c-myc activation. In the present study, we analyzed the region of c-myc most frequently associated with c-myc activation. In the present study, we analyzed the region of c-myc most frequently associated with c-myc activation. In the present study, we analyzed the region of c-myc most frequently associated with c-myc activation.

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The main finding of our study is that p53 mutations are frequently associated with NHL that display clinical progression and histologic transformation. Although the frequency of this association is apparently high (4 of 5), the number of cases studied is limited and therefore any conclusive assessment must await confirmation based on larger panels. In support of our findings, other investigators have preliminarily reported different frequencies of association between histologic progression and p53 mutations (7 of 38 cases) or overexpression (6 of 11), the latter being a consequence of mutation. In addition, Ichikawa et al have reported the association of p53 mutations with cases of advanced clinical stage and diffuse histology in NHL that may reflect histologic transformation because no p53 mutation was found in our panel of de novo diffuse large-cell lymphoma. Taken together, these observations identify p53 mutations as the first recurrent lesion detectable in association with histologic transformation of NHL. Given the presumed role of p53 in controlling cell proliferation and DNA replication, it is conceivable that the loss of p53 function may contribute to tumor progression directly, by providing FL cells with a high proliferative rate, and/or indirectly, by allowing the accumulation of additional genetic lesions. These lesions, such as 6q deletions, may occasionally become detectable as recurrent karyotypic abnormalities.

Finally, we note that our findings may have relevant clinical implications if confirmed in larger series. p53 mutations may represent a marker for the early diagnosis of transformation that can be detected at high specificity and high sensitivity using PCR-based techniques. Indeed, the presence of a p53 mutation in one pretransformation case displaying areas of diffuse infiltration in the context of a predominantly follicular pattern suggests that the molecular lesion associated with transformation may be detectable much earlier than a canonical diagnosis of transformation is made. The validation of this approach requires the demonstration that p53 mutations are associated with early appearing diffuse areas and clinical studies designed to test the prognostic value of early detection of mutation.

REFERENCES
p53 mutations are associated with histologic transformation of follicular lymphoma

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