RAPID COMMUNICATION

p53 Mutation Is Associated With Progression in Follicular Lymphomas

By Christian A. Sander, Takahiro Yano, Helen M. Clark, Cynthia Harris, Dan L. Longo, Elaine S. Jaffe, and Mark Raffeld

The majority of low-grade follicular lymphomas will eventually transform to an aggressive intermediate, or high-grade lymphoma. The molecular mechanisms responsible for this transformation have not been determined. We studied sequential biopsies from 34 patients with follicular lymphomas that underwent histologic transformation, for abnormalities of the p53 tumor suppressor gene by a combination of immunohistochemistry, single strand conformation polymorphism analysis (SSCP), and sequencing. We found overexpression of p53 in 10 of the 34 transformed aggressive lymphomas, 9 of which contained mutations identified by SSCP analysis and subsequent sequencing. Matched pretransformation low-grade follicular lymphoma biopsies were available for 7 of the 10 cases. None of six studied by immunohistochemistry showed overexpression of p53 and only 1 of 4 studied by SSCP/sequencing showed the presence of mutation in the pretransformation biopsy. Interestingly, an eighth p53 positive transformed lymphoma recurred with a clonally related, p53 negative low-grade lymphoma 5 years after the patient had achieved a complete remission. Immunohistochemistry also showed that several pretransformation biopsies from p53 positive transformed cases showed rare p53 positive cells and in one case we could document an increase in their number over time. Twenty-five additional low-grade follicular lymphoma biopsies were also examined. Three patients had lymphomas positive for p53 mutation. One of the three subsequently transformed within a year of the biopsy studied; the second patient had an earlier (unavailable) biopsy at a different site that showed transformed histology. The third patient was treated with ProMACE-MOPP combination chemotherapy and attained a complete remission. We conclude that (1) mutations of p53 are associated with histologic transformation in approximately 25% to 30% of follicular lymphomas and (2) p53 positive cells can be detected before histologic transformation, but do not comprise a significant percentage of the neoplastic cell population (identifiable by SSCP) until late in the disease, just before or after histologic progression. Finally, the data also suggest that p53 positive low-grade lymphomas are at risk for progression and that in this subset, aggressive therapy may be warranted.

This is a US government work. There are no restrictions on its use.
The p53 tumor suppressor gene is a particularly attractive candidate to be involved in progression of follicular lymphoma. It is mutated in a large variety of malignancies and is associated primarily with aggressive neoplasms.\textsuperscript{23-27} It is less frequently mutated in indolent neoplasms.\textsuperscript{28-31} There is accumulating evidence that p53 may be related to progression in a number of human tumors.\textsuperscript{32-37} Notably, in chronic myelogenous leukemia, p53 mutations have been associated almost exclusively with the blast transformation stage\textsuperscript{22} and, more recently, p53 has been shown to be involved in the progression of low grade astrocytomas to high-grade astrocytomas.\textsuperscript{35}

In lymphoid neoplasms, p53 mutations have been found mainly in aggressive lymphomas, primarily of small non-cleaved cell type.\textsuperscript{30} Mutation and overexpression of p53 protein have also been reported in acute lymphoblastic leukemia of T-cell phenotype and less commonly in other de novo aggressive lymphomas.\textsuperscript{38-42} Cases of indolent lymphoma have generally been reported to be negative for p53 mutation, including indolent follicular lymphomas and most cases of chronic lymphocytic leukemia (CLL). Interestingly, however, mutations have been reported in rare cases of CLL and in high-grade lymphomas that had evolved from CLL (Richter's transformation), suggesting that it may play a role in transformation of some cases of CLL.\textsuperscript{30,43}

To determine whether the p53 tumor suppressor gene might be involved in the histologic transformation of follicular lymphoma, we studied a series of sequential biopsies from 34 patients with follicular lymphomas that eventually transformed into aggressive lymphomas. Many of these patients had multiple biopsies spanning the time that they first presented with their low-grade follicular lymphoma through the time that they first showed histologic transformation to an aggressive lymphoma. These biopsies were studied by a combination of techniques including immunohistochemical staining for overexpression of the mutant p53 protein, single-strand conformation polymorphism analysis (SSCP) for identification of mutations within the p53 gene, and sequencing of PCR amplified p53 gene segments for confirmation and identification of the mutations. The data suggest that p53 mutations are involved in the histologic progression of a subset of patients with follicular lymphoma.

MATERIALS AND METHODS

Cases studied. Thirty-four patients seen at the National Institutes of Health with transformed follicular lymphomas were selected for this study, primarily based on the availability of frozen tissue for molecular analyses, and a pretransformation biopsy documenting a prior diagnosis of low-grade follicular lymphoma. Criteria for including a case as a transformed follicular lymphoma have been described previously.\textsuperscript{15} Briefly, to be considered transformed, a case had to have a previous histologically documented diagnosis of a low-grade follicular lymphoma (follicular small cleaved or follicular mixed small and large cell) and a current diagnosis of aggressive lymphoma of intermediate or high grade as defined by the International Working Formulation for Clinical Usage.\textsuperscript{44} For 17 of the 34 patients, frozen tissue for molecular studies was also available on one or more of the preceding indolent follicular lymphoma biopsies. A majority of the cases had paraffin-embedded tissue blocks available for biopsies from both the indolent and aggressive phases of their disease for immunohistochemical staining for p53 protein. Twenty-five unselected cases of low-grade follicular lymphoma were also included in this study. These biopsies all demonstrated either malignant lymphoma, follicular, small cleaved cell type or malignant lymphoma, follicular, mixed small, and large cleaved cell type. Four of the patients in this latter group had another biopsy showing aggressive histology; however, the transformed biopsy was not available for study. All patients in this study are part of ongoing clinical studies and have provided informed consent according to the guidelines of the Institutional Review Board of the National Institutes of Health. Single-strand conformation polymorphism analysis (SSCP). SSCP analysis for p53 was accomplished according to a modified version of a previously reported procedure.\textsuperscript{35} The oligonucleotide primers for exons 5 through 9 were synthesized by the solid-phase triester method and are identical to those used by Gaidano et al.\textsuperscript{36} Polymerase chain reactions (PCRs) were performed using 100 ng of genomic DNA, 0.4 \( \mu \)mol/L of each primer, 2.5 \( \mu \)mol/L dNTPs, 1 \( \mu \)Ci of (\( \alpha \)-\( ^{32} \)P) dCTP (specific activity 6,000 Ci/mmol), 10 mmol/L Tris-\( \mathrm{HCl} \) (pH 8.3), 1.5 to 3.0 mmol/L MgCl\(_2\), 50 mmol/L KCl, 0.01% gelatin, and 0.5 units of AmpliTaq DNA Polymerase (Perkin-Elmer Cetus, Norwalk, CT) in a final volume of 10 \( \mu \)L. After an initial denaturation for 5 minutes at 94°C, 35 cycles of denaturation (94°C for 1 minute), annealing (60°C for 1 minute), and extension (72°C for 1 minute) were performed on a DNA thermal cycler 480 (Perkin Elmer Cetus). An aliquot of each reaction mixture was diluted 1:25 with 0.1% sodium dodecyl sulfate (SDS) and 10 mmol/L EDTA, and further diluted 1:1 with sequencing stop solution (95% formamide, 20 mmol/L EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol FF (United States Biochemical Corp, Cleveland, OH)). Samples were boiled for 3 minutes, chilled on ice, and immediately loaded onto a 6% acrylamide gel containing 10% glycerol. To obtain optimal separation of the single-stranded conformers, the ratio of methylene-bis-acrylamide (bis) to acrylamide generally used was 1:99, although for the exon 5 fragment, the standard ratio of 1:10 was more effective. Gel electrophoresis was performed at 8 W for 12 to 15 hours or at 40 W for 6 to 8 hours at room temperature.

Autoradiography was performed with an intensifying screen for 4 to 24 hours at \( -70^\circ \)C. Sequencing strategies. To confirm the presence of a mutation, one of two sequencing strategies was used. For cases in which the tumor cells comprised a majority of the cellular population, a second set of PCRs was performed that incorporated a final asymmetric reaction. Direct sequencing of the single-stranded product was performed according to a previously published method.\textsuperscript{46} Briefly, an initial PCR was performed under the same conditions as described for the genomic SSCP with the following modifications. The volume of the reaction was increased to 50 \( \mu \)L, the concentration of dNTPs was increased to 200 \( \mu \)mol, and the radioactive nucleotide was omitted. The product was purified and subjected to a second "asymmetric" PCR reaction under the identical conditions of the first reaction except that the ratio of the two primers (primer 1 : primer 2) was 1:50 (6 ng:300 ng). The single-stranded product was annealed with 800 ng of primer 1 and the sequencing reaction was performed using \( ^{32} \)S-dATP and Sequenase according to the instructions supplied by the manufacturer (United States Biochemical).

Autoradiography was performed for 1 to 6 days at ambient temperature. This direct sequencing strategy was effective for cases 5b, 6b, 10a, 10b, and 11. Both strands were sequenced for all cases. For the remaining cases in which the asymmetric sequencing reaction could not be interpreted or was not attempted because of a high percentage of “contaminating” normal cells, a second PCR
was performed and the product subcloned into a plasmid vector as previously described using the CloneAmp system (GIBCO BRL, Gaithersburg, MD). In this system, DNA is PCR amplified using primers designed with uracil containing repeats at their sequences that are treated in the same way.

After treatment with uracil DNA glycosylase, 3' overhangs are generated allowing the fragment to be efficiently cloned into a vector supplied by the manufacturer, containing complementary sequences that are treated in the same way.

The primer pairs used for subcloning were identical to those used in the genomic SSCP with the following modification: the sense and antisense primers of each pair were synthesized with the following 12 nucleotide sequences added to their 5' ends: 5'-CAUCAUCAU-CAU...3', and 5'-CAUCAUCAUCAU...3', respectively. PCRs were performed in a final volume of 50 μL containing 500 ng of genomic DNA, 0.4 μmol/L of each primer, 200 μmol/L of dNTPs, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 0.01% gelatin, and 2.5 U of AmpliTaq DNA polymerase using the optimal concentration of MgCl₂ determined for each primer set. After an initial denaturation at 94°C for 5 minutes, 35 cycles of reaction were performed using a "hot start" procedure. Cycle parameters were 1 minute denaturation at 94°C, 1 minute annealing at 60°C, and 1 minute of extension at 72°C. Five to 10 ng of the PCR product was incubated with 25 ng of pAMP-1 plasmid vector and 1 U of uracil DNA glycosylase in a total volume of 20 μL and incubated at 37°C for 30 minutes. This mixture was used directly to transform competent bacteria (DH5α, GIBCO BRL, Gaithersburg, MD) and the transformants were selected for ampicillin resistance and screened for the correct size insert.

The subclones were further screened by SSCP analysis to determine whether the insert represented the normal or mutated allele. Approximately 1 pg of plasmid DNA was amplified under the identical conditions used for the genomic SSCP. The PCR products of 36 selected subclones were analyzed on 6% acrylamide gels as described for the genomic SSCP with amplified control DNAs that included the corresponding amplification product from the original tumor and a nonmutated control amplification product.

A number of subclones also displayed the same abnormal migration pattern as the original genomic DNA on the above SSCP analysis were selected for sequencing using the dye-deoxy chain termination procedure. For these reactions, we used the same primers that were used in the SSCP analyses and Sequenase (United States Biochemical). Briefly, 3 to 5 μg of plasmid DNAs were extracted with phenol/chloroform, purified on a Sephadex (3-50 column (Sephadex G-50 column (Select-D, G-50; 5 Prime → 3 Prime, Inc, West Chester, PA), and denatured in 0.2 N NaOH. The denatured DNAs were ethanol precipitated and resuspended in 10 μL of 1X Sequenase reaction buffer containing 100 ng of the sequencing primer. After incubation for 15 minutes at 37°C to allow primer annealing, the reaction was performed according to the manufacturer’s protocol using 32P-dATP. Autoradiography was performed for 1 to 6 days at ambient temperature.

**Immunohistochemical studies.** P53 overexpression was assessed using the DO7 monoclonal antibody (MoAb) (Dako Corp, Carpinteria, CA). This antibody recognizes both normal and mutant forms of p53. Because the half-life of normal p53 is short and the amount of normal p53 expressed is low, the detection of stable levels of p53 in a tumor cell suggests a p53 mutation. Before the application of the primary antibody, an antigen recovery technique was performed. The deparaffinized slides were placed into 50 mm Tris buffer pH 7.4, and microwaved (Model R4A80, Sharp Electronics, Mahwah, NJ) for 10 minutes at 700 W. Immunohistochemistry was then performed on an automated immunostainer (Ventana Medical Systems, Inc, Tucson, AZ) using the manufacturer’s paraffin slide protocol without digestive enzyme. The DO7 antibody was used at a concentration of 1:50 and applied for 32 minutes. A case was regarded as p53 positive if greater than 5% of the malignant cells stained with the antibody, although in practice all positive cases had 20% or more of their cells marking. Small numbers of p53 positive cells (generally less than 0.1%) were seen in some indolent lymphomas. When these occurred, they were noted to occur singly or in clusters.

**RESULTS**

**SSCP analysis of transformed follicular lymphomas.** To determine if the p53 tumor suppressor gene has a role in the histologic progression of indolent follicular lymphomas, we studied the biopsy samples from 34 patients with progressed follicular lymphomas. Thirty-two of the 34 cases were previously reported to contain BCL-2 rearrangements, consistent with their follicular center cell origin. The progressed or transformed biopsy samples (transformed phase) from all 34 patients, and the preceding indolent follicular lymphoma biopsy samples (indolent phase) from 17 of the 34 patients, were initially screened by SSCP analysis. The SSCP analysis covered the previously described mutation prone regions spanning exons 5 through 9.

The results of the SSCP analysis are summarized in Table 1 and shown in Fig 1, cases 1 through 11. Eleven of the 34 transformed phase follicular lymphomas showed altered electrophoretic mobility for 1 of 5 amplified fragments corresponding to exons 5 through 9. Altered electrophoretic mobility occurred in the exon 5 fragment in 3 cases, in the exon 6 fragment in 3 cases, in the exon 7 fragment in 4 cases, and in the exon 8 fragment in 1 case. Two of the exon 6 mobility alterations (cases 10 and 11) were subsequently shown to be polymorphisms (see below) and are not included as abnormalities in Table 1.

Five of the 17 matching indolent phase follicular lymphomas available for study were tumors in which the transformed lymphomas contained a p53 mutation. One of these five (case 9a) showed the identical p53 mutation seen in the corresponding transformed phase biopsy. However, this biopsy showed only rare p53 positive staining cells and will be discussed later. All of the remaining indolent phase lymphomas contained a p53 mutation as described below.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>SSCP +/Tested</th>
<th>IP +/Tested*</th>
<th>SSCP or IP +/Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transformed follicular lymphomas</td>
<td>9/34</td>
<td>10/28</td>
<td>10/34</td>
</tr>
<tr>
<td>Low-grade phase</td>
<td>1/5</td>
<td>0/7</td>
<td>1/8</td>
</tr>
<tr>
<td>With p53 positive TP</td>
<td>0/12</td>
<td>0/10</td>
<td>0/18</td>
</tr>
<tr>
<td>With p53 negative TP</td>
<td>1/21</td>
<td>1/21</td>
<td>1/21</td>
</tr>
<tr>
<td>Without evidence of progression</td>
<td>2/4</td>
<td>2/4</td>
<td>2/4</td>
</tr>
</tbody>
</table>

Abbreviations: IP, immunoperoxidase; FL, follicular lymphoma; TP, transformed phase.

* Immunoperoxidase (IP) studies were considered positive if >5% of the tumor cell nuclei stained with the DO7 antibody.
p53 MUTATION AND LYMPHOMA PROGRESSION

Fig 1. SSCP analysis of p53 exons in transformed follicular lymphoma cases. Genomic DNA fragments corresponding to exons 5 through 9 of the p53 gene were amplified by PCR in the presence of α-32P dCTP and the denatured product run under nondenaturing conditions in a 6% acrylamide gel containing 10% gelatin. All abnormal migration patterns are shown and occurred in exons 5 through 8. Lanes are labeled with case numbers (see Table 2). Lanes labeled C are nonmutated controls. Asterisks identify the abnormally migrating conformers. Cases 1 through 11 are transformed follicular lymphoma cases. Paired indolent and transformed samples are shown for cases 2 (2b and 2e), 4 (4a, 4b, and 4c), 6 (6a and 6c), 8 (8a and 8b), and 9 (9a, 9e, and 9f). The altered bands of cases 10 and 11 in exon 6 proved on sequencing to be the known polymorphism at position 213 (case 10, heterozygous; case 11, homozygous). Cases 13 through 15 are from “indolent” lymphoma cases (see text).

Interestingly, one p53 negative indolent phase follicular lymphoma (case 8b) was a recurrence that occurred 5 years after successful treatment of the p53 positive transformed aggressive lymphoma (8a). This suggests that although the p53 positive transformed lymphoma clone was successfully treated, residual p53 negative indolent lymphoma cells had survived, and eventually reappeared as a recurrence.

Figures 2A through 2D show examples of representative cases (see text).

Table 1. SSCP and Sequencing Analysis of Indolent and Transformed Phase Follicular Lymphomas

<table>
<thead>
<tr>
<th>Exon</th>
<th>Mutated Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2, 4, 6, 8, 9, 10, 11, 13, 15</td>
</tr>
<tr>
<td>6</td>
<td>1, 3, 4, 5, 7, 8, 10, 11, 13, 15</td>
</tr>
<tr>
<td>7</td>
<td>1, 3, 5, 6, 7, 8, 10, 11, 13, 15</td>
</tr>
<tr>
<td>8</td>
<td>1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 13, 15</td>
</tr>
</tbody>
</table>

Table 2. Immunohistochemical Analysis of Sequential Biopsies from p53 Mutated Cases

<table>
<thead>
<tr>
<th>Case</th>
<th>Indolent</th>
<th>Transformed</th>
<th>Overexpression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>13</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>14</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The results of this analysis is presented in Table 2 and selected sequences are illustrated in Fig 2 (panels A, C, and D). Seven of the SSCP abnormalities were the result of missense mutations in known mutation prone regions (cases 1 through 3, 5 through 7, and 9). Two (cases 4 and 8) contained small deletions. Case 4 had a 12-bp deletion eliminating codons 219 through 222 leaving the shortened transcript in frame, and case 8 had a 4-bp deletion that eliminated the last base pair of position 255 and all of codon 256. The latter deletion resulted in a frameshift and a truncated protein. The mobility shifts of cases 10 and 11 were found to result from the known exon 6 polymorphism at codon 213. Thus, a total of 10 of the 34 transformed cases (29%) showed overexpression of the p53 protein, including all 9 cases identified as having a p53 mutation by SSCP analysis.

Immunohistochemical analysis of sequential biopsies from p53 mutated cases. All available indolent phase and transformed phase biopsies from the 10 p53 positive transformed cases were collected and examined for overexpression of the p53 protein. This analysis is shown in Table 2 and is correlated with the SSCP results. These data indicate that all biopsies which showed transformed histology stained positively with the DO7 antibody, and that many of the preceding indolent phase biopsies exhibited rare positive cells that may occur singly, or less commonly in small clusters. Examples of representative cases are shown in Fig 3. Large numbers of p53 positive staining cells were only in the transformed phase biopsies, when the quantitatively...
Table 2. Analysis of Sequential Biopsies From p53 Positive Cases

<table>
<thead>
<tr>
<th>Case</th>
<th>Year</th>
<th>Diagnosis</th>
<th>p53</th>
<th>SSCP</th>
<th>Exon</th>
<th>Codon-Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transformed follicular lymphomas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>1988</td>
<td>DLC</td>
<td>+</td>
<td>+</td>
<td>5</td>
<td>158 CGG(Arg) → CAC(His)</td>
</tr>
<tr>
<td>2a.</td>
<td>1976</td>
<td>FSC</td>
<td>-</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2b.</td>
<td>1976</td>
<td>FSC</td>
<td>-/+†</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2c.</td>
<td>1978</td>
<td>FM</td>
<td>-/+†</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2d.</td>
<td>1983</td>
<td>DLC</td>
<td>+</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2e.</td>
<td>1985</td>
<td>FLC</td>
<td>+</td>
<td>+</td>
<td>5</td>
<td>173 GTG(Val) → ATG(Met)</td>
</tr>
<tr>
<td>3a.</td>
<td>1981</td>
<td>DLC</td>
<td>+</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3b.</td>
<td>1981</td>
<td>DLC</td>
<td>NA</td>
<td>+</td>
<td>5</td>
<td>176 TGC(Cys) → TTC(Phe)</td>
</tr>
<tr>
<td>4a.</td>
<td>1973</td>
<td>FM</td>
<td>-/+†</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4b.</td>
<td>1982</td>
<td>DLC</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4c.</td>
<td>1983</td>
<td>FM&amp;FSC</td>
<td>+</td>
<td>+</td>
<td>6</td>
<td>12-bp deletion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>219-222 CCCTATGAGCCG</td>
</tr>
<tr>
<td>5a.</td>
<td>1973</td>
<td>FSC</td>
<td>-/+†</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5b.</td>
<td>1975</td>
<td>FM</td>
<td>-</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5c.</td>
<td>1979</td>
<td>DLC</td>
<td>+</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5d.</td>
<td>1979</td>
<td>DLC</td>
<td>NA</td>
<td>+</td>
<td>7</td>
<td>238 TGT(Cys) → TAT(Tyr)</td>
</tr>
<tr>
<td>6a.</td>
<td>1979</td>
<td>FM</td>
<td>NA</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6b.</td>
<td>1985</td>
<td>DLC</td>
<td>+</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6c.</td>
<td>1986</td>
<td>DLC</td>
<td>NA</td>
<td>+</td>
<td>7</td>
<td>248 CGG(Arg) → CAG(Gln)</td>
</tr>
<tr>
<td>7a.</td>
<td>1969</td>
<td>FSC</td>
<td>-/+†</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7b.</td>
<td>1978</td>
<td>FM</td>
<td>-</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7c.</td>
<td>1982</td>
<td>DLC</td>
<td>+</td>
<td>+</td>
<td>7</td>
<td>249 AGG(Arg) → AGC(Ser)</td>
</tr>
<tr>
<td>8a.</td>
<td>1974</td>
<td>DLC</td>
<td>+</td>
<td>+</td>
<td>7</td>
<td>4-bp deletion, frameshift</td>
</tr>
<tr>
<td>8b.</td>
<td>1979</td>
<td>FM</td>
<td>-/+†</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9a.</td>
<td>1977</td>
<td>FSC</td>
<td>-/+†</td>
<td>+</td>
<td>8</td>
<td>275 TGT(Cys) → TTT(Phe)</td>
</tr>
<tr>
<td>9b.</td>
<td>1980</td>
<td>FM</td>
<td>-/+†</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9c.</td>
<td>1983</td>
<td>FM</td>
<td>-</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9d.</td>
<td>1984</td>
<td>F&amp;DLC</td>
<td>+</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9e.</td>
<td>1985</td>
<td>DLC</td>
<td>+</td>
<td>+</td>
<td>8</td>
<td>275 TGT(Cys) → TTT(Phe)</td>
</tr>
<tr>
<td>9f.</td>
<td>1985</td>
<td>DLC</td>
<td>+</td>
<td>+</td>
<td>8</td>
<td>275 TGT(Cys) → TTT(Phe)</td>
</tr>
<tr>
<td>10a.</td>
<td>1973</td>
<td>FM</td>
<td>-</td>
<td>+</td>
<td>6§</td>
<td>213 CGA(Arg) → CGG(Arg)§</td>
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<tr>
<td>10b.</td>
<td>1974</td>
<td>DM</td>
<td>-</td>
<td>+</td>
<td>6§</td>
<td>213 CGA(Arg) → CGG(Arg)§</td>
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<tr>
<td>11.</td>
<td>1987</td>
<td>DLC</td>
<td>-</td>
<td>+</td>
<td>6§</td>
<td>213 CGA(Arg) → CGG(Arg)§</td>
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<tr>
<td>12a.</td>
<td>1983</td>
<td>FM</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
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<tr>
<td>12b.</td>
<td>1988</td>
<td>DM</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-grade follicular lymphomas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>1984</td>
<td>FM</td>
<td>+</td>
<td>+</td>
<td>5</td>
<td>151 CCC(Pro) → TCC(Ser)</td>
</tr>
<tr>
<td>14a.</td>
<td>1973</td>
<td>FM</td>
<td>-</td>
<td>NA</td>
<td></td>
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<tr>
<td>14b.</td>
<td>1977</td>
<td>FM</td>
<td>-/+†</td>
<td>-</td>
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<td></td>
</tr>
<tr>
<td>14c. 1981</td>
<td>FM</td>
<td>+</td>
<td>+</td>
<td>Intron 5</td>
<td>Splice acceptor site AG → CG</td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>1988</td>
<td>FM</td>
<td>+</td>
<td>+</td>
<td>8</td>
<td>273 CGT(Arg) → CAT(His)</td>
</tr>
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Cases in bold lettering correspond to the transformed phases of the progressed follicular lymphomas.

Abbreviations: IP, immunoperoxidase; DLC, diffuse large cell lymphoma (malignant lymphoma, diffuse large cell); FSC, follicular small cleaved (malignant lymphoma, follicular, small cleaved cell); FM, follicular mixed (malignant lymphoma, follicular mixed small cleaved and large cell); FLC, follicular large cell (malignant lymphoma, follicular, large cell); DM, diffuse mixed (malignant lymphoma, diffuse, mixed small cleaved and large cell); NA, not available for analysis.

* Immunostaining was scored as follows: +, nuclear staining in >20% of cells; -/+†, rare positively staining cells occurring in clusters; -/+t, very rare positively staining cells occurring singly; -, no immunopositive cells.

§ Positive SSCP in cases 10 and 11 are shown to be caused by a known polymorphism at codon 213

1 Case 13 presented initially with follicular and diffuse lymphoma (unavailable for study) and case 14 progressed within one year of the p53 mutated biopsy (see text).

Less sensitive SSCP analysis was also positive. Intermediate percentages of p53 positive cells were generally not observed. However, in case 2 we could show a progressive increase in the percent of positive cell staining in the sequential pretransformation indolent phase lymphoma biopsies. These observations suggest that, in this case, the immunohistochemical analysis is identifying the earliest expansion of the p53 mutated clone destined to become the transformed aggressive lymphoma, perhaps after the occurrence of additional genetic alterations. The transformed
Fig 2. Sequence analysis of SSCP positive cases. Each of the four examples is accompanied by the corresponding SSCP analysis of either the genomic or subclone derived amplified PCR products that were used in the subsequent sequencing. Panel A shows a sequence derived from asymmetrically amplified genomic DNA of case 5b, and the corresponding genomic SSCP analysis. Panels B, C, and D show examples of sequences derived from subclones and the corresponding SSCP analyses of the subclones that were sequenced. The subclone SSCP analyses that accompany each sequence contain a normal control (C), the original genomic tumor DNA (T), and representative subclones showing either the mutated allele (M) or the normal allele (N). The following cases are illustrated: panel B, case 13; panel C, case 4c; and panel D, case 8a. The abnormality of each case is indicated. Each sequence is shown 5' (bottom) to 3' (top). Coding strands are shown for all cases.

Analysis of unselected indolent phase follicular lymphomas. In an attempt to investigate further the relationship between p53 positive cell accumulation and progression, we studied another 25 cases of indolent follicular lymphoma. Four of the 25 cases had another biopsy showing transformed histology; however, the transformed biopsies were not available for analysis. Three cases with indolent histology showed p53 mutation by SSCP/sequencing and overexpression of p53 protein by immunohistochemistry (Table 2, cases 13 through 15). Two of these (cases 13 and 15) were found to be the result of missense mutations, while one (case 14) had an A to C transversion within the splice acceptor site of intron 5 that, theoretically, should abrogate nor-
mal splicing of the intron. (The sequence of case 13 is illustrated in Fig 2, panel B). In 6 of the remaining 22 cases, we could find rare (<1 of 10⁵) p53 positive cells although none of these cases showed clustering of p53 positive cells (data not shown).

Notably, two of the three positive patients (13 and 14) were from the small subset of four patients that had had another biopsy showing transformed histology, but in which the transformed phase biopsy was not available for study. Patient 13 presented initially with follicular and diffuse lymphoma and recurred in an inguinal lymph node with the p53 positive indolent histology that was studied. After a brief period of “watch and wait,” local adenopathy recurred and he was treated with 3,000 rads of inguinal and pelvic irradiation to complete remission. He has been in remission 8 years. Patient 14 pursued an aggressive course despite the
low-grade histology and died approximately 1 year later with autopsy documented transformed large cell lymphoma. Of further interest was that this latter patient had two earlier indolent lymphoma biopsies, 4 and 8 years before the current biopsy. The earliest of these did not show evidence of p53 positive staining cells. However, the subsequent biopsy showed small numbers (0.1% to 1%) of large p53 positive staining cells. Four years later the current SSCP positive biopsy showed follicular mixed morphology with nearly all of the large cells staining for p53. The third patient, case 15, had stage IV follicular mixed lymphoma with extensive abdominal adenopathy at diagnosis, was randomized to an aggressive chemotherapy protocol (ProMACE-MOPP), and has been in complete remission since 1988.

**DISCUSSION**

We have examined sequential biopsy samples from 34 patients with transformed follicular lymphomas for mutation and overexpression of the p53 tumor suppressor gene. Ten of the 34 lymphomas exhibited overexpression of the p53 protein and mutations were identified in 9 of the 10 cases. None of the seven preceding indolent follicular lymphomas available for immunohistochemical study showed overexpression of the protein in a significant percentage of cells, although one (of five studied by SSCP analysis) was found to possess the identical mutation present in the transformed lymphoma (discussed below). Thus, it appears that approximately 25% to 30% of follicular lymphomas transform to aggressive histology via a pathway that involves mutation of p53 and that the mutated clone becomes predominant at, or around the time of progression.

Because we used both immunohistochemistry and single-strand conformation polymorphism analysis to screen for p53 mutation, we believe that we have identified most of the transformed lymphomas with mutations of p53. However, we cannot exclude the possibility of mutation occurring in a region of the gene outside of our primer sets that might not lead to overexpression of the gene product. In addition, cases that may have lost both alleles would not be identified. However, tumors with these characteristics are not common. The data also suggest that p53 mutation most likely is acquired during the histologically indolent phase of follicular lymphoma. The evidence for this comes from several observations. Rare p53 positive cells could be found in the majority of the pretreatment biopsies that progressed along the p53 dependent pathway. In the serial biopsies of case 2 we could show an increase in the number of p53 positive cells over time. Thirdly, the indolent phase of case 9 was p53 positive by SSCP at a time when immunohistochemistry showed only rare p53 positive cells. Fourthly, the transformed phase biopsies of cases 4c and 9d contained transition areas of indolent histology and transformed histology. In these composite regions, both the “indolent” follicular lymphoma and the aggressive transformed lymphoma were p53 positive. Finally, by studying additional indolent phase follicular lymphomas, we identified three more p53 positive lymphomas with low-grade follicular histology. These cases most clearly show that the presence of the mutation alone is not sufficient for aggressive histology and that the mutation is acquired when the lymphoma histology is still indolent and can disseminate before histologic transformation.

Case 9 is interesting in that the p53 mutated subclone became predominant early in the disease course, fully 7 years before histologic progression. This exceptional case was also the only mutated lymphoma biopsy in which there was discordance with the p53 immunostaining. We do not believe that the lack of immunostaining is due to technical considerations because staining was repeatedly attempted on two different tissue blocks and with other p53 antibodies (1801 and CM-1; data not shown) and because rare p53 positive cells could be identified (Fig 3D). Thus, the lack of immunostaining in the vast majority of the tumor cells in this biopsy suggests that p53 protein was not being overexpressed at this phase in the disease process despite the fact that a mutation was present, and provides an explanation why clinical and histologic progression did not occur. It is possible that at the time of this indolent phase biopsy the p53 mutation may still have been heterozygous and significant overexpression did not result. Clonal selection for the mutation may have occurred because of a dominant negative mechanism suggested for some p53 mutations. In fact, this particular mutation, involving codon 275, has been shown to have transforming activity in rat embryo fibroblast (REF) assays, an assay used to demonstrate dominant negative effects. Loss of heterozygosity may have occurred later leading to overexpression and ultimately to transformation. Unfortunately, because of the high percentage of normal cells in the biopsies of this patient, it was not possible to ascertain the state of p53 zygoty in these biopsies.

Three of 25 unselected indolent follicular lymphomas were found to be p53 positive. Two of the three were in a small subset of four cases that showed progressed histology in another temporally related biopsy. The third p53 positive indolent lymphoma patient achieved a long-lasting remission after treatment on an aggressive chemotherapy protocol (ProMACE/MOPP). These data further suggest that the finding of a p53 mutation in an indolent follicular lymphoma defines a subset of cases that are biologically unstable and at high risk for a transformation event. One can speculate that the third patient never progressed because she responded to ProMACE/MOPP combination chemotherapy.

Recently, an attractive model for p53 function has been postulated that attempts to explain its central role in tumorigenesis. This model proposes that p53 functions in cell cycle control at the G1-S checkpoint by delaying entry into S phase allowing any necessary DNA repair to occur. Tumors defective in p53 cannot repair DNA correctly, particularly strand breaks, and accumulate mutations at a high rate. This model is supported by in vitro experiments from a number of laboratories that have shown that both human and mouse cells homozygous for p53 mutation are genetically unstable and have much higher rates of mutation of introduced reporter genes than normal control cells. The model also predicts that the transforming event is not necessarily the p53 mutation itself, but rather other genetic alterations that confer the aggressive phenotype to the cell.

Our own data and the above concepts allow for the con-
struction of the following model for a p53-dependent progression pathway in follicular lymphoma (Fig 4). During the indolent phase of follicular lymphoma, the clone of cells bearing the t(14;18) translocation is slowly expanding throughout the lymphoid system (1). As the tumor proliferates, random mutations accumulate in the tumor cells including mutations in the p53 gene (2). The majority of the p53 mutations behave in a recessive manner and are silent. (Some of these mutations, such as the mutation at codon 275 [patient 9], may behave in a dominant negative manner and interfere with the tumor suppressor function of the normal p53 allele.) Eventually one of the heterozygous mutant cells will become homozygous through loss of the normal allele (3). When this happens, loss of the tumor suppressor function occurs, genomic instability ensues, and mutations begin to accumulate at a higher frequency. Some of these mutations will provide the tumor cell with a proliferative advantage and these new p53 positive subclones begin to replace the original slower growing clone. As the p53 mutant subclone becomes more numerous there is continuing selection for mutations that generate a more aggressive phenotype, and the follicular lymphoma enters a biologically unstable phase (4). Eventually a transforming mutation occurs and an aggressive high grade p53 positive lymphoma is the final outcome (5).

Although we found p53 mutations in close to 30% of progressed follicular lymphomas, 70% of cases do not have p53 mutations. The identification of p53 gene mutation probably defines a particular pathway by which progression can proceed. The functional defect that occurs as a result of the mutated p53 protein likely favors the accumulation of particular associated genetic lesions. In this regard, we have previously looked for the involvement of other potential progression related genes in progressed follicular lymphomas. It is of some interest that we find no overlap between cases previously reported to have progressed via MYC gene rearrangement and the present p53 positive cases. In addition, although mutant RAS genes are often found in association with mutant p53 in solid tumors, we have not found RAS mutations in the p53 positive progressed lymphomas (data not shown).

In conclusion, our data indicate that 25% to 30% of patients with follicular lymphoma will progress via a pathway involving p53 mutation. They also suggest that p53 positive follicular lymphomas with low-grade histology are at risk for progression and that aggressive treatment may be indi-

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**Fig 4.** Model for p53 dependent transformation of follicular lymphoma. Follicular lymphomagenesis is initiated when a pre-B cell undergoes t(14;18) translocation (step 0). Additional events are likely to be necessary to complete neoplastic development and result in the prototypic indolent follicular lymphoma (step 1). As the follicular lymphoma grows, random mutations occur, some of which will affect the p53 gene (step 2). These mutations are generally silent. Eventually, loss of heterozygosity occurs through the loss of the normal allele (step 3). This results in the loss of tumor suppressor function. The cell loses its ability to repair DNA efficiently and mutations accumulate at a high frequency. Some mutations provide the cell with a proliferative advantage, and the new p53 positive subclone begins to replace the original slower-growing clone. As the p53 positive subclone becomes more numerous (step 4), there is continuing selection for mutations that generate a more aggressive phenotype, and the indolent follicular lymphoma enters a biologically unstable phase. Eventually a transforming mutation occurs and an aggressive high-grade lymphoma results (step 5).
cated. Prospective studies of patients presenting with low-grade follicular lymphomas should help to assess the validity of this conclusion.

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