Post-Thymic T Cell Lymphomas Frequently Overexpress p53 Protein but Infrequently Exhibit p53 Gene Mutations

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We recently demonstrated that only one of 36 T-cell neoplasms contained p53 gene mutations. Although p53 gene mutations are known to result in overexpression of the p53 gene product, we also recently discovered that p53 protein overexpression does not correlate with p53 gene mutations, but does correlate with proliferation (r = 0.92), in anaplastic large cell lymphoma. In view of these findings, we investigated 34 non-human T-cell lymphotropic virus type I (HTLV-I) related post-thymic T-cell lymphomas immunohistochemically for p53 protein, using monoclonal antibody 1801, and for proliferation, using monoclonal antibody Ki-67, and quantitated the results with the CAS-200 computerized image analysis system. We evaluated the presence of mutations in conserved exons 5 to 9 of the p53 gene using single-strand conformation polymorphism analysis and DNA sequencing. p53 mutations were detected in three of 34 cases, including two that contained deletions. p53 protein overexpression was detected in 17 of 34 cases, including the three mutated cases, with reactivities ranging from 10% to 48%. However, many cases in which a structural alteration could not be detected demonstrated levels of p53 protein expression comparable to those cases that were mutated. Correlation of p53 protein expression and proliferation, as assessed by Ki-67 expression, in this group of lymphomas was poor (r = 0.34). Whether alternative mechanisms of p53 protein inactivation are causing phenotypic overexpression of the p53 protein in these malignant lymphomas is unknown, although preliminary studies do not support a major role for such mechanisms. Therefore, the etiology and the significance of p53 protein overexpression in the cases that lack a demonstrable mutation is unclear. Nevertheless, as in anaplastic large cell lymphoma, overexpression of the p53 gene product is not a reliable predictor of the presence of mutations in conserved portions of the p53 gene in non-HTLV-I associated post-thymic T-cell lymphoma. (Am J Pathol 1994, 144:573-584)

Originally identified as a tumor antigen that coimmunoprecipitated with the simian virus 40 large T antigen and subsequently as a dominantly transforming oncogene, the role of wild type p53 has evolved into that of a tumor suppressor gene whose protein product can inhibit cell growth by mediating its arrest in late G1 of the cell cycle. Functional inactivation of the p53 tumor suppressor gene has become one of the most frequently detected genetic alterations in all varieties of human malignancies including those of epithelial, mesenchymal, and hematopoietic origin. In accordance with the tumor suppressor gene model, mutation of one allele is often accompanied by deletion of the remaining allele, although this loss is not requisite.

The function of wild type p53 can be disrupted by numerous mechanisms including mutation or inactivation by binding with other cellular or virally encoded proteins. Both mechanisms can alter the posttranslational stability of the p53 protein, prolonging its half-life to several hours over its wild type form, which has a half-life of approximately 20 minutes. This leads to phenotypic overexpression of the p53 protein product, a property that has formed the basis for its study in a wide variety of tissues and tumors by immunohistochemical staining.

In a study performed in collaboration with our laboratory, a large panel of clinically and pathologically

Accepted for publication November 1, 1993.
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diverse non-Hodgkin's lymphomas (NHL) and lymphoid leukemias were screened for mutations in exons 5 to 9 (mutational hot spots) of the p53 locus.\textsuperscript{15} The highest mutational frequencies were found in certain categories of B-cell neoplasia, specifically Burkitt's lymphoma, B-cell chronic lymphocytic leukemia, and Richter's syndrome. We subsequently demonstrated a high incidence of p53 gene mutations among cases of adult T-cell leukemia/lymphoma.\textsuperscript{26} However, the findings from these and other studies have suggested that the incidence of p53 gene mutation in other categories of B- and T-cell NHL and lymphoid leukemia, including non-human T-cell lymphotropic virus type I (HTLV-I) associated post-thymic T-cell lymphomas, is very low.\textsuperscript{15,27-29} Indeed, Gaidano et al\textsuperscript{15} found evidence of p53 gene mutation in only one of 36 T-cell neoplasms.

In contrast, some immunohistochemical studies\textsuperscript{25,30-33} have suggested a significant incidence of p53 protein overexpression in various categories of lymphoid neoplasia, including CD30- (Ki-1) positive anaplastic large cell lymphoma (ALCL).\textsuperscript{34} We recently confirmed the high frequency of p53 protein overexpression in CD30-positive ALCL.\textsuperscript{35} However, despite high levels of p53 gene product in the tumor cells of 12 of 15 cases of ALCL, we found an actual mutation in exons 5 to 9 of the p53 locus in only one of 17 cases. The precise mechanism for p53 protein overexpression in these tumors is unknown. However, we found an excellent correlation ($r = 0.92$) between p53 protein expression and proliferation, as assessed by immunohistochemical staining with monoclonal antibody Ki-67. In view of this observation, we decided to investigate a separate and larger group of 34 post-thymic T-cell NHLs of heterogeneous morphology and proliferative capacity for evidence of p53 protein overexpression as determined by immunohistochemical staining of frozen tissue sections with anti-p53 monoclonal antibody 1801. We correlated these findings with the presence of p53 gene mutations as determined by the single-strand conformation polymorphism (SSCP) assay in conjunction with cloning and DNA sequencing.

**Materials and Methods**

**Pathological Specimens**

A panel of 34 clinically, morphologically, and immunophenotypically well-characterized non-(HTLV-I) associated post-thymic T-cell lymphomas were randomly selected from among cases processed in the surgical pathology laboratory of the Columbia Presbyterian Medical Center. These 34 cases included 25 morphologically heterogeneous peripheral T-cell lymphomas and nine cutaneous T-cell lymphomas (five mycosis fungoides and four nonmycosis fungoides type). Hematoxylin-and-eosin-stained sections were prepared from representative portions of each pathological specimen that had been fixed in 10% buffered formalin or B5 solution and embedded in paraffin. Additional remaining portions of each pathological specimen were embedded in a cryopreservative solution (OCT compound, Miles, Elkhart, ID) and stored at −70 C until needed. In some instances, a mononuclear cell suspension was prepared from portions of the pathological specimen by ficoll-hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation. The diagnosis of malignant lymphoma was based upon review of the histopathology and the results of immunophenotypic and/or genotypic analysis.

**DNA Extraction**

Before DNA extraction, a Giemsa-stained frozen tissue section or cytoplasm was prepared and examined to confirm the presence of morphologically identifiable neoplastic cells. Genomic DNA was extracted from cryopreserved tissue blocks and mononuclear cell suspensions using a salting-out procedure.\textsuperscript{36} The frozen tissue sections or cells were resuspended in 3 ml of nuclei lysis buffer containing 10 mmol/L Tris HCl, 400 mmol/L NaCl and 2 mmol/L ethylenediaminetetra-acetic acid (EDTA); 200 $\mu$L of 10% sodium dodecyl sulfate (SDS) (NaDODSO\textsubscript{4}), and 500 $\mu$L of proteinase K solution (1 mg proteinase K in 1% SDS and 2 mmol/L EDTA) were subsequently added. After overnight digestion at 37 C, 1 ml of saturated NaCl was added. This mixture was centrifuged at 2,500 rpm for 20 minutes and 2 volumes of ethanol were added to the supernatant to precipitate the DNA, which was washed several times in 70% ethanol. The tubes were spun in a microcentrifuge for 30 minutes, the supernatant transferred to a new tube, and the DNA quantitated spectrophotometrically.

**Southern Blotting**

Five-microgram aliquots of genomic DNA were digested with the appropriate restriction endonucleases according to the manufacturer's instructions (Boehringer-Mannheim, Indianapolis, IN), electrophoresed in 0.8% agarose gels, denatured with alkali, neutralized, and transferred to nitrocellulose filters according to Southern.\textsuperscript{37} The filters were
hybridized in 50% formamide/3× standard sodium citrate at 37 C, washed in 0.2× standard saline citrate/0.5% SDS at 60 C for 2 hours, and autoradiographed at −70 C for 16 to 48 hours, as previously described. The T-cell receptor β chain (TCR-β) gene was investigated by hybridization of EcoRI and BamHI digested DNAs to a DNA probe that hybridizes to the constant region of the TCR-β gene.  

Oligonucleotide Primers

The oligonucleotides used for polymerase chain reaction (PCR) amplification in this study were synthesized by the solid-phase trieter method. The sequences of the p53 primers used to amplify the p53 gene were derived from published sequences and are as follows: P5-5, 5'-TTCCCTCTCTGGGAGATCAG-3'; P5-3, 5'-ACCCCTTGGGACAGCCT-3'; P6-5, 5'-ACAGGGCTGGTTGCCCAGGGT-3'; P6-3, 5'-AGTTGCAAACCAGACCTCAG-3'; P7-5, 5'-ACCCTGGGCAACCAGCCCT-3'; P7-3, 5'-ACCCTGGGCAACCAGCCCT-3'; P8-5, 5'-GCAGTTATGCCTCAGATTC-3'; P8-3, 5'-AGTTGCAAACCAGACCTCAG-3'; P9-5, 5'-AGTTGCAAACCAGACCTCAG-3'; P9-3, 5'-AGTTGCAAACCAGACCTCAG-3'. The location of these primers has been previously published: primers P5-5, P5-3, P6-5, P7-5, P8-5, and P9-3 are derived from the corresponding intron/exon junction, and primers P5-3, P6-5, P7-3, P8-3, and P9-5 are derived from intron sequences flanking the corresponding exons.

SSCP Analysis

SSCP analysis was accomplished using an adapted version of a previously reported method. Each PCR reaction was performed with 100 ng of genomic DNA, 10 pmoles of each primer, 2.5 μmol/L dNTPs, 1 μCi of [α-35P]dCTP (NEN; specific activity, 3,000 Ci/mmol), 10 mmol/L Tris (pH 8.8), 50 mmol/L KCl, 1 mmol/L MgCl2, 0.01% gelatin, and 0.5 U Taq polymerase, in a final volume of 10 μl. Thirty cycles of denaturation (94 C), annealing (63 C for p53 exons 5, 6, and 9, 62 C for p53 exon 7, and 58 C for p53 exon 8), and extension (72 C) were done on an automated heat-block (DNA Thermal-Cycler, 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 (95% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). Samples were heated at 95 C for 5 minutes, chilled on ice, and immediately loaded onto a 6% acrylamide-TBE gel containing 10% glycerol. Gels were run at 4 to 8 watts for 14 to 16 hours at room temperature. The gels were fixed in 10% acetic acid, air-dried, and autoradiography was performed at −70 C with an intensifying screen for 6 to 24 hours.

Cloning and Sequencing of PCR Products

PCR products were cloned in the PCR 1,000 vector using the TA cloning system (Invitrogen Corp., San Diego, CA), following the manufacturer's instructions. DNA sequencing was performed directly from small-scale plasmid preparations after determining the presence of an insert. The Sequenase version 2.0 (United States Biochemical, Cleveland, OH) system was used, and a modification of the manufacturer's instructions was performed. Approximately 2 μg of plasmid DNA in a volume of 20 μl was alkali-denatured by adding 2 μl of 2 N NaOH, 2 mmol/L EDTA and incubating for 5 minutes at room temperature. The mixture was then neutralized with 8 μl 1 mol/L Tris HCl (pH < 5) and subsequently ethanol-precipitated. The DNA was resuspended in 7 μl of H2O, adding 1 μl of primer and 2 μl of 5X Sequenase sequencing buffer. After incubation at 37 C for 30 minutes, the following reagents were added: 2 μl of labeling mix dilution (1:5), 1 μl 0.1 mol/L dithiothreitol, 0.5 μl (4 μCi) [35S]dATP, and 2 μl Sequenase enzyme dilution (1:8). These labeling reactions were incubated at room temperature for 5 minutes, and subsequently 3.5 μl were aliquoted into a tube containing each of the four termination mixtures (ddGTP, ddATP, ddCTP, ddTTP). After a 5-minute incubation at 42 to 45 C, 4 μl of stop solution was added. The samples were denatured at 75 C for 5 minutes before loading into a 6% polyacrylamide/8 mol/L urea gel. Autoradiography was performed at room temperature for 16 to 24 hours.

Immunohistochemical Staining

The T-cell origin of these tumors was determined at the time of diagnosis by immunohistochemical staining of frozen and/or paraffin tissue sections using a three-step avidin-biotin immunoperoxidase technique or an immunoalkaline phosphatase anti-alkaline phosphatase method, and/or by direct and
indirect immunofluorescent flow cytometry of isolated cells in suspension using the FAScan fluorescent activated cell sorter (Becton-Dickinson, Mountain View, CA). Monoclonal antibodies (MAbs) used to immunophenotype the post-thymic T-cell lymphomas included anti-terminal deoxynucleotidyl transferase (TdT), OKT3 (CD3), OKT4 (CD4), OKT6 (CD1), OKT8 (CD8), OKT9 (CD71), OKT10 (CD38), OKT11 (CD2; Ortho Diagnostics, Raritan, NJ), T1 (CD5), interleukin-2R (CD25), BL9, HLA-DR (United Biomedical, Lake Success, NY), Leu2, (CD8), Leu3a (CD4), Leu4 (CD3), Leu9 (CD7), Leu14 (CD22), Leu22 (CD43), LeuM1 (CD15), LCA (CD45), L26 (CD20), kappa, lambda, epithelial membrane antigen (DAKO-PC, Dako Corp., Santa Barbara, CA), Ber-H2 (CD30; courtesy, Dr. Harald Stein, Berlin, Germany), and B1 (CD20; Coulter Immunology, Hialeah, FL).

To determine reactivity with the p53 protein, tissue sections were dried overnight at room temperature, post-fixed in 1:1 acetone chloroform solution, dried thoroughly, and refixed in 10% formalin. The sections were incubated overnight with the primary anti-p53 MAb 1801 (p53 Ab-2, Oncogene Science, Inc., Uniondale, NY), washed three times, and incubated with biotinylated horse anti-mouse immunoglobulin G (Vector Laboratories, Burlingame, CA). Peroxidase-conjugated avidin-biotin complex was applied and developed with diaminobenzidine. The sections were then counterstained with hematoxylin, dehydrated, and mounted with permount. The same method was used for staining with MAb Ki-67 (DAKO-PC, Dako Corp.) except that the sections were not dried overnight and were sequentially fixed in acetone, 1:1 acetone chloroform, and 10% formalin before incubation with the primary antibody for 30 minutes. Appropriate positive controls were selected for each antibody. A colon cancer containing a known mutation in the p53 gene was used as a positive control for MAb 1801, and a lymph node with follicular hyperplasia was selected for MAb Ki-67 because it labels proliferating germinal center cells. Both of these also served as negative controls, as benign colonic epithelium and nonproliferating interfollicular cells in the lymph node are nonreactive with the respective antibodies.

**Image Analysis**

Quantitative investigation with a computerized image analyzer (CAS-200, Cell Analysis System, Elmhurst, IL) was used to evaluate the amount of nuclear reactivity for p53 protein in the tumor cells as well as the number of Ki-67-positive cells (proliferation index). Frozen tissue section slides were analyzed after immunostaining using the CAS Quantitative Proliferation Index (QPI) software program, which provides an accurate and objective method for the assessment of proliferating cell populations when using immunohistochemical staining techniques with antibodies that have been shown to react with proteins present only during proliferation such as Ki-67. It detects total nuclear area and nuclear reactivity with the antibody utilized, so p53 nuclear staining is adequately measured using this program. For each antibody, 10 fields representative of the tumor were analyzed, and approximately 100 cells were counted in each field. These values were then averaged.

**Statistical Analysis**

Correlation of p53 protein expression and proliferation was determined by linear regression analysis of the 34 cases using the values obtained by the CAS-200 image analysis system. The results were depicted by plotting the values in a two-dimensional graph, with proliferation indices on the x axis and p53 protein expression on the y axis.

**Results**

**Clinical, Morphological and Immunophenotypic Characterization**

We analyzed 34 non-HTLV-I associated post-thymic T-cell lymphomas. The patients consisted of 18 males and 16 females ranging in age from 24 to 89 years, with a median age of 65 years. The lymph nodes were the primary site of involvement in 27; six patients presented with primary cutaneous lesions with the disease limited to the skin, and one patient presented with an intraparotid lesion. Ten patients had advanced stage disease at the time of diagnosis, including involvement of spleen, liver, lung, and bone marrow.

All 34 NHLs were shown to be T cell in origin by their expression of T-cell associated antigens and/or clonal TCR-β gene rearrangement. Aberrant expression of T-cell-associated antigens, including loss of one or more pan-T cell antigens (CD2, CD3, CD5, CD7), T-cell subset restriction (CD4+CD8− or CD4−CD8+), and/or anomalous T-cell subset expression (CD4+CD8− or CD4−CD8+) was observed in 28 of the 34 cases. Thirteen cases were
analyzed genotypically, and all 13 exhibited clonal TCR-β gene rearrangements.

**p53 Mutations**

The SSCP assay was used to screen the samples for mutations within exons 5 to 9 of the p53 gene. These exons encode the evolutionarily conserved portions of the gene. The vast majority of somatic p53 mutations that have been identified in all varieties of human malignancies occur in these regions, including the three “hotspots” at codons 175, 248, and 273. As reported previously, the SSCP assay is a sensitive and highly specific technique capable of detecting single base pair mutations. Figure 1 illustrates the results of the SSCP assays for the two exons, 5 and 8, in which abnormally migrating bands were observed in four cases, suggesting the presence of structural mutations. Abnormally migrating bands suggesting mutations were not detected in exons 6, 7, or 9. To confirm and further characterize the type of mutations present in these four cases, the fragments were reamplified, cloned, and sequenced. The results are shown in Figure 2. Case 14 had an A to G transition in the third nucleotide of codon 292, which does not lead to an alteration in the encoded amino acid. Hence, this is considered a genetic polymorphism and not a mutation. Case 15 contained a T to C transition in codon 270, resulting in a phenylalanine to serine change. Case 19 contained a 13-bp deletion spanning a segment in codons 288 to 292, resulting in a frameshift and truncated protein product. Case 28 showed a deletion/substitution mutation involving codons 174 to 175, leading to a frameshift and truncated protein product.

**p53 Expression**

Immunohistochemical staining for the p53 protein product was performed using monoclonal antibody 1801, which recognizes an epitope near the amino terminus of both wild type and mutant forms of the p53 gene product. This antibody has pro-

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**Figure 1.** SSCP analysis of exons 5 and 8 of the p53 gene. The panels show the results of the SSCP assays for the exons indicated. PCR-amplified, radiolabeled fragments of the exon are subject to electrophoresis, under non-denaturing conditions, in 6% acrylamide gels containing 10% glycerol. Each lane is marked with its corresponding case number. Lanes labeled ND denote amplified DNA fragments not denatured before electrophoresis, and lanes labeled PC indicate positive controls. Several bands showing identical patterns of mobility are found in cases containing the wild-type sequence, whereas bands of differing mobility are seen in the positive control lanes and in cases containing mutations (indicated by arrows). Abnormally migrating bands, consistent with an altered primary sequence, were detected in four cases, one (case 28) in exon 5 and three (cases 14, 15, and 19) in exon 8.
duced the most consistently reliable and reproducible immunohistochemical results in our laboratory. In benign or reactive lymph nodes, positive staining with this antibody has been either absent or present only in rare, activated cells in the follicular and interfollicular zones. A similar finding has been reported by other authors, who found occasional positively staining cells with this antibody in five of 14 reactive tonsils and lymph nodes.\(^{31}\) The pattern of nuclear staining was quantitated using the CAS-200 computerized image analysis system. A wide range of reactivity was found, varying from less than 2% to as high as 48% of the malignant cells (Table 1). Using an arbitrary threshold of 10% positive staining, which has been used by other authors,\(^{50}\) 17 of the 34 cases (50%) had insignificant levels of p53 expression. Sixteen of the remaining 17 cases contained 10% to 40% immunoreactive cells of variable staining intensity, and one case contained 48% strongly immunoreactive cells. All three cases exhibiting p53 gene mutations displayed p53 protein overexpression: case 19 displayed weak reactivity with 14% malignant cells and cases 15 and 28 displayed strong reactivity with 28% and 48% of the malignant cells, respectively. However, no structural alterations were found in the remaining 14 cases, many of which contained a proportion of malignant cells expressing levels of p53 protein comparable to the mutated cases. The summarized results of p53 expression and p53 gene mutation are shown in Table 2, and immunoreactivity for p53 protein in both mutated and nonmutated cases is illustrated in Figure 3.

We had recently demonstrated a similar discordance between p53 gene mutation and p53 protein expression among CD30- (Ki-1) positive ALCLs.\(^{35}\) The mechanism for p53 overexpression in the nonmutated cases of ALCL was unknown, but a previous report\(^{51}\) demonstrating p53 protein in actively proliferating benign and malignant tissues suggested that the observed p53 overexpression in this group of tumors may be related to their rapid rate of proliferation. When we compared proliferation, as assessed by Ki-67 reactivity, with p53 protein expression in these cases, we found a close correlation (r = 0.92) between the proportions of p53-positive and Ki-67-positive cells. In view of these findings, we analyzed these 34 post-thymic T-cell lymphomas for Ki-67 expression. As with p53 protein expression, the range of reactivities for Ki-67 was broad, varying from 4% to 44%. To determine whether proliferation and p53 protein overexpres-

Figure 2. Sequence analysis of p53 mutations. A through D show sequences for cases 14, 15, 19, and 28, respectively, next to the corresponding wild type sequence. Point mutations were found in cases 14 and 15 (A and B) and are indicated by arrows. In case 14, the mutation did not result in a change in the amino acid. Cases 19 and 28 contained deletions of 13 bp and 1 bp (G and D). In case 19, a 2-bp repeat, which is underlined, was found closely flanking the deleted segment. As one of the repeats is included in the missing segment, the exact site of the deletion cannot be determined. In case D, a single nucleotide deletion of a guanine residue, which is underlined, occurred at an iterated position in codon 174. In addition, a substitution/point mutation, indicated by the arrow, was found in the adjacent codon 175.
Table 1. Summary of Structural Analysis of Exons 5 to 9 of the p53 Gene and Relation to Expression of p53 Protein and Ki-67 in 34 Post-Thymic T-Cell Lymphomas

<table>
<thead>
<tr>
<th>Case No.</th>
<th>p53 Exons 5 to 9</th>
<th>p53 Expression*</th>
<th>Ki-67 positivity</th>
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<tr>
<td>1</td>
<td>WT</td>
<td>6%</td>
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<td>2</td>
<td>WT</td>
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<td>28%</td>
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<tr>
<td>34</td>
<td>M-exon 8</td>
<td>14%</td>
<td>9%</td>
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* Immunoperoxidase staining quantitated as percent of nuclear area of malignant cells stained.

WT: wild type; M: mutation.

Table 2. p53 Expression and Gene Mutation in 34 Post-Thymic T Cell Lymphomas

<table>
<thead>
<tr>
<th>No. of cases</th>
<th>%Cells positive</th>
<th>Brightness</th>
<th>p53 Mutated</th>
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<td>1/34</td>
<td>&gt;40</td>
<td>+++</td>
<td>1/34</td>
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Discussion

Among our 34 post-thymic T cell lymphomas, we found three cases containing mutations in the p53 gene that altered the predicted amino acid sequence of the protein product. This relatively low frequency of mutation confirms the results previously reported by Gaidano et al. who examined 36 T-cell neoplasms and found only one mutation among the 12 peripheral T-cell lymphomas that were included in their group. In all three of our cases, the mutations occurred in the central conserved domain of the p53 gene, which encodes sequences necessary for DNA binding. One of the cases had a point mutation in codon 270 leading to a change in the encoded amino acid from phenylalanine to serine. Although an identical mutation has not been previously described, a mutation involving this codon has been reported as a somatic mutation in a child with acute lymphoblastic leukemia. The remaining two cases had deletion mutations. One had a point mutation in codon 175, a known hotspot in human malignancies, complicated by a single nucleotide deletion in the preceding codon, leading to a frameshift and premature stop codon. The other case contained a 13-bp deletion involving codons 288 to 292, also leading to a frameshift and truncated protein product.

The fact that two of three mutations involved deletions is unusual. Recent studies of p53 gene mutations in hepatocellular carcinoma and esophageal carcinoma have found a much higher proportion (up to 50%) of deletions than previously recognized; however, missense mutations are still the most common and account for the majority of mutations. In a comprehensive review of the frequency and distribution of p53 mutations in human tumors, insertions/deletions were found to account for 10% of mutations. Structural analysis of these mutations suggested that these types of deletions arise through endogenous mechanisms during DNA replication. Deletions of single nucleotides occurred more often for GC rather than AT, and, in the majority of cases occurred at iterated positions. In all the cases that involved deletions of two or more nucleotides, 2- to 8-bp direct repeats were found within or closely flanking the deleted segment. The deletions most often removed one of the repeats. Less frequently, the deleted segment removed both repeats, imperfectly removed only part of a repeat, or removed only the intervening sequence in between the repeats. Both of our cases with deletions share these structural details and are consistent with these observations from this study.

Immunohistochemical staining for p53 protein expression using MAb 1801 detected overexpression in 50% of the cases. No large group of post-thymic T-cell lymphomas has been previously investigated for p53 expression, but significant p53 overexpres-
 Immunoperoxidase staining with anti-p53 monoclonal antibody 1801. A shows staining for case 28, in which 48% of the cells showed nuclear immunoreactivity for the p53 protein. A mutation was found in exon 5 in this case. B demonstrates p53 protein overexpression for case 7, in which 28% of the cells showed positive nuclear staining, but no mutation was detected in exons 5 to 9.

Correlation of p53 protein expression with proliferation. p53 protein expression, which is quantitated as percent of nuclear area staining, is compared to cell proliferation, as assessed by Ki-67 expression. When these values are plotted, only a poor correlation (r = 0.34) is found.

p53 protein expression has been found in high-grade B-cell NHLs, as well as in ALCL. Our cases represented a group of T-cell NHLs of heterogeneous morphology and proliferative capacity, and the overexpression was not associated with any particular grade of lymphoma. Despite the large number of cases with p53 protein overexpression, only three mutations were found by structural analysis of exons 5 to 9 that encode the most conserved portions of the gene where the vast majority of mutations have been documented using SSCP analysis. This is a highly sensitive assay that is capable of detecting point mutations as well as larger deletions. All three cases with p53 mutations were found to have overexpression of the p53 protein with reactivities varying from 14% to 48% of the cells. However, many of the nonmutated cases demonstrated comparable levels and intensities of staining.

p53 protein expression has been studied in a wide variety of tumors. It has been assumed in these studies that detectable overexpression of the p53 gene product is the phenotypic manifestation of a mutation prolonging the half-life of the protein. Some studies have corroborated this. However, other authors have found disparities between the frequency of cases overexpressing p53 protein as detected by immunohistochemical methods and...
those actually containing gene mutations.\textsuperscript{11,35,56} Overexpression of wild type p53 has been observed in rapidly proliferating cells\textsuperscript{54} and in some tumorigenic cell lines.\textsuperscript{57,58} In addition, in a study of p53 protein expression in various benign and neoplastic soft tissue lesions, immunoreactivity was found in 48% of the benign/reactive cases.\textsuperscript{59} Furthermore, in our study of CD30- (Ki-1) positive ALCLs, 80% of the cases showed overexpression of p53 protein, but only 6% of the cases exhibited a p53 mutation. When proliferation, as assessed by staining for Ki-67, was quantitated in these tumors, excellent correlation ($r = 0.925$) was found between p53 expression and proliferation. Similarly, we analyzed this group of 34 post-thymic T-cell neoplasms for Ki-67 expression and compared the results to p53 protein expression. The correlation for these lymphomas ($r = 0.34$) was poor, however, and, consequently overexpression in these cases cannot be explained by proliferation.

The reasons for the discrepancy between overexpression and the observed frequency of mutations in these NHLs is unknown. Mutations that inactivate the gene have been described in nonconserved regions outside of those exons we screened. In a comprehensive study of mutations occurring in the p53 locus, an estimated 6% are found outside of exons 5 to 9.\textsuperscript{60} This includes other exons as well as sites in introns that can inhibit normal splicing. It is possible that we may not have detected such mutations. On the other hand, it should be mentioned that some forms of mutation, particularly deletions that cause frameshifts, are known to lead to undetectable protein product, and it has been observed that in such cases, immunohistochemical staining can also underestimate the true frequency of mutation.\textsuperscript{56}

Alternatively, p53 transcriptional transactivation can be inhibited or inactivated by cellular mechanisms other than mutation. The p53 protein is known to bind to viral proteins such as the E6 protein of oncogenic strains of HPV\textsuperscript{19,20} or cellular proteins such as p90, the product of the mdm2 oncogene,\textsuperscript{61} and be functionally inactivated. However, preliminary studies in our laboratory do not support a major role for either of these mechanisms in the pathogenesis of NHLs in which there was unexplained overexpression of p53 protein. In selecting cases for this study, we specifically eliminated those that were associated with HTLV-I. Epstein–Barr virus (EBV), which has also been implicated in lymphomagenesis, has been shown to bind to p53.\textsuperscript{62} We looked for evidence of EBV infection by PCR, but we did not detect EBV sequences in any of the cases that had demonstrable p53 protein overexpression. In our study of ALCLs,\textsuperscript{36} EBV was found in two cases, neither of which showed p53 protein overexpression. Of the recognized cellular proteins that are known to bind to p53, the mdm-2 oncogene product does not seem to be overexpressed in a significant number of malignant lymphomas. We have screened 73 NHLs, including 18 of the 34 T-cell lymphomas included in this study, for amplification of the mdm-2 oncogene using Southern blot analysis. We found only one of them, a B-cell follicular lymphoma, to show a 10- to 20-fold amplification.\textsuperscript{63} Finally, exclusion of wild type p53 protein from the nucleus into the cytoplasmic compartment has been documented in some inflammatory breast cancers.\textsuperscript{64,65} This seems to inhibit effectively its function as a nuclear phosphoprotein. It is not certain, however, how any of these alternative mechanisms of p53 protein inactivation may affect its immunophenotypic expression. Furthermore, until all of these possibilities are investigated, there is no evidence thus far to suggest that such mechanisms may be active in this group of malignant lymphoma.

Unlike some categories of B-cell neoplasia,\textsuperscript{27} such as follicular center cell lymphomas, Burkitt's lymphoma, and AIDS-related lymphomas, very little is known about the pathogenesis of post-thymic T-cell lymphomas with the exception of HTLV-I related adult T-cell leukemia/lymphoma. We investigated these tumors for evidence of p53 mutation and found that only a small proportion of them contain mutations. Sequence analysis of these cases showed two of them to have deletions with structural features that suggest that they arose from endogenous events rather than exogenous mutagens. Immunohistochemical staining of these cases showed overexpression of the p53 protein in 50% of the cases, in most of which a structural alteration could not be detected. Such congruity between p53 gene mutation and p53 protein overexpression has been increasingly recognized in malignant lymphomas\textsuperscript{35} and in solid tumors\textsuperscript{11,56} as more authors have examined tumors genotypically to assess frequency of mutation as well as to characterize the exact nature and location of the mutation. The data from our study of ALCLs\textsuperscript{36} suggest that p53 protein overexpression may be related to rapid cell proliferation in tumors that lack a demonstrable mutation. This mechanism has been suggested by other authors.\textsuperscript{56} However, proliferation does not seem to be the explanation for p53 protein overexpression in
the absence of p53 gene mutation in the case of non-HTLV-I-associated post-thymic T-cell lymphomas. Presumably, therefore, other mechanisms by which p53 protein can be functionally inactivated may also lead to its overexpression. However, studies in our laboratory do not support a major role for such alternative mechanisms. Lastly, some immunohistochemical studies have found that p53 protein overexpression is not exclusively confined to neoplastic lesions and may be found in reactive tissues. In this regard, p53 protein overexpression may not necessarily indicate a disruption of normal function and may merely represent an epiphenomenon. Nevertheless, overexpression of the p53 gene product is not a reliable predictor of the presence of mutations in exons 5 to 9 of the p53 gene in non-HTLV-I related post-thymic T-cell lymphomas.

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