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High Levels of p53 Protein Expression Do Not Correlate with p53 Gene Mutations in Anaplastic Large Cell Lymphoma

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Strong immunohistochemical reactivity for p53 tumor suppressor gene product has been reported in a variety of different human malignancies including CD30- (Ki-1) positive anaplastic large cell lymphoma (ALCL). Although high levels of p53 protein have been interpreted as abnormal, rapidly proliferating benign and neoplastic lymphoid cells may have increased p53 expression in the absence of structural alterations. On the other hand, mutations in the p53 gene can lead to a lack of p53 protein production. Structural alterations of the p53 gene have not been documented in cases of ALCL and the mechanism for an abnormal pattern of p53 expression in these lymphomas has not been elucidated. Therefore, to determine whether an altered pattern of p53 expression correlates with mutations in the p53 locus in ALCL, we analyzed the expression of p53 protein immunohistochemically, compared it with the proliferation index using monoclonal antibody Ki-67, and assessed the presence of mutations in exons 5 though 9 of the p53 gene using a single-strand conformation polymorphism assay in a panel of 17 ALCLs. Furthermore, we studied the presence of allelic deletions of chromosome 17p by restriction fragment length polymorphism analysis. We found significant levels of p53 protein expression in 12 of the 15 cases studied, but identified mutations in only one of 17 cases. An allelic deletion in chromosome 17p was identified only in the one case containing a mutated p53 gene. Whereas the case containing structural alterations in the p53 gene did have strong p53 immunoreactivity, 11 cases that lacked p53 mutations in the regions examined also had significant levels of p53. Thus, our studies indicate that strong immunohistochemical reactivity for p53 is not a reliable indicator of the presence of structural alterations of p53 gene exons 5 through 9 in ALCL. (Am J Pathol 1993, 143:845–856)

The systematic utilization of the Ki-1 monoclonal antibody has led to the relatively recent identification of a previously unrecognized entity now designated CD30- (Ki-1) positive anaplastic large cell lymphoma (ALCL).[1–3] These malignant lymphomas are characterized by large, bizarre, and pleomorphic tumor cells containing abundant cytoplasm, one or more nuclei, prominent nucleoli, sometimes resembling Reed–Sternberg cells, and a high mitotic rate that preferentially infiltrate lymph node sinuses and involve certain extranodal sites, i.e., the skin.[3] Many tumors previously diagnosed as true histiocytic lymphoma, malignant histiocytosis, undifferentiated carcinoma, malignant fibrous histiocytoma, malignant melanoma, and various sarcomas are now thought to represent examples of ALCL. The lymphoid origin of these neoplasms has been demonstrated by their expression of B and T cell lineage restricted antigens and/or the presence of clonal immunoglobulin (Ig) or T cell receptor gene rearrangements. However, these analyses have shown that ALCLs are heterogeneous, and although the majority are of T cell origin, some are B cell or null cell neoplasms. Because of the reactivity of the Reed–Sternberg cells of Hodgkin's disease with anti-CD30 antibodies, as well as certain overlapping clinical and morphological features between Hodgkin's disease and ALCL, a relationship between these two diseases has been suggested, but the precise nature of this relationship remains controver-

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sial.[4–6] Recently, a large percentage of cases of Hodgkin's disease and ALCLs have been shown to contain immunohistochemical reactivity with antibodies recognizing the p53 tumor suppressor gene protein product.[7]

Tumor suppressor genes, namely, p53 and retinoblastoma, have been shown to play an important role in the pathogenesis of human neoplasms. It has been shown that disruption or loss of these genes relieves the cell from certain negative regulatory signals.[8] The normal p53 gene acts as a tumor suppressor gene in the murine model,[9] but point mutations within the coding region can convert the p53 gene from a recessive to a dominant oncogene.[10] Several lines of evidence support the notion that the loss or alteration of the p53 gene may contribute to growth deregulation of neoplastic cells. Several human neoplasms display a monoallelic loss of variable portions of the short arm of chromosome 17 and the 17p13.1 region, where the p53 gene maps,[11,12] is consistently lost in colon carcinoma.[13] Consistent with Knudson's model of tumor suppressor genes,[14] it has been found that the 17p13.1 loss is consistently associated with mutations of the residual p53 allele, which are thought to inactivate the remaining p53 function.[13,15,16] Furthermore, the vast majority of p53 mutations occur in one of four hotspots in exons 5 through 8, corresponding to evolutionarily conserved domains,[17] and representative mutants from each of these four regions lose the ability to bind p53-binding sites and to activate transcriptionally the expression of adjacent reporter genes, which is thought to be an important feature of the normal p53 protein product.[18–21] p53 mutations and/or allelic losses have been found in several types of human solid tumors, including colon, breast, lung, brain, and soft tissue.[13,15,16] Furthermore, p53 mutations have been recently identified in several lymphoid tumors, specifically T cell acute lymphoblastic leukemia cell lines,[22] B-cell acute lymphoblastic leukemia, B cell chronic lymphocytic leukemia and Burkitt's lymphoma,[23] follicular and diffuse B-cell lymphoma,[24] and adult T-cell leukemia/lymphoma.[25,26]

The p53 gene encodes a cell cycle-dependent nuclear phosphoprotein. The wild type p53 protein has a half-life of approximately 5 to 20 minutes.[27] However, the mutant p53 protein has an extended half-life of 4 to 8 hours.[28] Therefore, many tumor cells harbor a concentration of p53 that can be easily detected by immunoprecipitation,[29] and high levels of p53 expression, seen in both hematopoietic and nonhematopoietic neoplasms, have been interpreted as abnormal.[7,30–32] However, p53 protein products can be detected in benign and neoplastic hematopoietic cells that are actively proliferating.[33] Specifically, p53 immunoreactivity has been identified in 11 of 14 CD30-positive ALCLs.[7] The increased p53 expression observed in some ALCLs, as well as in other high grade non-Hodgkin's lymphomas, may be due to mutations in the p53 locus. Alternatively, this may be due to the rapidly proliferating nature of these neoplasms. To distinguish between these two possibilities, we analyzed 17 cases of ALCL for mutations in exons 5 through 9 of the p53 gene using a single-strand conformation polymorphism (SSCP) assay and assessed the presence of allelic deletions of chromosome 17p by restriction fragment length polymorphism (RFLP) analysis. We also determined the levels of p53 expression in these cases and its relationship to cellular proliferation by immunostaining with monoclonal antibodies (MAbs) to p53 and to Ki67, a proliferation-associated nuclear antigen, and quantitated these data using the CAS-200 computerized image analysis system. We found that all 17 cases of ALCL are rapidly proliferating and 12 displayed significant immunohistochemical reactivity for p53 which was comparable to that of Ki-67. However, only one of these cases exhibited mutations in the p53 gene in the regions examined, as well as an allelic deletion of chromosome 17 p53 sequences. Thus, strong immunohistochemical reactivity for p53 does not imply the presence of structural alterations of p53 gene exons 5 through 9 in ALCL.

Materials and Methods

Pathological Samples

A panel of 17 well-characterized cases of ALCL were selected from among cases processed in the surgical pathology laboratories of the Columbia Presbyterian and New York University Medical Centers. Heparinized peripheral blood and bone marrow aspiration samples and lymph node biopsy specimens were collected during the course of standard diagnostic procedures under sterile conditions and promptly delivered to the laboratory. Mononuclear cells were separated from the peripheral blood and bone marrow aspiration samples by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation. Representative portions of each tissue specimen were routinely fixed in buffered formalin, B5, or Bouin's, embedded in paraffin, and hematoxylin and eosin-stained sections were prepared. The remaining portions were embedded in a cryopreservative solution (optimum cutting temperature compound, Miles,

Elkhart, ID) and stored at −70 C. The diagnosis of ALCL was based upon correlative analysis of the clinical, histopathological, and immunophenotypic characteristics. The morphological features included infiltration by large pleomorphic cells with abundant cytoplasm and irregularly shaped nuclei containing one or more prominent nucleoli, a high mitotic rate, sinusoidal spread, the presence of multinucleated cells often resembling Reed–Sternberg cells, fibrosis, and numerous histiocytes intermingled with the tumor cells. Positive staining with monoclonal antibody Ber-H2[34] was required for the diagnosis of ALCL. Before DNA extraction, a Giemsa-stained frozen tissue section, or alternatively, a cytospin smear of suspended cells, was evaluated to determine the presence and proportion of neoplastic cells in each case. At least 20% of the cells in each of the cases were large and morphologically neoplastic.

DNA Extraction

Genomic DNA was extracted from cryopreserved mononuclear cell suspensions and tissue blocks using a salting-out procedure.[35] Briefly, the cells or frozen tissue sections were resuspended in 3 ml of nuclei lysis buffer containing 10 mmol/L TrisHCL, 400 mmol/L NaCl, and 2 mmol/L ethylene diaminetetraacetic acid (EDTA); 200 µl of 10% sodium dodecyl sulfate (SDS) ($NaDodSO_4$) and 500 µl of proteinase K solution (1 mg proteinase K in 1% SDS and 2 mmol/L EDTA) were subsequently added. After an 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. An alternative procedure was used when a very limited amount of tissue was available, which yielded sufficient DNA for polymerase chain reaction (PCR) analysis only.[36] Essentially, approximately three frozen sections were resuspended in 250 ul of a buffer containing 50 mmol/L KCl, 15 mmol/L Tris-Cl, 2.5 mmol/L $MgCl_2$, 0.5% Tween 20, and 100 µg/ml of fresh proteinase K. This mixture was incubated for 45 minutes at 56 C to digest the cells and then for 10 minutes at 95 C to inactivate the protease. The tubes were spun in a microcentrifuge for 30 minutes, the supernatant transferred to a new tube, and the DNA quantitated spectrophotometrically.

RFLP Analysis

Five-µgram aliquots of genomic DNA were digested with HinfI restriction endonucleases according to the manufacturer's instructions (Boehringer-Manheim, Indianapolis, IN), electrophoresed in 1% agarose gels, denatured with alkali, neutralized, and transferred to nitrocellulose filters according to Southern.[37] The filters were hybridized sequentially to two highly polymorphic markers for loci on chromosome 17, pYNZ22.1[38] and p144D6,[39] in 50% formamide/3× standard sodium citrate (SSC) 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.[40]

Oligonucleotide Primers

All of the oligonucleotides used for PCR amplification in this study were synthesized by the solid-phase triester method. Sequences of p53 primers derived from published sequences[23,41] are as follows: P5–5, 5′-TTCCTCTTCCTGCAGTACTC-3′; P5–3, 5′-ACCCTGGGCAACCAGCCCTGT-3′; P6–5, 5′-ACAGGGCTGGTTGCCCAGGGT-3′; P6–3, 5′-AGTTGCAAACCAGACCTCAG-3′; P7–5, 5′-GTGTTGTCTCCTAGGTTGGC-3′; P7–3, 5′-GTCAGAGGCAAGCAGAGGCT-3′; P8–5, 5′-TATCCTGAGTAGTGGTAATC-3′; P8–3, 5′-AAGTGAATCTGAGGC-ATAAC-3′; P9–5, 5′-GCAGTTATGCCTCAGATTCAC-3′; P9–3, 5′-AAGACTTAGTACCTGAAGGGT-3′. The exact location of these primers has been previously published.[23] Essentially, primers P5–5, P6–3, P7–5, P8–5, and P9–3 are derived from the corresponding intron/exon junction,[41] and primers P5–3, P6–5, P7–3, P8–3, and P9–5 are derived from intron sequences flanking the corresponding exon.[23]

SSCP Analysis

SSCP analysis was accomplished according to an adapted version of a previously reported method.[23,42] Briefly, PCRs were performed with 100 ng of genomic DNA, 10 pmoles of each primer, 2.5 umol/L deoxynucleoside triphosphates (dNTPs), 1 uCi of [α-32P] deoxycytidine triphosphate (dCTP) (NEN; specific activity, 3,000 Ci/mmol), 10 mmol/L Tris (pH 8.8), 50 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.01% gelatin, 0.5 U Taq polymerase, in a final volume of 10 ul. Thirty cycles of denaturation (94 C), annealing (63 C for p53 exons 5, 6, and 9, 62 C for

p53 exon 7, 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 ul) 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-Tris-borate-EDTA (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 1000 vector using the TA cloning system (Invitrogen Corporation, San Diego, CA), following the manufacturer's instructions. DNA sequencing was performed directly from a small-scale plasmid preparation[43] 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. Essentially, approximately 2 ug of plasmid DNA in a volume of 20 ul was alkali-denatured by adding 2 ul of 2 N NaOH, 2 mmol/L EDTA and incubating for 5 minutes at room temperature. The mixture was then neutralized with 8 ul 1 mol/L Tris-HCl (pH < 5) and subsequently ethanol precipitated. The DNA was resuspended in 7 ul of H_2O, adding 1 ul of primer and 2 ul of 5× Sequenase sequencing buffer. After incubation at 37 C for 30 minutes, the following reagents were added: 2 ul of Labeling Mix dilution (1:5), 1 ul 0.1 mol/L dithiothreitol, 0.5 ul (4 uCi) [^{35}S]-deoxyadenosine triphosphate (dATP), 2 ul Sequenase enzyme dilution (1:8). These labeling reactions were incubated at room temperature for 5 minutes and subsequently 3.5 ul were aliquoted into a tube containing each of the four Termination Mixtures (ddGTP, ddATP, ddTTP, ddCTP). After a 5-minute incubation at 42 to 45 C, 4 ul 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 immunophenotype of the ALCLs had been determined at the time of diagnosis by immunohistochemical staining of frozen 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).[44] MAbs used to immunophenotype the cases of ALCL included 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), Leu 22 (CD43), LeuM1 (CD15), LeuM5 (CD11c; Becton-Dickinson), LCA (CD45), L-26 (CD20), κ, λ, epithelial membrane antigen (DAKO-PC, Dako Corporation, Santa Barbara, CA), Ber-H2[34] (CD30; courtesy, Dr. Harald Stein, Berlin, Germany), and B1 (CD20; Coulter Immunology, Hialeah, FL).

To determine binding to the p53 protein, sections were incubated overnight with the primary anti-p53 MAb PAb1801[45] (p53 Ab-2, Oncogene Science, Inc., Uniondale, NY), washed three times, and incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA). Peroxidase-conjugated avidin-biotin complex was applied and developed with diaminobenzidine. 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 Corporation).

Image Analysis

Quantitative investigation with a computerized image analyzer (CAS-200, Cell Analysis System, Elmhurst, IL) was used to evaluate the number of p53-positive cells as well as the proliferation index (Ki-67-positive cells). Frozen tissue section slides were analyzed after immunostaining using the CAS Quantitative Proliferation Index software program, which provides an accurate and objective method for 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.[46,47] It detects total nuclear area and

nuclear reactivity with the antibody utilized, so p53 nuclear staining is adequately measured using this program.

Results

Clinical and Immunophenotypic Characterization

We investigated 17 cases of ALCL. The median age of the patients was 56 years with a range of 18 to 79 years. The male to female ratio was 14 to 3. Eleven patients had lymphadenopathy, one presented with a mediastinal mass, one with a paravertebral mass, one with a thigh mass, three with skin involvement, and one with ascites. At least seven patients had liver and/or spleen involvement, and two had lung nodules. Three of the patients (cases 15, 16, and 17) were HIV-positive.

The results of immunophenotypic analysis are shown in Table 1. Briefly, all the cases were CD30-positive, 70% were CD45-positive, 80% were epithelial membrane antigen-positive, and all the cases except one were CD15- (LeuM1) negative. A T cell origin was likely in nine cases, a B cell origin in five, and three cases had a null cell phenotype. In one of these three cases (case 16), the Ig heavy chain gene was discovered to be rearranged, and in another case (case 15), gene rearrangement studies showed a germline configuration for both the T cell receptor β chain and Ig heavy chain genes (data not shown).

p53 Mutations

The ALCL samples were analyzed for mutations within exons 5 through 9 of the p53 gene, which are the regions affected by mutations in the vast majority of other types of human tumors.[13,15–17] This was done by SSCP analysis, which allows the detection of single base pair mutations because the wild type and mutant radiolabeled PCR products show different migration patterns due to the formation of alternative secondary structures after denaturation and subsequent electrophoresis in a polyacrylamide gel under nondenaturing conditions. This method has been previously shown to be sensitive to the level of at least 1% and highly specific.[23] An abnormal migration pattern suggesting the presence of mutations was identified in three of the 17 cases of ALCL. Figure 1 shows the result of the SSCP analysis performed for p53 exons 5 and 6, where case 2 shows the appearance of abnormal bands in exon 5, and cases 4 and 12 had an abnormal migration pattern in exon 6, indicating the probable presence of mutations in these exons. No mutations were identified in exons 7, 8, or 9 (Table 2).

To confirm the results obtained by SSCP analysis and to determine the nature of these mutations, the fragments displaying an altered electrophoretic mobility were reamplified in a separate reaction, cloned, and sequenced (Figure 2). Cases 4 and 12 showed an A to G transition in codon 213, which does not lead to any amino acid substitution and has been previously reported as a polymorphism in

Table 1. *Immunophenotypic Profiles of 17 ALCLs*

		Miscellaneous antigens				T cell-associated antigens							B cell-associated antigens				
Case#	Lineage	CD30 BerH2	CD45 LCA	CD15 LeuM1	EMA	CD2 T11	CD3 T3	CD4 T4	CD5 Leu1	CD7 Leu9	CD8 T8	CD43 Leu22	CD19 B4 Leu12	CD20 L26 B1	CD22 BL9 Leu14	κ	λ
1	T	+	−	−	+	+	−	+	−		−	+			−	−	−
2	B	+			−		−	−	−		−	−		+	+		
3	?T	+	−	−	+							+		−			
4	T	+	−	−	+		−		+			−		−	−	−	−
5	B	+	+	−			−		−					+	+	−	−
6	T	+	+	−	+	−	−	+	−	−	−			−	−	−	−
7	T	+	+	−	+		+	−	−		+				−	−	−
8	T	+	+	−	+	−	−	+	−	−	−		−		−	−	−
9	B	+	+	−	−	−	−		−			−	+	+	+	−	−
10	T	+	+	−	+	+	+	−	−	+	−				−	−	−
11	B	+	+	−	+		−		−			−		+	−	−	−
12	B	+	+	−	−							−		−			
13	T	+	−	−	−							+		−			
14	N	+	−	−	+							−		−			
15	N	+	+	−	+							−		−			
16	N	+	+	−	+	−	−	−	−	−	−		−	−	−	−	−
17	T	+	−	+	+		+					+		−			

+: positive immunoreactivity in the tumor cells; −: negative immunoreactivity in the tumor cells.

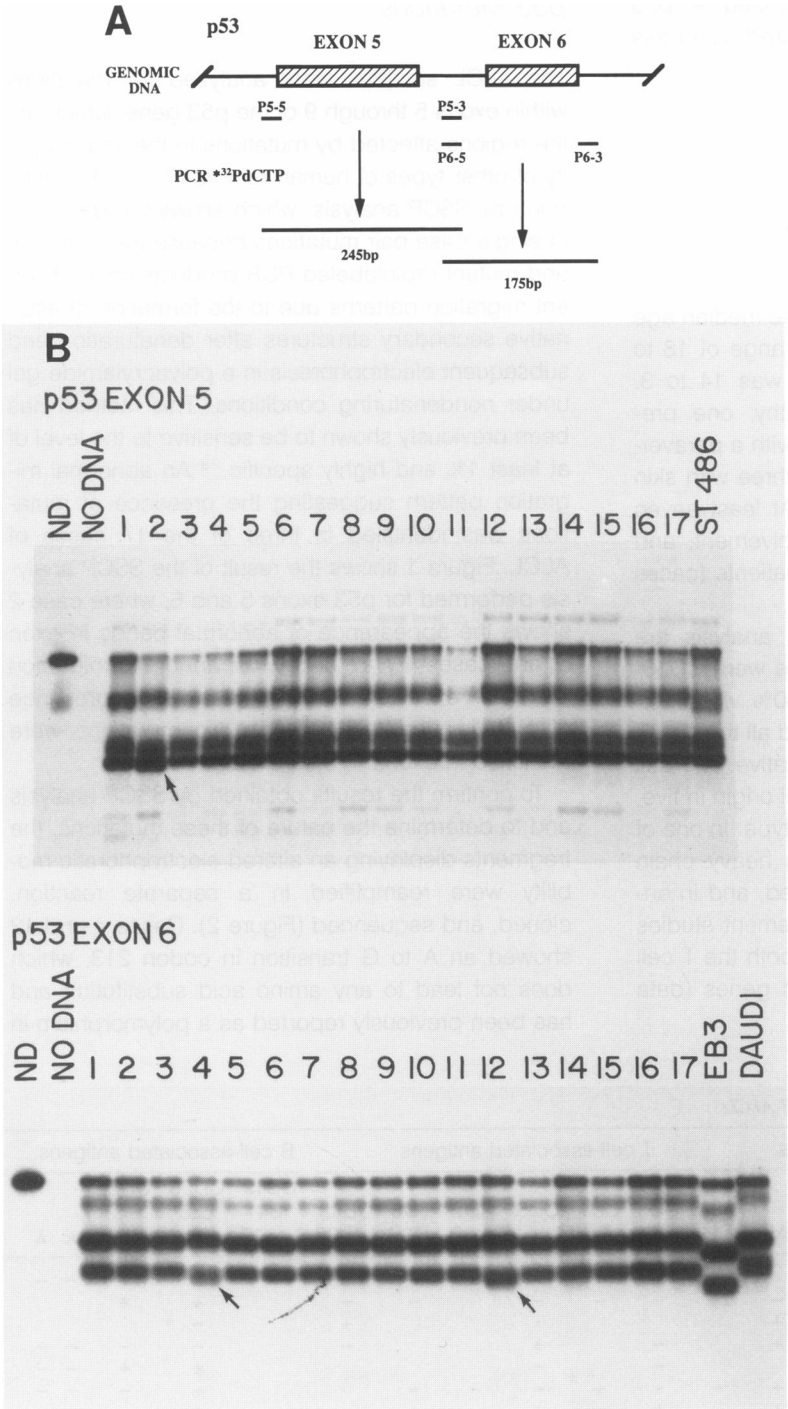

Figure 1. *SSCP analysis of exons 5 and 6 of the p53 gene.* **A** *shows a schematic diagram of p53 exons 5 and 6 with the location of the primers used and the resulting radiolabeled PCR products corresponding to these exons.* **B** *shows the actual results of this SSCP analysis. Radiolabeled, PCR-amplified fragments corresponding to exons 5 and 6 are shown in the upper and lower panels respectively, after denaturation and electrophoresis on a 6% acrylamide gel containing 10% glycerol.* **Lanes 1** *through* **17** *correspond to the case numbers, and the lane labeled ND is amplified DNA that was not denatured before electrophoresis. ST486, EB3, and DAUDI cell lines were used as positive controls for exons 5 and 6 as indicated, because they are known to contain mutations in these exons.[23] Several main bands were seen in most of the samples, which were identical for each exon in the cases containing the wild type sequence and different for the positive controls. Case 2 showed an abnormal migration for exon 5, whereas cases 4 and 12 showed a different pattern of bands for exon 6, and thus, these cases were interpreted as positive for mutations.*

this codon.[23] Case 2 had an A to G transition leading to a missense mutation in codon 132, with a resulting lysine to glutamic acid change.

RFLP Analysis

To assess the loss of heterozygocity in case 2, as well as to evaluate the possibility of p53 deletion in the nonmutated cases, we performed RFLP analysis for loss of 17p alleles. We used Southern blot analysis to assess the number of 17p loci, using two highly informative polymorphic probes that map near the p53 locus.[38,39] Using this method, a constitutive loss can be assessed within 95% confidence limits when both of the informative DNA probes show a loss of one 17p allele.[15] Figure 3

Table 2. *Summary of Structural Analysis of p53 and Expression of p53 and Ki-67 in ALCL*

Case #	p53 structure	p53 expression*	Ki-67 positivity*
1	WT	55%	56%
2	M-Ex 5	58%	59%
3	WT	15%	40%
4	Polymorphic	17%	20%
5	WT	47%	48%
6	WT	ND	ND
7	WT	40%	53%
8	WT	32%	42%
9	WT	12%	16%
10	WT	5%	20%
11	WT	36%	45%
12	Polymorphic	22%	36%
13	WT	60%	56%
14	WT	38%	45%
15	WT	5%	25%
16	WT	ND	ND
17	WT	5%	13%

* Immunoperoxidase staining as percentage of positive large neoplastic cells.
WT: wild type; M: mutated; ND: not done.

shows the pattern of 17p alleles from a control lymphoblastoid cell line and the 14 ALCL cases for which there was sufficient patient material to perform this analysis, using the two DNA probes pYNZ22 and p144D6. Probe p144D6 detects two different allelic systems, designated A and B in Figure 3, both being informative even though one of the frequently seen alleles in the system designated B comigrates with a constant band seen in all the cases. Whereas the lymphoblastoid cell line and all the nonmutated cases are heterozygous, containing two alleles with at least one probe, case 2 contains only one allele with both probes. These data confirm the loss of heterozygocity for 17p loci, and thus probably the p53 gene, in case 2.

p53 Expression

We performed immunohistochemical staining using anti-p53 MAbs to confirm the previously reported strong reactivity in ALCL and to correlate p53 expression with the presence or absence of p53 mutations. We used MAb PAb1801 anti-human p53 because we found this MAb to display stronger and more reproducible immunoreactivity than other commercially available anti-p53 antibodies (PAb240 and PAb421). The slides were analyzed for nuclear staining using the CAS-200 image analysis system, which allowed quantitation of positivity in the large neoplastic cells. The pattern of p53 staining in the ALCLs varied from 5% to 60% positivity among the neoplastic cells (Table 2). Three cases stood out in having only 5% of the neoplastic cells weakly positive for p53 immunoreactivity, so these cases were arbitrarily designated negative. Staining in more than 10% of the tumor cells has been considered the arbitrary threshold for positivity by other authors.[48] Staining was repeated in all these cases, and the same result was obtained despite appropriate controls and positive staining of the same tissue with other antibodies, such as Ki-67. As seen in Table 2, 12 of the 15 cases contained 12% to 60% p53-immunoreactive neoplastic cells and therefore were considered positive. In six of these cases, the immunoreactivity was strong and in the remaining cases it was moderate. However, only one of the p53-positive cases had mutations, and whereas this case had a large number of strongly positive cells, other nonmutated cases had comparable patterns of staining. Figure 4 shows p53 immunostaining in the mutated case (case 2, A) and in one of the nonmutated cases (case 13, C). Cellular detail can not be fully appreciated in frozen tissue sections used for immunohistochemical staining, and the neoplastic cells in case 13 are larger than those in case 2. However, the proportion of positive cells and the intensity of staining are comparable. Because the mechanism for p53 overexpression remains unexplained, we performed immunostaining with MAb Ki-67, which detects a proliferation-associated nuclear antigen, to determine if the p53-positive cases correspond to the more rapidly proliferating tumors (Table 2). The results of this staining for cases 2 and 13 are shown in Figure 4, B and D respectively. The results show a strong correlation between the percentage of p53- and Ki-67-positive cells. Statistical analysis confirmed this correlation, with a correlation coefficient of $r = 0.925$. These data suggest that p53 positivity may be related to the highly proliferative nature of these tumors.

Discussion

We found that one of 17 cases of ALCL contained mutations in the p53 gene in the regions studied, specifically a missense mutation in codon 132. Two structural features support the pathogenetic role of this mutation. The first one is the loss of the residual p53 allele in this case, as seen by RFLP analysis for chromosome 17p sequences, whereas the other 13 nonmutated cases examined remained heterozygous. This loss of heterozygocity supports the loss of function of both normal alleles as a model of activation,[14] as has been found in other types of tumors where the 17p13.1 loss is consistently associated with mutations of the residual p53 allele.[13,15,16] Second, this mutation results in an amino acid sub-

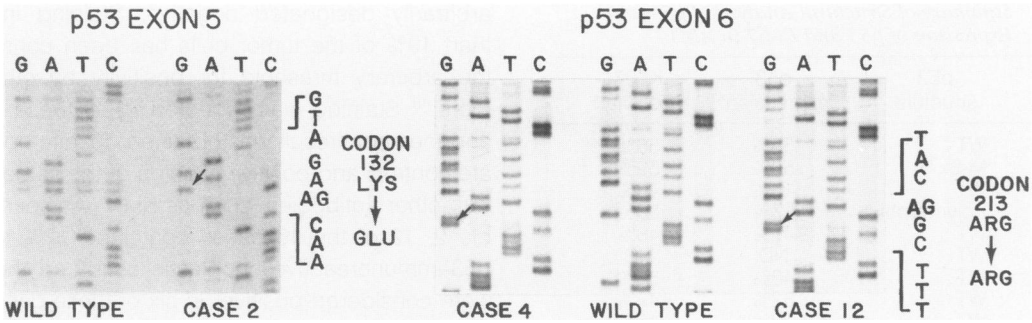

Figure 2. *Sequence analysis of p53 mutations. Sequences from exon 6 are shown for case 2, whereas the sequence for exon 6 is shown for cases 4 and 12. Each mutation shown is matched to a control wild type DNA. Arrows point to the mutated base pairs. The codon at which the mutations occur and the resulting change in the protein in case 2 are indicated. The nucleotide change in cases 4 and 12 is a silent mutation that does not result in any amino acid substitution.*

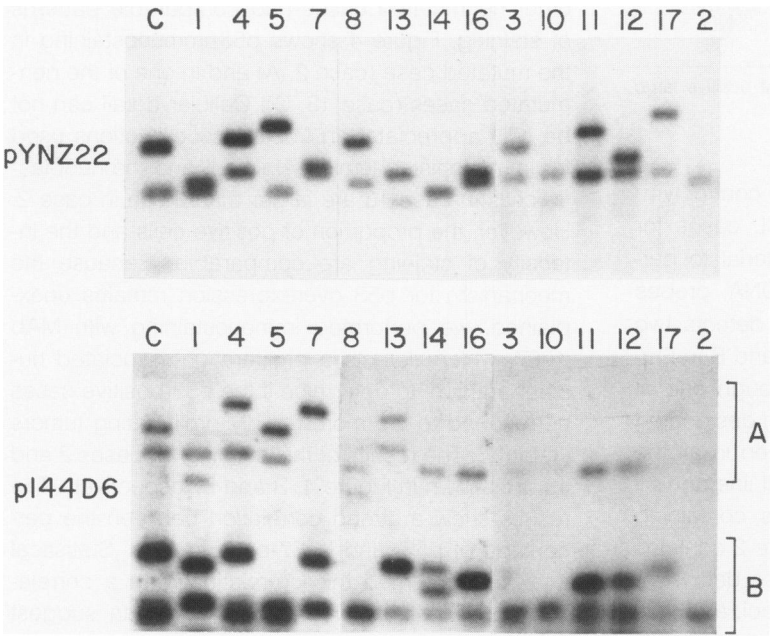

Figure 3. *RFLP analysis of p53 alleles. DNAs were digested with HinfI, separated on 1% agarose gels, and hybridized to probes pYNZ22 and p144D6 from chromosome p17. The numbers above the lanes represent the respective case number.* C *is a lymphoblastoid cell line used as a control.* A *and* B *denote two different allelic systems identified with probe p144D6. All the cases except 2 and 14 contain two alleles with probe pYNZ22, and all except 2, 11, 12, 16, and 17 contain two alleles with probe p144D6 in either system A or system B. This indicates that the only case showing only one allele with both probes is case 2.*

stitution in a region of the protein that is highly conserved throughout evolution.[17,49] Thus, structural alterations of the p53 gene may play a pathogenetic role in a minority of ALCLs.

Immunohistochemical staining revealed moderate to high levels of p53 protein expression in 12 of the 15 (80%) ALCLs studied here. This result is consistent with a previous report where high levels of p53 expression were detected in 78% of ALCLs.[7] In these studies, we used the PAb1801 anti-p53 monoclonal antibody,[45] which has been well characterized and we found provides specific and more sensitive staining than the other commercially available antibodies (PAb240 and PAb421).[50,51] Furthermore, even though PAb1801 detects both wild type and mutant p53 products, so do the other antibodies. MAb PAb421 can distinguish between wild type and some mutant forms of p53 in the nondenatured state, but it is not able to do so when immunohistochemical methods are used, as denaturation occurs with fixation.[51] We quantitated the staining using the CAS-200 image analysis system and found that the level of p53 immunoreactivity did not correlate with the presence of mutations, as several nonmutated cases contained levels of p53 comparable to that of the mutated case.

In the case containing p53 mutations, the high level of p53 expression can be explained as an increase in the half-life of the mutated protein.[28] However, the mechanism(s) responsible for the high level of expression in other cases of ALCL remained to be determined. Because it has been reported that high levels of p53 expression can be detected in benign and neoplastic hematopoietic cells that

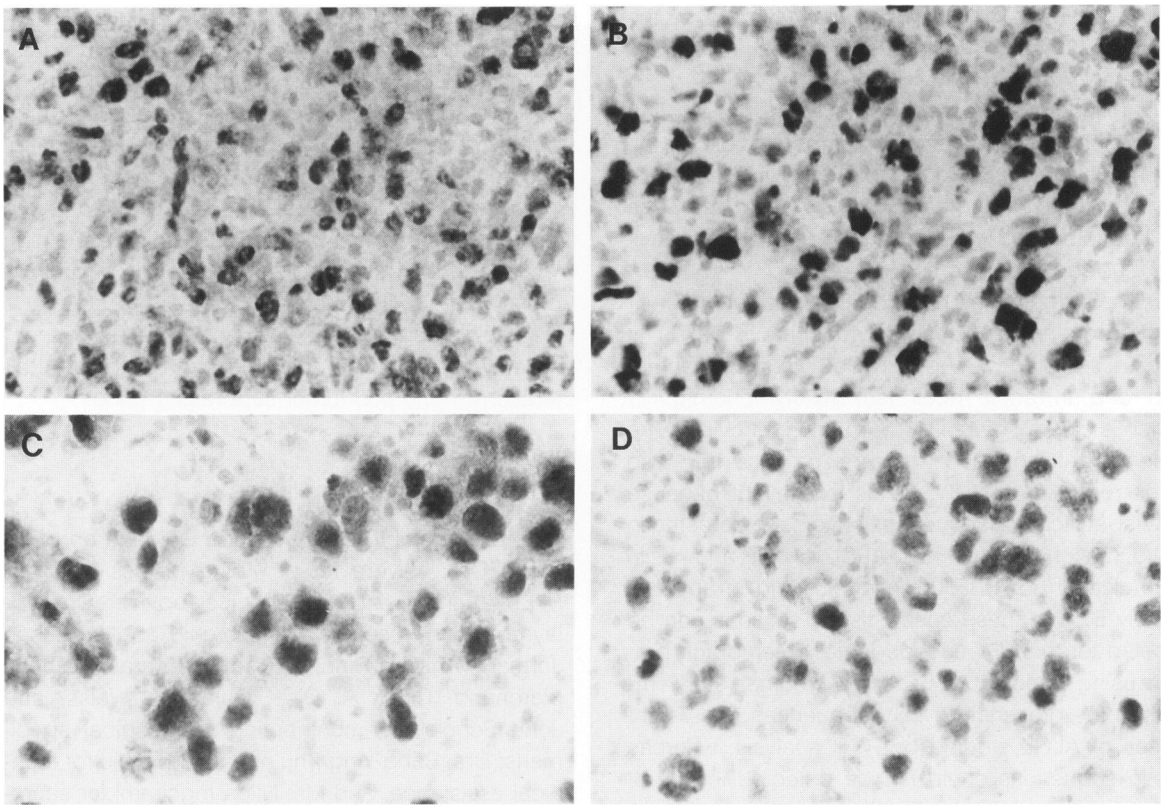

Figure 4. *Immunoperoxidase staining with anti-p53 and anti-Ki-67 monoclonal antibodies (400×).* **A** *and* **B** *show frozen tissue sections of case 2, containing p53 mutations, stained with the anti-p53 antibody PAb1801 and with anti-Ki-67, respectively.* **C** *and* **D** *show frozen tissue sections of a nonmutated case (case 13) stained similarly with PAb1801 and anti-Ki-67, respectively. Both cases show strong nuclear positivity with both antibodies in a large and comparable percentage of cells.*

are actively proliferating,[33] we investigated the possibility that these high levels are related to cell division. We determined the proliferation index by immunohistochemical staining with MAb Ki-67 and compared the percentage of p53- and Ki-67-positive cells. Double staining would probably have been a better method to determine whether the p53-positive cells correspond to those positive for Ki-67, but this type of study was not possible because both antigens are intranuclear and both antibodies are of the same isotype (IgGI). However, to have an objective quantitation and comparison of the percentage of positive cells with each antibody, this percentage was determined with the CAS-200 image analysis system. As seen in Table 2, the number of p53- and Ki-67-positive cells is very similar in most cases, with a statistically significant correlation in the expression of both antigens. Thus, we can conclude that in ALCLs expressing high levels of p53 protein product, the percentage of positive cells corresponds closely to the number of proliferating cells, and p53 expression in these tumors may be associated with cellular proliferation.

Whereas the expression of Ki-67 is known to be related to cell division, the mechanism that leads to p53 expression is less clear, and the relationship between these two events is not yet understood. Recent studies have shown that the wild type p53 plays a growth-controlling role, mediating the G1 arrest of the cell cycle, in response to X-ray or drug-induced damage.[52,53] The mechanism by which p53 is thought to carry out its function is binding of a p53 tetramer to a p53-binding site, leading to transcriptional activation of downstream genes which in turn negatively control cell growth.[18] In support of this model is the finding that p53 mutations in the regions most frequently found in human tumors lose the ability to bind p53-binding sites, and thus cannot activate the expression of adjacent reporter genes.[19–21] It is possible that in the p53-positive ALCL cells the levels are high due to stress, in a manner analogous to that of irradiated or drug-treated cells, as has been previously suggested.[18] Whether these high levels of wild type p53 actually have any effect on the G1 arrest and progression to the S phase, or whether the ALCL tu-

mor cells have abnormalities downstream in the cascade of events that lead to G1 arrest, remains to be determined.

Our findings do not exclude the possibility that other molecular mechanisms are responsible for the high p53 levels in ALCL. Of particular interest is the fact that p53 binds viral proteins, which has been well documented for SV40 large T antigen, adenovirus E1A and E1B, and HPV E6 and E7.[54–57] Furthermore, p53 mutations have been found with significant frequency in Burkitt's lymphoma,[23] which is often associated with Epstein–Barr virus (EBV) infection and in adult T-cell leukemia/lymphoma,[25,26] which is caused by the HTLV-I human retrovirus. The binding between p53 and viral proteins has also been reported for EBV.[58] This is relevant because EBV-specific DNA sequences have been detected in up to 32% of cases of nodal ALCL by PCR and *in situ* hybridization for EBER transcripts, and in approximately 50% of these cases, tissue sections can be labeled for the EBV-encoded latent membrane protein using specific MAbs.[3,59–60] Another study reported the presence of EBV sequences in all eight cases of ALCL studied by PCR analysis, whereas these sequences could not be detected in any of four of these cases by Southern blotting or *in situ* hybridization techniques.[61] Preliminary data from our laboratory suggest that two of our cases (cases 15 and 16) are positive for EBV sequences by PCR and Southern blotting, but both are from HIV-positive patients and do not correspond to the ones expressing high levels of p53 protein products.

Because p53 is considered to be a tumor suppressor gene, its structure and/or function may be abnormal in those few cases with little p53 immunoreactivity. Even though we explored the regions where p53 mutations are most frequently found, the possibility remains that certain types of mutations, such as those in the donor or acceptor splice sites, in the first four or last two exons, or in promoter or enhancer regions, could lead to low levels of p53 expression.[48] Thus, further characterization of the p53 gene structure, as well as the integrity and production of p53 messenger RNA, is necessary to determine whether the lack of p53 expression is relevant to the pathogenesis of ALCL.

In conclusion, we found significant levels of p53 expression in a large proportion (80%) of ALCLs. In these cases the percentage of tumor cells expressing p53 gene product correlates with the number of cells that are actively proliferating as determined by immunohistochemical staining with MAb Ki-67. Even though the pathological significance of this finding remains unclear, it suggests that p53 expression in ALCL cells is related to cellular proliferation. Furthermore, we have documented the presence of structural alterations of the p53 gene in one case of ALCL. Even though this case showed high levels of p53 protein detectable by immunostaining, it did not show a different staining pattern when compared with other ALCLs which are not mutated in the regions examined and also expressed high levels of p53. Thus, whereas high levels of p53 expression are found in a large number of ALCLs and may be pathogenetically relevant, these do not necessarily correlate with the presence of structural alterations in the p53 tumor suppressor gene.

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