The human T-cell lymphotropic virus type I (HTLV-I) is capable of inducing adult T-cell leukemia/lymphoma (ATLL). However, the long latency period between infection and development of ATLL, as well as the small fraction of the infected population that actually develops this disease, suggest that additional factors are involved in its pathogenesis. Therefore, we performed a molecular analysis of ATLL presenting in a nonendemic area that were shown to have HTLV-I sequences by polymerase chain reaction as well as clonal T-cell receptor β gene rearrangements. We analyzed these cases for alterations in some of the oncogenes and tumor suppressor genes frequently involved in hematopoietic neoplasia. Specifically, we used a single-strand conformation polymorphism assay to determine the presence of mutations in the p53 tumor suppressor gene, as well as the K-RAS, N-RAS, H-RAS, and c-myc oncogenes. In addition, we studied the c-myc gene for rearrangements by Southern blotting and assessed expression of the retinoblastoma (Rb) and p53 genes by immunostaining. Analysis of the c-myc gene and the RAS family of oncogenes did not show any alterations. Also, the Rb gene was expressed in all cases analyzed. However, we found mutations of the p53 gene in 3 of the 10 cases and these results were confirmed by sequence analysis. In two of these cases, we showed by restriction fragment length polymorphism analysis of chromosome 17p sequences that the p53 mutations were accompanied by a loss of heterozygocity. Also, these mutations correlated with an altered pattern of p53 expression. Thus, mutations in the p53 locus may be a cofactor for the development of ATLL in some cases, whereas the c-myc, Rb, and RAS genes do not appear to be involved in these neoplasms.

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Submitted May 5, 1992; accepted August 20, 1992.

G.G. is supported by a fellowship for AIDS research from ISS, Rome, Italy. These studies were partially supported by National Institutes of Health Grants No. CA48236 and EY06337 to D.M.K.

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0006-4971/92/8012-0020$3.00/0

found in several types of human solid tumors, including colon, breast, lung, brain, and soft tissue. Furthermore, p53 mutations have been recently identified in lymphoid tumors, specifically T-cell acute lymphoblastic leukemia (ALL) cell lines, B-ALL, B-chronic lymphocytic leukemia, and Burkitt lymphoma, as well as some B-cell lymphomas in advanced clinical stage.

Recently, missense mutations in the p53 tumor suppressor gene were detected in 4 of 10 cases of ATLL occurring in Japan, suggesting a role for p53 in the pathogenesis of HTLV-I-associated neoplasms. We previously reported the histologic appearance and an immunophenotypic characterization of six cases of ATLL occurring in a nonendemic region. Subsequently, we characterized an additional four cases, all proven to contain HTLV-I sequences and to exhibit the histologic appearance and an immunophenotypic consistency with ATLL. However, even though these 10 patients were seen in a nonendemic region (New York City), they all came from endemic regions, predominantly the Caribbean. Because mutations in the p53 gene in ATLL cases originating outside of Japan and alterations in oncogenes or other tumor suppressor genes in Japanese and non-Japanese ATLL have not been documented, we analyzed these cases for structural alterations and/or expression of some of the oncogenes and tumor suppressor genes that are sometimes involved in hematopoietic neoplasia, including p53, Rb, K-RAS, N-RAS, H-RAS, and c-myc. We found p53 gene mutations in 3 of the 10 cases of ATLL, confirming that they may be relevant for the development of ATLL in a subset of patients. We also found that the c-myc, RAS, and Rb genes do not appear to be involved in the pathogenesis of ATLL.

**MATERIALS AND METHODS**

**Pathologic samples.** A panel of 10 well-characterized cases of ATLL 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 (H&E) stained sections were prepared. The remaining portions were embedded in a cryopreservative solution (OCT compound; Miles, Elkhart, IN) and stored at −70°C. The diagnosis of ATLL was based upon correlative analysis of clinical characteristics, histopathology, cell surface markers, and Ig and TCR gene rearrangements, as well as the detection of HTLV-I sequences by polymerase chain reaction (PCR) and/or by Southern blot hybridization analysis.

**DNA extraction.** Genomic DNA was extracted from cryopreserved mononuclear cell suspensions and tissue blocks by digestion with proteinase K, extraction with phenol/chloroform, and precipitation with ethanol. An alternative salting-out procedure, not requiring organic extraction, was used in cases in which a limited amount of tissue was available. Briefly, the cells or frozen tissue sections were resuspended in 3 mL of nucleic lysis buffer containing 10 mmol/L Tris HCl, 400 mmol/L NaCl, and 2 mmol/L EDTA, 200 μL of 10% SDS (sodium dodecylsulfate, NaDodeSO4) 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 vol of ethanol was added to the supernatant to precipitate the DNA, which was washed several times in 70% ethanol.

**Southern blot hybridization analysis.** Five-microgram aliquots of genomic DNA were digested with the appropriate 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. The filters were hybridized in 50% formamide/3× standard sodium citrate (SSC) at 37°C, washed in 0.2× SSC/0.5% SDS at 60°C for 2 hours, and autoradiographed at −70°C for 16 to 48 hours, as previously described. The presence of HTLV-I sequences was determined by hybridization of Pst I-digested DNAs to a 3-kb DNA fragment containing sequences of the pol and env regions. The TCRβ gene was investigated by hybridization of EcoRI- and BamHI-digested DNAs to a DNA probe that hybridizes to the constant region of the TCRβ gene. The loss of heterozygosity of p53 loci was assessed by sequential hybridization of HindIII-digested DNAs to two highly polymorphic markers for loci on chromosome 17, pYN22.1 and p144D6. The c-myc oncogene was investigated by hybridization of EcoRI- and HindIII-digested DNAs to a third exon probe (MC413RC).

**Oligonucleotide primers.** All 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 are as follows: P5-5, 5'-TCTCCTCTTCTGCAGTAC- TCTC-3'; P5-3, 5'-ACCTGGAACACCGCCCAGGT-3'; P6-5, 5'-ACAGGCTTGTGGCCAGGGT-3'; P6-3, 5'-AGTTGCAACACGACCTCAG-3'; P7-5, 5'-GTGTTGGTGCTCTTAAAGTGGTGGC-3'; P7-3, 5'-GTGAGCCGAAAGCAGGAGGT-3'; P8-5, 5'-TATCCCTGAAGTGTGAATCCTAC-3'; P8-3, 5'-AGGATTGACATTGAGGCATAC-3'; P9-5, 5'-GCTCCTCAGGGCTTGGAGTACAGGCCCAGGAAAGGTGTC-3' (nucleotides 1640-1601). Synthetic oligonucleotide primers used for amplification of HTLV-I sequences have been previously reported. Essentially, we used the following primers from the gag region: SG 166, 5'-CTCGAGATACCTTTGCTCCTCCTTC-3'; SG 167, 5'-CCGGG GCCGAGGCCGGGAGCTGCTG-3' (nucleotides 1957-1934). The probe used for detection of the amplified HTLV-I gag product was SG 169, 5'-GCTCCTCAGGGCTTGGAGTACAGGCCCAGGAAAGGTGTC-3' (nucleotides 1640-1601). Synthetic oligonucleotide primers used for amplification of RAS genes are as previously reported; the sequences are as follows: N-RAS 12-13-5, 5'-CTGTTGATAAAGTCGTAGT-3'; N-RAS 12-13-3, 5'-GGTTGACATATTCATCATACT-3'; N-RAS 61-5, 5'-TTTATAGTGCTGAAACCTG-3'; N-RAS 61-3, 5'-ATACACAGAGAACCCCTGC-3'; H-RAS 12-13-5, 5'-CTGAAGAGGAGGTGAAAT-3'; H-RAS 12-13-3, 5'-ATACAGACAGAGAACCCCTGC-3'; K-RAS 12-13-5, 5'-CTGTCAGAATAATGACTGAAT-3'; K-RAS 12-13-3, 5'-TTGTGGATCATATCCTGCACC-3'; K-RAS 61-5, 5'-GTAATGGTGAAGAAGACTGCT-3'; K-RAS 61-3, 5'-ATACAAAGAAGCCCTGCC-3'. The 3' region of the first exon of the c-myc oncogene was amplified using the following primers: 2716-2735, 5'-GACAAGAACACTTACACC-3'; and 2871-2890, 5'-GTCCTCTACCTTGTTTCC-3'.

**Single-strand conformation polymorphism (SSCP) analysis.** The SSCP analysis was accomplished according to an adapted version.
of a previously reported method. Brieﬂy, PCRs were performed with 100 ng of genomic DNA, 10 pmol of each primer, 2.5 µmol/L dNTPs (for p53 and c-myc primers) or 50 µmol/L dNTPs (for RAS primers), 1 µCi of [α-32P]dCTP (NEN; speciﬁc activity, 3,000 Ci/mmol), 10 mM/L Tris (pH 8.8), 50 mM/L KCl, 1 mM/L MgCl2 (for p53, N-RAS 12-13, N-RAS 61, K-RAS 12-13, and H-RAS 12-13) or 1.5 mM/L MgCl2 (K-RAS 61 and c-myc), 0.01% gelatin, 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; 58°C for p53 exon 8; 58°C for all RAS ampliﬁcations; and 63°C for c-myc), and extension (72°C) were performed on an automated heat-block (DNA Thermal-Cycler; Perkin-Elmer, Norwalk, CT). The reaction mixture (2 µL) was diluted 1:25 in 0.1% SDS, 10 mM/L EDTA, and further mixed 1:1 with a sequencing stop solution containing 20 mM/L NaOH. Samples were heated at 85°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 W 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.

Enzymatic ampliﬁcation and detection analysis for HTLV-I sequences. The PCR was performed essentially as described for the SSCP analysis, using 1 mM/L MgCl2 and omitting the [32P]dCTP. The annealing temperature was 58°C. The reaction products were fractionated by agarose gel electrophoresis, visualized under UV light by ethidium bromide ﬂuorescence, and then assayed by Southern blot hybridization with a speciﬁc probe spanning the region between the primer pairs. The ﬁlters were prehybridized at 55°C for 30 minutes and hybridized with a 32P-end-labeled probe for 1 hour in a solution consisting of 3 mol/L tetramethyl ammonium chloride salt (TMAC), 1% SDS, 50 mM/L EDTA (pH 8.0), 1× Tris-buffered saline (TBS) solution, and 1 µg/mL salmon sperm DNA.59 Filters were washed twice in 2× SSC/1% SDS for 10 minutes, in 3 mM/L TMAC/1% SDS for 5 minutes at room temperature, and for 1 hour at 60°C and rinsed brieﬂy in 2× SSC. Autoradiography was performed at ~70°C with an intensifying screen for 1 to 3 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. The DNA sequencing was performed directly from the PCR products.

Quantitative investigation with a computerized image analyzer (CAS-200; Cell Analysis System, Elmhurst, IL) was used to evaluate the number of p53 antibodies used were PAb42162 and PAb24063 (p53 Ab-1 and Ab-3, respectively; Oncogene Science). The same method was used for staining with the Ki-67 antibody (DAKO-PC; Dako Corp). 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 sections or cytospin preparations were incubated overnight with the primary anti-p53 MoAb PAB18016 (p53 Ab-2; Oncogene Science, Inc, Uniondale, NY), washed three times, and incubated with biotinylated horse antiserum IgG (Vector). Peroxidase-conjugated avidin-biotin complex was applied and developed with diamobenzidine. Sections were then counterstained with hematoxylin, dehydrated, and mounted with permount. Other p53 antibodies used were PAb42162 and PAb24063 (p53 Ab-1 and Ab-3, respectively; Oncogene Science). The same method was used for staining with the Ki-67 antibody (DAKO-PC; Dako Corp).
RESULTS

Clinical, immunophenotypic, and genotypic characterization. We investigated 10 cases of ATLL, including 6 patients who have been previously reported. Complete clinical information was available on 9 of the 10 patients. The median age of the patients was 43 years, with a range of 33 to 88 years. The male to female ratio was 3 to 7. Sixty percent of the patients presented with hypercalcemia and 33% presented with lytic bone lesions. The outcome of these patients has been poor; nine of them died with a median survival of 6 months, and the remaining patient is alive 4 months after diagnosis. Interestingly, although all 10 patients presented in a nonendemic region (New York City), they were all discovered to have been born in HTLV-I–endemic regions, one in West Africa and the remainder in the Caribbean. Thus, none of the 10 patients represented a true “sporadic” case of ATLL.

Immunophenotypic analysis showed that each of the 10 cases expressed two or more of the pan–T-cell antigens CD2, CD3, and CD5, but all 10 cases were CD7+. All 10 cases were CD4+, and all of them also expressed one or more activation antigens such as CD25, CD38, CD71, and/or HLA-DR (Table 1).

The clonality of these neoplasms was determined by Southern blot hybridization analysis. As shown in Fig 1, all 10 cases exhibited clonal TCRβ gene rearrangements, indicating their T-cell origin, as well as the presence of a large clonal population.

We determined the presence of HTLV-I sequences in all 10 cases by PCR amplification, using oligonucleotide primers specific for sequences in the HTLV-I gag region. After amplification, the PCR products were fractionated in an agarose gel, and the presence of a 600-bp band was determined under UV light after ethidium bromide staining. The specificity of this band was assessed by Southern blot hybridization analysis. As shown in Fig 1, all 10 cases by PCR amplification, using oligonucleotide primers specific for sequences in the HTLV-I gag region. After amplification, the PCR products were fractionated in an agarose gel, and the presence of a 600-bp band was determined under UV light after ethidium bromide staining. The specificity of this band was assessed by Southern blot hybridization analysis. As shown in Fig 1, all 10 cases exhibited clonal TCRβ gene rearrangements, indicating their T-cell origin, as well as the presence of a large clonal population.

An analysis of the p53 gene. The ATLL samples were analyzed for mutations within exons 5 through 9 of the p53 gene, which are the regions most frequently affected by mutations in other types of human tumors. This was done by SSCP analysis, which has been reported to be highly sensitive and specific. Mutations were identified in 3 of the 10 cases of ATLL. Figure 3 shows the result of the SSCP analysis performed for p53 exons 6 and 8, in which cases 3 and 9 show the appearance of abnormal bands in exon 6 and case 10 displays an abnormal migration pattern in exon 8, indicating the probable presence of mutations in these exons. No mutations were identified in exons 5, 7, 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 (Fig 4). Case 3 had a 3-bp deletion in either codon 216, 217, or 218 (all containing a GTG sequence), leading to the loss of one of three consecutive valines. Case 9 had a C to T transition leading to a nonsense mutation in codon 213 and resulting premature protein termination. Case 10 had a G to A transition leading to a missense mutation in codon 281, with a resulting aspartic acid to asparagine change.

Direct sequencing of the PCR products was also performed for the three mutated cases to confirm the presence of these mutations and exclude the possibility of a cloning artifact. This method is also helpful in roughly determining the proportion of wild-type versus mutant alleles and thus assessing the heterozygocity of the mutant p53 allele in cases in which there is an almost pure tumor cell population. In cases 9 and 10, in which the vast majority of cells in the clinical samples are neoplastic, direct sequencing showed a mutant band that is more intense than the wild-type band, suggesting a loss of heterozygocity, with the neoplastic cells containing only the mutated allele (data not shown). Case 3 showed roughly the same intensity for the mutated and the normal bands; however, this sample contained approximately 20% normal lymphocytes as assessed by flow cytometry. Because the PCR is carried to saturation and is not a quantitative method, the results would be consistent with either p53 homozygocity or heterozygocity in the neoplastic cells. Unfortunately, we had insufficient clinical material from this patient to pursue this issue, eg, by sorting the neoplastic cell population or by performing other studies as for cases 9 and 10.

To assess the loss of heterozygocity in cases 9 and 10 more definitively, we performed restriction fragment length polymorphism (RFLP) analysis for loss of 17p allelic. We used Southern blot analysis to assess the number of 17p loci using two highly informative polymorphic probes that map near the p53 locus. 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. Figure 5 shows the pattern of 17p alleles from five different lymphoblastoid cell lines used as controls for heterozygocity (C1 through C5) and cases 9 and 10, using the two DNA probes, pYNZ22 and p144D6. Probe p144D6 detects two different allelic systems, designated A and B in Fig 5, 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. Although all the lymphoblastoid cell lines are heterozygous, containing two alleles with at least one probe, cases 9 and 10 contain one allele that is much more intense than the other one. The weakly hybridizing band is presumably derived from the normal cells present in the specimen. These data

<table>
<thead>
<tr>
<th>Table 1. Antigen Expression by ATLL</th>
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<tbody>
<tr>
<td>CD No.</td>
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<tr>
<td>--------</td>
</tr>
<tr>
<td>CD2</td>
</tr>
<tr>
<td>CD3</td>
</tr>
<tr>
<td>CD5</td>
</tr>
<tr>
<td>CD7</td>
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<tr>
<td>CD4</td>
</tr>
<tr>
<td>CD8</td>
</tr>
<tr>
<td>CD25</td>
</tr>
<tr>
<td>HLA-DR</td>
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</tbody>
</table>

*Greater than 25% of tumor cells positive.
Fig 1. Southern blot hybridization analysis for TCRβ gene rearrangements. DNAs were digested with EcoRI and hybridized to a constant region TCRβ gene probe. Numbers 1 through 10 each lane correspond to the 10 ATLL cases analyzed, whereas C indicates the germline control, for which DNA extracted from the HL60 cell line was used. The two main germline bands seen in lane C are of 12.0 and 4.2 kb (dashes). Cases 1, 2, 5, 7, 8, 9, and 10 show rearrangements and/or deletions of constant regions, whereas cases 3, 4, and 6 show new distinctive bands at positions other than the germline. Arrows indicate clonal rearrangement bands.

confirm the loss of heterozygocity for 17p loci, and thus probably the p53 gene, in the neoplastic cells for cases 9 and 10.

We performed immunostaining using the PAb1801 anti-p53 MoAb to determine the effect of these mutations on p53 protein expression. Staining was also performed with the PAb 421 and PAb240 anti-p53 antibodies, and although results were similar with PAb421, staining was weaker with PAb240 in all the cases examined. The pattern of p53 staining in the ATLL cases lacking p53 gene mutations was that of nuclear positivity of variable intensity in a relatively small, but nevertheless distinct, percentage of cells (Fig 6A and Table 2). These cells may correspond to those cells that are actively proliferating, as has been reported for mitogen-stimulated lymphocytes that progressively accumulate p53 during the G1, S, and G2 phases of the cell cycle. 66 To determine whether this is the case, the proliferation index was also assessed by immunostaining with the Ki-67 antibody. The percentages of p53- and Ki-67–positive cells were compared after quantitation with the CAS image analysis system (Table 2). 64,65 These studies show that the percentage of Ki-67–positive cells is significantly higher than that of p53-positive cells, suggesting that not all the dividing cells express p53 or that they express it at low levels, below our threshold for detection by immunohistochemistry. On the other hand, the majority of the cells in cases 3 and 10 containing p53 mutations exhibited nuclear positivity (Table 2), which was very strong in case 3 (Fig 6B) and of moderate intensity in case 10 (not shown). Case 9 failed to stain with all three anti-p53 antibodies (Fig 6C and Table 2). Lack of immunostaining as a result of nonsense mutations has been reported in lung cancer cell lines, including a case containing the identical C to T transition in codon 213 as in our case 9. 67 Thus, the three ATLL cases containing p53 gene mutations displayed abnormal patterns of p53 immunoreactivity compared with their wild-type counterparts.

Analysis of the RAS oncogene family. The possibility of mutations occurring in the RAS family of oncogenes was determined by SSCP analysis. Specifically, the region spanning codons 12-13 and the region spanning codon 61 for N-RAS, K-RAS, and H-RAS were amplified and studied separately. No mutations were identified in the eight cases studied using this type of analysis (Table 2).
mutations in this region. We did not find any mutations in the 3’ end of the c-myc first exon (data not shown).

Analysis of Rb gene products. The analysis of Rb gene products with monoclonal or polyclonal antibodies appears to be the most sensitive technique to identify the involvement of Rb in human tumors, as no primary Rb or Rb-derived cell lines have been reported to have normal expression of the Rb gene product.99 Thus, we performed immunohistochemical staining with an anti-Rb monoclonal antibody to determine the presence of Rb gene product. All the cases analyzed showed immunostaining with this antibody, making the involvement of the Rb gene in the ATLL cases examined in this study unlikely (Table 2).

DISCUSSION

We found that 3 of 10 cases of ATLL contain p53 mutations, one being an in-frame deletion around codon 217, the second a nonsense mutation in codon 213, and the third a missense mutation in codon 281. Certain structural features indirectly suggest a pathogenetic role for these mutations. First, one case, mutated in codon 281, contains an amino acid substitution in a region of the protein that is highly conserved through evolution.70 Second, the other two cases contain mutations in a region frequently mutated in other tumors, both hematopoietic45 and nonhematopoietic.42,67,71 Interestingly, the exact same nonsense mutation in codon 213 (GCA to TGA) found in one of our cases has been reported in three cases of Burkitt lymphoma,45 in a colon carcinoma,71 and in a lung carcinoma cell line.67 Third, we identified a loss of heterozygosity in the two mutated cases in which there was sufficient material to perform a restriction fragment length polymorphism (RFLP) analysis for chromosome 17p sequences.

Additional evidence for the relevance of p53 gene mutations in the pathogenesis of ATLL is our finding of an altered p53 pattern of expression in the cases examined. The p53 gene encodes a cell cycle–dependent nuclear phosphoprotein, and the wild-type p53 protein has a half-life of approximately 5 to 20 minutes.72 However, the

Analysis of the c-myc oncogene. Southern blot hybridization analysis was performed to determine if the c-myc oncogene was rearranged in these 10 cases of ATLL. Figure 7 shows that all 10 ATLL cases had the germline configuration when HindIII DNA digests were hybridized to the MC413RC probe. Similarly, rearrangements were not detected upon hybridization of this probe to EcoRI DNA digests (Table 2).

Mutations are found in the 3’ region of the c-myc first exon in the vast majority of Burkitt lymphomas,98 especially in endemic cases in which the translocation breakpoint is far from the c-myc locus; and may not be detected by Southern blot analysis. Therefore, we performed SSCP analysis in 8 of the 10 cases to assess the presence of

**Table 2. Summary of Structural Analysis and Expression of Oncogenes, Tumor Suppressor Genes, and a Proliferation-Associated Antigen in ATLL**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>p53</th>
<th>RAS</th>
<th>c-myc</th>
<th>p53* (%)</th>
<th>Ki-67* (%)</th>
<th>Rb</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>WT</td>
<td>WT</td>
<td>G</td>
<td>13</td>
<td>50</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>WT</td>
<td>G</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>M-Ex6</td>
<td>WT</td>
<td>G</td>
<td>57</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>WT</td>
<td>WT</td>
<td>G</td>
<td>12</td>
<td>54</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>WT</td>
<td>WT</td>
<td>G</td>
<td>7</td>
<td>84</td>
<td>+</td>
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<tr>
<td>6</td>
<td>WT</td>
<td>WT</td>
<td>G</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>7</td>
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<td>ND</td>
<td>+</td>
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<tr>
<td>8</td>
<td>WT</td>
<td>WT</td>
<td>G</td>
<td>7</td>
<td>84</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>M-Ex6</td>
<td>ND</td>
<td>G</td>
<td>&lt;1</td>
<td>49</td>
<td>+</td>
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<td>M-Ex8</td>
<td>ND</td>
<td>G</td>
<td>69</td>
<td>77</td>
<td>+</td>
</tr>
</tbody>
</table>

Abbreviations: WT, wild type; M, mutated; ND, not done; G, germline configuration; +, positive immunoreactivity.

*p53 and Ki-67 positivity are expressed as the percentage of positive cells in the total cell population as determined with the CAS image analysis system.
Fig 4. Sequence analysis of p53 mutations. Sequences from exon 6 are shown for cases 3 and 9, whereas the sequence for exon 8 is shown for case 10. Each mutation shown is matched to a control DNA. Arrows point to the mutated base pairs except for case 3 containing a 3-bp deletion. Coding strands are shown for exon 8; noncoding strands are shown for exon 6 sequences. The codon at which the mutation occurs and the resulting change in the protein are indicated.

Fig 5. 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. A and B denote two different allelic systems identified with probe p144D6. C1-C5 are different lymphoblastoid cell lines. All contain two alleles with probe pYNZ22, all except C3 contain two alleles with probe p144D6 in system A, and all except C4 contain two alleles with probe p144D6 in system B. Probes pYNZ22 and p144D6 detect one strong and one relatively weak band with DNA from cases 9 and 10, which is clearly seen with probe pYNZ22 but can also be seen with a longer exposure of the film after hybridization with probe p144D6. This indicates that the majoritiy of the cells in cases 9 and 10 contain only one 17p allele.

mutant p53 protein has an extended half-life of 4 to 8 hours. Therefore, many tumor cells harbor a concentration of p53 that can be easily detected by immunoprecipitation, and high levels of p53 expression have been interpreted as abnormal. On the other hand, p53 protein products can be detected in normal and neoplastic hematopoietic cells that are actively proliferating. This last observation may explain the p53 immunoreactivity in a certain number of cells in the nonmutated cases of ATLL. However, not all actively proliferating cells in these cases contain p53 levels sufficient for detection by immunostaining, because the proportion of p53-positive cells was lower than that of Ki-67-positive cells, as determined by quantitation using the Cell Analysis Systems (CAS) image analysis system. In contrast to the cases with no p53 mutations, the mutated cases showed one of two patterns. The case containing a nonsense mutation of codon 213 showed no p53 staining despite Ki-67 positivity in numerous cells, suggesting that either there are no p53 protein products or that they cannot be detected with any of the three p53 antibodies used even though one of these antibodies (PAb1801) reacts with an epitope located near the amino end of p53. This finding is consistent with the report of a lung carcinoma cell line that contains the identical mutation and similarly fails to show any immunostaining with monoclonal antibodies PAb1801 and PAb421. The second pattern observed was that of nuclear positivity in the vast majority of the tumor cells, which was strong in the case containing a 3-bp deletion around codon 217 and relatively weak in the case containing a missense mutation in codon 281. This pattern suggests an increased half-life as has been documented for other mutant p53 products. In agreement with our results, several staining patterns correlating with different types of p53 mutations have also been reported in lung carcinoma cell lines. In conclusion, even though the three cases containing p53 mutations found in this study have different staining patterns, they all are probably abnormal, suggesting that even though immunohistochemical staining for p53 is useful, definitive conclusions should not be drawn without analysis of the p53 genomic structure.
Fig 6. Immunoperoxidase staining with anti-p53 monoclonal antibodies (×400). (A) A frozen tissue section of case 1, with scattered cells demonstrating strong nuclear staining. (B) A cytospin preparation of case 3, showing strong nuclear reactivity in the majority of cells. (C) A frozen tissue section of case 9 demonstrating lack of reactivity with the anti-p53 antibody.
Epstein-Barr virus (EBV). They are both lymphotropic viruses capable of immortalizing lymphocytes in vitro. Neither of these viruses contain genes homologous to cellular oncogenes, and there is no evidence to suggest insertional mutagenesis. As with EBV, primary infection with HTLV-I is usually asymptomatic and only a very small proportion of infected individuals develop a lymphoid malignancy. It appears that, similar to EBV and Burkitt lymphoma, HTLV-I can stimulate lymphocyte proliferation early in the pathogenesis of ATLL, which in the case of HTLV-I is probably a function of the Tax protein. Interestingly, we have identified mutations in the p53 gene that have also been described with high frequency in Burkitt lymphomas, suggesting further similarities between the two diseases.

It has been postulated that p53 mutations occur as relatively late events in tumorigenesis and that they play an important role in tumor progression. However, p53 mutations are usually found in addition to activation of oncogene(s) in most tumors, and we can hypothesize that such oncogene activation is also necessary for the development of ATLL. Although practically all Burkitt lymphomas contain specific chromosomal translocations involving the c-myc oncogene, no comparable translocation has been identified in ATLL, and we have failed to detect any case in which c-myc is altered. We also explored the RAS family of oncogenes, because RAS mutations are associated with p53 alterations in solid tumors such as colorectal carcinoma and have also been reported in other hematopoietic neoplasms. However, no N-RAS, K-RAS, or H-RAS mutations were identified in the 10 ATLL cases examined here. Because the Rb gene has also been implicated in the pathogenesis of some hematopoietic malignancies, we assessed the presence of Rb gene products by immunostaining. All the cases analyzed showed positive staining with the anti-Rb antibody, making the involvement of the Rb gene in these cases of ATLL unlikely. Thus, although other genetic events besides HTLV-I infection and p53 mutations are probably involved in the pathogenesis of ATLL, further studies are required for their identification.

In conclusion, although no alterations were identified in the c-myc, N-RAS, K-RAS, or H-RAS oncogenes or in the expression of Rb gene products, we identified p53 mutations in 3 of 10 cases of ATLL. The finding of p53 mutations in ATLL is consistent with a previous report of 10 Japanese patients with ATLL, in which five were found to contain p53 mutations, four being missense mutations and one a silent mutation. Our data confirm the presence and approximate frequency of p53 mutations in ATLL. Our data also support the notion that p53 mutations are related to the development of ATLL in regions outside of Japan. Furthermore, p53 gene mutations are uncommon in non-HTLV-I-associated T-cell neoplasms, having been found in only 1 of 36 such cases when tissue samples rather than cell lines have been examined. This indirectly supports the role of p53 mutations in the development of ATLL rather than merely reflecting events occurring in all T-cell neoplasms. Thus, although our findings do not fully explain the mechanism by which HTLV-I leads to the development of ATLL in every case, they suggest that p53 mutations play an important role in the pathogenesis of this T-cell neoplasm and perhaps represent one step in the multistep process of tumorigenesis.

ACKNOWLEDGMENT

We are grateful to Dr Riccardo Dalla-Favera for his advice and access to his laboratory; Dr Rosemary Wieczorek for contributing some pathologic specimens; Drs Daniel Weiss (Good Samaritan Hospital, Suffern, NY), Steven McCormack (New York Eye and Ear Infirmary, New York, NY), Ellen Gold, Akim Ogundipe, Gunwant Kaur Guron, and Nurul Wahid (Columbia Presbyterian Medical Center, New York, NY) for clinical information; Drs Bang Ying Zhu and Jiguang Liu for technical assistance; and Nilda Inghirami for secretarial assistance.

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