The human T-cell lymphotropic virus type I (HTLV-I) is therefore, we performed a molecular analysis of gest that additional factors are involved in its pathogenesis. Moreover, we studied the c-myc gene for rearrangements by Southern graphic regions of the world where human T-cell lymphotropic virus type I (HTLV-I) infection is endemic, including southern Japan, sub-Saharan Africa, the Caribbean basin, and the southeastern United States. Clinically, ATLL is characterized by generalized lymph node, peripheral blood, and skin involvement by pleomorphic tumor cells with hyperlobated nuclei, lytic bone lesions, hypercalcemia, a rapidly progressive course, and a relatively short survival. HTLV-I has been recognized as the etiologic agent of ATLL. However, the mechanism by which HTLV-I induces ATLL is not yet understood. Unlike acutely transforming animal retroviruses, the HTLV-I genome does not encode a known oncogene. Furthermore, this retrovirus does not transform T cells by cis-activation of an adjacent proto-oncogene, because the provirus appears to integrate randomly within the host genome. Although various chromosomal abnormalities have been reported (trisomy 3, 6q-, and trisomy 7), the most consistent cytogenetic findings are frequent anomalies at band 14q11, the 14q11.3 region, and no specific oncogene has been mapped at this site of chromosomal breakpoints in ATLL.

It has been shown that one of the HTLV-I gene products, the Tax protein, transcriptionally activates the expression of certain cellular genes, including interleukin-2 (IL-2), the alpha chain of the IL-2 receptor (IL-2R), the c-fos and c-sis proto-oncogenes, and granulocyte-macrophage colony-stimulating factor (GM-CSF). The central role of some of these genes in normal T-cell activation and growth suggests that Tax activation of these cellular transcription units may represent an important mechanism by which HTLV-I initiates T-cell transformation. However, the long period of clinical latency that precedes the development of ATLL, the small percentage of infected patients that develop this malignancy, as well as the observation that leukemic cells from ATLL patients are monoclonal, suggest that additional cellular events are required for the development of the full malignant phenotype. Currently, the nature of these events is not fully understood.

Tumor suppressor genes, namely, p53 and retinoblastoma (Rb), 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 negative regulatory signals. The normal p53 gene acts as a tumor suppressor gene in the murine model, but point mutations within the coding region can convert the p53 gene from a recessive to a dominant oncogene. 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 monoclonal loss of variable portions of the short arm of chromosome 17, and the 17p13.1 region, where the p53 gene maps, is consistently lost in colon carcinoma. Consistent with Knudson’s model of tumor suppressor genes, 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. p53 mutations and/or allelic losses have been

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found in several types of human solid tumors, including colon, breast, lung, brain, and soft tissue.\textsuperscript{40,42,43} Furthermore, p53 mutations have been recently identified in lymphoid tumors, specifically T-cell acute lymphoblastic leukemia (ALL) cell lines,\textsuperscript{44} B-ALL, B-chronic lymphocytic leukemia, and Burkitt lymphoma,\textsuperscript{45} as well as some B-cell lymphomas in advanced clinical stage.\textsuperscript{46}

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.\textsuperscript{47} We previously reported the clinical, morphologic, and immunophenotypic characterization of six cases of ATLL occurring in a nonendemic region.\textsuperscript{48} Subsequently, we characterized an additional four cases, all proven to contain HTLV-I sequences and to be associated 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 by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation. Representative portions of each tissue specimen were prepared. The remaining portions were embedded in paraffin, and hematoxylin and eosin (H&E) stained sections were resuspended in 3 mL of nuclei lysis buffer containing 10 mmol/L Tris HCl, 400 mmol/L NaCl, and 2 mmol/L EDTA, 200 μL of 10% SDS (sodium dodecylsulfate, NaDodSO\textsubscript{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 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 restriction endonucleases according to the manufacturer’s instructions (Boehringer-Manheim, Indianapolis, IN), electrophoresed in 0.8% agarose gels, denatured with alkali, neutralized, and transferred to nitrocellulose filters according to Southern.\textsuperscript{51} The filters were hybridized in 50% formamide/× 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.\textsuperscript{52} 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.\textsuperscript{48} The TCR\textbeta gene was investigated by hybridization of EcoRI- and BamHI-digested DNAs to a DNA probe that hybridizes to the constant region of the TCR\textbeta gene.\textsuperscript{53} 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, pYNNZ2.1 and p144D6.\textsuperscript{48} The c-myc oncogene was investigated by hybridization of EcoRI- and HindIII-digested DNAs to a third exon probe (MC413RC).\textsuperscript{55}

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\textsuperscript{48,50} are as follows: P5-5, 5’-TTCTTCTTCTGCAG-TACTC-3’; P5-3, 5’-ACCTGGGCAACCAGCCGTT-3’; P6-5, 5’-ACAGGGCTGTTGGCCCAAGGT-3’; P6-3, 5’-AGTGTCACAACGACCTCAG-3’; P7-5, 5’-GTCTGTCTCAGTGGC-3’; P7-3, 5’-CTGACAGCCAGAGCAGGAGGT-3’; P8-5, 5’-TATCTCGAGTAAGTGATTAC-3’; P8-3, 5’-AAGTAGATCTGGCATAAC-3’. The 3’ region of the first exon of the TCR\gamma gene was investigated by hybridization of EcoRI- and HindIII-digested DNAs to a third exon probe (MC413RC).\textsuperscript{55}

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.\textsuperscript{47} An alternative salting-out procedure, not requiring organic extraction, was used in cases in which a limited amount of tissue was available.\textsuperscript{50} Briefly, the cells or frozen tissue sections were resuspended in 3 mL of nuclei lysis buffer containing 10 mmol/L Tris HCl, 400 mmol/L NaCl, and 2 mmol/L EDTA, 200 μL of 10% SDS (sodium dodecylsulfate, NaDodSO\textsubscript{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 vol of ethanol was added to the supernatant to precipitate the DNA, which was washed several times in 70% ethanol.
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-32PdCTP (NEN; specific activity, 3,000 Ci/mmol), 10 mmol/L Tris (pH 8.8), 50 mmol/L KCl, 1 mmol/L MgCl2 (for p53, N-RAS 12-13, N-RAS 61, K-RAS 12-13, and H-RAS 12-13) or 1.5 mmol/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 Cetus, Norwalk, CT). The reaction products (12 μL) was diluted 1:25 in 0.1% SDS, 10 mmol/L EDTA, and further mixed 1:1 with a sequencing stop solution containing 20 mmol/L NaOH. Samples were heated at 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 mmol/L MgCl2 and omitting the 32PdCTP. 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-labeled probe for 1 hour in a solution consisting of 3 mol/L tetramethyl ammonium chloride salt (TMAC), 1% SDS, 50 mmol/L EDTA (pH 8.0), 1× Denhardt’s solution, and 10 mg/mL salmon sperm DNA.59 Filters were washed twice in 2× SSC/1% SDS for 10 minutes, in 3 mmol/L TMAC/1% SDS, 50 mmol/L EDTA (pH <5) and subsequently ethanol precipitated. The DNA was resuspended in 7 μL of H2O, adding 1 μL of primer and 2 μL of 5× Sequenase sequencing buffer. After incubation at 37°C for 30 minutes, the following reagents were added: 2 μL of labeling mix solution (1:5), 1 μL of 0.1 mol/L DTT, 0.5 μL (4 μCi) 35SdATP, and 2 μL Sequenase enzyme dilution (1:8). These labeling reactions were incubated at room temperature for 5 minutes and, subsequently, 3.5 μL was aliquoted into a tube containing each of the four termination mixtures (ddGTP, ddATP, ddTTP, and ddCTP). After 5 minutes of 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% polycrylamide/8 mol/L urea gel. Autoradiography was performed at room temperature for 16 to 24 hours. Direct sequencing of PCR products was accomplished by performing a PCR as described for the SSCP analysis, omitting the 32PdCTP, and doubling the entire reaction. The PCR product was separated by electrophoresis in a low melting point agarose gel, purified, and then sequenced as previously described.57

Immunohistochemical staining. The immunophenotype of the T-cell neoplasms had been determined at the time of diagnosis by immunohistochemical staining of frozen tissue sections using a three-step avidin-biotin immunoperoxidase technique or an immunoperoxidase antialkaline phosphatase method and/or by direct and indirect immunofluorescent flow cytometry of isolated cells in suspension using the FACScan fluorescent-activated cell sorter (Becton Dickinson, Mountain View, CA).60 Monoclonal antibodies (MoAbs) used to immunophenotype the cases of ATLL included OKT3 (CD3), OKT4 (CD4), OKT6 (CD1), OKT8 (CD8), OKT9 (CD71), OKT10 (CD38), OKT11 (CD2; Ortho Diagnostics, Raritan, NJ), T1 (CD5), IL-2R (CD25), BL9, HLA-DR (United Biomedical, Lake Success, NY), Leu2 (CD8), Leu3a (CD4), Leu4 (CD3), Leu9 (CD7), LeuM1 (CD15), LeuMS (CD11c; Becton Dickinson), EMA (Dako Corp, Santa Barbara, CA), Ber-H2 (CD30; courtesy Dr Harold Stein, Berlin, Germany), and B1 (CD20; Coulter Immunology, Hialeah, FL).

Immunohistochemical staining to determine the presence of retinoic acid (Rh) protein was performed by a modification of the alkaline phosphatase antialkaline phosphatase method. Mononuclear cells and single cell suspensions of blood and bone marrow specimens were cytospun (Cytospin 2; Shandon, Pittsburgh, PA) on silane-coated slides and air dried overnight. In addition, serial 4-μm frozen sections of pathologic specimens were cut from cryopreserved tissue blocks on precoated slides (silane) and treated as described above. Samples were then fixed in acetone/chloroform (1:1) for 5 minutes, air dried, postfixed in 10% buffered formalin for 5 minutes, and then stained as previously described in detail.61 Briefly, sections were serially incubated with appropriately titered anti-Rh MoAbs (MAb-1; Triton Biosciences Inc, Alameda, CA) with F(ab’)2 goat antigoat IgG (Fcγ specific, 1:200; Organon Teknika, Malvern, PA), and alkaline phosphatase-anti-alkaline phosphatase complex (AAPAP; Dako Corp, Santa Barbara, CA), and then developed with Vector Red (Vector, Burlingame, CA) as a substrate.

In order to determine binding to the p53 protein, sections were incubated overnight with the primary anti-p53 MoAb PAb180161 (p53 Ab-2; Oncogene Science, Inc, Uniondale, NY), washed three times, and incubated with biotinylated horse antigoat IgG (Vector). Peroxidase-conjugated avidin-biotin complex was applied and developed with diaminobenzidine. 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; 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 cryostat preparations were analyzed after immunostaining using the CAS quantitative proliferation index (QPI) 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-67,54,55 It detects total nuclear area and nuclear reactivity with the antibody used, so p53 nuclear staining is adequately measured using this program.
RESULTS

Clinical, immunophenotypic, and genotypic characterization. We investigated 10 cases of ATLL, including 6 patients who have been previously reported.44 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.45 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 2, all 10 cases exhibited clonal TCRβ gene rearrangements, indicating their T-cell origin, as well as the presence of a large clonal population.

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.39,40,42,43 This was done by SSCP analysis, which has been reported to be highly sensitive and specific.45 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 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.39,44 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.42 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>
</tr>
<tr>
<td>CD2</td>
</tr>
<tr>
<td>CD3</td>
</tr>
<tr>
<td>CD5</td>
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<td>CD7</td>
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<td>CD4</td>
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<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.
MUTATIONS IN ATLL

南方印迹斑分析用于TCRβ基因重排。DNRs用EcoRI消化并用常染色体区TCRβ基因探针杂交。每个图例1到10分别对应10例ATLL病例，而C表示阳性对照，使用HL60细胞系中提取的DNA。图中两个主要的常染色体带为12.0和4.2 kb（短划线）。图5, 2, 5, 7, 8, 9, and 10显示了常染色体区的重排和/or删除，而图3, 4, and 6则显示了新出现的带在其他位置。箭头表示克隆重排带。

我们用PAb1801抗p53 MoAb对这些突变对p53蛋白表达的影响进行免疫组织化学检测。也用PAb421和PAb240抗p53抗体，尽管结果与PAb421相似，但PAb240的染色较弱。

图2.PCR扩增分析用于检测HTLV-I gag特异性序列。1到10例对应案例数字，M对应分子量标记（HindIII-digested lambda DNA和HaeIII-digested PhiX 174 DNA），带号（—）显示的是DNA的反应产物，在没有DNA的情况下，C是正常对照（HTLV-I-negative cell line HL60）。整个扩增反应均在每条带中使用。

图中10例显示了一个0.6-kb带（短划线），在乙醇染色琼脂糖凝胶（上层）和Southern印迹斑中使用放射性标记的合成寡核苷酸探针对HTLV-I gag区域进行杂交。
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. 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 one 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. Second, the other two cases contain mutations in a region frequently mutated in other tumors, both hematopoietic and nonhematopoietic. 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.72 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. However, the

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>Genotypic Analysis</th>
<th>p53 (%)</th>
<th>Ki-67 (%)</th>
<th>Rb</th>
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<tbody>
<tr>
<td>1</td>
<td>WT</td>
<td>G</td>
<td>13</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>G</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
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<td>WT</td>
<td>G</td>
<td>57</td>
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<tr>
<td>10</td>
<td>M-Ex 8</td>
<td>ND</td>
<td>69</td>
<td>77</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.
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. Certain analogies can be drawn between HTLV-I and the
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 cytopsin 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.

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.

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REFERENCES


Structural and functional analysis of oncogenes and tumor suppressor genes in adult T-cell leukemia/lymphoma shows frequent p53 mutations

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