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Genetic Alteration of p53 in Some Patients with Adult T-Cell Leukemia

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Abnormalities of p53 mRNA in adult T-cell leukemia (ATL) were analyzed using reverse transcription-polymerase chain reaction-single strand conformation polymorphism analysis. Mutations were present in two of 12 ATL patients studied, but not in 3 cell lines immortalized by human T cell leukemia virus type 1 (HTLV-1) infection *in vitro*. Direct sequencing analysis of the p53 gene from these two patients revealed missense point mutations at codon 153 (arginine to histidine) or codon 220 (cysteine to tyrosine), respectively. Immunohistochemical analysis revealed the elevated expression of p53 proteins in ATL cells from a patient carrying the mutated p53 gene at codon 158. Neither gross rearrangement of p53 gene nor abnormal size of mRNA for the gene was demonstrated by Southern or Northern blot analyses. Thus, there is a mutated p53 in some patients with ATL. The involvement of abnormalities in some suppressor oncogenes may play a role in the development of ATL.

Key words: p53 – Adult T-cell leukemia — Polymerase chain reaction — Single strand conformation polymorphism

p53 is a nuclear phosphoprotein that was first detected in simian virus 40 (SV40)-transformed cells, using an immunologic method.[1] The overexpression of mutant p53 protein can transform some cell lines and mutant murine p53 plus an activated ras can transform rat embryo fibroblasts.[2–4] These activities of mutant p53 seem to function in a dominant negative fashion. The wild-type p53 has the properties of a tumor suppressor. In rat embryo fibroblasts transformed by Ela plus the activated H-*ras* oncogene product, there is an inhibition of the transformed phenotype by wild-type p53.[5]

The p53 gene is on the short arm of chromosome 17,[6] and this portion is deleted in about 75% of human colorectal cancers.[7] Another study revealed that gross rearrangements of the p53 gene are present in human osteosarcomas.[8,9] Point mutations of the p53 gene have been found in lung cancers,[10,11] brain tumors,[11] breast cancers,[11] hepatocellular carcinomas,[12,13] esophageal cancers[14] and lymphomas.[15]

Adult T cell leukemia (ATL) is T cell malignancy strongly associated with human T cell leukemia virus type 1 (HTLV-1) infection.[16–18] However, the incidence of ATL per 1,000 HTLV-1 carriers per year was estimated to be 0.6–0.84, even in an ATL-endemic area in Japan.[19] Therefore, other factors in addition to HTLV-1 infection appear to be involved to the development of ATL. Multi-step carcinogenesis for ATL was suggested by stochastic analysis.[20] As there is a paucity of documentation on the possible involvement of suppressor oncogenes in ATL development, we analyzed for mutations of the coding region of p53 gene of 12 patients with ATL and 3 HTLV-1-infected cell lines. We used the PCR-SSCP and RT-PCR-SSCP (reverse transcription-polymerase chain reaction-single strand conformation polymorphism) method.[21,22] We found missense point mutations of the p53 gene in 2 of 12 ATL patients. There was no evidence of abnormality for the p53 gene in the 3 HTLV-1-infected cell lines.

The abbreviations used are: ATL, adult T-cell leukemia; HTLV-1, human T-cell leukemia virus type 1; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; SSCP, single strand conformation polymorphism.

MATERIALS AND METHODS

Patients and cell lines Twelve ATL patients (No. 1–12) and 3 HTLV-1-infected T cell lines (No. 13–15) were used in this study. Six of the 12 ATL patients were of the acute type (No. 3, 5, 6, 9, 11 and 12) and 6 were of the chronic type (No. 1, 2, 4, 7, 8 and 10), based on the criteria proposed by Kawano *et al.*[23] The 3 HTLV-1-infected cell lines were MT-2,[24] 1-K[25] and 35T− (No. 13–15, respectively). These cell lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum.

Peripheral blood mononuclear cells (PBMCs) from the patients were separated by Ficoll-Conray density gradient centrifugation of heparinized peripheral blood samples.

DNA and RNA preparation High-molecular-weight cellular DNA was isolated from PBMCs and cell lines by proteinase K digestion followed by phenol-chloroform extraction.[26] Total RNA was isolated from PBMCs and cell lines by the method of acid guanidine isothiocyanate phenol-chloroform extraction.[27]

PCR-SSCP and RT-PCR-SSCP analysis cDNA was synthesized from total RNA by reverse transcription.[28] In short, 1 μg of total cellular RNA was annealed with each specific primer (0.05 pmol), 4B and 6B (shown below), at 95°C for 2 min and at 55°C for 60 min. This annealed RNA was reverse-transcribed with 200 units of Moloney murine leukemia virus reverse transcriptase at 37°C for 60 min in a total volume of 14 μl.

The regions analyzed were designated as fragments C1 to C6, as shown in Fig. 1, and all the coding exons were covered. Exon 1 is a non-coding sequence.[29] The primer sets for analyzing fragment C1 are 1A (5′TCCACGACGGTGACACGCTT3′) and 1B (5′TCTGGGAGCTTCATCTGGAC3′), for fragment C2 they are 2A (5′ACTTCCTGAAAACAACGTTC3′) and 2B (5′GCAAAACATCTTGTTGAGGG3′), for fragment C3 they are 3A (5′TTGCATTCTGGGACAGCCAA3′) and 3B (5′CCTTCCACTCGGATAAGATG3′), for fragment C4 they are 4A (5′ACCATGAGCGCTGCTCAGAT3′) and 4B (5′TCAAAGCTGTTCCGTCCCAG3′), for fragment C5 they are 5A (5′CACCATCATCACACTGGAAG3′) and 5B (5′GAGTTCCAAGGCCTCATTCA3′), and for fragment C6 they are 6A (5′ATCCGTGGGCGTGAGCGCTT3′) and 6B (5′CTGACGCACACCTATTGCAA3′). The cDNA product using the 4B primer was used for the analysis of fragments C1, C2 and C3. The cDNA product using 6B primer was employed for the analysis of fragments C4, C5 and C6. Two primers sets (G5A and G5B, G6A and G6B) used for analyzing genomic fragments G5 and G6 were designed to cover exons 5 and 6, respectively (Fig. 1). The sequences of G5A, G5B, G6A and G6B are 5′TTCCTCTTCCTGCAGTACTC3′, 5′GCAAATTTCCTTCCACTCGG3′, 5′ACCATGAGCGCTGCTCAGAT3′ and 5′AGTTGCAAACCAGACCTCAG3′, respectively.

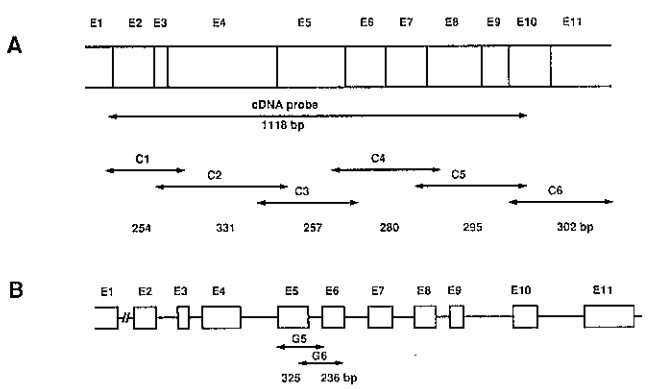

Fig. 1. Regions of p53 cDNA and genomic DNA corresponding to fragments analyzed by PCR-SSCP. (A) Six cDNA fragments (C1–C6) were analyzed by PCR-SSCP. These 6 fragments were designed to cover all the coding p53 cDNA. (B) Two genomic fragments (G5 and G6) were analyzed by PCR-SSCP. E1–E11 indicates exon 1–exon 11. The numbers shown under the fragments indicate the length of the nucleotides.

Each primer was labeled at the 5′ terminus with [γ-^{32}P]ATP, using T4 polynucleotide kinase. Using the labeled primers (each 0.1 μM), PCR was carried out in a DNA Thermal Cycler (Perkin-Elmer/Cetus) for 30 cycles. The reaction volume was 5 μl, containing 125 μM of each dNTP, 0.125 unit of Taq polymerase and 1 μl of cDNA or 0.05 μg of genomic DNA. The reaction cycle was as follows: primer annealing at 55°C for 30 s, polymerization at 72°C for 1 min, and denaturation at 94°C for 30 s. One-hundredth of each PCR product was analyzed by electrophoresis in 5% polyacrylamide gels (acrylamide/N,N - methylene - bis - acrylamide = 49/1). Before application to the gel, the reaction product was denatured at 80°C for 3 min.

Asymmetrical PCR and direct sequencing As the template for asymmetrical PCR, the corresponding fragment was eluted from the polyacrylamide gel.[30] For the asymmetric PCR,[30] the concentration of the primer used in the larger amount was 0.5 μM, and that of the other was 0.05 μM. PCR was carried out for 55 cycles, each cycle consisting of primer annealing at 55°C for 30 s, polymerization at 72°C for 1 min and denaturation at 94°C for 30 s. The PCR products were annealed with ^{32}P-labeled primers, then sequencing reactions were performed using the dideoxy chain termination method.

p53 cDNA probe The p53 cDNA probe of 1118 base pairs corresponding to positions 59 nucleotides upstream of ATG to codon 353 was prepared by PCR from the reverse transcription product of total cellular RNA isolated from normal human peripheral lymphocytes, using unlabeled primers.

Southern and Northern blot analysis Southern and Northern blotting were done as described.[31,32] The amounts of DNA and RNA used for Southern and Northern blot analysis were 5 μg and 10 μg per sample, respectively.

Immunohistochemical analysis The lymph node used for immunostaining of p53 was fixed in acetone and embedded in paraffin by the AMeX method.[33] For local-

ization of p53 in AMeX sections, the avidin-biotin-peroxidase complex (ABC) method was used and the immunostaining procedure was conducted as described.[33] The primary antibody was a mouse monoclonal antibody (PAb1801, Oncogene Science, Manhasset, NY) which recognizes both wild-type and mutant p53.[34] Biotinylated secondary antibody and ABC reagent were obtained from Vector Laboratories, Burlingame, CA. For visualization, the peroxidase reaction was developed in 3,3′-diaminobenzidine. Cells with definite nuclear staining were regarded as positive.

RESULTS

No gross genomic p53 rearrangement in ATL patients The genomic DNAs extracted from PBMCs of 11 ATL patients (except for No. 10 patient) were digested completely with restriction enzyme *Hin*d III or *Bgl* II. These samples were analyzed by Southern hybridization probing with ^{32}P-labeled p53 cDNA. All DNAs, after digestion with *Hin*d III, showed normal patterns of the p53 gene (Fig. 2). When digested with *Bgl* II, some samples showed polymorphic patterns (Fig. 2), attributed to an additional *Bgl* II site in intron 1.[35] These data show that there were no gross genomic rearrangements in ATL patients.

No remarkable changes of mRNA level of p53 gene The mRNAs of 12 ATL patients and 3 HTLV-1-infected cell lines were analyzed by Northern blotting. The ^{32}P-labeled p53 cDNA probe was used for this analysis. There were no remarkable differences in the expression levels of mRNA of p53 gene among these 15 samples (data not shown).

Screening of point mutaion of p53 gene using PCR-SSCP and RT-PCR-SSCP methods To look for point mutations in the p53 gene, the PCR-SSCP method was used. This method is rapid and convenient for detecting point mutations.[21, 22] The principle of this method is based on differences of the higher-order structures of single-stranded DNAs, which have different degrees of mobility in polyacrylamide gel on electrophoresis. Generally, one can observe two bands, indicating two separated strands, but if the DNA fragment has mutations, the bands of the mutated DNA will be shifted from the normal positions. We also analyzed 12 ATL patients and 3 HTLV-1-infected cell lines using the RT-PCR-SSCP method in all 6 fragments (C1-C6), covering the entire coding region of p53 gene (Fig. 1). Fragments C3 and C4 in cDNA of

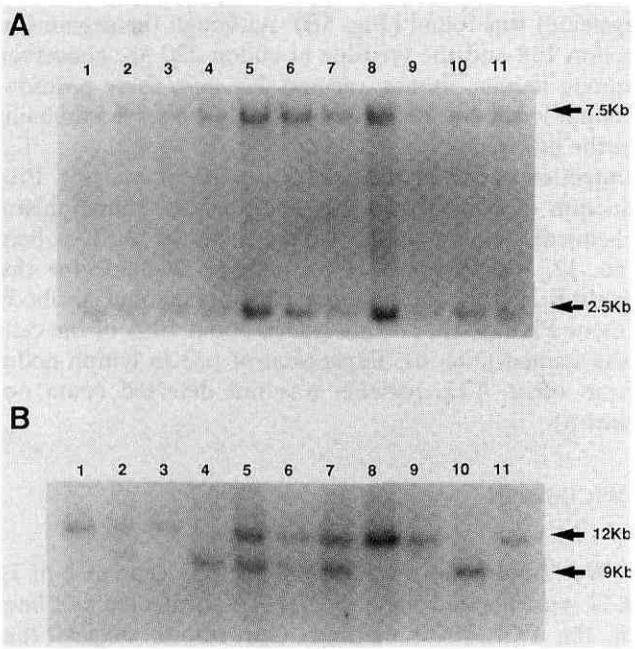

Fig. 2. Southern blot analysis of the p53 locus in ATL cases. Genomic DNAs (5 μg each) extracted from PBMCs of 11 ATL patients (No. 1–9, 11, 12; lane 1–11 respectively) were digested by *Hin*d III or *Bgl* II and subjected to Southern blot analysis with p53 cDNA probe, as described in "Materials and Methods," (A) *Hin*d III digestion. The 7.5 kb and 2.5 kb bands were detected in all cases. (B) *Bgl* II digestion. 12 kb and 9 kb bands were detected. 4 cases (lanes 2, 5, 6, 7) were heterozygous and 7 cases (lanes 1, 3, 4, 8, 9, 10, 11) were homozygous. This pattern is due to the polymorphic *Bgl* II site.

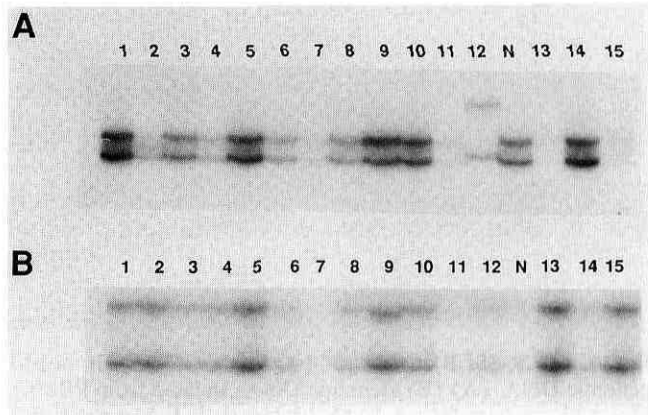

Fig. 3. RT-PCR-SSCP analysis of fragments C3 and C4 in p53 cDNA. Total cellular RNA extracted from PBMCs of 12 ATL patients (No. 1–12; lanes 1–12, respectively) and one healthy volunteer (lane N) and from 3 cell lines (MT-2, 1K and 35T−; lanes 13–15, respectively) were subjected to RT-PCR-SSCP analysis. The primers 3A and 3B, 4A and 4B were labeled at the 5′ end with ^{32}P and therefore all separated complementary strands should have been detected. (A) Fragment C3; Shifted bands were detected in lane 12. (B) Fragment C4; Shifted bands were detected in lane 9.

p53 gave two bands for each lane. These two bands correspond to the two strands of cDNA of fragments C3 and C4. The shifted bands of fragment C3 and C4, as compared to the fragments from normal p53 cDNA, were detected in lane 12 (patient No. 12) (Fig. 3A) and lane 9 (patient No. 9) (Fig. 3B), respectively. No shifted bands were detected in the analysis of fragments C1, C2, C5 and C6 (data not shown). In lane 12 (patient No. 12) of fragment C3, there are only two shifted bands and no normal bands, thereby indicating that expression of the normal counterpart of p53 gene is absent. In lane 9 (patient No. 9) of fragment C4, we could not evaluate loss of normal bands due to the small differences in mobility between normal and abnormal bands. We analyzed fragments G5 and G6 (Fig. 1) of genomic DNA by the PCR-SSCP method to search for allelic change in the p53 gene in these regions. Shifted bands were observed in lane 4 (patient No. 12, fragment G5) and lane 2 (patient No. 9, fragment G6) (Fig. 4). No band related to normal G5 and G6 DNA was observed in those lanes. Thus, the normal alleles of the p53 gene of samples with mutation (No. 9, No. 12) at p53 are either deleted or they are homozygous mutations.

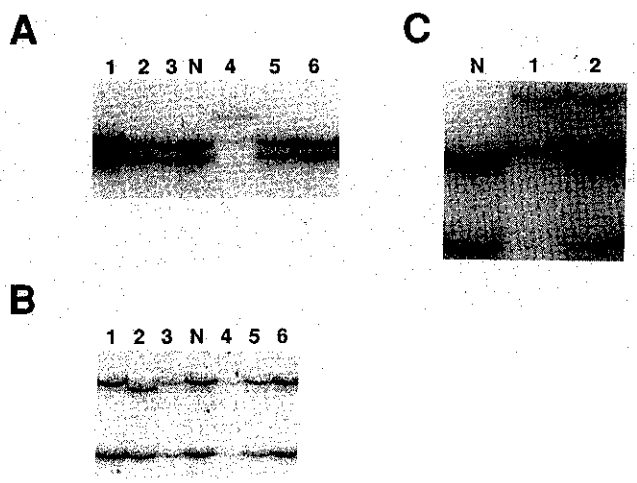

Fig. 4. PCR-SSCP analysis of fragments G5 and G6 in p53 genomic DNA. (A) (B) Genomic DNAs isolated from PBMCs of 4 ATL patients (No. 8, 9, 11, 12; lane 1, 2, 3, 4 respectively) and one healthy volunteer (lane N) and from 2 cell lines (MT-2; lane 5, 1K; lane 6) were subjected to PCR-SSCP analysis of fragments G5 (A) and G6 (B). Shifted bands were detected in lane 4 (No. 12 patient)(A), and lane 2 (No. 9 patient) (B). (C) Genomic DNAs, isolated from PBMCs of patient No.12 in the leukemic state (lane 1), patient No. 12 in partial remission (lane 2) and one healthy volunteer (lane N) were subjected to PCR-SSCP analysis of fragment G5. Two shifted bands were detected in lane 1, and two shifted bands and two non-shifted bands were detected in lane 2.

In the 3 HTLV-1-infected cell lines, no abnormality was found in those regions by either the RT-PCR-SSCP or PCR-SSCP method (Figs. 3 and 4).

Using the genomic DNA of No. 12 patient extracted from PBMCs at the time of partial remission (the PBMCs contained 50% normal lymphocytes and 50% atypical lymphocytes), a PCR-SSCP analysis was made of fragment G5 in which the mutation in No. 12 patient was detected (Fig. 4 C). In this patient (lane 2) there were 4 bands, two normal bands and two shifted ones. This result would suggest that p53 gene in the normal lymphocytes was normal and that the gene in the atypical lymphocytes was mutated. This mutation is probably specific to the leukemic cells in this patient.

Direct sequencing of the p53 gene To search for the mutated nucleotide in fragments G5 and G6 of the p53 gene, direct sequencing for the amplified G5 and G6 fragments from patients No. 12 and No. 9, respectively, was performed. The primers used in the asymmetrical PCR of fragment G5 and G6 were G5A plus G5B and G6A plus G6B. In p53 gene from patient No. 12, a point mutation at codon 158 (CGC to CAC, arginine to histidine) was found (Fig. 5A). In patient No. 9, a point mutation at codon 220 (TAT to TGT, tyrosine to cysteine) was found (Fig. 5B). Although the arginine at codon 158 and the tyrosine at codon 220 are conserved among human, mouse, rat and *Xenopus*, these positions are not located in the 5 known highly conserved domains in the p53 gene.[36]

Detection of p53 by immunohistochemical analysis Production of p53 protein was analyzed by immunohistochemical staining of the lymph nodes of ATL patient No. 12, which was only the sample available for this analysis in this study, using p53 monoclonal antibody (clone PAb1801). The nucleus in about 50% of the cells was stained (Fig. 6). Expression of p53 in lymph nodes from other ATL patients was not detected (data not shown).

DISCUSSION

We found point mutations of the p53 gene in 2 of 12 ATL patients and none of 3 HTLV-1-infected cell lines by the PCR-SSCP method. Our results suggest that mutations in p53 gene are involved in the development of ATL in some HTLV-1-infected subjects. The two patients with mutation of the p53 gene are both of the acute type, so point mutation of p53 might occur, if at all, as a late event of ATL progression. Since only a few percent of HTLV-1 carriers develop ATL during their lives,[19] factors other than HTLV-1 infection may be involved in the development of leukemia. Our results suggest that abnormalities of oncogene and/or suppres-

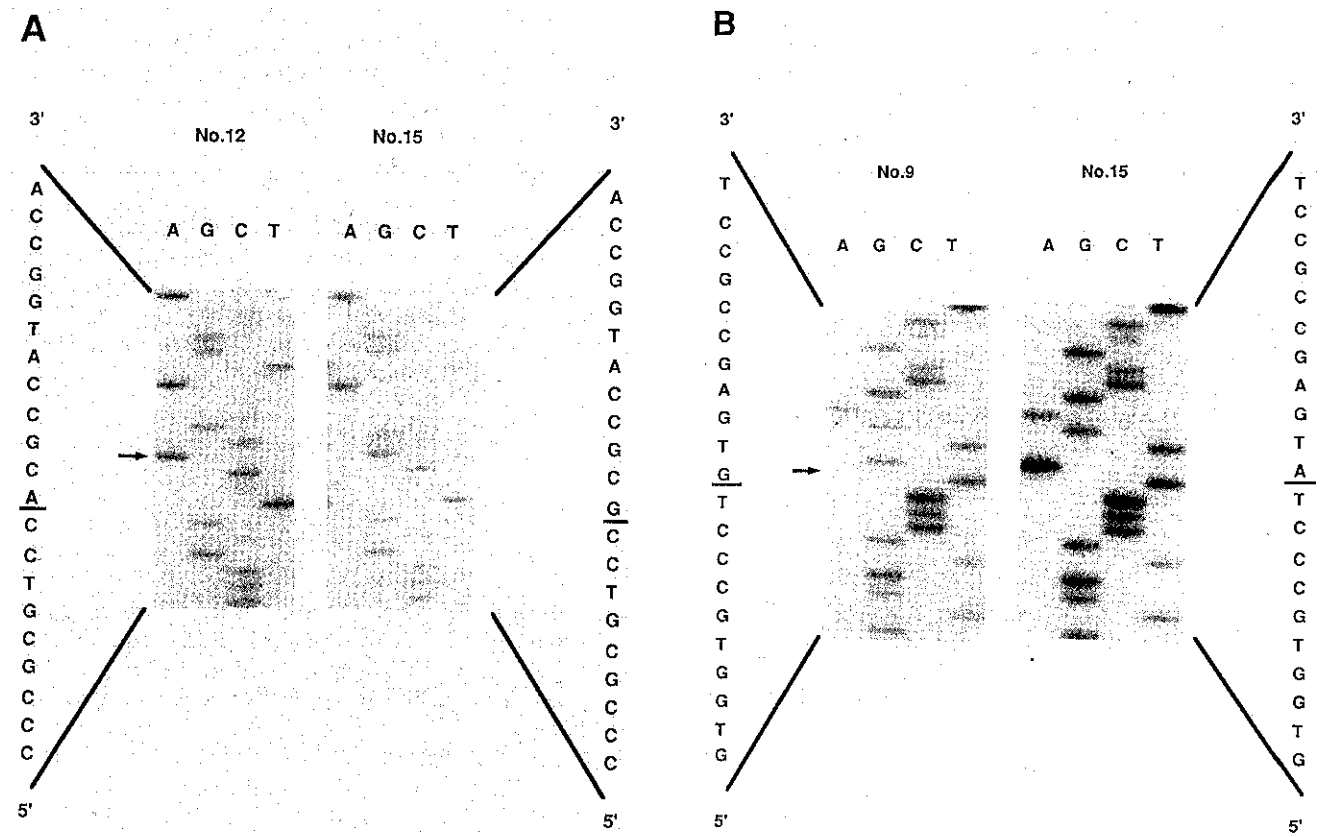

Fig. 5. Nucleotide sequence analysis of p53 genomic DNA. Using the same primer sets as for the PCR-SSCP analysis, asymmetrical PCR and direct sequencing were performed. (A) Point mutation was detected at codon 158 (CAC) in patient No. 12, and the wild-type sequence of codon 158 (CGC) was detected in 35T− (No. 15), which showed no abnormality in PCR-SSCP analysis. (B) Point mutation was detected at codon 220 (TAT) in patient No. 9, and the wild-type sequence of codon 220 (TGT) was detected in 35T− (No. 15).

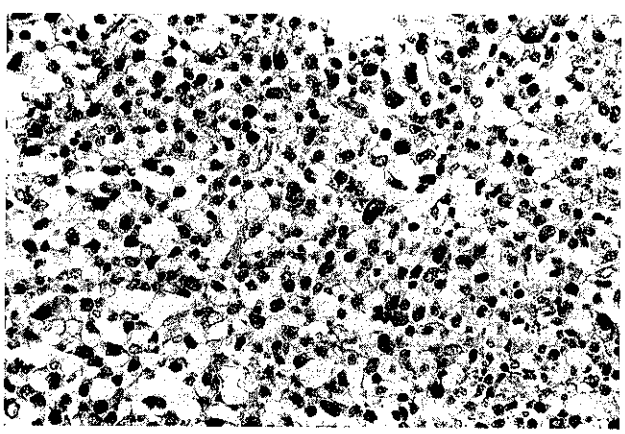

Fig. 6. Immunohistochemical staining of a lymph node from ATL patient No. 12, by anti-p53 monoclonal antibody PAb 1801. The lymph node fixed in acetone and embedded in paraffin was used. Cells with definite nuclear staining were regarded as being positive.

sor oncogenes might contribute to the development of ATL in HTLV-1-infected subjects and the involvement of p53 gene mutation is one such abnormality.

In the two patients only the mutated allele of p53 gene was noted, thereby indicating the absence of normal alleles in the ATL cells from these patients. This observation suggests that both allelic deletions and point mutations are involved in abnormalities of the p53 gene in these two patients. PCR-SSCP analysis of PBMCs from patient No. 12 in partial remission revealed both mutated and wild-type alleles, and hence the probable loss of the normal p53 gene in ATL cells of the patient, as a somatic mutation. We found no abnormalities in 3 HTLV-1-infected cell lines by RT-PCR-SSCP analysis. These cell lines were immortalized by HTLV-1 *in vitro*, and there is no evidence of transformed phenotype, such as tumorigenicity in nude mice.

Both point mutations of p53 genes produce an amino acid substitution, arginine (codon 158) to histidine in

patient No. 12, and cysteine (codon 220) to tyrosine in patient No. 9. Although these amino acids are not located in the five conserved domains proposed by Soussi *et al.*,[36] they are conserved among human, mouse, rat, and *Xenopus*, and may be assumed to be important for the functions of p53. Immunohistochemical analysis suggested that the level of expression of p53 in patient No. 12 was higher than in those with a normal p53 gene. These data imply a possible relationship between point mutation of the p53 gene and the pathogenesis of ATL. The mRNA level of patient No. 12 was not higher than those of other patients. Detection of p53 by immunohistochemistry in this patient was probably due to post-transcriptional regulation, such as elongation of the half life of p53.[37]

Involvement of factors other than HTLV-1 infection in the development of ATL in HTLV-1-infected subjects has been suggested. Abnormalities in cellular genes require further study.

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