Aberrant Expression of the p53 Tumor Suppressor Gene in Adult T-Cell Leukemia and HTLV-I-infected Cells

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By immunoprecipitation analysis, enhanced p53 expression was detected in 3 of 4 adult T-cell leukemia (ATL) cell lines, 1 of 3 HTLV-I-infected cell lines and 1 of 5 fresh ATL samples, compared with phytohemagglutinin-stimulated peripheral blood lymphocytes. Among these 5 high expressers, p53 missense mutations were indicated in 2 ATL cell lines and 1 fresh ATL sample by extensive p53 cDNA and genomic DNA polymerase chain reaction single-strand conformation polymorphism analysis. No mutation was found throughout the entire coding region of the remaining 2 high expressers (1 ATL and 1 HTLV-I-infected cell lines) and low expressers of p53 (2 HTLV-I-infected cell lines). Tax oncoprotein expression was found in these 2 high p53 expressers in which p53 mutation was not present, but not in low p53 expressers or cells carrying this mutation. The levels of p53 mRNA were similar among the samples regardless of p53 levels. Posttranscriptional mechanisms other than missense mutation would thus appear to increase p53 in the Tax-expressing cells but not in cells containing undetectable levels of Tax. No complex formation between p53 and Tax was observed.

Key words: p53 — Tax — HTLV-I — ATL

Adult T-cell leukemia (ATL) is caused by the human retrovirus HTLV-I.[1,2] The virus encodes the unique viral oncogene, *tax*,[3] having potential for co-transformation of primary rodent fibroblasts with the activated *ras* gene[4] and for immortalization of human T lymphocytes.[5] Besides viral infection, multiple genetic change is required for development of the disease.[6] Mutations and some viral oncoproteins inactivate the functions of the p53 tumor suppressor gene, which is pivotal to the neoplastic process in various human cancers and *in vitro* immortalization.[7] Mutation of p53 has been found in ATL at relatively low frequency.[8,9] In human cervical cancer cells, the incidence of p53 mutation has been shown to be rare, relative to the presence of human papilloma virus (HPV).[10] To determine the relation between the p53 gene and *tax* oncogene, we examined p53 mutation and the expression of p53 and Tax in ATL and HTLV-I-infected cells.

The levels of p53 expression were studied in ATL cells (SH, ATL-1 K,[11] MT-1,[12] HUT102[1]) and HTLV-I-infected cells (MT-2,[2] MF-1, MF-3) by immunoprecipitation and immunoblot analysis[13] using 3 monoclonal antibodies, PAb240,[13] PAb421 and PAb1801[14] (Onco-

gene Science, Manhasset, NY). PAb240 reacts with p53 in a mutant conformation but not with the wild type,[13] and PAb421 and PAb1801 react with the C- and N-terminal portions, respectively, of both the wild type and mutant p53. As shown in Fig. 1A, PAb421, PAb1801 and PAb240 immunoprecipitated high levels of p53 from ATL-1K and MT-1 cells, showing enhanced expression of the PAb240-reactive form of p53 (p53-240$^+$) in these cells. The previous study showed both MT-1 and ATL-1K cells to possess missense mutations at codons 176 and 278, respectively.[8] p53 was not detected in SH cells found to lack both p53 alleles by Southern blot hybridization (data not shown). Phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes (PBL) expressed low levels of p53 non-reactive to PAb240 (p53-240$^-$). Enhanced levels of p53-240$^-$ were observed in MT-2 and HUT102 (A and B), while MF-1 and MF-3 cells expressed it at essentially the same levels as PBL (C). Analysis of 5 ATL tumor samples (A2, A11, A12, A13, A14) showed the A13 cells to contain high levels of p53-240$^-$, while no p53 could be found in tumor samples A11, A12, A14 or A2 (data not shown). The A2 cells were previously shown to contain a 3-base in-frame deletion of codon 239 plus a missense mutation at codon 240 (Ser→ Cys).[8] Table I summarizes the conformations and levels of p53 in HTLV-I-infected and ATL cells. Northern blot analysis indicated the levels of p53 mRNA to be basically the same among HTLV-I-infected and ATL cell lines

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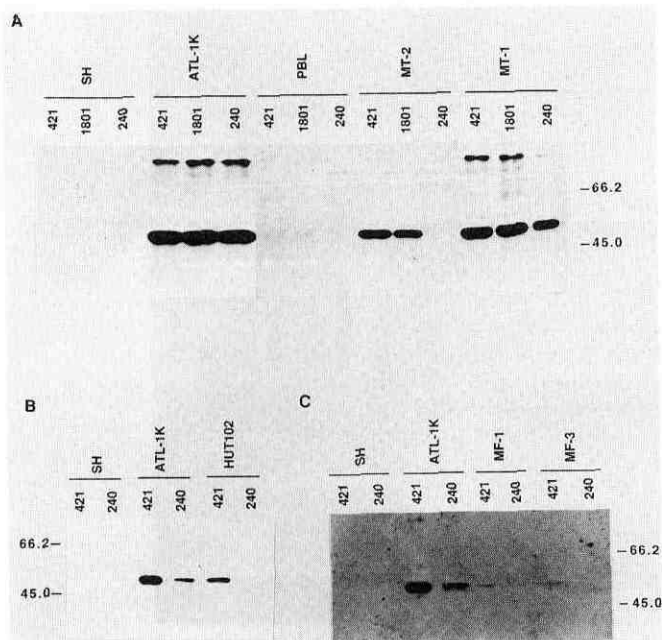
Fig. 1. Immunoblot analysis of p53 immunoprecipitates from ATL and HTLV-I-infected cell lines. p53 was immuno-precipitated with PAb421, PAb1801 and PAb240 from (A) SH, ATL-1K, MT-2, MT-1 and PHA-stimulated PBL, (B) HUT102, (C) MF-1 and MF-3. The immunoprecipitates were immunoblotted and sequentially reacted with a rabbit poly-clonal antibody against p53 (CM-1, Novocastra, Newcastle, UK) and peroxidase-conjugated goat anti-rabbit immuno-globulins.

Table I. p53 and Tax Expression in ATL and HTLV-I-infected Cells

	p53 expression type (% levels[a])		p53 mutation	Tax expression
PBL	240^-	(1)	$-$ [c]	Not tested
HTLV-I-infected cell lines				
MT-2	240^-	(30)	$-$ [b, c]	+
MF-1	240^-	(3)	$-$ [b, c]	−
MF-3	240^-	(2)	$-$ [c]	−
ATL cell lines				
HUT102	240^-	(34)	$-$ [b, c]	+
MT-1	240^+	(71)	$+$, $176^{[d]}$	−
ATL-1K	240^+	(100)	$+$, $278^{[d]}$	−
SH		(0)	$+$, loss of both alleles	−
ATL tumor samples				
A2		(0)	$+$, $239–240^{[d]}$	−
A11		(0)	$-$ [d]	
A12		(0)	$-$ [d]	
A13	240^-	(41)	$+$, $248^{[c]}$	
A14		(0)	$-$ [c]	−

240^+, PAb240-reactive form; 240^-, PAb240-nonreactive form. $+$, detected; $-$, not detected.
a) Densitometric reading of a band of PAb421 immuno-precipitate compared with that of ATL-1K.
b) cDNA PCR-SSCP and c) genomic PCR-SSCP analyses were performed.
d) Genomic PCR-SSCP and DNA sequencing analyses of MT-1, ATL-1K lines and A2, A11, and A12 cells were previously reported.[8]

(MT-2, HUT102, MF-1, MT-1, ATL-1K) irrespective of the levels of p53 and reactivity toward PAb240 (Fig. 2A and B). ATL tumor samples (A2, A13) contained slightly higher levels of p53 mRNA (B). The levels of p53 expression would thus appear to be regulated post-transcriptionally.
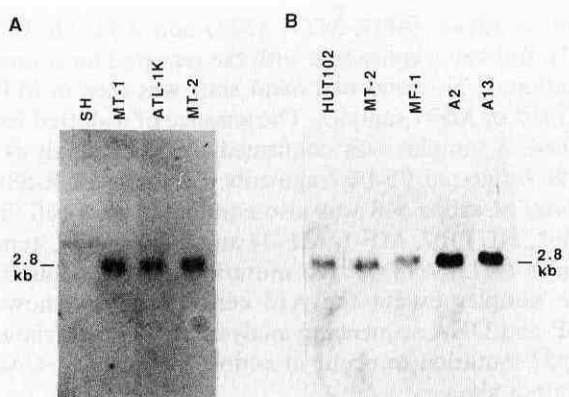
Missense mutation of the p53 gene is one mechanism for stabilizing this labile protein.[7] ATL and HTLV-I-infected cells were analyzed for the mutation by single strand conformation (SSCP) assay.[15] To examine the entire coding region of p53 cDNA sequence, cDNA was synthesized from cytoplasmic RNA and the N-terminal, middle and C-terminal regions of the p53 cDNA were amplified with primers of N1 and N2, P5 and P6, and C1 and C2, respectively (Fig. 3A). Each region was digested with a restriction enzyme to reduce the size of poly-merase chain reaction (PCR)-product suitable for detecting subtle changes by SSCP analysis. To maximize the sensitivity of the assay, at least 2 assays using differ-ent restriction enzymes were conducted for each PCR-amplified region. Genomic PCR SSCP analysis was carried out on exons 5–8 to confirm the results of cDNA SSCP analysis (data not shown). PCR-amplification with

Fig. 2. Northern blot analysis of p53 mRNA in ATL and HTLV-I-infected cells. Cytoplasmic RNA was extracted from (A) SH, MT-1, ATL-1K, and MT-2, (B) HUT102, MT-2, MF-1, and leukemic samples from A2 and A13 cases, Northern-blotted and hybridized with ^{32}P-labeled p53 cDNA from pR4-2.[18]
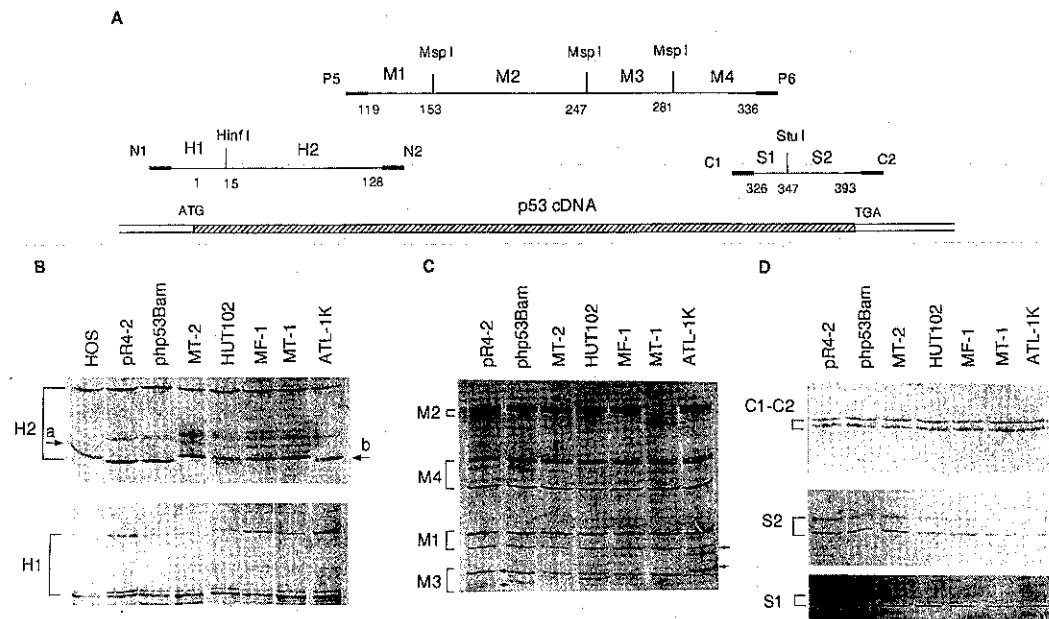
Fig. 3. PCR-SSCP analysis of p53 cDNA. A. Schematic representation of PCR products and restriction enzyme sites. Bold bars show primer locations. Numbers under horizontal bars representing PCR products indicate codons nearest the 3′ ends of primers and restriction enzyme sites. Sequences of PCR primers were as follows: N1, 5′-TTTCCACGACGGTGACACGC-3′ (sense); N2, 5′-GCAAAACATCTTGTTGAGGG-3′ (anti-sense); P5, 5′-GAGATCTTGCATTCTGGGACAGCC-3′ (sense); P6, 5′-GAGATCTCGGAACATCTCTGAAGCG-3′ (anti-sense); C1, 5′-AAGAAGAAACCACTGGATGG-3′ (sense); C2, 5′-CAGTGGGGAACAAGAAGTGG-3′ (anti-sense). B. SSCP analysis of the N-terminal region. Synthesis of cDNA and PCR-amplification was conducted using the GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT). N1-N2 fragments digested by *Hinf* I were analyzed in a 10–15% gradient (H2) and 20% polyacrylamide gels (H1) using the PhastGel system.[15] C. SSCP analysis of P5-P6 fragments. P5-P6 fragments digested with *Msp* I were separated in a 20% polyacrylamide gel. Bands with altered mobility are indicated by arrows. D. Analysis of the C-terminal region. Undigested C1-C2 fragments and *Stu* I-cleaved C1-C2 products were electrophoresed in 10–15% gradient (C1-C2) and 20% polyacrylamide gels (S1, S2), respectively.

N1 and N2 primers produced 466-bp fragments (codons 1–128). The region contained a polymorphism at codon 72,[16] and thus php53Bam (wild type)[17] and pR4-2 clones[18] with a sequence of CCC and HOS cDNA (human osteosarcoma cell line) with CGC at codon 72[19] were used as controls. PCR products were digested with *Hinf* I to produce 2 fragments of 103 bp (H1 fragment, codons 1–14) and 363 bp (H2 fragment, codons 15–128), and analyzed by SSCP using the PhastGel system (Pharmacia, Uppsala, Sweden) as previously described.[15] SSCP analysis failed to show an abnormal band shift for H1 and H2 fragments, but indicated a polymorphism (H2a, H2b) (Fig. 3B). MT-2, MF-1 and MT-1 and ATL-1K DNA showed homozygous bands of H2a and H2b, respectively. HUT102 DNA contained heterozygous bands. The polymorphism and absence of mutation were confirmed by SSCP analysis of *Pfl* MI-, and *Ava* I-cleaved DNA fragments and *Acc* II digestion, which cleaved the sequence of CGC but not CCC at codon 72 (data not shown).

The region encompassing codons 119–356 (699 bp) was PCR-amplified with P5 and P6 primers. Digestion of the PCR products with *Msp* I produced 4 fragments of 125 bp (codons 119–153), 283 bp (codons 154–247), 102 bp (codons 248–281) and 189 bp (codons 282–356), which were designated M1, M2, M3 and M4, respectively.[20] As shown in Fig. 3C, altered band-patterns were found in pR4-2 (M3), MT-1 (M2) and ATL-1K DNA (M3), this being consistent with the reported locations of mutations.[8] No abnormal band shift was seen in MT-2, HUT102 or MF-1 samples. The absence of a shifted band in these 3 samples was confirmed by SSCP analysis of *Sau96* I-digested P5-P6 fragments. Genomic PCR-SSCP analysis of exons 5–8 was also conducted on 4 cell lines (MT-2, HUT102, MF-1, MF-3) and 2 fresh ATL tumor samples (A13, A14).[12] No mutation could be found in these samples except the A13 cells (data not shown). SSCP and DNA sequencing analysis of A13 cells showed the p53 mutation to occur at codon 248 (CGG→CAG) (data not shown).
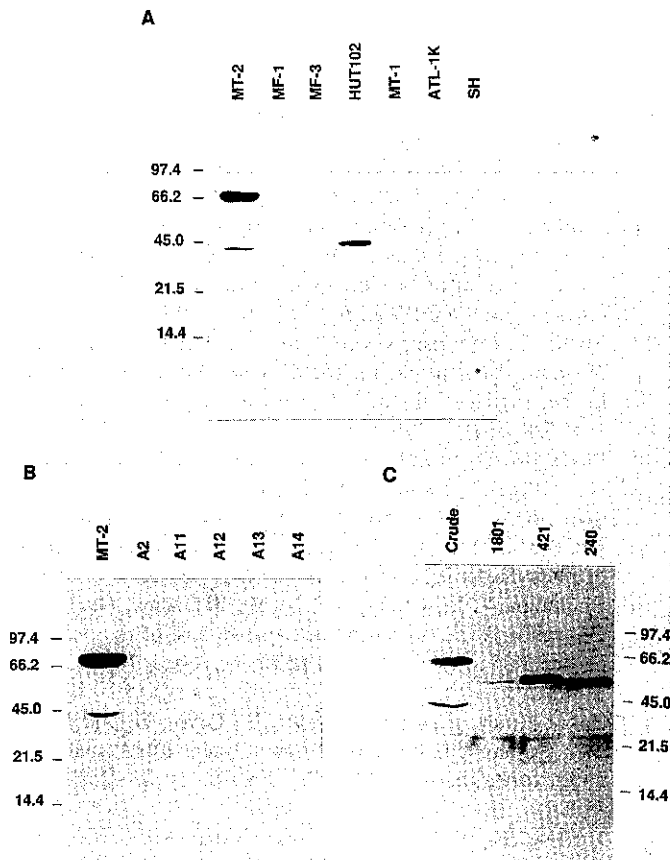
A

B   C

Fig. 4.   Tax expression in ATL and HTLV-I-infected cells. Protein extracts from the cell lines (A) and ATL tumor samples (B) (20 μg each) were separated by 10% SDS-PAGE, immunoblotted and reacted with Lt-4. C Crude protein extract and PAb1801-, PAb421-, and PAb240-immunoprecipitates from MT-2 cells were immunoblotted and probed by Lt-4. The 55 kD band represents heavy chains of mouse IgG.

The C-terminal region (256 bp, codons 326–393) was amplified with C1 and C2 primers. No band shift was found by SSCP analysis of undigested C1-C2 fragments, *Stu* I- (Fig. 3D), or *Ban* II-digested samples (data not shown).

Extensive SSCP analysis failed to detect p53 mutation in MT-2, HUT102, MF-1 or MF-3 cells. The absence of detectable p53 mutation was consistent with low levels of p53-240⁻ in MF-1 and MF-3 cells. However, the mechanisms for increasing p53-240⁻ levels in MT-2 and HUT102 have yet to be determined. Some viral oncoproteins have been found to stabilize wild type p53[7] and thus the levels of Tax expression were assayed by immunoblot analysis using the monoclonal antibody, Lt-4.[21] As shown in Fig. 4A and B, Tax was present in MT-2 and HUT102 cells, but not in low or non-expressers of p53

carrying no detectable p53 mutation or cells with the altered p53 gene (summarized in Table I). By Lt-4, gp68 of HTLV-I was also detected in MT-2 cells.[21] One possibility is that Tax increases the stability of p53 by forming a complex. Immunoprecipitates from MT-2 cells with PAb1801, PAb421 and PAb 240 were therefore immunoblotted and probed with Lt-4; the crude protein extract of MT-2 cells was used as a control (Fig. 4C). Tax was not detected in the p53 immunoprecipitates.

Extensive SSCP analysis did not show the p53 mutation in 3 HTLV-I-infected (MT-2, MF-1, MF-3) and 1 ATL cell lines (HUT102). SSCP assay detects more than 99% of p53 mutations when used in the analysis of 100–300 bp PCR fragments.[22] In this study, at least 2 separate SSCP analyses, each using different restriction enzymes, were conducted on PCR-amplified regions. The region where most mutations occur[7] was examined by SSCP analysis of cDNA and genomic DNA. Our previous study showed that analysis of cDNA and genomic DNA conducted in conjunction facilitated the detection of the p53 mutation, though only a marginal band shift was seen in a single assay.[20] It follows that MT-2, HUT102 and MF-1 cells probably express wild type p53.

The levels of p53 were 30–34 times greater in MT-2 and HUT102 cells and only 2–3 times greater in MF-1 and MF-3 cells than in PHA-stimulated PBL. Several differences were observed between the high (MT-2, HUT102) and low expressers of p53 (MF-1, MF-3); high expressers showed Tax expression and rapid *in vitro* growth independent of interleukin-2 (IL-2), while the low expressers lacked Tax expression and grew slowly, requiring IL-2 (data not shown). p53 mRNA levels were similar in these cells, thus showing that p53 levels are increased in high expressers (MT-2, HUT102) by post-transcriptional mechanisms. Tax may be involved in the stabilization of p53 or, alternatively, the co-expression of Tax and high levels of p53 may be an epiphenomenon. The half life of p53 was reported to be 2 h in HTLV-I-infected cells,[23] this being about 6 times that of human wild type p53 expressed in rat embryo fibroblasts.[7] This may favor the above-mentioned possibility of stabilization of wild type p53 by Tax oncoprotein. However, no complex formation between p53 and Tax was observed. Tax may increase the expression of a cellular gene(s), leading to the stabilization of p53 in a manner similar to that of the SV40 large T antigen.[7]

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