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Frequent Mutations in the p53 Tumor Suppressor Gene in Human Leukemia T-Cell Lines

JIAN CHENG[1] AND MARTIN HAAS[2]*

UCSD Cancer Center, Departments of Pathology[1] and Biology,[2] University of California, San Diego, La Jolla, California 92093

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Human T-cell leukemia and T-cell acute lymphoblastic leukemia cell lines were studied for alterations in the p53 tumor suppressor gene. Southern blot analysis of 10 leukemic T-cell lines revealed no gross genomic deletions or rearrangements. Reverse transcription-polymerase chain reaction analysis of p53 mRNA indicated that all 10 lines produced p53 mRNA of normal size. By direct sequencing of polymerase chain reaction-amplified cDNA, we detected 11 missense and nonsense point mutations in 5 of the 10 leukemic T-cell lines studied. The mutations are primarily located in the evolutionarily highly conserved regions of the p53 gene. One of the five cell lines in which a mutation was detected possesses a homozygous point mutation in both p53 alleles, while the other four cell lines harbor from two to four different point mutations. An allelic study of two of the lines (CEM, A3/Kawa) shows that the two missense mutations found in each line are located on separate alleles, thus both alleles of the p53 gene may have been functionally inactivated by two different point mutations. Since cultured leukemic T-cell lines represent a late, fully tumorigenic stage of leukemic T cells, mutation of both (or more) alleles of the p53 gene may reflect the selection of cells possessing an increasingly tumorigenic phenotype, whether the selection took place in vivo or in vitro. Previously, we have shown that the HSB-2 T-cell acute lymphoblastic leukemia cell line had lost both alleles of the retinoblastoma tumor suppressor gene. Taken together, our data show that at least 6 of 10 leukemic T-cell lines examined may have lost the normal function of a known tumor suppressor gene, suggesting that this class of genes serves a critical role in the generation of fully tumorigenic leukemic T cells.

p53 is a cell cycle-dependent nuclear phosphoprotein (11, 38) whose synthesis and accumulation correlate with cellular DNA synthesis (43). The p53 protein has been conserved during evolution, and the gene encoding it has been isolated from mammals, birds, *Xenopus laevis*, and fish (56). p53 proteins from these diverse species possess five conserved regions of high homology, suggesting that the protein plays an essential role in some basic cellular function. The p53 protein has a short half-life of 10 to 20 min in fibroblasts (22, 49) and of only 6 to 10 min in proliferating T cells (50, 51). In some tumor cells, the half life is extended to 4 to 8 h (25, 46). As a result, many tumor cells harbor concentrations of p53 protein that can be easily detected by immunoprecipitation or Western immunoblotting, while in nontumorigenic cells, p53 often is present in minute concentrations. The high concentration of p53 in tumor cells appears to be mostly due to stabilization of the protein (17, 28). Stabilization of the p53 protein has been shown to result from its complexing with viral proteins, such as simian virus 40 (SV40) large T antigen or adenovirus E1B (37, 38, 53), or from point, deletion, or insertion mutations (25). In the case of the human papillomavirus E6 protein, it has been suggested that binding of E6 protein with p53 may affect the rapid degradation of the p53 protein (60).

Constructs containing mutant p53 cDNAs, but not those containing wild-type p53 cDNAs, have been shown to immortalize rat chondrocytes (33) and rodent fibroblasts (52). Mutant p53 cDNA can complement EJ H-*ras* in the transformation of embryo fibroblasts (13, 15, 28, 32, 33, 47), whereas wild-type p53 cDNA constructs can act as suppressors of transformation in oncogene-mediated focus formation experiments (14, 16). Recent evidence (14, 16) suggests that the wild-type p53 gene is a negative regulator of cell proliferation whose inactivation is required for neoplastic transformation. In tumor cells, mutant p53 might exert a dominant negative effect (26, 27) by the formation of hetero-oligomers with the wild-type gene product (13, 16, 21, 52) though this has not been directly proven.

Missense mutations capable of modulating the cell cycle regulatory functions of p53 are frequently found in human neoplasia. The p53 protein has acquired a long half-life (which is most likely due to mutation) in significant numbers of human mammary carcinomas (6, 9), colon carcinomas (58), and leukemia/lymphomas (35, 40, 54). Allelic loss of the p53 locus which maps to human chromosome 17p13 has been found in carcinomas of the human breast (40), colon (3), and lung (57, 62); in astrocytomas (31); and in blast crisis of chronic myeloid leukemias (2, 4, 34, 41). In some cases, the remaining, nondeleted p53 allele was shown to be mutated (3). Complete sequencing of the p53 gene cDNA has been performed on 24 human colon, breast, and brain cancers, revealing missense point mutations in many of them (45).

The unusually rapid turnover of the p53 protein in proliferating normal T cells suggests that it serves a central, albeit unknown, role in T-cell physiology. Human leukemia cells show an increased expression of the p53 protein (35, 48), suggesting that the p53 gene may be mutated in these cells. Therefore, we decided to examine the state of the p53 gene in leukemic T cells and T-cell acute lymphoblastic leukemia (T-ALL) cells. While the leukemic T-cell lines showed no detectable genomic rearrangements at the p53 locus and produced p53 message of normal size, direct sequencing of polymerase chain reaction (PCR)-amplified p53 cDNA revealed missense and nonsense mutations in conserved regions of the gene in 5 of 10 cell lines. Previously, we have

* Corresponding author.

shown that the HSB-2 T-ALL cell line had lost both alleles of the retinoblastoma (Rb) gene (7). Taken together, our data show that 6 of 10 leukemic T-cell lines examined possess mutations in the p53 coding sequence or have lost part of the Rb coding sequence (7), suggesting that the class of tumor suppressor genes serves a critical role in the generation of fully tumorigenic leukemic T cells.

MATERIALS AND METHODS

Cell lines. Ten human leukemic T-cell lines were used in this study: the T-ALL lines Molt-4 (44), CEM (18), HSB-2 (1), BE-13 (19), 8402 (30), Jurkat (55), and Molt-16 (12); the malignant T-cell lymphoma line A3/Kawa (29); the undifferentiated T-cell lymphoblastoid line DU.528 (36); and the cutaneous T-cell lymphoma line HUT78 (20). The cells were grown in suspension culture in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. The T-ALL lines have the following number of chromosomes. Molt-4, CEM, BE-13, and 8402 were near-tetraploid with 4 apparently normal copies of chromosome 17 (7), HSB-2 is essentially diploid (7), Jurkat is tetraploid with 4 apparently normal no. 17 chromosomes (55), and Molt-16 has 45 chromosomes with multiple abnormalities but apparently normal no. 17 chromosomes. HUT78 is subtetraploid with multiple abnormalities but apparently normal no. 17 chromosomes (M. Minden, personal communication); DU.528 has an approximately normal number of chromosomes, with several translocations but normal no. 17 chromosomes (36); and A3/Kawa has 46 to 48 chromosomes and many cytogenetic abnormalities but no apparent abnormalities in no. 17 chromosomes (J. Minowada, personal communication). The two CEM sublines have 46 versus 88 to 92 chromosomes. Both lines are female and share an identical human leukocyte antigen type, suggesting that the tetraploid line originated by tetraploidization in culture.

Southern blot analysis. Southern blots were done as described previously (7).

RT-PCR. Total RNA was extracted from cultured cells by using the guanidinium-thiocyanate method (8). To synthesize single-stranded cDNA, 5 μg of RNase-free DNase-treated total RNA was mixed with 1 μg of oligo(dT) primer, 30 U of avian myeloblastosis virus reverse transcriptase (Seikaguku America, Inc.), and 40 U of RNasin (Promega Biotec, Madison, Wis.) in reaction buffer (100 mM Tris hydrochloride [pH 8.0], 80 mM KCl, 10 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates) and incubated for 90 min at 42°C. RNA was then hydrolyzed in 0.5 N NaOH for 30 min at 70°C, and single-stranded cDNA was precipitated with ethanol. The cDNA pellet was washed with 70% ethanol and dissolved in TE buffer. One-tenth of the cDNA was taken to do each amplification reaction, under conditions suggested by Perkin Elmer Cetus. Eight primers covering the whole p53 gene-coding region were used in the reverse transcription (RT)-PCR analysis. They include primer 1, 5′-GGA ATTCATGGAGGAGCCGCAGTCAGA-3′ (contains *Eco*RI site); primer 2, 5′-GTTCTAGAATGCAAGAAGCCCAGA C-3′ (contains *Xba*I site); primer 3, 5′-GGAATTCCGTCTG GGCTTCTTGCA-3′ (contains *Eco*RI site); primer 4, 5′-TCG GATCCAACCTCAGGCGGCTCATA-3′ (contains *Bam*HI site); primer 5, 5′-GGAATTCACTTTTCGACATAGTGTG-3′ (contains *Eco*RI site); primer 6, 5′-TTCTGCAGTGCTCG CTTAGTGCTCC-3′ (contains *Pst*I site); primer 7, 5′-GGAA TTCCACGAGCTGCCCCCAGGGA-3′ (contains *Eco*RI site); and primer 8, 5′-AGAAGCTTCTGTCTTGAACATGAG-3′ (contains *Hin*dIII site). Thirty-five cycles of denaturation (93°C, 1 min), annealing (at calculated temperature), and extension (72°C, 1 min) were carried out in a thermal cycler (Ericomp, San Diego, Calif.). One-tenth of each PCR reaction was analyzed by electrophoresis in 1.2 to 1.5% agarose gels.

Asymmetric PCR and direct sequencing. By using reverse transcriptase-generated cDNA as template, amplification reactions were carried out with 50 pmol of primer 3 and 1 pmol of primer 6 or 1 pmol of primer 3 and 50 pmol of primer 6. The reactions were subjected to 35 cycles, each consisting of 1 min at 94°C, 1 min at 63°C, and 90 s at 72°C. After the reaction, the 100 μl of PCR product was mixed with 100 μl of 4 M ammonium acetate and 200 μl of 2-propanol, left at room temperature for 10 min, and then spun for 10 min in a microcentrifuge. The pellet was washed with 70% ethanol and dissolved in TE buffer; then 20 to 50% of the precipitated DNA was subjected to sequence analysis by using primers 3, 4, 5, and 6 as the sequencing primers. Sequencing reactions were performed by using the dideoxy chain termination method.

RESULTS

Leukemic T cells show no gross genomic p53 rearrangements and express normal-sized p53 mRNA. High-molecular-weight DNA extracted from the 10 human leukemic T-cell lines was digested to completion with the restriction enzymes *Eco*RI, *Bam*HI, *Hin*dIII, and *Bgl*II; separated on agarose gels; blotted; and hybridized with a ^{32}P-labeled probe made from a 2.1-kilobase (kb) human complete p53 cDNA insert purified from the plasmid php53BAM (63). DNA from all 10 cell lines showed the normal genomic p53 hybridizing patterns when digested with *Eco*RI, *Bam*HI, or *Hin*dIII (data not shown). However, when digested with *Bgl*II, seven cell lines showed the common 12-kb and 3.7-kb bands; two cell lines showed the 12-kb, the 3.7-kb, as well as a 9-kb band (Fig. 1, lanes 2 and 7); and one cell line was homozygous for the 9-kb and 3.7-kb bands (Fig. 1, lane 11).

Previous studies (5) have demonstrated that in approximately 10% of human DNA samples, there exists a polymorphic allele in which an additional *Bgl*II site is found in the first noncoding p53 large intron, situated 3 kb downstream from the first *Bgl*II site. This polymorphism results in the loss of a 3-kb intronic fragment from the 12-kb *Bgl*II restriction fragment.

To detect smaller rearrangements which might go undetected in Southern blot analyses and to examine expression of the p53 gene in these leukemic T-cell lines, we used the RT-PCR method. Total RNA was prepared from each cell line, and single-stranded cDNA was synthesized with reverse transcriptase. Subregions of the p53 cDNA were then specifically amplified by using four pairs of overlapping PCR primers, covering the entire p53 cDNA coding region (Fig. 2). Each of the 10 cell lines studied showed the amplified fragments of p53 cDNA (Fig. 3). For each of the four pairs of PCR primers, the amplified fragments were of the expected size and migrated on agarose gels with mobilities identical to those of cDNA fragments amplified from RNA isolated from activated normal human peripheral blood T lymphocytes (7). Thus, each of the 10 leukemic T-cell lines examined expressed all of the sequences encoded by a normal human p53 gene and apparently synthesized normal-length p53 message. Similar RT-PCR analyses were performed on RNA extracted from HL-60 cells (61), which lack the p53 gene. The control experiment did not give rise to amplified p53 products (data not shown). In summary, the 10 leukemic

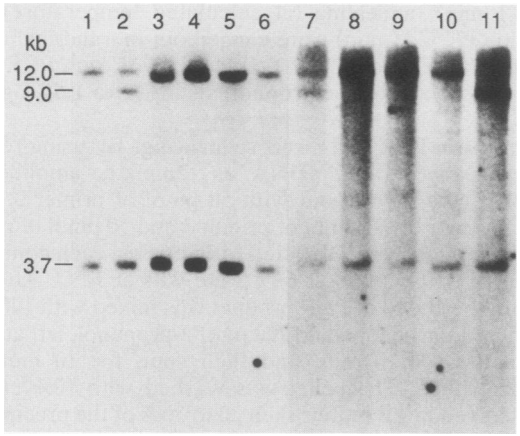

FIG. 1. Southern blot analysis of DNA extracted from 10 leukemic T-cell lines. DNAs (10 μg each) extracted from a normal human placenta (lane 1) and from the leukemic T-cell lines CEM (lane 2), Molt-4 (lane 3), HSB-2 (lane 4), 8402 (lane 5), BE-13 (lane 6), A3/Kawa (lane 7), DU.528 (lane 8), HUT78 (lane 9), Jurkat (lane 10), and Molt-16 (lane 11) were digested with restriction endonuclease *Bgl*II and transferred to nitrocellulose filters after electrophoresis in a 1% agarose gel. Filters were then hybridized with a ^{32}P-labeled complete p53 cDNA probe. The hybridized blot showed the 12-kb and 3.7-kb bands and a 9-kb polymorphic band. The CEM line (lane 2) and the A3/Kawa line (lane 7) are heterozygous, while Molt-16 (lane 11) is homozygous for the polymorphic *Bgl*II site.

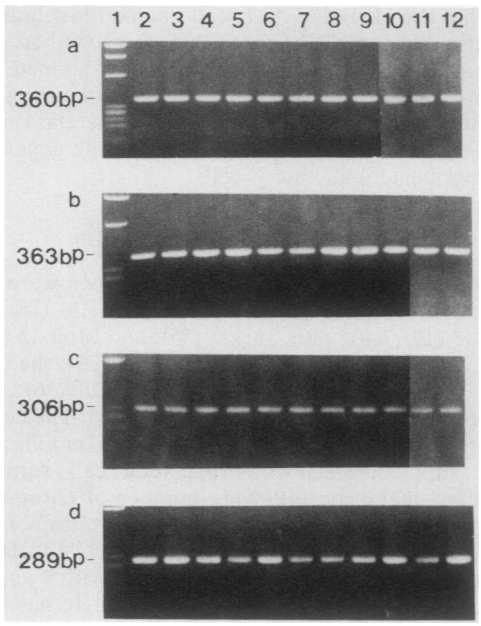

FIG. 3. Transcription of the p53 gene in T-cell leukemias, as analyzed by RT-PCR analysis. Total RNAs extracted from 10 leukemic T-cell lines and from activated human peripheral blood lymphocytes as normal control were reverse transcribed into cDNAs and amplified into four overlapping domains by PCR by using four pairs of primers. Aliquots of the PCR products were analyzed in agarose gels by using the following pairs of PCR primers: primers 1 and 2, generating 360-bp fragments (a); primers 3 and 4, generating 363-bp fragments (b); primers 5 and 6, generating 306-bp fragments (c); and primers 7 and 8, generating 289-bp fragments (d). Lane 1, Size markers of ΦX174 RF DNA-*Hae*III fragments; lane 2, human peripheral blood lymphocytes; lane 3, CEM; lane 4, Molt-4; lane 5, HSB-2; lane 6, 8402; lane 7, BE-13; lane 8, A3/Kawa; lane 9, DU.528; lane 10, HUT78; lane 11, Jurkat; lane 12, Molt-16.

T-cell lines examined lacked detectable genomic deletions of the p53 gene and transcribed the p53 gene into apparently normal-sized mRNA.

Nucleotide sequence analysis reveals frequent mutations of the p53 gene. Since both Southern blot and RT-PCR analyses failed to demonstrate any rearrangements or deletions in the p53 gene of the leukemic T-cell lines, we tested for point mutations in the p53 gene by sequence analysis of p53 cDNA. Comparison of the human, mouse, frog, and *Xenopus laevis* p53 sequences has revealed five highly conserved regions of amino acid sequence (56). In vitro studies have shown that point, deletion, and insertion mutations in the conserved regions of the murine p53 gene activate its ability to complement EJ H-*ras* in transformation assays of rodent embryo fibroblasts (17, 28). Furthermore, p53 mutations in human tumors are also mostly found in the evolutionarily conserved regions of the p53 gene (3, 45). Our sequence analysis of p53 cDNA in the leukemic T cells therefore focused on the conserved regions of the p53 gene.

For the asymmetric PCR reactions, we used unequal molar concentrations of amplification primers 3 and 6 (Fig. 2) to generate a 609-base pair (bp) single-stranded DNA fragment covering a region from codon 109 to codon 308, with cDNA as templates. The 609-bp fragment was sequenced by using primers 4 and 6 to sequence the sense strand and primers 3 and 5 to sequence the antisense strand (Fig. 2). A total of 11 point mutations were found among five cell lines

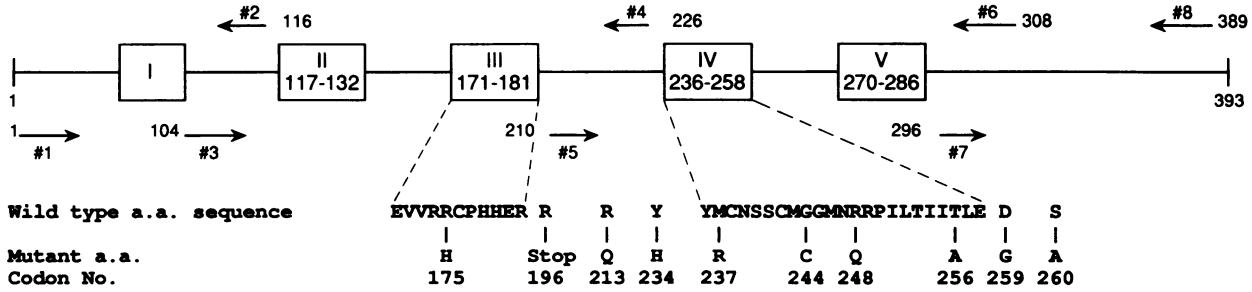

FIG. 2. Diagram of the structure of p53 cDNA. The five boxes represent the five domains of evolutionarily conserved amino acid sequences. The amino acid (a. a.) sequences from domains III and IV are shown. The eleven missense point mutations found in this study are indicated, as are the corresponding amino acid substitutions. The arrows show the approximate locations of the eight PCR primers used. The arrowheads point in the direction of nucleotide synthesis. The numbers refer to p53 cDNA codon numbers. The mutation in codon 196 was found both in the HUT78 and in the Jurkat cell lines (Table 1).

TABLE 1. p53 gene mutations in human T-cell leukemias

Cell line	Codons sequenced	Mutation site (codon)	Mutation nucleotide	Amino acid substitutions	Genotype
CEM	133-287	175	CGC→CAC	Arg→His	Heterozygous
		248	CGG→CAG	Arg→Gln	Heterozygous
A3/Kawa	135-304	213	CGA→CAA	Arg→Gln	Heterozygous
		234	TAC→CAC	Tyr→His	Heterozygous
Molt-16	126-301	237	ATG→AGG	Met→Arg	Heterozygous
		244	GGC→TGC	Gly→Cys	Heterozygous
Jurkat	126-306	196	CGA→TGA	Arg→Stop	Heterozygous
		256	ACA→GCA	Thr→Ala	Heterozygous
		259	GAC→GGC	Asp→Gly	Heterozygous
		260	TCC→GCC	Ser→Ala	Heterozygous
HUT78	135-294	196	CGA→TGA	Arg→Stop	Homozygous
Molt-4	135-296		ND[a]		
HSB-2	129-280		ND		
BE-13	124-282		ND		
DU.528	124-301		ND		
8402	131-294		ND		

[a] ND, Not detected.

(Table 1). All of the mutations are missense or nonsense mutations, and the majority of them are located in the highly conserved domains III and IV; the rest either fall between conserved domains III and IV or lie immediately downstream of domain IV (Fig. 2). One mutation, in codon 196, was found in two cell lines, HUT78 and Jurkat. A unique feature of the direct sequencing method is that it not only detects homozygous mutations, but it also is capable of identifying heterozygous mutations. Figure 4a shows the wild-type nucleotide sequence at codon 196(CGA), Fig. 4b shows a heterozygous point mutation (CGA→CGA/TGA) and Fig. 4c shows a homozygous point mutation at the same codon 196 (CGA→TGA).

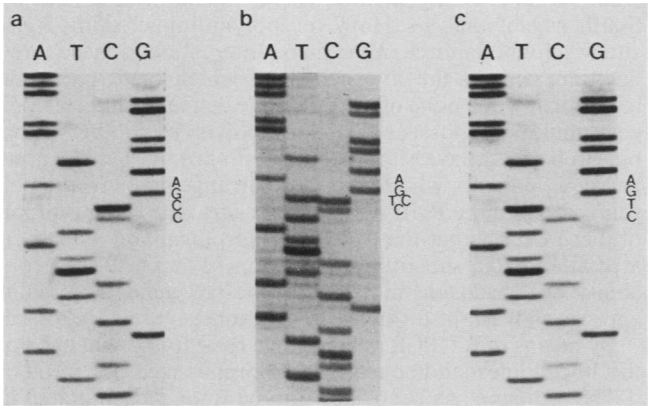

FIG. 4. Examples of direct PCR nucleotide sequence analyses showing a wild-type sequence, a heterozygous mutation, and a homozygous mutation of the same p53 codon in three different cell lines. Total RNA extracted from Molt-16, Jurkat, and HUT78 cells was reverse transcribed and amplified by the PCR by using 1 pmol of primer 3 and 50 pmol of primer 6. Each PCR product was precipitated with 2-propanol, and 20 to 50% of the precipitated DNA was subjected to sequence analysis using primer 5 as a sequence primer. (a) Molt-16 cells showing a wild-type sequence at codon 196 (CGA); (b) Jurkat cells showing a heterozygous mutation at codon 196 (CGA→CGA/TGA); (c) HUT78 cells showing a homozygous mutation at codon 196 (CGA→TGA).

Both alleles in CEM and A3/Kawa cells are mutated in p53. Among the 5 cell lines that were found to possess point mutations in p53, HUT78 is the only line that has one single, homozygous point mutation; the other four lines possess heterozygous mutations. Since some of the lines are tetraploid or subtetraploid and possess multiple cytogenetic abnormalities, the mutations in p53 that we have found might be located on one or on different alleles in each cell line. To address this question, we studied the CEM and A3/Kawa cell lines as examples. We have studied two different CEM sublines; one is essentially tetraploid, and the other is diploid. Both lines have cytogenetically normal no. 17 chromosomes (M. Haas and M. Bogart, unpublished data).

cDNA sequencing has shown that both CEM cell lines have two identical heterozygous mutations located in codon 175 (CGC→CGC/CAC) and in codon 248 (CGG→CGG/CAG) (Fig. 5a and b, respectively). The mutation in codon 175 abolishes an *Hha*I restriction site, while the mutation in codon 248 abolishes an *Msp*I restriction site. By using primer pairs 1 and 8, the p53 gene coding region was generated by PCR with CEM cDNA as template. The PCR-generated CEM-derived cDNA was digested with the restriction enzymes *Eco*RI and *Hin*dIII (which cut in primers 1 and 8, respectively) and subcloned into a pGEM-3Zf(−) vector. Plasmid DNA of 10 individual pGEM-CEM p53 cDNA clones was amplified by PCR by using primer pairs 3 and 4 (covering a region containing codon 175) and 5 and 6 (covering a region containing codon 248). cDNA prepared from activated human peripheral blood lymphocytes was similarly amplified as a wild-type control.

The 363-bp PCR fragments generated by primer pair 3 and 4 were digested with the restriction enzyme *Hha*I. As shown in the diagram (Fig. 5e), digesting the wild-type fragment with *Hha*I should result in bands of 153, 49, 18, and 143 bp, and digesting the mutant cDNA fragment with *Hha*I should result in the generation of bands of 153, 67, and 143 bp because of the loss of an *Hha*I site. The 306-bp PCR fragments generated by primer pair 5 and 6 were digested with restriction enzyme *Msp*I. The wild-type PCR fragment should give rise to three bands of 118, 102, and 86 bp (Fig.

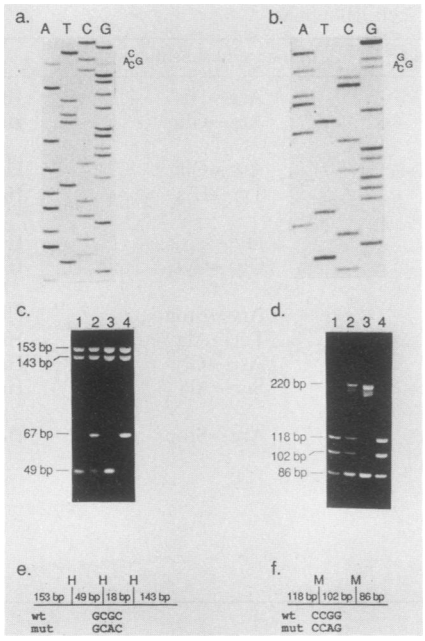

FIG. 5. Sequence analysis and allelic study of the CEM cell line. Total RNA extracted from CEM cells was reverse transcribed into cDNA and amplified by PCR by using 1 pmol of primer 3 and 50 pmol of primer 6. The PCR product was precipitated with 2-propanol, and 30% of the precipitated DNA was used for sequence analysis. (a) Heterozygous mutation at codon 175 (CGC→CGC/CAC) detected by using primer 3 as a sequence primer; heterozygous mutation at codon 248 (CGG→CGG/CAG) detected by using primer 5 as a sequence primer. CEM cDNA was amplified by PCR by using primers 1 and 8 and subcloned into the pGEM-3Zf(−) vector. Ten clones were picked and grown up. DNA of pGEM-CEM clones as well as cDNA of CEM cells and control cDNA from human peripheral blood lymphocytes was amplified by PCR. The resulting PCR products were digested with restriction enzymes, fractionated in 8% polyacrylamide gels, stained with ethidium bromide, and photographed under UV light. (c) DNA fragments amplified by primers 3 and 4 digested with *Hha*I; (d) DNA fragments amplified by primers 5 and 6 digested with *Msp*I. Lane 1, Human peripheral blood lymphocytes; lane 2, CEM cells; lane 3, pGEM-CEM clone 1; lane 4, pGEM-CEM clone 2. (e) Diagram of the 363-bp wild-type PCR fragment digested with *Hha*I; (f) diagram of the 306-bp wild-type PCR fragment digested with *Msp*I. wt, Wild type sequence; mut, mutant sequence.

5f), and the mutant form should only show two bands, of 220 and 86 bp, because of the loss of an *Msp*I site. Figure 5c and d show that p53 cDNA amplified from human peripheral blood lymphocytes (Fig. 5c and d, lanes 1) is wild type at both codons 175 and 248, while p53 cDNA amplified from CEM cells (Fig. 5c and d, lanes 2) is heterozygous at both codons 175 and 248.

To test whether the two point mutations in CEM cells reside on the same or on separate alleles, 10 pGEM-CEM p53 cDNA clones were studied. Each clone represents one copy of p53 cDNA from the CEM cell line. If both point mutations (at codons 175 and 248) are present on the same cDNA clone, there should be p53 cDNA clones that are wild type at both sites, and thus the mutations in question would be present on the same allele in CEM cells. If, on the other hand, the two point mutations were located in CEM cells on different alleles, p53 cDNA clones should each be mutant at either site, and there might not be a wild-type p53 allele in CEM cells. Two of ten pGEM-CEM p53 cDNA clones analyzed are shown in Fig. 5c and d. Clone 1 (Fig. 5c and d, lanes 3) is wild type at codon 175 (it has not lost the *Hha*I site) but is mutant at codon 248, as it has lost the *Msp*I site. Clone 2 (Fig. 5c and d, lanes 4) is mutant at codon 175 and has the wild-type sequence at codon 248. Among the 10 pGEM-CEM p53 cDNA clones studied, all were mutated either at codon 175 or at codon 248. Thus, both alleles of the CEM cell line have been mutated by different point mutations. Clones that were wild type at both locations were not found, suggesting that CEM cells carry two mutant p53 alleles and lack a wild-type p53 allele.

To exclude the possibility that the heterozygous mutations found in the CEM cells were due to the presence of more than one cell population in the CEM cell line, we cloned CEM cells by endpoint dilution. Each of six CEM single-cell clones tested possessed the same heterozygous point mutations at the genomic level (data not shown), suggesting that our CEM cell line is composed of a single population of cells and not a mixture of cells that differ at the p53 locus.

P53 cDNA from A3/Kawa cells was amplified in a method similar to that described above, subcloned, and isolated. Since one of the two heterozygous mutations found in this line (Table 1) did not alter a restriction enzyme site, the cDNA clones were directly sequenced. Of the five molecular clones sequenced, three had the wild-type sequence at codon 213 and the mutated sequence at codon 234, while two had the mutated sequence at codon 213 and the wild-type sequence at codon 234 (data not shown). No cDNA clones were found that were mutated or wild type at both codons 213 and 234. This indicates that like those found in the CEM cells, both alleles of the A3/Kawa cells possess a mutation in the p53 gene and the cells lack a wild-type allele.

DISCUSSION

By using Southern blot and RT-PCR analysis, we have found no gross rearrangements of the p53 gene in any of the 10 leukemic T-cell lines studied. This is in agreement with a study (59) of 80 patients with acute and chronic leukemias who had no rearrangement of the p53 gene, as analyzed by Southern blot analysis. However, polymorphism of the *Bgl*II site was found in three of the lines. Interestingly, these three lines are among the five cell lines which have p53 gene mutations, while none of the other five cell lines that lack p53 gene mutations possess this *Bgl*II polymorphic site. Other researchers (5) have shown that most chronic myelogenous leukemia patients who exhibited rearrangements in the p53 gene harbored the *Bgl*II polymorphic site. One interpretation of these data is that the *Bgl*II polymorphism and its associated amino acid substitution at codon 72 increase the probability of subsequent mutation of the p53 gene. This point appears to warrant further investigation.

By means of RT-PCR analysis, we have found that each of the 10 cell lines studied expresses normal-sized p53 mRNA.

The p53 gene has been shown to be frequently mutated in human solid tumors in the highly conserved region between codons 130 to 280 (3, 45). To look for mutations in this region of the leukemic T-cell lines, we used RT, asymmetric PCR, and direct sequencing methods. Direct sequencing of the PCR products has important advantages over sequencing of molecularly cloned cDNA. It has the capability of identifying two (or more) alleles in a heterozygous cell. Eight of the mutations found (Table 1) were repeatedly detected in both the sense and the antisense strands, and three others were found in two or more repeat-sequencing experiments, thus ruling out spurious PCR-induced mutations (23).

Our study shows that leukemic T cells express messages from both (or more) alleles, although different alleles may be expressed at different levels. Unequal levels of expression of different alleles may result in the generation of different concentrations of cDNA in the RT-PCR reaction, resulting in some cases in the lack of detection of a poorly expressed allele in the subsequent sequencing analysis. This was evident in our study, since some of the heterozygous mutations were detected in most, but not in all, independent repeats of PCR amplification and subsequent direct sequencing, with the same source of RNA.

The point mutations found in this study were located in or close to the evolutionarily highly conserved regions III or IV of the p53 gene, and each caused amino acid substitutions or a stop codon. Amino acid substitutions in the conserved regions of the p53 gene have been shown to convert it from an ostensible tumor suppressor-type gene into a transforming oncogene (17, 28). This suggests that the mutations that we have found in leukemic T-cell lines may contribute to the tumorigenic phenotype of the cells. While we have not yet tested whether the specific mutations found indeed endow the mutated alleles with transforming potential, we propose that mutations in p53 may play a role in the genesis or in the tumorigenic progression of leukemic T cells.

The missense and nonsense mutations found in five leukemic T-cell lines may have occurred either in vivo or during establishment of the cells in culture. The great majority of human leukemic T-cell lines have been established from patients in relapse. As we have proposed previously (24), relapse may involve the loss of a normal tumor suppressor function; hence, only cells that have lost a normal tumor suppressor function can be established (immortalized) in vitro. This scenario would argue that the mutations in p53 that we have found would have arisen in vivo, which is supported by our recent finding of p53 mutations in primary samples of T-ALL patients (J. Cheng, A. Yu, and M. Haas, unpublished data). The notion that mutations in p53 may occur in leukemic cells in vivo is also supported by the findings that in chronic myelogenous leukemia, the blast crisis is closely associated with alterations in the p53 gene (2, 34). It is thus quite conceivable that the p53 gene may continue to mutate in culture, and thus the results presented in this communication may be due to the combined effect of mutations that have occurred in vivo and in vitro.

Among the five leukemic T-cell lines that possess p53 mutations, only the HUT78 cell line has a homozygous mutation. The signals on Southern blots (Fig. 1 and data not shown) do not indicate an allelic loss in the HUT78 cell line, suggesting that following the loss of the wild-type allele, a duplication of the chromosome with the mutated allele took place. Alternatively, HUT78 cells might only express the mutant allele, while the other allele might not be expressed or might be expressed at a low level. Since the mutation at codon 196 creates a new *Dde*I restriction site (Table 1), we used the *Dde*I restriction enzyme to digest PCR-amplified genomic DNA of HUT78 cells. HUT78 DNA is completely cleaved by *Dde*I at codon 196, indicating the absence of a wild-type allele in HUT78 cells.

To address the question of whether the heterozygous mutations described here are localized on the same or on separate alleles, p53 cDNAs from CEM and A3/Kawa cells were subcloned and each clone was examined. We found that the two point mutations in CEM cells and in A3/Kawa cells are located on separate alleles. Thus, both p53 alleles of the CEM and the A3/Kawa lines may have been functionally inactivated by two different mutations, suggesting that the fully tumorigenic phenotype of leukemic T-cell lines is associated with the mutational inactivation of both (or more) p53 alleles.

TABLE 2. p53 and retinoblastoma gene abnormalities in leukemic T-cell lines

Cell line	p53 mutation[a]	Rb[b] protein[a]
CEM	+	−
A3/Kawa	+	−
Molt-16	+	−
Jurkat	+	−
HUT78	+	+
Molt-4	−	−
HSB-2	−	+
BE-13	−	−
DU.528	−	−
8402	−	−

[a] +, Positive for p53 mutation or for absence of $pp110^{RB}$; −, no p53 mutation detected or retinoblastoma protein expressed.
[b] Rb, Retinoblastoma.

Previously, we have demonstrated that the HSB-2 T-ALL cell line has a homozygous deletion of the retinoblastoma tumor suppressor gene (7). In the current experiments, we have found that the HUT78 line failed to express $pp110^{RB}$ (Table 2). Thus, HUT78 cells have a mutated p53 gene and lack $pp110^{RB}$. Taken together, at least 60% of the leukemic T-cell lines examined had lost the normal function of one or two tumor suppressor genes, suggesting that this class of genes serves a critical role in the generation of fully tumorigenic leukemic T cells. One would expect that the leukemic T-cell lines that harbor both wild-type p53 and wild-type retinoblastoma genes would be defective at other as yet unidentified tumor suppressor loci.

Further study is called for to see whether p53 mutation is also a common event in primary leukemic T-cell samples. Study of the p53 proteins in these T-cell leukemias also appears to be essential. Finally, it would be important to reintroduce the wild-type p53 gene into cell lines which have p53 gene mutations to assess the potential tumor-suppressive abilities of the normal p53 gene.

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