

Role of *p53* Mutations in Endocrine Tumorigenesis: Mutation Detection by Polymerase Chain Reaction-Single Strand Conformation Polymorphism¹

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ABSTRACT

To elucidate the molecular basis for endocrine tumorigenesis, *p53* mutations in human endocrine tumors were analyzed by using polymerase chain reaction-single strand conformation polymorphism. Exons 5 through 10 of the *p53* gene were studied in genomic DNAs from 134 primary endocrine tumors and 6 human endocrine cancer-derived cell lines. Mutations were detected and identified in 4 endocrine tumors, including one parathyroid adenoma and three thyroid carcinoma cell lines. The sites of these mutations were in exons 5 (codon 151 and 152) and 7 (codon 248 and 255). In all of three tumor cell lines, but not in a parathyroid adenoma, the normal allele encoding the *p53* gene was lost. However, *p53* mutations were not found in any other endocrine tumors or cell lines. Based upon these results, we concluded that the *p53* gene may play a role in the tumorigenesis of a limited number of parathyroid adenoma and thyroid cancers, and that the *p53* mutation with an allelic loss of the *p53* gene is an important factor in malignant tumorigenesis of the thyroid gland.

INTRODUCTION

It is widely accepted that both activation of protooncogenes and inactivation of tumor suppressor genes are involved in the tumorigenesis of various human tumors. However, the molecular and genetic basis for tumorigenesis in the relatively well-differentiated endocrine glands is not well understood. Aberrations of oncogenes including *ras*, *ret*, *gsp*, and *gip2* genes in only a few number of human endocrine tumors have been recently reported (1-3). The allelic loss of chromosome 11 has been identified in pituitary, parathyroid, and endocrine pancreatic tumors associated with multiple endocrine neoplasia type 1 (4-7), as well as in sporadic parathyroid adenomas (5). Our previous studies on thyroid tumors and pheochromocytomas failed to identify the segmental deletion of any chromosomes with a significant incidence (8, 9). Thus the molecular basis for tumorigenesis of the majority of endocrine tumors remains to be elucidated.

The *p53* gene acts as a tumor suppressor similar to the *Rb* gene, and its mutation is one of the most common genetic abnormalities in cancers (10). For example, *p53* mutations have been found in over one-half of breast, lung, and bladder cancers examined (11). On the other hand, *p53* mutations in endocrine tumors have been identified, to our knowledge, in a human follicular thyroid carcinoma cell line (12) and undifferentiated thyroid carcinomas (13).

In this study, we systemically screened *p53* mutations in 134 human endocrine tumors and 6 endocrine tumor-derived cell

lines to elucidate the molecular basis for endocrine tumorigenesis. The samples included tumors of the pituitary, thyroid, parathyroid, endocrine pancreas, adrenal cortex, and medulla. Exons 5 through 10 of the *p53* gene, *i.e.*, the regions encompassing the majority of *p53* mutations found in human cancers, were amplified by PCR,³ and screened by SSCP (14) for *p53* mutations.

MATERIALS AND METHODS

Human Tissue Samples. One hundred and thirty-four specimens from patients with endocrine tumors were studied. Seventy-nine were frozen samples and 55 were formalin-fixed and embedded in paraffin. The human endocrine tumors consisted of 27 pituitary adenomas, 55 thyroid tumors (22 follicular adenomas, 23 papillary carcinomas, 1 poorly differentiated papillary carcinoma, 8 medullary carcinomas, 1 anaplastic carcinoma), 13 parathyroid tumors (8 hyperplasia, 4 adenomas, 1 carcinoma), 11 endocrine pancreatic tumors (9 adenomas, 2 carcinomas), 11 adrenocortical adenomas, and 17 pheochromocytomas. Primary tumors together with the adjacent noncancerous tissue, or peripheral blood leukocytes, were obtained at the time of surgery.

Cells. Six cell lines established from human endocrine cancers, including 5 from thyroid and 1 from adrenocortical cancers, were studied. Two cell lines, 8505C and SW579 (15), were derived from poorly differentiated adenocarcinoma of the thyroid. Two cell lines, TCO-1 (16) and HTC/C3 (17), were derived from anaplastic carcinoma of the thyroid. One cell line, TT (18), was derived from medullary carcinoma of the thyroid. The cell line SW-13 (19) was derived from adenocarcinoma of the adrenal cortex. Two cell lines, 8505C and TCO-1, were supplied by the Japanese Cancer Research Resources Bank. The cell line HTC/3C was supplied by the RIKEN Cell Bank (Tsukuba, Japan). Three cell lines, SW579, TT, and SW-13, were supplied by the American Type Culture Collection. All cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum.

DNA Extraction. High molecular weight DNA was prepared from tissues and cell lines by proteinase K digestion and phenol/chloroform extraction as previously described (7). Genomic DNA was isolated from tissues embedded in paraffin as previously described (3). Briefly, the tumor regions in the tissues were located by microscopic observation of hematoxylin and eosin-stained samples. Sections 10 μ m in width were scraped from a glass microscope slide by a razor blade, placed into an Eppendorf centrifuge tube, deparaffinized by washing twice in xylene, twice in 95% ethanol, and finally dried under reduced pressure. The sample was treated with proteinase K (200 mg/liter) in 100 μ l of digestion buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5% Tween 20), at 37°C overnight. After inactivating proteinase K by incubating at 95°C for 10 min, the samples were used directly for PCR analysis.

PCR-SSCP Analysis. Oligonucleotide primers were synthesized by the phosphoramidite method by using a 392 DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). A set of PCR primers, flanking each exon of 5, 6, 7, 8, 9, or 10, were used to generate a single specific amplification product from each sample (20). The nucleotide sequence of the primers are summarized in Table 1. PCR was performed with the use of a thermal cycler (Astek, Fukuoka, Japan) with 50 ng of genomic DNA in a total volume of 5 μ l containing 0.5 μ l of [α -³²P]dCTP (3000 Ci/mmol; 10 mCi/ml) (21). Thirty cycles consisting of 1 min at 95°C for

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³ The abbreviations used are: PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism.

Table 1 Oligonucleotide primers (5'-3')

Exon	Upstream	Downstream
5	TTCCTCTTCTGTCAGTACTC	CAGCTGCTCACCATCGCTAT
6	CACTGATTGCTCTTAGGTCT	AGTTGCAAACCAGACCTCAG
7	GTGTTATCTCCTAGGTTGGC	CAAGTGGCTCCTGACCTGGA
8	CCTATCCTGAGTAGTGTTAA	TCCTGCTTGCTTACCTCGCT
9	TTGCTCTTTTCTAGCACTG	CCCAAGACTTCGTACTCTGAA
10	CTCTGTTGCTGCAGATCCGT	GCTGAGGTCACTCACCTGGA

denaturation, 1 min at 55°C for annealing, and 2 min at 72°C for extension, were performed. The PCR mixture (5 µl) was heated to 80°C with 245 µl of formamide dye mixture (95% formamide:20 mM EDTA:0.05% xylene cyanol; 0.05% bromophenol blue), then 1 µl of the mixture was applied to a 6% polyacrylamide gel containing 45 mM Tris-borate (pH 8.3) and 4 mM EDTA (14). Glycerol (5%) was also added when specified. Electrophoresis was performed at 40 W for 2 to 4 h with cooling by a fan at room temperature or at 4°C. The gel was dried on filter paper and exposed to an X-ray film for 10–24 h at –80°C with an intensifying screen. PCR-SSCP was repeated twice to ensure that the results were reproducible in each case showing a mobility shift.

Direct Sequencing. Abnormal bands detected by SSCP analysis were excised from the dried gel, placed in 100 µl of distilled water, and incubated at 37°C. An aliquot (1–3 µl) of the supernatant was then used as a DNA template in the PCR reaction. For direct sequencing, the primers were the same as those used in the original amplification, except that the 5' end of one primer contained M13, and the other contained the M13 reverse sequence. The double-stranded PCR product resulting from this amplification was purified by two centrifugal washes by using a SUPREC-2 microconcentrator (Takara Biomedicals, Kyoto, Japan). DNA sequences of the PCR products were determined by fluorescence-based dideoxy sequencing, using *Taq* polymerase in a thermal cycler, and fluorescently labeled M13 universal or reverse sequencing primers, followed by gel electrophoresis, data collection, and analysis on an Applied Biosystems Model 373A automated sequencer (Applied Biosystems, Inc.).

RESULTS

Screening of Point Mutations of the p53 Gene by PCR-SSCP. PCR-SSCP analysis of exon 5 from genomic DNA of a parathyroid adenoma, as shown in Fig. 1, disclosed extra bands with altered migration relative to those amplified from the patient's leukocytes. These extra bands were not found in the PCR

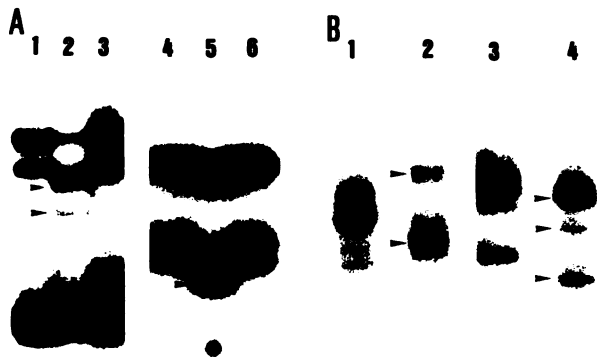


Fig. 1. PCR-SSCP analysis of exons 5 and 7 in p53 genomic DNA. DNA was PCR amplified with the primers for exon 5 or 7 of the p53 gene. Exons 5 and 7 are shown in A and B, respectively. A, Lane 1, patient leukocytes; Lane 2, parathyroid adenoma; Lane 3, papillary thyroid carcinoma; Lanes 4 and 6, normal leukocytes; and Lane 5, anaplastic thyroid carcinoma cell line HTC/C3. B, Lane 1, normal leukocytes; Lane 2, poorly differentiated thyroid carcinoma cell line 8505C; Lane 3, normal leukocytes; Lane 4, poorly differentiated thyroid carcinoma cell line SW579. Electrophoresis was performed in a 6% polyacrylamide gel without glycerol (A, Lanes 1–3, and B, Lanes 1 and 2) or with 5% glycerol (A, Lanes 4–6, and B, Lanes 3 and 4) both at the room temperature. Arrowheads denote the bands with altered migration relative to controls.

product from papillary thyroid carcinoma excised simultaneously from the same patient. Extra bands were also observed in PCR-amplified exon 5 from the anaplastic thyroid carcinoma cell line, HTC/C3 (Fig. 1A, Lane 5). PCR-SSCP of exon 7 from two other poorly differentiated thyroid carcinoma cell lines, 8505C and SW579, revealed several extra bands. In these 3 cell lines, there were no bands with migration similar to that of normal controls (Fig. 1A, Lane 5, 1B, Lanes 2 and 4), suggesting the loss of a normal p53 allele. This was confirmed by directly sequencing genomic DNA, of which the sequence of exon 5 from an anaplastic thyroid carcinoma cell line, HTC/C3, is shown in Fig. 2A, Panel 2 (other data not shown). In other endocrine tumors or cell lines, no extra bands with altered migration were detected by PCR-SSCP of exons 5 through 10 of the p53 gene.

Sequencing of the p53 Gene. Exons 5 and 7 of one parathyroid adenoma and three thyroid carcinoma cell lines with extra bands in SSCP analysis, were sequenced. As shown in Fig. 2, p53 mutations were identified at 4 different sites. In the parathyroid adenoma, codon 151 of CCC for proline was mutated to TCC for serine by a C to T transition of the first letter (Fig. 2A, Panel 1). In the anaplastic thyroid carcinoma cell line,

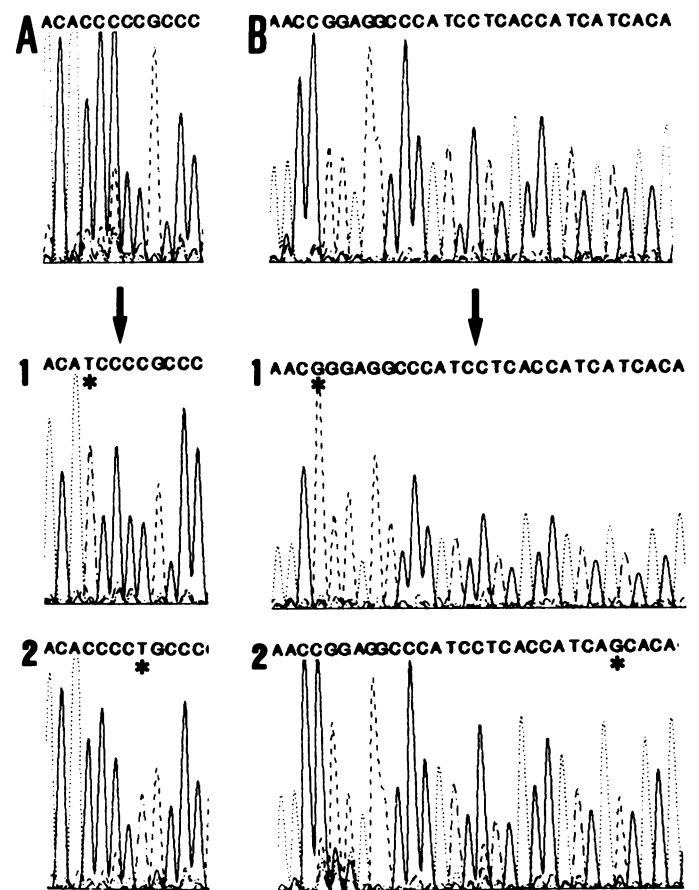


Fig. 2. Nucleotide sequence analysis of p53 genomic DNA. DNA fragments that showed a mobility shift on SSCP and DNA from normal leukocytes were PCR amplified and sequenced. A, sequences of variant SSCP alleles from a parathyroid adenoma (Panel 1) and the genomic direct sequence of the anaplastic thyroid carcinoma cell line HTC/C3 (Panel 2). The normal sequence of codons 150–153 is shown at the top; the middle and the bottom panels show the C to T transitions at codons 151 (Panel 1) and 152 (Panel 2), respectively. Mutated bases are indicated by *. B, sequence of the variant alleles of the poorly differentiated thyroid carcinoma cell lines 8505C (Panel 1) and SW579 (Panel 2). The normal sequence of codons 247–256 are shown at the top; the middle and the bottom panels show the C to G transversion at codon 248 (Panel 1) and the T to G transversion at codon 255 (Panel 2). Mutated bases are indicated by *.

HTC/C3, codon 152 of CCG for proline was mutated to CTG for leucine by a C to T transition of the second letter (Fig. 2A, Panel 2). In the poorly differentiated thyroid adenocarcinoma cell line, 8505C, the codon 248 of CGG for arginine was mutated to GGG for glycine by a C to G transversion of the first letter (Fig. 2B, Panel 1). In another poorly differentiated thyroid adenocarcinoma cell line, SW579, codon 255 of ATC for isoleucine was mutated to AGC for serine by a T to G transversion of the second letter (Fig. 2B, Panel 2). The 4 different amino acid changes that resulted in one parathyroid adenoma and three thyroid carcinoma-derived cell lines are summarized in Table 2.

DISCUSSION

Tumors, including cancers, are assumed to result from an accumulation of genetic alterations that disrupt normal cell growth and terminal differentiation. The mutational changes which are most commonly observed and presumed as the molecular basis for human cancerogenesis are point mutations of the p53 tumor suppressor gene (11). The sites for p53 mutations are dispersed to the relatively larger regions of over several hundred base pairs but are still limited mostly to exons 5 through 8 (11), therefore, most studies on p53 mutations were focused on these exons. Because over 95% of all p53 mutations have been located in exons 5 through 8, mutation screening of the p53 gene in exon 5 through 10 performed in this study should have covered the majority of p53 mutations.

Mutations in the p53 gene occur relatively late in the course of carcinogenesis in common nonendocrine cancers such as colon and gastric cancers as well as hepatomas (22-24). Although the presence of p53 mutations in human cancers does not signify the degree of malignancy and resulting prognosis of cancer, it generally correlates with the advanced stage. Contrary to this general rule, the fact that p53 mutations were also present in colon adenomas suggests that they also play a role in tumorigenesis preceding the later, and accordingly more malignant carcinoma stage (25, 26). In our study, a p53 mutation was found in one functioning parathyroid adenoma, but not in the thyroid papillary carcinoma excised simultaneously from a 53-year-old man. This p53 mutation in the parathyroid adenoma was found among 13 parathyroid tumors examined, which included 8 hyperplasia, 4 adenomas, and 1 carcinoma. These results suggest that the p53 mutation, although its incidence is rare in parathyroid tumors, may play a similar role in the tumorigenesis of parathyroid gland, to that in colon adenoma.

Recently Ito *et al.* (13) reported that p53 mutations were detected in 6 of 7 undifferentiated thyroid carcinomas, whereas p53 mutations were not detected in 10 differentiated thyroid papillary carcinomas, and suggested that p53 mutations played a crucial role in the progression to undifferentiated carcinomas.

This is inconsistent with the report by Wright *et al.* (12) that p53 mutations were not detected in 20 undifferentiated carcinomas. They detected p53 mutation in only one follicular thyroid carcinoma cell line. In our study, 1 of 3 anaplastic thyroid carcinomas (1 primary tumor and 2 cell lines) and 2 of 3 poorly differentiated thyroid carcinomas (1 primary tumor and 2 cell lines) showed p53 mutations, whereas p53 mutations were not found in 53 differentiated thyroid tumors (22 follicular adenomas, 23 papillary thyroid carcinomas, and 8 medullary thyroid carcinomas). These results suggest p53 mutations play a role in malignant progression.

The aberrations of the p53 gene so far reported in malignancies include a loss of one of the two alleles associated with a subtle structural change of a remaining allele. This type of aberration was detected in three thyroid carcinoma cell lines by PCR-SSCP and direct sequencing. All of the 3 cell lines established from malignant thyroid carcinomas having p53 mutations were associated with the allelic loss of the p53 gene, whereas only 1 parathyroid adenoma was heterozygous for the mutant and the wild-type intact p53 gene. Because no loss of heterozygosity was found at the 17p locus in primary thyroid tumors using p53 complementary DNA as a probe in our previous study (8), an allelic loss of the p53 gene seems not to occur at the significant frequency in endocrine tumors. Although it is possible that the genetic changes of the p53 gene in thyroid carcinoma cell lines have resulted from an *in vitro* selection culture, the mutation of the p53 gene with a concomitant allelic loss apparently determines malignant tumorigenesis of the thyroid gland leading to the formation of thyroid carcinoma.

In summary, our study has shown the following. (a) The incidence of p53 mutations in endocrine tumors is low. There were only 4, in 1 parathyroid adenoma and 3 thyroid carcinoma cell lines, of 134 human endocrine tumors and 6 endocrine carcinoma-derived cell lines examined. (b) The p53 mutation with an allelic loss of the p53 gene may be an important factor in malignant tumorigenesis of the thyroid gland, due to its presence in 3 of 5 thyroid cancer cell lines examined. To further explore the molecular basis for endocrine tumorigenesis in the remaining majority without the p53 mutations, other oncogene mutations including *ras*, *ret*, *gsp*, or *gip2* must be systemically studied.

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Table 2 Mutations of the p53 gene in primary endocrine tumors and cancer-derived cell lines

Sample	Codon	Nucleotide change	Amino acid change
Primary tumor			
Parathyroid adenoma	151	CCC → TCC	Pro → Ser
Cell line			
HTC/C3 (anaplastic thyroid carcinoma)	152	CCG → CTG	Pro → Leu
8505C (poorly differentiated thyroid carcinoma)	248	CGG → GGG	Arg → Gly
SW579 (poorly differentiated thyroid carcinoma)	255	ATC → AGC	Ile → Ser

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