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p53 Mutations in Human Adrenocortical Neoplasms: Immunohistochemical and Molecular Studies

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ABSTRACT

The mechanisms of tumorigenesis of adrenocortical neoplasms have not been elucidated as yet. However, loss of heterozygosity at chromosomal locus 17p has been consistently observed in adrenocortical cancer. p53 is a recessive tumor suppressor gene located on chromosome 17p. Mutations in the p53 gene play an important role in the tumorigenesis of diverse types of human neoplasms including breast and colon cancers. More than 90% of all mutations discovered in such tumors have been detected in 4 hot spot areas that lie between exons 5 and 8. In contrast to wild-type p53, mutant p53 accumulates intracellularly and can be easily detected by immunohistochemistry. We therefore investigated the frequency of p53 mutations in human adrenocortical neoplasms using molecular biology and immunohistochemistry techniques. Five patients with adrenocortical adenomas (5 female; ages 39–72 yr), 11 patients with adrenocortical carcinomas (8 female, 3 male; ages 15–50 yr), and two adrenocortical tumor cell lines were studied. After DNA extraction from frozen tumor tissue or paraffin-embedded material, exons 5 through 8 were amplified using the polymerase chain reaction and directly sequenced by the dideoxy termination method. Immunohistochemistry was performed on paraffin-embedded tumor specimens obtained during adrenalectomy using a monoclonal antibody reacting with both wild-type and mutant p53. Prevalence of mutations was adenomas, 0/5, carcinomas, 3/11, and adrenocortical cell lines, 2/2. Single point mutations were detected in 3 cases (exons 5, 6, and 7, respectively), and rearrangements of exon 7/8 and 8 were found in 2 cases. Immunohistochemistry detected strong nuclear and/or cytoplasmic p53 immunoreactivity in all adrenocortical carcinomas with point mutations of the p53 gene but not in adenomas and carcinomas with the wild-type sequence or with deletion/rearrangement of the p53 gene. We conclude that p53 plays a role in the tumorigenesis of adrenocortical carcinomas but is of less importance to benign adenomas. (*J Clin Endocrinol Metab* **78:** 790–794, 1994)

BENIGN adrenocortical adenomas are a frequent incidental finding in people over 50 yr of age with a prevalence of 0.5–2.0% (1). In contrast, adrenocortical carcinomas are rare and highly malignant tumors with an incidence of 1:1.7 million per year (2). The molecular and genetic basis of tumorigenesis and malignant transformation of adrenal tumors is not well understood.

The p53 tumor suppressor gene, located on 17p (3), encodes a 53-kilodalton, 393-amino acid nuclear phosphoprotein involved in the control of cellular proliferation (4). Acquired mutations in p53 are common tumor-specific genetic changes in humans, having been identified in most major cancer types (5). A variety of point mutations and deletions of p53 have been described in human tumors (6). Allelic loss of 17p is often accompanied by point mutations at four highly preserved areas within exons 5 to 8 of the remaining copy of the gene, thus eliminating all wild-type p53 (7). Because oncogenic p53 mutations increase the stability of p53 and result in elevated intracellular steady-state protein levels, p53 mutations may be identified by immunohistochemistry using p53 overexpression as a marker (8).

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Two lines of evidence suggest a role of p53 in the tumorigenesis of adrenocortical neoplasms. First, loss of heterozygosity at chromosomal locus 17p has been consistently demonstrated in adrenal carcinomas but not in adrenal adenomas (9). Second, germline mutations in p53 were recently identified in families with the Li-Fraumeni syndrome (10), a rare familial syndrome with an unusually high incidence of diverse cancers including adrenocortical carcinomas (11). We thus undertook a study to determine whether mutations in the p53 gene are involved in the tumorigenesis of sporadic adrenocortical neoplasms, using immunohistochemistry and molecular biology techniques.

Materials and Methods

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed paraffin-embedded tumor sections using the monoclonal antibody PAb 1801 (Ab 2, Oncogene Science, Manhasset, NY), which reacts specifically with an epitope between amino acids 32 and 79 detecting both wild-type and mutant p53 (8). Briefly, 5 μm sections were enzymatically digested with 0.1% trypsin and incubated with horse serum to block nonspecific binding of immunoglobulin G. After washing in phosphate buffered saline, sections were incubated with the primary antibody at a concentration of 10 μg/mL at room temperature for 60 min. Specific bound antibody was visualized by avidin-biotin complex immunoperoxidase staining according to the instructions of the manufacturer. Control sections were incubated with serially diluted primary antibody and with a negative control monoclonal antibody supplied by the manufacturer.

Positive staining was revealed as brown color. Hematoxylin was used as nonspecific counterstaining.

Isolation of DNA from tissue specimens

Adrenocortical tumor tissue was obtained at adrenalectomy from 16 patients. DNA was isolated from 11 adrenocortical carcinomas and 5 adrenocortical adenomas (clinical data, Table 1). In 7 cases, tumor DNA was extracted from paraffin-embedded sections, which were accumulated over a 30-year period. In 9 cases, both frozen (−80 C) tumor tissue and corresponding normal leukocyte DNA were studied. In addition, the DNA of the 2 currently available human adrenocortical tumor cell lines was studied. SW13, a nonsecretory human adrenocortical tumor cell line maintained in Ham's F 10 medium with 10% fetal bovine serum, was received from ATCC. NCI-H-295, a human steroid-producing adrenocortical tumor cell line maintained in HITES's medium with 2% fetal bovine serum, was kindly provided by Dr. E. Gazdar (12).

DNA preparation

DNA was isolated from paraffin-embedded specimens (13), leukocytes, or frozen tumor sections obtained at surgery (14), as previously described. Tissue samples were derived from a central portion of the tumor to minimize the possibility of contamination with normal tissue. Representative sections of the tumor material were examined by light microscopy and shown to consist of more than 90% tumor cells.

For paraffin-embedded specimens, 10-μm sections were carefully cut and placed in sterile Eppendorf tubes. New gloves, sterile forceps, and a microtome blade cleaned with xylene between each sample were used for every tumor block to avoid cross contamination and to minimize preparation artifacts (13). All paraffin-embedded tissue samples had been fixed in buffered formalin. In a previous study we have shown that polymerase chain reaction (PCR) products of 600-base pair length could be successfully amplified with these techniques using the same tumor blocks but a different oncogene (15).

PCR amplification of genomic DNA

PCR (16) was used to amplify DNA fragments including exons 5 through 8, which contain the four hot spot regions accounting for more than 90% of all mutations found in human tumors. Aliquots of 1 and 10 μL DNA preparation from paraffin-embedded tissues or 1 μg DNA from frozen tissue or leukocyte DNA were amplified in a 100 μL PCR mixture containing deoxynucleotide triphosphates (200 μmol/L each), upstream and downstream oligonucleotide primers (0.3 mmol/L each), 0.01% gelatin, 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 1.5 mmol/L $MgCl_2$, and 2.5 U Taq polymerase (Perkin Elmar Cetus, Norwalk, CT). The following primers were used: exon 5: 5′-TGT TCA CTT GTG CCC TGA CT-3′ and 5′-AGC AAT CAG TGA GGA ATC AG-3′; exon 6: 5′-TGG TTG CCC AGG GTC CCC AG-3′ and 5′-GGA GGG CCA CTG ACA ACC A-3′; exon 7: 5′-CTT GCC ACA GGT CTC CCC AA-3′ and 5′-AGG GGT CAG CGG CAA GCA GA-3′; exon 8: 5′ TTG GGA GTA GAT GGA GCC T-3′ and 5′-AGT GTT AGA CTG GAA ACT TT-3′ (17, 18). Special precautions were taken to avoid contamination of the PCR samples. In all PCR experiments, samples containing no DNA were included as negative controls. The amplification protocol consisted of denaturation at 95 C for 5 min followed by 30–40 cycles consisting of annealing at 55 C for 1½ min, primer extension at 72 C for 1½ min and denaturation at 94 C for 1 min. All amplified samples were examined by agarose gel electrophoresis to confirm successful amplification of each of the exons of p53.

Direct sequencing of the PCR product

Single-stranded DNA was synthesized using as template the product of the symmetric PCR described above. For the amplification of only one DNA strand selectively, a nested sense or an antisense primer (50 pmol) was added, and the asymmetric PCR was carried out for 20 cycles (19). Single-stranded DNA was separated from nonincorporated nucleotides and primers by filtration through Ultrapure MC filters (Millipore, Bedford, MA). The retentate was lyophilized at room temperature and redissolved before use in the DNA sequencing reaction. The following nested primers were used: exon 5: 5′-TTC AAC TCT GTC TCC TTC CT-3′ and 5′-CAG CCC TGT CGT CTC TCC AG-3′; exon 6: 5′-GCC TCT GAT TCC TCA CTG AT-3′ and 5′-TTA ACC CCT CCT CCC AGA GA-3′; exon 7: 5′-AGG CGC ACT GGC CTC ATC TT-3′ and 5′-TGT GCA GGG TGG CAA GTG GC-3′; exon 8: 5′-TTC CTT ACT GCC TCT TGC TT-3′ and 5′-AGG CAT AAC TGC ACC CTT GG-3′ (18). Using the dideoxy-chain-termination method of Sanger (20), the PCR product was sequenced (T7-DNA polymerase, Sequenase 2.0, United States Biochemicals, Cleveland, OH) with [α^{35}S]-dATP and run on a 6% denaturing polyacrylamide gel. Autoradiography was performed with Kodak XAR 2 films (Kodak, Rochester, NY).

To exclude cross contamination of mutated genes in the reaction buffers, primers, or genomic DNA preparation, each mutation was confirmed by sequencing both a second PCR sample and the coding and noncoding strands of the products.

Results

Expression of p53 in adrenocortical neoplasms

No p53 protein was detected by immunohistochemistry in any of the adrenocortical adenomas, in normal adrenal cortex adjacent to the adenomas (n = 3), or in 6 of 11 (55%)

TABLE 1. Clinical data of the patients studied

Patient no.	Sex	Age	Clinical presentation	Tumor size (cm)	Histology	DNA source
1	F	15	Cushing's syndrome, virilization	25 × 17 × 8	Liver metast., adrenal carcinoma	Frozen tissue
2	F	24	Nonsecretory tumor	1 × 2	Lung metast., adrenal carcinoma	Frozen tissue
3	F	14	Cushing's syndrome, virilization	7 × 4 × 2	Metastatic adrenal carcinoma	Frozen tissue
4	F	50	Conn syndrome	9 × 8 × 7	Adrenal carcinosarcoma	Frozen tissue
5	M	23	Virilization	6 × 5 × 2	Metastatic adrenal carcinoma	Paraffin
6	M	27	Feminization	36 × 21 × 15	Metastatic adrenal carcinoma	Paraffin
7	F	25	MEN I, Cushing syndrome	9 × 8 × 5	Adrenal carcinoma	Paraffin
8	F	29	Nonsecretory tumor	8 × 6 × 1	Brain metast., adrenal carcinoma	Paraffin
9	F	22	Cushing's syndrome	12 × 6 × 10	Adrenal carcinoma	Paraffin
10	F	27	Virilization	17 cm diam.	Adrenal carcinoma	Paraffin
11	M	37	Feminization	30 × 16 × 10	Metastatic adrenal carcinoma	Paraffin
12	F	72	Nonsecretory tumor	4 × 2 × 2	Adrenocortical adenoma	Frozen tissue
13	F	40	Conn syndrome	2 × 3 × 3	Adrenocortical adenoma	Frozen tissue
14	M	67	Cushing's syndrome	2.5 cm diam.	Adrenocortical adenoma	Frozen tissue
15	F	39	MEN I, Cushing's syndrome	4 × 4 × 3	Adrenocortical adenoma	Frozen tissue
16	F	49	Cushing's syndrome	2.5 cm diam.	Adrenocortical adenoma	Frozen tissue

F, female; M, male; metast., metastasis; diam., diameter.

TABLE 2. Mutational spectrum of p53 in human adrenocortical carcinomas and cell lines

Patient no.	p53 overexpression (immunohistochemistry)	DNA source	Mutation	Exon	Codon	Nucleotide exchange	Amino acid exchange
SW13	nd	Cell line	Point mutation	6	193	CAT → TAT	His → Tyr
NCI-H-295	nd	Cell line	Insertion	8			
4	++	Frozen tissue	Point mutation	7	248	CGG → TGG	Arg → Trp
5	++	Paraffin	Point mutation	5	151/152	CCC → GCC	Pro → Ala
						CCG → GCG	Pro → Ala
6		Paraffin	Deletion/rearrangment	7/8			

Arg, arginine; Trp, tryptophan; Pro, proline; His, histidine; Tyr, tyrosine; nd, not determined.

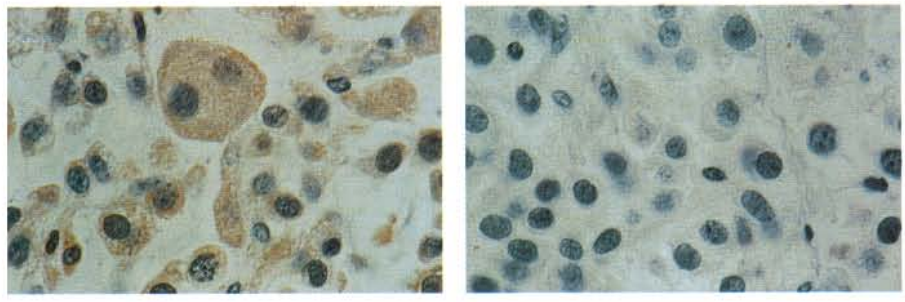

FIG. 1. (*Left*) p53 overexpression in an adrenocortical carcinoma (×20) showing strong cytoplasmic staining. (*Right*) Negative control section (×20).

carcinomas. Three adrenal cancer specimens (27%) had detectable cytoplasmic or nuclear p53 immunoreactivity but in only a small percentage of the malignant cells (<10%). Positively stained cells in this category were generally localized in one area of the section but were not closely clustered. Two carcinomas (18%; one metastatic adrenal carcinoma, one adrenosarcoma) exhibited strong cytoplasmic/nuclear p53 immunoreactivity throughout the tumor, suggesting very high levels of p53 expression in these tissues (Fig. 1).

Sequence of p53 exon 5 to 8 in adrenocortical tumors/cell lines

None of the adrenal adenomas had p53 mutations in exons 5 through 8. In contrast, 3 of 11 adrenal carcinomas (27%) and both adrenocortical tumor cell lines had p53 mutations of the highly conserved regions within exons 5–8. In three cases a point mutation was detected, which altered the coding region of the gene (Table 2 and Fig. 2). The mutations were confirmed by sequencing a second PCR sample of each tumor. In each case the mutation caused a nonconservative amino acid substitution resulting in the production of an abnormal p53 protein with a probably altered half-life and biological function. In two cases, the genomic sequence of p53 was grossly altered resulting in absence or truncation of the p53 protein. In one carcinoma, exon 7 was completely deleted and could not be amplified by means of PCR. Exon 8 of the same tumor showed a large 900-base pair insertion resulting in the amplification of an abnormal DNA fragment by PCR. In the second case, the NCI-H-295 adrenal cancer cell line, a 1200-base pair insertion was found in exon 8 (Fig. 3). The rearrangement of the p53 gene in this case was confirmed by Southern blot.

Discussion

Tumorigenesis has been assumed to result from molecular alterations that disrupt normal cell growth and terminal

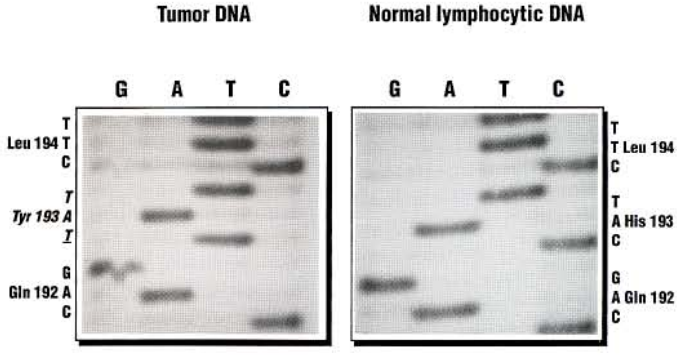

FIG. 2. (*Left*) Sequencing analysis of an amplified genomic fragment (exon 7) of the p53 gene from the adrecortical carcinoma cell line SW 13, showing a point mutation at codon 193. (*Right*) Lymphocyte DNA from a normal subject containing only the wild-type sequence of p53 for comparison.

differentiation. Mutations in the p53 tumor suppressor gene are common genetic alterations in human cancers and have been found mainly in 4 hot spot regions within exons 5 to 8 and between amino acids 132 and 286 (5, 6). Wild-type p53 is expressed in normal cells and appears to be involved in the control of cellular growth and proliferation, whereas alterations in the p53 gene, such as point mutations, allelic loss, rearrangements, and deletions result in tumor formation. Mutant p53 has transforming activity *in vitro* and fails to bind to the simian virus 40 tumor antigen (21). However, the mutants do form stable complexes with hsp70 (22), a constitutively expressed member of the heat shock protein family, and this may be the reason for the increased half-life and elevated steady-state levels of the mutant proteins in tumor cells (8).

Few studies have investigated the role of p53 in endocrine

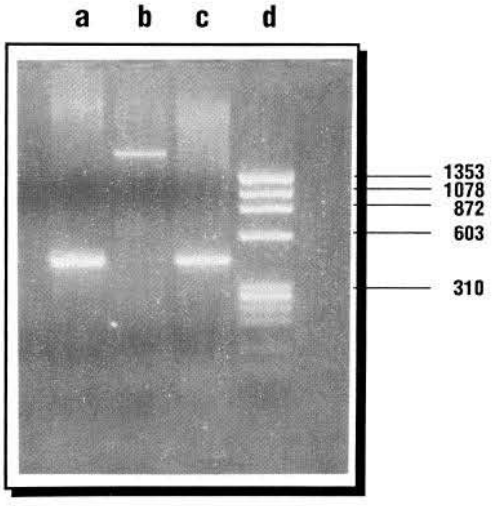

FIG. 3. PCR amplification of exon 8 showing an abnormal DNA fragment in the adrenocortical cancer cell line NCI H 295 (*lane b*) compared to normal amplification products in lymphocyte DNA from a normal subject or the adrenocortical cancer line SW13 (*lanes a and c*, respectively). *Lane d:* size marker ϕX174.

neoplasms. Yoshimoto *et al.* (23) studied 134 primary endocrine tumors, including pituitary adenomas, thyroid and parathyroid tumors, endocrine pancreatic tumors, pheochromocytomas, and adrenocortical adenomas using single-strand conformation polymorphism analysis. According to their results, p53 mutations play a role in a limited number of parathyroid adenomas and thyroid carcinomas but not in adrenocortical neoplasms. Interestingly, Yoshimoto *et al.* (23) did not detect a mutation in the SW 13 adrenal carcinoma cell line, which was shown in this study to have a mutation at codon 193 changing histidine to tryptophan. This difference may be explained by a lower sensitivity of single-strand conformation polymorphism analysis in detecting point mutations compared to direct sequencing of the PCR amplification product which was used in this study. Recently, Fagin *et al.* (24) reported a high incidence of p53 mutations in anaplastic thyroid carcinomas but not in more differentiated thyroid tumors. We identified mutations in 3 of 11 (27%) adrenocortical carcinomas and in both carcinoma cell lines. We found point mutations at codons 151/152, 193, and 248, which were close to or within the four highly conserved regions of the gene and have been previously reported in other human cancers. These mutations resulted in nonconservative amino acid substitution, likely to be of functional significance. In two other cases, large insertions or rearrangements of exons 7 and 8 were observed, altering the reading frame of the coding sequence of p53, resulting probably in the generation of altered mRNAs with absence or truncation of the p53 protein.

As expected, both adrenocortical tumor specimens containing point mutations showed p53 overexpression by immunohistochemistry, demonstrating a good correlation between the immunohistochemical data and the DNA abnormality. In contrast, in the carcinoma with a grossly abnormal p53 gene (case 6) p53 immunoreactivity was not detectable by immunohistochemistry, probably due to lack of p53 expression or expression of a truncated protein with altered immunogenity. The other tumors with scattered or absent p53 immunoreactivity showed only the wild-type sequence of p53.

Whereas p53 mutations are important in the carcinogenesis of adrenocortical cancer, they do not seem to play a major role in the formation of adrenal adenomas. It was recently suggested that G proteins, membrane-bound heterotrimeric polypeptides that couple receptor signals to second messenger systems like cAMP (25, 26), might be involved in adrenal tumor formation. Point mutations at 2 codons of the highly preserved α-chain of Gi, the adenylcyclase-inhibiting G protein, resulting in constitutively activated Gαi2, were reported in 2 of 10 adrenocortical adenomas, suggesting a potential role of this isoform in the genesis of these tumors (27). We reevaluated the frequency of Gαi2 mutations in 10 adrenocortical adenomas and were unable to confirm these findings (15). None of our tumors studied contained point mutations in the previously reported hot-spot regions. These findings, together with the low prevalence of p53 mutations in adrenal adenomas reported here, suggest that the mechanism of tumorigenesis in benign adrenocortical tumors involves oncogenic mutations of other cell constituents.

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