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P53 Gene Mutations in Pituitary Carcinomas

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Abstract Although p53 overexpression detected by immunohistochemistry has been reported in pituitary adenomas and carcinomas, genetic mutations in the p53 gene have not been previously detected in these tumors. We analyzed a series of eight pituitary adenomas and six pituitary carcinomas by immunohistochemistry, polymerase chain reaction amplification, and sequencing of p53 exon 5 through exon 8 for genetic mutations. Three carcinomas showed more than 20% expression of p53 protein in the tumor cells. One of these tumors with 60% overexpression of p53 protein had a mutation in codon 248, a common "hot spot" for p53 mutation, while the other carcinoma with 90% overexpression of p53 protein had a mutation in codon 135. All adenomas were negative for p53 mutations and had 15% of the cells expressing the p53 protein. Analysis of control tumors including four lung carcinomas with proven p53 mutations also had greater than 85% of the tumor cells overexpressing p53 protein. Two breast carcinoma cell lines with known p53 mutations, MBA-MD 231 and MBA-MD-486, also showed greater than 85% of the tumor cells overexpressing p53. These results show that p53 mutations are present in a subset of pituitary carcinomas and are usually associated with a high percentage of tumor cells overexpressing the p53 protein.

Keywords pituitary · p53 · carcinoma · gene mutation · immunohistochemistry

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Introduction

Pituitary tumors account for 10–15% of all intracranial tumor and occur mainly in adult age [1, 2]. Most tumors are benign, but some adenomas show recurrent and locally invasive behaviors. Pituitary carcinoma is very rare and account for less than 0.1% of pituitary tumors [3]. About 70% of pituitary carcinomas are reported hormonally active, and most produce prolactin (PRL) or adenocorticotropic hormone (ACTH). The accepted criterion for pituitary carcinoma diagnosis is cerebrospinal and/or systemic metastasis [4–6]. It is often difficult to predict the behavior of large and atypical pituitary adenomas.

The p53 protein has been detected in pituitary adenomas and carcinomas using immunohistochemical assay [3, 4, 7]. The frequency of p53 overexpression has been quite variable [3, 4, 7]. Thapar et al. and other investigators showed accumulation of immunohistochemically detectable p53 protein in clinically or biologically aggressive pituitary tumor [7–9]. However, in previous studies, mutation of p53 genomic deoxyribonucleic acid (DNA) was not detected in pituitary adenomas and carcinomas [10–12]. The reason for p53 accumulation in some pituitary adenomas and carcinomas has not been elucidated.

In the present study, we analyzed p53 gene mutations in 14 cases of pituitary PRL and ACTH tumors, including two PRL carcinomas and four ACTH carcinomas. p53 immunostaining was also performed for the pituitary tumors to

compare the p53 protein expression levels to the p53 gene mutation status. We found p53 mutations in two ACTH carcinomas, and this correlated with increased p53 protein expression.

Materials and Methods

Tissue Samples

Fourteen cases of formalin-fixed, paraffin-embedded pituitary tumor tissues were obtained from the archives of the Department of Laboratory Medicine and Pathology, Mayo Foundation (year 1988–2006), including five PRL and three ACTH adenomas and two PRL and four ACTH carcinomas (Table 1). The adenomas were randomly selected, while all cases of available carcinomas were used. All pituitary carcinomas had proven evidence of cerebrospinal and/or systemic metastasis. One ACTH carcinoma (case 11) was analyzed from both paraffin-embedded and fresh frozen tissues of the liver metastasis. The tumor from case 12 was from an autopsy case of an ACTH carcinoma with liver metastasis.

Four cases of lung adenocarcinoma tissues identified with mutation in p53 exons 5 and 7 detected by polymerase chain reaction (PCR) amplification and sequencing (unpublished data) and two breast carcinoma cell lines (MDA-MB-231 and MDA-MB-468, from ATCC, Rockville, MD) with known mutations in p53 exon 8 were used as positive controls. Normal liver tissue was used for p53 wild-type control.

Institutional Review Board permission was obtained for the study from the Mayo Clinic College of Medicine.

DNA Extraction

Genomic DNA was extracted from four to six 10-μm-thick paraffin sections of each pituitary tumor as previously reported [13]. Briefly, paraffin sections collected in 1.7-ml microtubes were deparaffinized, dehydrated, and air dried. The tissue pellet was resuspended in 480 μl of digestion buffer (20 mM Tris–HCl, 20 mM ethylenediamine tetraacetic acid, 1% sodium dodecyl sulfate, pH 7.5) and 20 μl of 25 μg/μl proteinase K (Roche Applied Science, Indianapolis, IN). The sample was then incubated at 55°C in a water bath overnight. After incubation, DNA was isolated with an equal volume of phenol–chlolroform–isoamylalcohol solution (22:24:1; Invitrogen, Carlsbad, CA), and the aqueous phase was collected for re-extraction with 1 vol of chloroform–isoamy alcohol (1:1). DNA samples were precipitated with 2 vol of ice-cold absolute ethanol and

Table 1 p53 mutation analysis and immunohistochemistry (IHC) in pituitary tumors

Case	Diagnosis	Age/sex	Tumor (%)	IHC[a] (%) p53	Mutation assay			
					Exon	Codon	Base change	Amino acid change
1	PRL adenoma	37/M	80	10				
2	PRL adenoma	44/F	90	2				
3	PRL adenoma	71/F	80	2				
4	PRL adenoma	16/F	80	15				
5	PRL adenoma (invasive)	45/M	70	15				
6	ACTH adenoma	28/F	80	0				
7	ACTH adenoma	46/F	90	5				
8	ACTH adenoma	32/F	80	0				
9	PRL carcinoma	44/F	70	1				
10	PRL carcinoma	N/A	90	20				
11	ACTH carcinoma	65/F	50	60	7	248	CGG>CAG	Arg>Gln
12	ACTH carcinoma	63/M	90	10				
13	ACTH carcinoma	57/M	60	90	5	135	TGC>TTC	Cys>Phe
14	ACTH carcinoma	52/F	60	0				
Controls								
1	Lung adenocarcinoma		90	95	5	158	CGC>CTC	Arg>Leu
2	Lung adenocarcinoma		30	95	5	159	GCC>CCC	Ala>Pro
3	Lung adenocarcinoma		80	90	7	248	CGG>CTG	Arg>Leu
4	Lung adenocarcinoma		90	85	7	245	GGC>GTC	Gly>Val
5	Breast-231 cells			95	8	280	AGA>AAA	Arg>Lys
6	Breast-486 cells			95	8	273	CGT>CAT	Arg>His

[a] p53 Immunohistochemistry (IHC) in pituitary tumors was performed by counting the percentage of cells with nuclear staining.

placed in the freezer (−20°C) for 2–4 h. After centrifugation at 12,000 rpm for 30 min, the supernatant was carefully removed. Finally, the DNA was resuspended in 100 to 500 μl of distilled water, and DNA concentration was measured by optical density with a spectrophotometer.

PCR Amplification

The sequences of the primers used for PCR amplification for p53 exons 5 to 8 and the resulting PCR product size are shown in Table 2. To maintain the PCR products size less than 200 bp, two sets of primer pair overlapping for each exon were designed and flanked each exon and include the intron–exon junction by using the Oligo-6 software program (Molecular Biology Insights, Cacade, CO).

PCR amplification was performed using the Thermocycler 9600 (PE Biosystems, Foster city, CA). The PCR mixture in a total 25 μl of volume contained 0.3 μM of each primer, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.5 U *Taq* polymerase (Invitrogen), 0.1 mM of deoxyribonucleotide triphosphates (Roche Diagnostics, Indianapolis, IN), and 250 ng genomic DNA. The PCR cycle profiles were as follows: initial denature at 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. After the final cycle, the elongation step was extended by 10 min at 72°C.

DNA samples from lung adenocarcinomas and breast carcinoma cell lines were used as p53 mutation-positive controls. Normal liver DNA was used for p53 wild-type control. Omission of template DNA was used for PCR negative control. After amplification, PCR products were separated on a 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

DNA Sequencing

PCR products amplified from p53 exons 5 through 8 were treated with ExoSAP-IT enzymes from the "PCR Products PRE-Sequencing Kit" (USB, Cleaveland, OH), in accordance with the manufacturer's protocol. Direct DNA sequencing was performed in the Molecular Biology Core at Mayo Clinic by using an ABI Prism 377 DNA sequencer (PE Aplied Biosystems, Foster City, CA). The sequencing results were analyzed with Mutation Surveyor software version 2.2.

Immunohistochemistry

Four-micrometer-thick paraffin tissue sections from pituitary tumors and lung adenocarcinomas were used for immunohistochemical staining as previously reported [14]. Briefly, deparaffinized sections were pretreated with 10 mM citrate buffer (pH 6.0) for 10 min at 750 W. A p53 monoclonal antibody (D-07, Dako, Santa Barbara, CA) was used at concentration 1/100. The avidin–biotin complex method with VECTASTIN Elite ABC-Peroxidase kit (Vector Laboratory, Burlingame, CA) was used to detect the immunoreactive signals. Diaminobenzidine was used as chromogen, and the hemotoxylin was used as a counterstain. Breast carcinoma cell line MDA-MB-231 and MDA-

Table 2 PCR primer sequences and location for p53 exons 5 to 8

Exon[a]		Sequences	Location[b]	Size (bp)
5a	F	5′-ACT TGT GCC CTG ACT TTC AAC-3′	1,510–1,530	126
	R	5′-CGG GGG TGT GGA ATC AAC-3′	1,618–1,635	
5b	F	5′-GTG CAG CTG TGG GTT GAT TCC-3′	1,606–1,626	171
	R	5′-CAA CCA GCC CTG TCG TCT CT-3′	1,757–1,776	
6a	F	5′-TCC CCA GGC CTC TGA TTC C-3′	1,784–1,802	134
	R	5′-GGG CAC CAC CAC ACT ATG TCG-3′	1,897–1,917	
6b	F	5′-TGC GTG TGG AGT ATT TGG AT-3′	1,862–1,881	126
	R	5′-CCA CTG ACA ACC ACC CTT AAC-3′	1,967–1,987	
7a	F	5′-TCT CCC CAA GGC GCA CTG-3′	2,453–2,470	110
	R	5′-CCG CCC ATG CAG GAA CTG TTA-3′	2,542–2,562	
7b	F	5′-TCC TAG GTT GGC TCT GAC TGT-3′	2,495–2,515	144
	R	5′-GTG TGC AGG GTG GCA AGT-3′	2,621–2,638	
8a	F	5′-TAC TGC CTC TTG CTT CTC TTT-3′	2,918–2,938	183
	R	5′-GCT TGC TTA CCT CGC TTA GT-3′	3,081–3,100	
8b	F	5′-CAA GAA AGG GGA GCC TCA CC-3′	3,041–3,060	155
	R	5′-TTG TTG GGC AGT GCT AGG AAA-3′	3,175–3,195	

[a] Two pair primer set (a and b) with overlap for each exon. *F* Forward, *R* reverse
[b] Gene location of PCR products (Genebank no. AF136270)

MB-468 were cultured, and cytospin slides were made for immunohistochemistry as described above.

The percentage of cells with nuclear staining for p53 was obtained by enumerating 500 cells using a 1-cm^2 grid in the ocular of the microscope. A minimum of ten fields were counted.

Results

Tumors

There were four PRL adenomas, one invasive PRL adenoma, and three ACTH adenomas. No patients with adenomas have had recurrent disease. In addition, there were two PRL carcinomas with metastasis to other sites in the brain. Two ACTH carcinomas had liver metastases (cases 11 and 12). One patient with a PRL carcinoma with brain metastasis and the two patients with ACTH carcinomas with liver metastasis died of disease. The other patients with carcinomas are alive with disease.

PCR and Sequencing

PCR amplification of p53 exon5 through 8 for 14 cases of pituitary tumors was performed along with control DNA samples (lung carcinomas or breast carcinoma cell lines) with known p53 mutations and the wild type (liver). A single band of each PCR product with respective molecular size was identified on the agarose gel. Direct sequencing analysis showed a point mutation in two ACTH carcinomas (case 11 and 13, Table 1). In case 13, a point mutation at codon 135 (TGC to TTC, Cys135Phe) in exon 5 was identified. In case 11, both formalin-fixed and paraffin-embedded tissues and fresh frozen tissue samples had the same sequencing results showing a point mutation at codon 248 (CGC to CAG, Arg248Gln) in exon 7 (Fig. 1). Two breast cell lines 468 and 231 had point mutations at codon 273 and codon 280 in exon 8, respectively. The four adenocarcinomas showed p53 point mutations in exon 5 and 7 (Table 1). Normal human liver tissue showed p53 wild-type sequences in p53 exon 5 to 8 (Fig. 1).

Immunohistochemistry of p53

Immunohistochemistry analysis showed p53 immunoreactivity with strong positive nuclear staining in 11 out of 14 cases of pituitary tumors (range 1–90%, Table 1). The two ACTH carcinomas with p53 mutation (cases 11 and 13) showed 60 and 90% positive immunostaining cells, respectively (Fig. 2), All lung adenocarcinoma tissues and breast carcinoma cells, which were used as positive controls, showed greater than 85% immunopositive cells.

Discussion

Analysis of pituitary adenomas and pituitary carcinomas showed p53 mutations in two of six carcinomas, but not in the eight adenomas examined. Our analyses showed good correlation between p53 mutation and overexpression of the p53 protein. Tumors expressing p53 in more than 50% of the cells were more likely to have mutations in the pituitary carcinoma group and in the control tumors with p53

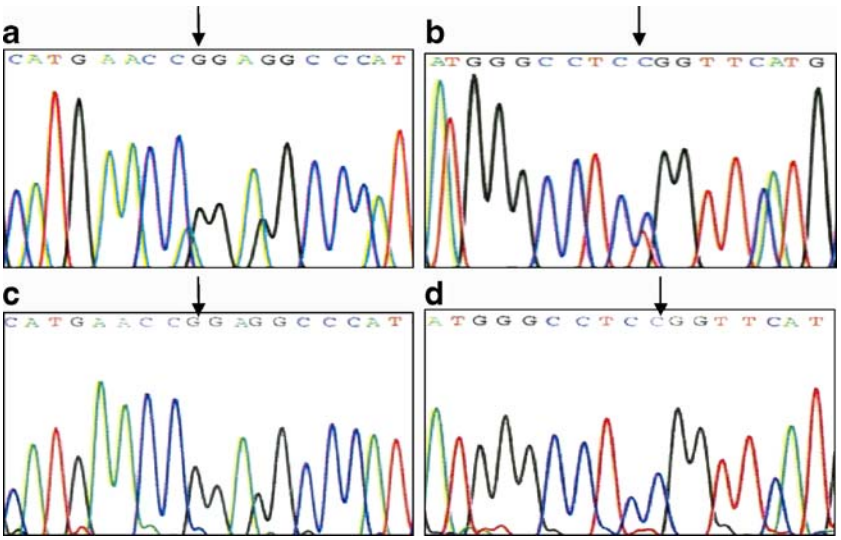

Fig. 1 DNA sequencing for p53 exon 7. A point mutation in exon 7 at codon 248 of the p53 gene (CGG to CAG) in a pituitary carcinoma from case 11 is detected in the forward (**a**) and reverse directions (**b**). Normal liver tissue showed a wild-type sequence in the forward (**c**) and reverse directions (**d**)

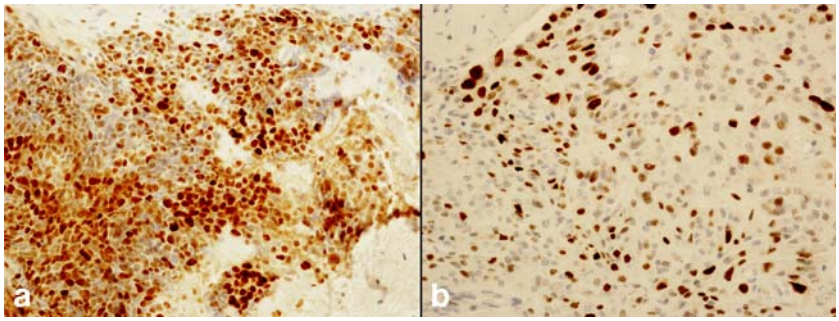

Fig. 2 Immunohistochemical staining for p53 in two pituitary carcinomas. **a** Case 13 with 90% of the tumor cells overexpressing p53 and **b** case 11 with 60% of the cells overexpressing p53 in the liver metastasis

mutations including lung carcinomas and two breast carcinoma cell lines.

The p53 gene located on the short arm of chromosome 17 is one of the most studied tumor suppressor genes. The p53 gene consists of 11 exons and encodes a 53-kDa DNA-binding protein, which functions as cell cycle regulator and is involved in arresting the cell cycle in the G1 phase of growth, gene transcription, cell proliferation, DNA synthesis and repair, cell differentiation, and induction of apoptosis [15]. p53 is the most commonly mutated gene in human cancers and is involved in up to 50% of clinical cancers, including colon, stomach, breast, lung, and lymphomas [16, 17]. The DNA-binding domain of p53 gene, which covers exons 5 through 8, is the target of 90% of the mutations found in human cancers.

Although p53 is an important tumor suppressor gene, the wild-type p53 protein is usually difficult to detect in cells because it is combined with mouse double minute 2 protein and digested by the ubiquitin system [17]. Wild-type p53 protein has a short half-life and is difficult to detect through standard immunohistochemical techniques. The mutant forms of p53 are stable with a longer half-life, which can be detected with standard immunohistochemistry [18, 19].

Pei et al. [12] did not detect p53 mutations in three ACTH and one PRL carcinoma. Our study is the first report showing p53 mutation in pituitary carcinomas. Other workers have not detected p53 mutations in pituitary adenomas or carcinomas in smaller series [10]. Because pituitary carcinomas are very rare cancers, the number of cases analyzed by Pei et al. [12] (n=4) and the number in our study (n=6) are small and may not reflect the true incidence of p53 mutations in pituitary carcinomas. Codon 248 (CGG-CAG) and codon 135 (TGC-TTC) are in the DNA-binding region of the p53 gene. This region is frequently involved in p53 mutations [18–20]. Both of these mutations can affect DNA binding [20, 21]. However, some p53 mutations may not lead to protein stabilization and increased immunoreactivity for p53 in tumors [7, 8, 20–24].

p53 protein overexpression has been detected in pituitary tumors by various investigators [7, 22–24]. Thapar et al. reported a higher percentage of p53 immunostaining in invasive pituitary adenomas and pituitary carcinomas compared to pituitary adenomas [7]. Although Thapar et al. found only a small percentage of PRL and ACTH tumor expressing a low level of p53, this study found a higher percentage of these tumors with low expression, which may be related to increased sensitivity in detecting immunoreactive tumor cells. These findings suggested that immunostaining for p53 could be used as a diagnostic marker to characterize aggressive pituitary adenomas as well as carcinomas when it is used in conjunction with Ki-67. One study reported p53 overexpression in up to 50% of Cushings adenomas, but genetic mutations were not detected by SSCP in these cases [21, 22].

The significance of p53 overexpression at the molecular level is uncertain. The prolonged half-life of p53 protein is probably related to structural stability. Feng et al. [25] and others described post-translational modification of the p53 protein as playing an important role in regulating p53 stability and activity including phosphorylation, acetylation, ubiquitination, neddylation, and methylation in response to DNA damage and other cellular stress [25].

In a recent study of a large series of pituitary adenomas and carcinomas, Scheithauer et al. [6] reported higher levels of p53 expression in premetastatic and metastatic pituitary tumors compared to the adenomas. There was a marked difference in Ki-67 labeling between these two groups with a 1.8 and a 8.2 labeling index, respectively, indicating that Ki-67 was more sensitive in separating these two groups than p53.

Our results suggest that immunostaining for p53 may be useful when the percent of positive cells or the labeling index is greater than 50%. A high labeling index is more likely to correlate with a mutation in the p53 gene. However, immunostaining by itself is not sufficient to determine if there is a mutation, and molecular analysis for mutations in p53 is necessary to confirm abnormal expression of the protein.

In conclusion, this is the first report to demonstrate p53 mutation in pituitary carcinomas and to show that high overexpression of p53 protein may correlate with mutation of the p53 gene.

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