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Mutation Analysis of the p53 Tumor Suppressor Gene using Paraffin-embedded Specimens of Human Transitional Cell Carcinomas by the Direct Sequencing Method

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A very small quantity of DNA was extracted from paraffin sections of 25 bladder and 2 ureteral cancer cases, and exons 5-8 of the p53 gene were amplified by the polymerase chain reaction. Mutations were detected by direct sequencing, and their relationships to clinicopathological factors were assessed. Point mutations of the p53 gene were observed in 2 of 27 specimens of transitional cell carcinoma. One was a novel nonsense mutation. The prognosis of patients with p53 gene mutations was poorer than those of patients without mutations. Analysis of the data showed that assessment of the potential malignancy of bladder cancer, combined with a conventional pathological diagnosis, allows a more precise prognosis. Direct sequencing of tissue specimens enables swift prognostic evaluation and prompt decisions concerning treatment.

Key words : Transitional cell carcinoma, p53 tumor suppressor gene, Direct sequencing, Specimen

INTRODUCTION

Recent advances in analytical methods in molecular biology have made possible elucidation of mechanisms underlying human tumorigenesis involving oncogenes or tumor suppressor genes in many types of carcinomas. Current belief holds that genetic alterations responsible for tumorigenesis include chromosomal deficiency and translocation, deletion, insertion, and point mutation. Loss or changes in gene function are believed to induce activation of oncogenes and inactivation of tumor suppressor genes. Abnormal cell proliferation is believed to be induced by loss of normal function of both alleles, a result of a point mutation or deficiency of the p53 tumor suppressor gene (p53 gene). These alterations may give clues to mechanisms of tumorigenesis in general. A recent study has shown that the p53 gene mutation is closely related to malignant development in various types of cancer [1].

In the field of urology, a relationship between bladder and prostatic cancer and structural changes in the p53 gene has been considered one of the mechanisms underlying malignant development [2]. The frequency of an abnormal p53 gene, however, is believed to be low in renal cell carcinoma and testicular tumors [3, 4]. In particular, with bladder cancer, some studies report a high occurrence of primarily point mutations in the p53 gene of high grade and high stage cases [5-8]. Based on these reports, both the clinicopathological findings and prognosis of bladder cancer appear to be related to the presence of p53 gene mutations.

The Polymerase chain reaction, followed by single-strand conformation polymorphism (PCR-SSCP) analysis, has been used conventionally on DNA extracted from frozen specimens, in the analysis of p53 gene mutations [5-7, 9]. Although this procedure is widely used, there are disadvantages in that the results give indirect evidence, and the methods of DNA extraction, purification, and analysis are complicated. With advances in molecular biology, gene

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mutation analysis has recently become possible. Here, we report one such method involving amplification of a target region by using DNA extracted from paraffin tissue sections and detecting the mutations by the most reliable method available, which is direct sequencing [10].

To our knowledge, this is the first analytical study of p53 gene mutations in transitional cell carcinomas, including bladder and ureteral cancers, using DNA purified from tissue sections. The efficacy of the method was also investigated to search for a new p53 gene point mutation.

MATERIALS AND METHODS

1. Materials

Twenty seven specimens of transitional cell carcinomas (25 bladder and 2 ureteral cancers) from 24 patients (Table 1) were analyzed. Nineteen of the 27 carcinomas were primary, and the remaining 8 were recurrent. Tissue specimens were obtained by total cystectomy in one of the 25 bladder cancer patients, and by transurethral resection of the bladder tumor (TUR) in the remaining 24. In the 2 ureteral cancer patients, tissue specimens were collected by nephroureterectomy. All tumor specimens were fixed in formaldehyde, embedded in paraffin, and evaluated pathologically. Classification of the clinical stages and histological grading of the bladder and ureteral cancers were based on The General Rules For The Bladder Cancer and Ureteral Cancer Study, formulated by the Japanese Society of Urology and the Japanese Society of Pathology [11]. The mean age of the 24 patients was 69.7 years. Twenty specimens (19 bladder cancers and 1 ureteral cancer) were obtained from male patients, and 7 specimens (6 bladder and 1 ureteral cancers) were collected from female patients. Based on histological grading, grades 1, 2, and 3 were observed in 2, 13, and 12 patients, respectively. According to tumor infiltration, stages pT1, pT2, and pT3 were observed in 17, 6, and 4 patients, respectively.

2. DNA extraction and purification from paraffin-embedded tissues

Genomic DNA was extracted and purified from tissues embedded in paraffin 3–26 months prior [12]. After 5 μm slices were cut from the tissue blocks, the paraffin was removed by xylene-ethanol treatment [9] [100% xylene for 5 min (5 times), 100% ethanol for 1 min (3 times), 95% ethanol for 1 min, 70% ethanol for 1 min, 50% ethanol for 1 min]. The tissue was then dissolved in distilled water and boiled for 15 minutes.

Table 1 Clinicopathological features of transitional cell carcinomas

Transitional cell carcinoma		No. of patients
Grade*		
	Grade 1	2
	Grade 2	13
	Grade 3	12
T factor*		
	T1	17
	T2	6
	T3	4
	Total No. patients	27
	Mean age	69.7 Years
	Male	20
	Female	7

No.: Number.
*Classified under the provision of the General Rule for Clinical and Pathological Studies on Bladder and Ureteral Cancer as desclibed in the text.

DNA was recovered by centrifugation.

3. Polymerase chain reaction (PCR)

Each region of exons 5 through 8, i.e., codons 126 to 306, considered hot spots for the p53 gene point mutation in human malignant tumors, was amplified by the PCR method for analysis of gene mutation. The primers used are shown in Table 2. The conditions used for the PCR were based on the report by Mayall *et al.* [12] One microliter of PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5–2.5 mM $MgCl_2$, 0.1% gelatin, 0.2% formaldehyde), 1 μl of 2.5mM each dNTPs, 1.25 U Taq polymerase (Takara Shuzo, Japan), 10 pmol of the primer corresponding to each exon, and less than 100 ng of the purified template DNA were added to the PCR tube, and the total amount was adjusted to 10 μl. PCR was then performed by 35 cycles of amplification with a DNA amplifier (PE Biosystems, USA) under the following conditions: 94℃ for 30 seconds, 50℃ for 40 seconds, and 72℃ for 60 seconds for each cycle (72℃ for 5 minutes in the final cycle). Amplification of the target region was confirmed by agarose gel electrophoresis. For the exon 5 and 8 regions, nested PCR was added by using 2 μl of PCR sample as template DNA, because amplification could not be confirmed by the first PCR. DNA was amplified in 25 cycles by using the following primers medial to those used in PCR: exon 5-sense, 5'-GTACTCCCCTGCCTCAACA-3'; antisense, 5'-CTCACCATCGCTATCTGAGCA-3'; exon 8-sense, 5'-AGTGGTAATCTACTGGGACGG-3'; antisense, 5'-ACCTCGCTTAGTGCTCCCTG, under the same conditions as those for PCR. As for the PCR products, the quantities of amplified products were determined by 2% agarose gel electrophoresis.

4. Determination of DNA sequence by the direct sequencing method

DNA sequence data were obtained by the dideoxy-chain termination method [13] with an Amplitaq FS Cycle Sequencing Kit (Perkin-Elmer Cetus, Norwalk, Connecticut) for the PCR-amplified p53 gene exons 5 through 8. First, each PCR product (30～90 ng) was transferred to 20 μl of a solution (2 μl of DNA, 0.8 μM primer, and 6 μl of water) containing 8 μl of Terminator Ready Reaction Mixture, and amplified for 25 cycles under the following conditions: 96℃ for 30 seconds, 50℃ for 15 seconds, and 60℃ for 4 minutes for each cycle. After cycle sequencing, the DNA was purified in columns and analyzed with an ABI PRISM™ 310 (The Parkin-Elmer Corporation). The DNA sequence data were compared with data on the p53 gene registered in Genebank.

RESULTS

1. DNA direct sequencing analysis

The DNA sequences of the p53 gene exons 5 through 8 were determined in all 27 specimens, and examined for the presence of gene mutations. The results are shown in Table 3. Point mutations were observed in patients 12 and 20. One of the two patients showed a nonsense mutation converting glutamine to a stop codon (Fig. 1-a), due to a mutation from G to T at codon 285 in exon 8. This point mutation has never been

Table 2 PCR primers used in mutation analysis of the p53 gene (exons 5 to 8)

Exon	Sequence of primers		Nucleotide positions
5	5' TTCCTCTTCCTACAGTACTCC 3'	Upstream	909–929
	5' GCCCCAGCTGCTCACCATCG 3'	Downstream	1103–1122
6	5' TTGCTCTTAGGTCTGGCCCC 3'	Upstream	1129–1148
	5' CAGACCTCAGGCGGCTCATA 3'	Downstream	1237–1256
7	5' TAGGTTGGCTCTGACTGTACC 3'	Upstream	1277–1297
	5' TGACCTGGAGTCTTCCAGTGT 3'	Downstream	1374–1394
8	5' TTCCTTACTGCCTCTTGCTT 3'	Upstream	1418–1439
	5' TGAGGCATAACTGCACCCTT 3'	Downstream	1539–1557

Table 3 Clinical profile of transitional cell carcinomas and mutations of the p53 gene

Patient	Sex	Age	Recurrences	Grade	T factor	p53 gene mutation			
						Exon	Codon	Sequence change	Amino acid change
1	F	74	FC	G3	PT1		—		
2	F	86	1	G3	pT3		—		
3	M	79	FC	G1	pT1		—		
4	M	53	1	G2	pT1		—		
5	M	73	FC	G2	pT1		—		
6	M	85	5	G3	pT3		—		
6	M	85	6	G3	pT3		—		
7	M	59	FC	G2	pT1		—		
8	M	60	1	G2	pT1		—		
8	M	60	2	G2	pT1		—		
9	M	67	FC	G3	pT1		—		
10	M	60	FC	G2	pT1		—		
11	M	76	FC	G2	pT1		—		
12	M	72	FC	G3	pT1		—		
12	M	72	1	G3	pT1	8	285	CAG-TAG	Glu-Stop
13	M	83	1	G3	pT2		—		
14	F	28	FC	G2	pT2		—		
15	M	64	FC	G2	pT1		—		
16	M	78	FC	G3	pT1		—		
17	F	53	FC	G2	pT1		—		
18	F	73	FC	G2	pT1		—		
19	F	80	FC	G3	pT3		—		
20	M	54	FC	G2	pT2	5	152	CCG-CCA	Pro-Pro
21	M	65	FC	G1	pT1		—		
22	M	59	FC	G2	pT2		—		
23	F	84	FC	G3	pT2		—		
24	M	86	FC	G3	pT2		—		

M: MALE; F: FEMALE; FC: First CASE; —: NO MUTATION

Table 4 Clinical profile of local recurrence bladder cancers and mutations of the p53 gene

Patient	Recurrences	Age	Sex	SURGERY	Grade	T factor	Duration (months)	Site of mutation of p53
12	FC	72	M	TUR	3	1	**	—
12	1	72	M	TUR	3	1	2	Exon 8
20	FC	54	M	TUR	2, CIS	1	**	Exon 5
20	1	55	M	TUR	2	1	9	*

FC: First CASE; M: male; TUR: transurethral resection of the bladder tumor
CIS: carcinoma *in situ*; —: no mutation **: first; *: Not DONE

reported in bladder cancer, and is a novel mutation. The other patient showed a silent mutation from G to A (Fig. 1-b) at codon 152 in exon 5. In the 2 patients with a point mutation, allele loss was indicated by the electropherogram.

2. Relationships between the p53 gene point mutation and clinicopathological factors

The relationship between gene mutation and clinicopathology were investigated in 25 bladder and 2 ureteral cancer patients. Mutation of the p53 gene was observed in 2 of the 27 transitional cell carcinoma specimens (7.4%). The mutation was observed in 1 of 10 infiltrating bladder cancers (10.0%) and 1 of 17 superficial bladder cancers (5.9%). According to the tumor nodes metastasis (TNM) classification, 1 of 17 T1 cases (5.9%) and 1 of 6 T2 cases (16.7%) showed a p53 gene mutation, whereas none of 4 T3 cases showed the mutation. All excised specimens were classified as grades 1 to 3. The p53 gene mutation was observed in 1 of 13 grade 2 and one grade 3 specimens, while neither of the 2 grade 1 specimens had the mutation. One grade 2 case was a carcinoma in situ (CIS). Comparison between genders revealed that the mutation was not observed in any of the 7 specimens from the female patients, but was observed in 2 of the 20 specimens (10.0%) from the male patients.

3. Clinical course of patients with the p53 gene mutation

The clinical course of 2 patients, in whom the p53 gene mutation was detected, is

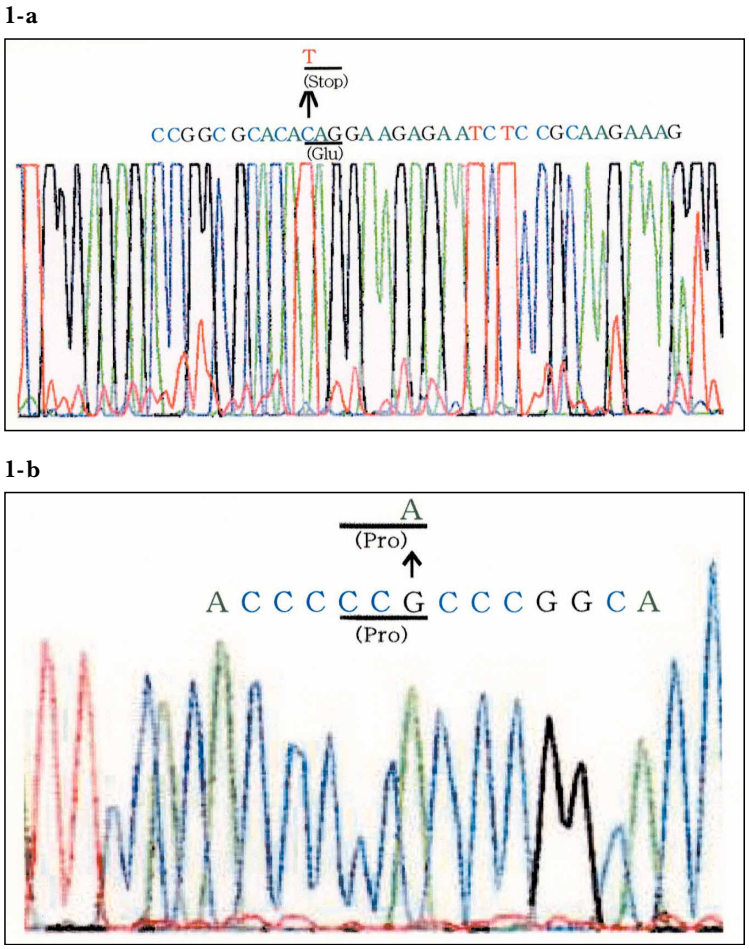

Fig. 1 The DNA sequence of the p53 gene in patients No. 12 and 20. The point mutatin of patient No. 12 is a substitution from CAG (glu) to TAG (stop) at codon 285 in exon 8 (Fig. 1-a) and the point mutation of patient No. 20 is a substitution from CCG (pro) to CCA (pro) at codon 152 in exon 5 (Fig. 1-b).

shown in Table 4. Patient No. 12 with a nonsense mutation in the exon 8 region, developed a local intracystic recurrence 2 months after TUR. In patient No. 20 with a silent mutation in exon 5, local intracystic recurrence, including CIS, was observed 9 months after TUR.

4. A relationship between the p53 gene mutation and the frequency of local intracystic recurrence

Local intracystic recurrence was observed 11 times in 5 of the 22 patients (22.7%) without a p53 gene mutation. Both patients with the gene mutation developed a local intracystic recurrence. These data suggest that the frequency of intracystic recurrence in the mutation-positive cases is higher than in the mutation-negative cases and that the potential malignancy also was higher in the positive cases.

DISCUSSION

The p53 gene, located in the short arm of chromosome 17 (17p13) in humans, is composed of 11 exons and codes 393 amino acids. The gene is a tumor suppressor gene, and is considered to play an important role in inactivating tumorigenesis and malignant development in various types of cancer [10, 14]. It functions via the p53 protein; when DNA is impaired, it controls transcription of the inhibitor p21 gene coding cyclin-dependent kinase, an essential enzyme in the cell cycle which controls progression from stage G1 to stage S and repairs damaged DNA. However, the gene is also believed to have a preventive function against cellular tumorigenesis by inducing apoptosis and eliminating cells with impaired DNA, when repair is impossible [10, 14, 15–17].

Some reports have shown that the p53 gene mutation is involved in the occurrence and development of many carcinomas [6]. Ninety percent of these mutation cases converge to regions Ⅲ, Ⅳ, and Ⅴ of the five conserved regions I through Ⅴ. The p53 gene mutation also shows high homology with p53 proteins in all species (Fig. 2).

In the field of urology, there is abundant evidence supporting a relation between the p53 gene mutation and bladder cancer. Many studies [5–8] have reported the high frequency of p53 gene mutation in exons 5 to 8 coding the conserved regions Ⅲ to Ⅴ, especially in high grade and high stage cases, as with other carcinomas. A relation between mutations in this region and CIS has been observed [18]. It has also been reported that the frequency of the p53 gene mutation is significantly high among recurrent superficial bladder cancers [5]. From these reports, the p53 gene mutation undoubtedly plays an important role in the recurrence and development of bladder cancer.

The occurrence of the p53 gene mutation in 114 cases of human bladder cancer is shown in Fig. 2 [8, 19–21]. Point mutations were observed in 107 (93.9%) of the 114 cases, showing that most of the gene mutation patterns are missense or nonsense mutations caused by single-base substitutions. According to the data in Fig. 2, a point mutation occurred in exon 4 in 3 cases (2.8%), exon 5 in 24 cases (22.4%), exon 6 in 12 cases (11.2%), exon 7 in 20 cases (18.7%), exon 8 in 48 cases (44.8%), and exon 10 in 1 case (0.09%). Thus, exons 5 to 8 were considered as hot spots for the mutation, the same as in other malignant human tumors. In particular, point mutations in exons 5 and 8 were observed in 67% of the hot spots. Based on tumor grading, frequencies of point mutation in the G1, G2, and G3 cases were 5.0%, 31.7%, and 68.3%, respectively. Based on tumor infiltration, frequencies of point mutation in the Ta, T1, T2, T3, and T4 cases were 5.1%, 13.8%, 15.5%, 46.6%, and 19.0%, respectively. These reports show that the frequency of p53 gene mutations tends to be higher in high grade and high stage bladder cancer cases. Our present analysis is also consistent with previous results demonstrating that the frequency of the p53 gene mutation tends to be higher in infiltrating bladder cancers. The two patients with p53 gene point mutations showed recurrence within a short period of time, and were G3 or exhibited pathological factors indicating a poor prognosis, including CIS. The frequency of local intracystic recurrence with the p53 gene mutation was higher than that for tumors without the mutation, suggesting that the p53 gene mutation is associated with potential malignancy in bladder cancer.

Among exons 5 to 8, hot spots for the p53 gene mutation, the mutations in exons 5 and 8 are seen to reflect malignancy and have

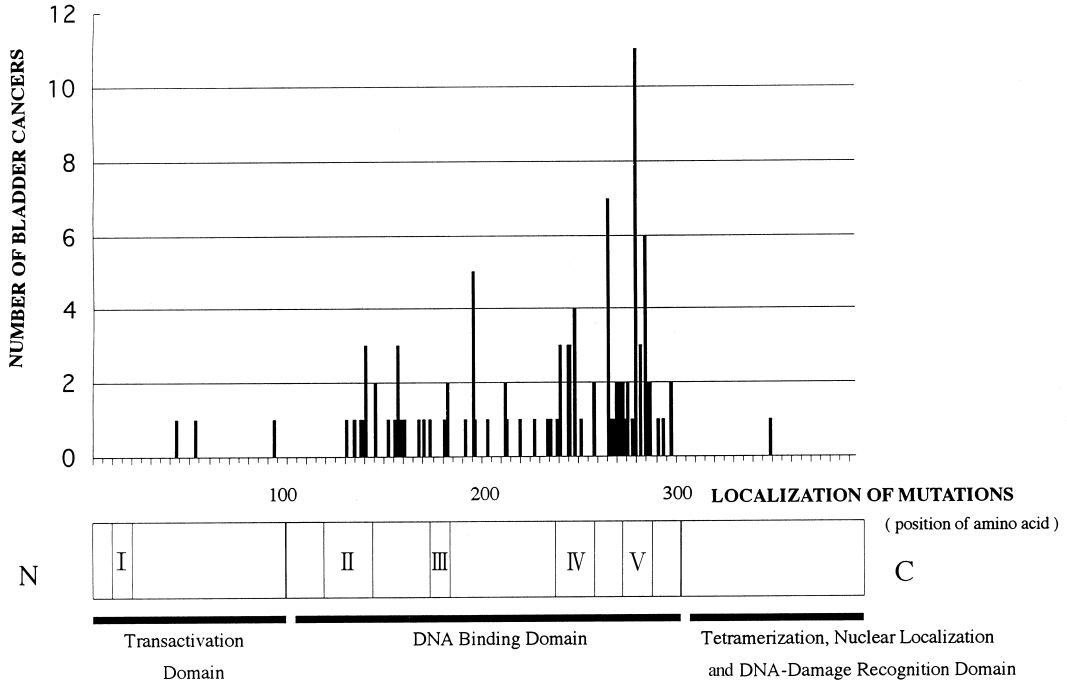

Fig. 2 Schematic representation of the p53 molecule and previously published mutations and mutational hot-spots in the p53 gene in bladder cancer. The human p53 protein consists of 393 amino acids with functional domains, including transactivation domains (amino acids 1–101), DNA binding domain (amino acids 102–292), tetramerization, nuclear localization and DNA-damage recognition domain (amino acids 293–393).

clinical significance in esophageal cancer [2]. A relationship between the p53 gene mutation in exons 5 to 8 and pathological factors were observed in 53 of the 114 bladder cancer cases showing p53 gene mutations [8, 19–21], and our data are from these 53 cases. According to grading histology and tumor infiltration, the frequency of gene mutation in exons 5 and 8 was high, overall accounting for 70% of the cases. In addition, 60% of the G3 cases and 42% of the T3 cases had a mutation in the exon 5 or 8 region. In our present analysis, point mutations were detected at codon 152 in exon 5 and codon 285 in exon 8 of the p53 gene, suggesting that mutations in these two regions are correlated with malignancy and have clinical significance in bladder cancer.

Furthermore, the present results show a single wave-form at the site of point mutation, as shown in Figure 1. This indicates loss of heterozygosity, which means that loss of one autosomal allele derived from a parent has already occurred. Loss of heterozygosity is believed to be the first step of p53 gene mutation in tumorigenesis. Our results support this hypothesis.

The methods of assessing p53 gene mutation include immunohistochemical staining of proteins, and the sequencing of DNA. Kajiyama *et al.* [22] who compared the results of immunohistochemical staining, PCR-SSCP, and the direct sequencing method in a patient with esophageal cancer, concluded that immunohistochemical staining and PCR-SSCP were relatively insensitive in detecting the mutation, and that information on the presence of mutations in each exon is useful in clinical analysis.

In this study we assessed mutation by PCR and the direct sequencing method, where the DNA sequence in exons 5 to 8 of the p53 gene was determined directly from pathological sections of human bladder cancer lesions. The prognosis of bladder cancer was correlated not only with the pathology but also with the presence of p53 gene mutations, suggesting that the present methods are predictive indicators of an increase in the malignant potential and recurrence of

bladder cancer, and that these methods are useful in making rapid diagnoses, thereby permitting rapid decisions for treating these tumors.

In this study, p53 gene mutations were analyzed from small samples used initially for pathological diagnoses. Assessment of the potential malignancy of bladder cancer using this method in conjuction with conventional pathology may allow more precise evaluation of the prognosis, thus facilitating subsequent treatment.

CONCLUSIONS

1) In 27 specimens of primary transitional cell carcinoma, obtained from thin paraffin sections, DNA was successfully amplified in exons 5 to 8 of the p53 tumor suppressor gene by PCR, with use of the nested PCR method for a part of the specimens. DNA sequences were determined by the direct sequencing method. Relationships between histopathological findings and the prognosis were assessed, and DNA sequences were determined in the exons of all specimens.

2) Mutations were observed in 2 of the 27 specimens, consisting of 1 of 10 infiltrating bladder cancer cases and 1 of 17 superficial bladder cancer cases.

3) The 2 patients with a p53 gene mutation underwent local intracystic recurrence within a short period, and they showed a poor clinical course. One of the mutations is novel, and codes a stop codon at this position.

4) Five of the 22 patients without the mutation had a total of 11 local intracystic recurrences. Both patients with the gene mutation revealed local intracystic recurrence. The frequency of local intracystic recurrence was higher in the group with the p53 gene mutation than in the group without the mutation.

5) Mutations in exons 5 and 8 of the p53 gene was correlated with malignancy, suggesting that the mutation has clinical importance and is useful for the determination of treatment and prognosis.

6) p53 gene analysis, including extraction of DNA from sections of paraffin tissue blocks, amplification of the target region by PCR, and direct sequencing, proved useful and effective.

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