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p16^{INK4} Gene Mutations Are Relatively Frequent in Ampullary Carcinomas

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A high incidence of gene mutations or deletions of p16^{INK4}, a cell cycle regulator which inhibits the activity of cyclin-dependent kinase 4/cyclin D complex and blocks the G1-to-S transition, has been reported in pancreato-biliary tract cancers. In order to investigate *p16*^{INK4} gene alterations in sporadic ampullary carcinomas, 17 sporadic ampullary carcinomas were examined. After histological diagnosis, DNA samples extracted separately from both cancerous and normal paraffin-embedded tissues were investigated. Loss of heterozygosity (LOH) was investigated utilizing 3 microsatellite markers on 9p21-22, and a mutational analysis was performed by cloning and sequencing. LOH was observed in 3 cases (17.6%) and somatic mutations with retention of heterozygosity were found in 7 cases (41.2%). Of note was that two mutations resulted in truncated incomplete proteins and one was a point mutation at the consensus site in the conserved ankyrin repeats, which would be crucial for function. Although two-hit inactivation was not evident in any of the mutation cases and further investigation would be needed to elucidate the role of altered p16^{INK4}, these results suggest that the *p16*^{INK4} gene mutations are relatively frequent and its inactivation might be important in ampullary carcinogenesis.

Key words: Ampullary carcinoma — *p16*^{INK4}

Ampullary carcinomas, arising from the papilla of Vater, represent about one percent of all epithelial malignancies and 5% of all carcinomas in the gastrointestinal tract.[1] Because it is relatively rare, the biological features of this tumor type have only received limited attention.[2,3] It was earlier demonstrated that alteration of the adenomatous polyposis coli (*APC*) and *p53* genes might be involved in the development of approximately 50% of ampullary carcinomas,[4,5] but the question of the involvement of other genes in the molecular mechanisms of carcinogenesis largely remains to be answered.

Recently, the *p16*^{INK4} gene, located on the short arm of chromosome 9 (9p21), has been reported to show frequent deletions in various tumors.[6–9] Identified as a regulatory protein in the cell cycle, p16^{INK4} inhibits the catalytic activity of the cyclin-dependent kinase 4 (CDK4)/cyclin D complex and blocks the G1-to-S transition.[6] Although p16^{INK4} has emerged as a major tumor suppressor, mutations of its gene did not appear to be particularly frequent in primary tumors with loss of heterozygosity (LOH) in 9p21, including tumors of the lung, bladder, head and neck, kidney and brain.[9–16] However, frequent deletions and point mutations have been reported in primary tumors such as esophageal, pancreatic and biliary tract cancers.[17–19] Thus, alterations of the *p16*^{INK4} gene might play a role in the development of ampullary carcinomas, which are classified as biliary tract tumors.

In the present study, we therefore examined alterations of the *p16*^{INK4} gene in a series of 17 primary ampullary carcinomas in order to determine its potential involvement in carcinogenesis.

MATERIALS AND METHODS

Materials A total of 17 cases of ampullary carcinoma, 5 obtained at autopsy in the Department of Pathology, the University of Tokyo from 1967 to 1995 (cases 1–5) and 12 surgically resected at Tokyo Kousei Nenkin Hospital from 1978 to 1992 (cases 6–17), were studied. Clinicopathological classification and stage grouping were done according to the General Rules for Surgical and Pathological Studies on Cancer of Biliary Tract[20] of the Japanese Society of Biliary Surgery. The patients were 12 males and 5 females, ranging from 51 to 85 years old (mean, 68.9). All but one (case 6) had advanced cancers, infiltrating beyond the sphincter of Oddi. Microscopically 13 of the tumors were well differentiated, 2 were moderately differentiated and 2 were poorly differentiated adenocarcinomas. The entire lesions were formalin-fixed and paraffin-embedded for conventional histological studies. The clinicopathological and detailed pathological features are summarized in Table I.

DNA extraction Genomic DNA was extracted from 15-μm-thick paraffin sections of the tumor and the surrounding normal tissues. On the basis of histopathological examination, tumor tissues, where at least 60% of micro-

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Table I. Clinicopathological Features of Ampullary Carcinoma Patients

Case	Age	Sex	Size (mm)	Macroscopy	Histology	Direct invasion		Metastasis		Stage	Origin
						pancreas	duodenum	liver	lymph node		
1	79	F	20×15×15	protruding	pap (W/D)	+	+	+	+	IV	common
2	55	F	30×30	protruding	tub (W/D)	+	+	+	+	IV	common
3	76	F	15×15	protruding	tub (P/D)	+	+	+	+	IV	common
4	80	M	30×20×20	ulcerated	tub (M/D)	+	+	+	+	IV	common
5	68	F	30×22×17	protruding	tub (W/D)	−	−	−	−	I	common
6	58	M	10×5	superficial	pap (W/D)	−	−	−	−	I	duo
7	73	M	15×8×8	ulcerated	pap (M/D)	+	+	−	+	III	bile
8	72	M	25×10×8	protruding	tub (W/D)	+	−	−	−	III	bile
9	51	M	13×6	ulcerated	pap (W/D)	+	+	−	−	III	bile
10	75	F	20×10	protruding	pap (W/D)	−	+	−	−	II	common
11	85	M	20×15×7	protruding	sig	−	+	−	−	II	common
12	76	M	11×6	protruding	tub (W/D)	−	−	−	−	I	common
13	72	M	7×6×3	protruding	tub (W/D)	−	+	−	−	III	common
14	63	M	8×6×3	protruding	tub (W/D)	+	+	−	+	III	common
15	67	M	30×20	ulcerated	pap (W/D)	−	+	−	−	II	common
16	52	M	43×33	ulcerated	pap (W/D)	+	+	−	−	II	common
17	70	M	30×20×14	protruding	pap (W/D)	−	+	−	−	II	common

duo and bile, origin from surface duodenal mucosa and intraduodenal bile duct, respectively; M, male; F, female; pap, papillary; tub, tubular; sig, signet ring cell; W/D, well differentiated; M/D, moderately differentiated; P/D, poorly differentiated. Case 17 contains small adenomatous foci. Clinicopathological classification and stage grouping are based on the General Rules for Surgical and Pathological Studies on Cancer of Biliary Tract (3rd edition) of the Japanese Society of Biliary Surgery.

scopical fields were occupied by neoplastic cells, were excised. Tumor tissues where neoplastic cells were supposed to amount to less than 50% were excluded to minimize non-tumor cell contamination. The excised tissues were deparaffinized in xylene, digested with proteinase K and extracted with phenol-chloroform. The genomic DNA was ethanol-precipitated, dried and finally dissolved in Tris-EDTA buffer (10 m*M* Tris-HCl (pH 8.0), 1 m*M* EDTA).

Polymerase chain reaction (PCR) We amplified almost the entire region of exons 1 and 2 of the $p16^{INK4}$ gene (from codons 17 to 152, 83.7% of the entire coding sequence) divided into 5 segments. Because template DNA was obtained from paraffin-embedded tissues and was supposed to be fragmented, semi-nested PCR was necessary in order to obtain a sufficient amount of products in each segment despite the relatively short length, ranging from 90 to 155 base pairs. The PCR primers used were as follows: 5′-CCTTCGGCTGACTGGCTGG-3′ (sense) and 5′-TCCCGCTGCAGACCCTCTAC-3′ (5′-TGCAGACCCTCTACCCACCT-3′) (antisense) for segment 1; 5′-CACCCTGGCTCTGACCATTCTGT-3′ (5′-GCTCTGACCATTCTGTTCTCTCT-3′) (sense) and 5′-GGGGTCGGCGCAGTTGGGCT-3′ (antisense) for segment 2; 5′-GCTGCTGCTGCTCCACGGC-3′ (sense) and 5′-CCAGGCATCGCGCACGTCCA-3′ (5′-CACGTCCAGCCGCGCCCC-3′) (antisense) for segment 3; 5′-CTGGACACGCTGGTGGTGCT-3′ (sense) and 5′-TGGTTACTGCCTCTGGTGCC-3′ (5′-ACTGCCTCTGGTGCCCCCC-3′) (antisense) for segment 4; 5′-CGGCTGCGGGGGGCACCA-3′ (sense) and 5′-GTACAAATTCTCAGATCATCAGTC-3′ (5′-AATTCTCAGATCATCAGTCCTCAC-3′) (antisense) for segment 5. The sequences in parentheses indicate the primers for semi-nested PCR. The PCR reaction was performed in a total volume of 25 μl, containing 2.5 μl of 10× Taq DNA polymerase buffer (100 m*M* Tris-HCl (pH 8.3), 500 m*M* KCl, 15 m*M* $MgCl_2$, 0.01% gelatin (w/v)), 4 μl of 2.5 m*M* dNTP, 0.75 μl of each primer (20 μM), 1.5 units of Taq DNA polymerase and 0.5 μg of template DNA, and consisted of 40 cycles of 30 s at 94°C, 30 s at 52°C and 30 s at 72°C.

DNA sequencing PCR products were subcloned into pBluescript SK(−) (Stratagene, La Jolla, CA) with a mixture containing at least 50 subclones being used as a template for DNA sequencing. PCR primers were used as sequencing primers. When mutations were observed, separate PCR products were analyzed again to confirm the original results. Then, DNA derived from the corresponding normal tissue was subjected to the same analysis to exclude germ line mutations.

LOH analysis LOH was analyzed using 3 dinucleotide repeat polymorphisms at 9p21-22, termed D9S162, *IFN*-A and D9S171.[21, 22] The extracted DNAs from the tumor portion and the corresponding normal tissue were amplified independently and simultaneously by PCR. Primer sequences were 5′-TACACACATAGACACAGACA-3′ (sense) and 5′-TTCCCACAACAAATCTCCTC-3′

(antisense) for D9S162, 5′-TCTAAATTTCTCCAGTC-TCA-3′ (sense) and 5′-GGAAGTTATTTAATCACA-GG-3′ (antisense) for *IFN*-A and 5′-TCATCTCTGTC-TGCTGCCTC-3′ (sense) and 5′-TTTTCTTGGGGCT-ACTTTATT-3′ (antisense) for D9S171. The antisense primer was radiolabeled at 37°C for 30 min in a total volume of 20 μl containing 10 μl of antisense primer (20 μM), 2.5 μl of [γ-^{32}P]dATP (3000 Ci/mmol, 10 Ci/ml), 2 μl of 10× polynucleotide kinase (PNK) buffer (500 mM Tris-HCl (pH 8.0), 100 mM $MgCl_2$, 100 mM 2-mercaptoethanol), and 1 μl of PNK (Toyobo, Osaka). PCR reaction was performed in the same way as described above, except for 0.75 μl of sense primer (20 μM) and 1.5 μl of [γ-^{32}P]dATP-labeled antisense primer (10 μM). Five microliters of the PCR product was diluted with 5 μl of a gel-loading buffer (98% formamide, 10 mM EDTA (pH 8.0), 0.025% xylene cyanol, and 0.025% bromophenol blue), heated at 95°C for 3 min, and loaded on a 6% polyacrylamide gel containing 7 M urea at 70W for 2 h. The gel was fixed to 3MM filter paper, dried and autoradiographed at −80°C overnight. Cases showing two distinct groups of bands were considered as heterozygous and underwent evaluation for LOH. Tumors presenting bands with relative intensity less than 50% compared to the normal counterpart were taken as positive for LOH.

RESULTS

LOH analysis By analyzing 3 dinucleotide repeat polymorphisms, LOH at 9p21-22 could be evaluated in 15 cases and was observed in a total of 3 cases (17.6%) (Fig. 1). The results are summarized in Table II.

Mutational analysis PCR and DNA-sequencing revealed a total of 7 somatic mutations (1 deletion, 1 nonsense and 5 missense mutations) out of the 17 cases (7/17, 41.2%).

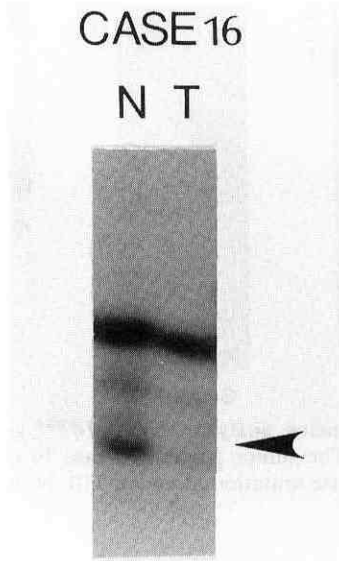

Fig. 1. LOH analysis of 9p21-22 at the D9S162 locus. PCR products of DNAs from normal and tumorous tissues were subjected to electrophoresis in a line in denaturing polyacrylamide gels. A representative LOH is indicated by an arrowhead in case 16. N, normal; T, tumor tissue.

Table II. Somatic Mutations of the *P16*^{INK4} Gene in Ampullary Carcinomas

Case	LOH			Mutation	
	D9S162	*IFN*-A	D9S171	codon	nucleotide change (amino acid change)
1	NI	−	−		
2	+	NI	NI		
3	−	−	NI		
4	−	NI	NI	35	GGG to AGG/GGG (Gly to Arg)
5	−	NI	NI	76	GCC to GC/GCC (C deletion)
6	−	NI	−		
7	−	NI	NI		
8	NI	NI	NI	99	CGG to CAG/CGG (Arg to Glu)
9	−	−	−		
10	NI	NI	NI	70	CCC to TCC/CCC (Pro to Ser)
11	−	NI	NI	56	AGC to AAC/AGC (Ser to Asn)
12	−	NI	NI		
13	−	−	NI	30	GCG to GTG/GCG (Ala to Val)
14	−	NI	NI	110	TGG to TGA/TGG (Try to Stop)
15	−	NI	NI		
16	+	NI	+		
17	+	NI	NI		

NI, not infomative; WT, wild type.

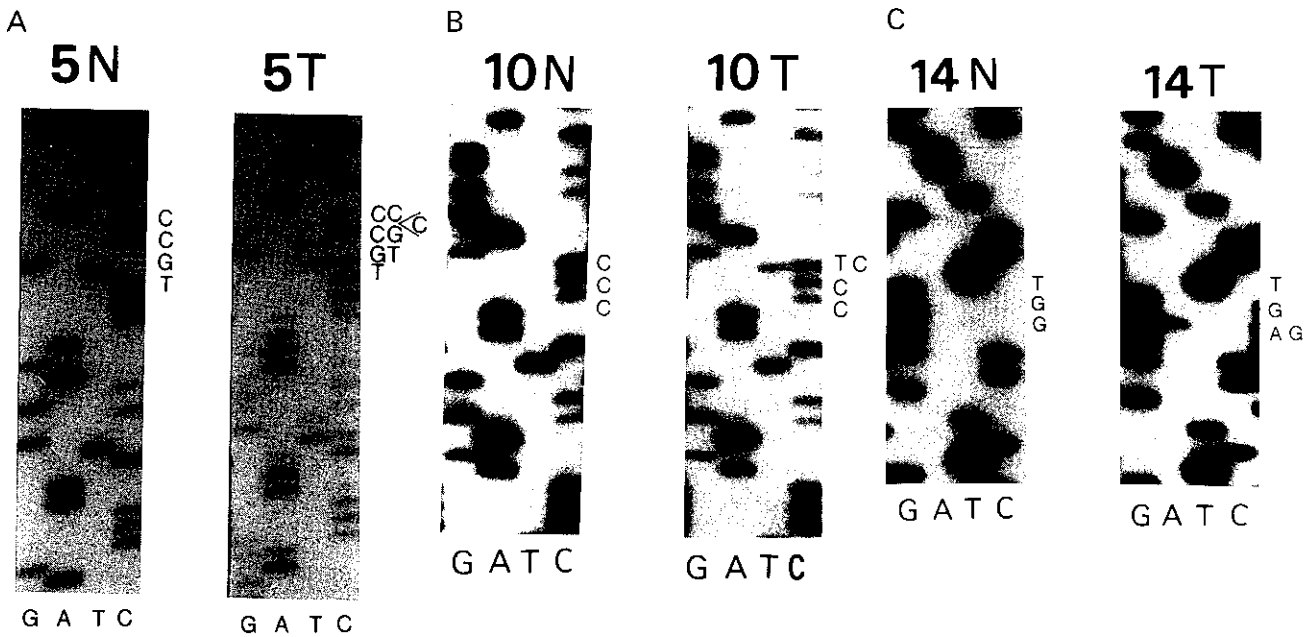

Fig. 2. Sequencing analysis of the *p16*^{INK4} gene. A, The tumor portion of case 5 shows a GCC-to-GC deletion mutation at codon 76. B, The tumor portion of case 10 contains a CCC-to-TCC missense mutation at codon 70. C, Case 14 has a TGG-to-TGA nonsense mutation at codon 110. N, normal; T, tumor; 5, 10 and 14; cases 5, 10, and 14, respectively.

The mutation sites were codons 30 (GCG to GTG/GCG, Ala to Val), 35 (GGG to AGG/GGG, Gly to Arg), 56 (AGC to AAC/AGC, Ser to Asn), 70 (CCC to TCC/CCC, Pro to Ser), 76 (GCC to GC/GCC, C deletion), 99 (CGG to CAG/CGG, Arg to Glu) and 110 (TGG to TGA/TGG, Try to Stop) (Fig. 2). None of the mutation cases revealed LOH. To rule out artifactual PCR mutations, the whole procedures were repeated to confirm the results. Furthermore, to exclude polymorphisms, corresponding normal tissues were subjected to investigation in each case. Nucleotide changes were noted only in DNAs from cancer tissues and were therefore concluded not to be polymorphisms. Of the 7 mutations, four were G-to-A transition mutations and two were C-to-G transversions, without any mutational hot spot. With regard to the relation to tumor phenotype, the *p16*^{INK4} gene mutations were found in 6 protruding and 1 ulcerative type tumors, which were microscopically diagnosed as 5 tubular adenocarcinomas, 1 papillary adenocarcinoma and 1 signet ring cell carcinoma. The results are summarized in Table II.

DISCUSSION

In the present investigation, LOH was noted in 3 cases and a subsequent search for point mutations revealed a total of 7 mutations (41.2%, 7/17) with retention of heterozygosity, two-hit inactivation not being evident in any of the cases. The frequency of the *p16*^{INK4} gene mutation (41.2%, 7/17) of primary ampullary carcinomas is higher than those reported previously for lung cancers (8.5% (6/71), 8.5% (6/71), 13.3% (2/15)),[10-12] malignant gliomas (34.4% (11/32), 14.3% (5/35)),[15,16] primary bladder tumors (9.1% (3/33)),[13] melanomas (14.9% (5/34)),[13] leukemias (0% (0/20), 0% (0/84)),[13,23] head-and-neck cancers (10.3% (7/68)),[14] gastric adenocarcinomas (0% (0/16))[24] and pancreatic cancers (34.4% (11/32)),[17] but lower than those for esophageal cancers (51.9% (14/27))[18] and biliary tract cancers (64% (16/25)).[19] In one previous study of biliary tract cancers, two ampullary carcinoma cases were included and point mutation was detected in one case.[19] The relatively high mutation rates of the *p16*^{INK4} gene common to all pancreato-biliary tract tumors might suggest the presence of an as-yet-unknown mutagen in the bile or pancreatic juice.

p16^{INK4} is, at present, thought to be generally inactivated by any of the following mechanisms: homozygous deletion of the gene, methylation of the promotor region, and mutation of the gene.[25] Because our study material was all in the form of paraffin-embedded samples, we could not detect loss of transcript and homozygous deletion in an appropriate manner. In this study, two of the 7 mutations we observed were a deletion mutation (at

codon 76) and a nonsense mutation (at codon 110), resulting in truncated incomplete proteins in which some of the ankyrin repeats, which are essential for p16^{INK4} protein-protein interactions,[6, 26] are absent or disrupted. p16^{INK4} includes 4 ankyrin repeats that are presumed to be crucial to its function. The other 5 missense mutations all resided in the four ankyrin repeats, and one (at codon 70) was at the ankyrin consensus. Considering the report that loss of the last ankyrin repeat or induced mutation at a consensus point (at codon 83) in the fourth ankyrin repeat almost completely abolished activity of p16^{INK4}, such mutations might be crucial.[26] Although the *p16*^{INK4} gene has been thought of as a tumor suppressor gene and two hits would therefore be necessary for inactivation, LOH analysis and sequencing studies revealed a normal allele along with the mutant forms in all the mutation cases. Possible explanations are that contamination of normal stromal cells occurred, that only a proportion of the tumor cells contained the mutated *p16*^{INK4} gene, or that p16^{INK4} operates in a dominant negative manner, like mutated *p53*. In the light of the similar mutation frequencies at each stage, stage I, 1/3 (33.3%), stage II, 2/5 (40%), stage III, 3/5 (60%), stage IV 1/4 (25%), the second hypothesis, which suggests that alteration of the gene is a late event in the course of tumor progression, seems unlikely. Furthermore, in a previous report on *p16*^{INK4} gene alteration in biliary tract cancers, LOH had been observed in only 1 of 16 (6.3%) *p16*^{INK4} mutated cases and other mechanisms of inactivation of the other allele, such as regulation of promoter activity or action in a dominant negative manner, were also suggested.[19] However, we think contamination by normal cells is probably the major factor. LOH cases without *p16*^{INK4} gene mutations, as found in lung, bladder, head and neck, kidney and brain tumors,[9–16] might suggest involvement of tumor suppressor genes other than the *p16*^{INK4} gene located at 9p21-22.

As for the relation between the mutation rates and clinicopathological parameters, mutations were observed more frequently in tubular adenocarcinomas (5/8, 62.5%) than papillary ones (1/8, 12.5%), and in protruding type tumors (6/11, 54.5%) than in ulcerated type ones (1/5, 20%). Mutations of the *p16*^{INK4} gene might be specifically associated with macroscopically protruding and histologically tubular adenocarcinomas.

In conclusion, although two-hit inactivation was not evident in any of the mutation cases and further investigation is needed to elucidate the role of altered p16^{INK4}, mutation of the gene is a frequent occurrence in primary ampullary carcinomas, and inactivation of p16^{INK4} might play a role in the development or progression of a subset of this neoplasm.

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