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Advances in Brief

Mutations of *p16*^{Ink4}/*CDKN2* and *p15*^{Ink4B}/*MTS2* Genes in Biliary Tract Cancers[1]

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Abstract

p16^{Ink4} and p15^{Ink4B} are cyclin-dependent kinase 4 inhibitors and link to the regulation of cell cycle in mammalian cells. The genes encoding these inhibitors are located at 9p21, which is a frequent site of allelic loss in various types of tumors. Twenty-five primary biliary tract cancers were examined for somatic mutations in *p16*^{Ink4}/*CDKN2*, *p15*^{Ink4B}/*MTS2*, *p53*, and K-*ras* genes and allelic loss of 9p21 by microsatellite analysis. Four biliary tract cancer cell lines were analyzed for homozygous deletions and point mutations. We found frequent homozygous deletions in *p16*^{Ink4}/*CDKN2* and *p15*^{Ink4B}/*MTS2* genes in the biliary tract cancer cell lines. Each cancer cell line had alteration of either *p16*^{Ink4}/*CDKN2*, *p15*^{Ink4B}/*MTS2*, or *p53* genes. In primary tumors, 16 of 25 (64%) biliary tract cancers had point mutations in the *p16*^{Ink4}/*CDKN2* gene. These include 14 missense and 2 silent mutations. The frequency of mutations in gall bladder cancer and hilar bile duct cancer were 80% (8 of 10) and 63% (5 of 8), respectively. Each of codons 1, 80, and 111 was changed in two cases of these cancers. One of three intrahepatic bile duct cancers, one of two common bile duct cancers, and one of two ampullary cancers had mutations in the *p16*^{Ink4}/*CDKN2* gene. In contrast, no mutation in the *p15*^{Ink4B}/*MTS2* gene, one base change in the K-*ras* gene, and one loss of heterozygosity at the *IFN*α locus in 25 cancers and one base change in the *p53* gene in 19 cancers were observed. These results suggest that *p16*^{Ink4}/*CDKN2*, rather than *p15*^{Ink4B}/*MTS2* or *p53* genes, and its inactivation may be important in biliary tract carcinogenesis.

Introduction

In spite of many clinical trials, the prognosis of biliary tract cancer is still poor. To improve the prognosis requires understanding of the biological features of biliary tract carcinogenesis. Progress of molecular biology has clarified the involvement of several gene alterations in carcinogenesis and cancer progression. In colorectal cancer, *APC*, K-*ras*, *p53*, *DCC*, and *MCC* are sequentially involved in carcinogenesis and tumor progression (1, 2). It is known that pancreatic cancer, which also shows the poorest prognosis in the cancers of alimentary tract, has frequent K-*ras* gene mutation (3). However, in biliary tract cancers, few reports have shown genetic changes, and no responsible oncogene or tumor suppressor gene has been reported. Involvement of K-*ras* in tumorigenesis of biliary tract cancers is unclear. Using PCR and direct sequencing analysis, point mutations of K-*ras* have been detected in only 5% of biliary tract cancer (4). In other reports, more frequent mutations (from 23 to 100%) of the K-*ras* gene have been found using methods based on modified PCR or PCR-denaturating gradient gel electrophoresis (5, 6). In hamsters, 25% of cholangiocarcinomas induced by *N*-nitrosobis(2-hydroxypropyl)amine have mutations of K-*ras* (7). *p53*, a major tumor suppressor gene, is frequently mutated in various kinds of cancer. The incidence of *p53* mutation in biliary tract cancers has not been reported.

Recently, frequent deletion of the *p16*^{Ink4}/*CDKN2* (*MTS1*[3]) gene in various tumors has been reported (8, 9). *p16*^{Ink4} has been identified as a regulatory protein in the cell cycle. *p16*^{Ink4} inhibits the catalytic activity of the complex of D-type cyclin and cyclin-dependent kinase 4 and blocks the G_1-to-S transition in the cells (10). Not only deletion but also frequent point mutations of the *p16*^{Ink4}/*CDKN2* gene in melanoma (8), esophageal cancer (11), and pancreatic cancer (12) have been reported. *p16*^{Ink4}/*CDKN2* has been reported to be mutated in a germline in some familial melanomas (13) and in a patient with dysplastic nevus syndrome (9). *p16*^{Ink4}/*CDKN2* has emerged as a major tumor suppressor. However, from the examination of primary tumor with LOH in 9p21, including lung, bladder, head and neck, kidney, brain, and breast, mutations of *p16*^{Ink4}/*CDKN2* were not so frequent (14). A lower frequency of *p16*^{Ink4}/*CDKN2* alterations in primary cancers than in cell lines has also been shown in bladder cancer (15) and squamous cell carcinoma of the head and neck (16). To clarify the role of *p16*^{Ink4}/*CDKN2* in tumorigenesis requires analysis of alterations of *p16*^{Ink4}/*CDKN2* in each primary tumor. *p15*^{Ink4B}/*MTS2*, which also belongs to the cyclin-dependent kinase 4 inhibitor family, has homology with *p16*^{Ink4}/*CDKN2* (8, 17) and is a neighbor of the *p16*^{Ink4}/*CDKN2* gene in 9p21 (8). Increased expression of *p15*^{Ink4B}/*MTS2* has been observed in G_1 arrest induced by treatment of transforming growth factor-β (17). It is possible that *p15*^{Ink4B}/*MTS2* is also a target of 9p21 deletion. Homozygous deletions, but no point mutation, of *p15*^{Ink4B}/*MTS2* in melanoma cell lines and primary brain tumors have been reported (8, 18).

In this study, to determine whether *p16*^{Ink4}/*CDKN2* and *p15*^{Ink4B}/*MTS2* are involved in biliary tract carcinogenesis, we examined alterations of *p16*^{Ink4}/*CDKN2*, *p15*^{Ink4B}/*MTS2*, *p53*, and K-*ras* genes and LOH at the *IFN*α and *D9S171* loci in 25 primary tumors of biliary tract cancer and four biliary tract cancer cell lines.

Materials and Methods

Tumor Specimens and Cell Lines. Primary tumors of biliary tract cancers were obtained from patients of the Tsukuba University Hospital. The tumor samples analyzed were 10 gall bladder cancers, 8 hilar bile duct cancers, 3 intrahepatic bile duct cancers, 2 common bile duct cancers, and 2 ampullary cancers. Four cell lines, including two bile duct cancer and two gall bladder cancer cell lines, were KMBC (19) from Dr. M. Kojiro (Kurume University, Kurume, Japan), and SK-ChA-1, Mz-ChA-1, and Mz-ChA-2 (20), were kind gifts from Dr. A. Knuth (Johaness-Gutenberg University, Mainz, Germany).

DNA Isolation and PCR. The homozygous deletion of *p16*^{Ink4}/*CDKN2* and *p15*^{Ink4B}/*MTS2* in cell lines and surgical specimens was analyzed by PCR. Briefly, genomic DNAs were isolated by proteinase K digestion and phenol-chloroform extraction. DNA fragments containing exon 1 of *p16*^{Ink4}/*CDKN2* and exon 2 of *p15*^{Ink4B}/*MTS2* were amplified from 0.1 μg of genomic DNA in 20 μl of PCR reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM

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[3] The abbreviations used are: MTS, multiple tumor supressor gene; LOH, loss of heterozygosity.

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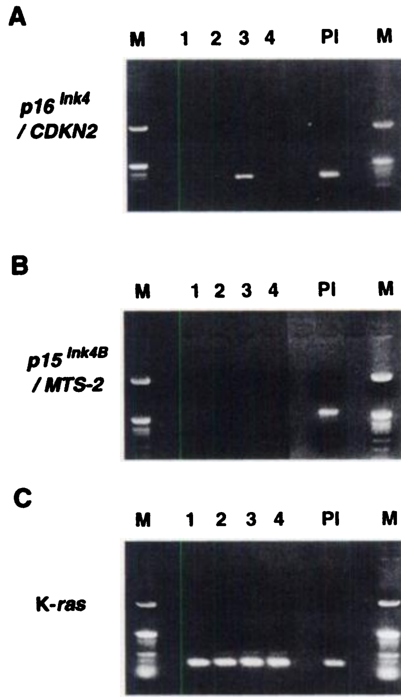

Fig. 1. Deletion analysis of exon 1 of the *p16*^Ink4^/*CDKN2* and *p15*^Ink4B^/*MTS2* genes. *Lane 1*, KMBC; *Lane 2*, SK-ChA-1; *Lane 3*, Mz-ChA-1; *Lane 4*, Mz-ChA-2. *Pl*, normal human placenta; *M*, size markers (*Hin*fI-digested pBR322). *A*, exon 1 of the *p16*^Ink4^/*CDKN2* gene. *B*, *p15*^Ink4B^/*MTS2* gene. *C*, exon 1 of the K-*ras* gene.

KCl, 2 mM $MgCl_2$, 0.1% gelatin, 0.5% Tween 20, 200 μM each of deoxyribonucleoside triphosphates, and 0.25 unit of Taq DNA polymerase (Wako, Osaka, Japan). Primer sets for the PCR amplification were as described previously (8). The PCR cycles for exon 1 of *p16*^Ink4^/*CDKN2* were four cycles of 1 min at 95°C, 1 min at 68°C, and 1.5 min at 72°C; four cycles of 1 min at 95°C, 1 min at 66°C, and 1.5 min at 72°C; 8 cycles of 1 min at 95°C, 1 min at 63°C, and 1.5 min at 72°C; and 40 cycles of 1 min at 95°C, 1 min at 60°C, and 1.5 min at 72°C. The PCR conditions for exon 2 of *p16*^Ink4^/*CDKN2* were 40 cycles of 1 min at 95°C, 1 min at 57°C, and 1.5 min at 72°C. The PCR conditions for exon 2 of *p15*^Ink4B^/*MTS2* were 40 cycles of 1 min at 95°C, 1 min at 57°C, and 1.5 min at 72°C. K-*ras* was also amplified as an internal control under the same conditions with *p15*^Ink4B^/*MTS2*. Primer sets for K-*ras* were as described (21). Amplified DNA fragments were analyzed on a 2% agarose gel. LOH of microsatellites, which locate at 9p21, *IFNα*, and *D9S171*, was examined in 15 cases. Oligonucleotide primers and conditions for PCR and electrophoresis for *IFNα* and *D9S171* were as described (22).

Sequencing. Mutations in the *p16*^Ink4^/*CDKN2* and *p15*^Ink4B^/*MTS2* genes were analyzed by direct sequencing. For confirmation of mutations, some of PCR products were subcloned and sequenced. PCR products of exons 1 and 2 of *p16*^Ink4^/*CDKN2* and exon 2 of *p15*^Ink4B^/*MTS2*, amplified from genomic DNAs of surgical specimens and cell lines, were used. The excess primers were removed using Suprec-02 (Takara Biomedicals, Kyoto, Japan), and direct sequencing was performed using a double-stranded DNA Cycle Sequencing System (GIBCO-BRL, Gaithersburg, MD) with each primer used for PCR amplification, end-labeled with [γ-^{32}P]ATP (5,000 Ci/mmol, Amersham, United Kingdom). Autoradiograms of the gels were analyzed with a Fujix BAS2000 imaging analyzer (Fuji Photo Film, Tokyo, Japan). To confirm the sequence changes in *p16*^Ink4^/*CDKN2*, PCR products derived from cases 3, 9, 11, 13, 18, 21, 22, and 25 were ligated in pCRII vector (Invitrogen, San Diego, CA), and a pool of such plasmid clones were sequenced with the M13 primers using an AmpliCycle Sequencing Kit (Perkin-Elmer, Foster, CA).

PCR/Single-stranded Conformation Polymorphism Analysis. Mutations of *p53* and *K-ras* genes were examined using PCR/single-stranded conformation polymorphism analysis (23) with slight modifications. Four exons (from exons 5 to 8) of the *p53* gene were amplified by PCR using oligonucleotide primers as follows: 5′-TTCAACTCTGTCTCCTTCCT-3′ (forward) and 5′-CAGCCCTGTCGTCTCTCCAG-3′ (reverse) for exon 5; 5′-GCCTCTGATTCCTCACTGAT-3′ (forward) and 5′-CGGAGGGCCACTGACAACCA-3′ (reverse) for exon 6; 5′-AGGCGCACTGGCCTCATCTT-3′ (forward) and 5′-TGTGCAGGGTGGCAAGTGGC-3′ (reverse) for exon 7; and 5′-TTCCTTACTGCCTCTTGCTT-3′ (forward) and 5′-AGGCATAACTGCACCCTTGG-3′ (reverse) for exon 8. Five μl of PCR mixture contained 50 ng of sample DNA, 2 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol, Amersham, United Kingdom), 0.5 μM each of primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.75 mM $MgCl_2$, 0.01% gelatin, 0.05% Tween 20, 25 μM each of four deoxyribonucleoside triphosphates, and 0.125 unit of Taq DNA polymerase (Wako, Osaka, Japan). After 30 cycles of reaction (1 min at 95°C, 1 min at 60°C, and 1.5 min at 72°C), PCR product was analyzed on a 6% polyacrylamide gel with or without 5% glycerol. Exons 1 and 2 of the K-*ras* gene were also amplified and analyzed as described (21). Migrating bands were excised, and DNAs were extracted. The DNA was sequenced as described above.

Results and Discussion

We examined *p16*^Ink4^/*CDKN2* and *p15*^Ink4B^/*MTS2* genes in biliary tract cancer cell lines and found high frequencies of homozygous deletion determined by failure to amplify exon 1 of *p16*^Ink4^/*CDKN2* and exon 2 of *p15*^Ink4B^/*MTS2*. Fig. 1*A* shows the deletion of exon 1 of the *p16*^Ink4^/*CDKN2* gene. Three of the four biliary tract cancer cell lines, KMBC, SK-ChA-1, and Mz-ChA-2, had homozygous deletions in *p16*^Ink4^/*CDKN2*, and *p15*^Ink4B^/*MTS2* was absent in all (Fig. 1*B*). Using placental DNA as a template, fragments of *p16*^Ink4^/*CDKN2* (340 bp) and *p15*^Ink4B^/*MTS2* (about 550 bp) were observed. The DNA fragments of exon 1 of K-*ras* (107 bp) were amplified in all samples, confirming the quality of the DNA samples (Fig. 1*C*). Homozygous deletions of *p16*^Ink4^/*CDKN2* and *p15*^Ink4B^/*MTS2* and the alterations of *p53* and K-*ras* genes in these four cell lines are summarized in Table 1. Gall bladder cancer cell line Mz-ChA-1 had neither deletion nor point mutation in the *p16*^Ink4^/*CDKN2* gene, but it had homozygous deletion of *p15*^Ink4B^/*MTS2* and point mutation of *p53*. The other three cell lines had homozygous deletion of *p16*^Ink4^/*CDKN2* and *p15*^Ink4B^/*MTS2*. Each cancer cell line had alterations of either *p16*^Ink4^/*CDKN2*, *p15*^Ink4B^/*MTS2*, or *p53*. Point mutations of *p53* were detected in one of two gall bladder cancer cell lines and one of two bile duct cancer

Table 1 *Alterations of p16*^Ink4^/*CDKN2, p15*^Ink4B^/*MTS2, p53, and K-ras genes in biliary tract cancer cell lines*

Cell lines	Origin	p16^Ink4^ [a]	p15^Ink4B^	p53 [b] Codon	p53 [b] mutation	K-ras [b] Codon	K-ras [b] mutation
KMBC	Bile duct cancer	Deleted	Deleted		WT	13	GGC → AGC (Gly → Ser)
SK-ChA-1	Bile duct cancer	Deleted	Deleted	294	GAG → CAG (Glu → Gln)	12	GGT → GTT (Gly → Val)
Mz-ChA-1	Gall bladder cancer	WT	Deleted	288	AAT → AAG (Asn → Lys)		WT
Mz-ChA-2	Gall bladder cancer	Deleted	Deleted		WT		WT

[a] WT, either no deletion or no point mutation in the *p16*^Ink4^/*CDKN2* gene.
[b] WT, the wild-type nucleotide sequences of *p53* and K-*ras* genes. Amino acid changes of *p53* and K-*ras* genes are shown in parentheses.

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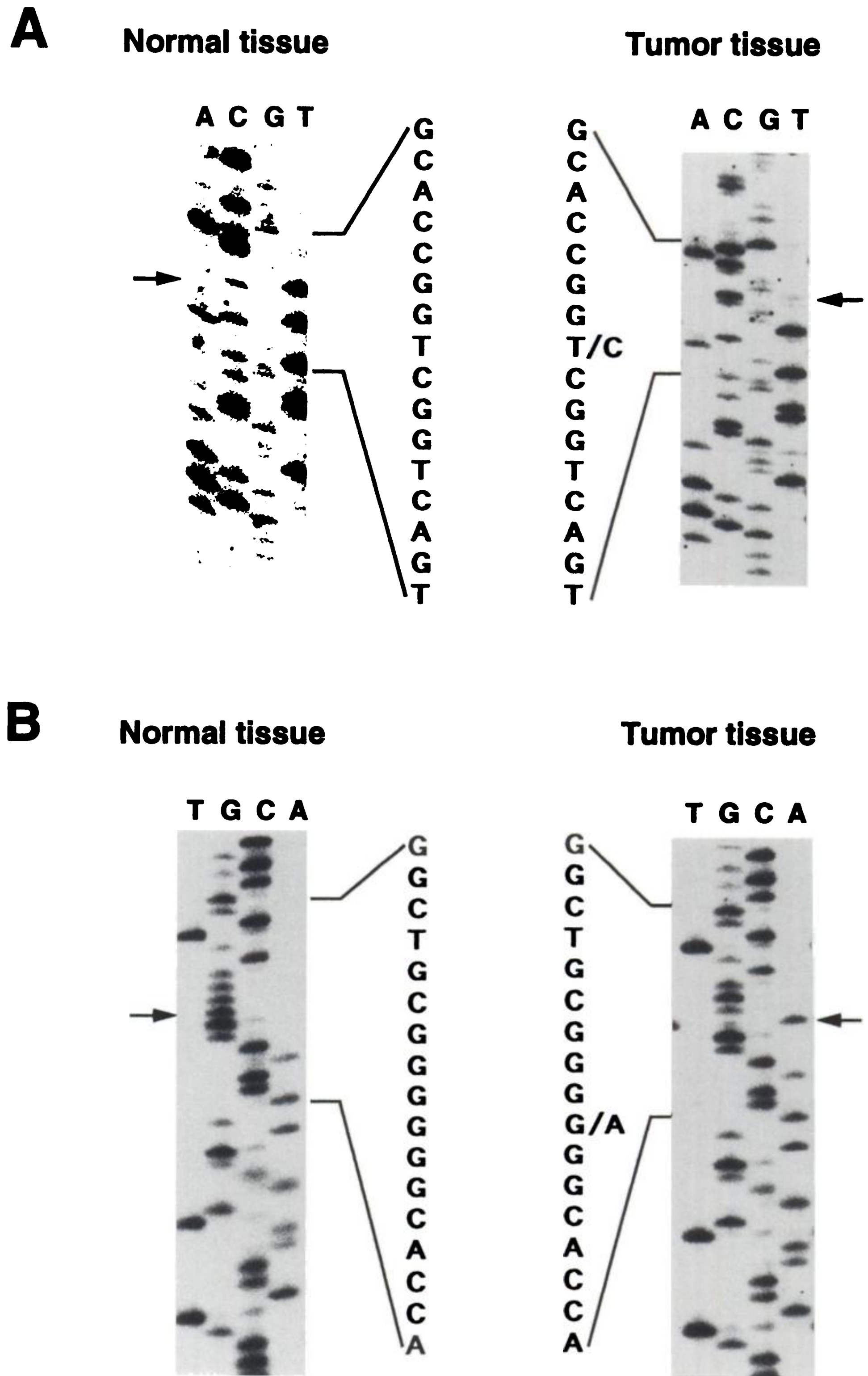

Fig. 2. *p16*^{Ink4}*/CDKN2* mutations in biliary tract cancers. *A*, case 13 had T to C transition in codon 8 of the *p16*^{Ink4}*/CDKN2* gene (exon 1). *B*, case 2 had G to A transition in codon 127 of the *p16*^{Ink4}*/CDKN2* gene (exon 2).

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cell lines. Point mutations of K-*ras* were detected in two bile duct cancer cell lines.

The high frequencies of deletions of *p16*^{Ink4}*/CDKN2* and *p15*^{Ink4B}*/MTS2* in cell lines suggest the possibility of these genes as tumor suppressor genes in biliary tract cancer. However, in the primary tumor samples of lung, kidney, brain (14), bladder (14, 15), head and neck (14, 16), melanoma (24), and breast (14, 25), a lower frequency of alterations of these genes has been reported. It is critical whether inactivation of *p16*^{Ink4}*/CDKN2* and *p15*^{Ink4B}*/MTS2* occurs in primary biliary tract cancer. To demonstrate the involvement of these genes in biliary tract cancer, we analyzed the intragenic mutations in surgical specimens. We analyzed 25 biliary tract cancer specimens by searching for point mutations of *p16*^{Ink4}*/CDKN2* and *p15*^{Ink4B}*/MTS2*, in addition to *p53* and K-*ras* genes. Fig. 2 shows representative results of sequencing. In case 13, T to C transition in codon 8 of *p16*^{Ink4}*/CDKN2* was detected (Fig. 2*A*), and in case

Table 2 *Point mutations of p16^Ink4^/CDKN2, p15^Ink4B^/MTS2, p53, and K-ras genes in biliary tract cancers*[a]

Cases	Origin	*p16*Ink4 Codon	*p16*Ink4 mutation	*p15*Ink4B	*p53* Codon	*p53* mutation	K-*ras* Codon	K-*ras* mutation
9	Gall bladder cancer	6	GAC → GAG (Asp → Glu)	WT		WT		WT
1	Gall bladder cancer	12	GCG → TCG (Ala → Ser)	WT		WT		WT
6	Gall bladder cancer	18	GAG → GAC (Glu → Asp)	WT		ND		WT
7	Gall bladder cancer	80	GAG → GAT (Glu → Asp)	WT		WT		WT
10	Gall bladder cancer	80	GAG → GAT (Glu → Asp)	WT		WT		WT
3	Gall bladder cancer	111	GAG → CAG (Glu → Gln)	WT		WT		WT
4	Gall bladder cancer	111	GAG → CAG (Glu → Gln)	WT		WT		WT
2	Gall bladder cancer	127	GGG → GGA (no change)	WT		WT		WT
5, 8	Gall bladder cancer		WT	WT		WT		WT
11	Hilar bile duct cancer	1	ATG → ATA (Met → Ile)	WT	222	CCG → CTG (Pro → Leu)		WT
12	Hilar bile duct cancer	1	ATG → ATA (Met → Ile)	WT		WT		WT
13	Hilar bile duct cancer	8	CTG → CCG (Leu → Pro)	WT		WT		WT
16	Hilar bile duct cancer	25	GAG → GAT (Glu → Asp)	WT		ND		WT
18	Hilar bile duct cancer	41	ATC → AGC (Ile → Ser)	WT		ND		WT
14, 15	Hilar bile duct cancer		WT	WT		WT		WT
17	Hilar bile duct cancer		WT	WT		ND		WT
21	Intrahepatic bile duct cancer	66	GAC → GTC (Asp → Val)	WT		WT	12	GGT → GTT (Gly → Val)
19, 20	Intrahepatic bile duct cancer		WT	WT		WT		WT
22	Common bile duct cancer	117	GAT → GAC (no change)	WT		WT		WT
23	Common bile duct cancer		WT	WT		WT		WT
25	Ampullary cancer	114	GGC → AGC (Gly → Ser)	WT		ND		WT
24	Ampullary cancer		WT	WT		ND		WT

[a] WT, the wild-type nucleotide sequence of *p16*^Ink4^*/CDKN2, p15*^Ink4B^*/MTS2 p53*, and K-*ras* genes. ND, sequence analysis has not been done. Amino acid changes of *p16*^Ink4^*/CDKN2, p53*, and K-*ras* genes are shown in parentheses.

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2, G to A transition in codon 127 of *p16*^Ink4^*/CDKN2* was detected (Fig. 2*B*).

Results of sequencing of primary tumors are summarized in Table 2. Sixteen of 25 (64%) cancer specimens contained point mutations of *p16*^Ink4^*/CDKN2*. Especially, gall bladder cancer showed the highest frequency (8 of 10) of point mutations of *p16*^Ink4^*/CDKN2*. The frequency of point mutation of *p16*^Ink4^*/CDKN2* in hilar bile duct cancer was 63% (5 of 8). Each one point mutation was detected in three intrahepatic bile duct cancers, two common bile duct cancers, and two ampullary cancers. In these 16 point mutations, 4 G to A transitions, 4 G to T transversions, and 3 G to C transversions were observed. Fourteen of 16 mutations were missense mutations, and 2 of 16 caused no amino acid change. Mutations at codons 1, 80, and 111 were found in two cases of these sample, respectively. From previous reports (8, 11, 12, 15, 16) and present data, it is difficult to find any mutational "hotspot" in the *p16*^Ink4^*/CDKN2* gene. To exclude polymorphisms in *p16*^Ink4^*/CDKN2*, we examined normal tissue and tumor tissue samples obtained from the same patient. As shown in Fig. 2, sequence changes were found in tumor tissues and not in the normal tissues. These data indicate that sequence changes were not polymorphisms.

On the other hand, no point mutation in exon 2 of *p15*^Ink4B^*/MTS2* was detected in surgical specimens of biliary tract cancers. We could not deny the involvement of the *p15*^Ink4B^*/MTS2* gene in biliary tract cancers, because deletion analysis of *p15*^Ink4B^*/MTS2* was not done in

the surgical specimens. Biliary tract cancers have larger numbers of interstitial cells than most other cancers. Contamination of many normal interstitial cells makes it methodologically difficult to detect homozygous allelic loss in biliary tract cancer specimens.

Point mutations detected in primary tumors were heterozygous in this study. If *p16*$^{\mathrm{Ink4}}$*/CDKN2* is a recessive tumor suppressor, the second allele must be inactivated by some other mechanisms, such as allelic loss. *IFNα* and *D9S171* are known as microsatellite markers located in 9p21, which are useful for the analysis of allelic losses in primary tumors. Fifteen cases that had point mutations in *p16*$^{\mathrm{Ink4}}$*/CDKN2* were analyzed with these markers. Only case 21 showed LOH in the *IFNα* locus and genetic instability in *D9S171* locus (data not shown). These data suggest that allelic loss of 9p21 surrounding *IFNα* and *D9S171* in primary tumors of biliary tract cancers are not frequent. It might be possible that one allele of the *p16*$^{\mathrm{Ink4}}$*/CDKN2* gene is inactivated by point mutation and the second allele is inactivated by other mechanisms, similar to regulation of promoter activity of the *p16*$^{\mathrm{Ink4}}$*/CDKN2* gene, or mutated *p16*$^{\mathrm{Ink4}}$*/CDKN2* may act in a dominant negative manner, similar to *p53* in carcinogenesis.

Point mutation of the *p53* gene was detected in 1 of 19 tumors (6%). A hilar bile duct cancer (case 11) had C to T transition in codon 222 of the *p53* gene. In this case, point mutation of codon 1 of the *p16*$^{\mathrm{Ink4}}$*/CDKN2* gene was also observed. A point mutation of the K-*ras* gene was detected in an intrahepatic bile duct cancer (4% of biliary tract cancers examined). This sample also had a point mutation of codon 66 of the *p16*$^{\mathrm{Ink4}}$*/CDKN2* gene. Frequencies of point mutation of *p53* and K-*ras* were much lower than that of *p16*$^{\mathrm{Ink4}}$*/CDKN2*. These results strongly suggest that the *p16*$^{\mathrm{Ink4}}$*/CDKN2* gene, rather than *p53* or K-*ras*, is possibly crucial in biliary tract carcinogenesis. It now remains to be clarified when the *p16*$^{\mathrm{Ink4}}$*/CDKN2* gene is altered during multistep carcinogenesis.

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