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CLINICAL RESEARCH

Two distinct pathways of p16 gene inactivation in gallbladder cancer

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Abstract

AIM: To examine the mechanism of inactivation of the p16 gene in gallbladder cancer, and to investigate p16 alterations and their correlation with clinicopathological features.

METHODS: Specimens were collected surgically from 51 patients with gallbladder cancer. We evaluated the status of protein expression, loss of heterozygosity (LOH), homozygous deletion and promoter hypermethylation using immunohistochemistry, microsatellite analysis, quantitative real-time polymerase chain reaction (PCR) and methylation-specific PCR, respectively. In addition, mutations were examined by direct DNA sequencing.

RESULTS: Homozygous deletions of the p16 gene exon2, LOH at 9p21-22, p16 promoter hypermethylation, and loss of p16 protein expression were detected in 26.0% (13/50), 56.9% (29/51), 72.5% (37/51) and 62.7% (32/51), respectively. No mutations were found. LOH at 9p21 correlated with the loss of p16 protein expression ($P < 0.05$). Homozygous deletion of the p16 gene, a combination LOH and promoter hypermethylation, and multiple LOH at 9p21 were significantly correlated with the loss of p16 protein expression ($P < 0.05$). LOH at 9p21 and promoter hypermethylation of the p16 gene were detected in 15.4% (2/13) and 92.3% (12/13) of the tumors with homozygous deletion of the p16 gene, respectively. P16 alterations were not associated with clinicopathological features.

CONCLUSION: Our results suggest that LOH and homozygous deletion may be two distinct pathways in the inactivation of the p16 gene. Homozygous deletion, a combination of LOH and promoter hypermethylation, and multiple LOH are major mechanisms of p16 inactivation in gallbladder cancer.

Key words: Gallbladder cancer; Homozygous deletion; Loss of heterozygosity; p16; Quantitative real time PCR

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INTRODUCTION

Gallbladder carcinoma is a highly malignant neoplasm with a poor prognosis, and most patients are diagnosed at an already advanced stage[1-3]. Gallbladder carcinoma is a relatively common cancer and is the sixth highest cause of cancer death in Japanese women.

Several genes have been implicated in the tumorigenesis of gallbladder cancers, including *K-ras*, cerbβ2, p53[4,5], $p16^{INK4a}$/CDKN2, and the fragile histidine triad (FHIT)[6]. Genetic alterations in the 9p21 chromosomal region have been linked to malignant progression.

The p16 gene, located on chromosome 9p21, encodes a critical negative regulator of cell cycle progression and is inactivated in various cancers. The p16 gene is an important tumor suppressor gene, which interacts strongly with cyclin-dependent kinases 4 and 6, and inhibits their ability to interact with cyclin D[7]. p16 induces cell cycle arrest at G1 and G2/M checkpoints, which blocks cells from phosphorylating retinoblastoma protein 1, and prevents cells from exiting the G1 phase of the cell cycle[8]. p16 can act as a negative regulator of normal cell proliferation. Inactivation of the p16 gene plays an important role in tumorigenesis. p16 inactivation by loss of heterozygosity (LOH) and point mutations has been reported in biliary tract cancers[9] and intrahepatic cholangiocarcinoma[10].

Aberrant promoter methylation is an important mechanism in silencing cancer-related genes during the process of carcinogenesis. Epigenetic inactivation of tumor suppressor genes has been commonly reported in various tumors[11]. Promoter hypermethylation, as

well as gene deletions and point mutations, has been shown to be a major mechanism of p16 inactivation[12,13]. Hypermethylation of the CpG islands of the p16 gene promoter region has been reported in various types of tumor.

The main modes of p16 gene inactivation in gallbladder carcinoma are known to include LOH, mutation and hypermethylation[12,14]. Homozygous deletion of the p16 gene has not previously been investigated in gallbladder cancer. Therefore, we sought to comprehensively study genetic and epigenetic alterations of p16, including homozygous deletion of the p16 gene, and the relationship between these abnormalities and clinicopathological features.

MATERIALS AND METHODS

Tissue specimens

Paraffin-embedded tissue samples were obtained from 51 patients who underwent surgical resection at Juntendo University School of Medicine, Japan, between April 1996 and April 2005. Gallbladder carcinoma patients consisted of 25 women and 26 men, ranging in age from 36 to 94 (mean, 65.1) years. Their tumors consisted of 46 adenocarcinomas and five adenosquamous carcinomas. The adenocarcinomas included 39 well-to-moderately differentiated and seven poorly differentiated tumors. Most of the patients had advanced gallbladder carcinoma, with invasion of the subserosa (n = 22, 43.1%) and serosa (n = 18, 35.3%), while the other 11 patients (19.6%) had early gallbladder carcinoma (mucosa or muscularis propria invasion). All histological slides were reviewed by M.T. and H.T. and were classified based on the WHO classification of gallbladder carcinoma. Medical records were available for all patients.

Immunohistochemical analysis

Immunohistochemistry was performed using anti-p16 (F-12; 1:500 dilution; Santa Cruz Biochemistry, Santa Cruz, CA, USA) and an automated slide staining system (NexES IHC; Ventana, AZ, USA), according to the manufacturer's instructions. P16 immunostaining was performed within 2 weeks of sectioning, because reactivity decreased over time after preparation. Normal lymphocytes and intrahepatic bile ducts were positive controls for p16. The percentage of positive nuclei was scored as follows: -, 0%-10%, + 1%-25%, ++, 25%-50%, and +++, > 50% positive cells. Scores of +, ++ and +++ were considered to represent positive immunostaining, while - was deemed to be negative.

DNA extraction

Formalin-fixed, paraffin-embedded tissue blocks were used. Serial 10-μm sections were cut from each block and stained with hematoxylin and eosin to locate the tumor and non-neoplastic tissue before DNA extraction. Sections were cut, deparaffinized, and microdissected with an 18-gauge needle. The microdissected tissues were digested overnight at 55℃ in buffer (1% Tween 20, 10 mmol/L Tris-HCl, pH 8, 1 mmol/L EDTA, and 100 μg/mL proteinase K). The lysates were heated at 95℃ for 10 min and stored at 4℃ until analyzed by polymerase chain reaction (PCR).

Methylation assay

DNA methylation was investigated using an EZ DNA methylation kit (Zymo Research, CA, USA), according to the manufacturer's protocol. Microdissected genomic DNA (1 ng) was denatured with M-dilution buffer at 37℃ for 15 min, followed by incubation with CT conversion reagent at 50℃ for 16 h in the dark. After treatment, the DNA was purified using M-binding buffer, incubated with M-desulphonation buffer at room temperature for 15 min, washed with wash buffer, and finally resuspended in M-elution buffer.

Primers for the p16 gene were 5'-TTATTAGAGGG TGGGGTGGATTGT-3' (sense) and 5'-CCACCTAAAT CAACCTCCAACCA-3' (antisense) for the unmethylated reactions, and 5'-TTATTAGAGGGTGGGGCGGATCG C-3' (sense) and 5'-GACCCCGAACCGCGACCGTAA-3' (antisense) for the methylated reactions, as described previously[15]. PCR reactions were started by denaturation at 95℃ for 5 min, followed by 40-45 cycles of 94℃ for 30 s, 65℃ (for methylated p16) or 60℃ (for unmethylated p16) for 45 s, and 72℃ for 60 s, with a final extension at 72℃ for 10 min. PCR products were analyzed by electrophoresis on a 3% agarose gel, and visualized by ethidium bromide staining. DNA from the Raji cell line was used as a positive control. Distilled water was used as a negative control.

LOH analysis

Paired normal and tumor DNA samples were amplified by hot-start PCR, using locus-specific flanking primer pairs for five fluorescently labeled microsatellite markers, D9S171-FAM, D9S1748-FAM, D9S942-NEX, D9S974-NED, and D9S1749-NED (Figure 1). Primer sequences were obtained from the NCBI UniSTS database (http://www.ncbi.nlm.nih.gov/). Markers mapping to the chromosome 9p21-22 region were used. D9S1748, D9S942 and D9S974 are within a coding sequence of the p16 gene. D9S1749 is telomeric to p16 and D9S171 is centromeric to p16. PCR was performed with an initial denaturation at 95℃ for 15 min, followed by 36 cycles of denaturation at 95℃ for 30 s, annealing at 58℃ (D9S171, D9S1748, D9S942, D9S974) or 55℃ (D9S1749) for 45 s, and extension at 72℃ for 60 s, and final extension at 72℃ for 10 min. After PCR, samples were diluted at 1:7 in formamide, heated to 95℃ for 2 min, chilled on ice, and analyzed with Genescan software on an ABI PRISM 310 genetic analyzer (PE-Applied Biosystems, Foster City, CA, USA). Allelic ratios in both normal and tumor samples were calculated and compared. The area under each peak, representing each allele in the microsatellite pair, was obtained. LOH was defined as a > 50% reduction in the tumor peak compared to that of the corresponding normal tissue. Additional bands that were not seen on normal DNA, but were observed in tumor samples, were considered evidence of microsatellite instability (MSI).

Detection of homozygous deletion of p16 exon2

Homozygous gene deletion and gene dosage of p16 exon2

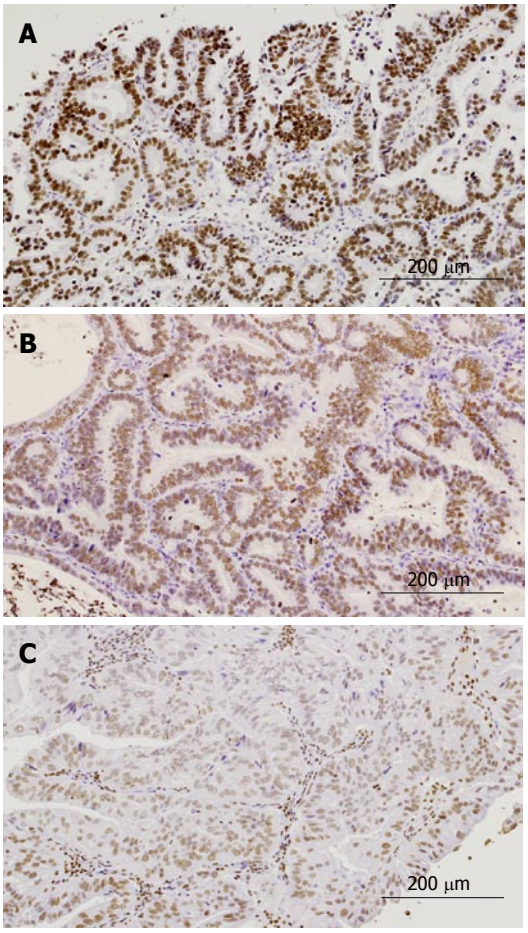

Figure 1 Immunohistochemical staining of p16. **A**: Tumor cells showing diffuse strong positive staining of p16; **B**: Tumor cells showing diffuse moderate staining; **C**: Tumor cells showing partly weak staining.

in gallbladder cancer were determined using a TaqMan-based real-time PCR method. Briefly, p16 gene exon2 and the GAPDH gene were amplified in a monoplex assay. The primer sequences for detecting p16 exon2 were 5'-AGCTTCCTTTCCGTCATGC-3' (sensze) and 5'-TCATGACCTGCCAGAGAGAA-3' (antisense). The primer sequences for detecting the GAPDH gene were 5'-GCATCCTGGGCTACACTGAG-3' (sense) and 5'-AG GTGGAGGAGTGGGTGTC-3' (antisense). The probe sequence for p16 gene exon2 was FAM-TGGCTCTG, and the probe sequence for the GAPDH gene was FAM-CTCCTCTG (the three FAM-labeled probes were manufactured by Roche Applied Science, Mannheim, Germany). The real-time PCR was performed in a 25 μL final volume containing 12.5 μL of Premix Ex Taq (Perfect Real Time; Takara, Kyoto, Japan), 50 ng DNA template, 10 μmol/L of each primer, 10 μmol/L Universal Library Probe, 0.5 μL ROX reference dye (50 ×), and 5.75 μL distilled water. The thermal cycling conditions on the ABI PRISM 7500 instrument were set to 10 s at 95℃, followed by 40 cycles of 5 s at 95℃, alternating with 34 s at 60℃. DNA from lymphocytes isolated from a paraffin block was used as a positive control. All reactions were done in triplicate. Normalized gene dosage ratios were interpreted as follows: 0-0.3, homozygous deletion; 0.31-0.69, under-representation (of the test gene, relative to the reference gene); 0.7-1.49, retention of copy number; and > 1.5 over-representation.

Table 1 The primer sequence of the p16 genes

Exon	Primer sequence	Size (bp)	Anneling temperature (℃)
Exon1α	F: 5'-GAGAGGGGGAGAGCAGGCAG-3' R: 5'-GCAAACTTCGTCCTCCAGAGT-3'	250	58
Exon2-1	F: 5'-AGCTTCCTTTCCGTCATGC-3' R: 5'-GCAGCACCACCAGCGTG-3'	203	56
Exon2-2	F: 5'-AGCCCAACTGCGCCGAC-3' R: 5'-CCAGGTCCACGGGCAGA-3'	147	58
Exon2-3	F: 5'-TGGACGTGCGCGATGC-3' R: 5'-GGAAGCTCTCAGGGTACAAATT C-3'	189	56
Exon3	F: 5'-CCGGTAGGGACGGCAAGAGA-3' R: 5'-CTGTAGGACCCTCGGTGACTGAT GA-3'	169	58

Mutation analysis

Mutation analysis was performed for the p16 gene (exons 1α, 2 and 3). Primer sequences and PCR conditions were as described previously[16,17] (Table 1). Amplification was performed using a Perkin Elmer GeneAmp 9600 Thermal cycler. After visualizing the PCR products in a 3% agarose gel, an aliquot (5 μL) of the PCR product was treated at 37℃ for 15 min with 1 μL ExoSAP-IT (GE Healthcare Biosciences, Piscataway, NJ, USA), followed by inactivation at 80℃ for 15 min. Part of this mix (6 μL) was directly sequenced using a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems Japan, Chiba) on an automated sequencer (ABI PRISM 3100 Genetic analyzer; Perkin-Elmer, Japan).

Statistical analysis

Frequency distributions were analyzed by the χ^2 test. Correlations were examined between alterations of p16 and p16 expression, or between alterations of p16 and clinicopathological parameters. $P < 0.05$ was deemed statistically significant.

RESULTS

Immunohistochemical analysis

Loss of p16 protein expression occurred in 62.7% (32/51) of gallbladder cancer patients. Staining for p16 was weak in 13.7% (7/51) of patients, moderate in 17.6% (9/51), and strong in only 9.8% (5/51) of patients (Figure 1). There was no significant difference in p16 protein expression according to patient age, gender, tumor stage, T factor, N factor or histology (Table 2).

p16 status

LOH at 9p21-22 was detected in at least one marker in 56.9% (29/51) of patients. LOH occurred in 11.8%, 27.5%, 11.8%, 13.7% and 39.2% of patients who were positive for the markers D9S171, D9S1748, D9S942, D9S974 and D9S1749, respectively. Approximately 29.4% of the cases presented with LOH in a single marker, 11.8% with LOH in two markers, and 15.7% with LOH in three or more markers. LOH at the three markers D9S1748,

Table 2 Association between p16 alterations and clinicopathological variables in gallbladder cancers

Clinicopathological variables	p16 protein expression			Methylation of $p16^{INK4a}$			LOH at 9p21-22			Homozygous deletion of p16 gene		
	Absent	Present	*P*	Absent	Present	*P*	Retension	LOH	*P*	Absent	Present	*P*
	n	*n*		*n*	*n*		*n*	*n*		*n*	*n*	
Age												
< 65	13	7	0.789	5	15	0.753	10	10	0.427	13	6	0.481
> 65	19	12		9	22		12	19		24	7	
Gender												
Female	14	11	0.329	6	19	0.588	10	15	0.657	20	4	0.148
Male	18	8		8	18		12	14		17	9	
Tumor type												
Adenocarcinoma	28	18	0.401	14	32	0.148	19	27	0.423	35	10	0.068
Adenosquamous	4	1		0	5		3	2		2	3	
Differentiation grade												
Well-Moderate	22	17	0.144	12	27	0.907	18	21	0.115	29	10	0.16
Poor	6	1		2	5		1	6		6	0	
Stage												
0 and ⅠA, ⅠB	15	13	0.135	9	19	0.407	15	13	0.097	20	7	0.99
ⅡA and ⅡB	17	6		5	18		7	16		17	6	
T factor												
Tis and T1	6	6	0.296	3	9	0.828	8	4	0.060	11	1	0.11
T2 and T3	26	13		11	28		14	25		26	12	
N factor												
N0	21	16	0.150	9	28	0.416	19	18	0.054	28	8	0.329
N1	11	3		5	9		3	11		9	5	

All *P* values were revealed by χ^2-test.

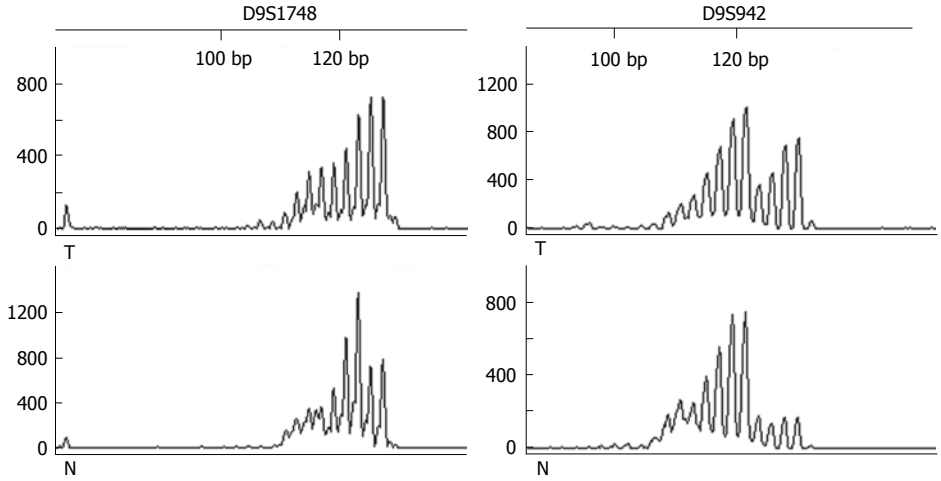

Figure 2 Representative example of the results of microsatellite analysis showing LOH at D9S1748 and LOH at D9S942 (right, case 25; left, case 45). The scales on the top and left side of each figure represent the size (bp) and the intensity, respectively. N: Normal; T: Tumor.

D9S942 and D9S974 within a coding sequence of the p16 gene was 37.3% in total. Representative examples of LOH at D9S942 and D91748 are shown in Figure 2.

Gene dosage of p16 exon 2 was successfully measured in 50 of 51 cases. Homozygous deletion of p16 exon2 was detected in 13 of 50 (26%) tumors. Overall the p16 gene was altered by homozygous deletion and LOH in 56.9% (29/51) of the tumors, indicating that alterations at this locus are involved in the vast majority of the tumors. In our analysis, LOH at 9p21-22 and homozygous deletion of p16 exon 2 were not associated with demographic variables such as age, gender, tumor histology and stage, T factor or N factor (Table 2).

p16 methylation status

Hypermethylation of the p16 gene was observed in 72.5% (37/51) of the patients (Figure 3). The relationship between p16 hypermethylation and various clinicopathological features was analyzed statistically. There was no significant correlation with the clinicopathological parameters assessed, including age, gender, tumor stage, T factor or N factor (Table 2). P16 methylation was found in 37 of 51 gallbladder cancer patients (72.5%), and loss of p16 protein expression was detected in 20 of the 37 tumors (54.1%) showing p16 hypermethylation. Our data showed that P16 protein expression was not significantly correlated with p16 hypermethylation.

p16 mutations

In tumors without homozygous deletions, exons 1α, 2 and 3 were amplified. In five cases, which were non-informative for the markers investigated, constitutive DNA was not available. No mutations were detected in exons 1α, 2 or 3.

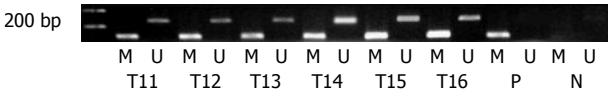

Figure 3 Methylation status of the $p16^{INK4a}$ gene by methylation-specific PCR. PCR products amplified by unmethylated (U) and methylated (M) specific primers. P: Positive control; N: Negative control. Methyaltion band is at 150 bp and unmethylated band is at 234 bp.

Table 3 Association between p16 protein expression and 9p21-22

	p16 protein expression		
	Negative	Positive	*P*
LOH (-)	12	10	
LOH (+)	20	9	0.292

OH was estiomated by allelic status at D9S171, D9S1748, D9S942, D9S974, D9S1749. LOH: Loss of heterozygosity.

Association between p16 protein expression and alterations of p16

We investigated the association between p16 protein expression and P16 alterations. LOH at 9p21-22 was not associated with the loss of p16 expression. However, LOH at three genes (D9S1748, D9S942 and D9S974) within a coding sequence of p16 was correlated with the loss of p16 expression ($P < 0.05$) (Tables 3 and 4). Furthermore, homozygous deletion of the p16 gene, a combination of LOH at 9p21 and promoter hypermethylation of the p16 gene, multiple LOH at 9p21 correlated with the loss of p16 protein expression ($P < 0.05$) (Table 5). Loss of p16 protein expression was detected in nine of 13 tumors with homozygous deletion. LOH at 9p21 was detected in only two of 13 cases with homozygous deletion, while promoter hypermethylation of the p16 gene was detected in 12 of 13 cases with homozygous deletion.

DISCUSSION

We examined the mechanisms of p16 inactivation and the relationship between p16 alterations and clinicopathological features in gallbladder cancer. p16 alterations have been evaluated individually in previous studies, but our study is believed to be the first attempt to evaluate homozygous deletion of the p16 gene. We sought to carry out a comprehensive study of p16 gene status, investigating gene dosage, allelic status, hypermethylation, mutation, protein expression, and clinicopathological features in gallbladder cancer.

Loss of p16 protein expression has been examined in various types of cancer, including gallbladder cancer. We examined p16 protein expression by immunohistochemistry in 51 cases of gallbladder cancer. Loss of p16 protein expression has been reported to range between 24 and 76% in gallbladder cancer[14,18-20,23]. In our study, the loss of p16 expression was detected in 32 of 51 (62.7%) patients with gallbladder cancer. In addition, the loss of p16 expression has been correlated with tumor progression or with decreased survival among patients with carcinoma of the lung, pancreas and esophagus, and malignant melanoma[21,22,24]. The correlation between p16 expression and clinicopathological factors is controversial. Ma *et al*[15] have reported that decreased expression of p16 is correlated with pathological grade and tumor progression in gallbladder carcinoma. However, Shi *et al*[16] have reported that loss of p16 protein expression is not significantly associated with any clinicopathological factors or survival. Quan *et al*[20] have reported that the loss of p16 expression is not associated with pathological grade. We also failed to find any association between the loss of p16 expression and clinicopathological parameters.

The mechanisms of inactivation of the p16 gene are homozygous deletion, LOH, promoter hypermethylation, rearrangement, and intragenic mutation.

Homozygous deletions are important for complete inactivation of tumor suppressor genes[13]. Previous investigators have evaluated homozygous deletion of the p16 gene in a small series of biliary tract and gallbladder cancer cell lines. Ku *et al*[25] have reported that homozygous deletion of the p16 gene was detected in three of six (50%) gallbladder cell lines. Yoshida *et al*[9] have reported that homozygous deletion of the p16 gene was detected in one of two gallbladder cell lines and in two biliary tract cell lines. Caca *et al*[26] have reported that homozygous deletion of the p16 gene was detected in eight of nine (88.8%) biliary tract cell lines, but homozygous deletion of the p16 gene was not detected in 21 biliary tract cancers. Homozygous deletion of the p16 gene has not previously been examined in gallbladder cancer. Our study is believed to be the first report to evaluate homozygous deletion of the p16 gene. We employed quantitative real-time PCR to evaluate homozygous deletion. In our series, homozygous deletion of the p16 gene was detected in 13 of 50

Table 4 Association between p16 protein expression and LOH at 9p21

	p16 protein expression		
	Negative	Positive	*P*
LOH (-)	16	16	
LOH (+)	16	3	0.0146

LOH was estiomated by allelic status at D9S1748, D9S942, D9S974.

Table 5 Association between p16 immunohistochemistry and p16 alterations in gallbladder cancers

	p16 immunohistochemistry		
	Positive	Negative	*P*
Retension	3	3	
Hypermethyaltion	9	6	
LOH	1	3	
LOH (multiple) + Hypermethylation	2	8	
Multiple LOH	0	3	
Homozygous deletion	4	9	$P < 0.05$

LOH: Loss of heterozygosity; Multiple LOH: LOH in 9p21 at more than two loci.

cases (26%). Previous studies have demonstrated that homozygous deletion of tumor suppressor genes plays an important role in the development and progression of some malignancies. However, in our series, homozygous deletion of the p16 gene was not associated with clinicopathological features.

Loss of p16 expression is correlated with homozygous deletion of the p16 gene in gallbladder cancer and other malignancies. Eight of nine biliary tract cell lines with homozygous deletion of the p16 gene showed loss of p16 expression, as reported by Caca *et al*[26]. In our series, loss of p16 expression correlated with homozygous deletion of the p16 gene in nine of 13 tumors. In four cases, homozygous deletion of the p16 gene did not correlate with p16 protein expression. Four tumors with homozygous deletion of the p16 gene displayed moderate to strong positive staining in immunohistochemistry. These tumors showed diffuse positive staining in some areas and partial or complete loss of p16 staining in other areas. The areas which showed loss of p16 expression were not captured during microdissection, and consequently the tumors were scored as having homozygous deletion. Previous reports have shown that the loss of p16 protein expression does not always correlate with homozygous deletion of the p16 gene[27,28].

Promoter hypermethylation of p16 leads to inactivation of the gene in various cancers. In gallbladder cancer, the frequency of p16 promoter hypermethylation ranges from 24% to 56%[12,29-31]. In our study, p16 hypermethylation was found in 72.5% (37/51) of the tumors. The frequency in our study was comparatively higher than that in previous studies. Previous studies have revealed that the frequency of p16 promoter hypermethylation is not associated with tumor progression and clinicopathological characteristics[30,31]. Similarly, we found that p16 hypermethylation was not associated with any clinicopathological features. Some investigators have demonstrated that p16 hypermethylation is correlated with the loss of p16 expression in intraductal carcinoma of the liver, lung cancer, hepatocellular carcinoma and esophageal cancer[32-35]. In the present study, 20 of 30 (66.6%) cases with p16 promoter hypermethylation showed a loss of p16 expression. However, there was no correlation between promoter hypermethylation and the loss of p16 expression.

We failed to detect any p16 mutations in the present study. Previous studies have shown a frequency of p16 mutation of 0%-80% in gallbladder and biliary tract cancer and cell lines[9,12,14,25]. Ueki *et al* have reported that 13 of 53 (24.5%) cases of gallbladder cancer showed non-silent p16 gene mutations. Kim *et al* have reported that p16 mutations were detected in four of 13 (30.7%) patients with gallbladder cancer. Yoshida *et al* have reported that eight of 10 cases of gallbladder cancer showed p16 point mutations. These studies did not examine homozygous deletion of the p16 gene. Ku *et al* have reported that homozygous deletion of the p16 gene was found in three of six biliary tract cell lines, but no p16 mutation was found in the remaining three biliary tract cell lines, which did not show homozygous deletion. Caca *et al*[26] have reported that p16 mutations were not found in three biliary tract cell lines and 21 biliary tract cancers, which did not show homozygous deletion of the p16 gene. In the present study, a p16 mutation was not found in any of the cases analyzed. These results suggest that the p16 mutation is associated with homozygous deletion of the p16 gene.

LOH at 9p21 has been detected in different types of tumors and cell lines[36,37]. The frequency of LOH at 9p21 in gallbladder carcinoma ranges from 38 to 60%[5,38,39]. Previous studies have demonstrated that LOH at 9p21 correlates with the loss of p16 expression in various types of cancer[22,40]. We also investigated the association between p16 protein expression and LOH at 9p21-22 in gallbladder cancer. Although an association between LOH at 9p21-22 and p16 protein expression was not found in our study, LOH at three genes (D9S1748, D9S942 and D9S974) which are located within a coding sequence of p16, correlated with loss of p16 protein expression. The mode of p16 silencing may be explained by a modification of Knudson's two-hit model[41]. In cases which show the loss of p16 protein expression, LOH or promoter hypermethylation may have occurred in only one allele, and other mechanisms may also have been involved in other alleles.

In conclusion, we investigated comprehensively the mechanisms of inactivation of the p16 gene in gallbladder cancer, and the association between p16 alterations and clinicopathologic features. Although the mutation of p16 is a rare event in gallbladder cancer, homozygous deletion, LOH and promoter hypermethylation were frequent events. LOH at 9p21 correlated with loss of p16 protein expression. In addition, homozygous deletion of the p16 gene, combination of LOH and promoter hypermethylation, and multiple LOH at 9p21 significantly correlated with loss of p16 protein expression ($P < 0.05$). LOH at 9p21 was detected in only two of 13 cases with homozygous deletion, while promoter hypermethylation of the p16 gene was detected in 12 of 13 cases with homozygous deletion. Promoter hypermethylation of the p16 gene may have occurred as an earlier event, followed by homozygous deletion as a later event in cases of homozygous deletion. LOH and homozygous deletion may be two distinct pathways for inactivating the p16 gene in gallbladder cancer.

Our results suggest that multiple alterations of the p16 gene imply multiple mechanisms for the inactivation of the p16 gene in gallbladder cancer. The mechanisms may be important for the diagnosis and treatment of this disease.

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