Alterations of *p16* and Prognosis in Biliary Tract Cancers from a Population-Based Study in China

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ABSTRACT

Purpose: **Biliary tract cancer is an uncommon malignancy with a poor survival rate. We evaluated *p16* gene alteration as a prognostic marker for this disease.**

Experimental Design: **We studied *p16* gene alterations by sequencing, methylation, and loss of heterozygosity of chromosome 9p in 118 biliary tract carcinomas, including 68 gallbladder cancers, 33 extrahepatic bile duct cancers, and 17 ampullary cancers. Survival was evaluated in 57 patients with gallbladder carcinomas, 27 with bile duct carcinomas, and 16 with ampullary carcinomas with and without somatic *p16* alterations detected by two different methods.**

Results: ***p16*** **gene alterations including silent mutations were present in 61.8% gallbladder cancers, 54.5% bile duct cancers, and 70.6% ampullary cancers. *p16* gene nonsilent mutations, *p16* methylation, and loss of chromosome 9p21-22 that targets *p14*, *p15*, and *p16* genes were present in 13 of 53 (24.5%), 8 of 54 (14.8%), and 32 of 44 (72.7%) gallbladder tumors; 5 of 25 (20.0%), 5 of 31 (16.1%), and 12 of 21 (57.1%) bile duct tumors; and 3 of 13 (23.1%), 6 of 15 (40.0%), and 8 of 16 (50.0%) ampullary tumors, respectively. The mean survival of patients with gallbladder cancers without *p16* alterations was 21.5 ± 14.8 months compared with 12.1 ± 11.4 months for patients with *p16* alterations ($P = 0.02$).**

Conclusions: **Alteration of *p16* gene alone or in combination with alterations of other tumor suppressor genes on chromosome 9p is a prognostic indicator in gallbladder carcinoma, with more favorable survival rates associated with carcinomas lacking *p16* gene alterations.**

INTRODUCTION

Biliary tract carcinomas are relatively uncommon in most parts of the world, although incidence is elevated in certain geographic locations and populations groups (1). From 1972 to 1994, biliary tract cancer was the most rapidly rising malignancy in Shanghai, the People's Republic of China, with a 119% increase in men and 124% increase in women (2). Reasons for the rising incidence of biliary tract cancers in Shanghai are unclear, but nutritional changes and improvements in diagnosis and classification may contribute to this trend.

The *p16* (INK4, MTS1, and CDKN2) tumor suppressor gene, which maps to chromosome 9p21-p22, is frequently inactivated in a wide variety of tumors by deletion, mutation, or CpG island methylation (3–7). *p14* (*ARF*) and *p15* genes are present in the same region of chromosome 9p as the *p16* gene (8). *p16* and *p14* transcripts are produced from two separate promoters and use alternative first exons (1α and 1β, respectively) joined through the same splice acceptor site to exon 2 coding sequences but in a different reading frames (9–10). *p16* is a cyclin-dependent kinase (CDK) inhibitor involved in regulation of the G_1 checkpoint in the cell cycle (11). The p16 protein blocks the G_1-S transition in the cells by binding to and preventing association of CDK4 and CDK6 with cyclin D1, leading to dephosphorylation of the retinoblastoma gene product and repression of transcription factor E2F (11, 12). The CDK4/cyclin D complex phosphorylates the product of the retinoblastoma gene, thereby promoting entry into the cell cycle. The *p16* gene is inactivated by homozygous or heterozygous deletion, mutations of coding and adjoining noncoding regions, or methylation of CpG island present at the 5′-end of the gene in a wide variety of primary cancers and tumor cell lines (5–7, 13–17). Loss of expression of p16 because of methylation or deletion or loss of functional p16 protein because of mutations result in unregulated CDK4 activity, leading to persistent retinoblastoma phosphorylation and therefore uncontrolled cellular proliferation.

p16 gene mutations (18, 19), loss of heterozygosity of chromosome 9p (19–22) and loss of p16 protein expression (23) are reported in primary biliary tract cancers and cell lines derived from these cancers. To clarify the role of *p16* inactivation in biliary tract carcinomas, we studied *p16* gene alterations in a population-based study of these tumors by sequencing, methylation of CpG island present at the 5′ of the *p16* gene, and loss of heterozygosity (LOH) of chromosome 9p. We also evaluated *p16* gene alteration status as a prognostic marker for biliary tract cancers.

MATERIALS AND METHODS

Patient Population. Patients with primary biliary tract cancer (ICD9 156) newly diagnosed between 1997 and 1999

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were identified through a rapid reporting system established between the Shanghai Cancer Institute and 30 collaborating hospitals in urban Shanghai. This reporting system recruited over 95% of patients with biliary tract cancers in Shanghai. A total of 118 patients with biliary tract carcinomas, including 68 gallbladder cancers, 33 bile duct cancers, and 17 ampullary cancers, were identified for analysis as part of a population-based case-control study. Eligibility criteria for recruitment of patients included the following: residents of urban Shanghai between 18 and 74 years of age diagnosed after April 1997 with gallbladder, extrahepatic bile duct, or ampullary carcinomas.

Tissue Specimens. Surgical pathology specimens were collected from patients with biliary tract cancers undergoing curative resection by pancreaticoduodenectomy or bile duct resection or biopsy of an advanced tumor. As part of the case-control study, 6 H&E-stained slides and 6 unstained slides (5 μ each) and 10 ml of peripheral blood were routinely collected for each patient from the surgical pathology and surgery departments of the participating hospitals. In addition, a structured questionnaire was used to elicit information on demographic, clinical, and epidemiological variables. The anatomical location where the tumor was taken was recorded in a diagram completed by the local pathologist at the participating hospital. The histopathological slides were reviewed by two pathologists from Shanghai and were independently reviewed by one of us (A. R.). Classification was based on the WHO classification of tumors of the biliary tract (24). Duodenal and pancreatic carcinomas were excluded from the study. Medical records were abstracted for all cancer cases. Patients' follow-up status was obtained by the Shanghai Cancer Institute by contacting the patients or their relatives from the date of diagnosis to March 2001.

DNA Preparation. Genomic DNA was extracted from tumor tissue by microdissection from three H&E-stained slides without a coverslip from formalin-fixed, paraffin-embedded blocks and prepared as described in previous studies (25). DNA from tumor tissue represented at least 50% tumor DNA. DNA from peripheral blood was used if available.

RNase Cleavage Assay. A RNase cleavage assay was used to screen for mutations of *p16* gene according to the manufacturer's instructions (Nonisotopic RNase cleavage assay; Ambion, Austin, TX). Exons 1 and 2 of the *p16* gene were amplified. Exon 1 of the *p16* gene was amplified using the forward and reverse amplification primers 5′-GAAGAAAGAGGAGGGCTG-3′ and 5′-GCGCTACCTGATTTCAATTC-3′, respectively. The cycling conditions were as follows: 95°C for 10 min; 37 cycles of 95°C for 30 s; and 55°C for 45 s. Exon 2 of the *p16* gene was amplified using the forward and reverse amplification primers 5′-ACACAAGCTTCCTTTCCGTC-3′ and 5′-TCTGAGCTTTGGAAGCTCTC-3′, respectively. The cycling conditions were as follows: 95°C for 10 min; 38 cycles of 95°C for 30 s; and 60°C for 45 s. The T7 promoter sequence was introduced at both ends of the PCR product by nested PCR using 2 μl of the PCR product and forward and reverse T7 primers containing the 20 bp of T7 promoter sequence at the 5′-end of each primer. The forward and reverse primers for nested PCR were 5′-TAATACGACTCACTATAGGGAGGGGCTGGCTGGTC-3′ and 5′-TAATACGACTCACTATAGGGTCCCCTGCAAACTTCGTC-3′ for exon 1, and 5′-TAATACGACTCACTATAGGGCTTCCTTTCCGTCATGCCG-3′ and 5′-TAATACGACTCACTATAGGGCTTTGGAAGCTCTCAGGGG-3′, respectively. The cycling conditions were as follows: 95°C for 10 min; 40 cycles of 95°C for 30 s; and 60°C for 45 s. The RNA product was made by *in vitro* transcription from the nested PCR product using T7 RNA polymerase. RNA was denatured at 95°C and hybridized at room temperature. The duplex RNA products were cleaved by RNase I and RNase T1 if mismatch nucleotide(s) were present in the duplex RNA. The cleaved product was directly loaded on 3% agarose gel and visualized by ethidium bromide. Samples from lanes with the shorter bands indicating a mismatch in nucleotide sequence or absence of bands indicating a technical failure or multiple mismatched nucleotide sequences were selected for DNA sequencing. No bands or aberrant bands were present in the RNase cleavage product of 18 carcinomas for exon 1 and 53 carcinomas for exon 2. All carcinomas with no bands or aberrant bands were sequenced. Forty carcinomas without any aberrant bands were sequenced, and no mutations were found. PCR amplification of exon 1, exon 2, or both exons failed for 26 carcinomas.

Sequencing of *p16*. DNA sequencing was performed using the PCR product and amplification primers using a commercial DNA sequencing kit according to the manufacturer's instructions (Sequitherm Excel; Epicenter Technologies, Madison, WI). The forward and reverse primers for sequencing were 5′-GCTGGCTGGTCACCAGAG-3′ and 5′-ACTTCGTCCTCCAGAGTC-3′ for exon 1, and 5′-CTTCCTTTCCGTCATGCGG-3′ and 5′-AGCTTTGGAAGCTCTCAGG-3′, respectively. Mutations were confirmed by reamplification of tumor DNA and sequencing in both directions. Germ-line mutations were excluded by sequencing DNA extracted from blood cells if available and by comparison with the previous reports of germline mutations (16–17).

Bisulfite Treatment of DNA and Methylation-Specific PCR. The methylation status of *p16* was determined by bisulfite treatment of DNA followed by methylation-specific PCR as described, with modifications (26). In brief, 1 μg of microdissected genomic DNA was denatured with 2 M NaOH at 37°C for 10 min, followed by incubation with 3 M sodium bisulfite (pH 5.0) at 50°C for 16 h in the dark. After treatment, DNA was purified using the DNA Cleanup Kit (Promega) as recommended by the manufacturer, incubated with 3 M NaOH at room temperature for 5 min, precipitated with 10 M ammonium acetate and 100% ethanol, washed with 70% ethanol, and finally resuspended in 20 μl of distilled water. The primers and PCR conditions for *p16* were the same as those reported by Herman *et al.* (26). In brief, 2 μl of bisulfite-treated DNA were used as template for PCR reactions using primers specific for methylated and unmethylated alleles. PCR products from methylated and unmethylated reactions were electrophoresed on 10% acrylamide gels and visualized by ethidium bromide staining (examples in Fig. 1*A*). Representative samples were reanalyzed to confirm the methylation status. DNA for bisulfite treatment was unavailable for 17 carcinomas.

LOH of Chromosome 9p. Fluorescent-labeled PCR amplification was performed using the markers on the short arm of chromosome 9 (D9S161, D9S259, D9S171, and D9S285, centromeric to telomeric). D9S161, D9S259, and D9S171 at 9p21 are centromeric to *p16* gene locus, and D9S285 at 9p22-9p23 is

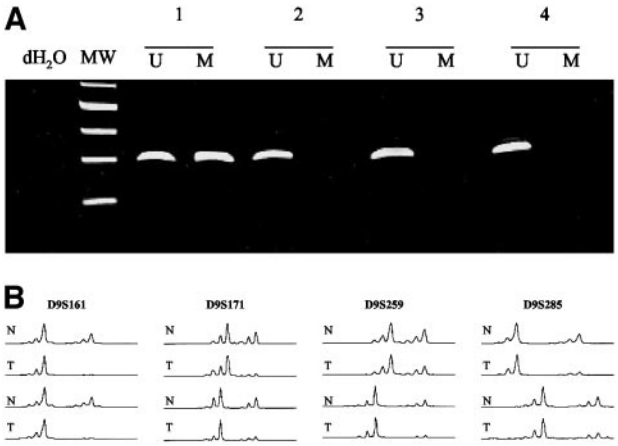

Fig. 1 *A*, methylation of *p16* in bile duct carcinomas by methylation-specific PCR. Four samples are shown in *Lanes 1–4*. M and U, represents PCR product from the methylated and unmethylated alleles, respectively; dH_2O, PCR reaction with deionized water; and MW, molecular weight markers. Sample 1 has a methylated allele but samples 2, 3 and 4 are not methylated. *B*, chromosome 9p loss by PCR amplification using fluorescent primers. The markers are indicated at the *top*. Examples of allelic loss are shown in two representative carcinomas for each marker. The ratio of the peak areas of the two alleles from the tumor DNA (T, second and fourth panels) was compared with the ratio of the peak area of the two alleles from the peripheral blood DNA (N, first and third panels). Samples showing difference of ≥50% were classified as loss of heterozygosity at that marker.

telomeric to *p16* gene locus. The fluorescent dye-labeled and unlabeled primers were obtained (Life Technologies, Inc., Gaithersburg, MD). The 5′-oligonucleotide was end labeled with 6-FAM (D9S161, D9S259) or HEX (D9S171, D9S285) fluorescent dyes. PCR was performed with 40 ng of DNA in reaction mix consisting of 1× GeneAmp PCR Gold buffer (Applied Biosystems, Foster City, CA), 2.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates, 0.83 μM of each primer, and 2 units of AmpliTaq Gold DNA polymerase (Applied Biosystems) in a final volume of 15 μl. PCR was performed using a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) with the following conditions: 95°C for 7 min; 3 cycles of 94°C for 1 min, 58°C for 30 s, and 72°C for 45 s; 42 cycles of 93°C for 45 s, 54°C for 30 s, 72°C for 40 s; and final extension at 72°C for 30 min. A 0.25-μl aliquot of each fluorescent-labeled PCR product was analyzed on an ABI 3700 Genetic Analyzer using GeneScan Analysis software (Applied Biosystems). Each sample included GeneScan 500 (TAMRA) size standard for accurate size calling. The ratio of the peak areas of the two alleles from the tumor DNA was compared with the ratio of the peak area of the two alleles from the peripheral blood DNA (examples in Fig. 1*B*). Samples showing difference of ≥50% were classified as LOH at that marker. Representative samples were reanalyzed to confirm the chromosome 9p loss status. Nontumor DNA from either slides or blood was unavailable for 27 patients, and 11 patients were noninformative for the markers used in this study.

Statistical Analysis. χ^2 or Fisher's exact tests were performed for comparison of frequency of each clinical or pathological characteristic. The *t* test was used for comparison of means. Overall survival time was measured from the date of surgery and compared using the product limit method (27). The simultaneous effects of more than one prognostic factors were estimated by multiple regression in the proportional hazards model (28). The overall survival time of patients with somatic *p16* alterations was compared with those without *p16* alterations according to at least two different assays using the log-rank statistic (Epistat Services, Richardson, TX). All three carcinomas with silent mutations without other *p16* alterations were excluded because these carcinomas had *p14* missense mutations.

RESULTS

The *p16* Gene Alterations. *p16* gene alterations including *p16* mutations (including silent mutations and/or *p14* gene mutations due to a shared exon), methylation of the CpG island in the 5′-region of the *p16* gene, and loss of chromosome 9p21-22 that targets *p14*, *p15*, and *p16* genes were present in 72 (61.0%) of 118 biliary tract carcinomas, including 42 of 68 (61.8%) gallbladder cancers, 18 of 33 (54.5%) bile duct cancers, and 12 of 17 (70.6%) ampullary cancers (Fig. 2). The frequency of these alterations in gallbladder, bile duct, and ampullary carcinomas were 13 of 53 (24.5%), 5 of 25 (20.0%), and 3 of 13 (23.1%) for nonsilent *p16* gene mutations, 8 of 54 (14.8%), 5 of 31 (16.1%), and 6 of 15 (40.0%) for *p16* methylation, and 32 of 44 (72.7%), 12 of 21 (57.1%), and 8 of 16 (50.0%) for LOH of chromosome 9p, respectively. LOH of chromosome 9p was more prevalent in gallbladder carcinomas and bile duct carcinomas than were *p16* mutations or methylation of CpG islands in the 5′-region of the *p16* gene ($P = 0.000001$ and $P = 0.02$, respectively). The frequency of LOH at D9S161, D9S259, D9S171, and D9S285 was 31 of 45 (68.9%), 23 of 31 (74.2%), 20 of 26 (76.9%), and 17 of 40 (42.5%) of informative cases, respectively.

p16 gene mutations are tabulated in Fig. 2. There were 36 mutations present in 18 gallbladder carcinomas, 6 bile duct carcinomas, and 3 ampullary carcinomas. The mutations included a noncoding mutation in the promoter region, 8 mutations in exon 1 and 27 mutations in exon 2 of *p16* gene. Twelve carcinomas had silent mutations in *p16* gene, but 9 were associated with other *p16* alterations. As exon 2 of *p16* gene also codes for exon 2 of *p14* gene in a different reading frame, all missense and silent mutations in exon 2 of *p16* gene were missense or silent mutations in exon 2 of *p14* gene. There were 21 missense and 5 silent mutations of the *p14* gene.

Among 32 coding single-base substitutions, all were present at G or C nucleotides; 30 (93.7%) were transitions (19 G to A and 11 C to T) and only 2 (6.3%) transversions (1 C to A and 1 G to T). Furthermore, 20 (62.5%) of the 32 mutations were present at non-CpG dinucleotides and 12 (37.5%) at CpG dinucleotides. Eleven of these 12 single-base substitutions at CpG dinucleotides were G-A or C-T transitions.

Three patients with gallbladder carcinomas, 2 with bile duct carcinomas, and 1 with ampullary carcinoma had two or more *p16* and/or *p14* mutations. In addition, of 98 carcinomas that were studied by two or more methods, 12 gallbladder carcinomas, 3 bile duct carcinomas, and 5 ampullary carcinomas had two or more *p16* alterations (Fig. 2). The prevalence of *p16* alterations was more frequent if all three methods were used: 24

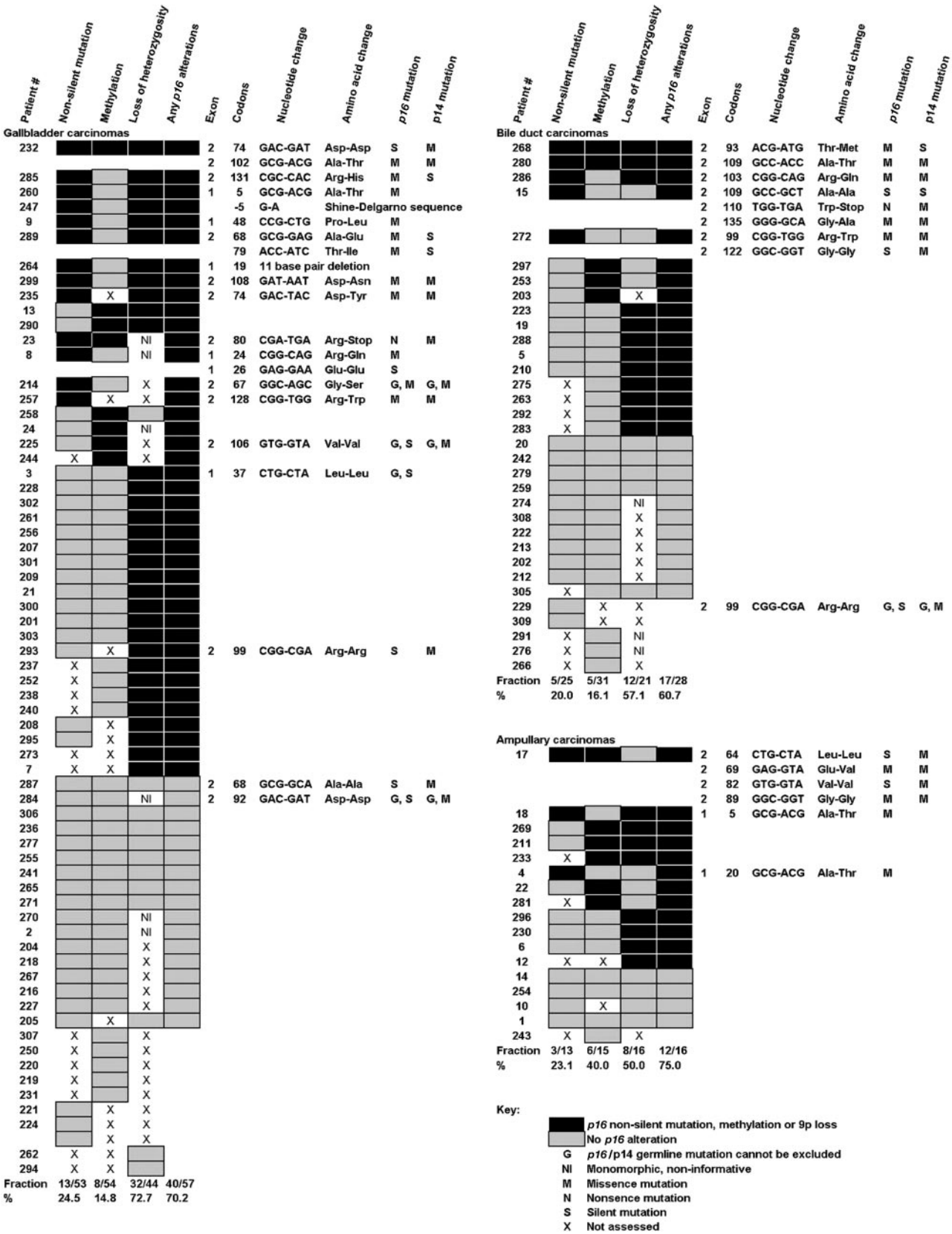

Fig. 2 *p16* alterations in 68 gallbladder, 33 bile duct, and 17 ampullary carcinomas. Carcinomas with any *p16* alterations had nonsilent *p16* mutation, *p16* methylation, or chromosome 9p loss; and carcinomas without any *p16* alterations had no alterations detected by at least two different methods.

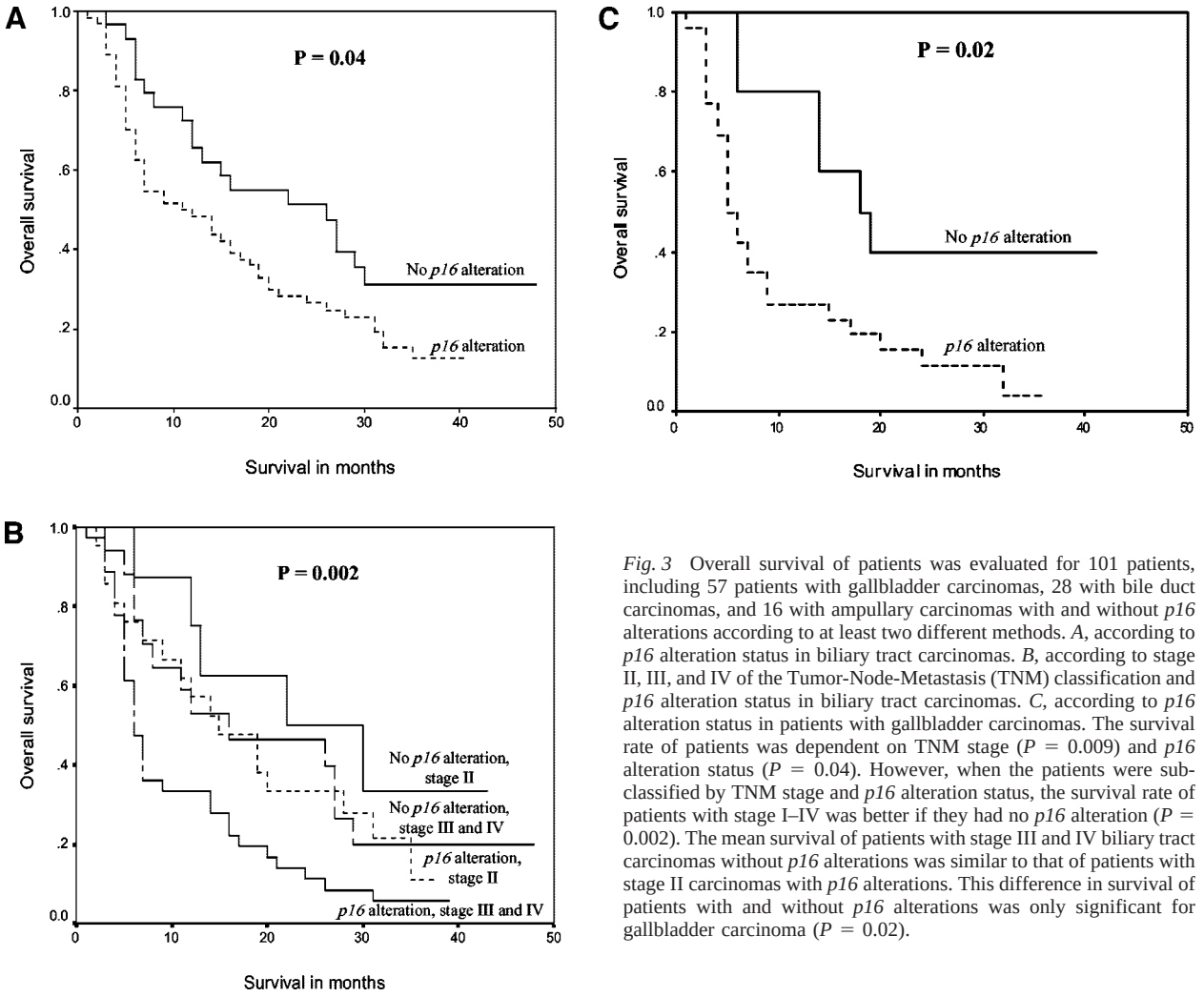

Fig. 3 Overall survival of patients was evaluated for 101 patients, including 57 patients with gallbladder carcinomas, 28 with bile duct carcinomas, and 16 with ampullary carcinomas with and without *p16* alterations according to at least two different methods. *A*, according to *p16* alteration status in biliary tract carcinomas. *B*, according to stage II, III, and IV of the Tumor-Node-Metastasis (TNM) classification and *p16* alteration status in biliary tract carcinomas. *C*, according to *p16* alteration status in patients with gallbladder carcinomas. The survival rate of patients was dependent on TNM stage ($P = 0.009$) and *p16* alteration status ($P = 0.04$). However, when the patients were subclassified by TNM stage and *p16* alteration status, the survival rate of patients with stage I–IV was better if they had no *p16* alteration ($P = 0.002$). The mean survival of patients with stage III and IV biliary tract carcinomas without *p16* alterations was similar to that of patients with stage II carcinomas with *p16* alterations. This difference in survival of patients with and without *p16* alterations was only significant for gallbladder carcinoma ($P = 0.02$).

of 31 (77.4%) gallbladder carcinomas, 12 of 16 (75.0%) bile duct carcinomas, and 9 of 12 (75.0%) ampullary carcinomas had *p16* alterations in patients whose carcinomas were examined by all three methods—DNA sequencing, methylation, and LOH.

Clinicopathological Characteristics and Survival Associated with *p16* Alteration Status. The clinicopathological characteristics of biliary tract carcinomas, *p16* alteration status, and survival times were evaluated for 101 patients, including 57 patients with gallbladder carcinomas, 28 with bile duct carcinomas, and 16 with ampullary carcinomas with and without *p16* alterations according to at least two different methods (Fig. 2). Two gallbladder carcinomas and one bile duct carcinoma with silent *p16* mutations (with *p14* mutations) without other *p16* alterations were excluded. None of 4 (0%) adenosquamous gallbladder carcinomas had *p16* alterations compared with 40 of 53 (75.5%) gallbladder carcinomas of other histological types ($P = 0.001$). The vital status, age or gender of patient, presence of cholelithiasis, and histological type of bile duct and ampullary carcinomas were not associated with *p16* status.

The *p16* Alteration Status and Survival. Patients whose carcinomas lacked *p16* alterations had a better mean survival compared with those with alterations. The mean survival of patients whose carcinomas lacked *p16* alterations was 20.5 ± 13.4 months, compared with 14.2 ± 11.9 months for those with alterations ($P = 0.04$; Fig. 3*A*).

As expected, clinical stage and histology of the carcinomas were significant prognostic factors. The overall mean survival of patients with stage I, II, III, and IV disease was 26.5 ± 11.5, 17.9 ± 12.1, 13.5 ± 12.0, and 12.0 ± 11.4 months, respectively ($P = 0.009$). The mean survival of patients with *p16* alterations *versus* those without was 26.5 ± 11.5 *versus* 29.2 ± 12.3 months for stage I carcinomas, 17.9 ± 12.1 *versus* 22.1 ± 12.5 months for stage II carcinomas, 13.8 ± 12.3 *versus* 18.3 ± 15.4 months for stage III carcinomas, and 12.0 ± 11.4 *versus* 17.5 ± 13.5 months for stage IV carcinomas, respectively ($P = 0.002$, Fig. 3*B*). Patients without *p16* alterations had a better prognosis at each stage, particularly those with stage II–IV tumors. Thus, the mean survival of stage III and IV patients with biliary tract cancers lacking *p16* alterations resembled that for stage II patients with *p16* alterations.

The histology of carcinomas was also associated with prognosis and with *p16* alteration status. The overall mean survival of patients with adenocarcinomas not otherwise speci-

Table 1 Prevalence of *p16* alterations compared with patient demographics and carcinoma characteristics in 57 gallbladder, 27 bile duct, and 16 ampullary carcinomas

	Gallbladder carcinomas		Bile duct carcinomas		Ampullary carcinomas	
	p16 Alteration					
	Absent ($n = 17$)[a]	Present ($n = 40$)[b]	Absent ($n = 11$)[a]	Present ($n = 17$)[b]	Absent ($n = 4$)[a]	Present ($n = 12$)
	n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Age (mean ± SD) yrs	63.5 ± 11.5	65.7 ± 11.0	65.1 ± 8.7	62.5 ± 8.9	65.5 ± 7.5	68.1 ± 5.6
Gender						
Female	12 (70.6)	30 (75.0)	4 (36.4)	5 (29.4)	0	5 (41.7)
Male	5 (29.4)	10 (25.0)	7 (63.6)	12 (70.6)	4 (100.0)	7 (58.3)
Cholelithiasis						
Present	12 (70.6)	28 (70.0)	4 (36.4)	7 (41.2)	1 (25.0)	3 (25.0)
Absent	5 (29.4)	11 (27.5)	6 (54.5)	10 (58.8)	3 (75.0)	9 (75.0)
Unknown	0	1 (2.5)	1 (9.1)	0	0	0
Histological type						
Adenocarcinoma						
Not otherwise specified	9 (52.9)[c]	29 (72.5)[c]	9 (81.8)	14 (82.4)	3 (75.0)	10 (83.3)
Papillary	2 (11.8)[c]	6 (15.0)[c]	0	1 (5.9)	1 (25.0)	0
Mucinous	1 (5.9)[c]	3 (7.5)[c]	1 (9.1)	0	0	1 (8.3)
Adenosquamous	4 (23.5)[c]	0[c]	1 (9.1)	1 (5.9)	0	0
Small cell carcinoma	1 (5.9)[c]	1 (2.5)[c]	0	1 (5.9)	0	1 (8.3)
Carcinosarcoma	0[c]	1 (2.5)[c]	0	0	0	0
Tumor-Node-Metastasis stage						
Stage I	4 (23.5)	7 (17.5)	1 (9.1)	0	0	0
Stage II	3 (17.6)	8 (20.0)	2 (18.2)	5 (29.4)	4 (100.0)	8 (66.6)
Stage III	3 (17.6)	9 (22.5)	3 (27.3)	2 (11.8)	0	4 (33.3)
Stage IV	7 (41.2)	16 (40.0)	4 (36.3)	10 (58.8)	0	0
Unknown	0	0	1 (9.1)	0	0	0
Vital status						
Alive	7 (41.2)	7 (17.5)	2 (18.2)	3 (17.6)	2 (50.0)	2 (12.5)
Dead	10 (58.8)	32 (80.0)	8 (72.7)	13 (76.5)	1 (25.0)	14 (87.5)
Unknown	0	1 (2.5)	1 (9.1)	1 (5.9)	1 (25.0)	0
Mean follow-up ± SD (mos)	21.5 ± 14.8[d]	12.1 ± 11.4[d]	18.0 ± 13.2	14.2 ± 12.2	22.5 ± 8.7	20.3 ± 11.8

[a] Nine gallbladder, four bile duct, and one ampullary carcinomas without *p16* alteration by only one method were excluded.
[b] Two gallbladder and one bile duct carcinomas with silent mutations without other *p16* alterations were excluded.
[c] $P = 0.001$; adenosquamous carcinomas of the gallbladder *versus* gallbladder carcinomas of other histological type.
[d] $P = 0.02$, log-rank statistic.

fied, papillary adenocarcinomas, mucinous adenocarcinomas, adenosquamous carcinomas, and small cell carcinomas was 13.9 ± 11.2, 26.9 ± 12.5, 25.6 ± 9.8, 26.8 ± 16.3, and 6.3 ± 3.4 months, respectively ($P = 0.00002$). The mean survival of patients with *p16* alterations *versus* those without was 13.3 ± 11.2 *versus* 15.6 ± 11.4 months for adenocarcinomas not otherwise specified, 23.3 ± 12.8 *versus* 35.3 ± 7.8 months for papillary adenocarcinomas, 24.5 ± 11.0 *versus* 30 months for mucinous adenocarcinomas, 6 *versus* 31.0 ± 14.2 months for adenosquamous carcinomas, and 4.7 ± 1.5 *versus* 11 months for small cell carcinomas, respectively ($P = 0.00007$). In the multiple regression proportional hazards model, only stage ($P = 0.001$) and *p16* alterations ($P = 0.045$) were independent predictors of survival.

The difference in survival associated with *p16* alterations status of carcinomas was present for all three subsites of biliary tract cancers but was statistically significant only for gallbladder carcinomas (Fig. 3*C* and Table 1). The mean survival of patients whose gallbladder carcinomas lacked *p16* alterations was 21.5 ± 14.8 months compared with 12.1 ± 11.4 months for those with alterations ($P = 0.02$). Patients whose gallbladder carcinomas lacked *p16* alterations had a better survival for clinical stages II–IV and each histological type, but the latter was not statistically significant. The mean survival of patients with *p16* alterations *versus* those without was 29.6 ± 8.0 *versus* 29.3 ± 12.3 months for stage I carcinomas, 11.4 ± 9.6 *versus* 26.5 ± 21.9 months for stage II carcinomas, 7.2 ± 8.1 *versus* 12.7 ± 11.6 months for stage III carcinomas, and 6.9 ± 5.2 *versus* 19.4 ± 16.0 months for stage IV carcinomas, respectively ($P = 0.0004$).

DISCUSSION

Few studies of the molecular genetics of biliary tract carcinomas have addressed clinical and pathological associations in well-characterized population samples. We therefore evaluated alterations of the *p16* gene in 118 gallbladder, extrahepatic bile duct and ampullary cancers from a population-based study. Patients with gallbladder carcinomas associated with *p16* gene alterations and/or alterations of other tumor suppressor genes on chromosome 9p had a poorer survival, stage for stage, compared with those without *p16* alterations.

It is possible that other genes such as *p14* or *p15* may contribute to the survival disadvantage associated with the pres-

ence of *p16* mutations or LOH at chromosome 9p in our study. As mutations in exon 2 of *p16* gene are also *p14* gene mutations in a different reading frames (9–10), missense or silent mutations in exon 2 of the *p16* gene were nonsense or missense mutations of the *p14* gene in our study. Similarly, *p14* and *p15* genes are present in the same region of chromosome 9p as the *p16* gene (8), and may be involved by allelic loss of chromosome 9p21–22 in our study. However, no *p15* mutations were present in a previous study of biliary tract carcinomas (18), but involvement of *p14* has not been evaluated. Overexpression of p14 induces G_1- and G_2-cell cycle arrest by a mechanism that is independent of CDKs (10). Chromosome 9p has additional tumor suppressor genes, which are deleted in a variety of other tumors (29), but their role in biliary tract cancer remains to be determined.

p16 gene mutations, methylation of CpG islands, and/or heterozygous deletions were present in 61.0% of the biliary tract carcinomas in our study. Homozygous deletion is another mechanism of *p16* inactivation, but detection requires pure tumor DNA from cell lines or tumor xenografts (14). In our study, nonamplification of *p16* gene was present in ten carcinomas by multiples PCR but also had LOH of chromosome 9p (data not shown). Other techniques such as real-time PCR or fluorescence *in situ* hybridization can be used to detect homozygous deletions in tumor tissue but require frozen tissue (30–33). Previous studies have reported *p16* mutations in biliary tract carcinomas (18, 19) and LOH of chromosome 9p in gallbladder carcinomas (19–22). Most of the *p16* mutations in the previous study were missense point mutations or silent mutations (18). Although these alterations predominated as well in our study, we also noted nonsense and silent mutations, along with a deletion resulting in frameshift mutation. Two or more mutations and/or alterations were present in 27 biliary tract carcinomas in our study, which probably represent inactivation of both alleles of the *p16* gene.

In our study, the *p16* alterations status of gallbladder carcinomas was associated with prognosis. Patients whose carcinomas lacked *p16* alterations had a better mean overall survival compared with those whose carcinomas had *p16* alterations. This difference in survival was associated with the stage of gallbladder cancer. In our study, the frequency of *p16* alterations was similar in patients with gallbladder, bile duct, and ampullary carcinomas, but *p16* alteration status was a significant prognostic factor only for patients with gallbladder carcinomas. In contrast, a recent study has shown loss or heterogeneous p16 protein expression was associated with poor survival among patients with bile duct and ampullary carcinomas (23). This difference in prognosis may be due to the geographical or racial differences in the study population and/or differences in the methodology of the two studies.

Comparison of *p16* gene alterations in biliary tract cancers *versus* other malignancies of the hepatopancreaticobiliary system suggests site-specific differences in the mechanisms of *p16* inactivation (34). Inactivation of *p16* is frequent in pancreatic malignancies and occurs usually by homozygous or intragenic deletions and occasionally by promoter region hypermethylation (35–37). However, in hepatocellular carcinomas and intrahepatic cholangiocarcinomas, *p16* is inactivated frequently by methylation of the promoter region rather than by homozygous deletions or gene mutations (38–41).

The majority of mutations present in our study have been described in previous compilations of *p16* gene mutations (16–17). However, there are interesting differences in the *p16* mutations in our study as compared with other studies. All single-base substitutions were present at C or G nucleotides, whereas 93.8% were transitions at G or C. In contrast, transitions at G or C appear to be responsible for 45% of the base substitutions in a number of malignancies, and for 57% of the base substitutions in melanomas (17). The type of mutation depends on the gene, site of tumor, and type of mutagenic exposure; for example, G to T transversions are common in the *p53* gene but not in *p16* in smoking-related lung carcinomas (16, 42). Distinctive mutational signatures from exposure to a specific carcinogens have been reported in the tandem base substitutions at dipyramidine sequences in UV-related skin cancers (42), G to T transversions at codon 249 of the *p53* gene in hepatocellular carcinomas due to aflatoxins (43), and G to T transversions in *p53* gene due to benzo(*a*)pyrene adducts in smoking-related lung carcinomas (44). C to T transitions due to deamination of 5-methylcytosine residues in methylated CpG dinucleotides can arise spontaneously or due to reactive oxygen species or nitric oxide (45–47). In our study, the majority of point mutations were present at non-CpG dinucleotides. G/C to A/T transitions are seen after exposure to alkylating agents, primarily because of O^6-methylguanine DNA adducts (48–50). O^6-Methylguanine-DNA methyltransferase protects against the mutagenic effects of alkylating agents by removing O^6-methylguanine adducts from the DNA, and this activity is modulated by *in vitro* and *in vivo* levels of O^6-methylguanine-DNA methyltransferase (50–53). It is possible that exposure to an alkylating agent may account for the increased G/C to A/T transitions in our study.

In conclusion, our study revealed that alterations of *p16* gene and/or other tumor suppressor genes on chromosome 9p are frequent in biliary tract cancers and may cause *p16* inactivation through gene mutations, methylation of the promoter region, and/or LOH of chromosome 9p. Patients with *p16* alterations in their gallbladder carcinomas have a worse survival when compared with those without *p16* alterations.

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REFERENCES

1. Fraumeni, J. F., Jr., Devesa, S. S., McLaughlin, J. K., and Stanford, J. L. Biliary tract cancer. *In:* D. Schottenfeld, J., and F. Fraumeni, Jr. (eds.), Cancer Epidemiology and Prevention, 2nd Ed., pp. 171–190. New York: Oxford University Press, 1996.

2. Hsing, A. W., Gao, Y-T., Devesa, S. S., Jin, F., and Fraumeni, J. F., Jr. Rising incidence of biliary tract cancers in Shanghai, China, 1972–1992. Int. J. Cancer, *75:* 368–370, 1998.

3. Nobori, T., Miura, K., Wu, D. J., Lois, A., Takabayashi, K., and Carson, D. A. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature (Lond.), *368:* 753–756, 1994.

4. Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S., III, Johnson, B. E., and Skolnick, M. H. A cell cycle regulator potentially involved in genesis of many tumor types. Science (Wash. DC), *264:* 436–440, 1994.

5. Okamoto, A., Demetrick, D. J., Spillare, E. A., Hagiwara, K., Hussain, S. P., Bennett, W. P., Forrester, K., Gerwin, B., Serrano, M., Beach, D. H., and Harris, C. C. Mutations and altered expression of $p16^{Ink4}$ in human cancer. Proc. Natl. Acad. Sci. USA, *91:* 11045–11049, 1994.

6. Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Burger, P. C., Baylin, S. B., and Sidransky, D. 5′ CpG island methylation is associated with transcriptional silencing of the tumor suppressor p16/CDKN2/MTS1 in human cancers. Nat. Med., *1:* 686–692, 1995.

7. Gonzalez-Zulueta, M., Bender, C. M., Yang, A. S., Nguyen, T., Beart, R. W., van Tomout, J. M., and Jones, P. A. Methylation of the 5′ CpG island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. Cancer Res., *55:* 4531–4535, 1995.

8. Carnero, A., and Hannon, G. J. The INK4 family of CDK inhibitors. Curr. Top. Microbiol. Immunol., *227:* 43–55, 1998.

9. Quelle, D. E., Zindy, F., Ashmun, R. A., and Sherr, C. J. Alternative reading frame or frames of *INK4a* tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell, *83:* 993–1000, 1995.

10. Stone, S., Jiang, P., Dayananth, P., Tavtigian, S. V., Katcher, H., Parry, D., Peters, G., and Kamb, A. Complex structure and regulation of the *p16* (*MTSI*) locus. Cancer Res., *55:* 2988–2994, 1995.

11. Serrano, M., Hannon, G. J., and Beach, D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature (Lond.), *366:* 704–707, 1993.

12. Weinberg, R. A. The retinoblastoma protein and cell cycle control. Cell, *81:* 323–330, 1995.

13. Cairns, P., Mao, L., Merlo, A., Lee, D. J., Schwab, D., Eby, Y., Tokino, K., van der Riet, P., Blaugrund, J. E., and Sidransky, D. Rates of *p16 (MTS1*) mutations in primary tumors with 9p loss. Science (Wash. DC), *265:* 415–416, 1994.

14. Cairns, P., Polascik, T. J., Eby, Y., Tokino, K., Califano, J., Merlo, A., Mao, L., Herath, J., Jenkins, R., Westra, W., Rutter, J. L., Buckler, A., Gabrielson, E., Tockman, M., Cho, K. R., Hedrick, L., Bova, G. S., Isaacs, W., Koch, W., Schwab, D., and Sidransky, D. Frequency of homozygous deletion at *p16/CDKN2* in primary human tumors. Nat. Genet., *11:* 210–212, 1995.

15. Herman, J. G., Merlo, A., Mao, L., Lapidus, R. G., Issa, J-P. J., Davidson, N. E., Sidransky, D., and Baylin, S. B. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res., *55:* 4525–4530, 1995.

16. Pollock, P. M., Pearson, J. V., and Hayward, N. K. Compilation of somatic mutations of the *CDKN2* gene in human cancers: non-random distribution of base substitutions. Genes Chromosome Cancer, *15:* 77–88, 1996.

17. Smith-Sorensen, B., and Hovig, E. CDKN2A ($p16^{Ink4a}$) somatic and germline mutations. Hum. Mutat., *7:* 294–303, 1996.

18. Yoshida, S., Todoroki, T., Ichikawa, Y., Hanai, S., Suzuki, H., Hori, M., Fukao, K., Miwa, M., and Uchida, K. Mutations of *$p16^{Ink4}$/CDKN2* and *$p15^{Ink4B}$/MTS2* genes in biliary tract cancers. Cancer Res., *55:* 2756–2760, 1995.

19. Imai, Y., Tsurutani, N., Oda, H., Nakatsuru, Y., Inoue, T., and Ishikawa, T. $p16^{INK4}$ gene mutations are relatively frequent in ampullary carcinomas. Jpn. J. Cancer, *88:* 941–946, 1997.

20. Wistuba, I. I., Sugio, K., Hung, J., Kishimoto, Y., Virmani, A. K., Rao, I., Fukao, K., Miwa, M., and Uchida, K. Allele-specific mutations involved in the pathogenesis of endemic gallbladder carcinoma in Chile. Cancer Res., *55:* 2511–2515, 1995.

21. Hidaka, E., Yanagisawa, A., Sakai, Y., Seki, M., Kitagawa, T., Setoguchi, T., and Kato, Y. Losses of heterozygosity on chromosome 17p and 9p/18q may play important roles in early and advanced phases of gallbladder carcinogenesis. J. Cancer Res. Clin. Oncol., *125:* 439–443, 1999.

22. Ahrendt, S. A., Eisenberger, C. F., Yip, L., Rashid, A., Chow, J. T., Pitt, H. A., and Sidransky, D. Chromosome 9p21 loss and p16 inactivation in primary sclerosing cholangitis-associated cholangiocarcinoma. J. Surg. Res., *84:* 88–93, 1999.

23. Ichikawa, K., Imura, J., Kawamata, H., Takeda, J., and Fujimori, T. Down-regulated p16 expression predicts poor prognosis in patients with extrahepatic biliary tract carcinomas. Int. J. Oncol., *20:* 453–461, 2002.

24. Albores-Saavedra, J., Henson, D. E., and Sobin, L. H. *In:* J. Albores-Saavedra, D. E. Henson, and L. H. Sobin (eds.), WHO Histological Typing of Tumors of the Gallbladder and Extrahepatic Bile Ducts, pp. 1–75. Berlin: Springer-Verlag, 1991.

25. Moskaluk, C. A., and Kern, S. E. Microdissection and polymerase chain reaction amplification of genomic DNA from histological tissue sections. Am. J. Pathol., *150:* 1547–1552, 1997.

26. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA, *93:* 9821–9826, 1996.

27. Kaplan, E. L., and Meier, P. Nonparametric estimation from incomplete observations. J. Am. Stat. Assoc., *53:* 457–481, 1958.

28. Cox, D. R. Regression models and life-tables. J. R. Stat. Soc. [B], *34:* 187–220, 1972.

29. Pollock, P. M., Welch, J., and Hayward, N. K. Evidence for three tumor suppressor loci on chromosome 9p involved in melanoma development. Cancer Res., *61:* 1154–1161, 2001.

30. Labuhn, M., Jones, G., Speel, E. J. M., Maier, D., Zweifel, C., Gratzl, O., Van Meir, E. G., Hegi, M. E., and Merlo, A. Quantitative real-time PCR does not show selective targeting of p14(ARF) but concomitant inactivation of both $p16^{INK4A}$ and $p14^{ARF}$ in 105 human primary gliomas. Oncogene, *20:* 1103–1109, 2001.

31. Nakamura, M., Watanabe, T., Klangby, U., Asker, C., Wiman, K., Yonekawa, Y., Kleihues, P., and Ohgaki, H. p14ARF deletion and methylation in genetic pathways to glioblastomas. Brain Pathol., *11:* 159–168.

32. Berggren, P., Kumar, R., Sakano, S., Hemminki, L., Wada, T., Steineck, G., Adolfsson, J., Larsson, P., Norming, U., Wijkström, H., and Hemminki, K. Detecting homozygous deletions in the CDKN2A($p16^{INK4a}$)/ARF($p14^{ARF}$) gene in urinary bladder cancer using real-time quantitative PCR. Clin. Cancer Res., *9:* 235–242, 2003.

33. Silva, J., Silva, J. M., Domingez, G., Garcia, J. M., Cantos, B., Rodriquez, R., Larrondo, F. J., Provencio, M., Espana, P., and Bonilla, F. Concomitant expression of *p16INK4a* and *p14ARF* in primary breast cancer and analysis of inactivation mechanisms. J. Pathol., *199:* 289–297, 2003.

34. Herman, J. G. $p16^{INK4}$: involvement early and often in gastrointestinal malignancies. Gastroenterology, *116:* 483–485, 1999.

35. Caldas, C., Hahn, S. A., da Costa, L. T., Redston, M. S., Schutte, M., Seymour, A. B., Weinstein, C. L., Hruban, R. H., Yeo, C. J., and Kern, S. E. Frequent somatic mutations and homozygous deletions of the *p16 (MTS1*) gene in pancreatic adenocarcinoma. Nat. Genet., *8:* 27–32, 1994.

36. Schutte, M., Hruban, R. H., Geradts, J., Maynard, R., Hilgers, W., Rabindran, S. K., Moskaluk, C. A., Hahn, S. A., Schwarte-Waldhoff, I., Schmiegel, W., Baylin, S. B., Kern, S. E., and Herman, J. G. Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas. Cancer Res., *57:* 3126–3130, 1997.

37. Ueki, T., Toyota, M., Sohn, T., Yeo, C. J., Issa, J-P. J., Hruban, R. H., and Goggins, M. Hypermethylation of multiple genes in pancreatic adenocarcinomas. Cancer Res., *60:* 1835–1839, 2000.

38. Hui, A. M., Sakamoto, M., Kanai, Y., Ino, Y., Gotoh, M., Yokota, J., and Hirohashi, S. Inactivation of $p16^{INK4}$ in hepatocellular carcinoma. Hepatology, *24:* 575–579, 1996.

39. Chaubert, P., Gayer, R., Zimmermann, A., Fontolliet, C., Stamm, B., Bosman, F., and Shaw, P. Germ-line mutations of the p16INK4

(MTS1) gene occur in a subset of patients with hepatocellular carcinoma. Hepatology, *25:* 1376–1381, 1997.

40. Matsuda, Y., Ichida, T., Matsuzawa, J., Sugimura, K., and Asakura, H. $p16^{INK4}$ is inactivated by extensive CpG methylation in human hepatocellular carcinoma. Gastroenterology, *116:* 394–400, 1999.

41. Tannapfel, A., Benicke, M., Katalinic, A., Uhlmann, D., Kockerling, F., Hauss, J., and Wittekind, C. Frequency of $p16^{INK4A}$ alteration and K-ras mutations in intrahepatic cholangiocarcinomas of the liver. Gut, *47:* 721–727, 2000.

42. Hollstein, M., Shomer, B., Greenblatt, M., Soussi, T., Hovig, E., Montesano, R., and Harris, C. C. Somatic point mutations in the *p53* gene of human tumors and cell lines: updated compilation. Nucleic Acids Res., *24:* 141–146, 1996.

43. Ozturk, M., Bressac, B., Puisieux, A., Kew, M., Volkmann, M., Bozcall, S., Bella Mura, J., de la Monte, S., Carlson, R., Blum, H., Wands, J., Takahashi, H., con Weizsacker, F., Galun, E., Siddhartha, K., Carr, B. I., Schroder, C. H., Erken, E., Varinli, S., Rustgi, V. K., Prat, J., Toda, G., Koch, H. K., Liang, X. H., Tang, Z-Y., Shouval, D., Lee, H-S., Vyas, G. N., and Sarosi, I. p53 mutation in hepatocellular carcinoma after aflatoxin exposure. Lancet, *338:* 1356–1359, 1991.

44. Denissenko, M. F., Pao, A., Tang, M-S., and Pfeifer, G. P. Preferential formation of benzo[*a*]pyrene adducts at lung cancer mutational hotspots in. p53. Science (Wash. DC), *274:* 430–432, 1996.

45. Jones, P. A., Buckley, J. D., Henderson, B. E., Ross, R. K., and Pike, M. C. From gene to carcinogen: a rapidly evolving field in molecular epidemiology. Cancer Res., *51:* 3617–3620, 1991.

46. Wink, D. A., Vodovotz, Y., Laval, J., Laval, F., Dewhirst, M. W., and Mitchell, J. B. The multifaceted role of nitric oxide in cancer. Carcinogenesis (Lond.), *19:* 711–721, 1998.

47. Souici, A-C., Mirkovitch, J., Hausel, P., Keefer, L. K., and Felley-Bosco, E. Transition mutation in codon 248 of the *p53* tumor suppressor gene induced by reactive oxygen species and a nitric oxide-releasing compound. Carcinogenesis (Lond.), *21:* 281–287, 2000.

48. Ito, T., Nakamura, T., Maki, H., and Sekiguchi, M. Roles of transcription and repair in alkylation mutagenesis. Mut. Res., *314:* 273–285, 1994.

49. Inga, A., Iannone, R., Monti, P., Molina, F., Bolognesi, M., Abbondandolo, A., Iggo, R., and Fronza, G. Determining mutational fingerprints at the human *p53* locus with a yeast functional assay: a new tool for molecular epidemiology. Oncogene, *14:* 1307–1313, 1997.

50. Allay, E., Veigl, M., and Gerson, S. L. Mice over-expressing human O^6-alkylguanine-DNA alkyltransferase selectively reduce O^6-methylguanine mediated carcinogenic mutations to threshold levels after *N*-methyl-*N*-nitrosourea. Oncogene, *18:* 3783–3787, 1999.

51. Jackson, P. E., Hall, C. N., O'Connor, P. J., Cooper, D. P., Margison, G. P., and Povey, A. C. Low O^6-alkylguanine DNA-alkyltransferase activity in normal colorectal tissue is associated with colorectal tumors containing a GC → AT transition in the *K-ras* oncogene. Carcinogenesis (Lond.), *18:* 1299–1302, 1997.

52. Psaroudi, M. C., and Kyrtopoulos, S. A. Toxicity, mutation frequency and mutation spectrum induced by dacarbazine in CHO cells expressing different levels of O^6-methylguanine-DNA methyltransferase. Mutat. Res., *447:* 257–265, 2000.

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