K-ras Mutation, p53 Overexpression, and Microsatellite Instability in Biliary Tract Cancers: A Population-based Study in China

Asif Rashid, Takashi Ueki, Yu-Tang Gao, Patrick Scott Houlihan, Charita Wallace, Bing-Sheng Wang, Ming-Chang Shen, Jie Deng, and Ann W. Hsing

Department of Pathology and Oncology Center, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205 [A. R., T. U.]; Department of Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [A. R., P. S. H., C. W.]; Shanghai Cancer Institute, Shanghai, China [Y.-T. G., J. D.]; Department of General Surgery, Zhongshan Hospital, Shanghai, China [B.-S. W.]; Department of Pathology, Shanghai Cancer Hospital, Shanghai, China [M.-C. S.]; and Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland 20892 [A. W. H.]

ABSTRACT

Purpose: The genetic alterations in biliary tract cancer and clinicopathological associations have not been studied in large population-based studies.

Experimental Design: We evaluated genetic alterations such as K-ras mutation, p53 overexpression, microsatellite instability (MSI), and alterations of the polyadenine tract present in the transforming growth factor β receptor type II (TGFβRII) gene in 126 biliary tract cancers: 75 gallbladder cancers, 33 bile duct cancers, and 18 ampullary cancers. These genetic alterations were compared with patient demographics and clinicopathological characteristics of the tumors.

Results: Mutation of the K-ras gene was present in 18 of 126 (14.3%) biliary tract cancers. K-ras mutation was present in 11 of 18 (61.1%) ampullary cancers, 5 of 33 (15.2%) bile duct cancers, and 2 of 75 (2.7%) gallbladder cancers (P = 0.000001). The mean survival of patients who had bile duct carcinomas with K-ras mutation was 3.0 ± 2.2 months compared with 15.5 ± 12.5 months for those without mutation (P = 0.03) but was not different for other tumor sites. p53 overexpression was present in 34 of 123 (27.6%) cancers. MSI-high (allelic shifts in 40% or more loci or alteration of the TGFβRII gene) was present in 4 of 126 (3.2%) biliary tract cancers without hereditary nonpolyposis colorectal cancer. MSI-high was more common in mucinous adenocarcinomas (P = 0.006) and in patients with early age of onset of cancer (P = 0.04).

Conclusions: The genetic alterations in biliary tract cancers are dependent on the tumor subsite, histology, and age of onset and are associated with prognosis.

INTRODUCTION

Biliary tract carcinomas are relatively uncommon in most parts of the world, although an increased incidence is present in certain geographical locations and certain areas (1). From 1972 to 1994, biliary tract cancer had the most rapid rise in incidence of any malignancy in Shanghai, the People’s Republic of China, with a 119% increase in men and a 124% increase in women (2). Reasons for the rising incidence of biliary tract cancers in Shanghai are unclear, although improvements in diagnosis and classification, as well as recent dietary and socioeconomic changes, may contribute to this trend. This rising incidence was present for all three sites of biliary tract carcinomas: gallbladder, extrahepatic bile duct, and ampulla of Vater.

The genetic alterations commonly observed in sporadic biliary tract cancers include mutations of the K-ras (3–13), p16 (14), APC (13, 15), β-catenin (16), and p53 (6, 15, 17, 18) oncogenes and tumor suppressor genes, p53 overexpression (19–22), and loss of heterozygosity of chromosomal arms (8, 23–25).

Molecular studies have identified cancers in which a primary genetic abnormality is the activation of a DNA mismatch repair gene, a condition that is termed MSI2 (also termed DNA replication error or ubiquitous somatic mutation; Refs. 27, 29, 30). MSI is caused by inactivation of one of a group of genes responsible for nucleotide mismatch repair, including hMSH2, hMLH1, PMS1, PMS2, hMSH6/GTBP, and hMSH3 (27, 29–34). HNPPC is usually attributable to a germ-line mutation in hMSH2 or hMLH1 (30–34). Alterations of the 10-bp polyadenine repeats within the TGFβRII gene are present in a vast majority of MSI-H colorectal cancers (35, 36). MSI of dinucleotide repeats in biliary tract cancers has been described (37–39), but alterations of TGFβRII have not been reported (37, 38).

Although these genetic events have been evaluated extensively in biliary tract cancers from hospital-based studies, these events have not been studied in large, prospectively defined, population-based studies. Therefore, we studied the K-ras mutation, p53 overexpression, and MSI, including alteration of the TGFβRII gene, in biliary tract cancers from a population-based...
study and correlated the findings with the patient demographics and with the clinicopathological characteristics of the tumors.

PATIENTS AND METHODS

Patient Population. Patients with primary biliary tract cancer (ICD9, 156) newly diagnosed between 1997 and 1999 were identified through a rapid-reporting system established between the Shanghai Cancer Institute and 30 collaborating hospitals in urban Shanghai. This reporting system recruited >95% of patients with biliary tract cancers in Shanghai. A total of 126 patients with biliary tract cancers were included in this analysis. These patients were identified as part of a large, population-based, case-control study. Eligibility criteria for recruitment of patients for this study 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. The study was approved by the National Cancer Institute Institutional Review Board.

Tissue Specimens. Surgical pathology specimens were collected from biliary tract cancer patients who were undergoing curative resection by pancreatoduodenectomy, 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 μm each) and 10 ml of peripheral blood were routinely collected from 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 the Shanghai Cancer Institute by contacting the patients or their relatives in the time interval from the date of diagnosis to March 2001.

DNA Preparation. Genomic DNA was extracted from carcinomas by microdissection from H&E-stained slides without coverslip obtained from paraffin embedded, formalin-fixed tissue and prepared as described in previous studies (41). The neoplastic cellularity of the areas selected for microdissection was >50%. DNA from peripheral blood was used but was unavailable from 28 patients.

Table 1: Prevalence of K-ras mutations compared with patient demographics and carcinoma characteristics in 126 biliary tract carcinomas

<table>
<thead>
<tr>
<th>K-ras mutation no. (%)</th>
<th>Absent</th>
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</tr>
</thead>
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<tr>
<td>Gallbladder (n = 73)</td>
<td></td>
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</tr>
<tr>
<td>Bile duct (n = 28)</td>
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<td>Ampulla (n = 7)</td>
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<td>All sites (n = 18)</td>
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<table>
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<th>Age (mean ± SD) yr</th>
<th>64.0 ± 9.3</th>
<th>64.0 ± 9.5</th>
<th>70.7 ± 3.4</th>
<th>64.9 ± 9.2</th>
<th>57.0 ± 22.6</th>
<th>57.4 ± 8.1</th>
<th>65.6 ± 6.3</th>
<th>62.4 ± 9.3</th>
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<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Female</td>
<td>55 (75.3)</td>
<td>9 (32.1)</td>
<td>3 (42.9)</td>
<td>67 (62.0)</td>
<td>1 (50.0)</td>
<td>2 (40.0)</td>
<td>3 (27.3)</td>
<td>6 (33.3) b</td>
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<td>4 (57.1)</td>
<td>41 (38.0)</td>
<td>1 (50.0)</td>
<td>3 (60.0)</td>
<td>8 (72.7)</td>
<td>12 (66.7) b</td>
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<td>51 (69.9)</td>
<td>11 (39.3)</td>
<td>3 (42.9)</td>
<td>65 (60.2)</td>
<td>1 (50.0)</td>
<td>1 (20.0)</td>
<td>2 (18.2)</td>
<td>4 (22.2) e</td>
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<tr>
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<td>20 (27.4)</td>
<td>17 (60.7)</td>
<td>4 (57.1)</td>
<td>41 (38.0)</td>
<td>1 (50.0)</td>
<td>3 (60.0)</td>
<td>9 (81.8)</td>
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<td>2 (7.1) d</td>
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<td>7 (6.5) d</td>
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<td>0</td>
<td>1 (9.1) d</td>
<td>1 (5.6) f</td>
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<td>1 (9.1) f</td>
<td>1 (5.6) f</td>
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<td>0</td>
<td>1 (5.6)</td>
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<td>5 (71.4)</td>
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<td>1 (50.0)</td>
<td>2 (40.0)</td>
<td>8 (72.7)</td>
<td>11 (61.1)</td>
</tr>
<tr>
<td>Stage III</td>
<td>14 (19.2)</td>
<td>5 (17.9)</td>
<td>2 (28.6)</td>
<td>21 (19.4)</td>
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<td>0</td>
<td>3 (27.3)</td>
<td>4 (22.2)</td>
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<tr>
<td>Stage IV</td>
<td>29 (39.7)</td>
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<td>Mean survival ± SD (mo)</td>
<td>14.2 ± 13.1</td>
<td>15.5 ± 12.5</td>
<td>21.0 ± 11.6</td>
<td>15.0 ± 12.8</td>
<td>17.0 ± 14.1</td>
<td>3.0 ± 2.2 e</td>
<td>21.3 ± 10.9</td>
<td>16.5 ± 12.1</td>
</tr>
</tbody>
</table>

a P = 0.000001, K-ras mutation in 6 of 75 (2.7%) gallbladder, 5 of 33 (15.2%) bile duct, and 11 of 18 (61.1%) ampullary carcinomas.
b P = 0.04, K-ras mutation in 6 of 73 (8.2%) female patients versus 12 of 53 (22.6%) male patients.
c P = 0.008, K-ras mutation in 4 of 69 (5.8%) patients with gallstones versus 13 of 54 (24.1%) patients without gallstones.
d Six mucinous, 1 intestinal, and 1 clear cell adenocarcinomas; K-ras mutation in one ampullary mucinous adenocarcinoma.
e Eight adenosquamous, 2 squamous, 3 small cell, 1 carcinosarcoma, and 1 undifferentiated carcinomas; K-ras mutation in one ampullary small cell carcinoma.
f TNM, Tumor-Node-Metastasis.
g P = 0.03, comparison of survival in patients with and without K-ras mutation by product-limit method.
Point Mutations of the K-ras Proto-oncogene. The first exon of K-ras was amplified, and all possible point mutations in codons 12 and 13 were tested by DNA sequencing, as described previously (42).

Immunohistochemistry for p53 Overexpression. Immunohistochemistry with mouse monoclonal antibody D07 (1:250 dilution) and standard techniques including antigen retrieval was used to determine p53 gene product overexpression, as in our previous studies (43). Overexpression of p53 was considered to be present when $\geq 50\%$ of the nuclei of tumor cells were stained by immunohistochemistry.

Microsatellite Markers and PCR Amplification. Fluorescent dye-labeled PCR amplification was performed using the markers recommended by the National Cancer Institute workshop (26). The fluorescent dye-labeled and unlabeled primers were obtained (Life Technologies, Inc., Gaithersburg, MD, or Applied Biosystems, Foster City, CA). The 5' oligonucleotide was end-labeled with 6-carboxyfluorescein (BAT-25, BAT-26, D17S250, and TGFβRII), HEX (D5S346), or TET (D2S123) fluorescent dyes. PCR was performed in 15-μl reaction volumes containing 40 ng of DNA, 9 μl of ABI Prism True Allele PCR premix (Applied Biosystems, Foster City, CA), and 5 pmol of each primer. PCR was performed using a GENEAMP PCR system 9700 thermal cycler (Applied Biosystems) using the following cycling conditions: denaturation at 95°C for 12 min; 10 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s; 32 cycles of 89°C for 15 s, 55°C for 15 s, 72°C for 30 s; and a final extension at 72°C for 10 min. A 1.0-μl aliquot of each fluorescent dye-labeled PCR product was combined with 12 μl of formamide and 0.5 μl of GENESCAN 400HD [ROX] size standard (Applied Biosystems) and analyzed on an ABI 3700 Genetic Analyzer using GeneScan Analysis software (Applied Biosystems).

Alteration of the TGFβRII Gene. Alteration in the 10-bp polyadenine tract present in the third exon of the TGFβRII gene was studied by PCR amplification of a 73-bp segment of TGFβ type II receptor gene (no. 2, Table 3). The PCR product from tumor has a 2-bp deletion. N, normal blood DNA; T, tumor DNA.

Fig. 1 Genetic alterations in biliary tract cancers. A, sequence of K-ras gene, exon 1, showing two cancers (A and B) with G to A transition at codon 12, second nucleotide (substitution of amino acid glycine with aspartic acid); and a G to A transition at codon 12, first nucleotide (substitution of amino acid glycine with serine). B, immunohistochemistry for p53 showing intense, nuclear staining of the neoplastic glands. C, PCR products from amplification of 73-bp segment of TGFβ type II receptor gene (no. 2, Table 3). The PCR product from tumor has a 2-bp deletion. N, normal blood DNA; T, tumor DNA. D, MSI-high carcinoma with allelic shifts in three dinucleotide markers (no. 4, Table 3). The dinucleotide markers are shown above the electropherogram. N, normal blood DNA; T, tumor DNA.
K-ras Mutation. Mutation of K-ras was present in 18 of 126 (14.3%) biliary tract cancers (Table 1 and Fig. 1A). Fifteen cancers had mutations in the second nucleotide of codon 12, and 9 were attributable to a G to A transition at this nucleotide, resulting in the substitution of aspartic acid for glycine. Mutation in K-ras was dependent on the subsite of tumor; mutation was present in 11 of 18 (61.1%) ampullary cancers but in only 5 of 33 (15.2%) bile duct cancers and 2 of 75 (2.7%) gallbladder cancers (P = 0.000001; Table 1).

The K-ras mutation status of the cancer was also associated with gender and gallstone status. K-ras mutation was present in 6 of 73 (8.2%) female patients but in 12 of 53 (22.6%) male patients (P = 0.04). Similarly, K-ras mutation was present in 4 of 69 (5.8%) patients with gallstones but in 13 of 54 (24.1%) patients without gallstones (P = 0.008). The associations of K-ras mutation status with gender or gallstones were not significant when the tumor site was taken into consideration. The mean survival of patients who had bile duct carcinomas with K-ras mutation was 3.0 ± 2.2 months compared with 15.5 ± 12.5 months for those without mutation (P = 0.03; Fig. 2; Table 1). The mean survival of patients with or without K-ras mutation was not different for gallbladder or ampullary carcinomas.

p53 Overexpression. Normal bile ducts, gallbladder, or duodenum had no staining for p53 by immunohistochemistry, but p53 overexpression was present in 34 of 123 (27.6%) biliary tract cancers (Fig. 1B). p53 overexpression was present in 30 of 100 (30.0%) adenocarcinomas not otherwise specified and papillary adenocarcinomas but was absent in 8 adenocarcinomas of other subtypes (P = 0.02; Table 2). p53 overexpression in cancer was not associated with other patient or tumor characteristics or with prognosis.

MSI. MSI-high was present in 4 of 126 (3.2%) biliary tract cancers (Table 3), and MSI-low was present in 1 of 126 (0.8%) biliary tract cancers without HNPCC. Alteration of the TGFβ/RII gene was present in 3 of 126 (2.4%) cancers (Fig. 1C), BAT-25 in one of 126 (0.8%), and BAT-26 in 0 of 126 cancers. Three of 98 (3.1%) cancers had allelic shifts in the dinucleotide markers. One cancer with alteration of TGFβ/RII gene and BAT-26 had shifts of all three dinucleotide markers. The other two cancers had shifts of three of the three dinucleotide markers (Fig. 1D) and one dinucleotide marker without alterations of the TGFβ/RII gene or BAT loci. DNA from peripheral blood was unavailable from two of three cancers with alteration of the TGFβ/RII gene, but frameshift mutations of the polyadenine tract were confirmed by DNA sequencing in all three cancers.

MSI-high in cancer was associated with histology of cancer and age of onset (Table 3). MSI-high was present in 2 of 6 (33.3%) mucinous adenocarcinomas but in only 2 adenocarcinomas not otherwise specified among 118 (1.7%) cancers of other types (P = 0.006; Fig. 3). MSI-high cancers were present in 2 of 11 (18.2%) patients <50 years of age but in only 2 of 115 (1.7%) patients 50 years or older (P = 0.04). The age of onset of cancer was 55.2 ± 8.9 years for patients with MSI-high cancers and 65.0 ± 8.9 years for patients with microsatellite-stable cancers (P = 0.04). The prognosis of patients with MSI-high cancers was heterogeneous; 1 patient with MSI-high cancer was alive for 37 months, 1 patient died 13 months after surgery, and the remaining 2 patients died but the survival period was unavailable.

DISCUSSION

The molecular genetics of biliary tract carcinomas has been studied extensively, but few studies have addressed clinical and
Genetic Alterations in Biliary Tract Cancers

Table 2  Prevalence of p53 overexpression compared with patient demographics and carcinoma characteristics in 123 biliary tract carcinomas

<table>
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<tr>
<th></th>
<th>Absent</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gallbladder</td>
<td>Bile duct</td>
</tr>
<tr>
<td>Age (mean ± SD) yr</td>
<td>66.1 ± 8.8</td>
<td>62.7 ± 9.9</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>36 (73.5)</td>
<td>7 (26.9)</td>
</tr>
<tr>
<td>Male</td>
<td>13 (26.5)</td>
<td>19 (73.1)</td>
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<td>Cholestasis</td>
<td>Present</td>
<td>35 (71.4)</td>
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<td>Histological type, Adenocarcinoma</td>
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<td>29 (59.2)</td>
<td>21 (80.8)</td>
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<td>Papillary</td>
<td>7 (14.3)</td>
<td>1 (3.8)</td>
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<tr>
<td>Other adenocarcinomas</td>
<td>5 (10.2)</td>
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<td>Other histological types</td>
<td>8 (16.3)</td>
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</tr>
<tr>
<td>Mean survival ± SD (mo)</td>
<td>13.6 ± 12.5</td>
<td>14.3 ± 12.1</td>
</tr>
</tbody>
</table>

*P = 0.02, p53 overexpression in 0 of 8 adenocarcinomas of other histological type versus 30 of 100 (30%) in adenocarcinomas not otherwise specified and papillary adenocarcinomas.

Table 3  Clinicopathological features of carcinomas with MSI-high

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Sex</th>
<th>Tumor site</th>
<th>Histological type of cancer</th>
<th>Shift of mononucleotide markers</th>
<th>Shift of dinucleotide markers</th>
<th>K-ras mutation</th>
<th>HNPCC</th>
<th>Survival status</th>
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<tr>
<td>1</td>
<td>69 yr</td>
<td>Male</td>
<td>Gallbladder</td>
<td>Mucinous</td>
<td>Present*b</td>
<td>NP</td>
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<td>Absent</td>
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<tr>
<td>2</td>
<td>65 yr</td>
<td>Female</td>
<td>Bile duct</td>
<td>Mucinous</td>
<td>Present*c</td>
<td>NP</td>
<td>Absent</td>
<td>Absent</td>
<td>Dead, unknown</td>
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<tr>
<td>3</td>
<td>38 yr</td>
<td>Male</td>
<td>Bile duct</td>
<td>NOSb</td>
<td>Present*a</td>
<td>NP</td>
<td>Absent</td>
<td>Absent</td>
<td>Dead, unknown</td>
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<tr>
<td>4</td>
<td>49 yr</td>
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<td>NP</td>
<td>Absent</td>
<td>Absent</td>
<td>Alive, 37 mo</td>
</tr>
</tbody>
</table>

*a TGFβRIIR.

b NOS, not otherwise specified; NP, not performed.

c BAT-26.

d Patient’s father had a lung carcinoma.

Pathological associations in prospectively defined patient populations. We, therefore, evaluated K-ras point mutations, p53 overexpression, and MSI, including alterations of the polyadenine tract of the TGFβRIIR gene, in 126 biliary tract cancers from a population-based study.

K-ras mutation was present in 14.3% of the biliary tract cancers in our study. The frequency of K-ras mutation was dependent on the subsite of cancer and was present in 61.1% of ampullary cancers but only 15.2% of bile duct and 2.7% of gallbladder cancers. The reported prevalence of K-ras mutation is 19–41% for ampullary carcinomas (5, 6, 9–11), 0–39% for bile duct carcinomas (3, 4, 7, 9, 12, 13), and 0–100% for gallbladder carcinomas (3, 7–9). These variations in K-ras mutation rates in biliary tract carcinomas may be attributable to the use of different assay techniques in these studies or to racial and geographical variations in the study populations. Similar to our study, the reported prevalence of K-ras mutation was lower when sequencing was used for detecting mutation (3, 5, 6, 8, 13), compared to when either restriction fragment length polymorphism, PCR mismatch amplification, or denaturing gradient gel electrophoresis was used to detect mutation (4, 7, 9, 10, 12). Racial or geographical variations in K-ras rates were reported in cholangiocarcinomas from Thailand and Japan (46). Similar to our study, a lower prevalence of K-ras mutation in gallbladder carcinomas has been reported (3, 8, 14); and the reported prevalence of K-ras mutation was lower in the gallbladder carcinomas compared with the bile duct carcinomas (3, 7, 9).

Similarly, lesions that are precursors of invasive carcinomas of the biliary tract show differences in K-ras mutations in the different subsites. Dysplasia and carcinoma in situ of the
K-ras mutations are frequent in gallbladder and bile duct carcinomas arising in patients with an anomalous junction of the pancreaticobiliary duct (49, 50), suggesting that a K-ras-dependent pathway also exists in the pathogenesis of gallbladder and bile duct tumors.

In our study, the lack of K-ras mutations in female patients and in patients with gallstones was attributable to a strong association between gallbladder cancer and gender and gallstone status. These associations were not present when K-ras mutation status was considered for each cancer subsite. In our study, K-ras mutation status was a prognostic factor for patients with bile duct carcinomas but not for patients with gallbladder or ampullary carcinomas. However, this finding was based on a limited sample site. Similarly, previous studies have reported poor survival of patients with K-ras mutation in biliary tract carcinomas (9) and extrahepatic bile duct carcinomas (12). In contrast, another study reported a lack of prognostic implications for the K-ras mutation in extrahepatic bile duct carcinomas (51).

p53 overexpression was present in 27.6% of biliary tract cancers. The reported frequency of p53 overexpression in the biliary tract ranges between 38 and 66% (19–22). p53 overexpression was present in adenocarcinomas not otherwise specified and of papillary type but not in other histological types of adenocarcinomas in our study. In a previous report, p53 overexpression and mutation were more common in “polypoid” macroscopic types of gallbladder cancers (17). p53 overexpression was not associated with other clinical or tumor characteristics or with prognosis. The lack of prognostic significance of p53 overexpression has been reported previously (52).

In our study, MSI-high was present in 3.2% of biliary tract cancers, including alteration of the polyadenine tract of the TGFβRII gene in 3 cancers. In contrast, previous studies of biliary tract cancers have reported MSI of dinucleotide repeats (37–39), but alterations of the TGFβRII gene have not been reported (37, 38). Alterations of the TGFβRII gene are frequent in colorectal and gastric MSI-high cancers but is infrequent in endometrial cancer (36).

In our study, MSI-high was associated with the mucinous histological subtype of biliary tract cancers and long survival in one of the two patients with follow-up. Similarly, MSI-high colorectal and pancreatic cancers are associated with a distinctive phenotype, genotype, and better survival (26, 53–57). HNPCC kindreds have increased incidence of biliary tract cancer (26), but there was no family history of HNPCC cancers in patients with MSI-high cancers in our study.

Geographical and/or racial differences have been reported in the frequencies of the genetic alterations of biliary tract cancers studied in the present study. However, these frequencies in our study are not markedly different from that reported in the literature and do not explain the rising incidence of biliary tract cancer in Shanghai, China.

In conclusion, genetic alterations in biliary tract cancer vary by subsite and histological type of tumor and by age of onset of the tumor. The frequency of the K-ras mutation is dependent on the subsite of the biliary tract cancer and associated with prognosis of bile duct cancers; p53 overexpression and MSI are associated with the histological type of tumor; and MSI is associated with early age of onset without HNPCC.
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