Double Cancer of Gall Bladder and Bile Duct not Associated with Anomalous Junction of the Pancreaticobiliary Duct System

Hiroshige Hori1, Tetsuo Ajiki1, Tsunenori Fujita1, Taro Okazaki1, Yasuyuki Suzuki1, Yoshikazu Kuroda1 and Takahiro Fujimori2

1Department of Gastroenterological Surgery, Kobe University Graduate School of Medical Sciences, Kobe and
2Department of Surgical and Molecular Pathology, Dokkyo University School of Medicine, Shimotsuga-gun, Tochigi, Japan

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Background: Simultaneous double cancers of the biliary tract are rare. Most of them are thought to be associated with pancreaticobiliary maljunction (PBM); however, the characteristics of tumours without PBM are still unclear.

Methods: Histology, immunoreactivity with carcinoembryonic antigen, carbohydrate antigen 19-9 and p53 and mutations in the K-ras gene were examined in tumours resected from cases of simultaneous double cancers of the biliary tract.

Results: Four cases of simultaneous double cancers of the biliary tract were identified among 108 patients with biliary tract cancer (3.7%). None of the four cases associated with PBM, and the results of histological, immunohistochemical and genetic examinations differed between the bile duct and gall bladder cancers in each case.

Conclusion: Even when they do not associate with PBM, double cancers in the biliary tract are more likely to be the result of multicentric development.

Key words: double cancer – gall bladder – bile duct – K-ras – p53

INTRODUCTION

Simultaneous double cancers in the biliary system are rare. Most are thought to be associated with pancreaticobiliary maljunction (PBM) owing to the action of the same carcinogen on the mucosa of the entire extrahepatic biliary system (1,2). With regard to biliary cancer cases without PBM, the presence of simultaneous double tumours poses the question of whether differentiation between independent primary cancers has occurred or different cancer foci have metastasized from a single tumour. From a clinical viewpoint, differentiation between these events is important since these two mechanistic origins imply different stages of disease, as well as different subsequent treatments and prognoses.

In order to track the origin of multiple cancers, altered steady state levels of p53 polypeptide or the presence or absence of mutations in K-ras can function as objective diagnostic adjuncts (3–8), since these abnormalities are due to genetic changes found frequently in a wide variety of malignancies, including biliary tract carcinomas (9,10). The patterns of immunohistochemical staining for carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9 can also be used to characterize biliary tract tumours (11–13).

We have treated four cases of simultaneous double cancers of the biliary tract without PBM. In this study, we compared several characteristics of these tumours, including immunohistochemical evaluation of CEA, CA 19-9 and p53 over-expression, as well as identification of mutations in K-ras. The results reported below suggest that most double cancers of the biliary tract have multicentric development, even in the absence of PBM.

PATIENTS AND METHODS

Patients

Of the 52 gall bladder and 56 bile duct cancer cases that were operated on at our hospital between 1980 and 2005, four cases of simultaneous double cancers of the biliary tract were identified. Histological diagnoses of these cases were based upon mapping examination of each entire biliary tract. Among these 108 biliary cancers, there were 12 cases with PBM; however, none of the four cases with simultaneous double cancers displayed anomalous junction of pancreaticobiliary duct system. The presence of PBM was confirmed by endoscopic retrograde cholangiopancreatography (14). Assessments of clinicopathological factors and staging were
in accordance with the General Rules for Surgical and Pathological Studies on Cancer of Biliary Tract (15).

**IMMUNOHISTOCHEMISTRY**

Tissue sections from paraffin-embedded tissue were de-paraffinized and incubated individually with anti-CEA antibody (Nichirei, Tokyo, Japan), anti-CA 19-9 antibody (Japan Turner, Tokyo, Japan) or anti-p53 antibody CM1 (1:2000, Novocastra Laboratory, Newcastle, England). Pre-treatment before staining was performed in double-distilled water by heating the immersed slides in a microwave oven for 10 min. Immunohistochemical staining was performed using labelled streptavidin–biotin complexes, as described previously (16). Immunohistochemical evaluation was based on both the distribution (percentage of positive cells) and the intensity of staining, as described previously (17). The patterns of CEA and CA 19-9 immunostaining were classified into three types (apical, cytoplasmic and stromal type) in accordance with previous reports (18,19). p53 protein expression was evaluated by the intensity and distribution of immunostaining, as reported previously by the authors (16). In the present study, p53 staining was confined to the nuclei of cancer cells, but not to those of dysplastic lesions.

**DNA EXTRACTION AND ANALYSIS OF K-RAS MUTATION**

DNA was extracted from paraffin-embedded tissue by microdissection, and DNA from dissected tissues was extracted using the DNA isolator PS kit (Wako Pure Chemical, Osaka, Japan) according to the manufacturer’s protocol. A two-step polymerase chain reaction (PCR)/restriction enzyme-based method was used to identify mutations in K-ras at codon 12. Sequences for respective sets of mismatched primers targeting codon 12 were described previously (20). For codon 12, the presence of either wild-type or mutant sequence was distinguished by fragments of 100 or 129 bp, respectively, by digestion to completion with the restriction enzyme Mva I (Takara, Kyoto, Japan). DNA encoding a mutation at codon 12 of K-ras was isolated from SW 480 colon cancer cells for use as a positive control, as described previously (20).

**RESULTS**

**CLINICOPATHOLOGICAL FINDINGS AND FOLLOW-UP**

Four cases of simultaneous double cancer of the biliary tract were identified among 108 patients with biliary tract cancers (3.7%). The clinicopathological findings from these four cases are summarized in Table 1. All cases were preoperatively diagnosed as bile duct carcinomas, and no gall bladder carcinoma was detected in any case before surgery. Biliary drainage was performed for Cases 1 and 3. Pancreatoduodenectomy with regional lymphadenectomy was performed in three cases (Cases 1–3), whereas bile duct resection and cholecystectomy was performed in the fourth (Case 4). By mapping the entire biliary tract, the tumours were separated completely (Fig. 1). There were intramucosal lesions both in the gall bladder and bile duct tumours in all four cases. Dysplastic lesions around cancer lesions were seen in the gall bladder of two cases (Cases 2 and 4), but among the four cases, no hyperplastic or metaplastic lesions were seen. Although three cases (Cases 2–4) were classified with similar histological subtypes (pap and tub1), Case 1 was the only case to display different histological subtypes (tub1 and por) in double biliary cancers and to have regional lymph node metastases. Histology of these lymph node metastases identified the cells as well-differentiated tubular adenocarcinoma. The patient defined as Case 1 has survived >5 years after resection, whereas patients described as Cases 2, 3 and 4 died of recurrence within 3 years after surgery.

Serum levels of CEA and CA19-9 are shown in Table 2. Values for Case 1 and 3 were obtained after biliary drainage. A mild elevation of CEA or CA 19-9 was seen in Cases 1, 2 and 3.

**IMMUNOHISTOCHEMICAL AND GENETIC FINDINGS**

A representative figure depicting immunohistochemical examination of CEA, CA19-9 and p53 is shown in Fig. 2.

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**Table 1. Clinicopathological features of four cases of double cancers of the biliary tract**

<table>
<thead>
<tr>
<th>Case</th>
<th>Year</th>
<th>Age</th>
<th>Sex</th>
<th>Tumour site</th>
<th>Tumour size (mm)</th>
<th>Gross feature</th>
<th>Histological type, depth</th>
<th>ly</th>
<th>v</th>
<th>n</th>
<th>Stage</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1987</td>
<td>77</td>
<td>F</td>
<td>BD, middle</td>
<td>15 × 12</td>
<td>Flat</td>
<td>tub1, ss</td>
<td>+</td>
<td>–</td>
<td>+*</td>
<td>III</td>
<td>Alive, 77 mo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GB, fundus-body</td>
<td>35 × 20</td>
<td>Nodular</td>
<td>tub3, ss</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1990</td>
<td>70</td>
<td>M</td>
<td>BD, middle-upper</td>
<td>75 × 19</td>
<td>Papillary</td>
<td>pap, fm</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>I</td>
<td>Dead, 34 mo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GB, body-neck</td>
<td>20 × 10</td>
<td>Flat</td>
<td>tub1, m</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1992</td>
<td>62</td>
<td>F</td>
<td>BD, middle</td>
<td>25 × 15</td>
<td>Nodular</td>
<td>tub1, se</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>III</td>
<td>Dead, 18 mo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GB, fundus-body</td>
<td>50 × 30</td>
<td>Flat</td>
<td>tub1, ss</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2000</td>
<td>62</td>
<td>F</td>
<td>BD, lower-upper</td>
<td>70 × 20</td>
<td>Papillary</td>
<td>tub1, ss</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>II</td>
<td>Dead, 32 mo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GB, fundus-body</td>
<td>10 × 10</td>
<td>Papillary</td>
<td>pap-tub1, mp</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BD, bile duct; GB, gall bladder; tub1, well-differentiated tubular adenocarcinoma; tub3, poorly differentiated tubular adenocarcinoma; pap, papillary adenocarcinoma; m, mucosal layer; mp, proper muscle layer; ss, subserosal layer; se, serosa exposed invasion; fm, fibromuscular layer; ly, lymphatic invasion; v, venous invasion; n, lymph node metastasis; *positive for #8, #12b1 and #12c; mo, months.
The identity of a K-ras mutation is shown in Fig. 3. Immunohistochemical and genetic findings are summarized in Table 3. The tumours from Case 1 displayed two different CEA patterns of immunostaining, but the same CA 19-9 pattern. In contrast, patterns of CEA and CA 19-9 immunostaining were distinctly different between tumour pairs resected from Case 2, 3 or 4. Within each case, the two tumours examined from Cases 1 and 2 showed the same pattern of p53 or K-ras, relative to each other, and the two tumours isolated from Cases 3 and 4 showed a different pattern of p53 or K-ras, relative to each other.

DISCUSSION

Of the biliary cancer cases in this study, 3.7% (a relatively high frequency) involved simultaneous double cancers of the biliary tract. On the basis of the histological type, protein staining and the presence of mutations in K-ras, the tumour pairs resected from Case 2, 3 or 4 were similar histologically, but immunohistochemical staining patterns and K-ras abnormalities were different.

There are two competing hypotheses to explain the pathogenesis of double cancers of the biliary tract: independent primary lesions (multicentric) or metastasis of the original cancer. Surgeons and pathologists have established several criteria to differentiate between primary tumours and metastases according to macroscopic appearance and histological characteristics (21,22). Many double simultaneous cancers have been detected clinically in cases with anomalous pancreaticobiliary ductal unions (1,2). Fujii et al. (23) reported that 62.5% of synchronous double cancers of biliary tract and 100% of metachronous double cancers of biliary tract were associated with PBM. Biliary cancer cases with PBM are thought to develop multicentrically, in part owing to the influence of pancreatic juice reflux on the mucosa of the entire biliary tract (24). Furthermore, Fahim et al. (25) reported that intraductal spread occurred in only ~4% of biliary tract carcinomas. Both of these findings support the hypothesis that double carcinomas of the biliary tract tend to derive from different primary lesions. In reality, however, determining whether double cancers are metastases or independent tumours can prove difficult. Immunohistochemical and genetic results from the four cases of double biliary tract carcinomas presented here suggest that, even in the absence of PBM, multicentric neoplastic development is common in the biliary tract double cancers than previously thought.

Genetic information from multiple neoplastic lesions provides additional criteria for differentiating between diagnoses of metastasis or independent primary neoplasms (3–8). Using genetic markers, multifocal polyclonal processes have been identified in lung (3,4) and head and neck cancers (8) (demonstrating so-called ‘field cancerization’), whereas analyses of p53 and c-erbB-2 expression in urothelial cancers revealed that multifocal carcinogenesis in the urothelium is generally due to seeding or intraepithelial metastatic spread of the original cancer cells (6,7).

With regard to double biliary cancers, only one study reports the use of genetic assays as an adjunct of differential diagnosis and suggests its importance for analysing LOH in bile duct double cancers (26). Although CEA or CA 19-9 staining patterns were informative in distinguishing double cancers, ras or p53 are more likely to be important for differentiating biliary tract double cancers, since CEA staining patterns have been reported to shift from apical to cytoplasmic types, and heterogeneous patterns of antigen localization may exist in one tumour (19).

Histologically, biliary tract cancers that occur in cases with PBM frequently show hyperplasia (27); nonetheless, our cases showed no such lesions. In addition, K-ras mutation and p53 abnormalities are more frequently detected in cancers with PBM than in those without PBM (28,29). Although some tumours in these four cases showed K-ras staining and gene abnormalities, the histological types of the tumours were different (tub1 and por). In contrast, double tumours from Case 2, 3 or 4 were similar histologically, but immunohistochemical staining patterns and K-ras abnormalities were different.

Figure 1. Diagram of the biliary tract in Cases 1–4. Bars show the positions of cancer lesions in each case.

Table 2. Serum tumour markers of double cancers of the biliary tract

<table>
<thead>
<tr>
<th>Case</th>
<th>CEA (ng/ml)*</th>
<th>CA19-9 (U/ml)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.7</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>6.0</td>
<td>65</td>
</tr>
<tr>
<td>3</td>
<td>2.1</td>
<td>44</td>
</tr>
<tr>
<td>4</td>
<td>4.6</td>
<td>23</td>
</tr>
</tbody>
</table>

*normal range: <5.0, **normal range: <37.
or p53 abnormalities, further studies with more cases will be necessary to identify the importance of genetic information in double cancers of the biliary tract without PBM.

In conclusion, in conjunction with existing pathological criteria, immunohistochemical and genetic analyses can provide valuable data for differentiating multiple cancers of the biliary tract. In the future, assays for mutations in other genes would increase the probability of identifying accurately the origins of multiple lesions of the biliary tract.

References


