Genetic Detection for Micrometastasis in Lymph Node of Biliary Tract Carcinoma

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ABSTRACT

The presence of regional lymph node metastasis is one of the most significant poor-prognosis factors in patients with biliary tract carcinoma. To establish a sensitive reverse transcription (RT)-PCR assay to detect micrometastases in lymph nodes of biliary tract carcinoma, we first investigated the optimal markers in biliary tract carcinoma. The expressions of the six candidates for a suitable RT-PCR marker [mammaglobin B, carcinoembryonic antigen (CEA), cytokeratin (CK) 20, prostate-specific antigen, MAGE-1, MAGE-3, and melanoma antigens (MAGE-1 and MAGE-3)] were evaluated in two bile duct cancer cell lines and human biliary tract carcinoma tissues. Of 32 carcinoma tissues, mammaglobin B, CEA, prostate-specific antigen, MAGE-1, MAGE-3, and CK 20 were expressed in 28 (88%), 26 (81%), 4 (13%), 5 (16%), 7 (22%), and 9 (28%), respectively. Mammaglobin B and CEA were considered to be good markers of the six candidates.

We then examined 209 lymph nodes obtained from 15 patients with biliary tract carcinoma by RT-PCR assay using both mammaglobin B and CEA and compared the results with those of histological examination. All of 20 histologically positive lymph nodes for metastasis displayed the PCR product(s) of marker genes. Of 189 histologically negative nodes, 24 (13%) nodes expressed mammaglobin B and/or CEA mRNA, suggesting the presence of micrometastasis. Our findings suggest that mammaglobin B and CEA could be useful RT-PCR markers for the detection of lymph node micrometastases in biliary tract carcinomas. Our RT-PCR assay allows accurate clinical staging necessary for patient stratification with respect to adjuvant therapy after surgery.

INTRODUCTION

Biliary tract carcinoma is one of the most difficult diseases to treat. For patients with these malignancies, only complete surgical resection provides the opportunity for cure and longer survival. However, despite recent advances in hepato-pancreato-biliary surgery, including safe major hepatectomy and extended lymph adenectomy with low perioperative mortality, the 5-year survival rate is still poor, even after radical resection of the tumors: 11 to 25% for extrapheathic bile duct cancer, 32 to 61% for gallbladder cancer, and <10% for intrahepatic cholangiocarcinoma (1–4).

Several clinicopathological characteristics, such as histological type of the lesion, its gross appearance, localization in the biliary tract system, and the mode of cancer invasion and metastasis, are considered to be important indicators of prognosis in biliary tract carcinoma (5–7). Among these indicators, lymph node involvement is significantly associated with poor prognosis (8–10). However, a subset of patients with early stage disease and no apparent evidence of lymph node involvement show a poor prognosis and die early of metastatic disease despite complete resection of the primary lesion. One possible reason for poor outcome in these patients is that they harbor occult metastases that could not be identified by conventional H&E staining at the time of surgical resection (11). Therefore, a more reliable and sensitive detection of tumor spread in biliary tract carcinoma should allow a better prognostic evaluation of patients and hence a better therapeutic approach in planning optimal management for patients with carcinoma of the biliary tract in terms of quality of life and treatment cost.

In recent years, many investigators have demonstrated the clinical significance of PCR technology using various molecular markers in the detection of solid tumors. For example, CEA and CK 20 have been used for the detection of colorectal cancer and gastric cancer (12, 13), mammaglobin for breast cancer (14, 15), MAGEs for melanoma (16), K-ras mutation for colon and pancreatic cancers (17, 18), and PSA for prostate and breast cancers (19, 20). PCR can detect tumor marker-expressing cells that are undetectable by other means in patients with localized or metastatic cancer (21).

With regard to prognosis of patients with malignancies,
micrometastases in lymph nodes are associated with a significant reduction in the 5-year survival rate of patients with stage II colorectal cancer (who show no lymph node metastases on H&E-stained sections; 22). Furthermore, adjuvant therapy against pancreatic cancer after surgery is more effective on minimal amounts of residual cancer such as lymphatic micrometastasis that could be detected only by molecular analysis (23). These data suggest that more precise staging based on the diagnosis of lymph node metastases by PCR may be a better predictor of recurrence and short-term survival than the conventional H&E-stained histological method and may serve as a criterion for clinical decision making. Thus, this study was conducted to develop a sensitive assay for detecting lymph node metastases in biliary tract carcinoma by RT-PCR.

**MATERIALS AND METHODS**

**Cell Lines.** Human biliary duct cancer cell lines, HuCCT-1 and HuH 28, were obtained from the Japanese Cancer Research Bank (Tokyo, Japan), maintained in RPMI 1640 containing 10% fetal bovine serum, and supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin.

**Tissue Samples and Patients.** Thirty-two human biliary tract carcinoma tissues, including 19 bile duct carcinomas, 7 gallbladder carcinomas, and 6 intrahepatic cholangiocarcinomas, were surgically obtained from patients who underwent curative surgery between July 1994 and April 1999 at Department of Surgery and Clinical Oncology, Osaka University Hospital, Osaka, Japan, and an affiliated hospital. All of the biliary tract carcinomas were confirmed to be adenocarcinomas by histological examination. We also collected a total of 209 lymph nodes dissected from 15 patients (9 patients with bile duct carcinoma, 2 patients with gallbladder carcinoma, and 4 patients with intrahepatic cholangiocarcinoma) who underwent curative surgery between August 1998 and April 1999. They consisted of nine males and six females with an average age of 64.9 years (range, 47–77 years). According to the tumor-node-metastasis classification of the International Union Against Cancer (24), patients were classified as follows: 1, stage I; 5, stage II; 5, stage III; and 4, stage IV. The follow-up period ranged from 5 to 14 months (mean, 10.5 months) after surgery. Nine patients were confirmed to have no metastatic lymph nodes by histological examination, and none had metastatic foci apart from the lymph nodes. Lymph node specimens were harvested from the peripancreatic tissue, hepatoduodenal ligament, hepatic hilar area, and para-aortic tissue. Their distributions varied among cases according to the location of the primary tumors and operative procedures (hepatectomy, extended cholecystectomy, or pancreaticoduodenectomy; 10, 25, 26). Each lymph node was cut into two pieces: one piece was formalin-fixed and paraffin-embedded for routine histological examination using H&E staining, and the other piece was stored for RT-PCR analysis. Tissue samples for molecular analysis were immediately frozen in liquid nitrogen after surgical resection at ~80°C until RNA extraction. As normal control nodes, we used 28 intra-abdominal lymph nodes from patients with cholelithiasis, benign bile duct strictures, chronic pancreatitis, and benign pancreatic tumors. Perioperative CEA and bilirubin levels in serum of these control patients were within normal limits. Informed consent was obtained from all of the patients before surgery.

**RNA Extraction and cDNA Synthesis.** RNA extraction was carried out with Trizol Reagent (Life Technologies, Vienna, Austria) in a single-step method, and purified total cellular RNA was quantitated and assessed for purity by UV spectrophotometry. cDNA was generated from 1 μg RNA with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI), as described previously (27).

**PCR.** The amplification of each specific RNA was performed in a 25-μl reaction mixture containing 2 μl of cDNA template, 1× PCR buffer (Perkin-Elmer Corp., Norwalk, CT), 1.5 mM MgCl₂, 0.8 mM deoxynucleotide triphosphates, 5 pmol of each primer, and 1 unit of Taq DNA polymerase (AmpliTaq Gold; Roche Molecular Systems, Inc., Nutley, NJ). The PCR primers used for detection of PBGD, MAGE-1, and MAGE-3, were described previously (16, 28, 29). The specific primers for mammaglobin B, CEA, PSA, and CK 20 were designed and used (sense, 5'-CTTGGACCAAAGCAATTGGGT-3' and antisense, 5'-TCTGAGCCAAACGAAGAGGG-3' for mammaglobin B; sense, 5'-TCTGGAACCTCTCGGTCCTCCTCAGC-3' and antisense, 5'-TGTAGCTGTGGTATGCTGTGAATG-3' for CEA; sense, 5'-CCATGACCTGTGTCGCAAGTGCA-3' and antisense, 5'-CGGGCAGGGCACATGTTGGTAGT-3' for PSA; and sense, 5'-GGTCCGACTACAGTGATTTACA-3' and antisense, 5'-CCTCAACGACGGCAGTGTAGTACCC-3' for CK 20; Refs. 30–33). These primers were designed to flank intronic sequences to avoid false-positive results attributable to amplification of contaminated genomic DNA. The PCR cDNA products of PBGD, mammaglobin B, CEA, PSA, MAGE-1, MAGE-3, and CK 20 were 127, 245, 160, 174, 421, 423, and 121 bp, respectively. The annealing temperature and cycles for PCR were set up as follows: one cycle of denaturing at 95°C for 12 min, followed by 40 cycles (95°C for 1 min, 72°C for 1 min, and 94°C for 1 min) for mammaglobin B, 35 cycles (95°C for 1 min, 72°C for 1 min, and 94°C for 1 min) for CEA, and 35 cycles (95°C for 1 min, 72°C for 1 min, and 94°C for 1 min) for PSA. The PCR conditions were set up in a GeneAmp PCR System 9600 (Perkin-Elmer). Aliquots (8 μl) from each reaction mixture were size fractionated on 2% agarose gel and visualized with ethidium bromide staining. To verify the integrity of each RNA sample, PBGD as the housekeeping gene was amplified. Specimens that failed to amplify PBGD were not considered.

**Immunohistochemistry.** Six sections, 4 μm thick, were prepared every 40 μm in each lymph node, deparaffinized in xylene, and rehydrated. Heat antigen retrieval was performed as described previously (34). Immunohistochemical staining was carried out in the Teck Mate Horizon automated staining system (Dako, Glostrup, Denmark), using the EnVision+ horseradish peroxidase kit (Dako; Refs. 35, 36). In the step of primary antibody reaction, slides were incubated with mouse anti-human CK antibody (clone AE1/AE3; final concentration: 0.87 μg/ml; Dako) for 20 min at room temperature. Sections of the corresponding primary tumor were used for positive controls in each staining procedure, and normal lymphocytes that were present in all of the slides served as the negative control. In addition, nonimmunized mouse IgG (Vector Laboratories, Burlingame, CA) or Tris-
buffered saline was used as a substitute for the primary antibody to differentiate false-positive responses from nonspecific binding of IgG or from the secondary antibody.

RESULTS

Expression of Six Markers in Two Bile Duct Cancer Cell Lines (HuCCT-1, HuH 28) and 32 Biliary Tract Carcinoma Tissues. The expression of six markers (mammaglobin B, CEA, PSA, MAGE-1, MAGE-3, and CK 20) in cell lines and carcinoma tissues were studied (Table 1). Mammaglobin B, PSA, MAGE-1, and MAGE-3 were expressed in two cell lines, whereas cDNA of CK 20 was not amplified in either of them. Among 32 carcinoma samples, mammaglobin B and CEA were expressed in 28 and 26 cases, respectively, whereas CK 20, PSA, MAGE-1, and MAGE-3 were expressed in <50% of them (9, 4, 5, and 7 cases, respectively). Thirty (94%) cases expressed mammaglobin B and/or CEA, whereas the remaining two cases did not display any band of these six markers. We, therefore, focused on mammaglobin B and CEA as markers for further examination. Twenty normal control lymph nodes obtained from patients with benign diseases were screened for mammaglobin B and CEA expression. All of the control lymph nodes were negative for both markers in our PCR condition (Fig. 1).

Detection of Carcinoma Cells by RT-PCR Assay in Regional Lymph Nodes. Lymph nodes from 15 patients with biliary tract carcinoma were examined by RT-PCR assay using mammaglobin B and CEA as marker genes. All of the corresponding primary tumor tissues were confirmed to express both the mammaglobin B gene and CEA gene (Fig. 2). Results of RT-PCR analysis were compared with those of an H&E-stained histological diagnosis in 209 lymph nodes (20 histologically positive nodes and 189 negative nodes; Table 2). We were able to detect at least one marker gene in all 20 nodes that were histologically positive for metastasis: 18 of 20 histologically positive nodes expressed both mammaglobin B and CEA, and the remaining 2 nodes expressed either mammaglobin B or CEA. Of the 189 negative nodes, 24 lymph nodes (13%) were found to express mammaglobin B and/or CEA. Of 24 PCR-positive but histologically negative nodes, 6 nodes displayed bands for two markers and 4 and 14 nodes displayed one band for CEA and mammaglobin B, respectively. Among the 15 patients analyzed, 7 patients (patients 2–6, 8, and 9) were positive for the presence of tumor cells in the lymph nodes by H&E-stained histological examination, and two more patients (1 and 7) were positive by RT-PCR assay (Table 3).

Immunohistochemical Examination in RT-PCR-positive but H&E-negative Lymph Nodes. To confirm the presence of cancer cells in lymph nodes with metastasis detected only by molecular analysis, we studied, immunohistochemically, 13 of 24 RT-PCR-positive but H&E-negative lymph nodes using the CK antibody AE1/AE3 (CK 1–10, 14–16, and 19). In all of the 15 patients whose lymph nodes were analyzed in this study, the primary tumors were positive for this antibody with strong intensity. Immunohistochemical examination revealed occult micrometastases in 5 of 13 H&E-stained histologically negative lymph nodes (Fig. 3). The remaining 11 nodes were excluded from this study because sufficient tissue was not available for further sectioning after routine H&E histological examination.
DISCUSSION

Lymph node metastasis is one of the most useful prognostic factors in various malignant neoplasms, including biliary tract carcinoma (8–10). Recent advances in molecular techniques allow us to assess the presence of small metastatic foci, so-called micrometastases, in lymph nodes that cannot be detected by conventional histological examination. Micrometastases often serve as markers of early systemic dissemination of malignant cells and may help identify patients who are at increased risk of early recurrence or shortened survival (22, 37). However, to our knowledge, there is no report concerning the detection of lymph node micrometastasis in biliary tract carcinoma by RT-PCR assay.

It is important to identify sensitive markers that can be used to indicate the presence of tumor cells, because the optimal marker would differ among the anatomical sites of tumor (21). In the present study, we first evaluated the expressions of six markers in two cell lines and 32 primary carcinoma tissues as candidates. Although CK 20, PSA, MAGE-1, and MAGE-3 are well-known RT-PCR markers in malignant neoplasms (13, 16, 19), they were detected in only a small number of biliary tract carcinomas. On the other hand, mammaglobin B and CEA were both expressed in the majority of biliary tract carcinomas. Tumors such as biliary tract carcinomas are heterogeneous because of the diversity of the original tissues, e.g., gallbladder, extrahepatic bile duct, and intrahepatic peripheral biliary epithelium. The levels of expression of specific mRNAs in cancer cells may vary among each different tumor (16, 38). Taking this property into account, we used both mammaglobin B and CEA as multiple markers of metastasis to improve the sensitivity of this assay. Indeed, the number of positive lymph nodes was increased markedly by using these two markers compared with the assay with either CEA or mammaglobin B alone. Our results demonstrated that CEA and mammaglobin B were optimal markers for biliary tract carcinoma.

Of the 189 histologically negative nodes, we could detect mammaglobin B and/or CEA in 24 lymph nodes by RT-PCR and diagnose them as metastasized, in addition to 20 H&E positive nodes. In this RT-PCR assay, the marker(s) detected in each node were not always the same as the markers that could be found in the corresponding primary tumor. In this context, we must consider that the amount of marker genes in each lymph node might depend not only on the number of tumor cells in the sample but also on the extent of their expression per single cancer cell. If a cancer cell produced low level expression of one marker gene, we could detect the micrometastases by the band of the other marker. Thus, it must be noted that the combination of these two markers significantly increased the detection sensitivity and allowed the establishment of a reliable assay without false-negative results.

In the present study, we also carried out immunohistochemical staining in a subset of RT-PCR-positive but histologically negative lymph nodes using the anticytokeratin antibody AE1/AE3 (CK 1–10, 14–16, and 19). Antibodies against CK 7 and CK 19 react for almost all biliary tract carcinomas with stronger intensity than the reaction of antibodies against CEA (39, 40). Nested or individual cancer cells were histologically detected in 5 of 13 nodes (38%). In the remaining 8 nodes, tumor cells could not be detected, partly because metastatic foci were localized between the sections or in the counterpart of the two halves. The results of this immunohistochemical study support our hypothesis that PCR-positive nodes possibly contain viable cancer cells that result in development of recurrence, even when conventional histological examination fails to find any evidence of metastasis.

CEA is widely used as a tumor marker for the molecular detection of gastrointestinal and breast cancer cells (12, 41, 42).
On the other hand, mammaglobin B was newly identified in 1998 as a member of the uterogene family, which shares high homology to mammaglobin (MGB1). Recently, mammaglobin and mammaglobin B have been considered as good markers for the detection of carcinoma cells in lymph nodes and peripheral blood in breast cancer patients (14, 30, 43, 44). Our report is the first to use the mammaglobin B gene for detecting cancer cells in gastrointestinal malignancies.

In 15 patients with biliary tract carcinoma, we were able to detect two more patients (patients 1 and 7) with lymph nodes positive for tumor cells by our RT-PCR assay. One of these two patients (patient 7) developed recurrence in the lymph node in the hilum of the right kidney 8 months after surgery, whereas the remaining patients who had no evidence of nodal involvement showed no signs of recurrence. Recurrence in this patient could be explained only by the presence of residual cancer cells, which were undetected by histological examination. Although this clinical outcome was noted within the short follow-up period and in a small number of patients, it seems that detection of lymph node micrometastases by our RT-PCR assay may provide a more accurate determination of prognosis in patients with biliary tract carcinoma than conventional histological diagnosis. These findings suggest that RT-PCR assay using both mammaglobin B and CEA may be a useful tool for the detection of lymph node micrometastasis from primary biliary tract carcinoma and for identification of patients who may need more intensive therapeutic intervention.

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