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Expression and Significance of Angiopoietin-1, 2 and Tie-2 Receptor in Human Extrahepatic Bile Duct Carcinoma: Correlation with Clinicopathological Factors

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Introduction

Cholangiocarcinoma is a high mortal malignancy, in that most patients present initially with unresectable disease, undergo palliative therapies, and die within 12 months. Given the small number of available studies and lack of randomized trials, there is no established role for neoadjuvant and adjuvant therapy associated with cholangiocarcinoma. Systemic (radio-) chemotherapy applied in a palliative setting has not been shown to prolong survival significantly. At present, complete surgical resection with histologically negative resection margins is the only cure for cholangiocarcinoma.

Cholangiocarcinoma is the second most common type of primary hepatic tumor and accounts for 3% of all gastrointestinal cancers. Among cholangiocarcinomas, 60-70% arise at the bifurcation of the hepatic ducts (Klatskin tumours), 20-30% in the distal extrahepatic common bile duct, and 5-10% are peripheral, arising within intrahepatic ducts of the liver parenchyma itself. Several international studies have shown an increasing incidence and mor-
tality rate for intrahepatic cholangiocarcinoma, and decreasing incidence and mortality for extrahepatic bile duct carcinoma. Some risk factors for cholangiocarcinoma include primary sclerosing cholangitis, liver fluke infestation, hepatolithiasis, and abnormalities of biliary anatomy; however, not only the mechanism of the carcinogenesis but also the biological aggressiveness of bile duct carcinoma has not been fully elucidated.

Angiopoietin (Ang)-1 and Ang-2 function as ligands for Tie-2 vascular endothelial-specific receptor tyrosine kinase, and as such are considered important growth factors during angiogenesis. Ang-1 acts as an agonist of Tie-2 receptor, stimulating Tie-2-mediated stabilization and maturation of vessels by promoting interactions between endothelial cells and supporting cells, as well as by stimulating endothelial cell migration in vitro. In contrast, Ang-2 is a context-dependent antagonist by binding to Tie-2 with an affinity similar to that of Ang-1, it blocks Ang-1-stimulated receptor phosphorylation in endothelial cells.

Tie-2 is a receptor tyrosine kinase that is expressed at high levels in embryos and plays a critical role in embryonic development. Experimental evidence from the targeted disruption of the Tie-2 gene suggests that Tie-2 plays a pivotal role in angiogenesis and vascular remodeling during development. Extra-endothelial expression of Ang-1, 2 and Tie-2 has also been documented recently, and increasing varieties of tumor cells, including gastric carcinoma cells, colorectal carcinoma cells, gastrointestinal stromal tumor, and glioma cells have been reported to express Ang-1, 2 and Tie-2. Also, in primary murine tumors and their metastases, soluble form of the extracellular domain of murine Tie-2 for Tie-2 inhibitor caused antitumor effect by inhibiting the tumor angiogenesis. These reports suggest that the Ang-Tie system may play a role in the progression of malignant tumors. To promote a better understanding of the Ang-Tie system, the objective of this study was to evaluate its role in the progression of human extrahepatic bile duct carcinoma.

Materials and Methods
Cases and Tissues
We studied 119 extrahepatic bile duct carcinomas, excluding those involving the gall bladder or ampulla. Specimens from Nagasaki University Hospital and the National Nagasaki Medical Center were obtained from patients between 1993 and 2007. All tumors were resected with clear or nearly clear margins. Cases were staged according to the TNM classification of the UICC. Bile duct adenocarcinomas was divided histologically into papillary- and tubular-type adenocarcinomas. Tubular-type adenocarcinomas were further classified according to their degree of differentiation (well, moderately or poorly differentiated). Examinations to identify lymphatic, venous and perineural invasions were performed on routine slides. To identify venous invasions, Elastica van Gieson staining was used in addition to hematoxylin and eosin. Invasions identified certainly were defined as "present", whereas not observed certainly were defined as "absent". Lymph node metastasis was defined as "present" when histologically proven, whereas not observed histologically were defined as "absent". Among the invasive cases, we also classified the degree of desmoplastic stromal reaction into "scirrhous" or "non-scirrhous", and tumor growth patterns as "non-infiltrative" or "infiltrative". Two independent pathologists (Y. Mihara and T. Nakayama) diagnosed all cases by examining the whole stepwise section of each extrahepatic bile duct. Those of questionable cases were omitted from the study.

Immunohistochemistry
Formalin-fixed and paraffin-embedded tissues were cut into 4 μm sections, deparaffinized in xylene and rehydrated in phosphate-buffered saline. Deparaffinized sections were preincubated with normal bovine serum to prevent nonspecific binding, and then incubated overnight at 4°C with an optimal dilution (0.1 μg/ml) of polyclonal goat antibody against Ang-1 (C-19) and Ang-2 (N-18), or polyclonal rabbit antibody against human Tie-2 (C-20) (all pur chased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Slides reacted with Ang-1 and Ang-2 antibodies were then incubated with biotinylated horse anti-goat immunoglobulin antibody; and those with Tie-2 were incubated with biotinylated horse anti-rabbit immunoglobulin antibody. Secondary antibody reaction products were viewed using diaminobenzidine (DAB; Dako Ltd., Glostrup, Denmark). Primary antibodies pre-absorbed with excess recombinant Ang-1, 2 or Tie-2, respectively (Santa Cruz Biotechnology, Inc.), were used as negative controls. Proliferated capillaries served as an internal positive control for Ang-1, 2 and Tie-2 immunostaining. Two independent investigators (Y. Mihara and T. Nakayama) analyzed all immunohistochemical results. Degree of Ang-1, 2 or Tie-2 expression was classified into two categories depending on the percentage of cells stained: (-) for 0% to 10% positive cells; and (+) for more than 10% positive carcinoma cells.

Cell Culture
HuCCT1, HuH28 and OZ cell lines, derived from human bile duct cancer, were obtained from the Human Health Resources Bank (Osaka, Japan). All cell lines were maintained in RPMI 1640 (Invitrogen Corp., Carlsbad, CA, USA) supplemented with heat-inactivated 10% fetal calf serum (Invitrogen Corp.) and 2 mM glutamine (Invitrogen Corp.), and incubated at 37 ºC in a humidified atmosphere containing 5% CO2.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)
Total RNA was prepared using the acid guanidine phenol method from five human bile duct carcinoma tissues and three human bile duct cancer cell lines, HuCCT1, HuH28 and OZ. Cellular RNA (1 μg) was incubated at 37 ºC for 1 hour in 50 μl of reverse transcriptase buffer containing 20 units of RNAsin (Promega Corp., Madison, WI), 100 pmol of random hexamer primers (Boehringer
Mannheim, Mannheim, Germany), and 400 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen Corp.). Reverse transcription was then terminated at 95 °C for 10 minutes, and 20% of the resultant cDNA was removed for PCR. PCR templates were amplified using 50 pmol of each primer and 2.5 units of Taq DNA polymerase. Primer sequences were as follows: human Ang-1 5'-GGGGGAGGTTGGACTGTAAT-3' (sense) and 5'-AGGGCACATTTGCACATACA-3' (antisense), human Ang-2 5'-GGATCTGGGGAGAGAGGAAC-3' (sense) and 5'-CTCTGCCGAGTCATCGTA-3' (antisense), human Tie-2 5'-CTGCAGTCATCGTA-3' (antisense), human β-actin 5'-TCCTCCCTGGAGAAGACTA-3' (sense) and 5'-AGTACTTGCGCTCAGGAGGA-3' (antisense). Primer pairs were designed to span introns in their respective human genes. Predicted amplification product sizes using these primer pairs are 362 bp (Ang-1), 535 bp (Ang-2), 389 bp (Tie-2) and 313 bp (β-actin). Samples were subjected to 30 cycles of PCR amplification (denaturation at 94 °C for 1 minute, annealing at 60 °C for 1 minute, and primer extension at 72 °C for 1.5 minutes) in a thermocycler. Equivolume aliquots of amplification reactions were resolved on 1.5% agarose gels, and DNA was visualized by ethidium bromide staining.

Statistical analysis
Stat View II (Abacus Concepts, Inc., Berkeley, CA, USA) was used for statistical analyses. Analyses comparing degrees of Ang-1, 2 and Tie-2 expression applied Mann-Whitney's U test and Chi-square for independent tests. Survival durations were calculated using the Kaplan-Meier method. A log-rank test was used to compare cumulative survival between patient groups. p<0.05 was considered significant for each analysis.

Results
Immunohistochemical staining of Ang-1, 2 and Tie-2
Immunohistochemical results from the bile duct carcinomas are summarized in Tables 1, 2 and Figure 1. Among 119 cases, 52 (43.7%), 50 (42.0%) and 89 (74.8%) cases showed positive staining.
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Expression of Ang-1, 2 and Tie-2 was variable in histological types. Adenosquamous carcinomas showed relatively high expression of Ang-2 (71.4%) (Table 1). Tie-2 expression was relatively high in all histological types of carcinomas except adenoendocrine and mucinous carcinoma (Table 1). In no case, Ang-1, Ang-2 or Tie-2 expression levels correlate with degree of histological differentiation.

Expression of Tie-2 inversely correlated with the stage of tumor invasion (p<0.05) (Table 1). There was also positive correlation between Tie-2 expression and degree of desmoplastic stromal reaction and tumor growth pattern (p<0.001 and p<0.05, respectively) (Table 2). No significant correlations between expression of Ang-1 or Ang-2 and grade of tumor invasion, stage, stromal reaction or tumor growth pattern were observed.

RT-PCR for Ang-1, 2 and Tie-2 in human bile duct carcinoma tissues and cultured cell lines

Ang-1, 2 and Tie-2 cDNA was amplified from the total RNA of all three cultured cell lines and all five tissues of human bile duct carcinoma (Figure 2), but expression levels of Ang-1, 2 and Tie-2

| Table 2. Immunohistochemical staining for Ang-1, 2 and Tie-2 and the relationships between desmoplastic reaction and tumor growth pattern. (114 cases) |
|---------------------------------|-------------------|-------------------|-------------------|
|                                | Ang-1             | Ang-2             | Tie-2             |
| Total carcinoma                | n + (43.9%)       | n + (57.9%)       | n + (74.6%)       |
| Desmoplastic stromal reaction  | NS                | NS                | NS                |
| Scirrhous                      | 61(55.3%)         | 30(49.2%)         | 25(41.0%)         |
| Non-scirrhous                  | 33(46.5%)         | 20(37.7%)         | 33(43.4%)         |
| Tumor growth pattern           | NS                | *                 | NS                |
| Infiltrative                   | 74(64.9%)         | 36(48.6%)         | 38(51.4%)         |
| Non-infiltrative               | 40(35.1%)         | 14(35.0%)         | 26(65.0%)         |
| **; p<0.05, ***; p<0.001, NS; not significant |

Figure 1. Ang-1, 2 and Tie-2 immunoreactivity in the extrahepatic bile duct carcinoma cells (A-C). Ang-1 and Ang-2 were expressed in the cytoplasm of carcinoma cell (A, B, respectively). Tie-2 was expressed in the cellular membrane and the cytoplasm of carcinoma cell (C). (Magnification; x400)

Figure 2. RT-PCR analysis of Ang-1, 2 and Tie-2 mRNA expression in human bile duct carcinoma tissues and cultured cell lines. Total RNA template was prepared from three cultured cell lines (Lane 1; HuCCT1, Lane 2; HuH28, Lane 3; OZ) and five human tissues of bile duct carcinoma (Lanes 4-8). Size markers (Lane M) consist of 100-bp DNA ladder markers (Invitrogen Corp.).
mRNAs appeared to be vary among the samples. Amplification of $\beta$-actin cDNA was used to control for differences in loading, and was detected in all samples.

**Relationships between Ang-1, 2 and Tie-2 expression and the survival rates**

Among the 119 cases, 54 cases were available for the investigation of their prognoses. The 5-year survival rate was 32.5%. A log-rank test showed no statistical difference between Ang-1 or Ang-2 expression and survival rate (Figure 3 A, B), but there was a significant difference between Tie-2-positive or -negative cases and survival among the patients of extrahepatic bile duct carcinoma (p<0.05) (Figure 3C).

**Discussion**

Mechanisms regulating tumorigenesis and biological aggressiveness in extrahepatic bile duct carcinomas have not been clarified. In the present study, we investigated the relationship between Ang-1, 2 and Tie-2 expression in carcinoma cells and clinicopathological factors of extrahepatic bile duct carcinoma using immunohistochemical and molecular techniques. The data presented here provide some evidence correlating Tie-2 expression with clinicopathological factors in bile duct carcinoma cells. Our results suggested the tumor progression by autocrine/paracrine effects of Ang-1, 2 and Tie-2 expression in carcinoma cells.

Although previous study showed faint expression of Ang-1, 2 and Tie-2 in normal mucosa of bile duct in the liver, we observed Ang-1, 2 and Tie-2 expression in normal bile duct mucosa in this study (Table 1). However, Ang-1 and Tie-2 expressions were increased in carcinoma cells compared to normal mucosa, and the difference was statistically significant (Table 1). Tie-2 expression was very high among in total cancer samples (p<0.0001), 80.0% of cases was Tie-2 positive in both Stage 0 and Tis grade. These findings suggested that Tie-2 may play a progressive role in carcinogenesis, and that Tie-2 expression could be a useful for differential diagnosis between normal epithelial cell and carcinoma cell of bile duct mucosa.

Factors that contribute to tumor angiogenesis play an important role in tumor progression, in that angiogenesis is essential for the nutrition, growth and metastasis of a tumor. Ang-1, a proangiogenic protein, serves as a chemical signal for endothelial cells to induce vascular maturation and stability during the angiogenesis. Positive expression of Ang-1, 2 and Tie-2 in tumor cells has been demonstrated in gastric cancer, colorectal cancer, gastrointestinal stromal tumors and glioma cells. Furthermore, there is a significant correlation between a high degree of tumor vascularity, poor prognosis and low survival rates in cases of extrahepatic bile duct carcinoma.

Tie-2 expression was seen in carcinoma cells as well as stromal fibroblasts. Almost all of carcinoma cells and stromal fibroblasts expressed both Ang-1 and Ang-2 (data not shown). Previous studies have demonstrated that Ang-1 stimulates a Tie-2-dependent pathway that modulates the activity of the cell-to-extracellular matrix adhesion, and Ang-1 has also been reported to bind to Tie-2 directly as a binding protein itself. Other reports show that Ang-1 is only expressed in fibroblasts co-cultured with carcinoma cell that express Ang-2, though neither the mono-cultured fibroblasts nor the cancer cells expressed both Ang-1 and Ang-2. Ang-Tie signaling has also been hypothesized to promote the growth of both carcinoma cells and stromal fibroblast via direct cell interactions.
Data in this study showed a positive correlation between Tie-2 expression, tumor growth pattern and degree of desmoplastic stromal reaction. Although our studies focused on autocrine/paracrine aspects of Ang-1, 2 and Tie-2 expression in carcinoma cells, we do not argue that fibroblasts may also promote cancer progression. Simultaneously, carcinoma cells could also promote fibroblast proliferation via the same interactions. Such mechanisms may suggest how the Ang-Tie pathway participates in the progression of desmoplasia.

Our analysis detected a significant correlation between the expression of Tie-2 and a good prognosis for patients with bile duct cancer (Figure 3C). Because Tie-2 expression was tend to be low in deeper invasive cases, and later stage cases (Table 1), we hypothesized that Tie-2 expression levels contribute to the good prognosis of human malignancies. However, no examination of Tie-2 expression has been reported previously in any prognostic studies on cancer, although some reports indicate that Ang-2 expression levels are valuable for clinical prognosis. A positive correlation between Ang-2 expression in breast cancers and shorter disease-free time and overall survival has been reported. Overexpression of Ang-2 also associates with a significantly worse prognosis for patients with hepatocellular carcinoma, non-small cell lung carcinoma or bladder cancer. However, tests of this study demonstrate no significant correlation between Ang-1 and Ang-2 expression and overall survival.

Gastrointestinal cancer, such as gastric or colorectal cancer, usually shows highly desmoplastic stromal reaction with deep invasion of cancer cell. However, the cancer of extrahepatic bile duct accompany desmoplasia in stroma and develops scirrhous feature in early stage of tumor invasion. In our study, many cases (10 of 23, 43.5%) in stage II showed scirrhous feature, and desmoplastic stromal reaction did not correlate with invasive grade. The correlation between survivals of the patients and stromal reaction of the extrahepatic bile duct cancer has been shown in recent report. However, both desmoplastic stromal reaction and tumor growth pattern did not correlate with survivals of patients in this study (data not shown).

Each clinicopathological factor; lymph node metastasis, lymph duct invasion, venous invasion or perineural invasion, did not correlate with the expression of Tie-2 in this study. However, the Tie-2 expression was showed relatively higher in absence cases of every factor except perineural invasion. The expression of Tie-2 significantly correlated with the grade of tumor stage that was diagnosed by the grade of tumor invasion and the presence of lymph node metastasis and distant metastasis (p<0.01, table 1). And tumor stage correlated with the prognosis of patients (p<0.0002, data not shown). These results suggested that the expression of Tie-2 may be an important factor in the tumor stage and prognosis.

We observed that Ang-1, 2 and Tie-2 were all highly expressed in human extrahepatic bile duct carcinoma cells, and that there was a significant correlation between Tie-2 expression and some clinicopathological factors. These findings suggest that Ang-Tie pathway plays a role in tumor progression and survival of patients with bile duct carcinoma. Further studies are needed to clarify the effect of Ang-Tie system on the prognosis of patients with bile duct cancer.

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