Acta Med. Nagasaki 53: 89−95

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

Yumi MIHARA,[1] Toshiyuki NAKAYAMA,[1] Atsushi NANASHIMA,[2] Tamotsu KUROKI,[3] Shinya ONIZUKA,[4] Masahiro ITO,[5] Yuki NARUKE,[1] Tomayoshi HAYASHI,[6] Hayato SANEFUJI,[7] Ichiro SEKINE[1]

[1] Department of Tumor and Diagnostic Pathology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

[2] Department of Surgical Oncology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

[3] Department of Transplantation and Digestive Surgery, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

[4] Department of Surgery, NHO Nagasaki Medical Center, Omura, Japan

[5] Department of Pathology, NHO Nagasaki Medical Center, Omura, Japan

[6] Department of Pathology, Nagasaki University Hospital of Medicine and Dentistry, Nagasaki, Japan

[7] Department of Pathology, Kitakyushu General Hospital, Kitakyushu, Japan

Extrahepatic bile duct cancer is a high mortal malignancy. Angiopoietin (Ang) and its receptor Tie, which are known to contribute to angiogenesis, have recently been reported to participate in the proliferation and differentiation of malignant tumor cells. The aim of this study is to investigate the expression and the significance of Ang-1, 2 and Tie-2 in extrahepatic bile duct carcinoma cells. We used immunohistochemistry to study 119 cases of surgically resected human extrahepatic bile duct carcinoma, and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) to confirm the expression of Ang-1, 2 and Tie-2 mRNA. Among these 119 cases, 52 (43.7%), 50 (42.0%) and 89 (74.8%) cases showed positive staining for Ang-1, 2 and Tie-2, respectively, in bile duct carcinoma cells. In 38 cases of normal mucosa, 6 (15.8%), 10 (26.3%) and 9 (23.7%) cases were positive for Ang-1, 2 and Tie-2, respectively. The positivity for Ang-1 and Tie-2 in normal mucosa was significantly different from all carcinomas ($p<0.01$ and $p<0.001$, respectively). We found no significant correlation between Ang-1 and Ang-2 expression and other clinicopathological factors such as histological differentiation, grade of tumor invasion or survival rate after surgery. In contrast, Tie-2 expression correlated significantly with degree of desmoplasia, cancer stage and survival of patients. RT-PCR analyses of five surgically resected tumor samples and three human bile duct cancer cell lines all showed positive expression of Ang-1, 2 and Tie-2 mRNAs. High expressions of Ang-1, 2 and Tie-2 in human extrahepatic bile duct carcinoma cells suggested that Ang-Tie system may be involved in the progression of human bile duct cancer.

ACTA MEDICA NAGASAKIENSIA 53: 89−95, 2008

Keywords: Angiopoietin-1; Angiopoietin-2; Tie-2; Human bile duct cancer; Prognosis

Introduction

Cholangiocarcinoma is a high mortal malignancy,[1] in that most patients present initially with unresectable disease, undergo palliative therapies, and die within 12 months.[2] 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.[3] Systemic (radio-) chemotherapy applied in a palliative setting has not been shown to prolong survival significantly.[4-6] 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,[7,8] and accounts for 3% of all gastrointestinal cancers.[7] 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.[9] Several international studies have shown an increasing incidence and mor-

Address correspondence: Toshiyuki Nakayama, M.D., Ph.D.,Department of Tumor and Diagnostic Pathology, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki, 852-8523 JAPAN
TEL: +81-(0)95-819-7107, FAX: +81-(0)95-819-7108, E-mail: toshi-n@nagasaki-u.ac.jp

Received January 9, 2009; Accepted February 9, 2009

tality rate for intrahepatic cholangiocarcinoma, and decreasing incidence and mortality for extrahepatic bile duct carcinoma.[8,10-12] Some risk factors for cholangiocarcinoma include primary sclerosing cholangitis, liver fluke infestation, hepatolithiasis, and abnormalities of biliary anatomy;[13,14] 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.[15-19] 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,[20] as well as by stimulating endothelial cell migration in vitro.[17,19] In contrast, Ang-2 is a context-dependent antagonist;[18,19] by binding to Tie-2 with an affinity similar to that of Ang-1, it blocks Ang-1-stimulated receptor phosphorylation in endothelial cells.[18]

Tie-2 is a receptor tyrosine kinase[21] that is expressed at high levels in embryos and plays a critical role in embryonic development.[15,16,22,23] 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.[16] 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,[24] colorectal carcinoma cells,[25] gastrointestinal stromal tumor,[26] and glioma cells[27] have been reported to express Ang-1, 2 and Tie-2.[28] Also, in primary murine tumors and their metastases, soluable form of the extracellular domain of murine Tie-2 for Tie-2 inhibitor caused antitumor effect by inhibiting the tumor angiogenesis.[29,30] 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.[31] 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 4oC 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 purchased 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,[32-34] 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 ℃ in a humidified atmosphere containing 5% CO_2.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared using the acid guanidine phenol method[35] from five human bile duct carcinoma tissues and three human bile duct cancer cell lines, HuCCT1, HuH28 and OZ.[32-34] Cellular RNA (1 μg) was incubated at 37℃ 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 leukemic virus reverse transcriptase (Invitrogen Corp.). Reverse transcription was then terminated at 95℃ 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'-CTCTGCACCGAGTCATCGTA-3' (antisense), human Tie-2 5'-CTGCAGTGCAATGAAGCATGC-3' (sense) and 5'-CTGCAGACCCAAACTCCTGAG-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 primers 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 ℃ for 1 minute, annealing at 60 ℃ for 1 minute, and primer extension at 72 ℃ 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 stain-

Table 1. Immunohistochemical staining for Ang-1, 2 and Tie-2 and the relationships between clinicopathological findings. (119 cases)

		Ang-1		Ang-2		Tie-2	
	n	+	-	+	-	+	-
		**		NS		***	
Normal mucosa	38	6(15.8%)	32(84.2%)	10(26.3%)	28(73.7%)	9(23.7%)	29(76.3%)
Total carcinoma	119	52(43.7%)	67(56.3%)	50(42.0%)	69(58.0%)	89(74.8%)	30(25.2%)
Histological differentiation		NS		NS		NS	
pap	12(10.1%)	6(50.0%)	6(50.0%)	6(50.0%)	6(50.0%)	8(66.7%)	4(33.3%)
tub/well	20(16.8%)	9(45.0%)	11(55.0%)	6(30.0%)	14(70.0%)	16(80.0%)	4(20.0%)
tub/mod	40(33.6%)	17(42.5%)	23(57.5%)	17(42.5%)	23(57.5%)	32(80.0%)	8(20.0%)
tub/por	37(31.1%)	16(43.2%)	21(56.8%)	15(40.5%)	22(59.5%)	28(75.7%)	9(24.3%)
Adenoendocrine	1(0.8%)	1(100.0%)	0(0.0%)	0(0.0%)	1(100.0%)	0(0.0%)	1(100.0%)
Adenosquamous	7(5.9%)	3(42.9%)	4(57.1%)	5(71.4%)	2(28.6%)	5(71.4%)	2(28.6%)
Mucinous	2(1.7%)	0(0.0%)	2(100.0%)	1(50.0%)	1(50.0%)	0(0.0%)	2(100.0%)
Tumor invasion		NS		NS		NS	
Tis	5(4.2%)	2(40.0%)	3(60.0%)	2(40.0%)	3(60.0%)	4(80.0%)	1(20.0%)
T1	10(8.4%)	3(30.0%)	7(70.0%)	5(50.0%)	5(50.0%)	8(80.0%)	1(10.0%)
T2	41(34.5%)	21(51.2%)	20(48.8%)	16(39.0%)	25(61.0%)	35(85.4%)	6(14.6%)
T3	63(52.9%)	26(41.3%)	37(58.7%)	27(42.9%)	36(57.1%)	42(66.7%)	21(33.3%)
Stage		NS		NS		*	
0	5(4.2%)	2(40.0%)	3(60.0%)	2(40.0%)	3(60.0%)	4(80.0%)	1(20.0%)
I	9(7.6%)	3(33.3%)	6(66.7%)	5(55.6%)	4(44.4%)	8(88.9%)	1(11.1%)
II	23(19.3%)	9(39.1%)	14(60.9%)	7(30.4%)	16(69.6%)	20(87.0%)	3(13.0%)
III	19(16.0%)	12(63.2%)	7(36.8%)	8(42.1%)	11(57.9%)46.	15(78.9%)	4(21.1%)
IVA	63(52.9%)	26(41.3%)	37(58.7%)	28(44.4%)	35(55.6%)	42(66.7%)	21(33.3%)
Lymph node metastasis		NS		NS		NS	
Present	50(42.0%)	24(48.0%)	26(52.0%)	23(46.0%)	27(54.0%)	35(70.0%)	15(30.0%)
Absent	69(58.0%)	28(40.6%)	41(59.4%)	27(39.1%)	42(60.9%)	54(78.2%)	15(21.7%)
Lymphatic invasion		NS		NS		NS	
Present	107(89.9%)	46(43.0%)	61(57.0%)	45(42.1%)	62(57.9%)	79(77.6%)	24(22.4%)
Absent	12(10.1%)	6(50.0%)	6(50.0%)	5(41.7%)	7(58.3%)	10(83.3%)	2(16.7%)
Venous invasion		NS		NS		NS	
Present	74(62.2%)	33(44.6%)	41(55.4%)	35(47.3%)	39(52.7%)	55(74.3%)	19(25.7%)
Absent	45(37.8%)	19(42.2%)	26(57.8%)	15(33.3%)	30(66.7%)	34(75.6%)	11(24.4%)
Perineural invasion		NS		NS		NS	
Present	96(80.7%)	40(41.7%)	56(58.3%)	39(40.6%)	57(59.4%)	74(77.1%)	22(22.9%)
Absent	23(19.3%)	12(52.2%)	11(47.8%)	11(47.8%)	12(52.2%)	15(65.2%)	8(34.8%)

*;$p<0.05$, **;$p<0.01$, ***;$p<0.0001$, NS; not significant

pap; papillary adenocarcinoma, tub/well; tubular adenocarcinoma well differentiated type, tub/mod; tubular adenocacinoma moderately differentiated type, tub/por, tubular adenocarcinoma poorly differentiated type, Adenoendocrine; Adenoendocrine carcinoma, Adenosquamous; Adenosquamous carcinoma, Mucinous;Mucinous adenocarcinoma

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	
	n	+	-	+	-	+	-
Total carcinoma	114	50(43.9%)	64(56.1%)	48(42.1%)	66(57.9%)	85(74.6%)	29(25.4%)
Desmoplastic stromal reaction		NS		NS		**	
Scirrhous	61(53.5%)	30(49.2%)	31(50.8%)	25(41.0%)	36(59.0%)	54(88.5%)	7(11.5%)
Non-scirrhous	53(46.5%)	20(37.7%)	33(62.3%)	23(43.4%)	30(56.6%)	31(58.5%)	22(41.5%)
Tumor growth pattern		NS		NS		*	
Infiltrative	74(64.9%)	36(48.6%)	38(51.4%)	31(41.9%)	43(58.1%)	60(81.1%)	14(18.9%)
Non-infiltrative	40(35.1%)	14(35.0%)	26(65.0%)	17(42.5%)	23(57.5%)	25(62.5%)	15(37.5%)

*; p<0.05, **; p<0.001, NS; not significant

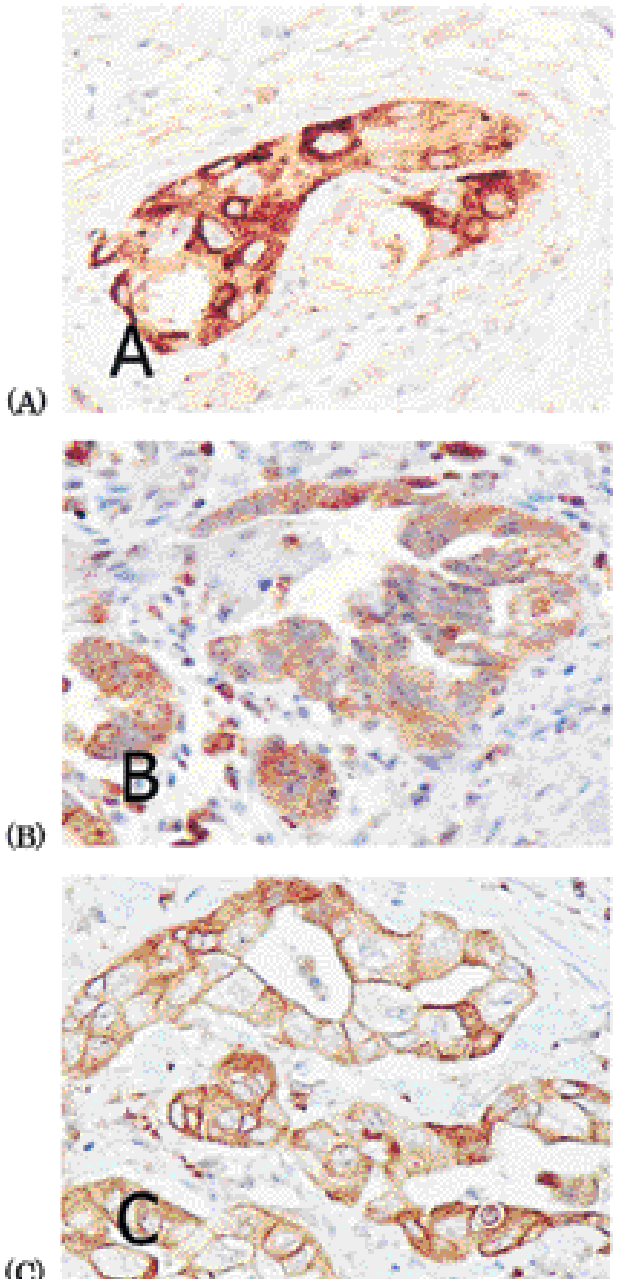

Figure 1. Ang-1, 2 and Tie-2 immunoreactivity in the extrahepatic bile duct carcinoma cells (A-C). Ang-1 and Ang-2 were expressed in the cytoplasm of carcinoma cell (A, B, respectively). Tie-2 was expressed in the cellular membrane and the cytoplasm of carcinoma cell (C). (Magnification; x400)

ing for Ang-1, 2 and Tie-2, respectively (Table 1). Ang-1 and Ang-2 were expressed in the cytoplasm of the carcinoma cells and Tie-2 was expressed in both membrane and cytoplasm of carcinoma cells (Figure 1). In 38 cases of normal mucosa, 6 (15.8%), 10 (26.3%) and 9 (23.7%) cases stained positively for Ang-1, 2 and Tie-2, respectively. The positivity of staining for Ang-1 and Tie-2 in normal mucosa was significantly different from all carcinomas ($p<0.01$ and $p<0.0001$, respectively).

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

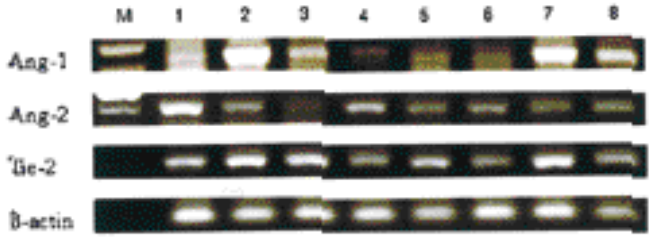

Figure 2. RT-PCR analysis of Ang-1, 2 and Tie-2 mRNA expression in human bile duct carcinoma tissues and cultured cell lines. Total RNA template was prepared from three cultured cell lines (Lane 1; HuCCT1, Lane 2; HuH28, Lane 3; OZ) and five human tissues of bile duct carcinoma (Lanes 4-8). Size markers (Lane M) consist of 100-bp DNA ladder markers (Invitrogen Corp.).

mRNAs appeared to be vary among the samples. Amplification of β-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).

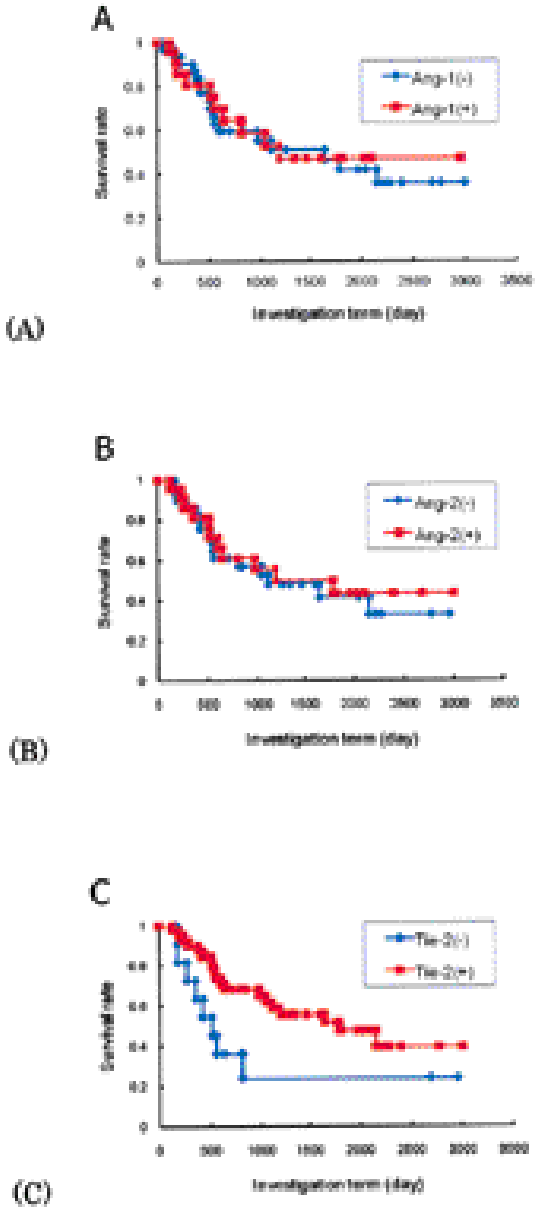

Figure 3. Kaplan-Meier survival curve after surgical therapy in patients of bile duct carcinoma (54 cases). A log-rank test showed no statistical difference between Ang-1 or Ang-2 expression and survival rate (A, B, respectively). Tie-2 positive patients had better prognosis ($p<0.05$, log-rank test) (C).

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 faintly expression of Ang-1, 2 and Tie-2 in normal mucosa of bile duct in the liver,[36] 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.[37] Ang-1, a proangiogenic protein, serves as a chemical signal for endothelial cells to induce vascular maturation and stability during the angiogenesis.[15] Positive expression of Ang-1, 2 and Tie-2 in tumor cells has been demonstrated in gastric cancer,[24] colorectal cancer,[25] gastrointestinal stromal tumors[26] and glioma cells.[27] 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.[38]

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.[39-42] 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.[43] 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.[44-47] A positive correlation between Ang-2 expression in breast cancers and shorter disease-free time and overall survival has been reported.[44] Overexpression of Ang-2 also associates with a significantly worse prognosis for patients with hepatocellular carcinoma, non-small cell lung carcinoma or bladder cancer.[45-47] However, tests of this study demon strated 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 developes scirrhous feature in early stage of tumor invasion.[48] 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.[49] However, both desmoplastic stromal reaction and tumor growth pattern did not correlated 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.

Acknowledgements

This study was supported in part by a grant from Nagasaki University. We are grateful to Mr. Toshiyuki Kawada (Nagasaki University Graduate School of Biomedical Sciences) for his excellent immunohistochemical and molecular biological assistance.

References

1. Khan SA, Thomas HC, Davidson BR, Taylor-Robinson SD. Cholangiocarcinoma. *Lancet* 366:1303-1314, 2005
2. Carriaga MT, Henson DE. Liver, gallbladder, extrahepatic bile ducts, and pancreas. *Cancer* 75 Suppl 1: 171-190, 1995
3. Malka D, Boige V, Dromain C, Debaere T, Pocard M, Ducreux M. Biliary tract neoplasms: update 2003. *Curr Opinion Oncol* 16: 364-371, 2004
4. Khan SA, Davidson BR, Goldin R, et al. Guidelines for the diagnosis and treatment of cholangiocarcinoma: consensus document. *Gut* 51 Suppl 6: vi1-19, 2002
5. Singhal D, van Gulik TM, Gouma DJ. Palliative management of hilar cholangiocarcinoma. *Surg Oncol* 14: 59-74, 2005
6. Thongprasert S. The role of chemotherapy in cholangiocarcinoma. *Ann Oncol* 16 Suppl 2: ii93-96, 2005
7. Vauthey JN, Blumgart LH. Recent advances in the management of cholangiocarcinomas. *Semin Liver Dis* 14: 109-114, 1994
8. Khan SA, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P, Thomas HC. Changing international trends in mortality rates for liver, biliary and pancreatic tumours. *J Hepatol* 37: 806-813, 2002
9. Nakeeb A, Pitt HA, Sohn TA, et al. Cholangiocarcinoma: a spectrum of intrahepatic, perihilar, and distal tumors. *Ann Surg* 224: 463-473, 1996
10. Patel T. Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. *Hepatology* 33: 1353-1357, 2001
11. Patel T. Worldwide trends in mortality from biliary tract malignancies. *BMC Cancer* 2: 10, 2002
12. Taylor-Robinson SD, Toledano MB, Arora S, et al. Increase in mortality rates from intrahepatic cholangiocarcinoma in England and Wales 1968-1998. *Gut* 48: 816-820, 2001
13. Ben-Menachem T. Risk factors for cholangiocarcinoma. *Eur J Gastroenterol Hepatol* 19: 615-617, 2007
14. Nagata E, Sakai K, Kinoshita H, Hirohashi K. Choledochal cyst: complications of anomalous connection between the choledochus and pancreatic duct and carcinoma of the biliary tract. *World J Surg* 10: 102-110, 1986
15. Suri C, Jones PF, Patan S, et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 87: 1171-1180, 1996
16. Sato TN, Tozawa Y, Deutsch U, et al. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 376: 70-74, 1995
17. Davis S, Aldrich TH, Jones PF, et al. Isolation of angiopoietin-1, a ligand for the Tie2 receptor, by secretion-trap expression cloning. *Cell* 87: 1161-1169, 1996
18. Maisonpierre PC, Suri C, Jones PF, et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277: 55-60, 1997
19. Witzenbichler B, Maisonpierre PC, Jones P, Yancopoulos GD, Isner JM. Chemotactic properties of angiopoietin-1 and -2, ligands for the endothelial-specific receptor tyrosine kinase Tie2. *J Biol Chem* 273: 18514-18521, 1998
20. Suri C, McClain J, Thurston G, et al. Increased vascularization in mice overexpressing angiopoietin-1. *Science* 282: 468-471, 1998
21. Dumont DJ, Gradwohl GJ, Fong GH, Auerbach R, Breitman ML. The endothelial-specific receptor tyrosine kinase, tek, is a member of a new subfamily of receptors. *Oncogene* 8: 1293-1301, 1993
22. Maisonpierre PC, Goldfarb M, Yancopoulos GD, Gao G. Distinct rat genes with related profiles of expression define a TIE receptor tyrosine kinase family. *Oncogene* 8: 1631- 1637, 1993
23. Sato TN, Qin Y, Kozak CA, Audus KL. Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. *Proc Natl Acad Sci USA* 90: 9355-9358, 1993

24. Nakayama T, Yoshizaki A, Kawahara N, et al. Expression of Tie-1 and 2 receptors, and angiopoietin-1, 2 and 4 in gastric carcinoma; immunohistochemical analyses and correlation with clinicopathological factors. *Histopathology* 44: 232-239, 2004
25. Nakayama T, Hatachi G, Wen CY, et al. Expression and significance of Tie-1 and Tie-2 receptors, and angiopoietins-1, 2 and 4 in colorectal adenocarcinoma: Immunohistochemical analysis and correlation with clinicopathological factors. *World J Gastroenterol* 11: 964-969, 2005
26. Nakayama T, Inaba M, Naito S, et al. Expression of angiopoietin-1, 2 and 4 and Tie-1 and 2 in gastrointestinal stromal tumor, leiomyoma and schwannoma. *World J Gastroenterol* 13: 4473-4479, 2007
27. Machein MR, Knedla A, Knoth R, Wagner S, Neuschl E, Plate KH. Angiopoietin-1 promotes tumor angiogenesis in a rat glioma model. *Am J Pathol* 165: 1557-1570, 2004
28. Shim WS, Ho IA, Wong PE. Angiopoietin: A Tie(d) Balance in tumor Angiogenesis. *Mol Cancer Res* 5: 655-665, 2007
29. Lin P, Polverini P, Dewhirst M, Shan S, Rao PS, Peters K. Inhibition of tumor angiogenesis using a soluble receptor establishes a role for Tie2 in pathologic vascular growth. *J Clin Invest* 100: 2072-2078, 1997
30. Lin P, Buxton JA, Acheson A, et al. Antiangiogenic gene therapy targeting the endothelium-specific receptor tyrosine kinase Tie2. *Proc Natl Acad Sci USA* 95: 8829-8834, 1998
31. Albores-Saavedra J, Scoazec JC, Wittekind C, et al. Carcinoma of the extrahepatic bile ducts. In *World Health Organization Classification of Tumours* (Hamilton SR, Aaltonen LA eds.; IARC Press, Lyon) pp. 206-214, 2000
32. Toyono T, Seta Y, Kataoka S, Toyoshima K. CCAAT/Enhancer-binding protein beta regulates expression of human T1R3 taste receptor gene in the bile duct carcinoma cell line, HuCCT1. *Biochim Biophys Acta* 1769: 641-648, 2007
33. Obama K, Ura K, Satoh S, Nakamura Y, Furukawa Y. Up-regulation of PSF2, a member of the GINS multiprotein complex, in intrahepatic cholangiocarcinoma. *Oncol Rep* 14: 701-706, 2005
34. Nagi P, Vickers SM, Davydova J, et al. Development of a therapeutic adenoviral vector for cholangiocarcinoma combining tumor-restricted gene expression and infectivity enhancement. *J Gastrointest Surg* 7: 364-731, 2003
35. van Dekken H, Pizzolo JG, Kelsen DP, Melamed MR. Targeted cytogenetic analysis of gastric tumors by in situ hybridization with a set of chromosome-specific DNA probes. *Cancer* 66: 491-497, 1990
36. Fabris L, Cadamuro M, Fiorotto R, et al. Effects of angiogenic factor overexpression by human and rodent cholangiocytes in polycystic liver diseases. *Hepatology* 43: 1001-1012, 2006
37. Porverini PJ. Angiogenesis in health and disease: insights into basic mechanisms and therapeutic opportunities. *J Dent Educ* 66: 962-975, 2002
38. Mobius C, Demuth C, Aigner T, et al. Evaluation of VEGF A expression and microvascular density as prognostic factors in extrahepatic cholangiocarcinoma. *Eur J Surg Oncol* 33: 1025-1029, 2007
39. Saharinen P, Eklund L, Miettinen J, et al. Angiopoietins assemble distinct Tie2 signaling complexes in endothelial cell-cell and cell-matrix contacts. *Nat Cell Biol* 10: 527-537, 2008
40. Lee OH, Xu J, Fueyo J, et al. Expression of the receptor tyrosine kinase in neoplastic glial cells is associated with Integrin beta1-dependent adhesion to the extracellular matrix. *Mol Cancer Res* 4: 915-926, 2006
41. Carlston TR, Feng Y, Maisonpierre PC, Mrksich M, Morla AO. Direct cell adhesion to the Angiopoietins mediated by integrins. *J Biol chem* 276: 26516-26525, 2001
42. Xu Y, Yu Q. Angiopoietin-1, unlike angiopoietin-2, is incorporated into the extracellular matrix via its linker peptide region. *J Biol Chem* 276: 34990-34998, 2001
43. Maruyama E, Sakamoto T, Azuma H, Ito Y, Katsuoka Y, Otsuki Y. Involvement of angiopoietins in cancer progression in association with cancer cell-fibroblast interaction. *Anticancer Res* 25: 171-177, 2005
44. Sfiligoi C, de Luca A, Cascone I, et al. Angiopoietin-2 expression in breast cancer correlates with lymph node invasion and short survival. *Int J Cancer* 103: 466-474, 2003
45. Mitsuhashi N, Shimizu H, Ohtsuka M, et al. Angiopoietins and Tie-2 expression in angiogenesis and proliferation of human hepatocellular carcinoma. *Hepatology* 37: 1105-1113, 2003
46. Takanami I. Overexpression of Ang-2 mRNA in non-small cell lung cancer: association with angiogenesis and poor prognosis. *Oncol Rep* 12: 849-853, 2004
47. Oka N, Yamamoto Y, Takahashi M, Nishitani M, Kanayama HO, Kagawa S. Expression of angiopoietin-1 and -2, and its clinical significance in human bladder cancer. *BJU Int* 95: 660-663, 2005
48. Albores-Saavedra J, Henson DE, Klimstra DS. Tumors of the gall bladder, extrahepatic bile ducts and ampulla of Vater. In *Atlas of Tumor Pathology, 3rd series* (Rosai J eds.; Armed Forces Institute of Pathology, Washington, D.C.) pp. 181-215, 2000
49. Sasaki R, Takeda Y, Funato O, et al. Significance of ductal margin status in patients undergoing surgical resection for extrahepatic cholangiocarcinoma. *World J Surg* 31: 1788-1796, 2007