of resected portal veins, have been reported by Ebata et al.[7] In contrast, caution should be exercised in when planning combined hepatic artery resection, because cancer invasion into adventitia of the hepatic artery occurs only about half of the patients despite clinically findings of apparent cancer invasion, as shown in our series; nevertheless, if the cancer encases the artery, resection for cure will require hepatic artery resection anyway.

In our series, combined vascular resection was carried out only in the patients with suspicion of cancer invasion to the vessels. We did not carry out an "en bloc" vascular resection such as reported by Neuhaus et al[6] who reported benefits of an "en bloc" portal vein resection in extended right hepatectomy. We are not sure whether this issue affects the results or not. As indicated by our results of multivariate analysis, both portal vein resection and hepatic artery resection are independent prognostic factors after operative resection in addition to both lymph node metastases and curative resection. Most studies reported previously[4,7,18] have indicated that combined vascular resection of the portal vein with hepatic artery resection was independently a factor in poor prognosis (in studies with both small and larger numbers of patients). Our study showed clearly that resections of the portal vein alone and of the hepatic artery were an independent prognostic factor after resection. In contrast, survival after both types of vascular resection differed from that of the non-vascular resection group and of the non-resection group. Portal vein resection alone seemed to confer a beneficial effect on prognosis of patients with hilar cholangiocarcinoma involving the portal vein, without increasing operative risk, however, combining hepatic artery resection with the portal vein resection was not of benefit in terms of survival, in comparison with the outcomes of unresectable patients. From our results, it seems that combined hepatic artery resection had no beneficial effect on prognosis and led to an increase in operative morbidity and mortality. In contrast, portal vein resection can be used aggressively in advanced cases of hilar cholangiocarcinoma in patients without lymph node metastases.

In conclusion, although portal vein resection is acceptable from a operative risk perspective and seems to improve the outcome in the selected patients with locally advanced hilar cholangiocarcinoma, hepatic artery resection could not be justified.

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Rapamycin, a specific inhibitor of the mammalian target of rapamycin, suppresses lymphangiogenesis and lymphatic metastasis

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Tumor lymphangiogenesis is now known to play a causal role in lymph node metastasis, and thus its inhibition would have great significance for the prevention of lymph node metastasis in cancer therapy. VEGF-C has recently been identified as a key molecule that involved in tumor lymphangiogenesis and lymphatic metastasis. However, the expressional regulation of VEGF-C is not fully understood. We investigated the role of mTOR, which is a downstream kinase of the phosphatidylinositol 3-kinase/Akt pathway, and the MAPK family (MEK1/2, p38, and JNK) in the regulation of VEGF-C and VEGF-A expression in B13LM cells, a lymphatic metastasis-prone pancreatic tumor cell line. We also investigated the antilymphangiogenic effect of rapamycin, a specific inhibitor of mTOR *in vivo* using male BALB/c nu/nu mice. VEGF-C expression was inhibited by the inhibitors for mTOR, p38, and JNK, but not by the inhibitor for MEK1/2, whereas VEGF-A expression was inhibited by all four of these inhibitors. The serum starvation-induced expression of VEGF-C was inhibited by rapamycin, whereas that of VEGF-A was incompletely inhibited. The metastatic experiment *in vivo* demonstrated that the number and the area of lymphatic vessels in the primary tumors were significantly decreased by rapamycin. Finally, the lymph node metastasis was significantly suppressed in rapamycin-treated mice. Our results suggest that mTOR, p38, and JNK play important roles in VEGF-C expression, and that rapamycin has an antilymphangiogentic effect and exerts the expected inhibition of lymphatic metastasis. (*Cancer Sci* 2007; 98: 726–733)

Metastatic dissemination is the final process in the progression of malignant tumors. Therefore, prevention of metastasis must be an ultimate goal for the treatment of malignant tumors. Spread of a malignant tumor from its primary site occurs by three main routes: vascular, lymphatic and transcoelomic. Among them, metastasis to the regional lymph nodes is often the earliest appearing metastasis, which significantly affects the prognosis of patients. In conjunction with recent advances in our understanding of the lymphatic system, accumulated experimental data have shown that tumor-induced lymphangiogenesis is an important mechanism promoting lymphatic metastasis.[1,2–4] A series of studies that investigated the relationship between the lymphatic vessel density in tumors and lymph node metastasis demonstrated that high lymphatic vessel density correlated with frequent lymph node metastasis[5–10] and with poor survival in multiple tumor types, including breast cancer, head and neck squamous cell carcinoma, and melanoma.[5,7,8]

VEGF-C has recently been identified as a key regulator in lymphangiogenesis.[11,12] VEGF-C has a VEGF-homologs region in the N-terminal and binds VEGFR-3, an fms-like tyrosine kinase receptor.[13] VEGF-C also binds to VEGFR-2 and can activate angiogenesis, but the higher affinity of VEGF-C for VEGFR-3 than for VEGFR-2 suggests that VEGF-C is a biologically relevant ligand of VEGFR-3.[14–16] Overexpression of VEGF-C in breast cancer cells promotes tumor lymphangiogenesis and increased lymph node metastasis.[1] The correlation between the expression of VEGF-C in tumor cells and lymph node metastasis is significant for a variety of tumor types, and the VEGF-C level in the primary tumor positively correlates with poor prognosis of patients.[17–24] Thus, the inhibition of VEGF-C expression in tumor cells could be a potential strategy for preventing lymph node metastasis.

The expressional regulation of VEGF-A has been well investigated. Hypoxia induces VEGF-A expression in an Akt-dependent pathway with downstream activation of HIF-1.[25] The MAPK family also mediates the signal transduction of VEGF-A expression in response to cell stresses.[26] Extracellular stimulation by growth factors has been shown to induce VEGF-C[27] and VEGF-A[28] at similar levels. However, the signal transduction involved in the expressional regulation of VEGF-C has not been fully established. PI3K and the MAPK family are involved in the signal transduction pathways of IGF-1-induced VEGF-C expression in lung carcinoma cells.[29] In contrast, IGF-1R signaling negatively regulates VEGF expression in prostatic cancer cells under conditions of androgen depletion.

Rapamycin is a lipophilic macrolide antibiotic that was initially developed as a fungicide and immunosuppressant.[30] Rapamycin acts as a specific inhibitor of mTOR, a serine/threonine kinase, that appears to be downstream of the PI3K/Akt signal pathway.[31] A complex of rapamycin and the FKBP-12 binds to mTOR and inhibits its activity.[32] The signaling through mTOR regulates phosphorylation and activation of its two major downstream components, p70S6K and eIF4E-binding protein 1 (4E-BP1).[33,34] Phosphorylation of p70S6K allows translation of ribosomal proteins.[35] Phosphorylation of 4E-BP1 regulates cap-dependent translation by enabling the formation of an active eIF4E complex.[36] mTOR plays a pivotal role in regulating the transcription initiation of many genes related to the process of oncogenic transformation and cancer progression.[37,38]

It has been noted that rapamycin and its derivatives exert a potent antitumor action on a variety of solid tumors.[39–42] The antiangiogenic effect of rapamycin is one of the mechanisms responsible for suppressing the tumor progression.[31,42] The mechanism responsible for the antiangiogenic effect of rapamycin is the inhibition of VEGF-A expression that is regulated by the Akt/mTOR pathway.[43] However, neither the effect of

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Abbreviations: 4E-BP1, initiation factor 4E-binding protein 1; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; eIF4E, initiation factor 4E; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HIF, hypoxia-inducible factor; FKBP, FK506-binding protein; IGF, insulin-like growth factor; IGF-1R, insulin-like growth factor receptor-1; PBS, phosphate buffered saline; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; RT-PCR, reverse transcriptase polymerase chain reaction; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; TGF-β tumor growth factor-β VEGF, vascular endothelial growth factor.

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rapamycin in regulating VEGF-C expression nor the effect of rapamycin on tumor-associated lymphangiogenesis has been determined. We investigated the role of signal pathways, including the mTOR pathway and MAPK pathways, in regulating the VEGF-C expression in tumor cells, and also investigated whether rapamycin reduces lymphatic metastasis using lymphatic-metastasis-prone murine pancreatic tumor cells. The main purpose of this study was to determine the effect of rapamycin on lymphangiogenesis and lymph node metastasis.

Materials and Methods

Mice. Male BALB/C *nu/nu* mice, 4 weeks old, were obtained from Japan SLC, Hamamatsu, Japan. All mice were maintained under specific pathogen-free conditions at the Center for Animal Experimentation, Chiba University Graduate School of Medicine. Regular laboratory food and tap water were made available *ad libitum*. All animal experiments were carried out under the guidelines of Chiba University.

Cell lines and culture conditions. Lymphatic metastasis-prone cells were established by *in vivo* selection of cells of a rat pancreatic tumor cell line, AR42J-B13, which were kindly provided by Professor Kojima (Gummma University, Maebashi, Japan). AR42J-B13 cells were inoculated subcutaneously into nude mice. After 3 weeks, mice were killed and the metastatic lymph nodes were resected. The resected lymph nodes were cut into small fragments *in vitro*. The fragmented tissues were then incubated with collagenase (1 mg/mL)(Wako, Osaka, Japan) for 24 h and washed with PBS. Harvested cells were cultured *in vitro* and re-inoculated into the nude mice for the second round of selection. The lymphatic metastasis-prone cell line, which was designated as B13LM, was obtained by repeating the procedure 10 times. B13LM cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) containing penicillin (50 unit/mL), streptomycin (50 μg/mL) (Invitrogen, Carlsbad, CA, USA), L-glutamine (2 mM), and 10% FBS (Biological Industries, Kibbutz Beit Haemek, Israel) at 37°C in 5%CO_2. For serum starvation, B13LM cells were cultured in the medium without FBS for 24 h.

Inhibition of signal transduction kinases. B13LM cells were cultured with serial concentrations of inhibitors for signal transduction kinases. Rapamycin (Cell Signaling Technology, Beverly, MA, USA) was added to the medium at a concentration of 0 nM, 1 nM, 10 nM, or 100 nM. U0126 (an inhibitor of MEK1/2) (Calbiochem, La Jolla, CA, USA), SB202190 (an inhibitor of p38) (Calbiochem), and SP600125 (an inhibitor of JNK) (Calbiochem) were added to the medium at a concentration of 0 μM, 1 μM, or 25 μM. Cells were treated by each inhibitor for 48 h and then prepared for Western blot or quantitative RT-PCR analysis.

Enzyme-linked immunosorbent assay. Concentrations of VEGF-C in the medium of the cultured B13LM cells were determined by ELISA (Bender MedSystems, Burlingame, CA, USA). The immunoassays were carried out and analyzed according to the manufacturer's instructions. B13LM cells were cultured in the medium with rapamycin (100 nM), then conditioned medium was used for ELISA. All samples and standards were run in duplicate.

Quantitative RT-PCR analysis. Total RNAs from B13LM cells were isolated using an RNeasy Mini kit (Quiagen, Tokyo, Japan) according to the manufacturer's instructions. One microgram of total RNA was subjected to a reverse transcription reaction, using a Ready To Go T-primed 1st strand cDNA synthesis kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). The cDNA from 33 ng of total RNA was used as a template. VEGF-A and VEGF-C mRNA levels were quantified by means of a LightCycler (Roche Diagnostics, Mannheim, Germany), using the double-strand-specific dye SYBE Green I and a HybProbe LightCycler RNA amplification kit specifically adapted for one-step RT-PCR in glass capillaries with a LightCycler instrument (Roche Diagnostics). The primer sequences used in this study were as follows: for rat VEGF-A, 5′-TATATCTTCAAGCCGTCCTG-3′ (forward) and 5′-TTGGTCTGCATTCACATCTG-3′ (reverse); rat VEGF-C, 5′-TGTCCAGCAAACTACGTGTG-3′ (forward) and 5′-ACTGGCAGGTGTCTTCATCC-3′ (reverse); rat β-actin, 5′-CTCCAGGATCTCACGCTCTA-3′ (forward) and 5′-AGAAGAAGCTGGGAAGAGAC-3′ (reverse). The cycling conditions were as follows: initial reverse transcription at 61°C for 30 min, denaturation at 95°C for 30 s, and 45 cycles of denaturation at 95°C for 1 s, annealing at 60°C for 15 s, and elongation at 65°C for 1 min with a ramp of 5°C/s (with fluorescence acquisition at the end of each elongation stage). The expression level of each mRNA was adjusted using the level of β-actin mRNA, and expressed as the ratio to β-actin mRNA.

Western blot analysis. B13LM cells were cultured in fully supplemented medium for 24 h, then cultured for 48 h with medium containing kinase inhibitors or cultured for 1 h, 2 h, 4 h, and 12 h without FBS. After this conditioning period, cells were homogenized in lysis buffer (1 mL RIPA buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM (EDTA), 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS], 100 μL Protease Inhibitor Cocktail [Sigma], and 10 μL Phosphatase Inhibitor Cocktail [Sigma]) and put on ice for 2 h. Protein concentration of each sample was determined by using a Bio-Rad Protein Assay kit (Bio-Rad, Laboratories, Hercules, CA, USA) according to manufacturer's instructions. Lysates containing 50 μg of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes (Nihon Eido, Tokyo, Japan). Nonspecific reactions were blocked for 4 h with TBS-T (10 mM Tris [pH 7.4], 100 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk. Then membranes were incubated overnight at 4°C with each antibody. After being washed with TBS-T containing non-fat dry milk, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibodies. The protein blots were visualized by chemiluminescence using ECL (Amersham).

Antibodies against Akt, phosphor-Akt (Ser473), eIF4E, phospho-eIF4E (Ser209), 4E-BP1, phospho-4E-BP1 (Thr70), p70S6K, phospho-p70S6K (Thr421/Ser424), mTOR, phospho-mTOR (Ser2448) were purchased from Cell Signaling Technology. Antibodies against VEGF-A and VEGF-C were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Experimental metastatic model. To establish subcutaneous tumors in mice, 5.0×10^6 B13LM cells were injected subcutaneously into the left inferior limb. Tumors were allowed to grow for 7 days and the establishment of subcutaneous tumors was confirmed before rapamycin treatment. Rapamycin (1.5 mg/kg per day)($n = 10$) or vehicle alone ($n = 11$) was intraperitoneally administrated to the mice every day from 8 days after the injection of tumor cells. The tumor volume ([major axis] × [minor axis]^2 × π/6) was measured every other day. Three weeks from the start of treatment, mice were killed. The subcutaneous tumors were removed and prepared for histological analysis. Lymph node metastasis was investigated, and the occurrence of metastasis was confirmed by microscope.

Histological and immunohistochemical analysis. Formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin and these sections were also used for immunohistochemical analysis. Immunostaining was carried out using the labeled streptoavidin-biotin-peroxidase (Dako Cytomation, Kyoto, Japan) and microwave antigen retrieval techniques. Goat polyclonal anti-LYVE-1 (1:100) was obtained from Santa Cruz Biotechnology. Diaminobenzidine tetrahydrochloride substrate was used to visualize the positive staining.

Evaluation of lymphatic vessels. Evaluation of lymphatic vessels in the vicinity of the subcutaneous tumors was carried out using

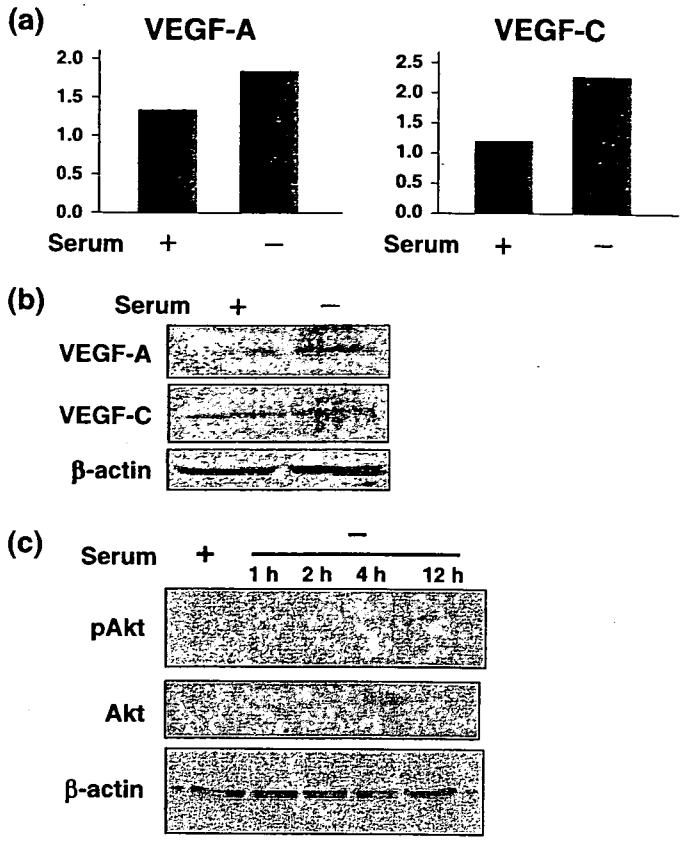

Fig. 1. The expression of vascular endothelial growth factor-A (VEGF-A) and VEGF-C in B13LM cells was evaluated by quantitative reverse transcription-polymerase chain reaction (RT-PCR) (a) and Western blot analysis (a representative data of two experiments) (b). Constitutive expression of VEGF-A and VEGF-C was observed in B13LM cells under a culture condition with 10% fetal bovine serum (FBS). The expression of VEGF-A and VEGF-C was up-regulated by culture in serum-free medium for 24 h (c) Western blot analysis of Akt and phosphor-Akt in B13LM cells. Proteins were prepared form B13LM cells that were cultured with 10% FBS (serum +) or without FBS for 1 h, 2 h, 4 h and 12 h (serum −). Phosphorylation of Akt (pAkt) was increased in serum-free medium.

LYVE-1 stains and computer-assisted quantitative analysis. Sections were scanned at low magnification, and 10 hot spot areas with the greatest numbers of positively stained vessels were identified in each section. Each hot spot was examined in turn at 400 × magnification and captured using an AxioCam MRc5 digital camera system (Carl Zeiss, Tokyo, Japan). The number of lymphatic vessels was counted, and the mean value for the 10 hot spot areas was determined for each section. The area of lymphatic vessel lumen was measured using an AxioVision 4.4 image analysis system (Carl Zeiss) by tracing the lymphatic vessel walls on a computer monitor. The mean value of the 10 hot spot areas was calculated and used as the value for each section.

Statistical analysis. Data are given as the mean ± standard deviation in quantitative experiments. The significance of the data was determined by unpaired Student's *t*-test for the evaluation of lymphangiogenesis, by a repeated-measure ANOVA test for the evaluation of tumor size, and by χ^2 test for the evaluation of lymph node metastasis. Values of P of less than 0.05 were considered statistically significant.

Results

Expression of VEGF-A and VEGF-C in B13LM cells. To investigate the role of the mTOR pathway in tumor lymphangiogenesis, we used cells of a lymphatic-metastasis prone tumor cell line, B13LM. We investigated the expression of VEGF-A and VEGF-C in B13LM cells by quantitative RT-PCR and Western blot analysis. B13LM cells constitutively expressed both VEGF-A and VEGF-C under normal culture conditions. To evaluate whether the expressional regulation of VEGF in response to cell stress remained intact in B13LM cells, we examined the expression of VEGF-A and VEGF-C under serum-starved conditions. The expressions of both VEGF-A and VEGF-C were up-regulated when the cells were cultured in serum-free medium for 24 h (Fig. 1a,b). Phosphorylation of Akt was increased in B13LM cells by the withdrawal of FBS from the cultured medium (Fig. 1c). Increased phosphorylation of Akt under the serum-starved conditions suggested that the PI3K/Akt pathway, which is upstream of mTOR, was activated by serum starvation in the B13LM cells.

Inhibition of the mTOR pathway in B13LM cells by rapamycin. We evaluated the inhibitory effect of rapamycin, a specific inhibitor of mTOR, in B13LM cells by analyzing the phosphorylation state of mTOR and the target molecules of mTOR. mTOR and its three downstream molecules, 4E-BP1, p70S6K, and eIF4E, were all phosphorylated in B13LM cells under the culture conditions containing 10% FBS, indicating the activity of the mTOR pathway in B13LM cells. Slight dephosphorylation of mTOR was observed in the B13LM cells treated with 1 nM of rapamycin, and mTOR appeared to be remarkably dephosphorylated in the B13LM cells treated with 100 nM of rapamycin. 4E-BP1, eIF4E and p70S6K were also dephosphorylated in B13LM cells treated with 1 nM of rapamycin. The reductions in the phosphorylation of these target molecules were considerable, although some phosphorylation remained (Fig. 2). These results indicated the efficacy of rapamycin for inhibiting the mTOR signal pathway in B13LM cells.

Inhibition of the expression of VEGF-C by rapamycin. We investigated whether the expressions of VEGF-A and VEGF-C were inhibited by rapamycin in B13LM cells *in vitro*. Dose-dependent reductions of VEGF-A and VEGF-C expression were observed when the cells were cultured with rapamycin for 48 h. The reduction of VEGF-C expression was more significant than that of VEGF-A expression (Fig. 3a). The secretion of VEGF-C was also reduced when B13LM cells were treated with rapamycin (Table 1). Next we investigated whether rapamycin can inhibit the serum starvation-induced induction of VEGFs. The mRNA expression of both VEGF-A and VEGF-C was increased under the normal culture conditions (Fig. 1a). Rapamycin repressed mRNA expression of both VEGF-A and VEGF-C of B13LM cells in normal culture conditions. Moreover, rapamycin repressed the serum starvation-induced expression of both of VEGF-A and VEGF-C, although the inhibition of VEGF-A mRNA was incomplete (Fig. 3b).

VEGF-C expression was inhibited by the inhibitors of p38 and JNK but not by the inhibitor of MEK1/2. We investigated the role of signal transduction pathways via three MAPK family members – MAPK kinase (MEK)1/2, p38, and c-Jun N-terminal kinase (JNK) – in the regulation of VEGF-C expression. We also investigated the regulation of VEGF-A expression by MAPK signaling, because MAPK signaling is known to be involved in the regulation of VEGF-A expression. The inhibitors of MEK1/2, p38, and JNK inhibited the expression of VEGF-A mRNA in a dose-dependent manner in B13LM cells. The inhibitors of p38 and JNK also inhibited the expression of VEGF-C mRNA, although a higher concentration of inhibitor was needed for effective inhibition of VEGF-C expression than for effective inhibition of VEGF-A. The inhibitory effect of the MEK1/2 inhibitor on the expression of VEGF-C mRNA was not clear (Fig. 4).

Rapamycin inhibited the intratumor lymphangiogenesis and the lymphatic metastasis in nude mice. We evaluated whether rapamycin plays a role in lymphangiogenesis and lymphatic metastasis

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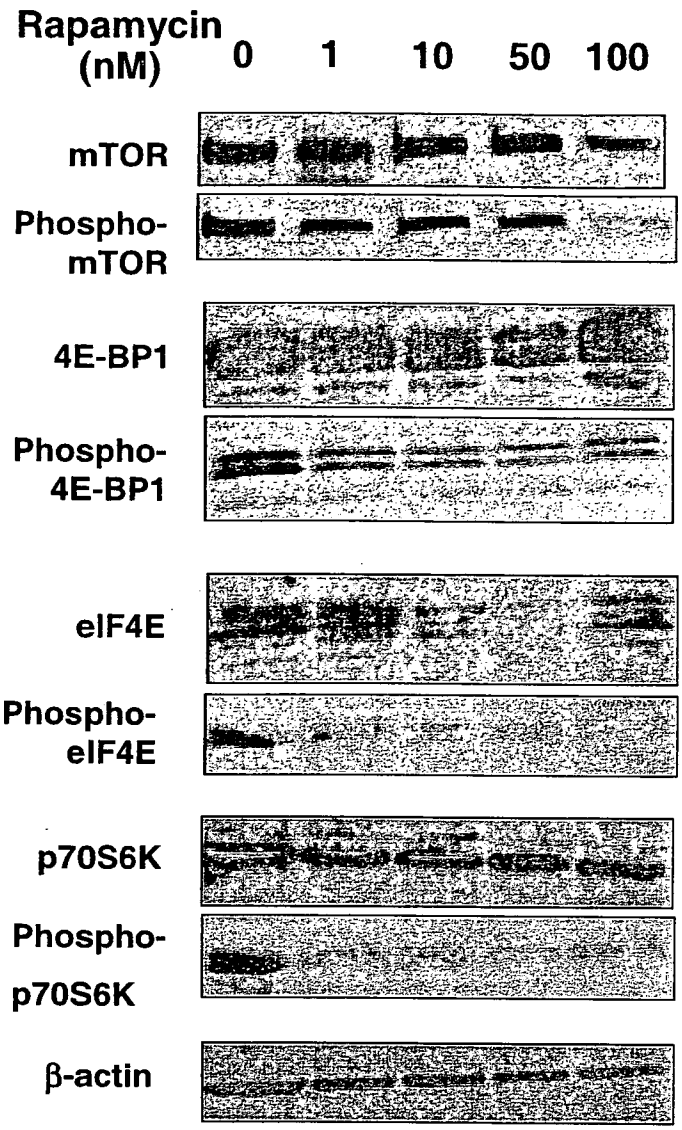

Fig. 2. Inhibition of the mammalian target of the rapamycin signal pathway by rapamycin in B13LM cells. Proteins were prepared from cells that were treated with a concentration gradient of rapamycin (0 nM, 1 nM, 10 nM, 50 nM, and 100 nM) for 48 h 50 mg of each protein was evaluated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) for the expression of the mammalian target of rapamycin, phosphor-mammalian target of rapamycin, initiation factor 4E-binding protein 1, phosphor-initiation factor 4E-binding protein 1, initiation factor 4E, phosphor-initiation factor 4E, p70S6K, and phospho-p70S6K. β-actin was also evaluated as a control. Slight dephosphorylation of mammalian target of rapamycin (mTOR) was observed in the B13LM cells treated with 1 nM of rapamycin. initiation factor 4E-binding protein 1, initiation factor 4E and p70S6K were also dephosphorylated in B13LM cells treated with 1 nM of rapamycin.

in vivo using a nude mouse model. The size of subcutaneous tumors in the rapamycin-treated mice became smaller than that of control mice at 20 days after the initiation of rapamycin treatment ($P = 0.009$), although it increased at the same rate as in the control mice until 16 days (Fig. 5a). To identify tumor-associated lymphatic vessels, we used immunohistochemistry using an antibody specific for mouse LYVE-1, which is a highly specific marker for mouse lymphatic vessels[44] (Fig. 5b). The computer-assisted quantitative analysis of lymphatic vessels revealed that rapamycin treatment reduced lymphangiogenesis in the xenografted tumors in nude mice. The number of lymphatic vessels was significantly decreased in the tumors of the rapamycin-treated mice compared with the control mice ($P < 0.001$). In addition, when we calculated the area of the lymphatic vessel lumen, it was significantly smaller in the tumors of the rapamycin-treated mice than in those of control mice ($P = 0.033$) (Fig. 5c). Finally, the occurrence of lymphatic metastasis was significantly decreased in the rapamycin-treated nude mice ($P = 0.049$). All control mice had one or two metastatic lymph nodes in the para-aortic area, whereas, three of 10 mice that were treated with rapamycin were free from lymph node metastasis and another one of the 10 had only one metastatic lymph node. Metastasis was not observed in lymph nodes in other areas, with the exception of the para-aortic area. No metastasis was observed in organs other than the lymph nodes. The mean size of the metastatic lymph nodes in mice with and without rapamycin treatment was 26.7 ± 21.3 mm^3 and 47.4 ± 77.8 mm^3, and the difference was not statistically significant (Table 2).

Discussion

Our experimental metastatic study using nude mice demonstrated that rapamycin has the potential to inhibit lymphatic metastasis of tumor cells, because the development of lymph node metastasis of B13LM cells was significantly reduced by intraperitoneal administration of rapamycin. Both the number and area of lymphatic vessels in the subcutaneous tumor were significantly smaller in the rapamycin-treated mice than in the control mice, indicating that *in vivo* rapamycin treatment inhibited the tumor-associated lymphangiogenesis.

VEGF-C and its receptor, VEFGR-3, are considered to be potent targets for the prevention of lymphatic metastasis, because the proliferating signals via VEGFR-3 promote lymphangiogenesis, and clinicopathological studies have indicated that VEGF-C/VEGFR-3 signal transduction has an important role in lymphatic metastasis.[1,17–24,45,46] Studies using experimental animal models have demonstrated that a decrease in lymphatic metastasis could be achieved by inhibiting VEGF-C/VEGFR-3 signal transduction.[47–49] Inhibition of the VEGF-C/VEGFR-3 interaction was experimentally realized by inhibitors including a soluble VEGFR-3 decoy receptor[47] and antagonistic antibody.[48] Tumor-derived VEGF-C is another therapeutic target, and VEGF-C-specific inhibition by small interfering RNA-mediated gene silencing has been shown to decrease lymphatic metastasis.[49] These results provide direct evidence of the significant role of the VEGF-C/VEGFR-3 signal transduction pathway in tumor-associated lymphangiogenesis and lymphatic metastasis. However, the methods of VEGF-C/VEGFR-3 inhibition in these studies were not immediately applicable to clinical therapy. The observed antimetastatic effect of rapamycin in our study thus has great meaning from a clinical point of view, because the analogs of rapamycin, such as CCI-779, have already progressed to the Phase III evaluation of antitumor activity.[50] In addition, it has been demonstrated that rapamycin and its derivatives have a potent antitumor action on a variety of solid tumors.[39–41] Furthermore, rapamycin and its analogs have been reported to increase the efficacy of a variety of chemotherapeutic agents, including cisplatin, doxorubicin, camptothecin, 5-fluorouracil, gemcitabin, and tamoxifen, in several types of cancers.[51–53]

The inhibitory effect of rapamycin on VEGF-C expression in B13LM cells is thought to be one of the mechanisms involved in the antilymphangiogenetic effect of rapamycin *in vivo*. There is a substantial body of evidence that tumor-derived VEGF-C plays a causal role in lymphangiogenesis and lymphatic metastasis.[1,17–24,45,46] However, because we used systemic administration of rapamycin in the present study, we cannot rule out the possibility that rapamycin affected the lymphatic endothelial cells. The decrease in the number of lymphatic vessels in the

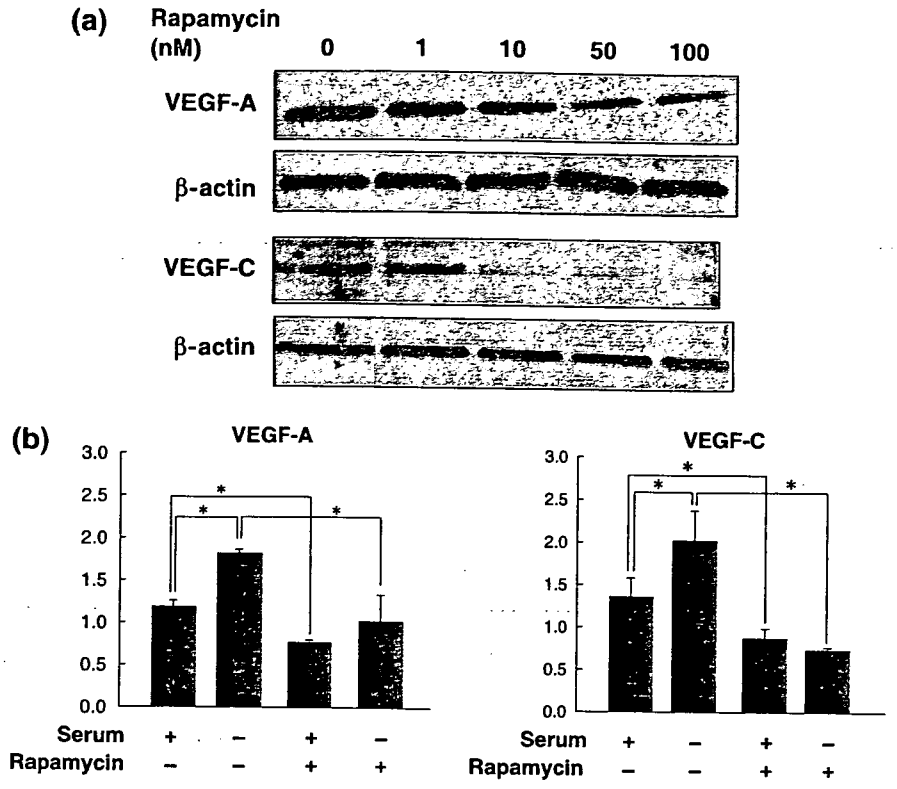

Fig. 3. Inhibition of vascular endothelial growth factor-A (VEGF-A) and vascular endothelial growth factor-C in B13LM cells by rapamycin. (a) Proteins were prepared from cells that were treated with a concentration gradient of rapamycin (0 nM, 1 nM, 10 nM, 50 nM, and 100 nM) for 48 h 50 μg of each protein was evaluated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) for the expression of VEGF-A and VEGF-C. β-actin was also evaluated as a control. The expressions of VEGF-A and VEGF-C were reduced by rapamycin in a dose-dependent manner. (a) Representative data of two experiments). (b) Cells were cultured in the medium with 10% (serum +), or with 0% (serum −) fetal bovine serum (FBS) for 24 h. The mRNA expressions of VEGF-A and VEGF-C were evaluated by quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. Serum starvation-induced expression of VEGF-A was observed, and was partially repressed by rapamycin treatment. In contrast, the serum starvation-induced expression of VEGF-C was almost completely inhibited by rapamycin treatment. Note that rapamycin inhibited the constitutive expression of VEGF-A and VEGF-C in the culture condition with 10% serum. (*$P < 0.05$).

Table 1. Inhibition of vascular endothelial growth factor-C (VEGF-C) secretion by rapamycin

	Rapamycin	
	(+)	(−)
VEFG-C (pg/mL)	510.6	131.8

rapamycin-treated tumor could be mediated by a direct effect of rapamycin on lymphatic endothelial cells, because the growth signal via VEGFR-3 activates the Akt-dependent pathway and promotes the proliferation of lymphatic endothelial cells.[54,55] However, it remains to be determined whether rapamycin directly inhibits the proliferation of lymphatic endothelial cells.

The mTOR pathway in B13LM cells was activated under a normal culture condition, which was not surprising because up-regulation of the mTOR pathway is observed in many human cancers.[56–58] Inhibition of the mTOR pathway by rapamycin reduced the expression of VEGF-C and, furthermore, inhibited the VEGF-C expression that was induced by serum starvation. Increased phosphorylation by withdrawal of FBS suggested that the Akt pathway in B13LM cells was activated by serum starvation, although PI3K/Akt pathway would be usually inactivated under the serum starved-conditions. These results indicate the involvement of the mTOR pathway in the expressional regulation of VEGF-C in B13LM cells. This is not in conflict with the results of a previous study that showed that the PI3K pathway plays an important role in IGF-1-induced VEGF-C expression,[29] because activation of the PI3K/Akt pathway leads to mTOR signaling.

The exact mechanism of how rapamycin inhibits mTOR function is not fully understood. Evidence has been presented that kinases, including Akt, can phosphorylate mTOR at Ser2448 and that such phosphorylation is likely to have a regulatory role.[59–61] On the other hand, the complex of rapamycin and FKBP-12 binds directly to mTOR and inhibits the mTOR-mediated phosphorylation of S6K1 and 4E-BP1.[32] Rapamycin also weakens the interaction of mTOR and raptor (a componet of the mTOR complex), which results in the inhibition of mTOR functions.[62] In the B13LM cells treated with 1 nM of rapamycin, the downstream molecules of mTOR, such as 4E-BP1, eIF4E and p70S6K were dephosphorylated, suggested that 1 nM of rapamycin sufficiently inhibited the mTOR pathway, although phosphorylation of mTOR (at Ser2448) remained. It was suspected from these results that the dephosphorylation of mTOR was not necessarily in the inhibitory mechanism of rapamycin in B13LM cells. A similar observation was previously observed in the hepatoma cell, Hep-G2, that was activated by insulin.[63]

The expression of both VEGF-A and VEGF-C was increased under the serum starved-conditions. However, the contribution of the mTOR pathway in the regulation of VEGF-A and VEGF-C to serum-starvation stimuli seemed not to be same because the efficacy of suppression by rapamycin was different. The serum-starvation-inducing expression of VEGF-C was completely suppressed by rapamycin, whereas the inhibition of serum-starvation-inducing VEGF-A by rapamycin appeared partially. Interestingly, a previous study using colon carcinoma cells suggested that extracellular signal-regulated kinase (ERK)1/2 activation, but not Akt activation, is required for the induction of VEGF-A by serum starvation.[26]

The size of the subcutaneous tumors in nude mice was reduced at day 20 by the rapamycin treatment. One possible explanation for the suppression of the growth of the tumor may be that tumor angiogenesis was inhibited, because rapamycin has an antiangiogenic effect. A previous study using an animal tumor model showed a similar inhibition of tumor growth, in which rapamycin was considered to mainly affect the endothelial cells; VEGF-induced endothelial cell proliferation rather than tumor-derived VEGF-A expression was inhibited by rapamycin.[41] In our previous study, rapamycin inhibited the B13LM cell proliferation *in vitro*. Thus, a direct effect of rapamycin on cell proliferation should be also considered.

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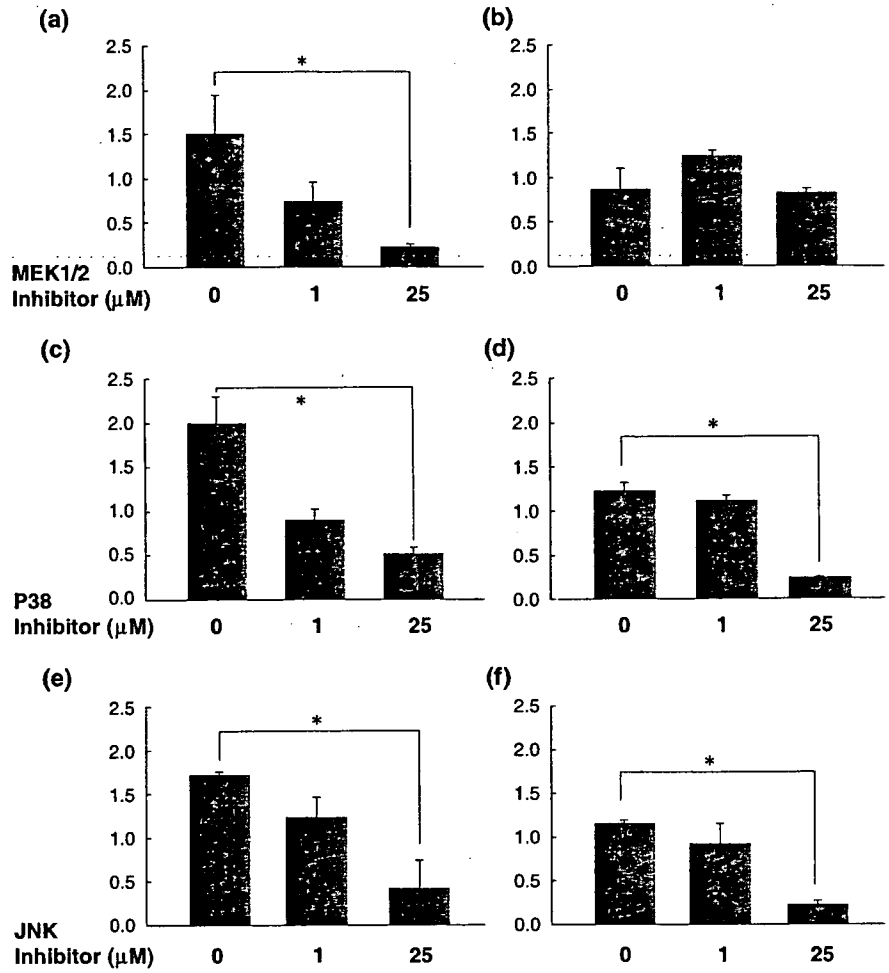
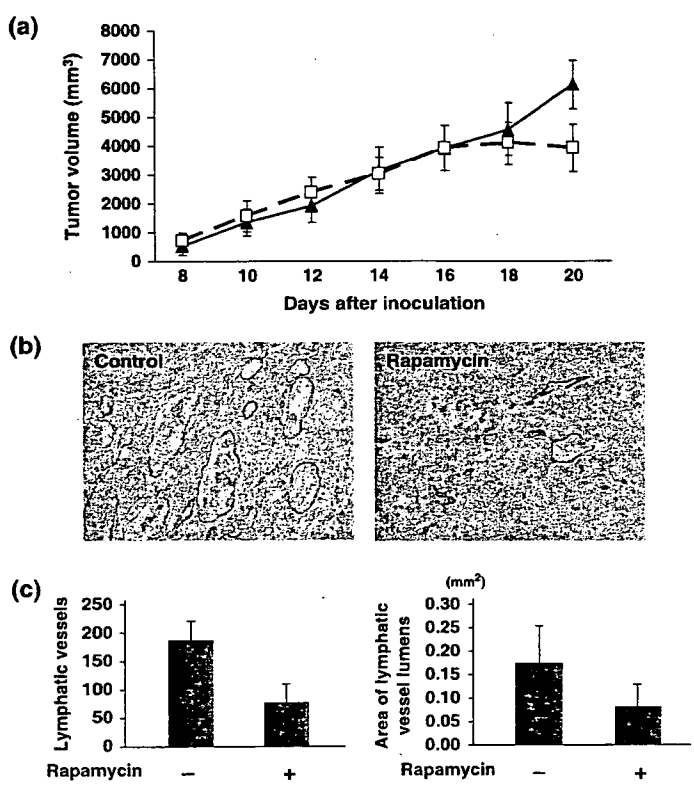


Fig. 4. mRNA expression of vascular endothelial growth factor-A (VEGF-A) (a, c, e) and VEGF-C (b, d, f) in B13LM cells treated by the MEK1/2 inhibitor (U0126) (a, b), p38 inhibitor (SB202190) (c, d), and JNK inhibitor (SP600125) (e, f). mRNA were prepared from cells treated with a concentration gradient of each inhibitor (0 nM, 1 nM, 25 nM) for 48 h. The expression level of each mRNA was adjusted using the level of β-actin mRNA, and expressed as ratio to β-actin mRNA. The inhibitors of MEK1/2, p38, and JNK inhibited VEGF-A expression in a dose-dependent manner. The inhibitors of p38 and JNK inhibited VEGF-C expression at their highest concentration (25 nM). However, the inhibitory effect of the MEK1/2 inhibitor was ambiguous. (*$P < 0.05$)



The stimulation by growth factors, including IGF-1, PDGF, EGF, and TGF-β, has been shown to induce mRNA expression of VEGF-C.[27] Stimulatory signals from these growth factors activate MAPK signal pathways, which in turn control the cell regulation, including proliferation, differentiation, and apoptosis. The results of our experiment using kinase inhibitors suggest that the signal transduction pathways of VEGF-A and VEGF-C are similar but not identical in B13LM cells. The inhibitors for JNK and p38 inhibited the expression of both VEGF-A and VEGF-C. However, it is notable that the inhibition of VEGF-C by the MEK1/2 inhibitor was not remarkable.

Fig. 5. Evaluation of the effect of rapamycin on intratumoral lymphangiogenesis and lymphatic metastasis using an experimental metastatic model in nude mice. 5.0×10.0^6 B13LM cells were injected subcutaneously into the left inferior limb. Tumors were allowed to grow for 7 days and then rapamycin (1.5 mg/kg per day) ($n = 10$) or the vehicle alone ($n = 11$) was intraperitoneally given to the mice every day. The subcutaneous tumor volume ([major axis] × [minor axis]^2 × $\pi/6$) was measured every other day (a). Inhibition of the tumor growth was observed in rapamycin-treated mice at day 20 ($P = 0.009$). After 3 weeks from the start of treatment, mice were killed. Lymphatic vessels in the subcutaneous tumors were evaluated using LYVE-1 immunostains and computer-assisted quantitative analysis. (b) Representative examples of LYVE-1 immunostains of the subcutaneous tumors (left, control mice; right, rapamycin-treated mice). The lymphatic vessels were less conspicuous in the tumor of rapamycin-treated mice than that of control mice. (c) Ten hot spots having the greatest number of LYVE-1-positive vessels were selected in each slide and evaluated. The number (left) and the area (right) of lymphatic vessels were significantly decreased in rapamycin-treated mice ($P < 0.001$ and $P = 0.033$, respectively).

Table 2. Results of the experimental metastasis model

Lymph node	Lymph node metastasis	Size of metastatic lymph node (mm^3)
Rapamycin	7/10*†	26.7 ± 21.3
Control	11/11	47.4 ± 77.8

†The numbers of metastatic lymph nodes in each mouse with and without rapamycin treatment were 0, 0, 0, 1, 1, 1, 1, 1, 1, 1 and 1, 1, 1, 1, 1, 1, 1, 2, 2, respectively. The size of the metastatic lymph node is given as the mean ± SD; *$P = 0.049$.

Binding of growth factors to their receptor-type tyrosine kinase leads to Grb2/SOS/Ras interaction, which activates MEK1/2. Activated MEK1/2 then activates ERK1/2. Interestingly, a previous study has shown that IGF-1-induced VEGF-C expression in lung carcinoma cells is both PI3K- and ERK-dependent, but PI3K has the predominant role.[29] Another study has shown that the induction of Ras oncoprotein in fibroblasts or fibrosarcoma cells induced VEGF-A mRNA, but did not induce VEGF-C mRNA.[27,54]

In summary, rapamycin inhibited the expression of VEGF-C, a potent growth factor for lymphatic endothelial cells, *in vitro*. Lymphatic vessels in the primary tumors were significantly reduced, and finally the lymph node metastasis was decreased in our experimental animal model using lymphatic metastatic-prone B13LM cells. Our results provide the first preclinical data that rapamycin has the potential to suppress tumor-related lymphangiogenesis and lymph node metastasis. Further understanding of the signal transduction of VEGF-C/VEGFR-3 axis could prove invaluable for improving treatments that target lymphangiogenesis.

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Status of Surgical Treatment of Biliary Tract Cancer

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Key Words

Biliary tract carcinoma · Bile duct carcinoma, hilar, middle, distal · Gallbladder carcinoma · Papilla of Vater carcinoma · Biliary surgery, 5-year survival · Disease stage

Abstract

Complete surgical resection of biliary tract carcinoma remains the best treatment. The Japanese Society of Biliary Surgery has organized a registry project and established a classification of biliary tract carcinoma. We report here the status of biliary surgery in Japan. For hilar bile duct carcinoma, major hepatectomy is needed to increase the resection rate, and total caudate lobectomy is required for curative resection. The 5-year survival rate was 39.1%. Middle and distal bile duct carcinomas were treated with pancreatoduodenectomy (PD) or pylorus-preserving PD (PPPD) or bile duct resection alone. The 5-year survival rate was 44.0%. The treatment of gallbladder carcinoma with pT1 lesions is cholecystectomy. The treatment of pT2 lesions is extended cholecystectomy or various hepatectomy with or without extrahepatic bile duct resection along with lymphadenectomy. Treatment of pT3 and pT4 lesions includes hepatectomy with or without bile duct resection, combined with vascular resection, extended lymphadenectomy, and autonomic nerve dissection. Several groups in Japan have performed hepatopancreatoduodenectomy. The 5-year survival rate of pT1, pT2, pT3, and pT4 were 93.7, 65.1, 27.3, and 13.8%. PD or PPPD is the standard operation for carcinoma of the papilla of Vater. The 5-year survival rate was 57.5%.

Introduction

Complete surgical resection of biliary tract carcinoma remains the best treatment for long-term survival. In Japan, the Japanese Society of Biliary Surgery (JSBS) is organized into 225 institutions. It performs registration of biliary tract carcinomas as one of its projects. In this project, the society has established guidelines for the treatment of cancer of the biliary tract based on the extent of involvement at each site. A total of 3,518 cases of biliary tract carcinoma were registered between 1998 and 2002; the site of carcinoma was the bile duct in 1,669, the gallbladder in 1,345, and the papilla of Vater in 504 cases. These cases were analyzed with regard to patient survival. We report the status of biliary surgery in Japan.

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Classification of Biliary Tract Carcinoma

As noted above, the JSBS established guidelines for the treatment of cancer of the biliary tract based on the extent of involvement at each site, according to the Classification of Biliary Tract Carcinoma currently used in Japan, and the 2nd English edition was published in 2004 [1]. The guidelines promoted in Japan for the treatment of biliary tract carcinoma are divided into three anatomical regions: the biliary duct, gallbladder, and papilla of Vater.

Bile Duct Carcinoma

Staging

Extrahepatic carcinomas have been subdivided into proximal or hilar, middle, and distal subgroups. The histological extent of tumor invasion around the bile duct (t category) in the classification of biliary tract carcinoma of JSBS is defined as the degree of tumor extension. According to the currently used Japanese classification of tumor invasion into the bile duct wall, serosal invasion is histologically divided into 5 stages, m, fm, ss, se, and si, in anatomical fashion. Furthermore, various types of direct invasion of the carcinoma into four structures present around the bile duct, i.e., invasion of the hepatic parenchyma (hinf), pancreatic parenchyma (panc), portal venous system (p), and arterial system (a), are graded from 0 to 3. Nodal involvement of carcinoma is classified into four groups. The stages of biliary tract carcinoma of JSBS are classified into five groups [1].

Hilar Bile Duct Carcinoma

Long-term survival with hilar bile duct carcinoma depends critically on complete resection with negative margins. The resection rate increases with the performance of major hepatectomy, and the likelihood of negative margins by performing it rises in hilar bile duct carcinoma. In addition, total caudate lobectomy is required for curative resection since caudate branches join the hilar bile duct [2, 3]. However, the risk of developing hepatic failure as a postoperative complication increases when major hepatectomy is performed. Therefore, when major hepatectomy is planned, portal vein embolization (PVE) is performed in many Japanese institutions. The main purpose of PVE is to induce compensatory hypertrophy of the future remnant liver and thus minimize postoperative liver dysfunction. In the recent literature from high-volume centers in Japan [4–11], the mortality rate ranged from 0 to 12%. The overall 3- and 5-year survival rates ranged from 26 to 54.8% and 23 to 40%, respectively. The proportion of stage IVa (5th edition of UICC) or over stage III (6th edition of UICC) ranged from 41 to 72%. A total of 255 cases of hilar bile duct carcinoma subjected to major hepatectomy were registered in the JSBS between 1998 and 2002 (table 1). The surgical procedure was left hepatectomy in 54 patients, extended left hepatectomy in 66 patients, left trisegmentectomy in 10 patients, right hepatectomy in 35 patients, extended right hepatectomy in 69 patients, right trisegmentectomy in 18 patients, and central bisegmentectomy in 3 patients. Bloc hepatic resection with pancreatoduodenectomy (HPD) was performed in 29 patients. Portal vein resection and reconstruction was performed in 167 patients. Resection of the right or left hepatic artery and reconstruction was performed in 78 patients. The overall the 1-, 2-, 3-, and 5-year survival rates of patients who underwent major hepatectomy were 77.3, 55.9, 46.7, and 39.1%, respectively. Patients were grouped according to the Japanese classification as stage I (n = 21, 8.2%), stage II (n = 45, 17.6%), stage III (n = 66, 25.9%), stage IVa (n = 86, 33.7%), or stage IVb (n = 37, 14.5%). The 5-year survival rate was 90.0% in stage I patients, 57.7% in stage II, 46.2% in stage III, 29.9% in stage IVa, and 17.0% in stage IVb patients (fig. 1). Compared with high-volume centers in Japan, the overall survival rate of the JSBS is good. This difference in survival rate is attributable to the higher percentage of cases of advanced cancer among those undergoing surgery at high-volume centers in Japan where aggressive surgery is often performed for advanced cancer.

Table 1. Surgical procedure for hilar bile duct carcinoma

Type of hepatectomy	Cases	With PD	With PV	With HA
Left hepatectomy	54	2	2	1
Extended left hepatectomy	66	9	6	3
Left trisegmentectomy	10	1	2	
Right hepatectomy	35	8	1	
Extended right hepatectomy	69	9	4	
Right trisegmentectomy	18	4	2	
Central bisegmentectomy	3			
Total	255	29	17	8

PD = Pancreatoduodenectomy; PV = resection and construction of the portal vein; HA = resection and construction of the hepatic artery.

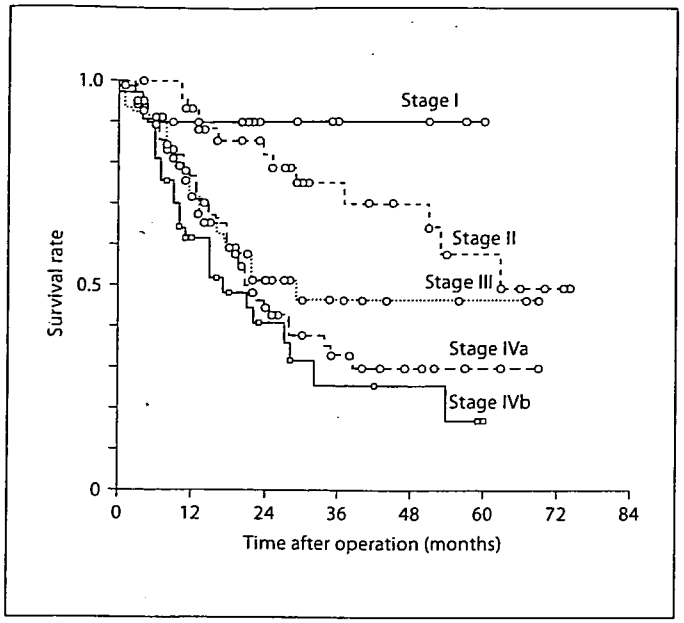

Fig. 1. Survival rates of hilar bile duct carcinoma according to stage.

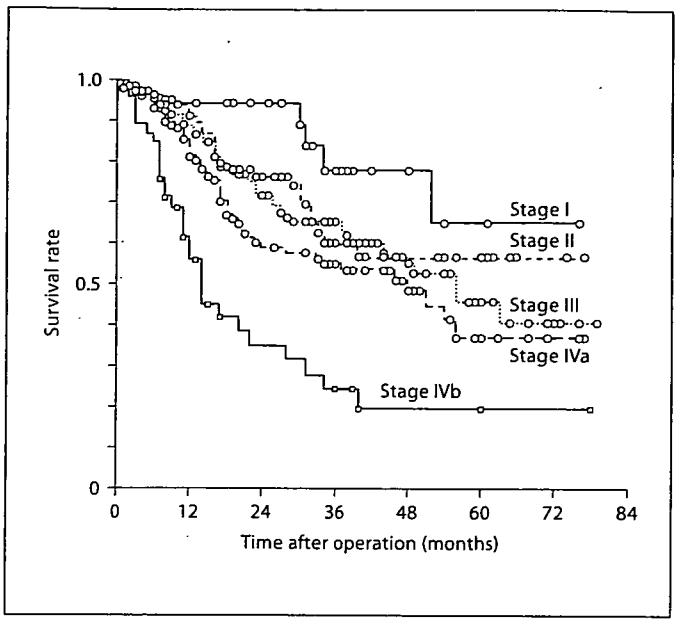

Fig. 2. Survival rates of middle and distal bile duct carcinoma according to stage.

Table 2. Studies of middle and distal bile duct carcinoma

Reference	Year	Location of tumor	Patients	3-Year survival, %	5-Year survival, %
Yamaguchi et al. [12]	1997	Distal	11	38	19
Yamaguchi et al. [12]	1997	Middle	11	33	11
Kayahara et al. [13]	1999	Middle and distal	50	47	35
Suzuki et al. [14]	2000	Middle and distal	99	50	37.4
Sasaki et al. [15]	2001	Middle and distal	59	42.6	33.6
Yoshida et al. [16]	2002	Distal	27	37	37
Sakamoto et al. [17]	2005	Middle and distal	55	52	24

Middle and Distal Bile Duct Carcinoma

Middle and distal bile duct carcinoma is treated with pancreatoduodenectomy (PD) or pylorus-preserving PD (PPPD) or bile duct resection alone. In Japan, some surgeons have advocated complete removal of the primary bile duct cancer with connective tissue clearance, including lymph nodes and neural plexus dissection. Recent results reported from high-volume centers in Japan are summarized (table 2) [12–17]. The overall 3- and 5-year survival rates ranged from 33 to 52% and 11 to 37.4%, respectively. 427 patients with middle and duct carcinoma, excluding those with insufficient data, who underwent PD or PPPD were registered in the JSBS between 1998 and 2002. The overall 1-, 2-, 3-, and 5-year survival rates were 83.5, 66.6, 57.9, and 44.0%, respectively. Patients were grouped according to the Japanese classification as stage I (n = 36, 8.4%), stage II (n = 82, 19.2%), stage III (n = 134, 31.4%), stage IVa (n = 130, 30.4%), or stage IVb (n = 45, 10.5%). The 5-year survival rates were 64.8% in stage I patients, 56.9% in stage II, 45.6% in stage III, 36.8% in stage IVa, and 19.5% in stage IVb (fig. 2).

Gallbladder Carcinoma

Staging

The histological extent of tumor invasion around the gallbladder (t category) in the classification of gallbladder carcinoma of the JSBS is defined as the degree of tumor extension. According to the currently employed Japanese

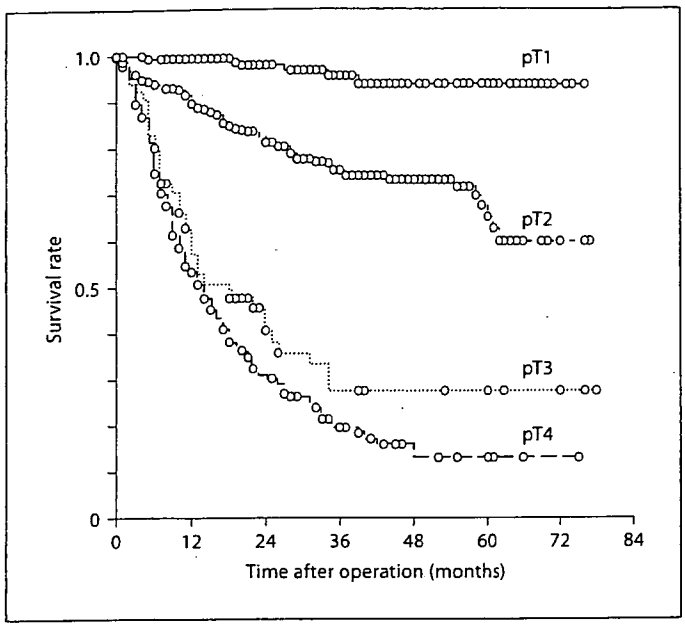

Fig. 3. Survival rates of gallbladder carcinoma according to depth of invasion.

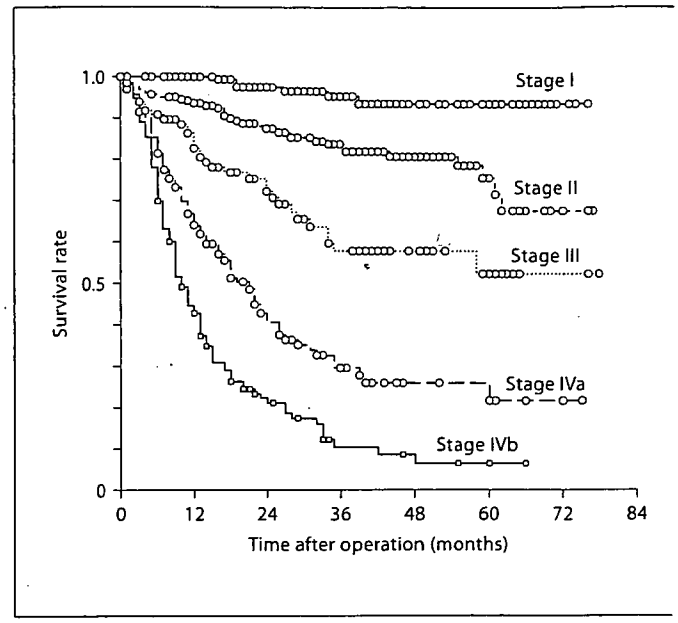

Fig. 4. Survival rates of gallbladder carcinoma according t stage.

classification of tumor invasion into the bile duct wall, serosal invasion is histologically classified into five stages, m, mp, ss, se, and si, in accordance with the anatomical structure. Furthermore, various types of direct invasion of carcinoma into four structures present around the bile duct are distinguished, i.e., invasion of the hepatic parenchyma (hinf), hepatoduodenal ligament (binf), portal venous system (p), and arterial system (a), which are graded from 0 to 3. Nodal involvement of gallbladder carcinoma is classified into four groups. The stages of biliary tract carcinoma of the JSBS are classified into five groups [1].

Surgery

Most surgeons agree that pT1 tumors are effectively treated with cholecystectomy. The 1-, 2-, 3-, and 5-year survival rates of pT1 patients (n = 160) registered with the JSBS were 99.4, 97.8, 95.4, and 93.7%, respectively (fig. 3). pT2 tumors often exhibit lymph node metastases. 30% (93/306) of the T2 tumors registered with JSBS were associated with lymph node metastases. Lymph node dissection is thus necessary for pT2 gallbladder carcinoma. Extended cholecystectomy or various types of hepatectomy with or without extrahepatic bile duct resection have been performed for pT2 patients in Japan. The range of liver resection required as part of radical surgery is still controversial. The 1-, 2-, 3-, and 5-year survival rates of T2 patients (n = 306) registered with the JSBS were 89.8 81.1, 75.2, and 65.1%, respectively (fig. 3). For pT3 an pT4 tumors, the surgical procedures currently used in Ja pan include various types of hepatectomy with or withou bile duct resection, combined vascular resection, extend ed lymphadenectomy, and autonomic nerve dissectior Several surgical groups in Japan have performed HPD fo locally advanced gallbladder carcinoma. The usefulnes of extrahepatic bile duct resection as part of radical sur gery for advanced gallbladder carcinoma is also still con troversial, particularly when there is no apparent extra hepatic bile duct involvement. The 1-, 2-, 3-, and 5-yea survival rates of pT3 patients (n = 66) registered with th JSBS were 57.7, 41.0, 27.3, and 27.3%, while those for pT patients (n = 228) were 53.6, 30.3, 19.5, and 13.8%, respec tively (fig. 3).

760 patients with gallbladder carcinoma, excludin those with insufficient data, who underwent resectio were registered in the JSBS between 1998 and 2002. Th overall 1-, 2-, 3-, and 5-year survival rates were 78.2, 66.: 58.9, and 52.6%, respectively. There are two papers cor cerning large numbers in Japan from different period. The 5-year survival rate of 1,686 patients with resectio between 1979 and 1988 was 30.1% [18], while that of 3,24 patients with resection between 1988 and 1998 (JSBS) wa 42% [19]. Patients were grouped according to the Jap nese classification as stage I (n = 160, 8.4%), stage

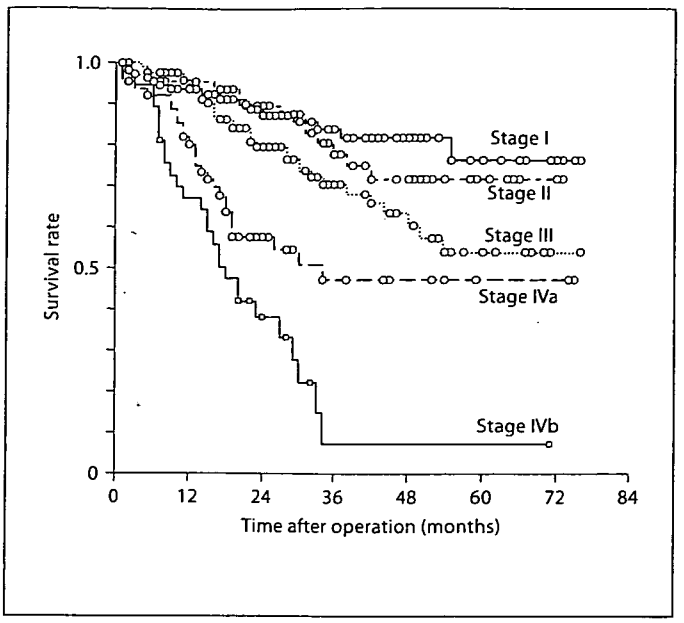

Fig. 5. Survival rates of carcinoma of the papilla of Vater according to stage.

Table 3. Studies of carcinoma of the papilla of Vater

Reference	Year	Patients	3-Year survival, %	5-Year survival, %
Kawarada et al. [20]	1993	89	63.6	57.4
Nakao et al. [21]	1994	26	57.7	52.3
Shirai et al. [22]	1996	35	60	
Kayahara et al. [23]	1997	36	66	56
Tanaka et al. [24]	2002	16	58.9	55

(n = 208, 19.2%), stage III (n = 98, 31.4%), stage IVa (n = 152, 30.4%), or stage IVb (n = 142, 10.5%). The 5-year survival rate was 93.6% in stage I patients, 80.8% in stage II, 52.6% in stage III, 21.5% in stage IVa, and 6.5% in stage IVb (fig. 4).

Carcinoma of the Papilla of Vater

Staging

The histological extent of tumor invasion around the papilla of Vater (t category) in the classification of gallbladder carcinoma of the JSBS is defined as the degree of tumor extension. Various types of histologically direct invasion of the carcinoma into two structures present around the papilla of Vater, i.e. the pancreatic parenchyma (panc) and duodenum (du), are graded from 0 to 3. Nodal involvement of gallbladder carcinoma is classified into four groups. The stages of biliary tract carcinoma of JSBS are classified into five groups [1].

Surgery

In most series, the resectability rate is higher than for other malignant tumors of the periampullary region. PD or PPPD is the standard operation for carcinoma of the papilla of Vater. Recent reports from high-volume centers in Japan are summarized (table 3) [20–24]. The overall 3- and 5-year survival rates ranged from 55 to 66% and from 40 to 60%, respectively. 404 patients with carcinoma of papilla of Vater, excluding those with insufficient data, who underwent PD or PPPD were registered in the JSBS between 1998 and 2002. The overall 1-, 2-, 3-, and 5-year survival rates were 89.3, 75.6, 66.0, and 57.5%, respectively. Patients were grouped according to the Japanese classification as stage I (n = 112, 27.7%), stage II (n = 65, 16.1%), stage III (n = 126, 31.2%), stage IVa (n = 64, 15.8%), or stage IVb (n = 37, 9.2%). The 5-year survival rates were 76.3% in stage I patients, 71.7% in stage II, 54.0% in stage III, 47.2% in stage IVa, and 7.4% in stage IVb (fig. 5).

Conclusion

We report the status of biliary surgery in Japan. Hilar bile duct carcinoma is one of the diseases on which Japanese biliary tract surgeons place particular emphasis. PVE performed during major hepatectomy and total caudate lobectomy have contributed to improving the outcome of treatment of hilar bile duct carcinoma. Middle and distal bile duct carcinomas are treated with PD or PPPD or bile duct resection alone. The treatment of gallbladder carcinoma with pT1 lesions is cholecystectomy. The treatment of pT2 lesions is extended cholecystectomy or various hepatectomy with or without extrahepatic bile duct resection, and lymphadenectomy. The treatment of pT3 and pT4 lesions includes various types of hepatectomy with or without bile duct resection combined vascular resection, extended lymphadenectomy, and autonomic nerve dissection. The usefulness of resection of the extrahepatic bile duct and the range of liver resection of gallbladder carcinoma are still controversial. Several groups in Japan perform HPD for locally advanced gallbladder carcinoma. PD or PPPD is the standard operation for carcinoma of the papilla of Vater.

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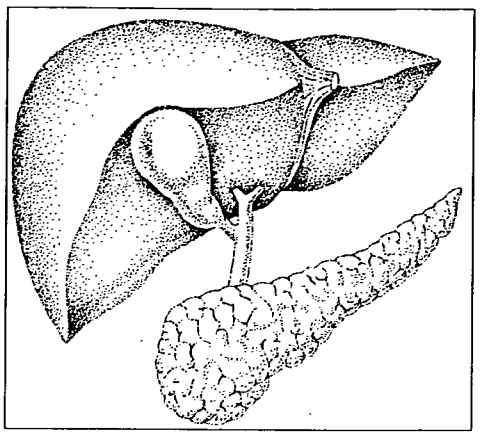

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Aggressive surgical approach for stage IV gallbladder carcinoma based on Japanese Society of Biliary Surgery classification

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Abstract

Background/Purpose. The role of aggresive surgery for stage IV gallbladder carcinoma remains controversial. Survival and prognostic factors were analyzed in patients with stage IV disease, based on the Japanese Society of Biliary Surgery (JSBS) classification, to identify the group of patients who could benefit from radical surgery.

Methods. A retrospective analysis was done of 79 patients with JSBS stage IV gallbladder carcinoma who had undergone surgical resection with curative intent at our institution. The standard procedures were anatomical S4a + S5 subsegmentectomy (n = 29) with extrahepatic bile duct resection and extended lymphadectomy, but when right Glisson's sheath and/or the hepatic hilum were involved, right extended hepatectomy (n = 34) or right trisegmentectomy (n = 3) was selected. To achieve a tumor-free margin combined pancreaticoduodenectomy was performed in 12 patients, and major vascular resection in 17 patients.

Results. In the patients with stage IV gallbladder carcinoma, the curative resection rate was 65.8% and the hospital mortality rate was 11.4%. The postoperative 5-year survival rate following curative resection was 13.7%. Univariate analysis indicated that curability, hepatoduodenal ligament invasion, nodal involvement, and vascular resection were significant prognostic factors. Neither hepatic invasion nor liver metastasis was a significant factor.

Conclusions. Aggressive surgical resection should be considered even in stage IV patients when hepatoduodenal ligament invasion and nodal involvement are absent or limited. Acceptable survival may be expected among such patients only when curative resection is achieved.

Key words Gallbladder carcinoma · Surgical resection · Stage IV · Hepatoduodenal ligament invasion · Liver metastasis

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Introduction

The majority of gallbladder carcinomas are diagnosed at advanced stages of the disease,[1–3] in spite of the recent advances in diagnostic modalities. This may be related to the lack of specific symptoms and signs,[1,4] and also the rapid growth of the tumor. Surgical approaches in resectable lesions remain the principal treatment for cure in gallbladder carcinomas, but prognosis is closely correlated with the extent of tumor invasion, suggesting that survival is stage-dependent.[1–8] In patients with early stages of the disease, surgical resection can achieve good outcome, but in advanced stages, the prognosis is apparently poor, even after radical resection.[2,3,8–12] Five-year survival rates in patients with advanced gallbladder carcinoma have been reported to range from 0% to 20%.[6,8,10–15]

In recent years, efforts have been made to increase the resectability of advanced gallbladder carcinoma, especially stage IV, by extending surgical procedures, such as extended right hepatectomy combined with pancreaticoduodenectomy and/or vascular resection and reconstruction.[16–18] According to the reports of Endo et al.[19] and Kondo et al.,[20] aggressive surgical approaches brought about some survival benefit among selected patients with advanced disease. On the other hand, extended surgical procedures carry a high risk of postoperative morbidity and mortality, especially in patients with obstructive jaundice.[15–21] The question therefore arises as to the indications for aggressive surgery in patients with stage IV disease, based on the balance between survival benefit and operative risk. At present, indications for radical resection of stage IV gallbladder carcinoma have not yet been fully defined.

The aim of the present study was to clarify which patients might benefit from radical surgery, and achieve long-term survival. We therefore analyzed our experience of surgical resection for stage IV gallbladder carcinoma, based on the Japanese Society of Biliary Surgery

(JSBS) staging system[22] (Table 1). Since 1980, we have performed aggressive surgical resections for 79 patients with stage IV disease (JSBS) with curative intent. In the present study, we retrospectively evaluated the effects of the aggressive surgery on prognosis, based on the medical records. Furthermore, as a strategy for analysis of tumor characteristics, stage IV tumors were divided into four subgroups, according to the extent of adjacent organ involvement, defined by the JSBS classification as follows: (1) hepatic-involvement type (pHinf 2-3, and pBinf 0-1), (2) biliary-involvement type (pBinf 2-3, and pHinf 0-1), (3) hepato-biliary type (pHinf 2-3 and pBinf 2-3), and (4) other. Surgical curability and postoperative morbidity and mortality were then investigated, and compared between the groups. The results of the present study may be helpful in evaluating candidates for aggressive surgical resection among stage IV patients in the future.

Patients and methods

Between 1980 and December 2005, 143 patients with gallbladder carcinoma underwent surgical resection with curative intent in the Department of General Surgery, Chiba University Hospital. During this period, surgical approaches were employed in all patients with gallbladder carcinoma unless there was involvement of the left and/or proper hepatic artery, peritoneal dissemination, or distant metastases. However, limited hepatic metastases or paraaortic lymph node metastases were resected with the primary tumor, but only if complete tumor resection was possible.

The latest International Union Against Cancer (UICC) staging system (sixth edition)[23] defines stage IV only by distant metastasis (M1), whereas stage IV as defined by the JSBS system comprises different extents of disease, as shown in Table 1. In the present study, the clinical stage of the disease was evaluated according to the JSBS classification. Among the patients with resected gallbladder carcinomas (n = 143), there were 7, 23, 34, and 79 with stage I, II, III, and IV, respectively. Therefore, the 79 patients classified as having stage IV (stage IVa, n = 42; and stage IVb, n = 37) were the subjects of this study, and their details were reviewed retrospectively. Patient age ranged from 44 to 83 years (mean, 67.8 years), and the ratio of men to women was 28: 51. None of the patients received chemotherapy before surgery. Furthermore, as a strategy for analysis of tumor characteristics, the 79 patients with stage IV tumors were stratified into subgroups according to the extent of adjacent organ involvement, defined by the JSBS, as follows: (1) hepatic-involvement type (pHinf 2-3, and pBinf 0-1), 32 patients; (2) biliary-involvement type (pBinf 2-3, and pHinf 0-1), 24 patients; (3) hepato-biliary type (pHinf 2-3 and pBinf 2-3), 17 patients; and (4) other, 6 patients. The surgical curability rates, as well as surgical morbidity and mortality rates, were then investigated, and compared between the groups.

The surgical procedures performed are listed in Table 2. Our standard procedures for advanced gallbladder carcinoma were anatomical S4a + S5 subsegmentectomy (n = 29) with extrahepatic bile duct resection and extended lymphadectomy including the N1 and N2 nodes (JSBS), and paraaortic node sampling. However, when right Glisson's sheath and/or the hepatic hilum was involved, right extended hepatectomy (n = 34) or right trisegmentectomy (n = 3) was performed. In addition, to achieve a tumor-free margin, pancreaticoduodenectomy was added in 12 patients, and major vascular resection and reconstruction of remaining liver was performed in 17 patients (portal vein for 14; hepatic artery for 4). The inferior vena cava was also partially resected and repaired by means of primary closure in 2 patients. "Curative resection", in which the surgical margin was histologically free from cancer involvement was achieved in 52 patients (65.8%), and noncurative resection was done in 27 patients (34.1%). Mean follow-up was 152 months (range, 6–288 months).

Survival curves were constructed using Kaplan-Meier analysis. Statistical assessment of survival was performed with the log-rank test. Cox univariate and multivariate analyses were performed to determine prognostic factors for survival. Surgical curability rates, as well as morbidity and mortality rates, were compared using the χ^2 test. A P value of less than 0.05 was considered significant.

Results

Survival

Cumulative survival curves according to stage of disease are shown in Fig. 1. Satisfactory results were achieved in patients with stage I and stage II diseases. The 5-year survival rates in patients with stage I and II were 86% and 49%, respectively. In contrast, the 5-year survival rate in patients with stage IV was only 9.1%, and a statistically significant difference was found when stage IV was compared to stage I and stage II ($P < 0.001$, respectively).

Cumulative survival curves for stage IV patients, according to curability, are shown in Fig. 2. The 5-year survival rate in patients who underwent curative resection (n = 52) was 13.7%, and a statistically significant difference was found between the curative and noncurative resection groups ($P = 0.023$). In addition, curative resection was possible more frequently in the hepatic involvement-type tumors, as compared to the other

Table 1. Classification systems for staging gallbladder carcinoma by the Japanese Society of Biliary Surgery (JSBS)

1. pT-category (primary tumor invasion)

pT_1:	m, mp	$pHinf_0$	$pBinf_0$	pPV_0/PV_0	pA_0/A_0
pT_2:	ss	$pHinf_{1a}$	$pBinf_0$	pPV_0/PV_0	pA_0/A_0
pT_3:	se	$pHinf_{1b}$	$pBinf_1$	pPV_0/PV_0	pA_0/A_0
pT_4:	any	$pHinf_{2,3}$	$pBinf_{2,3}$	$pPV_{1,2,3}/PV_{1,2,3}$	$pA_{1,2,3}/A_{1,2,3}$

1.1 Liver

$pHinf_0$: No direct invasion of the liver, or direct invasion limited to the muscularis propria of the gallbladder

$pHinf_1$: Direct invasion of the muscularis propria of the gallbladder and/or slight invasion of liver parenchyma (no more than 5 mm in depth)

$pHinf_2$: Direct invasion of the liver parenchyma, which invasion is 5 mm or more but not more than 20 mm in depth

$pHinf_3$: Direct invasion of the liver parenchyma, which invasion is 20 mm or more in depth

1.2 Hepatoduodenal ligament

$pBinf_0$: No invasion of the right margin of the hepatoduodenal ligament

$pBinf_1$: Invasion of the right margin of the hepatoduodenal ligament, but not to the left margin

$pBinf_2$: Invasion of the left margin of the hepatoduodenal ligament, but not to the entire ligament

$pBinf_3$: Invasion through the hepatoduodenal ligament

1.3 Portal veins

pPV_0: No invasion of portal veins

pPV_1: Invasion of the adventitia

pPV_2: Invasion of the media

pPV_3: Invasion of the intima and/or lumen

1.4 Hepatic arteries

pA_0: No invasion of hepatic arteries

pA_1: Invasion of the adventitia

pA_2: Invasion of the media

pA_3: Invasion of the intima and/or lumen

2. Lymph node metastasis

pN_0: No evidence of lymph node metastasis

pN_1: Metastasis in cystic duct and/or pericholedochal lymph node

pN_2: Metastasis in hepatoduodenal ligament except N1, superior retropancreas and/or along the common hepatic artery

pN_3: Metastasis in peripancreatic (except superior retropancreas), celiac, splenic, superior mesenteric, and/or paraaortic lymph nodes

3. Stage grouping

Final stage (fStage) should be classified according to the histopathological findings, in addition to the surgical findings.

	H0, P0, M (−)				
	pN0	pN1	pN2	pN3	H(+), P(+), M (+)
pT1	I	II			
pT2	II	III		IVa	
pT3			IVa		IVb
pT4	IVa				

types of tumors, and a statistically significant difference was found between the hepatic-involvement type (26/32) and the hepato-biliary type (8/17) of tumors (Table 3). However, the curative resection rate in the major vascular resection group ($n = 17$) was not significantly different from that in the non-vascular resection group, as shown in Table 4.

Prognostic factors

Univariate analysis of clinicopathological factors in the 79 stage IV patients revealed that operative curability ($P = 0.023$), hepatoduodenal ligament invasion (pBinf 1-2 vs pBinf 2-3; $P = 0.014$), vascular resection ($P = 0.016$), and lymph node metastasis ($P = 0.028$) were