Fascin is involved in tumor necrosis factor-α-dependent production of MMP9 in cholangiocarcinoma

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Fascin is an actin-binding protein involved in the cell motility. Recently, aberrant expression of fascin in carcinoma cells was reported to participate in their invasive growth in cooperation with proteinases such as matrix metalloproteinases (MMPs). This study examined the participation of fascin in the progression of cholangiocarcinoma (CC) with reference to MMPs and tumor necrosis factor-α (TNF-α). Expression levels of fascin and MMP2 and 9 were examined immunohistochemically in human non-neoplastic biliary epithelium (13 cases) and CC (87 cases). The relationship between fascin and MMP9-expression levels was examined using two CC cell lines (CCKS-1 and HuCCT1). It was also examined whether or not fascin was involved in TNF-α-induced overproduction of MMP9 in CC. Fascin and MMP9 were expressed in 49 and 53% of CC samples, respectively, and the expression of these genes was frequent in intrahepatic CC. Fascin expression was correlated significantly with MMP9 expression. In particular, these two molecules were expressed more intensely at the invasive fronts of CC. Fascin expression was an unfavorable prognostic factor for patients with intrahepatic CC. In vitro studies showed that TNF-α could induce the overexpression of fascin and MMP9 in two CC cell lines. A knockdown study of fascin by siRNA showed that TNF-α induced the overproduction of fascin, which in turn upregulated MMP9 expression. Overexpression of fascin may have an important function in the progression of CC, and fascin expression might be involved in the signaling pathway in TNF-α-dependent production of MMP9 in CC.

Fascin, an actin-bundling protein, has become of great interest because of its functional involvement in the cell motility of non-neoplastic and neoplastic cells.[1–3] Fascin was first isolated from sea urchin egg extracts[4] and then identified in Drosophila and human B-lymphocytes.[4–6] Fascin is expressed in mature dendritic cells and neurons during development and also in the adult.[7] Usually, fascin expression is not observed in non-neoplastic epithelia, but it is expressed in various epithelial neoplasms, including carcinomas of the pancreas, lung, esophagus, stomach, breast, skin, and ovary.[8–16] In addition, fascin expression is a poor prognostic factor for carcinomas of the lung, esophagus, stomach, breast, colon, and head and neck.[11–13,17–20] Interestingly, fascin overexpression was closely correlated with the invasiveness of esophageal carcinoma cells, and overproduction

of matrix metalloproteinase2 (MMP2) and MMP9 was involved in fascin-mediated carcinoma invasion.[21] Recently, it was also reported that fascin was overexpressed in cholangiocarcinoma (CC);[22–25] however, the clinicopathological significance of fascin expression in CC is still unclear. In particular, it has not been elucidated how fascin is involved in the development and progression of CC.

CC arising from the intrahepatic, hilar, and extrahepatic bile ducts is the second most common hepatobiliary malignancy. Once CC begins invasive growth, it invades aggressively into the surrounding tissue commonly associated with metastasis and finally resulting in a poor prognosis. Among factors involved in the tumor progression of CC, tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, seems to have a key function in promoting invasion by

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CC cells.[26–28] As for the roles of TNF-α in CC, it was shown earlier that TNF-α in proximity to the invasive front of CC was at least partly responsible for the increased migration of CC cells;[28] that is, the interaction of stromal cell-derived factor-1 released from fibroblasts and CXCR4 expressed on ICC cells may be actively involved in ICC migration, and TNF-α may enhance ICC cell migration by increasing the CXCR4 expression on CC cells. Tanimura *et al* reported that TNF-α can activate tumor invasion through the production of MMP9.[29,30] Multiple intracellular signaling, such as mitogen-activated protein kinase ((MAPK) or phosphatidyl-inositol 3-kinase (PI3K), were involved in TNF-α-dependent MMP9 secretion in CC;[29] however, detailed molecular mechanisms of TNF-α-dependent MMP9 secretion in CC has not been fully elucidated.

This study examined the clinicopathological significance of fascin expression in human cases of CC and then tried to test a hypothesis that fascin expression might be involved in TFN-α-dependent MMP production in CC using human materials of CC and two CC cell lines.

MATERIALS AND METHODS
Human Tissue Studies
Classification of the biliary tree and CCs
The biliary tree is divided into the extrahepatic bile duct (common hepatic and bile ducts), right and left hepatic ducts, and their branches. The intrahepatic large bile ducts refer to the first and second branches of the right and left hepatic ducts,[31] and the extrahepatic bile duct, right and left hepatic ducts, and intrahepatic large bile duct are referred to collectively as the large bile duct in this study.

In this study, CC cases were classified into three types based on their location: extrahepatic, hilar, and intrahepatic CCs. Extrahepatic CC is adenocarcinoma arising from extrahepatic bile ducts, and hilar CC is CC that is regarded to have arisen from the bifurcation of the common bile duct, and right and left hepatic ducts. Intrahepatic CC is adenocarcinoma originating from the finer branches of the intrahepatic bile ducts, and includes 'bile ductular carcinoma'.[32]

Patient selection and preparation of tissue specimens
A total of 87 cases of CC were obtained from the hepato-biliary disease files of the Department of Human Pathology, Kanazawa University Graduate School of Medicine and its affiliated hospitals in Japan between 1987 and 2006. This study consisted of 30 cases of extrahepatic CC, 25 cases of hilar CC, and 31 cases of intrahepatic CC. All CC cases used in this study were surgically resected cases. Thirteen cases treated surgically not for biliary malignancies and showing histologically normal liver were collected similarly and the large bile ducts of these livers were used as non-neoplastic biliary epithelium (control). Lesions of Vater's ampulla or the gall bladder were not included in this study. No CC cases had any preceding conditions of biliary tumors, such as primary sclerosing cholangitis or hepatolithiasis. All CCs were histologically conventional adenocarcinoma. Average ages, male/female ratios, and histological grades (well, moderately, and poorly differentiated adenocarcinoma) are shown in Table 1. The tissue specimens obtained from these patients after surgery were fixed in 10% neutral formalin and embedded in paraffin. One representative tissue block was chosen from each case for the immunohistochemical analysis.

Immunohistochemistry for fascin, MMP9, MMP2, and TNF-α
Immunohistochemical analyses for fascin, MMP9, MMP2, and TNF-α were performed using an EnVision + system (for fascin, MMP9, and MMP2) (Dako Cytomation, Glostrup, Denmark) or a Histofine Simple Stain MAX (for TNF-α) (Nichirei, Tokyo, Japan). Deparaffinized sections were heated in a microwave oven in 10 mM citrate buffer (pH 6.0) for 20 min (for fascin, MMP9, and MMP2) or pretreated in 0.1% trypsin buffer at 37°C (for TNF-α). After the blocking of endogenous peroxidase and incubation in Protein Block Serum-Free Solution (Dako Cytomation) for 20 min, the deparaffinized sections were incubated overnight at 4°C with primary antibodies: anti-fascin (clone 55K-2; 1:50; Dako Cytomation), anti-MMP9 (clone 56-2A4; 1:200; Daiichi Fine chemical, Toyama, Japan), anti-MMP2 (clone 42-5D11; 1:100; Daiichi Fine chemical), and anti-TNF-α (clone N-19; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were then incubated at room temperature for 1 h with goat anti-mouse immunoglobulins conjugated to peroxidase-labeled-dextran polymer (EnVision +, Dako Cytomation) or Histofine Simple Stain MAX PO(G) (Nichirei). The reaction products were developed by immersing the section in a 3,3′-diaminobenzidine tetrahydrochloride (DAB)

Table 1 Clinical features and histological differentiation of cases examined in this study

	Number of cases	Age average (range)	Gender male/female	Histology well/moderately/ poorly differentiated
Extrahepatic cholangiocarcinoma	30	72 (58–84)	16/14	11/15/4
Hilar cholangiocarcinoma	25	67 (35–79)	9/16	12/9/4
Intrahepatic cholangiocarcinoma	32	65 (39–85)	8/24	11/16/5
Non-neoplastic biliary epithelium	13	71 (44–84)	7/6	

solution containing 0.03% hydrogen peroxide. Nuclei were lightly counterstained with hematoxylin.

The expression of fascin, MMP9, and MMP2 was evaluated semiquantitatively according to the percentage of positive cells: 0, negative; 1, focally positive (1–10% positive cells in the lesion); 2, moderately positive (11–50%); and 3, markedly positive (more than 50%). Cases with moderate or marked expression patterns (scores 2 or 3) were considered positive cases in this study. Regarding TNF-α expression, cases with TNF-α-positive cells were determined positive, and the cellular sources of TNF-α were also examined. Staining levels were assessed independently by two observers (MO and YZ) and any discrepancies were resolved by consensus using a multiviewer microscope.

Culture Studies

Cell culture

Two human CC cell lines (CCKS-1 and HuCCT1) were used in this study. HuCCT1 was obtained from the Health Science Research Resources Bank (Osaka, Japan). CCKS-1 was established in our laboratory.[8] CCKS-1 and HuCCT1 were cultured with RPMI1640 (Roswell Park Memorial Institute 1640) (Life Technologies, Inc., Rockville, MD, USA) and D-MEM/F-12 (Dulbecco's modified Eagle's medium and nutrient mixture F-12, 1:1; Life Technologies, Inc.), respectively. Fetal bovine serum (10%) (Life Technologies, Inc.) and antibiotics-antimycotic (1%) (Life Technologies, Inc.) were also included in each medium.

TNF-α treatment

CC cell lines (CCKS-1 and HuCCT1) were cultured with TNF-α (PeproTech, London, UK) at concentrations of 0, 10, 50, and 100 ng/ml with serum-free medium for 12–48 h. After then, cultured cells were used for RT–PCR, western blotting, and gelatin zymography.

To examine the signaling pathway of TNF-α, inhibitory studies were performed using two neutralization antibodies to TNF-α receptors (TNF receptors 1 and 2 [TNFR1 and TNFR2]), an NF-κβ inhibitor (MG132), a PI3K inhibitor (LY294002), a mitogen-activated or extracellular signal-regulated protein kinase 1/2 (Erk1/2) inhibitor (U0126), and a p38 MAPK (p38 MAPK) inhibitor (SB203580). CC culture cells were incubated with TNFR receptors or inhibitors for 1 h before the treatment with TNF-α for 24 h. Antibodies used were an anti-TNFR1 monoclonal antibody (MAB625, R&D Systems, Minneapolis, MN) and an anti-TNFR2 monoclonal antibody (MAB226, R&D Systems). Four inhibitors were purchased from Sigma (St Louis, MO, USA).

RNA extraction and RT–PCR

RNA was isolated from CCKS-1 and HuCCT1 using an Qiagen RNAeasy kit (QIAGEN, Tokyo, Japan). Briefly, 1 μg of RNA was used to synthesize the first-strand cDNA using the Superscript system (Life Technologies, Inc.) in accordance with the manufacturer's instructions. RT–PCR reactions for

fascin, MMP9, and β-actin were performed. The oligonucleotide sequences, numbers of cycles, annealing temperatures, and product sizes of these primers are shown as follows: fascin, forward 5′-CTGGCTACACGCTGGAGTTC-3′, reverse 5′-CTGAGTCCCCTGCTGTCTCC-3′, 30 cycles, 60°C, and 492 bp; MMP9, forward 5′-GAAGATGCTGCTGTTCAGCG-3′, reverse 5′-ACTTGGTCCACCTGGTTCAA-3′, 40 cycles, 55°C, and 215 bp; and β-actin, forward 5′-CAAGAGATGGC CACGGCTGCT-3′, reverse 5′-TCCTTCTGCATCCTGTCGG CA-3′: 25 cycles, 52°C, and 275 bp. PCR consisted of each cycle at 94°C for 1 min, each annealing temperature for 2 min, and 72°C for 2 min. After PCR, 5 μl aliquots of the products were subjected to 1.5% agarose gel electrophoresis and stained with ethidium bromide. RT–PCR for the β-actin, a housekeeping gene, was used for a quantitative control.

Real-time quantitative PCR

Multiplex real-time PCR was performed for quantitative analyses, according to the standard protocol using the Taq-Man Universal PCR Master Mix (Applied Biosystems, Foster, CA, USA) and ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Specific primers and probes for fascin (Hs00602051 μml), MMP9 (Hs00957562 μml), and β-actin (Hs99999903 μml) were obtained from Applied Biosystems. The cycling conditions were as follows: incubation for 2 min at 50°C, for 10 min at 95°C, and 50 cycles of 15 s at 95°C and 1 min at 60°C. Expressions of fascin and MMP were normalized to β-actin. Each experiment was performed in triplicate, and the mean was adopted as the value in each experiment.

Western blot

Proteins were extracted from cultured cells using T-PER™ Tissue Protein Extraction Reagent (Pierce Chemical Company, Rockford, IL, USA) and were used for western blot analysis. Western blot analysis was carried out on 10% SDS–PAGE gel. The proteins in the gel were transferred electrophoretically onto a nitrocellulose membrane. The membranes were incubated with primary antibodies to fascin (the same antibody as used for immunohistochemistry), β-actin (clone AC-15, 1:5000, mouse monoclonal, Abcam Limited, Cambridge, UK). The expression of each protein was detected using second antibodies conjugated to peroxidase-labeled polymers (EnVision+ system, Dako Cytomation). DAB was used as the chromogen.

Gelatin zymography

MMP-9 expression was analyzed by gelatin zymography using Alexa Fluor 680-labeled gelatin.[33] This fluoro-zymography system can analyze not only conditioned medium but also cell lysates at high sensitivity. After 5×10^5/ml of CC cells were seeded into 6-well plates, these CC cells were treated with TNF-α for 24 h in serum-free medium. The conditioned medium or treated cells were incubated with SDS sample buffer and incubated for 30 min at 37°C. The

samples were separated on 10% SDS–PAGE containing 0.005% Alexa-labeled gelatin. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 2 h at room temperature to remove SDS, and then incubated in substrate buffer overnight at 37°C. The gel was scanned by an LI-COR Odyssey IR imaging system (Lincoln, NE, USA).

Transfection of fascin small interfering RNA

The knockdown of fascin was performed using small interfering RNA (siRNA). Fascin siRNA (h) (sc-35359) was purchased from Santa Cruz Biotech. Fascin siRNA (h) is a pool of three target-specific 20–25 nt siRNAs designed to knock down gene expression. A control siRNA (AllStars Neg. Control siRNA, QIAGEN) was used as a negative control. The CCKS-1 and HuCCT1 cells were plated in 35 mm dishes (5×10^5 cells) 1 day before transfection. The cells were transfected with fascin siRNA using FuGENE6 (Roche, Basel, Switzerland). Of 10 μM siRNA, 4 μL was diluted in OPTI-MEM (Life Technologies, Inc.). FuGENE6 was diluted with 500 μL of OPTI-MEM for 5 min, and the siRNA was added to the mixtures and incubated for 15 min at room temperature to enable transfection complex formation. The entire mixture was added to the 1 ml of culture medium with cells in 6 mm vessels. At 12 h after transfection, some groups of cells were treated with TNF-α as mentioned above. At 48 h after transfection, the cells were used for RNA or protein extraction. All in vitro experiments were performed at least twice and the reproducibility of the data was confirmed.

Statistical analysis

Overall survival was defined as that from the date of the operation to the date of death because of CC. Statistical analysis was performed using the Mann–Whitney U test. The correlation coefficient of two factors was evaluated using Spearman's rank correlation test. The survival of CC patients was compared using the Kaplan–Meier method, and differences between the survival curves were tested using the log-rank test. A P-value less than 0.05 was considered significant.

## RESULTS
Human Tissue Studies
Immunohistochemistry of fascin, MMP2, and MMP9 in CC

As shown in Figure 1, fascin was expressed in a fine granular pattern in the cytoplasm of CC cells. Non-neoplastic biliary epithelium was constantly negative for fascin. Fascin was expressed in 33% (10/30 cases) of extrahepatic, 52% (13/25 cases) of hilar, and 62% (20/32 cases) of intrahepatic CC (Figure 2). Intrahepatic CC expressed fascin more frequently than extrahepatic CC ($P = 0.023$). Fascin expression was not related to histological grade (positive case ratios: well differentiated adenocarcinoma, 41%; moderately, 53%; and poorly, 61%). Out of six cases with in situ carcinoma lesions around the invasive tumor, three cases showed fascin expression in in situ carcinoma lesions. Fascin was also expressed in lymphocytes, vascular endothelium, and fibroblasts in all neoplastic and non-neoplastic cases.

MMP9 expression was observed in 40% (12/30 cases) of extrahepatic, 56% (14/25 cases) of hilar, and 62% (20/32
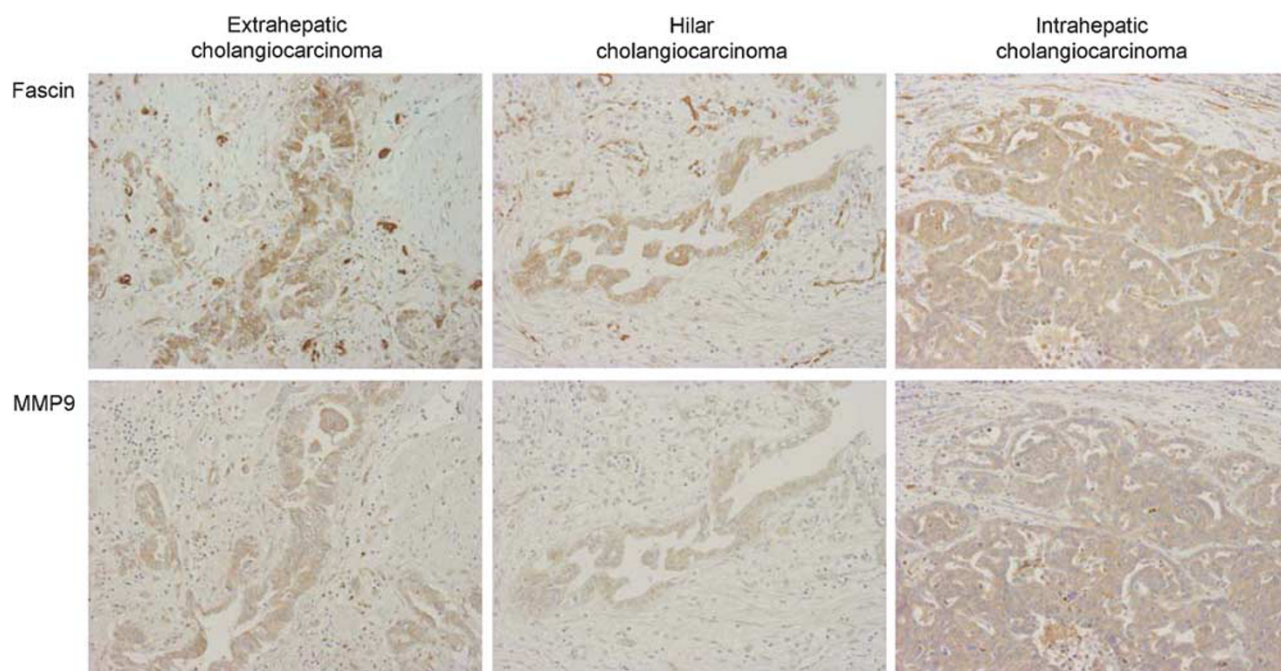
Figure 1 Immunohistochemical expression of fascin and MMP9 in extrahepatic, hilar, and intrahepatic cholangiocarcinoma (CC). Fascin and MMP9 were expressed in CC cells in a granular cytoplasmic pattern. Images of fascin and MMP9 are in almost the same field of CC. (All images, original magnification ×200.)
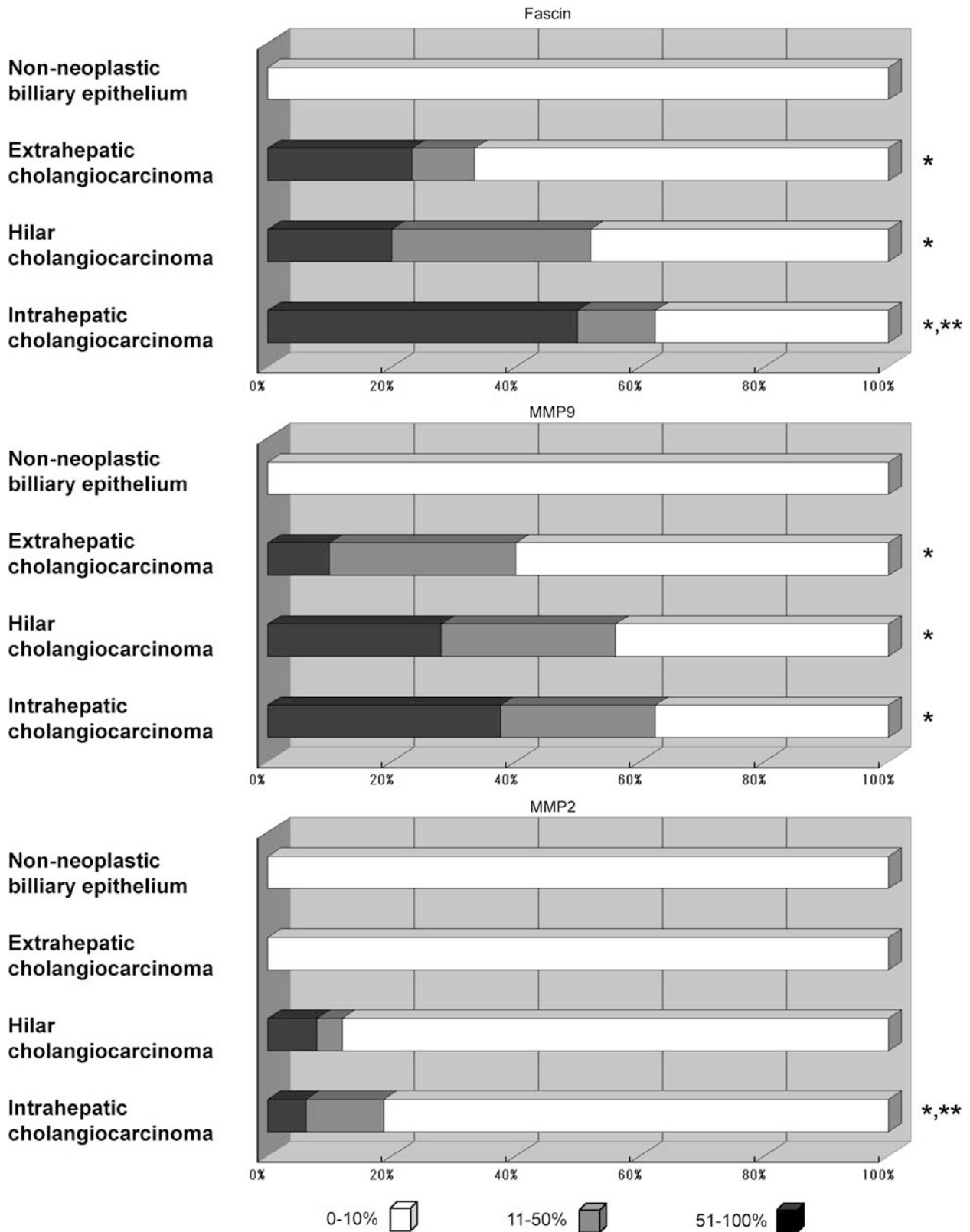
Figure 2 Frequencies and intensities of expressions of fascin, MMP9, and MMP2 in cholangiocarcinoma (CC). *, $P < 0.05$ *vs* non-neoplastic epithelium; **, $P < 0.05$ *vs* extrahepatic CC.

cases) of intrahepatic CC (Figure 2). MMP9 was expressed in the cytoplasm of CC cells as a fine granular pattern (Figure 1). Non-neoplastic biliary epithelium was negative for MMP9. Expression of MMP9 was not related to the histological grade of CC (positive case ratios: well-differentiated adenocarcinoma, 56%; moderately, 53%; and poorly, 46%). MMP9 expression in intrahepatic CC was slightly more frequent than in extrahepatic or hilar CC, although there was no significant difference.

MMP2 expression in CC was uncommon, and its expression was weak in CC cells, when compared with MMP9 (Figure 2). MMP2 expression was observed in only 8% (9/87 cases) of CC (three cases of hilar and six cases of intrahepatic CC). MMP2 expression was also observed in spindle-shaped stromal cells around CC but not in non-neoplastic biliary epithelium.

Correlation of fascin and MMP9 expressions in CC

Comparing the expression levels of fascin, MMP9, and MMP2 in each case, out of 43 cases with fascin expression, 37 cases (86%) also showed MMP9 expression. Fascin expression was significantly correlated with MMP9 expression ($r = 0.265$, $P = 0.018$). Next, the intensities of immunohistochemical staining of fascin and MMP9 were compared in the central part and invasive front in each tumor. As shown in Table 2, 70% of CC with fascin expression showed more intense fascin expression at the invasive fronts compared with the central parts of tumor. Similarly, 65% of CC showing MMP9 expression was found to have intense expression of MMP9 at the invasive fronts. Such preferential localization of fascin and MMP9 expression was correlated significantly ($P < 0.001$) (Table 3). In contrast, such a topological and positional relationship was not

Table 2 Intensities of (a) fascin and (b) MMP9 expressions in cholangiocarcinoma

(a)

	Cases with fascin expression	Location of fascin expression		
		Central > invasive front	Central = invasive front	Central < invasive front
Extrahepatic cholangiocarcinoma	10	0	3 (30%)	7 (70%)
Hilar cholangiocarcinoma	13	1 (8%)	3 (23%)	9 (69%)
Intrahepatic cholangiocarcinoma	20	0	6 (30%)	14 (70%)
Total	43	1 (2%)	12 (28%)	30 (70%)

(b)

|	Cases with MMP9 expression	Location of MMP9 expression		
		Central > invasive front	Central = invasive front	Central < invasive front
Extrahepatic cholangiocarcinoma	12	0	3 (25%)	9 (75%)
Hilar cholangiocarcinoma	14	0	5 (36%)	9 (64%)
Intrahepatic cholangiocarcinoma	20	0	8 (40%)	12 (60%)
Total	46	0	16 (35%)	30 (65%)

Table 3 Correlation of intensities of fascin and MMP9 expressions in cholangiocarcinoma based on an analysis of 37 cases with expressions of both fascin and MMP9

Predominant location		Fascin expression		
		Central > invasive front	Central = invasive front	Central < invasive front
MMP9 expression	Central > invasive front	0[a]	0	0
	Central = invasive front	1 (3%)[b]	8 (22%)	6 (16%)
	Central < invasive front	0	3 (8%)	19 (51%)

[a]Number of case(s).
[b]Percentage of case(s).
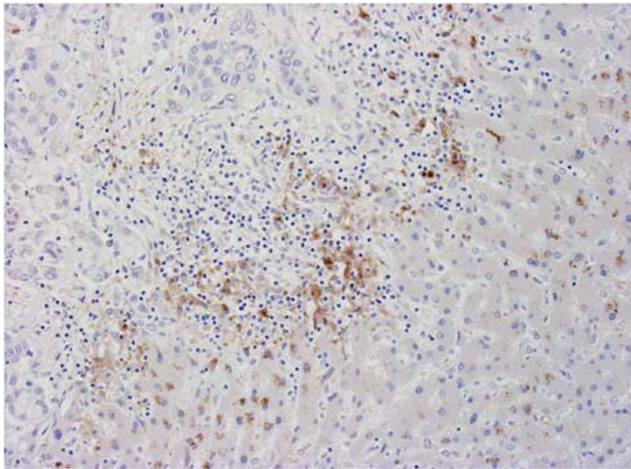
Figure 3 Immunohistochemistry of TNF-α in cholangiocarcinoma (a case of intrahepatic cholangiocarcinoma). TNF-α was expressed in macrophages at the invasive front of CC. (left upper, tumor; right lower, surrounding non-neoplastic liver tissue; original magnification ×200.)

observed between the expression patterns of fascin and MMP2.

Immunohistochemistry of TNF-α in CC

TNF-α expression was observed in 77% (23/30 cases) of extrahepatic, 64% (16/25 cases) of hilar, and 75% (24/32 cases) of intrahepatic CC (Figure 4). TNF-α was mainly expressed in Kupffer cells and macrophages within tumors or in the surrounding non-neoplastic tissues. Especially, macrophages positive for TNF-α were commonly observed at the invasive front of CC compared with the central part of CC (Figure 3). Same as our previous reports,[28] TNF-α was also focally expressed in cytoplasm of several carcinoma cells, although their expressions were less frequent and weaker compared with those in macrophages and Kupffer cells.

CC with fascin or MMP9 expression commonly showed TNF-α expression in macrophages or Kupffer cells. As shown in Table 4, 79% of CC with fascin expression also had TNF-α expression. Similarly, 83% of CC with MMP9 expression had macrophages positive for TNF-α.

Impacts of fascin expression on postoperative survival of CC patients

In a total of 87 cases of patients with CC, patients with fascin expression had poor postoperative prognosis compared with patients without fascin expression (P = 0.025, Figure 4a). In extrahepatic or hilar CC, fascin expression did not have an influence on postoperative survival (Figures 4b and c). In contrast, in intrahepatic CC, patients with fascin expression showed poor prognosis than patients without fascin expression (P = 0.007; Figure 4d).

Culture studies

The following culture studies were performed to examine the relationship between fascin and MMP9 expression patterns in CC, because histological studies showed that the expression of fascin and MMP9, but not MMP2, were significantly correlated with CC *in vivo*. The participation of TNF-α in the interaction of fascin and MMP9 was also examined.

### Expressions of fascin and MMP9 in CC cell lines

RT–PCR and western blot analysis showed that two CC cell lines expressed fascin mRNA and protein (Figures 5a and b). Fascin expression in CCKS-1 was more intense compared with HuCCT1. Similarly, MMP9 mRNA expression was easily identified in CCKS-1, whereas its expression was only slight in HuCCT1 (Figure 5a). Zymography showed MMP9 in both cell lines, whereas the active form of MMP9 was identified in only CCKS-1 (Figure 5c).

Alteration of fascin and MMP9-expression levels by TNF-α treatment

As shown in Figures 6a–c, TNF-α treatment dose-dependently upregulated fascin expression in CCKS-1 and HuCCT1. This upregulation was more evident in HuCCT1. In contrast, TNF-α treatment for 6 h induced overexpression of fascin in CCKS-1 in a dose-dependent manner, but it was not significant. This might be due to the finding that CCKS-1 had intense expression of fascin mRNA even before TNF-α treatment. In addition, TNF-α treatment significantly upregulated fascin expression time dependently in both cell lines (Figures 6d–f). According to the results of real-time PCR, fascin expression was almost peaked at 24 h after TNF-α treatment (Figure 6e). Similarly, TNF-α treatment induced overexpression of MMP9 in dose-dependent and time-dependent manners in CCKS-1 and HuCCT1 (Figure 6). The active form of MMP9 was also overproduced in both cell lines after TNF-α treatment (Figure 6f). A measure of 50 ng/ml concentration of TNF-α was enough to induce the overexpression of fascin and MMP9; therefore, this concentration of TNF-α treatment was applied in the following experiments.

Transfection of fascin siRNA

Next, we tried to determine appropriate conditions for fascin siRNA using CCKS-1, because this cell line, but not HuCCT1, had intense expression of fascin at basal culture conditions. Transfection of fascin siRNA successfully reduced fascin expression at mRNA and protein levels (Figures 7a and b). The knockdown of fascin expression at mRNA level was observed from 24 to 72 h after the transfection (Figure 7c). Similarly, the knockdown of fascin expression at protein level was identified 48 h after the transfection and continued until 120 h after transfection (Figure 7c).
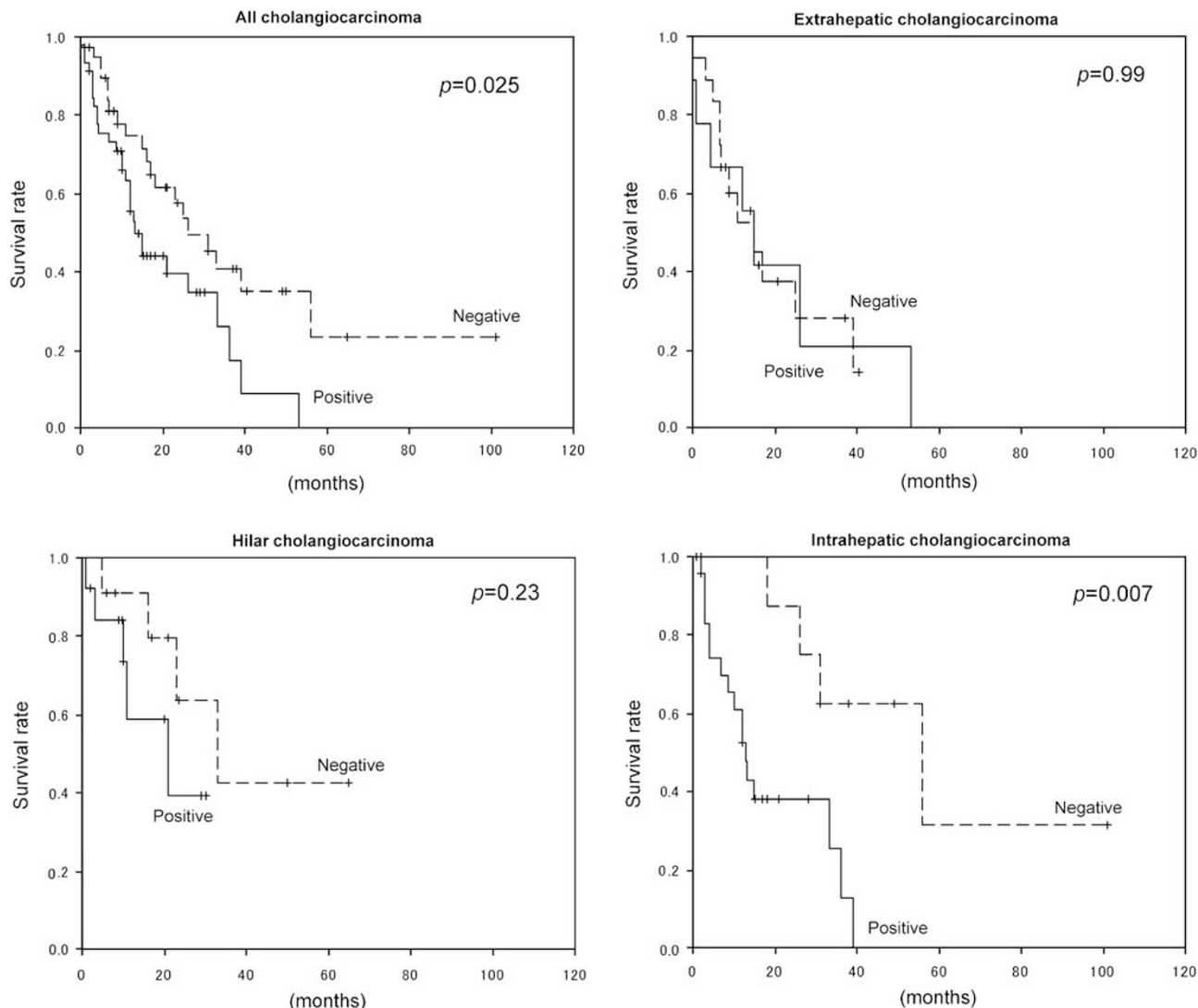
Figure 4 Postoperative survival rates of patients with extrahepatic, hilar, and intrahepatic cholangiocarcinoma (CC). When all cases were examined, the patients with fascin expression showed more unfavorable prognosis compared with those without fascin expression. However, fascin expression did not influence postoperative survival of patients with extrahepatic or hilar CC, when examined separately. In contrast, fascin expression was clearly an unfavorable prognostic factor in the patients with intrahepatic CC.

Table 4 Intensities of TNF-α, fascin, and MMP9 expressions in cholangiocarcinoma

|	Cases with TNF-α expression	Correlation of intensities of TNF, fascin, MMP9 expression	
		TNF-α/fascin positive cases	TNF-α/MMP9 positive cases
Extrahepatic cholangiocarcinoma	23/30 (77%)	9/10 (90%)	11/12 (91%)
Hilar cholangiocarcinoma	16/25 (64%)	8/13 (62%)	9/14 (64%)
Intrahepatic cholangiocarcinoma	24/32 (75%)	17/20 (85%)	18/20 (90%)
Total	63/87 (72%)	34/43 (79%)	38/46 (83%)

Alterations of fascin and MMP9-expression levels by treatment with TNF-α or fascin siRNA
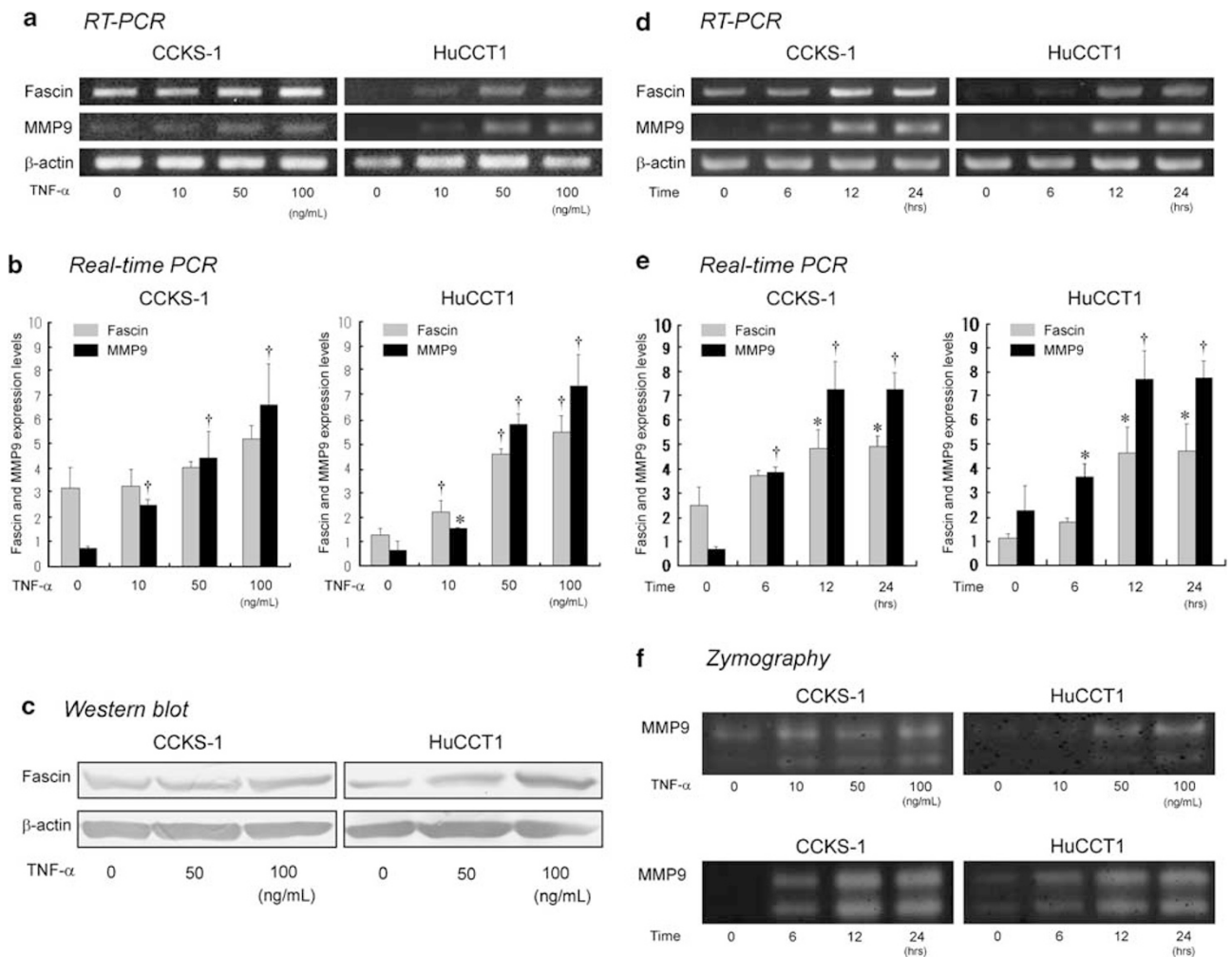
Figure 8 shows alterations of fascin and MMP9 mRNA-expression levels after treatment with TNF-α alone, with fascin siRNA alone, or with TNF-α and fascin siRNA. Without TNF-α treatment, fascin siRNA could significantly inhibit expression of fascin only in CCKS-1 but not HuCCT1. This might be due to the finding that HuCCT1 had only a slight
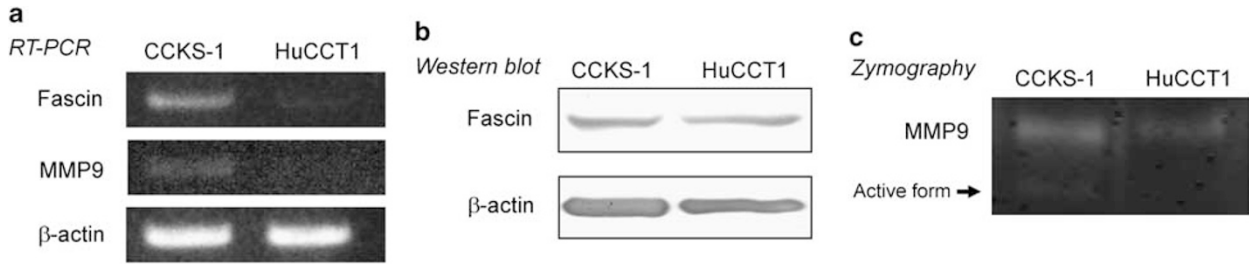
Figure 5 Expression of fascin and MMP9 in two cholangiocarcinoma (CC) cell lines (CCKS-1 and HuCCT1). (**a**) Fascin and MMP9 were expressed in both cell lines at an mRNA level (RT–PCR). CCKS-1 had more intense expression levels of fascin and MMP9 mRNAs compared with HuCCT1. (**b**) Western blotting showed fascin protein expression in both cell lines. (**c**) Zymography also showed MMP9 expression in two cell lines, although active form of MMP (arrow) was expressed in only CCKS-1.

Figure 6 Alterations of fascin and MMP9-expression levels in cholangiocarcinoma (CC) cell lines after TNF-α treatment. (**a**) TNF-α treatment (12 h) dose-dependently upregulated fascin and MMP9 expression at the mRNA level. (**b**) Real-time PCR clearly showed TNF-α-induced overexpressions of fascin and MMP were dose dependent. (**c**) Expression of fascin protein was increased by TNF-α treatment (48 h) in HuCCT1. In contrast, its expression was not significantly changed in CCKS-1, which had intense expression of fascin mRNA even before TNF-α treatment. (**d**) TNF-α (50 ng/ml) time-dependently upregulated fascin and MMP9 expression at the RNA level. (**e**) Real-time PCR also showed that TNF-α time-dependently upregulated expressions of fascin and MMP9, and they peaked around at 12 h after the treatment of TNF-α. (**f**) Zymography showed that TNF-α treatment dose-dependently and time-dependently upregulated expression of MMP9 protein including active forms. *, $P < 0.05$ *vs* without TNF-α treatment; †, $P < 0.01$ *vs* without TNF-α treatment.
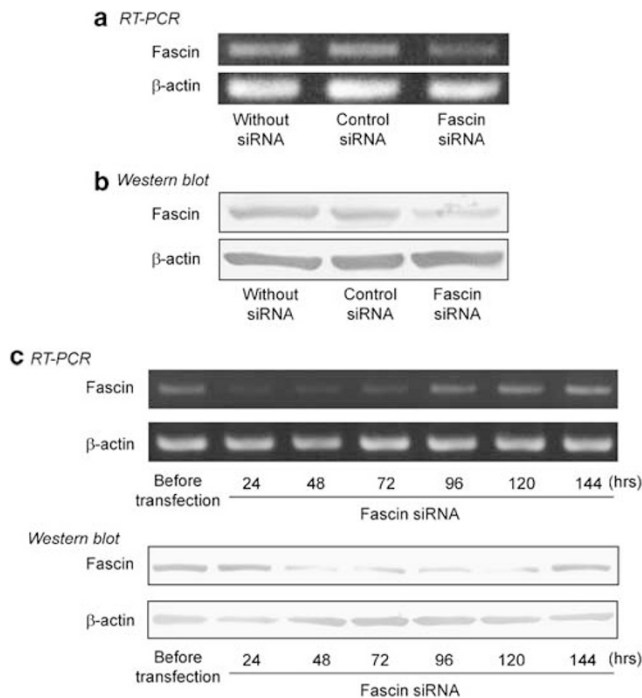
Figure 7 Expression of fascin in CCKS-1 after transfection of siRNA against fascin. (**a**, **b**) Transfection of fascin siRNA successfully induced a knockdown of fascin expression at mRNA and protein levels. (**c**) Knockdown of the fascin expression was observed from 24 to 72 h post transfection at mRNA level, and from 48 to 120 h post transfection at protein level.

expression of fascin mRNA at the normal culture condition. Fascin siRNA did not influence the expression level of MMP9 at the normal culture condition in both cell lines. TNF-α treatment (50 ng/ml) of CCKS-1 and HuCCT1 clearly induced the overexpressions of both fascin and MMP9 at mRNA and protein levels. Interestingly, knockdown of fascin expression by siRNA significantly inhibited TNF-α-induced overexpression of MMP9 mRNA in both cell lines, though TNF-α-induced overexpression of MMP9 was not completely inhibited by fascin siRNA alone in both cell lines (Figure 8). Overexpression of MMP9 protein including active forms was also inhibited by fascin siRNA.

Signaling pathway of TNF-α in CC cells
Figure 9 shows results of inhibitory studies using two neutralizing TNFR antibodies and four intracellular signaling inhibitors. Out of two TNFR antibodies, TNFR1 antibody significantly inhibited TNF-α-induced overexpressions of fascin and MMP9 in both cell lines (Figures 9a and b). In contrast, TNFR2 antibody significantly inhibited MMP9 overexpression only in CCKS-1, and its inhibition was less prominent compared with that by TNFR1 antibody. Those findings suggested that the signaling from TNF-α to the overexpression of fascin or MMP is mainly mediated by TNFR1.

Regarding the intracellular signaling, an NF-κβ inhibitor (MG132), an Erk1/2 inhibitor (U0126), and a p38 MAPK

inhibitor (SB203580) significantly inhibited TNF-α-induced overexpressions of fascin and MMP9 (Figures 9c and d). Each inhibitor alone could not completely inhibit fascin or MMP9 overexpression. In contrast, a PI3K inhibitor (LY294002) could not influence the expression levels of fascin and MMP9 during the treatment with TNF-α. Those findings suggested that NF-κβ, Erk1/2, and p38 MAPK are involved in TNF-α-induced overexpression of fascin and MMP9 in CC.

DISCUSSION
The main findings obtained in this study can be summarized as follows: (1) Fascin was expressed in 49% of CC, and its expression in intrahepatic CC was more common than that in extrahepatic and hilar CC. (2) MMP9 was expressed in 53% of CC. (3) Fascin expression was significantly correlated with MMP9 expression with respect to frequency and distribution in CC. In particular, these two molecules and TNF-α were more intensely expressed at the invasive fronts of CC. (4) Fascin expression is an unfavorable prognostic factor for the patients with intrahepatic CC. (5) TNF-α could induce overexpression of fascin and MMP9 expression in two CC cell lines. (6) Knockdown of fascin by siRNA inhibited TNF-α-induced overproduction of MMP9. (7) TNFR1, NFκB, Erk1/2, and a p38 MAPK are involved in the signal cascade of TNF-α-induced overexpression of MMP9. On the basis of these results, it was suggested that fascin is involved in the progression of CC, and MMP9 is one of the mediators of fascin-related tumor progression (Figure 10).

Invasive growth characterizes neoplasm, and it closely relates to metastatic potential and fatal outcome in patients. Cell movement and mesenchymal degradation are key mechanisms involved in tumor invasion.[34] For carcinoma cell movement, receptors on carcinoma cells sense chemokine gradients followed by actin polymerization at the leading edge of cells.[1] Next, an actin-rich cytoplasmic protrusion and cell–mesenchymal interaction enable the carcinoma cell to move. On the other hand, several proteases, such as MMPs, are produced by carcinoma cells themselves or by surrounding stromal cells. They degrade extracellular mesenchymal structures around the tumor.[35] It is well known that fascin is related to cell motility, although a possible involvement of fascin in mesenchymal degradation has not been well documented. This study showed that fascin was responsible for the overproduction of MMP9 in CC, raising a possibility that fascin relates not only to increased cell motility but also stromal degradation during the invasion of CC. That is, fascin can link two important events for carcinoma invasion, cell movement and mesenchymal degradation, in CC.

Until now, there has been only one report describing the relationship between fascin and MMP. Xie *et al* [21] examined a possible interaction of fascin and MMP-expression levels in esophageal carcinoma cells. When fascin expression in an esophageal carcinoma cell line was silenced by siRNA, the cultured cells changed morphologically with less protrusions
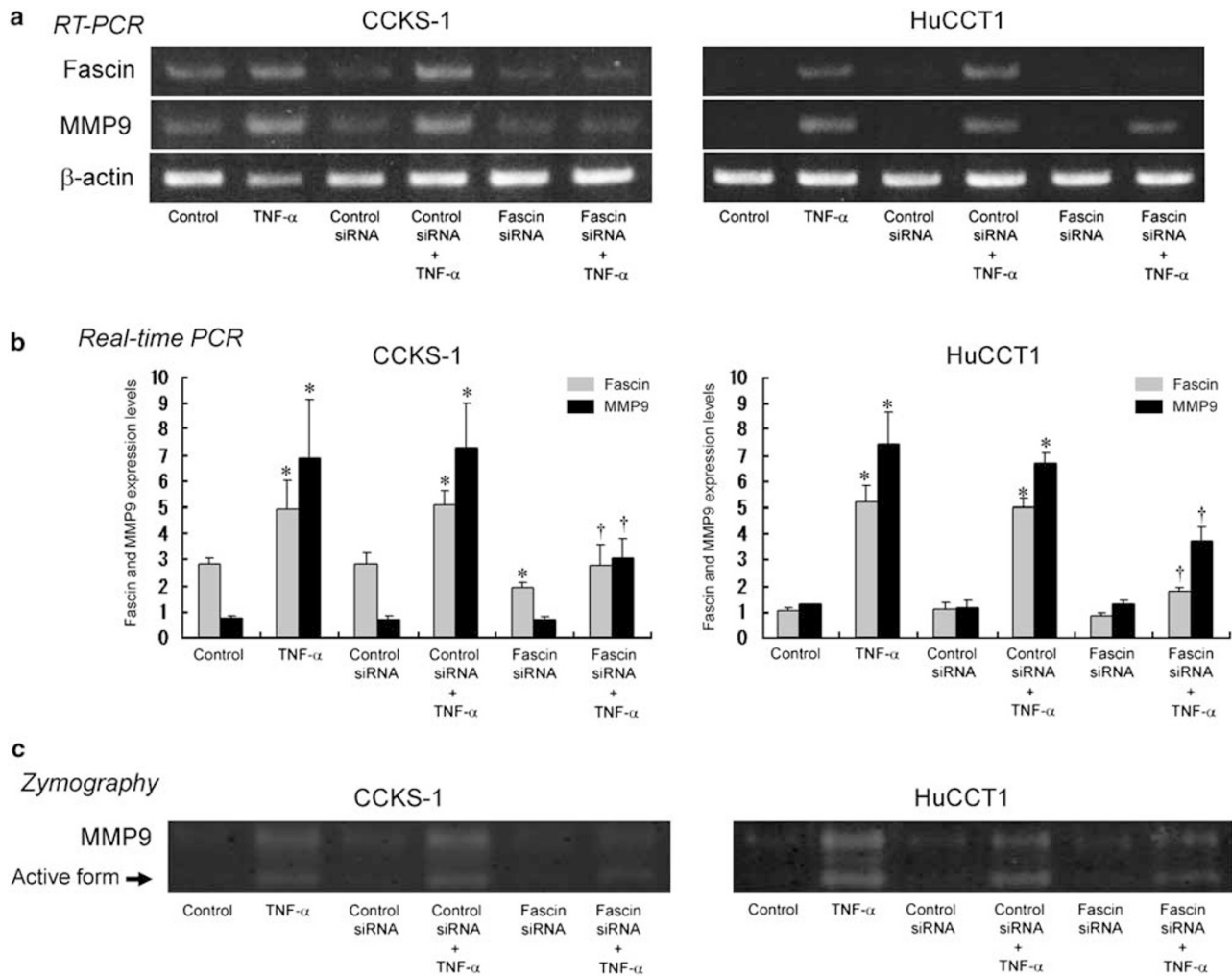
Figure 8 Alteration of fascin or MMP9 expression in cultured cholangiocarcinoma cell line after treatment with TNF-α alone, with fascin siRNA alone, or with TNF-α and fascin siRNA. At 12 h after transfection of siRNA, some groups of cells were treated with TNF-α (50 ng/ml). At 48 h after transfection, the cells were used for RNA or protein extraction. (**a**) In both CCKS-1 and HuCCT1, TNF-α treatment (50 ng/ml) induced the overexpression of both fascin and MMP9. (**b**) Real-time PCR showed that knockdown of the fascin expression by siRNA significantly inhibited TNF-α-induced overexpression of fascin and MMP9. (**c**) Zymography also showed that fascin siRNA inhibited overexpression of MMP9 protein including active forms (arrow). *, $P < 0.05$ *vs* control and control siRNA; †, $P < 0.05$ *vs* with TNF-α treatment and control siRNA with TNF-α treatment.

of the cellular membrane.[21] In addition, knockdown of fascin expression decreased the activation of MMP9 and MMP2 and finally resulted in less invasiveness. It was found in this study using two CC cell lines that fascin siRNA inhibited fascin-induced overexpression of MMP9, suggesting that fascin is one of the factors responsible for the induction of MMP9 in these CC cells. Furthermore, overexpression of MMP9 induced by TNF-α was also inhibited by fascin siRNA, implying that TNF-α-induced MMP9 overexpression is mediated by fascin. This may explain the correlated dense expression pattern of MMP9 and fascin in CC cells at the invasive fronts, as shown in this study. Interestingly, it was found in this study that macrophages positive for TNF-α were commonly observed at the invasive front of CC compared with the central part of CC. Such locally released TNF-α from macrophages around the invasive front of CC

may be responsible for such overproduction of fascin and then MMP9.

It is suggested that inflammatory cytokines have critical functions in the development and progression of CC.[25] Among many inflammatory cytokines, TNF-α has been proposed as an important, endogenous tumor promoter.[28–30] TNF-α can activate cell growth and stimulate the production of MMP9 in CC.[29,30] In addition, multiple intracellular signaling, such as NF-κβ, MAP kinases, PI3K, and focal adhesion kinase (FAK), may be involved in TNF-α-dependent MMP9 secretion in CC.[29,30,36] TNF-α is in turn controlled in the tumor environment by various factors such as other cytokines.[26,27] In addition, TNF-α can regulate several intracellular signaling and transcriptional factors. Probably, multiple signaling pathways may regulate TNF-α-dependent MMP9 production in CC, and it was found in this study that
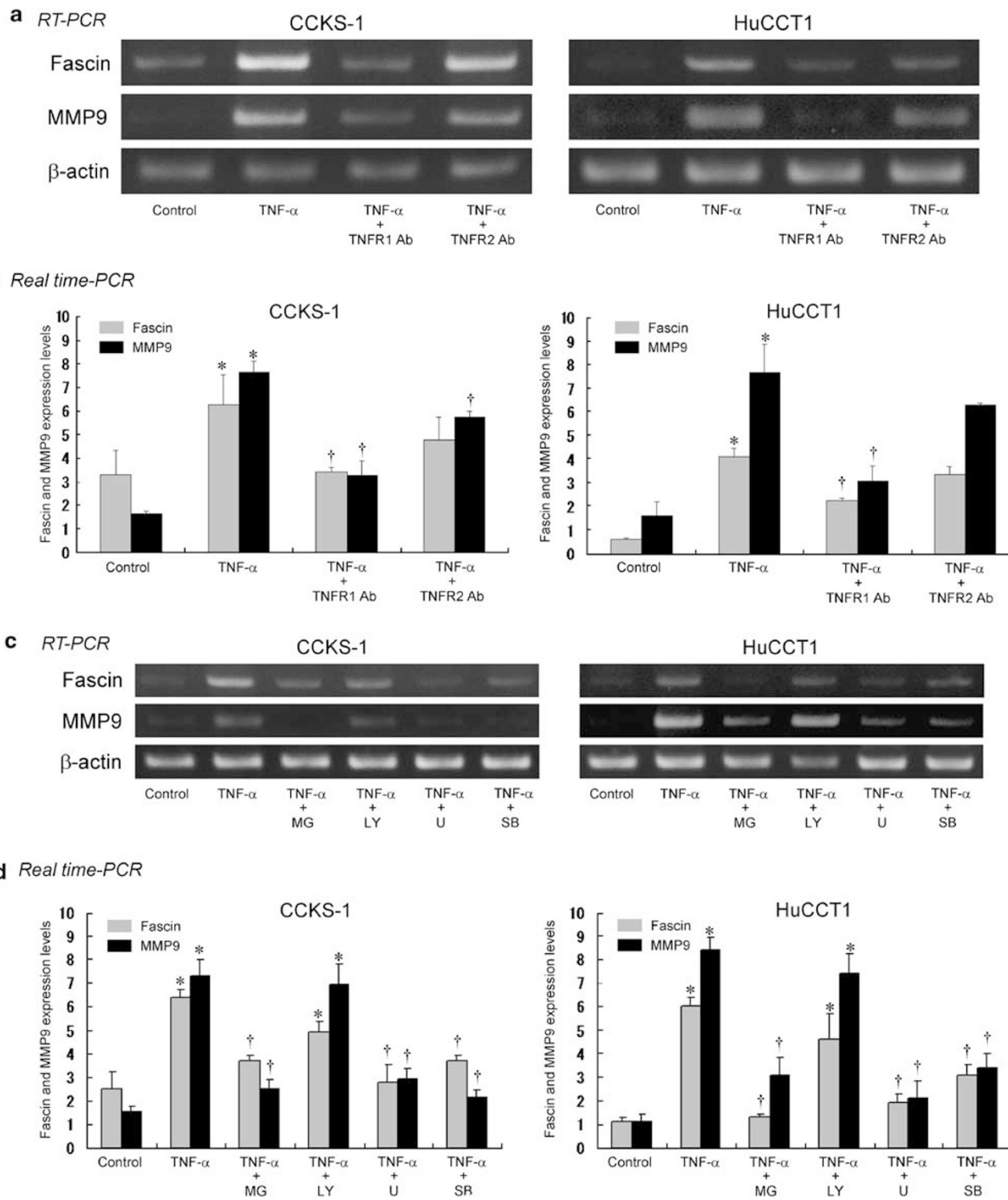
Figure 9 Inhibitory studies using TNFR antibodies and intracellular signal inhibitors. (**a**) TNFR1 antibody inhibited TNF-α-induced overexpressions of fascin and MMP9 more clearly compared with TNFR2 antibody. (**b**) Real-time PCR showed that TNFR1 antibody significantly inhibited overexpression of fascin and MMP9 in both CCKS-1 and HuCCT1. In contrast, TNFR2 antibody inhibited overexpression of MMP9 only in CCKS-1. (**c**) MG132 (an NF-κB inhibitor), U0126 (an Erk1/2 inhibitor), and SB203580 (a p38 MAPK inhibitor) inhibited TNF-α-induced overexpressions of fascin and MMP9. (**d**) Those inhibitions were significant based on findings of real-time PCR. In contrast, LY294002 (a PI3K inhibitor) did not influence TNF-α-induced overexpressions of fascin and MMP9 (MG, MG132; LY, LY294002; U, U0126; SB, SB203580). *, $P < 0.05$ *vs* control; †, $P < 0.05$ *vs* with TNF-α treatment.
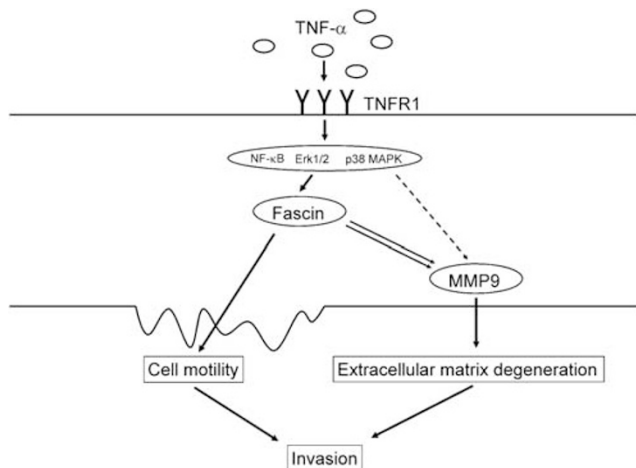
Figure 10 Schematic diagrams of the possible involvement of fascin in the activation process of cholangiocarcinoma invasion.

fascin is one of the mediators of this mechanism. Especially, the signaling cascade involving TNFR1, Erk1/2, p38 MAPK, and NFκB is important for fascin-mediated overexpression of MMP9 in CC.

Detailed mechanism controlling fascin expression is still ambiguous. There has been only one earlier report with regard to the relationship between fascin and TNF-α. TNF-α upregulates the expression of fascin in the mature dendritic cells.[37] In addition, fascin expression is controlled by β-catenin-TCF signaling in colon cancer.[38] In breast cancer, c-erbB-2 overexpression leads to transcriptional activation of the fascin gene.[39] This study suggested possible involvement of TNF-α in overexpression of fascin in CC. However, other cell signaling not related to TNF-α might be also involved in fascin expression in CC.

Interestingly, the frequencies of fascin expression were different between intrahepatic and extrahepatic/hilar CCs. It is noteworthy that the patients with intrahepatic CC showing fascin expressin had a poor postoperative prognosis in comparison with those without fascin exprssion, but such impact was not evident in those with extrahepatic/hilar CC, reflecting a different progression process between intrahepatic and extrheaptic/hilar CC. Furthermore, fascin and MMP9 expression were more extensive in the former and fascin expression was correlated with MMP-9 expression with respect to frequency and distribution in CC, supporting the view that fascin and MMP9 in CC are involved in the progression of intrahepatic CC and ultimately relates to the postoperative prognosis of the patients. Recently, it was suggested that carcinogenetic processes were different among CC arising from different-sized bile ducts,[40,41] and this may be causally related to such difference. Recently, there have been several reports that fascin is a useful biomarker of malignant tumors such as ovarian carcinoma and esophageal carcinoma.[42,43] This study showed that fascin could be added as a new biomarker of intrahepatic CC reflecting a poor

postoperative prognosis, and more detailed and comprehensive studies on the correlation between clinicopathological parameters and fascin expression in intrahepatic CC are warranted and mandatory.

In conclusion, the overexpression of fascin and MMP9 in CC cells may have an important function in the progression and invasion of CC, and fascin might be involved in the signaling pathway in TNF-α-dependent overproduction of MMP9 in CC. Analysis of the mechanisms for fascin-related MMP9 overproduction and interruption of this mechanism may lead to the development of novel therapeutic strategic approaches targeting fascin in CC, particularly intrahepatic CC. [17]

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