Ann Surg Oncol (2019) 26:907–917
https://doi.org/10.1245/s10434-018-07132-7

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Roles of Pin1 as a Key Molecule for EMT Induction by Activation of STAT3 and NF-κB in Human Gallbladder Cancer

Shinichiro Nakada, MD, Satoshi Kuboki, MD, Hiroyuki Nojima, MD, Hideyuki Yoshitomi, MD, Katsunori Furukawa, MD, Tsukasa Takayashiki, MD, Shigetsugu Takano, MD, Masaru Miyazaki, MD, and Masayuki Ohtsuka, MD

Department of General Surgery, Graduate School of Medicine, Chiba University, Chiba, Japan

ABSTRACT

Background. Despite developments in multidisciplinary treatment, the prognosis for advanced gallbladder cancer (GBC) still is poor because of its rapid progression. Epithelial–mesenchymal transition (EMT) plays a central role in promoting tumor invasion and metastasis in malignancies thorough signal transducer and activator of transcription-3 (STAT3) and nuclear factor κB (NF-κB) activation. Whereas Pin1 mediates STAT3 and NF-κB activation, the involvement of Pin1 in GBC progression is unclear.

Methods. Factors regulating Pin1-related STAT3 and NF-κB activation were evaluated using surgical specimens collected from 76 GBC patients, GBC cells, and orthotopic GBC xenograft mice.

Results. In the patients with GBC, high Pin1 expression in GBC was associated with aggressive tumor invasion and increased tumor metastasis, and was an independent factor for a poor prognosis. Pin1 expression was correlated with phosphorylation of STAT3(Ser727) and NF-κB-p65(Ser276), thereby activating STAT3 and NF-κB in GBC. Pin1-mediated STAT3 and NF-κB activation induced EMT in GBC. When Pin1 knockdown was performed in GBC cells, the phosphorylation of STAT3(Ser727) and NF-κB-p65(Ser276) was inhibited, and STAT3 and NF-κB activation was suppressed. Inactivation of STAT3 and NF-κB in Pin1-depleted cells decreased snail and zeb-2 expression, thereby reducing the rate of mesenchymal-like cells, suggesting that EMT was inhibited in GBC cells. PiB, a Pin1-specific inhibitor, inhibited EMT and reduced tumor cell invasion by inactivating STAT3 and NF-κB in vitro. Moreover, PiB treatment inhibited lymph node metastasis and intrahepatic metastasis in orthotopic GBC xenograft tumor in vivo.

Conclusions. Pin1 accelerates GBC invasion and metastasis by activating STAT3 and NF-κB. Therefore, Pin1 inhibition by PiB is an excellent therapy for GBC by safely inhibiting its metastasis.

Gallbladder cancer (GBC) is the most common malignancy among the bile duct cancers and the frequent cause of cancer-related death worldwide.[1,2] Despite recent improvement of the multidisciplinary therapy,[3,4] the prognosis for advanced GBC remains poor. Lymph node (LN) metastasis and intrahepatic metastasis are potent factors for a poor prognosis for GBC patients,[5] and the recurrence rate is extremely high even after curative resection.[1] Therefore, it is urgent to discover new therapeutic targets for preventing GBC metastasis.

Epithelial–mesenchymal transition (EMT) plays a central role in promoting tumor invasion and metastasis in many cancers.[6,7] Signal transducer and activator of transcription-3 (STAT3) and nuclear factor κB (NF-κB) are key enhancers to activate EMT-related transcriptional factors such as snail and zeb.[8,9] Therefore, STAT3 and NF-κB are important therapeutic targets for inhibiting EMT-mediated cancer progression.[10] However, therapies targeting STAT3 and NF-κB are not common because direct inhibition of these transcriptional factors induces severe adverse reactions.

Electronic supplementary material The online version of this article (https://doi.org/10.1245/s10434-018-07132-7) contains supplementary material, which is available to authorized users.

First Received: 25 August 2018;
Published Online: 4 January 2019

S. Kuboki, MD
e-mail: satoshi.kuboki@faculty.chiba-u.jp

Pin 1 is a peptidyl-prolyl cis/trans isomerase that binds and isomerizes specific phosphorylated serine or threonine residues in certain proteins.[11,12] Pin1 has a pivotal role in multiple oncogenic signal pathways because Pin1 regulates protein stability, enzymic activity, and protein localization.[13] Both STAT3 and NF-κB are target proteins of Pin1. Pin1 recognizes and binds to phosphorylated (p-)STAT3(Tyr705), and NF-κB-p65(Thr254) induces phosphorylation of NF-κB-p65(Ser276) and STAT3(Ser727), and accelerates STAT3 and NF-κB activation.[14–16] We have reported that Pin1 is associated with aggressive tumor progression and a poor prognosis in hepatocellular carcinoma (HCC) by mediating NF-κB activation.[17] However, no studies have evaluated Pin1 expression in any biliary tract cancers. Moreover, no investigations have focused on STAT3- or NF-κB-mediated tumor invasion and metastasis in any experimental models of GBC.

Therefore, this study aimed to investigate the roles of Pin1 in GBC progression, to evaluate the usefulness of Pin1 as a biomarker for predicting GBC behavior, and to show whether Pin1 is a novel therapeutic target in patients with GBC.

MATERIALS AND METHODS

Tissue Samples

This study investigated 76 patients with advanced GBC (≥ T2) who underwent R0 or R1 resection between 2003 and 2014 at our institution. In the current study, 3 of the 76 patients received right trisectionectomy, 21 received right hemihepatectomy, 47 received central inferior segmentectomy, and 5 received liver bed resection. All 76 patients received extrahepatic bile duct resection and regional lymph node dissection because we routinely perform these procedures for ≥ T2 GBC.

The 7th Union for International Cancer Control (UICC) classification was used to evaluate the clinicopathologic features of GBC. Some of the patients received neoadjuvant chemotherapy with gemcitabine and cisplatin and/or adjuvant chemotherapy with gemcitabine. Surgical samples were fixed in formalin, embedded in paraffin, and stained with hematoxylin–eosin (H&E). Normal gallbladder tissues were obtained from the patients with cholechystolithiasis.

Fully informed consent was obtained from all the patients. The study was performed in accordance with the guidelines of the Helsinki Declaration and approved by the Chiba University Human Research Committee.

Immunohistochemical Staining

Immunohistochemical staining was performed with anti-Pin1, anti-p-NF-κB-p65(Ser276), anti-p-STAT3(Ser727), anti-vimentin, anti-matrix metalloproteinase-9 (MMP-9) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-cytokeratin 19 (CK-19) (Abcam, Cambridge, UK) using the EnVision kit (Dako, Copenhagen, Denmark). The Pin1 percentage score was graded from 0 to 5 based on the percentage of Pin1-positive cells in GBC using ImageJ (NIH, Bethesda, MD, USA).

Staining intensity in GBC was scored by two independent clinical pathologists, and the average score was recognized as the Pin1 intensity score (range, 0–5). The total of the intensity score and the percentage score was defined as the Pin1 labeling index (range, 0–10).

Determination of the p-STAT3(Ser727)-labeling index and the p-NF-κB-p65(Ser276)-labeling index was evaluated by immunohistochemistry based on the percentage of positive nuclei in GBC. Overexpression of vimentin and MMP-9 was defined as 30% of cancer cells or more expressed vimentin or MMP-9 compared with cancer stroma.

Western Blotting

Western blotting was performed using whole-cell lysates, as previously described.[17] PhosphoBLOCKER (Cell Biolabs Inc., San Diego, CA, USA) was used to block nonspecific binding sites. Antibodies against Pin1, p-NF-κB-p65(Thr254), p-NF-κB-p65(Ser276), p-STAT3(Tyr705), p-STAT3(Ser727), vimentin, β-actin (Santa Cruz Biotechnology), zeb-2, and snail (Abcam, Cambridge, UK) were used for primary antibody. Immunoreactive proteins were detected by enhanced chemiluminescence and quantified by image analysis.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared by the method of Deryckere and Gannon,[18] and their DNA binding activity was analyzed by electrophoretic mobility shift assay (EMSA). Double-stranded consensus oligonucleotides to STAT3 (Santa Cruz Biotechnology) or NF-κB (Promega Corporation, Madison, WI, USA) were end-labeled with γ[^{32}P]ATP (Perkin Elmer Inc. Boston, MA, USA). Binding reaction products were separated in a polyacrylamide gel and analyzed by autoradiography.

Cell Culture

Human GBC cell lines (OCUG-1, NOZ-1033, TYGBK1, and TYGBK8 cells) purchased from the Japanese Collection of Research Bioresources (JCRB) Cell

Bank (Osaka, Japan) were distributed onto a 6-cm dish at a concentration of 5×10^4 cells/5 ml. Pin1-specific small interfering RNA (siRNA) or negative control siRNA was transfected into GBC cells using RNAiMAX Transfection Reagent (Fisher Scientific, Hampton, NH, USA). In some experiments, cells were incubated for 72 h in medium containing 20 μmol/l of PiB (Sigma-Aldrich, St. Louis, MO, USA) or 50 μmol/l of juglone (Sigma-Aldrich), competitive Pin1-specific inhibitors. Tumor cell invasion was evaluated using the Cell Invasion Assay Kit (Cell Biolabs Inc.). Cell lysates and nuclear extracts were prepared for Western blotting and EMSA.

Three-Dimensional Cell Culture

A three-layer gel complex was prepared for three-dimensional (3D) cell culture as previously described.[19] Briefly, collagen 1 solution was spread onto 4-well-chamber slides as a bottom layer and set in a 37°C oven for 60 min. Next, cells suspended in collagen 1 solution were added on the bottom layer and placed in a 37°C oven for 60 min to allow gelation of the middle layer. After culture medium was added onto this gel-cell complex in each well, the cells were further incubated for 7 days to allow cells to grow up in all directions. The shape of these cells was evaluated after 7 days of incubation. Spindle-shape cells and irregular type cells were defined as mesenchymal-like cells, and spheroid cyst cells were defined as epithelial-like cells.

Orthotopic GBC Xenograft Mouse Model

An orthotopic GBC xenograft mouse model was established based on the protocol previously reported.[20] Male CB-17 SCID mice (Charles River Laboratories Japan, Yokohama, Japan) weighing 20 to 25 g were used in these experiments. After laparotomy with the mice under general anesthesia, 1×10^7 cells/15 μl of NOZ-1033 cells mixed with 15 μl of Matriegel (BD Bioscience, Heidelberg, Germany) were injected into the gallbladder. The mice were treated intraperitoneally with 0 or 2.5 mg/kg of PiB solution every other day, starting after 7 days of inoculation.

The mice were killed 28 days after inoculation, and samples of GBC xenograft tumor, liver, and regional LNs were taken for analysis. Regional LN metastasis and intrahepatic metastasis were evaluated by H&E staining and immunohistochemical staining for CK-19. This project was approved by the Chiba University Animal Care and Use Committee and was in compliance with the guidelines published by NIH.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical comparisons for significance were performed with Student's *t* test, the Mann–Whitney *U* test, or the chi-square test. The Kaplan–Meier method was used to estimate survival, and statistical differences were analyzed by the log-rank test. Significant prognostic factors evaluated by univariate logistic regression were included in a multivariable analysis. Probability (*P*) values of 0.05 or lower were considered to be statistically significant. Statistical analyses were performed using the software JMP Pro 13 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Clinical Significance of Pin1 Expression in GBC Patients

When Pin1 expression was evaluated by immunohistochemistry (Fig. 1a), it was weak in normal gallbladder. However, increased Pin1 expression was seen in some GBC cases. Based on receiver operating characteristic (ROC) analysis in accordance with the 2-year survival (Fig. S1), the 76 GBC patients in this study were divided into two groups (cutoff value, 5; $P < 0.001$; area under the curve [AUC], 0.751). High Pin1 expression was significantly correlated with venous invasion ($P = 0.016$), perineural invasion ($P = 0.013$), LN metastasis ($P = 0.033$), distant metastases ($P = 0.030$), and intrahepatic metastasis ($P = 0.013$) (Table 1). Overall survival time and relapse-free survival time were significantly poorer for the GBC patients with high Pin1 expression (Fig. 1b, c). The uni- and multivariate analyses showed that high Pin1 expression in GBC was the independent poor prognostic factor ($P = 0.025$) (Table S1).

Pin1-Mediated STAT3 and NF-κB Activation-Enhanced EMT in GBC

Nuclear accumulation of p-STAT3(Ser727) and p-NF-κB-p65(Ser276), indicators of Pin1-related STAT3 and NF-κB activation (Fig. 1d, e), was significantly correlated with Pin1 expression in GBC (Fig. 1f, g). High vimentin expression was seen in 26 GBC cases (34.2%) by immunohistochemistry (Fig. 2a). Interestingly, the Pin1-labeling index, the p-STAT3(Ser727)-labeling index, and the p-NF-κB-p65(Ser276)-labeling index were higher in GBC with high vimentin expression (Fig. 2b–d). Accordingly, 22 GBC cases (28.9%) were defined as having high MMP-9 expression by immunohistochemistry (Fig. 2e), which was positively correlated with the Pin1-labeling

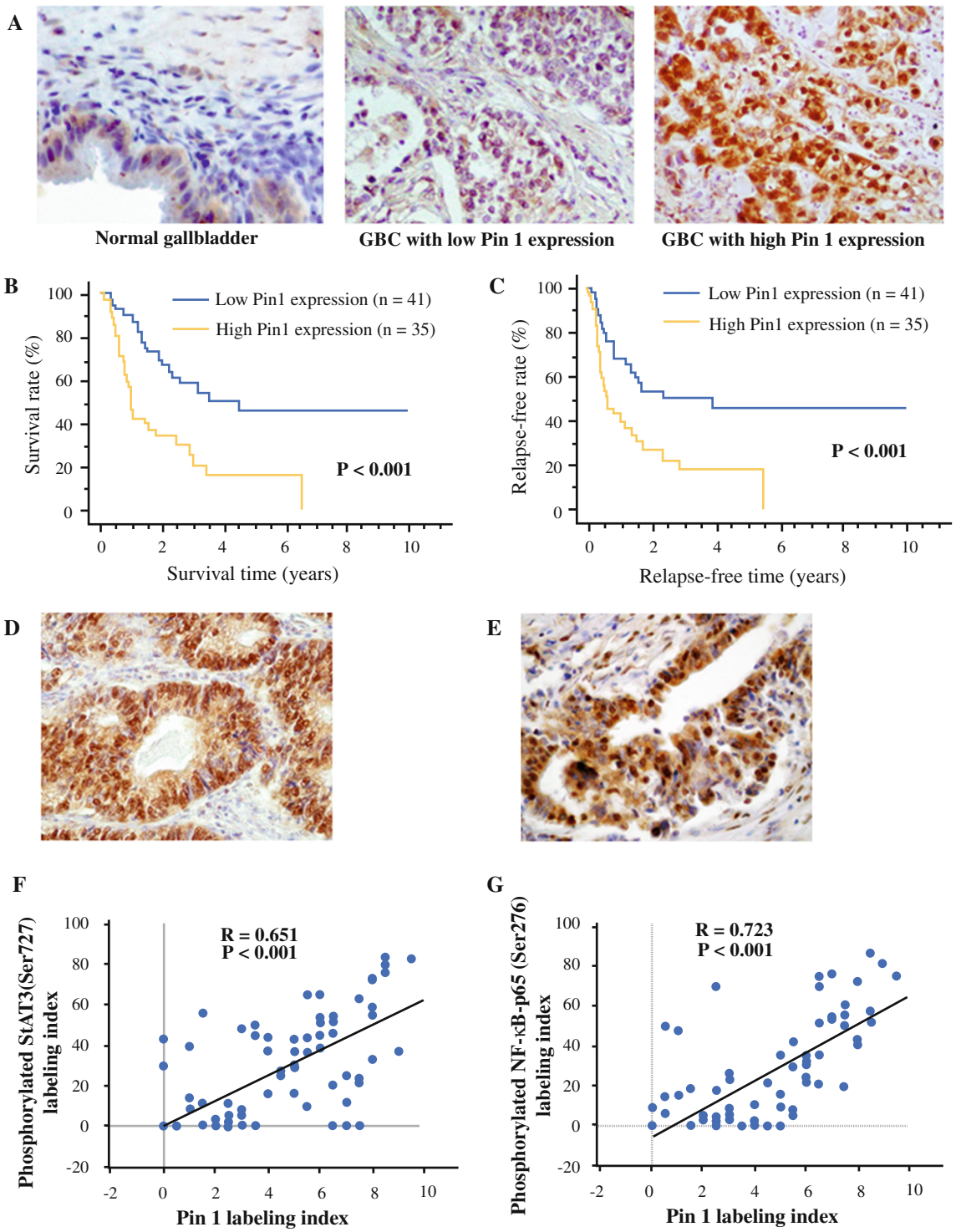


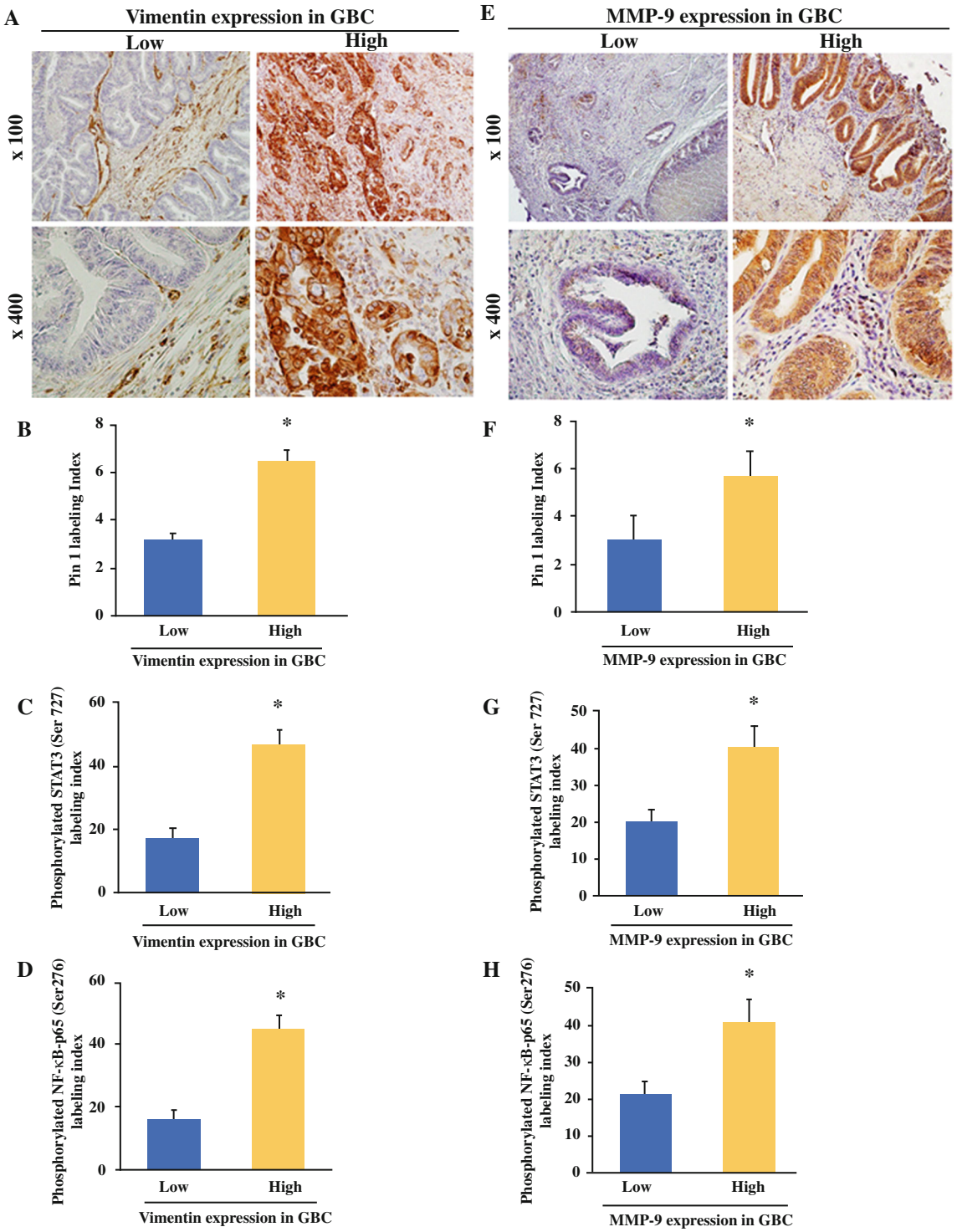
FIG. 1 **a** Immunohistochemical staining for Pin1 in normal gallbladder and in gallbladder cancer (GBC) with low and high Pin1 expression. The original magnification was × 100. **b** Overall survival ($P < 0.001$) and **c** relapse-free survival ($P < 0.001$) for 76 GBC patients in relation to Pin1 expression in GBC. Nuclear accumulation of **d** p-STAT3(Ser727) and **e** p-NF-κB-p65(Ser276) was evaluated by immunohistochemical staining. The original magnification was × 100. A significant correlation was seen between the Pin1-labeling index and **f** the p-STAT3(Ser727)-labeling index ($P < 0.001$) or **g** the p-NF-κB-p65(Ser276)-labeling index ($P < 0.001$). p-STAT3, phosphorylated signal transducer and activator of transcription-3; p-NF-κB, phosphorylated nuclear factor κB

TABLE 1 Relationship between Pin1 expression and clinicopathologic characteristics in gallbladder cancer (GBC)

	Pin1 expression in GBC		*P* value
	High	Low	
Patients (*n*)	35	41	
Age (mean years ± SEM)	66.8 ± 2.2	67.0 ± 1.3	0.823
Gender			
Male	14	16	0.931
Female	21	25	
Preoperative CEA levels (mean ng/ml ± SEM)	39.7 ± 22.2	20.6 ± 13.2	0.461
Preoperative CA19-9 levels (mean U/ml ± SEM)	389.5 ± 172.2	404.8 ± 218.4	0.904
ICG-15R (mean % ± SEM)	7.9 ± 0.7	7.9 ± 0.6	0.991
Neoadjuvant chemotherapy			
Done	2	5	0.330
None	33	36	
Adjuvant chemotherapy			
Done	20	21	0.605
None	15	20	
Operative procedure			
≥ Hemihepatectomy	10	14	0.602
Others	25	27	
Differentiation			
Well or moderately	25	35	0.137
Poorly	10	6	
Lymphatic invasion			
Positive	31	32	0.224
Negative	4	9	
Venous invasion			
Positive	28	22	0.016
Negative	7	19	
Perineural invasion			
Positive	26	19	0.013
Negative	9	22	
Pathologic T factor			
pT2	9	17	0.149
pT3 or pT4	26	24	
Lymph node metastasis			
Positive	27	22	0.033
Negative	8	19	
Distant metastasis			
Positive	11	5	0.030
Negative	24	36	
Intrahepatic metastasis			
Positive	7	1	0.013
Negative	28	40	
Curability			
R0	31	37	0.813
R1	4	4	

The 7th Union for International Cancer Control (UICC) classification was used for pathologic evaluation

SEM standard error of the mean, *CEA* carcinoembryonic antigen, *ICG* indocyanine green


A
Vimentin expression in GBC
Low
High
x 100
x 400
E
MMP-9 expression in GBC
Low
High
x 100
x 400
B
Pin 1 labeling Index
0
2
4
6
8
*
Low
High
Vimentin expression in GBC
F
Pin 1 labeling Index
0
2
4
6
8
*
Low
High
MMP-9 expression in GBC
C
Phosphorylated STAT3 (Ser 727) labeling index
0
20
40
60
*
Low
High
Vimentin expression in GBC
G
Phosphorylated STAT3 (Ser 727) labeling index
0
10
20
30
40
50
*
Low
High
MMP-9 expression in GBC
D
Phosphorylated NF-κB-p65 (Ser276) labeling index
0
20
40
60
*
Low
High
Vimentin expression in GBC
H
Phosphorylated NF-κB-p65 (Ser276) labeling index
0
10
20
30
40
50
*
Low
High
MMP-9 expression in GBC

◀**FIG. 2** **a** Immunohistochemical staining for vimentin in gallbladder cancer (GBC) with low and high vimentin expression. The original magnifications were × 100 and × 400. **b** The Pin1-labeling index, **c** the p-STAT3(Ser727)-labeling index, and **d** the p-NF-κB-p65(Ser276)-labeling index were significantly higher in GBC with high vimentin expression ($n = 50$) than in GBC with low vimentin expression ($n = 26$). The data are expressed as mean ± SEM. $^*P < 0.001$ compared with the low-vimentin-expression group. **e** Immunohistochemical staining for matrix metalloproteinase-9 (MMP-9) in GBC with low and high MMP-9 expression. The original magnifications were × 100 and × 400. **f** The Pin1-labeling index, **g** the p-STAT3(Ser727)-labeling index, and **h** the p-NF-κB-p65(Ser276)-labeling index were significantly higher in GBC with high MMP-9 expression ($n = 22$) than in GBC with in low MMP-9 expression ($n = 54$). The data are expressed as mean ± SEM. $^*P < 0.01$ compared with the low MMP-9 expression group. p-STAT3, phosphorylated signal transducer and activator of transcription-3; NF-κB, phosphorylated nuclear factor κB; SEM, standard error of the mean

index, the p-STAT3(Ser727)-labeling index, and the p-NF-κB-p65(Ser276)-labeling index in GBC (Fig. 2f–h).

Pin1 Expression in GBC Cells in Vitro

When Pin1 expression in GBC cells was evaluated by Western blotting, OCUG-1 and NOZ-1033 cells showed marked Pin1 expression, whereas Pin1 expression was low in TYGBK1 and TYGBK8 cells (Fig. 3a). Vimentin expression was high and E-cadherin expression was low in GBC cells with high Pin1 expression. In contrast, vimentin expression was rarely seen in GBC cells with low Pin1 expression, which expressed E-cadherin.

Effects of Pin1 Knockdown in GBC Cells in Vitro

When Pin1 knockdown was performed in OCUG-1 cells, expression of p-STAT3(Tyr705), p-STAT3(Ser727), p-NF-κB-p65(Thr254), and p-NF-κB-p65(Ser276) was significantly inhibited (Fig. 3b), thereby suppressing STAT3 and NF-κB activation (Fig. 3b–d). As the result, snail, zeb-2, and vimentin expression was decreased in Pin1-depleted OCUG-1 cells (Fig. 3e).

When cell morphology was evaluated by 3D cell culture, the rate of mesenchymal-like cells was significantly reduced after Pin1 knockdown (Fig. 3f). Accordingly, tumor cell invasion was inhibited in Pin1-depleted OCUG-1 cells (Fig. 3g). The effects of Pin1 knockdown were confirmed in NOZ-1033 cells, another GBC cell line with high Pin1 expression. Consistent with the findings in OCUG-1 cells, activation of STAT3 and NF-κB was significantly inhibited in Pin1-depleted NOZ-1033 cells (Fig. S2A and S2B), which reduced tumor cell invasion (Fig. S2C).

Stimulation with Pin1-Specific Inhibitors on GBC Cells in Vitro

When OCUG-1 cells were treated with a Pin1-specific inhibitor, STAT3 and NF-κB activation was significantly inhibited after treatment with 20 μmol/l of PiB (Fig. 4a). In addition, the rate of mesenchymal-like cells evaluated by 3D cell culture was reduced in OCUG-1 cells after PiB treatment (Fig. 4b). Consistent with these results, PiB treatment inhibited the invasiveness of OCUG-1 cells (Fig. 4c). The therapeutic effects of PiB on GBC cells were confirmed using NOZ-1033 cells.

Similar to its effects in OCUG-1 cells, treatment with 20 μmol/l of PiB significantly suppressed STAT3 and NF-κB activation in NOZ-1033 cells (Fig. S3A and S3B). Accordingly, tumor cell invasion was inhibited in PiB-treated NOZ-1033 cells (Fig. S3C).

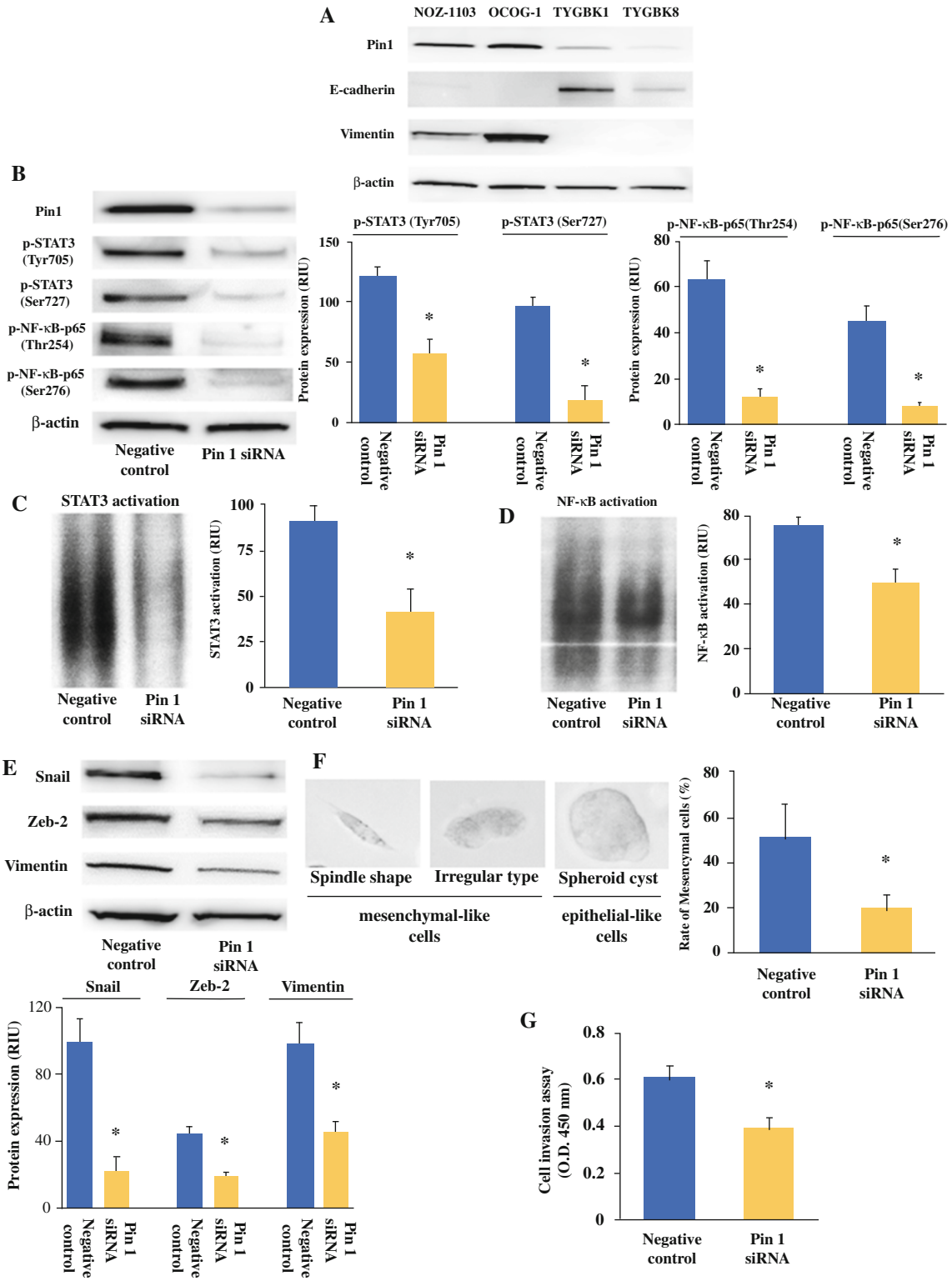
To confirm that these effects were Pin1-specific, we further stimulated OCUG-1 cells with juglone, another famous Pin1-specific inhibitor, and performed functional assays. Similar to the results for Pin1 siRNA and PiB, Pin1 inhibition by juglone significantly suppressed STAT3 and NF-κB activity (Fig. S4A and S4B), thereby inhibiting tumor cell invasion in OCUG-1 cells in vitro (Fig. S4C).

Treatment Efficiency of PiB for GBC Metastasis on Orthotopic GBC Xenograft Mice

Efficiency of 2.5 mg/kg PiB treatment of GBC progression in vivo was evaluated using orthotopic GBC xenograft mice. Advanced GBC (≥ T2) was found in 7 of 10 mice 28 days after inoculation in mock-treated mice (Fig. 4d). The tumor formation rate was similar between the mock-treated mice and the PiB-treated mice, as 6 of the 10 PiB-treated mice showed advanced GBC 28 days after inoculation. In some of the mice, regional LN metastasis and intrahepatic metastasis were found when the mice were killed (Fig. 4e). The PiB treatment significantly decreased the incidence of LN metastasis and intrahepatic metastasis (Fig. 4f). In addition, the p-STAT3(Ser727)-labeling index and the p-NF-κB-p65(Ser276)-labeling index assessed by immunohistochemistry were reduced after PiB treatment in orthotopic GBC xenograft tumors (Fig. 4g, h).

DISCUSSION

The poor prognosis of GBC is related to its characteristics such as strong invasiveness and frequent distant metastasis mediated by EMT. Both STAT3 and NF-κB are potent transcriptional factors for inducing EMT. However, their precise mechanisms for promoting GBC progression have not been understood.


A
NOZ-1103
OCOG-1
TYGBK1
TYGBK8
Pin1
E-cadherin
Vimentin
β-actin
B
Pin1
p-STAT3 (Tyr705)
p-STAT3 (Ser727)
p-NF-κB-p65 (Thr254)
p-NF-κB-p65 (Ser276)
β-actin
Negative control
Pin 1 siRNA
p-STAT3 (Tyr705)
p-STAT3 (Ser727)
p-NF-κB-p65(Thr254)
p-NF-κB-p65(Ser276)
Protein expression (RIU)
150
100
50
0
80
60
40
20
0
*
Negative control
Pin 1 siRNA
C
STAT3 activation
STAT3 activation (RIU)
100
75
50
25
0
D
NF-κB activation
NF-κB activation (RIU)
80
60
40
20
0
E
Snail
Zeb-2
Vimentin
β-actin
Negative control
Pin 1 siRNA
Snail
Zeb-2
Vimentin
Protein expression (RIU)
120
80
40
0
F
Spindle shape
Irregular type
Spheroid cyst
mesenchymal-like cells
epithelial-like cells
Rate of Mesencymal cells (%)
80
60
40
20
0
G
Cell invasion assay (O.D. 450 nm)
0.8
0.6
0.4
0.2
0

◀**FIG. 3** **a** Pin1, E-cadherin, and vimentin expression evaluated by Western blotting in several gallbladder cancer (GBC) cells in vitro. The effects of Pin1 knockdown on **b** phosphorylation of STAT3 and NF-κB-p65 were assessed by Western blotting, and **c** STAT3 or **d** NF-κB activation was assessed by EMSA in OCUG-1 cells in vitro. The data are expressed as mean ± SEM with $n = 3$ for negative control and $n = 4$ for Pin1 siRNA. $^*P < 0.05$ compared with negative control subjects. The effects of Pin1 knockdown on **e** snail, zeb-2, and vimentin expression were assessed by Western blotting and **f** cell morphology by 3D cell culture, and **g** and cell invasiveness was measured by cell invasion assay in OCUG-1 cells in vitro. The data are expressed as mean ± SEM with $n = 3$ for negative control and $n = 4$ for Pin1 siRNA. $^*P < 0.05$ compared with negative control subjects. STAT3, signal transducer and activator of transcription-3; NF-κB, nuclear factor κB; EMSA, electrophoretic mobility shift assay; SEM, standard error of the mean

To show the upstream pathway for STAT3 and NF-κB activation in GBC, we focused on Pin1. Pin1 overexpression is reported to be associated with increased NF-κB activation, which enhances malignant behavior in HCC, breast cancer, and glioblastoma.[16,17,21] However, these studies focused only on Pin1-mediated NF-κB activation in cancers. Moreover, no studies have evaluated the involvement of Pin1 in STAT3 activation in any malignancies.

The current study showed that Pin1-mediated STAT3 and NF-κB activation in GBC induces EMT and enhances tumor invasion in GBC by increasing snail and zeb-2. Our results suggest that phosphorylation of STAT3(Ser727) and NF-κB(Ser276) was induced by Pin1, which promoted nuclear translocation of STAT3 and NF-κB and enhanced their DNA binding, resulting in their activation. In addition, constitutive phosphorylation of STAT3(Tyr705) and NF-κB(Thr254), Pin1-specific binding sites, is necessary for Pin1-mediated activation of these transcriptional factors.

We further clarified that Pin1 expression in GBC is an excellent biomarker for predicting the malignant behavior in GBC patients because Pin1 overexpression in GBC was correlated with aggressive tumor invasiveness, enhanced tumor metastasis, and poor prognosis.

Based on our findings, personalized therapies targeting Pin1 are potentially effective for inhibiting tumor progression by controlling EMT in GBC patients with high Pin1 expression.

Currently, two major Pin1 inhibitors, PiB and juglone, are available. Juglone is the only natural Pin1 inhibitor known to have some anticancer effects.[22] In contrast, PiB is a synthesized Pin1-specific inhibitor, which selectively binds to the PPIase domains of Pin1 and inhibits the PPIase activities.[23] Therefore, in the current study, PiB was mainly used as a Pin1 inhibitor to evaluate Pin1-specific effects. Although PiB is known to inhibit tumor cell growth in several cancer cells in vitro,[17,24] it is unclear whether PiB shows inhibitory effects on tumor invasion or metastasis. In the current study, PiB inhibited EMT and tumor invasion through inactivation of STAT3 and NF-κB in GBC cells in vitro. Moreover, we treated GBC cells with juglone and confirmed that these effects of PiB on GBC cells are Pin1-specific because juglone also reduced tumor invasion by inhibiting STAT3 and NF-κB activation in OCUG-1 cells in vitro.

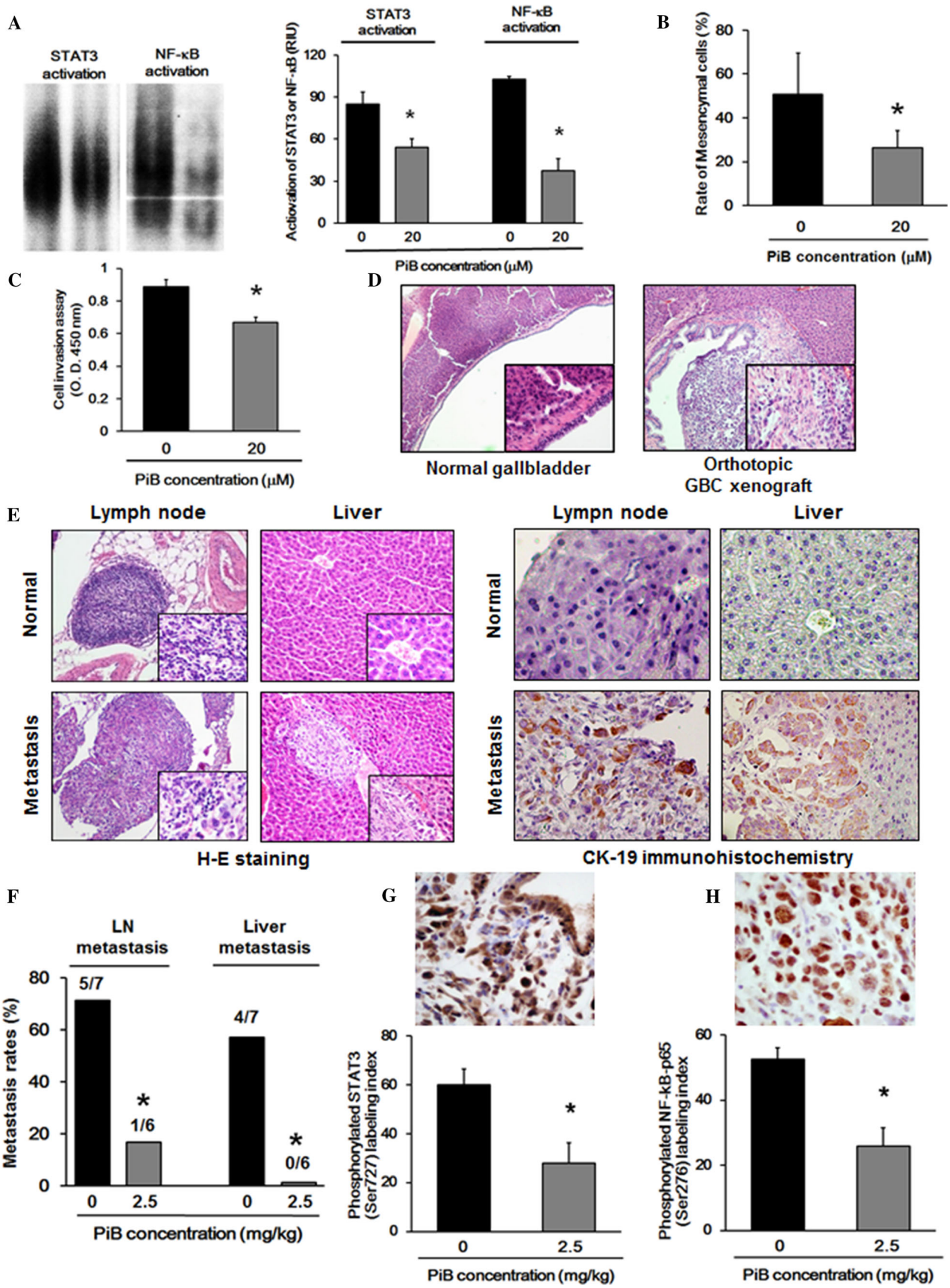
However, the adverse reactions of PiB are unknown because no previous investigators have treated any animals with PiB. In the current study, an orthotopic GBC xenograft mice model was used to evaluate the efficiency and safety of PiB treatment in vivo. Most previously established GBC xenograft mice models were subcutaneously transplanted heterotopic GBC xenograft mice models, which was inadequate for evaluating GBC-specific tumor progression such as regional LN metastasis or intrahepatic metastasis.[25]

Only a few studies have evaluated the invasion and metastasis of GBC using an orthotopic GBC xenograft mice model. Reported findings show that the tumor formation rate is 86–100%,[20,26,27] The LN metastasis rate is 69%,[27] and the intrahepatic metastasis rate is 50%[20] after 28 days of inoculation, similar to our results with mock-treated mice. Treatment with 2.5 mg/kg of PiB significantly inhibits Pin1-mediated STAT3 and NF-κB activation in orthotopic GBC xenograft tumor, thereby reducing the incidences of LN metastasis and intrahepatic metastasis without any adverse reactions.

Previous reports have suggested that deletion of STAT3 or NF-κB-p65 is lethal in mice. However, Pin1-knockout mice maintain minimal NF-κB activation and survive without any severe disadvantages.[28–30] Therefore, Pin1 inhibition by PiB is a safe and appropriate approach for inhibiting STAT3 and NF-κB activation in GBC. These in vivo data are consistent with the clinical data showing that LN metastasis and intrahepatic metastasis are increased in GBC patients with high Pin1 expression. Both LN metastasis and intrahepatic metastasis are potent risk factors for a poor prognosis for GBC patients. Therefore, controlling these factors by PiB is potentially an excellent therapy for GBC patients with high Pin1 expression.

CONCLUSIONS

Pin1 accelerates GBC progression by inducing tumor invasion and metastasis, and is associated with a poor prognosis for GBC patients after operation. Moreover, Pin1 inhibition by PiB is an excellent therapy for GBC patients by safely inhibiting its tumor progression through STAT3 and NF-κB inactivation.


A
STAT3 activation
NF-κB activation
STAT3 activation
NF-κB activation
Activation of STAT3 or NF-κB (RU)
120
90
60
30
0
*
*
0
20
0
20
PiB concentration (μM)
B
Rate of Mesencymal cells (%)
80
60
40
20
0
*
0
20
PiB concentration (μM)
C
Cell invasion assay (O. D. 450 nm)
1
0.8
0.6
0.4
0.2
0
*
0
20
PiB concentration (μM)
D
Normal gallbladder
Orthotopic GBC xenograft
E
Lymph node
Liver
Lympn node
Liver
Normal
Metastasis
Normal
Metastasis
H-E staining
CK-19 immunohistochemistry
F
LN metastasis
Liver metastasis
Metastasis rates (%)
80
60
40
20
0
5/7
*
1/6
4/7
*
0/6
0
2.5
0
2.5
PiB concentration (mg/kg)
G
Phosphorylated STAT3 (Ser727) labeling index
80
60
40
20
0
*
0
2.5
PiB concentration (mg/kg)
H
Phosphorylated NF-kB-p65 (Ser276) labeling index
60
40
20
0
*
0
2.5
PiB concentration (mg/kg)

◀**FIG. 4** Effects of 20 μmol/l PiB treatment on **a** STAT3 and NF-κB activation assessed by EMSA. **b** Cell morphology was evaluated by 3D cell culture, and **c** cell invasiveness was measured by cell invasion assay in OCUG-1 cells in vitro. The data are expressed as mean ± SEM with four per group. $*P < 0.05$ compared with 0 μmol PiB. **d** Orthotopic gallbladder cancer (GBC) xenograft tumor 28 days after inoculation. The original magnification was × 100. **e** Lymph node metastasis and liver metastasis 28 days after inoculation in orthotopic GBC xenograft mice evaluated by H&E staining and cytokeratin 19 (CK-19) immunohistochemistry. The original magnification was × 100. **f** The efficiency of 2.5 mg/kg PiB treatment of LN metastasis and liver metastasis in orthotopic GBC xenograft mice. The data are expressed as mean ± SEM with six to seven per group. $*P < 0.05$ compared with 0 mg/kg PiB. Effects of PiB treatment on **g** STAT3 and **h** NF-κB activation in orthotopic GBC xenograft tumor. The data are expressed as mean ± SEM with six or seven per group. $*P < 0.05$ compared with 0 mg/kg PiB. STAT3, signal transducer and activator of transcription-3; NF-κB, nuclear factor κB; EMSA, electrophoretic mobility shift assay; SEM, standard error of the mean

ACKNOWLEDGMENT This work was supported by JSPS KAKENHI (Grant No. 26462036) to Satoshi Kuboki.

DISCLOSURE There are no conflicts of interest.

REFERENCES

1. Ishihara S, Horiguchi A, Miyakawa S, Endo I, Miyazaki M, Takada T. Biliary tract cancer registry in Japan from 2008 to 2013. *J Hepatobiliary Pancreat Sci.* 2016;23:149–57.
2. Higuchi R, Ota T, Araida T, et al. Surgical approaches to advanced gallbladder cancer: a 40-year single-institution study of prognostic factors and resectability. *Ann Surg Oncol.* 2014;21:4308–16.
3. Valle J, Wasan H, Palmer DH, et al. Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. *N Engl J Med.* 2010;362:1273–81.
4. Kato A, Shimizu H, Ohtsuka M, et al. Downsizing chemotherapy for initially unresectable locally advanced biliary tract cancer patients treated with gemcitabine plus cisplatin combination therapy followed by radical surgery. *Ann Surg Oncol.* 2015;22:S1093–9.
5. Dutta U. Gallbladder cancer: can newer insights improve the outcome? *J Gastroenterol Hepatol.* 2012;27:642–53.
6. Thiery JP. Epithelial–mesenchymal transitions in tumour progression. *Nat Rev Cancer.* 2002;2:442–54.
7. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer.* 2003;3:362–74.
8. Min C, Eddy SF, Dherr DH, Sonenshein GS. NF-kappaB and epithelial to mesenchymal transition of cancer. *J Cell Biochem.* 2008;104:733–44.
9. Wendt MK, Balanis N, Carlin CR, Schiemann WP. STAT3 and epithelial–mesenchymal transitions in carcinomas. *JAKSTAT.* 2014;3:e28975.
10. Yu H, Jove R. The STATs of cancer: new molecular targets come of age. *Nat Rev Cancer.* 2004;4:97–105.
11. Yaffe MB, Schutkowski M, Shen M, et al. Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. *Science.* 1997;278:1957–60.
12. Lu KP, Liou YC, Zhou XZ. Pinning down proline-directed phosphorylation signaling. *Trends Cell Biol.* 2002;12:164–72.
13. Lu KP, Zhou XZ. The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease. *Nat Rev Mol Cell Biol.* 2007;8:904–16.
14. Lufei C, Koh TH, Uchida T, Cao X. Pin1 is required for the Ser727 phosphorylation-dependent Stat3 activity. *Oncogene.* 2007;26:7656–64.
15. Wakahara R, Kunimoto H, Tanino K, Kojima H, Shintaku H, Nakajima K. Phospho-Ser727 of STAT3 regulates STAT3 activity by enhancing dephosphorylation of phospho-Tyr705 largely through TC45. *Genes Cells.* 2012;17:132–45.
16. Ryo A, Suizu F, Yoshida Y, et al. Regulation of NF-kappaB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. *Mol Cell.* 2003;12:1413–26.
17. Shinoda K, Kuboki S, Shimizu H, et al. Pin1 facilitates NF-κB activation and promotes tumour progression in human hepatocellular carcinoma. *Br J Cancer.* 2015;113:1323–31.
18. Deryckere F, Gannon F. A one-hour minipreparation technique for extraction of DNA-binding proteins from animal tissues. *Biotechniques.* 1994;16:405.
19. Reichert M, Takano S, Heeg S, Bakir B, Botta GP, Rustgi AK. Isolation, culture and genetic manipulation of mouse pancreatic ductal cells. *Nat Protoc.* 2013;8:1354–65.
20. Egberts JH, Schniewind B, Schafmayer C, et al. Establishment of a novel orthotopic xenograft model of human gallbladder carcinoma. *Clin Exp Metastasis.* 2007;24:141–8.
21. Atkinson GP, Nozell SE, Harrison DK, Stonecypher MS, Chen D, Benveniste EN. The prolyl isomerase Pin1 regulates the NF-kappaB signaling pathway and interleukin-8 expression in glioblastoma. *Oncogene.* 2009;28:3735–45.
22. Henning L, Christner C, Kipping M, et al. Selective inactivation of parvulin-like peptidyl-prolyl cis/trans isomerases by juglone. *Biochemistry.* 1998;37:5953–60.
23. Uchida T, Takamiya M, Takahashi M, et al. Pin1 and Par14 peptidyl prolyl isomerase inhibitors block cell proliferation. *Chem Biol.* 2003;10:15–24.
24. Mantovani F, Tocco F, Girardini J, et al. The prolyl isomerase Pin1 orchestrates p53 acetylation and dissociation from the apoptosis inhibitor iASPP. *Nat Struct Mol Biol.* 2007;14:912–20.
25. Lin W, Jiang L, Chen Y, et al. Vascular endotherial growth factor-D promotes growth, lymphangiogenesis, and lymphatic metastasis in gallbladder cancer. *Cancer Lett.* 2012;314:127–36.
26. Mita Y, Ajiki T, Kamigaki T, et al. Antitumor effect of gemcitabine on orthotopically inoculated human gallbladder cancer cells in nude mice. *Ann Surg Oncol.* 2012;14:1374–80.
27. Du Q, Jiang L, Wang XQ, Pan W, She FF, Chen YL. Establishment of and comparison between orthotopic xenograft and subcutaneous xenograft models of gallbladder carcinoma. *Asian Pac J Cancer Prev.* 2014;15:3747–52.
28. Kuboki S, Sakai N, Clarke C, Schuster R, Blanchard J, Edwards MN, Lentsch AB. The peptidyl-prolyl isomerase, Pin1, facilitates NF-kappaB binding in hepatocytes and protects against hepatic ischemia/reperfusion injury. *J Hepatol.* 2009;51:296–306.
29. Lee TH, Tun-Kyi A, Shi R, et al. Essential role of Pin1 in the regulation of TRF1 stability and telomere maintenance. *Nat Cell Biol.* 2009;11:97–105.
30. Liou YC, Ryo A, Huang HK, et al. Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes. *Proc Natl Acad Sci USA.* 2002;99:1335–40.