

# Gadd45 $\beta$ silencing impaired viability and metastatic phenotypes in cholangiocarcinoma cells by modulating the EMT pathway

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**Abstract.** Growth arrest and DNA damage-inducible- $\beta$  (Gadd45 $\beta$ ) is a stress-response protein involved in a number of processes, including cell cycle control, DNA repair, survival and death control, and stress signaling, depending on its interactions. Gadd45 $\beta$  expression is dysregulated in numerous types of cancer, functioning as either a tumor promoter or a tumor suppressor. However, the functions of Gadd45 $\beta$  in cholangiocarcinoma (CCA), particularly in metastasis, has not been studied. The immunohistochemical analysis of Gadd45 $\beta$  expression revealed that 75% of histological specimens from patients with CCA expressed high levels of Gadd45 $\beta$ , and that high Gadd45 $\beta$  expression was associated with metastasis. The role of Gadd45 $\beta$  in CCA was examined using siRNA-mediated gene knockdown in HuCCA-1, a human CCA cell line established from a Thai patient. The effects of Gadd45 $\beta$  downregulation upon cell viability and death, invasion, migration, matrix metalloproteinase (MMP) activity and epithelial-mesenchymal transition (EMT) marker expression were investigated. Gadd45 $\beta$  knockdown impaired cell viability, which was associated with the induction of apoptosis. In addition, there was a marked reduction in invasion and migration, although MMP activity was unaffected. Impairment of these metastatic properties was accompanied by the decreased expression of EMT markers, including Slug, vimentin, claudin-1 and zona occludens protein 1, whereas E-cadherin expression was increased. The present study

suggests that Gadd45 $\beta$  is involved in regulating the viability and the metastatic potential of CCA cells, which may be mediated by the modulation of the EMT pathway.

## Introduction

Cholangiocarcinoma (CCA) is a cancer of the bile duct caused by the malignant transformation of cholangiocytes, the epithelial cells lining the bile duct. CCA is the second most common type of primary hepatic malignancy (1,2). Due to late diagnosis, resistance to chemo- and radiotherapy, and the high rates of local invasion and distant metastasis, the prognosis for CCA is very poor. The median survival time of patients with CCA is <2 years (3), and the 5-year survival rate of patients receiving curative resection is 0-40% (4). The prevalence of CCA is the highest in Asia, particularly in the northeastern region of Thailand (5). *Opisthorchis viverrini* infection is hypothesized to be a causal factor of CCA in Thailand, as it is markedly associated with the incidence of CCA (6).

In total, >90% of cancer-associated mortality is due to the local or distant metastasis of cancer cells (7). The epithelial-mesenchymal transition (EMT) is an important process in cancer metastasis, characterized by alterations in the gene expression and morphology of cells, which leads to a reduction of intercellular adhesion, and an increase in cell motility (8-11). This process is associated with a reduction of E-cadherin expression (12,13) and an increase in the expression of vimentin, an intermediate filament protein, leading to increased cell motility and promoting tumor metastasis (14,15).

Growth arrest and DNA damage-inducible- $\beta$  (Gadd45 $\beta$ ) is a stress-response protein; its expression is induced by physiological or environmental stress. The aberrant expression of Gadd45 $\beta$  in various types of cancer has implicated its involvement in tumorigenesis (16). Gadd45 $\beta$  belongs to the Gadd45 protein family (Gadd45 $\alpha$ , Gadd45 $\beta$ , Gadd45 $\gamma$ ) (17). Gadd45 $\beta$  may form a homodimer or heterodimer with the other Gadd45 proteins, or interact with a variety of other proteins, including proliferating cell nuclear antigen, cyclin-dependent kinase 1, p21, mitogen-activated protein kinase kinase 4, mitogen-activated protein kinase kinase 7 and p38 mitogen activated protein kinase (MAPK). The function of Gadd45 $\beta$  differs depending on the interacting molecules, including cell

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*Abbreviations:* CCA, cholangiocarcinoma; ZO-1, zona occludens protein 1; MMP, matrix metalloproteinase; EMT, epithelial-mesenchymal transition; siRNA, small interfering RNA

*Key words:* Gadd45 $\beta$ , cholangiocarcinoma, apoptosis, epithelial-mesenchymal transition, metastasis

cycle control, DNA repair, survival and death control, and stress signaling (18-22).

Cancer cells must survive and propagate in a strenuous environment of hypoxia, nutrient competition and oxidative stress (23). It is essential for cells to acquire the ability to thrive in these stressful conditions. The function of Gadd45 $\beta$  as a stress-response protein in cancer is paradoxical; the downregulation of Gadd45 $\beta$  via promoter methylation in hepatocellular carcinoma suggests that Gadd45 $\beta$  may act as a tumor suppressor (24), whereas the upregulation of Gadd45 $\beta$  in colorectal cancer was associated with recurrence and mortality of patients with colorectal cancer (25), suggesting a tumor-promoting role. When the Gadd45 $\beta$  gene from normal adjacent tissue was over-expressed in colorectal cancer cell lines, apoptotic cell death was induced (25). In addition, Gadd45 $\beta$  was identified as upregulated in the metastasis of uveal melanoma to the liver (26), and the silencing of Gadd45 $\beta$  in human embryonic carcinoma cells decreased viability and invasiveness (27), suggesting that Gadd45 $\beta$  may contribute to the malignant phenotypes of cancer. However, the function of Gadd45 $\beta$  in metastasis and EMT is not yet fully characterized.

In the present study, it was identified that patients with CCA exhibit increased Gadd45 $\beta$  expression in tumor tissue, and that a high level of Gadd45 $\beta$  expression was associated with metastasis. Gadd45 $\beta$  expression in a CCA cell line, HuCCA-1, was suppressed using siRNA-mediated gene silencing, and the effects on cell viability, survival and death signaling pathways, migration, invasiveness, and the EMT pathway were studied. The data of the present study thus indicated that Gadd45 $\beta$  expression promoted the viability, migration and invasion of the HuCCA-1 cells, traits required for successful metastasis.

## Materials and methods

*Ethical approval.* All procedures performed in the present study involving human participants were performed in accordance with the 1964 Declaration of Helsinki and its later amendments, or comparable ethical standards. The study was conducted with the approval of the Ethical Committee of Rajavithi Hospital (Bangkok, Thailand).

*Immunohistochemical (IHC) staining.* Paraffinized tissue samples from 28 patients with CCA who had undergone surgical treatment at Rajavithi Hospital between January 2010 and October 2010 were selected for retrospective analysis. Standard IHC technique was used for the detection of Gadd45 $\beta$  in paraffinized sections on glass slides. Polyclonal Gadd45 $\beta$  antibody (HPA029816-100UL; 1:500 dilution; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was hybridized with the sections overnight at 4°C, followed by incubation with biotinylated goat anti-rabbit IgG at RT for 1 h (E0432; dilution 1:500; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). Subsequently avidin-biotin-peroxidase conjugate was added (ABC Elite; Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature and the immunohistochemical reaction was developed with freshly prepared reagents from a Histofine SAB-PO kit (Nichirei, Inc., Tokyo, Japan). The samples were visualized at x400 magnification using an Olympus BX53 microscope (Olympus Corporation, Tokyo, Japan) and categorized into four grades according to the

intensity and percentage of positively stained cells: Negative or <5%, grade 0; weak or 5-25%, grade 1; moderate or 25-50%, grade 2; and strong or >50%, grade 3. IHC grade 0 (3 cases) and grade 1 (4 cases), which exhibited weak and/or <25% staining, were grouped as low expression, whereas grade 2 (5 cases) and grade 3 (16 cases), which exhibited moderate to strong and/or >25% staining, were grouped as high expression of Gadd45 $\beta$  for the purpose of statistical analysis.

*Cell line and culture condition.* The human CCA cell line, HuCCA-1 (28), developed from a Thai patient with CCA, was used for the study. The cells were provided by Professor Satitaya Sirisinha (Mahidol University, Bangkok, Thailand). These cells were grown in HAM's F-12 medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in 5% CO<sub>2</sub> humidified atmosphere.

*Small interfering (si)RNA-mediated Gadd45 $\beta$  gene silencing.* Commercial Gadd45 $\beta$  siRNA [GADD45 $\beta$  siRNA(h): sc-37416], a pool of 3 different siRNA duplexes to the Gadd45 $\beta$  gene (NCBI RefSeq. 12759252), was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Silencer<sup>®</sup> Negative control siRNA no. 1 (cat. no. 4404021; Ambion; Thermo Fisher Scientific, Inc.) was used as negative control siRNA. The cells were washed with PBS and detached using 0.25% Trypsin/EDTA (Gibco; Thermo Fisher Scientific, Inc.). Cells were seeded at 2x10<sup>5</sup> cells/well into a 6 well plate for 24 h, then transfected with 40 nM siRNA using Lipofectamine<sup>®</sup> RNAiMax reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The medium containing transfection reagents was changed for 10% FBS supplemented HAM's-F12 at 24 h after transfection.

*Western blot analysis.* At 48 h post-transfection, total cellular protein was extracted and prepared following a protocol described previously (29). Each protein sample of 40  $\mu$ g was separated via SDS-PAGE (12% gel; 120 V for 2 h) followed by an electroblotting transfer of the protein to a nitrocellulose membrane at 30 V at 4°C for 15 h. The membranes were blocked in 1.5% skimmed milk at room temperature for 1 h, the membranes were incubated with the primary antibodies at 4°C overnight on a rocking platform. The next day, the membranes were washed with TBS with Tween-20 prior to incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Following 3 washes with TBST for 10 min, ECL Plus Western Blotting Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to visualize the immunoreactive bands in G:Box ChemiXL 1.4 (Syngene; Synoptics, Cambridge, UK).

Rabbit polyclonal Gadd45 $\beta$  antibodies were purchased from Abcam (Cambridge, UK) and goat polyclonal GAPDH antibodies from Santa Cruz Biotechnology, Inc. The rest of the antibodies used in the study were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA); the details regarding all antibodies used in western blotting are included in Table I. Densitometry analysis was performed using ImageJ software (version 1.49v; National Institutes of Health, Bethesda, MD, USA) (30). The density of the protein of interest relative to GAPDH was determined in HuCCA-1 cells

Table I. The primary antibodies used for western blotting in the present study.

Antibody	Cat. no.	Clone no.	Type of antibody	Organism	Company	Dilution
Gadd45 $\beta$	ab105060		Polyclonal	Rabbit	Abcam	1:2,000
GAPDH	sc-48166	I-19	Polyclonal	Goat	Santa Cruz Biotechnology, Inc.	1:2,000
Cleaved PARP (Asp214)	#5625	D64E10	Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:1,000
Total PARP	#9542		Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:1,000
Cleaved caspase-3 (Asp175)	#9664	5A1E	Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:1,000
Full-length caspase 3	#9662		Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:1,000
Phospho-p38 MAPK (Thr180/Tyr182)	#9211		Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:1,000
p38 MAPK	#9212		Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:1,000
Phospho-Akt (Ser473)	#4058	193H12	Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:1,000
Akt	#9272		Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:1,000
Phospho-ERK1/2 (Thr202/Tyr204)	#9101		Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:1,000
ERK1/2	#9102		Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:1,000
Vimentin	#5741	D21H3	Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:1,000
Claudin-1	#13255	D5H1D	Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:1,000
$\beta$ -catenin	#8480	D10A8	Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:1,000
ZO-1	#8193	D7D12	Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:1,000
Slug	#9585	C19G7	Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:1,000
E-cadherin	#3195	24E10	Monoclonal	Rabbit	Cell Signaling Technology, Inc.	1:1,000

Gadd45 $\beta$ , growth arrest and DNA damage-inducible- $\beta$ ; PARP, Poly (ADP-ribose) polymerase; p38 MAPK, p38 mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; ZO-1, zona occludens protein 1.

transfected with Gadd45 $\beta$  or negative control siRNA. The activation of Akt, p38 MAPK and extracellular signal-regulated kinase (ERK)1/2 signaling pathways was examined using phospho-specific antibodies at 48 h after siRNA transfection. The densitometry measurements for total Akt, ERK1/2 and p38 MAPK were used for the normalization of protein loading. The phospho-protein/total protein density ratio was determined in HuCCA-1 cells transfected with Gadd45 $\beta$  siRNA or negative control siRNA. The phospho-protein/total protein density ratio of cells transfected with negative control siRNA was designated as 100%.

#### Cell viability assays

**Live/Dead<sup>®</sup> cell viability assay.** HuCCA-1 cells were grown on coverslips prior to transfection with siRNA. At 48 h post-transfection, cell viability was assessed using the Live/Dead<sup>®</sup> Viability/Cytotoxicity kit for mammalian cells (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, a working solution, containing 2  $\mu$ M calcein AM and 4  $\mu$ M ethidium homodimer (EthD-1), was prepared in PBS. The cells were washed with pre-warmed PBS, and 150  $\mu$ l of Live/Dead solution was applied to each cover slip. Following incubation at 37°C in a 5% CO<sub>2</sub> humidified atmosphere for 30 min, the reagents were removed and the cover slips were washed with PBS, mounted on the glass-slides, and viewed using a Nikon Eclipse TE2000-U microscope (Nikon Corporation, Tokyo, Japan) using B-2A (green, calcein) and G-2A (red, EthD-1) excitation filters. Images were recorded at x100 total magnification using ACT-1C software (version 1.02; Nikon Corporation). Live cells were stained with calcein-AM (green), whereas dead cells were stained with ethidium homodimer (red).

**WST-1 assay.** A total of 5,000 HuCCA-1 cells were seeded in 96-well plates for 24 h prior to siRNA transfection. At 24, 48 and 72 h post-transfection, 10  $\mu$ l of WST-1 reagent (Roche Diagnostics, Basel, Switzerland) was added into each well. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for 2 h. Then the absorbance at 450 nm was measured.

**Hoechst staining.** At 48 h post-transfection, the culture media was carefully removed to avoid the loss of detached cells. HuCCA-1 cells were then fixed in 2% w/v paraformaldehyde solution for 30 min at room temperature prior to permeabilization using 0.03% v/v Triton X-100 for 30 min. Nuclear staining was performed with Hoechst 333258 dye at room temperature (5  $\mu$ g/ml; Thermo Fisher Scientific, Inc.) for 2 h, followed by visualization at x400 total magnification with an Olympus IX83 live cell fluorescence microscope (Olympus Corporation).

**Gelatin zymography.** To analyze the activity of MMPs, the gelatinase activity of the enzymes secreted into the medium was determined using gelatin zymography assay, following a previously described protocol (29). The gelatinolytic activity of MMPs was observed as clear bands on the blue background of the Coomassie-stained gel. Densitometry analysis was performed using ImageJ software (30).

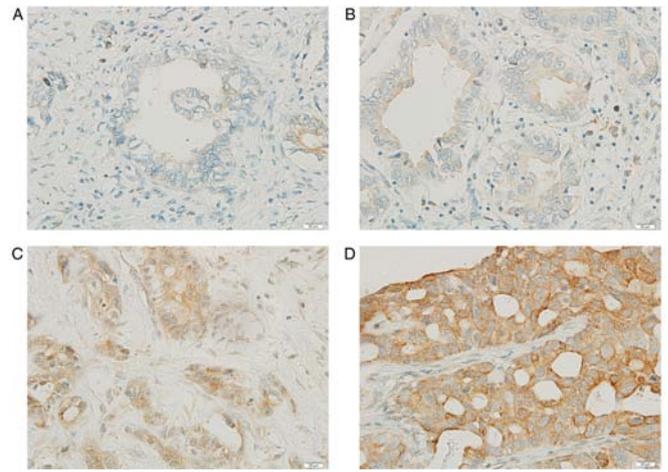


Figure 1. Gadd45 $\beta$  expression in CCA tissues, as determined by IHC. Paraffinized CCA tissue sections from patients were stained with a polyclonal anti-Gadd45 $\beta$  antibody. A total of 21/28 patients (75%) exhibited the high levels of Gadd45 $\beta$  expression. Representative images of IHC (A) grade 0 and (B) grade 1+, which were designated as low expression, and (C) grade 2+ and (D) grade 3+, which were designated as high expression, are presented. Gadd45 $\beta$ , growth arrest and DNA damage-inducible- $\beta$ ; CCA, cholangiocarcinoma; IHC, immunohistochemistry.

**Cell invasion and migration assays.** Cells were harvested at 48 h post-transfection for *in vitro* invasion and migration assays using transwell chambers, as described by Li *et al.* (31). Briefly, HuCCA-1 cells ( $1 \times 10^5$  cells/well) transfected with Gadd45 $\beta$  siRNA or negative control siRNA were seeded into the upper chamber of the transwells coated with Matrigel for the invasion assay and with no coating for the migration assay, and analyzed after 6 h of incubation at 37°C. HAM's F-12 supplemented with 10% FBS, used as chemoattractant, was added to the lower chamber. Cells were seeded in the upper chamber in serum free medium. Three independent experiments were performed in duplicate transwells.

**Statistical analysis.** Data is presented as the mean  $\pm$  standard deviation and every experiment was performed at least three times. The  $\chi^2$  test was used for clinicopathological data analysis, and experimental data analysis was performed using a paired t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**High levels of Gadd45 $\beta$  expression are associated with metastatic incidence in CCA.** Gadd45 $\beta$  expression in the histological specimens of CCA tissue from 28 patients was examined with IHC staining. The expression of Gadd45 $\beta$  was detected in all tumor tissues (n=28), and 75% (n=21) exhibited higher Gadd45 $\beta$  expression than the surrounding stroma (Fig. 1A-D). Although Gadd45 $\beta$  expression was previously reported to be moderate in the liver, gall bladder and pancreas (32), the expression level in cholangiocytes or CCA tissue has not previously been reported, to the best of our knowledge. A high level of Gadd45 $\beta$  expression was associated with the incidence of metastasis in patients ( $P=0.035$ ), although no statistically significant association was observed

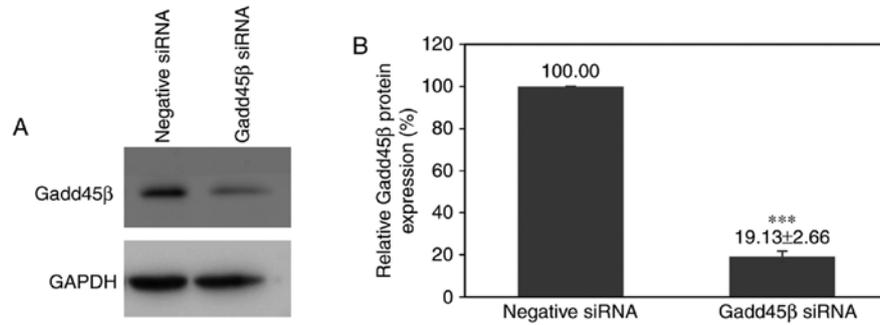


Figure 2. siRNA-mediated gene silencing efficiency in HuCCA-1 cells. (A) Representative images of the western blot analysis of Gadd45 $\beta$  silencing in HuCCA-1. (B) Densitometry analysis of the efficiency of Gadd45 $\beta$  silencing, using GAPDH as an internal control. Columns, mean of three independent experiments; bars, standard deviation. \*\*\*P=0.0004 compared with negative control siRNA transfection. siRNA, small interfering RNA; Gadd45 $\beta$ , growth arrest and DNA damage-inducible- $\beta$ .

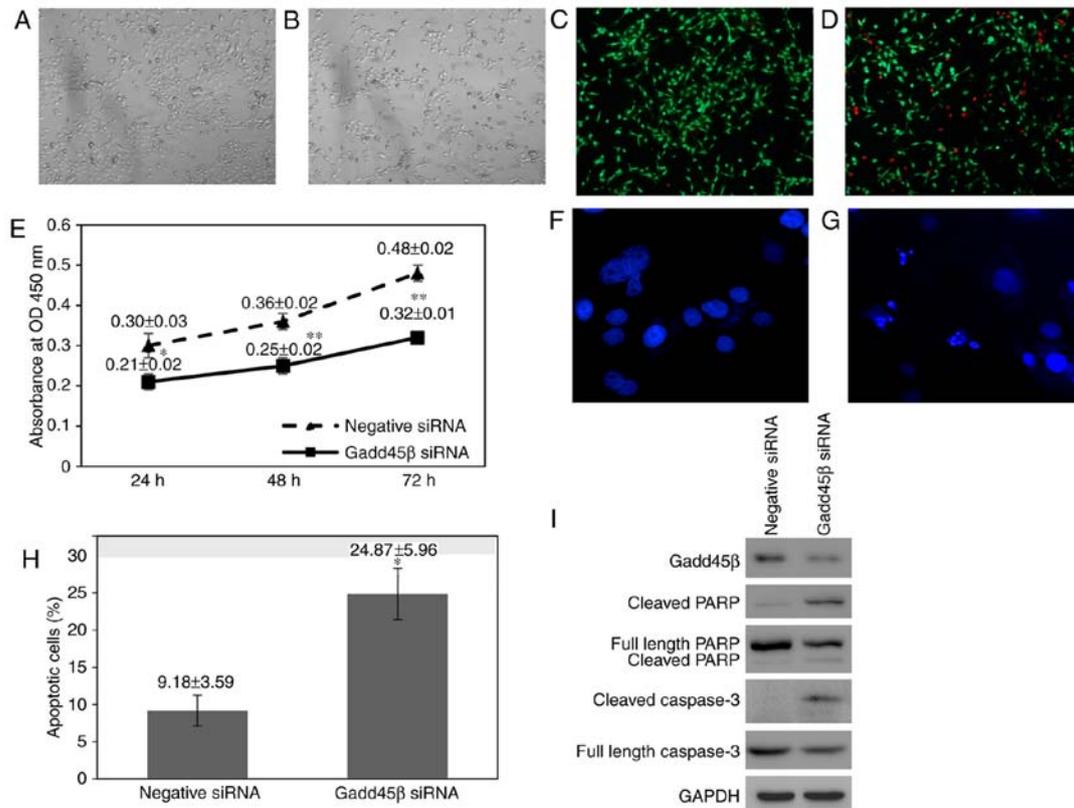


Figure 3. Gadd45 $\beta$  silencing impaired proliferation and induced apoptosis in HuCCA-1 cells. Light microscopic images of (A) negative control siRNA and (B) Gadd45 $\beta$  siRNA transfected HuCCA-1 cells at 48 h (x40 magnification). An evident decrease in the confluence of the Gadd45 $\beta$  siRNA transfected cells was noted, compared with negative control siRNA-transfected cells. In a Live/Dead Viability Assay, (C) negative control HuCCA-1 cells were relatively less likely to be dead (red) compared with (D) Gadd45 $\beta$ -silenced cells (x40 magnification). (E) WST-1 proliferation assay. Gadd45 $\beta$  siRNA transfection induced a significant reduction in HuCCA-1 cell proliferation. Data points, mean of three independent experiments; bars, standard deviation. \*P<0.05; \*\*P<0.005 compared with negative control siRNA transfection. Hoechst 333258 staining of HuCCA-1 cells transfected with (F) negative control siRNA and (G) Gadd45 $\beta$  siRNA (x400 magnification). The nuclei of live cells were homogeneously stained in blue, whereas those of apoptotic cells appeared condensed and fragmented. (H) Percentage of apoptotic cells in HuCCA-1 cells following Gadd45 $\beta$  silencing for 48 h. Columns, mean of three independent experiments; bars, standard deviation; \*P<0.05. (I) Representative images of the western blot analysis of caspase-3 activation and cleavage of PARP in HuCCA-1 following Gadd45 $\beta$  silencing for 48 h. Gadd45 $\beta$ , growth arrest and DNA damage-inducible- $\beta$ ; siRNA, small interfering RNA.

between the level of Gadd45 $\beta$  expression and age, sex, lymph node involvement or differentiation status (Table I).

*Gadd45 $\beta$  knockdown reduces the viability of HuCCA-1 cells.* To investigate the functional importance of Gadd45 $\beta$  in CCA, Gadd45 $\beta$  expression was suppressed using siRNA-mediated silencing in HuCCA-1, a CCA cell line that expresses moderate

levels of Gadd45 $\beta$ . Gadd45 $\beta$  siRNA transfection decreased the expression of Gadd45 $\beta$  in HuCCA-1 to 19.13±2.66% relative to negative control siRNA transfection (Fig. 2A and B).

During Gadd45 $\beta$  silencing experiments, a decrease in the confluence of Gadd45 $\beta$ -silenced HuCCA-1 cells was observed compared with negative control siRNA-transfected cells (Fig. 3A and B). Furthermore, there was an

increase in the number of cells floating in the medium of Gadd45 $\beta$ -silenced HuCCA-1 cells, indicating that silencing of Gadd45 $\beta$  perturbed the viability of HuCCA-1 cells (data not shown). A Live/Dead<sup>®</sup> cell viability assay confirmed that there was an increase in the number of dead cells in HuCCA-1 cells when Gadd45 $\beta$  was silenced (Fig. 3C and D). Cell viability was assessed using a WST-1 assay, in which the WST-1 reagent was added directly into the cells without removing the culture medium, preventing the loss of detached viable cells. Consistent with visual observation, Gadd45 $\beta$  silencing significantly decreased the viability of HuCCA-1 cells (Fig. 3E).

*Gadd45 $\beta$  silencing kills HuCCA-1 cells via apoptosis.* The mode of cell death in HuCCA-1 cells upon Gadd45 $\beta$  silencing was examined by staining the nuclear DNA with Hoechst 33258. Upon silencing of Gadd45 $\beta$ , HuCCA-1 cells were observed to exhibit nuclear condensation and DNA fragmentation, characteristics of apoptotic cells (33). The nuclei of live cells were homogeneously stained in blue, whereas those of apoptotic cells appeared condensed and fragmented. Gadd45 $\beta$ -silenced HuCCA-1 cells exhibited an increased rate of apoptotic cells compared with negative control cells (24.87 $\pm$ 5.96% vs. 9.18 $\pm$ 3.59%; Fig. 3F-H). Consistently, western blot analysis revealed that poly (ADP-ribose) polymerase (PARP) cleavage and caspase-3 cleavage were observed, indicating that HuCCA-1 cells died via apoptosis when Gadd45 $\beta$  was silenced (Fig. 3I) (34).

*Gadd45 $\beta$  silencing reduces Akt activity in HuCCA-1.* To determine which signaling pathways were involved, three of the principal pathways involved in the regulation of cell viability and proliferation, the Akt, ERK and p38 MAPK pathways, were studied. Akt is associated with cell proliferation, survival and apoptosis inhibition (35). p38 MAPK is a stress-activated protein kinase pathway important for cell proliferation, differentiation, survival and migration (36,37). ERK1/2 activation has been demonstrated to protect cells from apoptosis (38).

Gadd45 $\beta$  silencing resulted in a marked reduction of Akt phosphorylation ( $P=0.0237$ ), whereas no significant alteration in p38 MAPK or ERK1/2 phosphorylation was observed (Fig. 4A-D). The decrease in Akt activity may be responsible for the reduction of viability in HuCCA-1 upon Gadd45 $\beta$  silencing.

*Gadd45 $\beta$  silencing reduces the invasion and migration of HuCCA-1 cells.* A hallmark of malignancy is the ability to invade local tissues or to spread to distant sites (39). During this process, the cancer cells must acquire the ability to invade and migrate through connective tissue barriers, including the basement membrane, surrounding matrix and existing blood vessels (40). IHC data analysis of patient samples revealed that there was an association between the high expression of Gadd45 $\beta$  and metastasis (Table II). Furthermore, Gadd45 $\beta$  was demonstrated to be important for the viability of HuCCA-1 cells in the present study; viability is required by the cancer cells for successful metastasis (41). Therefore, the effect of Gadd45 $\beta$  silencing on the migration and invasion abilities of HuCCA-1 cells was investigated using *in vitro* transwell migration and invasion assays. Gadd45 $\beta$  silencing decreased

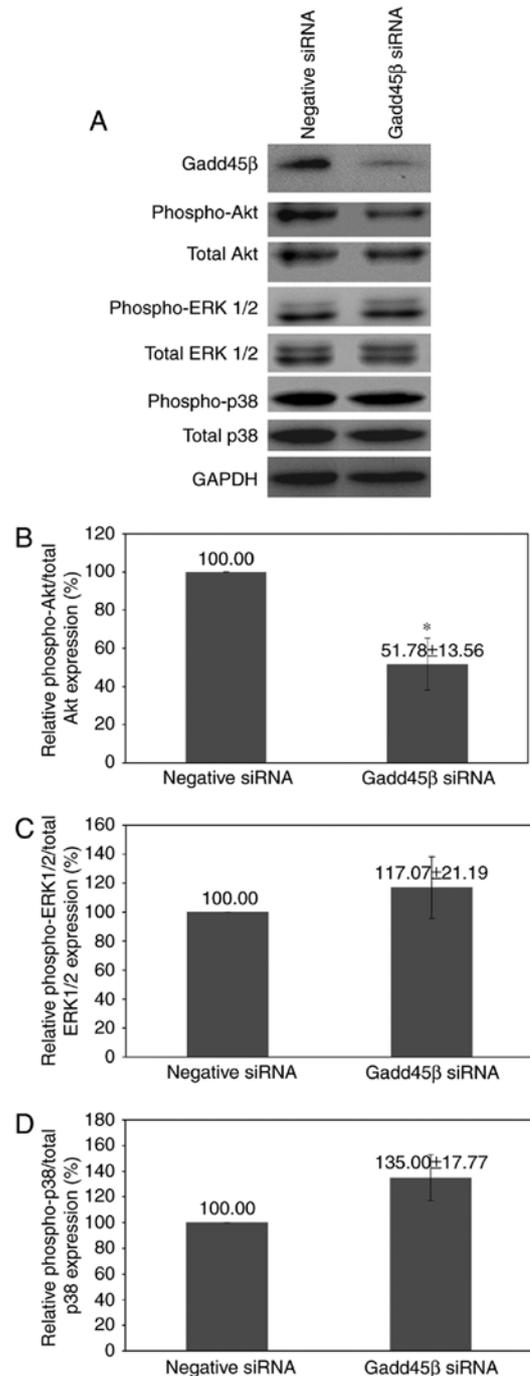


Figure 4. Effects of Gadd45 $\beta$  silencing upon major signaling pathways. (A) Representative images of the western blot analysis of p-Akt, p-p38 MAPK and p-ERK1/2 expression levels. Densitometry analysis for (B) p-Akt, (C) p-ERK1/2 and (D) p-p38 MAPK relative to the total form of each respective protein. Columns, mean of three independent experiments; bars, standard error of the mean; \* $P<0.05$ . Gadd45 $\beta$ , growth arrest and DNA damage-inducible- $\beta$ ; p38 MAPK, p38 mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase.

the migration of HuCCA-1 cells to 35.67 $\pm$ 5.60%, and invasion to 39.59 $\pm$ 11.09%, compared with the negative control siRNA (Fig. 5A and B).

Cancer cells may secrete matrix metalloproteinases (MMPs) or stimulate surrounding stromal cells to secrete MMPs, consequently leading to digestion of the extracellular matrix, promoting local invasion and metastasis (42,43).

Table II. Association between Gadd45 $\beta$  expression and the clinicopathological features of patients with cholangiocarcinoma.

Clinicopathological parameter	Total	Gadd45 $\beta$ expression		P-value
		Low	High	
Total, n (%)	28	7 (25.0)	21 (75.0)	
Age, years				0.378
Mean $\pm$ standard deviation	59.68 $\pm$ 10.41			
<60, n (%)	12 (42.9)	4 (14.3)	8 (28.6)	
$\geq$ 60, n (%)	16 (57.1)	3 (10.7)	13 (46.4)	
Sex, n (%)				0.512
Male	13 (46.4)	4 (14.3)	9 (32.1)	
Female	15 (53.6)	3 (10.7)	12 (42.9)	
Tumor size, cm				0.385 <sup>a</sup>
Mean $\pm$ standard deviation	2.90 $\pm$ 2.4			
<5, n (%)	25 (92.6)	7 (25.9)	18 (66.7)	
$\geq$ 5, n (%)	2 (7.4)	0 (0)	2 (7.4)	
Lymph node metastasis, n (%)				0.190
Absent	14 (50.0)	5 (17.9)	9 (32.1)	
Present	14 (50.0)	2 (7.1)	12 (42.9)	
Distant metastasis, n (%)				0.035 <sup>c</sup>
Absent	19 (67.9)	7 (25.0)	12 (42.9)	
Present	9 (32.1)	0 (0)	9 (32.1)	
Differentiation status, n (%)				0.696 <sup>b</sup>
Well	11 (52.4)	3 (14.3)	8 (38.1)	
Moderate	10 (47.6)	2 (9.5)	8 (38.1)	
Type of surgery, n (%)				0.076
R0	14 (50.0)	6 (21.0)	8 (28.6)	
R1	7 (25.0)	1 (3.6)	6 (21.4)	
R2	7 (25.0)	0 (0)	7 (25.0)	

<sup>a</sup>Tumor size data was not available in one case; <sup>b</sup>cell differentiation status data was not available in 7 cases; <sup>c</sup>P<0.05. Gadd45 $\beta$ , growth arrest and DNA damage-inducible- $\beta$ .

However, gelatin zymography analysis revealed that Gadd45 $\beta$  silencing did not significantly alter the activity of MMP-2 (P=0.5609) and MMP-9 (P=0.1886) secreted by HuCCA-1 cells (Fig. 5C-E).

#### *Gadd45 $\beta$ silencing reverses the EMT changes in HuCCA-1.*

Epithelial-mesenchymal transition is an important process during cancer metastasis, denoted by acquisition of mesenchymal phenotypes, including increased motility and invasiveness (8,9,44-46). Since Gadd45 $\beta$  silencing decreased the invasion and migration of HuCCA-1 cells, properties associated with EMT, including morphological changes and EMT marker expression, were examined. It was demonstrated that Gadd45 $\beta$  silencing may have induced HuCCA-1 cells to undergo morphological changes, from spindle-shaped and fibroblast-like in appearance, to a flattened epithelial-like phenotype (Fig. 6A and B). The expression patterns of the proteins involved in the EMT pathway were further investigated with western blotting. It was identified that Gadd45 $\beta$  silencing affected the expression

of multiple EMT markers (Fig. 7A-G). Epithelial marker E-cadherin expression was increased (P=0.0430), whereas the expression of the mesenchymal markers, Vimentin (P=0.0085) and Slug (P=0.0399), was decreased by Gadd45 $\beta$  silencing. However, there was no change in  $\beta$ -catenin expression (P=0.6006; Fig. 7A and D).

Notably, proteins associated with tight junctions were also affected by Gadd45 $\beta$  silencing. Claudin-1 and zona occludens protein 1 (ZO-1) constitute the tight junctions between cells (47,48); one of the mechanisms involved in cancer invasion is collective migration, in which the expression levels of tight-junction proteins are important to allow cells to move as a cluster (49). There was a decrease in claudin-1 (P=0.0100; Fig. 7A and F) and the ZO-1 expression (P=0.0087; Fig. 7A and G) upon Gadd45 $\beta$  silencing.

#### **Discussion**

The IHC staining of paraffin embedded tissue samples from patients with CCA revealed that the majority of CCA tissue

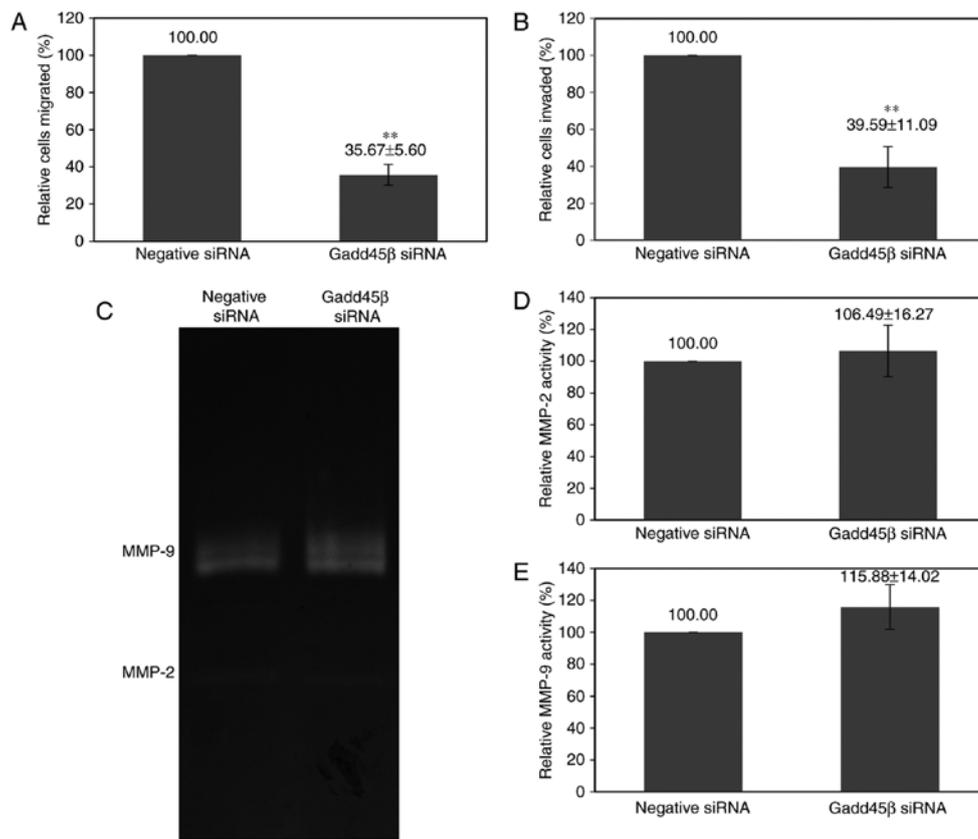


Figure 5. Gadd45 $\beta$  silencing decreased the *in vitro* migration and invasion of HuCCA-1, whereas MMP-2 and MMP-9 activity was not affected. Quantification of Transwell (A) migration and (B) invasion assays. The silencing of Gadd45 $\beta$  significantly decreased the *in vitro* invasion and migration activity of HuCCA-1 cells. (C) Gelatin zymography assay for secreted MMP-2 and -9. Densitometry analysis for (D) MMP-2 and (E) MMP-9. Columns, mean of three independent experiments; bars, standard deviation; \*P<0.05; \*\*P<0.01. Gadd45 $\beta$ , growth arrest and DNA damage-inducible- $\beta$ ; MMP, matrix metalloproteinase; siRNA, small interfering RNA.

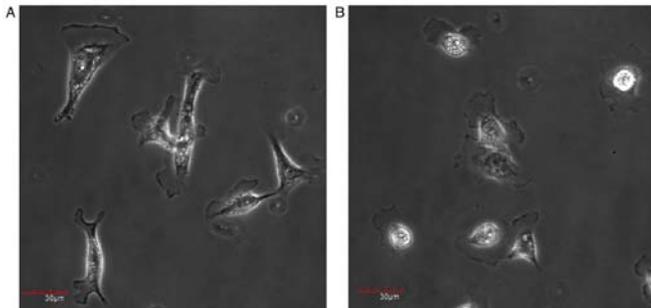


Figure 6. Effect of Gadd45 $\beta$  silencing upon cell morphology. Morphology of the HuCCA-1 cells at 48 h after (A) negative control siRNA and (B) Gadd45 $\beta$  siRNA transfection. HuCCA-1 cells changed in morphology from a spindle-shaped and fibroblast-like appearance to a flattened epithelial-like phenotype by Gadd45 $\beta$  silencing. Gadd45 $\beta$ , growth arrest and DNA damage-inducible- $\beta$ .

samples expressed increased Gadd45 $\beta$  relative to non-tumor tissue, and that the high level of Gadd45 $\beta$  in CCA was associated with an increased incidence of metastasis in patients. These findings suggest that Gadd45 $\beta$  may be of functional importance in CCA. As cancer cells are continuously exposed to stressful environments including hypoxia, competition for nutrients and oxidative stress during growth (23), Gadd45 $\beta$  appears to enable CCA cells to cope with stress and to thrive in the harsh tumor microenvironment.

In the present study, the functional importance of Gadd45 $\beta$  in CCA was determined using siRNA-mediated gene silencing in HuCCA-1, a cell line established from a patient with CCA. Gadd45 $\beta$  silencing decreased the proliferation and induced apoptosis in HuCCA-1 cells, as demonstrated by nuclear condensation and fragmentation, together with the activation of caspase-3 and PARP cleavage. The impairment of cell proliferation together with an increased rate of apoptosis in Gadd45 $\beta$ -silenced cells suggests that Gadd45 $\beta$  serves a pro-survival role in CCA. These data are in agreement with previous reports on genotoxic stress-induced apoptosis in hematopoietic cells of Gadd45 $\beta$ -deficient mice (50). Additionally, Gadd45 $\beta$  has been demonstrated to promote the survival of mouse embryo fibroblasts in response to tumor necrosis factor- $\alpha$  (51) and of B cells during Fas-induced apoptosis (52).

To study the underlying molecular mechanisms by which Gadd45 $\beta$  promotes viability in HuCCA-1, key cellular survival and death signaling pathways were studied, since the activation of death signals or the reduction of survival signals reduces the viability of cells. The results of the present study demonstrated that Gadd45 $\beta$  silencing significantly decreased the Akt activity, although p38 MAPK and ERK1/2 activity was unchanged. Activation of the Akt/PI3K pathway has been associated with growth and metastasis (53); therefore, the decrease in p-Akt may be responsible for the reduced growth

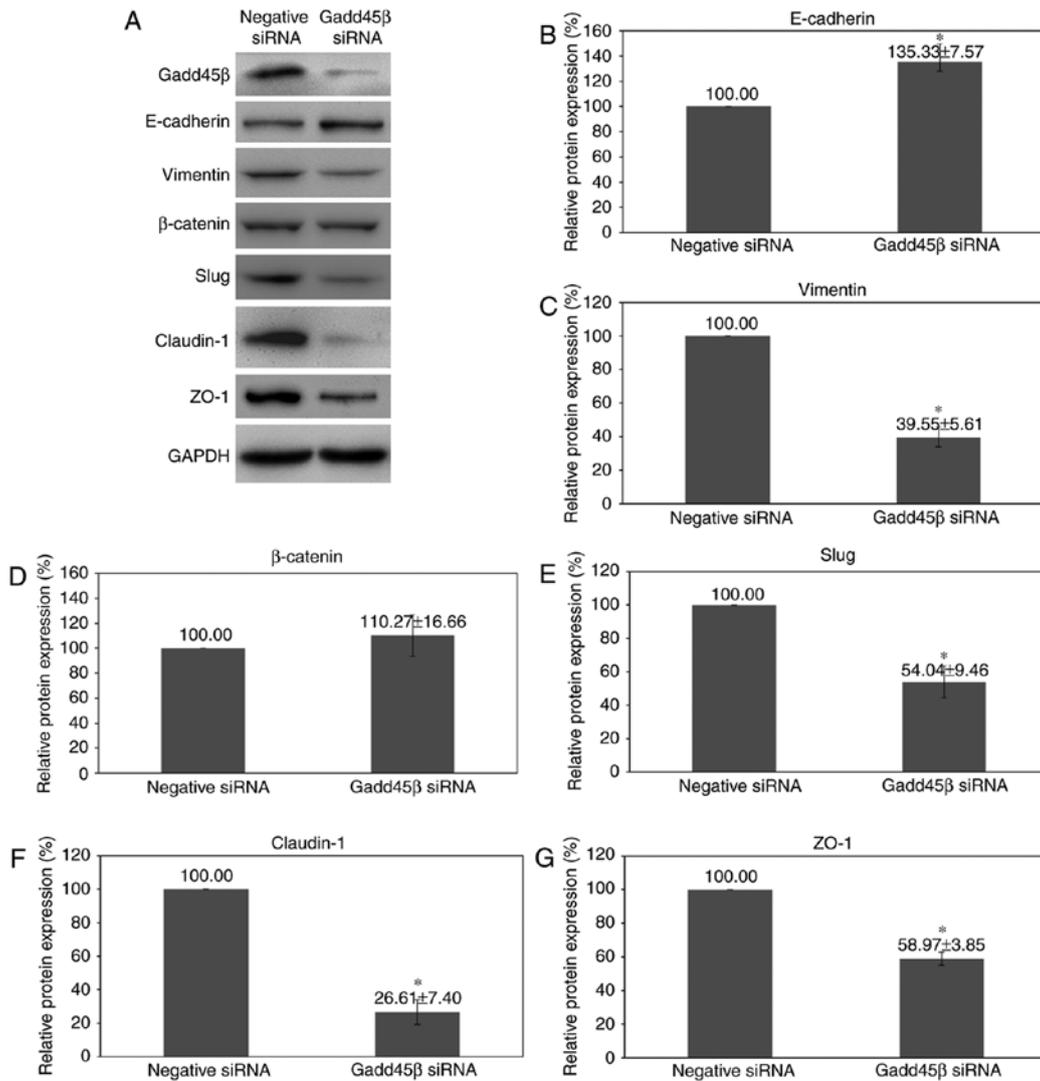


Figure 7. Effect of Gadd45 $\beta$  silencing upon EMT marker expression. (A) Representative images of western blot analysis for the expression of E-cadherin, vimentin,  $\beta$ -catenin, Slug, claudin-1 and ZO-1 at 48 h after Gadd45 $\beta$  silencing in HuCCA-1. Densitometry analysis of (B) E-cadherin, (C) vimentin, (D)  $\beta$ -catenin, (E) Slug, (F) claudin-1 and (G) ZO-1. Columns, mean of three independent experiments; bars, standard error of the mean. \* $P < 0.05$ . Gadd45 $\beta$ , growth arrest and DNA damage-inducible- $\beta$ ; EMT, epithelial-mesenchymal transition; ZO-1, zona occludens protein 1; siRNA, small interfering RNA.

and induction of apoptosis in HuCCA-1 cells upon Gadd45 $\beta$  silencing. The altered cell signaling activities appeared to shift the balance from pro-survival to pro-apoptotic following the silencing of Gadd45 $\beta$ .

EMT is an important biological process that allows malignant tumor cells to acquire migratory and invasive phenotypes; prerequisites for cancer invasion and metastasis. It is indicated by the acquisition of fibroblast-like morphology, with a reduction in intercellular adhesion and increased cell motility (11-18). The IHC data from the present study indicated that Gadd45 $\beta$  may be involved in the metastasis of CCA. Gadd45 $\beta$  silencing of HuCCA-1 cells induced a marked reduction in invasiveness and migration, although the activity of MMPs was not affected. A number of reports have implicated Gadd45 $\beta$  in the cell migration ability, an important characteristic of metastatic cancer cells. For example Salerno *et al* (54) demonstrated that the granulocytes of Gadd45 $\beta$ -deficient mice displayed the impairment of lipopolysaccharide-stimulated chemotactic migration. Furthermore, Kodama and Negishi (55) demonstrated that the ectopic expression of Gadd45 $\beta$  increased,

whereas siRNA-mediated silencing of Gadd45 $\beta$  decreased, pregnane X receptor-induced cell migration in hepatocellular carcinoma cells.

Examination of the expression of EMT markers revealed that Gadd45 $\beta$  silencing resulted in reduction in Slug expression, with a consequent increase in E-cadherin and decrease in vimentin expression. High Slug expression has been demonstrated to induce EMT in cancer cells (56) and the effects are orchestrated by an increase in vimentin expression (57). As Slug expression is negatively regulated by GS3K $\beta$  (58), which in turn is inactivated by pAkt (59), the decrease in Akt activity following Gadd45 $\beta$  silencing may have led to the downregulation of Slug expression, with the consequent suppression of vimentin expression and an increase in the invasiveness and migration of HuCCA-1 cells.

Furthermore, a decrease in the expression of the tight junction proteins, claudin-1 and ZO-1, was induced by Gadd45 $\beta$  silencing. Claudins and ZO-1 are integral proteins of the tight junctions that seal adjacent epithelial cells (47,48). Although the conventional roles of claudins and ZO-1 are to

control paracellular ion flux and maintain cell polarity, accumulating evidence has demonstrated that these proteins also exhibit non-junctional functions, including in cell motility, and may function in the EMT, apoptosis resistance, invasion and metastasis of cancer cells (60-62). Claudins have been demonstrated to promote collective migration, where groups of cells move in a coordinated manner and remain connected via cell-cell junctions. Additionally, claudin expression has been associated with increased MMP activity and cell survival (62). Claudin-1 expression has been demonstrated to be associated with invasive and metastatic phenotypes in a range of types of cancer including colon, liver, oral squamous cell carcinoma and melanoma (63). ZO-1 is a component of the 'junctional plaque' at the cytoplasmic surface of the tight junctions, which links integral membrane proteins with the cytoskeleton (64). It was identified in a previous study that ZO-1 can translocate from the cell membrane to the nucleus and may be involved in cell signaling (65). The upregulation of ZO-1 has been reported in melanoma, and the silencing of ZO-1 led to a marked reduction in the rate of cell invasion (66). Thus, the identified association between a decrease in claudin-1 and ZO-1 expression with impaired cell invasion and migration in the present study appears to be consistent with these studies.

In conclusion, the silencing of Gadd45 $\beta$  significantly decreased the viability, metastatic phenotypes and EMT markers of HuCCA-1 cells, suggesting that Gadd45 $\beta$  serves a function in the metastatic process in CCA. This is in accord with the IHC data of patients with CCA, in which Gadd45 $\beta$  expression was associated with metastasis. However, a limitation of this study was that the experiments were performed in only one CCA cell line. Further studies should be performed in more CCA cell lines with more stable knock down systems, as transient siRNA transfection has a limited gene silencing duration. The study of Gadd45 $\beta$  expression, function and Gadd45 $\beta$ -mediated signaling pathways in CCA will be important in understanding cancer biology, in which the cancer cells are dynamically evolving with accumulated mutations. Since the cancer cells must thrive in a competitive environment, a stress response protein like Gadd45 $\beta$  may be critical. The understanding or manipulation of Gadd45 $\beta$ -dependent survival signaling and EMT pathways may be beneficial as a potential therapeutic option, adjuvant to the conventional therapies for CCA.

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