Periostin activates integrin α5β1 through a PI3K/AKT-dependent pathway in invasion of cholangiocarcinoma

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Abstract. Periostin (PN) is mainly produced from stromal fibroblasts in cholangiocarcinoma (CCA) and shows strong impact in cancer promotion. This work aimed to investigate the mechanism that PN uses to drive CCA invasion. It was found that ITGα5β1 and α6β4 showed high expression in non-tumorigenic biliary epithelial cells and in almost all CCA cell lines. PN had preferential binding to CCA cells via ITGα5β1 and blocking this receptor by either neutralizing antibody or si*ITGα5* could attenuate PN-induced invasion. After PN-ITGα5β1 binding, intracellular pAKT was upregulated whereas there was no change in pERK. Moreover, PN could not activate AKT in condition of treatment with a PI3K inhibitor. These data provide evidence that PN-activated invasion of CCA cells is through the ITGα5β1/PI3K/AKT pathway. Strategies aimed to inhibit this pathway may, thus, provide therapeutic benefits.

Introduction

Cholangiocarcinoma (CCA), caused by liver fluke infection, is a major public health problem in the Northeastern and Northern parts of Thailand. It is a slow growing cancer with rapid metastasis and a high mortality rate (1). The incidence of CCA has increased worldwide with the highest rate in Thailand where around 93-100 per 100,000 peoples have been diagnosed with this disease (2). The pathogenesis of CCA has been demonstrated mainly by genetic mutations of bile duct epithelial cells themselves as the result of chronic inflammation which can also create a local environment enriched with cytokines and growth factors (3). In addition to cancer cells, the role of cancer-associated fibroblasts has been revealed in cancer pathogenesis by production of several mitogenic and pro-invasive factors into the tumor microenvironment (4). Several current reports indicate the crucial involvement of stromal fibroblasts in the progression of CCA via certain types of secreted tumorigenic substances (5-7). These substances can prime cancer cells in a paracrine mode to develop tumorigenic intracellular signaling pathways resulting in the increased cell proliferation, growth and invasion.

Almost all of the CCA stromal fibroblasts have been revealed to be α-smooth muscle actin (α-SMA)-positive cells (5,8). The fibroblasts were transformed into activated fibroblasts or myofibroblasts and were incorporated into the tumor and produced extracellular matrix proteins that led to tumor fibrosis (8). The recent work from the authors of the present group suggests that levels of α-SMA positive CCA stromal fibroblasts were correlated with poor patient survival (5). CCA fibroblasts isolated from CCA tissues have exhibited the ability to promote tumor cell proliferation and invasion (5,6). The whole gene expression analysis of CCA fibroblasts compared to normal fibroblasts has reported several differential expressed genes encoded to produce tumorigenic secreted proteins including periostin (PN) (6). The findings reported here are in support of several groups of researchers, wherein PN has been confirmed to express in CCA tissues solely from stromal fibroblasts (6,9,10). It has been proposed as a potential marker of poor prognosis in CCA patients (6). Though *in vitro* studies revealed the function of PN in induction of CCA cell proliferation, growth, and invasion, little is known regarding the mechanism of PN-induced CCA cell invasion that is a crucial phenomenon to activate CCA metastasis.

PN is an extracellular matrix (ECM) protein with multifunctional roles in tumorigenesis and tumor progression at each step of the transformation of normal into malignant cells and metastatic tumors (11). It has been proposed as a marker associated with cancer aggressiveness in pancreatic cancer (12,13), gastric cancer (14), breast cancer (15), thyroid carcinoma (16) and non-small cell lung cancer (17). In CCA, the impact of fibroblast-derived PN is convincing by its ability to activate cancer cell proliferation and invasion (6). To activate biological

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functions of cells, PN has been investigated for its ability to bind to integrin (ITG) receptors. In epithelial ovarian carcinoma, PN bound with ITGs αvβ3 and αvβ5 promoted cancer cell motility (18). The interference of these two ITGs by specific anti-ITG antibodies had an effect on the ability of PN to mediate cell adhesion in head and neck squamous cell carcinoma (19). For intracellular signaling, PN potently promotes metastatic growth of colon cancer by augmenting cell survival via the AKT/PKB pathway in colon cancer (20). Similarly, PN from pancreatic cancer cells activated ITGβ4 and promoted invasiveness of cancer cells through the PI3K pathway (21). But in vascular smooth muscle cells, PN was demonstrated to induce cell migration through ITGs αvβ3 and αvβ5 and the focal adhesion kinase pathway (22). In breast cancer, PN enhanced angiogenesis, in part, from the up-regulation of the vascular endothelial growth factor receptor on endothelial cells through the ITGαvβ3-focal adhesion kinase (FAK)-mediated signaling pathway (23). In addition, PN could induce epithelial-mesenchymal transition characteristics resulting in tumor metastasis through cross-talk between ITGαvβ5 and the epidermal growth factor receptor signaling pathways (24). It seems reasonable then to conclude that PN, in the dependent context, can activate specific ITG-mediated signal pathways and different biological responses.

Even though, it was found that the invasive property of CCA cells stimulated by PN was reduced after transient knockdown *ITGα5* (6), little is known regarding the intracellular signaling pathway activated by PN through ITGα5-mediated cell invasion. In the present study, the ITGs expression in CCA cell lines were explored and showed abundance of ITGs α5β1 and α6β4. The adhesion assay revealed the propensity of CCA cells to bind PN via ITGα5β1. Using cells with a low level of functional ITGα5β1 by treating these cells with either si*ITGα5β1* or neutralizing anti-ITGα5 antibody, the results showed that the PI3K/AKT-mediated, but not the ERK-mediated signaling pathway was involved in PN-stimulated CCA invasion. Since, PN is abundant in CCA tissues, understanding the role of PN in induction of cancer cell invasion may provide important information to attenuate metastasis and help identify the possible therapeutic targets.

Materials and methods

CCA cell culture. Human CCA cell lines KKU-M055, KKU-100, KKU-M139, KKU-M156, KKU-M213, KKU-M214 and KKU-OCA17 were kindly donated from Associate Professor Banchob Sripa, Khon Kaen University, Thailand. KKU-M213, KKU-M214 and KKU-OCA17 originated from well differentiated CCA tissues; KKU-M055, KKU-M139 and KKU-M156 from moderate CCA; and KKU-100 was isolated from poorly differentiated tissue (25). The non-tumorigenic immortalized bile duct epithelial cell MMNK1 was kindly provided by Professor Naoya Kobayashi, Department of Surgery, Okayama University Graduate School of Medicine and Dentistry, Japan (26). CCA cells and MMNK1 were cultured in Ham F-12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen) and anti-fungal agent. Cells were cultured in a humidified 5% CO_2 incubator at 37˚C. Cells were passaged by 0.25% trypsin-EDTA and those of more than 90% viability were used in further experiments.

Measurement of ITG expression pattern in CCA cell lines by real-time PCR. Total-RNA was extracted from all CCA cell lines and MMNK1 using PerfectPure RNA Cultured Cell Kit (5 Prime, Gaithersburg, MD, USA) according to the manufacturer's instructions. The cDNA was synthesized using SuperScript™ III First-Strand Synthesis System (Invitrogen) according to the instructions. Expression levels of *ITGs αv*, *α5*, *α6*, *β1*, *β3*, *β4* and *β5* were determined by SYBR-Green-based real-time PCR in Light Cycler® 480 II machine (Roche Applied Sciences, Indianapolis, IN, USA). The *β-actin* served as an internal control to adjust the amount of starting cDNA. The expression of each *ITG* was calculated by the $2^{-\Delta C_P}$ equation. In this case, $\Delta C_p = C_p(ITG) - C_p(\beta\text{-}actin)$. The sequences of genes used in this study were retrieved from PubMed (www.ncbi.nln.nih.gov) and primers were designed using Primer 3 software. A list of primers is summarized (Table I).

Flow cytometry analysis of ITG expression. For detection of the actual ITG α5β1 and α6β4 levels in biliary epithelial cells, cell pellets of around $1x10^6$ were fixed in 2% formaldehyde for 15 min at room temperature. The fixed cells were incubated with 1:50 goat anti-human ITGα5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in a washing solution which was HAM/F-12 containing 2% (v/v) FBS, 1% (w/v) bovine serum albumin (BSA) and 10 mM NaN_3 for 2 h at room temperature. Cells in the washing solution were centrifuged at 400 x g for 3 times and 5 min each to remove the excess primary antibody and this was followed by staining with 1:2,000 donkey anti-goat IgG-Alexa 488 (Invitrogen) diluted in the washing solution for 1 h at room temperature with light protection. For ITGα6β4 detection, 1:100 mouse anti-human ITGβ4 (Millipore, Temecula, CA, USA) diluted in washing solution was incubated with cells for 1 h at 4˚C and followed by 1:100 rabbit anti-mouse IgG-FITC (Dako, Carpinteria, CA, USA) diluted in washing solution for 30 min at 4˚C with light protection.

The ITGα5 and ITGβ4 signals were determined in the FL-1 channel of a Becton Dickinson FACSort (Becton Dickinson, Franklin Lakes, NJ, USA) and data analysis was performed by CellQuest software (Becton Dickinson). The relative mean fluorescence intensity (MFI) of CCA cell lines was normalized to that of the negative control stained with secondary antibody only. Two independent experiments were performed.

Immunocytochemistry of ITGα5β1 and α6β4 in CCA cell lines. Immunocytochemistry was employed to localize ITGα5β1 and ITGα6β4 on the cell membrane. KKU-M213 ($2x10^4$ cells) were cultured on sterile cover slips placed in a 24-well plate for 48 h. Cells were fixed in 4% paraformaldehyde for 15 min and blocked with 1% BSA for 30 min at room temperature. Then the cells were incubated with 1:50 goat anti-human ITGα5 (Santa Cruz Biotechnology) for 2 h at room temperature and subsequently stained with 1:500 donkey anti-goat IgG-Alexa 488 (Invitrogen) for 1 h at room temperature with light protection.

For localization of the ITGβ4, cells were plated onto glass cover slips at a density of $4x10^4$ cells in 24-well plate. After 48 h, cells were washed twice with 1X PBS and then blocked with blocking solution (10% FBS containing 1X PBS) for

Table I. The primers used in this study.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Size (bp)	Accession no.
ITGαv	TGACTGGTCTTCTACCCGC	CTCACAGATGCTCCAAACCA	121	NM_002210
ITGα5	AGTTGCATTTCCGAGTCTGG	CTCTGGGAGCACCAGATACAA	223	NM_002205
ITGα6	GGCCTTATGAAGTTGGTGGA	CTCTGGGAGCACCAGATACAA	144	NM_000210
ITGβ1	TCCCTGAAAGTCCCAAGTGT	TTTCCTGCAGTAAGCATCCA	143	NM_033666
ITGβ3	TGGTCCTGCTCTCAGTGATG	TGAAGGTAGACGTGGCCTCT	180	NM_000212
ITGβ4	TCTCCTACCGCACACAGGA	CTTCACCTGCAGCTCTTTCC	110	NM_001005619
ITGβ5	CTCCACTCTGGGAAACCTGA	AGGACGGTCAGGTTGGACTT	188	NM_002213
β-actin	CACACTGTGCCCATCTACGA	CTCCTTAATGTCACGCACGA	162	X00351

30 min at room temperature. Cells were further incubated with 1:500 mouse anti-human ITGβ4 (Millipore) for 2 h at room temperature and then incubated in 1:2,000 goat anti-mouse IgG-Cy3 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA).

The nuclei were stained with 1:1,000 Hoechst 33258 (Invitrogen) for 30 min at room temperature. The fluorescence signal was observed under the LSM 510 Meta laser scanning confocal microscope (Carl Zeiss, Jena, Germany) at the Division of Medical Molecular Biology, Office for Research and Development, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Neutralization of ITGα5β1 and ITGβ4 on CCA cells. To ensure roles of ITGα5β1 and ITGβ4 on PN-mediated CCA cell adhesion and invasion, neutralizing antibody specific to the ITGα5β1 heterodimer and ITGβ4 subunits was employed to block intact ITGα5β1 and ITGβ4 on the cell membrane of CCA cells. KKU-M213 CCA cells were trypsinized and washed with 1X PBS two times. Cell pellets of around $1x10^5$ cells were incubated with 1:200 anti-human ITGα5β1 (Millipore) or 1:200 mouse anti-human ITGβ4 (Millipore) at 37˚C for 1 h. Cells in the antibody solution were centrifuged at 400 x g for 5 min to remove excess antibody. The ITG-blocked cells were then collected to explore their responses to PN-induced invasion and adhesion. The number of either adhered or invaded cells induced by PN were compared with and without antibody blocking conditions. Two independent experiments were performed.

Adhesion assay of CCA cell lines on PN-coated surface. Recombinant PN (rPN) (1 μg) (Biovendor, Heidelberg, Germany) was coated on a 96-well plate surface at 37˚C for 2 h. Cells with or without exposure to neutralizing antibodies against ITGα5β1 or ITGβ4 (Millipore) were then added to each well and incubated at 37˚C for 1 h. Unattached cells were removed by rinsing twice with serum-free media. The number of adherent cells was determined by an MTS assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The percentage of PN-induced cell adhesion was normalized to that of cells attached on 1% BSA-coated wells. Two independent experiments were performed.

Invasion assay. To investigate the effect of rPN on the invasion of parental KKU-M213 cells, si*ITGa5*-treated cells, and ITGα5β1-blocked cells; $2x10^4$ cells of each condition were suspended in 100 ng/ml rPN containing complete medium and cultured in the upper chamber of the Matrigel™ invasion chamber (BD Biosciences, San Jose, CA, USA) for 24 h. Invaded cells were fixed in 5% (v/v) glutaraldehyde and then hematoxylin and eosin staining was performed. The number of invaded cells was counted under an inverted microscope by two independent investigators using x100 magnification fields. The assays were done in replicates of three independent experiments. Numbers of invaded cells was compared to those without rPN treatment.

Western blot analysis of pAKT and pERK. Cells with or without ITGα5 silencing and cells in the presence or absence of 100 μM LY294002, a PI3K inhibitor (Calbiochem, San Diego, CA, USA) or 30 μM U0126, an ERK inhibitor (Tocris Bioscience, MO) were induced by 100 ng/ml rPN for 30 and 120 min. Then the levels of pAKT and pERK1/2 were determined using western blot analysis. Cell pellets were collected after centrifugation of cell suspensions at 400 x g for 5 min in a refrigerated centrifuge. The cell pellets were rinsed by cold 1X PBS 2 times before lysed in 1X sample buffer containing 50 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.05% (w/v) bromophenol blue. Cell lysate was boiled for 10 min and centrifuged to remove the undissolved proteins and cell debris at 8,000 x g for 1 min. Cell extracts were then separated in 10% SDS-PAGE and transferred onto PVDF membranes (Amersham, Buckinghamshire, UK). Membranes were blocked in 5% skim milk containing TBST for 1 h at room temperature. Rabbit anti-human pAKT (Thr308) (Santa Cruz Biotechnology) at the dilution of 1:1,000 and rabbit anti-human pERK1/2 (Cell Signaling Technology Inc., Danvers, MA, USA) at the dilution of 1:2,000 were used as primary antibodies for incubation with the membrane for 1 h at room temperature. The 1:2,000 goat anti-rabbit IgG-HRP (Abcam, Cambridge, MA, USA) was used as the secondary antibody and incubated for 1 h at room temperature. The immunoreactive signals were visualized by enhanced chemiluminescense (Pierce, Rockford, IL, USA). The β-actin protein level was used as an internal control to determine the equal amount of loading proteins.

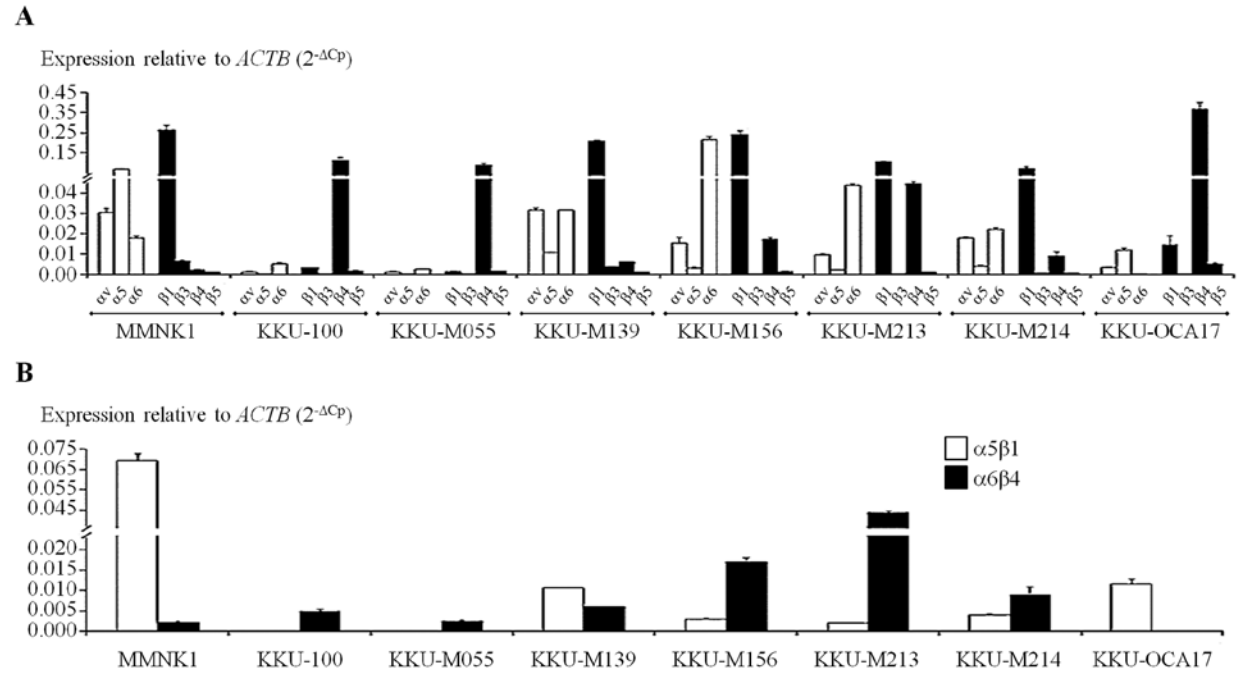

Figure 1. (A) Expression profile of ITG α- and β-subunits in immortalized bile duct epithelial cell MMNK1, KKU-K100, KKU-M055, KKU-M139, KKU-M156, KKU-M213, KKU-M214 and KKU-OCA17 CCA cell lines. (B) The possible expressions of different ITGαβ heterodimers are predicted. Bars represent mean ± SD of triplicate experiments.

Statistical analysis. The values from different independent experiments were expressed as mean ± SD. The significance of the different data sets was determined by the Student's t-test. A P-value of ≤0.05 was defined as statistically significant.

Results

Integrin expression profile in CCA cell lines. ITGs that were previously reported in CCA, and in the related cancer, hepatocellular carcinoma, and those that have been revealed as PN receptors including α-subunits: αv, α5 and α6; and β-subunits: β1, β3, β4 and β5 were explored. Real-time PCR using 50 ng starting cDNA exhibited different levels of each subunit in CCA cell lines and MMNK1 cells. Among α-subunits, α6 was expressed at the highest level in almost of cell types except KKU-OCA17 (Fig. 1A). In addition, α5-subunit showed the highest level among other α-subunits in MMNK1, but a moderate expression level was found in KKU-M139, KKU-M213 and KKU-M214. For the β-subunit, the results revealed that ITGβ1 had the highest expression level in KKU-M139, KKU-M156, KKU-M213, KKU-M214 and MMNK1, whereas KKU-K100, KKU-M055 and KKU-OCA17 had highest level of ITGβ4.

Since certain type of α-subunit ITG can be paired with a specific type of β-subunit, the predicted level of intact ITGs were presented based on the minimal level of their counterpart of either α-subunit or β-subunit. It has been shown that ITGα5 can bind only to β1 while ITGβ4 can only bind with α6 (27). Moreover, ITGαv can bind to several β-subunits including β1, β3, β5, β6 and β8. So the level of ITGβ3 may determine the maximal level of ITGαvβ3. Using the same concept, the amount of ITGβ5 would roughly present the possible maximal level of ITGαvβ5. Whereas, for ITGα5β1 and ITGα6β4, the expression of α5-subunit and β4-subunit may determine the maximal levels. Hence, the possible maximal levels of ITGs αvβ3, αvβ5, α5β1 and α6β4 are shown (Fig. 1B). The expressions of both α5β1 and α6β4 ITGs were found in all cell types. Some cell types had ITGα5β1 as the predominant expression level including MMNK1 and KKU-M139, but KKU-M156, KKU-M213 and KKU-M214 CCA cells showed predominant ITGα6β4 expression. Cells with high levels of both ITGα5β1 and ITGα6β4 were KKU-M139, KKU-M156, KKU-M213 and KKU-M214. Interestingly, KKU-M055 showed very low levels of ITGα5β1 as well as KKU-100.

Expressions of membrane ITGs α5β1 and α6β4 on CCA cell lines. To confirm the actual protein expression levels of ITG α5β1 and α6β4, FACS analysis was performed. The results revealed different amounts of these two ITGs among different types of CCA cell lines. For ITGα5β1, the relative mean fluorescence intensity (MFI) showed similar levels of expression in all cell lines with the highest signal in KKU-M213 (Fig. 2A). Most of CCA cells originated from well differentiated cancers including KKU-M213 and KKU-M214 expressed high levels of ITGα6β4 similar to KKU-M139 which was derived from moderate differentiation of squamous cell types (Fig. 2B). Almost all cell lines derived from moderately differentiated types (KKU-M055 and KKU-M156) and the poorly differentiated type (KKU-100) had low expression levels of ITGα6β4. In contrast, the expression of ITGα6β4 was found higher in KKU-M139, KKU-M213 and KKU-M214 than other cells, which is concordant to the results of mRNA levels. Interestingly, the highest level of ITGα6β4 (relative MFI=12.4±1.6) and ITGα5β1 (relative MFI=2.0±0.57) were detected in KKU-M213 (Fig. 2B).

PN mediates cell adhesion through ITGsα5β1 and α6β4 receptors. To demonstrate the impact of ITGα5β1 and ITGα6β4 in

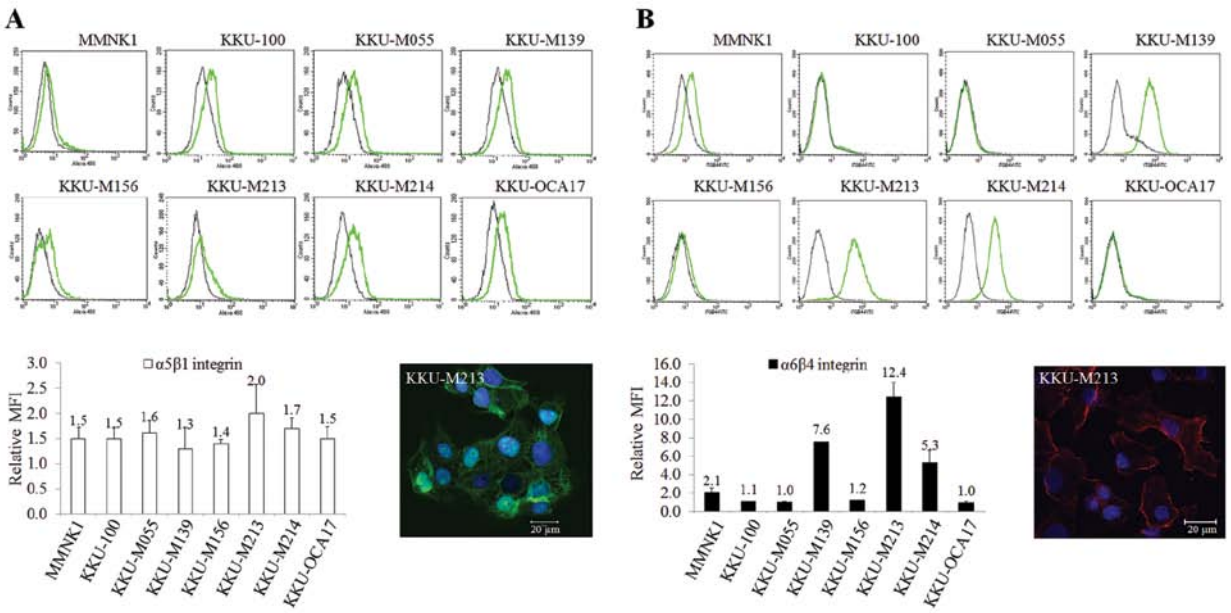

Figure 2. Flow cytometry analysis of the intact ITGs (A) α5β1 and (B) α6β4 CCA cell lines. Mean fluorescence intensity (MFI) of ITGα5β1 shows in relative signals compared to unstained cells without adding the first antibodies presented as grey in the graph. Bar graphs represent mean ± SD of relative MFI from three independent experiments. Immunofluorescence staining indicates the membranous pattern of both ITGs on the membrane of KKU-M213 cells.

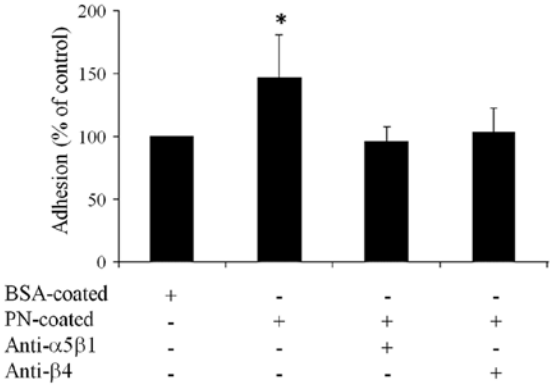

Figure 3. Adhesion assays of KKU-M213 cells in different culture coating surfaces with and without treatment with neutralizing anti-ITGs antibodies. Cells adhered onto BSA-coated plate were used as controls. Bars represent mean ± SD of two independent experiments. An asterix represents statistical significance (P<0.05) compared to the control BSA coating surface.

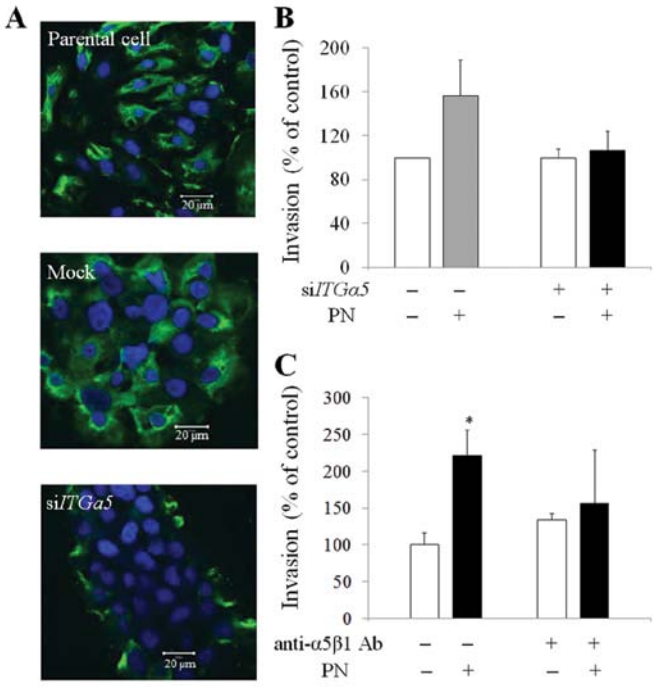

Figure 4. Invasion assay of KKU-M213 cells with and without ITGα5β1. (A) Cells were treated with si*ITGα5* and the reduction of ITGα5β1 is shown by fluorescence microscopy. (B) PN-induced invasion of si*ITGα5*-treated cells in comparison to that in mock cells are shown. (C) Percents of invaded KKU-M213 cells when ITGα5β1 is blocked with anti-α5β1 antibody and untreated cells are shown in conditions with and without PN stimulation. Invasion of cells without si*ITGα5* or antibody treatments was used as the control. Bars represent mean ± SD of duplicate or triplicate experiments. *P<0.05 compared to the control. The graph shows percentage of invaded cells exposed to PN compared to that of no PN treatment which was assumed to be 100%.

PN-activated tumorigenic function of CCA cells, KKU-M213 cells having the highest level of both ITGs were used to perform the adhesion assay. PN-coated culture plates were utilized to explore the binding efficiency of the cancer cells with and without functional ITG receptors on the cell membrane after incubation with the neutralizing antibodies. The results showed that CCA cells could intrinsically bind to PN-coated surface more than to BSA-coated surfaces or negative controls with statistical significance (Fig. 3). The binding efficiency was reduced to a similar level of the negative control when cells were blocked either with the intact ITGα5β1 or ITGα6β4 by specific neutralizing antibodies.

PN-mediated CCA invasion via ITGα5β1. To confirm whether cells with unavailable ITGα5β1 could be induced by PN, si*ITGα5*-treated and anti-ITGα5β1 antibody-treated cells were performed invasion assay with and without PN treatment. The

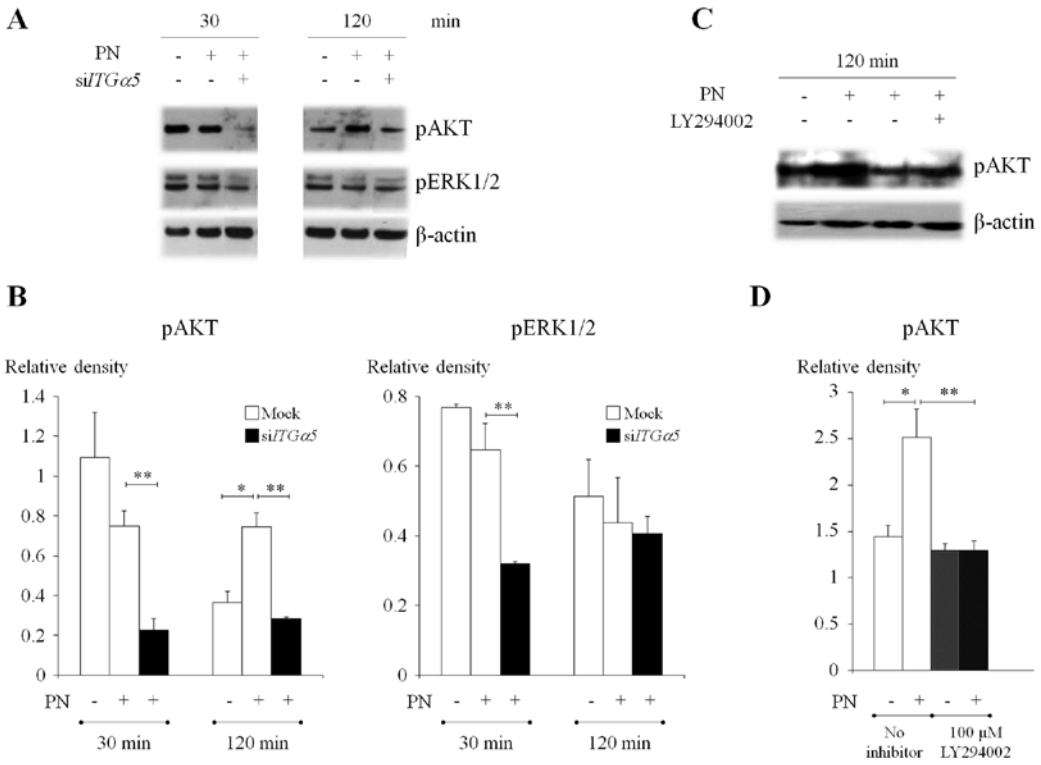

Figure 5. PN induces pAKT through PI3K pathway. (A) Western blot analysis shows level of pAKT and pERK1/2 in si*ITGα5*-treated cells and mock cells after being treated with 100 ng/ml of rPN for 30 and 120 min. The β-actin level indicates equal amounts of total protein. (B) Densitometry measurement of pAKT and pERK1/2 after normalization with the corresponding intensity of β-actin. (C) The KKU-M213 cell line was treated with rPN in the presence or absence of 100 μM LY294002, a PI3K inhibitor, for 120 min. (D) Densitometry measurement of pAKT in cells treated with PI3K inhibitor after normalization with the corresponding intensity of β-actin. The * and ** represent P<0.05 of either pAKT or pERK in mock cells treated and untreated with rPN; and mock and si*ITGα5*-treated cells after stimulation with rPN, respectively.

intact ITGα5β1 receptor on the membrane of CCA cells was detected by immunocytochemistry and showed that cells exposed to si*ITGα5* successfully inhibited the expression of ITGα5β1 on the membrane of cancer cells as compared to the intrinsic expression in parental and mock cells (Fig. 4A). The decreased PN-induced invasive capability of the *ITGα5*-knockdown cells was revealed (Fig. 4B). Significant increases of PN-induced invasion was observed in mock cells (156±18%) as compared to cells without PN stimulation. The results revealed that *ITGα5*-knockdown cells showed decreased PN-induced invasive capability (106±18% of invaded cells) when compared to negative controls without PN. Moreover, in cells blocking ITGα5β1 with neutralizing anti-ITGα5β1 antibody, the results showed in a similar way that PN could not induce KKU-M213 cell invasion if there was no ITGα5β1 available on the cell membrane (Fig. 4C).

PN induces pAKT through activation of PI3K pathway in CCA cells. In order to investigate the intracellular signaling pathway activated by PN via ITGα5β1, cells with normal levels and ITGα5β1-knockdown cells were treated with recombinant PN. The results revealed that exogenous PN could significantly induce phosphorylation of AKT in KKU-M213 parental cells at 120-min post-treatment compared to control cells without PN treatment (Fig. 5A and B). PN could not activate pAKT in cells with transient knockdown of ITGα5, pERK1/2, however, did not change as a result of PN stimulation when compared between cells with and without ITGα5β1 (Fig. 5A and B). These results suggested that upon PN stimulation via ITGα5β1, AKT, but not ERK, was activated.

To confirm the signaling pathway of PN-induced AKT, the pharmacological inhibitor of PI3K (LY294002) which is the upstream molecule of AKT, was applied to KKU-M213 CCA cells and the level of AKT phosphorylation was determined under the conditions with and without PN stimulation. The results showed depletion of pAKT level in responding to LY294002 treatment which confirmed the antagonist effect of this inhibitor to pAKT (Fig. 5C and D). Interestingly, PN could not induce pAKT in the PI3K-inhibited cells. Hence, in cells activated with PN, the pAKT was significantly higher in parental cells than in cells pretreated with PI3K inhibitor.

Discussion

The progression of cancer is no longer recognized as an independent aberrant event occurring only in cancer cells. Substances surrounding the cancer cells secreted from a variety of cells in the tumor microenvironment and signaling pathways induced by cancer and other cells, and cancer cell-substance interactions are thought to play crucial roles. The role of tumor-associated fibroblasts as the most abundant cell types in the tumor microenvironment and the major source of growth factors and extracellular matrix substances in tumorigenesis and tumor progression have been reported as important contributors (4,28). In the current situation drug resistance of cancer treatment is a common phenomenon

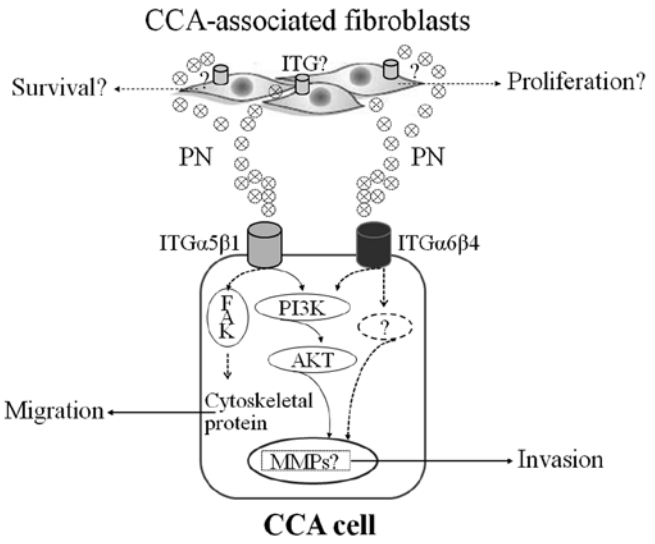

Figure 6. Proposed mechanism of PN-induced CCA migration and invasion. CCA-associated fibroblasts are the major source for PN production in CCA microenvironment. The fibroblast-derived PN acts as a ligand of ITGα5β1 and α6β4 receptors. Upon binding of PN and ITGα5β1, the PI3K/AKT signaling pathway is activated and invasiveness property is driven in CCA cells. It is possible that PN may also activate the PI3K/AKT pathway resulting in cell proliferation and survival of CCA fibroblasts in an autocrine manner. Base on these effects, CCA fibroblasts may be used as a therapeutic target for CCA patients. The dashed lines were not investigated in this study.

in cancer patients due to the vulnerability of cancer cells to undergo genetic changes as the result of their rapid proliferation rate. Cancer fibroblasts are therefore suitable to target since they exhibit less genetic instabilities (29). Based on this concept, several studies have been performed and the obtained information has been summarized in several review articles to confirm the possibility of targeting cancer fibroblast as an additional treatment strategy in cancer patients (4,30-32).

Different cancer fibroblasts have their unique properties in the production of certain substances (6,33,34). Fibroblasts isolated from CCA tissues can produce a variety groups of tumorigenic substances which play important roles in induction of cancer cell proliferation (5). Unpublished data from the present research team has revealed migration induction when CCA cells were treated with the conditioned-medium from primary cultured-CCA fibroblasts. The gene expression profile of CCA fibroblasts has been performed by this group and the increased expression of tumor-related genes in CCA-derived fibroblasts has been reported (6). Among these genes, PN has been confirmed with high expression in the microenvironment of CCA tissues with relation to the short survival time of the patients together with tumorigenic induction in cancer cells *in vitro* including cell proliferation, growth, and invasion (6). Herein, the underlying mechanism of how CCA cells respond to PN-driven invasion has been explored. ITGs, as the receptors for PN (18,21,22), were explored in CCA cells. Adhesion assays indicated that ITGα5β1 and ITGα6β4 were the receptors for PN and the invasion assay confirmed that ITGα5β1 was involved in PN-induced CCA cell invasion and PI3K/AKT was the signaling pathway underlying this mechanism.

Previous reports on ITG expression in CCA cells indicated that α1 had no expression whereas ITGs α2, α3, α6, β1 and β4 were expressed in almost all CCA cell lines (35-37). This is consistent with the present findings that all CCA cell lines expressed high level of ITGα6, though the synthesis of ITGs α2 and α3 were not included in the current study. Notably, the expression of ITGα5 is not uniformly expressed in CCA (36). The result showed the expression of ITGα5 in some CCA cells; in particular the cells with previously reported high responses to PN-induced invasion such as KKU-M213 CCA cell lines (6). Though the expression of ITGαv has mostly been reported as PN receptor, no evidence was found in CCA cells. The current results revealed that ITGαv could be detected in almost all CCA cell lines but at a lower level than that of ITGs α5 and α6. These results imply that in addition to ITGs α2, α3, and α6 previously reported, CCA cells could express αv and α5. It is difficult to predict the level of ITGαvβ3 and αvβ5 because ITGαv can bind to several β-subunits of ITG including β1, β3, β5, β6 and β8 (27). ITGα5, however, can form heterodimers only with ITGβ1 (27,38). It can then be concluded that ITGα5β1 may be one of the existing ITGs on the CCA cell membrane and may have an important impact in cancer progression after the stimulation by stromal PN.

For β-subunit ITGs, the present results are similar to previous reports that ITGs β1 and β4 were expressed in almost all CCA cells (35,36). ITGβ4 was found at higher levels in CCA cells as compared to those in MMNK1 immortalized non-tumorigenic biliary cells. Though in the previous report (35), ITGβ4 was detected in normal and proliferating biliary epithelial cells but was an inconsistent finding in CCA. This study provides conclusive evidence of the presence of ITGβ4 in CCA cells. Since ITGβ4 can bind only to ITG α6, it is likely that the level of ITGβ4 can be roughly determined by the level of ITGα6β4 presented on CCA cell membranes. In addition, this study indicated β3 and β5, generally form heterodimers with ITGαv, as the ITGs with low expression in CCA cells. These results therefore suggested that CCA cells express high levels of ITGs α5, α6, β1 and β4. It is suggested that the heterodimers of ITG α5β1 and α6β4 may be the major ITG receptors expressed on CCA cell membrane. The flow cytometry analysis and immunofluorescence staining confirmed the presence of these two ITGs on the membrane of KKU-M213 cells. The favorable binding of PN on ITGs α5β1 and α6β4 indicated by the lower numbers of cells bound onto the PN-coated surface after treatment of cells with the specific neutralizing antibody. The ITGα6β4 was able to interact with PN as well as ITGα5β1 at similar levels. Hence, it can be concluded that PN may influence the progressive tumor behavior of CCA cells though either ITGα5β1 or ITGα6β4.

PN has been demonstrated to activate cellular responses via different ITGs depending on cell type context, for example, ITGαvβ3 in non-small cell lung cancer (39) and ITGα6β4 in pancreatic cancer (21). Previous work by this group reports that si*ITGα5*-treated cells have lower PN-induced cell proliferation and invasion compared to the parental cells (6) and shows in the first report presenting the association of PN and ITGα5β1 in tumor promotion of CCA cells. It is possible that the interaction of PN and ITGα5β1 may be the unique phenomenon of PN in promotion of CCA. Hence, in this current report, we explored the signaling pathway starting from ITGα5β1 in the induction of cancer invasion by PN. Similar to the data on the PN-activated signaling pathway reported previously (21,39), PN could acti-

vate cell invasion via the stimulation of AKT-dependent, but not ERK, pathway in CCA cells. It is well known that activation of the PI3K-AKT-dependent pathway is essential in the regulation of several different biological functions including cell survival, growth and proliferation, invasion and migration in a ligand specific manner (40-42). ITGα5β1 plays an important role in metastasis, invasion and poor prognosis of some cancers (43). Cancer cells with high expression of ITGα5β1 showed an increased invasiveness into 3D collagen matrices through enhancement of the contractile force (44). In addition, the role of ITGα5β1 in stimulation of cell invasion has been revealed through activation of matrix metalloproteinase 2 (MMP2) in breast cancer (45). The fact that PN-ITGα5β1 interaction stimulates the enhancement of cell contractile force and some MMP expressions is the possible underlying mechanisms of how PN helps tumor cells to invade and finally metastasize. Unpublished data by the present authors showed that PN induced MMP9 and MMP13 from CCA cells and activated cancer cells to migrate (data not shown) which may be the effect of PN-mediated change of cytoskeletal proteins through FAK (22). Finally, the actual downstream signaling pathway after AKT activation is of particular interest because the proper inhibitor can be proposed to apply for the attenuation of cancer progression with minimal side-effects (46).

In summary, fibroblasts are not passive bystanders in tumor environment. The current trend in cancer research is the inclusion of the cancer fibroblast as a major contributor of disease progression and suggests the inhibition of fibroblast-derived tumor-promoting factors as the first line approach. This study provides evidence for the contribution of CCA-associated fibroblast-derived PN in the activation of tumorigenic properties of CCA cells through receptor ITGs (Fig. 6). PN secreted mainly from fibroblasts binds to either ITGα5β1 or ITGα6β4 and can facilitate the invasiveness property of CCA cells. The ITG5α5β1-mediated PI3K-dependent or FAK (22) signaling pathways are activated eventually regulating several cellular responses in particular MMP production and cell migration. The obtained knowledge implies the potential of using an anti-PN antibody (47), anti-ITGα5β1 antibody (48), and PI3K/AKT inhibitors (49,50) to attenuate CCA progression driven by fibroblasts. Understanding the exact mechanisms responsible for fibroblast-associated cancer progression is a challenge for the future as the alternative and synergistic cancer-targeted therapy in CCA patients.

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References

1. Sirica AE: Cholangiocarcinoma: molecular targeting strategies for chemoprevention and therapy. Hepatology 41: 5-15, 2005.
2. Sriamporn S, Pisani P, Pipitgool V, Suwanrungruang K, Kamsa-ard S and Parkin DM: Prevalence of *Opisthorchis viverrini* infection and incidence of cholangiocarcinoma in Khon Kaen, Northeast Thailand. Trop Med Int Health 9: 588-594, 2004.
3. Wise C, Pilanthananond M, Perry BF, Alpini G, McNeal M and Glaser SS: Mechanisms of biliary carcinogenesis and growth. World J Gastroenterol 14: 2986-2989, 2008.
4. Franco OE, Shaw AK, Strand DW and Hayward SW: Cancer associated fibroblasts in cancer pathogenesis. Semin Cell Dev Biol 21: 33-39, 2010.
5. Chuaysri C, Thuwajit P, Paupairoj A, Chau-In S, Suthiphongchai T and Thuwajit C: Alpha-smooth muscle actin-positive fibroblasts promote biliary cell proliferation and correlate with poor survival in cholangiocarcinoma. Oncol Rep 21: 957-969, 2009.
6. Utispan K, Thuwajit P, Abiko Y, *et al*: Gene expression profiling of cholangiocarcinoma-derived fibroblast reveals alterations related to tumor progression and indicates periostin as a poor prognostic marker. Mol Cancer 9: 13, 2010.
7. Sirica AE, Campbell DJ and Dumur CI: Cancer-associated fibroblasts in intrahepatic cholangiocarcinoma. Curr Opin Gastroenterol 27: 276-284, 2011.
8. Terada T, Makimoto K, Terayama N, Suzuki Y and Nakanuma Y: Alpha-smooth muscle actin-positive stromal cells in cholangiocarcinomas, hepatocellular carcinomas and metastatic liver carcinomas. J Hepatol 24: 706-712, 1996.
9. Darby IA, Vuillier-Devillers K, Pinault E, *et al*: Proteomic analysis of differentially expressed proteins in peripheral cholangiocarcinoma. Cancer Microenviron 4: 73-91, 2010.
10. Dumur CI, Campbell DJ, DeWitt JL, Oyesanya RA and Sirica AE: Differential gene expression profiling of cultured neu-transformed versus spontaneously-transformed rat cholangiocytes and of corresponding cholangiocarcinomas. Exp Mol Pathol 89: 227-235, 2010.
11. Ruan K, Bao S and Ouyang G: The multifaceted role of periostin in tumorigenesis. Cell Mol Life Sci 66: 2219-2230, 2009.
12. Erkan M, Kleeff J, Gorbachevski A, *et al*: Periostin creates a tumor-supportive microenvironment in the pancreas by sustaining fibrogenic stellate cell activity. Gastroenterology 132: 1447-1464, 2007.
13. Fukushima N, Kikuchi Y, Nishiyama T, Kudo A and Fukayama M: Periostin deposition in the stroma of invasive and intraductal neoplasms of the pancreas. Mod Pathol 21: 1044-1053, 2008.
14. Li JS, Sun GW, Wei XY and Tang WH: Expression of periostin and its clinicopathological relevance in gastric cancer. World J Gastroenterol 13: 5261-5266, 2007.
15. Puglisi F, Puppin C, Pegolo E, *et al*: Expression of periostin in human breast cancer. J Clin Pathol 61: 494-498, 2008.
16. Puppin C, Fabbro D, Dima M, *et al*: High periostin expression correlates with aggressiveness in papillary thyroid carcinomas. J Endocrinol 197: 401-408, 2008.
17. Takanami I, Abiko T and Koizumi S: Expression of periostin in patients with non-small cell lung cancer: correlation with angiogenesis and lymphangiogenesis. Int J Biol Markers 23: 182-186, 2008.
18. Gillan L, Matei D, Fishman DA, Gerbin CS, Karlan BY and Chang DD: Periostin secreted by epithelial ovarian carcinoma is a ligand for alpha(V)beta(3) and alpha(V)beta(5) integrins and promotes cell motility. Cancer Res 62: 5358-5364, 2002.
19. Kudo Y, Ogawa I, Kitajima S, *et al*: Periostin promotes invasion and anchorage-independent growth in the metastatic process of head and neck cancer. Cancer Res 66: 6928-6935, 2006.
20. Bao S, Ouyang G, Bai X, *et al*: Periostin potently promotes metastatic growth of colon cancer by augmenting cell survival via the Akt/PKB pathway. Cancer Cell 5: 329-339, 2004.
21. Baril P, Gangeswaran R, Mahon PC, *et al*: Periostin promotes invasiveness and resistance of pancreatic cancer cells to hypoxia-induced cell death: role of the beta4 integrin and the PI3k pathway. Oncogene 26: 2082-2094, 2007.
22. Li G, Jin R, Norris RA, *et al*: Periostin mediates vascular smooth muscle cell migration through the integrins alphavbeta3 and alphavbeta5 and focal adhesion kinase (FAK) pathway. Atherosclerosis 208: 358-365, 2010.
23. Shao R, Bao S, Bai X, *et al*: Acquired expression of periostin by human breast cancers promotes tumor angiogenesis through up-regulation of vascular endothelial growth factor receptor 2 expression. Mol Cell Biol 24: 3992-4003, 2004.

24. Yan W and Shao R: Transduction of a mesenchyme-specific gene periostin into 293T cells induces cell invasive activity through epithelial-mesenchymal transformation. J Biol Chem 281: 19700-19708, 2006.
25. Sripa B, Leungwattanawanit S, Nitta T, *et al*: Establishment and characterization of an opisthorchiasis-associated cholangiocarcinoma cell line (KKU-100). World J Gastroenterol 11: 3392-3397, 2005.
26. Maruyama M, Kobayashi N, Westerman KA, *et al*: Establishment of a highly differentiated immortalized human cholangiocyte cell line with SV40T and hTERT. Transplantation 77: 446-451, 2004.
27. Gahmberg CG, Fagerholm SC, Nurmi SM, Chavakis T, Marchesan S and Gronholm M: Regulation of integrin activity and signalling. Biochim Biophys Acta 1790: 431-444, 2009.
28. Angeli F, Koumakis G, Chen MC, Kumar S and Delinassios JG: Role of stromal fibroblasts in cancer: promoting or impeding? Tumour Biol 30: 109-120, 2009.
29. Qiu W, Hu M, Sridhar A, *et al*: No evidence of clonal somatic genetic alterations in cancer-associated fibroblasts from human breast and ovarian carcinomas. Nat Genet 40: 650-655, 2008.
30. Pietras K and Ostman A: Hallmarks of cancer: interactions with the tumor stroma. Exp Cell Res 316: 1324-1331, 2010.
31. Gonda TA, Varro A, Wang TC and Tycko B: Molecular biology of cancer-associated fibroblasts: can these cells be targeted in anti-cancer therapy? Semin Cell Dev Biol 21: 2-10, 2010.
32. Rasanen K and Vaheri A: Activation of fibroblasts in cancer stroma. Exp Cell Res 316: 2713-2722, 2010.
33. Rosenthal E, McCrory A, Talbert M, Young G, Murphy-Ullrich J and Gladson C: Elevated expression of TGF-beta1 in head and neck cancer-associated fibroblasts. Mol Carcinog 40: 116-121, 2004.
34. Paland N, Kamer I, Kogan-Sakin I, Madar S, Goldfinger N and Rotter V: Differential influence of normal and cancer-associated fibroblasts on the growth of human epithelial cells in an in vitro cocultivation model of prostate cancer. Mol Cancer Res 7: 1212-1223, 2009.
35. Volpes R, van den Oord JJ and Desmet VJ: Integrins as differential cell lineage markers of primary liver tumors. Am J Pathol 142: 1483-1492, 1993.
36. Enjoji M, Sakai H, Nakashima M and Nawata H: Integrins: utility as cell type- and stage-specific markers for hepatocellular carcinoma and cholangiocarcinoma. In Vitro Cell Dev Biol Anim 34: 25-27, 1998.
37. Patsenker E, Wilkens L, Banz V, *et al*: The alphavbeta6 integrin is a highly specific immunohistochemical marker for cholangiocarcinoma. J Hepatol 52: 362-369, 2010.
38. Hynes RO: Integrins: bidirectional, allosteric signaling machines. Cell 110: 673-687, 2002.
39. Ouyang G, Liu M, Ruan K, Song G, Mao Y and Bao S: Upregulated expression of periostin by hypoxia in non-small-cell lung cancer cells promotes cell survival via the Akt/PKB pathway. Cancer Lett 281: 213-219, 2009.
40. Fresno Vara JA, Casado E, de Castro J, Cejas P, Belda-Iniesta C and Gonzalez-Baron M: PI3K/Akt signalling pathway and cancer. Cancer Treat Rev 30: 193-204, 2004.
41. Sarker D, Reid AH, Yap TA and de Bono JS: Targeting the PI3K/AKT pathway for the treatment of prostate cancer. Clin Cancer Res 15: 4799-4805, 2009.
42. Yadav V and Denning MF: Fyn is induced by Ras/PI3K/Akt signaling and is required for enhanced invasion/migration. Mol Carcinog 50: 346-352, 2011.
43. Roman J, Ritzenthaler JD, Roser-Page S, Sun X and Han S: alpha5beta1-integrin expression is essential for tumor progression in experimental lung cancer. Am J Respir Cell Mol Biol 43: 684-691, 2010.
44. Mierke CT, Frey B, Fellner M, Herrmann M and Fabry B: Integrin alpha5beta1 facilitates cancer cell invasion through enhanced contractile forces. J Cell Sci 124: 369-383, 2011.
45. Morozevich G, Kozlova N, Cheglakov I, Ushakova N and Berman A: Integrin alpha5beta1 controls invasion of human breast carcinoma cells by direct and indirect modulation of MMP-2 collagenase activity. Cell Cycle 8: 2219-2225, 2009.
46. Castillo SS, Brognard J, Petukhov PA, *et al*: Preferential inhibition of Akt and killing of Akt-dependent cancer cells by rationally designed phosphatidylinositol ether lipid analogues. Cancer Res 64: 2782-2792, 2004.
47. Kyutoku M, Taniyama Y, Katsuragi N, *et al*: Role of periostin in cancer progression and metastasis: inhibition of breast cancer progression and metastasis by anti-periostin antibody in a murine model. Int J Mol Med 28: 181-186, 2011.
48. Almokadem S and Belani CP: Volociximab in cancer. Expert Opin Biol Ther 12: 251-257, 2012.
49. Li Z, Tan F, Liewehr DJ, Steinberg SM and Thiele CJ: In vitro and in vivo inhibition of neuroblastoma tumor cell growth by AKT inhibitor perifosine. J Natl Cancer Inst 102: 758-770, 2010.
50. Pal SK, Reckamp K, Yu H and Figlin RA: Akt inhibitors in clinical development for the treatment of cancer. Expert Opin Investig Drugs 19: 1355-1366, 2010.