CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Biological materials

3.1.1.1 Primary culture fibroblasts

All of primary culture fibroblast samples including one Cf and two Lfs were established as previously described (Chuaysri et al., 2009). In brief, hepatectomized livers from CCA patients undergone surgery at Srinagharind Hospital, Khon Kaen University were freshly used to isolate fibroblasts from cancerous and non-cancerous areas. The sample collection was ethically approved by Human Ethic Committee, Khon Kaen University with the approval license number HE490143. The primary culture fibroblast from cancerous tissue was designated Cf, whereas Lfs were isolated from non-cancerous tissues at the most distant area from the edge of cancerous tissues. Cf and Lf1 were derived from the same CCA patient whereas Lf2 was isolated from the other patient. These patients had been diagnosed papillary type adenocarcinoma. Cf and 2 Lfs were confirmed the fibroblastic phenotype without any epithelial cell contamination (Chuaysri et al., 2009). Cf was confirmed the increased expression of *α-SMA* which indicated activated fibroblasts. Moreover, Cf was confirmed the tumorigenic effect on CCA cells and non-tumorigenic human biliary epithelial cells by increasing the proliferation more than that of Lfs by both contact and non-contact manners (Chuaysri et al., 2009). Cfs and Lfs were grown in 10% FBS containing DMEM with 20% epidermal growth factor. The numbers of passage of fibroblasts used in this experiment was not more than 10 to ensure the phenotypes at the origin. Cells with more than 95% viability quantitated by trypan blue staining were used in the experiments.

3.1.1.2 Non-tumorigenic and tumorigenic biliary epithelial cell lines

CCA cell lines including KKU-M213, KKU-OCA17, KKU-M214, KKU-M156, KKU-M055, and KKU-100 were established and kindly donated by Associate Professor Dr. Banchob Sripa, Department of Pathology, Faculty of Medicine, Khon Kaen University. They were cultured in complete medium which was 10% FBS containing HAM F-12 supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 5 µg/ml amphotericin at 37°C in 5% CO_2 incubator. Cells were subpassaged with 0.25% trypsin- EDTA when reached 90-100% confluence. Cells of more than 95% viability were used in further experiments. Cells were kept in 10% DMSO and 20% FBS in liquid nitrogen as storage. The numbers of cell passage used in the whole experiment was between 30 to 80.

Immortalized non-tumorigenic bile duct epithelial cell, MMNK1 was kindly donated by Professor Naoya Kobayashi, Department of Surgery, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan. The established method and characterization of this cell were previously reported (Maruyama et al., 2004). MMNK1 was cultured in 10% FBS containing HAM F-12 supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 5 µg/ml amphotericin B at 37°C in 5% CO_2 incubator. Cells of more than 95% viability were used for the experiments. Cells were kept in 10% DMSO and 20% FBS in liquid nitrogen as cell storage. The number of cell passage used in the whole experiment was between 30 to 40.

3.1.2 Chemical materials

All chemicals used in this project are cell culture and molecular biology grade products and purchased from the standard companies (Table 3-1). Some important instruments are listed (Table 3-2).

Table 3-1 Lists of the important chemical materials

Chemicals	Companies
Cell culture	
HAM F-12	Invitrogen, Carlsbad, CA, USA
DMEM	Invitrogen, Carlsbad, CA, USA
Fetal bovine serum (FBS)	Invitrogen, Carlsbad, CA, USA
Epidermal growth factor	CytoLab Ltd., Rohovot, Israel
Penicillin, Streptomycin	Invitrogen, Carlsbad, CA, USA
0.25% Trypsin-EDTA	Invitrogen, Carlsbad, CA, USA
Gene expression profiling study	
RNeasy Micro Kit	Qiagen, Valencia, CA, USA
Affymetrix GeneChip Human Genome U133 plus 2.0 Array	Affymetrix, Santa Clara, CA, USA
T7-oligo (dT) primer and Superscript II reverse transcriptase Two-Cycle cDNA Synthesis Kit	Affymetrix, Santa Clara, CA, USA
MEGAscript T7 Kit	Ambion, Inc., Woodward St Austin, TX, USA
Biotin-labeled cRNA using GeneChip IVT Labeling Kit	Affymetrix, Santa Clara, CA, USA
Streptavidin-phycoerythrin (SAPE)	Molecular Probes, Eugene, OR, USA
Goat biotinylated anti-streptavidin	Vector Laboratories, Burlingame, CA, USA
Conventional PCR	
Taq polymerase	Invitrogen, Carlsbad, CA, USA
dNTPs	Invitrogen, Carlsbad, CA, USA
Invasion assay	
Matrigel™ invasion chamber	BD Biosciences, San Jose, CA, USA
Neutralizing of ITGα5β1	
Mouse anti-human ITGα5β1 Ab	Chemicon Inc., Temecula, CA, USA
Flow cytometry	
BD FACSFlow™	BD Biosciences, San Jose, CA, USA
RNA extraction and real time PCR	
RNeasy Mini kit	Qiagen, Valencia, CA, USA
PerfectPure RNA Culture Cell Kit	5 PRIME Inc., Gaithersburg, MD, USA
First strand cDNA synthesis kit (AMV)	Roche Molecular Biochemicals, Mannheim, Germany
Superscript III First-Strand Synthesis kit	Invitrogen, Carlsbad, CA, USA
LightCycler® 480 SYBR Green I Master	Roche Applied Science, Mannheim, Germany

Table 3-1 Lists of the important chemical materials (Cont.)

Chemicals	Companies
Western blot and immunostaining	
Polyvinylidene fluoride (PVDF) membrane	Amersham, Buckinghamshire, UK
Rabbit anti-human PN Ab	Biovendor, Heidelberg, Germany
Mouse anti-human α-SMA Ab	Sigma, St Louis, MO, USA
Mouse anti-human phospho-AKT Ab	Cell signaling Technology, Inc, Danvers, MA, USA
Rabbit anti-human phospho-ERK1/2 Ab	Cell signaling Technology, Inc, Danvers, MA, USA
Goat anti-human ITGα5 Ab	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Mouse-anti human β-actin Ab	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Goat anti-rabbit IgG-HRP	Abcam, Cambridge, MA, USA
Anti-rabbit Envision^{+} System-HRP labeled polymer	Dako, Carpinteria, CA, USA
Rabbit anti-mouse IgG-HRP	Zymed, Sanfrancisco, CA, USA
Donkey anti-goat IgG-Alexa 488	Invitrogen, Carlsbad, CA, USA
Goat anti-rabbit-IgG-Cy3	Invitrogen, Carlsbad, CA, USA
Goat anti-mouse IgG-Alexa 488	Invitrogen, Carlsbad, CA, USA
Hoechst 33258	Invitrogen, Carlsbad, CA, USA
Enhance chemiluminescense (ECL)	Pierce, Rockford, IL, USA
Cell proliferation assay/ Colony formation assay	
Recombinant PN	Biovendor, Heidelberg, Germany
CellTiter 96® AQ_{ueous} One Solution Reagent (MTS)	Promega, Medison, WI, USA
Propidium iodide (PI)	Invitrogen, Carlsbad, CA, USA
DNase-free RNaseA	Amresco, Solon, OH, USA
MetaPhor® agarose	Cambrex Bio Science, Rockland, ME, USA
Small interfering RNA transfection	
Lipofectamine 2000	Invitrogen, Carlsbad, CA, USA
OptiMEM I	Invitrogen, Carlsbad, CA, USA
siITGα5	Santa Cruz Biotechnology, Santa Cruz, CA, USA
1. 5'-gucagaauuucgagacaaa-3'	
2. 5'-caccaacaagagagccaaa-3'	
3. 5'-ccacugaccagaacuagaa-3'	

Table 3-2 Lists of the important instruments used in this study

Instruments	Companies
Gene expression profiling study	
Agilent RNA 6000 Nano Kit	Agilent Technologies, Waldbronn, Germany
GeneChip Fluidics Station 400	Affymetrix, Santa Clara, CA, USA
GeneChip Scanner 3000	Affymetrix, Santa Clara, CA, USA
Gene Spring G.X.7.3 software	Agilent Technologies, Waldbronn, Germany
Conventional PCR	
GeneAmp® PCR System 9700	Applied Biosystem, Foster City, CA, USA
RNA extraction and real time PCR	
ND-1000 Spectrophotometer	NanoDrop Technologies, Inc., Wilmington, DE, USA
ABI 7500 machine	Applied Biosystem, Foster City, CA, USA
LightCycler® 480	Roche Applied Science, Mannheim, Germany
Western blot analysis	
TE70 Semi-dry transfer unit	Amersham Biosciences, Buckinghamshire, UK
Immunofluorescence	
LSM 510 Meta laser scanning confocal microscope	Carl Zeiss, Jena, Germany
Flow cytometry	
Becton Dickinson FACSort and CellQuest software	Becton Dickinson, Franklin Lakes, NJ, USA
Cell proliferation assay	
Anthos HTII ELISA reader	Anthos Mikrosysteme GmBH, Krefeld, Germany
Statistical analysis	
SPSS version 16.0	SPSS Inc., Chicago, IL, USA

3.2 Methods

3.2.1 Gene expression profiling study and data analysis

Total RNA was extracted from 3-5 x 10^5 cells of Cf, Lf1 and Lf2, using RNeasy Micro Kit as the manufacturer's instruction. The quality of RNA was assessed by an Agilent RNA 6000 Nano Kit. RNA integrity number (RIN) of more than 9.0 was accepted for the good quality of RNA to perform gene expression analysis.

Affymetrix GeneChip Human Genome U133 plus 2.0 Array containing 38,500 human genes/EST sequences was used following Affymetrix's instruction.

RNA amplification and RNA labeling, and hybridization are 2 main steps of this method (Fig 3-1). To amplifiy RNA, Superscript II reverse transcriptase Two-Cycle cDNA Synthesis Kit was used. Briefly, 100 ng of total RNA was converted to cDNA using T7 promoter-oligo (dT) primer and incubated with Superscript II reverse transcriptase at 42°C for 1 h. The RNA was removed from RNA-cDNA heteroduplex by RNase H activity and the second-strand cDNA was synthesized by incubated with DNA polymerase I at 16°C for 2 h. Complementary DNA was then used to be the template for *in vitro* transcription (IVT) to get complementary RNA (cRNA) using MEGAscript T7 Kit by incubating all of the double-stranded cDNA with T7 RNA polymerase at 37°C for 16 h. The cRNA was then purified using GeneChip Sample Cleanup Module and used as the template for the second cycle of RNA amplification.

The amplified cRNA was purified and then labeled with biotin using GeneChip IVT Labeling Kit. Twenty μg of biotin-labeled cRNA was added in fragmentation buffer and heated for 35 min at 94°C. Fifteen μg of fragmented cRNA was hybridized to the Affymetrix GeneChip Human Genome U133 plus 2.0 Array for 16 h at 45°C. The GeneChip arrays were subjected to wash and stain with streptavidin-phycoerythrin (SAPE) using GeneChip Fluidics Station 400. To amplify the signals, the arrays were further stained with goat biotinylated anti-streptavidin. The array data were scanned by a GeneChip Scanner 3000 and analyzed by Affymetrix microarray suite, version 5.0.

Raw data from the GeneChips were used to analyze the expression levels of each gene in Cf represented as fold change normalized by that in Lf. To reduce the different genetic background of patients to whom Lf2 was originated from to that of the patient in which Cf and Lf1 were originated, genes up-regulated in Cf compared to those in both Lf1 and Lf2 were determined as common up-regulated genes whereas genes which were down-regulated in Cf compared to those in both Lfs were determined as common down-regulated genes. Gene ontology was used to categorize the common up-and down-regulated genes regarding to their biological functions by Gene Spring G.X.7.3 software. In addition, all of arrays were checked the intensity of the control sequences prior to perform data analysis. Only the data

with detected signals designated as P (Presence) were included to analyze whereas those with undetected ones represented as A (Absence) were excluded out of the analysis.

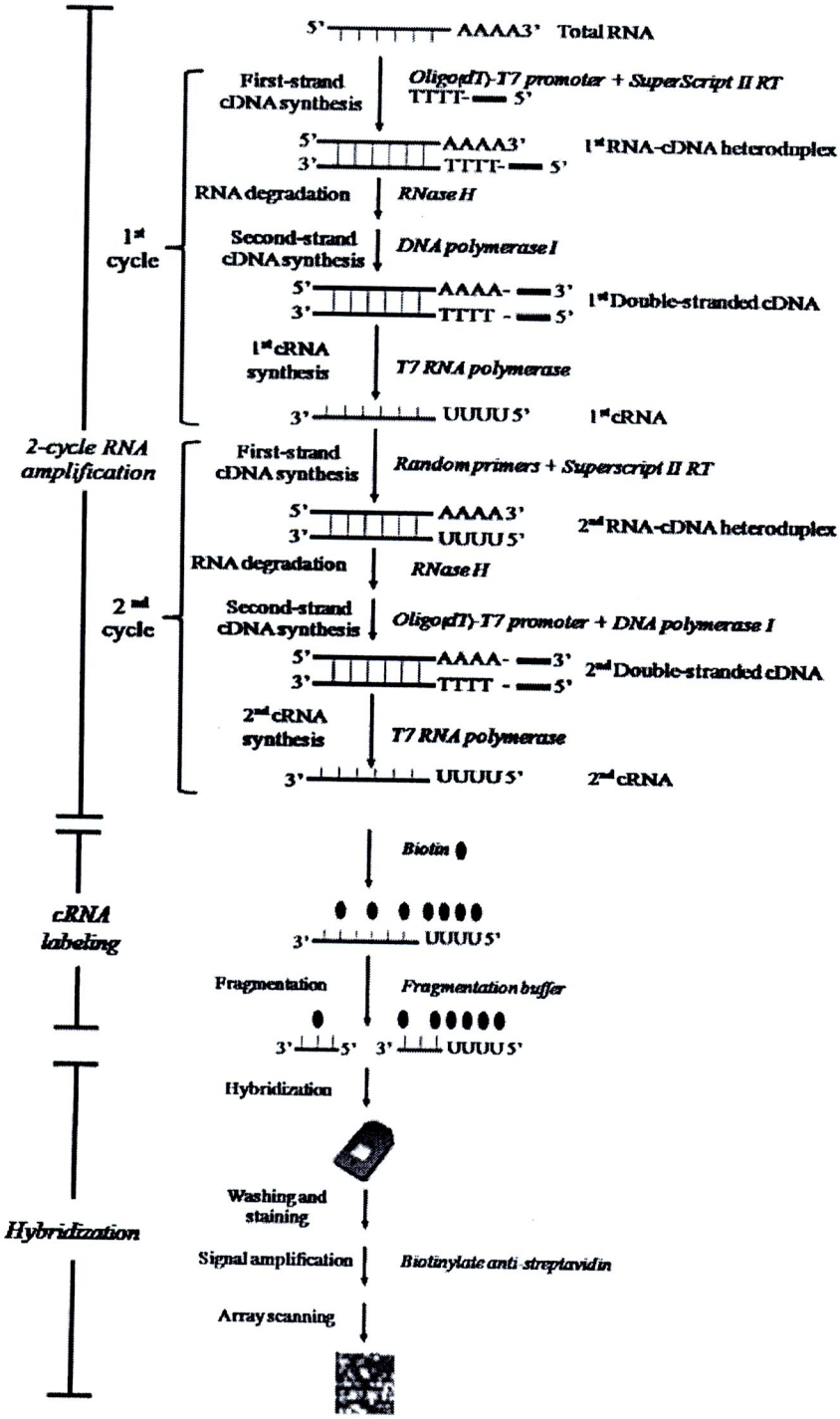

Figure 3-1 Schematic protocols for RNA amplification, labeling and hybridization in gene profiling study.

3.2.2 Semi-quantitative real time PCR

To confirm the altered expressions of genes selected from microarray result, semi-quantitative real time PCR was performed. Total RNA was extracted from different biological preparations of Cf and Lfs using RNeasy Mini kit followed RNeasy's protocol. One μg of total RNA was used to synthesize cDNA using first strand cDNA synthesis kit (AMV) according to the company's instruction. The expression levels of genes were determined by SYBR Green-based real time PCR in 25-μl reaction containing 50 ng of cDNA, 0.04 μM each primer, 2 U Taq polymerase and 1X SYBR Green I buffer. The reaction was performed in ABI 7500 machine with different cycles for different genes as summarized (Table 3-3).

The nucleotide sequences of genes tested in this study were retrieved from PubMed (www.ncbi.nln.nih.gov) and the primers for each gene were designed by Primer 3 program (Table 3-4). The expression of *β-actin* was measured and served as an internal control to adjust the amount of starting cDNA of different samples. The relative expression of gene in Cf compared to that of Lf was calculated by the following equation:

$$\text{Relative gene expression} = 2^{-\Delta\Delta C_T}$$

In this case, $\Delta C_T = C_T$ (gene of interest) - C_T (*β-actin*)

$$\Delta\Delta C_T = \Delta C_T \text{ (Cf)} - \Delta C_T \text{ (Lf)}$$

Hence, fold change of up-regulated gene = $2^{-[\Delta C_T \text{ (Cf)} - \Delta C_T \text{ (Lf)}]}$, whereas fold change of down-regulated gene = $2^{-[\Delta C_T \text{ (Lf)} - \Delta C_T \text{ (Cf)}]}$.

Table 3-3 PCR condition for gene validation using real time PCR

Gene	Denaturing	Annealing	Extension	Total cycle
ADAM12	95°C, 15 sec	55°C, 30 sec	72°C, 45 sec	40
AREG	95°C, 15 sec	50°C, 1 min	72°C, 1 min	50
AGN2	95°C, 15 sec	58°C, 1 min	72°C, 1 min	50
ER	95°C, 15 sec	50°C, 1 min	72°C, 1 min	50
JAGL1	95°C, 15 sec	55°C, 30 sec	72°C, 45 sec	40
LAMA5	95°C, 15 sec	58°C, 1 min	72°C, 1 min	50
NOV	95°C, 15 sec	58°C, 1 min	72°C, 1 min	50
PDGF-A	95°C, 15 sec	50°C, 1 min	72°C, 1 min	50
PN	95°C, 15 sec	50°C, 1 min	72°C, 1 min	50
RL	95°C, 15 sec	55°C, 1 min	72°C, 1 min	50
SCG2	95°C, 15 sec	58°C, 1 min	72°C, 1 min	50
β-actin	95°C, 15 sec	55°C, 30 sec	72°C, 45 sec	40

Note: *ADAM12*, a disintegrin and matrix metalloproteinase 12; *AREG*, amphiregulin; *ANG2*, angiopoietin 2; *ER*, epiregulin; *JAGL1*, jagged soluble form; *LAMA5*, laminin alpha 5; *NOV*, nephroblastoma over expressed; *PDGF-A*, platelet-derived growth factor-alpha; *PN*, periostin; *RL*, reelin; *SCG2*, secretogranin 2; *β-actin*, beta-actin

Table 3-4 Primer sequences for real time PCR

Gene	Forward primer 5′-3′	Reverse primer 5′-3′	Size (bp)	Accession no.
ADAM12	tttgggggtcaacagttttc	agagctgggttccctttgt	191	NM_003474
AREG	tggggaaaagtccatgaaaa	tttcgttcctcagcttctcc	174	NM_001657
AGN2	ccacctgaggaactgtctcg	ggtcttgctttggtccgtta	191	NM_001147
ER	catatgggagaaggggagt	aagtgcaattacagagtgcaaaa	166	NM_001432
JAGL1	gcctgccttaagtgaggaaa	gccaagaacaacacatcaaaga	169	U77914
LAMA5	gtgatgaaaagcgggaatgt	acctccacagagcgagtcat	221	BC003355
NOV	tgcaattccaagaaaatatcactg	cttggatttggagcttggaa	167	NM_002514
PDGF-A	acacgagcagtgtcaagtgc	tctggttggctgctttaggt	250	X03795
PN	cactctttgctcccaccaat	tcaaagactgctcctcccata	157	AY140646
RL	tgctgaatttggggctactt	gggagatagggtcttcatcca	198	NM_005045
SCG2	cccgaagaatgatgataccc	aaatgttgggatttgcttgg	195	NM_003469
β-actin	cacactgtgcccatctacga	ctccttaatgtcacgcacga	162	X00351

3.2.3 Human CCA tissues and immunohistochemistry

Fifty-two cases of CCA tissues were obtained from patients undergone hepatectomy at Srinagarind Hospital, Department of Surgery, Faculty of Medicine, Khon Kaen University. The obtained tissues were embedded in the paraffin and collected at Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University using the protocol approved by the Human Research Ethics Committee, Khon Kaen University. The age, sex, tumor size, histological type and staging data were retrieved from the medical charts and pathological records (Appendix B). Tumor size was assessed as the smallest diameter in the fresh specimen. Staging was classified as stage 1-4 using TNM classification of tumors of the liver and intrahepatic bile duct cancer, World Health Organization Classification of Tumours 2000 (Kleihues and Sobin, 2000). Histological types were graded as (1) Papillary adenocarcinoma shows a well-differentiated, distinctive papillary growth pattern lining with columnar or cuboidal tumor cells around the fibrovascular stalks. (2) Well-differentiated shows a uniform glandular structure lining with basally situated nuclei. (3) Moderately differentiated shows moderately distorted glandular or tubular patterns with cribriform formations and/or a cord-like pattern. (4) Poorly differentiated shows markedly distorted tubular structures with cellular pleomorphism.

Benign liver tissues were characterized as chronic inflammation by other causes rather than CCA such as cavernous haemangioma, chronic cholecystitis, and biliary cystadenoma (Appendix B).

For immunohistochemical staining, antigens in the paraffin-embedded tissues were retrieved in 10 mM citrate buffer pH 6.0 at 95°C for 40 min in heated water bath and endogenous peroxidase was blocked in 3% H_2O_2 for 5 min. Non-specific binding was blocked with 2% bovine serum albumin (BSA) for 20 min at room temperature. Rabbit anti-human PN polyclonal antibody at the dilution of 1:10,000 was applied to the sections and incubated overnight at room temperature. Then anti-rabbit Envision+ System-HRP labeled polymer was applied and incubated for 30 min at room temperature. The immunoreactive signal was developed by diaminobenzidine (DAB) and counterstained with hematoxylin. The signal was observed under light microscope.

PN expression of intratumoral fibroblasts on the histological sections was semiquantitatively scored on the basis of the percentage of PN-positive fibroblasts and the immunostaining intensity. The number of positive fibroblast cells were classified as <10% (negative); 10-25% (+1); 26-50% (+2); and >50% (+3). The intensity of PN expression in fibroblasts was scored as no staining, 0; weak staining, 1; intermediate or focal weak and focal intense staining, 2; intense staining, 3. The interpretation of PN expression was performed by multiplying the scores of the percent positive cell (0-3) and the scores of staining intensity (1-3) to reach the final score of 0-9. The results were then categorized as follows; low expression, score 1-4; and high expression, score 5-9. All samples were anonymized and independently scored by one pathologist and 2 investigators. In case of disagreement, the slides were reexamined and a consensus was reached by at least 2 observers. The expression of PN in 4 cases of hepatocellular carcinoma tissues and 8 cases of benign liver tissues were also explored using the same protocol. The expression level of PN in CCA were compared to that in these 2 related conditions. In addition, clinicopathological correlation to PN level was analyzed by suitable statistical tests.

3.2.4 Double immunofluorescence staining of CCA tissues

In order to localize PN and α-SMA expression in CCA tissues, double immunofluorescence staining was performed. The 1:200 mouse anti-human α-SMA

monoclonal antibody and 1:500 rabbit anti-human PN polyclonal antibody diluted in 1X PBS were used as primary antibodies and incubated with the tissues overnight at room temperature. The secondary antibodies including 1:2,000 goat anti-mouse IgG-Alexa 488 and 1:500 goat anti-rabbit IgG-Cy3 diluted in 1X PBS were used corresponding to their specific primary antibodies. Tissue sections were rinsed with 1X PBS before adding the secondary antibodies and incubated for 30 min at room temperature with light protection. Tissues were then washed out the excess secondary antibodies by 1X PBS. To stain the nucleuses, 1:500 Hoechst 33258 diluted in 1% BSA was added onto the tissues and incubated for 30 min at room temperature with light protection. The stained tissues were mounted with 50% glycerol containing 1X PBS. The fluorescence signal was observed under the LSM 510 Meta laser scanning confocal microscope at the Division of Medical Molecular Biology, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University. The fluorescence signal was stabilized at 4°C with protection from light until 1 month.

3.2.5 Total protein extraction and western blot analysis

Cells at the mid-exponential phase of the growth curve and having more than 95% cell viability were trypsinized as standard protocol. Cell pellet was collected after centrifugation the cell suspension at 400 g for 5 min in refrigerated centrifuge. The cell pellet was rinsed by cold 1X PBS for 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. The ratio of number of cells and volume of sample buffer was 5 x 10^5cells per 100 µl. Cell lysate was boiled for 10 min and centrifuged to get rid the undissolved proteins and cell debris at 8,000 g for 1 min. The 20 µl of cell lysate was then separated in 10% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. The electrophoresis was performed at 120 V for 1 h 30 min to seperate protein and at 38 mA (48 cm^2 PVDF membrane) for 1 h to blot proteins onto PVDF membrane using semi-dry blotting. Membrane was blocked in 5% skim milk containing TBST (150 mM NaCl, 6 mM Tris pH 7.4 and 0.1% (v/v) Tween 20) at 4°C overnight. The 1:500 rabbit anti-human PN antibody was added to the membrane and incubated at 4°C

overnight. Membrane was washed with TBST for 3 times for 5 min each before adding 1:1,500 goat anti-rabbit-IgG-HRP and incubated for 1 h at room temperature.

For phospho-AKT (pAKT) and phospho-ERK1/2 (pERK1/2) detections, the total protein was separated in 8% SDS-PAGE at 120 V for 1 h 30 min. Mouse anti-human pAKT monoclonal antibody at the dilution of 1:1,000 and rabbit anti-human pERK1/2 polyclonal antibody at the dilution of 1:2,000 were used as primary antibodies by incubating with the membrane for 1 h at room temperature. The 1:1,000 rabbit anti-mouse IgG-HRP and 1:2,000 goat anti-rabbit IgG-HRP were used as secondary antibodies against their specific first antibodies and incubated for 1 h at room temperature.

The immunoreactive signals of PN, pAKT and pERK1/2 were visualized by enhance chemiluminescense (ECL). The β-actin protein level was used as an internal control to determine the equal amount of loading proteins.

3.2.6 Cell proliferation assay

CCA cell lines were plated into 96-well plate using 1,000 cells/well and let them settle down in complete medium for 24 h. Cells were washed twice with 1X PBS and then 1% FBS containing HAM F-12 was added and incubated for 12 h to arrest cells in G1 phase of the cell cycle. Recombinant PN (rPN) with various concentrations including 1, 10 and 100 ng/ml was added and incubated with cells for 6, 12 and 24 h. At the ending time, the viable cells in each condition were determined using 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium solution (MTS) according to the manufacturer's instruction. Briefly, 20 µl of MTS solution was added into each well which already contained 100 µl of culture medium. Then, the plates were incubated for 2 h at 37°C in 5% CO_2 incubator to let the dehydrogenase enzyme in the viable cells to change the color of MTS tetrazolium (yellow) to formazan product (purple). The amount of soluble formazan was measured after adding 25 µl of 10% SDS to stop the enzymatic reaction. The absorbance at 490 nm was measured using an Anthos HTII ELISA reader and converted to number of viable cells using standard curve of each cell type (Appendix D). The number of viable cells in the condition of treatment with rPN was compared to those without treatment. The assays were performed in triplicate and two independent experiments were repeated.

To explore the effect of rPN on cells with transient knockdown *ITGα5*, cells with or without transfection of si*ITGα5* were treated with 100 ng/ml rPN and incubated for 12 h. Cell viability was measured by MTS assay as mentioned above. The assays were performed in triplicate and two independent experiments were repeated.

3.2.7 Cell cycle analysis

To address the effect of PN on the distribution of CCA cells in cell cycle, cell cycle distribution analysis was performed by flow cytometry as previously described (Thuwajit C et al., 2004). PN-treated CCA cell lines were trypsinized and 1 x 10^6 cells were collected by refrigerated centrifugation at 400 g for 5 min and washed with cold 1X PBS for 2 times. After that, ice cold 70% EtOH was added and chilled at -20°C for at least 15 min or overnight until used (if needed). The suspension was centrifuged and cell pellet was washed with cold 1X PBS. The 100 µl of 1X PBS containing 0.2 µl of 50 mg/ml DNase-free RNaseA were added and incubated in heat box at 37°C for 30 min to eliminate RNA. Cell pellet was collected after centrifugation and 50 µl of 1 mg/ml PI solution was added and incubated on ice with light protection for at least 30 min. Cells were then analyzed their distributions in the cell cycle by Becton Dickinson FACSort and CellQuest software. Numbers of CCA cell lines in G0/G1 and S+G2/M phases were measured in the condition of rPN treatment compared to those without rPN. Cells cultured in 0% and 10% FBS containing media were used as the negative and positive controls, respectively.

3.2.8 Colony formation assay

To ensure the capability of PN to induce cancer cell growth *in vitro*, the anchorage growth-independent and anchorage growth-dependent were performed by colony formation assay with and without soft agar, respectively. For colony formation assay without soft agar, CCA cell lines including KKU-M213 (250 cells/well) and KKU-M156 (125 cells/well) were seeded and cultured in 6-well plate for 24 h to let cells adhered to the culture plate. One hundred ng/ml of rPN diluted in 1% FBS containing medium was added and incubated in CO_2 incubator for 12 d. For soft agar colony formation assay, 1 ml of 0.5% MetaPhor® agarose was plated onto 6-well plate as the lower layer. After the lower layer agar was polymerized, 250 CCA

cells diluted in 0.35% MetaPhor® agarose containing 100 ng/ml rPN were added on-top of lower layer agar and cultured for 12 d. Cells were fixed with 5% (v/v) glutaraldehyde and 0.5% (w/v) crystal violet in 40% (v/v) methanol for 30 min each. Cell growth was estimated by counting number of colonies having more than 30 cells under inverted microscope and compared the numbers of colony between those of treated and untreated with rPN. The experiments were performed in replicate wells.

3.2.9 Invasion assay

To investigate the effect of rPN on CCA cell invasion, 2 x 10^4 cells of KKU-M213 and 4 x 10^4 cells of KKU-M156 were suspended in 100 ng/ml rPN containing complete medium and cultured in the upper chamber of the Matrigel™ invasion chamber for 24 h. Invaded cells were fixed with 5% (v/v) glutaraldehyde and 0.5% w/v crystal violet in 40% (v/v) methanol for 30 min each. The number of invaded cells was counted under an inverted microscope by two independent investigators using 100x magnification field. The assays were done in replicate and three independent experiments were performed. Numbers of invaded cells in the condition of no PN were measured and compared to those of treated with rPN.

3.2.10 Small interfering RNA against receptor ITGα5

CCA cells of around 1.5 x 10^5 cells were seeded into 6-well culture plate and cultured in complete medium for 24 h. Cells were switched to antibiotics-free 10% FBS containing medium for 24 h before transfection with 100 pmole si*ITGα5* (3 strands mixture) using Lipofectamine 2000 as manufacturer's instruction. In this case, 5 μg lipofectamine containing OptiMEM I were incubated with cells for 6 h, then switched to the complete medium to recover cells to grow in normal condition. The decreasing of *ITGα5* expression in both mRNA and protein levels were confirmed by real time PCR and immunocytochemistry, respectively. The efficiency of knockdown was normalized using *β-actin* as an internal control. The transient knockdown *ITGα5* CCA cell lines were then explored their responses to PN-induced proliferation, invasion, and pAKT and ERK1/2 detection by MTS, invasion assay and western blot analysis, respectively, with the methods mentioned above.

3.2.10.1 Real time PCR of *ITGα5*

To confirm the transient knockdown of *ITGα5* after transfection with si*ITGα5,* real time PCR of *ITGα5* was performed. Total RNA was

extracted from CCA cell lines in both with and without transfection followed protocol of PerfectPure RNA Culture Cell Kit. Four μg of total RNA was used to synthesize cDNA using Superscript III First-Strand Synthesis kit according to the company's instruction. The expression level of *ITGα5* was determined by SYBR Green-based real time PCR in 10-μl reaction containing 50 ng of cDNA, 1X LightCycler® 480 SYBR Green I Master, 0.5 μM each primer and 3.5 mM $MgCl_2$. The reaction was performed in LightCycler® 480 system at the Division of Medical Molecular Biology, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University.

The nucleotide sequences of human *ITGα5* were retrieved from PubMed (www.ncbi.nln.nih.gov) and the primers were designed by Primer 3 program (Table 3-5). The expression of *β-actin* was measured and served as an internal control to adjust the amount of starting cDNA of different samples. The relative expression of *ITGα5* in cells with si*ITGα5* treatment compared to that of the parental cells without si*ITGα5* treatment was calculated as the following equation:

$$\text{Relative gene expression} = 2^{-\Delta\Delta C_T}$$

$$\text{In this case, } \Delta C_T = C_T \text{ (}ITG\alpha5\text{)} - C_T \text{ (}\beta\text{-}actin\text{)}$$

$$\Delta\Delta C_T = \Delta C_T \text{ (cells with si}ITG\alpha5 \text{ transfection)} - \Delta C_T \text{ (parental cells)}$$

Table 3-5 Primer and PCR condition for *ITGα5* detection

Forward primer 5′-3′	**Reverse primer 5′-3′**	**Size (bp)**	**Accession no.**
agttgcatttccgagtctgg	ccaaacaggatggctaggat	223	NM_002205
	PCR condition		
Denaturing	95°C, 10 sec		
Annealing and extension	60°C, 45 sec		
Amplification cycle	60		

3.2.10.2 Flow cytometry for measurement of ITGα5

For detection of the actual ITGα5 level in cell treated with and without si*ITGα5*, cell pellet of around 1 x 10^6 was fixed with 2% formaldehyde for 15 min at room temperature or overnight at 4°C (if needed). The fixed cells were

incubated with 1:50 goat-anti human ITGα5 polyclonal antibody diluted in 100 µl washing solution which was HAM F-12 containing 2% (v/v) FBS, 1% (w/v) BSA and 10 mM NaN_3 for 2 h at room temperature. Cells were centrifuged in 1 ml washing solution at 400 g for 3 times and 5 min each to get rid of the excess primary antibody and followed by staining with 1:2,000 donkey-anti goat IgG-Alexa 488 diluted in 100 µl washing solution for 1 h at room temperature with light protection. Cells were centrifuged in 1 ml washing solution at 400 g for 2 times and 5 min each to get rid of the excess secondary antibody. The ITGα5 signal was determined in FL-1 channel of Becton Dickinson FACSort and data analysis was performed by CellQuest software. The relative Mean Fluorescence Intensity (MFI) of CCA cell lines was normalized to that of the negative control stained with only secondary antibody. The MFI of ITGα5 in cells with and without si*ITGα5* transfection were compared. Two independent experiments were performed.

3.2.10.3 Immunocytochemistry for ITGα5 expression

Immunocytochemistry was employed to localize the position of ITGα5 on the cell membrane. KKU-M213 (2 x 10^4 cells) were cultured on sterile cover slip placed in 24-well plate for 48 h. Cells were fixed with 4% paraformaldehyde for 15 min and blocked with 1% BSA for 30 min at room temperature. Then cells were incubated with 1:50 goat anti-human ITGα5 diluted in 1% BSA for 2 h at room temperature and subsequently stained with 1:500 donkey-anti goat IgG-Alexa 488 diluted in 1% BSA for 1 h at room temperature with light protection. Nucleus was stained with Hoechst 33258. The fluorescence signal was observed under the LSM 510 Meta laser scanning confocal microscope at the Division of Medical Molecular Biology, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University.

3.2.11 Neutralization of ITGα5β1 on CCA cells

To ensure role of ITGα5 on PN-induced CCA cell invasion, neutralizing antibody specific to ITGα5β1 heterodimer was employed to block intact ITGα5β1 on the cell membrane of CCA cells. KKU-M213 CCA cells were trysinized and washed with 1X PBS for 2 times. Cell pellet around 1 x 10^5 cells were incubated with 200 µl of 1:100 anti-human ITGα5β1 antibody diluted in serum-free media at 37°C for 1 h. Without clumping phenomenon caused by cell-antibody complex

observed in hemecytometer under light microscope, cells in antibody solution were centrifuged at 400 g for 5 min to get rid of excess antibody. ITGα5β1-blocked cells were then collected to explore their responses to PN-induced invasion using invasion assay protocol described above. The numbers of invaded cells induced by PN were compared between with and without anti-ITGα5β1 blocking condition. The experiment was performed in duplicated wells.

3.2.12 Statistical analysis

Statistical analyses in this study were performed using SPSS version 16.0. The correlation of PN expression and pathological parameters of CCA patients was analyzed by the $\chi 2$-test. Patient survival was calculated from the time of surgical resection to death and the survival curves were constructed according to Kaplan-Meier, with a Log-Rank test. A multivariate analysis was performed by the Cox Proportional Hazard Regression model. The significance of the different sets of data was determined by the Student's t-test. A P value of equal to or less than 0.05 was statistically significant.