รายงานวิจัยฉบับสมบูรณ์

การศึกษาบทบาทของ metastasis suppressor protein RECK (Reversion-inducing cysteine-rich protein with Kazal motifs) ในการยับยั้งการเจริญเติบโตและการแพร่กระจายของเซลล์มะเร็งท่อน้ำดี

Role of metastasis suppressor RECK (Reversion-inducing cysteine-rich protein with Kazal motifs) in the inhibition of cellular proliferation and invasion in cholangiocarcinoma cell lines

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1 มีนาคม 2555

สัญญาเลขที่ **RSA5280007**

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สังกัด

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สนับสนุนโดย

สำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

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Nisana Namwat, Ph.D.

Abstract

Project Code : RSA5280007

Project Title : Role of metastasis suppressor RECK (Reversion-inducing cysteine-rich protein with Kazal motifs) in the inhibition of cellular proliferation and invasion in cholangiocarcinoma cell lines

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Cholangiocarcinoma (CCA), a primary cancer of the bile duct epithelium, primarily associated with chronic infection with the liver fluke, *Opisthorchis viverrini* (Ov) that causes long-standing inflammation, the hallmark of carcinogenesis. Thus molecular mechanisms of CCA genesis and progression need to be studied in order to identify molecular targets for chemoprevention and treatment. Our study aimed to investigate the molecular pathways and the roles of biomolecules including the metastasis suppressor RECK (reversion-inducing cysteine rich protein with Kazal motifs) and miR-21 (an oncogenic microRNA), which involve in chronic inflammation associated CCA progression.

Our data showed that chronic inflammation persistently occurred in CCA tissues presented by increasing of iNOS expression. The status of inflammation-related cytochrome P40, CYP2A6 and CYP2E1, were aberrant according to an increase of oxidative stress. Liver tissues of Ov plus NDMA treated hamsters showed high RECK expression in hyperplastic biliary duct epithelia, low RECK expression in precancerous lesions and no RECK expression in CCA. In human specimens, RECK was highly expressed in normal biliary cells, whereas intrahepatic CCA showed low levels of expression. Down-regulation of RECK correlated with tumor metastasis ($P < 0.01$) and shorter patient survival ($P < 0.02$). RECK expression levels were inversely correlated with MMP-2 and MMP-9 expression ($P < 0.05$). SiRNA RECK-depleted M139 CCA cells exhibited increased MMP-2/-9 gelatinase activities and invasiveness. Non-selective COX inhibitor, aspirin (500 μM), demonstrated myriad effects in human CCA cell line, including growth suppression, enhanced phosphorylation of Akt/Erk/c-Jun, elevation of RECK expression, inhibition of MMP-2/-9 activity, and enhanced invasiveness.

Besides the altered profile of RECK, miR-21 expression was significantly upregulated in liver tissues of Ov plus NDMA treated hamsters at 2 months (hyperplastic lesion) and at 6 months when CCA had developed. MiR-21 was also upregulated in human CCA tissues and CCA cell. In patient's CCA tissues which miR-21 has been increased, we found that miR21 targeting genes such as the programmed cell death 4 (PDCD4) and RECK, had weaker staining in CCA tissues compared to the normal biliary duct. Knocking down miR-21 caused the reduction of CCA cells growth and migration ability. Cells with suppressing miR-21 showed an increase in expressions of many genes including STK40, KLHL15, GPR64, ZTBT47, CYBD1, FBX2, FNIP1, SOX7, APAF1, TNRC6B, TPRG1L, SH3GLB1, SOX6, GPR180 and ANKRD46, which possibly controls cancer growth and metastasis.

Our data suggest that the molecular biology network of miR-21 might play role in the genesis Ov-associated CCA. Modulation of aberrantly expressed miR-21 and miR-210 may be a useful strategy to inhibit tumor cell phenotypes or improve response to chemotherapy including prevention of carcinogenesis.

Keywords : cholangiocarcinoma, RECK, metastasis, NSAIDs, miR-21

บทคัดย่อ

รหัสโครงการ : RSA5280007

ชื่อโครงการ : การศึกษาบทบาทของ metastasis suppressor protein RECK (Reversion-inducing cysteine-rich protein with Kazal motifs) ในการยับยั้งการเจริญเติบโตและการแพร่กระจายของเซลล์มะเร็งท่อน้ำดี

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มะเร็งท่อน้ำดี เป็นโรคมะเร็งปฐมภูมิของเซลล์เยื่อบุผิวท่อน้ำดี สาเหตุเกิดจากการติดเชื้อพยาธิใบไม้ตับทำให้เกิดการอักเสบเรื้อรังซึ่งนำไปสู่กระบวนการก่อมะเร็ง การศึกษากลไกในระดับโมเลกุลของมะเร็งท่อน้ำดีจะทำให้ทราบโมเลกุลเป้าหมายที่จะนำมาประยุกต์ในการป้องกันและรักษาได้ วัตถุประสงค์ในการศึกษาครั้งนี้ เพื่อค้นหาวิถีและบทบาทของโปรตีน RECK (reversion-inducing cysteine rich protein with Kazal motifs) ซึ่งเป็น metastasis suppressor และไมโครอาร์เอ็นเอ miR-21 ซึ่งเป็น oncogenic microRNA โดยเกี่ยวข้องกับกระบวนการก่อมะเร็งท่อน้ำดีที่สัมพันธ์กับการอักเสบเรื้อรัง

ผลการศึกษาแสดงพบว่า ในเนื้อเยื่อมะเร็งท่อน้ำดีมีการเพิ่มการแสดงออกของโปรตีน iNOS ซึ่งเกี่ยวข้องกับกระบวนการอักเสบเรื้อรัง สภาวะการอักเสบเรื้อรังสัมพันธ์กับการทำงานที่ผิดปกติของเอนไซม์ ไซโทโครม พี 450 ชนิด CYP2A6 และ CYP2E1 ซึ่งเกี่ยวข้องกับการคะตะไลส์สารก่อมะเร็งชนิด NDMA การทดลองในแฮมสเตอร์ที่เหนี่ยวนำให้เกิดมะเร็งท่อน้ำดีด้วยการติดเชื้อพยาธิใบไม้ตับ Ov ร่วมกับให้สารก่อมะเร็ง NDMA พบว่า โปรตีน RECK มีระดับเพิ่มขึ้นในเซลล์เยื่อบุผิวท่อน้ำดีที่เปลี่ยนแปลงเป็น hyperplasia จากนั้นจะมีปริมาณลดลงในระยะที่เป็น precancerous lesions และไม่มีการแสดงออกเลยในระยะที่เป็นมะเร็ง การศึกษาในเนื้อเยื่อมะเร็งของผู้ป่วยมะเร็งท่อน้ำดียืนยันว่าโปรตีน RECK มีระดับการแสดงออกลดลงอย่างมีนัยสำคัญเมื่อเปรียบเทียบเนื้อเยื่อตับบริเวณข้างเคียงและสัมพันธ์กับสภาวะการแพร่กระจาย ($P < 0.01$) และอัตราการรอดชีพที่สั้นลง ($P < 0.02$) ระดับการแสดงออกของโปรตีน RECK สัมพันธ์กับการแสดงออกของเอนไซม์ MMP-2 และ MMP-9 ($P < 0.05$) การทดลองโดยการใช้ siRNA ยับยั้งการทำงานของ RECK mRNA ในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีชนิด KKU-M139 พบว่าทำให้เพิ่มการทำงานของ MMP-2 และ MMP-9 ในการย่อยเจลาทิน และเพิ่มการบุกรุกของเซลล์มะเร็งอีกด้วย การใช้ยาต้านอักเสบชนิด aspirin ขนาด 500 μM ต่อเซลล์มะเร็ง พบว่าสามารถยับยั้งการเจริญเติบโต ลดปริมาณฟอสเฟตโปรตีนชนิด Akt/Erk/c-Jun เพิ่มปริมาณโปรตีน RECK ยับยั้งการทำงานของเอนไซม์ MMP-2/-9 และยับยั้งการบุกรุกของเซลล์มะเร็งในหลอดทดลองได้

การศึกษานี้ได้ศึกษาเกี่ยวกับ ไมโครอาร์เอ็นเอชนิด miR-21 ที่สามารถควบคุมการแสดงออกของยีน RECK โดยพบว่า การแสดงออกของ miR-21 เพิ่มขึ้นในเนื้อเยื่อตับของแฮมสเตอร์ที่ถูกเหนี่ยวนำให้เป็นมะเร็งท่อน้ำดี ในระยะ 2 เดือน (hyperplastic lesion) และ 6 เดือน (CCA) ยังพบว่า miR-21 เพิ่มขึ้นในเนื้อเยื่อมะเร็งท่อน้ำดีของผู้ป่วยและมีความสัมพันธ์กับการลดลงของโปรตีน RECK และ PDCD4 การศึกษาด้วย siRNA ยับยั้งการทำงานของ miR-21 ในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี พบว่า สามารถยับยั้งการเจริญเติบโตและการแพร่กระจายของเซลล์มะเร็งได้ นอกจากนี้ยังมีผลต่อระดับการแสดงออกที่เพิ่มขึ้นของยีนจำนวนหลายชนิดได้แก่ STK40, KLHL15, GPR64, ZTBT47, CYBD1, FBX2, FNIP1, SOX7, APAF1, TNRC6B, TPRG1L, SH3GLB1, SOX6, GPR180 and ANKRD46 ซึ่งเกี่ยวข้องในการควบคุมการเจริญเติบโตและการแพร่กระจายของเซลล์มะเร็ง

การศึกษาครั้งนี้สรุปได้ว่าเครือข่ายการทำงานของ miR-21 มีผลต่อการควบคุมกระบวนก่อมะเร็งท่อน้ำดีและการแพร่กระจายของเซลล์มะเร็ง หากสามารถจัดการลดระดับการทำงานของโปรตีนที่อยู่ภายใต้เครือข่ายของ miR-21 ได้ ก็น่าจะมีความเป็นไปได้ในการป้องกันและรักษาโรคมะเร็งท่อน้ำดีให้มีประสิทธิภาพดีขึ้นได้ในอนาคต

คำหลัก : มะเร็งท่อน้ำดี RECK กระบวนการแพร่กระจาย ยาแอสไพริน miR-21

INTRODUCTION

Cholangiocarcinoma (CCA) is a primary bile duct cancer that is associated with chronic infection with the liver fluke *Opisthorchis viverrini* [1]. The incidence of CCA is increasing globally and carries a high mortality [2]. A hamster model of CCA has provided valuable insights into pathogenesis, showing that tumor formation is induced by infection with O. viverrini in combination with oral intake of a carcinogenic *N*-nitroso compound (N-nitrosodimethylamine; NDMA) [3]. Notably, NDMA, a product of nitrosation, was detected in urine of *O. viverrini*-infected subjects and appeared to be associated with lymphoproliferative responses to liver fluke antigen [4]. Nitric oxide (NO) production and endogenous nitrosation were demonstrated within the inflamed bile ducts of humans with opisthorchiasis [1]. NO is generated from L-arginine by NO synthase (NOS), which is induced by pro-inflammatory cytokines, and acts as a mediator of host protection in opposition to invading parasites [5, 6]. NO is also addressed as a central molecule linking *Opisthorchis viverrini* infection and CCA progression via chronic inflammation [7-9]. Satarug and coworkers showed that subjects infected with *O. viverrini* had a significant increase in cytochrome P450 2A6 activity, the major liver enzyme that biotransforms NDMA into a carcinogen [10]. Chronic inflammation in O. viverrini infected persons is correlated with elevated levels of oxidative and nitrative DNA adducts detected in urine [11] and this phenomenon is persistently found in tumor tissues of CCA patients [12]. Increased CYP2A6 gene expression and activity are also found in hepatocellular, bronchogenic and oesophageal carcinomas, all of which are associated with chronic inflammation [13-15]. High expression of CYP2A5 (an orthologue of human CYP2A6) in mouse hepatomas is associated with an increase in coumarin 7-hydroxylation, a marker activity of CYP2A5, and the corresponding mRNA, suggesting that regulation of CYP2A5 in hepatomas is pretranslational [16]. Overexpression of CYP2A6 in both mRNA and protein has also been found in human colorectal adenocarcinomas, carcinomas in adenoma, and adenomas than control tissue, signifying that it might play role in tumorigenesis and tumor progression [17]. CYP2E1, the major xenobiotic metabolizing enzyme that catalyzes ethanol [18], is expressed in low levels in hepatocellular carcinoma, and is correlated with an aggressive phenotype [19, 20]. Low CYP2E1 activity has been shown to be related to oxidative stress during aging and ischemia/reperfusion in rats [21, 22].

At present, only surgical resection of all detectable tumor leads to an improvement in five-year survival. However, complete resection is often impossible and typically results in subsequent local recurrence and metastasis. In order to prevent post-surgical recurrence,

chemotherapy is a preferred adjunctive treatment. Thus molecular mechanisms of CCA genesis and progression need to be studied in order to identify tumor biomarkers for chemoprevention and treatment.

The 110-kDa metastasis suppressor RECK (reversion-inducing cysteine rich protein with Kazal motifs) was initially discovered by its ability to induce reversion in v-Ki-ras-activated fibroblasts [23]. RECK is a protease inhibitor-like molecule that is anchored to the plasma membrane, and can be detected in a wide variety of normal human tissues [23]. The key action of RECK is to regulate matrix metalloproteinases (MMPs) involved in the breakdown of extracellular matrix (ECM) and angiogenesis [24]. RECK regulates MMP-2 and MMP-9 by inhibiting secretion and proteolytic activity of these enzymes [24]. TGF-β signaling in activated pancreatic stellate cells promotes ECM accumulation via a mechanism that preserves the protease inhibitory activity of RECK [25].

Down-regulation of RECK has been found in many types of solid tumors such as pancreas, breast, lung, colorectal, prostate, stomach, liver, and bone [26], and frequently is correlated with a poor prognosis [27]. Down-regulation of RECK has been reported in a hamster carcinogenesis model of oral cancer [28]. Suppression of RECK mRNA expression has been reported in human hilar CCA [29]. Repression of RECK levels is associated with an increase in MMP-2 and MMP-9 activities [30]. Suppression of RECK gene expression in cancer is possibly mediated via the oncogenic Ras signaling pathway through inhibition of the Sp1 promoter site of the RECK gene [31].

NSAIDs, including O-acetylsalicylate (aspirin, a non-selective COX inhibitor) and NS-398 (a COX-2 inhibitor), as well as trichostatin A (TSA, a histone deacetylase inhibitor), can up-regulate RECK gene expression in CL-1 human lung cancer cell line and suppress MMP-2 activity [32, 33]. Thus, these agents may possess anti-metastasis effects through up-regulation of RECK expression.

MicroRNAs (miRNAs, miRs) are endogenous regulatory RNA molecules that modulate gene expression. Alterations of miRNA expression can contribute to tumor growth by modulating the expression of critical genes involved in carcinogenesis. The extensive researches of miRNA profile have shown to be a useful tool for diagnosis and prognosis in many cancers. Many studies demonstrated specific miRNA profiles in CCA in both CCA cell lines and human CCA tissues. They found the altered expressions of oncogenic and tumor suppressor miRNAs that involved in regulation of human CCA when compared with the normal cells. One of the most abundant microRNAs that highly expresses in all types of cancer is microRNA-21 (miR-21) including CCA, strongly suggesting for its functions in the control of

growth and metastasis [34] [35]. In addition, the direct targets which play roles in tumor suppression and metastasis inhibition of these miRNAs have been identified, for instance, phosphatase and tensin homolog (PTEN, Programmed cell death protein 4 (PDCD4) and Metalloproteinase inhibitor 3 (TIMP3) as direct targets of oncogenic miR-21 and the evidence has been uncovered in CCA [36]. Beside this, miR-21 was overexpressed in CCAs and played roles in cell proliferation and anti-apoptosis functions. Recently RECK have been proved that it is a direct target of miR-21, found in a human glioma cell line [37]. It is noted that the regulatory network of miR-21 and its direct target (especially RECK) in Ov-associated CCA has not been discovered. Modulation of aberrantly expressed miR-21 may also be a useful strategy to inhibit cell proliferation and metastasis for improving the CCA prevention and treatment.

OBJECTIVES

1. To determine whether human CCA exhibits altered levels of CYP2A6 and CYP2E1 expression and activity, and to assess whether the progression of CCA in association with prolonged inflammation affects liver CYP2A6 and CYP2E1 status.
2. Little is known concerning the role of RECK in Ov-associated CCA. This study was also to investigate RECK expression during Ov-induced CCA development in the hamster model, as well as to assess the prognostic value of RECK expression in Ov-associated human intrahepatic CCA. An additional aim was to investigate the functional effects of modulation of RECK by siRNA and an NSAID (aspirin) in CCA cell lines.
3. The miR-21 expression profiles associated with cholangiocarcinogenesis in hamster model have not yet been investigated. The altered miR-21 expression profile was provided for the discovery of the interaction between miRNA and mRNA network by which miRNAs regulate Ov-associated CCA.

METHODS

Liver samples

Liver samples were obtained from CCA patients (8 men and 5 women) ranging in age from 44-64 years who underwent therapeutic hepatectomy. CCA was confirmed histologically. The inclusion criteria included non-metastatic tumors seen as a single mass. The liver was divided and 2 cm long slices were obtained from 2 distinct zones: adjacent area of tumor (J) and farther zone (N). The control group (n=10) was comprised of liver tissue obtained from

cadaveric donors whose kidneys were harvested for transplantation. The Ethics Committee of the Faculty of Medicine, Khon Kaen University approved the study protocol, according to Helsinki criteria (HE43172). Informed consent for obtaining and using liver tissue was obtained from all patients and donors' relatives.

Animals and tumor induction

Induction of CCA in male Syrian golden hamsters was performed by Ov metacercariae infection combined with *N*-nitrosodimethylamine (NDMA) treatment as previously described [3]. The animal experiments were conducted according to the guidelines of the National Committee of Animal Ethics. The protocol was approved by the Animal Ethics Committee of the Faculty of Medicine, Khon Kaen University, Thailand. In brief, out-bred Syrian golden hamsters (*Mesocricetus auratus*), ranging from 6 to 8 weeks of age and weighing approximately 100 g, were arbitrarily divided into 2 groups: Group 1 remained untreated; Group 2 was infected with 50 Ov metacercariae and treated with NDMA (Sigma, St. Louis, MO) (12.5 ppm given in drinking water) for 8 weeks. Each hamster received approximately 0.166-0.04 mg/day of NDMA. After treatment, five animals per group were sacrificed at weeks 1, 4, 12 and 24. Liver tissue from each animal was fixed in 10% (v/v) neutral buffered formalin and embedded in paraffin for histological and immunohistochemical examinations.

Patients and samples

Forty paraffin-embedded and 24 frozen intrahepatic CCA specimens from primary tumors of patients, collected during 2002 to 2004, were obtained from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki and its later revision. Human Research Ethics Committee, Khon Kaen University (#HE43201 and #HE471214), approved the research protocol.

Cell culture, chemicals and reagents

Three types of cell lines were studied: (1) human intrahepatic CCA cell lines M055, M139, M156 and OCA17 established at Khon Kaen University Liver Fluke and Cholangiocarcinoma Research Center; (2) human bile duct carcinoma cell lines HucCT1 and EGI-1, from the National Cancer Center of Singapore; and (3) human cholangiocyte MMNK1 cell line (transduced with SV40T and hTERT) [22] from Prof. Naoya Kobayashi (Okayama

University, Japan). All cell lines were cultured in Ham's F12 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37^0C with 5% CO_2. *O*-acetylsalicylate was purchased from Sigma (St. Louis, MO), rabbit polyclonal anti-RECK and anti-MMP-9 antibodies from Abcam (Cambridge, MA), mouse monoclonal anti-MMP-2 antibodies from Calbiochem (San Diego, CA), mouse monoclonal antibodies to Cyclin D1and CDK4 from Cell Signaling (Boston, MA), and peroxidase-conjugated Envision secondary antibody from DAKO (Glostrup, Denmark). All other chemicals used were of analytical grade. Cancer PhosphoELISArray multi-analyte kit (FEM-5001H) was from SABioscience Corporation (Frederick, MD).

Preparation of liver microsomes

Human livers were immediately excised after resection, snap frozen and stored at -80 $^{\circ}$C before use. Microsomes were isolated by differential centrifugation as described in Tassaneeyakul et al. [38].

CYP2A6 and CYP2E1 activities

CYP2A6 activity was assessed in liver microsomes by measuring coumarin-7-hydroxylation and the product quantified by luminescence spectrometry as described in Koenigs et al. [24]. The CYP2E1 activity was determined by the assay for chlozoxazone-6-hydroxylation primarily as described in Tassaneeyakul et al.[38].

Liver CYP2A6 and CYP2E1 protein levels

Ten μg of microsomes was added to 0.125 mM PMSF, 0.5 mM EDTA and 2% (w/v) deoxycholic acid prior to separation and quantification using western blot analysis as described by Towbin et al [25]. Blots were then developed using a polyclonal antibody to hepatic CYP2A6 (1:2000; Gene Tex, San Antonio, TX) and CYP2E1 (1:2000; provided by Prof. John Miners, Department of Clinical Pharmacology, Flinders University, Australia). The detection of immobilized specific antigen on membranes was performed using an indirect method with horseradish peroxidase (HRP) labeled secondary antibody. The bands were detected by enhanced chemiluminescence (ECL). Relative quantification of immunoreactive CYP2A6 and CYP2E1 in immunoblots was accomplished with an UltroScan XL device (Pharmacia LKB Biotechnology, Uppsala, Sweden). Gel-to-gel variation was standardized by loading pooled microsomes into each gel. Intensity signals of each sample were corrected to those of the pooled microsomes before being compared to other samples.

RNA extraction and RT-PCR reaction

Total RNA was extracted from liver samples using an RNAeasy Mini Kit (QIAgen, Hilden, Germany), following the manufacturer's instruction and quantified by spectrophotometry. Total RNA (2.5 μg) was reverse-transcribed using the oligo$(dT)_{15}$ primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). First-strand cDNAs were synthesized at 42°C for 60 min. PCR was carried out in Perkin Elmer Gene Amp PCR system 2400 (PE Applied Biosystems, Foster City, CA) using CYP2A6 primers (forward: TGCCATCAAGGATAGGCAAG, reverse: AATGCTGCAAAATGGCACAC) or CYP2E1 primers (forward: AATGCTGCAAAATGGCACAC, reverse: CATGAGGGAGTTGTACATCTGAA) and GAPDH primers (forward: CAACAGCCTCAAGATCATCAGC, reverse: TTCTAGACGGCAGGTCAGGTC). The amplification was initiated by a one cycle at 94°C for 3 min followed by 28 cycles of denaturation at 94°C for 1 min each, a 1-min annealing at 58°C and polymerization at 72°C for 1 min, with a final 10-min extension at 72°C. PCR products were separated on 1.2% agarose gels containing 100 ng/ml ethidium bromide. Gels were visualized and photographed and the DNA bands were analyzed with gelwork 1D advance analysis software. (Amersham Pharmacia Biotech, Piscataway, USA). The relative amount of mRNA of CYP2A6 or CYP2E1 was expressed as a ratio to GAPDH mRNA.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated from cells with Trizol reagent (Invitrogen, Carlsbad, CA), and 2 μg of RNA was reverse transcribed into cDNA using RevertAid M-MuLV reverse transcriptase (Fermentas, Burlington, CA) and random hexamers (Promega, Madison, WI). qPCR was performed with TaqMan Gene Expression Assay reagent kits (Applied Biosystems, Foster City, CA) in an ABI7500 Real Time PCR System (Applied Biosystems). The reaction mixture (20 μl) contained 10 μl of 2X Taqman mastermix (Applied Biosystems), 1 μl of TaqMan Gene Expression Assay (RECK: Hs01019179_m1; GAPDH: 436317E), 4 μl of diethylpyrocarbonate (DEPC)-treated water and 1:3 diluted cDNA. qPCR procedures were performed according to the manufacturer's instructions. Expression levels of target genes were calculated relative to that of GAPDH.

qRT-PCR for miRNA expression detection

MiRNAs are validated by TaqMan qRT-PCR analysis. For this, the cDNA from each candidate miRNA is synthesized by using the miRNA specific primers supplied by the

manufacturer (Applied Biosystems). Briefly, 100 ng of total RNA is taken for cDNA synthesis (TaqMan® MicroRNA RT Kit; Applied Biosystems) of each miRNA in a final volume of 20 µl by using the miRNA specific primers. U6 snRNA is used as an internal control. Relative expression is calculated using the $\Delta\Delta C_T$ method.

Immunohistochemical staining

Target proteins were detected on the paraffin-embedded sections using standard immunohistochemistry protocols. In brief, 4-µm-thick sections were incubated with appropreciate dilution of anti-human antibody for 1 h in a humidified chamber at room temperature. Sections were incubated with peroxidase-conjugated Envision secondary antibodies and peroxidase activity was visualized with DAB solution. Hematoxylin was employed for counterstaining. Staining frequency of proteins was semi-quantitatively scored based on percentage of positive cells as follows: 0% = negative; 1%-25% = +1; 26%-50% = +2; and >50% = +3. The intensity of protein staining was scored as weak = 1, moderate = 2, and strong = 3. For determination of biliary epithelial hyperplasia and dysplasia, hyperplastic ducts were defined as having increased cell numbers, enlargement of duct size and pseudopapillary protrusion, while dysplastic ducts were defined as having cells with multilayered nuclei, increased nuclear-cytoplasmic ratio and micropapillary projections.

Western blot analysis of protein content in cell culture

Cells (approximately $4x10^5$) were washed twice with cold phosphate-buffered saline and lysed with 30 mM Tris containing 7 M urea, 2 M thiourea, 4%(w/v) CHAPS and protease inhibitor cocktail (Roche, Mannheim, Germany) with homogenization for 5 min. Following centrifugation at 12,000 x *g* for 10 min at 4^0C, 10 µg of cell lysate were electrophoresed in 10% SDS-polyacrylamide gel, transferred to polyvinylidene fluoride membrane (Whatman, Dassel, Germany), then incubated with primary antibodies at 4^0C overnight and secondary antibodies at room temperature for 1 h. Peroxidase activity was detected using Enhanced Chemiluminescence Plus solution (GE Healthcare, UK). Band intensity was quantified with ImageQuant Imager and ImageQuant analysis software (GE Healthcare, UK)

Transient knock down by RNA interference

CCA cells were seeded in a 75 cm^2 flask for 48 h prior to transfection with human specific siRNA or anti-miR™ miRNA inhibitor (Ambion) according to the manufacturer's instructions. Specificity was validated using siRNA-containing random sequence (Ambion). In

brief, 1 h before transfection, cells (3×10^5) were trypsinized and resuspended in HAM-F12 medium supplemented with 10% FBS without antibiotics and set aside at 37^0C in water bath. Five μl aliquots of siPORT NeoFX Transfection Agent and 95 μl aliquots of OPTI-MEM® I medium (Invitrogen) were mixed together and the solution was incubated for 10 min at room temperature. Next, 7.5 μl of 10 μM stock concentration siRNA or scrambled siRNA was diluted into 92.5 μl OPTIMEM I medium and mixed gently. Diluted siPORT NeoFX Transfection Agent and diluted siRNA were combined and mixed by pipetting and further incubated for 10 min at room temperature to allow transfection complexes to form before being dispensed into 6-well culture plates. A 2.3 ml aliquot of 2×10^5 cell suspension was subsequently overlayed onto the transfection complexes and gently mixed. The final concentration of siRNA was 30 nM. Cells were cultured for 48 h to achieve complete transfection. Cell growth was determined by trypan blue staining and the level of RECK mRNA and protein was determined by qRT-PCR assay and western blotting, respectively.

Gelatin zymography

Cells were pre-treated with siRNA or aspirin in serum-free media for 48 h. Conditioned medium from an equal number of cells was collected, concentrated using Vivaspin MW50CO columns (GE Healthcare, UK) and separated by electrophoresis in 10% acrylamide gel containing 0.1% gelatin (Bio basic inc., Ontario, CA). The gel was washed twice with 2.5% Triton X-100 solution at room temperature, then once in distilled water for 10 min. The gel was soaked in developing buffer (0.5 M Tris-HCl (pH 8.8), 50 mM $CaCl_2$, 0.2% (w/v) NaN_3) at 37°C for 20 h, and then stained overnight with the following solution:(0.1% (w/v) Coomassie Brilliant Blue, 30% (v/v) methanol, and 10% (v/v) acetic acid). Destaining was then performed with the same solution except without Coomassie Brilliant Blue. Gelatinolytic activity of MMP was visualized as a clear band against a dark background of stained gelatin.

Cell invasiveness assay

A total of 4×10^4 siRECK/antimiR-21 transfected or aspirin treated cells were seeded onto the upper chamber of Transwell® pre-coated Matrigel-culture inserts (8 μm pore size, Becton-Dickinson, Franklin Lakes, NJ), and HAM-F12 medium supplemented with 10% (v/v) fetal bovine serum was placed in the lower chamber. After incubation at 37ºC for 18 h, cells in the upper surface of the filter were scraped off, and cells which migrated to the underside of the filter were fixed with absolute ethanol for 30 min, stained with Hematoxylin solution, and then

counted under a microscope. Mean value of all low-power fields (100 magnifications) was determined. Assays were done in triplicate and two independent experiments were conducted.

Cell growth assay

Sulforhodamine B (SRB) assay was used to determine growth inhibition as described previously [39]. In brief, CCA cell lines (5×10^4 cells/ml) at exponential growth phase were treated with 0.25% (v/v) trypsin and seeded in triplicate into 96-well flat-bottom microtiter plates and incubated for 24 h at 37^0C in a humidified 5% CO_2 atmosphere. A100 μl aliquot of medium containing aspirin (500 μM) or DMSO (≤ 1%, v/v) as control was added to the 96-well plates, which were then incubated at 37^0C for 72 h. The culture medium was removed and 200 μl aliquot of 10% (w/v) ice-cold TCA was added to each culture well. Plates were incubated at 4^0C for 60 min and TCA-treated cells were stained for 30 min with 0.4% (w/v) SRB in 1% (v/v) acetic acid for 30 min, and subsequently washed with 1% (v/v) acetic acid. Plates were left to air dry and the protein-bound dye was solubilized with 200 μl of 10 mM Tris base (pH 10.5) for 60 min. Absorbance was measured at 540 nm using a microplate reader (Tecan Austria GmbH, Austria). The concentration of drug required to inhibit cell proliferation by 50% (IC_{50}) was determined.

PhosphoELISArray assay

The assays were conducted using Cancer PhosphoELISArray multi-analyte kit according to the manufacturer's protocols. In brief, 50 μl aliquots of samples, and positive and negative controls were added into the appropriate wells of the plate containing pre-coated phosphoproteins (Akt S473, total Akt, Erk 1/2 T202/Y204, total Erk 1/2, c-Jun S63 and total c-Jun) and incubated for 2 h at room temperature. Wells were washed and a 50 μl aliquot of Detection Antibody Solution was added into each well. The plate was incubated for 1 h at room temperature, wells were washed and 50 μl aliquots of HRP-linked secondary antibody were added to each well, followed by incubation for 1 h. After washing the wells, 50 μl aliquots of Development Solution were added to each well and the plate was incubated for 20 min in the dark. Then, a 50 μl aliquot of Stop Solution was added and absorbance at 450 nm was measured within 30 min.

mRNA array and data analysis

KKU-M214 and KKU-100 cells were transfected with antimiR-21 at a final concentration of 30 nM. Total RNAs were isolated from cells 24 h post-transfection using QIAgen mini

RNAesy kit (QIAgen). The mRNA expression profile was performed using human genome Affymetrix oligo array core facility service (Singapore). Each sample was analyzed once, and the core facility data preprocess, normalization and filtering were done. Ratios were defined as marginal signal intensity when there was a substantial amount of variation in the signal intensity within the pixels from 800 to 1,500. Candidates of miR-21 targets were determined by several algorithms including miBridge (licensing software), TargetScan, PicTar and miRTarbase.

Statistical analysis

Target gene expression in human CCA tissues and adjacent non-tumorous tissues were compared using Wilcoxon matched pair test. Clinicopathological characteristics were compared with target gene expression (high and low) using Chi^2 or Fisher's exact probability test. Kaplan-Meier method was used to calculate survival curves, and Log-rank test was performed to compare differences in the survival rates of patients who were subjected to surgery. A multivariate analysis was performed by the Cox proportional hazard regression model. Target gene expression level, MMP activities and invasive ability of cells were compared by independent *t*-test and one-way ANOVA. All analyses were performed using SPSS software (version 15.0). $P \leq 0.05$ was considered significant.

RESULTS AND DISCUSSIONS

Part I: Hepatic cytochrome P450 2A6 and 2E1 status in peri-tumor tissues of patients with *Opisthorchis viverrini*-associated CCA

Results for Part I

Pathological and clinical characteristics of CCA patients for Cytochrome P450 assessment

Thirteen CCA patients (8 men and 5 women) ranging in age from 45-61 years were included in the study (Table 1.1). Cadaveric donors (n=10) provided normal liver controls. Histology of CCA liver tissues were classified as poorly differentiated (PD, n=1), well-differentiated (WD, n=7), papillary adenocarcinoma (PP, n=3), moderately differentiated (MD, n=1) and mucinous cyst adenocarcinoma (MCA, n=1). All were clinically diagnosed in the late stage (stage III-IV). Infection was ruled out for all liver cases. Liver enzymes including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) measured in CCA livers were increased over the normal ranges. Expression of CYP2A6 and

CYP2E1 were unrelated to patient age, sex, histological grade, tumor stage and hepatic enzyme levels

Table 1.1 Pathological and clinical characteristics of CCA patients

Subject	Age (Year)	Gender	Histogical findings	Alanine amino transferase (ALT)	Aspartate amino transferase (AST)	Alkaline phosphatase (ALP)
CCA patients (n=13)	53 ± 8	Male = 8 Female = 5	WD = 7 PD = 1 PP = 3 MCA = 1 MD = 1	45 ± 24	58 ± 41	155 ± 106

Normal range: ALT = 4-36 U/L, AST = 12-32 U/L, ALP = 42-121 U/L

PD = poorly differentiated, WD = well-differentiated, PP = papillary adenocarcinoma, MD = moderately differentiated, MCA = mucinous cyst adenocarcinoma

CYP2A6 and CYP2E1 activities, protein levels and mRNA expressions

CYP2A6 activity was significantly increased in J and N zones of CCA livers when compared to those of cadaveric donors (Fig. 1.1A). CYP2E1 activity was markedly decreased in J and N zones of liver CCA compared to cadaveric donors (Fig. 1.1D). Liver microsomal CYP2A6 protein levels were higher in both J and N zones of CCA patients (Fig. 1.1B), whereas CYP2E1 protein levels were markedly lower in J zones when compared to those of cadaveric donors (Fig. 1.1E). The mRNA expression level of CYP2A6 was significantly increased in both J and N zones of CCA liver (Fig. 1.1C) whereas the CYP2E1 mRNA expression level was not altered in both zones when compared to those of cadaveric donors (Fig. 1.1F).

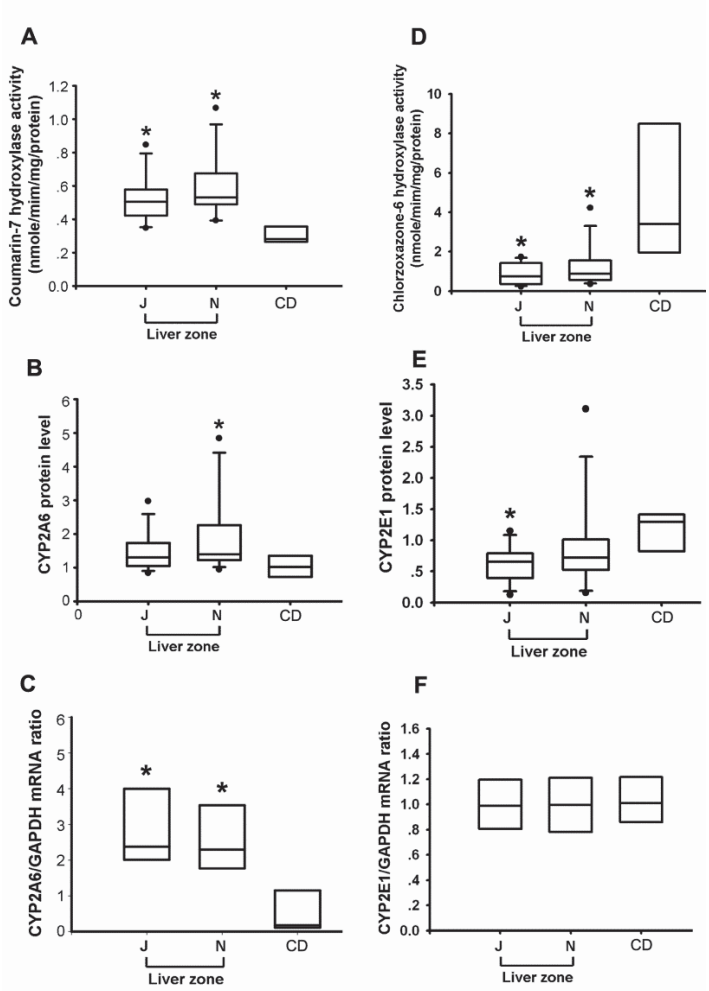

Fig. 1.1 Box plots represent enzyme activities (A, D), protein levels (B, E) and mRNA levels (C, F) of CYP2A6 and CYP2E1, respectively, in J and N zones of peri-tumor tissues of CCA compared to cadaveric donors. CD = cadaveric donors. Data are expressed as the mean + S.D. *P<0.05 compared with liver tissues of cadaveric donors.

Degree of inflammation in CCA liver

Inflammatory cells in the J and N zones of CCA liver tissues were stained with hematoxylin and eosin and graded as severe (numerous inflamed cells: 3+/4+), moderate (few inflamed cells: 2+) and mild (rare inflamed cells: 1+). The most severe inflammation was found in the J zone. Inflammation decreased to moderate in the farther (N) zone (Fig. 1.2A, 1.2B). Positive iNOS staining was found in inflammatory cells infiltrating the J zones (Fig. 1.2C).

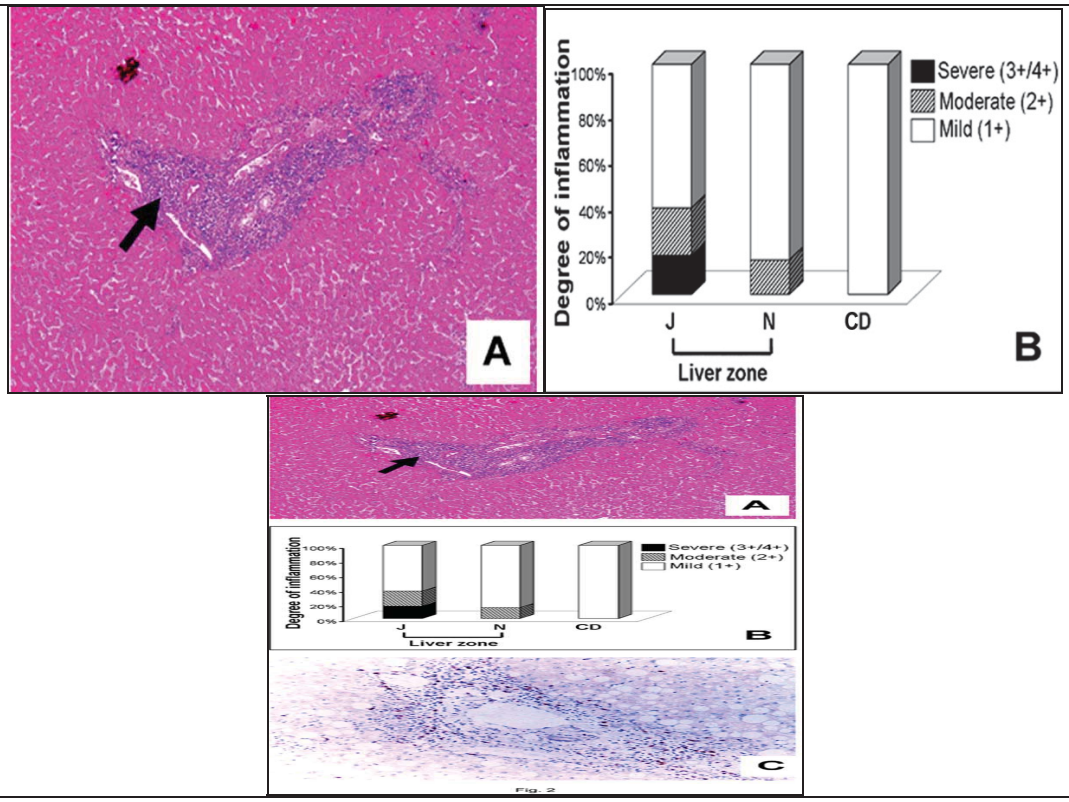

Fig. 1.2 Inflammatory cells in peri-tumor tissues of CCA stained with hematoxylin and eosin (A). Degree of inflammation was calculated by counting the number of inflammatory cells (B). CD = cadaveric donors. iNOS staining in inflammatory cells (C) infiltrating into peri-tumor tissue.

Discussion for Part I

Our study demonstrated enhanced levels of CYP2A6 but suppressed levels of CYP2E1 in liver tissue surrounding CCA. We also found increased CYP2A6 mRNA and protein expression as well as enzyme activity, whereas CYP2E1 was decreased in protein level and enzyme activity but not in mRNA expression. CCA patients examined in this study had altered levels of hepatic enzymes where the inflammatory cells are infiltrated (J and N zones) and showed the positive cells of inducible NOS (iNOS) expression. Our findings agree with previous studies in *O. viverrini* infected hamsters [40] and in *O. viverrini* infected humans with intrahepatic biliary fibrosis [10] which showed that CYP2A6 levels are elevated in the inflammatory sites where NO, are reached a high local concentration [41]. Increase in CYP2A6 expression and activity found in this study, which is also investigated in other cancers, could be possibly involved in tumorigenesis and tumor progression [13, 17]. Nevertheless, the mechanisms by which inflammation in hepatic tissues surrounding CCA suppress CYP2E1

activity remain unknown. Wauthier and colleagues has demonstrated that a decrease in CYP2E1 activity in aged rats is most probably due to post-translational modifications of CYP2E1 proteins and it may be correlated with an accumulation of oxidative damage [21, 42]. Therefore, homeostasis of CYP2E1 appears to be under tight regulatory control, and this enzyme may play a crucial role in tumorigenesis. Furthermore, low levels of CYP2E1 in cancer may cause more aggressive tumor behavior and increased risk of recurrence as in HCC [19]. An explanation for this finding is that overexpression of CYP2E1 generates oxidative stress and induces cytotoxicity, so a high level of CYP2E1 might be cytotoxic to tumor cells and inhibit rapid proliferation, thus resulting in a better prognosis. In our study, expression levels of CYP2A6 and CYP2E1 did not correlate with clinicopathological findings due to the small sample sizes. We conclude that in CCA patients increased CYP2A6 expression and activity and decreased CYP2E1 activity in liver tissue surrounding CCA where inflammatory cells have infiltrated may promote tumor progression. The findings suggest that reducing the degree and severity of inflammation that occurs in opisthorchiasis and/or CCA could be a strategy to prevent the progression of CCA.

Part II: Down-regulation of reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) is associated with enhanced expression of matrix metalloproteinases and CCA metastases

Results for Part II

Histopathological changes

Hamster liver tissues were collected at different times following treatment with Ov plus NDMA. Hyperplastic lesions were seen at week 4 and precancerous lesions were seen at week 12. Carcinomas were seen at week 26 in all Ov plus NDMA-treated hamsters, consistent with a previous report [21]. No histopathological changes in bile duct epithelial cells were observed in the control group.

RECK, MMP-2 and MMP-9 expression in hamster liver tissues during CCA development

Immunohistochemical staining of RECK were seen in hamster biliary cells of the control group, whereas MMP-2 and MMP-9 staining was not found (Fig. 2.1a, 2.1b, 2.1c). RECK staining was intense at the plasma membrane and cytoplasm of bile duct epithelia after Ov plus NDMA administration at week 1 (Fig. 2.1d) and at week 4 (Fig. 2.1g) when hyperplastic biliary

ducts were prominently found. At week 12 when precancerous lesions had developed, RECK staining became less intense (Fig. 2.1j). At week 24 when CCA developed, no staining of RECK was observed in the cancerous tissues (Fig. 2.1m), whereas intense staining remained in most of surrounding hepatocytes (Fig. 2.1p). No MMP-2 or MMP-9 staining was seen in bile ducts of control and Ov plus NDMA treated-groups at week 1 (Fig. 2.1e and 2.1f) and week 4 (Fig. 2.1h and 2.1i). Positive staining was observed, however, in biliary, stromal and infiltrating cells of precancerous tissues at week 12 (Fig. 2.1k and 2.1l) and in CCA tissues at week 24 (Fig. 2.1n and 2.1o).

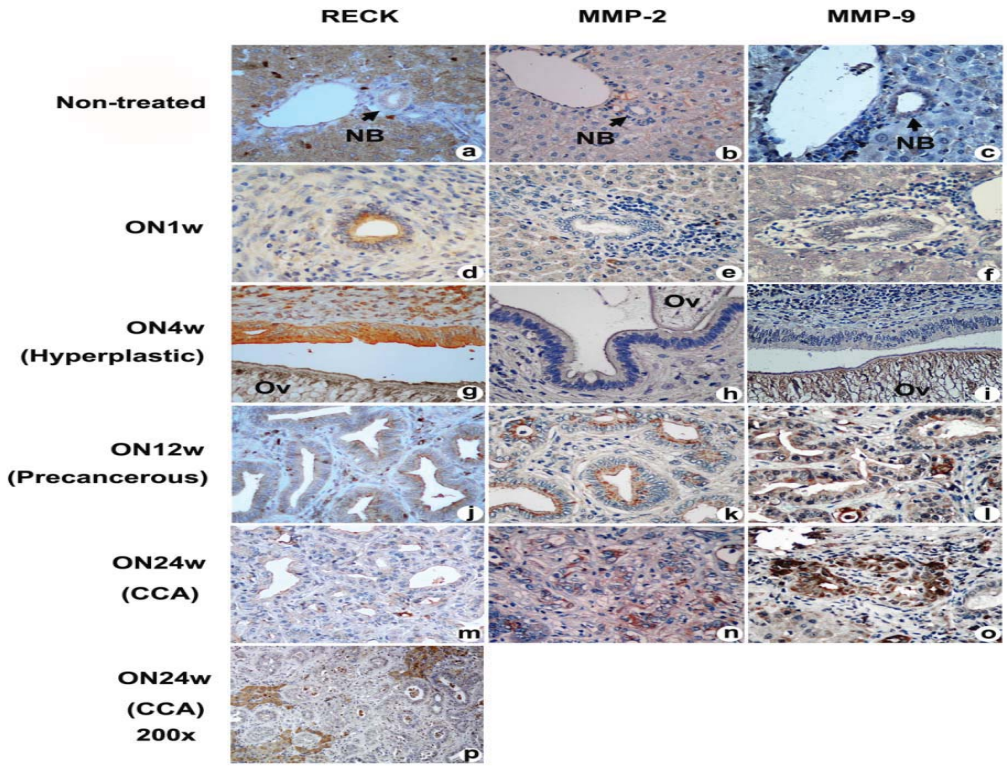

Fig 2.1 Immunohistochemical staining of RECK, MMP-2 and MMP-9 in liver treated with Opisthorchis viverrini (Ov) and N-nitrosodimethylamine (NDMA)

RECK mRNA expression in human CCA tissues

Analysis by qRT-PCR of 24 matched cases of human intrahepatic CCA tissues demonstrated that RECK mRNA expression was significantly down-regulated in tumor tissues as opposed to their adjacent non-tumorous tissues (P = 0.0042, Wilcoxon matched pair test) (Figure 2.2A).

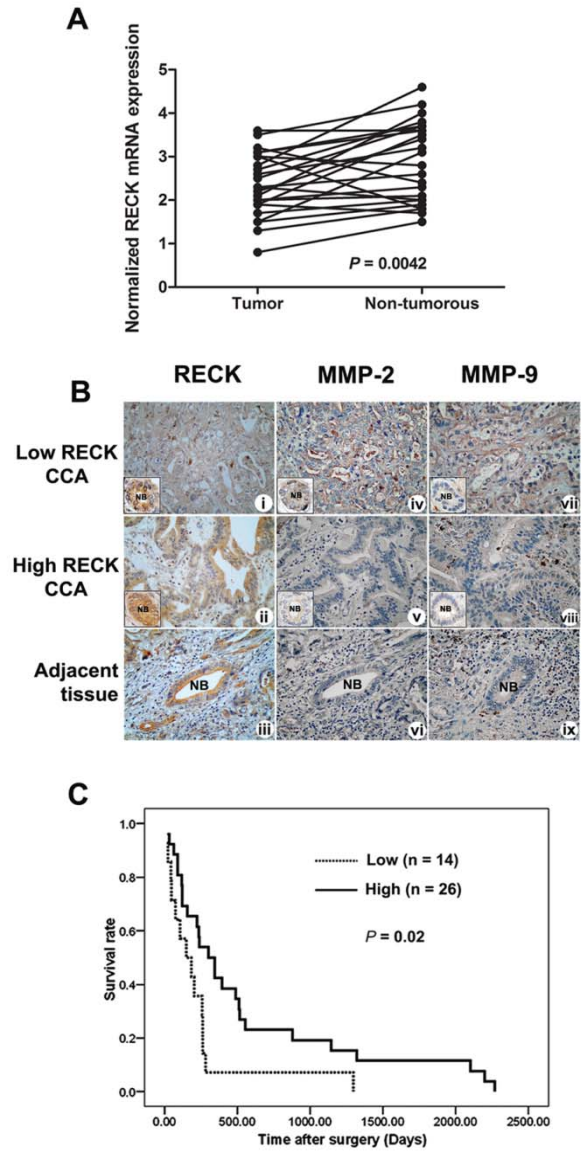

Fig. 2.2 RECK mRNA level in 24 matched pair cases of human intrahepatic CCA (A); The correlation of RECK mRNA expression in tumor tissues was compared with their adjacent non-tumor tissues (n =24, P = 0.004, Wilcoxon matched pair test); Immunohistochemical analysis of RECK, MMP-2 and MMP-9 expression in human intrahepatic CCA tissues (B)

Immunohistochemical analysis of RECK, MMP-2 and MMP-9 in human CCA tissues

Among the liver sections of 40 patients with intrahepatic CCA examined, 14 (35%) specimens weakly stained for RECK protein (Fig. 2.2B-i) in the low RECK mRNA expression group, and 26 (65%) gave moderate to strong staining for tumor cells (Fig. 2.2B-ii) in the high RECK mRNA expression group. All normal bile duct epithelia residing in adjacent tissues of tumor sections were strongly positive for RECK staining (insets in Fig. 2.2B-i and Fig. 2.2B-ii

and in Fig. 2.2B-iii). RECK staining was also seen in hepatocytes, some infiltrating cells and stromal cells.

MMP-2 staining was clearly observed in the cytoplasm of cancer cells, while there was no staining in stromal or inflammatory cells. Staining for MMP-2 showed 20 positive (50%) (Fig. 2.2B-iv) and 20 (50%) negative cases (Fig. 2.2B-v) in CCA patients. MMP-9 staining was detected in some carcinoma cells and almost all of the stromal cells. MMP-9 staining was abundant in inflammatory cells and in infiltrating surrounding tumor tissues. MMP-9 staining in CCA patients was positive (Fig. 2.2B-vii) in 14 (35%) and negative (Fig. 2.2B-viii) in 26 cases (65%) of cancer cells. All normal bile duct epithelia residing in adjacent tissues of tumor sections were negative for MMP-2 (insets in Fig. 2.2B-iv and Fig. 2.2B-v and in Fig. 2.2B-vi) and MMP-9 (insets in Fig. 2.2B-vii and Fig. 2.2B-viii and in Fig. 2.2B-ix). Low score of RECK protein is significantly correlated with high protein scores for MMP-2 (P = 0.02) and MMP-9 (P = 0.007) in tumor cells (Table 2.1).

Table 2.1 Association between expression of RECK, MMP-2 and MMP-9 in tumor tissues of CCA patients demonstrated by immunohistochemical staining

		RECK IHC score		
	n	Low	High	*P*
MMP-2 expression				
Negative	20	3	17	
Positive	20	11	9	0.02*
MMP-9 expression				
Negative	26	5	21	
Positive	14	9	5	0.007*

Fisher's exact probability was used in comparison of variables that had 2 categories. **P* value equal to or less than 0.05 is considered statistically significant. IHC, immunohistochemical staining

Correlation of RECK protein score with clinicopathological features of CCA patients

A comparison of prognosis in 40 cases of CCA indicated that the 5-year survival rate as determined by Log rank test was shorter for patients (n = 14, 35%) with low RECK expression compared to patients with high RECK expression (P = 0.02) (Fig. 2.2C). Fisher's exact test indicated a significant inverse correlation between RECK expression and metastasis-to-blood vessel status (P = 0.01) (Table 2.2). Age, sex, histological grade, lymph node metastasis and

tumor staging did not show significant differences between these two groups (Table 2.2). To determine its independent prognostic value with respect to the overall survival of patients, RECK protein score was included in the Cox's proportional hazard model with the various clinicopathological factors (listed in Table 2.3). Using multivariate analysis, RECK protein score was an independent and significant variable predictor of favorable prognosis. The hazard ratio for death based on this variable was 0.36 (95% CI, 0.16 to 0.85; P = 0.02).

Table 2.2 Correlation between the expression of RECK and clinicopathological findings

Factor	RECK expression (cases)			
	n	Low (weak staining)	High (moderate-to-strong staining)	*P*
Age				
≤55 years	20	6	14	
>55 years	20	8	12	0.74
Sex				
Male	26	9	17	
Female	14	5	9	> 0.05
Histological type				
Non papillary	27	10	17	
Papillary	13	4	9	> 0.05
Tumor staging				
I –II	19	5	14	
III-IV	21	9	12	0.33
Metastasis to blood vessel				
Negative	28	6	22	
Positive	12	8	4	0.01*

Fisher's exact probability was used in comparison of variables that had 2 categories. **P* value equal to or less than 0.05 is considered statistically significant.

Table 2.3 Multivariate analysis by Cox proportional hazard regression model for the evaluation of prognostic factors.

Variable (No. of patients)	**No. of patients that died (5-yr survival cut-off)**	**Hazard ratio (HR)**	**95% confidence interval (CI)**	**P**
Age in years				
≤55 years	20	1		
>55 years	20	0.77	0.37-1.59	0.48
RECK IHC score				
Low	14	1		
High	26	0.364	0.16-0.85	0.02*
Sex				
Female	14	1		
Male	26	1.96	0.91-4.23	0.08
Histological type				
Non papillary	27	1		
Papillary	13	0.67	0.32-1.38	0.28
Tumor staging				
I –II	19	1		
III-IV	21	1.57	0.76-3.22	0.22

**P value equal to or less than 0.05 is considered statistically significant.*

RECK protein levels in CCA cell lines and their invasive ability compared to immortalized cholangiocyte cells

Western blot analysis was performed to detect the RECK protein level in CCA cell lines. Six CCA cell lines (M055, M139, M156, OCA17, HucCT1 and EGI-1) tested had significantly lower levels of RECK protein as determined by Western blotting when compared with immortalized MMNK1 cells (Fig. 2.3A). Conversely, the gelatinase activities of MMP-2 and -9 of M139, OCA17 and HucCT1 cells were higher than those of MMNK1 cells (Figure 2.3B).

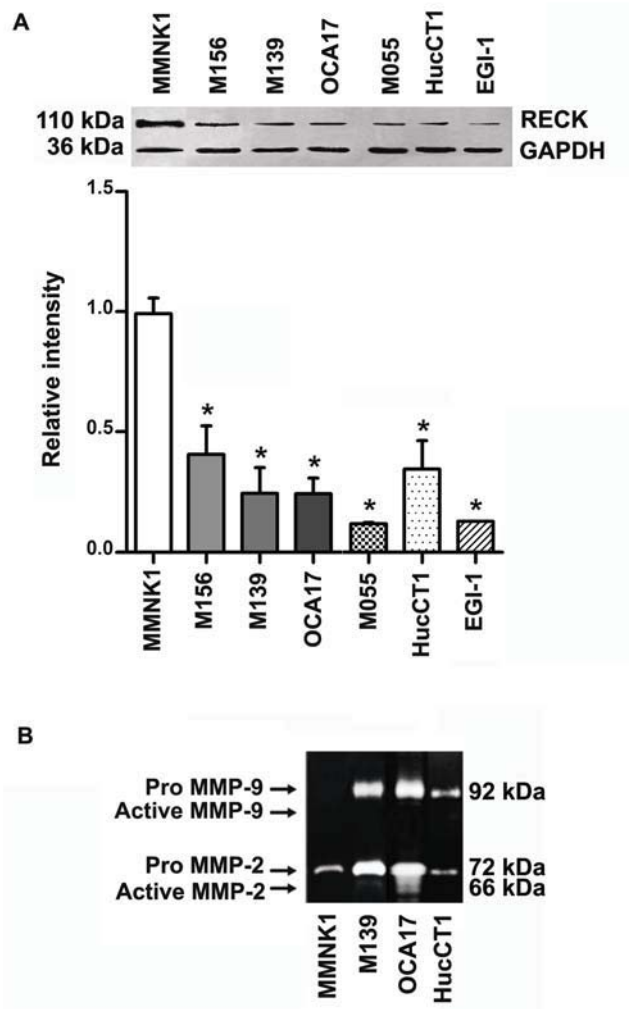

Fig. 2.3 RECK protein content in 7 human cell lines (2.3A) and the gelatinase activities of secreted MMP-2 and MMP-9 in M139 cells (2.3B).

Effect of RECK status on viability and invasiveness of CCA cells

M139 cells transfected with siRNA directed against RECK mRNA showing 70% suppression of RECK protein (Fig. 2.4A) did not affect cell viability (Fig. 2.4B). Levels of the cell cycle proteins cyclin D1 and Cdk4 were not altered upon siRNA treatment (data not shown). However, RECK-depleted M139 cells had significantly elevated levels of MMP-2 and -9 gelatinase activities ($P < 0.05$) (Fig. 2.4C) and increased invasiveness ($P = 0.03$) (Fig. 2.4D).

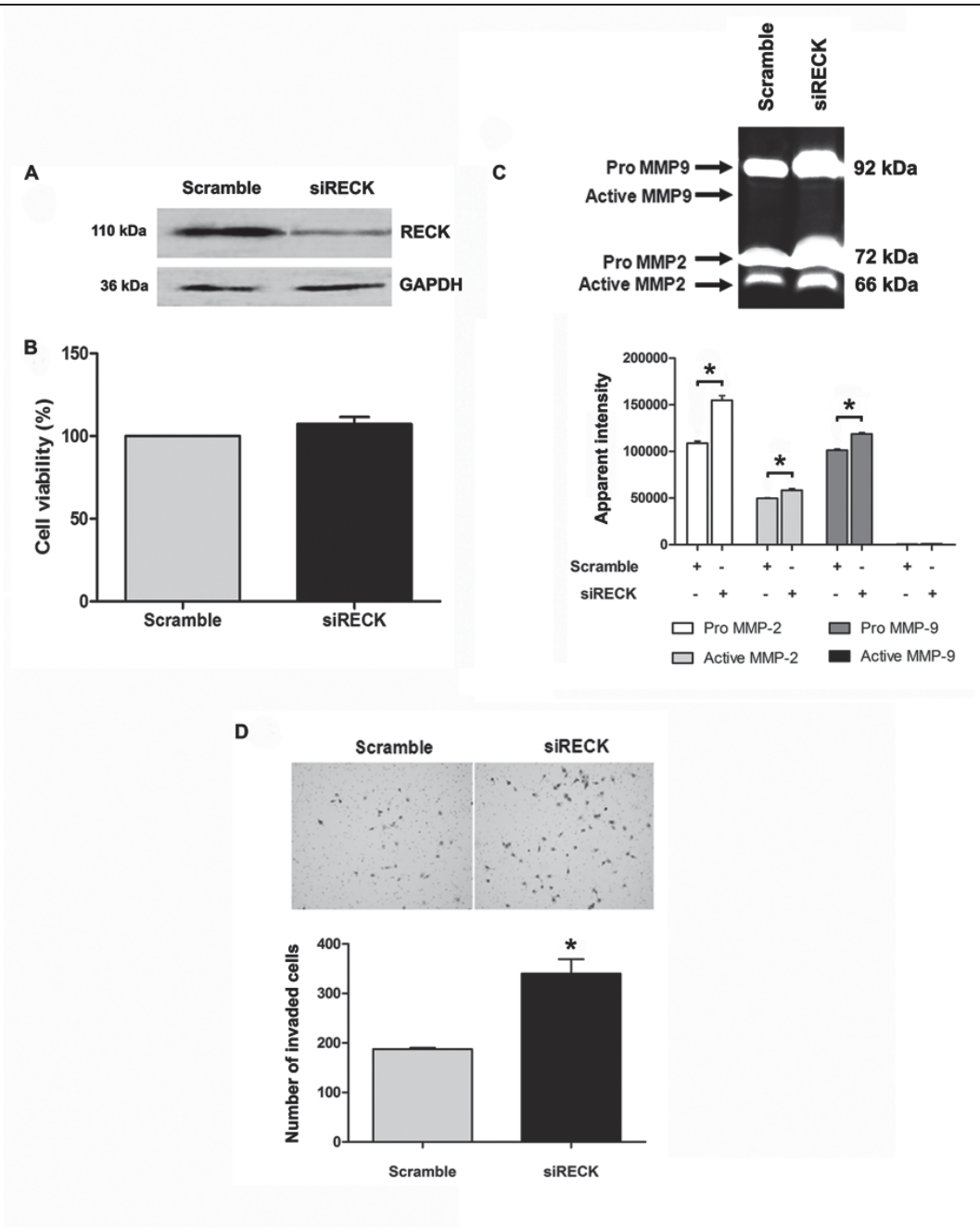

Fig. 4 Namwat et al

Fig. 2.4 Effect of siRNA-mediated knockdown of RECK on RECK content (2.4A), cell viability (2.4B), MMP-2 and MMP-9 activities (2.4C) and invasiveness (2.4D) in the human M139 CCA cell line.

Effect of aspirin treatment on RECK status, MMP-2 and MMP-9 activities and invasiveness

NSAIDs are able to up-regulate RECK gene expression in the CL-1 human lung cancer cell line and suppress MMP-2 activity [19, 20]. Thus, we treated CCA cell lines with 500 μM aspirin for 48 h prior aspirin treatment led to elevated levels of RECK compared with untreated controls (Fig 2.5A) in all 6 CCA cell lines tested (M139, OCA17, M156, M055, HucCT1 and EGI-1). As expected, gelatinase activities of both pro- and active forms of MMP-2 and -9 were inhibited in aspirin-treated M139 cells (Fig. 2.5B). In addition, there was a corresponding reduction in cell invasiveness (Fig. 2.5C).

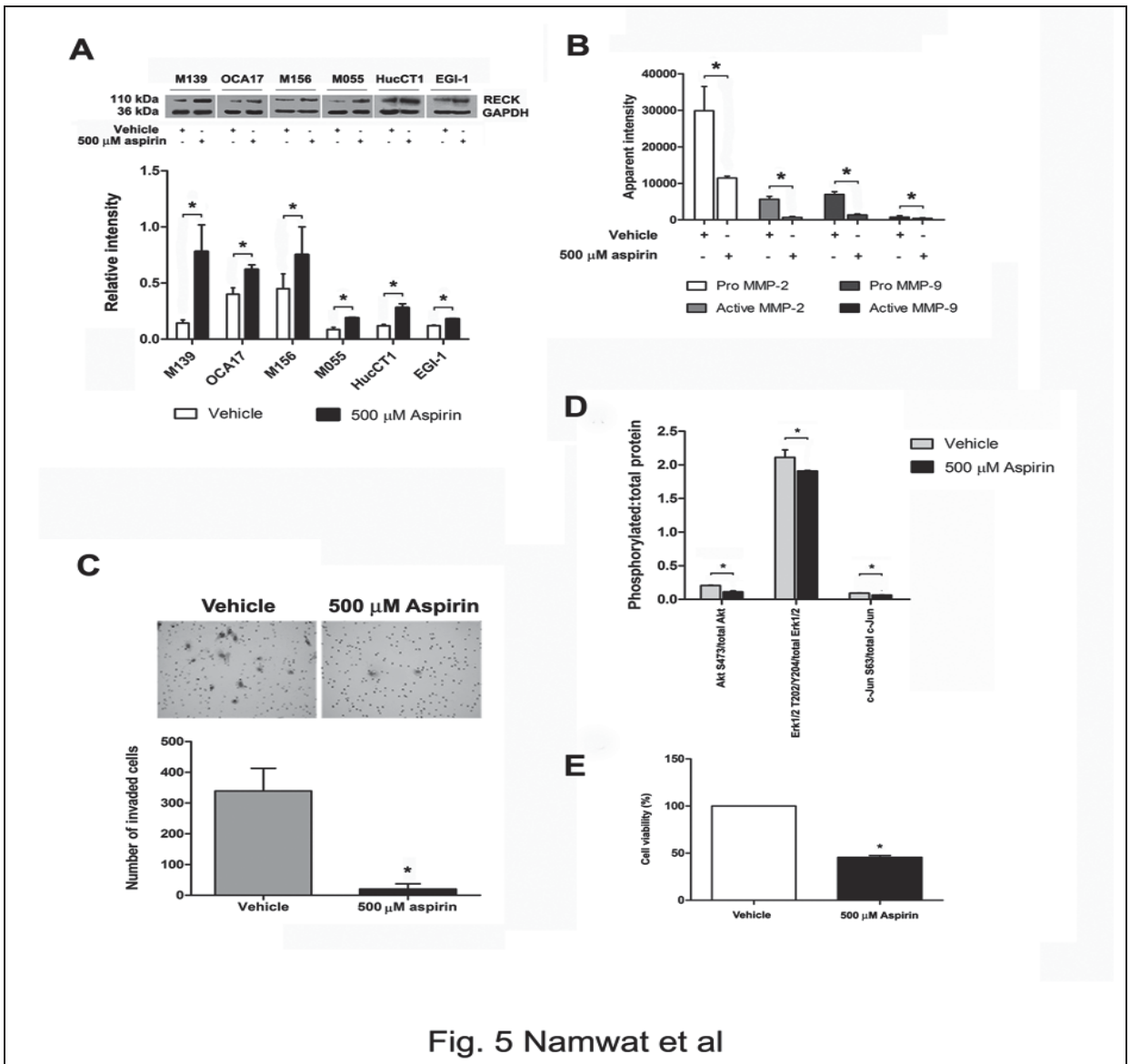

Fig. 5 Namwat et al

Fig. 2.5 Effect of aspirin treatment on RECK content, activities of secreted MMP-2 and MMP-9, invasiveness, phosphorylation status of Akt/Erk/c-Jun and viability of CCA cell lines.

Effect of aspirin treatment on phosphorylation of Akt/Erk/c-Jun and growth of CCA cells

Down-regulation of RECK gene expression in cancer is believed to involve the oncogenic Ras signaling pathway [18]. In order to determine whether aspirin's effects on RECK gene expression in CCA cells are related to this kinase signaling pathway, PhosphoELISArray was used to determine levels of total Akt, Erk1/2, c-Jun and their respective phosphorylated forms in 500 μM aspirin-induced RECK expressing M139 cells. This demonstrated a significant reduction in the ratio of phosphorylated to total forms of Akt, Erk and c-Jun (Fig. 2.5D). In addition, 500-μM aspirin treatment reduced growth of M139 cells by 50% (Fig. 2.5E).

Discussion for Part II

Down-regulation of RECK gene expression is clearly demonstrated in many types of cancer and is strongly associated with high expression/activity of MMPs-2 and -9. MMPs secreted by cancer cells and/or adjacent stromal cells are positively associated with tumor progression including poor tumor differentiation, invasiveness, poor prognosis, metastasis, and shorter patient survival time [43]. CCA is recognized as a highly metastatic tumor, possibly due to its ability to secrete MMP-2 and MMP-9 [44]. In addition, the presence of MMP-2 in Ov plus NDMA-induced hamster CCA is associated with periductal fibrosis and tumor progression [45].

The present study demonstrates that Ov plus NDMA-induced hamster CCA had diminished RECK content as the cells progressed from precancerous to cancerous stages. Concomitant with the development of cancer was an increase in secreted MMP-2 and MMP-9. These patterns of down-regulation of RECK and increase of MMP-2 and -9 expressions have also been reported in hamster buccal pouch carcinomas [28].

In the 24 cases of human CCA examined in this study, RECK mRNA expression levels were lower in tumor tissue than in adjacent non-tumor tissue. Although moderate-to-strong immunohistochemical staining of RECK was found in 26/40 cases examined, the intensities of staining were not stronger than those observed for normal biliary cells in adjacent non-tumorous tissues. RECK protein levels are negatively correlated with those of MMP-2 and MMP-9 in intrahepatic CCA. Patients with high RECK protein level scores tended to have less invasive CCA than those with low RECK scores and this is correlated with good prognosis as indicated by tumor metastasis to blood vessels and longer survival time. Previous studies, in other cancers, showed similar results in the correlation between RECK and MMP-2/-9 expression as

demonstrating in this study. Patients with hilar CCA [29] and gastric cancer [46] show a significant negative correlation between RECK and MMP-9 expression. In addition, RECK expression in patients with colorectal cancer [28], pancreatic cancer [9] and hepatocellular carcinoma [8] is markedly correlated with MMP-2.

Restoration of RECK expression in malignant cells results in suppression of their invasive and metastatic abilities [4]. Over expression of RECK leads to a decrease in MMP-9 mRNA level, and conversely knockdown of RECK expression enhances MMP-9 mRNA level in human fibrosarcoma (HT1080) cells, both with endogenous and over expressed RECK [30]. This study demonstrated that in human M139 CCA cells, siRNA knockdown of RECK also causes an increase in both secreted MMP-2 and MMP-9 and enhances in vitro invasive ability.

NSAIDs are able to modulate RECK expression in tumor cells [32]. Pharmacological concentrations of aspirin induced RECK expression in a number of human CCA cell lines, including M139. Using the latter cell line, aspirin treatment resulted in a concomitant decrease in secreted MMP-2 and MMP-9, and in cell invasiveness. Down-regulation of RECK in malignant cells has been implicated with activation of oncogenic Ras signaling, including Raf/Mek/Erk and Mekk/Sek/Jnk pathways [47, 48]. It has been recently reported that the microRNA miR-21 up-regulated by Ras/Erk signaling can suppress RECK expression in tumor cells [49]. In aspirin-augmented RECK expressing M139 cells, Erk, Akt and c-Jun phosphoforms were suppressed. In HeLa cells, aspirin inhibits Erk and Akt activities by reducing phospho-Erk and phospho-Akt and induces apoptosis [50]. Therefore, aspirin can induce RECK expression mediated by the inhibition of the Ras signaling pathway in CCA cells, possibly due to the re-activation of transcription initiation at the Sp1 promoter site of the RECK gene [31]. In addition, aspirin blocks NMDA-induced activation of c-Jun and NF-kappaB in cortical cell cultures [51]. Activation of c-Jun induces cellular invasion of breast cancer cells [52].

Long-term daily use of adult-strength aspirin is associated with modest reductions in overall cancer incidence in populations in which colorectal, prostate, and breast cancers, are common [53]. Nevertheless, the concentration of this drug may need to be precisely evaluated as high doses of aspirin may have undesirable side effects. To demonstrate the ability of RECK over expression in inhibiting metastasis in vivo, experiments on xenografts in mice will have to be performed.

In summary, our results demonstrate that in CCA RECK inhibits cell migration via suppression of secretion of MMPs. In human CCA tissues, low RECK levels are correlated with poor prognosis as indicated by tumor metastasis to blood vessels and shorter survival

time. In several human CCA cell lines, pharmacological concentrations of aspirin up-regulated RECK expression and suppressed MMPs secretion. Before advocating the use of aspirin (or other NSAIDs) as adjuvant therapy in chemotherapy of CCA patients, however, further studies of the efficacy and safety of this approach will need to be conducted.

Part III: Role of miR-21 in *Opisthorchis viverrini*-associated CCA

Results for Part III

The miR-21 is upregulated in liver tissue as CCA develops

The miR-21 expression was found to be elevated after Ov plus NDMA administration in hamsters on week 1 and thereafter. The miR-21 levels were remarkably upregulated in hamsters from 3 weeks until 24 weeks when the CCA developed (Fig. 2). The miR-21 level was elevated in hyperplasia (ON2M) -> dysplasia (ON3M, ON4M) and the level was highest at tumorous stage (ON6M). The miR-21 level was increased in Ov or NDMA treated group but the level was lower than that in Ov+NDMA (Fig. 3.1).

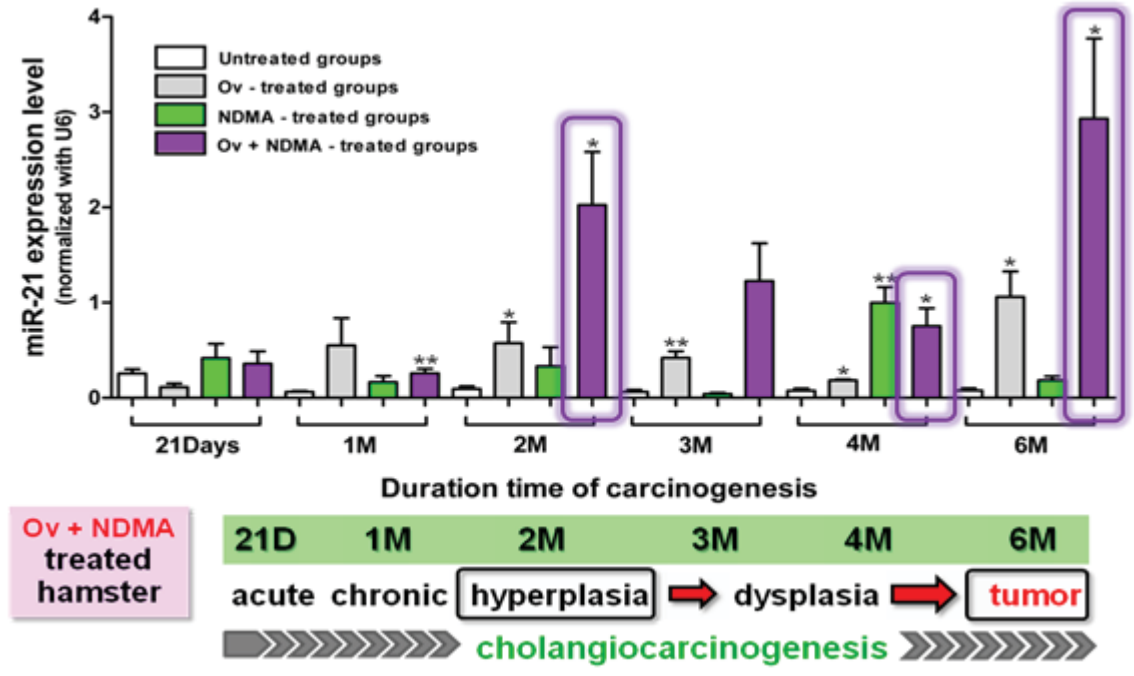

Fig. 3.1 MiR-21 expression in Ov plus NDMA induced CCA model.

The miR-21 is upregulated in human CCA tissues

Analysis by qRT-PCR of 30 matched cases of human intrahepatic CCA tissues demonstrated that miR-21 expression was significantly up-regulated in tumor tissues as opposed to their adjacent non-tumorous tissues (P < 0.001, Wilcoxon matched pair test) (Figure 2).

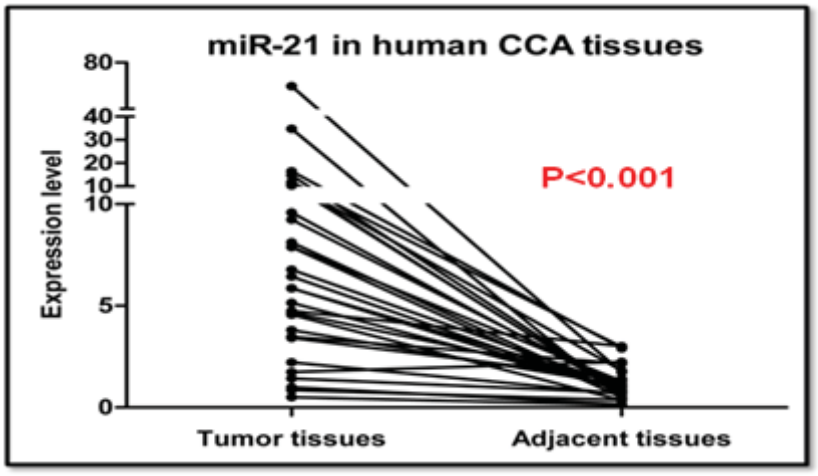

Fig. 3.2 MiR-21 expression in tumor and non-tumor tissues of CCA patients.

The miR-21 expression pattern was inversely correlated with RECK and PDCD4 proteins in human CCA

Among 24 cases, the result demonstrated that high level of miR-21 was appeared in cases with weak staining of RECK and PDCD4 (P=0.032). On the other hand, low level of miR-21 was appeared in cases with strong staining of RECK and PDCD4 (P=0.032) (Fig 3.3).

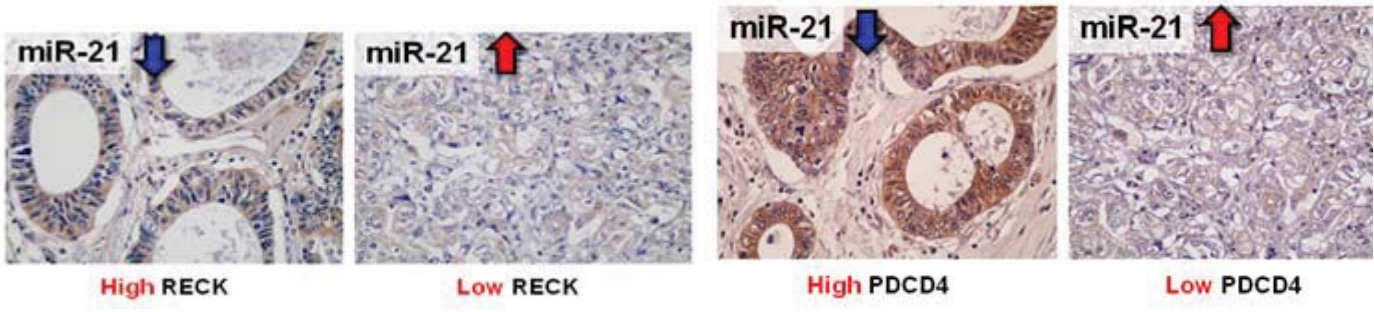

Fig 3.3 RECK and PDCD4 staining in tumor tissues of CCA patients (24 cases). The Fisher Exact test showed PDCD4 (P=0.032).

Knocking down miR-21 suppressed CCA cell growth and migration ability

CCA cell lines, KKU-M214 and KKU-100, were transfected with anti-miR-21. After 48 hr incubation, the decreased level of miR-21 was observed (Fig 3.4). Cell viability was decreased in both cell lines whereas the reduced migration ability was observed in KKU-100 but not in KKU-M214.

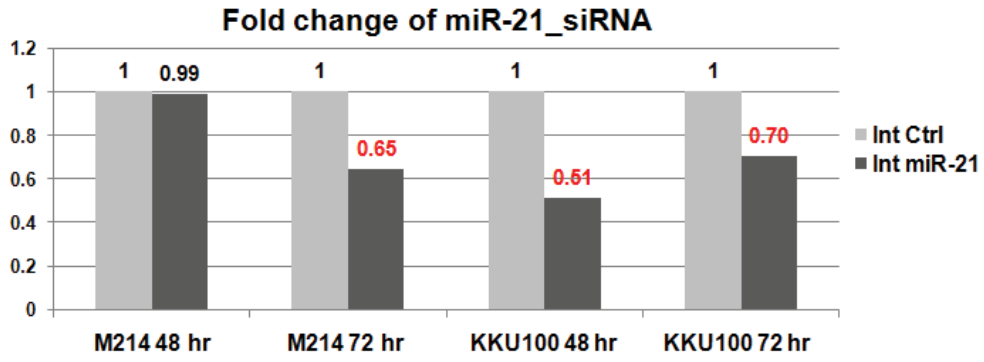

Fig 3.4 The decreased level of miR-21 in transient knock down CCA cell lines, KKU-M214 and KKU-100

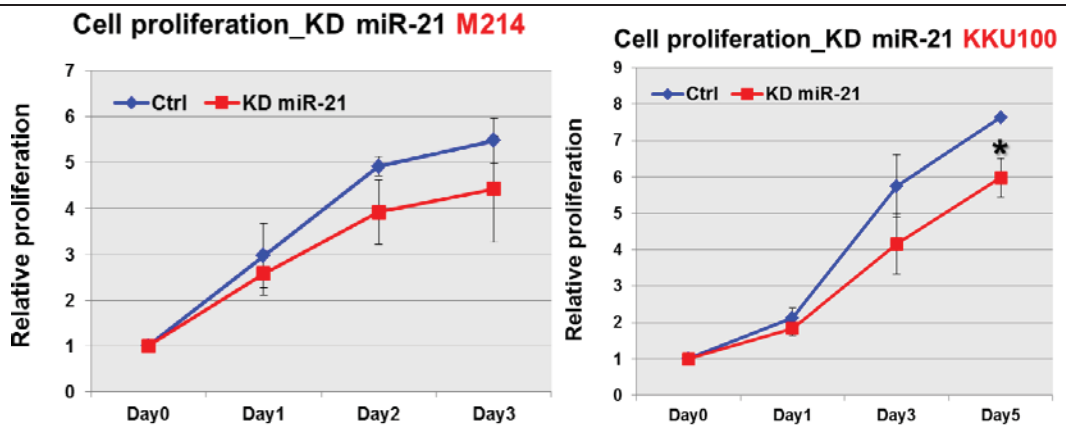

Fig 3.5 Decrease in cell proliferation in transient knock down CCA cell lines, KKU-M214 and KKU-100

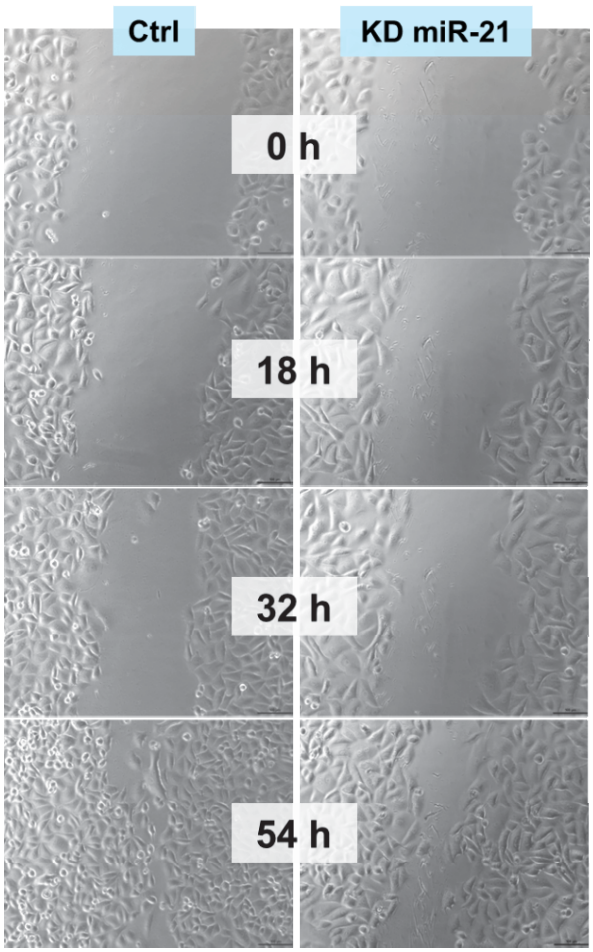

Fig 3.6 Decrease in the migration ability in transient knocking down CCA cell lines, KKU-100

Knocking down miR-21 in CCA cells elevated the levels of predicted target mRNAs

After 24 hr transient knocking-down of miR-21 in 2 CCA cell lines, KKU-M214 and KKU-100, the mRNA level of target genes was determined. Target genes were previously found to be downregulated in CCA tissues (unpublished data) were selected and predicted by MiRanda software. Highly changed target mRNAs, STK40, KLHL15, GPR64, ZTBT47 and CYBD1, were found in both cell lines. Moderately changed target mRNAs, FBX2, FNIP1 and SOX7, were found in both cell lines. CD44 was increased only in M214 (Fig. 3.7).

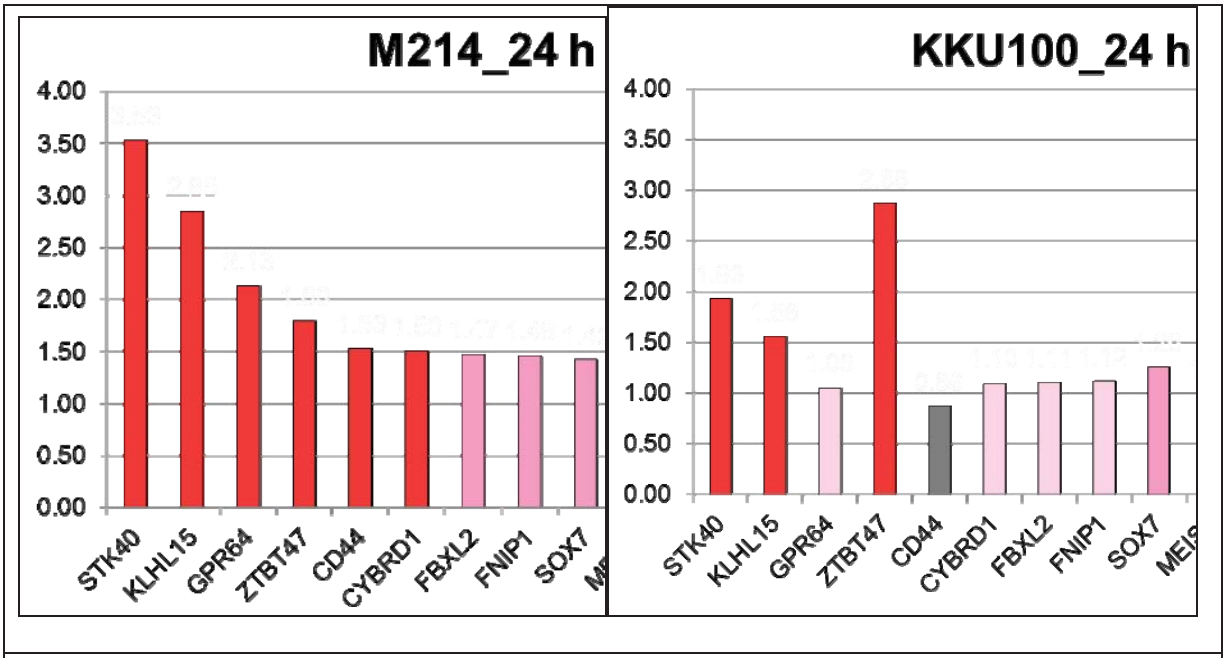

Fig. 3.7 Altered expression of target mRNAs upon 24-hr miR-21 knocking down in KKU-M124 and KKU-100 cell lines

Identification of potential direct targets of miR-21 by mRNA microarray

It is known that animal miRNAs regulate gene expression by inhibiting translation and/or by inducing degradation of target. In our study, most modulated genes on in the mRNA differential expression profiles changed by less than two-fold may still be miRNA targets; we defined differentially expressed genes as no less than 1.00-fold change. Candidate up-regulated mRNAs upon knocking down was analysed for miR-21 direct targets using several algorithms including miBridge (licensing software) combining with freely published prediction softwares that predict at 3'UTR seed region including TargetScan, PicTar and miRTarbase. Top

24 candidates were listed in Table 3.1. RECK, TIMP3 and PDCD4 were further verified by qRT-PCR for their expression level.

Table 3.1 Regulated mRNAs in CCA cells after miR-21 knockdown determined by Affymatrix array

Item No.	Gene Name	Description	Fold change		Prediction program			
			KKU-100	KKU-M214	miBridge	TargetScan	PicTar	miRTarBase
1	**ANKRD46**	**Ankyrin repeat domain 46**	**1.22**	**1.28**	✓			
2	**RECK**	**Reversion-inducing-cysteine-rich protein with kazal motifs**	**1.20**	**1.01**		✓	✓	
3	CDC25A	Cell division cycle 25 homolog A (*S. pombe*)	1.15	1.07				✓
4	FAM63B	Family with sequence similarity 63, member B	1.12	1.13		✓		
5	C17orf39	Chromosome 17 open reading frame 39	1.10	1.07		✓	✓	
6	BCL2	B-cell CLL/lymphoma 2	1.10	1.04				✓
7	GLT8D3	Glycosyltransferase 8 domain containing 3	1.09	1.08		✓		
8	TPRG1L	Tumor protein p63 regulated 1-like	1.07	1.06	✓			
9	**SOX6**	**SRY (sex determining region Y)-box 6**	**1.07**	**1.02**	✓		✓	
10	SNTB2	Syntrophin, beta 2 (dystrophin-associated protein A1, 59kDa, basic component 2)	1.06	1.02		✓		
11	**PDCD4**	**Programmed cell death 4 (neoplastic transformation inhibitor)**	**1.06**	**1.04**		✓	✓	
12	**TNRC6B**	**Trinucleotide repeat containing 6B**	**1.05**	**1.05**	✓	✓	✓	
13	PCBP2	Poly(rC) binding protein 2	1.05	1.08		✓	✓	
14	ZCCHC3	Zinc finger, CCHC domain containing 3	1.05	1.05		✓	✓	
15	KLF9	Kruppel-like factor 9	1.05	1.04			✓	
16	MSH2	MutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)	1.05	1.01				✓
17	**GPR180**	**G protein-coupled receptor 180**	**1.04**	**1.02**	✓			
18	NCAPG	Non-SMC condensin I complex, subunit G	1.02	1.00				✓
19	**APIP**	**APAF1 interacting protein**	**1.02**	**1.01**	✓			✓
20	**SH3GLB1**	**SH3-domain GRB2-like endophilin B1**	**1.01**	**1.01**	✓			
21	MPRIP	Myosin phosphatase Rho interacting protein	1.01	1.04		✓	✓	
22	PTEN	Phosphatase and tensin homolog	1.01	1.05	✓			✓
23	PCBP1	Poly(rC) binding protein 1	1.01	1.01				✓
24	**TIMP3**	**TIMP metallopeptidase inhibitor 3**	**1.00**	**1.08**		✓	✓	

Verification of TIMP3, RECK and PDCD4 miR-21 targets by qRT-PCR

After 24 hr transient knocking-down of miR-21 in 2 CCA cell lines, KKU-M214 and KKU-100, the mRNA level of TIMP3, RECK and PDCD4 was verified by qRT-PCR. Increase in the mRNA level in both cell lines was observed for RECK expression.

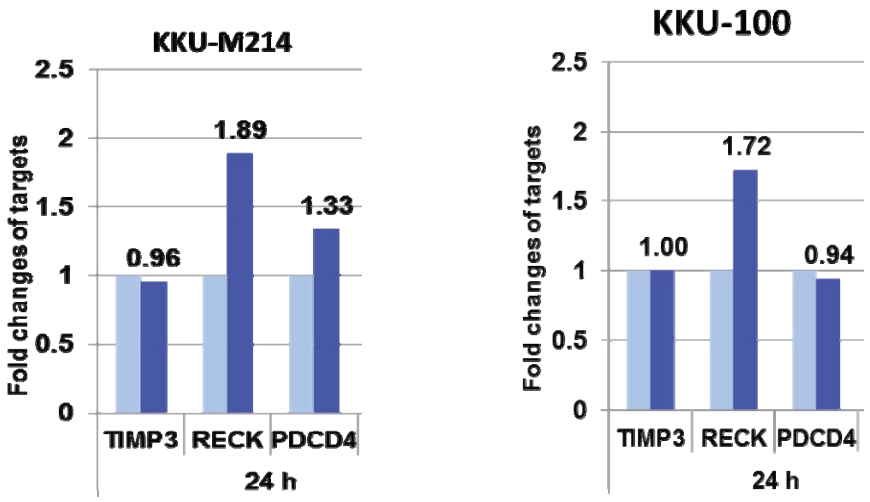

Fig. 3.8 Expression of target mRNAs upon 24-hr miR-21 knocking down in KKU-M124 and KKU-100 cell lines verified by qRT-PCR

Discussions for Part III

miR-21 is a key molecule in a wide range of cancers, and identifying its functional role in CCA has possibly biological implications. In CCA model, it indicates that miR-21 upregulation in early stage of carcinogenesis may play important role in the development of CCA. From animal to human study, the expression of miR-21 was also increased in tumor tissues of CCA patients. The expression of miR-21 was inversely correlated with RECK (metastasis suppressor) and PDCD4 (cell death protein), the miR-21 targets, suggesting that miR-21 may regulate tumor cell growth and metastasis via suppressing such molecules. We show here that knockdown of miR-21 suppresses cell proliferation and migration ability of KKU-M214 and KKU-100 cell lines. Besides, knockdown of miR-21 increases RECK mRNA level. Therefore, our study strongly conclude that RECK was down-regulated in CCA, both animal and human, may strongly be mediated by miR-21 function. Regulatory network of miR-21 in CCA cells is also discovered from this study. New entries of miR-21 targets were analyzed by several methods.

Basically, many miRNAs with their 39-end interaction sites in the 59-UTRs turn out to simultaneously contain 59-end interaction sites in the 39-UTRs. Based on these finding, Lee and co-workers demonstrate combinatory interactions between a single miRNA and both end regions of an mRNA using model systems [54]. They further show that genes exhibiting large-scale protein changes due to miRNA overexpression or deletion contain both UTR interaction sites predicted. They provide the predicted targets of this new miRNA target class, miBridge, as an efficient way to screen potential targets, especially for nonconserved miRNAs, since the target search space is reduced by an order of magnitude compared with the 39-UTR alone. Using miBridge (licensing software) combining with freely published prediction softwares that predict at 3'UTR seed region including TargetScan, PicTar and miRTarbase, we discover several directed targets of miR-21 including APAF1, TNRC6B, TPRG1L, SH3GLB1, SOX6, GPR180 and ANKRD46 that are upregulated upon knock down of miR-21 in CCA cells. Indirect downstream effectors of miR-21 are also investigated such as STK40, KLHL15, GPR64, ZTBT47, CYBD1, FBX2, FNIP1, SOX7 that are elevated upon miR-21 knock down. Indeed, those miR-21 targets need to be further determining the clinical significance and the role in CCA, and it may provide the potential targets for CCA chemoprevention and treatment in the future.

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CONCLUSIONS AND PERSPECTIVES

Alteration of CYP2A6 and CYP2E1 is caused by the chronic inflammation in CCA

We conclude that in CCA patients increased CYP2A6 expression and activity and decreased CYP2E1 activity in liver tissue surrounding CCA where inflammatory cells have infiltrated may promote tumor progression. The findings suggest that reducing the degree and severity of inflammation that occurs in opisthorchiasis and/or CCA could be a strategy to prevent the progression of CCA.

Pattern of RECK expression is clinically significant in CCA.

In hamster CCA, the expression of RECK protein is high in normal-large bile duct epithelia and hyperplastic lesion. RECK protein is decreased when the carcinogenesis reaches to precancerous stage and markedly suppressed in tumorous stage. In human CCA, RECK

expression is down-regulated in both mRNA and protein level when compared to non-tumorous tissues. RECK protein staining in hamster CCA carcinogenesis is opposite to MMP-2 and MMP-9 staining. In human CCA, RECK protein score is negatively correlated with MMP-2 and MMP-9 protein score. Low RECK protein score is correlated with shorter survival rates and metastasis to blood vessels. Our data indicates that the suppression of RECK plays the crucial role in CCA metastasis by associating with ECM degradation, functions of MMP-2 and -9.

RECK functions as an MMP inhibitor.

Functional analysis of RECK by siRNA transfection to the M139 cell reveals that the RECK depleted cell increases in gelatinase activities of both MMP-2 and MMP-9. The RECK depleted M139 cell also elevates the in vitro invasion. Suppression of RECK does not effect on cell growth. This results imply that RECK regulates the cancer cell metastasis by preventing ECM degradation and eventually preventing cellular invasion. We can conclude that RECK is a metastasis suppressor for CCA.

Modulation of RECK expression by NSAIDs can inhibit the in vitro metastasis of CCA cells.

Aspirin elevate RECK expression levels in most CCA cell lines. At a certain concentration (500 μM), aspirin can suppress MMP-2/-9 activities and cellular invasion of RECK-induced CCA cells. This data indicates that restoration or artificial increase of RECK in tumor cells may be the new therapeutic methods for inhibiting the ability of cancer cells to invade, metastasize, and recruit blood vessels.

The regulation of RECK expression is possibly mediated by the phosphorylations of Akt/Erk/c-Jun.

Aspirin-induced RECK can suppress the phosphorylation of Akt/Erk/c-Jun observed for the M139 cell. Aspirin also inhibits growth, gelatinase activities and invasion in M139 cells. Among those aspirin-induced RECK CCA cells, aspirin does not either suppress the phosphorylation of those proteins or growth in OCA17 cells. It is however noted that aspirin cannot suppress gelatinase activities in OCA17 cells (data not shown). This data implies that the regulation of RECK expression in CCA cells may be divided into 2 mechanisms; RAS-dependent (via Akt/Erk/c-Jun) and RAS-independent pathways (may be possibly mediated by FAK?). Therefore, the mechanism of aspirin suppressed invasion may not only be due to the inactivation of RAS pathway.

Modulation of RECK as a therapeutic methods for inhibiting CCA metastasis

Our results suggest that RECK inhibits CCA migration and metastasis via suppression of MMPs activity. Based on this observation, RECK expression level in cancer tissue may be used for a predictor of metastasis evaluation. Moreover, application of RECK modulation approach is also one of the candidates. Aspirin and other NSAIDs that can up-regulate RECK expression and can inhibit MMPs activity may be proposed as potential chemotherapeutic agents to suppress CCA metastasis. However, better understanding in the specific pathway is expected to improve the identification of patients who most likely will be beneficial for treatment with targeted therapeutic drugs. Based on our results, RECK is involved in cancer cell metastasis via an inhibition of MMPs activity. Therefore, using drug modulators of metastasis suppressor protein RECK in combination with targeted therapeutic drugs is a good choice for challenging. To our present knowledge, aspirin is a safe drug of choice for cancer chemoprevention as reported in colorectal carcinoma, therefore, modulation of RECK by aspirin could provide a potential strategy to improve the prognosis of CCA patients by inhibiting the cancer metastasis.

The regulatory network of miR-21 plays important role in CCA

Our study demonstrates that miR-21 overexpressed in Ov plus NDMA induced CCA and in human CCA tissues. Knocking down of miR-21 causes the inhibition of CCA cell growth and metastasis *in vitro*. Direct targets of miR-21 have been identified, presumably involve in cell differentiation, cell growth, apoptosis and metastasis. Modulation of miR-21 and its downstream targets could serve as the good strategy to apply in CCA prevention and treatment.

Suggestions for further study

1. In this study, siRNA strategy was used for finding the candidate functions of RECK in CCA cell lines. We found that RECK might be strongly implicated in CCA carcinogenesis and tumor metastasis processes especially in ECM degradation and cellular invasion. Therefore, searching for RECK inducers such as RTK or kinase or NF-ΚB inhibitors is the new challenging.

2. Our results showed that RECK is down-regulated in CCA cells. Moreover, CCA patients who have high RECK expression have more survival rate than CCA patients who have low RECK expression. So, If RECK expression can be up-regulated or restored in cancer cells, it might be the good way to improve a survival rate of CCA patients. This finding can open

further investigation on the role of RECK in CCA. *In vivo* study by introducing of CCA cells with stably overexpressing RECK into xenograft mice should be explored in the future of this investigation.

3. Clinical significances and functional assay of miR-21 targets should be further investigated in CCA patients. The data may provide the benefit of novel targets and prognostic values in CCA.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 **Namwat N,** Puetkasichonpasutha J, Loilome W, Yongvanit P, Techasen A, Puapairoj A, Sripa B, Tassaneeyakul W, Khuntikeo N, Wongkham S. Down-regulation of reversion-inducing-cysteine-rich protein with Kazal motifs RECK is associated with enhanced expression of matrix metalloproteinases and cholangiocarcinoma metastases. J Gastrol (2011) 46:664-675. Impact factor = 3.61

1.2 Yongvanit P. Phanomsri E. **Namwat N**, Kampan J. Tassaneeyakul W. Loilome W. Puapairoj A. Khuntikeo N. Hepatic cytochrome P450 2A6 and 2E1 status in peri-tumor tissues of patients with *Opisthorchis viverrini*-associated cholangiocarcinoma. Parasitol Int (2012) 61:162-166. Impact factor = 2.259

1.3 Chusorn P, **Namwat N**, Loilome W, Pairojkul C, Bin T, Yongvanit. The role of miR-21 in *Opisthorchis viverrini*-associated cholangiocarcinoma. *Manuscript in preparation*

2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

2.1 ได้วิทยาศาสตรมหาบัณฑิต สาขาวิชาชีวเคมีทางการแพทย์ จำนวน 2 คน ได้แก่

2.1.1 น.ส.เอมอร พนมศรี ตำแหน่งนักวิทยาศาสตร์ผู้ชำนาญการ ประจำภาควิชาพยาธิวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

2.1.2 น.ส.จันทร์เพ็ญ พืชกสิชลพสุธา กำลังศึกษาต่อในระดับปริญญาเอก ณ คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

2.2 ได้นักศึกษาบัณฑิตศึกษาระดับปริญญาเอก สาขาวิชาชีวเคมีทางการแพทย์ จำนวน 1 คน

2.2.1 น.ส.พรชีรา ชูสอน นักศึกษาระดับปริญญาเอกทุนโครงการปริญญาเอกกาญจนาภิเษก ทำวิทยานิพนธ์เกี่ยวกับ บทบาทของ miR-21 ในโรคมะเร็งท่อน้ำดี (อยู่ระหว่างเตรียม manuscript จำนวน 2 เรื่อง)

3. อื่น ๆ

3.1 Nisana Namwat, Porncheera Chusorn, Watcharin Loilome, Sopit Wongkham, Anchalee Techasen, Anucha Puapairoj, Narong Khuntikeo, Puangrat Yongvanit. MiR-21 Overexpression Controls Tumor Cell Growth and Metastasis in Cholangiocarcinoma of Northeastern Thailand. New trends in health care ประชุมวิชาการประจำปี คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ครั้งที่ 27 ประจำปี 2554 4-6

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