รายงานวิจัยฉบับสมบูรณ์

แบบแผนการแสดงออกและบทบาทของ Wnt/β-catenin ในโรคมะเร็งท่อน้ำดี

Expression profile and role(s) of Wnt/β-catenin signaling pathway in cholangiocarcinoma

โดย ผศ.ดร. วัชรินทร์ ลอยลม และคณะ

มิถุนายน พ.ศ. 2556

สัญญาเลขที่ MRG5480034

รายงานวิจัยฉบับสมบูรณ์

แบบแผนการแสดงออกและบทบาทของ Wnt/β-catenin ในโรคมะเร็งท่อน้ำดี

(Expression profile and role(s) of Wnt/β-catenin signaling pathway in cholangiocarcinoma**)**

โดย

ผศ.ดร.วัชรินทร์ ลอยลม มหาวิทยาลัยขอนแก่น

รศ.ดร. พวงรัตน์ ยงวณิชย์ มหาวิทยาลัยขอนแก่น

ผศ.นพ. อนุชา พัวไพโรจน์ มหาวิทยาลัยขอนแก่น

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา สำนักงานกองทุนสนับสนุนการวิจัย และ มหาวิทยาลัยขอนแก่น

ACKNOWLEDGEMENT

Many thanks to my collaborators, Dr. Nisana Namwat, Dr. Anucha Puapairoj, Ms. Pornpun Buangkanjana (Ms.C), Ms. Supak Yothaisong (Ms.C) and Ms. Hasaya Dokduang (Ph.D student) to help me get this work accomplished. I also thank my mentor, Dr. Puangrat Yongvanit for her helpful advice to bring me a success for this project. Grateful appreciation is also expressed to all members of the department of Biochemistry, Faculty of Medicine, Khon Kaen University for supporting throughout my work.

The acknowledgement is also given to the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, for facilitating me to get the success of this project. Finally, a sincere gratitude would be expressed for the Thailand Research Funds, Office of the Higher Education Commission and Khon Kaen University for providing me the financial support throughout this project.

Watcharin Loilome, Ph.D

บทคัดย่อ

รหัสโครงการ : MRG5400834

ชื่อโครงการ : แบบแผนการแสดงออกและบทบาทของ Wnt/β-catenin ในโรคมะเร็งท่อน้ำดี

ชื่อนักวิจัย :ผศ.ดร.วัชรินทร์ ลอยลม

E-mail Address: watloi@yahoo.com, watclo@kku.ac.th

ระยะเวลาโครงการ : กรกฎาคม 2554-มิถุนายน 2556

มะเร็งท่อน้ำดีซึ่งถือเป็นปัญหาทางสาธารณสุขที่สำคัญของประเทศไทยโดยเฉพาะในภาคตะวันออกเฉียงเหนือ แนวทางรักษามะเร็งท่อน้ำดีในปัจจุบันได้แก่การผ่าตัด ซึ่งเป็นการรักษาแบบ curative และเมื่อใช้ร่วมกับการใช้ยาเคมีบำบัด พบว่าให้ผลดีกว่าการผ่าตัดเพียงอย่างเดียว แต่ก็ยังได้ผลไม่เป็นที่น่าพอใจนักต่อการเพิ่มอัตราการอยู่รอดของผู้ป่วย ดังนั้นการศึกษาเพื่อหาแนวทางใหม่ๆในการรักษามะเร็งท่อน้ำดีจึงถือเป็นสิ่งที่ท้าทายสำหรับนักวิจัยและแพทย์ โดยการรักษาโรคมะเร็งในปัจจุบันเน้นไปที่การใช้ยาที่ออกฤทธิ์จำเพาะต่อโมเลกุลของมะเร็งที่เรียกว่าการรักษาแบบมุ่งเป้าหมาย (targeted therapy) และโปรตีนในกลุ่มไคเนส (kinase proteins) ได้ถูกนำมาใช้เป็นเป้าหมายของยารักษาโรคมะเร็งหลายชนิดและมีแนวโน้มที่ได้ผลดี ดังนั้นวัตถุประสงค์ของการวิจัยนี้เพื่อศึกษาแบบแผนการกระตุ้นการทำงานของเอนไซม์ไคเนส และทดสอบประสิทธิภาพของการใช้สารยับยั้งต่อกลุ่มเอนไซม์ไคเนส ในการยับยั้งการเจริญเติบโตของเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี

จากผลการตรวจสอบแบบแผนการกระตุ้นการทำงานของโปรตีนไคเนสโดยวิธี phospho RTK array และ phospho kinase array ในเนื้อเยื่อและเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี พบว่าในเนื้อเยื่อผู้ป่วยและเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี มีแบบแผนการกระตุ้นการทำงานของตัวรับและโปรตีนไคเนสได้หลาย pathway ประกอบด้วย PI3K-Akt, Ras-MAPK, JAK-STATและ Wnt-β-catenin ตามลำดับ ซึ่งแต่ละ pathway มีบทบาทในการควบคุมการเจริญเติบโตและการอยู่รอดของมะเร็งท่อน้ำดีและน่าจะใช้เป็นเป้าหมายของยาในการรักษาผู้ป่วยมะเร็งท่อน้ำดีในลักษณะที่เป็นการรักษาแบบมุ่งเป้า (targeted therapy) ได้ และได้ทำการทดสอบประสิทธิภาพของยาในกลุ่ม kinase inhibitor หลายๆชนิดต่อการยับยั้งการเจริญเติบโตของเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีและพบว่ายา 2 ชนิดคือ sorafenib และ sunitinib ซึ่งเป็น multi-targeted kinase inhibitor มีประสิทธิภาพสูงกว่ายาตัวอื่นๆในกลุ่มที่ทำการทดสอบ

นอกจากนี้ได้ศึกษาแบบแผนการแสดงออกของโปรตีนที่เป็นองค์ประกอบของวิถี PI3K/AKT ซึ่งประกอบด้วย PI3K regulatory subunit (p85α), PI3K catalyic subunit (p110α), AKT, p-AKT, mTOR, p-mTOR, GSK-3β, p-GSK-3β, PTEN และ p-PTEN ในเนื้อเยื่อผู้ป่วยมะเร็งท่อน้ำดี นอกจากนี้ยังศึกษาผลของการใช้สารยับยั้งวิถี PI3K/AKT ได้แก่ pan-class I PI3K inhibitor (NVP-BKM120) และ dual-PI3K/mTOR inhibitor (NVP-BEZ235) ต่อการเจริญเติบโตของเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี พบว่าร้อยละ 97 ของตัวอย่างของผู้ป่วยมะเร็งท่อน้ำดีที่ศึกษามีการสูญเสียการทำงานของ PTEN ทั้งโดยการที่ไม่การแสดงออกและถูกกดการทำงานโดยการเติมหมู่ฟอสเฟต พร้อมทั้งพบการแสดงออกที่สูงขึ้นของ PI3K คิดเป็นร้อยละ 90 ของผู้ป่วยมะเร็งท่อน้ำดี ส่งผลให้มีการกระตุ้นวิถี PI3K/AKT สูงขึ้นในตัวอย่างผู้ป่วยมะเร็งท่อน้ำดี เป็นผลให้เกิดการพยากรณ์โรคที่ไม่ดีโดยเฉพาะการแพร่กระจายของของเซลล์ฒะเร็ง ยิ่งไปกว่านั้นยังพบว่า dual PI3K/mTOR inhibitor NVP-BEZ235 มีฤทธิ์ในการยับยั้งการเจริญเติบโตของเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีได้

และจากการศึกษาแบบแผนการแสดงออกของโปรตีนที่เป็นองค์ประกอบของวิถี Wnt/β-catenin รวมถึงบทบาทที่เกี่ยวข้องกับการเกิดมะเร็งท่อน้ำดี พบว่าการแสดงออกของโปรตีนในกลุ่ม Wnt ที่พบว่ามีความสัมพันธ์กับโรคมะเร็งชนิดต่างๆจำนวน 9 ชนิดด้วยเทคนิค quantitative real time RT-PCR ในตัวอย่างตับของผู้ป่วยมะเร็งท่อน้ำดีพบว่ามีโปรตีน 5 ชนิด ได้แก่ Wnt1, Wnt3a, Wnt5a, Wnt7b และ Wnt8b ที่มีการแสดงออกที่เพิ่มสูงขึ้นในเนื้อเยื่อมะเร็งเมื่อเปรียบเทียบกับเนื้อเยื่อตับปกติจากผู้ป่วยรายเดียวกันอย่างมีนัยสำคัญ ในทางตรงกันข้ามพบว่าWnt3 นั้นมีการแสดงออกที่ลดลงอย่างมีนัยสำคัญ สำหรับการแสดงออกในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีนั้นพบว่าเซลล์เพาะเลี้ยงชนิด M156, M213 และ M214 มีการแสดงออกของ Wnt7a และ Wnt7b ในระดับที่สูง ในขณะที่ไม่พบการแสดงออกของโปรตีนสองชนิดนี้ในเซลล์ M055 ส่วนโปรตีน Wnt ชนิดอื่นๆพบว่ามีการแสดงออกในระดับที่ต่ำ สำหรับการศึกษาการแสดงออกของโปรตีน Wnt 4 ชนิด และโปรตีน β-catenin โดยวิธี immunohistochemistry พบการแสดงออกของโปรตีน Wnt3a, Wnt5a และ Wnt7b ในเซลล์มะเร็งท่อน้ำดีคิดเป็น 92.5%, 77.5% และ 100% ของจำนวนตัวอย่างทั้งหมด 40 รายตามลำดับ ในขณะที่พบว่าโปรตีน Wnt3 มีการแสดงออกส่วนใหญ่ที่ kupffer cell และกลุ่มเซลล์อักเสบ โดยที่พบการแสดงออกของโปรตีน Wnt3 ในเซลล์มะเร็งท่อน้ำดีคิดเป็น 40% นอกจากนี้ยังพบว่าการแสดงออกของโปรตีน Wnt5a มีความสัมพันธ์กับอัตราการรอดชีวิตของผู้ป่วยที่สั้นลง สำหรับการแสดงออกของโปรตีน β-catenin นั้นพบว่าเซลล์มะเร็งมีการแสดงออกของโปรตีนชนิดนี้ลดลงที่บริเวณผนังเซลล์ โดยพบว่า 72.5% มีการแสดงออกของโปรตีนนี้ที่บริเวณไซโตพลาสซึมและพบว่าการแสดงออกของ β-catenin มีความสัมพันธ์ในเชิงบวกกับการแสดงออกของโปรตีน Wnt3a และ Wnt5a อย่างมีนัยสำคัญ (P = 0.017 และ 0.001, ตามลำดับ) บ่งชี้ถึงโมเลกุลที่ทำหน้าที่ส่งสัญญาณควบคุมโปรตีน β-catenin ในมะเร็งท่อน้ำดี นอกจากนี้ในการศึกษาได้ทำการตรวจสอบการแสดงออกของ Wnt3 ในเซลล์ macrophage หลังจากทำการกระตุ้นด้วย LPS ซึ่งพบว่า LPS สามารถกระตุ้นให้มีการแสดงออกของ Wnt3 เพิ่มสูงขึ้นทั้งในระดับ mRNA และโปรตีน นอกจากนี้ยังพบว่าน้ำเลี้ยงเซลล์ macrophage ที่ถูกกระตุ้นด้วย LPS สามารถกระตุ้นโปรตีน β-catenin ในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีได้ และในการศึกษาการทำงานของโปรตีน β-catenin ในมะเร็งท่อน้ำดีนั้น ได้ทำการแทรกแซงการทำงานของ β-catenin ในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีด้วย siRNA ซึ่งพบว่ามีผลยับยั้งการเจริญเติบโตของเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีอย่างมีนัยสำคัญและสัมพันธ์กับการลดลงของยีนที่เป็นเป้าหมายของ β-catenin ซึ่งได้แก่ cyclin D1 อีกด้วย

ซึ่งผลการศึกษาทั้งหมดข้างต้นสนับสนุนว่าการแสดงออกที่ผิดปกติหรือการทำงานที่ผิดปกติของโปรตีนไคเนสหลายชนิดมีบทบาทในการควบคุมการเจริญเติบโตและการอยู่รอดของเซลล์มะเร็งท่อน้ำดี และการยับยั้งการทำงานของโปรตีนไคเนสเหล่านี้ก็ส่งผลยับยั้งการเจริญเติบโตและกระตุ้นการตายของเซลล์มะเร็งท่อน้ำดีได้ ดังนั้นจึงน่าจะนำมาใช้เป็นกลยุทธ์หนึ่งในการรักษาผู้ป่วยมะเร็งท่อน้ำดีทั้งชนิดที่ใช้เป็นยาเดี่ยวหรือใช้ร่วมกับยาชนิดอื่นเพื่อปรับปรุงประสิทธิภาพในการรักษาผู้ป่วยมะเร็งท่อน้ำดี

คำหลัก : มะเร็งท่อน้ำดี โปรตีนไคเนส การรักษาแบบมุ่งเป้า PI3K/Akt Wnt/β-catenin

Abstract

Project Code : MRG5480034

Project Title : Expression profile and role(s) of Wnt/β-catenin signaling pathway in cholangiocarcinoma

Investigator: Watcharin Loilome, Ph.D.

E-mail Address: watloi@yahoo.com, watclo@kku.ac.th

Project Period: July 2011- June 2013

Improving therapy for patients with cholangiocarcinoma (CCA) presents a significant challenge. This is made more difficult by a lack of a clear understanding of potential molecular targets, such as deregulated kinases. In this work, we profiled the activated kinases in CCA in order to apply them as the targets for CCA therapy.

The present study, human phospho- receptor tyrosine kinases (RTKs) and phospho-kinase array analyses revealed that multiple kinases are activated in both CCA cell lines and human CCA tissues that included cell growth, apoptosis, cell to cell interaction, movement and angiogenesis RTKs. Predominately the kinases activated downstream were those in the PI3K/Akt, Ras/MAPK, JAK/STAT and Wnt/ β-catenin signaling pathways. Western blot analysis confirms that Erk1/2 and Akt activation were increased in CCA tissues when compared to their normal adjacent tissue. The inhibition of kinase activation using multitargeted kinase inhibitors, sorafenib and sunitinib led to significant cell growth inhibition and apoptosis induction via suppression of Erk1/2 and Akt activation while drugs with specificity to a single kinase showed less potency.

Additionally, we also determined the expression levels of several key components of PI3K signaling and evaluated whether NVP-BEZ235, a novel dual PI3K/mTOR inhibitor could inhibit CCA cell growth. Immunohistochemistry for p85α, p110α, AKT, p-AKT (T308), mTOR, p-mTOR (S2448), GSK-3β, p-GSK-3β (S9), PTEN and p-PTEN (S380, T382/383) was performed in 30 CCA patients. Western blotting was used to analyze PTEN and p-PTEN expression in the cell lines. The effects of NVP-BEZ235 on CCA cells were evaluated using a growth inhibition assay, flow cytometer, migration assay. Increased activation of PI3K/AKT signaling was reproducibly observed in the CCA tissues. The expression of p85α, mTOR and GSK-3β was significantly correlated with metastasis. Interestingly, PTEN suppression by loss of expression or inactivation by phosphorylation was observed in the majority of patients. Furthermore, NVP-BEZ235 effectively inhibited CCA cell growth and migration through reduced AKT and mTOR phosphorylation and significantly induced G1 arrest without apoptosis induction, although, increase autophagy response was observed.

Furthermore, the quantitative real time RT-PCR demonstrated that Wnt3a, Wnt5a and Wnt7b mRNA were significantly higher in CCA tissues than adjacent non-tumor tissues and normal liver tissues, whereas Wnt3 was significantly lower in human CCA tissues. Immunohistocemical staining revealed that Wnt3a, Wnt5a and Wnt7b were positive in 92.5%, 77.5% and 100% of CCA tissues studied. Additionally, Wnt3 expression was mainly found in inflammatory cells, whereas cancer cells showed 40% positive staining. Furthermore, the expression of Wnt5a related to poor survival of CCA patients (P=0.007). Reduced membrane expression of β-catenin was observed in tumor area and 72.5% of CCA cases showed cytoplasmic β-catenin expression. Moreover, to test whether inflammation conditions involved in CCA development at least in part by activating Wnt/β-catenin signaling pathway, we investigated the expression of Wnt3 in macrophage cell line upon LPS stimulation. The result showed that LPS can induce the expression of Wnt3 both mRNA and protein levels in macrophage cell line. Moreover, conditioned media from LPS-induced activated macrophage can promote β-catenin accumulation in CCA cell lines. Furthermore transiently suppression of β-catenin by siRNA significantly induced growth inhibition of CCA cells, with an associated decrease in cyclin D1 expression. In conclusion, the present study reports the abundant expression of Wnt protein family and β-catenin in CCA as well as the role of inflammatory condition in Wnt/β-catenin activation in CCA cell lines. Importantly, abrogation of β-catenin expression caused significant CCA cell growth inhibition. Thus, Wnt/β-catenin signaling pathway may contributes to CCA cell proliferation and might be able to serve as a potential target for inhibiting CCA cell growth.

Taken together, the data demonstrate that multiple protein kinases are activated which lead to stimulation of several signaling cascades in CCA. Therefore, targeting protein kinases is a promising strategy to improve CCA therapy.

Keywords : cholangiocarcinoma, protein kinases, targeted therapy, PI3K/Akt**,** Wnt/β-catenin

INTRODUCTION

Cholangiocarcinoma (CCA), a malignant tumor arising from the bile duct epithelium, worldwide shows the highest recorded incidence in Northeastern Thailand [1]. CCA initially progresses slowly, but the prognosis is very poor because it is difficult to diagnose until the tumor is in an advanced and more aggressive stage. The current treatment for advanced CCA is maximal surgical resection, chemotherapy and radiation therapy [2]. However, most of the patients are unsuitable for surgery and apparent complete resection is often followed by metastasis or local recurrence [3]. Moreover, chemotherapy and radiation therapy does not improve the patient's survival rate [4]. In this study, we sought to identify the kinase regulated pathways that might be the basis for better understanding and eventually improving CCA therapy.

We have previously demonstrated that a regulatory subunit 1 alpha of protein kinase A type I (PRKAR1A) was overexpressed in the liver fluke-induced hamster and human CCA, indicating an association of PRKAR1A with CCA. We have also shown that inhibition of PRKAR1A by using siRNA resulted in growth inhibition and apoptosis induction of CCA cell lines. Moreover, using cAMP analogues or PKA inhibitors have shown significantly reduced CCA cell growth, suggesting PRKAR1A as a possible target for CCA treatment [5, 6]. These results lead us to focus more on the roles of protein kinases in CCA.

Protein kinases are enzymes that catalyze the transfer of phosphate from ATP to serine/ threonine or tyrosine residues of target proteins, and are key mediators of cancer related signaling cascades, such as those involved in cell proliferation, differentiation, apoptosis and metabolism [7]. However, overexpression and/ or mutations in kinases can lead to abnormal constitutive activation of cellular signaling cascades that contribute to carcinogenesis and tumor progression [8-10]. Kinase inhibitors are one of the most successful classes of anticancer drugs [11-13]. Therefore, for this study our goal is to profile the activated protein kinases in CCA and provide evidence that might support the use of certain protein kinases as the potential targets for CCA treatment.

OBJECTIVES

To profile the activated kinases in CCA in order to apply them as the targets for CCA therapy.

Part I: Survey of activated kinase proteins reveals potential targets for cholangiocarcinoma treatment

METHODS

Human CCA tissue specimens

Twenty pairs of fresh frozen human liver tissues including CCA and their adjacent tissues were obtained from CCA patients who admitted at a surgical ward of Srinagarind Hospital, Khon Kaen University. The informed consent was obtained from each subject before surgery. The protocol of specimen collection and study were approved by the Ethic Committee for Human Research, Khon Kaen University (#HE521209). Tissue samples were collected according to CCA gross types including 10 cases of mass forming type and 10 cases of mixed mass forming with periductal infiltrating type.

Cell lines and cell culture

Four human CCA cell lines including M156, M214, OCA17 and KKU100 were cultured in Ham's F-12 medium (Gibco®; Invitrogen, Carlsbad CA, USA) supplemented with 2 mg/ml sodium bicarbonate, 10% inactivated fetal bovine serum (Gibco®; Invitrogen, Carlsbad CA, USA), 100 U/mL penicillin, and 100 µg/ mL streptomycin. All cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Antibodies and small molecule kinase inhibitors

Antibodies were as follows: phospho- Akt, Akt, phospho- p44/42 mitogen activated protein kinase (p-MAPK; p-Erk1/2 Thr202/Tyr204) and p44/42 MAPK (Erk1/2) (Cell signaling Technology, Danvers, MA, USA) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA).

Small molecule kinase inhibitors were as follows: Gefitinib and Vanditinib (AstraZeneca, UK), Erotinib (Genentech Inc., CA, USA), Imatinib (Novartis, NY, USA), Sunitinib and PDGFRA inhibitor (Pfizer, NY, USA), Sorafenib (Nexavar®, Bayer, Germany), PD173074, SU5402, SU4984, SU11274 (Calbiochem, CA, USA)

Phospho- RTKs and phospho- kinase arrays

Profiling of receptor tyrosine kinases, downstream kinases and their protein substrates were analyzed using human phospho- RTKs and phospho- kinase arrays (ARY- 001 and ARY- 003, R&D systems, Minnepolis, MN, USA) according to the manufacturer's instructions. Briefly, human CCA tissues and human CCA cell lines were lysed with human phospho- RTKs lysis buffer containing proteinase cocktail inhibitors (Roche, Branford, CT). The suspension was then pipetted up and down on ice to complete cell lysis for 30 min, followed by centrifugation at 14,000xg, 4°C for 5 min. Protein lysates were collected and protein concentration was determined by Bradford method**.** After that**,** protein lysates (1,500 µg for pools of human CCA tissues or 750 µg for CCA cell lines) were incubated overnight with antibody array membranes containing 42 phospho- RTKs and 46 phospho- kinases printed in duplication. The membranes were then incubated with cocktail- detection antibody

conjugated with streptavidin- horse- radish peroxidase. The signals were detected by ECL Prime Western Blotting Detection System (GE Healthcare, UK) and quantified using Image Quant™ Imager (GE Healthcare, UK).

Western blot analysis

A protein extract (50 μg) was solubilized in SDS buffer and boiled for 5 min at 95°C. Samples were electrophoresed on 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% skimmed milk in Tris- buffered saline (TBS) for 1 h and incubated with primary antibody at 4°C overnight. After rinsing TBS containing 0.1% polyoxyethylene sorbitan monolaurate (Tween-20 or TBS-T), membranes were then incubated with horseradish peroxidase- conjugated secondary antibody at room temperature for 1h. After rinsing with TBS-T, membranes were exposed to the ECL Prime Western Blotting Detection System (GE Healthcare, UK). Human GAPDH was used as a loading control.

Cell proliferation assay

Cell proliferation was examined by the alarmarBlue® assay (Invitrogen, Carlbad, CA, USA). In brief, 5×10^2 cells/ml were seeded in black clear bottom 96 wells- plates (BD Falcon™, CA, USA) and incubated overnight. Then 20 μl of 1x alarmarBlue® was added and the volume in each well was made up to 200 μl with culture media containing a designed concentration of each kinase inhibitors. After 72 h of incubation, fluorescence was measured on a Perkin Elmer Wallac 1420 Multilable counter (Perkin Elmer, Turku, Finland) with a 540 nm excitation filter and a 590 nm emission filter. An experiment was done in triplicate.

Apoptosis assay

Apoptosis study was determined using the caspase- Glo® 3/7 assay (Promega, USA) according to manufacturer's instructions. Briefly, cells 5×10^3 cells/ml was plated in 100 μl of media and incubated overnight. Then, a range concentration of each drug was added. After incubated for 24 h, caspase- Glo® 3/7 reagent (50μl) was added and mixed to each well then incubated 1 h at room temperature. Luminescence was measured on a Victor3 multiwell plate reader (Perkin Elmer). Experiments were performed twice in three replicates per experiment.

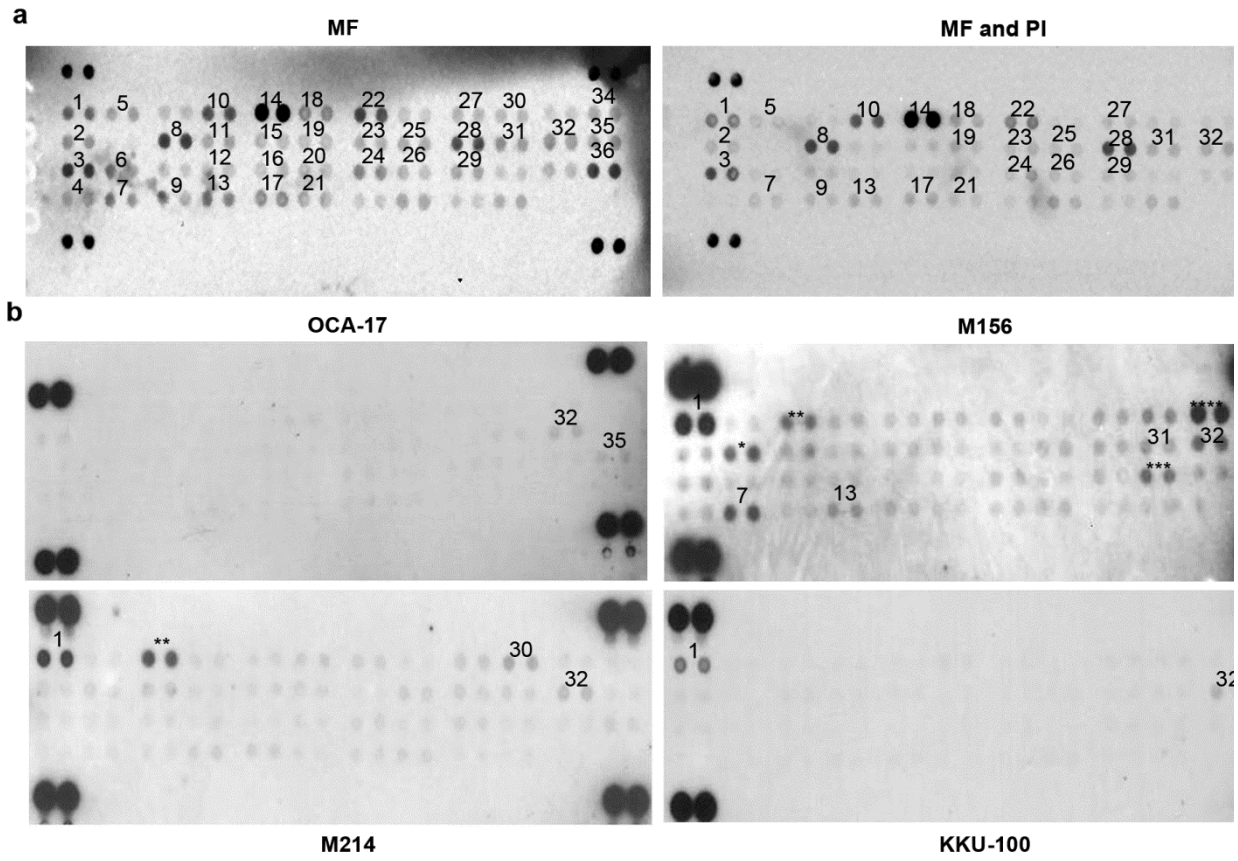
Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software. Results from cell proliferation and apoptosis experiments were represented as mean ± SD and the significance of differences was addressed by Two-way ANOVA test. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Activation of multiple RTKs in cholangiocarcinoma

To identify the activated RTKs in CCA, pool protein lysates of twenty human CCA tissues (10 cases of mass forming and 10 cases of mixed mass forming with periductal infiltrating type) and four CCA cell lines (M156, M214, OCA-17 and KKU-100) were incubated with human phospho- RTKs antibody arrays which can detect relative phosphorylation levels of 42 RTKs simultaneously. The results demonstrated that multiple RTKs are activated in human CCA tissues and CCA cell lines as shown in **Fig.1a**, **1b** and **Table 1**, respectively. The activated RTKs pattern in CCA tissues and cell lines are somewhat different; the activated RTKs in CCA tissues were p-EGFR, p-ErbB4, p-FGFR1, 2, 3, p-ISR, p-MSPR, p-Dtk, p-Mer, p-MCSFR, p-c-RET, p-ROR1, p-ROR2, p-Tie1, p-Tie2, p-VEGFR3, p-Musk, p-EphA3, p-EphA6, p-EphB1, p-EphB2, p-EphB4 and p-EphB6, respectively while the activated RTKs identified in 4 CCA cell lines were p-EGFR, p-ErbB3, p-IGFR, p-Axl, p-HGFR, p-MSPR, p-ROR2, p-EphA2, p-EphA4, p-EphA7 and p- EphB2. Only ROR2 receptor activation was commonly found in CCA tissues and cell lines.

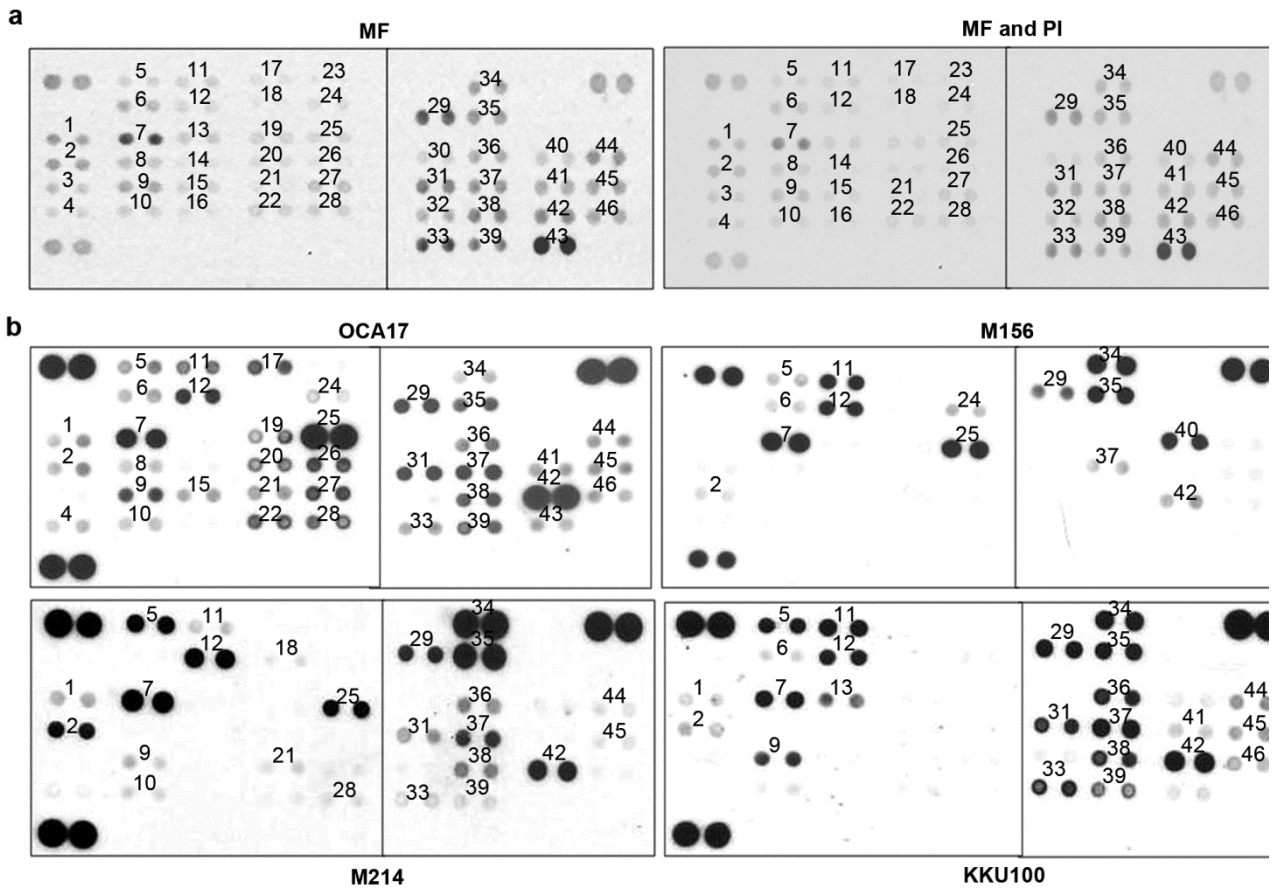


1. p-EGFR 2. p-Mer 3. p-Tie2 4. p-EphR(EphA6) 5. p-ErbB2 6. p-TrkA 7. p-EphR(EphA7) 8. p-MSPR 9. p-EphR(EphB1)
10. p-ErbB4 11. p-PDGFR1-α 12. p-TrkC 13. p-EphR(EphB2) 14. p-FGFR-1 15. p-PDGFR-β 16. p-VEGFR-1
17. p-EphR(EphB4) 18. p-PDGFR-2 19. p-SCFR 20. p-VEGFR-2 21. p-EphR(EphB6) 22. p-FGFR-3 23. p-Flt-3
24. p-VEGFR-3 25. p-MCSF-R 26. p-MuSK 27. p-InsulinR 28. p-c-RET 29. p-EphR(EphA1) 30. p-IGF-IR 31. p-ROR1
32. p-ROR2 33. p-EphR(EphA3) 34. p-Dtk 35. p-Tie 36. p-EphR(EphA1)
* p-HGFR, ** p-ErbB3, *** p-EphR(EphA2), ****p-Axl

Fig. 1 Profiling of activated receptor tyrosine kinases and downstream kinases in CCA. Antibody kinase array detects phophorylated RTKs in human CCA tissues (a) and CCA cell lines (b). The lower panels are the profiles created by quantifying the mean spot pixel density which normalized by subtracted from the density of negative controls. Array signal were analyzed using Image Quant™ Imager.

Co- activation of non- receptor kinases in cholangiocarcinoma

To assess of the phosphorylation status of the cytoplasmic non-receptors in CCA, we used human phospho- kinase antibody array that allowed simultaneous detection of 46 phospho- kinases. As demonstrated in **Fig.2a and 2b**, the co- activation of several non-receptor kinases determined in human CCA tissues and cell lines were appeared as a similar pattern. The result revealed that pathways including PI3K/Akt, Ras/MAPK, JAK/STAT and Wnt/β-catenin signaling were activated in both human CCA tissues and cell lines **(Table 1)**.



1. p-mTOR(S2448) 2. p-Src(Y419) 3. p-Fyn(Y420) 4. p-Hck(Y411) 5. p-p38α(T181/Y182) 6. p-MEK1/2(S128/S222,S222/S26)
7. p-CREB(S133) 8. p-Lyn(Y397) 9. p-Yes(Y426) 10. p-Chk(T68) 11. p-Erk1/2(T202/Y204,T185/Y187) 12. p-MSK1/2(S376/S360)
13. p-HS27(S78/S82) 14. p-Lck(Y394) 15. p-Fgr(Y412) 16. p-FAK(Y387) 17. p-JNK pan(T183/Y185, T21/Y223) 18.p-AMPKα1(T17
19. p-AMPKα2(T172) 20. p-STAT2(Y689) 21. p-STAT3(Y705) 22. p-STAT6(Y641) 23. p-GSK-3α/β(S21/S9) 24. p-Akt(S473)
25. p-β-catenin 26. p-STAT5a(Y699) 27. p-STAT5b(Y699) 28. p-STAT5a/b(Y699) 29. p-Akt(T308) 30. p-p-70 S6 kinase(T421/S424
31. p--70 S6 kinase(T389) 31. p-p-70 S6 kinase(T421/S424) 32. p-p-70 S6 kinase(S299) 33.p-STAT1(Y701) 34. p-p53(S15)
35. p-p53(S15) 36. p-p-53(S392) 37. p-RSK1/2/3(S380) 38. p-RSK1/2(S221) 39. p-STAT4(Y693) 40. p-p-27(T198) 41. p-p-27(T157
42. p-c-Jun(S63) 43. p-eNOS(S1177) 44. p-Paxillin(Y118) 45. p-PLCγ(Y783) 46. p-Pyk2(Y402)

Fig. 2 The downstream kinases phosphorylation detects in human CCA tissues (a) and CCA cell lines (b). The lower panels are the profiles created by quantifying the mean spot pixel density which normalized by subtracted from the density of negative controls. Array signal were analyzed using Image Quant™ Imager.

Table 1 Activated RTKs and downstream kinases in CCA

Sample types	RTKs	Downstream kinases			
		PI3K/ Akt pathway	**RAS/MAPK pathway**	**JAK/ STAT pathway**	**Wnt/β-catenin pathway**
CCA cell lines	EGFR, ErbB3, IGFR, Axl, HGFR, MSPR, ROR2, EphA2, EphA4, EphA7, EphB2	p53 , AKT, mTOR, HSP27, P27, Chk-2, eNOS	p38 alpha, ERK1/2, JNK pan, MEK1/2, MSK1/2, AMPKα1,AMPKα2, CREB, Paxillin, Src, Lyn, Yes, Fgr, Hck, RSK1/2/3, RSK1/2, c-JUN, PLCγ-1	STAT1, STAT2, STAT3, STAT4, STAT5a, STA5b, STAT6	β-catennin
CCA tissues	EGFR, ErbB4, FGFR1,2,3, ISR, MSPR, Dtk, Mer, MCSFR, c-RET, ROR1, Tie1, Tie2, VEGFR3, Musk, EphA3, EphA6, Ephb1,EphB2, EphB4, EphB6	p53 , AKT, mTOR, HSP27, P27, Chk-2, eNOS, p70 S6 kiase, GSK-3 α/β	p38 alpha, ERK1/2, JNK pan, MEK1/2, MSK1/2, AMPKα1,AMPKα2, CREB, Paxillin, Src, Lyn, Yes, Fgr, Hck, RSK1/2/3, RSK1/2, c-JUN, PLCγ-1	STAT1, STAT2, STAT3, STAT4, STAT5a, STA5b, STAT6	β-catennin

Verification of kinase activation in CCA by western blot analysis

Among those of kinase array results, we verified the activation level of Akt and Erk1/2 which are respective key molecules in PI3K/Akt and MAPK signaling pathways using western analysis. The result showed that activated Akt and Erk1/2 were strongly detected in CCA tissues when compared to their non- tumorous adjacent tissues (**Fig. 3**).

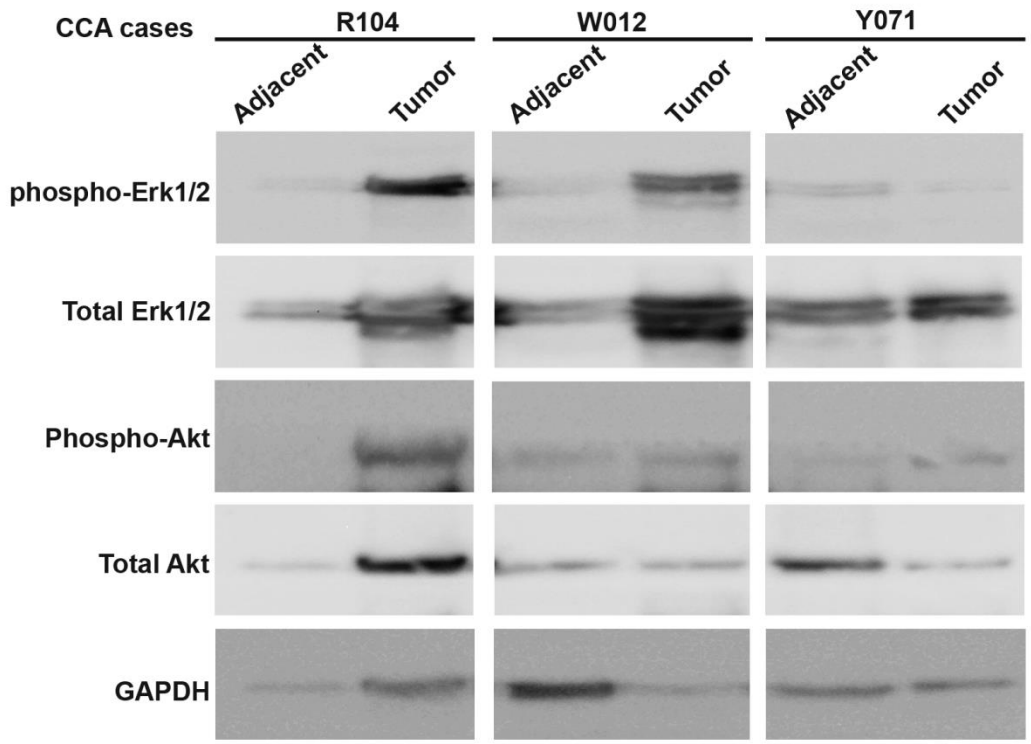


Fig. 3 An increasing of kinases activation in CCA. Representative western blot analysis confirmed the increase in the phosphorylation status of Erk1/2 (T202/Y204) and Akt (S473) in human CCA tissues compared to their normal adjacent tissues.

Targeting kinases using small molecule kinase inhibitors

As several kinases were activated in CCA, we then examined whether kinase inhibitors would affect CCA cell growth. Four CCA cell lines, M156, M214, OCA-17 and KKU-100, were treated with small molecule kinase inhibitors with a range of concentrations and cell proliferation were determined by the alarmarBlue® assay. Incubation with small molecule kinase inhibitors for 72 h led to CCA cell growth inhibition as shown in **Table2**. Among those small molecule kinase inhibitors, it was demonstrated that sorafenib and sunitinib (multi- kinases inhibitors) were the most potent inhibitors that suppressed CCA cell growth with the lowest IC_{50} values (M156 = 3.72±0.69 μM and 10.37±3.55 μM, M214 = 1.64±0.04 μM and 5.12±3.37 μM, OCA-17 = 3.12±1.15 μM and 6.81±0.33 μM, KKU-100 = 6.24±2.80 μM and 12.84±3.91 μM, respectively) when compared with other compounds (**Table 2**).

Apoptotic induction of sorafenib and sunitinib in CCA cells

From the above result, we next evaluated effect of sorafenib and sunitinib on CCA cell growth and apoptosis. Four CCA cell lines; M156, M214, OCA-17 and KKU-100 were incubated with the designed concentrations of sorafenib and sunitinib (0.01μM, 0.1μM, 1μM, 10 μM and 100μM) then cell growth and apoptosis were determined. The results demonstrated that sorafenib and sunitinib significantly inhibited CCA cell proliferation in a dose- dependent manner (**Fig. 4a**). Sorafenib (10μM and 100 μM) and sunitinib (10 μM) significantly induced caspase3/7 activity at the concurrent concentration of growth inhibition in CCA cells as shown in **Fig.4b**. We also determined the effect of those inhibitors suppressing CCA cell growth on the function of Erk1/2 and Akt. After treatment of CCA cells with 10 μM of sorafenib and sunitinib for 24 h, western blot analysis showed that sorafenib and sunitinib markedly inhibited the phosphorylation of Erk1/2 and Akt as shown in **Fig. 4c**.

Table 2 Growth inhibitory activities of protein kinase inhibitors on CCA cell lines.					
Protein kinases inhibitors	**Targets**	**IC_{50} (μM)**			
		M156	**M214**	**OCA-17**	**KKU-100**
Gefitinib	EGFR	15.93 (±2.87)	23.13(±3.95)	24.63 (±4.85)	45.59(±0.72)
Vandetinib	EGFR, VEGFR, RET	19.23 (±5.96)	9.06 (±1.60)	23.72 (±4.16)	17.85 (±0.19)
Erotinib	EGFR	>100	ND	>100	ND
Gleevec	PDGFR, c-KIT, ABL	68.72 (±12.98)	35.74(±1.95)	37.34 (±13.97)	>100
Sorafenib	PDGFR, VEGFR, c-KIT, c-Raf-1, b-Raf	3.72 (±0.69)	1.64(±0.04)	3.12 (±1.15)	6.24 (±2.80)
Sunitinib	PDGFR, VEGFR, c-KIT, FLT3, CSF-1R, RET	10.37 (±3.55)	5.12(±3.37)	6.81 (±0.33)	12.84 (±3.91)
PD173074	FGFR, VEGFR	16.20 (±2.52)	3.89(±3.95)	12.52 (±3.90)	56.32 (±2.95)
SU5402	FGFR	>100	ND	>100	ND
SU4984	FGFR	>100	ND	>100	ND
SU11274	c-MET	11.87 (±6.34)	5.99(±0.18)	6.45 (±5.17)	21.05 (±2.49)
PDGFR inhibitor	PDGFRA	3.41 (±1.45)	7.45(±0.58)	6.23 (±0.08)	44.44 ±4.31)

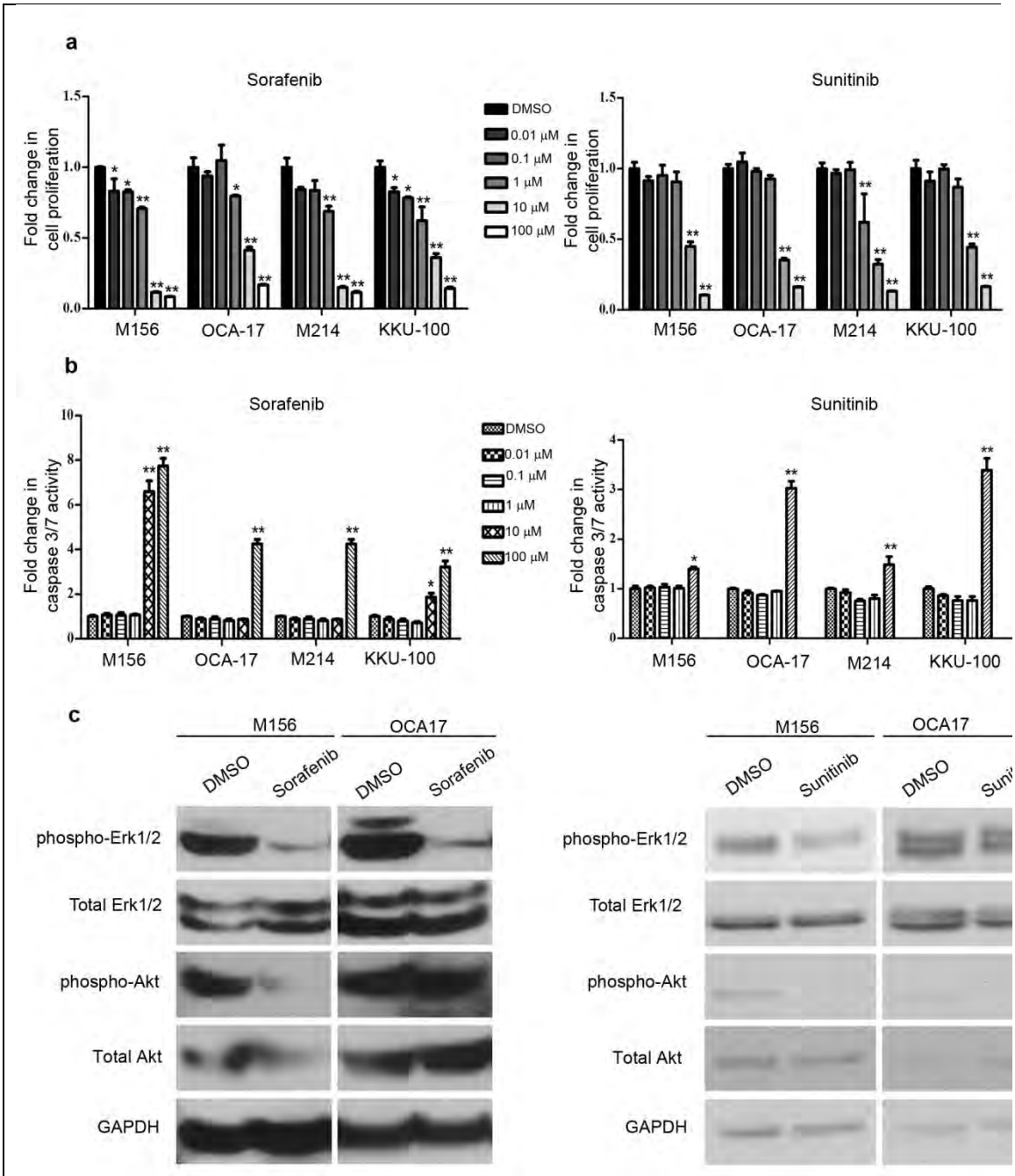


Fig.4 Effects of sorafenib and sunitinib on CCA cell growth and apoptosis. (**a)** CCA cell's proliferation was reduced when treatment with the indicated concentration of sorafenib and sunitinib and cell proliferation was detected with alamarBlue® assay. **(b)** Sorafenib and sunitinib enhance CCA cell apoptosis which was detected by caspase3/7 activity assay. (**c)** Western blot analysis showed the decrease Erk1/2 (T202/Y204) and Akt (S473) phosphorylation in CCA cell lines after treatment with 10 μM of sorafenib and sunitinib. Data of cell proliferation and apoptosis are represented as mean ± SD of three independent experiments. **P*- value < 0.05 compared to untreated cells and ***P*- value <0.001compared to untreated cells.

DISCUSSION

Activation of kinase signaling pathways is involved in the stimulation of multiple cellular processes, while abnormal regulation of kinases is implicated in neoplastic transformation and tumor growth in many types of cancers including CCA. Loilome and coworkers have reported the altered gene expression pattern in the carcinogenesis model of liver fluke- associated CCA [6] Among those upregulated genes, signal transduction genes including protein kinase A regulatory subunit 1 alpha (PRKAR1A) and myristoylated alanine- rich protein kinase C substrate (MARCKS) were detected in CCA tissues. PRKAR1A is a regulatory subunit of PKA type I by which its activation is prominently found in many cancers [14-18].

Increasing of PRKAR1A expression in both mRNA and protein levels also observed for human CCA tissues and cell lines. Silencing PRKAR1A expression and targeting PRKAR1A by specific PKA inhibitors lead to growth inhibition and apoptotic induction of CCA cells. These evidences support an important role of PRKAR1A in control of CCA cell growth [19]. In addition, MARCKS (a substrate of PKC) implicating in cell adhesion, secretion, and motility via the regulation of actin cytoskeletal structure was found to be overexpressed in both the carcinogenesis model of liver fluke- associated CCA and patients' CCA tissues. Moreover, stimulation of CCA cells with a PKC activator, TPA, induced phosphorylation of MARKS and promoted CCA cell migration suggesting the role of MARCKS on CCA cell motility and potentially regulated the metastasis of biliary cancer cells [20]. The results from these studies convince us to study other kinases in CCA in order to identify new drug targets for CCA therapy.

In our study, we surveyed the activated kinase proteins status in CCA using phospho- RTKs and phospho- kinase arrays. We profiled the activated receptor kinases in CCA cells and human CCA tissues. Activated receptor kinases included EGFR, MSPR and EphB2 were found in both CCA cells and tissues. EGFR can be activated by various ligands such as EGF and TNF-α leading to the activation of several downstream target molecules including Ras-Raf-MEK-ERK1/2, STAT3 and STAT5 which involve in regulating cancer cell proliferation and differentiation. In addition, the activation of PI3K-Akt-mTOR cascade contributes in pro- survival pathway of cancers [21, 22]. MSPR or RON receptor can be stimulated by the macrophage stimulating protein (MSP) and hepatocyte growth factor (HGF) which can mediate cell growth, survival and movement via the activation of PI3K/Akt cascade, C-Src/MAPK and FAK [23]. EphB2 is a member of Eph RTKs family which binds with ephrin A5 ligand and can interact with Src, Abl and Arg that are implicated with cell growth stimulation [24]. The above data imply the crucial roles of these RTKs on controlling of growth, survival and movement of CCA.

Interestingly, activated receptor kinases including FGFR family (FGFR1, 2, 3), VEGFR 3, Tie1, Tie2 and Ephrin family (EphA3, EphA6, EphB1, EphB4, EphB6) which implicate in cell growth, survival, tissue microenvironment and angiogenesis via the stimulation of several signaling cascades are only detected in human CCA bulk tissue [24-28]. The result indicates the interaction between tumor and its microenvironment via these molecules may involve in the CCA progression.

We also profiled the non-receptor kinase phosphorylation in CCA. The multiple kinases observed included those in the PI3K/Akt, Ras/MAPK, JAK/STAT and Wnt/β-catenin pathways (summarized in **Table1**). Basically, the non-receptor kinases are the downstream kinase signaling cascades that are triggered via RTKs residing at the cell membrane. Our results indicate that the activation of non-receptor kinases is related with the activation of RTKs. Moreover, we have confirmed the existence of activated kinases in kinase arrays using western blot analysis and found that activated Erk1/2 and Akt were highly expressed in CCA tissues when compared to their normal adjacent tissue (**Fig. 3**). These results reveal the crucial roles that kinases play in CCA whereby receptor kinases act as the primary mediators of the cellular responses by linking ligand binding to downstream signaling cascades and result in mediation of CCA carcinogenesis and progression. In addition, our previous study [19] demonstrated that abrogate PRKAR1A protein expression resulting in growth inhibition of CCA cells which is correlated with a decrease in several signaling cascade' s phosphorylation including MAPK , PI3K/Akt ,JAK/STAT and Wnt/β-catenin pathways, suggesting that CCA required the activation of the multiple kinase signaling pathways to mediate growth and development. Taken together, the involvement of kinases and their exact roles in CCA should be further identified in order to evaluate them as drug targets for CCA treatment.

Recently, the mode of activation of kinases in CCA has been elucidated. Ong and coworkers [27] have recently reported the analyses of whole genome exome sequencing of Ov-associated tumors and their normal adjacent tissues. They have revealed that several somatic mutated genes were detected in liver fluke-associated CCA including Tp53, KRAS, SMAD4, MLL3, ROBO2, RNF43, PEG3 and GNAS which are implicated in histone modification, genomic instability and G protein signaling pathways. However, there were no reports about somatic mutation of kinase genes. In addition, our preliminary data of kinase microarrays analysis revealed the upregulation of EphrinA2 and ErbB3 genes in liver fluke-associated human CCA tissues and IHC staining demonstrated the expression of ErbB3 around 50% (positive 10 in 20 cases) in the corresponding CCA tissues studied (unpublished data). This information suggests that overexpression of kinase genes may be a common mechanism involved in the pathological activation of kinases signaling pathways in CCA.

We also investigated whether targeting kinases using small molecule kinase inhibitors would inhibit CCA cell growth and induce apoptosis. Upon treatment of CCA cells with various types of small molecule kinases inhibitor, it was shown that multi- targeted kinase inhibitors, sorafenib and sunitinib, had higher potency on CCA cell growth inhibition than other single target inhibitors (**Table 2**). Less potency of single target kinase inhibitors on CCA cell growth inhibition might be resulted from the resistant mechanisms of CCA cells which use growth signaling from the alternate pathways consistent with phospho- kinase array data that demonstrated the activated of multiple kinases signaling pathway in CCA. The above information suggests that targeting multiple kinases should be more effective than targeting the single kinase. We therefore evaluated if sorafenib and sunitinib could inhibit CCA cell growth as well as induce apoptosis.

The growth of CCA cell lines was significantly decreased after treatment with the indicated concentration of sorafenib and sunitinb in a dose dependent manner. In addition, high concentration of sorafenib (10μM and 100 μM) and sunitinib (10 μM) could markedly enhance caspase dependent apoptosis in CCA cells.

Sorafenib is a small molecule inhibitor of several kinases including PDGFR, VEGFR, c-KIT, c-Raf-1 and b-Raf-1 which have been approved by the FDA to use for advance renal cancer [29] and hepatocellular carcinoma treatment [30] while sunitinib is an oral multi- kinase inhibitor which block the activation of PDGFR, VEGFR, c-KIT, FLT3, CSF1R, RET and are now used for treatment of pancreatic cancer [31], renal and gastrointestinal stromal cancer [32]. In CCA, pre-clinical study has been reported the potential of sorafenib on growth suppression and apoptosis enhancement both *in vitro* and *in vivo* by inhibiting STAT3 activation via the induction of SHP2 phosphatase enzyme [33]. Nowadays, the usefulness of sorafenib for bile duct cancer treatment has been investigated in a phase II clinical study. This study reveals that a single dose of sorafenib has low activity in CCA patients. Nevertheless, patients having a good performance status show good progression of free survival and mild side effect. Therefore, combination of sorafenib with cytotoxic drugs remains further investigation [34].

Boonjaraspinyo and co- workers [35] have found the overexpression of PDGFR in hamster CCA carcinogenesis model. They also confirmed and reported the upregulation and point mutation of PDGFR in human CCA tissues. In addition, they demonstrated that suppressed PDGFRA gene by sunitinib malate resulting in decreasing of PDGFB, Akt, PDPK1 and STAT3 mRNA expression as well as CCA cell growth inhibition [36]. Recently, a phase II clinical study of sunitinib as second line treatment for metastatic biliary tract cancer has been reported. Patients with advanced biliary tract cancer who failed from the first line chemotherapy were administered with a daily dose of sunitinib (37.5 mg). The results showed acceptable

outcome with manageable toxicity [37]. Moreover, our results also demonstrated that sorafenib and sunitinib can suppress MAPK and Akt signaling in CCA cells. Taken together, this information indicates that protein kinases are involved in CCA development and can be used as therapeutic targets for CCA treatment.

Part II: Increased activation of PI3K/AKT signaling pathway is associated with cholangiocarcinoma metastasis and PI3K/mTOR inhibition presents a possible therapeutic strategy

METHODS

Human CCA specimens

The 30 paraffin-embedded tissues collected from primary tumors of CCA patients were obtained from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand. Informed consent was obtained from each patient prior to surgery and the Ethics Committee for Human Research at Khon Kaen University approved the research protocols (#HE561035).

Cell lines and cell culture

Human CCA cell lines including KKU-OCA17, KKU-100, KKU-M055, KKU-M139, KKU-M156, KKU-M213 and KKU-M214 were obtained from CCA patient, which were established at Khon Kaen University Liver Fluke and Cholangiocarcinoma Research Center. All cell lines were cultured in Ham's F-12 medium (Gibco/BRL, Grand Island, NY, USA) supplemented with 44 mM $NaHCO_3$, penicillin (100 units/ml), streptomycin (100 mg/ml) and 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2.

Antibodies and inhibitor

Antibodies used for this study were as follows: anti-PI3K (p85α), PI3K (p110α), AKT, p-AKT(S473), p-AKT (T308), mTOR, p-mTOR (S2448), GSK-3β, p-GSK-3β (S9), PTEN were purchased from Cell Signaling Technology (Cell Signaling Technology, Danvers, MA, USA.), p-PTEN (S380, T382/383) and LC3B were purchased from Abcam (Abcam, Cambridge, UK) and anti-β-actin antibody was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). The dual PI3K/mTOR inhibitor, NVP-BEZ235 was kindly supplied by Novartis Pharma AG (Basel, Switzerland). The inhibitor was dissolved in dimethylsulfoxide (DMSO) (Amresco, Solon, OH, USA) at a stock concentration of 10 mM and stored at -20°C until used.

Immunohistochemistry staining and scoring

Paraffin-embedded tissues were sectioned for immunohistochemistry (IHC) staining according to standard methods. Briefly, the sections of CCA tissues were deparaffinized and rehydrated through a graded series of aqueous ethanol solutions. Next, microwave antigen retrieval was performed with sodium citrate buffer for 10 min. Then, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in phosphate buffered saline (PBS) for 30 min and non-specific binding was blocked by 10% skim milk in PBS for 1 h. The tissue sections were incubated overnight with the primary antibody against designed target proteins at 4°c. After that, sections were incubated with peroxidase-conjugated Envision™ secondary antibody (DAKO, Denmark) for 1 h. After washing, peroxidase-labeled polymer, 0.1% diaminobenzidinetetrahydrochloride (DAB) solution was used for the signal development and then counterstained with Mayer's hematoxylin. The stained sections were observed under a light microscope by using the high magnification power ×200 and ×400 (Axioscope A1, Carl Zeiss, Jena, Germany).

The IHC scoring system was used for quantitation of results. Grading of staining depended upon the intensity and frequency of staining in tumor area. Intensity of protein expression was classified into four groups: 0, negative; +1, weak expression; +2, moderate expression; +3, strong expression, respectively. The frequency of staining was divided into four groups: 0, negative; +1, 1–25%; +2, 26–50%; +3, >50%, respectively. Staining score were calculated by multiplying - intensities and frequencies in each case which are classified into two groups: low expression levels are < 4 and high expression levels are ≥ 4 [38, 39].

Growth inhibition assay

CCA cells (5 x 10^3) in 100 µl media were seeded in 96 well plates (Costar, Corning, NY, USA) and incubated overnight. Then, the cells were treated with different concentrations of NVP-BEZ235 ranging from 1 to 10^5 nM. After 48 hours of incubation, cell numbers were estimated using the sulforhodamine B (SRB) assay (Sigma-Aldrich, St. Louis, MO, USA). The optical densities were read using a microtiter plate reader (Sunrise, TECAN Trading, Switzerland) at 540 nm. The experiments were performed in triplicate. The percentage of growth inhibition (%GI) was calculated by using the equation: %GI = (1 – (*Nt*/*Nc*) x 100, where *Nt* and *Nc* represent the absorbance in treated and control cultures respectively. IC_{50}, the drug concentration causing cells GI of 50% was determined by interpolation from dose response curves, as previously described [40].

Flow cytometry analysis

CCA cells were seeded at 1.5×10^5 cells in 6 well plates, cultured overnight and then cells were treated with 10, 100 and 1000 nM of NVP-BEZ235 or control medium for 48 hours. After that, the cell cycle distribution was detected by staining

DNA with propidium iodide (Invitrogen, Paisley, U.K.) while apoptosis and necrosis was detected by using Annexin-V-FLUOS staining kit (Roche, Penzberg, Germany), respectively. The cell cycle distribution and apoptosis were determined by flow cytometry (BD FACSCanto™ II, BD Biosciences, CA, USA) and analyzed by BDFACSDiva™ software (BD Biosciences, CA, USA).

Western blot analysis

Cells were treated with NVP-BEZ235 at a concentration as 10, 100 and 1000 nM or control medium for 48 hours, respectively. Cell lysates were electrophoresed and transferred to polyvinylidenedifluoride membranes (Millipore, Bedford, USA). Membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) at room temperature for 1 h and incubated with primary antibody at 4 ^{o}C overnight. After rinsing with TBS containing 0.1% polyoxyethylenesorbitanmonolaurate (Tween-20) (TBST), membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) at room temperature for 1 h. After rinsing with TBST, membranes were exposed to the ECL Prime Western Blotting Detection System (GE Healthcare Bio-Science, UK). The immunoblot and intensity were analyzed by the ImageQuant^{TM} analysis system (GE HealthcareBio-Science, UK). Human β-actin was used as a loading control.

Wound healing assay

CCA cells were seeded and grown to confluence in the fibronectin-coated 12 well plates (Costar, Corning, NY, USA). The *in vitro* wound healing assay was made by scraping the middle of the cell monolayer using a sterile micropipette tip. Floating cells were removed by extensive washing with sterile PBS. After that, cells were cultured with control medium (1% of DMSO) and 10 nM of NVP-BEZ235 in culture medium. The experiments were performed in duplicate well for both conditions. Photographs were taken using a phase-contrast microscope with a digital camera (Axiovert 40, Carl Zeiss, Germany) at 0, 12 and 24 hours after wounding. Degrees of cell spreading between groups were observed.

Statistical analysis

Statistical analyses were performed by SPSS software version 17 (IBM Corporation, NY, USA). The correlation between IHC scores of each protein in CCA tissues was calculated using the Pearson's correlation coefficient. The association of protein expression in CCA tissues and patients' clinico-pathological factors were assessed by Fisher's exact test. Results of cell growth inhibition were presented as mean ± SEM of a representative experiment. The cell cycle analysis was represented as mean ± SD, and the significance of differences was addressed by student's t-test. A *P*-value <0.05 was considered statistically significant.

RESULTS

Altered expression and association of PI3K/AKT signaling pathway molecules with CCA clinicopathological characteristics

A total of 30 CCA tissues were studied, 70% were male and 30% were female. The age of patients ranged from 37 to 71 years old (median=58 years old). The patients were at an advanced stage with 50% presenting with metastasis. In this study, the histological types were classified as papillary type CCA (47%) and non-papillary type CCA (53%). The results of immunohistochemical staining revealed no or very weak positive staining of all proteins investigated in normal bile duct epithelia while the increased expression was observed in pre-cancerous and cancer cells, respectively. The percent expression of p85α, p110α, AKT, p-AKT (T308), mTOR, p-mTOR (S2448), GSK-3β and p-GSK-3β (S9) in CCA tissues is 27%, 90%, 50%, 57%, 67%, 20%, 40% and 27%, respectively (Fig. 5a, Table 3). Moreover, expression levels of the p110α; catalytic subunit of PI3K has a significant correlation with its regulatory subunit; p85α and its downstream targets as shown in Table 4. Interestingly, the expression of p85α, mTOR and GSK-3β was significantly correlated with metastasis (P=0.035, P=0.008 and P=0.025, respectively) (Table 5 and 6). Additionally, elevated expression of AKT, p-AKT and GSK-3β were significantly correlated with non-papillary type of CCA (P=0.028, P=0.030 and P=0.007, respectively) (Table 5 and 6). Furthermore, there was a trend towards significance between p-AKT expression and metastasis status of patients (P=0.065). In addition, the expression levels of p110α also showed a trend associated with non-papillary (P=0.073), with 67% of these cases are metastatic CCA. Interestingly, we found loss of PTEN expression in the majority of CCA patients (70%) as shown in Table 3. Furthermore, we observed that within 89% of the PTEN positive cases, their PTEN were inactivated by phosphorylation. In addition, western blot analysis revealed the PTEN inactivation by phosphorylation in all CCA cell lines studied (Fig. 5b). Therefore, loss of PTEN expression as well as inactivation by phosphorylation seems to be the major cause of PI3K/AKT overactivation in this series of CCA.

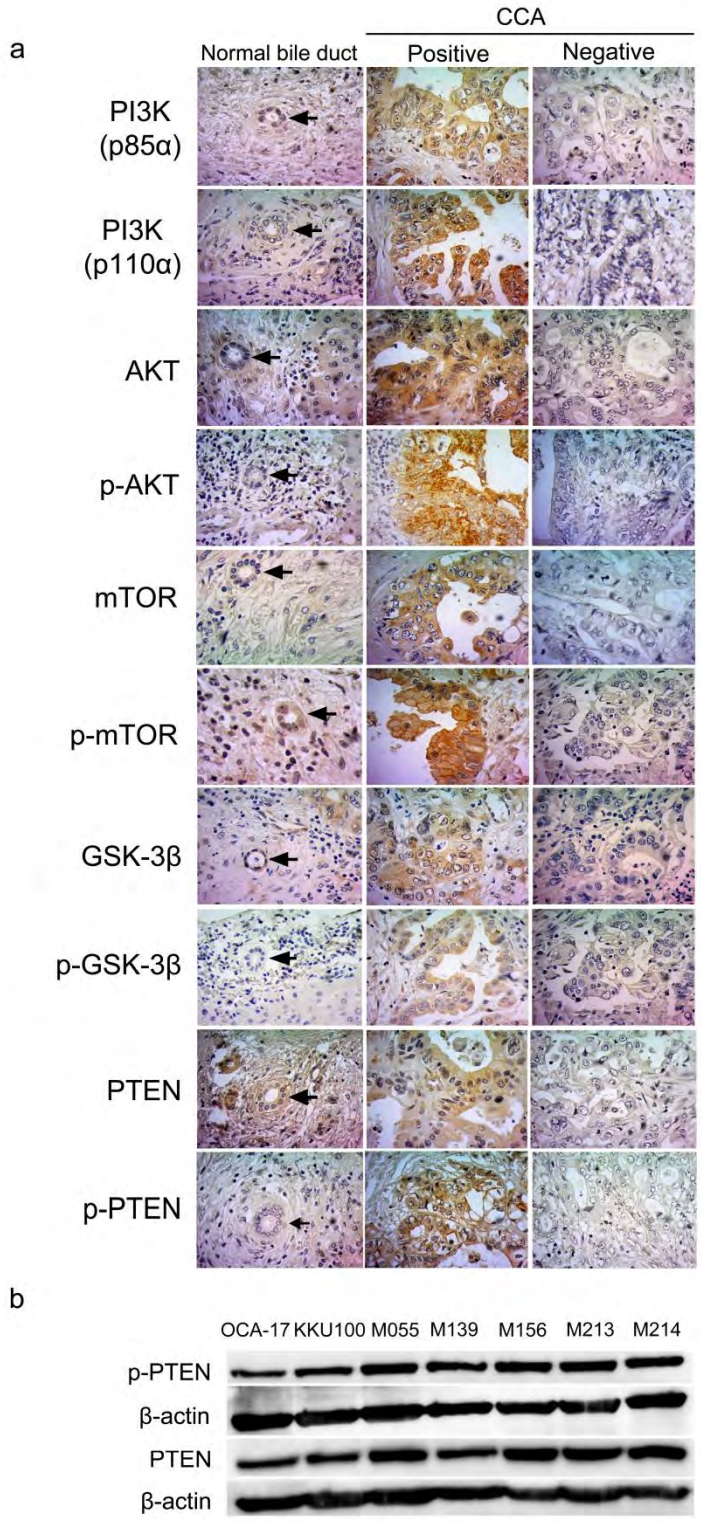


Fig. 5 Expression of several key components of PI3K/AKT signaling pathway. (a) The expression of p85α, p110α, AKT, p-AKT (T308), mTOR, p-mTOR (S2448), GSK-3, p-GSK-3β (S9), PTEN and p-PTEN (S380, T382/383) in normal adjacent bile duct and human CCA tissues by immunohistochemistry staining. Original magnification is ×400. (b) Western blot analysis of the expression of PTEN and p-PTEN in a panel of CCA cell lines.

Table 3 Summary of the PI3K/AKT signaling pathway components expression in human CCA tissues.

Molecules	Total cases	Results			
		Positive			Negative (%)
		High	Low	Total (%)	
1. p85α	30	3	5	8 (27%)	22 (73%)
2. p110α	30	18	9	27 (90%)	3 (10%)
3. Total AKT	30	6	9	15 (50%)	15 (50%)
4. p-AKT (T308)	30	5	12	17 (57%)	13 (43%)
5. Total-mTOR	30	8	12	20 (67%)	10 (33%)
6. p-mTOR (S2448)	30	1	5	6 (20%)	24 (80%)
7. Total-GSK3	30	6	6	12(40%)	18 (60%)
8. p-GSK3 (S9)	30	3	5	8 (27%)	22 (73%)
9. Total-PTEN	30	4	5	9 (30%)	21 (70%)
10. p-PTEN (S380, T382/383)	9	5	3	8 (89%)	1 (11%)

Table 4 Correlation coefficients between immunohistochemistry scores of the PI3K/AKT signaling pathway components in human CCA tissues.

		p110α	AKT	p-AKT	mTOR	p-mTOR	GSK-3β	p-GSK-3β	PTEN	p-PTEN
P85α	Correlation coefficient *P*	0.535 0.002*	.593 0.001*	0.619 0.000*	0.619 0.000*	0.667 0.000*	0.597 0.000*	0.564 0.001*	0.621 0.000*	0.283 0.460
P110α	Correlation coefficient *P*		0.465 0.010*	0.528 0.003*	0.464 0.010*	0.351 0.057	0.452 0.012*	0.282 0.132	0.332 0.073	0.473 0.199
AKT	Correlation coefficient *P*			0.580 0.001*	0.681 0.000*	0.270 0.148	0.835 0.000*	0.681 0.000*	0.678 0.000*	0.200 0.606
p-AKT	Correlation coefficient *P*				0.715 0.000*	0.323 0.082	0.564 0.001*	0.770 0.000*	0.728 0.000*	0.228 0.555
mTOR	Correlation coefficient *P*					0.478 0.008*	0.686 0.000*	0.664 0.000*	0.828 0.000*	0.807 0.009*
p-mTOR	Correlation coefficient *P*						0.083 0.665	0.234 0.213	0.439 0.015*	0.452 0.222
GSK-3β	Correlation coefficient *P*							0.637 0.000*	0.625 0.000*	0.326 0.392
p-GSK-3β	Correlation coefficient *P*								0.751 0.000*	0.369 0.328
PTEN	Correlation coefficient *P*									0.754 0.019*

Table 5 Correlation between PI3K subunits, AKT and their active form expression in CCA tissues and clinicopathological findings.

Clinicopathologic factors	No. of patients	p110α			p85α			AKT			p-AKT (T308)		
		-/Low	High	*P*	-	+	*P*	-	+	P	-	+	*P*
Age (years)													
≤ 58	14	6	8	0.765	10	4	1.000	5	9	0.143	7	7	0.491
> 58	16	6	10		12	4		10	6		6	10	
Gender													
Female	9	4	5	1.000	7	2	1.000	5	4	1.000	3	6	0.691
Male	21	8	13		15	6		10	11		10	11	
Histological types													
Non-paillary	16	4	12	0.073	10	12	0.266	5	11	0.028	4	12	0.030*
Papillary	14	8	6		6	2		10	4	*	9	5	
Metastasis													
Non-metastasis	15	8	7	0.136	14	1	0.035	9	6	0.273	9	6	0.065
Metastasis	15	4	11		8	7	*	6	9		4	11	

Table 6 Correlation between mTOR and their active form and GSK-3β and their inactive form expression in human CCA tissues and clinicopathological findings.

Clinicopathologic factors	No. of patients	mTOR			p-mTOR			GSK-3β			p-GSK-3β		
		-	+	*P*	-	+	*P*	-	+	P	-	+	*P*
Age (years)													
≤ 58	14	3	8	0.105	12	2	0.657	7	7	0.296	11	11	0.689
> 58	16	11	8		12	4		11	5		3	5	
Gender													
Female	9	3	8	0.419	7	2	1.000	5	4	1.000	5	4	0.195
Male	21	11	8		17	4		13	8		17	4	
Histological types													
Non-paillary	16	4	12	0.156	13	3	1.000	6	10	0.007	10	6	0.22
Papillary	14	7	7		11	3		12	2	*	12	2	6
Metastasis													
Non-metastasis	15	9	6	0.008*	13	2	0.651	12	3	0.025	12	3	0.682
Metastasis	15	2	13		11	4		6	9	*	10	5	

NVP-BEZ235 inhibits CCA cell growth through blocking the PI3K/mTOR activity

We examined further if NVP-BEZ235, a dual PI3K/mTOR inhibitor, could inhibit CCA cell proliferation. Seven CCA cell lines (OCA17, KKU100, M055, M139, M156, M213, and M214) were treated with a range of concentration of the small molecule and cell proliferation was assessed by SRB assay. The results showed that NVP-BEZ235 effectively suppressed CCA cell growth at one nanomolar (nM) and in a dose-dependent manner (Fig. 6a). The IC_{50} values (mean±SD) of NVP-BEZ235 in seven CCA cell lines; OCA17, KKU100, M055, M139, M156, M213, and M214 were 48±19, 15±4, 5±2, 135±48, 2±1, 30±5 and 45±5, respectively. Additionally, western blot analysis revealed that NVP-BEZ235 was able to inhibit the phosphorylation of S473 AKT and S2448 mTOR in two representative cell lines in a dose-dependent response (Fig. 6b), suggesting that NVP-BEZ235 blocks the PI3K/AKT pathway resulting in inhibition of CCA cell proliferation. Moreover, flow cytometry analysis demonstrated that NVP-BEZ235 induced significantly G1 arrest as well as reduced S phase progression of CCA cell lines, for M139 at 1000 nM and M214 at 100 and 1000 nM (Fig. 6c).

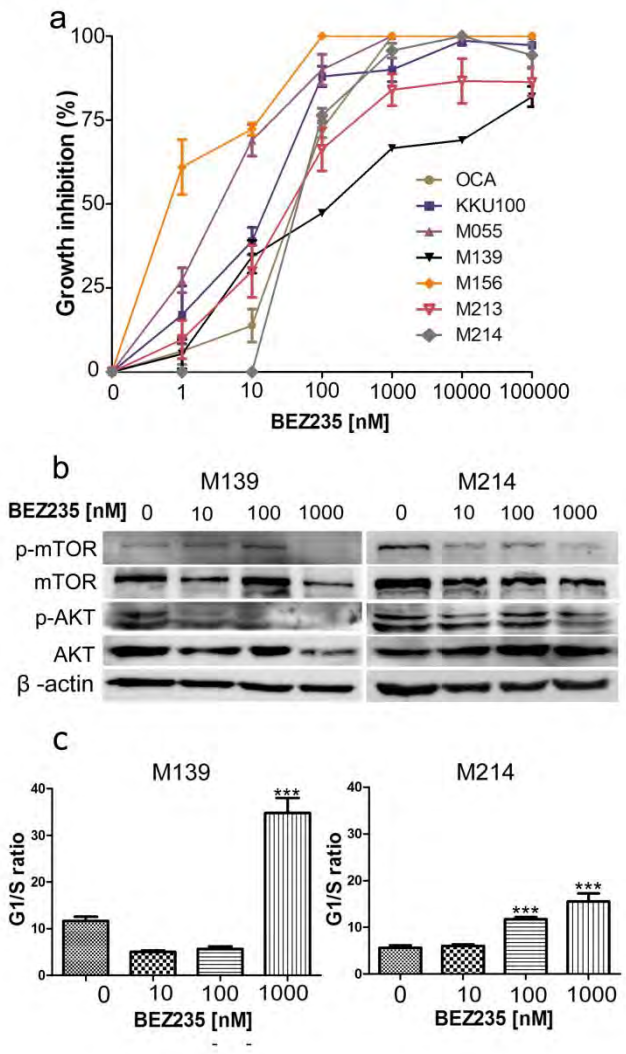


Fig.6 NVP-BEZ235 inhibits CCA cell proliferation. (a) Seven CCA cell lines were treated with NVP-BEZ235 with the indicated concentration for 48 hours and cell proliferation was assessed using SRB assay. Data are shown as mean ± SEM of one representative experiment. Each culture was done in three independent experiments. (b) Effects of NVP-BEZ235 on reducing of AKT and mTOR phosphorylation in CCA cells. Cells were treated with NVP-BEZ235 as indicated concentration for 48 hours. Cell extracts were subjected to immunoblotting analysis for AKT, p-AKT, mTOR and p-mTOR, respectiviely. (c) NVP-BEZ235 causes cell cycle arrest at G1 phase. Cells were treated with NVP-BEZ235 for 48 hours and subjected to propidium iodide staining analysis. The results were shown as the mean ± SD from three independent experiments.*, $P<0.05$ compared with control cells.

NVP-BEZ235 induces autophagy without remarkable effect on apoptosis in CCA cells

To test if NVP-BEZ235 inhibited cell growth, at least in part by inducing apoptosis, we performed flow cytometry using Annexin-V-FLUOS staining. The results indicated that NVP-BEZ235 has no remarkable effect on apoptosis induction (Fig. 7a). Although, NVP-BEZ235 decreased phosphorylation of AKT and mTOR which function as a key negative regulator for autophagy, the type-II programmed cell death. We therefore examined the effect of NVP-BEZ235 on autophagy induction. The results revealed that NVP-BEZ235 induces LC3B (LC3-II) protein, a hallmark of cells undergoing autophagy in a dose- and time-dependent manner in treated CCA cell lines (Fig. 7b).

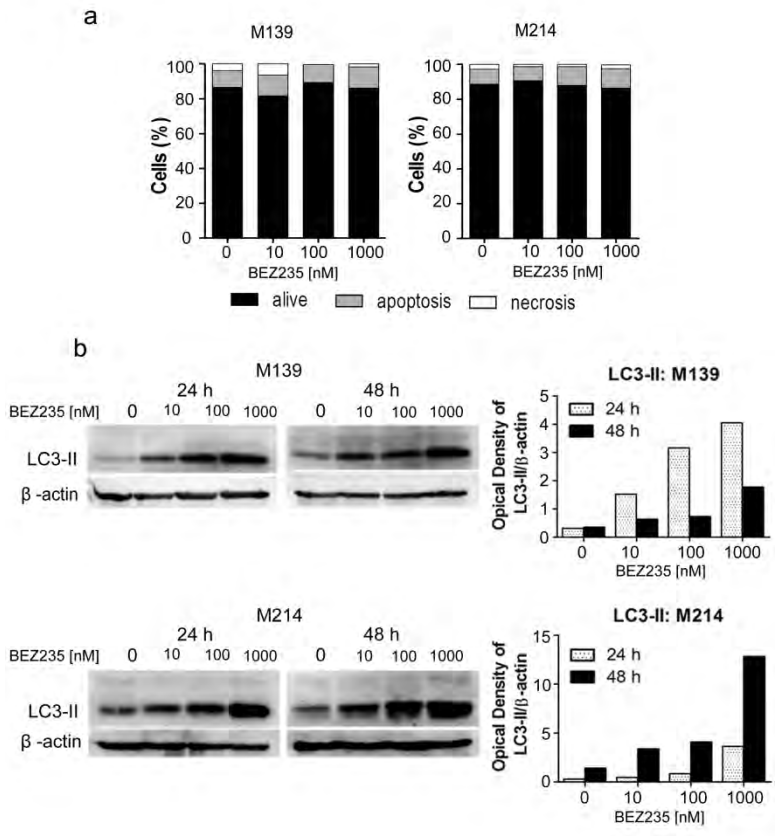


Fig. 7 NVP-BEZ235 induces autophagy without remarkable effect on apoptosis in CCA cells. (a) Cells were treated with NVP-BEZ235 as indicated concentration for 48 hours, then apoptosis cells were analyzed by using Annexin-V-FLUOS staining kit and flow cytometry. (b) NVP-BEZ235 induces autophagy in CCA cell lines. Cells were treated with NVP-BEZ235 for 24 and 48 hours, respectively. Protein extracts were analyzed by western blot with specific antibodies against LC3B (LC3-II), an autophagic marker. The apparent intensity of bands on the membranes was estimated using densitometry.

NVP-BEZ235 inhibited migration of CCA cells

The above IHC data indicated the association of several key components of PI3K pathway with the metastasis status of the CCA patients studied. Therefore, we sought to determine if NVP-BEZ235 could inhibit CCA cell migration, an important step in the metastatic process. We performed an *in vitro* wound healing assay in conditioned culture medium with or without NVP-BEZ235. The results showed that the migration of the M139 and M214 cells was reduced by treating cells with 10 nM of NVP-BEZ235, a concentration which has no effect on cell proliferation (Fig. 8).

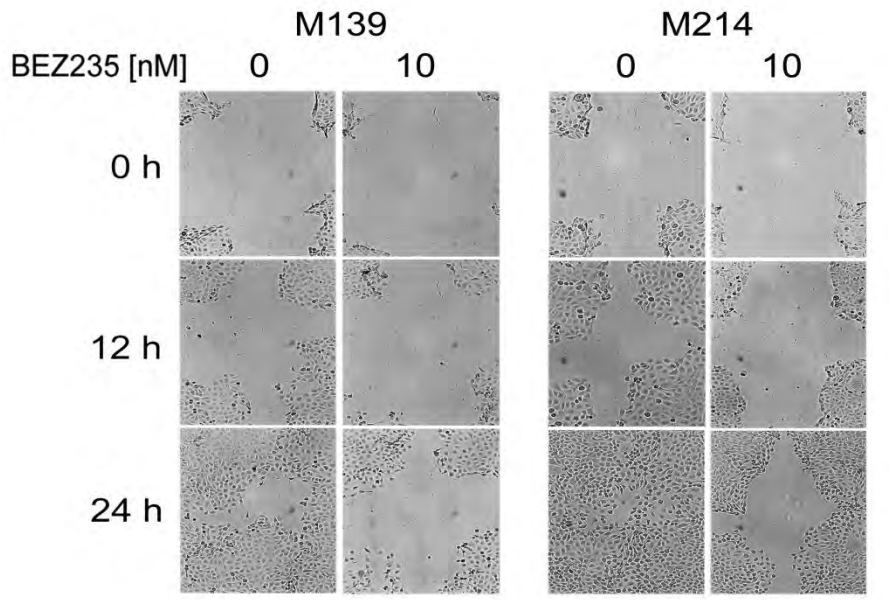


Fig. 8 NVP-BEZ235 reduced CCA cell migration. The effect of NVP-BEZ235 on the migration of CCA cell lines was determined by *in vitro* wound healing assay. The extent of wound closure was observed and photographed using a phase-contrast microscope with a digital camera at 0, 12 and 24 hours incubation.

DISCUSSION

Although, an increased activation of PI3K/AKT pathway along with PTEN loss [41, 42] has been reported in CCA, until now there has been no comprehensive study of PI3K, AKT, p-AKT, mTOR, p-mTOR GSK-3β, p-GSK-3β together with PTEN and its inactive form, p-PTEN in human CCA tissues. A previous study revealed that altered expression of p110α, the catalytic subunit of class IA PI3K, by amplification, overexpression or somatic mutation has been found in various human tumors [43]. In this current work, we report the overexpression of p110α in human CCA tissues. Moreover, our results demonstrated the significant positive correlation between expression levels of p110α with its regulatory subunit, p85α, and its downstream targets. However, expression of p85α showed fewer positive cases than p110α. Although, p110α could be directly activated by RAS proteins [44] which are usually altered in CCA. A high frequency of KRAS proto-oncogene mutation was found in 4–60% among Japanese and Thai CCA patients [45-48] and 100% of English patients [49]. Moreover, there are studies that indicate elevated expression of mutated KRAS have been correlated with a more aggressive phenotype of CCA [50-52]. Furthermore, our data revealed that the expression of p85α was significantly associated with metastasis. Phosphorylation at T308 by PDK1 and S473 by mTORC2 is essential for maximal activation of AKT. Although, phosphorylation of T308 in AKT is essential and necessary for the cell transforming activity and for signaling [53], possibility as a result of changing in conformation that allows substrate binding and phosphorylation [54]. However, several studies focused on S473 rather than T308 phosphorylation, probably caused by relatively effective of S473 antibodies, particularly in immunohistochemistry resulting in analyzing p-AKT (T308) in few studies. Thus, this study aimed to reveal the expression of p-AKT (T308) in CCA. Previous studies demonstrated an activation of the PI3K/AKT pathway in human CCA tissues which more than 50% of cases expressed p-AKT and correlated with poor prognosis [41, 42, 55-58]These data consistent with our results as shown that 57% of the cases were positive for p-AKT (T308) and correlated with poor prognosis. Our results showed a few cases, 20% positive for p-mTOR with no correlation to any clinical data. Chung and coworkers reported that p-mTOR expression was elevated in 83.7% in extrahepatic CCA tissues [41]. Additionally, the altered expression of GSK-3β which was previously reported to be a tumor suppressor in various types of cancer [59-61]. There is a single study that demonstrated the increased levels of p-GSK-3β (S9), an inactive form in a mouse model of CCA carcinogenesis [42]. In this study, we report the expression of GSK-3β and its inactive form, p-GSK-3β (S9) in human CCA tissues. Our IHC results revealed the strong positive staining intensity in tumors when compared with adjacent normal bile ducts. We found that about 40% of cases

expressed GSK-3β, which correlated with metastasis and histological type of the poor prognosis. Our finding was supported by growing evidence which indicated that GSK-3β can positively regulate cell survival and proliferation [62], as well as promote tumor development and progression. Moreover, inhibition of GSK-3β can sensitize tumor cells to chemotherapeutic drug as shown in pancreatic cancer [63]. In addition, we found only 8 of 30 cases were positive for p-GSK-3β, an inactive form of GSK-3β. Furthermore, the expression of PTEN which defined as a tumor suppressor and major negative regulator of PI3K/AKT signaling was investigated. We found loss PTEN expression in the majority of CCA patients (70%) and consistent with the study of Chung and coworkers, which showed the absence of PTEN expression in extrahepatic cholangiocarcinoma patients [41]. Moreover, we found that among PTEN positive cases, their PTEN was inactivated through phosphorylation. The results are consistent with the study of Xu and coworkers [42].

We analyzed for the first time in CCA the therapeutic potential of a PI3K/mTOR inhibitor, NVP-BEZ235, an imidazo[4,5-c]quinoline derivative that inhibits activity by binding to the kinase ATP-binding cleft. This compound equally targets wild-type, mutated p110 and mTOR [64] as well as in cells with loss of PTEN expression [65]. We tested the growth inhibition effect of NVP-BEZ235 on a panel of CCA cell lines, which showed PTEN inactivated by phosphorylation. We found that NVP-BEZ235 could suppress CCA cell growth in the one nanomolar range of IC_{50} values in a dose-dependent manner. Moreover, the agent exerted its anti-cancer activity by reducing AKT and mTOR phosphorylation. Furthermore, flow cytometric analysis demonstrated that NVP-BEZ235 was capable of inducing G1 cell cycle arrest while lacking significant apoptosis induction. On the other hand, NVP-BEZ235 activates the CCA cells undergoing autophagy, a type-II cell death which indicated by increased LC3-II, an autophagic marker level, in a dose and time dependent manner. In many cancers NVP-BEZ235 had no apparent apoptosis induction [65-69]. Liu and coworker demonstrated previously the cell growth inhibition as well as autophagy induction of NVP-BEZ235 in human glioma cells [68]. This is not surprising because NVP-BEZ235 target is mTORC1, an autophagic blocker resulting in autophagy induction. Although, many studies demonstrate the proapoptotic properties of NVP-BEZ235, in breast cancer [64], waldenstrom macroglobulinemia [70] and hepatocellular carcinoma [71]. Additionally, it is known that autophagy is a cellular response to stress conditions, which is implicated in both pro-survival and pro-death processes in normal cells, as well as in cancer. It is also defined as a mechanism that may enable tumor cells to survive antineoplastic therapy. In addition, there are several lines of evidence demonstrating that many anti-cancer agents can also induce autophagy [72-75]. There are studies suggesting that NVP-

BEZ235 alone was not sufficient to induce apoptosis; hence this compound should be combined with other therapeutic approaches, such as MEK inhibitor [76] or radiotherapy [66, 67]. Furthermore, there are some studies mentioned that combined NVP-BEZ235 with autophagy inhibition can enhance its anti-cancer efficacy by driving the cancer cells to undergo apoptosis [77, 78]. Since our IHC analysis demonstrated the expression of several key components in PI3K/AKT pathway is correlated with metastasis in CCA patients, we tested if NVP-BEZ235 could inhibit CCA cell migration. As shown in Figure 4, treated CCA cells with 10 nM of NVP-BEZ235 resulting in reduced CCA cell migration. This result suggests that this pathway plays a role in metastasis of CCA. This is supported by the study of Menakongka and colleagues who reported that hepatocyte growth factor (HGF) promotes CCA cell invasiveness through PI3K pathway [79]. Furthermore, there are several reports that are consistent with our finding that NVP-BEZ235 can inhibit cancer cell migration [66, 69]. Moreover, Manara and co-workers also reported that NVP-BEZ235 suppressed cell metastasis of Ewing sarcoma xenografts *in vivo* mouse models [69]. Therefore, the PI3K pathway may be used as a potential target for treatment or prevention of metastasis in CCA.

Taken together, we demonstrated the constitutive activation of PI3K/AKT pathway in CCA patients as well as CCA cell lines. The possible mechanisms are PI3K overexpression as well as PTEN inactivation by both loss of expression and phosphorylation resulting in poor prognosis in human CCA studied. Therefore, this particular pathway represents a possible useful target for CCA treatment. This work also indicates that inhibition of PI3K and mTOR activity by a novel inhibitor, NVP-BEZ235 has anti-proliferation as well as migration inhibition effects on CCA cells. However, this small inhibitor failed to induce apoptosis of CCA cells while autophagy induction could be observed. Based on these findings, NVP-BEZ235 might be applied in a therapeutic strategy for CCA, however, using this particular drug in combination with other therapy should be considered.

Part III: Activated macrophages promote Wnt/β-catenin signaling pathway in cholangiocarcinoma cells

METHODS

Human CCA specimens

The 40 paraffin-embedded and 48 frozen intrahepatic CCA tissues were selected from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand. The informed consentwas obtained from each subject before surgery and Human Research Ethics Committee, Khon Kaen University has approved the research protocol (#HE43201, #HE471214 and #HE521209).

Human CCA Cell lines

Five respective CCA cell lines, KKU-M055, KKU-M139, KKU-M156, KKU-M213 and KKU-M214 were developed from primary tumor of patients, who were admitted to Srinagarind Hospital with the primary diagnosisof CCA. CCA cell lines were established by Associate Professor Dr. Banchob Sripa; Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University.

All cell lines were cultured in HAM-F12 (Gibco/BRL, Grand Island, NY) supplemented with 10% inactivated fetal bovine serum, 2 mg/ml sodium bicarbonate and 1% antibiotic-antimycotic solution (Life Technologies, Inc., Gaithersburg, MD). All cultured cell lines were incubated at 37°C in a humidified incubator maintained with an atmosphere of 5% CO_2. Subculture was done when the cell reached the confluent stage and the media were changed once every two to three days.

U937 human macrophage cells

Human promonocytic leukemia U937 cells were purchased from the American Type Culture Collection (ATCC). U937 cells were maintained in suspension culture in RPMI-1640 supplemented with 10%(v/v) heat-inactivated fetal bovine serum in a humidified atmosphere containing 5% CO_2.

Antibodies

Antibodies used for this study were as follows: Rabbit anti-Wnt3 polyclonal antibody, Rabbit anti-Wnt3a polyclonal antibody, Rabbit anti-Wnt5a polyclonal antibody, and Rabbit anti-Wnt7b polyclonal antibody were purchased from Abcam Company (Abcam, USA), while Mouse anti-β-catenin polyclonal antibody was from BD Biosciences (USA).

Total RNA extraction and cDNA synthesis

Total RNA was isolated from liver tissues and cell lines using TRIZOL® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. A 5µg aliquot was reverse transcribed using RevertAid M-MuLV reverse transcriptase (Fermentas, Burlington, CA) and random hexamer (Promega, Madison, WI) as a primer which were mixed together and then heated at 70°C for 5 min. After that, reaction mixture containing the first-stranded cDNA synthesis buffer (1x; 75 mM KCl, 50mMTris-Cl pH 8.3, 3mM $MgCl_2$), 0.5 mM each dNTPs and 200 units reverse transcriptase (Fermentas, Burlington, CA) was added. Reverse transcription was carried on using a DNA thermocycler (GeneAmp PCR system 2400, Applied Biosystems, CA). The thermal conditions were at 25 °C for 10 min, 42°C for 1 h, and 70°C for 10 min, respectively.

Quantitative real-time polymerase chain reaction (qPCR)

qPCR of Wnts and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was performed using a Taqman gene expression assay kit and the ABI 7500 real time PCR system (Applied Biosystems, Foster City, CA). The TaqMan® Probes were labeled with quencher and reporter dyes. The reporter dye of targeted TaqMan® Probes in this experiment was labeled with FAM dye whereas GAPDH probe was labeled with VIC dye. PCR reactions were performed using FastStart Universal Probe Master Mix (Roche, Switzerland) following the manufacturer's protocol in duplication for each sample. Briefly, each 20 µl/reaction contained 10 µl of 2X Taqman master mix, 1 µl of Taqman probe and 9 µl of 1:6 diluted cDNA. After that the PCR reaction was performed in 2 stages PCR reaction including (i) stage 1 at 95°C for 10 min for beginning the hot start DNA polymerase, and (ii) stage 2 at 95°C for 10 sec for denaturing DNA to single strand and unfolding secondary form to the primary structure of DNA then the temperature was down to 60°C for 1 min for annealing of primer and DNA as well as DNA polymerase chain reaction; this stage was performed for 50 cycles. Furthermore, during DNA polymerase chain reaction the specific probe was digested then released of quencher dyes resulting of activation of reporter dye and then the fluorescence signal was detected from the activated reported dye. The relative quantification of the gene expressions was done using the comparative cycle threshold (C_T) method and GAPDH expression as the endogenous control. C_T was calculated from the reporter dye florescence signal in the middle log phase of the signal chromatogram. The calculation formula is as below.

Relative Expression Quantification (REQ) = $2^{-\Delta CT}$

Where;

ΔC_T = Average C_T (target) – Average C_T (endogenous)

And, Target =interesting genes, Endogenous = GAPDH

Immunohistochemistry

Wnts and β-catenin were detected on the formalin-fixed, paraffin-embedded sections using standard immunohistochemistry protocols. The paraffin sections were deparaffinized then hydrated by submerging respectively in xylene and ethanol with stepwise decreasing concentration. The sections were treated with 3% H_2O_2 in PBS to block the endogenous peroxidase. After blocking with 5% skimmed milk, the sections were incubated with 1:500 rabbit anti-Wnt3 antibody (Abcam, MA, USA) at 4ºC overnight, 1:200rabbit anti-Wnt3a antibody (Abcam, MA, USA) at 4ºC overnight, 1:50 rabbit anti-Wnt5a antibody (Abcam, MA, USA) at 4ºC overnight, 1:100 rabbit anti-Wnt7b antibody (Abcam, MA, USA) at 4ºC overnight, 150 mouse anti-β-catenin antibody (BD Biosciences, USA) at 4ºC overnight. After that, sections were incubated with peroxidase-conjugated Envision™ secondary antibody (DAKO, Glostrup, Denmark). Peroxidase activity was observed using DAB as the substrate. The sections were counterstained with hematoxylin, dehydrated with stepwise increasing concentration of ethanol, cleared with xylene and mounted with permount. The staining frequency of proteinwas semiquantitatively scored on the basis of the percentage of positive cells as: 0% = negative; 1% to 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 duct was defined as bile duct with increasing cell numbers, enlargement of duct size and pseudopapillary projection, while dysplastic duct was defined as bile duct composed of cells with multilayer nuclei, increasing nuclear-cytoplasm ratio and containing micropapillary projection.

Stimulation of U937 cells with LPS

U937 macrophage cells were cultured in a 6-well plate 1.5×10^5 cells/well overnight. Then, cells were treated with1 or 2 µg/ml LPS for 12, 24 and 48 h. Phosphate buffered saline (PBS)-treated cells were used as negative controls. After stimulation, cells were processed for qRT-PCR.

Production of conditioned media

U937 macrophage cells were plated in 6-well culture plates 1×10^5 cells/well overnight and were left unstimulated or stimulated with 2 µg/ml LPS for 48 h. After treatment, conditioned media were collected under sterile conditions, centrifuged (2000rpm, 5minutes, 4°C) to remove cell debris, and stored at -80°C. The supernatant from the LPS-treated U937 macrophages was designated as activated macrophage-conditioned media (AMCM) and that from the untreated control cells as non-activated macrophage-conditioned media (NAMCM).

Stimulation of CCA cells with conditioned media from macrophages

Three CCA cells, M139, M213 and M214 were cultured in a 6-well plate 1×10^5 cells/well overnight. Then, cells were treated with NAMCM for 24 h and AMCM for 12 and 24 h. Untreated-CCA cells were used as controls. After stimulation, cells were subjected to western blot analysis.

Transient knockdown of β-catenin using siRNA

High endogenous β-catenin expression, M214 CCA cells ($2x10^5$ cells) were seeded into a 6-well plate for 24 h before transfection. Cells were transfected either with siRNA specific for human β-catenin mRNA (siRNA ID: s438; Ambion, Foster City, CA, USA) or validated non-targeting siRNAs (scramble controls) (Ambion, Foster City, CA, USA) or no siRNA sequence at all, using Lipofectamine Transfection Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instruction. Specifically, 5 µl of lipofectamine was diluted in Opti-MEM I reduced serum media then incubate for 5 min at room temperature. siRNA was added to diluted lipofectamine to get a total volume of 500 µl. Then the solution was mixed gently and incubated for 20 min at room temperature. Then 500 µl of siRNA/lipofectamine complex was added to each well containing 2.5 ml Opti-MEM I reduced serum medium and mix gently by rocking the plate back and forth. Then, cells were incubated at 37°C in 5% CO_2 incubator. After 24 h of incubation, the medium was replaced with 2.5 ml of complete medium and further incubated for 24 h. After that cells were subjected for further analysis.

Protein extraction and determination

Cells were washed with PBS and then lysed with radioimmuno-precipitation assay (RIPA) buffer containing Protease K inhibitor cocktail, 0.5 MNaF, 0.2 M $NaVO_4$, 1M Tris-HCl pH 7.5, 0.5 M EDTA, 2.5 MNaCl, 10% NP-40, 10% SDS and deionized water. Cell lysate was then centrifuged at 13,000 xg for 10 min at 4°C. The supernatant was transferred to a new tube and determined for protein content using the Pierce BCA™ Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Twenty-five µl of each cell lysate was mixed with 200 µl of BCA working reagent, and then incubated at 37°C for 30 min. The absorbance was read at 540 nm against the blank. The concentration of protein in the sample was estimated from a standard curve that was constructed by using serial dilution starting from 2 mg/ml of standard bovine serum albumin.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and western blot analysis

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins extract from the cell lysates were solubilized in 4X SDS buffer containing DTT and boiled at 95°C for 10 min, then cooled down

withice. Fifty μg of protein extract was electrophoresed by 10% SDS-PAGE. Then, proteins in electrophoresed polyacrylamide gel were transferred onto a polyvinylidenedifluoride (PVDF) membrane (Bio-Rad, Hercules, CA). Prior to transferring, PVDF membrane was soaked and agitated in 100% methanol for 15 sec then soaked in the transferring buffer at room temperature for at least 5 min with gentle agitating. The electrotransferring of proteins onto a PVDF membrane was performed at 41mAmp for 1.5 h and incubated overnight with 5% non-fat dry milk. The blots were then probed with primary antibodies including 1:1000 mouse anti-β-catenin antibody (BD Biosciences, USA), 1:1000 mouse anti-cyclin D1 (Cell signaling, MA, USA), 1:200 mouse anti-CDK4 (Cell signaling, MA, USA), and 1:20000 mouse anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA) as an internal control. The membranes were incubated with primary antibodies at 4°C for 1 h. or overnight and secondary antibodies at room temperature for 1 h. Proteins were detected by the Enhanced Chemiluminescence Plus solution (GE Healthcare, UK). The apparent density of the bands on the membranes was captured by ImageQuant Imager and analysed using ImageQuant analysis software (GE Healthcare, UK).

Statistical analysis

Wnt mRNA levels in human CCA tissues and normal liver tissues were reported as mean ± SD. Difference expression between groups was examined using Mann-Whitney U-test and Wilcoxon signed rank test. Clinico-pathological characteristics and protein staining were compared using the χ^2 or Fisher's exact probability test. The Kaplan-Meier method was used to calculate the survival curves, and the log-rank test was performed to compare differences in the survival rates of patients who were subjected to curative surgery. All analyses were done using SPSS software (version 15.0). A $P < 0.05$ was considered as significance.

RESULTS

Patient characteristics

Of the 48 intrahepatic CCA patients examined, 33 (70.2%) were males and 14 (29.8%) were females. A ratio between male to female is 2.3:1. The mean age was 57±9.1 years (rang, 37-74 years). Most of the patients were in an advanced stage, with 44.7% metastasis. In this series, the histological type was classified as poorly differentiated- (6.4%), moderately differentiated- (19.1%), well differentiated- (46.8%) and papillary (27.7%) –type CCA.

Wnt mRNAexpression profiles in human CCA tissues

The mRNA expression level of 9 Wnt genes which the altered expression has been previously reported in various types of cancer including Wnt1, Wnt2, Wnt3, Wnt3a, Wnt5a, Wnt7a, Wnt7b, Wnt8b, and Wnt10b, respectively was investigated in 47 human CCA as well as in adjacent non-tumorous tissues of the same patient and normal liver of 9 cadaveric donors. The relative Wnt mRNAs expression level determined using real-time RT-PCR is shown in Figure 9. Our data demonstrated that there was no differential expression of Wnt1 among the studied groups, whereas the expression of Wnt2 and Wnt7a were significantly increased in adjacent non-tumor and tumor tissues when compared with cadaveric donor. In addition, Wnt3a, Wnt5a and Wnt7b expression was significantly increased in tumor tissues when compared with adjacent tissues and liver tissues from a cadaveric donor, respectively. Conversely, Wnt3 expression is significantly decreased in tumor tissues when compare with cadaveric donors and adjacent tissues. The Wnt8b mRNA level in the tumor was significantly increased compared to adjacent tissues. Furthermore, the expression of Wnt10b in adjacent tissues was significantly increased when compared with liver tissues from a cadaveric donor. However, there was no correlation between Wnts mRNA expression and clinic-pathological as well as survival data.

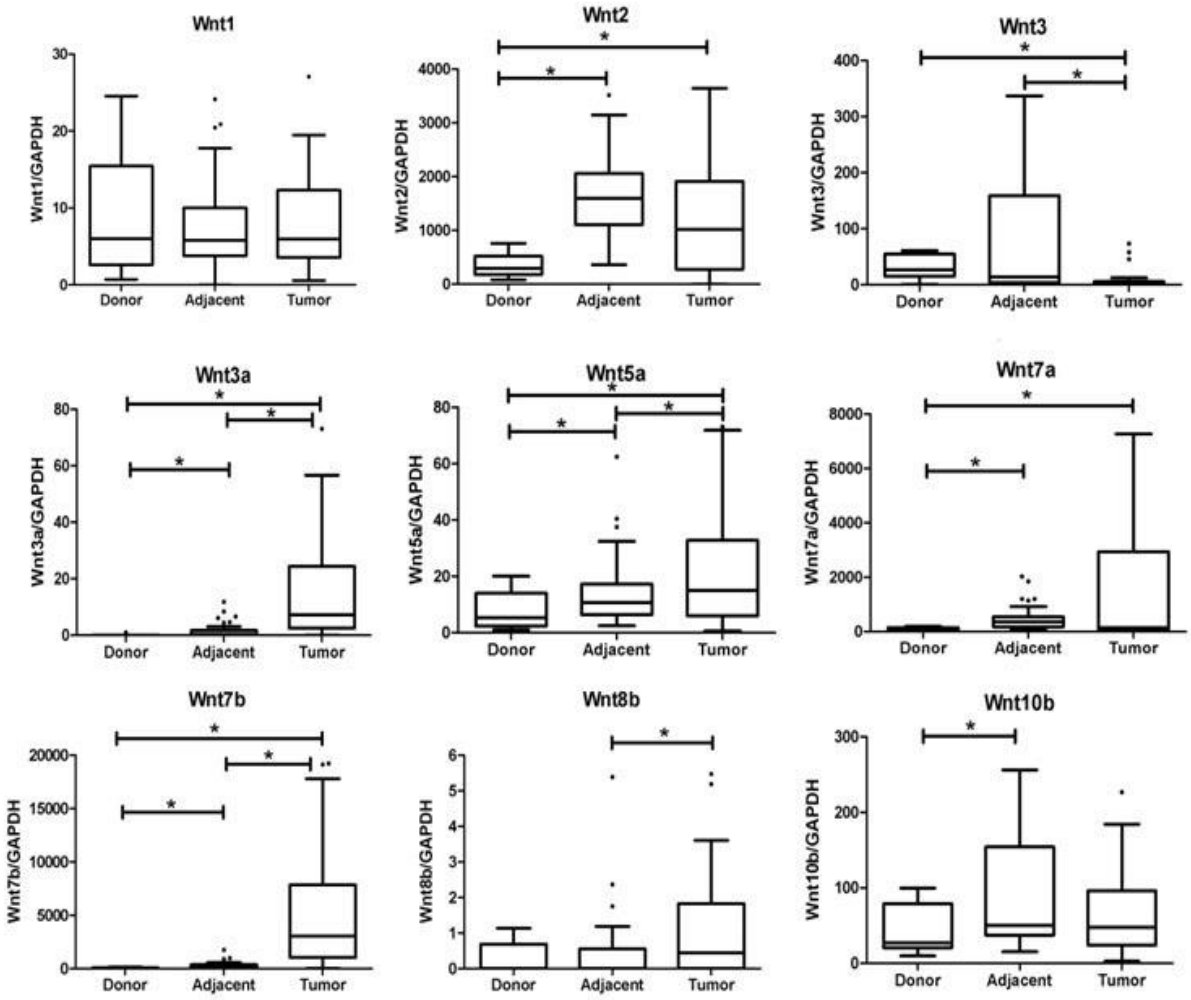


Fig. 9 The relative Wnt mRNAs expression level in human CCA tissues determined using real-time RT-PCR

Immunohistological analysis of Wnt protein and β-catenin in human CCA tissues and their significant correlations

For a better understanding of Wnt/β-catenin signaling pathway activation in CCA, immunohistochemistry analysis was performed to determine the expression and localization of Wnt proteins and β-catenin in human CCA tissues. Based on the real time RT-PCR result, we have chosen Wnt3 which down-regulated in CCA and three Wnts including Wnt3a, Wnt5a and Wnt7b which significantly increased in tumor tissues when compared with adjacent tissues and liver tissues from a cadaveric donor, respectively (Fig. 10a and Fig. 10b).

The normal bile duct from cadaveric donors showed negative staining of Wnt3. All normal bile duct epithelia residing in an adjacent tissue of tumor sections and hepatocytes were no staining. For 40 intrahepatic CCA patients examined, 24 (60%) were negative stained for Wnt3 protein whereas 16 (40%) were weakly to moderately positive for tumor cells. Wnt3 staining was also found in the hyperplastic bile duct. Interestingly, Wnt3 strongly positive staining was seen in kupffer cells and inflammatory cells residing in tumor and adjacent non-tumor tissues. We further investigated the specific cell type of inflammatory cells which expressed Wnt3 in CCA tissues. Double immunofluorescence analysis was performed to co-localized Wnt3 and MAC387 which is macrophage marker. Double immunostaining showed a part of Wnt3-positive cells expressed MAC387. This result suggested that some of inflammatory cells which expressed Wnt3 are the macrophages. However, no statistic significant correlation was noted between Wnt3 expression in tumor cells and inflammatory cells with clinico-pathological findings.

Additionally, the immunohistochemical staining demonstrated that Wnt3a proteins were positive in cytoplasm of tumor cells in 37 of 40 CCA cases (92.5%) whereas only 3 of 40 cases (7.5%) showed negative staining. Strongly positive of Wnt3a was found in bile duct with hyperplasia as well as cancer cells. Normal bile duct in non-tumor area showed low expression of Wnt3a, while hepatocytes, kupffer cells and inflammatory cell showed negative staining (Fig. 10a). No significant correlation was observed between Wnt3a expression and clinico-pathological findings.

Furthermore, the normal bile duct from a cadaveric donor showed barely positive staining of Wnt5a. Of the 40 intrahepatic CCA patients explored, 9 (22.5%) were negative stained for Wnt5a and 31 (77.5%) were weakly to strongly positive for tumor cells. Normal bile duct epithelia showed a weak cytoplasm staining for Wnt5a, whereas hepatocytes and inflammatory cells were no staining (Fig. 10a). No correlation was observed between Wnt5a expression and clinico-pathological findings

of CCA patients. Cumulative survival was compared between CCA patients. The log-rank analysis indicated that CCA patients with Wnt5a positive had significantly lower survival than those with Wnt5a negative (P = 0.007) as demonstrated in Fig. 10c.

In addition, all of CCA patients exhibited Wnt7b positive staining in cytoplasm of tumor cells. Low and high cytoplasmic Wnt7b expression were observed in 25 (65.8%) and 13 cases (34.2%) of CCA patients, respectively. Moreover, nuclear staining was mostly found in bile duct with hyperplasia as well as tumor cells. In addition, Wnt7b staining was also seen in the cytoplasm of hepatocytes and some infiltrating cells (Fig. 10a). No significant correlation was observed between Wnt7b expression and clinico-pathological findings or survival data.

Moreover, β-catenin expression in CCA is presented in Fig. 10b. Membrane expression of β-catenin was observed in hepatocytes, normal bile duct epithelium in non-tumor area as well as hyperplastic ducts. β-catenin expression was not found in inflammatory and stromal cells. In tumor area, immunohistochemical analysis revealed the reduced membranous expression of β-catenin. The remaining 29 (72.5%) tumors had a cytoplasmic β-catenin expression. Moreover, aberrant nuclear expression for β-catenin was observed in cholangiocarcinoma cells. Correlation between the expression of β-catenin with clinico-pathological factors and Wnt proteins expression was investigated. No significant correlation was observed between β-catenin expression and clinico-pathological findings. In addition, cumulative survival was compared between CCA patients with negative and positive for cytoplasmic β-catenin. The log-rank analysis indicated that there was a trend toward a correlation of cytoplasmic β-catenin expression and poor survival rate of CCA patients but no statistically significant association of the survival curves (P = 0.100) (Fig. 10c).

Moreover, significantly, β-catenin expression was correlated with Wnt3, Wnt5a and Wnt7b expression (P = 0.041, 0.016 and 0.005, respectively), indicating the upstream regulatory molecules of β-catenin in CCA (Table. 7).

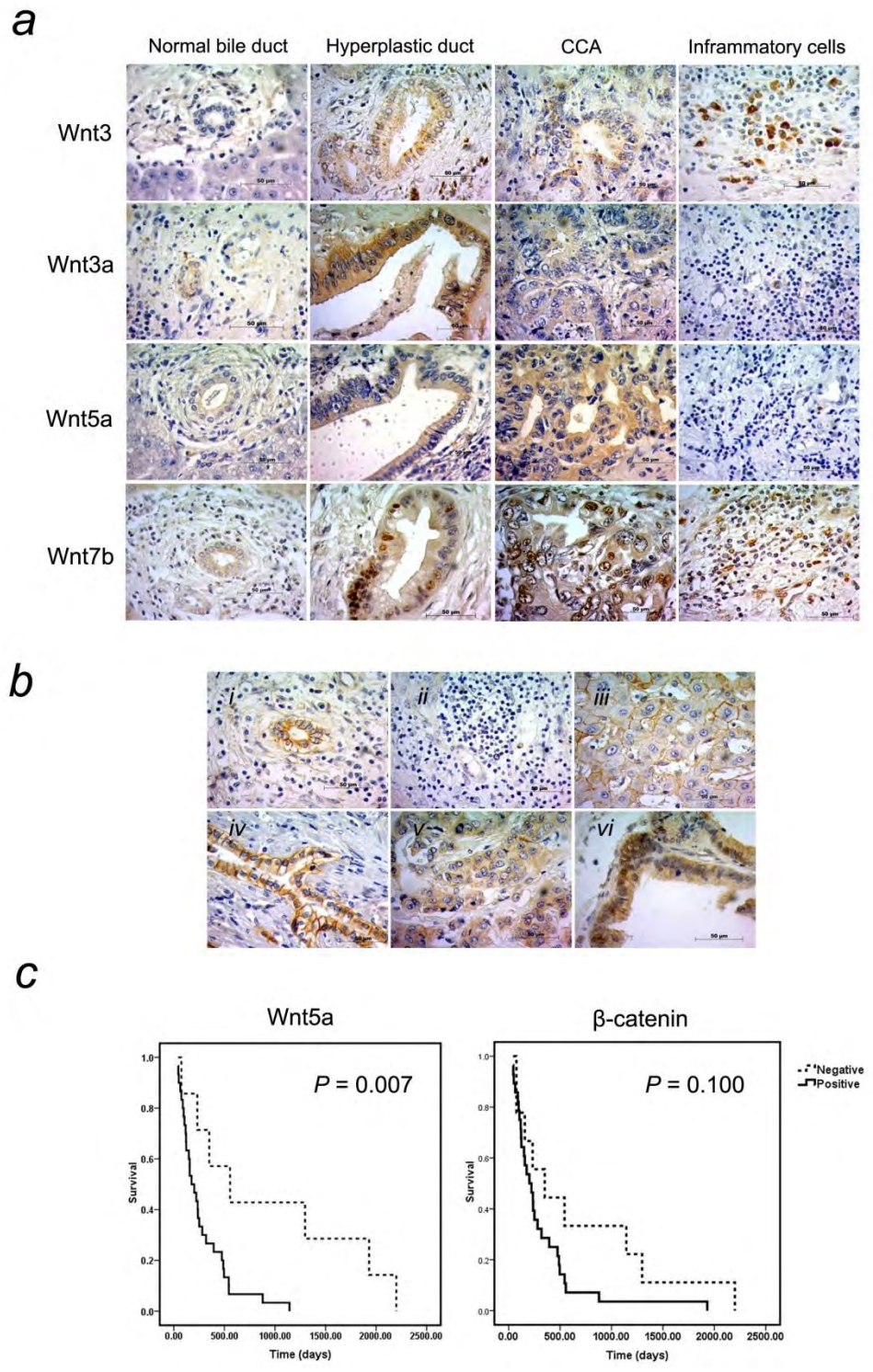


Fig. 10 Immunohistochemical staining of (a) Wnt3, Wnt3a, Wnt5a and Wnt7b as well as (b) β-catenin in human cholangiocarcinoma tissues. (c) The log-rank analysis demonstrated a correlation between wnt5a and β-catenin with a survival of patients.

Table 1 Correlation of β-catenin expression and clinico-pathological data of CCA patients

Variable	No. of patients	β-catenin score		
		Low	High	*P* value
Age (Year)				
≤ 56	21	16	5	0.186
> 56	19	10	9	-
Gender				
Female	11	9	2	0.270
Male	29	17	12	-
Histological grading				
Papillary	8	6	2	0.689
Non-papillary	32	20	12	-
Metastasis stage				
Negative	20	12	8	0.741
Positive	20	14	6	-
Wnt3 expression				
Negative	24	19	5	0.041*
Positive	16	7	9	-
Wnt3a expression				
Negative	3	3	0	0.539
Positive	37	23	14	-
Wnt5a expression				
Negative	9	9	0	0.016*
Positive	31	17	14	
Wnt7b expression				
Low expression	25	20	5	0.005*
High expression	13	4	9	

Lipopolysacharide (LPS) induced Wnt3 expression in human macrophage cell line

As demonstrated by immunohistochemistry staining that Wnt3 is predominantly expressed in kupffer cells and inflammatory cells residing in tumor and adjacent non-tumor tissues. We therefore hypothesized those inflammatory cells as well as inflammation reaction could mediate β-catenin activation. To prove this hypothesis, we investigated the expression of Wnt3 in macrophage cell line upon lipopolysaccharide (LPS) stimulation. Then, quantitative real time RT-PCR and western blot analysis were performed to determine mRNA and protein levels of Wnt3 in U937 human macrophage cell line**.** For real time RT-PCR assay**,** cells were treated with 1 and 2 μg/ml of LPS for 12, 24 and 48 h, respectively. Wnt3 mRNA level in LPS-induced cells were found to be increased when compared to their corresponding untreated cells and reached the highest expression level at 48 h of induction with 2 μg/ml of LPS as shown in Fig. 11a. For western blot analysis, cells were treated with 2 μg/ml LPS for 48 h and were harvested for Wnt3 protein analysis. The result showed that the protein expression of Wnt3 in LPS induced-cells was elevated when compared to non-treated control cells (Fig. 11b).

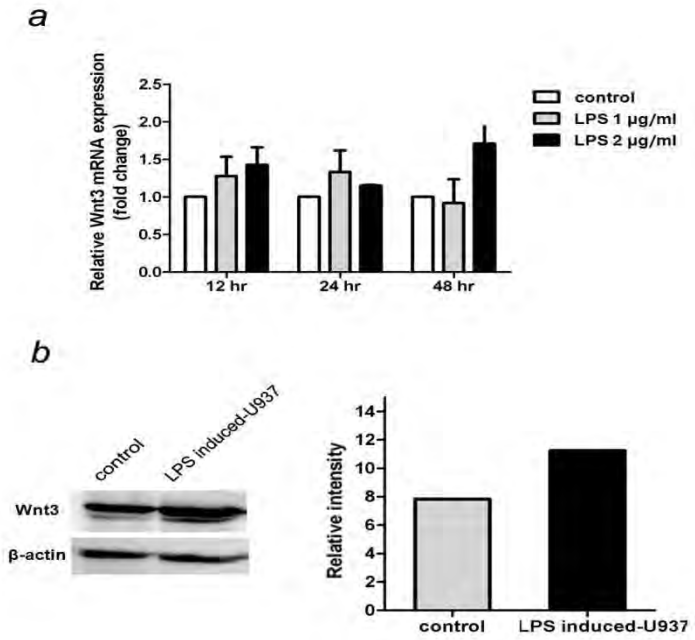


Fig. 11 Wnt3 mRNA and protein expression in LPS treated-macrophage cell line. (A) Relative real time RT-PCR was performed to detect Wnt3 mRNA level upon being treated with LPS. The data were represented the results of two independent experiments. Each bar represents the mean±S.D. (B) Western blot analysis showed the increased of Wnt3 protein level in LPS-induced U937 cells. Apparent intensity of bands on the membranes was analyzed by ImageQuant analysis software**.**

Activated macrophage conditioned media (AMCM) induced β-catenin activation in CCA cell lines

In order to confirm that factors which secreted from activated macrophage could promote β-catenin activation in CCA cell lines, U937 human macrophage cells were untreated or treated with 2 μg/ml of LPS for 48 h to become activated macrophages. Then, conditioned media from both non-activated macrophages (NAMCM) and conditioned media from activated macrophages (AMCM) were collected. After that, CCA cell lines (M139, M213 and M214) were treated with RPMI (negative control), 2 μg/ml of LPS, NAMCM and AMCM for 12 and 24 h, respectively. After that, western blot analysis was performed to investigate the protein level of β-catenin in CCA cell lines as shown in Fig. 12a. The results presented that treatment with AMCM resulted in increasing of β-catenin proteins levels at 24 h in both M139 and M213 compared to their controls, respectively. In M214 cells, AMCM treatment led to increasing of β–catenin at 12 and 24 h after treatment. In contrast, no change of β–catenin expression was observed in all CCA cells after LPS or NAMCM treatment. Moreover, cytosolic and nuclear β-catenin in M214 were detected by western analysis (Fig. 12b). The result revealed that treatment of M214 with AMCM for 24 h resulted in elevation of β-catenin level in the nucleus of cell. This change correlated with decreasing in cytosolic β-catenin. This result indicated that factors which secreted from activated macrophage can promote β-catenin activation in CCA cell lines.

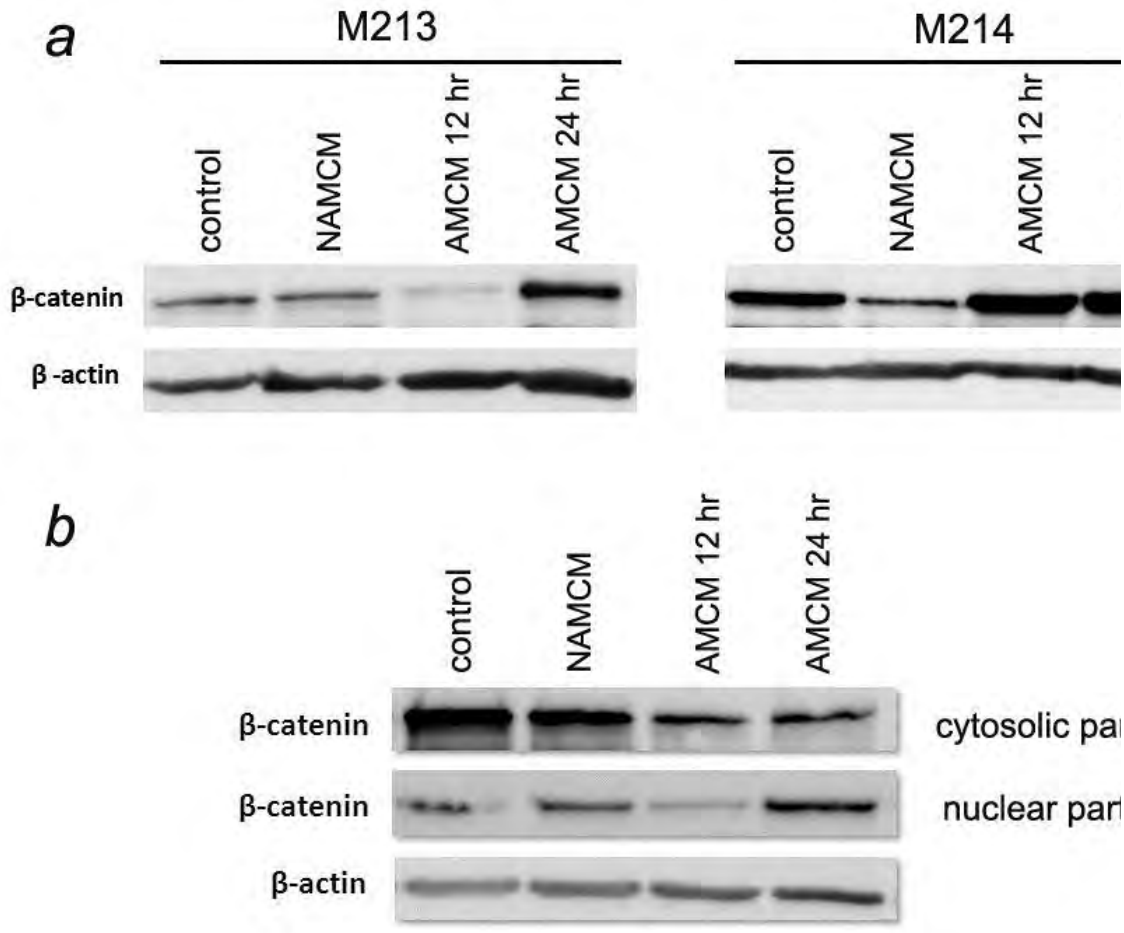


Fig. 12 β-catenin activation in CCA cell lines after treatment with AMCM. Three CCA cell lines (M139, M213 and M214) were treated with 2 μg/ml LPS, conditioned media from untreated macrophage (NAMCM), LPS-activated macrophages (AMCM) or left untreated (control) at the indicated time. Western blot analysis was performed to determine the protein levels of β-catenin from whole cell lysates of three CCA cell lines (a). Cytosolic and nuclear β-catenin were investigated in M214 cells (b). β -actin was used to check for equal loading of the gel. AMCM = activated macrophage conditioned media, NAMCM = non-activated macrophage condition media.

Knocking down of β-catenin by siRNA reduced CCA cell growth

We then further evaluated whether β-catenin is involved in CCA cell growth. M214 CCA cell line was transiently transfected either with β-catenin siRNA to suppress β-catenin protein translation or with the scrambled siRNA as a control. After 48 h of transfection, the western blot analysis revealed 90% suppression of β-catenin protein by siRNA (Fig. 13a). The effect of β-catenin knockdown on cell viability was determined by trypan blue counting. The result showed that β-catenin suppression causes significantly decreased the growth rate of M214 cell when compared with scramble siRNA treated-cells (P = 0.02, Fig. 13b).

We also evaluated whether the decrease in β-catenin protein in M214 cells following siRNA treatment could affect the transcriptional regulatory function of β-catenin. Western blot analysis was then performed to determine the effect of down-regulation of β-catenin on its targets, cyclin D1 and regulatory partners, cdk4 in M214 cells. Our results showed that siRNA-mediated down-regulation of β–catenin expression caused decreased of cyclin D1, but not effected cdk4 expression (Fig. 13a).

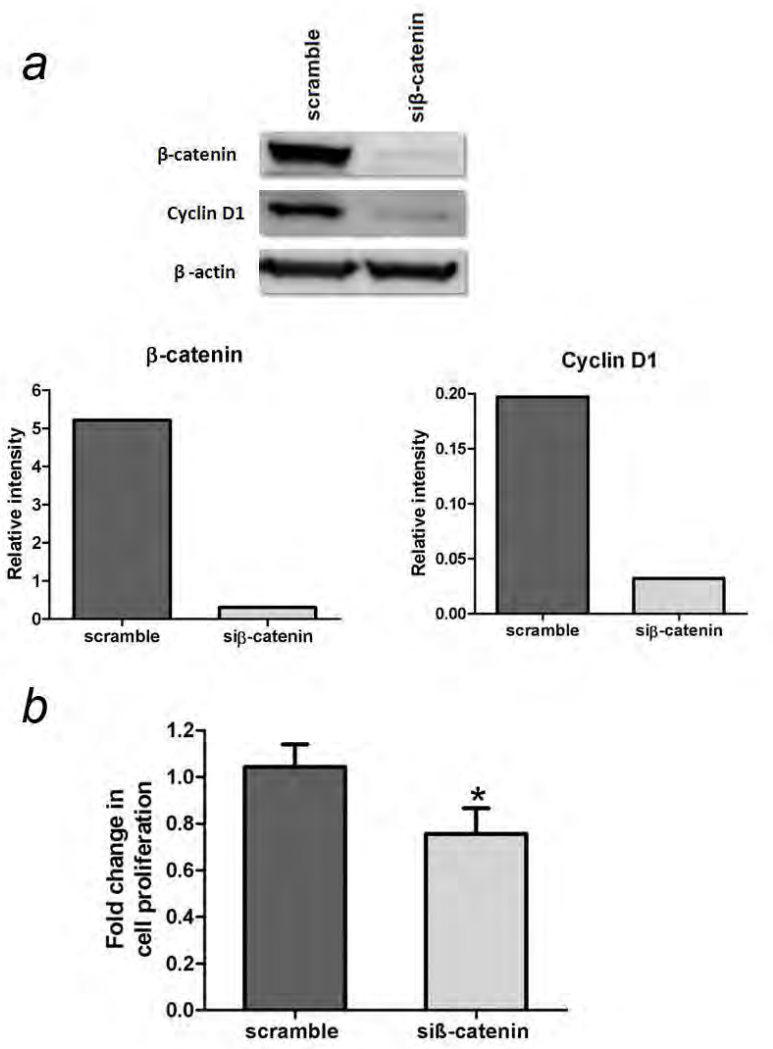


Fig. 13 Effect of siRNA-mediated knockdown of β-catenin in M214 CCA cell line. Western blot analysis showed a markedly decreased of β-catenin protein level after 48 h of transfection (a). β-catenin siRNA transfection was significantly inhibited CCA cell proliferation (b). *$P < 0.05$. Scramble = cells treated with non-specific siRNA; siβ-catenin = cells treated with siRNA specific to β-catenin

DISCUSSION

Accumulated evidences have indicated that Wnt/β-catenin signaling pathway is associated with many tumor types [80]. In humans, the Wnt family is composed of 19 structurally related molecules. The members exhibit unique expression patterns and distinct functions in development [81]. There are several studies which have shown aberrant expression of Wnt molecules in various types of human cancers [82-84]. The present study is the first observation of Wnt expression profile in CCA. Profiling Wnt genes by real time RT-PCR indicated that 5 Wnt genes including Wnt2, Wnt3a, Wnt5a, Wnt7a and Wnt7b were significantly overexpressed in CCA patients, compared with cadaveric donor tissues whereas Wnt3 expression was down-regulated in cancer tissues. In CCA cell lines, most of them showed a high level of expression of Wnt7a and Wnt7b, respectively. Our findings indicated that aberrant expression of the Wnt gene family was found in CCA.

We then further investigated the expression and localization of 4 Wnt proteins in CCA patients. In the 40 cases of human CCA examined in the present study, most of them showed the positive staining of Wnt3a, Wnt5a and Wnt7b in tumor cells. In contrast, Wnt3 was predominantly expressed in kupffer cells as well as inflammatory cells while only 40% of cancer cells showed positive Wnt3 staining. Moreover, the cumulative survival analysis demonstrated that CCA patients with Wnt5a positive expression had a significantly shorter survival time than those with Wnt5a negative expression. A similar finding was reported in ovarian cancer. This indicates that Wnt5a in tumor cells might be able to serve as a prognostic marker for CCA. However, no correlation was observed in the Wnt proteins expression and other clinico-pathological features.

β-catenin is a key mediators of Wnt/β-catenin pathway. In normal state, β-catenin is primarily located in the membrane and the cytoplasm of cells and the expression is kept at low levels [85]. The role of free cytoplasmic β-catenin has been revealed to be the co-activator for a family of transcription factors, TCF/LEF, which results in activating expression of TCF/LEF-regulated target genes [86]. The present study revealed the immunolocalization of β-catenin in CCA patients. Our results showed that loss of membranous β-catenin was observed in tumor. Moreover, most of CCA patients (72.5%) had a cytoplasmic β-catenin expression and nuclear translocation of β-catenin was also found in cancer cells. These results support the other studies which have been reported the accumulation of cytoplasmic and nuclear β-catenin in CCA [87, 88]. Sugimashi and coworkers demonstrated that reduced membranous expression of β-catenin is associated with non-papillary intrahepatic CCA which have a more malignant behavior and that nuclear translocation of β-catenin results in oncogenicevents. These results

suggested that the aberrant cytoplasm and nuclear expression of β-catenin might be involved in the carcinogenesis and progression of CCA. In addition, we also found a significant correlation of abnormal β-catenin expression with Wnt3, Wnt5a and Wnt7b expression. Our findings suggested that Wnt3, Wnt5a and Wnt7b might be the key upstream molecules that activate Wnt/ β-catenin signaling pathway in CCA.

Additionally, a number of studies have established that inflammation contributes to many types of malignancies, including CCA. Consistently, previous study in CCA demonstrated that the presence of *O. viverrini* antigen was associated with heavy inflammatory cell infiltration, particularly with mononuclear cells [89]. In the present work, our immunohistochemistry result presented that inflammatory cells surrounding tumor cells showed strongly Wnt3 expression. Therefore, we postulated that inflammatory conditions could modulate β-catenin activation in CCA. In this study, we used the human U937 macrophage cell line to evaluate whether LPS can induce Wnt3 expression. The result showed that treatment with 2 ug/ml of LPS for 48 h stimulated Wnt3 mRNA and protein expression in macrophage cell line. Therefore, this information revealed that inflammation condition induced by LPS could induce Wnt3 expression in macrophage.

We then further studied whether LPS induced Wnt3 expression in macrophage could mediate β-catenin activation in CCA cells. There are many studied demonstrated that the crosstalk between the tumor cells and macrophages was mediated by soluble factors [90]. Therefore, in this work CCA cells were exposed to conditioned media from LPS induced macrophages then β-catenin activation was investigated by western blot analysis. We could demonstrate that conditioned media from LPS induced macrophages was able to increase β-catenin protein level of CCA cells, whereas conditioned media from non-activated macrophages did not, suggesting that factors secreted by activated macrophages were responsible for β-catenin activation in CCA cells. These observations can be supported by our earlier study showing that activated macrophages showed increasing of Wnt3 expression. It implies that Wnt3 could be one factor which secrete from activated macrophages and mediate cross-talking between carcinoma cells and stromal cells resulting in β-catenin activation in CCA cells. Unfortunately, we could not detect Wnt3 protein in conditioned media of LPS activated macrophage which probably due to the very low level of protein.

Furthermore, the increased levels of β-catenin frequency found in both premalignant and malignant cells are associated with increased rates of cellular proliferation [91, 92]. In this study, we demonstrated that β-catenin is involved in regulating of CCA cell proliferation. M214 CCA cell line transfected with siRNA targeting β-catenin showed less cell viability. Moreover, our current data revealed

that siRNA-mediated down-regulation of β-catenin can inhibit the expression of growth-related-gene, cyclin D1, which is a specific target gene of β-catenin. Our results are consistent with the previous studies that suppressed β-catenin by siRNA could successfully induce growth inhibition in a variety of cancers both *in vitro* and *in vivo* ([93, 94]. However, our results demonstrated that suppression of β-catenin had reduced about 20% on CCA cell growth which implies that inhibition only β-catenin may not be enough for inhibiting CCA cells growth. This was consistent with the previous study which indicated that other signaling pathways participate in controlling of CCA cell growth [19]. Therefore, blocking only β-catenin may result in activation of an alternative pathway giving rise to some advantage to cell growth so blocking two or more pathway would be more effective therapeutic approach to treatment of CCA.

CONCLUSIONS AND PERSPECTIVES

Our data demonstrate that multiple protein kinases are activated which lead to stimulation of several signaling cascades in CCA. We also illustrate an increasing of kinase activation including Erk1/2 and Akt in CCA tissues compared with their non-tumorous adjacent tissues. These results indicate the involvement and important role of kinases in CCA that potentially contribute to the CCA progression. Importantly, we depict that the abrogation of kinase inhibitors leads to suppression of CCA cell growth. In addition, multi-targeted kinase inhibitors, sorafenib and sunitinib show the best potential on CCA cell growth inhibition as well as apoptosis induction by suppressing Erk1/2 and Akt activation. These results indicate that certain protein kinases are promising as drug targets for CCA treatment and targeting multi- kinases should be further investigated as an approach to improve CCA therapy.

In addition, we demonstrated the constitutive activation of PI3K/AKT pathway in CCA patients as well as CCA cell lines. The possible mechanisms are PI3K overexpression as well as PTEN inactivation by both loss of expression and phosphorylation resulting in poor prognosis in human CCA studied. Therefore, this particular pathway represents a possible useful target for CCA treatment. This work also indicates that inhibition of PI3K and mTOR activity by a novel inhibitor, NVP-BEZ235 has anti-proliferation as well as migration inhibition effects on CCA cells. However, this small inhibitor failed to induce apoptosis of CCA cells while autophagy induction could be observed. Based on these findings, NVP-BEZ235 might be applied in a therapeutic strategy for CCA, however, using this particular drug in combination with other therapy should be considered.

Moreover, our results also indicate that many Wnt genes were abundant expressed in CCA. The expression can also be found in inflammatory cells and LPS

can induce Wnt up-regulation in macrophages. It implies that Wnt/β-catenin pathway may involve in inflammatory-associated CCA. In addition, abrogation of β-catenin expression induced growth inhibition of CCA cells. Therefore, suppression of Wnt/β-catenin signaling could be a potential target for inhibiting CCA cell growth.

Based on the above information, we are now investigating the expression and also the role(s) of the remain kinases which got from kinase array data including JAK/STAT and Ephrin signaling pathway in order to find the best target for CCA treatment. Furthermore, the efficiency of various kinase inhibitors as well as those natural extracted compounds is now testing both *in vitro* and *in vivo* to find the new strategy for CCA prevention and treatment.

REFERENCES

1 Sripa B, Pairojkul C: Cholangiocarcinoma: Lessons from thailand. Current opinion in gastroenterology 2008;24:349-356.

2 Khan SA, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P, Thomas HC: Changing international trends in mortality rates for liver, biliary and pancreatic tumours. J Hepatol 2002;37:806-813.

3 Shaib Y, El-Serag HB: The epidemiology of cholangiocarcinoma. Seminars in liver disease 2004;24:115-125.

4 de Groen PC, Gores GJ, LaRusso NF, Gunderson LL, Nagorney DM: Biliary tract cancers. The New England journal of medicine 1999;341:1368-1378.

5 Loilome W, Wechagama P, Namwat N, Jusakul A, Sripa B, Miwa M, Kuver R, Yongvanit P: Expression of oxysterol binding protein isoforms in opisthorchiasis-associated cholangiocarcinoma: A potential molecular marker for tumor metastasis. Parasitology international 2012;61:136-139.

6 Loilome W, Yongvanit P, Wongkham C, Tepsiri N, Sripa B, Sithithaworn P, Hanai S, Miwa M: Altered gene expression in opisthorchis viverrini-associated cholangiocarcinoma in hamster model. Molecular carcinogenesis 2006;45:279-287.

7 Posada J, Cooper JA: Molecular signal integration. Interplay between serine, threonine, and tyrosine phosphorylation. Molecular biology of the cell 1992;3:583-592.

8 Kolibaba KS, Druker BJ: Protein tyrosine kinases and cancer. Biochimica et biophysica acta 1997;1333:F217-248.

9 Krause DS, Van Etten RA: Tyrosine kinases as targets for cancer therapy. The New England journal of medicine 2005;353:172-187.

10 Zwick E, Bange J, Ullrich A: Receptor tyrosine kinase signalling as a target for cancer intervention strategies. Endocrine-related cancer 2001;8:161-173.

11 Cohen P: Protein kinases--the major drug targets of the twenty-first century? Nature reviews 2002;1:309-315.

12 Faivre S, Djelloul S, Raymond E: New paradigms in anticancer therapy: Targeting multiple signaling pathways with kinase inhibitors. Seminars in oncology 2006;33:407-420.

13 Steeghs N, Nortier JW, Gelderblom H: Small molecule tyrosine kinase inhibitors in the treatment of solid tumors: An update of recent developments. Annals of surgical oncology 2007;14:942-953.

14 Bradbury AW, Carter DC, Miller WR, Cho-Chung YS, Clair T: Protein kinase a (pk-a) regulatory subunit expression in colorectal cancer and related mucosa. British journal of cancer 1994;69:738-742.

15 Cho-Chung YS: Role of cyclic amp receptor proteins in growth, differentiation, and suppression of malignancy: New approaches to therapy. Cancer Res 1990;50:7093-7100.

16 Cho-Chung YS, Pepe S, Clair T, Budillon A, Nesterova M: Camp-dependent protein kinase: Role in normal and malignant growth. Critical reviews in oncology/hematology 1995;21:33-61.
17 Miller WR, Watson DM, Jack W, Chetty U, Elton RA: Tumour cyclic amp binding proteins: An independent prognostic factor for disease recurrence and survival in breast cancer. Breast cancer research and treatment 1993;26:89-94.
18 Tortora G, Ciardiello F: Protein kinase a as target for novel integrated strategies of cancer therapy. Annals of the New York Academy of Sciences 2002;968:139-147.
19 Loilome W, Juntana S, Namwat N, Bhudhisawasdi V, Puapairoj A, Sripa B, Miwa M, Saya H, Riggins GJ, Yongvanit P: Prkar1a is overexpressed and represents a possible

therapeutic target in human cholangiocarcinoma. Int J Cancer 2011;129:34–44.
20 Techasen A, Loilome W, Namwat N, Takahashi E, Sugihara E, Puapairoj A, Miwa M, Saya H, Yongvanit P: Myristoylated alanine-rich c kinase substrate phosphorylation promotes cholangiocarcinoma cell migration and metastasis via the protein kinase c-dependent pathway. Cancer science 2010;101:658-665.
21 Sibilia M, Kroismayr R, Lichtenberger BM, Natarajan A, Hecking M, Holcmann M: The epidermal growth factor receptor: From development to tumorigenesis. Differentiation; research in biological diversity 2007;75:770-787.
22 Yarden Y: The egfr family and its ligands in human cancer. Signalling mechanisms and therapeutic opportunities. Eur J Cancer 2001;37 Suppl 4:S3-8.
23 Danilkovitch A, Leonard EJ: Kinases involved in msp/ron signaling. Journal of leukocyte biology 1999;65:345-348.
24 Surawska H, Ma PC, Salgia R: The role of ephrins and eph receptors in cancer. Cytokine & growth factor reviews 2004;15:419-433.
25 Folkman J, Shing Y: Angiogenesis. The Journal of biological chemistry 1992;267:10931-10934.
26 Karkkainen MJ, Petrova TV: Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. Oncogene 2000;19:5598-5605.
27 Ong CK, Subimerb C, Pairojkul C, Wongkham S, Cutcutache I, Yu W, McPherson JR, Allen GE, Ng CC, Wong BH, Myint SS, Rajasegaran V, Heng HL, Gan A, Zang ZJ, Wu Y, Wu J, Lee MH, Huang D, Ong P, Chan-on W, Cao Y, Qian CN, Lim KH, Ooi A, Dykema K, Furge K, Kukongviriyapan V, Sripa B, Wongkham C, Yongvanit P, Futreal PA, Bhudhisawasdi V, Rozen S, Tan P, Teh BT: Exome sequencing of liver fluke-associated cholangiocarcinoma. Nature genetics 2012;44:690-693.
28 Puri MC, Rossant J, Alitalo K, Bernstein A, Partanen J: The receptor tyrosine kinase tie is required for integrity and survival of vascular endothelial cells. The EMBO journal 1995;14:5884-5891.

29 FDA: Fda approval letter for use of sorafenib in advanced renal cancer, US Food and Drug Administration., 2005,
30 FDA: Fda approval letter for use of sorafenib for hepatocellular carcinoma, US Food and Drug Administration, 2007,
31 FDA: Fda aproves sutent for rare type of pancreatic cancer, US National Cancer Institute, 2011,
32 Rock EP, Goodman V, Jiang JX, Mahjoob K, Verbois SL, Morse D, Dagher R, Justice R, Pazdur R: Food and drug administration drug approval summary: Sunitinib malate for the treatment of gastrointestinal stromal tumor and advanced renal cell carcinoma. The oncologist 2007;12:107-113.
33 Blechacz BR, Smoot RL, Bronk SF, Werneburg NW, Sirica AE, Gores GJ: Sorafenib inhibits signal transducer and activator of transcription-3 signaling in cholangiocarcinoma cells by activating the phosphatase shatterproof 2. Hepatology (Baltimore, Md 2009;50:1861-1870.
34 Bengala C, Bertolini F, Malavasi N, Boni C, Aitini E, Dealis C, Zironi S, Depenni R, Fontana A, Del Giovane C, Luppi G, Conte P: Sorafenib in patients with advanced biliary tract carcinoma: A phase ii trial. British journal of cancer 2010;102:68-72.
35 Boonjaraspinyo S, Wu Z, Boonmars T, Kaewkes S, Loilome W, Sithithaworn P, Nagano I, Takahashi Y, Yongvanit P, Bhudhisawasdi V: Overexpression of pdgfa and its receptor during carcinogenesis of opisthorchis viverrini-associated cholangiocarcinoma. Parasitology international 2012;61:145-150.
36 Boonjaraspinyo S, Boonmars T, Wu Z, Loilome W, Sithithaworn P, Nagano I, Pinlaor S, Yongvanit P, Nielsen PS, Pairojkul C, Khuntikeo N: Platelet-derived growth factor may be a potential diagnostic and prognostic marker for cholangiocarcinoma. Tumour Biol 2012
37 Yi JH, Thongprasert S, Lee J, Doval DC, Park SH, Park JO, Park YS, Kang WK, Lim HY: A phase ii study of sunitinib as a second-line treatment in advanced biliary tract carcinoma: A multicentre, multinational study. Eur J Cancer 2012;48:196-201.
38 Namwat N, Puetkasichonpasutha J, Loilome W, Yongvanit P, Techasen A, Puapairoj A, Sripa B, Tassaneeyakul W, Khuntikeo N, Wongkham S: Downregulation of reversion-inducing-cysteine-rich protein with kazal motifs (reck) is associated with enhanced expression of matrix metalloproteinases and cholangiocarcinoma metastases. J Gastroenterol 2011;46:664-675.
39 Jamnongkan W, Techasen A, Thanan R, Duenngai K, Sithithaworn P, Mairiang E, Loilome W, Namwat N, Pairojkul C, Yongvanit P: Oxidized alpha-1 antitrypsin as a predictive risk marker of opisthorchiasis-associated cholangiocarcinoma. Tumour Biol 2013;34:695-704.
40 Sun SY, Yue P, Dawson MI, Shroot B, Michel S, Lamph WW, Heyman RA, Teng M, Chandraratna RA, Shudo K, Hong WK, Lotan R: Differential effects of synthetic nuclear

retinoid receptor-selective retinoids on the growth of human non-small cell lung carcinoma cells. Cancer Res 1997;57:4931-4939.

41 Chung JY, Hong SM, Choi BY, Cho H, Yu E, Hewitt SM: The expression of phospho-akt, phospho-mtor, and pten in extrahepatic cholangiocarcinoma. Clin Cancer Res 2009;15:660-667.

42 Xu X, Kobayashi S, Qiao W, Li C, Xiao C, Radaeva S, Stiles B, Wang RH, Ohara N, Yoshino T, LeRoith D, Torbenson MS, Gores GJ, Wu H, Gao B, Deng CX: Induction of intrahepatic cholangiocellular carcinoma by liver-specific disruption of smad4 and pten in mice. J Clin Invest 2006;116:1843-1852.

43 Bader AG, Kang S, Zhao L, Vogt PK: Oncogenic pi3k deregulates transcription and translation. Nat Rev Cancer 2005;5:921-929.

44 Ong SH, Hadari YR, Gotoh N, Guy GR, Schlessinger J, Lax I: Stimulation of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins. Proc Natl Acad Sci U S A 2001;98:6074-6079.

45 Furubo S, Harada K, Shimonishi T, Katayanagi K, Tsui W, Nakanuma Y: Protein expression and genetic alterations of p53 and ras in intrahepatic cholangiocarcinoma. Histopathology 1999;35:230-240.

46 Petmitr S: Cancer genes and cholangiocarcinoma. Southeast Asian J Trop Med Public Health 1997;28 Suppl 1:80-84.

47 Tada M, Omata M, Ohto M: High incidence of ras gene mutation in intrahepatic cholangiocarcinoma. Cancer 1992;69:1115-1118.

48 Wattanasirichaigoon S, Tasanakhajorn U, Jesadapatarakul S: The incidence of k-ras codon 12 mutations in cholangiocarcinoma detected by polymerase chain reaction technique. J Med Assoc Thai 1998;81:316-323.

49 Levi S, Urbano-Ispizua A, Gill R, Thomas DM, Gilbertson J, Foster C, Marshall CJ: Multiple k-ras codon 12 mutations in cholangiocarcinomas demonstrated with a sensitive polymerase chain reaction technique. Cancer Res 1991;51:3497-3502.

50 Ahrendt SA, Rashid A, Chow JT, Eisenberger CF, Pitt HA, Sidransky D: P53 overexpression and k-ras gene mutations in primary sclerosing cholangitis-associated biliary tract cancer. J Hepatobiliary Pancreat Surg 2000;7:426-431.

51 Isa T, Tomita S, Nakachi A, Miyazato H, Shimoji H, Kusano T, Muto Y, Furukawa M: Analysis of microsatellite instability, k-ras gene mutation and p53 protein overexpression in intrahepatic cholangiocarcinoma. Hepatogastroenterology 2002;49:604-608.

52 Ohashi K, Nakajima Y, Kanehiro H, Tsutsumi M, Taki J, Aomatsu Y, Yoshimura A, Ko S, Kin T, Yagura K, et al.: Ki-ras mutations and p53 protein expressions in intrahepatic cholangiocarcinomas: Relation to gross tumor morphology. Gastroenterology 1995;109:1612-1617.

53 Hart JR, Vogt PK: Phosphorylation of akt: A mutational analysis. Oncotarget 2011;2:467-476.
54 Pearce LR, Komander D, Alessi DR: The nuts and bolts of agc protein kinases. Nature reviews Molecular cell biology 2010;11:9-22.
55 Chen Y, Xu J, Jhala N, Pawar P, Zhu ZB, Ma L, Byon CH, McDonald JM: Fas-mediated apoptosis in cholangiocarcinoma cells is enhanced by 3,3'-diindolylmethane through inhibition of akt signaling and flice-like inhibitory protein. Am J Pathol 2006;169:1833-1842.
56 Javle MM, Yu J, Khoury T, Chadha KS, Iyer RV, Foster J, Kuvshinoff BW, Gibbs JF, Geradts J, Black JD, Brattain MG: Akt expression may predict favorable prognosis in cholangiocarcinoma. J Gastroenterol Hepatol 2006;21:1744-1751.
57 Schmitz KJ, Lang H, Wohlschlaeger J, Sotiropoulos GC, Reis H, Schmid KW, Baba HA: Akt and erk1/2 signaling in intrahepatic cholangiocarcinoma. World J Gastroenterol 2007;13:6470-6477.
58 Tanno S, Yanagawa N, Habiro A, Koizumi K, Nakano Y, Osanai M, Mizukami Y, Okumura T, Testa JR, Kohgo Y: Serine/threonine kinase akt is frequently activated in human bile duct cancer and is associated with increased radioresistance. Cancer Res 2004;64:3486-3490.
59 Luo J: Glycogen synthase kinase 3beta (gsk3beta) in tumorigenesis and cancer chemotherapy. Cancer Lett 2009;273:194-200.
60 Thiel A, Heinonen M, Rintahaka J, Hallikainen T, Hemmes A, Dixon DA, Haglund C, Ristimaki A: Expression of cyclooxygenase-2 is regulated by glycogen synthase kinase-3beta in gastric cancer cells. The Journal of biological chemistry 2006;281:4564-4569.
61 Wang Y, Lam JB, Lam KS, Liu J, Lam MC, Hoo RL, Wu D, Cooper GJ, Xu A: Adiponectin modulates the glycogen synthase kinase-3beta/beta-catenin signaling pathway and attenuates mammary tumorigenesis of mda-mb-231 cells in nude mice. Cancer Res 2006;66:11462-11470.
62 Shin S, Wolgamott L, Yu Y, Blenis J, Yoon SO: Glycogen synthase kinase (gsk)-3 promotes p70 ribosomal protein s6 kinase (p70s6k) activity and cell proliferation. Proc Natl Acad Sci U S A 2011;108:E1204-1213.
63 Shimasaki T, Ishigaki Y, Nakamura Y, Takata T, Nakaya N, Nakajima H, Sato I, Zhao X, Kitano A, Kawakami K, Tanaka T, Takegami T, Tomosugi N, Minamoto T, Motoo Y: Glycogen synthase kinase 3beta inhibition sensitizes pancreatic cancer cells to gemcitabine. J Gastroenterol 2012;47:321-333.
64 Serra V, Markman B, Scaltriti M, Eichhorn PJ, Valero V, Guzman M, Botero ML, Llonch E, Atzori F, Di Cosimo S, Maira M, Garcia-Echeverria C, Parra JL, Arribas J, Baselga J: Nvp-bez235, a dual pi3k/mtor inhibitor, prevents pi3k signaling and inhibits the growth of cancer cells with activating pi3k mutations. Cancer Res 2008;68:8022-8030.
65 Maira SM, Stauffer F, Brueggen J, Furet P, Schnell C, Fritsch C, Brachmann S, Chene P, De Pover A, Schoemaker K, Fabbro D, Gabriel D, Simonen M, Murphy L, Finan P, Sellers W,

Garcia-Echeverria C: Identification and characterization of nvp-bez235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. Mol Cancer Ther 2008;7:1851-1863.

66 Fokas E, Yoshimura M, Prevo R, Higgins G, Hackl W, Maira SM, Bernhard EJ, McKenna WG, Muschel RJ: Nvp-bez235 and nvp-bgt226, dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitors, enhance tumor and endothelial cell radiosensitivity. Radiat Oncol 2012;7:48.

67 Konstantinidou G, Bey EA, Rabellino A, Schuster K, Maira MS, Gazdar AF, Amici A, Boothman DA, Scaglioni PP: Dual phosphoinositide 3-kinase/mammalian target of rapamycin blockade is an effective radiosensitizing strategy for the treatment of non-small cell lung cancer harboring k-ras mutations. Cancer Res 2009;69:7644-7652.

68 Liu TJ, Koul D, LaFortune T, Tiao N, Shen RJ, Maira SM, Garcia-Echevrria C, Yung WK: Nvp-bez235, a novel dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor, elicits multifaceted antitumor activities in human gliomas. Mol Cancer Ther 2009;8:2204-2210.

69 Manara MC, Nicoletti G, Zambelli D, Ventura S, Guerzoni C, Landuzzi L, Lollini PL, Maira SM, Garcia-Echeverria C, Mercuri M, Picci P, Scotlandi K: Nvp-bez235 as a new therapeutic option for sarcomas. Clin Cancer Res 2010;16:530-540.

70 Roccaro AM, Sacco A, Husu EN, Pitsillides C, Vesole S, Azab AK, Azab F, Melhem M, Ngo HT, Quang P, Maiso P, Runnels J, Liang MC, Wong KK, Lin C, Ghobrial IM: Dual targeting of the pi3k/akt/mtor pathway as an antitumor strategy in waldenstrom macroglobulinemia. Blood 2010;115:559-569.

71 Masuda M, Shimomura M, Kobayashi K, Kojima S, Nakatsura T: Growth inhibition by nvp-bez235, a dual pi3k/mtor inhibitor, in hepatocellular carcinoma cell lines. Oncol Rep 2011;26:1273-1279.

72 Ertmer A, Huber V, Gilch S, Yoshimori T, Erfle V, Duyster J, Elsasser HP, Schatzl HM: The anticancer drug imatinib induces cellular autophagy. Leukemia 2007;21:936-942.

73 Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S: Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ 2004;11:448-457.

74 Saiki S, Sasazawa Y, Imamichi Y, Kawajiri S, Fujimaki T, Tanida I, Kobayashi H, Sato F, Sato S, Ishikawa K, Imoto M, Hattori N: Caffeine induces apoptosis by enhancement of autophagy via pi3k/akt/mtor/p70s6k inhibition. Autophagy 2011;7:176-187.

75 Shinojima N, Yokoyama T, Kondo Y, Kondo S: Roles of the akt/mtor/p70s6k and erk1/2 signaling pathways in curcumin-induced autophagy. Autophagy 2007;3:635-637.

76 Faber AC, Li D, Song Y, Liang MC, Yeap BY, Bronson RT, Lifshits E, Chen Z, Maira SM, Garcia-Echeverria C, Wong KK, Engelman JA: Differential induction of apoptosis in her2 and

egfr addicted cancers following pi3k inhibition. Proc Natl Acad Sci U S A 2009;106:19503-19508.

77 Fan QW, Cheng C, Hackett C, Feldman M, Houseman BT, Nicolaides T, Haas-Kogan D, James CD, Oakes SA, Debnath J, Shokat KM, Weiss WA: Akt and autophagy cooperate to promote survival of drug-resistant glioma. Sci Signal 2010;3:ra81.

78 Xu CX, Zhao L, Yue P, Fang G, Tao H, Owonikoko TK, Ramalingam SS, Khuri FR, Sun SY: Augmentation of nvp-bez235's anticancer activity against human lung cancer cells by blockage of autophagy. Cancer Biol Ther 2011;12:549-555.

79 Menakongka A, Suthiphongchai T: Involvement of pi3k and erk1/2 pathways in hepatocyte growth factor-induced cholangiocarcinoma cell invasion. World J Gastroenterol 2010;16:713-722.

80 Logan CY, Nusse R: The wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 2004;20:781-810.

81 Maiese K, Li F, Chong ZZ, Shang YC: The wnt signaling pathway: Aging gracefully as a protectionist? Pharmacol Ther 2008;118:58-81.

82 Huang CL, Liu D, Ishikawa S, Nakashima T, Nakashima N, Yokomise H, Kadota K, Ueno M: Wnt1 overexpression promotes tumour progression in non-small cell lung cancer. Eur J Cancer 2008;44:2680-2688.

83 Uraguchi M, Morikawa M, Shirakawa M, Sanada K, Imai K: Activation of wnt family expression and signaling in squamous cell carcinomas of the oral cavity. J Dent Res 2004;83:327-332.

84 Yamamoto H, Oue N, Sato A, Hasegawa Y, Matsubara A, Yasui W, Kikuchi A: Wnt5a signaling is involved in the aggressiveness of prostate cancer and expression of metalloproteinase. Oncogene 2010;29:2036-2046.

85 Polakis P: The many ways of wnt in cancer. Curr Opin Genet Dev 2007;17:45-51.

86 Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, Grosschedl R, Birchmeier W: Functional interaction of beta-catenin with the transcription factor lef-1. Nature 1996;382:638-642.

87 Lim K, Han C, Xu L, Isse K, Demetris AJ, Wu T: Cyclooxygenase-2-derived prostaglandin e2 activates beta-catenin in human cholangiocarcinoma cells: Evidence for inhibition of these signaling pathways by omega 3 polyunsaturated fatty acids. Cancer Res 2008;68:553-560.

88 Sugimachi K, Taguchi K, Aishima S, Tanaka S, Shimada M, Kajiyama K, Tsuneyoshi M: Altered expression of beta-catenin without genetic mutation in intrahepatic cholangiocarcinoma. Mod Pathol 2001;14:900-905.

89 Sripa B, Kaewkes S: Localisation of parasite antigens and inflammatory responses in experimental opisthorchiasis. Int J Parasitol 2000;30:735-740.

90 Kaler P, Augenlicht L, Klampfer L: Macrophage-derived il-1beta stimulates wnt signaling and growth of colon cancer cells: A crosstalk interrupted by vitamin d3. Oncogene 2009;28:3892-3902.

91 Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW: Activation of beta-catenin-tcf signaling in colon cancer by mutations in beta-catenin or apc. Science 1997;275:1787-1790.

92 Polakis P: Wnt signaling and cancer. Genes Dev 2000;14:1837-1851.

93 Udit N V, RamaM S, AureliaSchmaltieg C, BG R: Small interfering rnas directed against β-catenin inhibit the *in vivo* and *in vitro* growth of colon cancer cells. Clin Cancer Res 2003

94 Wang JS, Zheng CL, Wang YJ, Wen JF, Ren HZ, Liu Y, Jiang HY: Gene silencing of beta-catenin by rnai inhibits cell proliferation in human esophageal cancer cells in vitro and in nude mice. Dis Esophagus 2009;22:151-162.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 Hasaya Dokduang, Sirinun Juntana, Anchalee Techasen, Nisana Namwat, Puangrat Yongvanit, Narong Khuntikeo, Gregory J. Riggins, **Watcharin Loilome*.** Survey of activated kinase proteins reveals potential targets for cholangiocarcinoma treatment. Tumor Biology. Impact factor = 2.1 (Inpress)

1.2 Supak Yothaisong, Hasaya Dokduang, Anchalee Techasen, Nisana Namwat, Puangrat Yongvanit, Vajarabhongsa Bhudhisawasdi, Anucha Puapairoj, Gregory J. Riggins, **Watcharin Loilome***. Increased activation of PI3K/AKT signaling pathway is associated with cholangiocarcinoma metastasis and PI3K/mTOR inhibition presents a possible therapeutic strategy**.** Tumor Biology. Impact factor = 2.1 (Inpress)

1.3 **Watcharin Loilome** *• Pornpan Bungkanjana• Anchalee Techasen• Nisana Namwat• Puangrat Yongvanit• Anucha Puapairoj• Narong Khuntikeo• Gregory J. Riggins. Activated macrophages promote Wnt/β-catenin signaling pathway in cholangiocarcinoma cells. *Manuscript in preparation*

*Corresponding author

2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

2.1 ได้วิทยาศาสตรมหาบัณฑิต สาขาวิชาชีวเคมีทางการแพทย์และชีววิทยาโมเลกุล จำนวน 2 คน ได้แก่

2.1.1 น.ส.พรพรรณ บึงกาญจนา นักเทคนิคการแพทย์ โครงการวิจัยโรคติดเชื้อระบาดใหม่ จ. นครพนม

2.1.2 น.ส.สุพักตร์ โยไธสง กำลังศึกษาต่อในระดับปริญญาเอก สาขาชีวเคมีทางการแพทย์และชีววิทยาโมเลกุล ที่ภาควิชาชีวเคมี คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

2.2 ได้นักศึกษาบัณฑิตศึกษาระดับปริญญาเอก สาขาวิชาชีวเคมีทางการแพทย์และชีววิทยาโมเลกุล จำนวน 1 คน ได้แก่

2.2.1 น.ส.หัสยา ดอกดวง นักศึกษาระดับปริญญาเอกทุนโครงการมหาวิทยาลัยวิจัย ทำวิทยานิพนธ์เกี่ยวกับ บทบาทโปรตีนไคเนสเพื่อใช้เป็นเป้าหมายในการรักษาโรคมะเร็งท่อน้ำดี

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

3.1 Pornpan Bungkanjana, Anchalee Techasen, Nisana Namwat, Puangrat Yongvanit and Watcharin Loilome. Wnt expression profile in human cholangiocarcinoma. ประชุมวิชาการประจำปี คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ครั้งที่ 27 “New trends in health care” ประจำปี 2554 4-6 ตุลาคม 2554 Precongress : 1-3 ตุลาคม 2554 ณ ห้องบรรยาย คณะแพทยศาสตร์ (เสนอผลงานแบบโปสเตอร์ ได้รางวัลรองชนะเลิศ อันดับที่ 1 ประเภทนักศึกษาบัณฑิต)