ROLE OF YKL-40 IN CHOLANGIOCARCINOMA AND ANTI-TUMOR ACTIVITY OF PIPERLONGUMINE ON CHOLANGIOCARCINOMA CELLS

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บทบาทของ YKL-40 ในมะเร็งท่อน้ำดีและการต้านมะเร็งของไปเปอร์ลองกูมิน ในเซลล์มะเร็งท่อน้ำดี

นางสาวสุนิสา ทองสม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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มะเร็งท่อน้ำดีเป็นมะเร็งที่มีความรุนแรงอย่างมากซึ่งพบบ่อยในแถบเอเชียตะวันออกเฉียงใต้ โดยเฉพาะในภาคตะวันออกเฉียงเหนือของประเทศไทย มะเร็งท่อน้ำดีเป็นมะเร็งที่วินิจฉัยได้ยากในระยะแรกของโรคเพราะไม่แสดงอาการจำเพาะและไม่มีตัวบ่งชี้ชีวภาพที่จำเพาะ ด้วยการตอบสนองไม่ดีต่อการรักษาโดยใช้รังสีและเคมีบำบัด ดังนั้นตัวบ่งชี้ชีวภาพและยาสำหรับเคมีบำบัดชนิดใหม่เป็นสิ่งที่จำเป็นเร่งด่วน ปัจจุบันมีความพยายามที่จะศึกษาการใช้ตัวบ่งชี้ชีวภาพในการช่วยประกอบการวินิจฉัยมะเร็งท่อน้ำดี แต่ ณ ปัจจุบัน ยังไม่มีตัวบ่งชี้ชีวภาพใดที่ให้ผลจำเพาะต่อมะเร็งท่อน้ำดีเท่าที่ควร โปรตีน YKL-40 เป็นไกลโคโปรตีนที่หลั่งออกมาจากเซลล์และมีการแสดงออกสูงในผู้ป่วยมะเร็งที่มีการพยากรณ์โรคที่ไม่ดี ในการศึกษาครั้งนี้ผู้วิจัยศึกษาว่าโปรตีน YKL-40 เป็นตัวบ่งชี้ชีวภาพสำหรับการวินิจฉัยหรือพยากรณ์โรคสำหรับมะเร็งท่อน้ำดีได้หรือไม่ และศึกษาบทบาทของมะเร็งท่อน้ำดีที่ไม่เคยมีรายงานมาก่อน การแสดงออกของโปรตีน YKL-40 ถูกตรวจสอบในตัวอย่างของผู้ป่วยมะเร็งท่อน้ำดีชนิดพลาสมา 57 ตัวอย่าง และเนื้อเยื่อมะเร็งท่อน้ำดี 34 ตัวอย่าง โดยใช้วิธี enzyme-linked immunosorbent assay (ELISA) และอิมมูโนพยาธิวิทยา พบว่าโปรตีน YKL-40 ในพลาสมามีค่าสูงขึ้นอย่างมีนัยสำคัญในผู้ป่วยมะเร็งท่อน้ำดี ค่ามัธยฐาน 169.5 ng/mL เมื่อเปรียบเทียบกับคนปกติ ค่ามัธยฐาน 46.9 ng/mL ผลการวิเคราะห์ทางสถิติพบว่าโปรตีน YKL-40 ที่เพิ่มสูงขึ้นมีความสัมพันธ์กับการรอดชีวิตที่สั้นลงในผู้ป่วยมะเร็งท่อน้ำดี แต่การศึกษาพบว่าการแสดงออกของโปรตีน YKL-40 พบมากในเซลล์ตับและเนื้อเยื่อเกี่ยวพันบริเวณรอบ ๆ เซลล์มะเร็งแต่มีการแสดงออกน้อยในเซลล์มะเร็งท่อน้ำดี ผลการศึกษานี้แสดงให้เห็นว่าโปรตีน YKL-40 สามารถใช้เป็นตัวบ่งชี้ชีวภาพเพื่อพยากรณ์โรคมะเร็งท่อน้ำดีได้ ในการศึกษาบทบาทหน้าที่ของโปรตีน YKL-40 ในรูปแบบออโตไครน์และพาราไครน์ รีคอมบิแนนท์โปรตีน YKL-40 (rYKL-40) ถูกแสดงออกในระบบ mammalian cells และถูกทำให้บริสุทธิ์เพื่อใช้ตรวจสอบหน้าที่แบบพาราไครน์ โปรตีน rYKL-40 ส่งเสริมการเจริญเติบโตและการเคลื่อนที่ของเซลล์มะเร็งท่อน้ำดี ในทำนองเดียวกันการเพิ่มการแสดงออกของโปรตีน YKL-40 ในเซลล์มะเร็งก็ให้ผลไปในทางเดียวกันคือเพิ่มการเจริญเติบโต การเจริญเติบโตแบบ anchorage-dependent และการเคลื่อนที่ของเซลล์มะเร็งท่อน้ำดี การทดสอบการยึดเกาะของเซลล์มะเร็งท่อน้ำดีแสดงให้เห็นถึงปฏิสัมพันธ์ระหว่างเซลล์กับตัวรับบนผิวเซลล์ผ่านวิถีทาง Akt/Erk นอกจากนี้โปรตีน rYKL-40

แสดงให้เห็นถึงการจับกับ Chitohexaose (GlcNAc6) และการรวมตัวกันเป็นโครงสร้างเชิงซ้อนระหว่าง GlcNAc6 กับ YKL-40 น่าจะมีผลยับยั้งการเจริญเติบโตและการเคลื่อนที่ของเซลล์มะเร็งท่อน้ำดีตามความเข้มข้นของ GlcNAc6 การศึกษานี้บ่งชี้ว่าโปรตีน YKL-40 มีบทบาทสำคัญในการส่งเสริมการเจริญเติบโตและการแพร่กระจายในมะเร็งท่อน้ำดี จากการศึกษาดังที่ได้กล่าวมาข้างต้นสนับสนุนว่าโปรตีน YKL-40 เป็นตัวบ่งชี้ชีวภาพที่ใช้เพื่อการพยากรณ์โรค และน่าจะเป็นยีนเป้าหมายเพื่อใช้ในการรักษามะเร็งท่อน้ำดีต่อไป มะเร็งท่อน้ำดีเป็นมะเร็งที่มีการเจริญเติบโตช้าและมีลักษณะการพยากรณ์โรคที่ไม่ดีเนื่องจากการตอบสนองต่อการรักษาด้วยยาเคมีบำบัดน้อยมากไปกว่านั้น การเป็นมะเร็งซ้ำยังพบได้บ่อยและอัตราการรอดชีพในระยะ 5 ปีก็ค่อนข้างน้อย ในการศึกษานี้เป็นการศึกษาครั้งแรกของยาไปเปอร์ลองกูมินที่เป็นสารอัลคาลอยด์ที่เหนี่ยวนำให้เซลล์มะเร็งท่อน้ำดีตาย แบบตามเวลาและปริมาณยาที่เพิ่มขึ้นผ่านการกระตุ้นของ Caspase-3 และ PARP ไปเปอร์ลองกูมินทำให้เซลล์มะเร็งท่อน้ำดีตายด้วยการเพิ่มการสะสม ROS ในเซลล์และชักนำให้เซลล์หยุดชะงักการแบ่งตัว การทดสอบด้วยสารต้านอนุมูลอิสระ N-acetyl-L-cysteine (NAC) หรือ reduced-glutathione (GSH) สามารถขัดขวางการออกฤทธิ์ของไปเปอร์ลองกูมินได้อย่างสมบรูณ์ สุดท้ายการศึกษาครั้งนี้แสดงให้เห็นว่าไปเปอร์ลองกูมินกระตุ้นการตายของเซลล์มะเร็งท่อน้ำดีโดยผ่านวิถี JNK และ ERK นอกจากนี้ในการศึกษาครั้งนี้พบว่าความสามารถในการต้านอนุมูลอิสระที่แตกต่างกันของเซลล์บ่งบอกความไวในการรักษาด้วยไปเปอร์ลองกูมิน จากผลดังกล่าวข้างต้นแสดงให้เห็นว่าไปเปอร์ลองกูมินเป็นยาสำหรับเคมีบำบัดที่มีศักยภาพในการรักษามะเร็งท่อน้ำดี

จากผลการศึกษาทั้งหมดในการศึกษานี้โปรตีน YKL-40 มีบทบาทสำคัญในกระบวนการส่งเสริมพัฒนาของมะเร็งท่อน้ำดี ดังนั้นการยับยั้งโปรตีน YKL-40 น่าจะเป็นเป้าหมายใหม่ในการรักษามะเร็งท่อน้ำดี นอกจากนี้การรักษาด้วยไปเปอร์ลองกูมินแบบเฉพาะหรือรักษาร่วมกับยาเคมีบำบัดที่ใช้อยู่ ณ ปัจจุบัน อาจจะเป็นแนวทางการรักษารูปแบบใหม่ในมะเร็งท่อน้ำดี

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SUNISA THONGSOM : ROLE OF YKL-40 IN CHOLANGIOCARCINOMA AND ANTI-TUMOR ACTIVITY OF PIPERLONGUMINE ON CHOLANGIOCARCINOMA CELLS. THESIS ADVISOR : PROF. WIPA SUGINTA, Ph.D. 184 PP.

ROLE OF YKL-40 IN CHOLANGIOCARCINOMA AND ANTI-TUMOR ACTIVITY OF PIPERLONGUMINE ON CHOLANGIOCARCINOMA CELLS

Cholangiocarcinoma (CCA) is a devastating cancer and is found more frequently in Southeast Asia and particular in Northeast of Thailand. CCA is difficult to diagnose at early stage since there are no specific symptom and biomarker, and responds poorly to current radio- and chemo-therapy. Therefore, novel biomarker and treatment strategies for CCA are urgently needed. YKL-40 is a secreted glycoprotein and highly expressed in cancer patients with poor prognosis. In present study, we investigated whether YKL-40 is a biomarker for CCA diagnosis or prognosis and we also examined the roles of YKL-40 in CCA that have never been reported. YKL-40 expression was determined plasma and tumor tissues from CCA patients by ELISA and immunohistochemistry. YKL-40 plasma concentration was significantly increased in CCA patients comparing with healthy subjects and elevated plasma YKL-40 level was particularly associated with short survival in CCA patients. However, YKL- 40 is rarely expressed in CCA tumor cells, but highly expressed in liver cells and connective tissue at intratumoral stroma. Next, we demonstrated the role of YKL-40 on autocrine and paracrine functions to promoted CCA progression. Adding purified recombinant YKL-40 (rYKL-40) significantly enhanced growth, and

migration of CCA cells. YKL-40 overexpression in CCA cells showed similar effects in proliferation, anchorage-independent growth, and migration. Cell adhesion assay suggested that YKL-40 interact with cell-surface receptor involving the Akt/Erk mediated pathway. In addition, blocking YKL-40 effects by GlcNAc6 seemed to inhibit proliferation and migration of CCA cells in a concentration manner. Taken together, our results support the proposal of YKL-40 as a new potential prognostic biomarker and new molecular targeted therapy for CCA. CCA is considered a slow-growing tumor and characterized by very poor prognosis due to rare response to current chemotherapeutic agents. The current study showed for the first time that piperlongumine (PL), a biologically-active alkaloid, markedly induced death in CCA cell lines in a dose- and time-dependent manner through the activation of caspase-3 and PARP. PL mediated CCA cell death via inducing ROS accumulation in CCA cells and promoting G2/M phase arrest, and pre-treatment with the antioxidant NAC or GSH completely abolished those effects of PL. Finally, our finding demonstrated that PL can trigger CCA cell death through ROS mediated JNK/ERK activation. Furthermore, the different antioxidant capacity of CCA cell lines also indicates the susceptibility of the cells to PL treatment. Gather all evidences obtained from the present study, YKL-40 plays an important role in tumor progression. Thus, the inhibition of YKL-40 may be as a novel therapeutic strategy for CCA. Moreover, treatment with PL or combination with current chemotherapeutic agents can be a new chemotherapeutic protocol for the treatment of CCA.

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Academic Year 2016 Advisor's signature _____

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AKT	a three letter name (not an abbreviation); “p” stands for phosphorylated (active) form
BAD	BCL-2-associated death promoter
BAX	BCL-2-associated protein X
BCA	Bicinchoninic acid
BCL-2	B cell lymphoma gene 2
bp	base pair(s)
BSA	Bovine serum albumin
CCA	Cholangiocarcinoma
cDNA	DNA complementary to RNA
CI	Confidence interval
CMV	Cytomegalovirus
DAB	3,3′-diaminobenzidine-tetrahydrochloride
DAPI	4’, 6-diamidino-2-phenylindole
DCFH-DA	Dichlorodihydrofluorescein diacetate
DI	Deionized
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphates

LIST OF ABBREVIATIONS (Continued)

ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked-immunosorbent assay
ERK1/2	Extracellular Signal-Regulated Kinases 1 and 2
FBS	Fetal bovine serum
G148	Geneticin
G2/M	G2-mitosis transition
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GlcNAc	N-acetylglucosamine
GSH	L-glutathione reduced
HEK	Human embryonic kidney
HP	Degree of magnification of the high power objective
HRP	Horseradish peroxidase
HRP	horseradish peroxidase
IHC	Immunohistochemical analysis
JNK	Jun N-terminal Kinase
kb	kilobase(s) or 1,000 bp
kD	Kilodalton(s)
miR	microRNA
mRNA	messenger RNA
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenil)-2H-tetrazolium

LIST OF ABBREVIATIONS (Continued)

n	subsample size
NAC	N-Acetylcysteine
Ni-NTA	Ni-nitrilotriacetic acid
NP-40	Nonidet P-40
Opti-MEM	Reduced-Serum Medium is an improved Minimal Essential Medium
OV	*Opisthorchis viverrini*
P value	Probability value
p21	21 kilodalton cyclin-dependent kinase inhibitor
p53	53 kilodalton tumor suppressor protein
PARP	poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PBST	phosphate-buffered saline tween
PCR	Polymerase chain reaction
pERK	phosphorylated (active) extracellular-signal-regulated kinase
PL	Piperlongumine
PI	Propidium iodide
pJNK	phosphorylated (active) C-Jun NH2-terminal kinase
pTNM	pathological tumor-node-metastasis staging
qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RNase A	Ribonuclease A

LIST OF ABBREVIATIONS (Continued)

ROS	Reactive oxygen species
rpm	revolutions per minute
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
SEM	Standard error of the mean
Ser33/37/Thr41	Serine 33 / Serine 37 / Threonine 41
SRB	Sulforhodamine B
STAT	Signal transducer and activators of transcription
SV40	Simian Virus 40
TCA	Trichloroacetic acid
v/v	Volume/volume
w/v	Weight/volume
ZnPP	Zinc protoporphyrin IX

CHAPTER I

INTRODUCTION

1.1 Significance of research

Worldwide incidence and mortality rate from cholangiocarcinoma (CCA) - a aggressive malignancy of bile duct epithelium-.is increasing. CCA is found more frequently in Southeast Asia and is a major health problem in the Northeast of Thailand, particularly in areas where *Opisthorchis viverrini* (OV) infection is endemic. Because chronic infection with this liver fluke has been shown strong risk factor for CCA development. CCA is normally difficult to diagnose until the disease becomes advanced or disseminated, at which the prognosis is poor. Surgical resection is a standard treatment and is the only effective therapeutic option for CCA. However, this treatment is applicable in less than 50% of cases, because most of CCA patients are diagnosed at late stages. Currently, several advance techniques with high resolution are used for diagnosis of CCA, such as ultrasonography, computerized tomography (CT) scanning, magnetic resonance imaging (MRI), and biopsy. However, they are limited to the minority of patients due to high cost and low accessibility in the local hospitals. Detection of serum biomarkers including CEA, CA19-9, ALP, MUC5AC, and CA-S12 for CCA screening test is non-invasive and alternative method to diagnose this cancer. Nevertheless, each biomarker still provides different diagnostic and prognostic values. To increase both specificity and sensitivity of CCA diagnosis and prognosis either utility of a combination of

biomarkers or a highly specific CCA biomarkers is required.

YKL-40 (also called chitinase-3-like 1; CHI3L1) is a chitinase-like protein, which is a member of family-18 glycosyl hydrolases that lacks chitinase activity. YKL-40 is identified in 1989 to be secreted in vitro in a large amount by the human osteosarcoma cell line MG63. Human YKL-40 is expressed by variety of cells including macrophages, neutrophils, epithelial cells, synovial cells, chondrocytes, smooth muscle cells, malignant tumors, and tumor cell lines. Elevated plasma YKL-40 concentrations have been seen in patients with inflammation diseases including ischemic cardiovascular diseases, diabetes, asthma, chronic obstructive pulmonary disease, rheumatoid arthritis, inflammatory bowel disease, pneumonia, and liver fibrosis, and solid tumors such as breast cancer, colorectal cancer, endometrial cancer, non-small cell lung cancer, ovarian cancer, cervical cancer, and prostate cancer. High expression level of YKL-40 has been reported to be strongly associated with poor prognosis of various types of cancers. Therefore, YKL-40 has been recently suggested as a new prognostic marker for cancer diseases. In addition, YKL-40 has been shown to participates in the tumor progression by promoting proliferation, migration and invasion of tumor cells and indirectly by promoting angiogenesis in the tumor microenvironment. Thus, YKL-40 has impact on cancer development and progression and also has attention as independent prognostic marker for cancer. However, the significance of YKL-40 in CCA; expression level, and its role in CCA development and progression has not been reported.

The majority of patients with CCA develop local recurrence and/or metastases, and treatment of these patients is therefore an important issue. Chemotherapy has been used in an attempt to control the disease, and improve

survival and quality of life in patients with irresectable, recurrent and metastastic cholangiocarcinoma. Various chemotherapeutic agents, either alone or in combination, have been tested. However, CAA is highly resistant to available chemotherapeutic agents and confers a 5-year relative survival rate of less than 5 %. Therefore, it necessary to search for highly potent chemotherapeutic agents against this malignancy. Recently, the strategies for anti-cancer therapy is focus on reduction–oxidation (REDOX) signaling pathways that play a major role in cancer formation and especially in responses to radiotherapy and chemotherapy. It has been suggested that induction of oxidative stress by exogenous ROS generation therapy has an effect on selectively killing cancer cells without affecting normal cells. Piperlongumine (PL) is a natural alkaloid of the long pepper (*Piper longum*) that can selectively kill cancer cells but not normal cells. PL has an inhibitory effect on tumor proliferation, migration, invasion and angiogenesis through selective accumulation of ROS in cancer cells and activation of p38, JNK, Erk, Akt, promoting protein glutathionylation, or suppressing NFκB activities. These cytotoxic effects of PL have been documented in several type of cancers such as hepatocellular carcinoma, glioblastoma, breast, gastric, head and neck and, ovarian cancer. However, there is no any report on the effect of PL in CCA treatment. This information motivated us to explore the cytotoxicity and mechanism of PL in CCA.

In this study, we investigated 1) the expression levels of YKL-40 in CCA and the association between YKL-40 expressions (both plasma level and tissue expression) and clinicopathological features or survival of CCA patients 2) the biological effects of YKL-40 on malignant phenotypes including proliferation, migration invasion and chemosensitivity and 3) the anti-cancer effects of PL on the

CCA cell lines and also explored the underlying mechanisms of PL-induced CCA cell death. The outcome of this study may lead to 1) a new prognostic marker for CCA, 2) the knowledge gained may help to explain the role of YKL-40 during cholangiocarcinogenesis, and 3) a novel chemotherapeutic drug to improve the outcome of CCA treatment.

1.2 Research objectives

The aim of this thesis focused on 1) YKL-40 expression and its role in CCA and 2) anti-cancer effect of PL in CCA cell lines.

1. To investigate YKL-40 expression in CCA tissues and the association of YKL-40 expression with the clinicopathological features and the survival of CCA patients.

2. To investigate the plasma levels of YKL-40 in CCA patients and control groups.

3. To investigate the association of plasma YKL-40 levels and the clinicopathological features and the survival of CCA patients.

4. To investigate the biological functions of YKL-40 in CCA cell lines.

5. To investigates the anti-cancer effects of PL and its cytotoxic mechanism in CCA cell lines.

1.3 Scope of the study

According to the research objective, this study was divided in to two parts as follow:

Part I: ROLE OF YKL-40 IN CHOLANGIOCARSINOMA

YKL-40 expression in tissues was determined by immunohistochemistry. The correlation between YKL-40 expression and clinicopathological features and survival was analyzed. The levels of plasma YKL-40 in blood from CCA patients and heathy subjects were determined by ELISA. The comparison of plasma YKL-40 among two groups and the association of plasma YKL-40 and clinicopathological features and survival were analyzed. The biological effects of YKL-40 were performed in CCA cell lines using recombinant YKL-40 protein and exogenous YKL-40 expression model. The phenotypic changes of CCA cell lines were observed in terms of proliferation, migration, invasion and chemosensitivity. The molecular mechanism of YKL-40 in cholangiocarcinogenesis was performed by western blot analysis.

Part II: ANTI-TUMOR ACTIVITY OF PIPERLONGUMINE ON CHOLANGIOCACINOMA CELLS

Five human CCA cell lines, cholangiocyte cell line and mouse fibroblast cell lines were used to determine the anti-proliferation effect of PL. PL-induced apoptosis in CCA cell lines was evaluated by apoptosis assay and cell cycle analysis using flow cytometer. ROS accumulation in CCA cell lines was analyzed by flow cytometer. The molecular mechanism of PL-induced CCA cell death was investigated by western blot analysis. Expression profiles of antioxidant related genes in CCA cell lines were determined by quantitative RT-PCR.

CHAPTER II

LITERATURE REVIEW

2.1 Cholangiocarcinoma (CCA)

Cholangiocarcinoma (CCA) is an epithelial cancer originating from the bile ducts with features of cholangiocyte differentiation (de Groen *et al.*, 1999). Anatomically, CCA is classified into extrahepatic and intrahepatic forms of the disease. The extrahepatic form is more common in worldwide, accounting for up to 90% of CCAs (Lim and Park, 2004). CCA is an adenocarcinoma with different variants being recognized, including tubular adenocarcinoma, papillary adenocarcinoma, intestinal-type adenocarcinoma, and mucinous adenocarcinoma. Histologically, CCA is classified according to grade as being well, moderately, or poorly differentiated, with the classic diagnosis being well-to moderately-differentiated ductal adenocarcinoma. Intrahepatic CCA is further classified by growth characteristics or macroscopic into three subtypes including (1) mass-forming type, (2) periductal infiltrating type, and 3) intraductal growth type. Mass-forming type is the most commonly encountered type being the desmoplastic infiltrating nodular or diffusely infiltrating varieties (Sirica, 2005). This type of CCA has very poor prognosis and is extremely aggressive with symptoms unobservable until there is a blockage of the bile duct by the tumor.

2.1.1 Epidemiology and Etiology of CCA

The causes of CCA are still unknown. However, chronic inflammation together with partial obstruction of bile duct flow (Gores, 2003; Sirica *et al.*, 2002) manifest as high risk conditions for CCA development. Chronic inflammation including primary sclerosing cholangitis, hepatolithiasis, Caroli's disease, and congenital choledochal cysts, were thought to be relevant factors for CCA development in Western countries (Gores, 2003). Whereas, Liver fluke infections by *Opisthorchis viverrini* (OV) or *Clonorchis sinensis* is endemic mainly in Thailand , Laos PDR, Malaysia Japan, Korean, and Vietnam (Kullavanijaya *et al.*, 1999; Sithithaworn *et al.*, 1994). It was strongly showed the association with CCA development in this geographical area (Thamavit *et al.*, 1978). In 1994, OV is categorized by the International Agency for Research in Cancer (IARC) to be type I carcinogen (IARC, 1994). Based on the information above suggests that OV infection is a major risk factor of CCA especially in Eastern countries.

CCA is rare worldwide but the incidence rate is steadily increasing. For example, an estimated incidence of 3,000 cases of primary bile duct cancer is reported in 2002 in the United States (American Cancer Society Cancer Facts and Figure: www.cancer.org 2003). In the United Kingdom, CCA is now notified as one of the most common primary liver tumor-related causes of death (Taylor-Robinson *et al.*, 2001). Similar to Japan, Western Europe and Australia also showed the increased mortality rate of this cancer in between 1979 to 1998 (Khan *et al.*, 2002). However, the highest incidence is still found in Southeast Asia, especially northeast of Thailand (Khon Kaen province) (Green *et al.*, 1991) where the prevalence of OV infection and CCA are the highest in the country (Vatanasapt *et al.*, 1990). It was showed 89.7 per

100,000 male and 67.2 per 100,000 female (Vatanasapt *et al.*, 2002). These incidences were at least 30-fold higher than those of people in the regions without OV infection. The most of CCA patients were between 32 and 78 years old (Carriaga and Henson, 1995; Elkins *et al.*, 1990; Parkin *et al.*, 1993), with the peak age of 40-60 years having intrahepatic CCA (Uttaravichien *et al.*, 1999).

2.1.2 Pathogenesis of Opisthorchiasis association with CCA

CCA has been proposed to be the result of multi-stage process. OV infection is the major risk of CCA in Thailand. However, the understanding of CCA development in association with OV infection remains unclear. The possible mechanism of CCA development has been proposed as a four-stage cascade (Figure 1) (Coussens and Werb, 2002; Holzinger *et al.*, 1999; Hussain *et al.*, 2003; Ohshima and Bartsch, 1994; Okada, 2002). Firstly, exposure to risk factor(s) may lead to chronic inflammation and/or cholestasis through the biochemical and/or mechanical processes. The resident inflammatory cells are possibly involved in carcinogenesis. Pro-inflammatory cytokines, namely TNF-α, interferon (IFN)-γ, IL-1β, -6 and others, are produced by various activated inflammatory cells, which could induce and activate various oxidant-generating enzymes leading to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) during inflammation. Secondly, genotoxic events may be the consequence of ROS and RNS overproduction by inflammatory cells. Nitric oxide and oxygen radicals can inactivate, leading to impaired functions of biomolecules, such as lipids, proteins, DNA, and carbohydrates within the cells, via direct oxidative and nitrosative damages. Recently, two biomarkers of DNA damage, including 8-oxo-7, 8-dihydro 2'-deoxyguanisine (8-oxodG) and 8-nitroguanine, have been demonstrated in the liver of OV infected

hamsters . Repeated infection with OV could mediate oxidative and nitrosative DNA damages and may play a role in the initiation and/or promotion steps of cholangiocarcinogenesis (Pinlaor *et al.*, 2004). In addition, free radicals caused by inflammatory cytokine can induce many subsequent events, such as activation of gene expression, alteration of detoxification gene expression and activation of carcinogen metabolism to form ultimate carcinogens, which enhance DNA damage. Moreover, overproduction of NO has been reported to be immunosuppressive for lymphocyte proliferation. Thirdly, dysregulation of DNA repair and apoptosis. Genotoxic events with DNA damage usually lead to either DNA mismatch repair mechanism or, if the damage is beyond repaired, cell death occurs through apoptosis. Finally, the histological change to malignant could occur through the "hyperplasia-dysplasia-carcinoma" model, as suggested for colon and gastric cancers (Holzinger *et al.*, 1999).

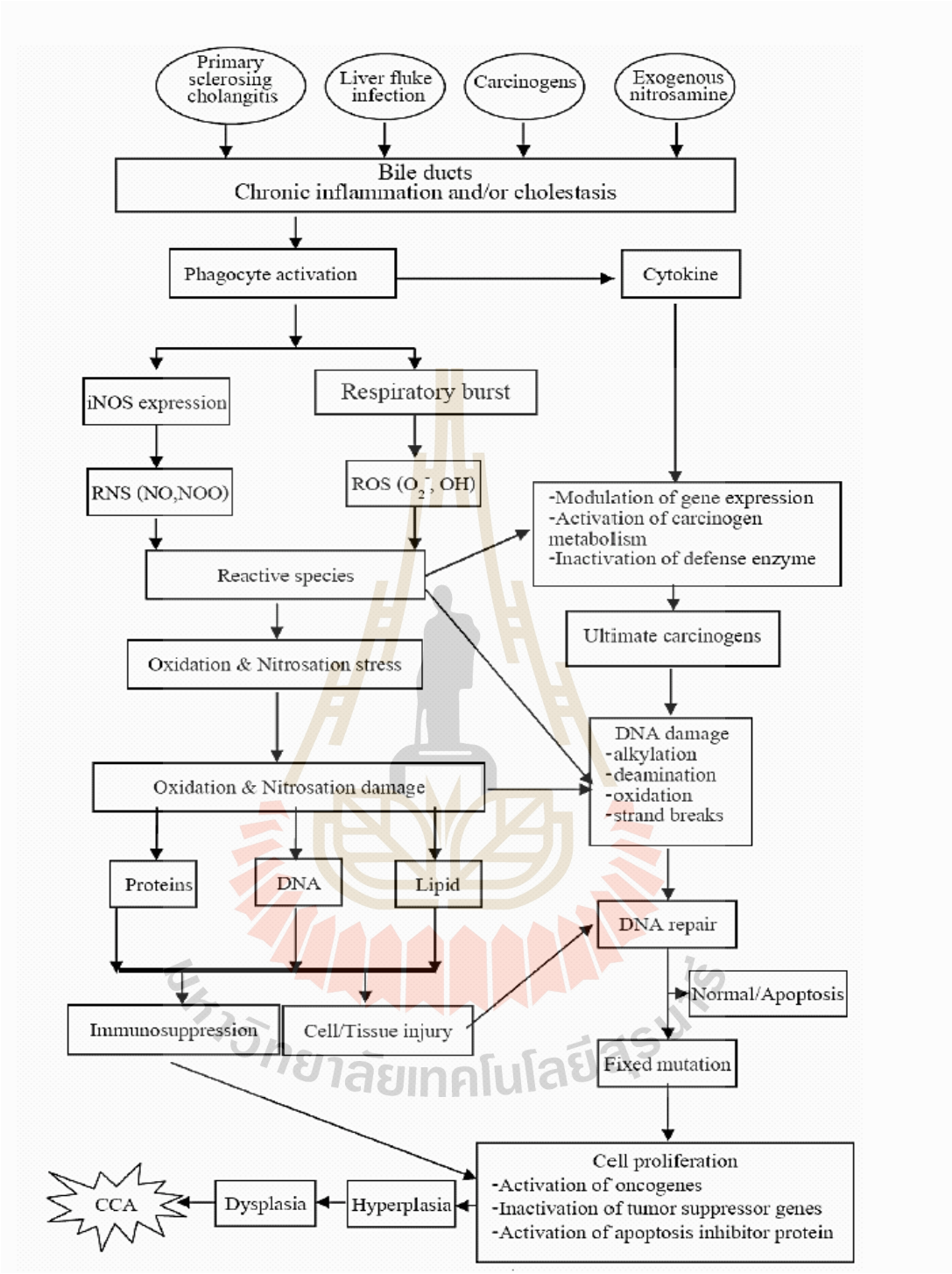

Figure 2.1 The possible carcinogenesis mechanism of CCA (modified from (Holzinger *et al.*, 1999; Hussain *et al.*, 2003; Ohshima and Bartsch, 1994)).

2.1.3 Tumor microenvironment promoting CCA progression

CCA is originated from the neoplastic transformation of the epithelial cells of the intrahepatic or extrahepatic bile ducts. The molecular mechanisms underlying the development, growth, and metastatic diffusion of this cancers are still undefined. Recent attention has been paid to the origin of CCA from the neoplastic transformation of resident hepatic stem cells (Nomoto *et al.*, 2006; Sell and Dunsford, 1989). The microenvironment has been shown to play a role in neoplastic transformation, progression, metastasis and invasion of cancer cells. Also, resistance to radiotherapy and chemotherapy is influenced by the interaction between the cancer cells and the tumor microenvironment (Leyva-Illades *et al.*, 2012). Tumor microenvironment (TME) contains many distinct cell types, including fibroblasts, carcinoma-associated fibroblasts (CAFs), myofibroblasts, smooth muscle cells, endothelial cells and their precursors, pericytes, neutrophils, eosinophils, basophils, mast cells, T and B lymphocytes, natural kill cells, and antigen presenting cells (APC) such as macrophages and dendritic cells. Moreover, the TME contains non-cellular components, including extracellular matrixes, growth factors, proteases, protease inhibitors and other signaling molecules that play important roles in stromal reactions in TME (Koontongkaew, 2013) (Figure 2.2). Therefore, it is a very important factor in the regulation of tumor angiogenesis, invasion, and metastasis (Hezel and Zhu, 2008; Orimo and Weinberg, 2006; Tlsty, 2001).

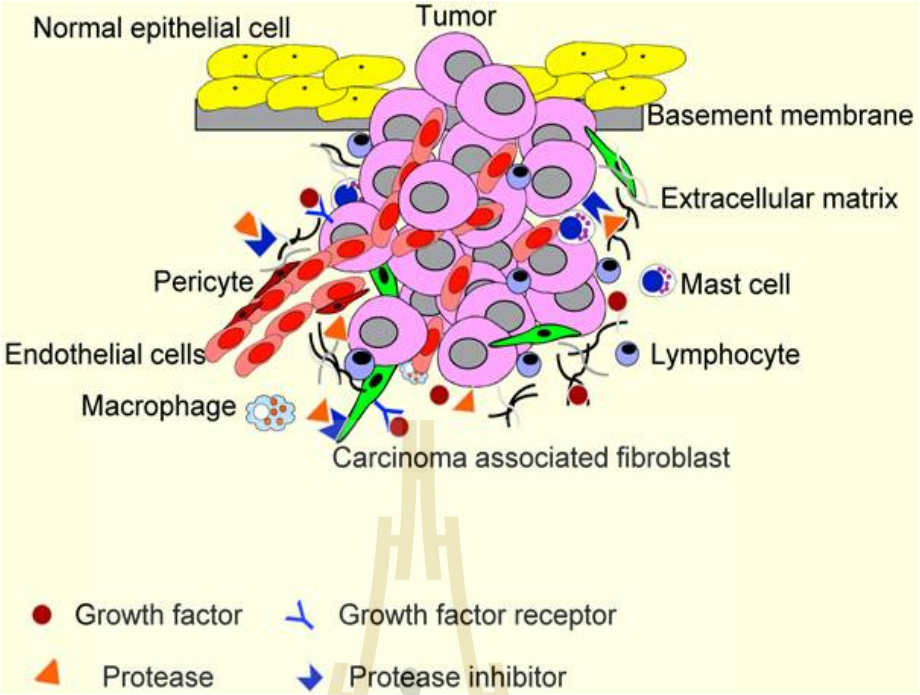

Figure 2.2 The tumor microenvironment (TME). The TME comprises different stromal cells in addition to tumor cells. These include vascular or lymphatic endothelial cells, supporting pericytes, fibroblasts, and both innate and adaptive infiltrating immune cells (Koontongkaew, 2013).

There is evidence to show that the interaction between the cancer cells and stromal cells of the microenvironment is bi-directional and dynamic (Lorusso and Ruegg, 2008). Neoplastic cells can secrete certain factors that recruit and activate stromal cells into the tumor microenvironment in a paracrine fashion. Stromal cells that have been recruited and activated can then release those factors into the extracellular milieu that further stimulate or inhibit tumor growth. Vascular endothelial cell proliferation and recruitment leading to the formation of new blood vessels provide the tumor with the nutrient supply necessary for its growth and

metastasis. Cancer-associated fibroblasts can stimulate angiogenesis as well as promote tumor growth and invasion. Tumor-associated macrophages (TAMs) also contribute to tumor growth, invasion, and metastasis by producing various mediators in many cancers. Finally, the proliferation of lymph endothelial cells leading to the increase in lymphatic vessel density can promote tumor metastasis (Leyva-Illades *et al.*, 2012) (Figure 2.3). In CCA, stromal-derived growth factor-1 (SDF-1) secreted by the embryonic lung fibroblast cell line WI-38 interacting with its receptor CXCR4 expressed in 2 human CCA cell lines (HuCCT1 and CCKS-1) and lead to promoted CCA cell migration (Ohira *et al.*, 2006). Recent studies have shown that myofibroblast-derived platelet-derived growth factor protects CCA cells from tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) induced cell death via a hedgehog dependent mechanism both *in vitro* and *in vivo* (Fingas *et al.*, 2011). Moreover, the high density of M2 phenotype tumor-associated macrophages in tissues has been suggested to act as an independent risk factor of disease-free survival and treating macrophages *in vitro* with the supernatant from CCA cells leads to macrophage polarization toward the M2 phenotype and secretion of down-regulation via STAT3, VEGF-A, IL-10 and TGFβ. Collectively, the tumor microenvironment seems to play an important role in the growth, progression, and metastatic invasion of CCA (Leyva-Illades *et al.*, 2012).

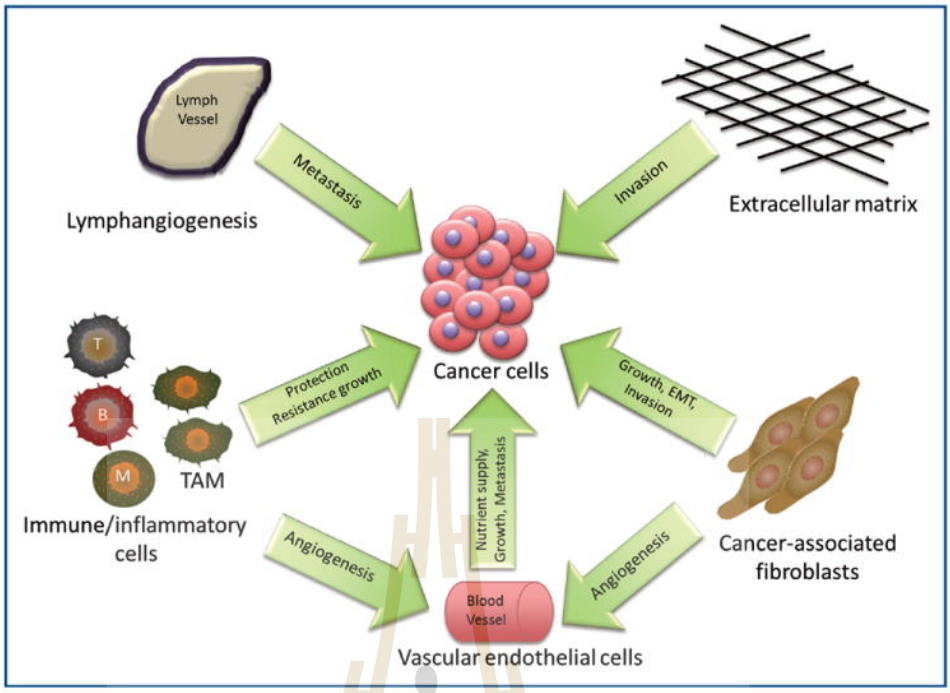

Figure 2.3 Effects of stromal support cells on tumor growth and metastasis. B (B-cell), EMT (epithelial-mesenchymal transition), M (monocyte), T (T-cell), TAM (tumor associated macrophage) (Leyva-Illades *et al.*, 2012).

2.1.4 CCA biomarkers

CCA is a highly lethal malignancy with very poor prognosis. CCA is extremely aggressive with symptoms unobservable until there is a blockage of the bile duct by the tumor (Leyva-Illades *et al.*, 2012). CCA is clinically silent, with symptoms only developing at advanced stages. Surgical resection or liver transplantation is the only effective therapeutic option for CCA, but this is applicable in less than 50% of cases, because this cancer is mostly diagnosed at late stages (Blechacz and Gores, 2008; Gatto and Alvaro, 2010). For many years, efforts have been made to identify, in serum or biological fluid (Alvaro *et al.*, 2007; Ramage *et al.*,

1995), biomarkers with adequate diagnostic accuracy for CCA, which could also be useful for the population screening or for the surveillance of pathologies at risk, including primary sclerosing cholangitis (PSC) (risk factors for CCA) (Bjornsson *et al.*, 1999; Ramage *et al.*, 1995). Although several prognostic molecular markers, such as serum tumor markers, are attractive because of the easiness of samples obtaining and relatively low cost, their septicity for a particular type of cancer is always controversial. To date, CCA biomarkers have been the objects of extensive investigation to aid CCA diagnosis but, unfortunately, none of these markers has reached adequate specificity for CCA (Gatto and Alvaro, 2010). However, there are a number of biomarkers in serum for demonstrating CCA, which can also be used as potential markers for monitoring, prognosis and recurrence of the cancer (Table 2.1). So far, carbohydrate antigen (CA 19-9) is the most widely used serum marker for CCA, although it is also elevated in pancreatic cancer, gastric cancer, and primary biliary cirrhosis (Charbel and Al-Kawas, 2011; Silsirivanit *et al.*, 2012). Taken together, CCA biomarker with high sensitivity and specificity is needed.

Table 2.1 Prognostic and monitoring markers for CCA (Silsirivanit *et al.*, 2012).

Biomarkers	Indicated level	Association
CA19-9	≥ 100 U/ml	- Poor prognosis
		- Low survival rate
CYFRA21-1	≥ 2.7 ng/ml	- Large tumor size
		- Number of tumors
		- Lymphatic invasion
		- Low survival rate
MUC5AC	WB positive	- Large tumor size
	OD. ELISA > 0.074	- TNM stage IVA-B
		- Low survival rate
CA-S121	OD. ELISA > 0.23	- Decrease after tumor removal
		- Low survival rate
IL-6		- Decreased after photodynamic therapy
RCAS1		- Decrease after tumor removal
		- Increase when tumor recurrence

2.1.5 Treatment of CCA

As mention earlier, cholangiocarcinoma is an aggressive cancer that carries a poor prognosis. The majority of patients with CCA develop local recurrence and/or metastases, and treatment of these patients is therefore an important issue. The options for the treatment of CCA are limited and associated with high rates of perioperative mortality, recurrence, and short survival times (Macias, 2014). The first step for treatment is to assess operative indication. Therefore, after CCA diagnosis and staging, the resectability has been evaluated for the treatment options, resectable or unresectable (Miyazaki *et al.*, 2015). Tumor resection is the only potential cure for CCA. Curative resection, or resection of tumor-free surgical margins (R0), remains the best chance for long-term survival, and lymph node status is the most important prognostic factor following R0 resection (DeOliveira *et al.*, 2007). However,

resectability rates have been quite low and variable, as most patients present at an advanced stage. Intrahepatic cholangiocarcinoma resectability rates range from 18 to 70%, with a 1-year survival rate of 35%-86%, 3-year survival of 20%-52%, a 5-year survival rate after surgery of 20-40% and the median survival following diagnosis of cholangiocarcinoma is 12-37.4 months (Morise *et al.*, 2010). For patients that are not surgical candidates for curative resection, liver transplantation, chemotherapy and targeted radiation are considerations (Anderson *et al.*, 2004; Gatto and Alvaro, 2010). Transplantation is an emerging therapy for unresectable CCA without evidence of metastatic disease, is a consideration in patients with perihilar extrahepatic cholangiocarcinoma (Rosen *et al.*, 2008). The 5-year survival rate for perihilar cholangiocarcinoma patients receiving a liver transplant is greater than 70% (Heimbach *et al.*, 2006). Moreover, adjunctive chemotherapy and targeted radiation in addition to surgery are considerations, though there is no obvious evidence for the benefit of adjuvant therapy for resected bile duct cancers (Anderson *et al.*, 2004; Miyazaki *et al.*, 2015). Various chemotherapeutic agents, either alone or in combination, have been tested (Macias, 2014). 5-Fluorouracil (5-FU)-based regimens were among the first reported in biliary tract cancers and regimens based on gemcitabine have also generated promising results. The most used agent has been 5-fluorouracil, which has a partial response rate of about 12%. Gemcitabine has a similar response rate. Although fluoropyrimidines and doxorubicin have been reported to have response rates as high as 30-40%, partial responses lasting from weeks to months have been observed in only 10-35% of trials (Thongprasert, 2005). The combination chemotherapy with gemcitabine and cisplatin is recommended as the first-line chemotherapy for patients with CCA in Japan (Miyazaki *et al.*, 2015).

Combining cisplatin with gemcitabine has been shown to increase progression-free survival when compared with gemcitabine alone, with a median survival time of 11.2 months without any significant increases in side effects (Okusaka *et al.*, 2010). However, there is no good standard treatment till now and further investigation of new chemotherapeutic agents and novel targeted therapy is warranted.

2.2 YKL-40

Chitinases (EC.3.2.1.14) hydrolyze the β-1, 4-linkages in chitin, an abundant N-acetyl-β-D-glucosamine polysaccharide that is a structural component of protective biological matrices such as insect exoskeletons and fungal cell walls. The family 18 glycosyl hydrolases (GH18) is an ancient gene family widely expressed in archea, prokaryotes and eukaryotes (Merzendorfer and Zimoch, 2003). Mammals are not known to synthesize chitin or to metabolize it as a nutrient, it was generally assumed that mammals lacked chitinases, yet the human genome encodes eight well-documented genes for proteins now classified as GH 18 members. Currently, more than seven GH 18 in mammals have been identified in mice and humans. Acidic mammalian chitinase (AMCase), chitotriosidase (CHIT1), oviductin, YKL-40, and YKL-39 have been described in human, whereas YM-1, YM-2, AMCase, oviductin, and breast regression protein 39 (BRP-39) have been described in mice (Boot *et al.*, 2001; Chang *et al.*, 2001; Johansen *et al.*, 1992). Chitotriosidase, and acidic mammalian chitinase are the only 2 of these proteins demonstrating chitinolytic activity, while none of the other mammalian chitinases show enzymatic activity despite the retention and conservation of the substrate-binding cleft of the chitinases (Boot *et al.*, 2001; Chang *et al.*, 2001; Fusetti *et al.*, 2003; van Aalten *et al.*, 2001).

Therefore, the latter proteins chitinases are called chitinase-like-lectins (Chi-lectins) or mammalian chitinase-like proteins (CLPs). However, the roles of mammalian chitinase like proteins (CLPs) have only recently begun to be elucidated. Acidic mammalian chitinase inhibits chitin-induced innate inflammation; augments chitin-free, allergen-induced Th2 inflammation; and mediates effector functions of IL-13. On the other hand, YKL-40 inhibits oxidant-induced lung injury, augments adaptive Th2 immunity, regulates apoptosis, stimulates alternative macrophage activation, and contributes to fibrosis and wound healing (C. G. Lee *et al.*, 2011). In present study, we focus on YKL-40.

The glycoprotein YKL-40 was identified in 1989 to be secreted *in vitro* in a large amount by the human osteosarcoma cell line MG-63 (Johansen *et al.*, 1992). The protein was named YKL-40 based on its structure: three NH_2-terminal amino acids – tyrosine (Y), lysine (K), and leucine (L), and the molecular weight (40 kDa) gave the number (Johansen *et al.*, 1992). Although, the protein has several names: YKL-40 (Johansen *et al.*, 1992), human cartilage glycoprotein-39 (HCgp39) (Hakala *et al.*, 1993), breast regressing protein 39 Kd (BRP-39) (Morrison and Leder, 1994), 38-kDa heparin-binding glycoprotein (gp38k) (Shackelton *et al.*, 1995), chitinase-3-like-1 (CHI3L1) (Rehli *et al.*, 1997), chondrex (Harvey *et al.*, 1998), and a 40 kDa mammary gland protein (MGP-40) (Mohanty *et al.*, 2003). The *YKL-40* gene consists of 10 exons located within 8 kb of DNA on human chromosome 1q32.1, and encodes a single polypeptide chain of 383 amino acids (Hakala *et al.*, 1993; Shackelton *et al.*, 1995) with an isoelectric point of about 7.6 (Renkema *et al.*, 1998).

2.2.1 YKL-40 structure

YKL-40 is a highly phylogenetically conserved chitin-binding glycoprotein, and belongs to the glycosyl hydrolase family 18 (B Henrissat, 1999), a mammalian member of the chitinase protein family, with no chitinase activity (Hakala *et al.*, 1993; Hu *et al.*, 1996; Johansen *et al.*, 1993; Rehli *et al.*, 1997; Renkema *et al.*, 1998; Shackelton *et al.*, 1995) but does bind chitin (Renkema *et al.*, 1998). The three-dimensional structures of human YKL-40 (Fusetti *et al.*, 2003; Houston *et al.*, 2003) display the typical fold of family 18 glycosyl hydrolases (B. Henrissat and Davies, 1997). The structure is divided into two globular domains: a big core domain which consists of a $(\beta/\alpha)_8$ TIM barrel domain structure with a triose-phosphate isomerase (TIM) barrel fold, and a small α/β domain composed of five antiparallel β-strands and one α-helix that is inserted in the loop between strand β7 and helix α7 of the TIM barrel (Figure 1). This gives the active site of YKL-40 a groove-like character (Fusetti *et al.*, 2003). The glutamate and aspartate at the end of the conserved DXXDXDXE sequence motif are essential for catalysis of the family 18 chitinases (Bokma *et al.*, 2002; De Ceuninck *et al.*, 2001; Watanabe *et al.*, 1993; Watanabe *et al.*, 1994). In human YKL-40, the catalytic glutamic acid is mutated to leucine (L, residue 140) and the catalytic aspartic acid mutated to alanine (A, residue 138). Theses mutations led to a complete lack of hydrolytic activity of YKL-40 (Houston *et al.*, 2003). YKL-40 is N-glycosylated at asparagine (Asn, residue 60). Glycosylation is a unique feature of YKL-40 as the residue corresponding to Asn 60 does not exist in chitinases and is mutated to proline in other CLPs (Fusetti *et al.*, 2003). YKL-40 binds to heparin (Shackelton *et al.*, 1995) and amino acid sequence analysis reveals that YKL-40 contains one heparin-binding motif (GRRDKQH, residues 143-149).

This putative heparin-binding site is located in a surface loop (Fusetti *et al.*, 2003). It has been suggested that heparan sulfate is likely acts as a specific ligand of YKL-40 (Fusetti *et al.*, 2003; Johansen *et al.*, 1997) and unsulfated fragments of heparan sulfate can be accommodated in the binding groove of YKL-40 (Fusetti *et al.*, 2003)

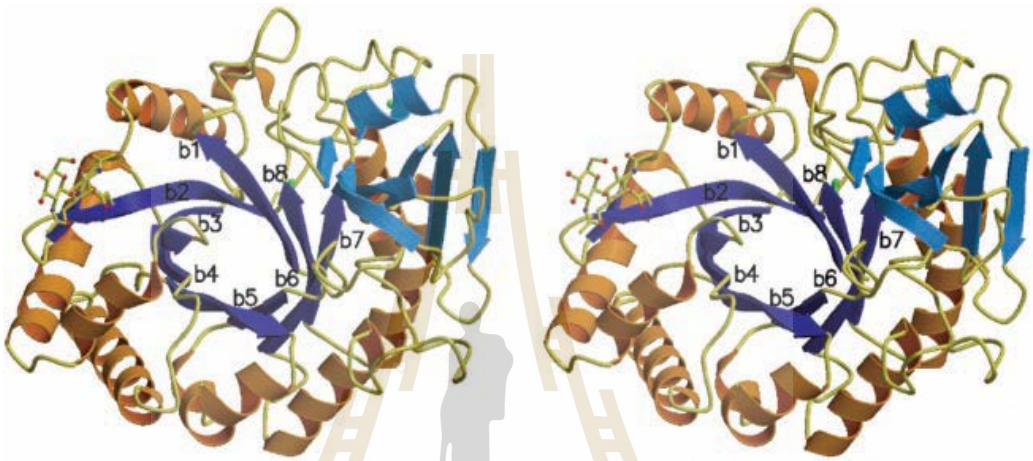

Figure 2.4 Stereo view of the YKL-40 structure. The $(\beta/\alpha)_8$ barrel domain is colored blue and yellow. The $\alpha + \beta$ domain is represented in light blue. The N-glycosylation at residue Asn_{60} is shown as *ball-and-stick*. The β-strands of the $(\beta/\alpha)_8$ barrel are labeled *b1–b8* (Fusetti *et al.*, 2003).

2.2.2 YKL-40 expression and secretion

YKL-40 is expressed and secreted from a variety of cells, including macrophages, neutrophils, chondrocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, hepatic stellate cells, colonic, ductal, airway epithelial cells, and cancer cells (Johansen, 2006; C. G. Lee *et al.*, 2011; Libreros *et al.*, 2013a). Although, YKL-40 is not expressed under physiological conditions, an induction of

this molecule is observed in patients with inflammation diseases and cancer. YKL-40 concentrations can be measured in conditioned media of human cell cultures and in human serum, EDTA plasma, and synovial fluid by an in-house radioimmunoassay (RIA) or by commercial enzyme-linked immunosorbent assay (ELISA) (Johansen, 2006). The median plasma level of YKL-40 was 40 ng/mL (2.5-97.5% reference levels: 14-155 ng/mL) with no difference between sexes, but plasma YKL-40 increases with age within and across individuals from the general population, with the mean increasing being 1.5 ng/mL/year (Figure 2.5) (Bojesen *et al.*, 2011; Schultz and Johansen, 2010). Based on the results from the large study of healthy subjects, they suggest that an elevated plasma YKL-40 is defined as an age-corrected plasma YKL-40 concentration higher than the 95th or the 97th percentile of plasma. YKL-40 in healthy age-matched subjects and age-stratified or age-adjusted reference levels are important when YKL-40 test results are evaluated (Bojesen *et al.*, 2011; Schultz and Johansen, 2010). In addition, most of the circulating YKL-40 in healthy subjects probably originates from activated macrophages and neutrophils (Johansen, 2006).

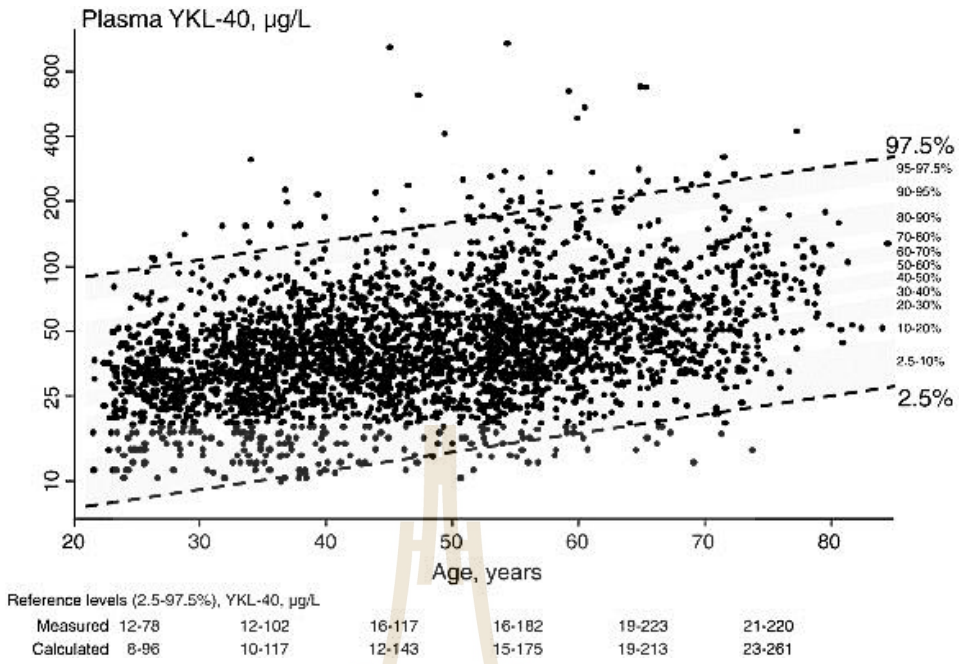

Figure 2.5 Individual plasma concentrations of YKL-40 as a function of age in 3,130 healthy participants. Age (in years) percentile strata of plasma YKL-40 (µg/L) calculated by percentile = 100 / (1 + (YKL-40^{-3}) x (1.062^{age}) x 5,000). Calculated and measured age-stratified YKL-40 reference levels (2-97.5%) by 10 year age groups are shown beneath the x-axis (Bojesen *et al.*, 2011).

The limitation of YKL-40 detection has been documented that levels of YKL-40 are sensitive to different handling conditions. The time interval between drawing of blood and centrifugation of blood stored at room temperature must be less than 3 hours for serum and 8 hours for EDTA plasma samples. Otherwise significant and not disease related elevations of YKL-40 are found in the serum and EDTA plasma samples left on the clot for a longer time when compared with YKL-40 concentrations in serum and EDTA plasma samples centrifuged within 1 hour after venipuncture. If the blood is stored at 4°C before centrifugation, YKL-40

concentrations are stable in serum for 24 hours and in EDTA plasma for 72 hours. After the blood cells are removed, YKL-40 is stable in plasma stored up to five days at room temperature (Hogdall *et al.*, 2000), up to nine days at 4°C (Harvey *et al.*, 1998), and at -80°C for at least 14 years (Schultz and Johansen, 2010). YKL-40 concentrations in corresponding serum and EDTA plasma with a YKL-40 serum/EDTA plasma ratio of 1.4 (Hogdall *et al.*, 2000; Johansen *et al.*, 1993). This is probably caused by a small release of YKL-40 from activated neutrophils during the coagulation process (Johansen, 2006).

2.2.3 YKL-40 and diseases

During the past decade, dysregulation of YKL-40 has been noted in a wide variety of human diseases characterized by acute inflammation, chronic inflammation, cancer and tissue remodeling (Table 2.2). Therefore, YKL-40 has been proposed to be a therapeutic target for those of the diseases (Johansen, 2006; Johansen *et al.*, 2007b; Schultz and Johansen, 2010).

Table 2.2 Human diseases associated with the dysregulation of YKL-40 expression (C. G. Lee *et al.*, 2011).

Acute infections
Purulent meningitis
Pneumonia
Chronic inflammatory conditions
Asthma/allergy
Atherosclerosis
Types 1 and 2 diabetes mellitus
Rheumatoid arthritis/osteoarthritis
Systemic lupus erythematosus
Inflammatory bowel disease
Chronic obstructive lung disease
Cancers (primary and/or metastatic)
Osteosarcoma, glioblastoma, breast, colon/rectum, ovary, lung, prostate, kidney, malignant melanoma
Others
Alcoholic liver cirrhosis
Liver fibrosis
Schizophrenia
Giant-cell arteritis

2.2.3.1 Elevated YKL-40 associated with non-malignant diseases

High plasma YKL-40 levels are also found in patients with non-malignant diseases. It is, therefore, very important to take into account any co-morbidity in patients with cancer, since a high plasma YKL-40 level may also originate from non-malignant cells (Schultz and Johansen, 2010). Several studies have shown that YKL-40 levels are elevated in patients with inflammatory diseases; infectious diseases, cardiovascular diseases, diabetes, rheumatic diseases, lung diseases, inflammatory bowel diseases, and liver fibrosis (Johansen, 2006; Schultz and Johansen, 2010). Also, high plasma YKL-40 is associated with poor prognosis in patients with inflammatory diseases. *In vivo*, YKL-40 is expressed by a subpopulation of macrophages in tissues with inflammation, such as atherosclerotic plaques (Boot *et al.*, 1999), arteritic vessels (Johansen *et al.*, 1999), inflamed synovial

membranes (Baeten *et al.*, 2000), sarcoid lesions (Johansen *et al.*, 2005), and peritumoral macrophages (Junker *et al.*, 2005). YKL-40 can be regarded as an acute phase protein, as its plasma concentration increases in some patients with non-malignant diseases, such as rheumatoid arthritis, severe osteoarthritis, severe bacterial infections, inflammatory bowel disease (IBD), and liver cirrhosis, liver fibrosis, and fatty liver (Johansen, 2006). It has been shown significantly elevated YKL-40 levels of more than 50% in patients with type 2 diabetes mellitus (DM), where YKL-40 correlates positively with insulin resistance and with parameters of the lipid profile, but showed no correlation with highly sensitive C-reactive protein (hsCRP) (Rathcke *et al.*, 2006). Also, patients with type 1 diabetes have elevated plasma YKL-40 compared to healthy subjects, and increasing plasma YKL-40 levels are associated with increasing levels of albuminuria (Rathcke *et al.*, 2009). Plasma YKL-40 has been suggested as an emerging biomarker in patients with cardiovascular disease and diabetes (Rathcke and Vestergaard, 2009). Plasma YKL-40 levels are associated with the severity of asthma measured by clinical variables, including forced expiratory volume in 1 second (FEV_1), and with thickness of the subepithelial basement membrane in biopsy specimens of the lung (Chupp *et al.*, 2007; Johansen *et al.*, 2005). Therefore, plasma YKL-40 has been suggested as a potential new biomarker in patients with inflammatory lung diseases (C. G. Lee and Elias, 2010; Ober and Chupp, 2009). In addition, plasma YKL-40 was significantly related to the degree of liver fibrosis, and staining of YKL-40 antigen was higher in areas with fibrosis, particularly in areas with active fibrogenesis (Johansen *et al.*, 2000).

2.2.3.2 Elevated YKL-40 associated with malignant diseases

Elevated serum/plasma levels of YKL-40 have been reported in many cancers including colorectal cancer (Cintin *et al.*, 2002), breast cancer (Johansen *et al.*, 2003), lung cancer (Junker *et al.*, 2005), acute leukemia (Bergmann *et al.*, 2005), endometrial and cervical cancers, and ovarian cancer (Dehn *et al.*, 2003; Dupont *et al.*, 2004; Hogdall *et al.*, 2003). In addition, several studies also reported that elevated plasma levels of YKL-40 were measured in patients having metastasis when compare to patients having only primary tumor (Table 2.3 and Figure 2.6).

Table 2.3 Levels of plasma YKL-40 in cancer patients.

Diagnosis	***n***	**Serum YKL-40 (ng/mL)**	**Reference**
Primary breast cancer	271	57*** (22-688)	Johansen *et al.* (2003)
Metastatic breast cancer, all	54	80*** (20-560)	Johansen *et al.* (1995)
Soft tissue	10	59 (29-433)	
Bone	25	75*** (21-560)	
Viscera	19	157*** (20-468)	
Metastatic breast cancer, all	100	65*** (20-430)	Jensen *et al.* (2003)
Nodes and skin only	36	51 (20-267)	
Bone	28	61*** (24-310)	
Viscera	36	110*** (21-430)	
Locally advanced breast cancer	45	149* (25-1,021)	Yamac *et al.* (2008)

Table 2.3 Levels of plasma YKL-40 in cancer patients. (Continued)

Diagnosis	***n***	**Serum YKL-40 (ng/mL)**	**Reference**
Colorectal cancer, all	603	86*** (27-1,298)	Cintin *et al.* (1999)
Dukes A	58	73** (27-295)	
Dukes B	223	86*** (27-604)	
Dukes C	175	77*** (27-582)	
Dukes D	147	119*** (27-1,298)	
Glioblastoma multiforme	45	130*** (38-654)	Tanwar *et al.* (2002)
Lower grade gliomas	20	101*** (50-225)	
Ovarian cancer, all	50	94*** (20-517)	Dupont *et al.* (2004)
Ovarian cancer, stages I-II	31	75*** (20-517)	Dupont *et al.* (2004)
Ovarian cancer, stage III	47	168*** (32-1,808)	Hogdall *et al.* (2003)
Ovarian cancer, relapse	73	94*** (20-1,970)	Dehn *et al.* (2003)
Epithelial ovarian cancer	42	220* (72-496)	Zou *et al.* (2010)
Small cell lung cancer, all	131	82*** (23-1,188)	Johansen *et al.* (2004)
Local disease	59	71* (23-417)	
Extensive disease	72	101*** (27-1,188)	
Acute myeloid leukemia	77	116*** (15-3,637)	Bergmann *et al.* (2005)
Metastatic melanoma	110	95*** (20-184)	Schmidt *et al.* (2006)
Hepatocellular Carcinoma	158	166*** (16-1,247)	Zhu *et al.* (2012)
Endometrial cancer	34	137*** (22-1,738)	Diefenbach *et al.* (2007)

NOTE: Values are median (range). *, P<0.05; **, P<0.01, and ***, P<0.001 compared with controls (Mann-Whitney test).

Several independent studies demonstrated that high YKL-40 plasma concentrations are associated with poor prognosis of cancer patients and high pre-treatment plasma YKL-40 is an independent prognostic biomarker of short overall survival in patients with different types of adenocarcinoma (Table 2.4). This has been found in patients with localized or advanced breast (Coskun *et al.*, 2007; Jensen *et al.*, 2003; Johansen *et al.*, 2003; Johansen *et al.*, 1995; Yamac *et al.*, 2008), colorectal (Cintin *et al.*, 1999; Cintin *et al.*, 2002), endometrial (Diefenbach *et al.*, 2007), non-small cell lung (Thom *et al.*, 2010), ovary (Dehn *et al.*, 2003; Dupont *et al.*, 2004; Hogdall *et al.*, 2003), cervix (Mitsuhashi *et al.*, 2009) and prostate (Brasso *et al.*, 2006; Johansen *et al.*, 2007a; Kucur *et al.*, 2008) adenocarcinoma both at time of first cancer diagnosis and at time of relapse.

Table 2.4 Plasma level of YKL-40 is an independent prognostic variable of overall survival in cancer patients.

Diagnosis	**Hazards ratio (95% CI)**	**P value**	**Reference**
Primary breast cancer (n = 271)	1.8 (1.0-3.1)	0.04	Johansen *et al.* (2003)
Metastatic breast cancer (n = 100)	2.6 (1.6-4.1)	0.0002	Jensen *et al.* (2003)
Colorectal cancer (n = 603)	1.4 (1.1-1.8)	0.007	Cintin *et al.* (1999)
Ovarian cancer stage III (n = 47)	4.0 (1.5-10.3)	0.005	Hogdall *et al.* (2003)
Recurrent ovarian cancer (n = 73)	2.3 (1.3-4.1)	0.006	Dehn *et al.* (2003)
Small cell lung cancer (n = 131)	1.9 (1.1-3.4)	0.02	Johansen *et al.* (2004)
Metastatic prostate cancer (n = 129)	1.3 (1.0-1.7)	0.02	Brasso *et al.* (2006)
Metastatic melanoma (n = 110)	1.9 (1.2-2.8)	0.004	Schmidt *et al.* (2006)
Acute myeloid leukemia (n = 78)	1.4 (1.1–1.7)	0.0002	Bergmann *et al.* (2005)
Head- and neck cancer (n = 138)	2.2 (1.4–3.4)	0.0006	Roslind *et al.* (2008)
Anaplastic astrocytoma (n = 38)	2.2 (1.0–4.9)	0.05	Hormigo *et al.* (2006)
Glioblastoma (n = 75)	1.4 (1.1–1.9)	0.02	Hormigo *et al.* (2006)
Hepatocellular carcinoma (n = 158)	2.0 (1.1-3.5)	0.02	Zhu *et al.* (2012)

NOTE: These results are from multivariate Cox regression analyses applying routinely used prognostic variables. These cancer patients were scored as having elevated serum YKL-40 if it was higher than the upper 95th percentile confidence limit of serum YKL-40 in healthy subjects adjusted for age.

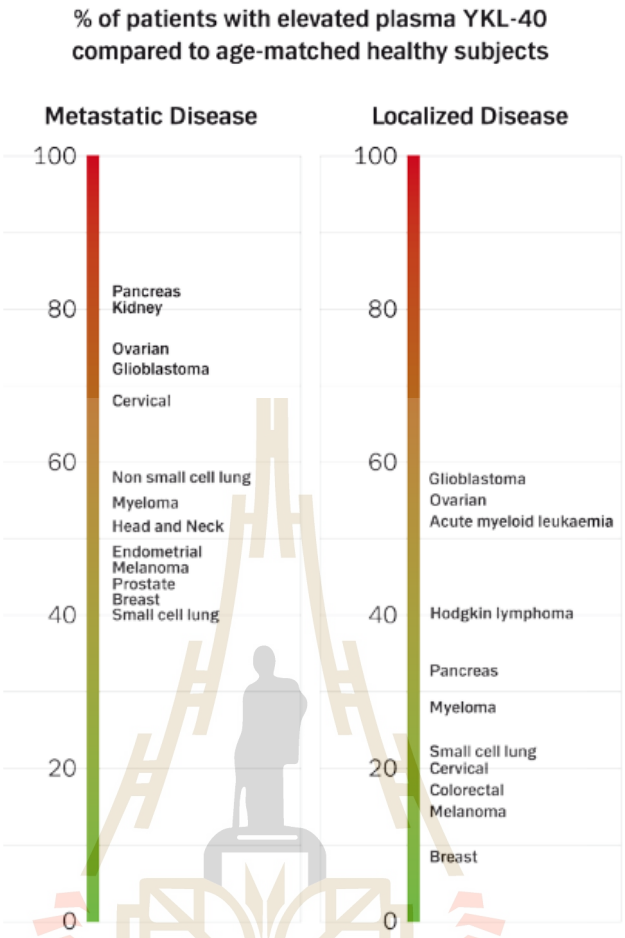

Figure 2.6 The percentage of patients with elevated plasma YKL-40 is calculated as the number of patients in the different studies with plasma YKL-40 higher than the age-adjusted 95th percentile of plasma YKL-40 in healthy subjects (Schultz and Johansen, 2010).

2.2.4 Biological function of YKL-40 in cancer progression

Elevated plasma levels of YKL-40 have been reported in patients with a variety of cancers and chronic inflammatory conditions, and are associated with disease severity, poor prognosis and shorter survival (Johansen, 2006; Schultz and Johansen, 2010). In cancer development and inflammatory conditions, YKL-40 expression is produced by cancer cells, tumor-associated macrophages and inflammatory cells and seems to be involved in cell proliferation, differentiation, anti-apoptosis, angiogenesis and invasiveness via remodeling of the extracellular matrix (ECM) (Francescone *et al.*, 2011) (Francescone *et al.*, 2011; Nigro *et al.*, 2005; Nishikawa and Millis, 2003; Recklies *et al.*, 2002). The functional role of YKL-40 is not completely understood. So far, a few evidence demonstrated that YKL-40 overexpression enhanced the proliferation rate in cancer cell lines, which were derived from colon cancer and gliomas (Kawada *et al.*, 2012; Ku *et al.*, 2011). It has been also shown that YKL-40 played a role in promoting tumor attachment via actin cytoskeletal rearrangement by increasing of myosin light chain 2 (MLC2) phosphorylation and tumor invasion via production of matrix metalloproteinase 2 (MMP2). In addition, increasing YKL-40 expression in gliomas promoted tumor resistance to chemotherapy agents, such as cisplatin, ectoposide and doxorubicin (Ku *et al.*, 2011). *In vivo* study in gliomas, showed a positive association between YKL-40 and activated AKT and MAPK pathways (Pelloski *et al.*, 2007). They hypothesized that YKL-40 acted as a secretory protein and may serve as an extracellular signal, inducing increased downstream activity of Ras (Pelloski *et al.*, 2006), or may be used as a surrogate measurement of Ras/PI3-K activation (Pelloski *et al.*, 2007).

YKL-40 is known to be a growth factor for connective tissue cells and an adhesion and migration factor for endothelial cells (Malinda *et al.*, 1999; Nishikawa and Millis, 2003; Recklies *et al.*, 2002). A recent study identified that YKL-40 was capable of stimulating angiogenesis of microvascular endothelial cells in breast cancer which YKL-40-induced angiogenesis was dependent on the interaction between membrane receptors syndecan-1 (Syn-1) and integrin αvβ3 (Shao *et al.*, 2009). Shao and co-worker demonstrated that angiogenesis was promoted by YKL-40 via activation of the mitogen-activated protein kinase (MAPK)/ERK pathway (Shao *et al.*, 2009). Kawada and co-worker also confirmed that YKL-40 induced interleukin (IL)-8 and monocyte chemotactic protein-1 (MCP-1) secretion leading to activation of MAPK signaling pathway, in particular ERK and JNK but not p38 for promoting endothelial cell migration and tube formation. Moreover, vascular endothelial growth factor (VEGF) is believed to be a primary promoter of angiogenesis (Yancopoulos *et al.*, 2000). YKL-40 also induced coordination of membrane receptor syndecan-1 and integrin αvβ5, and triggered a signaling cascade through focal adhesion kinase (FAK) to ERK-1 and ERK-2, leading to elevated VEGF and enhanced angiogenesis in glioblastoma (Francescone *et al.*, 2011). NF-κB pathway was also activated by YKL-40, and led to enhanced secretion of IL-8 and TNF-α in SW480 human colon cancer cells (C. C. Chen *et al.*, 2011). These inflammatory chemokines appear to mediate macrophage recruitment and tumor angiogenesis in development of colorectal cancer. Gather all information together, YKL-40 seems to play important roles in promoting tumor development and progression.

It has been demonstrated that YKL-40 acted as a promoter of angiogenesis in cancer including activating MAPK/ERK pathway, and also activated the PI3K/Akt pathway in the connective-tissue cells, including fibroblasts, chondrocytes and synovial cells, and modulated expression levels of chemokines and metalloproteases in inflammatory fibroblasts, as well as enhanced chemotaxis of endothelial cells (Kawada *et al.*, 2012; Ling and Recklies, 2004; Recklies *et al.*, 2002). Therefore, the signaling pathway of YKL-40 in regulating angiogenesis and cellular proliferation has been purposed that, YKL-40 may contribute to tumor invasion and metastasis through the regulation of cancer cell proliferation and differentiation (Figure 2.7) (C. G. Lee *et al.*, 2011). Although membrane receptors specific for YKL-40 binding have not yet been identified, the heparin-binding affinity of YKL-40 appears to be essential for its activity (Beauvais *et al.*, 2004; Fusetti *et al.*, 2003; Shao *et al.*, 2009). However, it has been reported that YKL-40 binds the heparin sulfates of syndecan-1and activates (phosphorylates) focal adhesion kinase (FAK) together with integrin αvβ3 (Shao *et al.*, 2009). This induces tumor angiogenesis through the subsequent activation of MAPK/ERK (Shao *et al.*, 2009), by increased secretion of chemotaxis of macrophages, IL-8 and MCP-1(Kawada *et al.*, 2012), and angiogenic factors VEGF . FAK can also activate the phosphatidylinositol 3 kinase (PI3K)/Akt signaling pathways that are critical for cell survival, differentiation, and fibrogenesis (Francescone *et al.*, 2011; Kallergi *et al.*, 2007). Moreover, YKL-40 initiates a PI3K/Akt signalling cascade in connective-tissue cells, which leads to increased cell proliferation (Recklies *et al.*, 2002) and anti-apoptosis (C. G. Lee *et al.*, 2009).

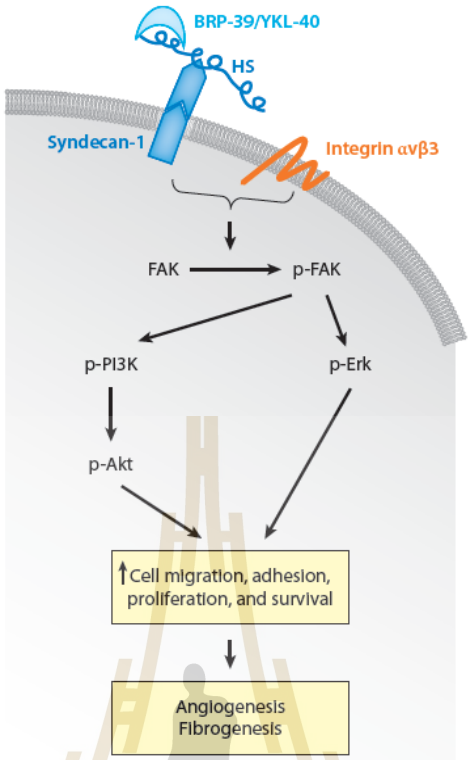

Figure 2.7 The proposed signaling pathways of YKL-40 in regulating angiogenesis and cellular proliferation (C. G. Lee *et al.*, 2011).

In addition, functional inhibition of YKL-40 has been reported in several studies. Using a monoclonal anti-YKL-40 antibody (mAY) as a neutralizing antibody has been shown the blocking of tumor angiogenesis and metastasis in osteoblastoma cells (MG-63) and brain tumor cells U87 (Faibish *et al.*, 2011). The activation of the membrane receptor VEGF receptor 2 (Flk-1/KDR) and intracellular signaling mitogen-activated protein (MAP) kinase extracellular signal-regulated kinase (Erk1 and Erk2) of HMVECs induced by YKL-40 was abolished by mAY, which may contribute to reduced tube formation *in vitro* and angiogenesis *in vivo*. Moreover, treatment with chitin microparticles significantly decreases the expression

of YKL-40 and pro-angiogenic molecules in the “metastatic” lung, inhibited tumor growth and angiogenesis in primary tumor site but also reduced lung metastasis (Libreros *et al.*, 2013b; Libreros *et al.*, 2012). These studies suggest that therapeutic targeting of YKL-40 function in cancer emerges as a novel approach to alleviate tumor progression.

2.3 Piperlongumine (PL)

As mention earlier, CCA is difficult to diagnose, and responds poorly to current radio- and chemo-therapy. In addition, drug resistance or drug inefficacy remain major obstacles in the treatment of CCA (Hezel and Zhu, 2008). Thus, a new chemotherapeutic agents or/and novel targeted therapy for CCA is still needed. To date, as much as 80% of the cancer therapeutic agents currently used in clinical trials for cancer were either natural products or their derivatives (Demain and Vaishnav, 2011). Piperlongumine (PL) (also known as piplartine) is a biological active pyridine alkaloid that was first isolated from *Piper longum* L. (Chatterjee and Dutta, 1967), commonly referred to as the long pepper. A pepper plant found in Southern India and Southeast Asia, and PL exists in the fruits and roots of the plant (S. E. Lee, 2000). The chemical structure of PL was made based on analyses of mass spectrometry (MS), infrared (IR), ultraviolet–visible (UV–vis) and proton nuclear magnetic resonance (1H NMR) spectra. The structure is shown in Figure 2.8, where the double bond is conjugated to the pyridinone carbonyl (Boll *et al.*, 1984).

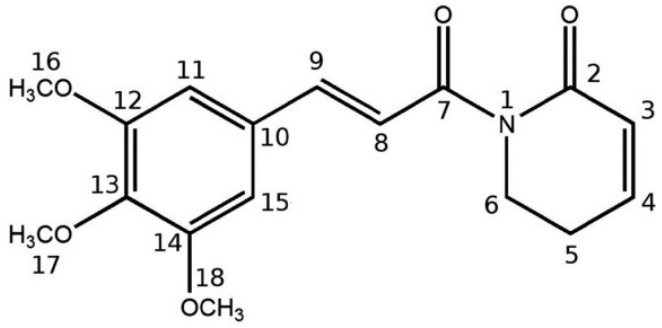

Figure 2.8 Structure of piperlongumine (5,6-dihydro-1-](2E)-1-oxo-3-(3,4,5-trimethoxyphenyl)-2-propenyl[-2(1H)-pyridinone). The compound is a chalcone-type molecule, consisting of two ring systems linked by a α-β-unsaturated carbonyl chain. One of the rings is aromatic and substituted with three methoxy groups, while the second is a piperidinone-type ring containing a conjugated alkene (Bezerra *et al.*, 2013).

Piperlongumine has been used widely in traditional medicine, including the Indian Ayurvedic system of medicine, traditional Chinese medicine, Tibetan medicine, and the folk medicine of Latin America. Multiple pharmacological activities have been reported including cytotoxic, genotoxic, anti-tumor, anti-angiogenic, anti-metastatic, anti-platelet aggregation, antinociceptive, anxiolytic, anti-depressant, anti-atherosclerotic, anti-diabetic, anti-bacterial, anti-fungal, leishmanicidal, trypanocidal, and schistosomicidal activities (Bezerra *et al.*, 2013). However, the most recent indications of PL implied its anti-tumor effect. PL has been shown to selectively target a wide spectrum of cancer cells such as colon, melanocyte, lung, breast, pancreatic, nasopharyngeal, bladder, renal, and prostate (Bezerra *et al.*, 2005; Bokesch *et al.*, 2011; Jin *et al.*, 2014; Jyothi *et al.*, 2009; Kong *et al.*, 2008; Raj *et al.*, 2011) and transformed cells overexpressing oncogenes (e.g., ERBB2 and/or

HRAS), but not normal cells (Raj *et al.*, 2011). PL selectively kills cancer cell by 1) induction of oxidative stress, induces genotoxicity, and lead to apoptotic cell death (Jin *et al.*, 2014; Raj *et al.*, 2011; Roh *et al.*, 2014; Xiong *et al.*, 2015) and 2) inhibition of ubiquitin-proteasome system via its proteasome inhibitory activity (Jarvius *et al.*, 2013). Taken together, selective induction of oxidative stress in cancer cells by PL represents a novel therapeutic strategy for cancers treatment especially treatment in CCA.

2.3.1 Anti-cancer effect of piperlongumine through ROS generation

Reactive oxygen species (ROS), produced by various biochemical and physiological oxidative processes in the body, are also associated with numerous physiological and pathophysiological processes (Nogueira and Hay, 2013). ROS are byproducts of a normal cellular metabolism and play vital roles in the stimulation of signaling pathways, such as intracellular signal transduction, metabolism, proliferation, and apoptosis (Finkel, 2000; Jabs, 1999). At low concentrations, ROS exhibit beneficial effects by regulating intracellular signaling and homeostasis; at high levels, however, ROS play a major role in the damage of proteins, lipids, and DNA (Acharya *et al.*, 2010). Cancer cells have increased levels of ROS and antioxidant enzymes compared with normal cells due to their high metabolism (Nogueira and Hay, 2013). Thus, cancer cells are more susceptible than normal cells to agents that induce further oxidative stress or impair the antioxidant response. With these cancer properties, PL has been showed to selectively kills cancer cells by directly binds to and inhibits the antioxidant enzyme glutathione S-transferase pi 1 (GSTP1) and carbonyl reductase 1 (CBR1), which are critical in the regulation of ROS homeostasis, resulting in elevated intracellular ROS and a DNA damage response, subsequently

leading to mitochondrial apoptosis (Raj *et al.*, 2011). To support the selective anti-cancer effect of PL, several types of cancers have been studied the cytotoxic activity of PL *in vitro* and *in vivo* experiments (Table 2.5). The most of studies indicate that PL selectively kills tumor cell in the micromolar range (μM) and had little cytotoxicity effect on normal cells. Base on the information presented here, PL would be a potential chemotherapeutic agent to treat CCA. In present study, we investigated the anti-cancer effect of PL on CCA cell lines.

Table 2.5 In vitro cytotoxic effects of piperlongumine against normal and tumor cell lines.

Cell line	**Histotype**	**IC_{50} μM**	**References**
MCF7	Breast carcinoma	~10	Jin *et al.* (2014)
BT474	Breast carcinoma	~7	
HepG2	Hepatocellular carcinoma	~10-20	Y. Chen *et al.* (2015)
Huh7	Hepatocellular carcinoma	~10-20	
LM3	Hepatocellular carcinoma	~10-20	
PC-3	Prostate carcinoma	~10	Kong *et al.* (2008)
LNCaP	Prostate carcinoma	>30	
LN229	glioblastoma multiforme	~10-20	J. M. Liu *et al.* (2013)
U87 MG	glioblastoma multiforme	~10-20	
8MG BA	glioblastoma multiforme	~10-20	
IMR32	Neuroblastoma	>25	Jyothi *et al.* (2009)
CEM	Leukemia lymphocytic	4.4	Bezerra *et al.* (2005)

Table 2.5 In vitro cytotoxic effects of piperlongumine against normal and tumor cell lines (Continued).

Cell line	**Histotype**	**IC_{50} µM**	**References**
HL-60	Leukemia promyelocytic	5.3	Bezerra *et al.* (2005)
K-562	Leukemia myeloid	5.7	Bezerra *et al.* (2007)
HT29	Colon carcinoma	~7.5	Basak *et al.* (2016)
HCT116	Colon carcinoma	~7.5	
SW620	Colon carcinoma	~7.5	
MCF10A	Immortalized breast epithelial	>14	Jin *et al.* (2014)
L-02	Normal hepatocyte	>20	Chen *et al.* (2015)
PBMC	Normal lymphocytes	>31.5	Bezerra *et al.* (2007)
PAE	Normal aortic endothelial	>15	Raj *et al.* (2011)
76N	Normal breast epithelial	>15	
HKC	Normal keratinocytes	>15	
HDF	Normal skin fibroblasts	>15	

2.3.2 Molecular mechanism of piperlongumine-induced cell death

Generally, ROS induce cellular signaling in cancer were mediated by many pathways, including ROS-mediated regulation of the mitogen-activated protein (MAP) kinase/Erk cascade, phosphoinositide-3-kinase (PI3K)/Akt-regulated signaling cascades, as well as the IκB kinase (IKK)/nuclear factor κ-B (NF-κB)-activating pathways (Liou and Storz, 2010). The accumulation of ROS leads to oxidative stress, resulting in upregulation of unfolded or misfolded proteins which trigger a cellular adaptive procedure known as ER stress (Marciniak *et al.*, 2004). As mention earlier, PL has been showed to be toxic selectively to tumor cells *in vitro* and *in vivo*, and ROS is the major downstream player of PL's action in many types of cancer (Raj *et al.*, 2011). However, molecular mechanism underlying its anti-tumor action was exclusive in between cancer types. Chen and co-workers reported that PL activates MAPKs signaling pathways by activated ROS-ER-MAPKs-CHOP signaling, inhibited cell migration/invasion which preferentially suppress hepatocellular carcinoma (HCC) (Y. Chen *et al.*, 2015). PL induces apoptotic and autophagic death of the primary myeloid leukemia cells from patients via activation of ROS-p38/JNK pathways (Xiong *et al.*, 2015). Moreover, effect of PL was showed to induce G2/M phase arrest in the cell cycle of several cancer cells such as, human prostate cancer cells (PC‑3) (Kong *et al.*, 2008), gastric cancer cell, (AGS and HGC27) (Duan *et al.*, 2016), ovarian cancer cells (OVCAR3) (Gong *et al.*, 2014). Later, Kong *et al.* (2008) have been demonstrated that PL induced G2/M cell cycle arrest followed by mitrochondrial-dependent apoptosis, as observed by chromatin condensation, internucleosomal DNA fragmentation, and loss of mitochondrial membrane potential (Kong *et al.*, 2008). Next, the effect of PL on extracellular signal-regulated kinase

(ERK1/2) was also examined. Randhawa *et al.* (2013) showed that PL induced apoptosis in colon cancer HT-29 cells through the MEK/ERK pathway, and further induced apoptosis as observed by apoptotic blebbing, chromatin condensation, and caspase-3 cleavage. Activation of ERK signaling leads to transcriptional events such as enhanced gene expression of p53 and p53 up-regulated modulator of apoptosis (PUMA) and decreased expression of the anti-apoptotic protein Bcl-2 (J. Liu *et al.*, 2008; Tang *et al.*, 2002). Furthermore, PL was found to suppress NF-κB transcription factor by directly interacts with IκBα kinase (IKK) through interaction with its cysteine 179 and inhibited its activity. Inhibition in NF-κB activity downregulated of proteins involved in cell survival (Bcl-2, Bcl-xL, c-IAP-1, c-IAP-2, survivin), proliferation (c-Myc, cyclin D1), inflammation (COX-2, IL6), and invasion (ICAM-1, -9, CXCR-4, VEGF), which are major mediators involved in tumor invasion and metastasis (Han *et al.*, 2014).

In summary, PL kills cancer cells by triggering different pathways, including apoptosis, necrosis, autophagy or cell-cycle arrest (Bezerra *et al.*, 2007; Duan *et al.*, 2016; Kong *et al.*, 2008; Wang *et al.*, 2013; Xiong *et al.*, 2015). These PL effects were mediated by ROS though several signals including activation of p38/JNK (Xiong *et al.*, 2015), MAPKs-CHOP (Y. Chen *et al.*, 2015), MEK/ERK (Randhawa et al., 2013) and NF-kB (Zheng *et al.*, 2016) signaling.

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CHAPTER III

ROLE OF YKL-40 IN CHOLANGIOCARSINOMA

Abstract

YKL-40, a chitinase-like glycoprotein, is expressed at a high level in cancer patients with poor prognosis. Its exact function is unknown and is the subject of current investigation. Here, we report the correlation of plasma YKL-40 levels with clinicopathological features of cholangiocarcinoma (CCA), a lethal bile duct cancer, particularly prevalent in Northeastern Thailand. Statistical analysis of plasma YKL-40 concentrations in 57 CCA patients and 41 normal healthy subjects gave a median value of 169.5 ng/mL for CCA patients compared with 46.9 ng/mL for the control subjects ($P<0.0001$). There was no significant association of plasma YKL-40 levels with patient age, tumor grade, or histology type. However, Kaplan-Meier analysis suggested that the elevated plasma YKL-40 level was particularly associated with short survival in CCA patients ($P=0.038$). Immunohistochemical examination of 34 CCA tissues revealed low expression of YKL-40 in CCA cells, but high expression in adjacent intratumoral stroma, liver, and connective tissues. Univariate analysis showed significant association of the intratumoral YKL-40 expression in CCA tissues with the non-papillary type CCA. Addition of recombinant YKL-40 (rYKL-40) in the culture medium and transient and stable expression of YKL-40 in CCA cell lines were shown to promote the growth, migration, and drug resistant of the tumor cells. Co-exposure of rYKL-40 with chitohexaose (GlcNAc6) reversed the effect of rYKL-40

induced CCA cell growth and migration in a dose-dependent manner. In addition, the study showed that YKL-40 interacted with a cell-surface receptor involved in the Akt/Erk-mediated pathway. In conclusion, our results support the proposal of YKL-40 as a new candidate prognostic biomarker for cancer diseases and it also plays an important role in tumor progression. Thus, the inhibition of YKL-40 may be as a novel therapeutic strategy for CCA.

3.1 Introduction

Cholangiocarcinoma (CCA) is a relatively rare adenocarcinoma of mutated epithelial cells that originate in the intrahepatic and extrahepatic bile ducts. CCA is one of the most aggressive forms of cancer, with a high mortality rate (Khan *et al.*, 2002; Sirica, 2005; Taylor-Robinson *et al.*, 2001), and is endemic in East and Southeast Asian countries (Shin *et al.*, 2010). The incidence is particularly high in Northeastern Thailand, with 85 in 100,000 cases. Definite risk factors for CCA are primary sclerosing cholangitis, choledochal cysts, congenital liver malformations, hepatolithiasis, and hepatitis C virus infection (Bragazzi *et al.*, 2011). However, long-term exposure to dietary nitrosamines from fermented fish together with chronic infection with the liver fluke Opisthorchis viverrini (OV) are known to be the leading factors for Thai CCA patients (Burak *et al.*, 2004; Sripa and Pairojkul, 2008; Srivatanakul *et al.*, 1991). Although symptoms of advanced-stage CCA include abnormal liver function tests, abdominal pain, jaundice, and weight loss, early-stage CCA is most often asymptomatic, making early diagnosis of this cancer ineffective. Currently, several serum biomarkers, including CEA, CA19-9, ALP, MUC5AC, and CA-S121, have been used in the CCA screening test (Wongkham and Silsirivanit

(2012). However, such biomarkers still provide different diagnostic and prognostic values. To increase the accuracy of CCA diagnosis and improve prognosis, either a highly specific CCA biomarker or a panel of screening and prognostic biomarkers is required.

YKL-40 (also called chitinase-3-like 1 or CHI3L1) is a 40-kDa glycoprotein originally secreted by monocytes, macrophages, neutrophils, cultured chondrocytes, and synovial cells (Hakala *et al.*, 1993; Johansen *et al.*, 2001; Nordenbaek *et al.*, 1999). YKL-40 is classified in the carbohydrate-active enzyme (CAZy) database (www.cazy.org) as a chitinase-like protein that belongs to a family of 18 catalytically inactive glycoside hydrolases (GH-18) (Fusetti *et al.*, 2003; Renkema *et al.*, 1998). The overall structure of YKL-40 is similar to that of GH-18 glycoside hydrolases (Olland *et al.*, 2009; Perrakis *et al.*, 1994; Ranok *et al.*, 2015; Songsiriritthigul *et al.*, 2008), comprising a typical $(\beta/\alpha)_8$ TIM barrel domain that contains a chitin-binding groove, responsible for binding to chitooligosaccharides (Houston *et al.*, 2003). In humans, YKL-40 has key functions in the control of cell proliferation and survival, as well as promoting cancer cell proliferation and tumor angiogenesis (Francescone *et al.*, 2011; Junker *et al.*, 2005; Kawada *et al.*, 2012). Prominent expression of YKL-40 has been reported in cancer cell lines (Francescone *et al.*, 2011; Kawada *et al.*, 2012; Ku *et al.*, 2011), and high serum YKL-40 levels have been detected in patients with several types of cancer (Kucur *et al.*, 2008; Thom *et al.*, 2010; Wang *et al.*, 2012; Xiao *et al.*, 2011; Yamac *et al.*, 2008). Earlier reports associated the highest levels of serum YKL-40 in cancer patients with bad prognosis and poor overall survival. Hence, YKL-40 has received much attention as an independent prognostic biomarker for cancer (Johansen *et al.*, 2006; Schultz and Johansen, 2010).

In this study, we systematically employed several types of statistical analysis to evaluate the correlations between plasma YKL-40 concentrations and YKL-40 expression tumor tissues in CCA patients with clinicopathological parameters, in order to address the significance of elevated YKL-40 levels in CCA. We also demonstrated, the biological effects of YKL-40 on cancer progression in CCA cell lines using recombinant YKL-40 (rYKL-40) and exogenous YKL-40 expression representing the paracrine and autocrine action of YKL-40 in CCA. Based on the results obtained from this study, we provide a further outlook on whether YKL-40 could be used as a useful prognostic biomarker for the monitoring of recurrent CCA and a potential target for cancer treatment.

3.2 Experimental and methods

3.2.1 Ethics

Written informed consent was obtained from all patients. The study has been approved by the Ethics Committee for Human Research, Suranaree University of Technology (EC-55-17).

3.2.2 Subjects and samples

Plasma of healthy subjects (n=41) and CCA patients (n=57) were prepared freshly from whole blood specimens that were collected and treated with heparin to prevent clotting. The CCA plasma samples were obtained from the Specimen Bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand, and all had histologically proven to be CCA. The reference range for plasma YKL-40 was determined in 41 healthy individuals (18 woman and 39 men) who visited Suranaree University of Technology

Medical Center for annual health checkups and were sex- and age-matched (median age 56 years, range 34-72 years) with the patients with CCA. These individuals did not receive medication and had no signs or symptoms of cancer or chronic diseases. All plasma samples were stored at -40 °C until used. For immunochemical study, only 34 paraffin-embedded tissue blocks out of 57 CCA patients were available and were then tested for intracellular expression of YKL-40 in CCA tissues. Age, gender, histological grading, and pathological tumor-node-metastasis staging (pTNM) (according to the American Joint Committee on Cancer classification and staging system (Edge and Compton, 2010) were evaluated by reviewing the medical charts and pathological records.

3.2.3 Chemicals and antibodies

All chemicals and antibodies used in this study were presented in Table 3.1

Table 3.1 List of chemicals and antibodies.

Chemicals	**Company**
Plasmid Maxi kit	Geneaid Biotech (New Taipei, Taiwan)
Human Cartilage glycoprotein 39/YKL40 ELISA Kit	BlueGene Biotech Co., Ltd. (Shanghai, China)
EnVision™/HRP (Rabbit)	(Dako) (California, USA)
Anti-His Affinity Resin (Ni-NTA)	GenScript (New Jersey, USA)
Novagen® BCA Protein Assay Kit	EMD Millipore (Massachusetts, USA)

Table 3.1 List of chemicals and antibodies (Continued).

Chemicals	**Company**
3,3-diaminobenzidine-tetrahydrochloride (DAP), bovine serum albumin (BSA), sodium dodecyl sulphate (SDS), and 2-β-mercaptoethanol	Sigma-Aldrich (Missouri, USA)
Fetal bovine serum (FBS), amphotericin B, 0.25% trypsin-EDTA, and Opti-MEM Reduced Serum Medium	Gibco (New York, USA)
Serum-free medium (ISF-1)	Biochrom (Berlin, Germany)
iProof High-Fidelity DNA Polymerase, Nitrocellulose membrane	Bio-Rad (California, USA)
Developer, replenisher, and half-speed blue x-ray film	Kodak (New York, USA)
Lipofectamine™2000, Lipofectamine™3000, G418 (Geneticin), TRIzol reagent, SYBR safe DNA gel stain, and complementary DNA synthesis kit	Invitrogen (California, USA)
Pre-stained protein ladder	GeneDirex (Keelung, Taiwan)
Skimmed milk, Tryptone, yeast extract powder, and agar powder	Himedia Laboratories (Mumbai, India)

Table 3.1 List of chemicals and antibodies (Continued).

Chemicals	**Company**
Skimmed milk, Tryptone, yeast extract powder and agar powder	Himedia Laboratories (Mumbai, India)
Phenylmethyl sulfonyl fluoride (PMSF), nonidet P-40, and Triton X-100	USB Corporation (Ohio, USA)
Tween-20	Scharlau chemie SA (Barcelona, Spain)
Antibodies	**Company**
Rabbit anti-human YKL-40 polyclonal antibody	Quidel (San Diego, CA, USA)
Phospho-Akt (Ser473) rabbit monoclonal antibody, total Akt antibody, rabbit monoclonal antibody, p44/42 MAPK (Erk1/2), rabbit monoclonal antibody, and phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) rabbit monoclonal antibody	Cell Signaling Technology (Massachusetts, USA)
Rabbit polyclonal β-Catenin, Frizzled, and Cyclin D1 antibody	Santa Cruz (Texas, USA)
HRP labelled polymer anti rabbit and HRP labelled rabbit anti mouse IgG	Dako
HRP labelled goat anti rabbit IgG	GenScript (New Jersey, USA)

3.2.4 Determination of YKL-40 expression in CCA patients

3.2.4.1 Determination of plasma YKL-40 concentrations by competitive ELISA

Plasma samples were collected and handled according to standard operating procedures. YKL-40 concentrations in heparin-treated plasma were determined in duplicate. Concentrations of YKL-40 in plasma were measured using a commercially available Human Cartilage glycoprotein 39/YKL40 ELISA Kit (BlueGene Biotech). Concentrations of standard YKL-40 protein were determined based on the principle of competitive binding described in the manufacturer's instructions. In brief, each well of a 96-well plate was pre-coated with 50 µL of the YKL40-HRP-conjugated polyclonal antibody. One hundred microliters of twofold dilutions of the standard YKL-40 solution or plasma sample were added, and the plate was incubated for 1 h at 37 °C. The wells were washed five times, and then the antigen-antibody reaction was initiated by adding 100 µL of the substrate solution, 15 min at 25 °C in the dark. After termination of the reaction by adding 50 µL of the stop solution, product formation was monitored by measuring absorbance at a wavelength of 450 nm (A450) using a MultiRead 400 Microplate Reader (Anthos Labtec Instruments, Walls, Austria). A calibration curve of A450 versus of YKL-40 concentration (0-250 ng/mL) was constructed and used to determine the YKL-40 concentration in each sample. The detection limit of this commercial ELISA assay was found to be 0.1 ng/mL, with estimated intra- and inter-assay coefficients of variation of <10%. Concentrations of plasma YKL-40 from all CCA samples were measured and analyzed simultaneously with protein standards, to minimize the inter-assay coefficient of variation.

3.2.4.2 Determination of cellular expression of YKL-40 in CCA tissue by immunohistochemistry

The immunohistochemical experiments were carried out at room temperature. Specimens were fixed in 10% neutral formalin buffer, embedded in paraffin, and cut into 5-μm-thick sections. Immunohistochemical staining was performed with rabbit anti-human YKL-40 polyclonal antibody (Quidel) and detected by the immunoperoxidase method. In brief, each paraffin section was deparafinized, and the endogenous YKL-40 antigen was retrieved by boiling in a microwave oven for 10 min in 0.1 M citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol for 30 min, and non-specific binding blocked with normal horse serum (dilution 1:20) for 20 min. The section was then incubated with rabbit anti-human YKL-40 polyclonal antibody (dilution 1:100) overnight at room temperature and subsequently incubated with EnVision™/HRP, Rabbit (Dako) according to the manufacturer's instructions. The stained section was visualized with 3,3-diaminobenzidine-tetrahydrochloride (DAB) (Sigma-Aldrich) and counterstained with hematoxylin. The intensity of YKL-40- positive tumor cells was scored as follows: 0, no staining; 1+, light brown; and 2+, intense brown. All stained specimens were evaluated by two experts with no knowledge of prognosis or clinicopathological variables. To validate our evaluation method, we performed kappa statistical analysis available in SPSS 16.0, and the obtained kappa value of 0.932 indicated almost perfect agreement of the data presented by two different sources (Landis and Koch, 1977). For statistical analysis of the data, the scores 0 and 1+ were categorized as negative expression, while the score 2+ as positive expression.

3.2.5 Human cell culture

Two human CCA cell lines, KKU-055 and KKU-213 were derived from primary tumors of CCA patients, were supplied by the Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Thailand. CCA cells were cultured in Ham's F12 Nutrient Mixture supplemented with 10% fetal bovine serum (FBS) (Gibco), gentamycin (50 μg/mL) (Sigma-Aldrich), and amphotericin B (0.25 μg/mL) (Gibco). HEK-293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, gentamycin (50 μg/mL) (Sigma-Aldrich), and amphotericin B (0.25 μg/mL) (Gibco). All cell lines were cultured in a humidified 5% CO_2 incubator at 37 °C. Cells with 70-80% confluence were trypsinized with 0.25% trypsin-EDTA (Gibco) and subcultured in the same media. Some aliquots of cells were transferred to freezing medium containing 10% (v/v) DMSO and 50% (v/v) FBS and stored in a liquid nitrogen tank for next use. CCA cell lines were used for gene expression and functional studies of YKL-40.

3.2.6 Whole cell lysate preparation and western blot analysis

To prepare whole-cell lysates, 80% cells confluence in 6-well plates were washed twice with phosphate-buffered saline (PBS) and then lysed with 50 μL NP-40 lysis buffer [50 mM Tris–HCl, pH 8.2, 150 mM NaCl, 1% (v/v) NP-40, 5% (v/v) glycerol, and protease inhibitor cocktail (Roche, Indianapolis, IN, USA) at the recommended concentration] and incubated on ice for 30 min. After centrifugation at 16,000 xg for 30 min at 4 °C, protein concentrations were determined with the BCA Protein Assay Kit (Pierce Biotechnology).

For Western blot analysis, cell lysate (20 μg) was resolved on SDS-polyacrylamide gel electrophoresis (PAGE) gel (10% glycine gel) and the separated

proteins transferred to nitrocellulose membranes. Membranes were blocked with 5% (w/v) skimmed milk in PBS containing 0.05% (v/v) Tween-20 (0.05% PBST) for 1 h at room temperature, then incubated overnight at 4 °C with primary antibody diluted in 1% (w/v) skimmed milk with 0.05% PBST. After washing with 0.05% PBST for 10 min x 3 times, the blotted membranes were incubated for an additional 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies [horse anti-mouse (Vector Laboratories, Burlingame, CA, USA) or goat anti-rabbit IgG (GenScript)] at 1:5,000 dilution in 0.05% PBST containing 1% (w/v) skimmed milk. Labeled proteins were visualized using PicoEPD Western Blot Detection Reagent (Elpis Biotech, Daejeon, Korea). The relative intensities of protein bands on the immunoblots were recorded using a D50 SLR digital camera (Nikon, Tokyo, Japan), and the images of the ECL developed blots quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

3.2.7 Production of recombinant YKL-40 (rYKL-40)

The nucleotide sequence of the human *CHI3L1* gene encoding YKL-40 was retrieved from the GenBank database under accession number M80927. A full-length cDNA corresponding to this gene was designed to be cloned into the pCMV/hygro vector, so as to be suitably expressed in a mammalian cell expression system. The pCMV/hygro-His/*CHI3L1* construct was generated and synthesized by a commercial source (Sino Biological Inc., Beijing, China). The recombinant YKL-40 protein (designated rYKL-40) produced from this construct contained an additional six histidine residues at the C-terminus, to aid its purification. For expression in HEK-293T cells, the transfected cells (1.5×10^6 cells/10 mL medium/10-cm dish) were grown overnight at 37 °C with 5% CO_2. Lipofectamine™2000 (Invitrogen) and

the recombinant cDNA were diluted in serum-free medium (ISF-1) (Biochrom) and then combined in a ratio of 1:1 to obtain a final volume of 3 mL. The transfected cells were grown for 48 h at 37 °C in a 5% CO_2 incubator, and afterwards the culture medium containing secreted rYKL-40 was harvested by centrifugation at 4,500 rpm at 4 °C for 10 min, and the cells discarded. For protein purification, one-tenth volume of 10X binding buffer (500 mM NaH_2PO_4, 1.5 M NaCl, and 100 mM imidazole, pH 8.0) was added into 15 mL of the culture medium and then mixed with 100 µL of Ni-NTA beads (GenScript). The culture medium/bead suspension was rotated at 4 °C for 1 h to allow the rYKL-40 to bind to the magnetic beads, before centrifugation at 4,500 rpm for 10 min. The beads were collected and then transferred into a 1.5-mL tube and washed four times, each with 0.5 mL wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 0.05% Tween-20, pH 8.0) containing 20 mM imidazole, and centrifuged at 4,000 rpm for 2 min. The bound rYKL-40 protein was eluted with 200 µL of 250 mM imidazole in wash buffer, and the beads removed by centrifugation at 4,000 rpm for 2 min at 4 °C. The purity and specificity of the rYKL-40 protein was determined by 12% SDS-PAGE analysis and western blot analysis. The protein concentration was determined by Novagen® BCA Protein Assay Kit (EMD Millipore, Billerica, MA, USA). The freshly purified rYKL-40 was stored at -80 °C until used.

3.2.8 Generation of the new recombinant YKL-40 construct for expression in CCA cells

To generate the recombinant YKL-40 construct for transient expression in CCA cell lines, the full-length *CHI3L1* cDNA (1,867 bp) encoding 383 amino acid residues of YKL-40 obtained as described above was amplified from a human cDNA template obtained from GenScript by PCR technique using the forward primer 5´ -GCGGATCCGTCATGGGTGTGAAGGCGTCTCAA-3´ and the reverse primer 5´-GACTCGAGCGTTGCAGCGAGTGCATCCTT-3´. Sequences underlined indicate the restriction sites *Bam*HI and *Xho*I, respectively. PCR amplification was performed with iProof High-Fidelity DNA Polymerase (Bio-Rad) according to the manufacturer's instruction. The amplified DNA was cloned into pGEM-T-Easy (Promega, Madison, WI, USA) and then subcloned to pCMV6-entry (Origene, Singapore) that contained cytomegalovirus (CMV) and SV40 promotors for mammalian expression system. The correct nucleotide sequence of the newly generated construct was confirmed by automated sequencing (First BASE Laboratories Sdn Bhd, Selangor Darul Ehsan, Malaysia) and double digestion with *Bam*HI and *Xho*I (Figure 3.1). The pCMV6/CHI3L1 construct was further used to generate transient or stable expression in two CCA cell lines (KKU-055 and KKU-213).

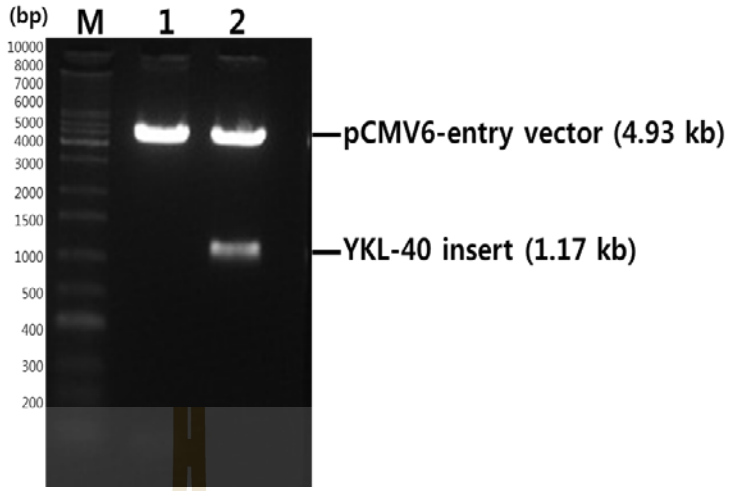

Figure 3.1 Double digestion of pCMV6/CHI3L1 construct. pCMV6/CHI3L1 construct was cut with BamHI and XhoI and determined the product sized using. 1% agarose gel electrophoresis. M: Marker, 10 kb DNA ladder; Lane1: pCMV6-entry vector control (no gene insert); Lane 2: pCMV6/YKL-40 digested by BamHI and XhoI restriction enzymes, showing 2 bands of pCMV6-entry vector (4.93 kb) and *CHI3L1* gene insert (1.17 kb).

3.2.9 Generation of YKL-40 overexpression in CCA cell lines

3.2.9.1 Transient YKL-40 overexpression in CCA cell lines

To generate CCA cell lines with transient YKL-40 expression, transfection of the recombinant plasmid into the target cells was performed following the protocol recommended by the manufacturer (Invitrogen). Briefly, KKU-055 and KKU-213 cells (4.0×10^5 cells/2 mL medium/6-well plates) were grown overnight at 37 °C with 5% CO_2. Lipofectamine™3000 (Invitrogen) and 2 µg of pCMV6/CHI3L1 vector or pCMV6-entry empty vector were diluted in Opti-MEM Reduced Serum Medium (Gibco) with a ratio of 1:1 to obtain a final volume of

250 µL. After 24 h, CCA cell lines were harvested and used in subsequent experiment. The expression of YKL-40 in CCA cell lines were confirmed by Western blot analysis.

3.2.9.2 Stable YKL-40 overexpression in CCA cell lines

To generate stable cell expressing YKL-40 expression, transfection condition and procedure was similar with transient YKL-40 expression. Briefly, The KKU-055 and KKU-213 cells were transfected with pCMV/YKL-40 vector or empty vector control using Lipofectamine™3000 (Invitrogen) as the delivery vehicle. After 48 h of transfection, CCA cell expressing YKL-40 (pCMV/YKL-40 vector) or pCMV empty vector were selected with the additional 700 µg/mL of G418 (Invitrogen) into the medium for 2 weeks. YKL-40 expression in the stable cell lines were checked the expression at both mRNA and protein levels.

3.2.9.3 RNA Extraction and reverse transcription

CCA cell expressing YKL-40 were checked the expression level comparing with those of control cells by reverse transcription PCR (RT-PCR). First, total RNA was extracted from CCA cell expressing YKL-40 or empty vector control cells at 80% confluence in 6-well plates using 1 mL TRIzol reagent (Invitrogen). After that, 200 µL of chloroform was added and then subsequently centrifuged at 12,000 xg for 15 min. The aqueous phase (upper) with 500 µL was transferred and precipitated with 500 µL of isopropanol for 10 min. RNA pellet was collected by centrifugation at 12,000 xg for 15 min and washed with 75% ethanol and left air dried for 10 min. The obtained RNA was finally resuspended in 20-40 µL double sterile distilled water. The purity and concentration of the extracted RNA were checked by Nano Drop (NanoDrop Technologies, Delaware, USA) at the

wavelength of 260 and 280 nm. RNA integrity was checked by visualizing rRNA bands (28S and 18S) by 1% agarose gel electrophoresis (Figure 3.2). cDNA synthesis was performed using Superscript VILO cDNA synthesis kit (Invitrogen). Briefly, total RNA was converted to cDNA by reverse transcription reaction which contained superscript reverse transcriptase and random hexamer primers. All reactions were set up on ice to avoid premature cDNA synthesis and to minimize the risk of RNA degradation. Reverse transcription reaction was performed in a 20 µL containing 2 µg of total RNA. Total reaction was incubated for 60 min at 42 ℃ and kept at -20 ℃ until analysis.

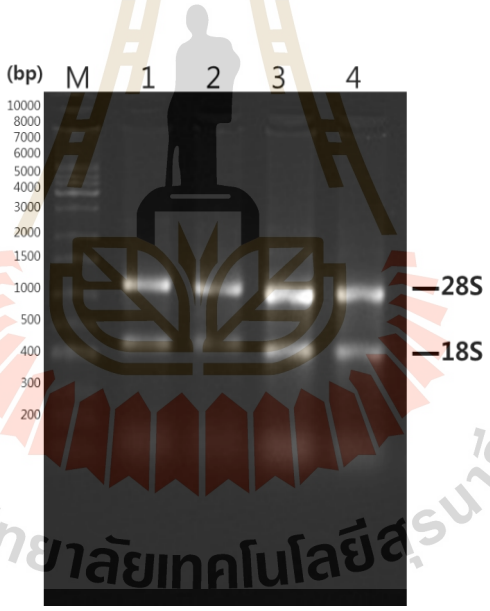

Figure 3.2 RNA integrity. Total RNA was extracted from stable CCA expressing YKL-40 and empty vector control cells, M is DNA molecular size marker (NEB Quick-Load® 2-Log DNA Ladder (0.1-10.0 kb)). The 18S and 28S ribosomal RNA after RNA extraction are clearly visible in each RNA samples. Lane1: KKU-055 empty vector control; Lane2: KKU-055 expressing YKL-40; Lane3: KKU-213 empty vector control cells; and Lane4: KKU-213 expressing YKL-40.

3.2.9.4 Polymerase chain reaction (PCR)

PCR amplification was performed with GoTaq® DNA Polymerase (Promega) according to the manufacturer's instruction. The reaction mix for the final PCR was composed of 100 µM dNTPs and 0.05 units/µL GoTaq DNA polymerase in 1X PCR Buffer. Each reaction was performed in single tubes in a volume of 20 µL PCR mix. The cycling conditions were as follows: an initial denaturation step at 94 ℃ for 3 min, 25 cycles (94 ℃ -30 s, 56 ℃ -30 s, 72 ℃ -1 min) and a final extension step lasting 5 min at 72 ℃. Primers were designed for the amplification of cDNA from CCA cell expressing YKL-40 or empty vector control. The sequences of primers included: YKL-40 forward primer 5´-CACCCTAATCAAGGAAATGAA -3´ and reverse 5´-CCAGGTGTTGGGATATCTTG -3´, and human β-actin forward 5´-CTTCCTTCCTGGGCATGGAG-3´, reverse 5´-GAGCAATGATCTTGATCTTCAT-3´. 10 µL of the PCRs were separated by electrophoresis in a 2% agarose gel (Figure 3.6).

3.2.9.5 Immunofluorescence microscopy

Stable YKL-40 expressed in CCA cell lines was confirmed by fluorescent staining after selected with G148 for 2 weeks. Briefly, the stable KKU-055 and KKU-213 expressing YKL-40 were growth on cover slip for 24 h. Cells grown on cover slips were fixed by 4% paraformaldehyde for 30 min, and penetrated by Triton X-100 for 15 min, then blocked by BSA for 30 min. Rabbit polyclonal YKL-40 antibody (1:100, Quidel) was incubated at room temperature for 1 h. After rinsed by PBS, Goat Anti-Rabbit Alexa Fluor® 488 (1:400, Invitrogen) was incubated for 30 min. Slides were mounted by VECTASHIELD Mounting Medium

with DAPI (Vector Labs). Images were captured by inverted fluorescence microscope (Olympus IX71), controlled in Metamorph software (Molecular Devices)

3.2.10 Investigating the biological role of YKL-40 in CCA cell lines

3.2.10.1 Monolayer wound healing assay

CCA cells with exogenous YKL-40 expression and control were seeded into 24-well plates (2.5×10^5 cells/well) and cultured overnight in Ham's F12 Nutrient Mixture medium supplemented with 10% (w/v) FBS, streptomycin (100 μg/mL), and amphotericin B (50 μg/mL) in a humidified atmosphere of 5% CO_2 at 37 °C. After the cells were grown to confluence (> 90%), two separate wounds were generated per well by scratching the surface of the confluent monolayer using a sterile 1-mL pipette tip. Average wound widths along the scrape lines were estimated from six representative fields using the program ImageJ (The National Institute of Health, Bethesda, MD, USA) after the cells were further incubated for 18 and 24 h.

3.2.10.2 Transwell migration assay

Effects of YKL-40 on the migration of CCA cells were investigated. For adding rYKL-40, KKU-213 cells (1×10^5 cells) were seeded into the upper chambers of a 24-well Transwell plate with 8.0-μm pore polycarbonate filters (SPL Life Sciences, Pocheon, Korea). The lower chambers contained 600 μL of complete medium with or without rYKL-40. After incubation for 6 h at 37 °C under 5% CO_2, migrating cells were fixed in 3.7% (v/v) formaldehyde, permeabilized with 100% ethanol, and crystal violet staining performed. Numbers of the migrating cells were counted under an inverted microscope by two independent investigators using a magnification field of ×100. The numbers of migrating cells averaged from

experiments carried out in triplicate were compared to those without rYKL-40 treatment.

3.2.10.3 Cell proliferation assay

CCA cell lines (2.5×10^3 cells/well) were seeded onto a 96-well plates in the presence and absence of rYKL-40 (80 ng/mL). The effects of rYKL-40 on CCA cell proliferation were determined by the colorimetric cell viability (MTS) assay using CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI, USA). In vitro reduction of a tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenil)-2H-tetrazolium (MTS) by metabolically active cells yielded a colored formazan that was released into the culture medium. Color development in this assay, measured at 490 nm, was directly proportional to the number of viable cells and determined from day 0 to day 3.

3.2.10.4 Cell adhesion assay

Purified rYKL-40 (1 µg) was coated on the wells of a 96-well plate by incubation at 37 °C for 2 h. The cells were seeded at a density of 5×10^4 cells/wells and incubated at 37 °C for 1 h. Unattached cells were removed by rinsing twice with serum free medium. Numbers of adherent cells were determined by the MTS assay (Promega) as described above. The percentage of the rYKL-40-induced cell adhesion was normalized to that of cells attached on 1% bovine serum albumin (BSA)-coated wells using the equation: Adhesion (% of control) = (A_{490} of YKL-40 coated) / (A_{490} of 1% BSA coated) × 100

3.2.10.5 Cell cytotoxicity assay

Anti-apoptotic effect of YKL-40 was investigated. Briefly, the CCA expressing YKL-40 or empty vector control were seeded to 5×10^3 cells/well in 96-well plates for 24 h, and then treated with specified concentrations of ciaplatin, doxorubicin and piperlongumine. Cell viability was determined by a sulforhodamine B (SRB) assay. After 48 h of drug treatment, the media was discarded and cells were fixed with ice-cold 10% (v/v) TCA in deionized (DI) water at 4 ℃ for 1 h. The TCA was then removed and the cells were washed 5 times with DI water, followed by staining with 0.4% (w/v) SRB dissolved in 1% (v/v) acetic acid for 30 min at room temperature. Unbound dye was removed by 5 washes with 1% (v/v) acetic acid, after which the cells were solubilized with 10 mM unbuffered Tris-base (pH 10.0) and shaken on plates for 10 min. The absorbance was measured at 564 nm using a Synergy HTX Multi-Mode Reader (BioTek Industries, Inc., VT, USA). The number of viable cells was directly proportional to the protein bound-dye formation.

3.2.10.6 Cell cycle analysis

To determine the DNA contents, KKU-055 expressing YKL-40 or empty vector control cells (4.5×10^5) were seeded in 6-well cultured plates for 24 h. The cells were treated with various concentration of cisplatin for 24 h and harvested. After washing with PBS, cells were fixed with ice-cold 70% ethanol and incubated overnight at 4 ℃. The cells were then washed twice with PBS to removed ethanol and labeled with propidium iodide (PI) by incubating with PI solution, PI (20 µg/mL), 0.1% (v/v) Triton-X 100, and RNase A (20 µg/mL), in the dark for 30 min. Stained cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Automated quantification of the DNA content histograms was

done with Flowing software (Cell Imaging Core, Turku Centre for Biotechnology, Abo Akademi University, Finland).

3.2.10.7 Soft agar colony formation assay

This experiment was carried out with 6-well plates. One milliliter medium with 0.5% agarose was used as bottom gel. Stable KKU-055 erexpressing YKL-40 or vector control cell were trypsinized and counted. Trypsinized cells were diluted to a volume that would yield 5,000 cells per plate and cells were mixed with cooled agarose to yield a final agarose concentration of 0.7% and were poured on bottom gel. Triplicate plates were prepared for each cell line. Plates were incubated at 37 °C with 5% CO_2 for 14 days. Following incubation, plates were stained with 0.5 mL of 0.005% crystal violet (Sigma, St. Luis, MO), and colonies were counted using a dissecting microscope.

3.2.11 Statistical analysis

The statistical analysis of the CCA data was carried out using SPSS Statistics 16.0 (IBM, NY, USA) or GraphPad Prism 5.0 (GraphPad Software, CA, USA) software. Plasma YKL-40 concentrations from the CCA group were compared with those of the control group using the Mann–Whitney U test. Correlation between plasma YKL-40 concentrations, YKL-40 expression in CCA tissues, and clinicopathological features were evaluated using the chi-squared test. The Kaplan–Meier method was used to calculate survival curves, and the log-rank test was performed to compare differences in the survival rates of CCA patients. A Cox proportional-hazard regression model was used to identify the independent prognostic factors. Data obtained from functional studies on YKL-40 including cell proliferation

and migration assay were expressed as the mean ±SEM and were compared using Student's t-tests, with a P value of <0.05 taken as statistically significant.

3.3 Results

3.3.1 Demographics of CCA patients and controls

The characteristics of CCA patients are summarized in Table 3.2. Plasma samples from 57 patients (39 male and 18 female) with various stages of CCA were examined. The mean age of the CCA patients recruited to the study was 56 years (range 34-72 years) and was similar to that of the controls, which also had a mean age of 56 years (range 35-60 years). The ratio of females to males was 1 to 2.2. Plasma samples were obtained before surgical resection for CCA, and none of the patients received radiotherapy or chemotherapy prior to the surgery. Tumor staging was based on the American Joint Committee on Cancer classification (Edge and Compton, 2010). The survival of each CCA patient was recorded from the date of surgery to the date the patient succumbed to the disease or to 15 August 2011.

Table 3.2 Association between plasma YKL-40 concentrations and clinicopathological features in 57 CCA patients.

Characteristics	Number	Plasma YKL-40 levels		P value
		Normal (%)	Elevated (%)	
Age (years)				
<56	27	7 (25.9)	20 (74.1)	0.820
≥56	30	7 (23.3)	23 (76.7)	
Sex				
Male	39	9 (23.1)	30 (76.9)	0.720
Female	18	5 (27.8)	13 (72.2)	
Tumor stage				
I–II	12	4 (33.3)	8 (66.7)	0.498
III	17	5 (29.4)	12 (70.6)	
IV	28	5 (17.9)	23 (82.1)	
Histology type				
Papillary	14	5 (35.7)	9 (64.3)	0.264
Non-papillary	43	9 (20.9)	34 (79.1)	

The dichotomized data were analyzed using the chi-squared test. The median overall survival of the patient group was 224 days, and the 1-year survival rate was 29.8%.

3.3.2 Association of plasma YKL-40 concentrations and clinicopathological features

The competitive ELISA assay showed a median plasma YKL-40 concentration of 57 CCA patients of 169.5 ng/mL (range 36.45-543.4 ng/mL), which was significantly higher than those of 41 healthy controls with a median of 46.92 ng/mL (range 11.2-116.7 ng/mL) ($P<0.0001$) (Figure 3.3). To further evaluate the significance of plasma YKL-40 concentrations with the clinical characteristics of the CCA patients, the 25th percentile of the YKL-40 values in the CCA subjects, giving the median of 100.7 ng/mL, was used as the cutoff value. The dichotomized plasma YKL-40 concentrations in normal (n = 14) versus elevated (n = 43) concentrations are summarized in Table 3.2. There was no significant correlation between the plasma levels of YKL-40 with clinicopathological features of CCA patients tested. Although the Mann–Whitney U test did not suggest a significant correlation between the YKL-40 level and tumor grade, the median plasma YKL-40 concentration of the patients with advanced stage CCA (stage IV; median 177.9 ng/mL) appeared to be notably higher than that of patients with early stages (stages I–II; median 143.5 ng/mL) (Figure 3.4).

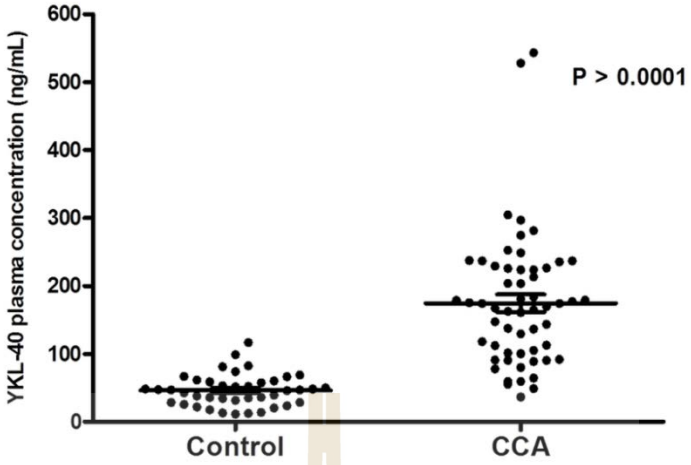

Figure 3.3 YKL-40 plasma concentrations in CCA pateints and healthy control. Absolute value plot showing plasma YKL-40 values (ng/mL) and range in CCA patients (*n* = 57) and normal subjects (*n* = 42). The horizontal solid line represents the median values analyzed by the Mann–Whitney U test at P<0.0001

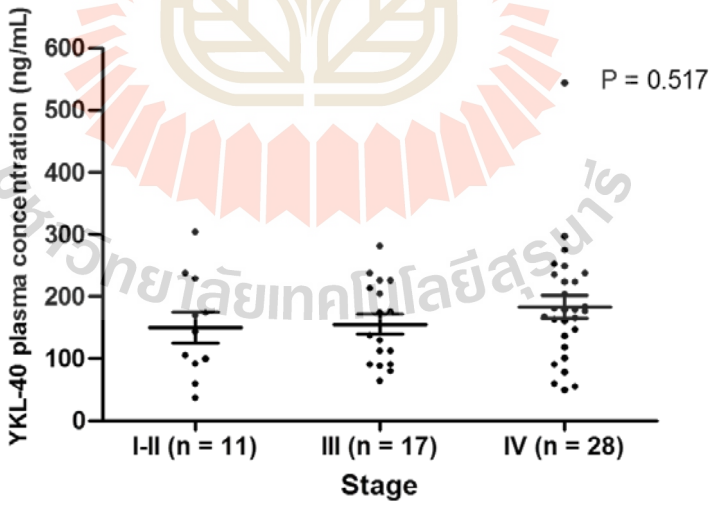

Figure 3.4 A relationship between plasmaYKL-40 concentration and tumor stage (P=0.517) of CCA patients. No significant differences were detected among the three groups of tumor stages. However, the concentration of plasma YKL-40 was slightly increased in the late stage (IV) of the disease

3.3.3 Association of plasma YKL-40 concentrations and overall survival

The Kaplan-Meier analysis suggested a positive correlation between overall survival and plasma YKL-40 concentration. No patient with perioperative death (survival <30 days) was included in the analysis. As shown in Figure 3.5, patients with elevated plasma YKL-40 concentrations had significantly shorter overall survival than patients with normal plasma YKL-40 concentrations (median overall survival, 207 vs. 336 days; P=0.038, log-rank test). The overall survival rates were 42.8 and 25.6% at 1-year post-surgery for patients with elevated and normal YKL-40 plasma concentrations, respectively. Age and gender had no influence on survival. In addition, overall survival was significantly decreased in the patients with advanced tumor stage (stage IV) and non-papillary type CCA (Table 3.3).

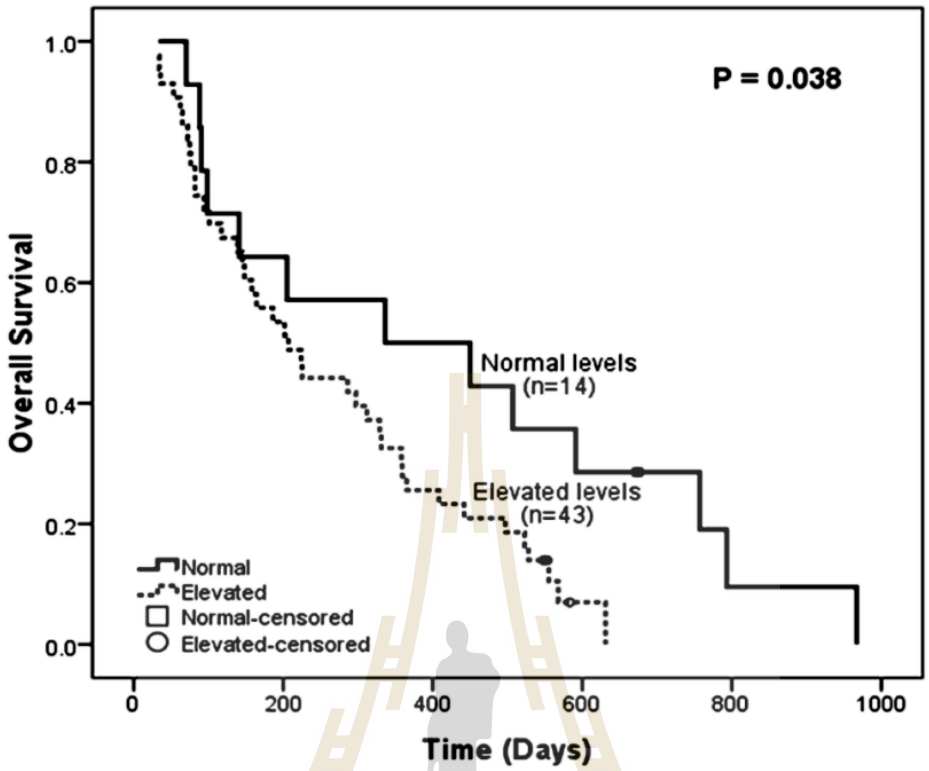

Figure 3.5 Kaplan-Meier analysis of plasma YKL-40 levels and overall survival in CCA patients. The YKL-40 concentrations were dichotomized according to the 25th percentile of the total CCA values, giving a cutoff value between normal and elevated YKL-40 levels of 100.7 ng/mL. Elevated YKL-40 was significantly associated with poorer overall survival in CCA patients (P=0.038)

3.3.4 Univariate and multivariate analyses of overall survival and clinicopathological parameters

Several clinicopathological parameters have been shown to correlate with the overall survival time of cancer patients. We further performed univariate and multivariate Cox proportional hazard regression analyses to evaluate which parameters apart from plasma YKL-40 concentration influenced the overall survival of the CCA patients. Univariate Cox regression analysis suggested that three clinicopatholigical parameters, namely tumor stage ($P<0.0001$), histological type ($P=0.005$), and plasma YKL-40 concentration ($P=0.038$), were associated with overall survival (Table 3.3). However, further multivariate Cox regression analysis indicated that, of these three parameters, only tumor grade was an independent predictor of overall survival, with a hazard ratio of 2.817 (95% CI, 1.428-5.559, between IV and III or I–II, $P=0.003$) (Table 3.3).

Table 3.3 Multivariate Cox regression analyses of potential prognostic factors in CCA patients.

Variable	Univariate analyses		Multivariate analysis		
	HR	**P value**	**HR**	**95% CI**	**P value**
Age					
<56	1	0.208	1.046	0.569 – 1.926	0.884
≥56	0.696 (0.394 – 1.227)				
Sex					
Male	1				
Female	1.020 (0.570 – 1.823)	0.948	1.022	0.558 – 1.875	0.943
Tumor Stage					
I-II	1				
III	1.856 (0.836 – 4.122)	0.124			
IV	3.298 (1.771 – 6.141)	<0.0001	2.817	1.428 – 5.559	**0.003**

Table 3.3 Multivariate Cox regression analyses of potential prognostic factors in CCA patients (Continued).

Variable	Univariate analyses		Multivariate analysis		
	HR	**P value**	**HR**	**95% CI**	**P value**
Histology Type					
Papillary	1				
Non-Papillary	2.647 (1.309 – 5.356)	**0.005**	1.870	0.868 – 4.026	0.110
Plasma YKL-40					
Normal	1				
Elevated	2.117 (1.027 – 4.363)	**0.038**	1.642	0.780 – 3.455	0.192

P-values rendered in bold are statistically significant. HR relative hazard ratio, CI confidence interval

3.3.5 Intratumoral expression of YKL-40 and clinicopathological features

Immunohistochemical staining of 34 CCA tissue samples showed that YKL-40 was rarely expressed in CCA tumor cells, but highly expressed in liver cells and infiltrating inflammatory cells, especially macrophages and connective tissue at intratumoral stroma (Figure 3.6(A)-(C)). Cellular YKL-40 expression was identified in 22 of 34 CCA tissues (64.70%). Score distributions were as follows: score 0 in 12 (35.3%) patients, 1+ in 5 (14.7%) patients, and 2+ in 17 (50%) patients. The positive staining of YKL-40 intratumoral expression was categorized as cytoplasmic or membranous/cytoplasmic reactivity. The presence of membranous/cytoplasmic YKL-40 protein was noted in 5/34 cases (14.7%) and was predominantly apical where a lumen was present (Figure 3.6(F)). Since YKL-40 expression in CCA tissues was found in various types of cell, we quantitatively analyzed the correlation between YKL-40 expression in CCA cells alone and the clinicopathological features, using a univariate analysis. A high level of intratumoral YKL-40 was associated with the non-papillary type which was a poor prognosis parameter (P=0.005) (Table 3.4). There was no statistically significant correlation between YKL-40 intratumoral expression and age, gender, or tumor stage. There was also no significant difference between YKL-40 intratumoral expression and patient overall survival (Figure 3.7).

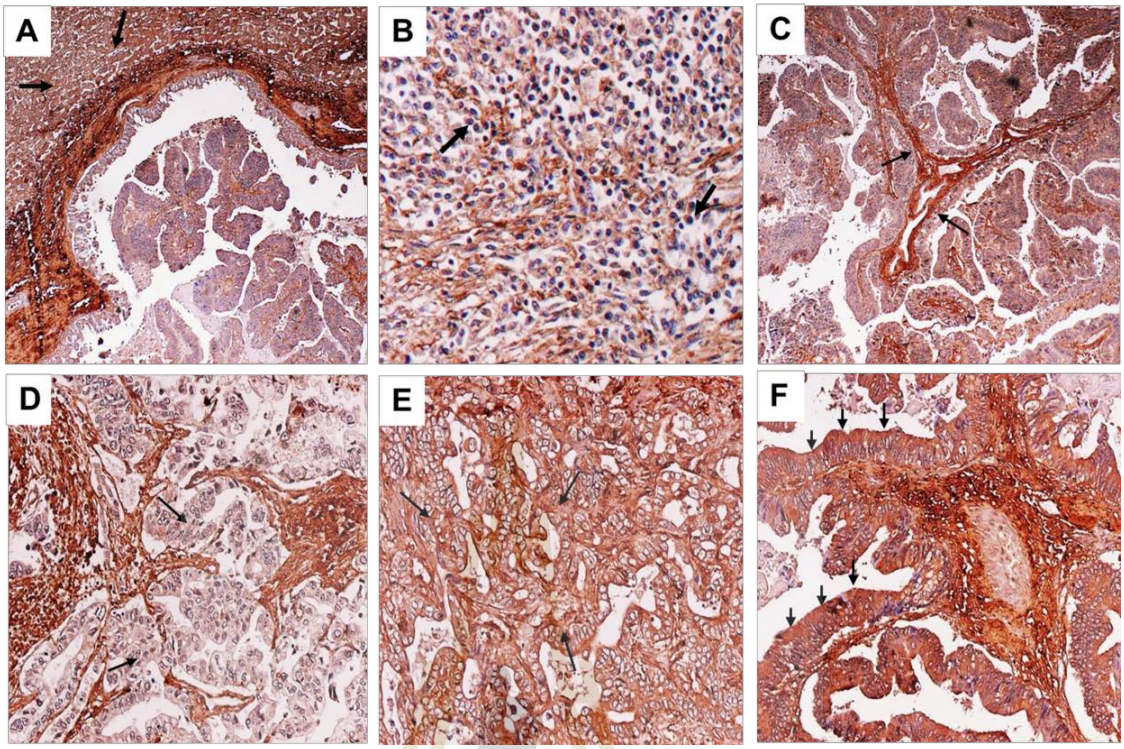

Figure 3.6 Distribution and expression of YKL-40 in CCA tissues detected by immunohistochemical staining. (A) YKL-40 expressed in liver cells (×4 HP). (B) Infiltrating macrophages (×10 HP). (C) Connective tissue at intratumoral stroma (×4 HP). (D) Negative YKL-40 immunostaining in CCA tumor cells (×10 HP). (E) YKL-40 positive staining of cytoplasmic immunoreactivity in CCA tumor cells (×10 HP). (F) YKL-40 positive staining of membranous/cytoplasmic immunoreactivity in CCA tumor cells, especially the luminal surfaces and apical sites (black arrows) (×10 HP).

Table 3.4 Intratumoral expression of YKL-40 in cholangiocarcinoma tissues in relation to clinicopathological features of the CCA patients.

Variable	Number	YKL-40 intratumoral staining (%)		*P* value
		Negative	Positive	
Age (years)				
<56	19	4 (21.1)	15 (78.9)	0.051
≥56	15	8 (53.3)	7 (46.7)	
Sex				
Male	19	7 (36.8)	10 (63.2)	0.832
Female	15	5 (33.3)	12 (66.7)	
Tumor Stage				
II	17	4 (41.2)	13 (58.8)	0.473
III & IV	17	6 (29.4)	10 (70.6)	
Histology Type				
Papillary	12	8 (66.7)	4 (33.3)	**0.005**
Non-Papillary	22	4 (18.2)	18 (81.8)	

The dichotomized data were analyzed using the chi-squared test. *P*-values rendered in bold are statistically significant (P<0.05)

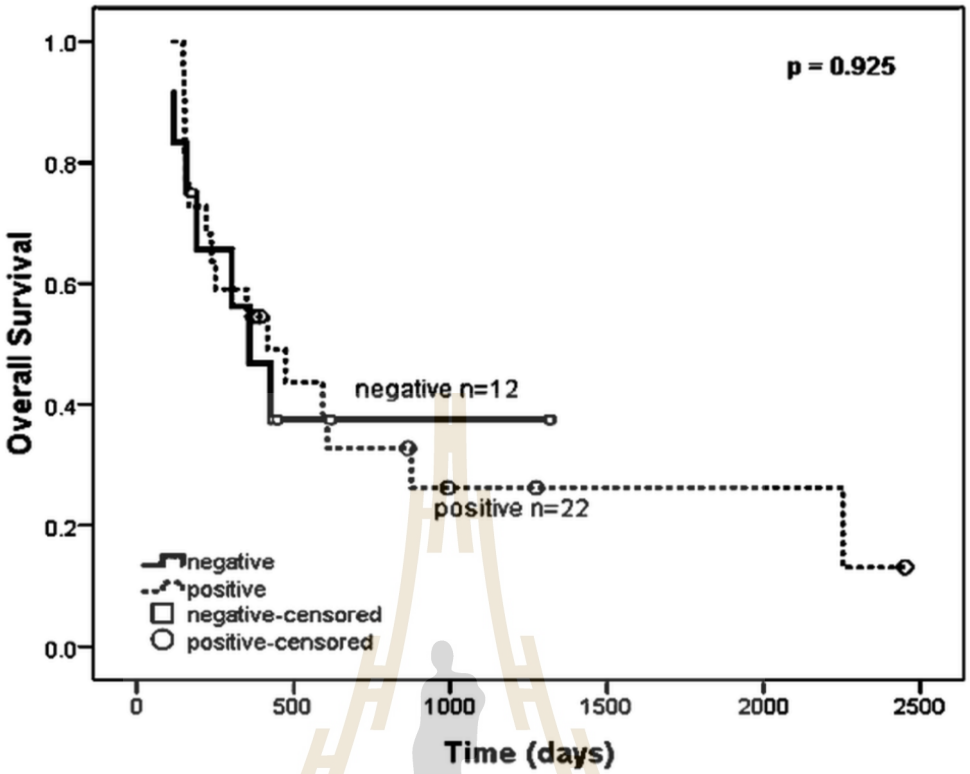

Figure 3.7 The level of YKL-40 expression was not associated with patient overall survival. Patient survival data were analyzed using a Kaplan–Meier survival curve. A log-rank test did not show a significant difference between groups with positive or negative YKL-40 expression.

3.3.6 Effects of YKL-40 in CCA cell lines

3.3.6.1 Paracrine effect of YKL-40 in CCA

In this series of experiments, we examined the effects of rYKL- 40 on the growth and migration of two CCA cell lines (KKU-055 and KKU-213), grown in the presence of recombinant YKL-40 (rYKL-40). rYKL-40 have been determined the purity and specificity by 12% SDS-PAGE analysis (Figure 3.8) and Western blot analysis (Figure 3.9), respectively. The results (Figure 3.10) showed that rYKL-40 significantly enhanced the growth of KKU-055 (P=0.005) and KKU-213 (P=0.038) after 72 h of incubation. A Transwell migration assay was subsequently carried out to determine whether YKL-40 affected the migration of CCA cells. The results (Figure 3.11) showed that the KKU-M213 cells treated with 80 ng/mL of rYKL-40 had a 1.8-fold increase in migration as compared to the untreated cells (P=0.036). Note that the CCA cells were allowed to migrate for only 6 h, to ensure that no cell division occurred during the time of investigation.

To investigate whether YKL-40 mediated cell migration through cell-surface receptors, the KKU-213 cell with high expression of integrin receptors was used in the cell adhesion assay (Utispan *et al.*, 2010). KKU-213 cells were allowed to attach for 1 h, we observed that the intrinsic binding capacity was increased by 1.5-fold in cells induced with rYKL-40, compared with that of the non-induced cells (P=0.007) (Figure 3.12).

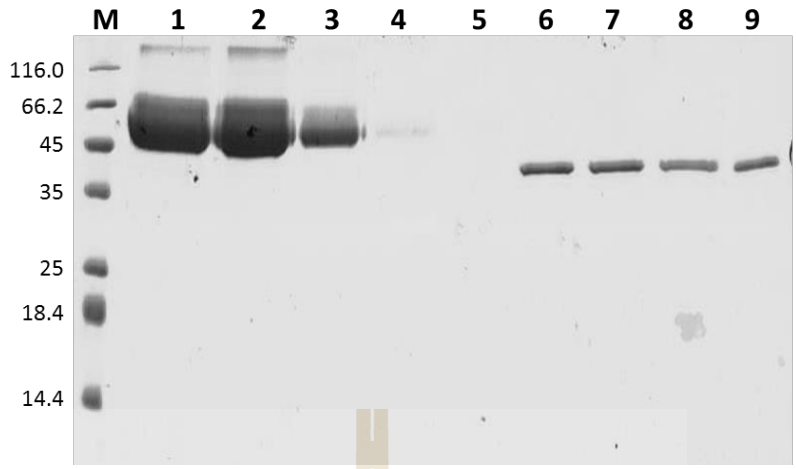

Figure 3.8 Purification of rYKL-40 from HEK293T cell. M: Protein marker (kDa); Lane 1: HEK293T cell culture medium after overexpress with YKL-40 for 48 h; Lane 2: Flow-through (HEK293T cell culture medium after incubated with Ni-NTA resin for 1 h); Lane 3: wash 1 (LEW containing 10 mM imidazole); Lane 4: wash 2 (LEW containing 10 mM imidazole); Lane 5: wash 3 (LEW containing 10 mM imidazole); Lane 6: Elution 1 (LEW containing 150 mM imidazole); Lane 7: Elution 2 (LEW containing 150 mM imidazole); Lane 8: Elution 3 (LEW containing 150 mM imidazole); Lane 9: rYKL-40 after desalting.

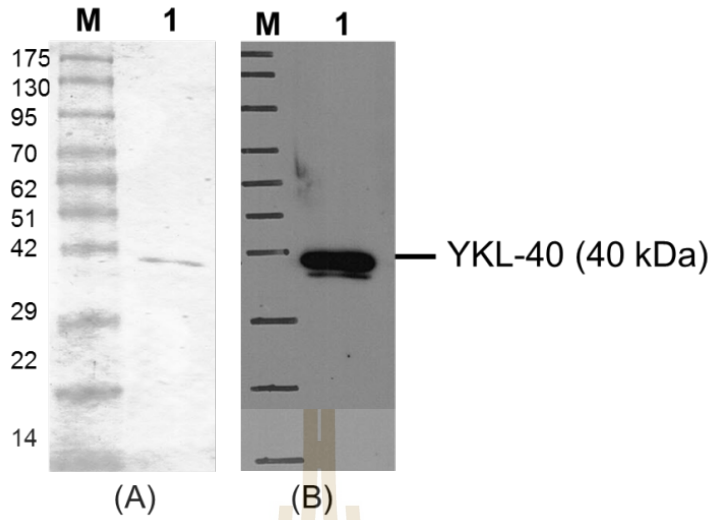

Figure 3.9 rYKL-40 specific with rabbit anti human YKL-40 antibody (Quidel). (A) 10% SDS-PAGE analysis. (B) Western blot analysis. M: Protein marker (kDa); Lane 1: rYKL-40 (0.5 µg).

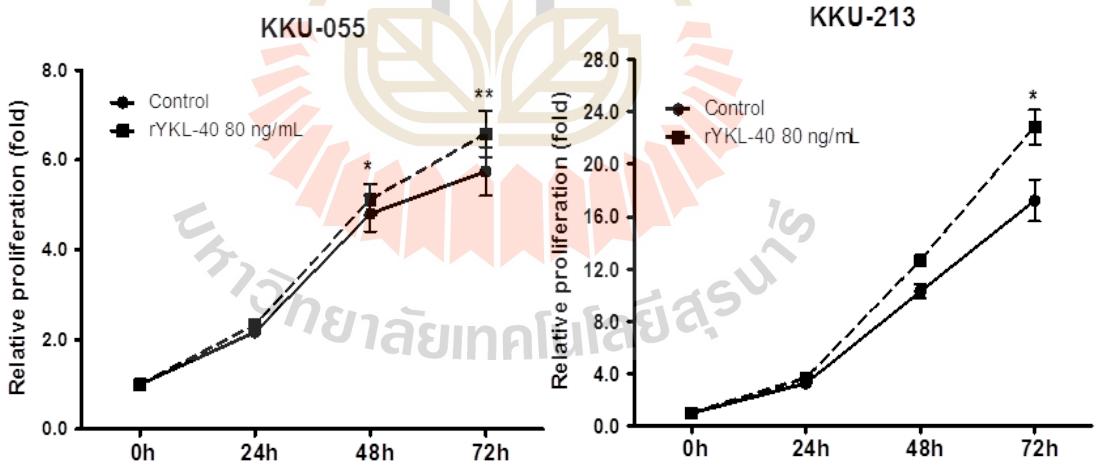

Figure 3.10 Effect of rYKL-40 on cell proliferation. The MTS assay was performed on two cell lines, KKU-055 and KKU-213, in medium supplemented with rYKL-40 (80 ng/mL) and without rYKL-40 (control). Error bars represent ±SEM (triplicate experiments). *P<0.05, **P<0.01.

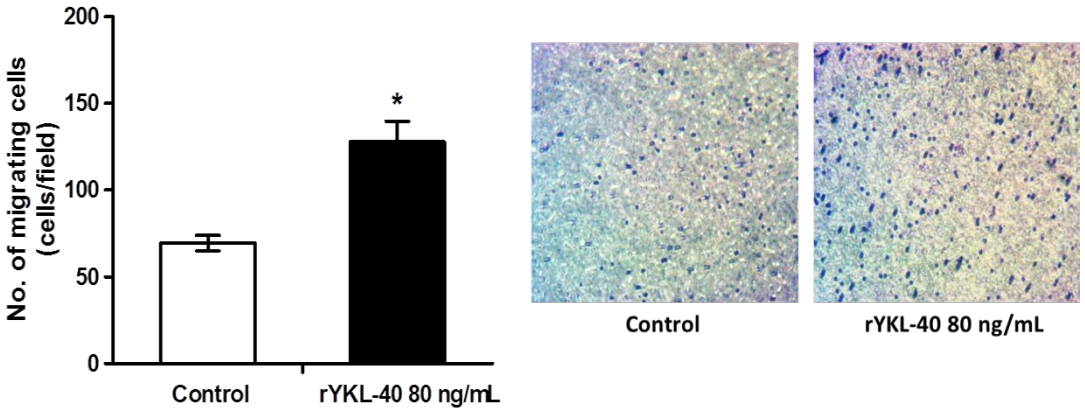

Figure 3.11 Effect of rYKL-40 on cell migration. In the migration assay, KKU-213 cells were loaded into the upper chamber of a well in a transwell plate and rYKL-40 protein (80 ng/mL) was introduced into the lower chamber. Numbers of migrating cells were counted (left panels), and representative images are shown for each condition (right panels). Error bars represent ±SEM (triplicate experiments). *P<0.05.

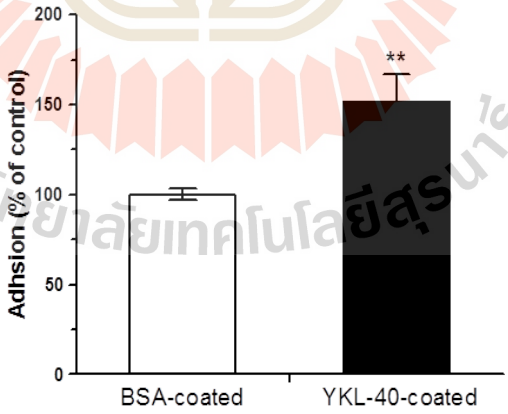

Figure 3.12 Adhesion effect of KKU-213 cell on rYKL-40-coated surface. After removing unbound cells, adherent cells were quantified by the MTS assay. Cells adhering to BSA-coated plates were used as a control. Error bars represent ±SEM (triplicate experiments). **P<0.01.

Next experiment, to verify that YKL-40 is a key factors in culture medium which activates CCA cell growth and migration. In this study, chitohexaose ($GlcNAc_6$) was used as YKL-40 inhibitor to neutralize the action of YKL-40. Since, long length of chitooligosaccharides can tightly interacts with carbohydrate-binding site of YKL-40 and induces a significant conformational change (Houston *et al.*, 2003). As shown in Figure 3.13, co-exposure of rYKL-40 80 ng/mL with chitohexaose (GlcNAc6) significantly reversed the effect of rYKL-40 –induced CCA cell growth (KKU-055, KKU-213, and KKU-214) at 50 μM of GlcNAc6 when incubated for 72 h and migration (KKU-213, $P<0.05$) at at 10 and 50 μM of GlcNAc6 (Figure 3.17) in a dose-dependent manner.

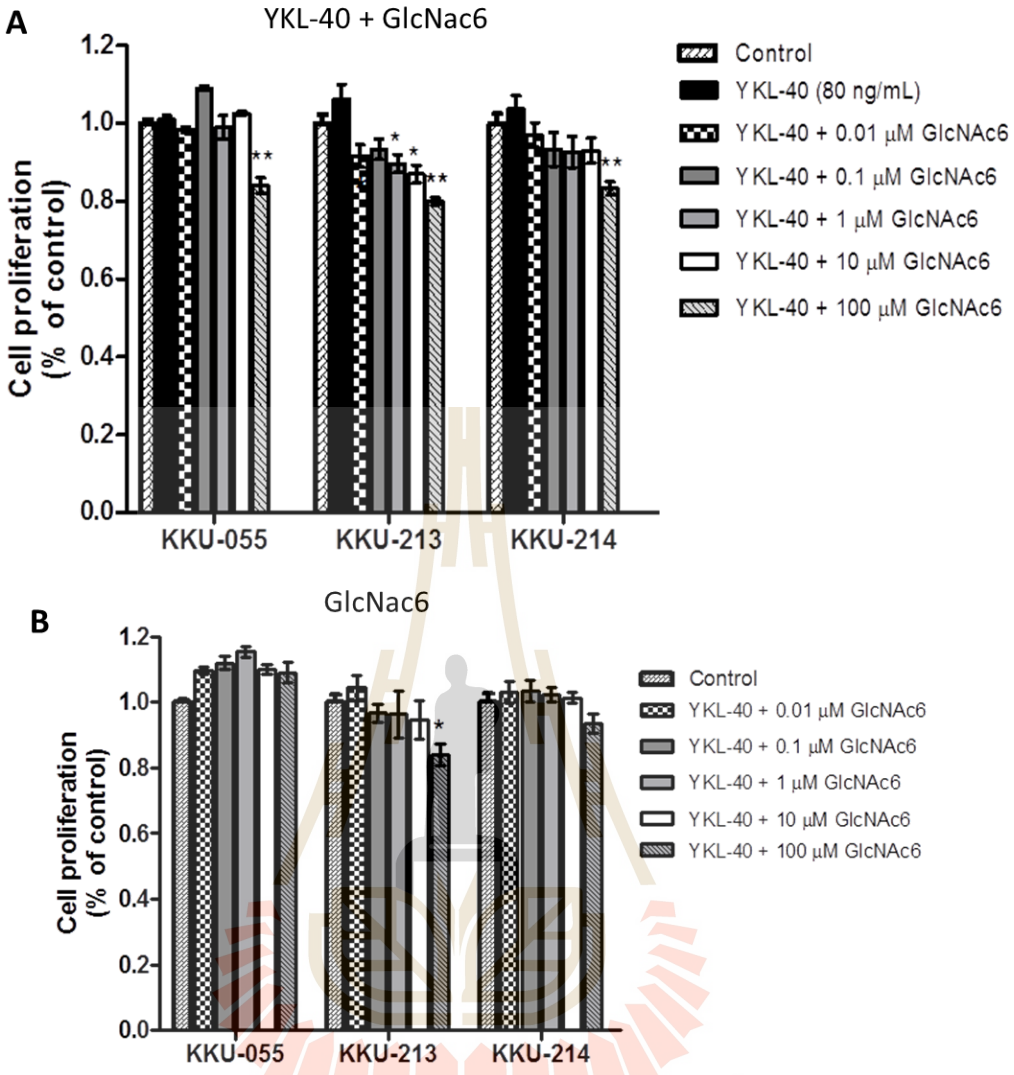

Figure 3.13 Co-exposure of rYKL-40 and chitohexaose (GlcNAc6) reversed CCA cell proliferation. The SRB assay was performed on cell lines, KKU-055 and KKU-213, and KKU-214 in medium supplemented with rYKL-40 (80 ng/mL) and with/or without GlcNAc6, and 0 ng/mL rYKL-40 was used as control. Error bars represent ±SEM (triplicate experiments). *P<0.05, **P<0.01.

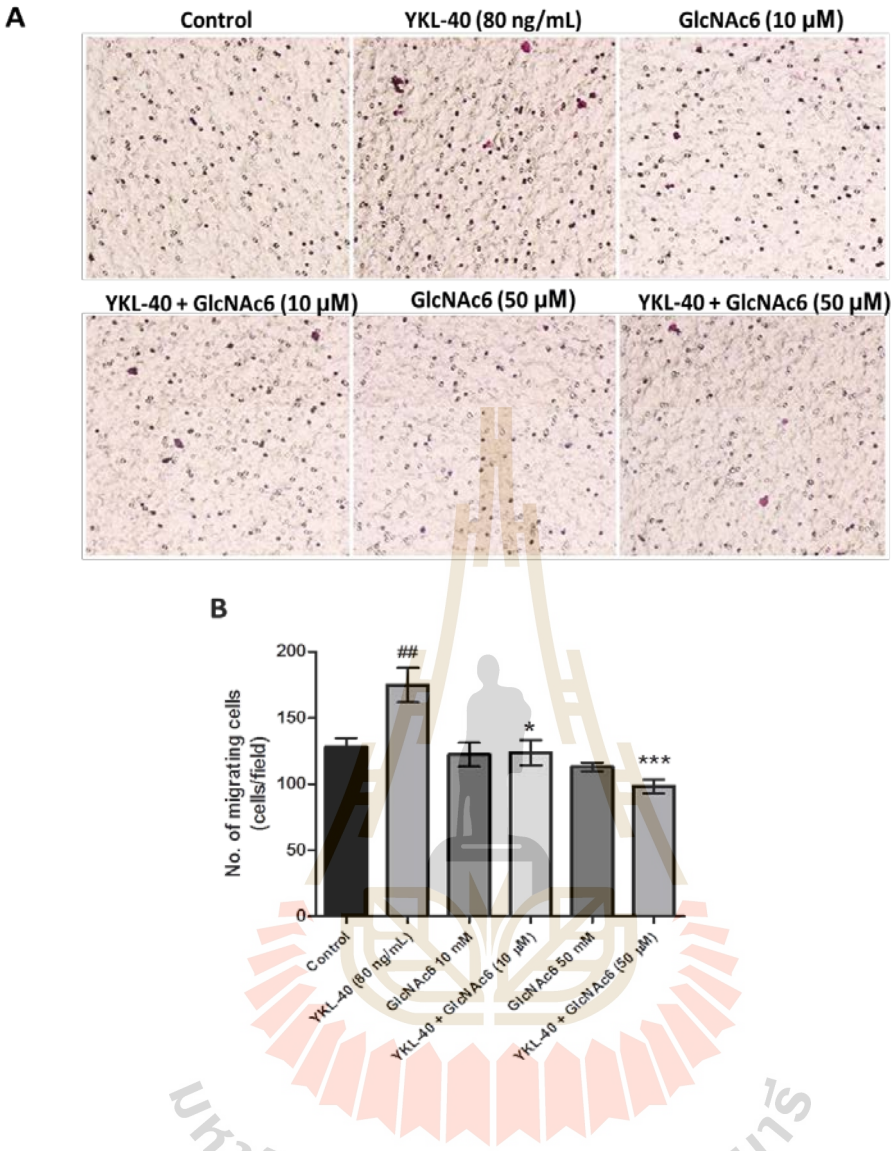

Figure 3.14 Co-exposure of rYKL-40 and chitohexaose (GlcNAc6) reversed CCA cell migration. In the migration assay, KKU-213 cells were loaded into the upper chamber of a well in a transwell plate and rYKL-40 protein (80 ng/mL) or rYKL-40 combined with GlcNAc6 was introduced into the lower chamber, and 0 ng/mL rYKL-40 was used as control. Numbers of migrating cells were counted (A), and representative images are shown for each condition (B). Error bars represent ±SEM (triplicate experiments). *P<0.05 ***P<0.001 vs. control, and ##P<0.01 vs. YKL-40 (80 ng/mL).

In the last set of experiments, the KKU-213 cells were treated with 80 ng/mL of rYKL-40 and incubated for up to 48 h. The results showed that exogenous YKL-40 induced considerable phosphorylation of Akt at 24 h and Erk1/2 at 24 h and 48 h, compared with those of the control cells without rYKL-40 treatment (Figure 3.15).

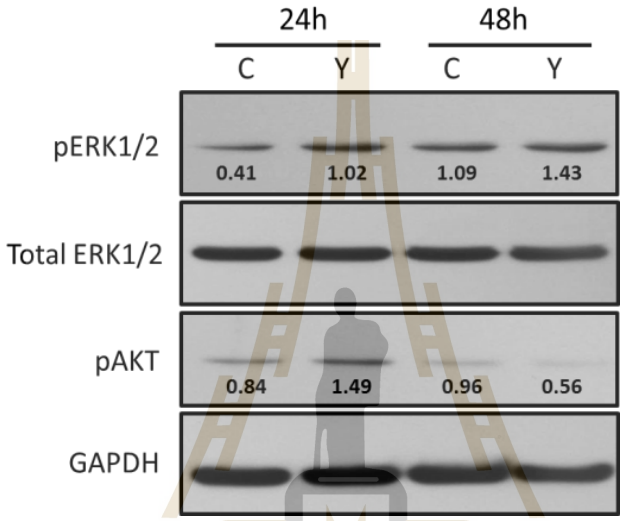

Figure 3.15 YKL-40 activates cell growth and migration through ERK/AKT signaling. The numerical value indicates the intensity of the pAKTor pERK1/2 signal, which was normalized to those of the "housekeeping" protein GAPDH. C indicates KKU-213 samples without rYKL-40 treatment and Y indicates samples treated with 80 ng/mL rYKL-40.

3.3.6.2 Autocrine effect of YKL-40 in CCA

We subsequently investigated the effects of YKL-40 expression on cell growth, migration, anchorage-independent growth, and chemosensitivity. Two cell lines, KKU-055 and KKU-213, were transfected with the pCMV6/CHI3L1 construct or pCMV6-entry vector as control cells. Western blot analysis demonstrated after transfection that both cell lines could express exogenous YKL-40, while no expression was seen in the cells transfected with the pCMV6-entry vector (Figure 3.16). Cell proliferation was tested using MTS assay, and the results showed that the exogenous expression of YKL-40 was significantly visible in KKU-055 cells after 48 h of cell growth (P=0.0178), while no expression of YKL-40 was seen in KKU-213 cells (Figure 3.17). Transwell migration assay and monolayer wound healing were carried out to determine whether expression of YKL-40 in CCA cells affected cell migration. As shown in Figure 3.18, both transwell migration and monolayer wound healing assays showed that expression of YKL-40 significantly enhanced the migration ability of KKU-213 cells as compared with those of the control. However, this malignant phenotype was not observed with KKU-055 cells overexpressed YKL-40.

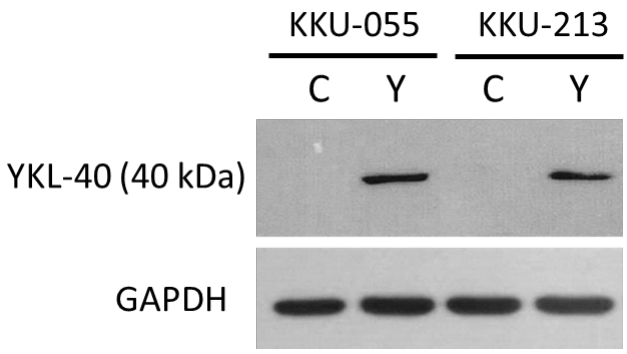

Figure 3.16 Western blot analysis for exogenous YKL-40 expression in CCA cell lines. C represents as the control of KKU-055 and KKU-213 (transfected with pCMV6-entry vector); Y represent as exogenous YKL-40 expression in KKU-055 and KKU-213 at 40 kDa (transfected with pCMV6/CHI3L1 vector).

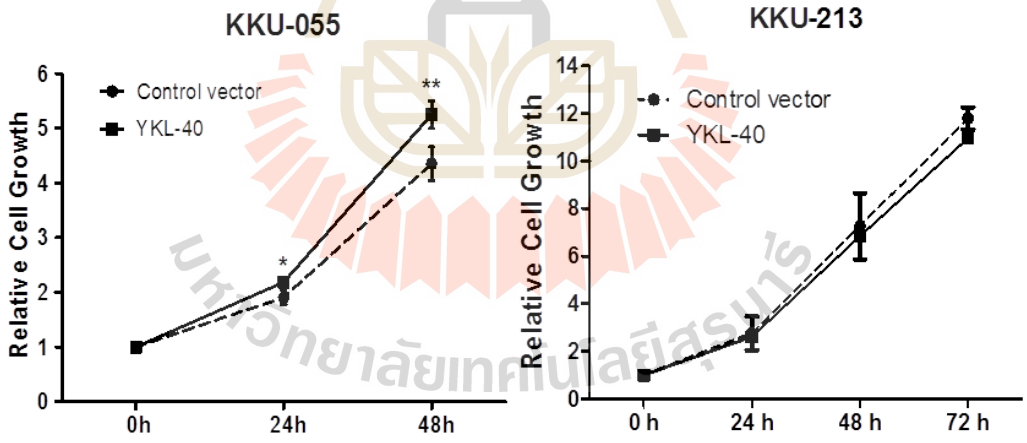

Figure 3.17 Effect of exogenous YKL-40 expression on cell proliferation. The MTS assay was performed on two cell lines, KKU-055 and KKU-213 with pCMV6/CHIL3 or control vector. Error bars represent ±SEM from experiments carried out in triplicate; *P<0.05, **P<0.01.

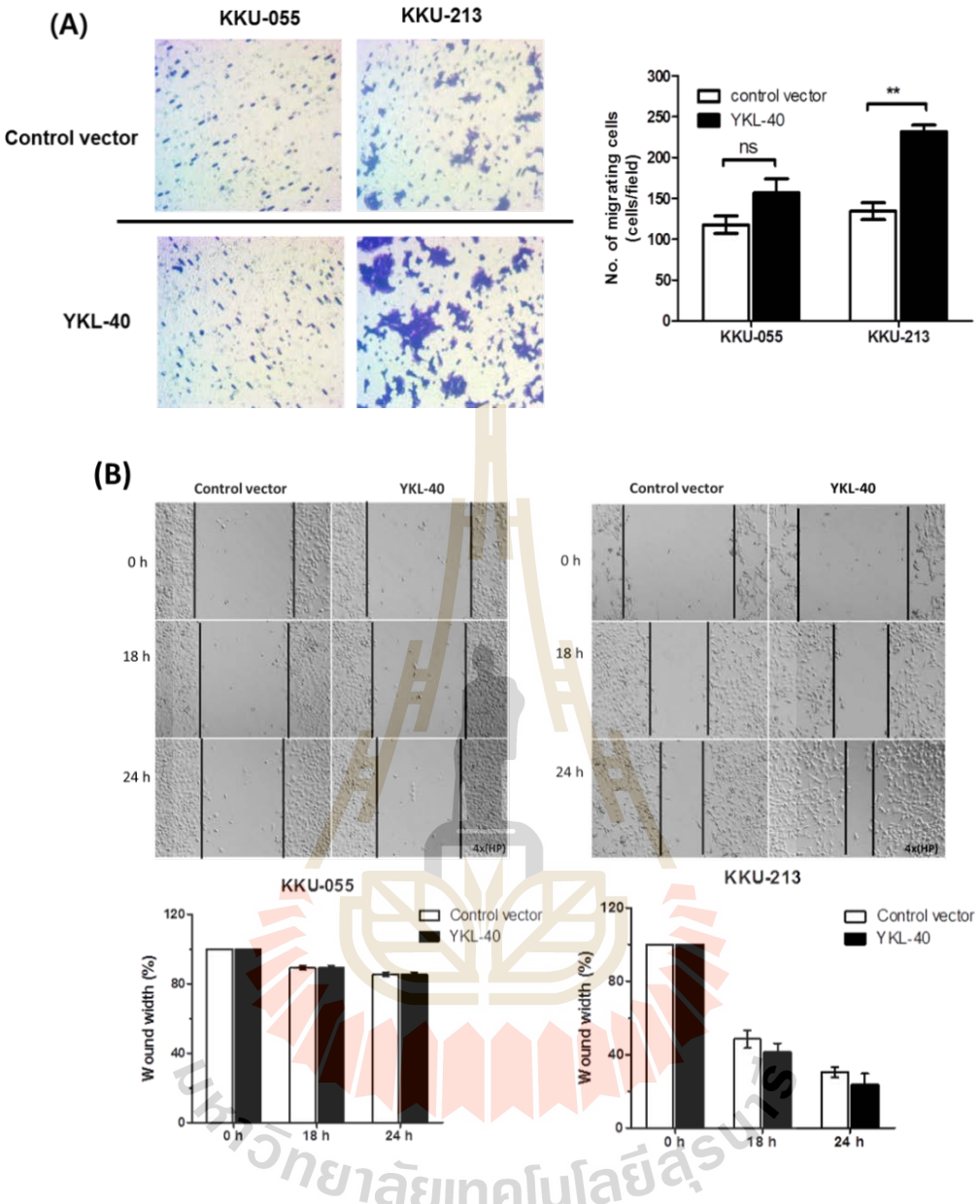

Figure 3.18 Effect of exogenous YKL-40 expression on cell migration. KKU-M055 and KKU-M213 with pCMV6/CH1L3 or control vector were used to determine the migration ability by (A) transwell migration and monolayer wound healing assay. Bar charts represent the numbers of migrating cells (A on right panel) and average wound widths of each assays (B on lower panel). Error bars represent ±SEM from experiments carried out in triplicate; *P<0.05.

To investigate the oncogenicity of YKL-40 in CCA, we assessed whether YKL-40 promotes anchorage-independent growth of CCA cells. Stable YKL-40 overexpressing KKU-055 cells were generated by selecting with 700 µg/mL of G418 for 2 weeks and confirmed the expression of YKL-40 after drug selection at both mRNA and protein levels (Figure 3.19 and 3.20, respectively). Overexpression of YKL-40 enhanced the colony-forming ability of KKU-055 cells compared with that of control, which do not express YKL-40 (Figure 3.21). Stable YKL-40 overexpressing KKU-055 cells exhibited the increment of size and number of colonies formed in soft agar at 14 days.

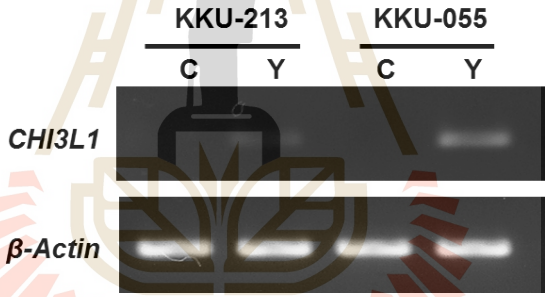

Figure 3.19 YKL-40 mRNA expression in stable YKL-40 overexpressing CCA cells. C represents as the control of KKU-055 and KKU-213 (transfected with pCMV6-entry vector); Y represent as exogenous YKL-40 expression in KKU-055 and KKU-213 at 40 kDa (transfected with pCMV6/CHI3L1 vector).

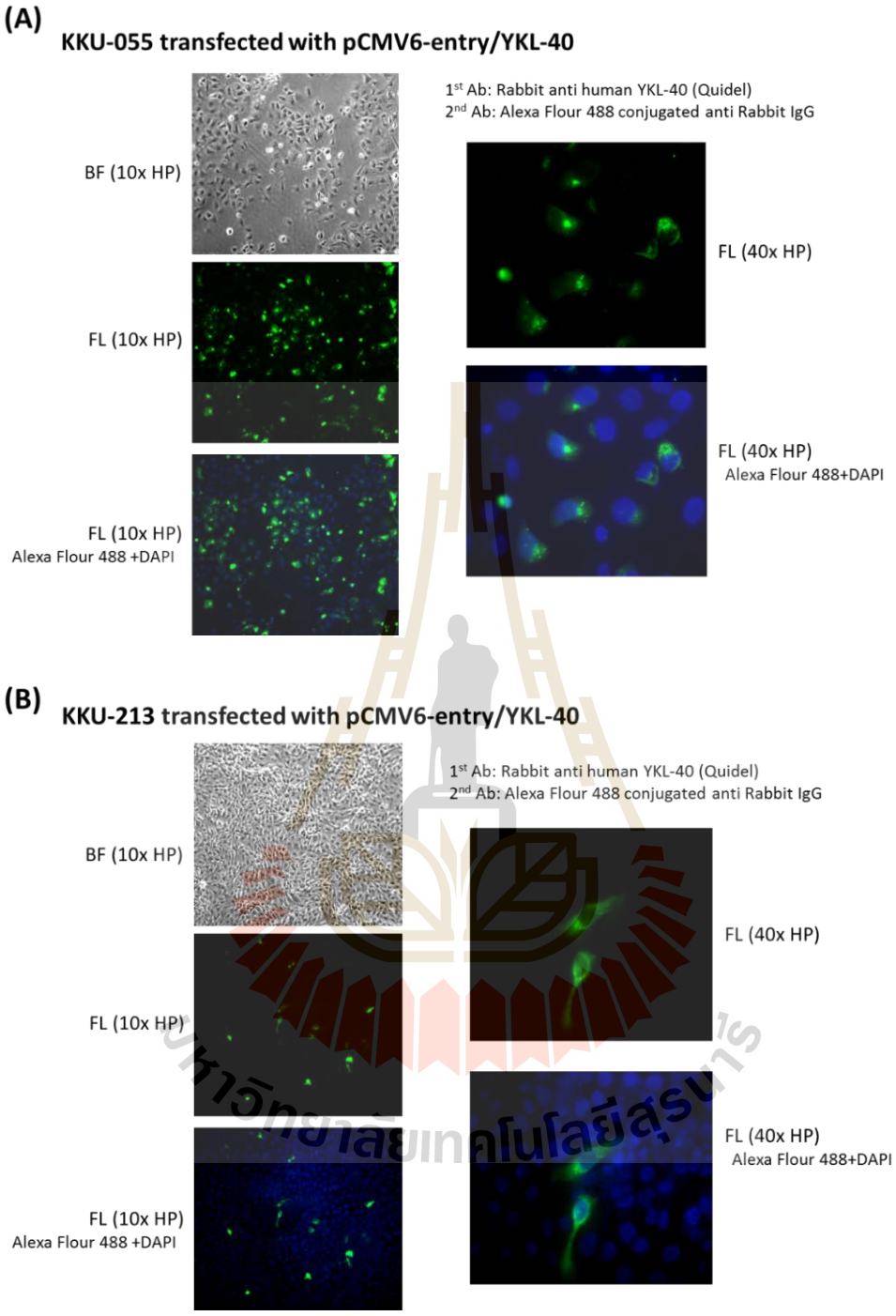

Figure 3.20 Immunofluorescent staining of stable YKL-40 overexpressing CCA cells. Immunofluorescence (FL) and corresponding bright field (upper) images are presented in both (A) KKU-055 and (B) KKU-213 that were stained with antibody for YKL-40 (green color) and DAPI (blue color).

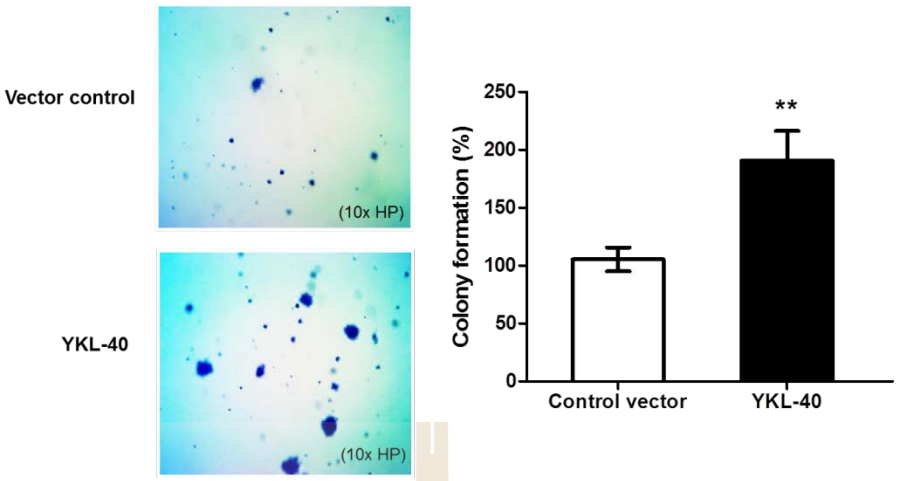

Figure 3.21 Effects of YKL-40 expression on anchorage-independent growth. Stable YKL-40-overexpressing KKU-055 cells were seeded in soft agar and cultured for 2 weeks. Photographs were taken at a magnification of ×20. (Left panel). Colonies are counted (Right panel) and represented as mean ± SEM. **P<0.01.

Next, we tested whether high expression of YKL-40 influences the chemosensitivity of CCA cells. Stable YKL-40 overexpressing KKU-055 cells and control cells were treated with cisplatin (0-20 µM), docxorubicin (0-2 µM) and piperlongumin (0-20 µM) for 24 hr. The IC_{50} values of these three drugs for stable YKL-40 overexpressing KKU-055 cells were 18.40, 0.33, and 5.19 µM respectively, which higher than those vector control (IC_{50}=8.55, 0.27, and 6.34 µM respectively). Sensitivity to cisplatin was reduced in YKL-40 overexpressing cells. Cell cycle analysis also demonstrated that subG1 phase population was increased after treated with 10 µM of cisplatin for 24 h (%SubG1 = 23.98) compared with that of control (%SubG1 = 9.42) (Figure 3.22(B)). This result suggested that high YKL-40 expression may promote the chemoresistance mechanism in CCA cells (Figure 3.22(A)).

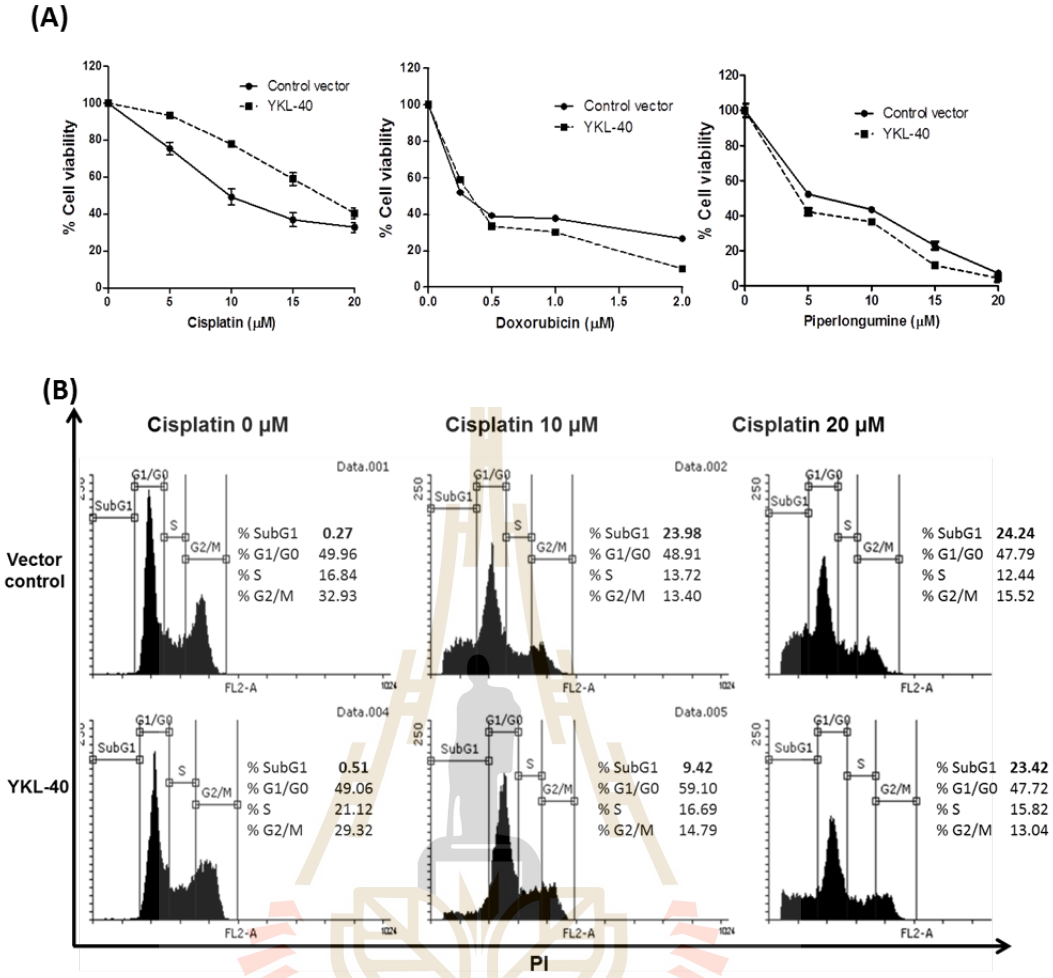

Figure 3.22 Overexpression of YKL-40 in KKU-055 cells prmotes chemoresistance mechanism to cisplatin. (A) Cells were treated with cisplatin, doxorubicin and piperlongumine for 24 h, cells viability was determined using SRB assay. (B) Cell cycle analysis using flow cytometer showed overexpressed YKL-40 reduced subG1 phase population when treated with cisplatin. Error bars represent ±SEM from the experiments done in triplicate.

3.4 Conclusion and discussion

In this study, we assayed the plasma concentrations of YKL- 40 in 57 CCA patients and also the cellular expression of the *CHI3L1* gene in 34 CCA tissue samples. Statistical analysis clearly suggested that elevated plasma YKL-40 level was associated with short overall patient survival, but not with other clinical–pathological factors, including patient age, gender, tumor grade, and histology type. The results reported here agree with several independent studies that showed elevated serum/plasma YKL-40 concentrations in a wide range of cancers (Cintin *et al.*, 2002; Hogdall *et al.*, 2003; Johansen *et al.*, 2003; Thom *et al.*, 2010). The baseline YKL-40 concentration in the healthy groups in different cancer types was similar (median range 27–97 ng/mL) (Dehn *et al.*, 2003; Jensen *et al.*, 2003; Tarpgaard *et al.*, 2014; Zhu *et al.*, 2012). The median value of pre-operative plasma YKL-40 levels in our cancer groups (170 ng/mL) is slightly higher than the median values reported for other cancer types (78-150 ng/mL) (Bernardi *et al.*, 2012; Brasso *et al.*, 2006; Fan *et al.*, 2014; Hogdall *et al.*, 2009). However, one should take into account that the reported values are deviated among cancer types, depending on sample preparation (plasma or serum) and pathological (tumor stages) and physiological (age and sex) conditions of cancer patients during their hospital visits for blood collection. Even the prognostic values of YKL-40 within the same sample groups may be deviated, since some patients have advanced or disseminated disease and even some have received chemo and/or radiation therapy. In our study, the YKL-40 levels were evaluated from blood samples of CCA patients taken before surgical resection. Note that we did not include chemo/radiotherapy parameters in our prognosis analysis, since not all patients were assigned with the same treatment protocol. For examples, 38 of 57

CCA patients received operation, while 14 of which were offered to have adjuvant chemotherapeutic treatment, but only 5 of 14 CCA patients had completed full cycles of chemotherapy.

In our study, we also found that not all patients with CCA had elevated serum/plasma YKL-40 levels compared with the healthy age-matched controls, implying that YKL-40 may not be secreted directly by CCA tumor cells. Our immunohistochemical data showed the predominant localization of YKL-40 to be in infiltrating inflammatory cells that spread all over non-tumor areas surrounding the CCA cells. These results indicate that expression of YKL-40 is not tissue-type-specific, but more cell-type-specific. Our data complement a previous study that showed intense expression of YKL-40 in various adult tissues, especially in cells exhibiting high metabolic activity, including mast cells, polymorphic nuclear granulocytes, endocrine cells, exocrine epithelial cells, basal layer squamous epithelial cells, endothelial cells, and neurons (Ringsholt *et al.*, 2007). The role of YKL-40 in these cells might be related to increased metabolism of cells during tissue inflammation/remodeling. For CCA, elevated plasma YKL-40 levels in patients with stage IV CCA may suggest some role of YKL-40 in stimulating local inflammation of tissues lining the bile duct. Our further immunohistochemical study suggested a low level of expression of YKL-40 in CCA tissues, but high-level expression in adjacent non-cancerous tissues, including intratumoral stroma, liver, and connective tissues. This result implies that induced YKL-40 secretion occurred as an indirect activator of CCA.

In cancer cells, YKL-40 has been shown to enhance proliferation, differentiation, metastasis potential, apoptosis, and angiogenesis (De Ceuninck *et al.*,

2001; Johansen *et al.*, 2006; Recklies *et al.*, 2002). However, the exact role of YKL-40 in cancer progression is unknown. YKL-40 could potentially act as a tumor growth factor, as we observed that culture medium supplemented with rYKL-40 activated the proliferation of two CCA cell lines (KKU-213 and KKU-055). In addition, studies of transient expression of YKL-40 in CCA cell lines also suggested the promotion of growth of the studied CCA cell lines. A similar growth-stimulating effect has been reported previously with glioma c1ells (Ku *et al.*, 2011) and cervical cancer cells (Ngernyuang *et al.*, 2014). YKL-40 has also been demonstrated to act as a chemoattractant that enhanced the migration, adhesion, and spreading of vascular smooth muscle cells (VSMC) and umbilical vein endothelial cells (HUVEC) (Nishikawa and Millis, 2003), while similar effects have been observed for several cancer cells (Kawada *et al.*, 2012; Ku *et al.*, 2011; Ngernyuang *et al.*, 2014; Shao *et al.*, 2009). In our study, we demonstrated that YKL-40 not only promoted CCA cell growth but also increased CCA cell migration. YKL-40 promotion of migration has been shown for colorectal cancer (Kawada *et al.*, 2012), glioma (Ku *et al.*, 2011; Singh *et al.*, 2011), and cervical cancer (Recklies *et al.*, 2002).

Although the cellular receptors mediating the biological effects of YKL-40 remain to be identified, there is evidence for YKL-40 interaction with signaling components on the cell membrane (Johansen *et al.*, 2006). Singh and coworkers demonstrated that expression of YKL-40 regulated NFI-X3 and STAT3 (known regulators of gliogenesis), controlling migration and invasion of both astrocytes and glioma cells (Nishikawa and Millis, 2003). YKL-40 also showed angiogenic properties by interacting with many known membrane receptors in endothelial cells, such as syndecan-1 and integrins αvβ3 and/or αvβ5, so as to mediate cell growth and

migration (Francescone *et al.*, 2011; Shao *et al.*, 2009). In CCA, syndecan-1 is expressed as a cell-surface, transmembrane, heparin sulfate-containing proteoglycan that interacts with various effectors during the initiation of cell adhesion (Harada *et al.*, 2003). In addition, CCA cells also show high-level expression of integrins, the transmembrane receptors that are the bridges for cell–cell and cell–extracellular matrix interaction receptor (Utispan *et al.*, 2012; Utispan *et al.*, 2010). Our cell adhesion assay showed a positive response of CCA cells to rYKL-40, which induced both cell migration and the phosphorylation of two intracellular kinases, Akt and Erk1/2, in these same cell lines, suggesting that YKL-40 interacts with membrane receptors that may stimulate a downstream pathway involved in the cancer cell migration. However, identification of specific receptors for YKL-40 in CCA cells is a subject for future study. Moreover, our study also demonstrated that YKL-40 decreased the chemosensitivity to cisplatin but not changed in doxorubicin and piperlongumine. This finding agrees with the study in small cell lung cancer that cancer patients with high serum YKL-40 showed a poorer response to chemotherapy than those patients with low serum YKL-40 (Xu *et al.*, 2014). Taken together, our findings provide evidence that elevated plasma YKL-40 levels correlate significantly with poor prognosis of CCA and play an indirect role in promoting cancer progression, acting as an oncogenic factor that stimulates the proliferation, anchorage dependent growth and migration of CCA cells, through a membrane receptor that regulates the Akt/Erk pathway.

3.5 References

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CHAPTER IV

ANTI-TUMOR ACTIVITY OF PIPERLONGUMINE ON CHOLANGIOCARCINOMA CELLS

Abstract

Cholangiocarcinoma (CCA) is an aggressive, metastatic bile duct cancer. CCA is difficult to diagnose, and responds poorly to current radio- and chemo-therapy. Piperlongumine (PL) is a naturally-occurring small molecule selectively toxic to cancer cells by targeting reactive oxygen species (ROS). In this study, we demonstrated the potential anticancer activity of PL in CCA. PL markedly induced death in CCA cell lines in a dose- and time-dependent manner through the activation of caspase-3 and PARP. PL also stimulated ROS accumulation in CCA. Co-exposure of PL with the ROS scavenger N-acetyl-L-cysteine and glutathione reduced completely blocked PL-induced apoptosis in CCA cell lines. Up-regulation of p21 via the p53-independent pathway in PL-treated CCA cells led to G2/M phase arrest and cell apoptosis. In addition, the study showed that PL trigger CCA cell lines death through JNK-ERK activation. Furthermore, the different antioxidant capacity of CCA cell lines also indicates the susceptibility of the cells to PL treatment. Our findings reveal that PL exhibits anti-tumor activity and has potential to be used as a chemotherapeutic agent against CCA.

4.1 Introduction

Cholangiocarcinoma (CCA) is a lethal cancer arising from the biliary epithelia and it is the sixth most common cancer in the world and CCA accounts for around 10–25% of primary liver cancers (Jepsen *et al.*, 2007; Kamangar *et al.*, 2006). A high local prevalence of CCA has been particularly reported in Northeast Thailand (Sripa and Pairojkul, 2008). CCA is considered a slow-growing tumor, that is difficult to diagnose at an early stage and that has a poor prognosis. Surgical resection is a potentially curative therapy for only early detected cases, but most patients are diagnosed at a late stage so are ineligible for this treatment. The median survival of patients with a potentially curative surgery is about 5 years if the surgical margin status is negative. Patients undergoing a post-operative adjuvant chemotherapy with 5-Fluorouracil (5-FU)-based regimen have a significant survival advantage with an increasing median survival time (Bhudhisawasdi *et al.*, 2012). Nonetheless, the 5-year survival rate of CCA has not increased despite various treatments and remains at 5-10%; as current regimens of chemotherapy are not definitive especially for patients with recurrent or unresectable CCA (Rizvi and Gores, 2013). Therefore, it is necessary to search for highly potent chemotherapeutic agents against this malignancy.

Piperlongumine (PL) is a biologically-active alkaloid isolated from the long pepper *Piper longum L*. (Chatterjee and Dutta, 1967). PL has been demonstrated to have anti-bacterial, anti-angiogenic, and anti-diabetic activities (Bezerra *et al.*, 2013). Recently, PL was shown to have anti-tumor activity through its inducing high levels of reactive oxygen species (ROS), resulting in in vitro and in vivo cell death (Raj *et al.*, 2011) by activating several mechanisms including p38/JNK (Xiong *et al.*, 2015),

MAPKs-CHOP (Chen *et al.*, 2015), NF-kB (Zheng *et al.*, 2016) pathways. PL can also selectively kill numerous transformed cell types without affecting normal cells; including in head and neck (Roh *et al.*, 2014), hepatocellular carcinoma (Chen *et al.*, 2015), breast (Lee *et al.*, 2015), gastric (Duan *et al.*, 2016), and lung cancers (Zheng *et al.*, 2016). The selective accumulation of ROS in cancer cells by PL induction, therefore, represents a novel therapeutic strategy for cancer treatment, especially for CCA.

In the current study, we evaluated anti-tumor activity of PL in human CCA cell lines. Subsequently, we investigated anti-apoptosis mechanisms of PL on CCA, and possible PL-resistance mechanism by evaluating the expression levels of different antioxidant genes.

4.2 Experimental and methods

4.2.1 Chemicals and antibodies

Piperlongumine (PL) was purchased from Tocris Bioscience (Bristol, UK). N-acetyl-L-cysteine (NAC), dimethyl sulfoxide (DMSO), propidium iodide (PI), 2´,7´-dichlorodihydrofluorescein diacetate (DCFH-DA), trichloroacetic acid (TCA), Zinc-protoporphyrin IX (ZnPP; HO-1 inhibitor), L-glutathione reduced (GSH), L-ascorbic acid and sulforhodamine B (SRB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MG-132 was purchased from Merck. SP600125 (JNK pathway inhibitor) and U0126 (ERK pathway inhibitor) were obtained from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies were purchased from Cell Signaling Technology including total JNK (46/54 kDa), Thr183/Tyr185 phosphorylated SAPK/JNK (p-JNK) (46/54 kDa), total Akt (60 kDa), Ser 473

phosphorylated Akt (p-Akt) (60 kDa), Caspase-3 (17/19/35 kDa), poly (ADP-ribose) polymerase (PARP) (116/89 kDa), Bcl-2 (28 kDa), and Bax (20 kDa). Antibody against β-Actin (42 kDa) and mouse anti-rabbit IgG-HRP secondary antibody were purchased from Sigma-Aldrich. Goat anti-rabbit IgG-HRP secondary antibody was purchased from GenScript (Piscataway, NJ, USA).

4.2.2 Cell line and cell culture

Human CCA cell lines (KKU-055, KKU-100, KKU-139, KKU-213, and KKU-214) were established from CCA patients at Srinagarind Hospital, Khon Kaen University, by Professor Banchob Sripa. Certificates of analysis were obtained from the Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan. The immortalized normal cholangiocyte cell line with SV40T and hTERT, MMNK-1, was generated and supplied by Prof. Naoya Kobayashi. These cell lines were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco/Invitrogen, Grand Island, NY, USA), 1% (v/v) penicillin-streptomycin (Gibco). The mouse fibroblast cell line, NIH-3T3, was bought from the American Type Culture Collection (ATCC; Manassas, VA, USA). It was maintained in DMEM/HAM-F12 (1:1 ratio) with 10% (v/v) fetal bovine serum (FBS), 2 mM L-Glutamine (Gibco/Invitrogen, Grand Island, NY, USA), 1% (v/v) penicillin-streptomycin (Gibco). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells with 70-80% confluence were trypsinized with 0.25% trypsin-EDTA (Gibco) and subcultured in the same media. Some aliquots of cells were transferred to freezing medium containing 10% (v/v) DMSO and 50% (v/v) FBS and stored in a liquid nitrogen tank for later use.

4.2.3 Piperlongumine and other drug treatments

A stock concentration of 10 mM PL was dissolved in dimethyl sulfoxide (DMSO) and stored in aliquots at -20 ℃ until its use. PL with designated final concentrations (0-20 µM) was diluted with cell culture media for subsequent experiments. The vehicle control was DMSO with the same final concentration used in preparation of PL the working solutions. The ROS scavenger NAC, GSH and ascorbic acid were used at 3 mM, other specific inhibitors SP600125/ SB203580/ U0126/ ZnPP were used at 10 µM and MG132 was used at 0.75 µM. The cells were pre-incubated with the inhibitors for 1 h then treated with PL (10 µM or 20 µM) for 24 or 48 h.

4.2.4 Cell viability assay

Cell viability was determined by a sulforhodamine B (SRB) assay capable of determining cell density based on the measurement of cellular protein content; performed per Voigt with slight modifications (Voigt, 2005). Briefly, the CCA cell lines were seeded to 5×10^3 cells/well in 96-well plates for 24 h, and then treated with specified concentrations of PL. After 48 h, the media was discarded and cells were fixed with ice-cold 10% (v/v) Trichloroacetic acid (TCA) in deionized (DI) water at 4 ℃ for 1 h. The TCA was then removed and the cells were washed 5 times with DI water, followed by staining with 0.4% (w/v) SRB dissolved in 1% (v/v) acetic acid for 30 min at room temperature. Unbound dye was removed by 5 washes with 1% (v/v) acetic acid, after which the cells were solubilized with 10 mM unbuffered Tris base (pH 10.0) and shaken on plates for 10 min. The absorbance was measured at 564 nm using a Synergy HTX Multi-Mode Reader (BioTek Industries, Inc., VT,

USA). The number of viable cells was directly proportional to the protein bound-dye formation.

For the treatment of PL in the presence of the ROS scavenger NAC, GSH, ascorbic acid or other inhibitors, the cells were pre-treated with antioxidant or inhibitor for 1 h before adding the PL. The % cell viability was calculated as (A564 in treatment wells) / (A546 in control wells) × 100. Each experiment was performed three times independently. The half maximal inhibitory concentration (IC_{50}) values were calculated using CompuSyn software (Chou, 2006) (ComboSyn Inc.; Paramus, NJ, USA).

4.2.5 Cell cycle analysis

To determine the cell cycle distribution, cell lines ($2.0\text{-}4.5\times10^5$ cells/well) were seeded in 6-well cultured plates for 24 h. The cells were treated with various concentration of PL for 24 h and harvested. After washing with PBS, cells were fixed with ice-cold 70% ethanol and incubated overnight at 4 °C. The cells were then washed twice with PBS to remove ethanol and labeled with propidium iodide (PI) by incubating with PI solution containing, PI (20 µg/mL), 0.1% (v/v) Triton-X 100, and RNase A (20 µg/mL), in the dark for 30 min. Stained cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Automated quantification of the DNA content histograms was done with Flowing software (Cell Imaging Core, Turku Centre for Biotechnology, Abo Akademi University, Finland). For the PL treatment with the ROS scavenger NAC: the cells were pre-treated with 3mM NAC for 1 h before being exposed to PL. Then cell cycle analysis was performed after treatment for 24 h.

4.2.6 Apoptosis analysis

The apoptotic cells were measured using Alexa Fluor® 488 annexin V/Dead Cell Apoptosis kit (Invitrogen-molecular probes, Eugene, OR, USA) according to the manufacturer's instructions. Briefly, cell lines (2.5 - 4.5×10^5 cells/well) were seeded in a 6-well plate and grown to 70% confluence. After being treated with various concentrations of PL for 48 h, the cells were collected and washed twice with PBS. The pellet was then re-suspended in 100 µL of annexin-binding buffer and incubated with 5 µL of Alexa Fluor® 488 annexin V and 1 µL of propidium iodide (PI) working solution for 15 min in the dark. Annexin-binding buffer (400 µL) was then added, and the cells were determined using a FACS calibur flow cytometer (Becton Dickinson). The results were analyzed using Flowing Software (Cell Imaging Core, Turku Centre for Biotechnology).

4.2.7 Measurement of intracellular ROS

The intracellular accumulation of reactive oxygen species (ROS) was assessed by flow cytometry using the fluorescent probe 2'-7'-dicholofluorescin diacetate (DCFH-DA, Sigma-Aldrich). Briefly, cell lines (2.5 - 4.5×10^5 cells/well) were seeded in 6-well plates for 24 h. Then cells were treated with PL at 0, 10, and 20 µM for 2 h. After treatment, the cells were washed with PBS twice and incubated with 10 µM of DCFH-DA in a humidified 5% CO_2 incubator at 37 °C for 30 min. After incubation, the DCFH-DA was removed, and the cells were washed with PBS. The cells were trypsinized and re-suspended with complete media in order to check the fluorescent intensity of the DCFH-DA using flow cytometry.

4.2.8 Quantitative real time PCR (qPCR)

To determine the level of basal mRNA expression of antioxidant genes in five CCA cell lines, the cells were seeded in 6-well plates and allowed to growth for 24 h. Total RNA was isolated using TRIzol reagent (Invitrogen) and 1 µg of total RNA was reverse transcribed to single-stranded cDNA using Reverse Transcription Master Premix (Elpis Biotech, Daejeon, Korea) at 42 °C for 1 h. The reverse transcription product served as a template for qPCR. All primers were purchased from Macrogen, (Seoul, Korea) and the sequences of primers were show in Table 4.1. The qPCR was performed in a LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany) using SYBR green master mix. The PCR was performed in a final volume of 20 µL containing 1 µL of primer mixture, 10 µL of HiPi Real-Time PCR 2x Master Mix (Elpis Biotech), 1 µL of cDNA template, and 18 µL of nuclease-free distilled water. Real-time PCR cycles included: initial denaturation at 94 °C for 4 min; 94 °C for 10 sec, annealing at 60 °C for 20 sec, and extension at 72 °C for 30 sec through 40 cycles. The specificity of each of the PCR products was confirmed by melting curve analysis. Relative mRNA expression was obtained after normalization with endogenous human β-actin. Duplicate wells were run for each experiment and the experiments were performed in triplicate.

To investigate mRNA expression levels of antioxidant genes in CCA cell lines treated with PL, the cells were plated in 6-well plates for 24 h and treated with PL at 10 µM or DMSO for 6, 12, and 24 h. Then RNA isolation was performed and qPCR as described. The fold change in mRNA expression was calculated by comparing the β-actin-normalized threshold cycle numbers (Ct) in the PL-treated cells compared to the DMSO-treated cells using the $2^{-\Delta\Delta Ct}$ method.

Table 4.1 List of primer sequences.

Primers	Forward primer sequence (5′→ 3′)	Reverse primer sequence (5′→ 3′)	Product size (bp)
PARK7	CGAGCTGGGATTAAGGTCA	CATATGGTCCCTCTTTTTTGC	121
HMOX1	CAACATCCAGCTCTTTGAGGA	GGGCAGAATCTTGCACTTTG	119
NQO1	GATATTCCAGTTCCCCCTGC	TTCTTACTCCGGAAGGGTCC	132
TXN	GAGAGCAAGACTGCTTTTCA	CAGAGAGGGAATGAAAGAAAG	124
GSTP1	TACACCAACTATGAGGCGGG	AGCGAAGGAGATCTGGTCTC	132
SOD2	GTTGGCCAAGGGAGATGTTAC	AGCAACTCCCCTTTGGGTTC	226
β-actin	CTTCCTTCCTGGGCATGGAG	GAGCAATGATCTTGATCTTCAT	204

4.2.9 Western blot analysis

CCA cell lines were cultured in 6-wells plates for 24 h, then treated with PL 10 µM for 0, 3, 6, 12, and 24 h. Afterward, the cells were collected and prepared whole cell lysates. To prepare whole-cell lysates, the cells were washed twice with PBS and then lysed with 50 µL NP-40 lysis buffer and incubated on ice for 30 min. After centrifugation at 16,000 xg for 30 min at 4 °C, protein concentrations were determined with the BCA Protein Assay Kit (Pierce Biotechnology). Equal amounts of total proteins (25 µg) were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was probed with each primary antibody at a dilution of 1:1,000 at 4 °C overnight, and 1:5,000 HRP-conjugated secondary antibody for 1 h at room temperature. To ensure equal protein loading, β-actin was used as an internal control. Proteins probed with specific primary antibodies were visualized with an enhanced chemoluminescence reagent (SuperSignal West Pico Chemiluminescent Substrate: Thermoscientific, IL, USA) and the bands were captured using ImageQuant LAS 4000 (GE Healthcare, Piscataway, NJ, USA).

4.2.10 Statistical analysis

All experiments were performed at least 3 times, and all results expressed as a mean ± the standard error. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). The unpaired t-test was used for a between group statistical analyses. $P<0.05$ was considered statistically significant.

4.3 Results

4.3.1 Piperlongumine inhibits growth of human CCA cell lines by inducing apoptosis

To investigate the effects of piperlongumine (PL) on human CCA cell lines, we performed a cell viability test using the SRB assay on 5 human CCA cell lines, (viz., KKU-055, KKU-100, KKU-139, KKU-213, and KKU-214), and two immortalized cell lines (viz, MMNK-1 and NIH3T3) treated with various concentration of PL (0, 2.5, 5, 10, and 15 µM) for 48 h. Cell growth was suppressed with PL treatment in all cell lines in dose-dependent manners (Figure 4.1 (A)). KKU-055 was most sensitive whereas KKU-100 and NIH3T3 was least sensitive in among all cell lines. The IC_{50} values of PL using CompuSyn software at 48 h were 4.21, 5.28, 6.26, 8.83, and 15.92 µM for CCA cell lines (KKU-055, KKU-213, KKU-214, KKU-139, and KKU-100, respectively) and were 5.75 and 12.74 µM for MMNK1 and NIH3T3. Based on the IC_{50} values, 10 and 20 µM were considered effective doses for subsequent experiments since those concentrations were sufficient to suppress growth of all CCA cell lines to <50% of the control. The time-dependent cytotoxic effect of PL on KKU-055 and KKU-213 was demonstrated (Figure 4.1 (B)).

We further investigated the possibility of apoptosis as a mechanism of PL cytotoxicity. First, we examined the caspase-dependent pathway markers and non-caspase protease in CCA cell lines in the presence of 10 µM of PL, at various times. Western blot analyses showed that the activation of caspase 3 and BAX in KKU-055 indeed increased in response to the PL treatments especially at 12 and 24 h but this effect was not seen in KKU-100. In addition, cleavage of the non-caspase protease, PARP, was also observed in a time dependent manner (3, 6, 12, and 24 h).

Moreover, there was no activation of caspase 3 and cleavage PARP in NIH3T3 treated with PL at 10 μM. Taken together, PL inhibited growth and induced apoptosis in the most of CCA cell lines except KKU-100 and NIH3T3 by activation of the caspase pathway (Figure 4.1 (C) and Figure 4.2).

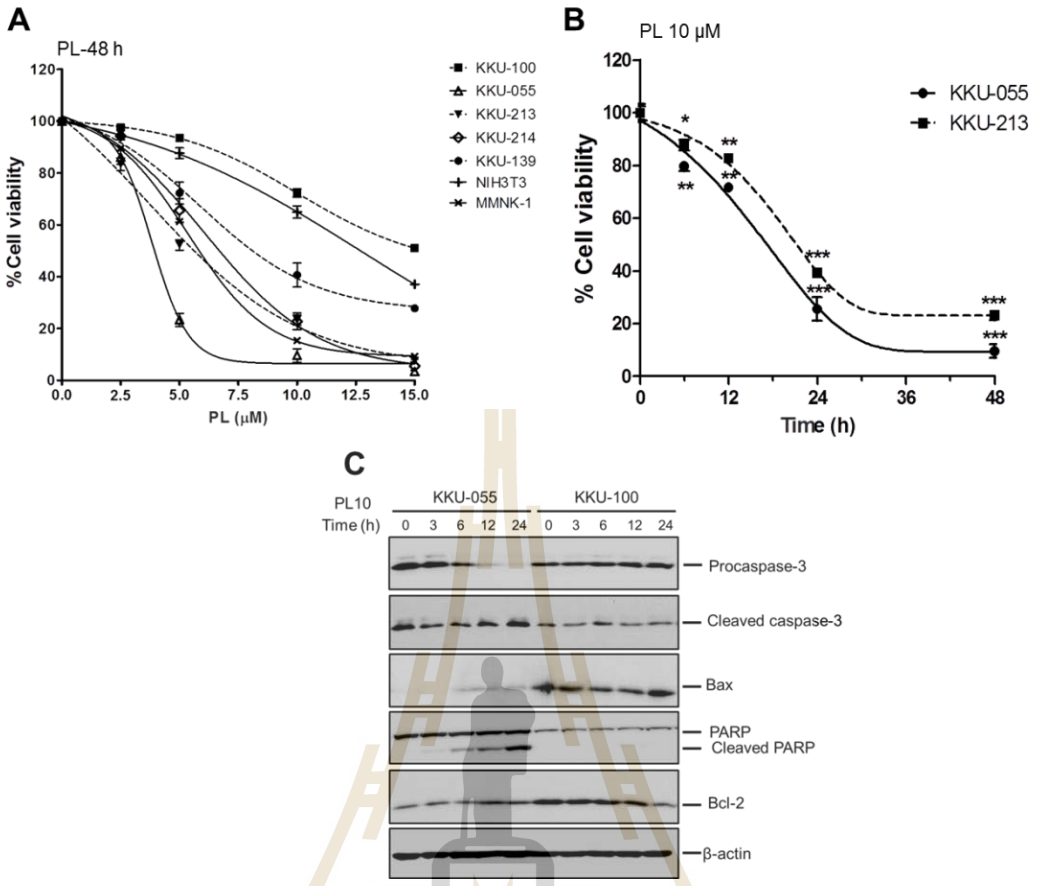

Figure 4.1 Piperlongumine (PL) inhibits cell proliferation and induces cell apoptosis via activation of caspace-3 and PARP. (A) Five CCA cell lines (KKU-M055, KKU-M213, KKU-M214, KKU-M139, and KKU-100) and two immnortalized cell lines (MMNK1 and NIH3T3) were treated with various concentration of PL for 48 h. (B) Representative IC_{50} values of PL were used to treated KKU-M055 and KKU-M213 for 6, 12, 18, and 24 h. Percentages of cell viability relative to control (without PL) are shown as mean ±SD from three independent experiments. *P<0.05. (C) KKU-M055 and KKU-100 were treated with PL at 10 μM for 0, 3, 6, 12, and 24 h. Activation of caspase-3, PARP, and expression levels of Bax, and Bcl-2 proteins were examined using western blot analysis. *β*-actin was used as an equal loading control for normalization.

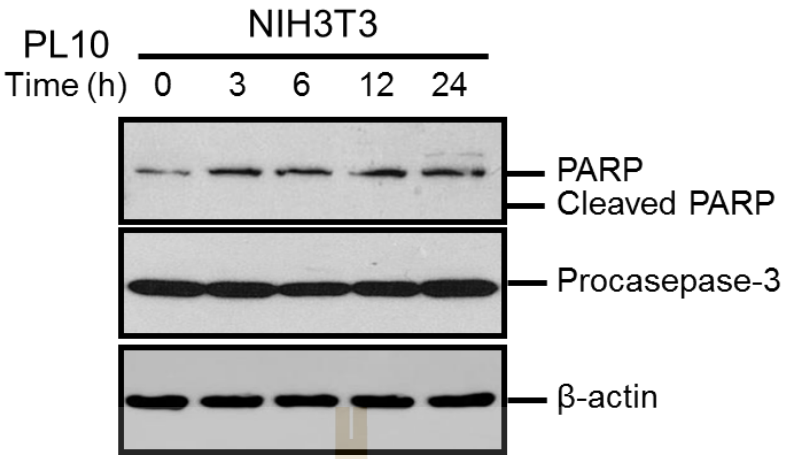

Figure 4.2 Western blot analysis of PL treated with NIH3T3 cell. NIH3T3 was treated with PL at 10 μM for 0, 3, 6, 12, and 24 h. Activation of caspase-3, and PARP were examined using western blot analysis. *β*-actin was used as an equal loading control for normalization.

4.3.2 Piperlongumine increases ROS accumulation in CCA cells

We subsequently investigated whether PL induces CCA cell death by generating and accumulating ROS in the cells, and that—as previous studies have demonstrated—ROS accumulation is required for PL's anti-tumor activity in various cancer cells (Chen *et al.*, 2015; Jin *et al.*, 2014; Q. R. Liu *et al.*, 2014; Raj *et al.*, 2011). The fluorescent intensity of DCFH-DA—a specific ROS indicator—was used to determine the ROS accumulation in CCA cell lines by flow cytometry. The fluorescent intensity of DCFH-DA was increased in KKU-055 and KKU-214 in a dose-dependent manner (10 and 20 μM) at 2 h of PL treatment (Figure 4.3). Statistical analyses demonstrated that the fluorescent intensity of DCFH-DA was significantly increased in KKU-055 and KKU-214 at 10 and 20 μM but the effect of

PL on ROS accumulation in KKU-100 was significant at 20 μM of PL treatment. Pre-incubation with 3 mM of N-acetyl-cysteine (NAC)—a selective ROS scavenger—absolutely abolished ROS generation and accumulation in all CCA cells at 2 h of PL treatment at 20 μM (Figure 4.3). Pre-treatment with NAC completely reversed the effect of PL-induced cell death in CCA cells at 24 h of PL treatment at 10 and 20 μM (Figure 4.4 (A)). Similar ROS scavenging effect was found in pre-treatment with GSH, but not by ascorbic acid (VitC) (Figure 4.4 (B)). Statistical analyses showed that the percentages of cell viability were significantly increased in KKU-055 cell with pre-incubation of NAC or GSH followed with PL treatment at 10 and 20 μM, but not by ascorbic acid (Figure 4.4 (C)). The results of the present study suggest that PL-induced CCA cells death are mediated by ROS generation.

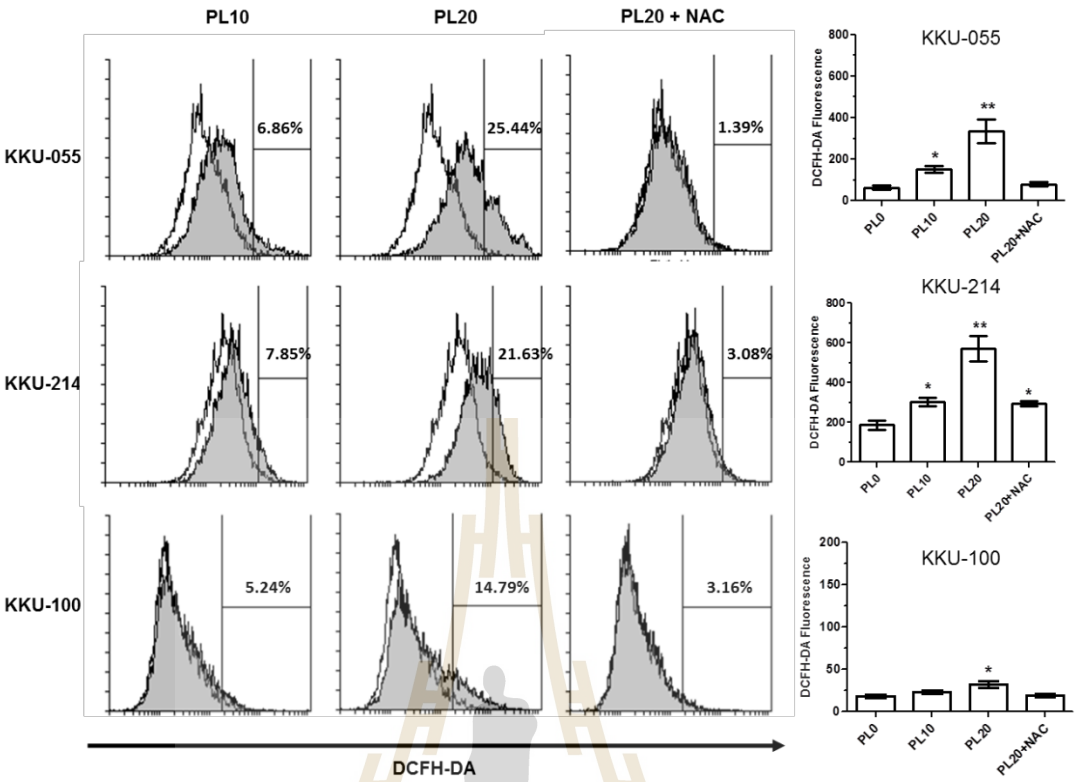

Figure 4.3 Piperlongumine induces ROS accumulation in CCA cell lines. Three CCA cell lines (KKU-M055, KKU-M214, and KKU-100) were treated with PL at 10 and 20 µM or DMSO for 2 h. Cell lines were also pretreated with NAC (3 mM) for 1 h before treating with PL (20 µM) for 2 h. ROS levels were measured by flow cytometry using DCFH-DA. Left panel shows the comparison of DCFH-DA fluorescence between CCA cell lines with DMSO treatment (open histogram) and PL-treated CCA cells (closed histogram). Right panel represents the mean DCFH-DA fluorescence intensity using Flowing software. *P<0.05, **P<0.01 relative to control (DMSO).

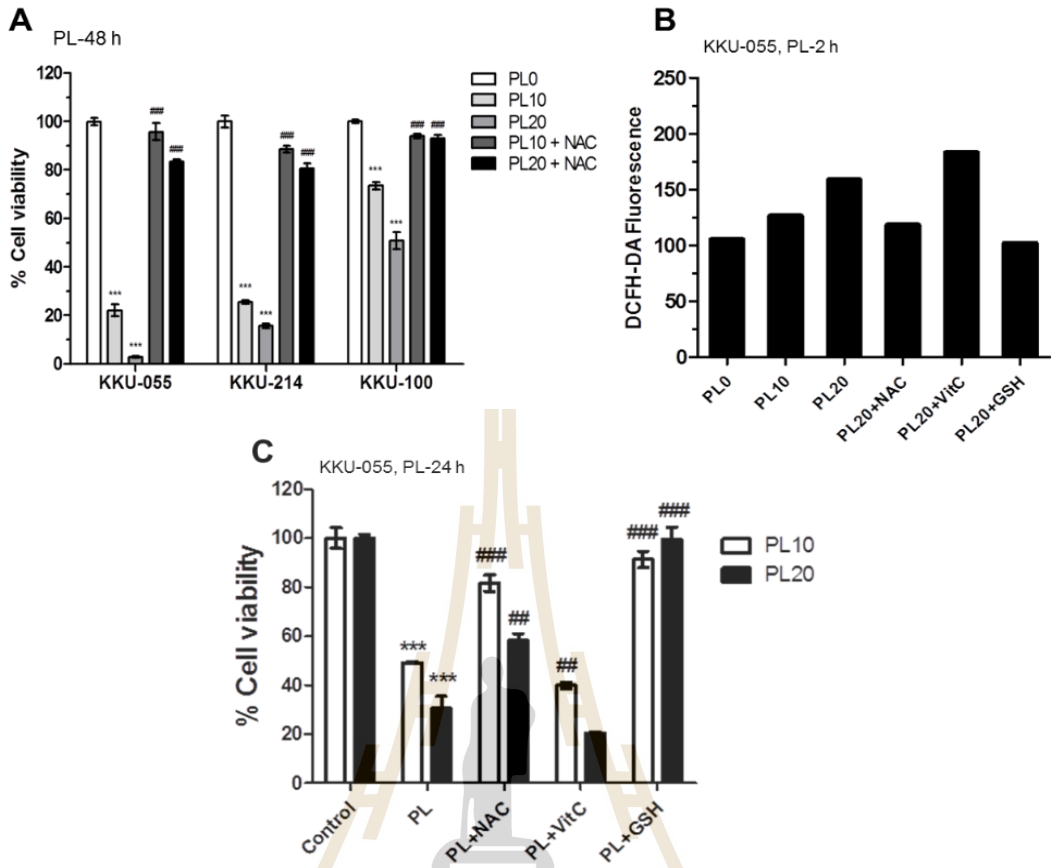

Figure 4.4 PL-effect in CCA is mediated by ROS generation. (A) KKU-M055, KKU-M214, and KKU-100 were grown in 96-well plates for 24 h then pretreated with NAC and followed by PL treatment at 10 and 20 μM for 48 h. (B) KKU-055 was pretreated with NAC, GSH, or ascorbic acid (VitC) for 1 h before treatment with PL (10 and 20 μM) for 2 h. ROS levels were measured by flow cytometry using DCFH-DA. (C) KKU-055 was grown in 96-well plates for 24 h then pretreated with NAC, GSH, or ascorbic acid and followed by PL treatment at 10 and 20 μM for 48 h. Cell viability was measured by SRB assay. All ROS inhibitors were used at 3 mM and pretreated 1 h before treatment with PL. **P<0.01, ***P<0.001 relative to control (DMSO); ##P<0.01, ###P<0.001 relative to PL at 10 or PL at 20 μM. All data values represented mean ± SEM from three independent experiments.

4.3.3 Piperlongumine induces G2/M phase arrest and cell apoptosis

In this series of experiments, we examined whether the effect of PL led to an alteration in the cell cycle and promoted cell apoptosis in CCA. Cell cycle analysis revealed a major accumulation of G2/M cell populations in KKU-055 (from 36 to 63%) and KKU-214 (from 8 to 49%) in a dose-dependent manner (5 and 10 μM) at 24 h of PL treatment (Figure 4.5). There was, however, no alteration in the cell cycle of KKU-100 after PL treatment (Figure 4.5). In addition, the effect of PL was evidently enhanced at 48 h of PL treatment by increasing cell population in the sub-G1 phase in KKU-055 (Figure 4.6). Pre-treatment with NAC completely reversed the effect of PL-induced cell cycle progression at the G2/M phase in KKU-055 (Figure 4.6). Further, the number of apoptotic cells as shown by annexin V and PI staining (Annexin V and PI positive cells) was significantly increased in KKU-055 (from 3 to 34%) in a dose-dependent manner (2.5, 5, 10 μM) at 48 h of PL treatment and the effect was blocked by co-exposure to NAC (Figure 4.7).

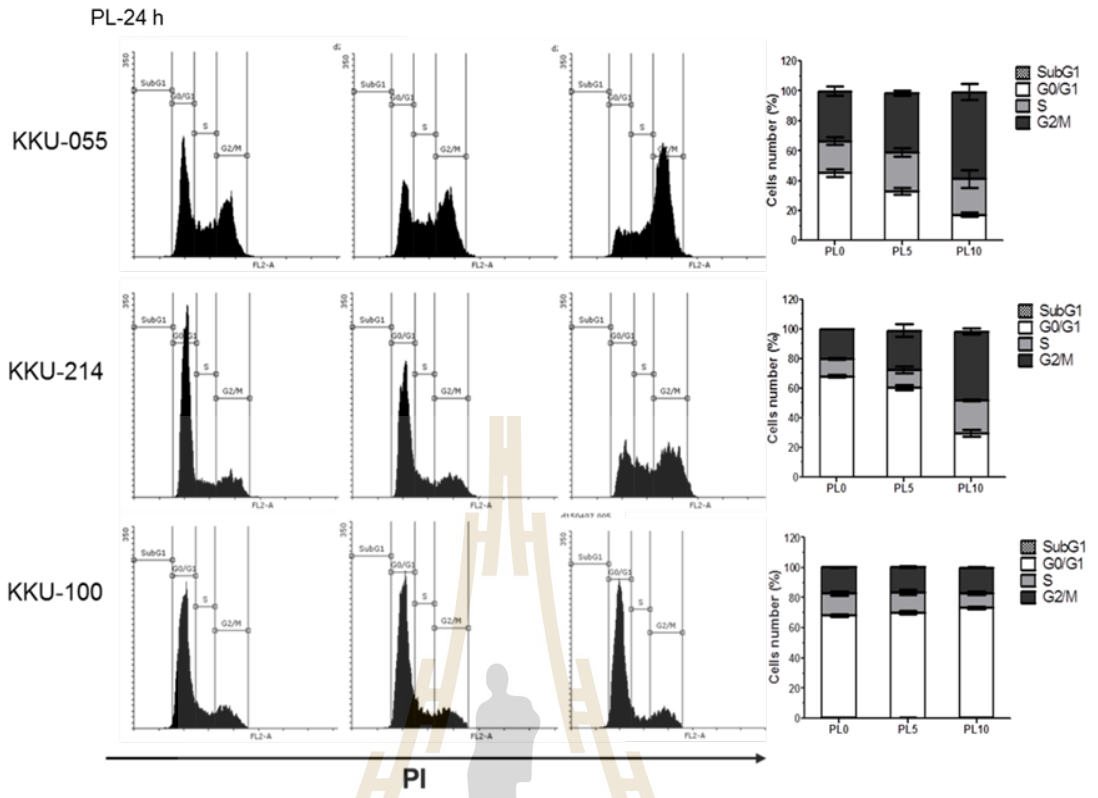

Figure 4.5 PL induces G2/M phase arrest in CCA cell lines. KKU-M055, KKU-M214, and KKU-100 were treated with 0, 5, 10 μM of PL or DMSO for 24 h. DNA content was analyzed by flow cytometer using PI staining (Left panel, representative pictures of cell cycle analysis; Right panel, the summary of percentages of the cells in sub G1, G0/G1, S, and G2/M phases). The experiments were done three times independently and all values are means ±SEM.

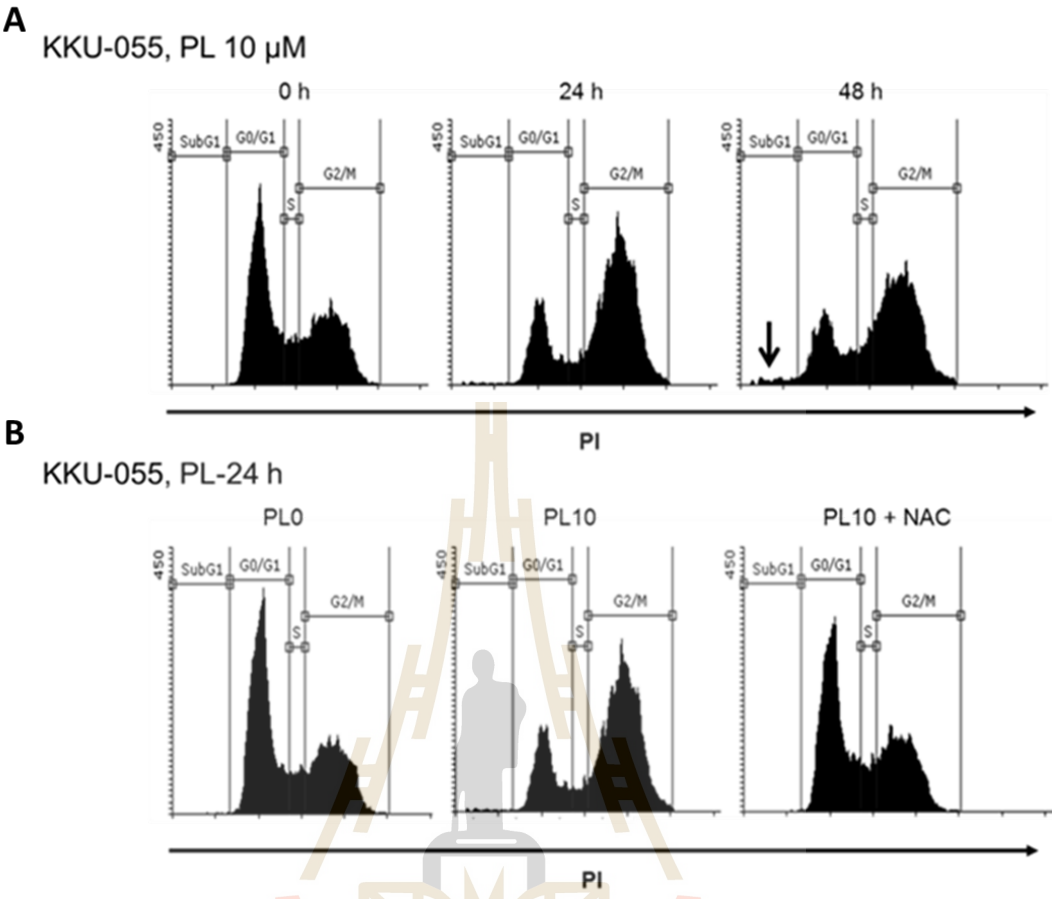

Figure 4.6 PL induced G2/M phase arrest and increased subG1 population in KKU-055. DNA content was analyzed by flow cytometer using PI staining. (A) Cell cycle analysis for KKU-055 treated with PL at 10 μM for 24 and 48 h. The arrow indicated the sub-G1 population. (B) Cell cycle analysis for KKU-055 treated with PL at 10 μM or pretreated with 3 mM NAC for 1 h.

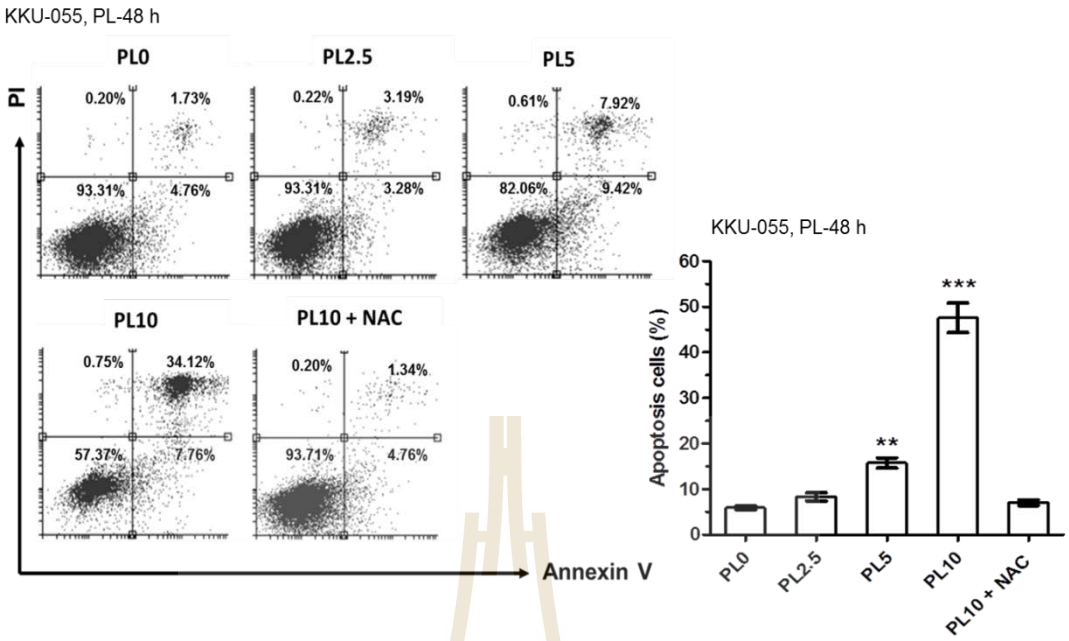

Figure 4.7 PL induces CCA cell apoptosis. KKU-M055 was treated with various concentration of PL and was also pretreated with NAC (3mM) for 1 h before treatment with PL (10 µM) for 48 h. Apoptotic cells were evaluated using Annexin V-AlexaFluor 488/PI staining and analysis using flow cytometry. Representative dot plots demonstrated the percentage of early apoptotic cells on lower right quadrant and late apoptotic cells on upper right quadrant) at various concentrations of PL (Left panel, representative pictures of cell apoptosis analysis; Right panel, the summary of the percentage of apoptotic cells in PL-treated KKU-M055 at various concentrations). **P<0.01; ***P<0.001 relative to control (DMSO). All values represented mean ±SEM of three independent experiments.

4.3.4 Piperlongumine induces cell death through ROS-mediated JNK/ERK activation

Reactive oxygen species (ROS) have been reported to participate in the regulation of apoptosis through the activation of ERKs, JNKs, and p38; via mitogen-activated protein kinases (MAPKs) pathways (Son *et al.*, 2011). Next, we determined the effect of PL on JNK and ERK activation. Western blot analysis showed a dosage-dependent increase in both p-JNK and p-ERK in KKU-055 (5, 10, and 20 μM) and KKU-100 (10 and 20 μM) at 1 h after PL treatment. Pre-treatment at 10 μM with NAC completely blocked PL-induced activation of JNK and ERK in both KKU-055 and KKU-100. Our present study demonstrated that the cell viability of KKU-100 was affected at the high concentrations tested (viz., 10 and 15 μM PL) (Figure 4.1). In addition, activation of Akt has been demonstrated to promote cellular growth and anti-proliferation in several types of cancer (Majewski *et al.*, 2004). Thus, we further assessed whether Akt activation also plays a role in regulating apoptotic cell death in CCA during PL treatment. PL increased the expression level of p-Akt and Akt downstream effector, Bad, in only KKU-100 in a dose-dependent manner and that effect was blocked by co-exposure to NAC. In addition, the up-regulation of anti-apoptotic protein Bcl-2 was clearly detected in KKU-100 in a dose-dependent manner. This result indicates that KKU-100 has a high ability to suppress PL-induced cell death by activating Akt and Bcl-2. Since, Roh *et al.* (2014) demonstrated that PL has anti-cancer activity regardless of p53 mutational status. We further showed that p21 expression in KKU-055 was moderately increased after PL treatment, however, there was no change of p53 expression in both CCA cells after PL treatment (Figure 4.8 (A)). We then confirmed the association between ROS elevation and JNK-ERK

activation in PL-induced CCA apoptosis. The specific inhibitor for JNK (SP600125) or ERK (U0126) was co-incubated with PL at 10 μM in KKU-055 and cell viability was determined using the SRB assay. The treatment with SP600125 significantly attenuated PL-reduced cell viability while treatment with U026 slightly prevented PL-induced cell apoptosis in KKU-055 (Figure 4.8 (B)). Taken together, the results suggest that PL induces CCA cell death through the activation of JNK and ERK.

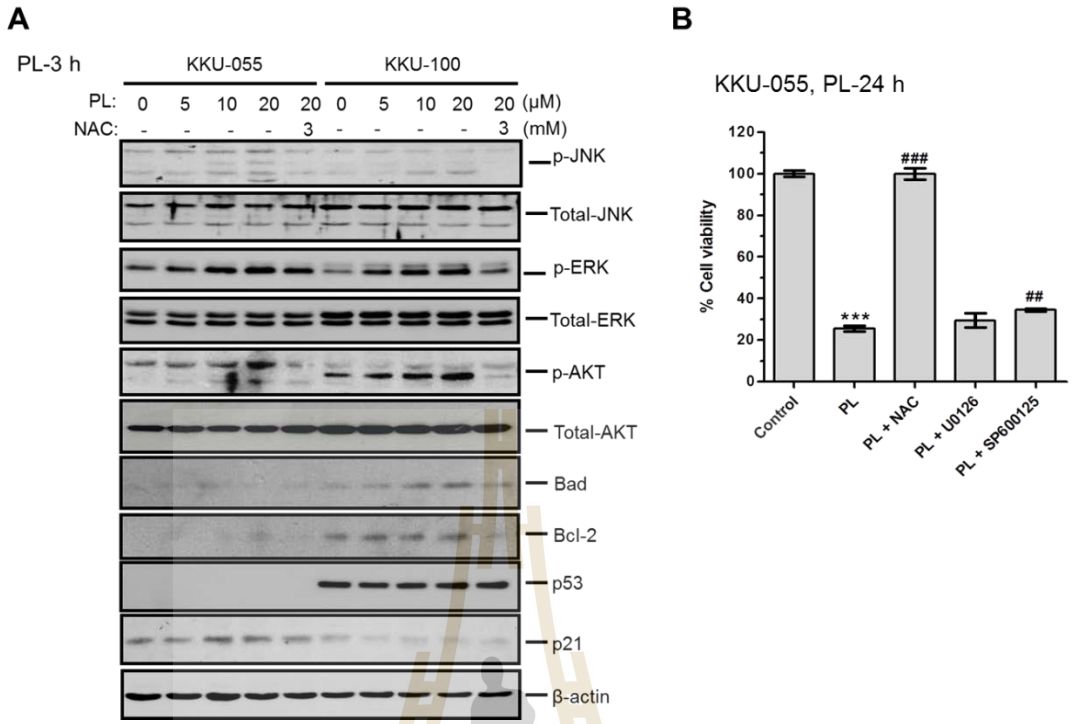

Figure 4.8 Piperlongumine triggers cell death in CCA via ROS-JNK-ERK pathways. (A) KKU-055 and KKU-100 were treated with various concentration of PL for 3 h and also pretreated with NAC (3mM) for 1 h before treating with PL (20 μM) for 3 h. The whole cell lysates were subjected to western blotting and probed with an antibody specific to JNK, ERK, Akt, Bad, Bcl-2, p53, p21 and β-actin. (B) Effect of NAC, ERK (U0126), or JNK (SP600125) inhibitors on the PL-induced cell death. KKU-055 was pretreated with each inhibitor for 1 h and then treated with PL at 10 μM for 24 h. Cell viability was measured by SRB assays. All values represented mean ±SEM of three independent experiments. ***P<0.001 relative to control; ##P<0.01 or ###P<0.001 relative to PL at 10 μM.

4.3.5 Differential responses of CCA cells to Piperlongumine depend on their antioxidant activity

Since antioxidants provide crucial survival and proliferation signaling to cancer cells under oxidative stress (Gorrini *et al.*, 2013); we, therefore, examined whether the effect of PL can alter the expression of antioxidant genes in CCA cell lines. We first determined the mRNA expression levels of 5 antioxidant genes including protein deglycase DJ-1 (*PARK1*), thioredoxin (*TXN*), NAD(P)H-quinone oxidoreductase 1 (*NQO1*), heme oxygenase-1 (*HO-1*), superoxide dismutase 2 (*SOD2*), and glutathione S-transferase P1 (*GSTP1*) in 5 CCA cell lines by RT-qPCR. High antioxidant capacity was detected in KKU-100, which had high expression levels of all 6 antioxidant genes (including PARK1, TXN, NQO1, HO-1, SOD2, and GSTP1), while at least 3 genes (i.e., PARK1, TXN, and GSTP1) showed high expression in KKU-055, KKU-213, KKU-214 and KKU-139 (Figure 4.9). High expression levels of NQO1, HO-1, and SOD2 were found only in KKU-100; this result suggests that these three genes may be important antioxidant genes which counteract elevated ROS levels from PL treatment in CCA. We investigated the mRNA expression level of NQO1, HO-1 and SOD2 in KKU-214 and KKU-100 treated with 10 μM PL or DMSO for 6, 12, and 24 h using RT-qPCR. Expression of NQO1, HO-1, and SOD2 were up-regulated by PL in both CCA cells, but HO-1 expression was shown to have strong antioxidant capacity to PL (Figure 4.10 (A)). Subsequently, pre-incubation with ZnPP (HO-1 inhibitor) significantly decreased cell viability in KKU-100 at 24 h of PL treatment at 10 and 20 μM (Figure 4.10 (B)).

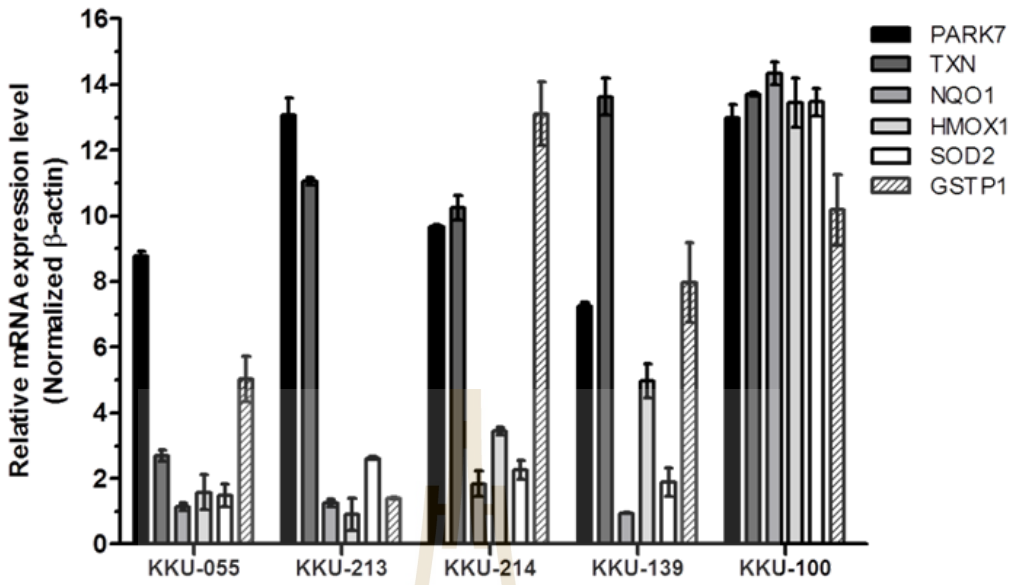

Figure 4.9 Antioxidant profiles of five human CCA cell lines. The mRNA expressions of 5 antioxidant genes in all cell lines were analyzed by qRT-PCR and normalized using β-actin as reference gene.

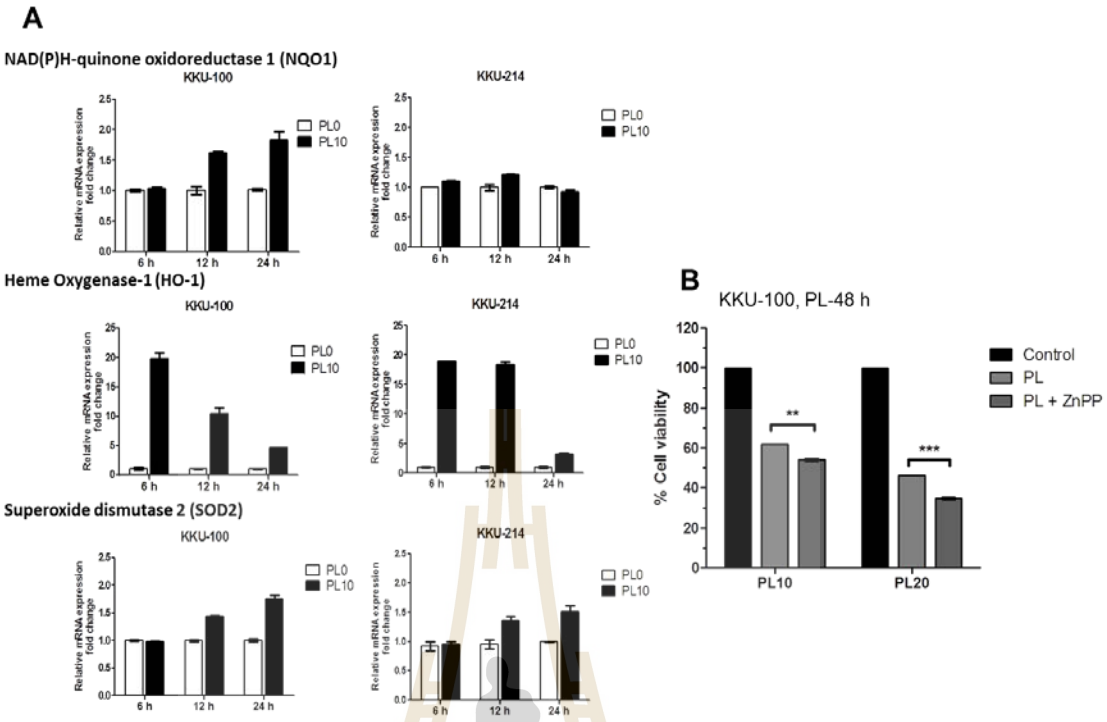

Figure 4.10 Antioxidant capacity determines the differential response of CCA cell lines to piperlongumine. KKU-100 and KKU-M214 were treated with PL at 10 µM or DMSO (control) for 6, 12, and 24 h and then were determined the mRNA expressions of NQO1, HO-1, and SOD2 by qRT-PCR. The relative mRNA expression of these three genes between with or without PL treatment was calculated using $2^{-\Delta\Delta Ct}$. (B) KKU-100 was treated with PL (at 10 and 20 µM) in the absence or presence of ZnPP for 24 h. Cell viability was measured by SRB assays. The data are presented as the mean ±SEM of three independent experiments.

4.4 Conclusion and discussion

The induction of oxidative stress by exogenous ROS generation treatment can now be considered an effective cancer therapy because this approach selectively kills cancer cells without affecting normal cells (J. Liu and Wang, 2015). Piperlongumine (PL) is a biologically active plant alkaloid, which functions as a ROS inducer in anticancer therapy. PL has been shown to increase ROS levels and induce selective cell death in several cancer cells but not in normal cells (Raj *et al.*, 2011). The anti-tumor effects of PL and its molecular mechanisms in CCA, however, still need to be elucidated. In our current study, we demonstrated that PL markedly limited the growth of CCA cell lines by inducing G2/M phase arrest. PL activated ROS accumulation, leading to CCA cell apoptosis via the activation of the JNK/ERK pathway. In addition, whenever there was less response of CCA cells to PL, it was associated with increasing survival; signaled by Akt activation and up-regulated antioxidant capacity especially the activity of heamoxygenase-1 (HO-1).

PL inhibited growth in various CCA cell lines including KKU-055, KKU-213, KKU-214, and KKU-139 with an IC50 of less than 10 μM. This finding agrees with several independent studies in which PL has the ability to suppress growth of a variety of cancers (including CCA) with a similar effective dose (i.e., head and neck (Roh *et al.*, 2014), hepatocellular carcinoma (Chen *et al.*, 2015), breast (Lee *et al.*, 2015), gastric (Duan *et al.*, 2016), and lung cancers (Zheng *et al.*, 2016). PL, however, had little cytotoxicity against the highly malignant KKU-100 which is identified as a poorly differentiated CCA. This is perhaps due to the presence of high antioxidant capacity in KKU-100 (Figure 4.9). Lee et al. recently demonstrated that HO-1 is a key cytoprotective protein involved in determining the selective effect of PL on

cancer cell apoptosis in breast cancer (Lee *et al.*, 2015). This evidence agrees with our result that high expression of HO-1 was significantly demonstrated in KKU-100 and treatment with ZnPP (HO-1 inhibitor) significantly decreased the ability of PL to reduce cell viability in KKU-100. In case of sensitivity to PL, several studies have been reported that PL has no effect on primary culture cells but the increment of sensitivity to PL will be associated with the degree of cell transformation (Raj *et al.*, 2011). This evidence agrees with our study, in which MMNK1was more sensitive than NIH3T3. This perhaps due to 1) origin of MMNK1 is SV40T-transduced human liver OUMS-21 cells and is transduced another time with retroviral vector encoding hTERT to improve the immortalization and life span, and 2) developed bile duct-like structure in the spleen of immunodeficient mice (Maruyama *et al.*, 2004) whereas NIH3T3 is a tetraploid cell lines, which immortalize spontaneously. Furthermore, our result showed that PL increased ROS in CCA cells, while ROS accumulation led to suppressed tumor growth and subsequently induced cell apoptosis via the activation of caspase-3 and PARP in a dose-dependent manner. In addition, the ROS scavenger NAC and GSH completely reversed the effect of PL on anti-proliferation and PL-induced apoptosis. These results indicate that the anti-tumor activity of PL was primarily caused by increasing ROS generation.

It has been demonstrated that ROSs cause a wide range of adaptive cellular responses ranging from transient growth arrest to permanent growth arrest, to apoptosis or to necrosis, depending on the level of ROS (Boonstra and Post, 2004). In the current study, PL clearly increased ROS accumulation and cell cycle arrest at the G2/M phase in both KKU-055 and KKU-214, which were more susceptible to the PL treatment than KKU-100 at 24 h. Prolonged exposure to PL at 48 h enhanced the

numbers of the sub-G1 cell population and cell apoptosis in KKU-055 and that that effect was completely reversed by co-exposure to NAC. Our findings are consistent with studies on gastric cancer and oral squamous cell carcinoma in which PL activated cell apoptosis by inducing G2/M arrest (Duan *et al.*, 2016; Roh *et al.*, 2014). Several studies have demonstrated that elevation of ROS causes G2/M phase arrest via up-regulation of p21 in the p53-independent pathway. The function of p21 can directly inhibit the cyclin dependent kinase Cdc2, which is the essential protein for entry into mitosis (Taylor and Stark, 2001). This effect of ROS during the G2 phase is accompanied by an increase in both ERK and p38 activity (Bijur *et al.*, 1999; Y. W. Chung *et al.*, 2002; Thorn *et al.*, 2001; Zhang *et al.*, 2001). In the current study, p53 independent expression of p21 was moderately increased after PL treatment and a similar observation was made vis-à-vis the expression of p-ERK in KKU-055, suggesting that the increasing level of p21 via ERK activation may contribute to PL-induced G2/M phase arrest.

Generally, excessive generation of ROS in cells leads to activation of MAPK pathways, including ERKs, JNKs, or p38 MAPKs (Son *et al.*, 2011). In the present study, we provided convincing evidence that PL-induced apoptosis in CCA regulated via activating the JNK/ERK-signaling pathway. Our data demonstrated that PL induced prominent activation of JNK and ERK in a dose-dependent manner and this activation was suppressed by the ROS scavenger NAC. In addition, our results confirmed that PL induced both JNK and ERK in CCA through treatment with specific inhibitors. Moreover, the activation of Akt (which promotes cellular growth and proliferation as well as inhibition of apoptosis (Majewski *et al.*, 2004)) and increased Bcl-2 expression were observed in only KKU-100 after PL treatment. This

result might be the reason why KKU-100 had a low response to PL treatment and this finding is supported with the studies of CCA in which over-expression of Akt is associated with poor prognosis and the inhibition of Akt activation via the phosphoinocitide 3-kinase (PI3K) inhibitor results in increases apoptosis in the CCA resistant cells (J. Y. Chung *et al.*, 2009; Wilson *et al.*, 2015; Yoon *et al.*, 2011). Taken together, our findings in the present study show (a) that PL induced apoptosis in CCA via ROS-mediated JNK-ERK activation and (b) that the susceptibility of CCA to PL treatment may depend on the activation of Akt.

We next investigated whether PL induces the expression of antioxidant genes; thereby removing the cytotoxicity effect of PL. We identified the high level of expression of all 6 antioxidant genes including *PARK1*, *TXN*, *NQO1*, *HO-1*, *SOD2*, and *GSTP1* in KKU-100, while at least 3 genes (viz., *PARK1*, *TXN*, and *GSTP1*) showed high expression in KKU-055, KKU-213, KKU-214, and KKU-139. Up-regulation of NQO1, HO-1, and SOD2 were detected in both KKU-100 and KKU-214 after PL treatment. HO-1 expression had a strong response to PL and co-exposure with ZnPP (an HO-1 inhibitor) significantly decreased cell viability in KKU-100 after PL treatment. These results suggest that HO-1 may play a critical role in cyto-protection in CCA cell lines against PL treatment. Our findings agree with several studies on HO-1 vis-à-vis CCA; in that high expression of the HO-1 protein is associated with poor survival of CCA patients and suppression of HO-1 increases the sensitivity of CCA cell lines to chemotherapeutic agents, which enhances CCA cell apoptosis via the p53-dependent pathway (Kongpetch *et al.*, 2012; Kongpetch *et al.*, 2016). The mechanism underlying PL-induced antioxidant gene expression in CCA,

however, remains unclear. Further investigation is required to clarify the role of antioxidant genes upon PL treatment in CCA.

In conclusion, we demonstrated that PL exhibits anti-tumor activity against CCA cell lines by inducing ROS production and enhancing G2/M phase arrest and cell apoptosis. The study also revealed that PL can trigger CCA cell death via JNK and ERK activation. Moreover, we demonstrated for the first time that the sensitivity of CCA cell lines to PL treatment relies on their antioxidant activity and the status of the Akt activation pathway. Our data suggests that PL is a potential chemotherapeutic drug for treating CCA.

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CHAPTER V

CONCLUSION

Cholangiocarcinoma (CCA) is characterized by very poor prognosis and virtually no response to current treatment strategies. Surgical resection is the only effective treatment but is not frequently applicable because late diagnosis. In addition, resistance to common chemotherapy is characterized as CCA behavior. CCA Biomarkers for screening program and for follow-up of categories at risk are under investigation. For all these considerations, CCA should merit much more scientific attention.

In this study, the roles of YKL-40 in CCA and the potential used of piperlongumine (PL) as anti-cancer agent for CCA were emphasized. In chapter III, the elevated plasma level of YKL-40 in CCA patients was evident. The elevated plasma YKL-40 level was only associated with short overall patient survival. YKL-40 rarely expressed in CCA cells, but highly expressed in liver cells and infiltrating inflammatory cells, especially macrophages and connective tissue at intratumoral stroma. With these evidences implied that YKL-40 may be a key molecule in the process of CCA progression. The functional studies were also showed the role of YKL-40 on autocrine and paracrine functions to promoted CCA growth and metastasis. The study reported in Chapter IV highlighted the anti-cancer activity of PL on CCA cell line. PL killed all CCA cell lines and two immortalized cell lines, which KKU-055 was most sensitive whereas KKU-100 and immortalize NIH3T3

cells was least sensitive in among all cell lines. PL induced CCA cell death through the activation of apoptosis pathway and induction of cell cycle arrest. The death activation signal was mediated by PL-induced ROS generation and activation of JNK-ERK pathway. Lastly, the sensitivity of CCA cell lines to PL treatment may depend on their antioxidant activity and the status of the Akt activation pathway.

All together, this study has demonstrated the significance of YKL-40 in CCA progression. The prognostic value of plasma YKL-40 was highlighted. In addition, PL has anti-cancer activity in CCA and can be used as alternative or adjuvant chemotherapeutic agents for treating CCA.

APPENDIX

APPENDIX

PUBLICATION

Publication output

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ORIGINAL ARTICLE

YKL-40/chitinase-3-like protein 1 is associated with poor prognosis and promotes cell growth and migration of cholangiocarcinoma

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Abstract YKL-40, a chitinase-like glycoprotein, is expressed at a high level in cancer patients. Its exact function is unknown and is the subject of current investigation. Here, we report the correlation of plasma YKL-40 levels with clinicopathological features of cholangiocarcinoma (CCA), a lethal bile duct cancer, particularly prevalent in Northeastern Thailand. Statistical analysis of plasma YKL-40 concentrations in 57 CCA patients and 41 normal healthy subjects gave a median value of 169.5 ng/mL for CCA patients compared with 46.9 ng/mL for the control subjects ($P < 0.0001$). There was no significant association of plasma YKL-40 levels with patient age, tumor grade, or histology type. However, Kaplan-Meier analysis suggested that the elevated plasma YKL-40 level was particularly associated with short survival in CCA patients ($P = 0.038$). Immunohistochemical examination of 34 CCA tissues revealed low expression of YKL-40 in CCA cells, but high expression in adjacent intratumoral stroma, liver, and connective tissues. Univariate analysis showed significant association of the intratumoral YKL-40 expression in CCA tissues with the non-papillary type CCA. Addition of rYKL-40 in the culture medium and transient expression of YKL-40 in CCA cell lines were shown to promote the growth and migration of the tumor cells, and that YKL-40 interacted with a cell-surface receptor involved in the Akt/Erk-mediated pathway. In conclusion, our results support the proposal of YKL-40 as a new candidate prognostic biomarker for cancer diseases.

Keywords Chitinase-3-like protein 1 · Cholangiocarcinoma · GH-18 glycosyl hydrolases · Prognostic marker · YKL-40

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Abbreviations

pAkt	Phosphorylated (active) AKT/PKB (protein kinase B) kinase
BSA	Bovine serum albumin
CCA	Cholangiocarcinoma
pERK	Phosphorylated (active) extracellular signal-regulated kinase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HRP	Horseradish peroxidase
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline tween
rpm	Revolutions per minute

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Introduction

Cholangiocarcinoma (CCA) is a relatively rare adenocarcinoma of mutated epithelial cells that originate in the intrahepatic and extrahepatic bile ducts. CCA is one of the most aggressive forms of cancer, with a high mortality rate [1-3], and is endemic in East and Southeast Asian countries [4]. The incidence is particularly high in Northeastern Thailand, with 85 in 100,000 cases. Definite risk factors for CCA are primary sclerosing cholangitis, choledochal cysts, congenital liver malformations, hepatolithiasis, and hepatitis C virus infection [5]. However, long-term exposure to dietary nitrosamines from fermented fish together with chronic infection with the liver fluke *Opisthorchis viverrini* (OV) are known to be the leading factors for Thai CCA patients [6-8]. Although symptoms of advanced-stage CCA include abnormal liver function tests, abdominal pain, jaundice, and weight loss, early-stage CCA is most often asymptomatic, making early diagnosis of this cancer ineffective. Currently, several serum biomarkers, including CEA, CA19-9, ALP, MUC5AC, and CA-S121, have been used in the CCA screening test [9, 10]. However, such biomarkers still provide different diagnostic and prognostic values. To increase the accuracy of CCA diagnosis and improve prognosis, either a highly specific CCA biomarker or a panel of screening and prognostic biomarkers is required.

YKL-40 (also called chitinase-3-like 1 or CHI3L1) is a 40-kDa glycoprotein originally secreted by monocytes, macrophages, neutrophils, cultured chondrocytes, and synovial cells [11-13]. YKL-40 is classified in the carbohydrate-active enzyme (CAZy) database (www.cazy.org) as a chitinase-like protein that belongs to a family of 18 catalytically inactive glycoside hydrolases (GH-18) [14, 15]. The overall structure of YKL-40 is similar to that of GH-18 glycoside hydrolases [16-19], comprising a typical $(\beta/\alpha)_8$ TIM barrel domain that contains a chitin-binding groove, responsible for binding to chitooligosaccharides [20]. In humans, YKL-40 has key functions in the control of cell proliferation and survival, as well as promoting cancer cell proliferation and tumor angiogenesis [21-23]. Prominent expression of YKL-40 has been reported in cancer cell lines [21, 24, 25], and high serum YKL-40 levels have been detected in patients with several types of cancer [26-30]. Earlier reports associated the highest levels of serum YKL-40 in cancer patients with bad prognosis and poor overall survival. Hence, YKL-40 has received much attention as an independent prognostic biomarker for cancer [31, 32].

In this study, we systematically employed several types of statistical analysis to evaluate the correlations between plasma YKL-40 concentrations in CCA patients with clinicopathological parameters, in order to address the significance of elevated YKL-40 levels in CCA. We also demonstrated, using a mammalian cell line system, the biological effects of YKL-40 on cell proliferation and migration of CCA. Based on the results obtained from this study, we provide a further outlook on whether YKL-40 could be used as a useful prognostic biomarker for the monitoring of recurrent CCA.

Materials and methods

Ethics

Written informed consent was obtained from all patients. The study has been approved by the Ethics Committee for Human Research, Suranaree University of Technology (EC-55-17).

Subjects and samples

Plasma of healthy subjects ($n = 41$) and CCA patients ($n = 57$) were prepared freshly from whole blood specimens that were collected and treated with heparin to prevent clotting. The CCA plasma samples were obtained from the Specimen Bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand, and all had histologically proven to be CCA. The reference range for plasma YKL-40 was determined in 41 healthy individuals (18 woman and 39 men) who visited Suranaree University of Technology Medical Center for annual health checkups and were sex- and age-matched (median age 56 years, range 34-72 years) with the patients with CCA. These individuals did not receive medication and had no signs or symptoms of cancer or chronic diseases. All plasma samples were stored at −40 °C until used. For immunochemical study, only 34 paraffin-embedded tissue blocks out of 57 CCA patients were available and were then tested for intracellular expression of YKL-40 in CCA tissues. Age, gender, histological grading, and pathological tumor-node-metastasis staging (pTNM) (according to the American Joint Committee on Cancer classification and staging system [33] were evaluated by reviewing the medical charts and pathological records.

Determination of plasma YKL-40 concentrations by competitive ELISA

Plasma samples were collected and handled according to standard operating procedures. YKL-40 concentrations in heparin-treated plasma were determined in duplicate. Concentrations of YKL-40 in plasma were measured using a commercially available Human Cartilage glycoprotein 39/YKL40 ELISA Kit (BlueGene Biotech Co., Ltd., Shanghai, China). Concentrations of standard YKL-40 protein were determined based on the principle of competitive binding described in the manufacturer's instructions. In brief, each well of a 96-well plate was pre-coated with 50 μL of the YKL40-HRP-conjugated polyclonal antibody. One hundred microliters of twofold dilutions of the standard YKL-40 solution or plasma sample were added, and the plate was

incubated for 1 h at 37 °C. The wells were washed five times, and then the antigen-antibody reaction was initiated by adding 100 μL of the substrate solution, 15 min at 25 °C in the dark. After termination of the reaction by adding 50 μL of the stop solution, product formation was monitored by measuring absorbance at a wavelength of 450 nm (A_{450}) using a MultiRead 400 Microplate Reader (Anthos Labtec Instruments, Walls, Austria). A calibration curve of A_{450} versus of YKL-40 concentration (0- 250 ng/mL) was constructed and used to determine the YKL-40 concentration in each sample. The detection limit of this commercial ELISA assay was found to be 0.1 ng/mL, with estimated intra- and inter-assay coefficients of variation of <10 %. Concentrations of plasma YKL-40 from all CCA samples were measured and analyzed simultaneously with protein standards, to minimize the inter-assay coefficient of variation.

Determination of cellular expression of YKL-40 in CCA tissue by immmunohistochemistry

The immunohistochemical experiments were carried out at room temperature. Specimens were fixed in 10 % neutral formalin buffer, embedded in paraffin, and cut into 5-μm-thick sections. Immunohistochemical staining was performed with rabbit anti-human YKL-40 polyclonal antibody (Quidel, San Diego, CA, USA) and detected by the immunoperoxidase method. In brief, each paraffin section was deparafinized, and the endogenous YKL-40 antigen was retrieved by boiling in a microwave oven for 10 min in 0.1 M citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked using 3 % hydrogen peroxide in methanol for 30 min, and non-specific binding blocked with normal horse serum (dilution 1:20) for 20 min. The section was then incubated with rabbit anti-human YKL-40 polyclonal antibody (dilution 1:100) overnight at room temperature and subsequently incubated with EnVision™/HRP, Rabbit (Dako, Carpinteria, CA, USA) according to the manufacturer's instructions. The stained section was visualized with 3,3′-diaminobenzidine-tetrahydrochloride (DAB) (Sigma-Aldrich, St. Louis, MO, USA) and counterstained with hematoxylin. The intensity of YKL-40-positive tumor cells was scored as follows: 0, no staining; 1+, light brown; and 2+, intense brown. All stained specimens were evaluated by two experts with no knowledge of prognosis or clinicopathological variables. To validate our evaluation method, we performed kappa statistical analysis available in SPSS 16.0, and the obtained kappa value of 0.932 indicated almost perfect agreement of the data presented by two different sources [34] For statistical analysis of the data, the scores 0 and 1+ were categorized as negative expression, while the score 2+ as positive expression.

Human cell culture

Two human CCA cell lines, KKU-M055 and KKU-M213, derived from primary tumors of CCA patients, were supplied by the Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Thailand. CCA cells were cultured in Ham's F12 Nutrient Mixture supplemented with 10 % fetal bovine serum (FBS), streptomycin (100 μg/mL), and amphotericin B (50 μg/mL). HEK-293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % FBS, streptomycin (100 μg/mL), and amphotericin B (50 μg/mL). All cell lines were cultured in a humidified 5 % CO_2 incubator at 37 °C.

Cloning, expression, and purification of recombinant YKL-40 (rYKL-40)

The nucleotide sequence of the human *CHI3L1* gene encoding YKL-40 was retrieved from the GenBank database under accession number M80927. A full-length cDNA corresponding to this gene was designed to be cloned into the pCMV/hygro vector, so as to be suitably expressed in a mammalian cell expression system. The *CHI3L1*/pCMV/hygro-His construct was generated and synthesized by a commercial source (Sino Biological Inc., Beijing, China). The recombinant YKL-40 protein (designated rYKL-40) produced from this construct contained an additional six histidine residues at the *C*-terminus, to aid its purification. For expression in HEK-293T cells, the transfected cells (1.5×10^6 cells/10 mL medium/10-cm dish) were grown overnight at 37 °C with 5 % CO_2. Lipofectamine™ 2000 and the recombinant cDNA were diluted in serum-free medium (ISF-1) (Biochrom, Berlin, Germany) and then combined in a ratio of 1:1 to obtain a final volume of 3 mL. The transfected cells were grown for 48 h at 37 °C in a 5 % CO_2 incubator, and afterwards the culture medium containing secreted rYKL-40 was harvested by centrifugation at 4500 rpm at 4 °C for 10 min, and the cells discarded. For protein purification, one-tenth volume of 10× binding buffer (500 mM NaH_2PO_4, 1.5 M NaCl, and 100 mM imidazole, pH 8.0) was added into 15 mL of the culture medium and then mixed with 100 μL of Ni-NTA beads (GenScript USA Inc., NJ, USA). The culture medium/bead suspension was rotated at 4 °C for 1 h to allow the rYKL-40 to bind to the magnetic beads, before centrifugation at 4500 rpm for 10 min. The beads were collected and then transferred into a 1.5-mL tube and washed four times, each with 0.5 mL wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 0.05 % Tween-20, pH 8.0) containing 20 mM imidazole, and centrifuged at 4000 rpm for 2 min. The bound rYKL-40 protein was eluted with 200 μL of 250 mM imidazole in wash buffer, and the beads removed by centrifugation at 4000 rpm for 2 min at 4 °C. The purity of the rYKL-40 protein was determined by SDS-PAGE analysis, and the protein

concentration was determined by Novagen® BCA Protein Assay Kit (EMD Millipore, Billerica, MA, USA). The freshly purified rYKL-40 was stored at −80 °C until used.

Generation of the new recombinant YKL-40 construct for transient expression in CCA cells

To generate the recombinant YKL-40 construct for transient expression in CCA cell lines, the full-length *CHI3L1* cDNA (1867 bp) encoding 383 amino acid residues of YKL-40 obtained as described above was amplified from a human cDNA template obtained from GenScript by PCR technique using the forward primer 5′-GCGGATCCGTCATGGGTGTGAAGGCGTCTCAA-3′ and the reverse primer 5′-GACTCGAGCGTTGCAGCGAGTGCATCCTT-3′. Sequences underlined indicate the restriction sites *Bam*HI and *Xho*I, respectively. PCR amplification was performed with iProof High-Fidelity DNA Polymerase (Bio-Rad Laboratories, Redmond, WA, USA) according to the manufacturer's instruction. The amplified DNA was cloned into pGEM-T-Easy (Promega, Madison, WI, USA) and then subcloned to pCMV6-entry (Origene, Singapore) that contained cytomegalovirus (CMV) and SV40 promotors for mammalian expression system. The correct nucleotide sequence of the newly generated construct was confirmed by automated sequencing (First BASE Laboratories Sdn Bhd, Selangor Darul Ehsan, Malaysia). The pCMV6/*CHI3L1* construct was further used to generate transient expression in two CCA cell lines (KKU-M055 and KKU-M213). Transfection of the recombinant plasmid into the target cells was performed following the protocol recommended by the manufacturer (Invitrogen). Briefly, CCA cells (4.0×10^5 cells/2 mL medium/6-well plates) were grown overnight at 37 °C with 5 % CO_2. Lipofectamine™ 3000 and the recombinant cDNA were diluted in Opti-MEM Reduced Serum Medium (Gibco™) with a ratio of 1:1 to obtain a final volume of 250 μL. The transfected cells were grown for 24 h at 37 °C in a 5 % CO_2 incubator. Afterwards, the transfected cells were used for proliferation and migration assays.

Monolayer wound healing assay

The transfected CCA cells were seeded into 24-well plates (2.5×10^5 cells/well) and cultured overnight in Ham's F12 Nutrient Mixture medium supplemented with 10 % (*w*/*v*) FBS, streptomycin (100 μg/mL), and amphotericin B (50 μg/mL) in a humidified atmosphere of 5 % CO_2 at 37 °C. After the cells were grown to confluence (>90 %), two separate wounds were generated per well by scratching the surface of the confluent monolayer using a sterile 1-mL pipette tip. Average wound widths along the scrape lines were estimated from six representative fields using the program ImageJ (The National Institute of Health, Bethesda, MD, USA) after the cells were further incubated for 18 and 24 h.

Transwell migration assay

Effects of rYKL-40 on the migration of CCA cells were investigated. KKU-M213 cells (1 × 105 cells) were seeded into the upper chambers of a 24-well Transwell plate with 8.0-μm pore polycarbonate filters (SPL Life Sciences, Pocheon, Korea). The lower chambers contained 600 μL of complete medium with or without rYKL-40. After incubation for 6 h at 37 °C under 5 % CO_2, migrating cells were fixed in 3.7 % (*w*/*v*) formaldehyde, permeabilized with 100 % ethanol, and crystal violet staining performed. Numbers of the migrating cells were counted under an inverted microscope by two independent investigators using a magnification field of ×100. The numbers of migrating cells averaged from experiments carried out in triplicate were compared to those without rYKL-40 treatment.

Cell proliferation assay

CCA cell lines (2.5×10^3 cells/well) were seeded onto a 96-well plate in the presence and absence of rYKL-40 (80 ng/mL). The effects of rYKL-40 on CCA cell proliferation were determined by the colorimetric cell viability (MTS) assay using CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI, USA). In vitro reduction of a tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenil)-2H-tetrazolium (MTS) by metabolically active cells yielded a colored formazan that was released into the culture medium. Color development in this assay, measured at 490 nm, was directly proportional to the number of viable cells and determined from day 0 to day 3.

Assay of adhesion to rYKL-40 protein-coated surfaces by CCA cell lines

Purified rYKL-40 (1 μg) was coated on the wells of a 96-well plate by incubation at 37 °C for 2 h. The cells were seeded at a density of 5×10^4 cells/wells and incubated at 37 °C for 1 h. Unattached cells were removed by rinsing twice with serum-free medium. Numbers of adherent cells were determined by the MTS assay (Promega) as described above. The percentage of the rYKL-40-induced cell adhesion was normalized to that of cells attached on 1 % bovine serum albumin (BSA)-coated wells using the equation:

$$\text{Adhesion}(\%\text{ of control}) = (A_{490}\text{ of YKL-40 coated} - A_{490}\text{ of 1\% BSA coated}) \times 100.$$

concentration was determined by Novagen® BCA Protein Assay Kit (EMD Millipore, Billerica, MA, USA). The freshly purified rYKL-40 was stored at −80 °C until used.

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The transfected CCA cells were seeded into 24-well plates (2.5×10^5 cells/well) and cultured overnight in Ham's F12 Nutrient Mixture medium supplemented with 10 % (*w*/*v*) FBS, streptomycin (100 μg/mL), and amphotericin B (50 μg/mL) in a humidified atmosphere of 5 % CO_2 at 37 °C. After the cells were grown to confluence (>90 %), two separate wounds were generated per well by scratching the surface of the confluent monolayer using a sterile 1-mL pipette tip. Average wound widths along the scrape lines were estimated from six representative fields using the program ImageJ (The National Institute of Health, Bethesda, MD, USA) after the cells were further incubated for 18 and 24 h.

Transwell migration assay

Effects of rYKL-40 on the migration of CCA cells were investigated. KKU-M213 cells (1 × 105 cells) were seeded into the upper chambers of a 24-well Transwell plate with 8.0-μm pore polycarbonate filters (SPL Life Sciences, Pocheon, Korea). The lower chambers contained 600 μL of complete medium with or without rYKL-40. After incubation for 6 h at 37 °C under 5 % CO_2, migrating cells were fixed in 3.7 % (*w*/*v*) formaldehyde, permeabilized with 100 % ethanol, and crystal violet staining performed. Numbers of the migrating cells were counted under an inverted microscope by two independent investigators using a magnification field of ×100. The numbers of migrating cells averaged from experiments carried out in triplicate were compared to those without rYKL-40 treatment.

Cell proliferation assay

CCA cell lines (2.5×10^3 cells/well) were seeded onto a 96-well plate in the presence and absence of rYKL-40 (80 ng/mL). The effects of rYKL-40 on CCA cell proliferation were determined by the colorimetric cell viability (MTS) assay using CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI, USA). In vitro reduction of a tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenil)-2H-tetrazolium (MTS) by metabolically active cells yielded a colored formazan that was released into the culture medium. Color development in this assay, measured at 490 nm, was directly proportional to the number of viable cells and determined from day 0 to day 3.

Assay of adhesion to rYKL-40 protein-coated surfaces by CCA cell lines

Purified rYKL-40 (1 μg) was coated on the wells of a 96-well plate by incubation at 37 °C for 2 h. The cells were seeded at a density of 5×10^4 cells/wells and incubated at 37 °C for 1 h. Unattached cells were removed by rinsing twice with serum-free medium. Numbers of adherent cells were determined by the MTS assay (Promega) as described above. The percentage of the rYKL-40-induced cell adhesion was normalized to that of cells attached on 1 % bovine serum albumin (BSA)-coated wells using the equation:

$$\text{Adhesion}(\%\text{ of control}) = (A_{490}\text{ of YKL-40 coated} - A_{490}\text{ of 1\% BSA coated}) \times 100.$$

The median overall survival of the patient group was 224 days, and the 1-year survival rate was 29.8 %.

Association of plasma YKL-40 concentrations and clinicopathological features

The competitive ELISA assay showed a median plasma YKL-40 concentration of 57 CCA patients of 169.5 ng/mL (range 36.45-543.4 ng/mL), which was significantly higher than those of 41 healthy controls with a median of 46.92 ng/mL (range 11.2-116.7 ng/mL) ($P < 0.0001$) (Fig. 1). To further evaluate the significance of plasma YKL-40 concentrations with the clinical characteristics of the CCA patients, the 25th percentile of the YKL-40 values in the CCA subjects, giving the median of 100.7 ng/mL, was used as the cutoff value. The dichotomized plasma YKL-40 concentrations in normal ($n = 14$) versus elevated ($n = 43$) concentrations are summarized in Table 1. There was no significant correlation between the plasma levels of YKL-40 with clinicopathological features of CCA patients tested. Although the Mann-Whitney U test did not suggest a significant correlation between the YKL-40 level and tumor grade, the median plasma YKL-40 concentration of the patients with advanced stage CCA (stage IV; median 177.9 ng/mL) appeared to be notably higher than that of patients with early stages (stages I-II; median 143.5 ng/mL) (Fig. S1).

Association of plasma YKL-40 concentrations and overall survival

The Kaplan-Meier analysis suggested a positive correlation between overall survival and plasma YKL-40 concentration. No patient with perioperative death (survival <30 days) was included in the analysis. As shown in Fig. 2, patients with elevated plasma YKL-40 concentrations had significantly shorter overall survival than patients with normal plasma YKL-40 concentrations (median overall survival, 207 vs. 336 days; $P = 0.038$, log-rank test). The overall survival rates were 42.8 and 25.6 % at 1 year post-surgery for patients with elevated and normal YKL-40 plasma concentrations, respectively. Age and gender had no influence on survival. In addition, overall survival was significantly decreased in the patients with advanced tumor stage (stage IV) and non-papillary type CCA (Table 2).

Univariate and multivariate analyses of overall survival and clinicopathological parameters

Several clinicopathological parameters have been shown to correlate with the overall survival time of cancer patients. We further performed univariate and multivariate Cox proportional hazard regression analyses to evaluate which parameters apart from plasma YKL-40 concentration influenced the overall survival of the CCA patients. Univariate Cox regression analysis suggested that three clinicopatholigical parameters, namely tumor stage ($P < 0.0001$), histological type ($P = 0.005$), and plasma YKL-40 concentration ($P = 0.038$), were associated with overall survival (Table 2). However, further multivariate Cox regression analysis indicated that, of these three parameters, only tumor grade was an independent predictor of overall survival, with a hazard ratio of 2.817 (95 % CI, 1.428-5.559, between IV and III or I-II, $P = 0.003$) (Table 2).

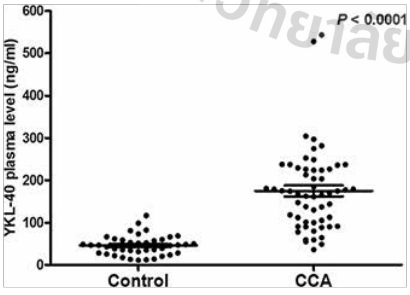

Fig. 1 Absolute value plot showing plasma YKL-40 values (ng/mL) and range in CCA patients ($n = 57$) and normal subjects ($n = 42$). The *horizontal solid line* represents the median values analyzed by the Mann-Whitney U test at $P < 0.0001$

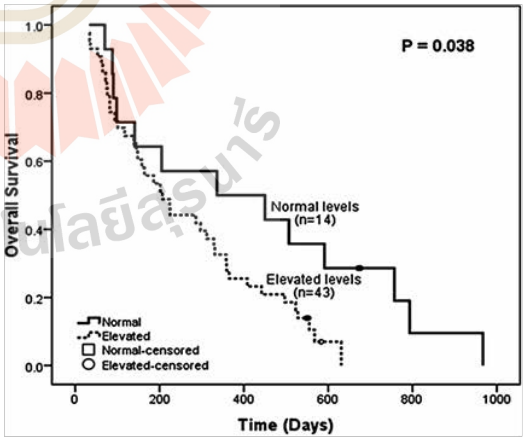

Fig. 2 Kaplan-Meier analysis of plasma YKL-40 levels and overall survival in CCA patients. The YKL-40 concentrations were dichotomized according to the 25th percentile of the total CCA values, giving a cutoff value between normal and elevated YKL-40 levels of 100.7 ng/mL. Elevated YKL-40 was significantly associated with poorer overall survival in CCA patients ($P = 0.038$)

Table 2 Multivariate Cox regression analyses of potential prognostic factors in CCA patients

Variable	Univariate analysis		Multivariate analyses		
	HR	*P* value	HR	95 % CI	*P* value
Age					
<56	1				
≥ 56	0.696 (0.394-1.227)	0.208	1.046	0.569-1.926	0.884
Sex					
Male	1				
Female	1.020 (0.570-1.823)	0.948	1.022	0.558-1.875	0.943
Tumor stage					
I-II	1	0.124			
III	1.856 (0.836-4.122)				
IV	3.298 (1.771-6.141)	<0.0001	2.817	1.428-5.559	0.003
Histology type					
Papillary	1				
Non-papillary	2.647 (1.309-5.356)	0.005	1.870	0.868-4.026	0.110
Serum YKL-40					
Normal	1				
Elevated	2.117 (1.027-4.363)	0.038	1.642	0.780-3.455	0.192

P-values rendered in bold are statistically significant

HR relative hazard ratio, *CI* confidence interval

Intratumoral expression of YKL-40 and clinicopathological features

Immunohistochemical staining of 34 CCA tissue samples showed that YKL-40 was rarely expressed in CCA tumor cells, but highly expressed in liver cells and infiltrating inflammatory cells, especially macrophages and connective tissue at intratumoral stroma (Fig. 3a–c). Cellular YKL-40 expression was identified in 22 of 34 CCA tissues (64.70 %). Score distributions were as follows: score 0 in 12 (35.3 %) patients, 1+ in 5 (14.7 %) patients, and 2+ in 17 (50 %) patients. The positive staining of YKL-40 intratumoral expression was categorized as cytoplasmic or membranous/cytoplasmic reactivity. The presence of membranous/cytoplasmic YKL-40 protein was noted in 5/34 cases (14.7 %) and was predominantly apical where a lumen was present (Fig. 3f). Since YKL-40 expression in CCA tissues was found in various types of cell, we quantitatively analyzed the correlation between YKL-40 expression in CCA cells alone and the clinicopathological features, using a univariate analysis. A high level of intratumoral YKL-40 was associated with the non-papillary type which was a poor prognosis parameter ($P = 0.005$) (Table 3). There was no statistically significant correlation between YKL-40 intratumoral expression and age, gender, or tumor stage. There was also no significant difference between YKL-40 intratumoral expression and patient overall survival (Fig. s2).

Effects of YKL-40 on growth and migration of CCA cell lines

In this series of experiments, we examined the effects of YKL-40 on the growth and migration of two CCA cell lines (KKU-M055 and KKU-M213), grown in the presence of rYKL-40 (Fig. 4a). The results (Fig. 4b) showed that rYKL-40 significantly enhanced the growth of KKU-M055 ($P = 0.005$) and KKU-M213 ($P = 0.038$) after 72 h of incubation. A Transwell migration assay was subsequently carried out to determine whether YKL-40 affected the migration of CCA cells. The results (Fig. 4c) showed that the KKU-M213 cells treated with 80 ng/mL of rYKL-40 had a 1.8-fold increase in migration as compared to the untreated cells ($P = 0.036$). Note that the CCA cells were allowed to migrate for only 6 h, to ensure that no cell division occurred during the time of investigation.

To investigate whether YKL-40 mediated cell migration through cell-surface receptors, the KKU-M213 CCA cell line with high expression of integrin receptors was used in the cell adhesion assay [35]. When KKU-M213 cells were allowed to attach for 1 h, we observed that the intrinsic binding capacity was increased by 1.5-fold in cells induced with rYKL-40, compared with that of the non-induced cells ($P = 0.007$) (Fig. 4d). In the last set of experiments, the KKU-M213 cells were treated with 80 ng/mL of rYKL-40 and incubated for up to 48 h. The results showed that exogenous YKL-40 induced

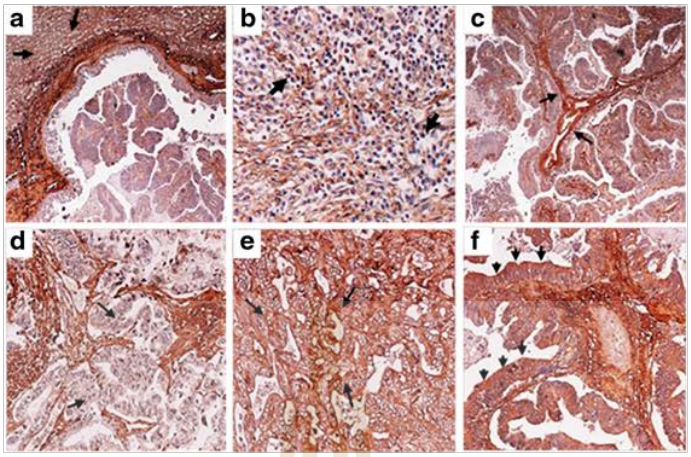

Fig. 3 Distribution and expression of YKL-40 in CCA tissues detected by immunohistochemical staining. a YKL-40 expressed in liver cells (×4 HP). b Infiltrating macrophages (×10 HP). c Connective tissue at intratumoral stroma (×4 HP). d Negative YKL-40 immunostaining in CCA tumor cells (×10 HP). e YKL-40 positive staining of cytoplasmic immunoreactivity in CCA tumor cells (×10 HP). f YKL-40 positive staining of membranous/cytoplasmic immunoreactivity in CCA tumor cells, especially the luminal surfaces and apical sites (*black arrows*) (×10 HP)

considerable phosphorylation of Akt at 24 h and Erk1/2 at 24 and 48 h, compared with those of the control cells without rYKL-40 treatment (Fig. 4e).

Effects of transient expression of YKL-40 on CCA growth and migration

We subsequently investigated the effects of YKL-40 expression on cell growth and migration. Two cell lines, KKU-M055 and KKU-M213, were transfected with the pCMV6/*CHI3L1* construct. Western blot analysis demonstrated after transfection that both cell lines could express YKL-40, while no expression was seen in the cells transfected with the pCMV6 empty vector (Fig. 5a). Cell proliferation was tested using MTS assay, and the results showed that the expression of YKL-40 was significantly visible in KKU-M055 cells after 48 h of cell growth ($P = 0.0178$), while no expression of YKL-40 was seen in KKU-M213 cells (Fig. 5b). Monolayer wound healing was carried out to determine whether expression of YKL-40 in CCA cells affected cell migration. As shown in Fig. 5c, the YKL-40 expression enhanced the mobility of KKU-M213 at 18 and 24 h of incubation as compared with that of the control. However, this malignant phenotype was not observed with KKU-M055 cells transfected with the pCMV6/*CH13L1* plasmid.

Discussion

In this study, we assayed the plasma concentrations of YKL-40 in 57 CCA patients and also the cellular expression of the *CHI3L1* gene in 34 CCA tissue samples. Statistical analysis clearly suggested that elevated plasma YKL-40 level was associated with short overall patient survival, but not with other clinical-pathological factors, including patient age, gender, tumor grade, and histology type. The results reported here agree with several independent studies that showed elevated serum/plasma YKL-40 concentrations in a wide range of cancers [27, 36-38]. The baseline YKL-40 concentration in the healthy groups in different cancer types was similar (median range 27-97 ng/mL) [39-42]. The median value of

Table 3 Intratumoral expression of YKL-40 in cholangiocarcinoma tissues in relation to clinicopathological features of the CCA patients

Variable	Number	YKL-40 intratumoral staining (%)		*P* value
		Negative	Positive	
Age (years)				
<56	19	4 (21.1)	15 (78.9)	0.051
≥56	15	8 (53.3)	7 (46.7)	
Sex				
Male	19	7 (36.8)	10 (63.2)	0.832
Female	15	5 (33.3)	12 (66.7)	
Tumor stage				
II	17	4 (41.2)	13 (58.8)	0.473
III and IV	17	6 (29.4)	10 (70.6)	
Histology type				
Papillary	12	8 (66.7)	4 (33.3)	0.005
Non-papillary	22	4 (18.2)	18 (81.8)	

The dichotomized data were analyzed using the chi-squared test. *P* values rendered in bold are statistically significant ($P < 0.05$)

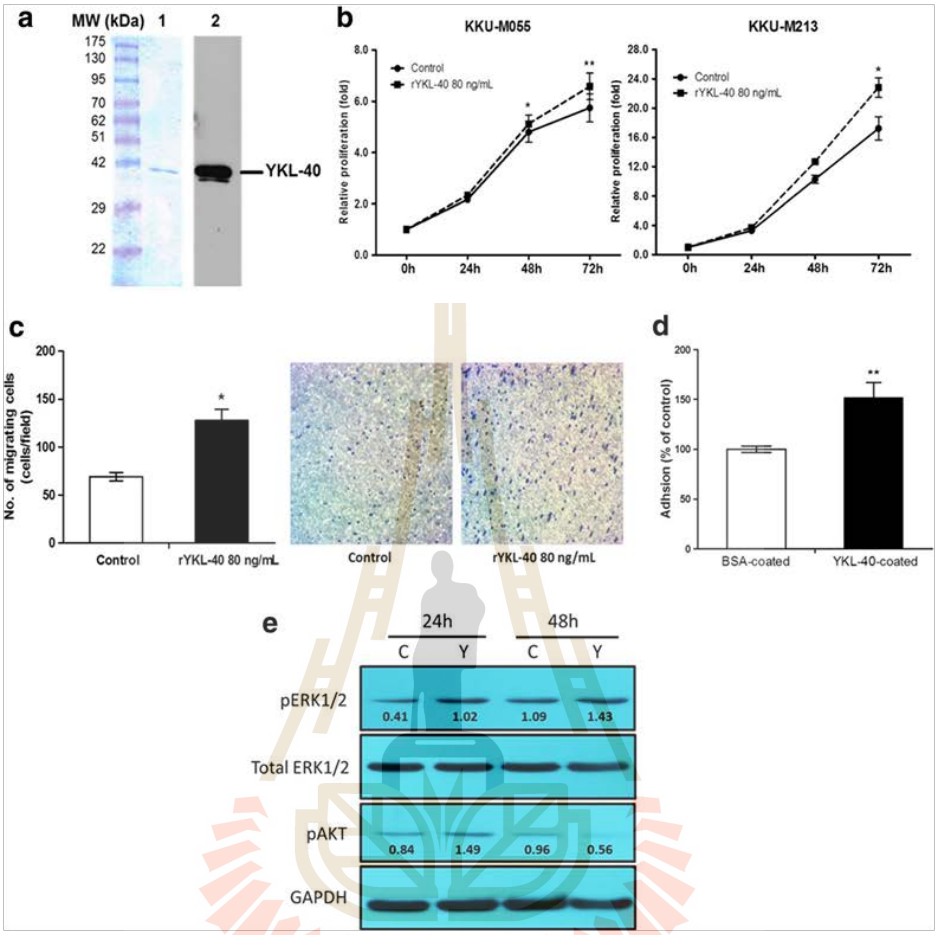

Fig. 4 Functional analysis of YKL-40 in CCA cell lines. a rYKL-40 expression in HEK-293T culture medium. Lane: *M*, molecular weight markers; *1*, Coomassie staining of purified rYKL-40; *2*, immunoblotting of the purified rYKL-40, detected by anti-YKL-40 polyclonal antibody. b Effect of rYKL-40 on cell proliferation. The MTS assay was performed on two CCA cell lines, KKU-M055 and KKU-M213, in medium supplemented with rYKL-40 (80 ng/mL). c Effect of rYKL-40 on cell migration. In the migration assay, KKU-M213 cells were loaded into the upper chamber of a well in a Transwell plate and rYKL-40 protein (80 ng/mL) was introduced into the lower chamber. Numbers of migrating cells were counted (*left panel*), and representative images are shown for each condition (*right panels*). d Assay of cultured KKU-M 213 cell adhesion to a rYKL-40-coated surface. After removing unbound cells, adherent cells were quantified by the MTS assay. Cells adhering to BSA-coated plates were used as a control. e Western blot analysis showing the intensity of pAKT and pERK1/2 in KKU-M213 cells after treatment with 80 ng/mL of rYKL-40 for 24 and 48 h. The numerical value indicates the intensity of the pAKT or pERK1/2 signal, which was normalized to that of the ‘housekeeping’ protein GAPDH. *C* indicates KKU-M213 samples without rYKL-40 treatment and *Y* indicates samples treated with 80 ng/mL rYKL-40. *Error bars* represent ±SEM (triplicate experiments). $^{*}P<0.05$, $^{**}P<0.01$

pre-operative plasma YKL-40 levels in our cancer groups (170 ng/mL) is slightly higher than the median values reported for other cancer types (78-150 ng/mL) [43-46]. However, one should take into account that the reported values are deviated among cancer types, depending on sample preparation (plasma or serum) and pathological (tumor stages) and physiological (age and sex) conditions of cancer patients during their hospital visits for blood collection. Even the prognostic values of YKL-40 within the same sample groups may be deviated, since some patients have advanced or disseminated disease and even some have received chemo and/or radiation therapy. In our study, the YKL-40 levels were evaluated from blood samples of CCA patients taken before surgical resection. Note that we did not include chemo/radiotherapy parameters in our prognosis analysis, since not all patients were assigned with the same treatment protocol. For examples, 38 of 57 CCA

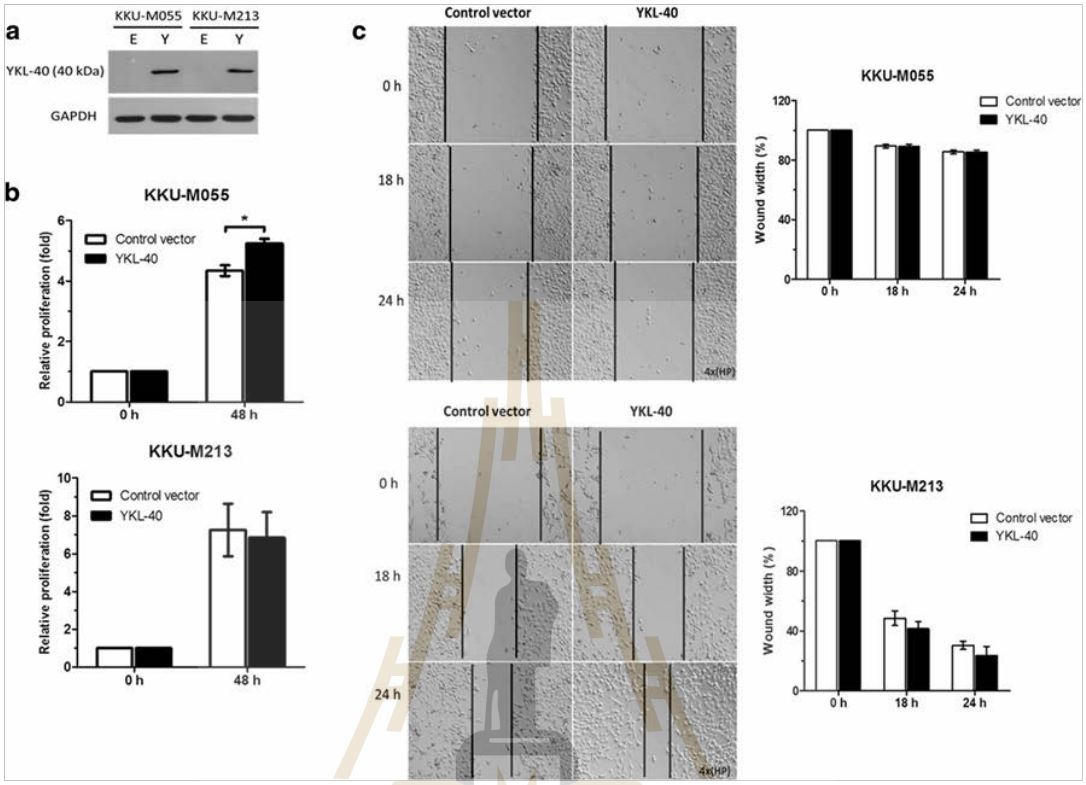

Fig. 5 Transient expression of YKL-40 activates CCA cell proliferation and migration. a Western blot analysis displaying YKL-40 expression in KKU-M055 and KKU-M213 after being transfected with the recombinant plasmid pCMV6/*CH1L3* for 24 h. *E* and *Y* represent control vector and the newly generated construct, respectively. GAPDH was used as sample loading control. b Cell proliferation of KKU-M055 and KKU-M213 with pCMV6/*CH1L3* or control vector was determined by MTS assay at 0 and 48 h. c Monolayer wound of KKU-M055 and KKU-M213 transfected with pCMV6/*CH1L3* or control vector was created by scratching the surface of the transfected cells as described in text. Healing was observed at different time points. *Left panel*, the representative images of wound healing at 0, 18, and 24 h. *Right panel*, plots of average wound widths at different times of growth after the scratches. *Error bars* represent ±SEM from triplicate experiments. $^{*}P < 0.05$, *t* test

patients received operation, while 14 of which were offered to have adjuvant chemotherapeutic treatment, but only 5 of 14 CCA patients had completed full cycles of chemotherapy.

In our study, we also found that not all patients with CCA had elevated serum/plasma YKL-40 levels compared with the healthy age-matched controls, implying that YKL-40 may not be secreted directly by CCA tumor cells. Our immunohistochemical data showed the predominant localization of YKL-40 to be in infiltrating inflammatory cells that spread all over non-tumor areas surrounding the CCA cells. These results indicate that expression of YKL-40 is not tissue-type-specific, but more cell-type-specific. Our data complement a previous study that showed intense expression of YKL-40 in various adult tissues, especially in cells exhibiting high metabolic activity, including mast cells, polymorphic nuclear granulocytes, endocrine cells, exocrine epithelial cells, basal layer squamous epithelial cells, endothelial cells, and neurons [47]. The role of YKL-40 in these cells might be related to increased metabolism of cells during tissue inflammation/remodeling. For CCA, elevated plasma YKL-40 levels in patients with stage IV CCA may suggest some role of YKL-40 in stimulating local inflammation of tissues lining the bile duct. Our further immunohistochemical study suggested a low level of expression of YKL-40 in CCA tissues, but high-level expression in adjacent non-cancerous tissues, including intratumoral stroma, liver, and connective tissues. This result implies that induced YKL-40 secretion occurred as an indirect activator of CCA.

In cancer cells, YKL-40 has been shown to enhance proliferation, differentiation, metastasis potential,

apoptosis, and angiogenesis [31, 48, 49]. However, the exact role of YKL-40 in cancer progression is unknown. YKL-40 could potentially act as a tumor growth factor, as we observed that culture medium supplemented with rYKL-40 activated the proliferation of two CCA cell lines (KKU-M213 and KKU-M055). In addition, studies of transient expression of YKL-40 in CCA cell lines also suggested the promotion of growth of the studied CCA cell lines. A similar growth-stimulating effect has been reported previously with glioma cells [25] and cervical cancer cells [50]. YKL-40 has also been demonstrated to act as a chemoattractant that enhanced the migration, adhesion, and spreading of vascular smooth muscle cells (VSMC) and umbilical vein endothelial cells (HUVEC) [51], while similar effects have been observed for several cancer cells [23-25, 50]. In our study, we demonstrated that YKL-40 not only promoted CCA cell growth but also increased CCA cell migration. YKL-40 promotion of migration has been shown for colorectal cancer [24], glioma [25, 52], and cervical cancer [49].

Although the cellular receptors mediating the biological effects of YKL-40 remain to be identified, there is evidence for YKL-40 interaction with signaling components on the cell membrane [31]. Singh and coworkers demonstrated that expression of YKL-40 regulated NFI-X3 and STAT3 (known regulators of gliogenesis), controlling migration and invasion of both astrocytes and glioma cells [51]. YKL-40 also showed angiogenic properties by interacting with many known membrane receptors in endothelial cells, such as syndecan-1 and integrins αvβ3 and/or αvβ5, so as to mediate cell growth and migration [21, 23]. In CCA, syndecan-1 is expressed as a cell-surface, transmembrane, heparan sulfate-containing proteoglycan that interacts with various effectors during the initiation of cell adhesion [53]. In addition, CCA cells also show high-level expression of integrins, the transmembrane receptors that are the bridges for cell-cell and cell-extracellular matrix interaction receptor [35, 54]. Our cell adhesion assay showed a positive response of CCA cells to rYKL-40, which induced both cell migration and the phosphorylation of two intracellular kinases, Akt and Erk1/2, in these same cell lines, suggesting that YKL-40 interacts with membrane receptors that may stimulate a downstream pathway involved in the cancer cell migration. However, identification of specific receptors for YKL-40 in CCA cells is a subject for future study. Taken together, our findings provide evidence that elevated plasma YKL-40 levels correlate significantly with poor prognosis of CCA and play an indirect role in promoting cancer progression, acting as an oncogenic factor that stimulates the growth and migration of CCA cells through a membrane receptor that regulates the Akt/Erk pathway.

Concluding remarks

In agreement with previous studies on other types of cancer, elevated YKL-40 levels have been observed in the plasma of CCA patients. The highest levels of plasma YKL-40 were detected in CCA patients with the most advanced stage (stage IV), with poor prognosis and short overall survival. The results obtained from our study strongly support the recent proposal that YKL-40 could be used as candidate biomarker for cancer diseases. From the point of view of CCA diagnosis, we recently developed a capacitive immunosensor that is highly specific for plasma YKL-40 detection [55] and therefore could be used for measurement of YKL-40 levels in cancer patients. We propose that YKL-40 could serve as a useful prognosticator, helping to improve prognosis of this lethal cancer, if included in the CCA screening program together with other currently used biomarkers.

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Authors' contributions ST carried out all the experiments, performed data analysis, and prepared the manuscript draft. WC generated the human *CHI3L1* construct. AS, SW, and BS provided patients' plasma samples and paraffin-embedded tissues and clinical data related to the CCA patients. HC provided guidance on Transwell migration assay. WS was a grant holder, conceived the idea of research, provided advice on gene isolation, cloning, expression, and purification of rYKL-40, and revised and approved the manuscript. CT coordinated cancer studies, provided guidance on statistical analyses, immunohistochemistry, cell proliferation, and migration assays, and took part in manuscript preparation.

Compliance with ethical standards Written informed consent was obtained from all patients. The study has been approved by the Ethics Committee for Human Research, Suranaree University of Technology (EC-55-17).

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Conflicts of interest None

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