ROLE OF RING FINGER PROTEIN 43 (RNF43) IN CHOLANGIOCARCINOMA

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บทบาทของ Ring finger protein 43 (RNF43) ในมะเร็งท่อน้ำดี

นางสาวพัชรี จันทร์ถาวร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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ROLE OF RING FINGER PROTEIN 43 (RNF43) IN CHOLANGIOCARCINOMA

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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โปรตีน Ring finger protein 43 หรือ RNF43 คือ E3 ubiquitin-protein ligase ทำหน้าที่ควบคุมยับยั้งกระบวนการ Wnt signaling โดยจะนำโปรตีน ubiquitin มาจับกับโปรตีนเป้าหมายคือ Wnt receptor บนผิวเซลล์ได้อย่างจำเพาะ ซึ่งมีผลทำให้เกิดการชักนำการย่อยสลายโปรตีน Wnt receptor โดยการนำโปรตีนเป้าหมายนี้เข้าสู่เซลล์ด้วยวิธีเอนโดไซโทซิสและถูกย่อยด้วยไลโซโซม ในปัจจุบันหลายการศึกษาพบยีน *RNF43* มีคุณสมบัติและหน้าที่ในการควบคุมการเจริญเติบโตของเซลล์มะเร็ง และยังพบว่าในมะเร็งหลายชนิดรวมถึงมะเร็งท่อน้ำดี มีการกลายพันธุ์ของยีนนี้ด้วยความถี่ที่สูง อย่างไรก็ตาม ลักษณะการแสดงออกของยีนหรือบทบาทหน้าที่ของโปรตีนนี้ในการส่งเสริมการเจริญและพัฒนาของมะเร็งท่อน้ำดียังไม่เคยมีปรากฏหรือมีการศึกษามาก่อน จากข้อมูลที่กล่าวมาข้างต้น ทางผู้วิจัยจึงทำการศึกษาเกี่ยวกับการแสดงออกของยีน *RNF43* และศึกษาความสัมพันธ์ของการแสดงออกของยีนกับสถานการณ์การกลายพันธุ์ของยีน (somatic mutation) อาการทางพยาธิคลินิกและการพยากรณ์โรคของผู้ป่วยมะเร็งท่อน้ำดี ผลการศึกษาพบการแสดงออกของอาร์เอนเอและโปรตีนของยีน *RNF43* ลดลงในเนื้อเยื่อมะเร็งท่อน้ำดี ผู้ป่วยมะเร็งท่อน้ำดีที่มีการกลายพันธุ์ของยีนนี้ไม่สามารถตรวจพบโปรตีน RNF43 ในเนื้อเยื่อมะเร็งท่อน้ำดี ซึ่งแสดงให้เห็นว่ายีน *RNF43* สูญเสียบทบาทหน้าที่เมื่อเกิดการกลายพันธุ์ของยีน ขณะที่ผู้ป่วยมะเร็งท่อน้ำดีที่มีการแสดงออกของโปรตีน RNF43 ลดลงหรือไม่สามารถตรวจพบได้จะมีความสัมพันธ์กับการพยากรณ์โรคที่ไม่ดีอย่างมีนัยสำคัญทางสถิติ แต่ไม่พบความสัมพันธ์กับอาการทางพยาธิคลินิกต่าง ๆ และการวิเคราะห์ทางสถิติพบว่า การแสดงออกของโปรตีน RNF43 ที่ลดลงยังเป็นตัวพยากรณ์โรคที่เป็นอิสระต่อปัจจัยอื่น ๆ ที่มีผลต่อการรอดชีวิตของผู้ป่วยมะเร็งท่อน้ำดีอีกด้วย นอกจากนี้ทางผู้วิจัยได้ศึกษาบทบาทหน้าที่ของยีน *RNF43* ในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี พบการแสดงออกของโปรตีน RNF43 ที่เพิ่มมากขึ้นมีผลลดการเจริญเติบโตของเซลล์มะเร็งท่อน้ำดีอย่างมีนัยสำคัญ โดยผ่านการยับยั้งกระบวนการส่งสัญญาณภายในเซลล์ Wnt signaling การยับยั้งดังกล่าวเกิดขึ้นจากการลดระดับของโปรตีน frizzled หรือ Wnt receptors การเพิ่มขึ้นของกระบวนการย่อยสลายโปรตีน β-catenin และนำมาสู่การยับยั้งการแสดงออกของโปรตีน cyclin D1 ที่มีบทบาท

หน้าที่ในการส่งเสริมการแบ่งตัวของเซลล์และถูกควบคุมการแสดงออกด้วยกระบวนการส่งสัญญาณภายในเซลล์ Wnt signaling

จากผลการศึกษาทั้งหมดของงานวิจัยครั้งนี้ สรุปได้ว่า RNF43 มีบทบาทหน้าที่เกี่ยวข้องกับการเจริญพัฒนาของมะเร็งท่อน้ำดี และการตรวจวิเคราะห์หาความผิดปกติของยีนนี้ในผู้ป่วยมะเร็งท่อน้ำดี น่าจะเป็นประโยชน์ในการเลือกวิธีการรักษาอย่างเหมาะสมให้กับผู้ป่วยมะเร็งท่อน้ำดีต่อไป

สาขาวิชาเคมี
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ลายมือชื่อนักศึกษา Patchanee Jantha
ลายมือชื่ออาจารย์ที่ปรึกษา _____

PATCHAREE JANTHAVON : ROLE OF RING FINGER PROTEIN 43 (RNF43) IN CHOLANGIOCARCINOMA. THESIS ADVISOR : CHUTIMA TALABNIN, Ph.D. 84 PP.

ROLE OF RING FINGER PROTEIN 43 (RNF43) IN CHOLANGIOCARCINOMA

Ring finger protein 43 (RNF43) is an E3 ubiquitin-protein ligase that negatively regulates Wnt signaling pathway by selectively ubiquitinating frizzled receptors at the plasma membrane and promotes its endocytosis and lysosome degradation. RNF43 is proposed as a tumor suppressor gene and is frequently mutated in several types of malignancy including cholangiocarcinoma (CCA). However, the significant of its expression and the exact molecular mechanisms of RNF43 in the CCA development and progression have not been reported. In this study, we determined RNF43 expression in CCA tissues and investigated the correlation between RNF43 expression and *RNF43* mutation status, clinicopathological features and prognosis of CCA patients. RNF43 reduced expression in both messenger RNA and protein levels in CCA tissues. The absent protein expression of RNF43 was significantly correlated with somatic mutation status, confirming that all *RNF43* somatic mutations in CCA are inactivating. Overall survival was worst in patients with down-regulation of *RNF43* expression. Univariate and multivariate analyses revealed that RNF43 protein expression was an independent prognostic factor. However, there was no statistically significant association between RNF43 messenger RNA and protein expression with any clinicopathological features. Further, the functional analysis revealed that

overexpression of RNF43 significantly decreased cell proliferation in CCA cell lines, which were dependent in part on the inactivation of Wnt signaling pathway via reducing frizzled receptors and activating β-catenin phosphorylation to suppress Cyclin D1 expression. These results suggest that RNF43 is involved in development and progression of CCA and the identification of genetic alteration status of RNF43 may provide specific therapeutic strategies for CCA patient treatment.

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Student's signature Patchanee Jantharen

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APC/C	Anaphase-promoting complex/Cyclosome
ATP	Adenosine triphosphate
BD	Bile duct
BSA	Bovine serum albumin
CCA	Cholangiocarcinoma
cDNA	Complementary DNA
CI	Confidence interval
DAB	3,3′ -diaminobenzidine-tetrahydrochloride
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-ligase enzyme
E. coli	*Escherichea coli*
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FZD	Frizzled receptor
HRP	Horseradish peroxidase
IARC	International Agency for Research on Cancer

LIST OF ABBREVIATIONS (Continued)

Ig	Immunoglobulin
IHC	Immunohistochemical analysis
IPMN	Intraductal papillary mucinous neoplasms of pancreas
K	Lysine amino acid
kb or bp	kilobase or base pair
kD	Kilodalton
LB	Luria broth
LGR4	Leucine-rich repeat containing G-protein coupled receptor 4
LRP5/6	Low-density lipoprotein-related protein 5 or 6
mRNA	messenger RNA
miR	microRNA
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenil)-2H-tetrazolium
NIH	National Institutes of Health
NO	Nitric oxide
NR	Not reported
OV	*Opisthorchis viverrini*
PA	Protease-associated domain
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethyl sulfonyl fluoride

LIST OF ABBREVIATIONS (Continued)

qPCR	The quantitative real-time PCR
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RNF43	RING finger protein 43
ROI	Reactive oxygen intermediate
R-spo	R-spondin
Ser33/37/Thr41	Serine 33 / Serine 37 / Threonine 41
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel Electrophoresis
TCF4	T-cell factor 4
TCF/LEF	T-cell factor/lymphoid enhancer factor
TM	Transmembrane domain
TSG	Tumor suppressor gene
Ub	Ubiquitin
3′UTR	Three prime untranslated region
ZNRF3	Zinc and Ring Finger 3

CHAPTER I

INTRODUCTION

1.1 Significance of research

Cholangiocarcinoma (CCA) is a fatal cancer that develops along the biliary tract. CCA is a rare tumor world-wide and high incidence rate of this malignancy is found in Southeast Asia, and particularly in the Northeast Thailand. This is due to strong association between CCA and chronic inflammation by food-borne liver fluke *Opisthorchis viverrini* (Ov) that is classified to be group 1 carcinogen by WHO's International Agency for Research on Cancer. CCA is a highly metastasis with poor prognosis and responds poorly to current chemo- and radiotherapy. Surgical resection is the treatment of choice for early stage of the disease but most patients are diagnosed at advance stage and some is inoperable. Thus surgical resection is considered as palliative treatment for the CCA patients.

Genetic mutations are associated with the formation and progression of cancer, and it also has significant clinical implications from diagnosis to therapy. Recently, the active mutation in well-known cancer genes (*TP53, Kras* and *SMAD4)* and novel mutated genes (*MLL3, RNF43, GNAS* and *PEG3)* have been documented in patients with Ov-related CCA using whole exome sequencing technique. Alteration of these mutated genes involves in seven biological processes or pathways; genome stability, transforming growth factor beta (TGF-b)/SMAD4 signaling, KRAS and G protein signaling, epigenetic regulation, WNT signaling, cell cycle control and AKT and

PI3K signaling. Of particular concerning among biological pathway is Wnt signaling which has several newly mutated genes (*RNF43* and *PEG3*) involved and that have never been demonstrated the role in cholangiocarcinogenesis. *RNF43* or Ring finger protein 43 is an E3 ubiquitin-protein ligase that proposed to inhibit Wnt signaling by 1) interacting with the Wnt receptors of the frizzled, and 2) sequestering TCF4 to the nuclear membrane. *RNF43* is frequently mutated in several types of cancers, including CCA. It has been demonstrated in approximately 3-10% of both Ov and non Ov-related CCA. In addition, poorer survival was observed in CCA patients with inactive *RNF43* mutations. However, the significance of *RNF43* in CCA; expression level and its role in Wnt signaling has not been reported in CCA.

In present study, we investigated the 1) RNF43 protein and mRNA expression and the correlation with *RNF43* somatic mutation status, and clinicopathological features and 2) the biological roles of *RNF43* in CCA. The finding obtained from the current study may provide the crucial roles of *RNF43* in Wnt signaling during development and progression of CCA and the *RNF43* expression may have a potential for diagnostic and monitoring CCA patients.

1.2 Literature reviews

1.2.1 Significance of cholangiocarcinoma (CCA)

Cholangiocarcinoma (CCA) is bile duct cancer that arises from the ductular epithelium of the biliary tree, either within the liver or more commonly from the extrahepatic bile ducts (Olnes and Erlich, 2004). CCA can be classified anatomically into intrahepatic or extrahepatic tumors. In the case of intrahepatic tumors is further classified by growth characteristics or macroscopic into mass-

forming, periductal infiltrating or intraductal growth type (Bhudhisawasdi et al., 2012; Khan et al., 2005) (Figure 1.1). Mass-forming type arises from peripheral intrahepatic small bile duct that manifests as a large, white tumor with dense fibrosis and high metastasis. Periductal infiltrating type is the most common type of CCA in the hilar area and demonstrates very small fibrotic tumors which cause segmental dilatation of the bile ducts. Intraductal growth type is the least common but it has a better prognosis than other types and is characterized by intraductal growth with little or no extension beyond the bile duct walls (Han et al., 2002). Histologically, CCA is classified further according to grade as being well, moderately, or poor differentiated, with the classic diagnosis being well-to moderately-differentiated ductal adenocarcinoma. CCA is a devastating malignancy that presents late, is notoriously difficult to diagnose, and is associated with a high mortality. Surgical treatment is the only treatment of choice for this tumor because of its late clinical presentation and the lack of effective non-surgical therapeutic modalities (Leyva-Illades et al., 2012).

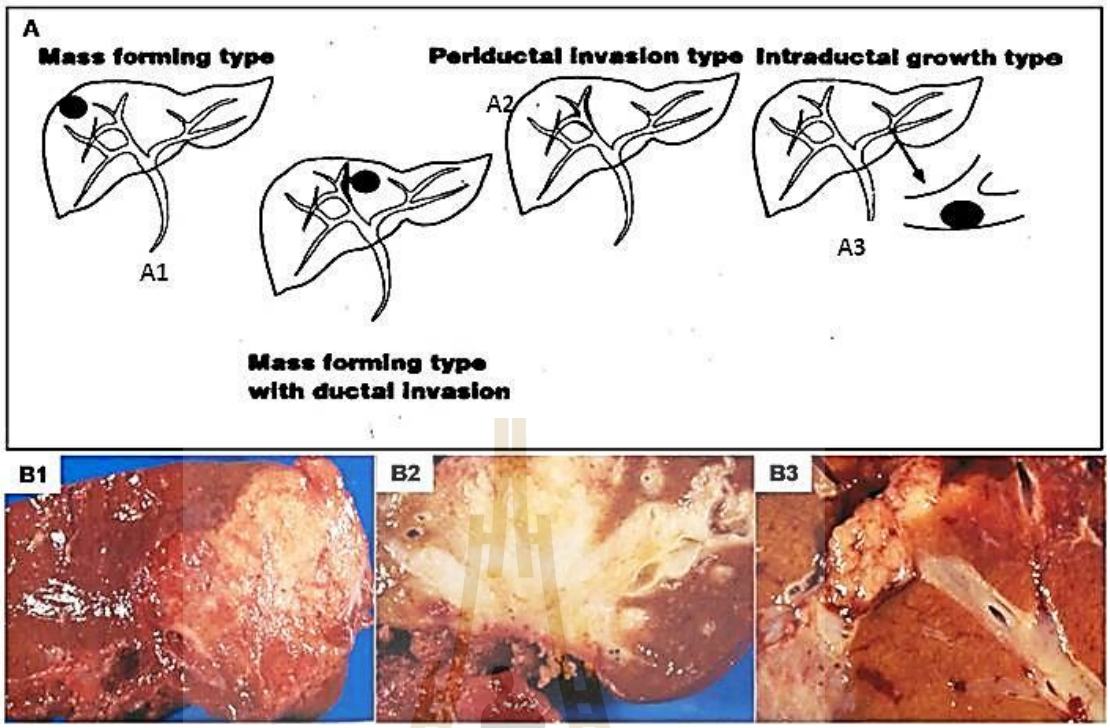

Figure 1.1 Intrahepatic cholangiocarcinoma is classified into mass-forming (A1, B1), periductal infiltrating (A2, B2) and intraductal growth (A3, B3) (Bhudhisawasdi et al., 2012).

1.2.1.1 Epidemiology and Etiology of CCA

CCA is a rare tumor world-wide but high incidence rates have been reported in Southeast Asia, especially in Northeast Thailand (Parkin et al., 1993). CCA is the second-most common primary liver cancer and accounts for an estimated 15% of primary liver cancer worldwide; however, its prevalence varies widely by region, from 5% in Japan (Primary liver cancer in Japan 1990) to 20% in Pusan (Busan), Korea (Jung et al., 1993) and up to 90% in Khon Kaen, Thailand (Parkin et al., 1993).

The etiology of CCA are still unknown, although chronic inflammation and cellular injury within bile ducts, together with partial obstruction of bile duct flow (Gores, 2003; Sirica et al., 2002) manifest as high risk conditions for CCA development. Well established risk factors in Western country include primary sclerosing cholangitis, hepatolithiasis, and choledochal cysts, whereas less-established potential risk factors include inflammatory bowel disease, hepatitis C virus, hepatitis B virus, cirrhosis, diabetes, obesity, alcohol consumption and smoking (Rizvi and Gores, 2013). In Eastern countries, liver fluke, *Opisthorchis viverrini* (Ov) infection is the major risk factor of CCA in Thai, Lao, and Malaysian cases, while *Clonorchis sinesis* infection is prominent in Japanese, Korean, and Vietnamese cases (Kullavanijaya et al., 1999; Sithithaworn et al., 1994). In addition, these two liver flukes are listed as a human carcinogen in 1994 and 2007, respectively by International Agency for Research on Cancer (IARC) (IARC, 1994;2012).

1.2.1.2 Pathogenesis of Ov- associated CCA

Pathological consequences of Ov infection occur mainly in the bile duct within liver, extrahepatic bile ducts and gallbladder, and have been described in both humans and animal models. The severity of the pathology appears to be associated with both intensity and duration of infection (Sripa, 2003). The main histopathologic features of Ov both in man and animal model are inflammation, epithelial desquamation, epithelial and adenomatous hyperplasia, goblet cell metaplasia, periductal fibrosis, and granuloma formation (Figure 1.2).

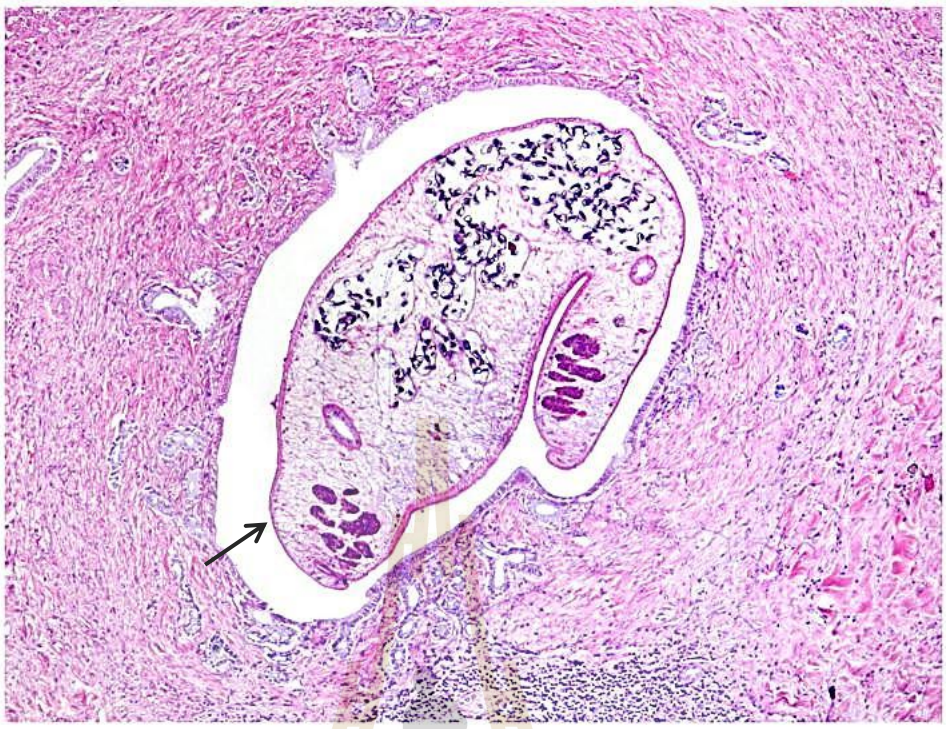

Figure 1.2 Histopathology of Ov- infected human liver showing periportal inflammation, adenomatous hyperplasia and periductal fibrosis (Sripa and Pairojkul, 2008). The adult parasite is seen in the bile duct lumen (black arrow).

Ov- induced CCA is a result of chronic inflammation. Several mechanisms by which Ov infection may enhance cholangiocarcinogenesis have been proposed (Figure 1.3). Biliary epithelial desquamation may be due to mechanical irritation caused by the liver fluke and/or its metabolic products; however, immunopathological processes may contribute to the long standing hepatobiliary damage. With liver fluke infection, inflammation, periductal fibrosis and proliferative responses, including epithelial hyperplasia, goblet cell metaplasia and adenomatous hyperplasia, may represent predisposing lesions that enhance susceptibility of DNA to carcinogens. Several nitric oxide compounds and their precursors occur at low levels in fermented food, such as preserved mud fish paste, pla-ra, that is a common food additive in

Northeast Thailand. In addition, endogenous nitric oxide caused by Ov infection and inflammation has been observed in animals and humans. Both exogenous and in-situ nitrosamine formation may lead to DNA alkylation and deamination in predisposed and inflamed tissues (Sripa et al., 2007). Moreover, excess nitric oxide and other reactive oxygen intermediates produced by inflammatory cells during infection might exert direct cytotoxic and mutagenic effects and increase cell proliferation. It has been reported the excess of nitric oxide- mediated DNA damage in biliary epithelium of hamsters infected with Ov (Kawanishi et al., 2006; Pinlaor, Hiraku, et al., 2004), and repeated infections with the liver fluke result in enhanced biliary DNA damage (Pinlaor, Ma, et al., 2004). Additionally, due to lower rates of apoptosis of the infected biliary epithelium, after several successions of replication, genetic alterations may become fixed, which can lead to malignant transformation (Sripa et al., 2007).

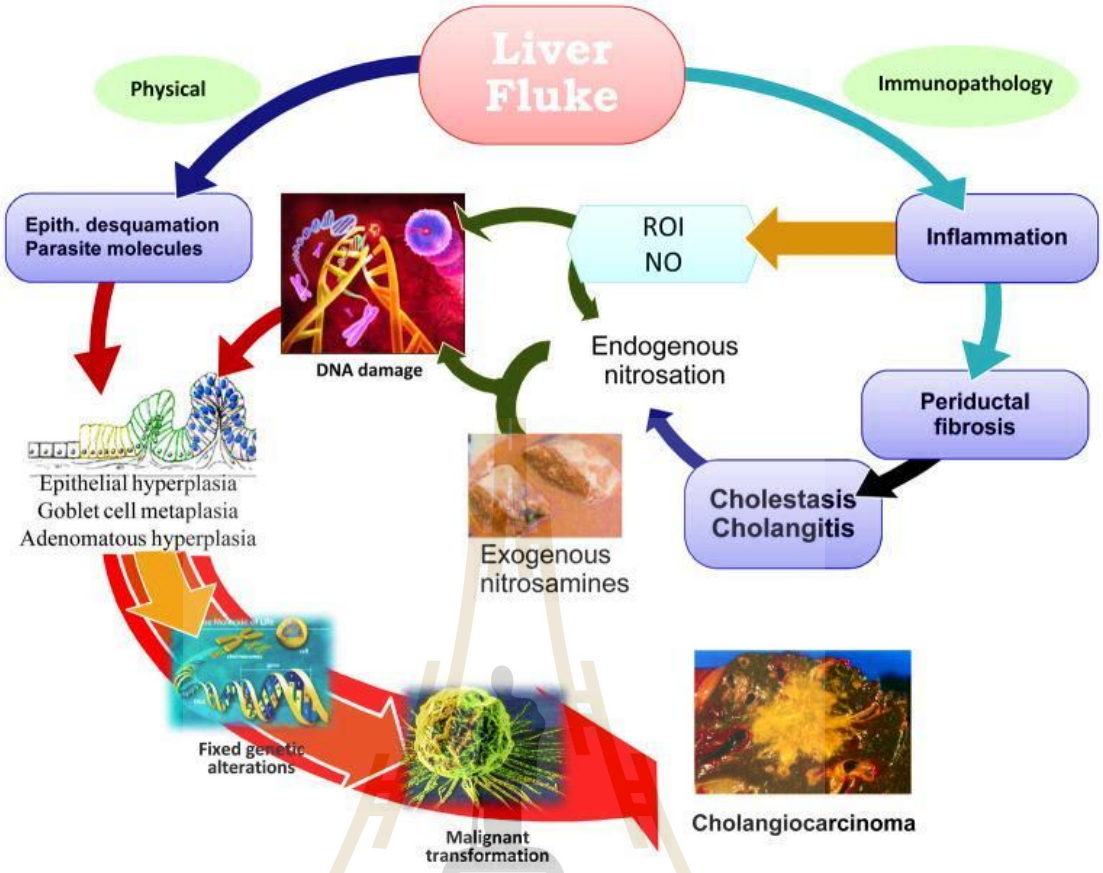

Figure 1.3 Proposed mechanisms of Ov- induced CCA. The liver fluke induces damage to the bile duct tissue at least by two pathways: mechanical (by parasite sucker/excretory-secretory products) and immune pathology to parasite antigens. Inflammation-induced oxidative DNA damage occurs concurrent with biliary epithelial proliferation driven by parasite molecules. Damaged DNA/genes after successive replication become fixed and lead to malignant transformation. NO, nitric oxide; ROI, reactive oxygen intermediates (Sripa and Pairojkul, 2008).

1.2.1.3 Mutational landscape of Ov-associated CCA

Chronic Ov infection and inflammation are proposed to contribute genetic alteration in Ov-related CCA (Sripa and Pairojkul, 2008). In recent years, high-throughput next-generation sequencing has enabled comprehensive mutational

profiling of Ov-associated CCA. The CCA-related mutant genes, including *TP53* (44%), *KRAS* (16.7%) and *SMAD4* (16.7%) are identified using exome-capturing resequencing. Besides known mutations, several novel mutated genes are found, including inactivating mutations in *MLL3* (in 14.8% of cases), *ROBO2* (9.3%), *RNF43* (9.3%) and *PEG3* (5.6%), and activating mutations in the *GNAS* oncogene (9.3%) (Table 1.1) (Ong et al., 2012). While non-Ov related CCA reported the recurrent somatic mutations in *BAP1* and *ARID1A* (Chan-On et al., 2013). Comparisons between Ov and non–Ov–related CCAs demonstrate statistically significant differences in mutation patterns: *BAP1*, *IDH*1 and *IDH2* are more frequently mutated in non– Ov related CCAs, whereas *TP53* mutations show the reciprocal pattern (Chan-On et al., 2013) (Table 1.1). Furthermore, the commonly mutated genes in Ov-related CCA can be functionally grouped into seven biological pathways, including genome stability, transforming growth factor beta (TGF-β)/SMAD4 signaling, KRAS and G protein signaling, epigenetic regulation, Wnt signaling, cell cycle control, and AKT/PI3K signaling (Jusakul et al., 2015) (Figure 1.4). *TP53* is involved in genomic stability and it has been reported the TP53 mutations in mice cause the progression of epithelial hyperplasia of bile duct to malignant intrahepatic CCA (Farazi et al., 2006). *KRAS* is involved in RAS/RAF pathway alteration. In addition, both *TP53* and *KRAS* mutations have been shown to play an important role in CCA tumorigenesis. While newly mutated genes are involved in the Wnt signaling pathway, *RNF43* and *PEG3* are negative regulators of Wnt with role in regulating genomic stability though p53 regulation. In contrast, the chromatin modifiers including *BAP1*, *ARID1A*, *PBRM1* and *IDH* are highly mutated in non-Ov related CCA. These indicate that histone dysregulation is a major

biological pathway which is involved in carcinogenesis of non-Ov related CCA. Taken together, this information indicates that different causative etiologies may induce distinct somatic alterations, even within the same tumor type (Chan-On et al., 2013).

Based on the information of genomic alteration in CCA, *RNF43* is a novel mutated gene, which is detected in both Ov and non-Ov related CCA. It has also shown that the presence of *RNF43* somatic mutations associates with poor patient survival. These data motivate us to study the *RNF43* expression and its role in Ov-related CCA.

Table 1.1 Commonly mutated genes in Ov-related and non-Ov-related CCA (Jusakul et al., 2015).

Genes	Ov-related CCA (%)		Non-Ov-related CCA (%)	
	Thailand (n=54)[a]	Thailand (n=108)[b]	Singapore + Romania (n=86)[b]	Europe (n=32)[c]
TP53	44.4	39.8	9.3	6
KRAS	16.7	13.9	11.6	3
SMAD4	16.7	19.4	5.8	0
CDKN2A	5.6	NR	NR	3
MLL3	14.8	13.0	3.5	NR
ROBO2	9.3	5.6	2.3	NR
GNAS	9.3	5.6	0	NR
RNF43	9.3	7.4	3.5	NR
PTEN	3.7	NR	0	6
BAP1	NR	2.8	10.5	25
ARID1A	NR	17.6	10.5	19
IDH1/2	NR	2.8	9.3	19
PBRM1	NR	NR	NR	17

[a](Ong et al., 2012); [b](Chan-On et al., 2013); [c](Jiao et al., 2013)

CCA, cholangiocarcinoma; NR, not reported.

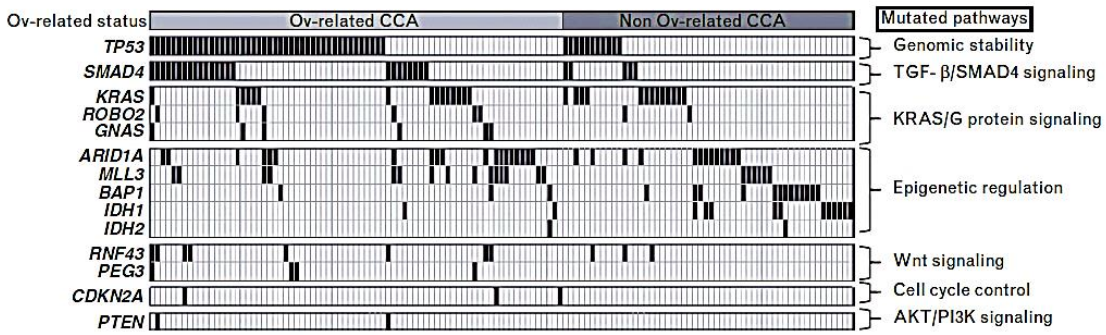

Figure 1.4 Somatic mutations and mutated pathways in Ov-related and non-Ov-related CCA. Left column indicates genes validated in Ong et al., 2012 and Chan-On et al., 2013. Top row indicates Ov-related status. Samples with or without mutations are labeled in black or white, respectively. (Jusakul et al., 2015)

1.2.2 Ring finger protein 43 (RNF43)

RNF43 gene consists of 10 exons located within 63 kb of DNA on human chromosome 17q22, and encodes a single polypeptide chain of 783 amino acids and the molecular weight 85 kDa with an isoelectric point of about 8.10. The protein encoded by this gene is a RING-type E3 ubiquitin ligase and is predicted to contain a signal peptide, a transmembrane domain, a protease-associated domain, an ectodomain, and a cytoplasmic RING domain (Figure 1.5). RNF43 is proposed as an E3 ubiquitin-protein ligase that accepts ubiquitin from an E2 ubiquitin-conjugating enzyme and directly transfers the ubiquitin to targeted substrate proteins for protein degradation (Figure 1.6).

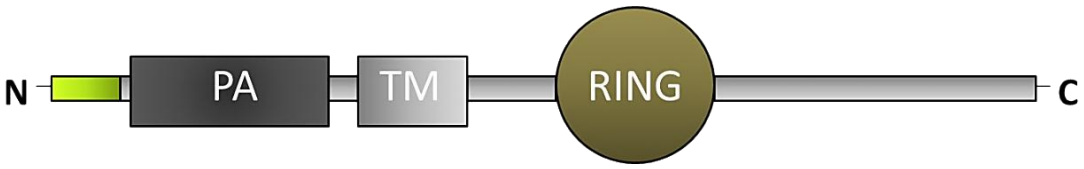

Figure 1.5 Schematic overview of RNF43. The green line denotes the signal peptide. PA, protease-associated domain; TM, transmembrane domain; RING, RING domain.

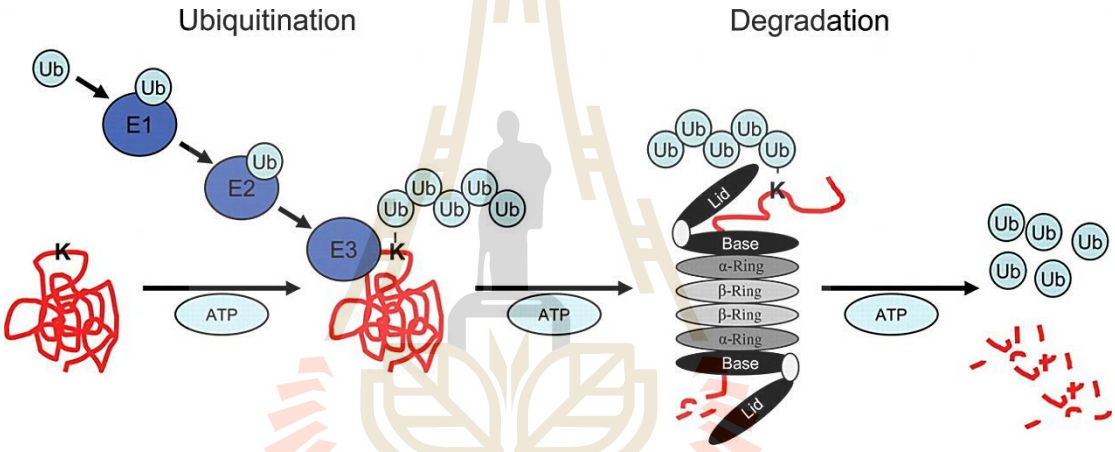

Figure 1.6 Overview of the ubiquitin - proteasome pathway. Ubiquitination, also known as ubiquitylation, regulates degradation of cellular proteins by the ubiquitin proteasome system, controlling a protein's half-life and expression levels. Briefly, Ubiquitin (Ub) is a small 8-kDa protein, which is first transferred to the ubiquitin-activating enzyme, E1, in an ATP-dependent manner. This activated ubiquitin is then transferred to the ubiquitin-conjugating enzyme, E2. Finally, the ubiquitin is covalently attached to the target protein by E3 ubiquitin ligase, leading to the formation of a polyubiquitin chain. The polyubiquitylated protein is recognized by the 26S proteasome, and is destroyed in an ATP-dependent manner (Carrier et al., 2010).

1.2.2.1 The role of RNF43 in Wnt signaling pathway

Wnt signaling pathway is a powerful signaling that plays a crucial role in cell fate determination, survival, proliferation and movement in variety of tissues (Moon et al., 2002). To trigger a cellular response and activate intracellular signal transduction, Wnt ligands are secreted lipoglycoprotein ligands that bind to receptors of frizzled (FZD) family and several co-receptors including low-density lipoprotein-related protein 5 or 6 (LRP5/6) (Klaus and Birchmeier, 2008). Then, it results in the disassembly of the destruction complex consisting of axin, adenomatous polyposis coli (APC), and GSK3β and stabilization of β-catenin in the cytoplasm. Cytoplasmic β-catenin accumulates and is eventually translocate into nuclear where it interacts with TCF/LEF family transcription factors and various co-factors to induce the expression of various selected genes including *CCND1* (Cyclin D1) (Shtutman et al., 1999), *C-myc* and *CD44* (Sansom et al., 2004), MMP-7 (Crawford et al., 1999), EphB/ephrin-B (Batlle et al., 2002), *ZNRF3* and *RNF43* (Hao et al., 2012). (Figure 1.7)

RING finger protein 43 (RNF43) is an E3 ubiquitin ligase originally found in stem cells (LGR5 positive cells) and proposed to play a role in negative feedback regulation of Wnt signaling by ubiquitinating frizzled receptors at the plasma membrane and, consequently promote its endocytosis and lysosome degradation (Koo et al., 2012). It has been demonstrated that abnormalities of this E3 ubiquitin ligase in particular Wnt signaling pathway are involved in various diseases including cancers. For example, the deletion of *ZNRF3/RNF43* increases Wnt stimulation through β-catenin/TCF activity (Jiang et al., 2015) and this activation leads to induce rapidly growing adenomas in animal model (Koo et al., 2012). In addition, Wnt signals can

also be boosted in the presence of R-spondin (R-spo) and their receptor leucine-rich repeat containing G-protein coupled receptor 4 (LGR4) through direct inhibition of two membrane-bound E3 ligases (RNF43 and ZNRF3) (Carmon et al., 2014; Hao et al., 2012) (Figure 1.8). Furthermore, the role of RNF43-mediated inhibition of Wnt signaling pathway is not limited at plasma membrane by selectively ubiquitinating frizzles receptors. Loregger et al. have demonstrated that RNF43 is also localized at the nuclear envelope of human intestinal crypt and colon cancer cells. It can physically interact with T cell factor 4 (TCF4) and lead to translocation of TCF4 to the nuclear membrane, resulting in inhibition of TCF4 transcriptional activity (Loregger et al., 2015).

In summary of the data so far provide an explanation for the role of RNF43 in regulating Wnt signals in normal condition by 1) interacting with the Wnt receptors of the frizzled, and 2) sequestering TCF4 to the nuclear membrane.

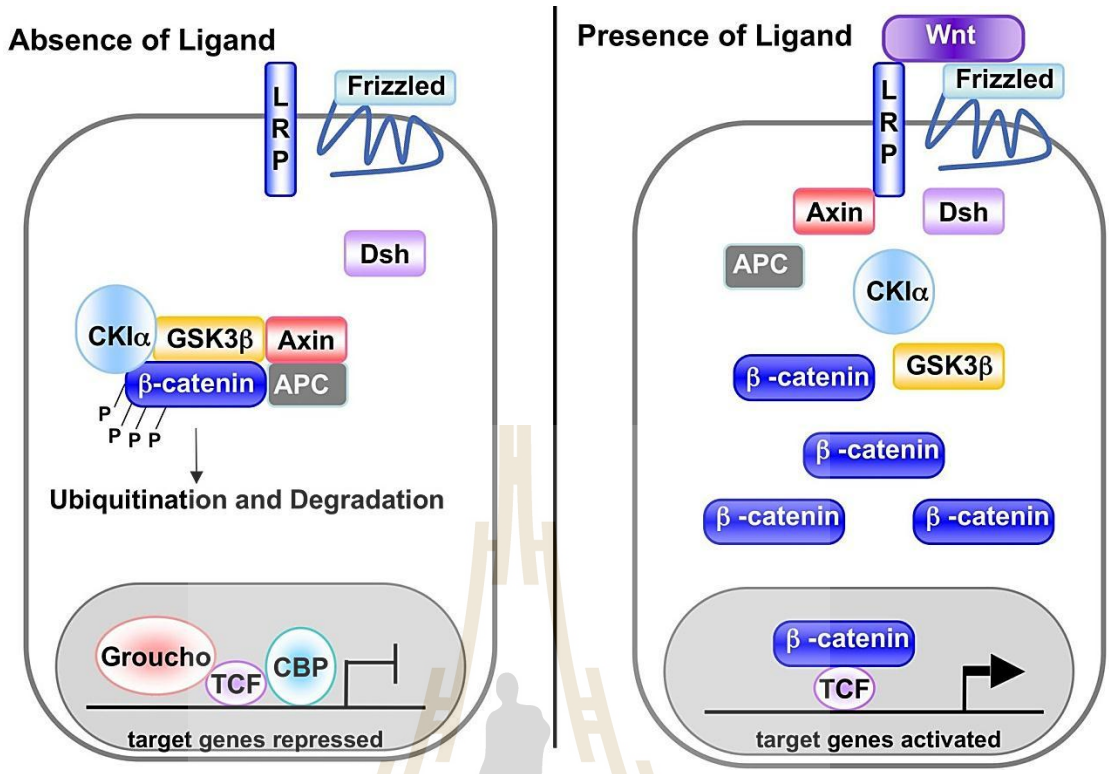

Figure 1.7 A canonical Wnt signaling pathway. Left: In the absence of Wnt ligand, action of the destruction complex (CKIα, GSK3β, APC, Axin) creates a hyperphosphorylated β-catenin, which is a target for ubiquitination and degradation by the proteasome. Right: Presence of Wnt ligand, binding of Wnt ligand to a frizzled/LRP-5/6 receptor complex leads to stabilization of hypophosphorylated β-catenin, which interacts with TCF/LEF proteins in the nucleus to activate transcription (Eisenmann, 2005).

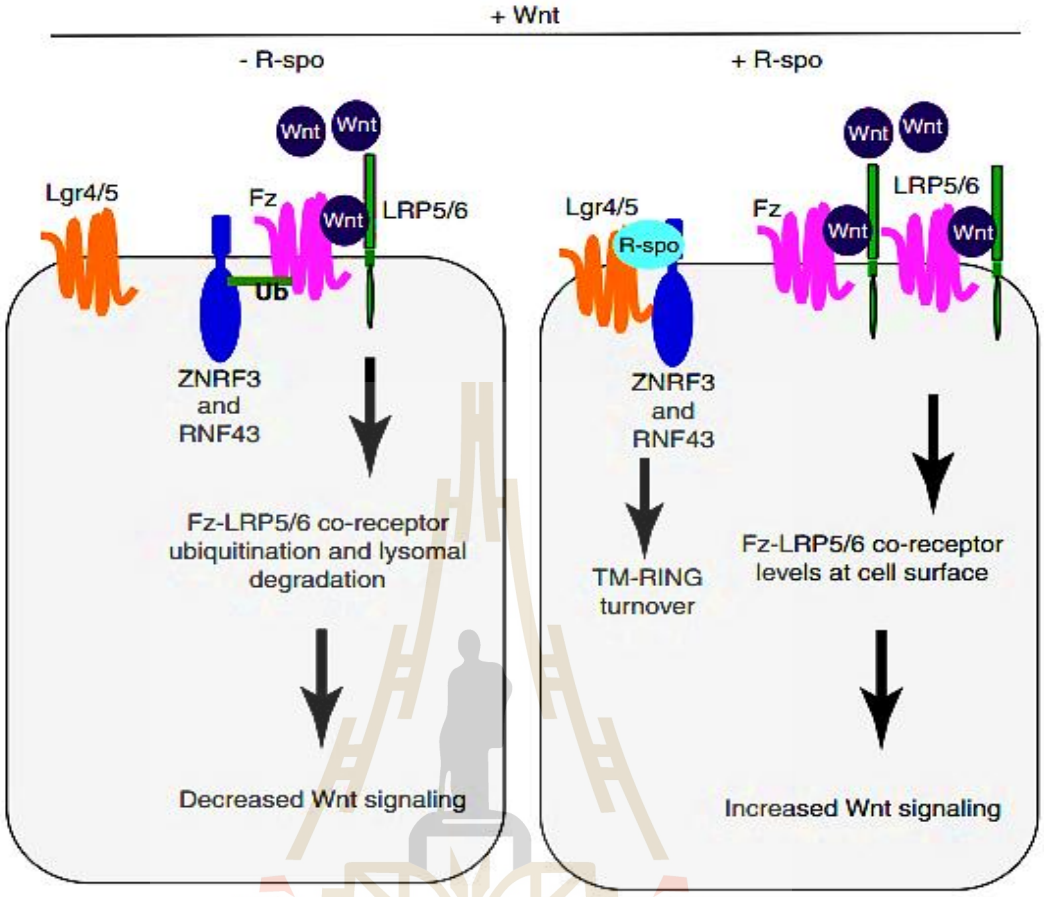

Figure 1.8 Model for R-spondin (R-spo), LGR4/5, and ZNRF3/RNF43 interactions in the modulation of Wnt/β-catenin signaling. Left: In the presence of Wnt ligand and the absence of R-spo, the transmembrane E3 ubiquitin ligase proteins ZNRF3 or RNF43 (TM-RING) ubiquitinate frizzled–LRP5/6 co-receptor proteins, resulting in internalization and lysosomal degradation of the TM-RING–frizzled–LRP complex. Right: In the presence R-spo, R-spo simultaneously binds to the extracellular domains of ZNRF3/RNF43 and LGR4/5, and induces auto-ubiquitination in an E3 ligase-dependent manner and membrane clearance of ZNRF3/RNF43, resulting in increased level of frizzled-LRP5/6 receptors at the cell surface (Fearon and Spence, 2012).

1.2.2.2 The role of RNF43 in cancers

Oncogenic transformation is characterized by dysregulated cell growth signals that lead to limitless autonomous proliferation, insensitivity to anti-growth or pro-apoptotic signals, dysregulation of the cell cycle and genomic instability (Hanahan and Weinberg, 2011). RING finger E3 ligases are implicated in all of these steps. It has been reported that some are bona fide oncogenes, whereas others are products of tumor suppressor genes (TSGs) (Table 1.2). For example, the RING finger E3 function in TSG, the anaphase-promoting complex/cyclosome (APC/C) consists of at least 13 subunits which have multisubunit E3s that regulate the cell cycle. The frameshift and point mutations of APC/C in colon cancer cell lines and tumors can cause the inappropriate progression of the cell cycle, genomic instability and aberrant chromosome segregation (Wang et al., 2003). In contrast, the mouse double minute 2 homolog (MDM2) is an oncoprotein and E3 activity which bind directly to P53 (TSG) means of inactivating P53 and escaping growth control in cancer (Marine and Lozano, 2010).

Table 1.2 RING finger E3s as oncogenes and tumor suppressor genes (Lipkowitz and Weissman, 2011).

E3 function	**Role in cancer**	
	A tumor suppressor gene	**An oncogene**
Cell Cycle	APC/C ligases COP1 *(RFWD2)* *FBXW7*	β-TrCP (*FBW1A*) EMI1 *(FBXO5)* *SKP2*
Genomic integrity	*BARD1* *BRCA1* FANC core complex	*CUL7* *MDM2* MDMX *(MDM4)* PARC *(CUL9)* PIRH2 *(RCHY)*
Signal transduction	TRC8 *(RNF139)*	CBLs *FBXW5* Hakai *(CBLL1)*
Hypoxia	*VHL*	SIAHs
Metastasis		gp78 *(AMFR)*

β-TrCP, β-transducin repeat containing protein; AMFR, autocrine motility factor receptor; APC/C, anaphase-promoting complex/cyclosome; BARD1,BRCA1-associated RING domain 1; COP1, constitutive photomorphogenesis protein 1 homologue; CUL, cullin; EMI1, early mitotic inhibitor 1; FANC, Fanconi anaemia; FBXW, F-box/WD-repeat containing protein; RFWD2, RING finger and WD repeat domain protein 2; RNF139, RING finger protein 139; SKP2, S-phase kinase-associated protein 2; VHL, von Hippel–Lindau tumor suppressor.

RNF43 is a newly implicated RING finger E3 ligase in cancer. It is frequently mutated in several types of malignancy, including CCA (Table 1.3). However, the loss or gain-of-function mutation in *RNF43* is still unclear since it can act as either an oncogene or a tumor suppressor gene depending on the tumor type. In CCA, *RNF43* was thought to act as a tumor suppressor gene because CCA patients with inactive *RNF43* mutations are associated with poorer survival (Ong et al., 2012). Similar finding has demonstrated in glioma and gastric cancer where reduced *RNF43* expression is associated with poor prognosis (Niu et al., 2015; Xi et al., 2015). In contrast, overexpression of *RNF43* in hepatocellular (Table 1.4) reveals oncogenic function because the high expression of *RNF43* mediates cancer cell proliferation and is associated with positive vascular invasion and advanced tumor stage (Xing et al., 2013). However, the significance of its expression and clinical correlation in CCA has not been reported.

The significant role of *RNF43* in regulating Wnt signaling pathway have been documented in stem cells by targeting frizzled receptor for protein degradation (Koo et al., 2012). This evidence was also demonstrated in pancreatic ductal adenocarcinoma that inactivating mutations of *RNF43* confer Wnt dependency by increasing membrane expression of frizzled (Jiang et al., 2013). It has been also demonstrated that RNF43 can interact with NEDL1 (Novel ubiquitin-protein isopeptide ligase for Dishevelled-1) and suppresses p53-mediate transcriptional activity in colorectal cancer (Shinada et al., 2011). Similar findings have demonstrated in hepatocellular carcinoma that suppression of *RNF43* induces the accumulation of G1-S phase cell cycle arrest leading to cell apoptosis via p53 dependent pathway and inhibits cell migration and invasion (Xing et al., 2013). In

contrast, suppression of *RNF43* significantly promotes cell proliferation in gastric cancer (Niu et al., 2015) and epithelial-to-mesenchymal transition change and metastasis in colorectal cancer (Wang et al., 2016). However, its role in CCA remains unknown.

Taken together, RNF43 is implicated in all steps of tumor development and progression. The loss or gain-of-function in *RNF43* can act as either an oncogene or a tumor suppressor gene depending on the tumor type.

Table 1.3 *RNF43* is frequently mutated in cancers.

Cancers	Percentage of *RNF43* mutations	Number of mutations			References
		Missense	Nonsense, Splice sites, Frameshift	Synonymous	
Intraductal papillary mucinous neoplasms of pancreas ;IPMN (Japan)	14 % (8/57)	2	6	-	(Sakamoto et al., 2015)
Pancreatic cancer including IPMN (USA)	31.2 % (10/32)	2	8	-	(Wu et al., 2011)
Endometrial cancer	22 % (49/222)	9	39	1	(Giannakis et al., 2014)
Mucinous ovarian carcinomas	21 % (6/29)	-	6	-	(Ryland et al., 2013)
Colorectal cancer	18.9 % (35/185)	7	26	2	(Giannakis et al., 2014)
Cholangiocarcinoma	9.3 % (5/54)	3	2	-	(Ong et al., 2012)

Table 1.4 RNF43 protein expressions in cancers.

Cancers	RNF43 protein expression	References
Hepatocellular carcinoma	High (81%; 80/98)	(Xing et al., 2013)
Intraductal papillary mucinous neoplasms	High (70%; 124/176)	(Sakamoto et al., 2015)
Glioma	High (61%; 141/229)	(Xi et al., 2015)
Gastric carcinoma	Low (57%; 44/77)	(Niu et al., 2015)

1.3 Thesis objectives

The aim of this thesis focused on *RNF43* expression and its role in CCA.

1. To investigate the RNF43 protein expression in 50 CCA tissues.

2. To investigate the *RNF43* mRNA expression level in 28 frozen CCA tumors including the adjacent nontumorous tissue of the same patients.

3. To investigate the association of *RNF43* expression (mRNA or protein levels) and *RNF43* somatic mutation status, clinicopathological features and survival of patients.

4. To investigate the biological functions of RNF43 in CCA cell lines.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Specimens

2.1.1.1 Tumor tissues and clinical data

Fifty paraffin-embedded blocks and 28 of 50 frozen tissues from CCA patients were obtained from the specimen bank of Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand.

Written informed consent was obtained from each subject. The Khon Kaen University Ethics Committee for Human Research and the Ethics Committee for Human Research (HE521209), Suranaree University of Technology, approved the protocol for sample collection (EC-57-25). The clinicopathological features of the included cases are listed in Table 3.1. The *RNF43* mutation status was obtained from our previous study (Ong et al., 2012). None of the patients received radiotherapy or chemotherapy before the surgery. The survival of each CCA patient was recorded from the date of surgery until the patient died of the disease or to October 27, 2014. The follow-up time was at least 4 years, with median of 10 months and range of 1 to 83 months.

In case of paraffin embedded tissues were used for immunohistochemistry study and frozen tissues were used for performing RNA extraction and checking the

expression level.

2.1.1.2 Human cholangiocarcinoma cell lines

Human CCA cell lines including KKU-055, KKU-100, 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. Four CCA cell lines were cultured in Ham's F12 nutrient mixture medium with 10% (v/v) fetal bovine serum (FBS) (Gibco, New York, USA), 40 µg/mL of gentamicin, and 0.25 µg/mL of amphotericin B (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. CCA cell lines were used for gene expression and functional studies of *RNF43*.

2.1.2 Chemicals and antibodies

All chemicals and antibodies used in this study were presented in Table 2.1

Table 2.1 List of chemicals and antibodies.

Chemicals	**Company**
Plasmid Maxi kit	Geneaid Biotech (New Taipei, Taiwan)
Liquid DAB+substrate chromogen system	Dako (California, USA)
Nitrocellulose membrane	Bio-Rad (California, USA)
Developer, replenisher and half-speed blue x-ray film	Kodak (New York, USA)
SYBR safe DNA gel stain and complementary DNA synthesis kit	Invitrogen (California, USA)
Prestained protein ladder	GeneDirex (Keelung, Taiwan)
Skimmed milk, Tryptone, yeast extract powder and agar powder	Himedia Laboratories (Mumbai, India)
Bovine serum albumin (BSA), Sodium dodecyl sulphate (SDS) and 2-β-mercaptoethanol	Sigma-Aldrich (Missouri, USA)
Phenylmethyl sulfonyl fluoride (PMSF) and Triton X-100	USB Corporation (Ohio, USA)
Dimethyl sulfozide (DMSO)	Bio Basic Inc (Ontario, Canada)
Tween-20	Scharlau chemie SA (Barcelona, Spain)

Table 2.1 List of chemicals and antibodies (Continued).

Antibodies	**Company**
Rabbit polyclonal RNF43 antibody	Sigma-Aldrich
Rabbit polyclonal Phospho-β-catenin (Ser33/37/Thr41) antibody	Cell Signaling Technology (Massachusetts, USA)
Rabbit polyclonal β-Catenin, Frizzled, and Cyclin D1 antibody	Santa Cruz (Texas, USA)
Mouse monoclonal β-Actin and Histone H1 antibody	Santa Cruz
HRP labelled polymer anti rabbit and HRP labelled rabbit anti mouse IgG	Dako
HRP labelled goat anti rabbit IgG	GenScript (New Jersey, USA)

2.1.3 Oligonucleotide primers

The primer sequences used for *RNF43* and *β-actin* expression were designed in-house by Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primers were purchased from First BASE Laboratories Sdn Bhd (Selangor, Malaysia) (Table 2.2).

Table 2.2 List of primer sequences.

Primers	Forward primer sequence (5'→ 3')	Reverse primer sequence (5'→ 3')	Product size (bp)
β-actin	GAT CAG CAA GCA GGA GTA TGA CG	AAG GGT GTA ACG CAA CTA AGT CAT AG	92
RNF43	TCT GTC TGG AGG AGT TCT CT	CTC CCT CTG TGA TGT TGA AC	138

2.1.4 Construction of RNF43-DDK-Myc expression vector and plasmid preparation

RNF43/pCMV6/neomycin-DDK-Myc construct and PCMV6-entry vector were purchased from Origene (Maryland, USA) (Figure 2.1). *Escherichea coli* (*E. coli*) strain DH5α were purchased from Qiagen (Manchester, UK) and used for plasmid amplification. RNF43/pCMV6/neomycin-DDK-Myc construct and PCMV6-entry vector were transformed into *E. coli* strain DH5α and let the cells grown in Luria broth (LB, 10 g/L tryptone, 5 g/L yeast extract, 5 g/L sodium chloride) agar plate containing 25 μg/mL kanamycin for 16 h. A single colony was picked into 500

µL of LB media containing kanamycin incubated at 37°C for 6 h with shaking at 200 rpm. Then, 0.5 mL of starter cultures were inoculated into 200 mL of LB containing kanamycin in a flask and incubated at 37°C with shaking until the OD_{600} reached 0.5-0.6. The bacterial cultures were cooled at 4°C, and then were purified using Plasmid Maxi kit (Geneaid Biotech). In brief, bacterial cultures were harvested by centrifugation at 3,000 xg for 15 min to form a cell pellet, followed by resuspending the cell pellet with buffer. Cells were lysed and neutralized, then transferred to the equilibrated Plasmid Maxi column and allowed column to empty by gravity flow. Then, the DNA binding columns were washed and eluted with a high salt buffer, followed by precipitation step of pure plasmid DNA. Finally, double digestion was performed in RNF43/pCMV6 construct and PCMV6-entry vector using EcoRI and XhoI restriction enzymes to verify the purified plasmid.

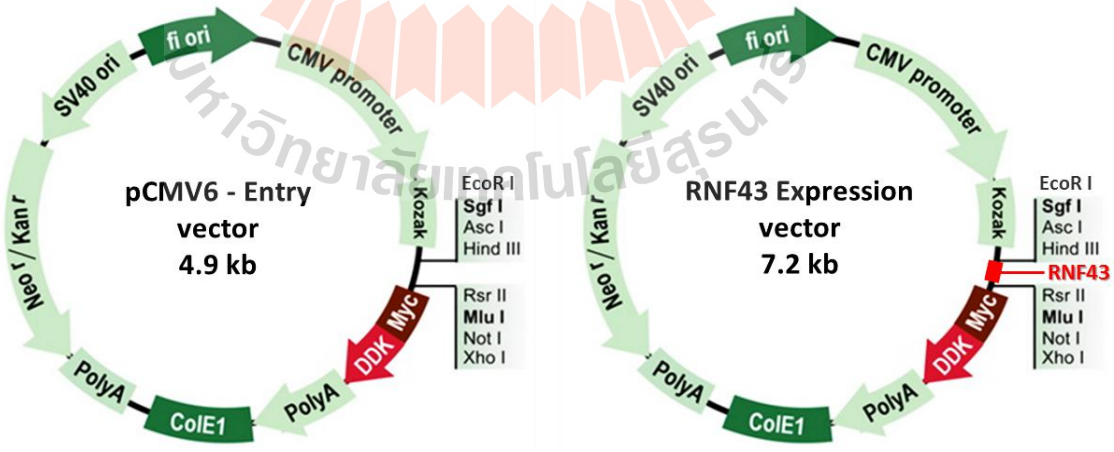

Figure 2.1 Mapping of pCMV6-entry (left) and RNF43 constructed (right) vector.

2.1.5 Instrumentation

The instruments were used in this experiment, including of a shaking incubator (MRC ltd, Holon, Israel), a Thermomixer comfort and centrifuge 5415R (Eppendorf AG, Hamburg, Germany). High-speed microcentrifuge CF16RXII (Hitachi, Tokyo, Japan), a microcentrifuge Denville 26OD (Denville Scientific, New Jersey, USA), a PCR 9700 thermocycler (PE Applied Biosystems, California, USA). CX21 light microscope and CKX41 inverted microscope (Olympus, New York, USA). Biosafety laminar flow class II (Esco Lifesciences, Thailand), Forma series II water jacket CO_2 incubator and Forma -80°C ULT freezer (Thermo Fisher Scientific, Massachusetts, USA). Myrun DNA electrophoresis system (Cosmo Bio, Tokyo, Japan), A vertical mini protein gel electrophoresis system and The mini Trans-blot cell (Bio-Rad).

2.2 Methodology

2.2.1 Determination of RNF43 protein and mRNA expression

2.2.1.1 Immunohistochemical analysis

Specimens were fixed in 10% neutral formalin buffer, embedded in paraffin, and cut into 5-µm-thick sections. Immunohistochemical staining was performed using an immunoperoxidase method, with rabbit anti-human RNF43 polyclonal antibody (Sigma-Aldrich;HPA008079). In brief, each section was deparafinized and rehydrated by submerging in xylene and ethanol (respectively) with decreasing concentration. The antigens were unmarked by heating each section (in 0.1 mol/L citrate buffer, pH 6.0) in a microwave oven. After inactivating the endogenous peroxidase (0.5% H_2O_2 in methanol, 30 min, room temperature) and

blocking nonspecific binding (20% normal horse serum 30 min, room temperature), sections were incubated with primary rabbit anti-human RNF43 polyclonal antibody (dilution 1:100) at room temperature for overnight, then incubated with HRP labelled polymer anti rabbit (Dako), according to the manufacturer's instructions. The section was visualized with 3,3′ -diaminobenzidine-tetrahydrochloride, Liquid DAB+ (Dako), and counterstainedwith hematoxylin.

Staining specificity was evaluated for all the staining batches, using a negative control staining condition (i.e., no primary antibody) and CCA tissue sections from the 2 CCA patients (codes W039 and T157 that showed positive staining in tumor cells, normal bile ducts, and hepatocytes). Because there were no differences in staining intensity for each positive staining sample, the immunohistochemical results were evaluated as a frequency of RNF43-positive cells at the tumor area—classified into 4 scoring categories (0, negative; 1+, 1% - 10%; 2+, 11% - 50%; and 3+, >50%). Two researchers without any knowledge of the prognosis or clinicopathological variables evaluated the specimens. In the statistical analysis, the scores 0 and 1+ were categorized as "low expression"; and the scores 2+ and 3+, as "high expression."

2.2.1.2 RNA Extraction

Total RNA was extracted from tumor tissues and adjacent nontumorous tissues of the same patient using the TRIzol reagent (Invitrogen). In brief, frozen tissues were ground in liquid nitrogen with a mortar and pestle. Tissues were then transferred into a 1.5 ml microtube containing of 1 ml TRIzol reagent and 200 µl of chloroform 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. 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 agarose gel electrophoresis.

2.2.1.3 The complementary DNA (cDNA) synthesis

RNA was converted to cDNA by reverse transcription using Superscript VILO cDNA synthesis kit (Invitrogen) which contained 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 template RNA, 5x buffer and 10x enzyme mix. Total reaction was incubated for 60 min at 42°C. The cDNA was diluted with sterile distilled water to make final concentration of cDNA at 10 ng/ µl for subsequent analysis or kept at -20°C.

2.2.1.4 Quantitative real-time polymerase chain reaction analysis

Expression levels of *RNF43* gene were determined by SYBR-Green–based real-time polymerase chain reaction (PCR) in Light Cycler 480 II machine (Roche Applied Science, Indiana, USA). The PCR condition is summarized in Table 2.3. *β-Actin* served as the internal control for adjusting the amount of starter cDNA. Melting curve analysis was observed the presences of primer dimer formation and non-specific PCR product (Figure 2.2). The relative expression of *RNF43* gene was calculated using the $2^{-\Delta CT}$ equation for each tumor tissue or adjacent nontumorous tissue. The RNF43 messenger RNA (mRNA) expression of tumor tissues

compared to the adjacent nontumorous tissues of the same patients was 1.5-fold different.

Table 2.3 The thermal cycling profiles of qPCR in Roche Lightcycler 480 instrument.

Reagent and condition	***RNF43* or *β-actin***
Reaction volume	20 µl
2X SYBR green master mix	10 µl
10 µM Forward and Reverse primer	1 µl
Template cDNA (10 ng/ µl)	5 µl
Sterile distilled water	4 µl
PCR cycle	1 cycle at 95°C 300 sec
	40 cycle at 95°C 10 sec
	58°C 10 sec
	72°C 10 sec
Melting curve analysis	1 cycle at 95°C 5 sec
	65°C 60 sec
PCR efficiency	2

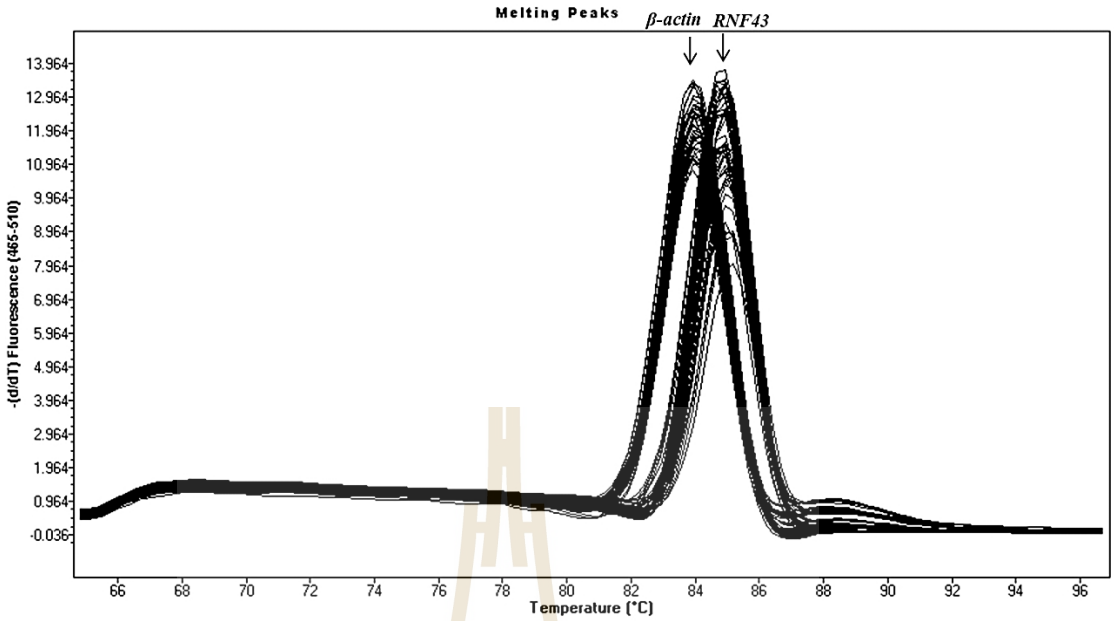

Figure 2.2 Melting peaks of *β-actin* and *RNF43* by melting curve analysis originated from an experiment on a Roche Lightcycler 480 instrument. The RNF43 amplicon has a melting temperature around 85°C, and (β-actin) internal control has a melting temperature around 84°C.

2.2.2 Investigating the biological role of RNF43 in CCA cell lines

2.2.2.1 Cell culture and transfections

CCA cell lines (KKU-055, KKU-213, KKU-214 and KKU-100) were cultured in Ham's F12 nutrient mixture medium (Gibco) supplemented with 10% FBS, 40 µg/mL gentamicin, and 0.25 µg/mL amphotericin B (Gibco) in a humidified atmosphere of 5% CO_2, at 37°C. To generate CCA cell lines with transient RNF43 expression, CCA cell lines were transfected with 2.5 µg of RNF43/pCMV6 construct or pCMV6 empty vector using Lipofectamine® 3000 (Invitrogen) according to the manufacturer's protocol. After 48 h, CCA cell lines were harvested, measured RNF43 mRNA or proteins expression and used in subsequent experiments.

2.2.2.2 Cell proliferation assay

Transfected cells were seeded (1-3 x 10^3cells/well) in a 96-well plate in the presence and absent of Wnt3a at 5 nM and were incubated for 24 and 48 h. The effects of RNF43 overexpression on CCA cell proliferation upon Wnt stimulation were measured using the colorimetric cell viability (MTS) assay using CellTiter 96 Aqueous One Solution Reagent (Promega, Wisconsin, USA). In vitro reduction of a tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carb oxymethoxyphenyl)-2-(4-sulfophenil)-2H-tetrazolium (MTS) by metabolically active cells yielded a colored formazan that was released into the culture medium. In brief, 20 µl/well MTS reagent was added into each well and then incubated for 2 h at 37°C in a humidified, 5% CO_2 atmosphere. Color development in this assay was measured at 490 nm using a microplate spectrophotometer (Bio-Rad).

2.2.2.3 Transwell migration assay

Transfected cells (4 x 10^4 CCA) suspending in 200 µl serum-free Ham's F12 were seeded into the upper chambers of a transwell apparatus with a 8-µm pore size of polycarbonate membrane (Corning Inc., New York, USA). Then 600 µl of Ham's F12 containing 10% FBS was added into the lower chambers as a chemoattractant. After incubation for 15 h (KKU-213) or 22 h (KKU-214), cells on the upper membrane surface were wiped off, and the cells that migrated across the pore of polycarbonate membrane were fixed with 100% methanol and stained with 0.5% crystal violet. The number of migrating cells was then counted (6 - 9 low power fields with 4x magnifications for each membrane) by ImageJ software (National Institutes of Health (NIH) Image). The percentage of migration was obtained from the following equation.

$$\% \text{ Migration} = \frac{\text{Mean of test cell migration number}}{\text{Mean of control cell migration number}} \quad X\ 100\ \%$$

2.2.2.4 Stimulation of recombinant human Wnt3a

Determination of intracellular proteins in *RNF43* overexpressing cells and control cells, Wnt3a at 5 nM (R&D Systems, Minnesota, USA) or bovine serum albumin (BSA) was used to activate Wnt signaling pathway for 24 h. The culture medium was then removed and the cells were washed with phosphate-buffered saline (PBS). Then the cells were lysed for protein collection and western blot analysis.

2.2.2.5 Whole cell lysate preparation

Cells were washed with PBS and lysed with lysis buffer pH 7.5 (20 mM Tris-base, 150 mM NaCl, 1% Triton X-100, and protease inhibitors) and incubated on ice for 30 min. Cell lysates were clarified by centrifugation at 16,000 xg for 20 min at 4°C. Protein concentration was determined with Novagen® BCA protein assay kit (EMD Chemicals, Darmstadt, Germany).

2.2.2.6 Nuclear extraction

Cells were collected and washed with PBS. Cells were lysed in hypotonic buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol [DTT], 0.25% NP-40, and protease inhibitors), and incubated on ice for 10 min. After centrifuging at 6000 rpm for 4 min at 4°C, the nuclear pellet was lysed in nuclear lysis buffer (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, and protease inhibitors), and incubated on ice for 15 min. Nuclear extracts were obtained by centrifugation at 14,000 rpm for 5 min.

2.2.2.7 Western blot analysis

Protein samples were mixed with 4x non-reducing Laemmli sample buffer to get the final concentration of 1x buffer and were boiled for 5 min by heat block (Labnet, New Jersey, USA). Then, protein samples were resolved by SDS-polyacrylamide gel electrophoresis on a 10% separating gel, transferred onto nitrocellulose membranes by wet/tank electroblotting systems, and incubated with primary antibodies overnight at 4°C after blocking with 5% low-fat milk. Secondary antibodies goat anti-rabbit IgG (GenScript) or rabbit anti-mouse IgG (Dako) conjugated with horseradish peroxidase were used for signal visualization with SuperSignal® West Pico Chemiluminescent Substrate kit (Thermo Fisher Scientific). Intensities of the signals examined were quantified using ImageJ software (NIH) and normalized by the internal loading control. Antibodies used in this study were RNF43 (Sigma-Aldrich), Phosphorelated β-catenin (Cell Signaling), β-catenin, Frizzled, Cyclin D1, Histone H1, and β-actin (Santa Cruz).

2.2.2.8 Statistical analysis

The statistical analysis of the CCA data was carried out using SPSS statistical software version 16.0.1 (SPSS, Illinois, USA). RNF43 mRNA expression from tumor tissues were compared with those of adjacent nontumorous tissues those of the control group using the independent Student *t* test or the Mann-Whitney *U* test. Cross-tabulations and chi-squared test were used to analyze the associations between RNF43 mRNA or protein expression and the clinicopathological features of CCA patients, and *RNF43* mutations. The Kaplan-Meier survival analysis was used to estimate overall survival and comparison between groups done using the log-rank test. The statistical significance was accepted if P value ≤ 0.05.

The data obtained from functional studies on RNF43 including cell proliferation and migration assay were expressed as the mean $\pm$ SD and were compared using Student's *t*-tests, with a *P* value of ≤ 0.05 taken as statistically significant.

CHAPTER III

RESULTS

3.1 RNF43 protein expression in cholangiocarcinoma tissues

Immunohistochemical staining of 50 CCA tissues showed that, in nontumorous area, RNF43 protein was expressed in the nuclei of hepatocytes and normal bile duct epithelium (Figure 3.1). By comparison, RNF43 protein expression varied in the tumor tissues (CCA cells), high expression was identified in 36% (n = 18) with specific diffuse nuclear staining for the RNF43, whereas the remaining 64% (n = 32) with focal or negative staining were evaluated as a low or absent expression.

We further quantitatively analyzed the correlation between RNF43 protein expression in CCA cells and the clinicopathological features, using a univariate analysis. There was no statistically significant association between RNF43 protein expression and age, sex, histologic types, tumor stages, vascular or lymphatic invasion, or somatic mutation (Table 3.1). However, the absent RNF43 protein expression was examined in 5 CCA patients with *RNF43* somatic mutations (including 3 missense and 2 nonsense mutations, Supplementary Table 1).

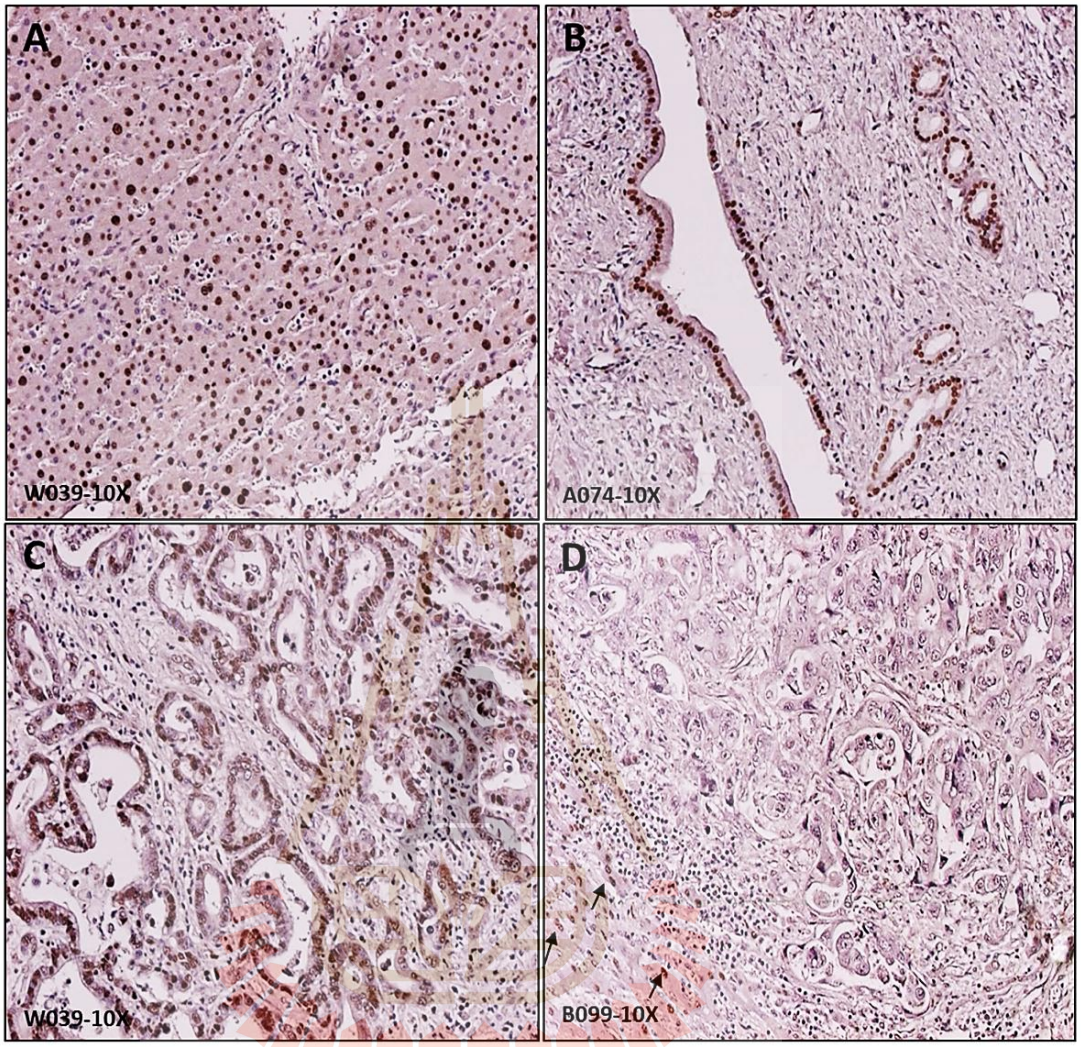

Figure 3.1 Immunohistologic analysis indicates high RNF43 protein expression in nontumorous area: in the nuclei of hepatocytes (A), normal bile duct epithelium (B), and tumor area (C) CCA cells with high expression and absence of RNF43 proteins (D). Arrows indicate hepatocytes at junction area. Original magnification, ×100.

Table 3.1 Association between RNF43 protein expression and clinicopathological features (n = 50).

Variables	RNF43 protein expression		*P**
	Low (n = 32)	High (n = 18)	
Age (y)			
< 56	14	6	0.470
≥ 56	18	12	
Sex			
Male	4	6	0.077
Female	28	12	
Histologic type			
Papillary type	12	9	0.390
Nonpapillary type	20	9	
Stage			
I - III	16	9	0.942
IV	16	9	
Lymphatic invasion			
Present	24	12	0.529
Absent	8	6	
Vascular invasion			
Present	23	12	0.700
Absent	9	6	
RNF43 somatic mutation			
Mutant	5	0	0.077
Wild type	27	18	

* $P \leq 0.05$, considered statistically significant.

3.2 Down-regulation of RNF43 protein expression associated with poor patient survival

The Kaplan-Meier analysis provided an estimate of the overall survival stratified by RNF43 protein expression (absent or low versus high) (Figure 3.2). One patient died perioperatively (survived < 30 days), so was excluded from the analysis. Patients with absent or low expression of RNF43 had significantly shorter overall survival than patients with high RNF43 expression (median overall survival, 237 ± 31 versus 475 ± 97 days; P = 0.016, log-rank test). Several clinicopathological parameters have been shown to correlate with the prognosis of CCA patients. We, thus, assessed whether RNF43 protein expression was a confounding factor—underlying the clinical conditions—by performing univariate and multivariate Cox proportional hazard regression analysis. The results showed that age, sex, and RNF43 protein expression were significant prognostic factors associated with overall survival (Table 3.2). The multivariate Cox regression model for survival, which controlled for age, sex, histology types, tumor stages, lymphatic invasion, vascular invasion, and RNF43 protein expression of the patients, indicated that only RNF43 protein expression (between low and high) was potentially an independent predictor of CCA survival (hazard ratio, 1.961 [95% confidence interval, 0.981-3.920]; P = 0.057) (Table 3.2).

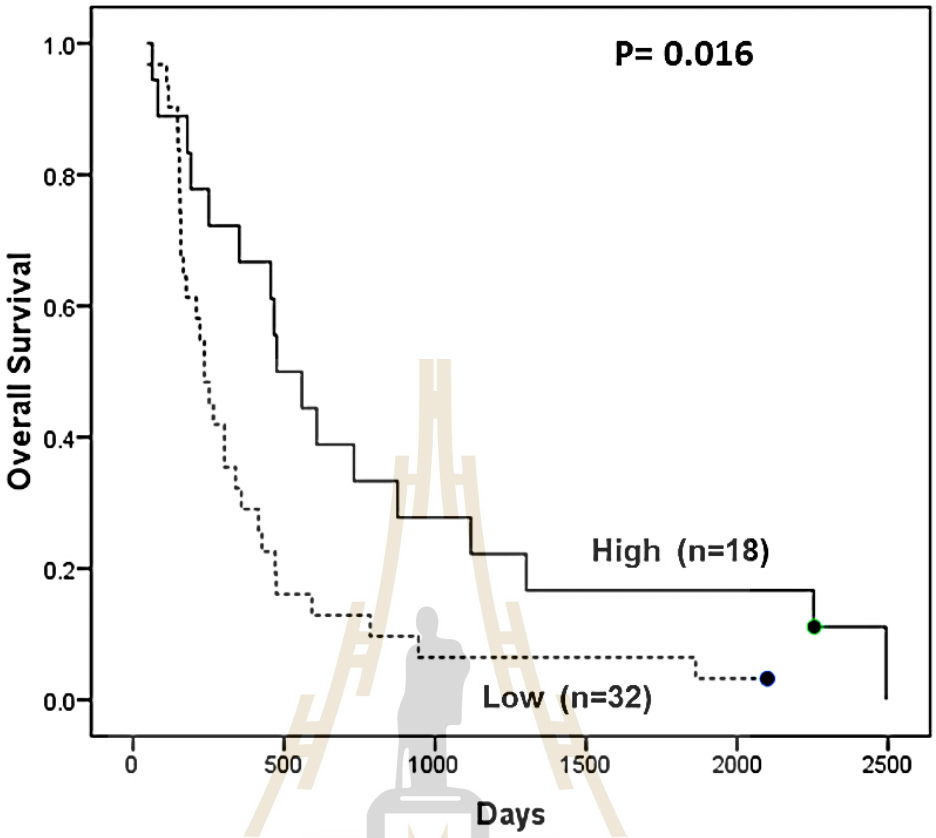

Figure 3.2 Cumulative overall survival determined using Kaplan-Meier analysis for CCA patients with high protein expression of RNF43: they had significantly better survival than those with low protein expression of RNF43. Black circles indicate censor cases.

Table 3.2 Univariate and multivariate analyses of factors associated with survival.

Variables	Univariate analysis [a]		Multivariate analysis [a]	
	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P* [b]
Age: ≥ 56 *vs* < 56 years	2.143 (0.986 – 4.656)	0.049	1.746 (0.754 – 4.042)	0.193
Sex: male *vs* female	1.210 (0.666 – 2.198)	0.053	1.206 (0.640 – 2.270)	0.562
Histologic type: papillary *vs* nonpapillary	1.739 (0.945 – 3.198)	0.071	1.336 (0.656 – 2.720)	0.424
Tumor stage: VI *vs* I-III	0.951 (0.529 – 1.709)	0.865	0.803 (0.396 – 1.628)	0.542
Lymphatic invasion: present vs absent	1.067 (0.553 – 2.061)	0.846	0.756 (0.339 – 1.688)	0.495
Vascular invasion: present *vs* absent	1.748 (0.920 – 3.320)	0.083	1.822 (0.881 – 3.767)	0.106
RNF43 protein expression: low vs high	2.142 (1.137 – 4.036)	0.016	1.961 (0.981 – 3.920)	0.057

Abbreviation: CI, confidence interval.

[a] Cox proportional hazards regression.

[b] Partial likelihood ratio test.

3.3 Genetic alterations of *RNF43* in cholangiocarcinoma

To address the association between genetic alteration status (mRNA expression level, and mutations) and RNF43 protein expression, we obtained 28 frozen CCA tumors from the total 50 CCA cases including the adjacent nontumorous tissue of the same patient and determined the *RNF43* mRNA expression levels using the quantitative real-time PCR (qPCR) assay. The median *RNF43* mRNA expression of 28 CCA tumors was 6.88 (range 2.11-35.62) which was lower than those of 28 adjacent nontumorous tissue with a median of 10.20 (1.30-45.88). However, there was no statistically significant difference between these two tissues. We then further analyzed the difference of *RNF43* mRNA expression between CCA tumor and adjacent nontumorous tissues of the same patient to minimize the background which due to use of bulky tissues. Down-regulation of *RNF43* mRNA expression in CCA tissues was identified in 43% (n = 12), whereas remaining CCA tissues had up-regulated (25%; n = 7) or no change (32%; n = 9) in the *RNF43* mRNA expression (Figure 3.3). The relationship between the mRNA expression and the protein expression or clinicopathological features was observed using a univariate analysis. There was no statistically significant association between *RNF43* mRNA expression and protein expression or the clinicopathological features tested (Table 3.3). In addition, Kaplan-Meier analysis demonstrated that CCA patients with down-regulation of *RNF43* mRNA expression were had significantly shorter survival compared with those of the patients with up-regulation of *RNF43* mRNA expression (Figure 3.4; $P = 0.037$).

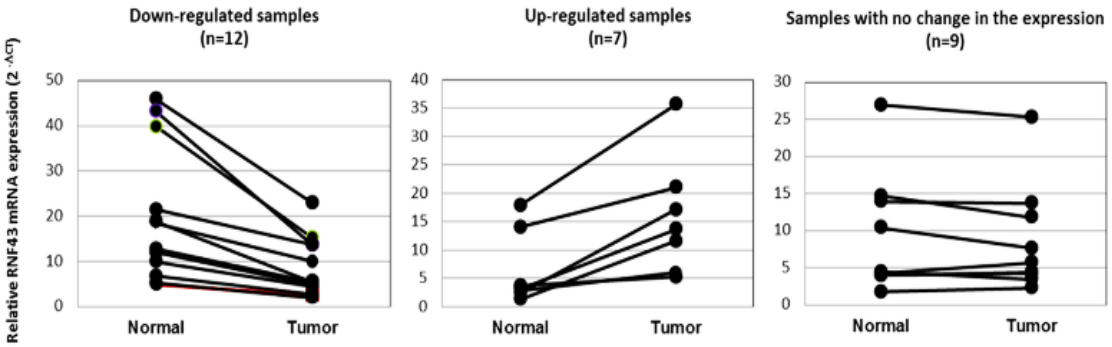

Figure 3.3 *RNF43* mRNA expression level down-regulated in cholangiocarcinoma. The expression level of *RNF43* was analyzed by qPCR and normalized using *β-actin* as reference gene. *RNF43* expression of the 28 CCA cases was grouped into 3 categories based on the fold change between tumor tissues and adjacent nontumorous tissues of the same patient.

Table 3.3 Association between *RNF43* mRNA expression and clinicopathological features (n = 28).

Variables	*RNF43* mRNA expression			*P**
	Down-regulated (n = 12)	Up-regulated (n = 7)	No change (n = 9)	
Age (y)				
< 56	2	2	1	0.657
≥ 56	10	5	8	
Sex				
Male	9	3	3	0.134
Female	3	4	6	
Histologic type				
Papillary type	2	3	3	0.442
Nonpapillary type	10	4	6	
Stage				
I - III	4	4	4	0.595
IV	8	3	5	
Lymphatic invasion				
Present	6	2	2	0.380
Absent	6	5	7	
Vascular invasion				
Present	3	4	3	0.364
Absent	9	3	6	
RNF43 protein expression				
Absent or low	8	4	6	0.792
High	3	3	3	

* $P \leq 0.05$, considered statistically significant.

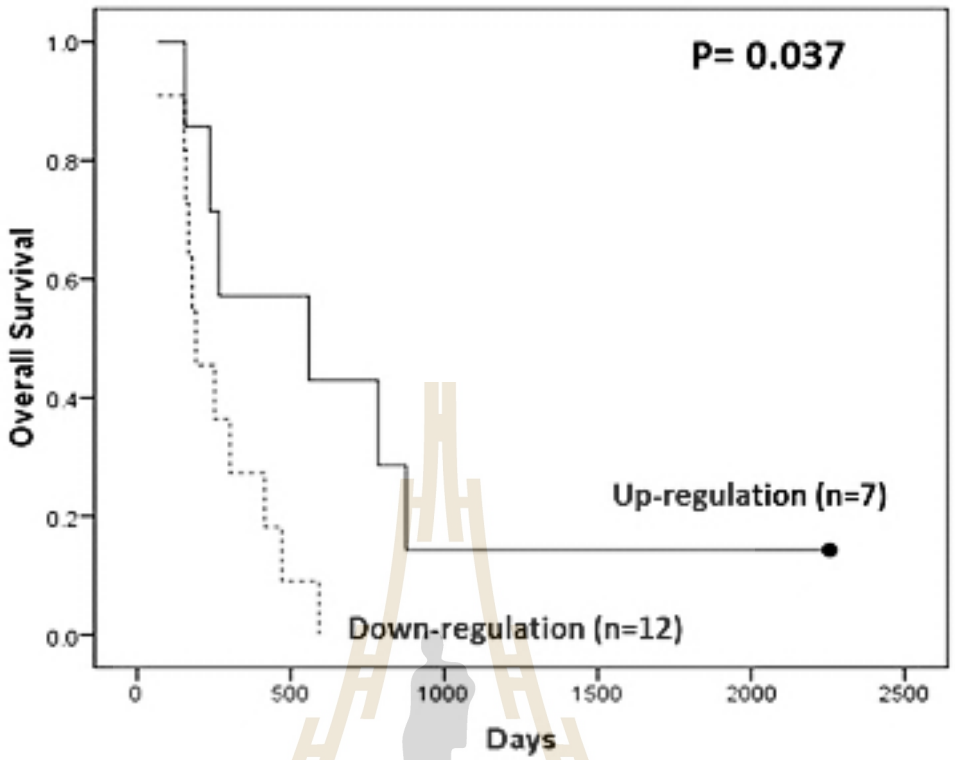

Figure 3.4 Cumulative overall survivals between CCA patients with *RNF43* mRNA up-regulation and down-regulation determined using Kaplan-Meier analysis. CCA patients with up-regulation: they had significantly better survival than those with down-regulation of *RNF43* mRNA. Black circles indicate censor cases.

3.4 The functional analysis of RNF43 in CCA cell lines

3.4.1 Endogenous *RNF43* mRNA expression in CCA cell lines

We investigate the role of RNF43 in vitro. Firstly, the endogenous *RNF43* mRNA and protein expression were determined in CCA cell lines including KKU-055, KKU-100, KKU-213 and KKU-214 using qPCR and Western blot analysis. High expression of *RNF43* mRNA was demonstrated in both KKU-214 and KKU-213 while KKU-055 and KKU-100 had a relatively low endogenous mRNA level of RNF43 (Figure 3.5). However, the endogenous RNF43 protein was not detectable in all CCA cell lines (Figure 3.6). This is perhaps due to low levels of endogenous *RNF43* mRNA in all CCA cell lines which have been detected at late stage of PCR cycle (PCR cycle range at 29-35). In addition, down-regulation of *RNF43* may occur in the particular patient who donated the CCA tissues for establishing CCA cell lines.

To study the biological role of *RNF43* in these CCA cell lines via overexpression model, CCA cell lines were transfected with RNF43/pCMV6 construct or pCMV6 empty vector using lipofectamine 3000 and incubated for 48 h. Western blot analysis demonstrated that the exogenous RNF43 protein was expressed in KKU-055, KKU-213, and KKU-214 with RNF43/pCMV6 construct except KKU-100 (Figure 3.7) when compared to those cells with control vector. The result of protein expression is consistent with the *RNF43* mRNA expression for all three CCA cell lines (Figure 3.8).

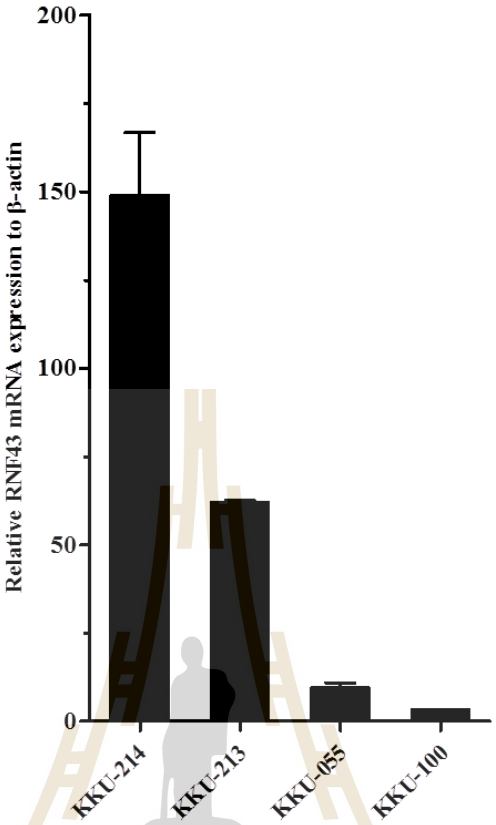

Figure 3.5 The endogenous *RNF43* mRNA expression in four human CCA cell lines. The relative expression level of *RNF43* was analyzed by qPCR and normalized using *β-actin* as reference gene. Data are presented as mean ± SD of two dependent experiments.

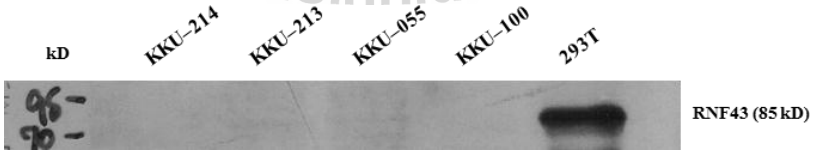

Figure 3.6 The endogenous RNF43 protein expression in four human CCA cell lines; KKU-214, KKU-213, KKU-055, and KKU-100. The whole cell lysates were subjected to Western blotting. 293T cell (originally derived from human embryonic kidney cell containing the SV40 T-antigen) expressing RNF43 was used as a positive control. kD; kilodalton

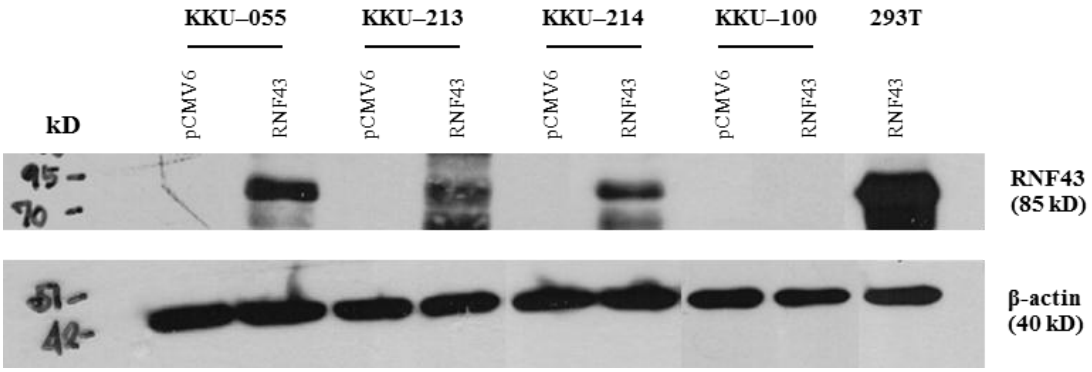

Figure 3.7 The exogenous RNF43 protein expression in four human CCA cell lines KKU-055, KKU-213, KKU-214, and KKU-100. Whole cell lysates was used to determine the protein level of RNF43 in four human CCA cell lines that transfected with RNF43/pCMV6 construct or pCMV6 emtry vector for 48 h. 293T cell expressing RNF43 was used as a positive control. The β-actin level indicates equal amounts of total protein. kD; kilodalton

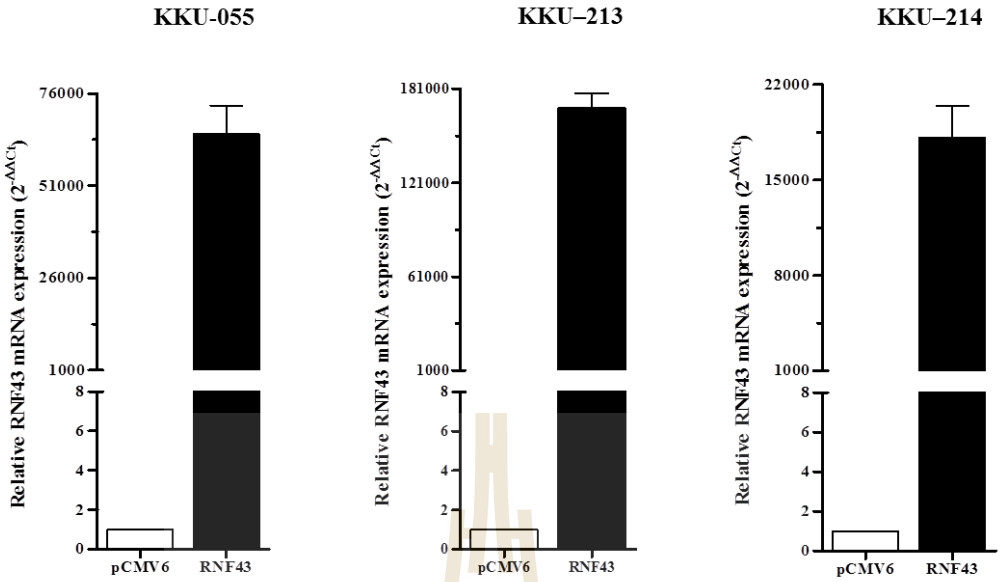

Figure 3.8 The exogenous *RNF43* mRNA expression in three human CCA cell lines KKU-055, KKU-213, and KKU-214. The total RNA was extracted after transfection for 48 h and the *RNF43* mRNA expression was performed using qPCR analysis. *β-actin* was used as reference gene. The fold change of *RNF43* mRNA expression was calculated by $2^{-\Delta\Delta Ct}$. Data are presented as mean ± SD of two dependent experiments.

3.4.2 The restoration of RNF43 gene expression reduces the proliferation rate of CCA cell line upon Wnt3a stimulation.

We investigated whether the effect restoration of RNF43 expression in CCA cell lines alter the rate of cell proliferation and migration upon Wnt stimulation. Firstly, KKU-055 was selected to determine the role of *RNF43* in vitro because it had a relatively low endogenous level of RNF43 among all CCA cell lines and showed high effeciency of transfection with low cytotoxicity. The results showed that exogenous RNF43 expression was expressed in KKU-055 (Figure 3.7). However, there was no changed on cell proliferation and migration in KKU-055 expressing RNF43 as compared to control cells (Figure 3.9 and 3.10). To study the potential function of RNF43 in CCA ,we changed to use KKU-213 and KKU-214 instead as an in vitro model. Although, KKU-213 and KKU-214 showed high endogeous mRNA level of RNF43, however, the protein level was not detectable. Thus, these two CCA cell lines were forced to express RNF43 using RNF43/pCMV6 construct (Figure 3.7). We examined the effect of RNF43 expression on the growth of the two CCA cell lines. The results showed that RNF43 expression reduced proliferation rate of both cell lines after 24 and 48 h of incubation. Moreover, stimulation with Wnt3a at 5 nM significantly decreased the growth of KKU-213 ($P < 0.001$) and KKU-214 ($P < 0.05$) expressing RNF43 in a time-dependent manner (after 24 and 48 h of incubation) where Wnt3a stimulation had no influence to rate of cell proliferation in CCA cell lines (Figure 3.11 and Supplement Figure 1 and 2). A Transwell migration assay was subsequently carried out to determine whether RNF43 expression affects the migration of CCA. The number of migrating cells in KKU-213 or KKU-214 expressing RNF43 was found to be relatively lower than those cell with control vector

(Figure 3.12). However, there was no statistically significant observed between RNF43 overexpression and cell migration.

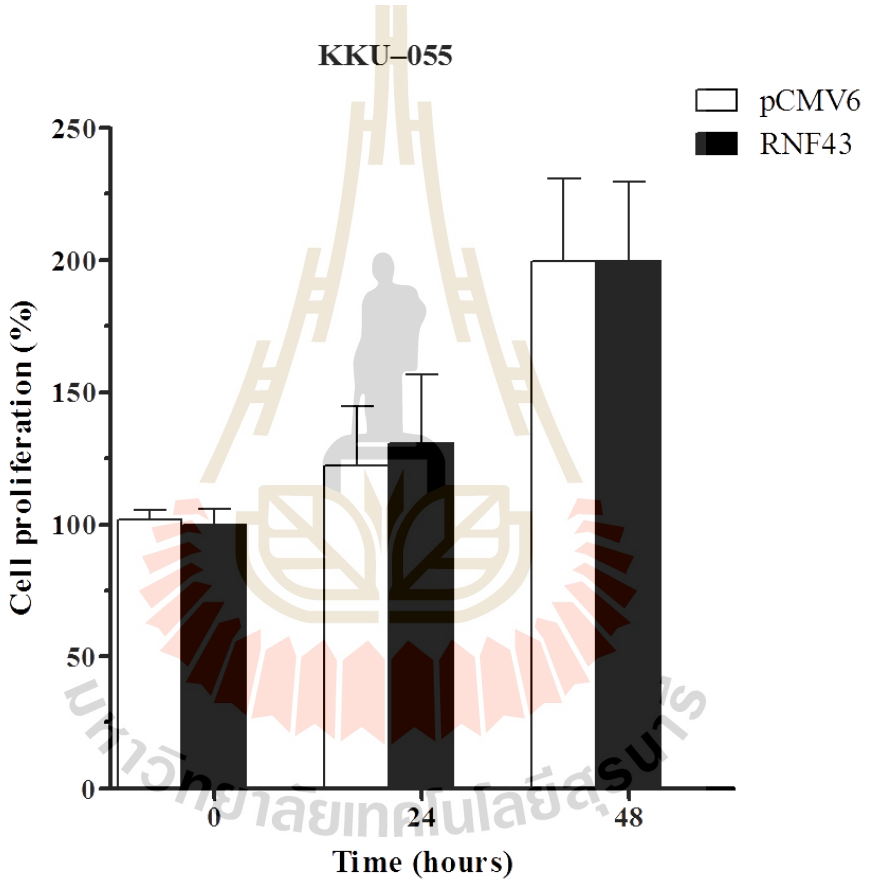

Figure 3.9 Effects of RNF43 on cell proliferation in KKU-055 cells. The MTS assay was performed to measure the cell viability in KKU-055 expressing RNF43 or pCMV6 empty vector at 24 and 48 h. Percentage of cell proliferation was normalized to absorbance of KKU-055 transfected with pCMV6 empty vector at 0 h. Data represent the mean ± SD of two independent experiments.

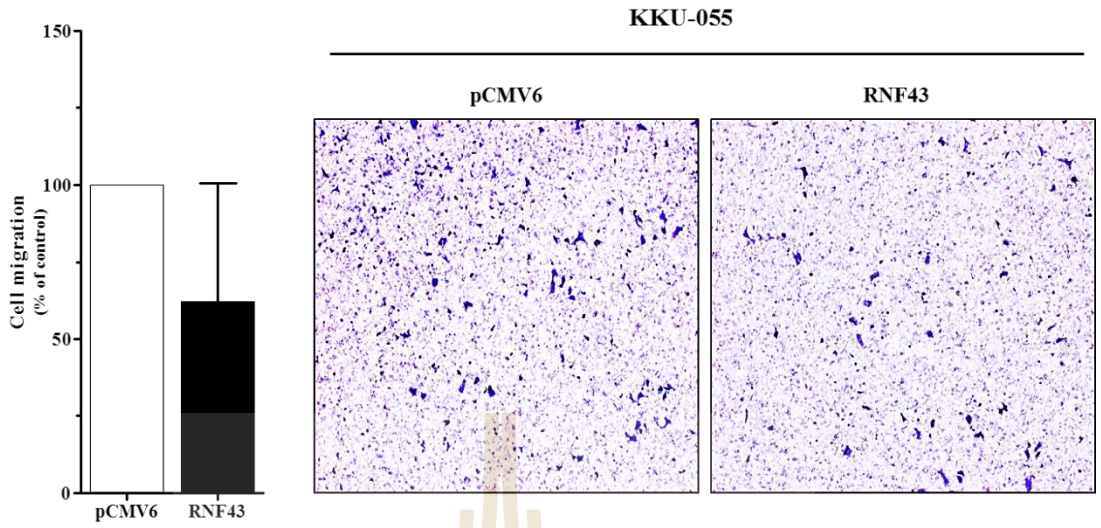

Figure 3.10 Effects of RNF43 on cell migration in KKU-055 cell. KKU-055 expressing RNF43 or pCMV6 empty vector were loaded into the upper chamber of a well in a Transwell plate and incubated for 22 h. Number of migrating cells were counted (Left panels) from six random microscopic field and representative image of migrating cell in the chamber are shown for each condition (Right panels). Data represent the mean ± SD of three independent experiments and the percentage of cell migration was normalized to that of KKU-055 transfected with pCMV6 empty vector which was assumed to be 100%.

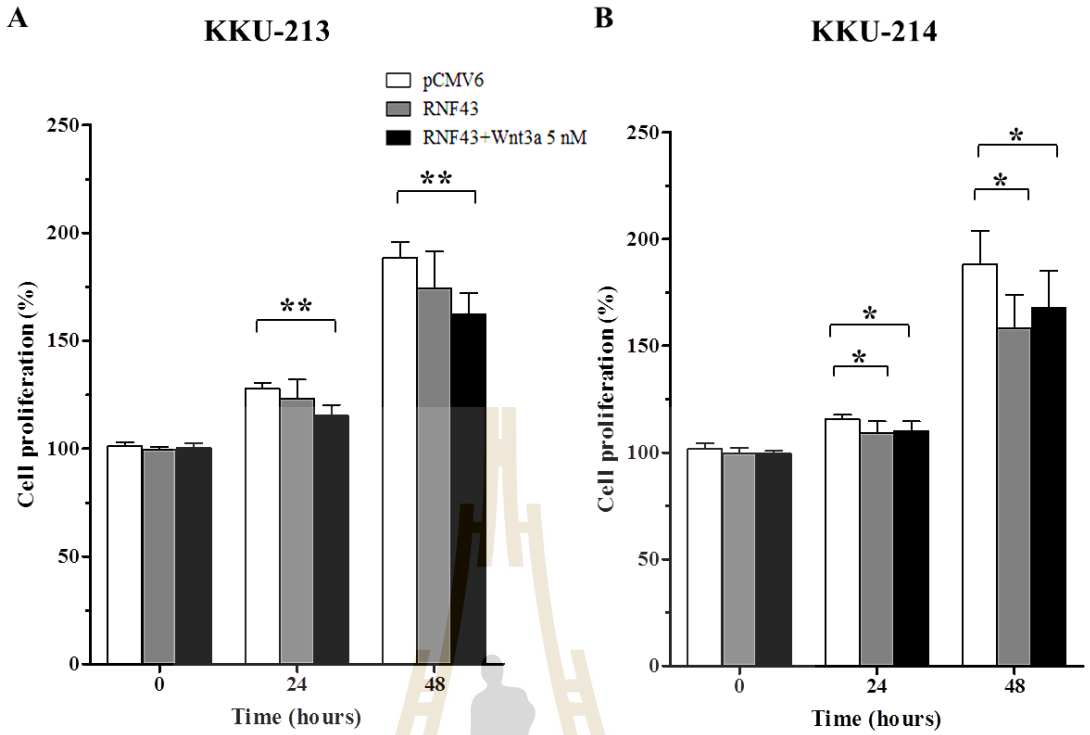

Figure 3.11 Overexpression of RNF43 reduces the growth of CCA cell lines. The MTS assay was performed to measure the cell viability in KKU-213 (A) and KKU-214 (B) expressing RNF43 or pCMV6 empty vector at 24 and 48 h. Percentage of cell proliferation was normalized to absorbance of KKU-213 or KKU-214 transfected with pCMV6 empty vector at 0 h. Data represent the mean ± SD of two independent experiments. $*P < 0.05$, $**P < 0.001$, *t*-test.

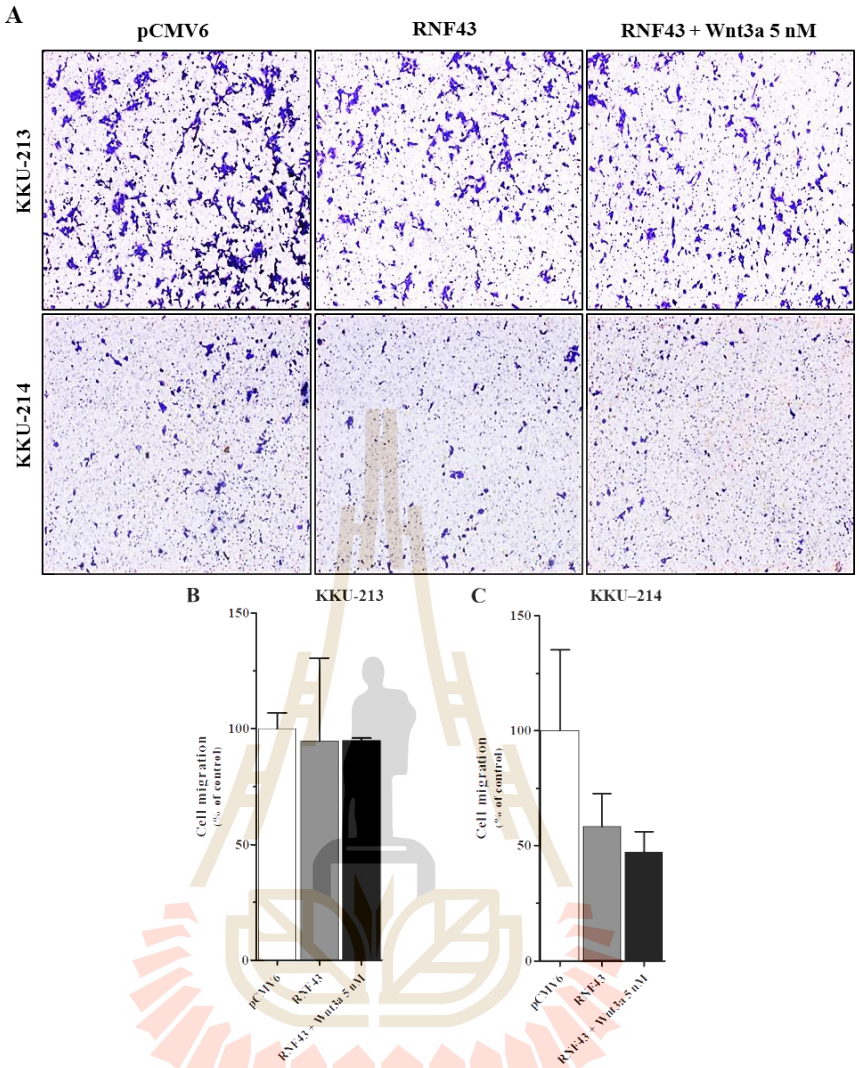

Figure 3.12 Overexpression of RNF43 does not alter the migration ability of CCA cell lines. The two CCA cell lines (KKU-213 and KKU-214) expressing RNF43 or pCMV6 empty vector were loaded into the upper chamber of a well in a Transwell plate and incubated for 15 h (KKU-213) and 22 h (KKU-214). Number of migrating cells were counted (Lower panels) from six random microscopic fields and representative images of migrating cell in the chamber are shown for each condition (Upper panels). Data represent the mean ± SD of three independent experiments and the percentage of cell migration was normalized to that of KKU-213 or KKU-214 transfected with pCMV6 empty vector which was assumed to be 100%.

3.4.3 The change in RNF43 expression affects the activation of Wnt signaling

To study the molecular mechanism of RNF43-reduced the growth of CCA cell lines, Western blot analysis was performed to determine the change in expression level of RNF43 and Wnt signaling proteins. Our data demonstrated that exogenous RNF43 protein was detected in the whole cell lysate from both KKU-213 and KKU-214 cell lines expressing RNF43 in comparison with control vector. Subcellular fractionation demonstrated that exogenous RNF43 protein was prominently expressed in the nucleus fraction when compared to cytoplasmic fraction. Forced overexpression of RNF43 was able to suppressed the activaton of Wnt signaling in both CCA cell lines (KKU-213 and KKU-214), evidenced by reducing the level of frizzled protein, increased the phosphorylation of β-catenin (Ser33/37/Thr41) and decreased the protein level of cyclin D1. Similar result was observed on KKU-213 and KKU-214 expressing RNF43 under Wnt3a stimulation (Figure 3.13). Therefore, these results suggested that decreased the protein level of frizzled and cyclin D1, major downstream targets of Wnt signaling, induced by RNF43 might play a key role in negative regulation of proliferation in CCA.

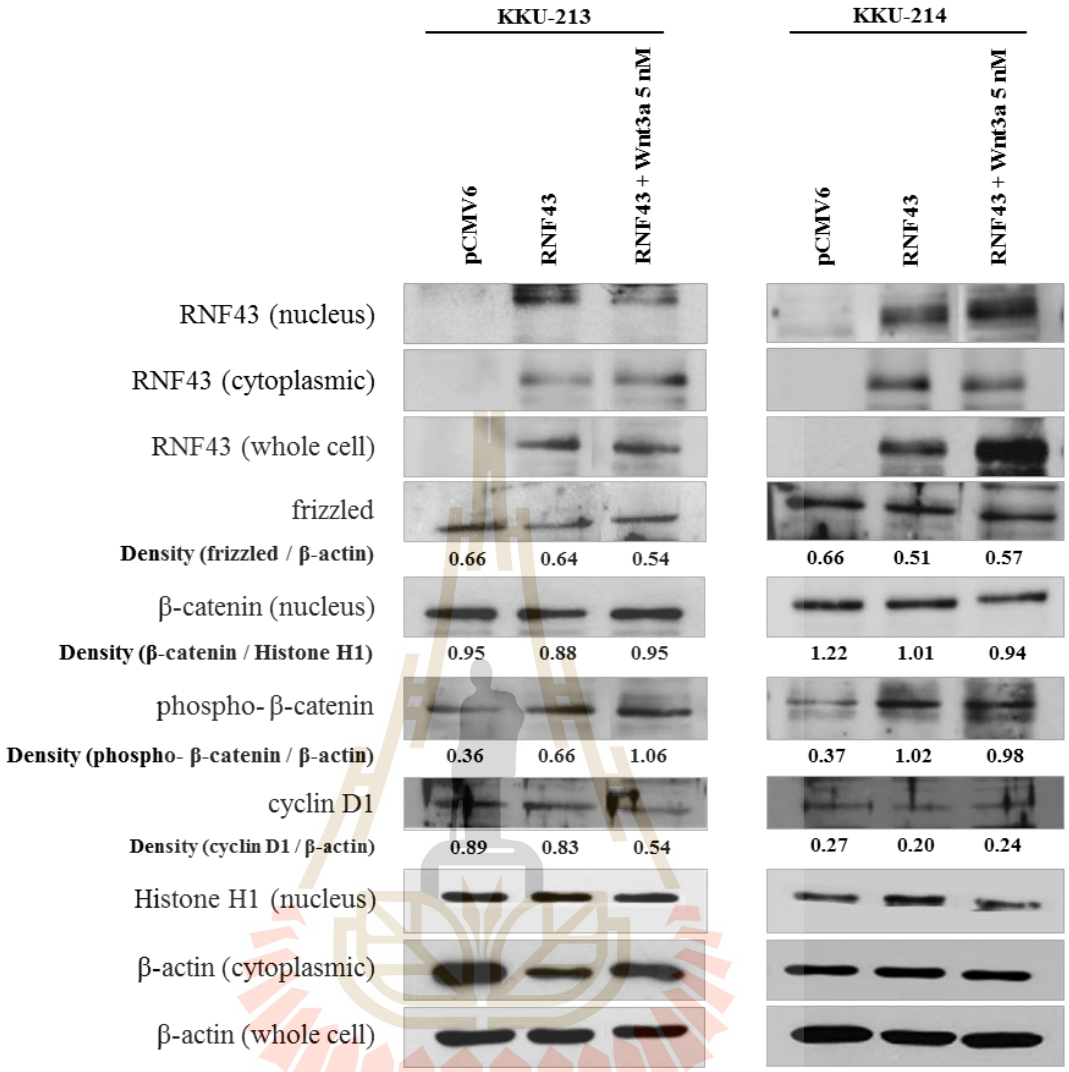

Figure 3.13 Overexpression of RNF43 suppresses the activation of Wnt signaling pathway. KKU-213 and KKU-214 were transiently transfected with RNF43/pCMV6 construct or pCMV6 empty vector for 48 h. and then treated with 5 nM of Wnt3a for 24 h. The whole cell lysates were subjected to Western blotting and probed with an antibody specific to RNF43, frizzled, phosphorylated β-catenin, cyclin D1 and β-actin while the protein level of RNF43, β-catenin were also performed in nuclear protein fractions. Histone H1 and β-actin were used as a loading control to indicate the equal amount of total protein from nucleus fractions and whole cell lysate, respectively. Intensity of the immunoreactive bands were measured by ImageJ software (NIH).

CHAPTER IV

DISCUSSION AND CONCLUSION

RING finger ubiquitin-protein ligases (E3s) are the most abundant class of E3 that mediate protein ubiquitination. They regulate crucial cellular functions, such as the cell cycle, DNA repair, cell signaling and responses to hypoxia. It has become evident that many RING finger E3s are implicated in malignancy. Some are validated oncogenes (such as *MDM2*) or tumor suppressor genes (such as *BRCA1* and von Hippel–Lindau tumor suppressor (*VHL*)) because of their role in regulating crucial cell functions (Lipkowitz and Weissman, 2011). Ring finger protein 43 or RNF43 belongs to the family of E3 finger ubiquitin-protein ligases. It has been demonstrated that the loss-of-function mutation in *RNF43* acts as either an oncogene or a tumor suppressor gene depending on the tumor type. In CCA, *RNF43* was thought to act as a tumor suppressor gene because CCA patients with inactive *RNF43* mutations are associated with poorer survival (Ong et al., 2012). In the present study, we determined the expression level of RNF43 in both mRNA and protein levels in CCA tissues and its role in CCA cell lines. Most of the CCA tissues showed predominantly down-regulated RNF43 expression from both mRNA and protein levels using qPCR and immunohistochemistry technique, respectively. The expression results from mRNA and protein levels of RNF43, which showed the same trend, although no statistically significant expression of *RNF43* mRNA was found in CCA tissues. This is perhaps due to use of bulky CCA tissues, which were possibly contaminated with

both hepatocytes and stromal cells. Down-regulation of RNF43 protein expression was significantly associated with less survival of CCA patients. Univariate and multivariate analyses revealed that RNF43 protein expression was a potential independent prognostic factor of CCA. Our findings are thus consistent with a study of glioma and gastric cancer, in which decreased expression of RNF43 was associated with a poor prognosis (Niu et al., 2015; Xi et al., 2015). However, there was no statistically significant association between RNF43 protein expression and age, sex, histologic types, tumor stages, vascular or lymphatic invasion, or somatic mutation. According to RNF43 protein expression in the CCA tissues—especially the 5 CCA patients with 3 missense and 2 nonsense mutations, which showed absent RNF43 protein expression in tumor cells—we, nevertheless, observed protein expression in the normal bile duct epithelium. This result confirmed the prediction of our previous study (Ong et al., 2012) that the 3 missense mutations are functionally damaged. Since, we frequently noted the down-regulation of RNF43 rather than the detection of any RNF43 somatic mutations. This result implies us that epigenetic alteration might account for the reduced expression in addition to a somatic mutation. Wang et al. recently demonstrated that miR550a-5p directly targets the 3′UTR of RNF43, thereby regulating the protein expression of RNF43 (Wang et al., 2016). Taken together, the results so far imply that down-regulation of RNF43 may play a role in cholangiocarcinogenesis.

RNF43 is proposed as a negative Wnt regulator by reducing frizzled receptor surface expression (Koo et al., 2012). In the current study, we provided convincing evidence that RNF43 decreased cell proliferation in CCA via regulating frizzled receptors in Wnt signaling pathway. Our data demonstrated that down-regulation of

RNF43 was observed in all CCA cell lines but KKU-055 had a relatively low endogenous expression of RNF43 among all CCA cell lines. This is perhaps due to down-regulation of *RNF43* may occur in the particular patient who donated the CCA tissues for establishing CCA cell lines. RNF43 expression was restored in KKU-055, KKU-213 and KKU-214 by transfecting with RNF43/pCMV6 construct. MTS demonstrated that restoration of RNF43 expression reduced proliferation rate of both KKU-213 and KKU-214. However, there was no change on the growth of KKU-055 expressing RNF43. This result implies that the effect of RNF43 on cell growth is a cell-type specific. Moreover, the significance of RNF43 on cell growth was evidently observed upon Wnt3a stimulation in particular of KKU-213 expressing RNF43 where stimulation of Wnt3a on parental CCA cells did not show the influence on the growth of CCA cell lines (Supplementary Figure 1 and 2). This result agrees with the studies in pancreatic and gastric cancers in which RNF43 expression inhibits cell proliferation and lead to induces cell apoptosis (Niu et al., 2015) while the overexpression of *RNF43* in hepatocellular carcinoma reveals oncogenic function by promoting cell proliferation and invasion (Xing et al., 2013). In addition, the role of RNF43 on cell migration and invasion has also been observed for several cancers. Wang et al. demonstrated that the decreased protein expression of RNF43 is regulated by miR550a-5p which directly targets the 3′UTR of RNF43. This process leads to increase cell migration in colorectal cancer (Wang et al., 2016). In CCA, transwell migration assay indicates that RNF43 does not play a significant role in CCA cell migration. Hence, it can be concluded that RNF43 has a role in CCA progression by reducing the growth of CCA cells.

RNF43 has been demonstrated to inactivate Wnt signals via 1) interacting with the Wnt receptors of the frizzled, and 2) sequestering TCF4 to the nuclear membrane (Jiang et al., 2013; Koo et al., 2012; Loregger et al., 2015). Inactivation mutation of RNF43 has been identified in CCA patients with poor prognosis (Ong et al., 2012). Moreover, down-regulation of RNF43 mRNA or protein was associated with genetic alteration status including somatic mutation and polymorphism (Talabnin et al., 2016). However, functional study of RNF43 *in vitro* or *in vivo* had not been conducted in CCA. Here, we show that forced overexpression of RNF43 was able to suppressed the activation of Wnt signaling in CCA cell lines (KKU-213 and KKU-214), evidenced by reducing the level of frizzled protein, increased the phosphorylation of β-catenin (Ser33/37/Thr41) and decreased the protein level of cyclin D1. Upon Wnt3a stimulation significantly reduced the protein level of cyclin D1 in particular of KKU-213 expressing RNF43. This result is consistent with the finding from MTS assay that showed markedly reduction of cell growth in KKU-213 expressing RNF43 with Wnt3a stimulation. These results indicates that RNF43 decreased cell growth by reducing the membrane level of frizzled in CCA cell line and then promotes the inactivation of Wnt signaling pathway by increasing β-catenin protein degradation. Similar observation has been previously reported in pancreatic ductal adenocarcinoma (Jiang et al., 2013). Since, the function of RNF43 is also proposed in nucleus by interacting with TCF4 in the nucleus and then tethers TCF4 to the nuclear membrane, thus silencing TCF4 transcriptional activity and inactivation of Wnt signaling (Loregger et al., 2015). In CCA, RNF43 was mainly expressed in nuclei of tumor cells in CCA tissues and RNF43 overexpressing CCA cell lines. Thus, further investigation is required to clarify the role of nuclear RNF43 in CCA.

In conclusion, this study confirms the frequent down-regulation of RNF43 in CCA samples based on qPCR and IHC studies. CCA patient with reduced RNF43 mRNA or protein expression was associated with poor prognosis. RNF43 protein expression was identified as an independent prognosis factor of overall survival in CCA. The suppressive role of RNF43 in regulating Wnt signaling was observed to control the growth of CCA cells. Present study suggests that RNF43 in CCA has a tumor suppressive function and the identification of genetic alteration status of RNF43 may provide specific therapeutic strategies for patient treatment.

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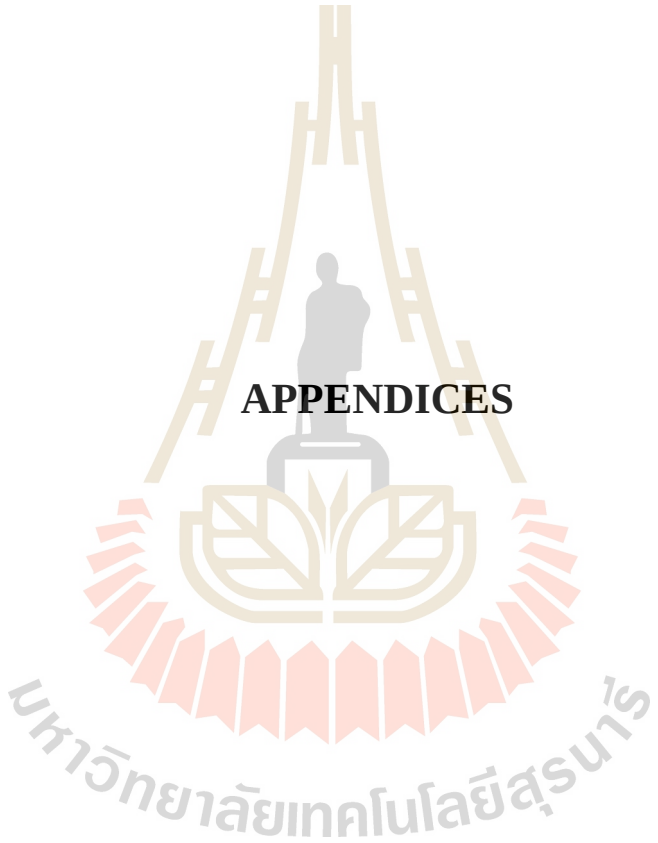
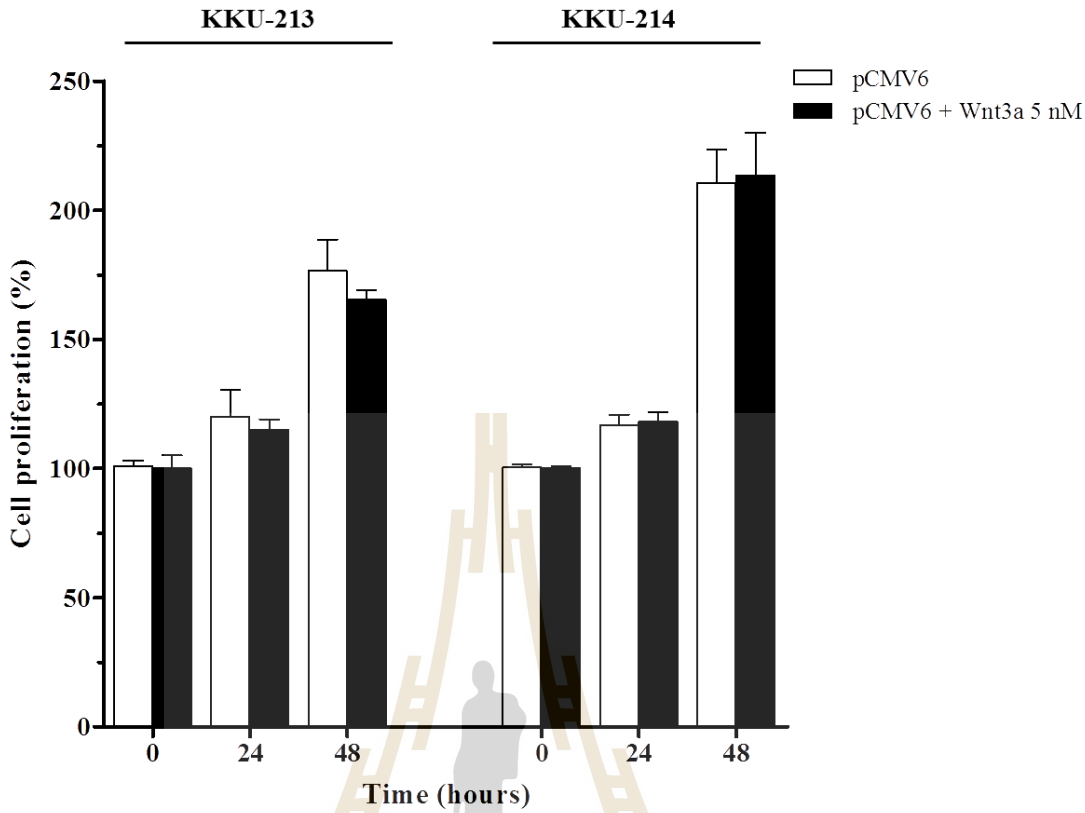
APPENDICES

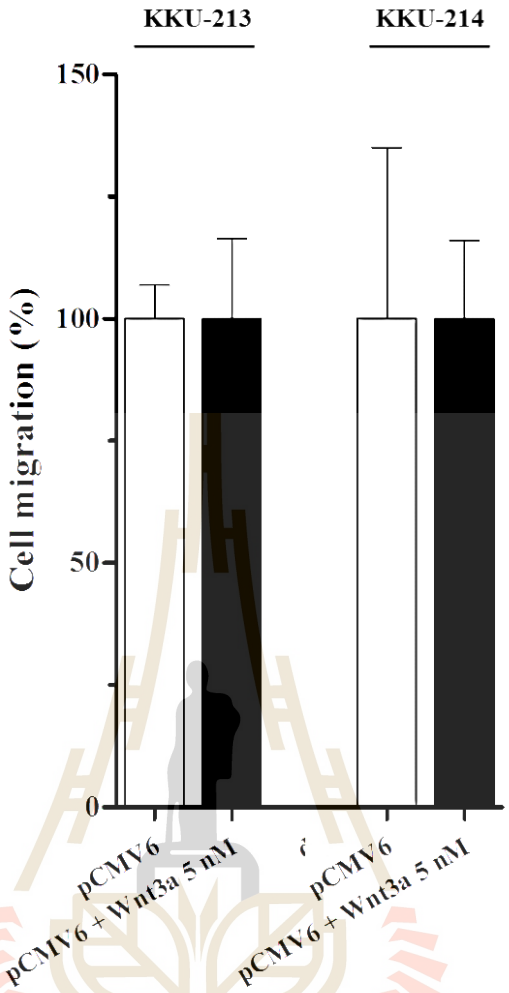


APPENDIX A

SUPPLEMENTARY DATA



Supplementary Figure 1 Effect of Wnt3a stimulation on cell proliferation. KKU-213 and KKU-214 cells were transiently transfected with pCMV6 empty vector. The MTS assay was performed to measure cell viability of KKU-213 (Left) and KKU-214 (Right) after incubation for 24 and 48 h. Percentage of cell proliferation was normalized to absorbance of KKU-213 or KKU-214 transfected with pCMV6 empty vector at 0 h. Data represent the mean ± SD of two independent experiments.



Supplementary Figure 2 Effect of Wnt3a stimulation on cell migration. KKU-213 and KKU-214 were transiently transfected with pCMV6 empty vector. The transfected cells were loaded into the upper chamber of a well in a Transwell plate and incubated for 15 h (KKU-213, Left) and 22 h (KKU-214, Right). Number of migrating cells were counted from six random microscopic field. Data represent the mean ± SD of two independent experiments and the percentage of cell migratin was normalized to that of KKU-213 or KKU-214 transfected with pCMV6 empty vector which was assumed to be 100%.

Supplementary Table 1 *RNF43* somatic mutations and RNF43 protein expression in 5 CCA.

CCA	Histological type	Stage	*RNF43* mutation	Immunohistochemistry Result
A074	WD tubular adenocarcinoma	IVA	Missense (p.T204R)	Negative (+ve on normal and Dilatation of BD)
A159	Papillary carcinoma	IIIB	Nonsense (p.R113X)	Negative (+ve on normal and Dilatation of BD)
R104	WD tubular adenocarcinoma	III	Missense (p.C119R)	Negative
U044	Papillary carcinoma	IIIB	Missense (p.N167I)	Negative
W012	WD tubular adenocarcinoma	III	Nonsense (p.Q723X)	Negative (+ve on normal and Dilatation of BD)

Abbreviation BD; Bile Duct

APPENDIX B

RESEARCH PUBLICATIONS AND PRESENTATIONS

1) Research Publication

Talabnin, C., Janthavon, P., Thongsom, S., Suginta, W., Talabnin, K., and Wongkham, S. (2016). Ring finger protein 43 expression is associated with genetic alteration status and poor prognosis among patients with intrahepatic cholangiocarcinoma. **Human Pathology**, 52, 47–54. doi.org/10.1016/j.humpath.2015.12.027 (IF2014 = 2.79)

2) Poster presentation

Janthavon, P., Thongsom, S., Suginta, W., Wongkham, S., and Talabnin, C. Ring Finger Protein 43 (RNF43) Expression and Its Role in Cholangiocarcinoma. The 10th International Symposium of The Protein Society of Thailand (PST), July 15-17, 2015. Bangkok, Thailand.

3) Extracurricular Activities

1. Attending "The 7th Asia Oceania Human Proteome Organization (AOHUPO) Congress and The 9th International Symposium of The Protein Society of Thailand (PST)", August 6-8, 2014. Bangkok, Thailand.
2. Attending "Frontier in Cancer Research (FCR) I: Systems Biology For Cancer Research", May 7-8, 2015. Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.
3. Attending "The 10th International Symposium of The Protein Society of Thailand (PST)", July 15-17, 2015. Bangkok, Thailand.
4. Attending "The 41st Congress on Science and Technology of Thailand (STT41)", November 6-8, 2015. Suranaree University of Technology, Nakhon Ratchasima,Thailand

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