MECHANISM OF RING FINGER PROTEIN 43 (RNF43) IN CHOLANGIOCARCINOMA

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กลไกการทำงานของริงค์ฟิงเกอร์โปรตีน 43 (RNF43) ในมะเร็งท่อน้ำดี

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

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กระบวนการกระตุ้นที่ผิดปกติของวิถีวิงค์เบต้าแคดทีนิน (Wnt/β-catenin) นำมาซึ่งการเจริญพัฒนาของมะเร็งหลากหลายชนิดรวมไปถึงมะเร็งท่อน้ำดี มะเร็งท่อน้ำดีเป็นมะเร็งที่มีความผิดปกติในกระบวนการส่งสัญญาณของวิถีวิงค์เบต้าแคดทีนินค่อนข้างมาก โดยมักเกิดจากยีนในวิถีนี้มีการกลายพันธุ์หรือมีการแสดงออกสูงเพิ่มขึ้น ซึ่งความผิดปกติทั้งสองลักษณะนี้พบมีความสัมพันธ์กับการเจริญเติบโตและการรุกรานของมะเร็งที่เพิ่มขึ้น ริงค์ฟิงเกอร์โปรตีน 43 (RNF43) เป็นเอนไซม์ที่ทำหน้าที่ในการนำยูบิควิติน (ubiquitin) มาจับกับโปรตีนเป้าหมายเพื่อยับยั้งการเกิดกระบวนการของวิถีวิงค์เบต้าแคดทีนิน การกลายพันธุ์ของยีนริงค์ฟิงเกอร์โปรตีน 43 สามารถพบได้ในผู้ป่วยมะเร็งท่อน้ำดีส่วนใหญ่ และการแสดงของยีนริงค์ฟิงเกอร์โปรตีน 43 ที่ลดลงมีความสัมพันธ์กับอัตราการรอดชีวิตที่ลดลงด้วย จากข้อมูลดังกล่าวในการศึกษานี้ผู้วิจัยมีเป้าหมายในการศึกษาผลกระทบในด้านการยับยั้งของยีนริงค์ฟิงเกอร์โปรตีน 43 เมื่อมีการแสดงออกที่เพิ่มมากขึ้นกับกระบวนการส่งสัญญาณวิถีวิงค์เบต้าแคดทีนิน โดยเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีจากผู้ป่วยมะเร็งท่อน้ำดีคือเซลล์ KKU-213B ถูกนำมาใช้ในการศึกษาครั้งนี้ โดยเซลล์ถูกชักนำให้เกิดการแสดงออกของยีนริงค์ฟิงเกอร์โปรตีน 43 ในปริมาณมากด้วยการใช้ดีเอ็นเอพาหะที่เรียกว่า pCM6-*RNF43* construct vector ก่อนและเซลล์ที่มีการแสดงออกของยีนริงค์ฟิงเกอร์โปรตีน 43 ในปริมาณมากขึ้นนี้ถูกนำมาทดสอบต่อด้วยการวิเคราะห์การเจริญเติบโตของเซลล์ การเคลื่อนที่ของเซลล์ การตอบสนองต่อยา และการแสดงออกของยีนเป้าหมายที่ถูกความคุมด้วยวิถีวิงค์เบต้าแคดทีนิน ผลการศึกษาผู้วิจัยแสดงให้เห็นว่าโปรตีนเบต้าแคดทีนิน (β-catenin) ถูกสะสมอยู่ที่ไซโตรพลาสซึมของเซลล์เมื่อมีการแสดงออกที่เพิ่มขึ้นของยีนริงค์ฟิงเกอร์โปรตีน 43 จากผลการศึกษานี้บ่งชี้ให้เห็นว่าเกิดการยับยั้งวิถีวิงค์เบต้าแคดทีนิน และผลการศึกษานี้ถูกสนับสนุนด้วยการพบการแสดงออกที่ลดลงของกลุ่มยีนเป้าหมายที่ถูกความคุมด้วยวิถีวิงค์เบต้าแคดทีนิน *(AXIN2, BIRC5, CCND1, MMP7, ABCB1)* ขณะที่การวิเคราะห์การเจริญเติบโตของเซลล์พบว่าการแสดงออกที่เพิ่มขึ้นของยีนริงค์ฟิงเกอร์โปรตีน 43 มีผลยับยั้งการเจริญเติบโตของเซลล์มะเร็งท่อน้ำดีได้ ณ เวลา 48 และ 72 ชั่วโมง การเคลื่อนที่ของเซลล์มะเร็งที่ลดลงก็ถูกพบเมื่อมีการแสดงออกของยีนเพิ่มขึ้นริงค์ฟิงเกอร์โปรตีน 43 เช่นกัน มากไปกว่านั้นยังพบว่ามีการตอบสนองต่อยาเคมีบำบัดที่เพิ่มขึ้นอีกด้วย ดังนั้นจากผลการศึกษาในครั้งนี้แสดงให้เห็นว่ายีนริงค์ฟิงเกอร์โปรตีน 43 มีบทบาทสำคัญใน

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MECHANISM OF RING FINGER PROTEIN 43 (RNF43) IN CHOLANGIOCARCINOMA

Aberrant activation of the Wnt/β-catenin signaling pathway is a step in the progression of various cancer. Earlier studies have reported cholangiocarcinoma (CCA) is a Wnt high tumor, and various genes involved in the progression of this cancer are upregulated. RING finger protein 43 (RNF43) is a ubiquitin E3 ligase which negatively regulates Wnt/ β-catenin signaling. Interestingly, an inactivating mutation of RNF43 was found in a majority of CCA patients and low expression of RNF43 was closely related to a lower CCA patient survival rate. In this study, we studied the inhibition effect by which RNF43 overexpression suppresses Wnt/β-catenin signaling pathway. Human CCA cell line KKU-213B was used as a model and RNF43 transfection was carried out with the pCM6-*RNF43* expression vector using Lipofectamine 3000. Phenotypic alterations, such as cell proliferation, cell migration and the sensitivity of the chemotherapeutic drugs responses, were examined following RNF43 overexpression. Additionally, quantitative PCR was used to determine Wnt/β-catenin target genes changes. Here, we observed that β-catenin was accumulated in the cytoplasmic fraction after RNF43 overexpression, indicating successful Wnt/β-catenin inhibition. Cell viability was significantly diminished after 48 and 72 h of proliferation. Cell migration as determined by wound healing and transwell cell migration assay was also obviously decreased. Additionally, 5-Fluorouracil (5-FU) chemotherapeutic drug

response was increased following RNF43 overexpression. Downregulation of numerous Wnt/β-catenin target genes, including *AXIN2, BIRC5, CCND1, MMP7,* and *ABCB1* upon *RNF43* overexpression was further support for *RNF43* inhibition of this pathway. Our findings provide evidence for the important role of RNF43 in suppressing tumor progression via Wnt/β-catenin signaling pathway cascade.

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LIST OF ABBREVIATION

5-FU	5-Fluorouracil
ABC	ATP-binding cassette
ABC-B1	ATP-binding cassette sub-family B member 1
ABC-C1	ATP binding cassette subfamily C Member 1
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
AXIN2	Axis inhibition protein 2
BAX	BCL2 Associated X, apoptosis regulator
BCA	Bicinchoninic acid
BCL2	B-cell lymphoma-2
BIRC5	Baculoviral inhibitor of apoptosis repeat-containing 5
CCA	Cholangiocarcinoma
CCND1	Cyclin D1
CDH1	Cadherin-1/ E-cadherin
CDH2	Cadherin-2/ N-cadherin
cDNA	Complementary DNA
CK1α	Casein kinase 1α
c-MYC	Cellular- Myelocytomatosis
CRD	Cysteine-rich domain
CRISPR	Clustered regularly interspaced short palindromic repeats

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CHAPTER I

INTRODUCTION

1.1 Significance of Research

Epithelial cell malignancy that arises from the biliary tree is commonly known as cholangiocarcinoma (CCA), and is classified into three major sub-groups, including intrahepatic, perihilar and distal (Razumilava and Gores, 2014a; Bertuccio et al., 2019). There are significant variances of CCA incidence based on the geographical area, due to the various risk factors (Pratap, Raza, and Pratap 2018). Although there were significant discrepancies in some regions, in the past few decades, the overall average morbidity and mortality of ACC has increased (Banales et al., 2020; Bertuccio et al., 2019; Bergquist and Von Seth, 2015).

Undetectable CCA and the absence of specific symptoms contribute to poor prognosis, consequently, there is a parallel prevalence and mortality rates (Banales et al., 2020; Pratap, Raza, and Pratap, 2018). Curative surgery is commonly used to treat CCA patients, but is limited to the early stage (Patel, 2011; Razumilava and Gores, 2014a; Martinez-Becerra et al., 2012). On the other hand, early diagnostic in CCA patients remains painful, therefore, the majority of the patients are diagnosed with advanced stages of CCA, at which point it is impossible to undergo surgical restorative treatment (Patel, 2011; Banales et al., 2020). Gemcitabine, cisplatin, 5-fluorouracil (5-FU) are the chemotherapy regiments commonly used to treat unresectable patients (Chong and Zhu, 2016; Yao, Kunam, and Li, 2014). However, unresectable therapies are related to poor outcome (Razumilava and Gores, 2014a; Simile et al., 2019).

According to previous studies based on whole exome sequencing, 206 somatic mutations were found in 187 genes from patients with CCA related to *Opisthorchis viverrini* (Ong et al., 2012). At least 15 genes were found to be highly mutated, including newly implicated genes, such as *MLL3, ROBO2, RNF43* and *PEG3*. *RNF43* is one of the genes that is directly involved in Wnt signaling activity and is reported to be mutated as much as 9.3% in *Opisthorchis viverrini*–related CCA patients. Interestingly, according to the finding by Boulter et al. (2015), CCA is a Wnt high tumor, in which many of Wnt signaling related genes were reported to be mutated.

The Wnt/β-catenin signaling pathway is negatively regulated by a ring finger E3 ligase, RNF43 (Tsukiyama et al., 2015). RNF43 is reported to be able to inhibit the progression of a variety of cancers, such as gastric carcinoma (Niu et al., 2015; Gao et al., 2017), pancreatic ductal adenocarcinoma (Jiang et al., 2013), and ovarian cancer (Nguyen et al., 2019). Additionally, based on the previous study by Talabnin et al., (2016), both the mRNA and protein expression levels of RNF43 in most CCA tissues were downregulated. Interestingly, low mRNA expression of RNF43 and undetectable RNF43 protein were highly correlated with lower patient survival rates. This finding indicates the importance of RNF43 to supress the progression of cancers (Niu et al., 2015; Talabnin et al., 2016). Therefore, we suspect RNF43 is important in maintaining cell physiological and plays a significant role to supress tumor progression.

In this study, in order to understand the functional role and mechanism of the RNF43 in CCA, overexpression of the RNF43 was performed in CCA cell lines. The phenotypical changes upon RNF43 overexpression were observed, including cell proliferation, cell metastasis and the sensitivity of the cells to chemotherapeutic drugs.

1.2 Literature Review

1.2.1 Cholangiocarcinoma (CCA)

Cholangiocarcinoma is a neoplasm that emerges from the epithelial cells of the biliary tree within or below the hilum. Based on anatomical of origin, CCA is further grouped into three major group; intrahepatic CCA (iCCA), perihilar CCA (pCCA) and distal CCA (dCCA) (Razumilava and Gores, 2014a; Bertuccio et al., 2019; Khan, Tavolari, and Brandi, 2019). iCCA found within the liver which located proximally to the second-degree bile duct, whereas the cystic duct is the anatomical distinction between pCCA and dCCA. (Figure 1.1) (Bergquist and Von Seth, 2015; Khan et al., 2019; Razumilava and Gores, 2014).

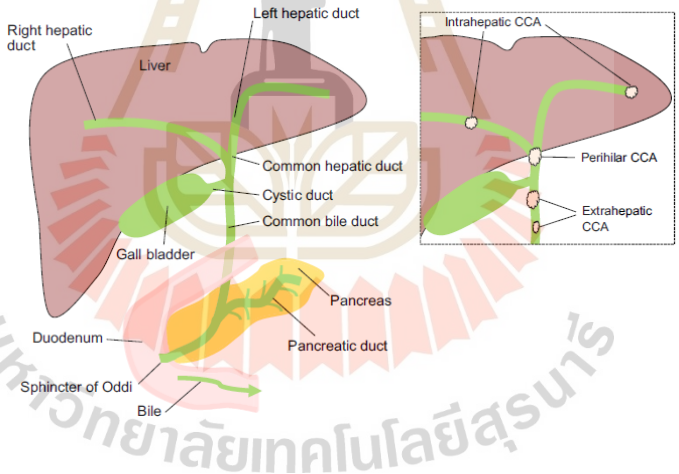

Figure 1.1 Schematic representation of Cholangiocarcinoma types based on their localization. CCA is classified as intrahepatic, perihilar or extrahepatic, according to its position relative to the bifurcation of the hepatic ducts (Zabron, Edwards, and Khan, 2013).

Undetectable CCA and the absence of specific symptoms contribute to poor prognosis, consequently, there is a parallel prevalence and mortality rates (Banales et

al., 2020; Pratap, Raza, and Pratap, 2018). Curative surgery is commonly used to treat CCA patient but limited to the early stage (Patel 2011; Razumilava and Gores, 2014b; Martinez-Becerra et al., 2012). On the other hand, early diagnostic in CCA patients remains painful, therefore, the majority of the patients are diagnosed with the advance stage of CCA which often impossible to undergo surgical restorative treatment (Patel, 2011; Banales et al., 2020). Gemcitabine, cisplatin, 5-fluorouracil (5-FU) are the chemotherapy regiments commonly used to treat unresectable patients (Chong and Zhu, 2016; Yao et al., 2014). However, unresectable therapies are related to poor outcomes Therefore, CCA patients are remain unable to receive proper surgical treatment and not well enough to expose to the chemotherapy and radiation therapy (Patel, 2011; Razumilava and Gores 2014a; Marin et al., 2018; Simile et al., 2019).

1.2.2 Epidemiology and risk factor of CCA

According to reports, in the global scale, CCA accounts for 10-15% of all primary liver cancers and 3% of gastrointestinal cancers in humans, and it is the second most common type of cancer after hepatocellular carcinoma. (HCC) (Banales et al., 2020; Bergquist and Von Seth, 2015). The 5-year survival rate reported to be as low as 10% and this attributed to 10-12% hepatobiliary malignancies which contribute for 13% cancer-related-death globally (Bergquist and Von Seth, 2015; Rizvi and Gores, 2013). The incidence rate of CCA varies greatly depending on its geographical profile, which reflects the distribution of environmental and genetic risk factors (Bergquist and Von Seth, 2015; Kirstein and Vogel, 2016). Globally, CCA cases are varies and this delineating the differences in genetic and environmental risk factors. Although geographic variations show significant differences of bile CCA, the worldwide incidence of CCA has been rising over the few past decades. CCA is more

prevalent in Asian countries than the United States and Europe (Table 1.1). Northeast Thailand has the highest incidence of CCA, with 80-90 cases per 100,000 people, whereas Costa Rica and Israel have the lowest incidence with 0.3 cases per 100,000 people (Labib, Goodchild, and Pereira, 2019; Zabron et al., 2013). Based on the geographical distribution, the highest incidence of CCA in Thailand is mainly occurring in the north (19.3%) and northeast (15.7%). This number is much higher compared to the central (3.8%) and southern region (0%) (Sripa et al., 2007) (Figure 1.2).

Table 1.1 CCA incidence in various countries.

REGION	Age-standardized incidence rate/100 000 population
Thailand - North East	85
Thailand - North and Central	14.5
Thailand - South	5.7
China, Shanghai	7.6
Hong Kong	2.3
Taiwan	4.7
South Korea, Gwangju	8.8
South Korea, Busan	7.1
Japan, Osaka	3.5
Japan, Hiroshima	3.1
Italy	3.4
Germany	3
Austria	2.7
United Kingdom	2.2
United States	1.6
Singapore	1.5
Denmark	1.3
France	1.3
Philippines	1.2
Finland	1.1
Poland	0.7
Spain	0.5
Switzerland	0.5
Australia	0.4
Canada	0.4
New Zealand	0.4
Puerto Rico	0.4
Costa Rica	0.3
Israel	0.3

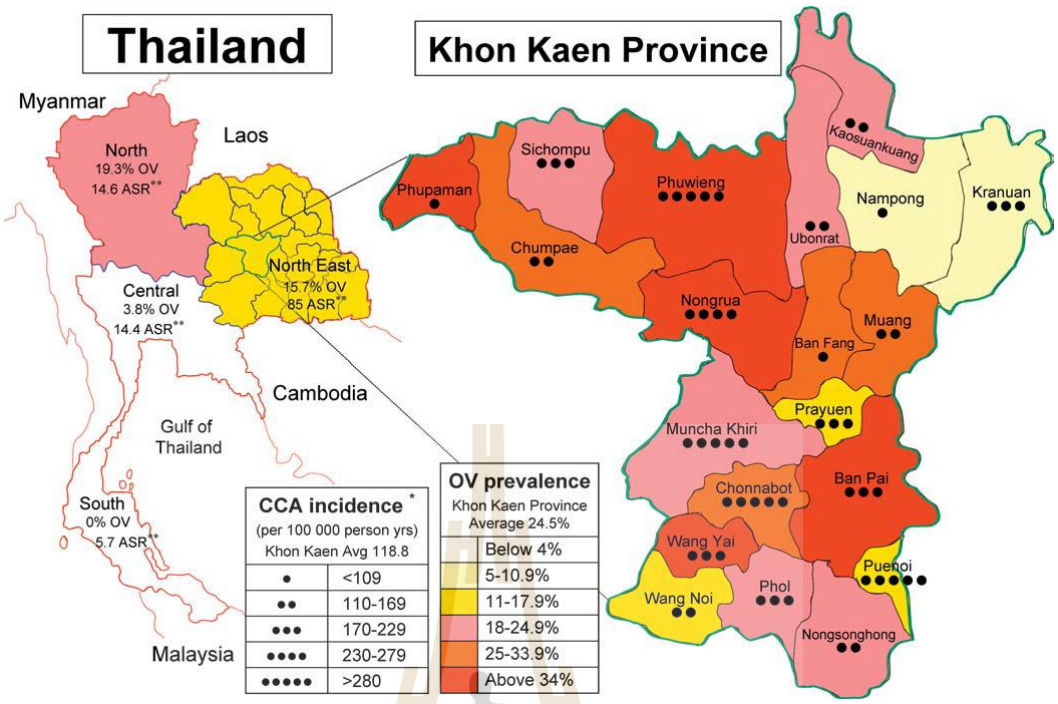

Figure 1.2 Geographical distribution of CCA in Thailand.

CCA encompasses various group of malignancies followed by certain phenotypic and molecular signature changes. Current evidence supports the idea that CCA heterogeneity is the result of a complex interplay between the patients' genetic background and various risk factors. Several risk factors have been identified in CCA which may vary depending on the geographic region including: (1) parasitic infections; (2) primary sclerosing cholangitis; (3) cholelithiasis; (4) biliary disorder; (5) hepatitis B and C; (6) lifestyle-related etc. (Khan et al., 2019; Kirstein and Vogel, 2016). Additionally, Labib et al., summarized several other risk factors causing chronic inflammation and cholestasis (Table 1.2). The inflammation and cholestasis have been reported to be involved in a cell proliferation, genetic and epigenetic mutations and lead to cholangiocarcinogenesis (Labib et al., 2019).

Table 1.2 Risk factor of cholangiocarcinoma (Labib, Goodchild, and Pereira, 2019).

Risk factors of cholangiocarcinoma	
Cholestatic liver diseases	Primary Sclerosing Cholangitis (PSC)
	Fibropolycystic liver diseases
	Congenital hepatic fibrosis
	Caroli disease
	Choledochal cysts
	Biliary hamartomas
Liver cirrhosis (any aetiology)	
Biliary stone disease	Cholecystolithiasis
	Hepatolithiasis
	Choledocholithiasis
Infections	Liver flukes
	Hepatitis B and C
	Chronic typhoid disease
	Recurrent pyogenic cholangitis
	Human Immunodeficiency Virus (HIV)
Inflammatory disorders	Inflammatory bowel disease
	Chronic pancreatitis
	Gout
	Thyrotoxicosis
Toxins	Alcohol
	Tobacco
	Thorotrast (contrast agent)
	Chemical toxins, e.g., dioxins, vinyl chloride,
Metabolic conditions	Diabetes
	Obesity
	Non-Alcoholic Fatty Liver Disease (NAFLD)
Genetic disorders	Lynch syndrome (Hereditary Non-Polyposis Colorectal Cancer)
Other	Intraductal Papillary Neoplasms of the Bile duct (IPNB)

The infection of liver fluke infections is a major risk factor for CCA cases in Asian regions, including *Opisthorchis viverrini*, *Clonorchis sinensis* and *Schistosomiasis japonica*. It has been suggested that cancer of the bile duct or CCA endemically in Thailand is primarily infected by *O. viverrini*. Almost one in six of

this liver flukes infect over its lifetime (Smout et al., 2013). This parasite infestation is mainly due to the dietary lifestyle by consuming raw fish which contains the metacercariae. Once *O. viverrini* ingested by human, it resides in the intra- and extrahepatic bile ducts, causing epithelial cell inflammation and fibrosis. When the parasites reach the adult stage, they cause hyperplasia and adenomatous formation of the bile duct epithelium, and this state considered as the chronic phase. During this stage, periductal fibrosis and scarring are developed and become the most prominent feature in the chronic infection stage. The life cycle of *O. viverrini* is illustrated in figure 3. *O. viverrini* has two intermediate host, including fresh-water snail and freshwater fish, while mammals represent the final host of this parasite (Al-Bahrani et al., 2013; Plentz and Malek, 2015).

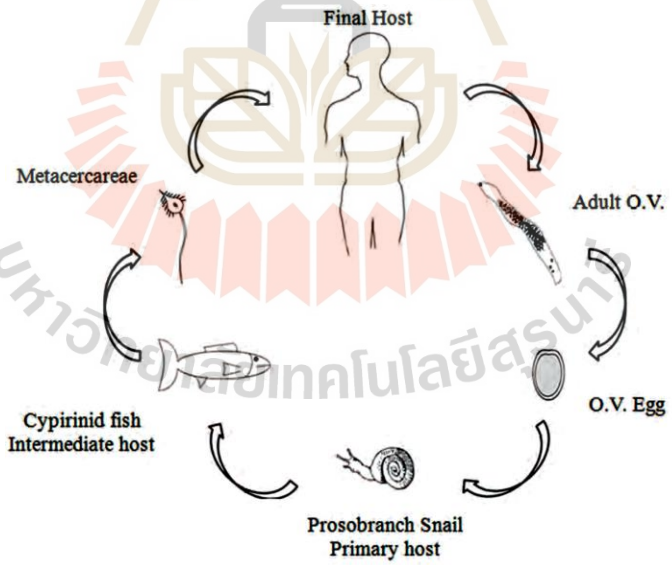

Figure 1.3 Life cycle of *Opisthorics vivverrini* (Al-Bahrani et al., 2013).

Even though liver fluke infection is the major risk factor in Asia specially Thailand, the mortality rates for CCA due to the liver fluke infections are varied in the different region of Thailand. For example, in Northeast Thailand mortality

rate for CCA is 43.6/100,000 and *O. viverrini* prevalence is 16%, meanwhile in South Thailand where the prevalence of *O. viverrini* is nearly zero and the mortality rate for CCA is only 4/100,000. Another interesting finding by (Bhamarapravati and Vajrasthira, 1978), there are the administration of nitrosamine to the animal model which infected with *O. viverrini*, and it determines the development of biliary cancer. Nitrosamines are commonly found in the fermented foods which are the dietary staple in Northeast Thailand. This exogenous co-factor involved in the constant cell division with the mechanically driven tissue injury. This mechanism is illustrated in hamster with fluke-induced wounding followed by sub-carcinogenic nitrosamine. The hamsters showed significant biliary lesion development (Smout et al., 2013). These findings suggest that *O. viverrini* is a major risk factor of CCA cases in Thailand and categorized as the fluke associated CCA.

1.2.3 Wnt signaling

Over than four decades ago, the wingless gene was discovered during mutagenesis screening of *Drosophila melanogaster*, affects its various developmental patterning processes. Subsequently, further genetic screening identified Wnt family as the key mediators of patterning decision during embryonic development. The name Wnt is formed from the combination name of the Drosophila segment polarity gene wingless and the name of its vertebrate homolog, integrated or int-1. There is a connection between Wnt pathway to cancer which implicated by the discovery of int1 (Wnt1) activation either by proviral insertion into the Wnt locus or transgenic overexpression in mice which resulted in mammary hyperplasia and tumor (Komiya and Habas, 2008; Zhan, Rindtorff, and Boutros, 2017).

Wnt pathway is commonly categorized as canonical (β-catenin dependent) or non-canonical (β-catenin independent) pathway which further distinguished as Planar Cell Polarity (PCP) and Wnt/Ca^{2+} pathway (Komiya and Habas, 2008). Wnt signaling pathway begin with the interaction of Wnt ligand and Frizzled (Fzd) receptor. At the level of Fzd, Wnt signaling further divided into three major Wnt pathways: Wnt/ β-catenin, Wnt/PCP and Wnt/Ca^{2+}. The activation of canonical Wnt/ β-catenin signaling cascade involving the interaction of Wnt ligands, Fzd receptor, and its co-receptor Low-density lipoprotein receptor- related protein 5,6 (LRP5/6). Whereas the activation of non-canonical Wnt signaling, both Wnt/PCP and Wnt/Ca^{2+} majorly depend on the interaction of Wnt ligands, Fzd receptor and/or Receptor tyrosine kinase-like orphan receptor (ROR1/ROR2/RYK) (Komiya and Habas, 2008; Martin-Orozco et al., 2019; S. Patel et al., 2019). Both canonical and non-canonical Wnt signaling are tightly regulated to maintain the stability of the cell through various mechanisms. Specifically, canonical Wnt/ β-catenin signaling cascade is responsible for cell proliferation, self-renewal, and cell differentiation. On the other hand, non-canonical Wnt signaling cascade maintains stem cell and cell movement (Duchartre, Kim, and Kahn 2016; Martin-Orozco et al., 2019).

According to the many studies of genetic and biochemistry, the common components of Wnt pathway in both canonical and non-canonical Wnt signaling are Wnt ligands and Fzd receptor. Wnt ligand family is a secreted lipid-modified glycoprotein consist of 19 different cysteine rich glycoprotein which exhibit specific function and highly conserved in invertebrates and mammals. Glycosylation and palmytoilation of Wnt ligand take place in the endoplasmic reticulum (ER). O-acetyltransferase Porcupine (PRCN) has been reported to be the major protein

responsible for palmytoilation. Modified Wnt ligand further secreted under the regulation of transmembrane protein Evenness interrupted WNTless (Evi/Wls) and transported via Golgi apparatus. Fzd receptor is a seven-transmembrane-span protein with topological homology to G-protein coupled receptor which serve as binding sites for Wnt ligands. Fzd receptor is a common Wnt receptor in canonical and non-canonical Wnt signaling pathway (Komiya and Habas, 2008; Martin-Orozco et al., 2019; S. Patel et al., 2019). The first complex step of Wnt signaling pathway begin with Wnt ligand secretion (Mikels and Nusse, 2006). Secreted Wnt ligands further activates Wnt signaling pathways by binding to the N-terminal extra-cellular cysteine-rich domain of the Fzd receptor family.

In canonical Wnt/ β-catenin signaling pathway, the interaction between Wnt ligand, Fzd receptor and LRP 5/6 co-receptor transduces signal to cytoplasmic phosphoprotein Dishevelled (Dvl). This event triggers series of events that disturb the destruction complex (Figure 1.4). Destruction complex contains the two scaffold proteins Axis Inhibition Protein 1 (AXIN1) and Adenomatous Polyposis Coli (APC), and Ser/Thr kinases including Glycogen Synthase Kinase 3β (GSK3β) and Casein Kinase 1 (CK1α, ε, γ, δ). Under unstimulated condition when Wnt signaling is inactivated, destruction complex renders Wnt signaling cascade by continuously synthesizing, ubiquitinating and degrading Wnt signaling key player, β-catenin (Komiya and Habas, 2008; Martin-Orozco et al., 2019). Phosphorylated β-catenin is recognized by E3 ubiquitin ligase β-Trcp which targets β-catenin for proteasomal degradation. As a result, β-catenin is not translocated into the nucleus and the activation of Wnt target genes are repressed (MacDonald, Tamai, and He, 2009).

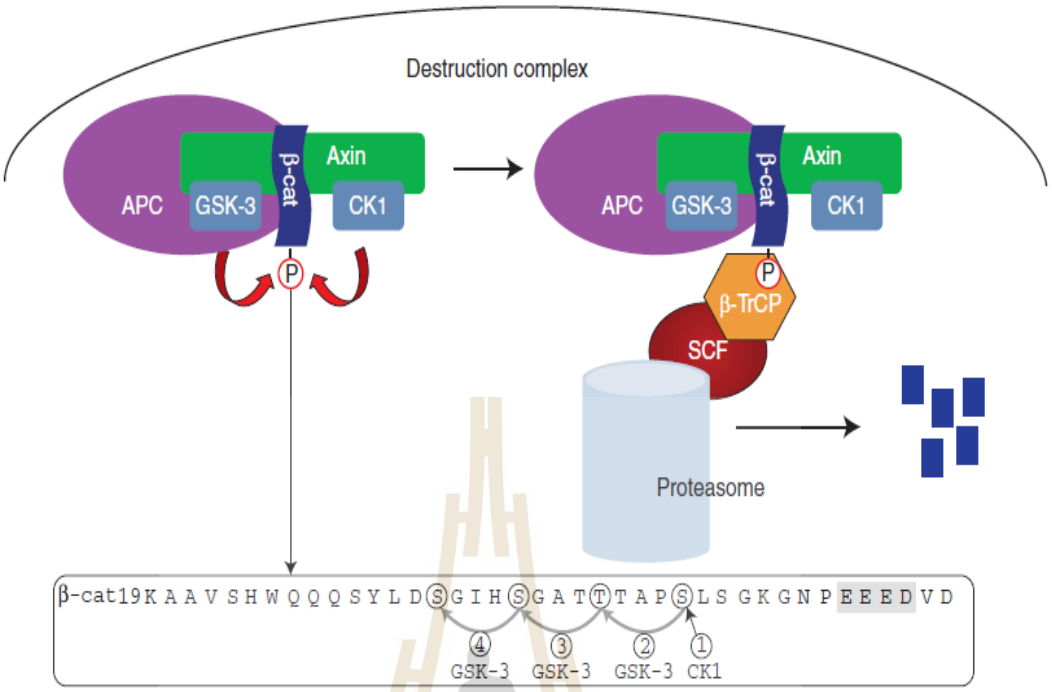

Figure 1.4 Overview of destruction complex. Wnt signaling activity is controlled by multiprotein *"*destruction complex*"* that includes the tumor suppressor Axin and APC, Ser/Thr kinases GSK-3 and CK1, protein phosphatase 2A (PP2A), and the E-3 ubiquitin ligase β-TrCP (Stamos and Weis 2013).

It is important to understand that CK1α and GSK3β play a distinct role in Wnt signaling. At the level of destruction complex, in the absence of Wnt activation signal, both kinases continuously phosphorylating β-catenin. However, upon Wnt ligand stimulation, CK1α and GSK3β mediate LGR 5/6 phosphorylation which recruits Dishevelled (Dvl) protein to the plasma membrane followed by polymerization and activation of Wnt signaling (Komiya and Habas 2008). The direct interaction between Fzd and Dvl is the mediator of Wnt/ β-catenin signaling pathway. In the activation of Wnt signaling and formation of Dvl polymer in the plasma membrane stabilizes β-catenin and inactivates destruction complex. The accumulation of stabilized β-catenin

translocated into the nucleus and forms active complex with lymphoid enhancer factor (LEF) and T-cell factor (TFC) proteins (Figure 1.5a). This transcriptional switch leads to a change of multiple cellular process, regulating cell differentiation and cell proliferation (Zhan, Rindtorff, and Boutros, 2017; Buechling et al., 2011; Mikels and Nusse, 2006; Komiya and Habas, 2008; Tsukiyama et al., 2015; Noll et al., 2016).

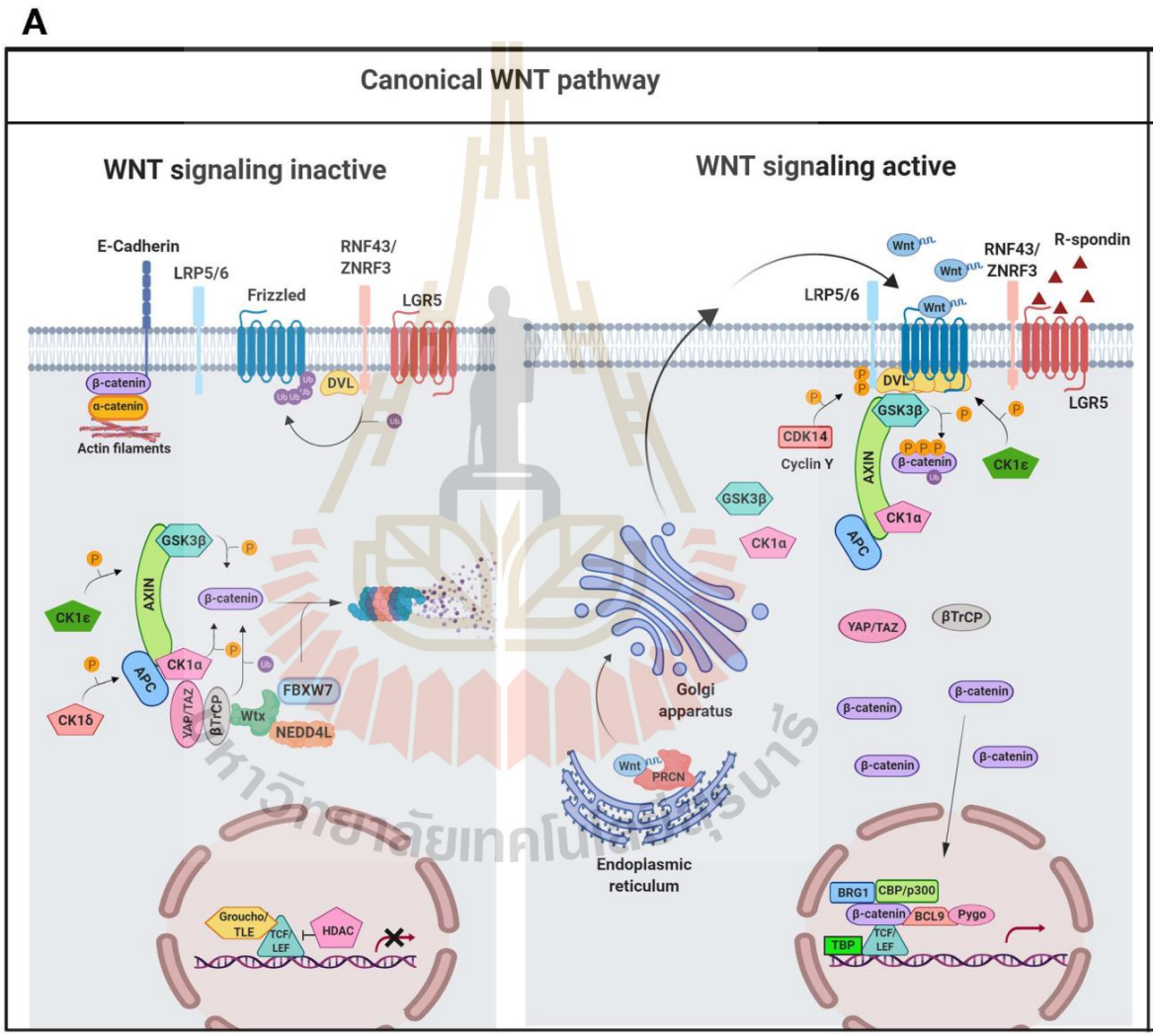

Figure 1.5 Schematic illustration of (A) canonical Wnt signaling pathway.

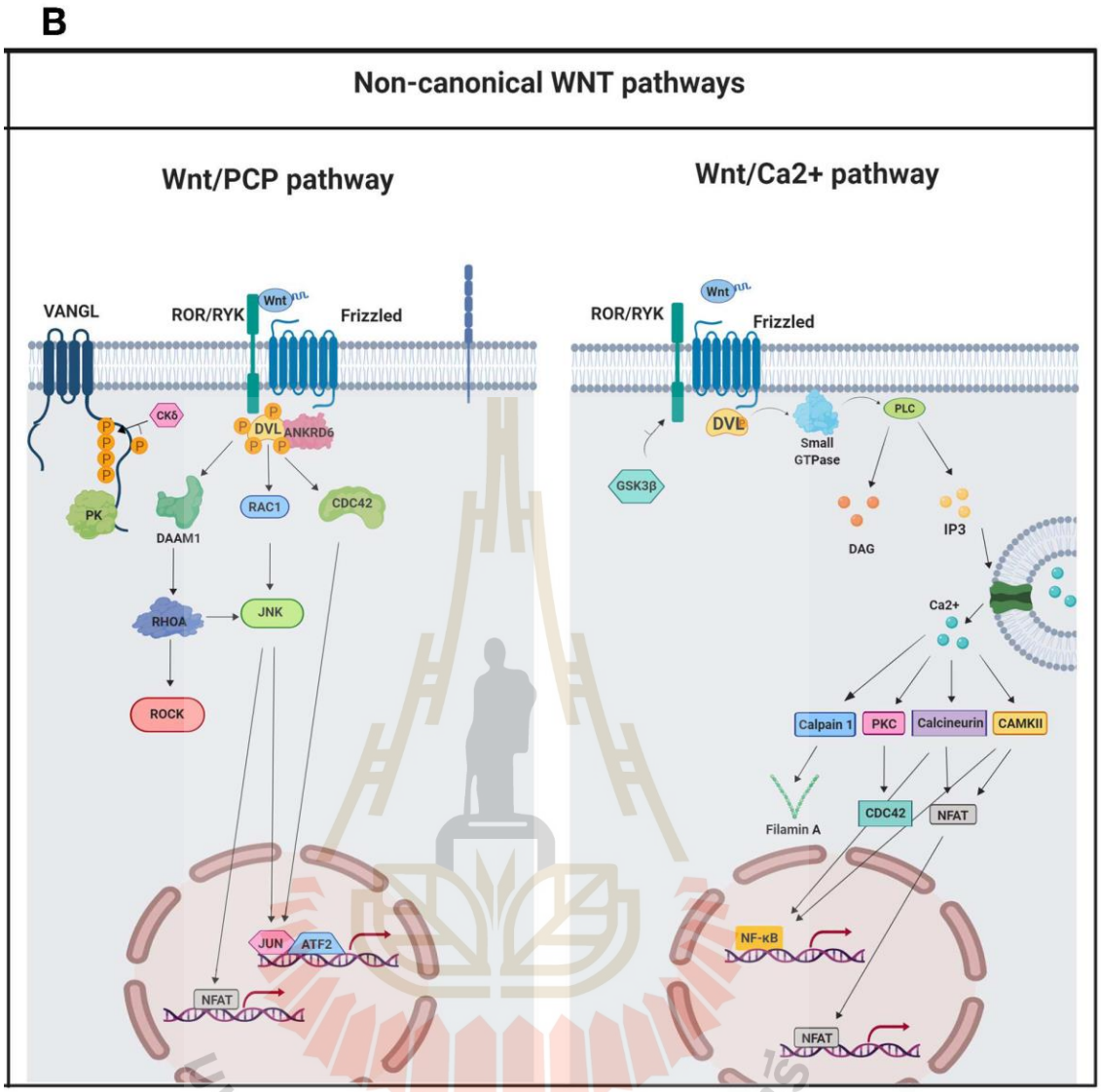

Figure 1.5 and (B) non-canonical Wnt signaling pathway (Martin-Orozco et al., 2019) (Continued).

The activation of non-canonical Wnt signaling pathway or also referred as β-catenin-independent pathway also begin with the interaction of Wnt ligand and Fzd receptor, without the participation of LRP 5/6 co-receptor. Two widely studied non-canonical Wnt pathways are including Wnt/PCP and Wnt/Ca^{2+}. The major distinction of these two pathways is the type of Wnt ligand, receptor, and co-receptor along with downstream receptor they paired with.

In Wnt/PCP pathway (Figure 1.5b, left panel), Wnt ligand and Fzd receptor activates signaling cascade by inducing the phosphorylate of VANGL2 by ROR/RYK, followed by activation of JUN kinase (JNK) by RHOA and RAC. JNK activation induce transcription of AP1 (Jun-ATF-2) and the nuclear factor of activated T cell (NFAT), followed by the regulation of cell polarity during morphogenesis. The activation of Wnt/Ca^{2+} (Figure 1.5b; right panel) begin with Wnt ligand and Fzd receptor binding which activates Phospholipase C (PLC). This activation stimulates the release of various molecules including diacylglycerol (DAG) and 1,4,5-triacylphosphate (IP3) which release Ca^{2+} ion and activates effector molecules protein like kinase C (PKC), calmodulin-dependent kinase II (CAMKII) and calcineurin (Cn). Subsequently, these kinases activate various transcription factor such as NFAT and nuclear factor kappa B (NFκB). The activity of this signaling is important in the regulation of cell migration, cell adhesion and embryonic development (Martin-Orozco et al., 2019; S. Patel et al., 2019).

1.2.4 Wnt signaling pathway in cancer

Abnormal regulation of Wnt signaling pathways is commonly found as the hallmark of cancer and other degenerative diseases. The activation of both canonical and non-canonical Wnt signaling associated with various types of liquid and solid tumor. Activation of canonical Wnt/ β-catenin signaling as well as non-canonical Wnt signaling have been reported in various types of cancer via distinct mechanisms. Although both mechanisms are reportedly to be involve in the progression of cancer, non-canonical Wnt signaling pathway is less studied. One of significant study of the involvement of non-canonical Wnt signaling is the role of a specific non-canonical Wnt signaling Wnt5a and its receptor Fzd6. Upregulation and binding activity of these two

proteins activate PKC and other Wnt/ Ca^{2+} pathway in glioblastoma (Hirano et al., 2014), and gastric cancer (Kurayoshi et al., 2006; Corda and Sala, 2017) as well as the activation of Wnt/ PCP pathway in breast cancer (Anastas et al., 2012).

Conversely, canonical Wnt/ β-catenin signaling is often found to be activated in numerous cancers. For example, in colon and rectum cancer another Wnt receptor Colon cancer is one of the most studied cancer with the activation of Wnt signaling via APC mutations. Human intestinal organoids were used to study the carcinogenesis of CRC after APC truncation by genome editing of APC using the CRISPR/Cas9 (Tianzuo Zhan et al., 2019). Mouse model with reversible knockdown of APC via shRNA demonstrated tumor progression could be repressed when APC is restored. This finding underlined the importance of continuous Wnt signaling in maintaining tumor progression. Additionally, the mutations of a secreted protein which also play role in mediating the activation Wnt/ β-catenin signaling known as R-spondin also commonly found in colon cancer (Yang and Tang, 2000; Seshagiri et al., 2012). R-spondin specifically interact with Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) which also highly expressed and act as a marker of intestinal stem cells. In colon cancer, LGR5 significantly enhanced along with the deletion of APC in these cells (Barker et al., 2009). Aside from colon cancer, the important role of LGR5 receptor and its ligand R-spondin, have also been demonstrated in several tumor subtypes of colorectal cancer, pancreatic ductal adenocarcinoma, and endometrial cancer. (Mikels and Nusse, 2006; Zhan, Rindtorff, and Boutros, 2017). Wnt activity is also found to be substantially increased in most of leukemias. The most common acute leukemias in adults are frequently characterized by chromosomal translocation. This progression is marked by the translocation products such as AML1-

ETO, MLL-AF9 which positively affect canonical Wnt signaling. β-catenin showed to be essential for the progression of leukemias initiating cells (LIC) and its self-renewal (Beghini et al., 2012). In more than 50% of breast cancer patients, Wnt signaling is also found to be activated and linked to the poor survival of the patients. Canonical Wnt activation was reportedly inhibit GSK3β kinase activity which initiates epithelial mesenchymal transition (EMT) transcriptional program in breast cancer cell (Wu et al., 2012).

Cholangiocarcinoma (CCA) is a Wnt-high tumor marked by high Wnt activation. According to findings by Boulter et al. (2015) canonical Wnt pathway is activated in human CCA marked by the overexpression of multiple Frizzled receptor as well as the Wnt ligands including WNT7B and WNT10A (Figure 1.6). The persistent activation of Wnt signaling in CCA is occur during CCA development. While in most of other gastrointestinal cancer and colon cancer, β-catenin and APC frequently found to be mutated, unlike those cancer, β-catenin and APC in liver-fluke associated CCA are unfrequently mutated. Whereas E3 ubiquitin ligase RNF43 is regularly mutated in liver-fluke associated CCA. Therefore, liver-fluke associated CCA could be susceptible to Wnt-inhibition based therapy since no commonly described mutation found downstream inhibition point (Boulter et al., 2015).

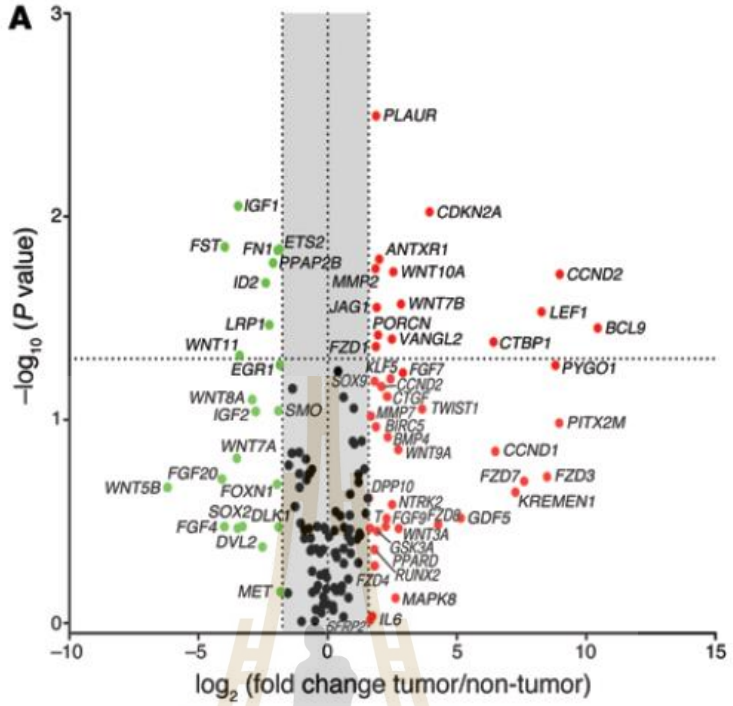

Figure 1.6 mRNA expression of WNT pathway genes and WNT target genes in CC versus patient-matched non-cancerous tissue (n = 11). Represented as a 3-fold change; P <0.05 (Boulter et al., 2015).

1.2.5 RING finger protein 43 (RNF43) in normal cell

The RING finger domain was first studied in the human protein RING and first identified in the early 1900's in the protein encoded by the Really Interesting New Gene (RING). The RING finger domain contains eight metal-binding residues which coordinate two zinc atoms in an interleaved pattern for folding and biological actions. RING finger proteins play critical roles in mediating the transfer of ubiquitin (Ub) to both of the heterologous substrates as well as the RING finger protein themselves. This protein act by mediating diverse protein-protein interaction and one of their main biological functions is catalysing ubiquitylation as a ubiquitin protein isopeptide ligase.

The *RNF43* gene is made up of ten exons that are located within 63 kb of DNA on human chromosome 17q22 and encode a single polypeptide chain of 783 amino acids with a molecular weight of 85 kDa and an isoelectric point of about 8.10. This gene encodes a RING-type E3 ubiquitin ligase with a signal peptide, a transmembrane domain, a protease-associated domain, an ectodomain, and a cytoplasmic RING domain (Figure 1.7). RNF43 is suggested to become an E3 ubiquitin-protein ligase that accepts ubiquitin from an E2 ubiquitin-conjugating enzyme and transfers it directly to targeted substrate proteins for protein degradation (Figure 1.8).

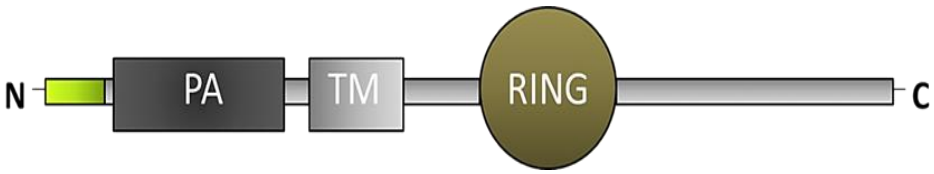

Figure 1.7 RNF43 Schematic Overview The signal peptide is denoted by the green line. PA stands for protease-associated domain; TM stands for transmembrane domain; and RING stands for RING domain.

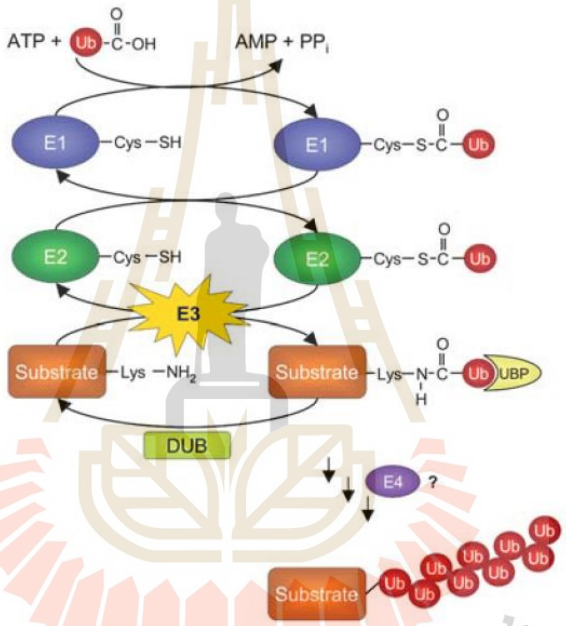

Figure 1.8 Ubiquitin (Ub) is covalently attached to substrate proteins in a three-step process involving the sequential actions of the enzymes E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase). Multiple ubiquitin moieties are attached by E3, possibly with the assistance of an E4, resulting in the formation of a poly-ubiquitin chain (Passmore and Barford, 2004).

RNF43 is highly associated with the Wnt receptor complex and inhibits canonical Wnt/β-catenin and non-canonical Wnt signaling through distinct mechanism. Inhibition Wnt signaling by RNF43 is necessary to prevent dysregulation of Wnt signaling and supress tumorigenesis Nevertheless, inhibition of RNF43 in both canonical Wnt/β-catenin and non-canonical Wnt signaling take place at the level of Fzd receptor. RNF43 plays a role in mediating the ubiquitination, endocytosis, and subsequent degradation of Wnt receptor complex component Fzd (Tsukiyama et al., 2015; Serra and Chetty, 2018). In canonical Wnt/β-catenin signaling RNF43 interacts with the complex consist of Wnt receptor, Fzd and LRP5/6 receptors, mediating the ubiquitination of specific lysine residues in the cytoplasmic loops of these receptors (Figure 1.9). Wnt receptor removal by ubiquitination subsequently followed by Wnt signaling inactivation. Oppositely, in order to activate Wnt signaling, a secreted protein agonist of the canonical Wnt signaling, the ligand R-spondin removes RNF43 in the cell surface by autoubiquitination via the formation of tertiary complex consist of LGR4/5. The removal of RNF43 results in re-accumulation of Wnt at the cell surface with the consequent Wnt signaling enrichment (Serra and Chetty, 2018).

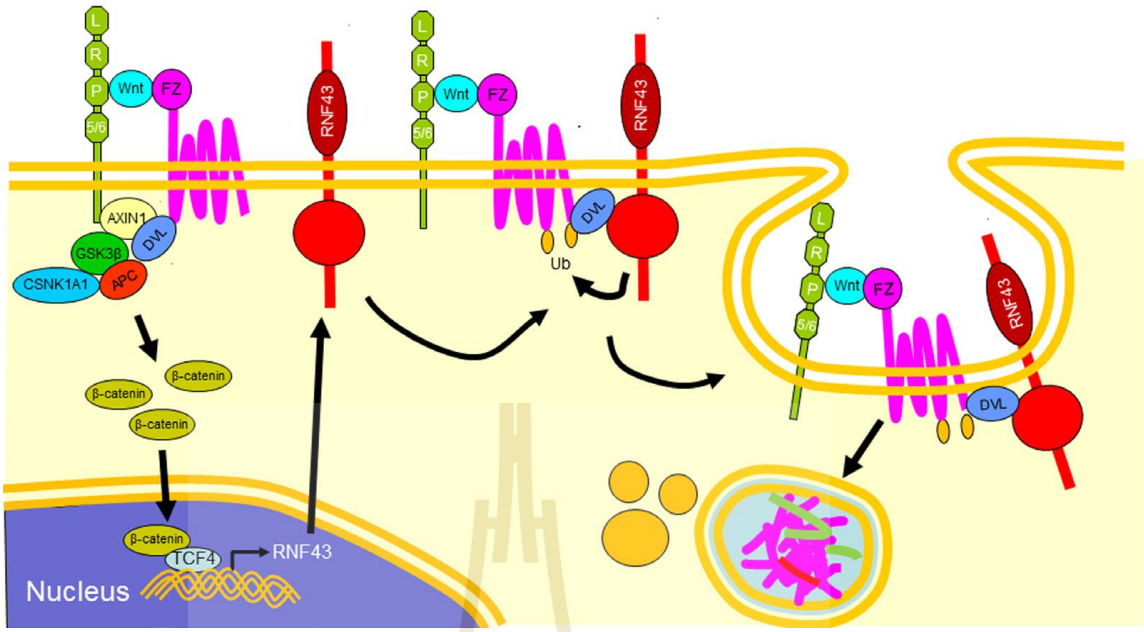

Figure 1.6 The interaction between RNF43 and Frizzled results in ubiquitination, endocytosis, and subsequent degradation of Frizzled, a component of the Wnt receptor complex. Wnt proteins interact with Frizzled and LRP5/6 to activate Wnt/- TCF4 and induce RNF43 expression (Serra and Chetty, 2018).

In addition to canonical Wnt/β-catenin signaling, RNF43 has also been shown to physically interact with T cell factor 4 (TCF4), tethering TCF4 to the nuclear membrane and suppressing its transcriptional activity. TCF4 represents a key transcriptional factor that mediates canonical Wnt signaling, which plays a central role in embryonic development and maintains tissue homeostasis (Kendziorra et al., 2011). RNF43 also known to interact with NEDD-4 like ubiquitin-protein ligase 1 (NEDL1) and regulates p53-mediated transcription (Sugiura, Yamaguchi, and Miyamoto, 2008). Furthermore, RNF43 facilitates the ubiquitination of p53 and inhibited nucleoprotein-driven activation of p53 transcription (Figure 1.10) (Shinada et al., 2011a; Sugiura,

Yamaguchi). Taken together, *RNF43* gene contributes to the regulation of *p53*, and the dysregulation of *RNF43* may contribute to the development of tumorigenesis.

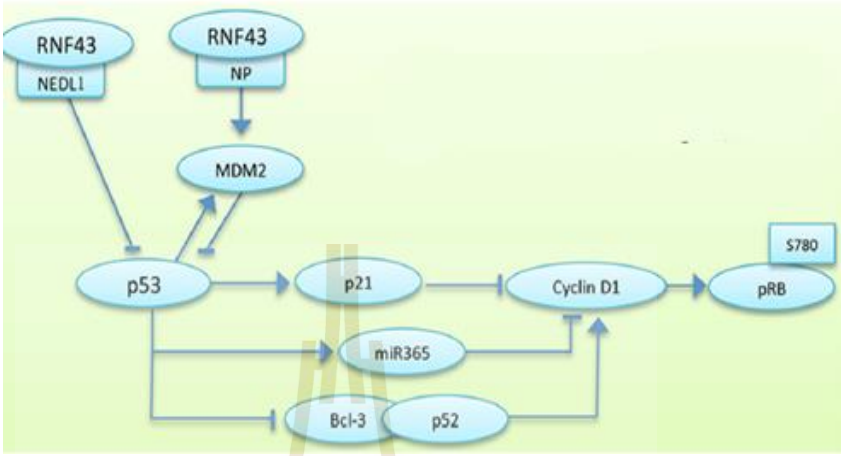

Figure 1.7 RNF43-regulated p53 cell cycling pathway model. The arrow denotes positive regulation, while the perpendicular lines denote negative regulation (Xie et al., 2015).

On the other hand, the regulation of non-canonical Wnt signaling activity by RNF43 simply occur in the level of Fzd and highly depends on Dvl2. In normal trafficking pathway, RNF43 maintain the level of non-canonical Wnt signaling by preventing the interaction between Dvl2 and Fzd by directly binds to Dvl2 (Tsukiyama et al., 2015).

1.2.6 RNF43 in Cancer

Transmembrane E3 ubiquitin ligase RNF43 negatively regulates Wnt signaling by promoting Fzd degradation in canonical Wnt/β-catenin signaling or binds to Dvl2 in non-canonical Wnt signaling (Tsukiyama et al., 2015; Serra and Chetty 2018). Under normal condition, RNF43 directly interacts with Fzd receptor and Dvl2 and inhibit Wnt signaling via protein-associated (PA) domain of RNF43. The major

difference of canonical and non-canonical Wnt signaling is the region of PA domain of the RNF43 (Figure 1.11). To inhibit canonical Wnt/β-catenin signaling activation, extracellular PA domain of RNF43 at the N-terminus is necessary to bind with Fzd. While cytoplasmic C-terminus PA domain is required to supress tumorigenesis by interact with Dvl2 in non-canonical Wnt pathway (Tsukiyama et al., 2015). Therefore, mutation at this point is extremely critical to determine the fate of tumorigenesis.

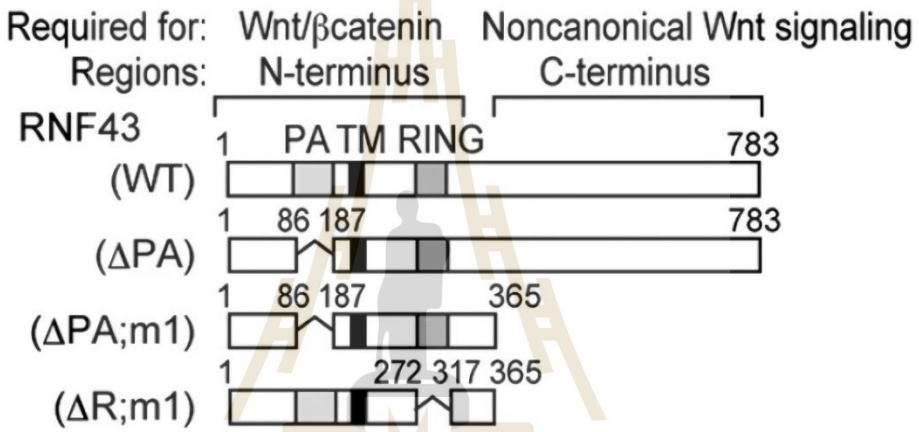

Figure 1.8 RNF43 regulates canonical and non-canonical Wnt signaling through distinct mechanism. N-terminal domain is important to regulate canonical Wnt/ β-catenin signaling by interacting with Fzd. C-terminus domain of RNF43 interact with Dvl to non-canonical Wnt signaling (Tsukiyama et al., 2015).

Numerous studies related to RNF43 mutation frequencies have been reported over the past years. RNF43 act of mechanism in cancer cell can be either be tumor suppressor or oncogene, depends on the types of cancer (Table 1.3). Nevertheless, RNF43 mutation frequencies are often found in cancers indicating its importance as tumor suppressor.

Table 1.3 RNF43 mutations and frequencies in various cancers.

Cancers	RNF43 act of mechanism	Mutation frequency	References
Pancreatic cyst			
Intraductal papillary mucinous neoplasm (IPMN)	Inactivate mucinous type (Tumor suppressor)	75%	(J. Wu et al., 2011)
Mucinous cystic neoplasm (MCN)		37.5%	
Cholangiocarcinoma (CCA)			
O. viverrini associated	Poor patient survival related to the low RNF43 expression. (Tumor suppressor)	9.3%	(Ong et al., 2012; Chan-On et al., 2013; Talabnin et al., 2016)
Non-*O. viverrini* associated		3.5%	
Gastric cancer			
Microsatellite-stable (MSS)	Inactivation of RNF43 result in deregulation of Wnt activity	4.8%	(K. Wang et al., 2014)
Microsatellite instability (MSI)		54.6%	
Colorectal adenocarcinomas			
NHS and HPFS data set	Highly mutated, increasing tumor progression	18.9%	(Giannakis et al., 2014)
TCGA dataset		17.6%	
Endometrial carcinomas	Frequent mutation conferred fitness advantage to the tumor	18.1%	(Giannakis et al., 2014; Ryland et al., 2013)
Pancreatic carcinomas with acinar differentiation	Low mutation RNF43 is found in this cancer	4%	(Jiao et al., 2014)

The changes at the genetic level are mainly manifested by various phenotypic characteristic by which tumor cells are able to escape the anti-tumor signals, to acquire resistance to the treatment, invade and create further secondary tumor, to replicate limitlessly. Resisting cell death, maintaining proliferative signaling, evading growth suppressors, inducing angiogenesis, enabling replicative immortality, and

activating invasion and metastasis are typical phenotypic changes seen in common cancers.(Hanahan and Weinberg, 2011).

According to various studies, the alteration of RNF43 expression level both mRNA and protein, significantly correlated to clinicopathological outcomes. For example, in human gastric carcinoma Niu et al., (2015) found a significant downregulation of RNF43 in mRNA level compared to the normal mucosae. This finding highly correlated to the histological differentiation (Figure 1.12; p=0.001), T-stage cancer (P<0.001), depth of invasion (P<0.001), lymph node metastasis (P<0.001), pTNM stage (P<0.001) and survival (P=0.021). These results indicate that low expression level of RNF43 are typically correlated with the severity level of malignant and metastasis (Niu et al., 2015).

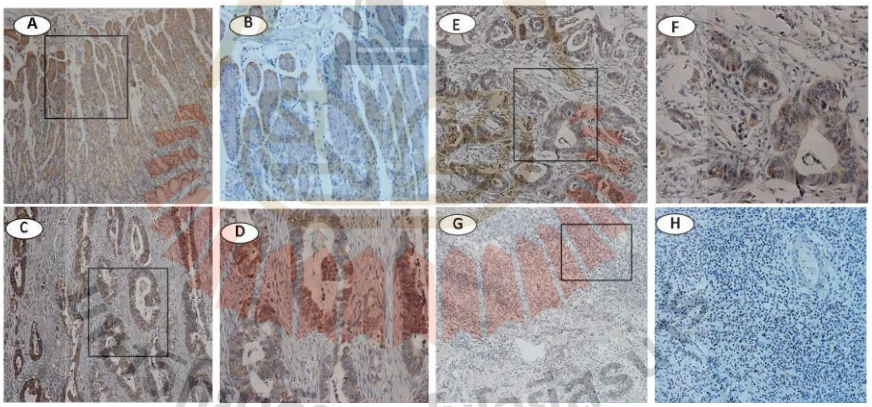

Figure 1.12 RNF43 immunohistochemistry in gastric carcinoma and adjacent normal tissues. RNF43 staining in adjacent normal tissue: (a) strong positive x100, (b) strong positive x200; highly differentiated gastric carcinoma tissue: (c) moderately positive x200, (d) moderately positive x400; moderately differentiated gastric carcinoma tissue: (e) weakly positive x200, (f) weakly positive x400; poorly differentiated gastric carcinoma tissue: (g) negative x100, (h) negative x200 (Niu et al., 2015).

Worse prognosis in gastric cancer patients with low mRNA and protein expression level were also reported by Gao et al., (2017). Notably low expression level of RNF43 is significantly correlated with various clinicopathological features such as distant metastasis (P=0.030), pTNM stage (P=0.033) and Laurent classification (P=0.001). Furthermore, low RNF43 expression may predict a patient's overall survival (Figure 1.13) Additionally, two different studies were confirmed the functional role of RNF43 inhibiting chemoresistance in gastric cancer. One study tried to overexpress RNF43 in human cell line model, followed by chemotherapeutic drugs 5-FU and oxiplatin. The result showed that RNF43 overexpression increase drug response marked by significant decrease of cell viability. Another study confirm depletion of RNF43 increase cell chemoresistance via DNA damage response (DDR) indicated by reduced activation of DDR and apoptosis (Gao et al., 2017; Neumeyer et al., 2021).

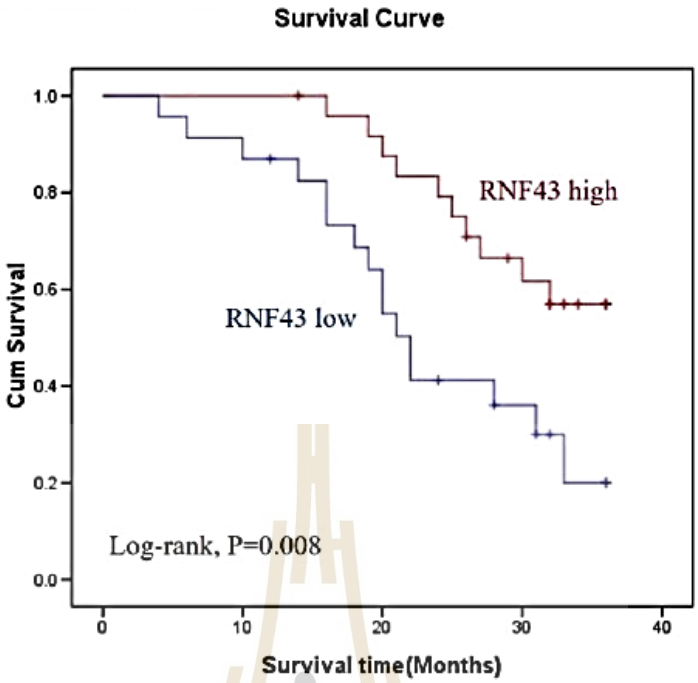

Figure 1. 9 Kaplan-Meier analysis depicted the correlation of poor overall survival rate of gastric carcinoma cancer and the downregulation of RNF43 (Gao et al., 2017).

Even though in the majority of cancer RNF43 is mutated and downregulated, the high expression of RNF43 was reported to promote cancer progression in colorectal cancer. Based on finding by Yagyu et al., (2004) the expression of RNF43 was elevated in majority of colon cancer. Further studies demonstrated functional role of RNF43 as the oncogene. In colorectal cancer, RNF43 has the ability to interact with NEDL1 and suppress p53-mediated transcriptional activity (Shinada et al., 2011). In hepatocellular carcinoma, suppression of RNF43 induces the accumulation of G1-S phase cell cycle arrest and leads to cell apoptosis via a p53 dependent pathway. Moreover, cell migration and invasion were inhibited under RNF43-knockdown condition (Xing et al., 2013). Taken together, RNF43 is implicated in malignancy which may involve in all steps of tumor development and progression.

1.2.7 RNF43 in CCA

Wnt/ β-catenin signaling activation has long been linked to tumor progression, with many downstream Wnt components found to be mutated in cancer. Treating cancer harbouring Wnt downstream mutations remains challenging due to lack of tractable targets. However, targeting upstream of Wnt signaling pathway may be promising options including LRP6, Frizzled, Porcupine as well as ZNRF3/RNF43 which degrade Frizzled. Therefore, understanding defined mutations in patient population that are dependent on ligand-induced Wnt/β-catenin signaling is necessary. According to the finding by Ong et al. (2012), there were numerous genes found to be mutated in liver-fluke associated CCA, including 10 newly implicated genes such as *MLL3, ROBO2, RNF43* and *PEG3* (Table 1.4). These genes were obtained from the identification and validation of 206 somatic mutation in 187 genes.

Table 1.4 Mutated genes found in *O. viverrini* – related CCA identified by whole-exome sequencing (Ong et al., 2012).

Gene	*O. viverrini*– associated CCA (*N* = 54)	PDAC (*N* = 114)	HCV-associated HCC (*N* = 95)
TP53	44.4% (24)	85%	33.7%
KRAS	16.7% (9)	100%	0/10
SMAD4	16.7% (9)	27%	0/10
CDKN2A	5.6% (3)	25%	0/10
MLL3	14.8% (8)	7.9%	0/10
ROBO2	9.3% (5)	0/24	0/10
GNAS	9.3% (5)	0/24	0/10
RNF43	9.3% (5)	0/24	0/10
PEG3	5.6% (3)	1/24	0/10
XIRP2	5.6% (3)	0/24	0/10
PTEN	3.7% (2)	0/24	0/10
RADIL	3.7% (2)	0/24	0/10
NDC80	3.7% (2)	0/24	0/10
PCDHA13	3.7% (2)	0/24	0/10
LAMA2	3.7% (2)	0/24	0/10
CTNNB1	0/8	0/24	20%
ARID2	0/8	0/24	7.4%
DMXL1	0/8	0/24	4.2%
NLRP1	0/8	0/24	4.2%

Among 10 newly implicated genes, *RNF43* incited particular interest, because RNF43 was found to be involved in the activity of the Wnt signaling pathway. According to the previous study by Talabnin et al. (2016), most of the CCA patients were expressing lower RNF43 mRNA and protein levels. The experiments were confirmed the association of low RNF43 protein level with the overall poor survival of the patients. (Figure 1.14).

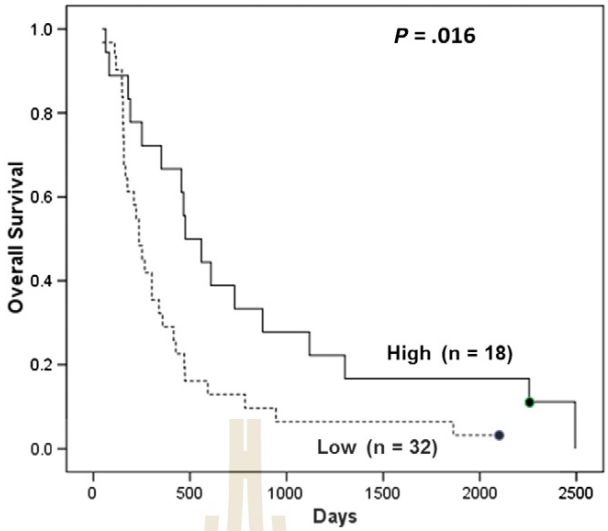

Figure 1.14 Kaplan-Meier analysis was used to determine the overall survival of CCA patients with different levels of RNF43 protein expression (low versus high). According to the findings, patients with high RNF43 protein expression outlived those with low protein expression (Talabnin et al., 2015).

The genetic alteration also found to be associated with the RNF43 expression. They found one significant association of the *RNF43* polymorphism between rs2257205 and the decrease of RNF43 mRNA expression (Figure 1.15). However, this result was not directly associated with level of RNF43 protein level expression. They assume that this perhaps due to the location of this polymorphism that is situated in the coding sequence of the exon 3 rather than the regulatory region. Therefore, no significant protein expression changes were observed.

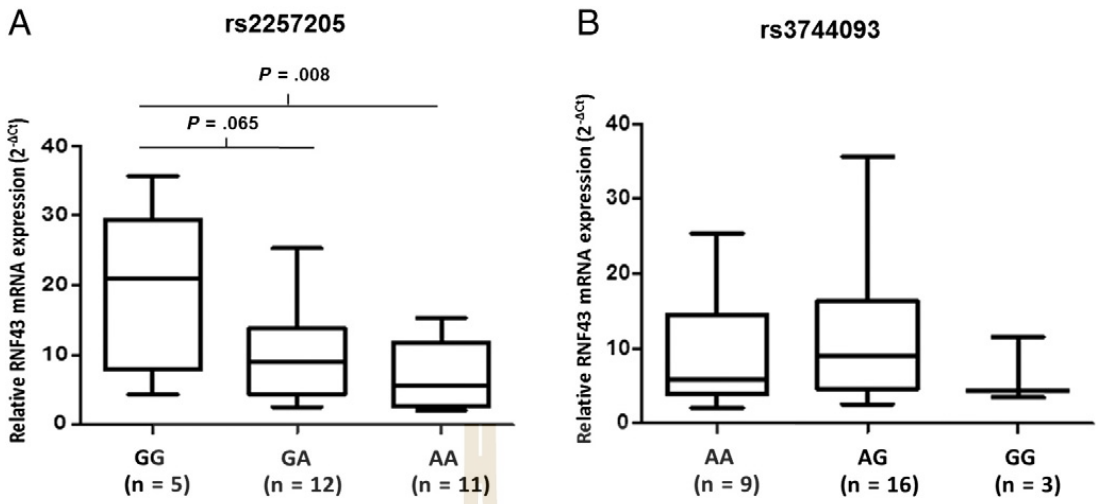

Figure 1.15 RNF43 polymorphism influenced the RNF43 mRNA level. (A) P = .065 between GA and GG genotypes; (B) P = .008 between AA and GG genotypes (Talabnin et al., 2015).

Although the mechanisms underlying this phenomenon remains unclear, this genetic alteration may serve as the prognostic marker of CCA. Hence, further studies are required to elucidate the role of RNF43 in CCA.

1.3 Thesis Objectives

The objectives of this thesis work were:

1.3.1 To determine the biological function of RNF43 by observing the phenotypic changes of CCA cell lines after RNF43 overexpression.

1.3.2 To investigate the effect of forced RNF43 expression on the Wnt signaling pathway in CCA.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Human cholangiocarcinoma cell lines

As previously described by Sripa et al., (2005) four human CCA cell lines (KKU055, KKU-100, KKU-213 and KKU-213B) were established from tumors of patients with CCA and liver-fluke infection admitted to Srinagarind Hospital, Khon Kaen University (Khon Kaen, Thailand). The Japanese Collection of Research Bioresources Cell Bank provided certificates of analysis. Cells were grown in DMEM Nutrient Mixture (cat. no. 12100-046; Gibco; Thermo Fisher Scientific, Inc.; USA) supplemented with 1% penicillin-streptomycin (cat. no. 15140-122; Gibco; Thermo Fisher Scientific, Inc.; USA) and 10% FBS (cat. no. 10270-098; Gibco; Thermo Fisher Scientific, Inc.; USA). Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. Cells with 70-80% confluence at 24 h were trypsinized with 0.25% trypsin-EDTA (cat. no. 25200-072; Gibco; Thermo Fisher Scientific, Inc.; USA) and sub-cultured in the same media. Mycoplasma testing with MycoAlert mycoplasma detection kit (cat. no. LT07-418; Lonza Rockland, Inc.; USA) was conducted for the cell lines used.

2.1.2 Chemicals, oligonucleotide primers and antibodies

All chemicals, primers and antibodies used in this experiment are listed in tables 2.1 to 2.3.

2.1.2.1 Chemicals

Table 2.1 Chemical list.

Chemical	Source	Identifier
DMEM Nutrient Mixture	Gibco; Thermo Fisher Scientific, Inc.; USA	12100-046
1% penicillin-streptomycin	Gibco; Thermo Fisher Scientific, Inc.; USA	15140-122
10% FBS	Gibco; Thermo Fisher Scientific, Inc.; USA	10270-098
0.25% trypsin-EDTA	Gibco; Thermo Fisher Scientific, Inc.; USA	25200-072
MycoAlert mycoplasma detection kit	Lonza Rockland, Inc.; USA	LT07-418
Plasmid Mini prep		
Endo Free Maxi Plasmid Kit	Tiangen Biotech; Beijing	4992194
Lipofectamine® 3000	Invitrogen, Thermo Fisher Scientific, Inc.; USA	3000-008
Opti-MEM	Gibco; Thermo Fisher Scientific, Inc.; USA	22600-050
5-Fluorouracil	Boryung Pharmaceutical Co., Ltd; South Korea	1C 229/39
Trichloroacetic acid (TCA)	Merck; Sigma-Aldrich, Inc.; USA	T0699
Sulforhodamine B	Merck; Sigma-Aldrich, Inc.; USA	3520-42-1
Triton-X 100	PanReac, AppliChem	A4975,0500
TRIzol® Reagent	Invitrogen; Thermo Fisher Scientific, Inc	15596026
SensiFAST™ cDNA Synthesis Kit	Bioline; Meridian Bioscience	BIO-65053
LightCycler® 480 SYBR Green I Master	Roche; Roche Molecular Systems, Inc	04707516001
cOmplete™ ULTRA Tablets, Mini, EASYpack Protease Inhibitor Cocktail	Roche; Roche Molecular Systems, Inc	5892970001
Pierce™ BCA Protein Assay Reagent A	Thermo Scientific, Inc; USA	23225
Immobilon Forte Western HRP substrate	Merck Millipore; Darmstadt; Germany	WBLUF0100

2.1.2.2 Oligonucleotide primers

Table 2.2 Oligonucleotide primer list.

Target gene	Forward	Reverse
RNF43	5’-tctgtctggaggagttctct-3’	5’-ctccctctgtgatgttgaac-3’
AXIN2	5’-cagatccgagaggatgaagaga-3’	5’-agtatcgtctgcgggtcttc-3’
BIRC5	5’-ctgctgtggaccctactg-3’	5’-aactgcgtctctgccaggac-3’
CDH1	5’- caccacgggcttggattttg-3’	5’- tgggggcttcattcacatcc-3’
CDH2	5’- atcctgcttatccttgtgctg-3’	5’- gtcctggtcttcttctcctcc-3’
c-MYC	5’- tgaggagacaccgcccac -3’	5’- caacatcgatttcttcctcatcttc-3’
MMP-2	5’ tgaccaagggtacagcctgt 3’	5’ agaggccccatagagctcct 3’
MMP-7	5’-tgtatggggaactgctgaca-3’	5’-gcgttcatcctcatcgaagt-3’
MMP-9	5’ ctctggaggttcgacgtgaa 3’	5’ ggctttctctcggtactgga 3’
CCND1	5’-aactacctggaccgcttcct-3’	5’-ccacttgagcttgttcacca-3’
ABC-B1	5’-ctcagacaggatgtgagttggt-3’	5’-acagcaagcctggaacctat-3’
ABC-C1	5’-ctgggcttatttcggatcaa-3’	5’-tgaatgggtccaggttcatt-3’
ACTB	5’-gatcagcaagcaggagtatgacg-3’	5’-aagggtgtaacgcaactaagtcatag-3’

RNF43, Ring finger protein 43: *AXIN2*, axis inhibition protein 2; *BIRC5*, baculoviral inhibitor of apoptosis repeat-containing 5; *CDH1*, Cadheri-1;(E-cadherin) *CDH2*, Cadherin-2 (N-Cadherin*); c-MYC*, cellular myelocytomatosis; *MMP-2, -7, -9,* Matrix metalloprotease-2, -7, -9*; CCND1*, Cyclin D1; *ABC-B1* (MDR1), ATP-binding cassette sub-family B member 1(Multidrug resistance mutation 1); *ABC-C1* (MRP1); ATP Binding Cassette Subfamily C Member 1 (Multidrug resistance-associated protein 1).

2.1.2.3 Antibodies

Table 2.3 Antibody list.

Antibody	Source	Identifier
Mouse anti-β actin	Santa Cruz Biotechnology	Cat# sc-47778
Mouse anti Histone H1	Santa Cruz Biotechnology	Cat# sc-8030
Rabbit anti RNF43	Sigma-aldrich	Cat# HPA008079
Rabbit anti Frizzled	Santa Cruz Biotechnology	Cat# sc-293261
Rabbit anti Cyclin D1	Santa Cruz Biotechnology	Cat# sc-8396
Rabbit anti TCF4	Cell signaling	Cat# 2569
Rabbit anti β-catenin	Santa Cruz Biotechnology	Cat# sc-7963
Rabbit anti Lamin B1	Cell signaling	Cat# 12586
Rabbit anti Cleaved Caspase-3	Cell signaling	Cat# 9664
Rabbit anti Caspase-3	Cell signaling	Cat# 9662
Rabbit anti BAX	Proteintech	Cat# 50599-2-Ig
Mouse anti BCL2	Proteintech	Cat# 12789-1-AP
Rabbit anti PARP 1	Proteintech	Cat# 13371-1-AP
Rabbit anti-phosphorylated β-catenin	Cell signaling	Cat# 9561
Mouse IgG, HRP-linked whole Ab (from sheep)	Amersham ECL	Cat# NXA931
Rabbit IgG, HRP-linked whole Ab (from donkey)	Amersham ECL	Cat# NA934

2.1.3 RNF43-DDK-Myc expression vector

RNF43/pCMV6/neomycin-DDK-Myc construct vector (cat. no. PS100001; Origene; OriGene Technologies, Inc.) and PCMV6-entry vector were transformed into *Escherichea coli* (*E. coli*) strain DH5α (Qiagen, Hilden, Germany) and 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. The starter culture was prepared by picking a single colony and inoculating it into 500 μL of LB media containing kanamycin, followed by incubation at 37°C with shaking at 200 rpm for 3-4 h. The starter cultures were inoculated into 200 mL of LB containing kanamycin in a flask and incubated at 37°C for 20-22 h with shaking until the OD_{600} reached 0.5- 0.6.

Plasmid was collected by harvest of the bacterial cultures and application of the Endo Free Maxi Plasmid Kit (cat no. 4992194; Tiangen Biotech; Beijing). In brief, the cell pellet was collected by centrifuging the bacterial culture at 3,000*xg* for 15 min, followed by resuspending the cell pellet with buffer. Cells were lysed and neutralized, then transferred to the equilibrated Plasmid Maxi column and the column was allowed to empty by gravity flow. Then, the DNA binding columns were washed and eluted with a high salt buffer, followed by precipitation of the pure plasmid DNA. Plasmid amplification products were verified by PCR and both plasmid single digestion by *EcoRI* restriction enzyme and double digestion by *EcoRI* and *XhoI* restriction enzyme.

2.2 Methods

2.2.1 RNF43 overexpression

The pCMV6-entry/RNF43 expression vector (cat. no. PS100001; Origene; OriGene Technologies, Inc.) was transfected into CCA cell lines by using Lipofectamine® 3000 reagent (cat. no. 3000-008; Invitrogen, Thermo Fisher Scientific, Inc.; USA). Cells were seeded on either 6-well plates or 24-well plates (3-4 x 10^5/well), depending on the following experiment, and incubated for overnight. The amount of DNA transfected into the cells for both empty plasmid (250 ng/µl) and RNF43 containing plasmid (195 ng/µl) was 2.5 µg. Transfection was followed by 24 h incubation with Opti-MEM (cat. no. 22600-050; Gibco; Thermo Fisher Scientific, Inc.; USA) with no media replacement at 37°C and with 5% CO_2.

2.2.2 Cell proliferation and drug treatment

Cell proliferation was performed by seeding the 1 x 10^4 cells in a 96 well plate with the complete DMEM Nutrient Mixture (cat. no. 12100-046; Gibco; Thermo Fisher Scientific, Inc.; USA) supplemented with 1% penicillin-streptomycin (cat. no. 15140-122; Gibco; Thermo Fisher Scientific, Inc.; USA) and 10% FBS (cat. no. 10270-098; Gibco; Thermo Fisher Scientific, Inc.) and incubated for 0, 24, 48 to 72h. Three rounds of serial replating were performed for each experiment.

Drug sensitivity assay was performed by using various commonly used chemotherapeutic drugs for CCA patient including: 5-Fluorouracil (5-FU) (cat. no. 1C 229/39; Boryung Pharmaceutical Co., Ltd; South Korea), Gemcitabine (cat no. 1C 32/53; Fresenius Kabi Oncology Ltd; India) and Cisplatin (cat no. 1C 253/39; Boryung Pharmaceutical Co., Ltd; South Korea). Transfected cells (1 x 10^4 cells in 96-well plate) were incubated for overnight. On the following day, the cells were treated with 5-FU with 2-fold serial diluted concentrations 0, 3.125, 6.25, 12.5, 25, and 50 µM. Cells were further incubated for 48 h and 72 h and maintained with 5.0% CO_2 at 37°C. Each experiment was performed with three replications.

Sulforhodamine B (SRB) sssay was used to determine cell viability after the treatment. Initially, cells in 96-well plate were fixed in 100 µL of 10% (v/v) Trichloroacetic acid (TCA) (cat. no. T0699; Merck; Sigma-Aldrich, Inc.; USA) for at least 3 h at 4°C. Cells were washed and subsequently stained with 0.4% (w/v) Sulfrhodamine B (cat. 3520-42-1; Merck; Sigma-Aldrich, Inc.; USA) for 30 minutes at room temperature. Cells were rinsed with 1% (v/v) acetic acid 5 times followed by dilution in 10 mM Tris base solution (pH 10.5) and the plate shaken on an orbital shaker

for 10 min to solubilize the protein-bound dye (approximately 10 min). The absorbance was further measured in a by microplate reader at 562 nm.

2.2.3 Colony formation assay

Transfected cells were seeded at the density 2.000 cells per well in a 6-well plate with complete DMEM media in the presence of 0, 3.125 and 6.25 μM 5-FU. The cells were treated for 48 h prior to further incubation for 10 to 14 days and maintained with 5.0% CO_2 at 37°C. Crystal violet staining was used to visualize the colony formed. The cells were washed with 1x phosphate buffered saline (PBS) three times. Methanol was used to fix the cell for at least 2 hours at -20°C, followed by staining by 0.5% (w/v) Crystal violet in 100% methanol for 1 h at room temperature. Cells were washed with tap water and dried. The observation was made by observing the colony formation and captured by camera. Colony area was measured by ImageJ software version 1.53a (National Institute of Health, USA) and calculated by comparing colony area for each drug concentration relative to the colony with 0 μM 5-FU.

2.2.4 Wound healing assay

RNF43 was overexpressed in $4x10^5$ cells in 6-well plate, which were incubated for 24 h. On the following day, a vertical wound was made down through the cell mono layer by a 200 μl pipette tip with certain force against the tissue culture plate to avoid damage. The old media was discarded followed by additional new complete DMEM. The initial observation was performed by record the picture right after the wound was made. The cells were further incubated at 37°C and with 5% CO_2. Wound was further recorded at 16, 20, and 24 h incubation times. The experiment data were obtained from triplicate. Wound closure was calculated by comparing the wound area size relative to

the initial wound area and calculated by ImageJ software version 1.53a (National Institute of Health, USA).

2.2.5 Transwell cell migration assay

Approximately 600 µl of complete DMEM supplemented with 1% penicillin-streptomycin and 10% FBS was added into the 24-well plate. A total of 3 x 10^4 cells in 200 µl DMEM media without FBS were added into the Transwell insert apparatus with 8-µm pore size of polycarbonate membrane (cat no. 3422; Corning Inc.; USA) and securely moved by forceps to 24-well cell plate filled with complete DMEM. The cells were further incubated at 37°C and with 5% CO_2 for 16 h and 24 h. Migrated cells in the transwell insert were fixed in 600 µL of 10% (v/v) TCA and stained by 0.4% (w/v) SRB. Cells were rinsed with 1% (v/v) acetic acid. Cells were further observed under the microscope and images captured by camera. To further quantify cell migration, dilution of migrated cells by 10 mM Tris-base solution (pH 10.5) was performed. The plate was placed on an orbital shaker for 10 min to solubilize the protein-bound dye. The absorbance was further read by microplate reader at 562 nm.

2.2.6 Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the cells by using TRIzol® Reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc, USA), followed by cDNA synthesis with the SensiFAST™ cDNA Synthesis Kit (cat. no. BIO-65053; Bioline; Meridian Bioscience, USA). Quantitative real-time (RT) PCR was analyzed according to LightCycler® 480 SYBR Green I Master (cat. no. 04707516001; Roche; Roche Molecular Systems, Inc., USA) and performed using LightCycler ® 480 Real-Time PCR System (Roche; Roche Molecular Systems, Inc.). Target genes were amplified

with the primers listed on Table 2.2 Gene expression values were normalized against the house-keeping gene β-actin. Relative gene expressions were determined by the $2^{-\Delta\Delta CT}$ and $2^{-\Delta CT}$ methods.

2.2.7 Protein collection

Protein samples were collected from various sources as follows:

Whole cell lysate

Whole cell protein lysate from cell line culture collection was performed by washing cell culture with 1X PBS followed by adding lysis buffer (150 mM NaCl; 50 mM Tris-HCl pH 7.4; 1% sodium deoxycholate; 0.1% SDS; and 1X cOmplete™ ULTRA Tablets, Mini, EASYpack Protease Inhibitor Cocktail (cat no. 5892970001; Roche, Indianapolis; USA). The cells were scraped with a rubber policeman and transferred into the 1.5 µl Eppendorf tube followed by 20 minutes centrifugation at 16,000 rpm at 4°C. Cell lysate on the top layer was collected in a new 1.5 ml sterile Eppendorf tube and stored at -80°C.

Cytoplasmic and nuclear fractions

To collect the protein from cytoplasmic and nuclear fractions, cells were initially washed with 1X PBS. CHEMICON®'s Nuclear Extraction Kit (cat no. 2900, Merck Millipore, Darmstadt, Germany) was used to collect the protein by adding ice cold 1x Cytoplasmic Lysis Buffer (10x Cytoplasmic lysis buffer, 1 M DTT and 1/1000 protease inhibitor cocktail) and incubating on ice for 15 minutes. The cells were scraped and moved to a new 1.5 ml tube and centrifuge at 8.000*xg*, 20 minutes at 4°C. The supernatant, which contain the cytoplasmic fraction, was transferred into new 1.5 ml tube and store at -80°C. The pellet was resuspended by 1x Nuclear extraction buffer

(1x nuclear extraction buffer, 1 M DTT and 1/1000 protease inhibitor) and incubated on ice for 30 minutes. Nuclear suspension was centrifuged at 16.000*xg* for 5 minutes. The supernatant, which contains the nuclear fraction, was collected into 1.5 ml tube and store at -80°C.

Collected protein was quantified by Pierce™ BCA Protein Assay Reagent A (cat no. 23225; Thermo Scientific, Inc; USA). Protein was diluted in 1x PBS (1:20) mixed with BCA working reagent (BCA solution and 4% cupric sulfate) and incubated in 60°C for 15 minutes. The absorbance at 562 nm was read by microplate reader.

2.2.8 SDS-PAGE and Western blotting

Protein separation by SDS-PAGE was performed according to the standard laboratory protocols. Separated protein was further electro-transferred to an Amersham Protran 0.45 NC nitrocellulose Western blotting membranes (cat. no. 10600003, GE Healthcare, Amersham, UK). Protein detection was observed by using antibody listed on the Table 2. Blot was visualized by using Immobilon Forte Western HRP substrate (cat. no. WBLUF0100, Merck Millipore, Darmstadt, Germany) and Amersham™ Hyperfilm™ (cat. no. 28906838, GE healthcare, Amersham, UK).

2.2.9 Statistical analysis

To compare two groups of samples, Student's t-test to compare two groups of samples, one-way ANOVA and Dunnett test for multiple comparisons, and two-way ANOVA and Bonferroni correction for comparisons among groups with different time points. To indicate statistical significance the following annotation were used: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Statistical parameters,

including the exact values of n and precision measure (mean ± SEM) and statistical significance, are reported in the figures and figure legends. Statistical analysis and graph construction were performed using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA).

CHAPTER III

RESULT

3.1 Basal *RNF43* gene expression and RNF43 protein expression, and RNF43 overexpression in CCA

In order to confirm the correlation between the low expression of RNF43 with the progression of CCA, several CCA cell lines were used to determine the basal *RNF43* mRNA and RNF43 protein expression level. According to our integrated analysis in four different CCA cell lines (KKU-055, KKU-100, KKU-213A and KKU-213B), both *RNF43* mRNA and RNF43 protein showed exceptionally low expression level (Figure 3.1). To understand the functional involvement of *RNF43* gene in CCA, we used transient transfection system to overexpress *RNF43* gene. The transfection was carried out by Lipofectamine 3000 with pCMV6-RNF43 for *RNF43* gene overexpression, and pCMV6-Entry as the control. Overexpression of *RNF43* was successfully performed in the KKU-213B cell line (Figure 3.2). We also found that the majority of RNF43 protein was expressed in the nuclear fraction, rather than in the cytoplasm. Accordingly, our results demonstrate all CCA cell lines were obviously express low *RNF43* mRNA and RNF43 protein expression levels, which is in line with previous findings in CCA tissue (Talabnin et al., 2016)

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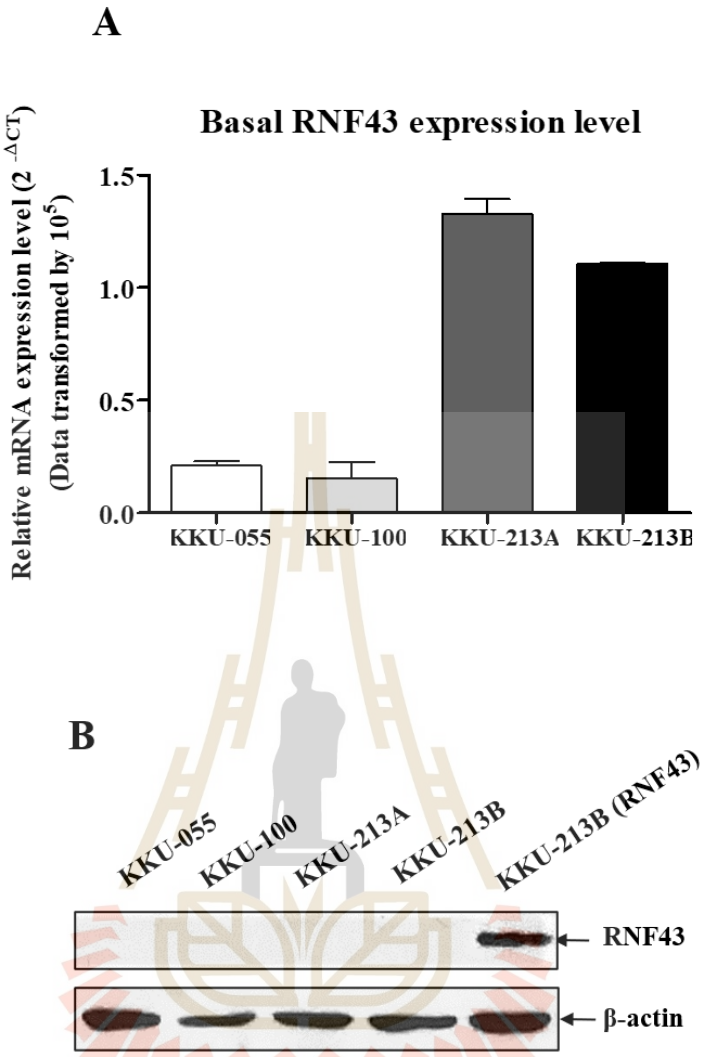

Figure 3.1 Basal RNF43 expression in various CCA cell lines*:* (A) q-RT PCR and (B) western blot were performed to determine basal expression of *RNF43* mRNA and RNF43 protein expression level of various CCA cell lines. *ACTB* (β-actin) was used as the reference gene. mRNA expression level was determine using $2^{-\Delta\Delta CT}$. A CCA cell line with RNF43 overexpression was used as a positive control to determine RNF43 protein.

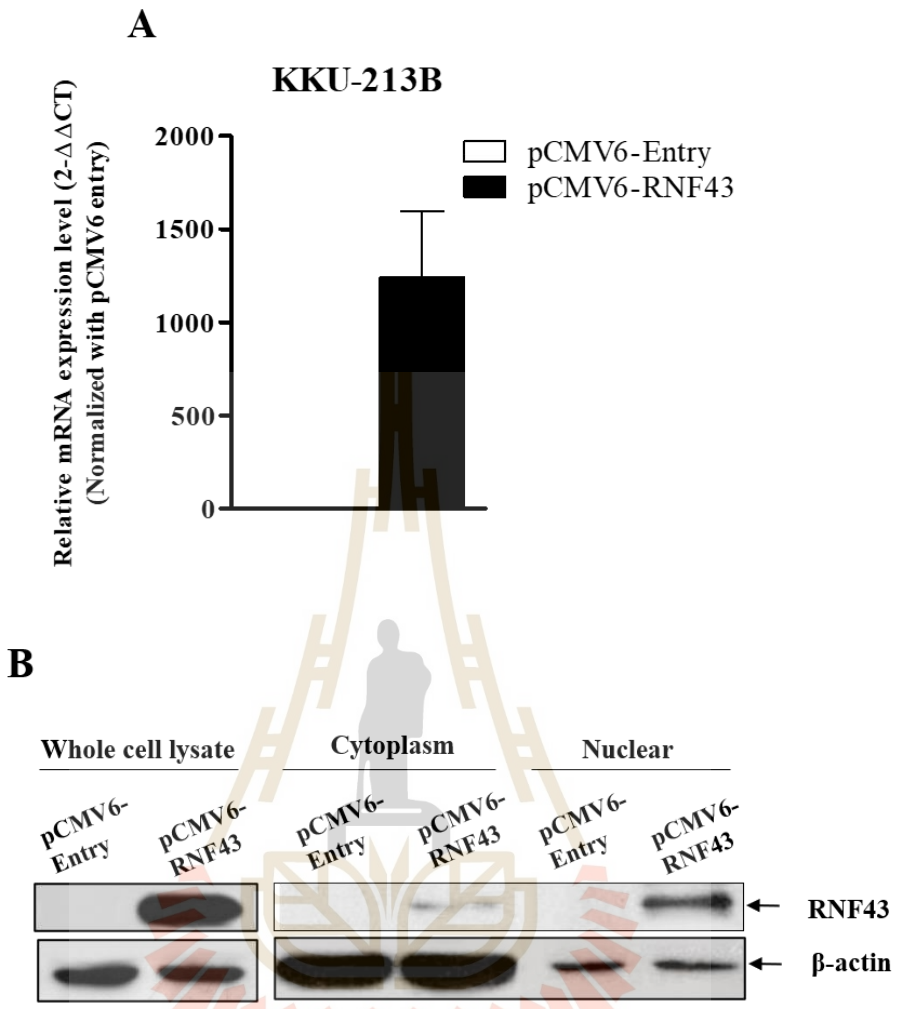

Figure 3.2 *RNF43* overexpression. (a) q-RT PCR and (b) western blot were used to determine the level of RNF43 in KKU-213B after transfection. *ATCB1* (β-actin) was used as the reference gene. Relative mRNA expression level was determine using $2^{-\Delta\Delta CT}$ normalized to pCMV6-Entry. Bar graph represents mean ± SEM of three independent experiments.

3.2 *RNF43* overexpression inhibits Wnt/β-catenin signaling pathway subsequently altered downregulation of various Wnt target genes

Since the translocation of β-catenin from the cytoplasm into the nucleus is an indispensable event to determine Wnt/β-catenin signaling activation **(**Loregger et al., 2015**)**, we further studied the localization of β-catenin in KKU-213B overexpressing *RNF43* The result showed that overexpression of *RNF43* accumulates the β-catenin protein expression in the cytoplasm and blocks translocation into the nucleus indicated by less RNF43 observed in the nuclear fraction (cytoplasmic and nuclear β-catenin localization protein fold change 1.2 and 0.9, respectively. $*P<0.05$) (Figure 3.3). Additionally, the nuclear-to-cytoplasmic ratio (N/C ratio) of β-catenin protein expression was significantly lower in the cell overexpressing *RNF43*l when compared to that of the control (*$*P<0.05$;* protein fold change 0.78 and 1, respectively) (Figure 3.4).

Next, various Wnt/ β-catenin target genes were also examined to further confirm the activity of Wnt/ β-catenin signaling. The result showed there were remarkable downregulations of all 6 Wnt target genes in KKU-213B following *RNF43* overexpression (Figure 3.5). Interestingly, the downregulation of the Wnt target genes upon RNF43 overexpression has been reported to involve in various aspects of tumor progression such as anti-apoptosis and proliferation (*BIRC5*, *CCND1*, *CMYC)*, migration and invasion (*MMP7)*, and drug resistance, *(ABCB1)*. Moreover, downregulation of *AXIN2*, a specific Wnt/β-catenin target gene, further confirm that the downregulation of Wnt target genes was occur via the inhibition of Wnt/β-catenin

signaling pathway by RNF43. Taken together, these results indicated that RNF43 blocks the key protein β-catenin to translocate into the nucleus, leading to inactivation of Wnt/ β-catenin signaling via suppressing transcriptional level of known Wnt target genes.

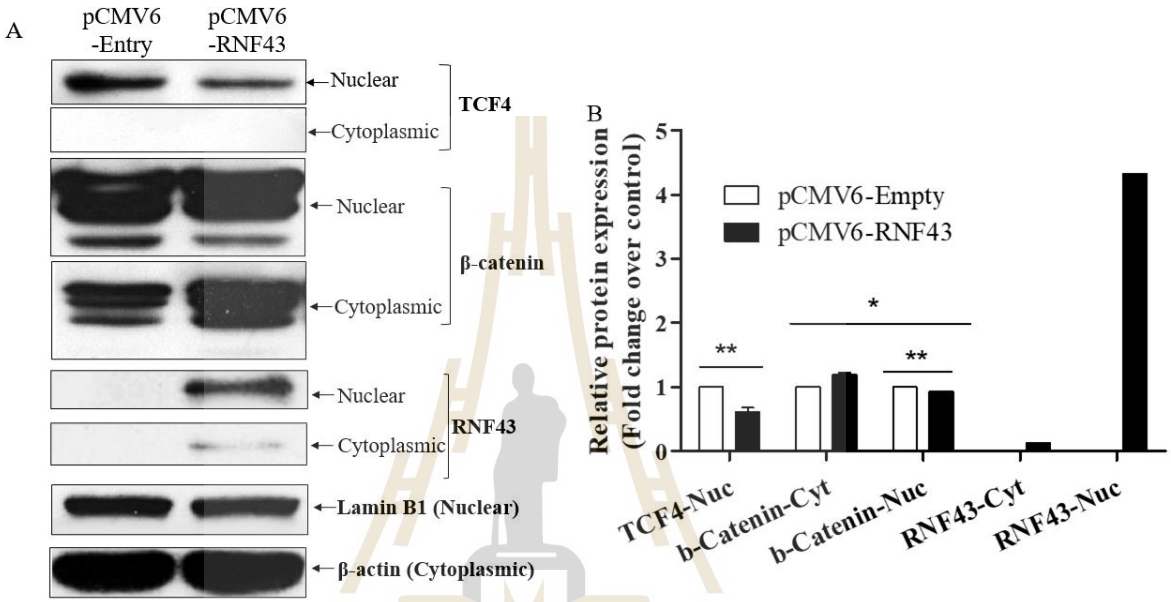

Figure 3. 3 Localization of various key Wnt/ β-catenin proteins. (A) Western blot analysis showed the accumulation of β-catenin in the cytoplasm in KKU-213B overexpression *RNF43*. The majority of RNF43 and TCF4 proteins are expressed in the nuclear fraction. (B), Protein measurement and calculation were performed by ImageJ and GraphPad Prism 5. Relative protein expression was normalized to pCMV6-Entry-transfected KKU-213B. The bar graph represents mean ± SEM of three independent experiments. **P*<0.05, ***P*<0.001.

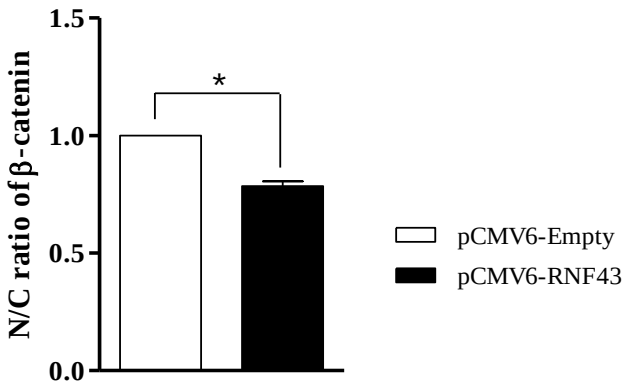

Figure 3.4 Ratio of nuclear/cytoplasmic β-catenin (N/C) of *RNF43*-overexpressing KKU-213B compared to the control. The N/C ratio indicates the amount of β-catenin translocated into the nucleus versus β-catenin remains in the cytoplasm. Relative protein expression was normalized to pCMV6-Entry-transfected KKU-213B. The bar graph represents mean ± SEM of three independent experiments. **P*<0.05.

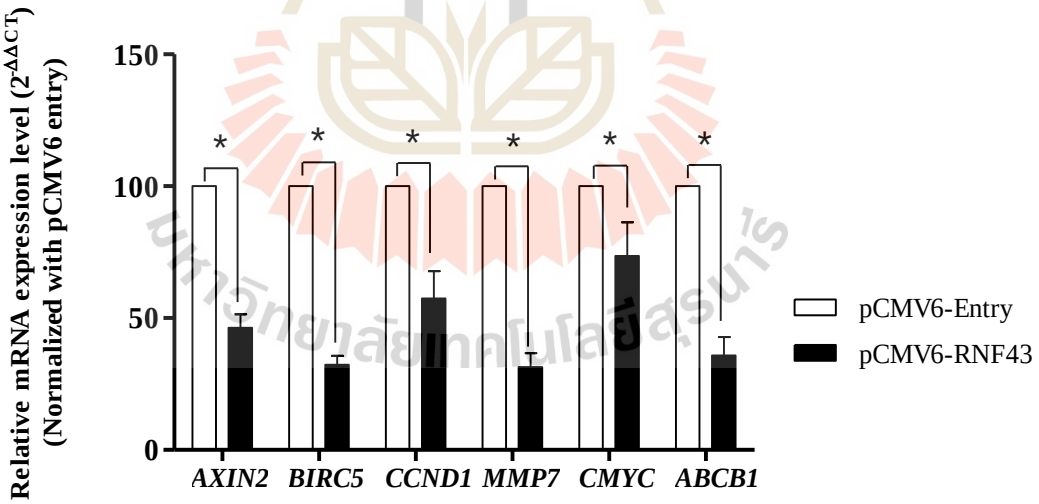

Figure 3.5 Downregulation of various Wnt target genes treated with *RNF43* overexpression. Relative mRNA expression level was determined using $2^{-\Delta\Delta CT}$ normalized to cell transfected with pCMV6-Entry. The bar graph represents mean ± SEM of three independent experiments. **P*<0.05.

3.3 *RNF43* overexpression reduces CCA cell proliferation

In order to understand the functional role of RNF43 in mediating the suppression of proliferative cell growth, we studied the effect of RNF43 by overexpressing *RNF43* in KKU-213B cell line. Overexpression was performed by pCMV6-RNF43 and pCMV6-Entry transfection, followed by incubation for 24 h, 48 h and 72 h to observe its proliferative activity. The result showed that KKU-213B overexpressing *RNF43* have a significantly lower cell proliferation rate, when compared to that of the control cells transfected with pCMV6-Entry plasmid (Figure 3.6). Efficient *RNF43* overexpression substantially decrease cell growth after 48 h (186% vs 166% for pCMV6-Entry vs pCMV6-RNF43, respectively. $*P<0.05$) and 72 h of incubation (338% vs. 317% for pCMV6-Entry vs pCMV6-RNF43, respectively ($*P< 0.05$). Additionally, downregulation of *CCND1*(Cyclin D1) and *CMYC* (c-MYC) following *RNF43* overexpression were further observed. These findings confirm that cell growth was specifically decreased by RNF43 via Wnt/ β-catenin signaling pathway inhibition (Figure 3.7).

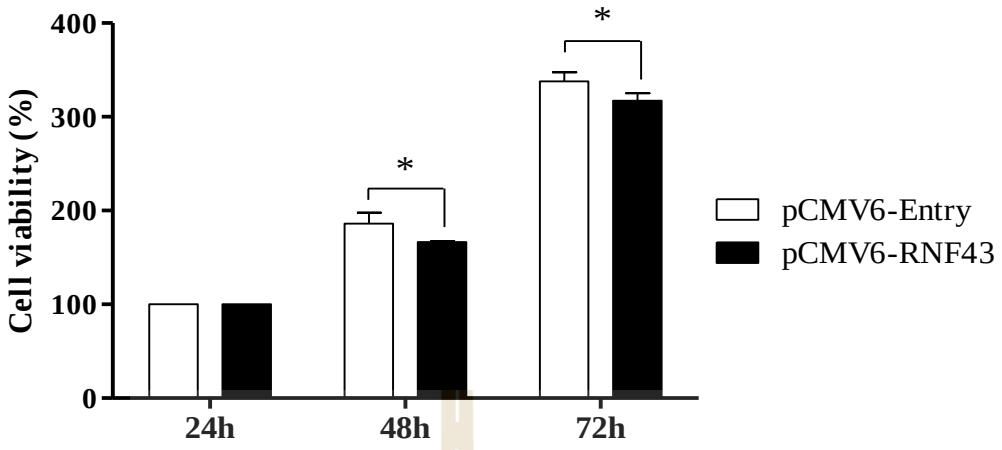

Figure 3. 6 *RNF43* overexpression supressed cell proliferation. Percent cell viability was normalized to absorbance of KKU-control at 0 h. Bar graph represents mean ± SEM of three independent experiments. *$P<0.05$.

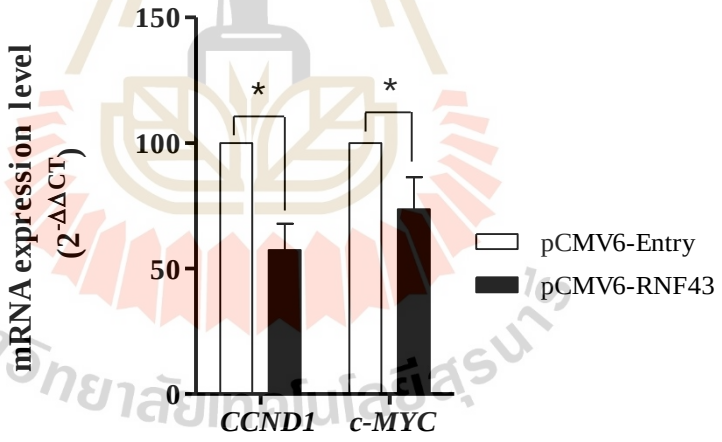

Figure 3. 7 Downregulation of Wnt/ β-catenin as well as proliferative marker genes *CCND1* and *c-MYC* upon *RNF43* overexpression was measured by qRT PCR. Relative mRNA expression level was determine using $2^{-\Delta\Delta CT}$ normalized to pCMV6-Entry. Bar graph represents mean ± SEM of three independent experiments. *$P<0.05$.

3.4 RNF43 overexpression enhances 5-fluorouracil sensitivity

Cell proliferation is directly or indirectly supported by various classical hallmarks of cancers, including one of the current greatest challenges in cancer, the drug resistance mechanism (Hanahan and Weinberg 2011; Nussinov, Tsai, and Jang 2017).

To unravel the possible RNF43 mechanism in suppressing drug resistance, we performed drug treatment to *RNF43*-overexpressed cells. Initially, we tried to compare various common chemotherapeutic drugs, such as Gemcitabine and Cisplatin. However, we were unable to observe noticeable effect of *RNF43* on the chemoresistance of these two regiments (Figure 3.8) in KKU-213B. Then, we focused on 5-FU as the chemotherapeutic agent used to study *RNF43* effect in KKU-213B with various gradient concentrations (0, 3.125, 6.25, 12.5, 25 and 50 μM) and treated for 48 and 72 h. Our data showed, overexpression of *RNF43* in KKU-213B decreased cell viability which indicated the improvement of drug sensitivity of the cells (Figure 3.9). Although statistically there was no significant effect of *RNF43* in KKU-213B, *RNF43* overexpression moderately increased 5-FU drug response. Better drug response was indicated by lower cell viability after treatment compared to the control. This effect was even more pronounced in the cell with longer treatment time (48 h compared to 72 h).

In order to corroborate this finding, we further investigated cell survival by performing colony formation assay. pCMV6-RNF43 and pCMV6-Entry transfected cells were treated with 5-FU (0, 3.125, 6.25, 12.5, 25 and 50 μM) for 48h prior to 14 days of prolonged incubation time. The result showed that the colony in the cells which transfected with pCMV6-RNF43 were less formed compared to that of the control (Figure 3.10). Furthermore, various drug resistance genes were downregulated

following *RNF43* overexpression, including *ABCB1* and *ABCC1* which are known as the major contributors to chemoresistance in cancers including CCA by mediating drug efflux (Figure 3.11).

Next, to define the role of RNF43 in mediating cell death, we examined various apoptotic markers after RNF43 overexpression. Our result showed that RNF43 overexpression slightly altered the protein expression level of apoptotic marker proteins, Caspase-3, and the anti-apoptotic protein, Bcl2, (Figure 3.12). Moreover, there was a significant downregulation of apoptotic inhibitor *BIRC5* (Figure 3.11). Collectively, we conclude that overexpression of RNF43 enhances 5-FU chemotherapeutic drug susceptibility via WNT/ β-catenin pathway inhibition.

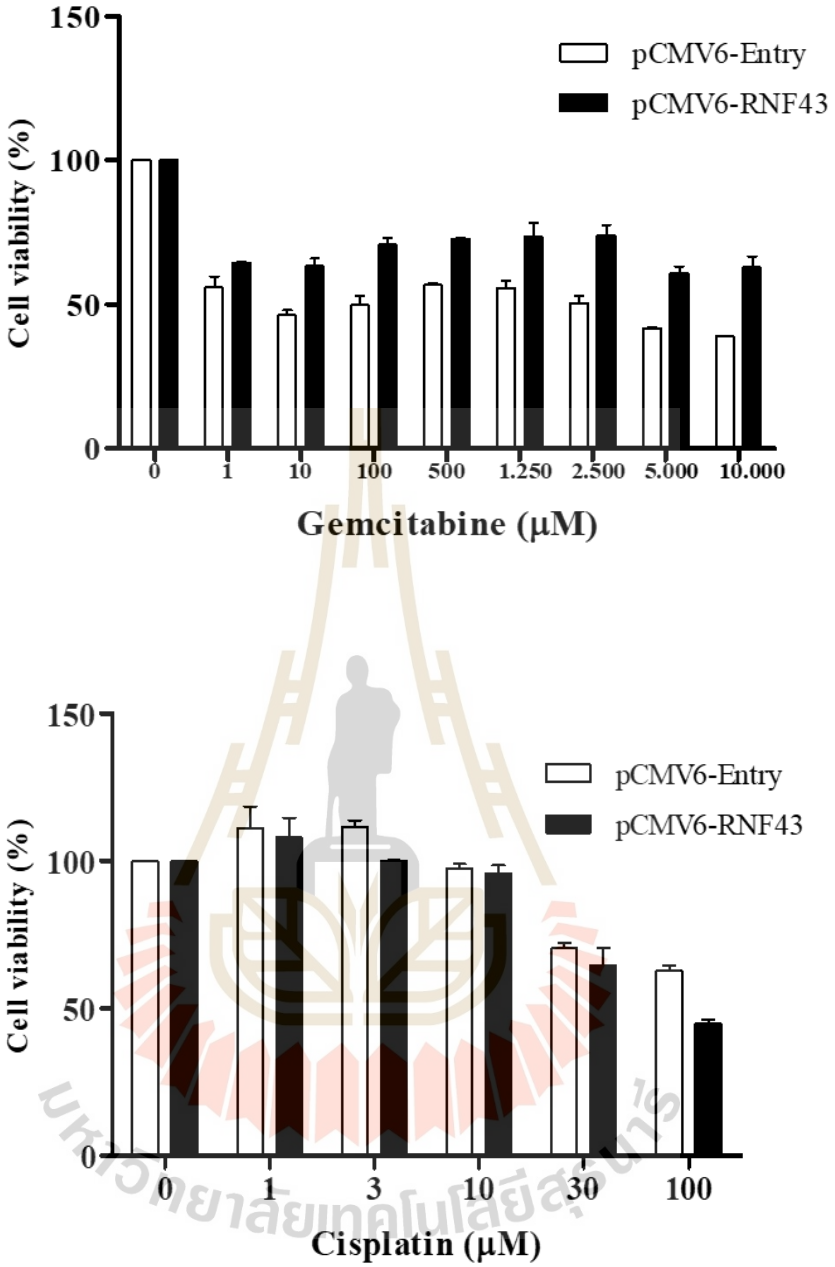

Figure 3.8 Gemcitabine and cisplatin was used to determine the effect of RNF43 to the chemotherapeutic drug response of KKU-213B. Gemcitabine was used to treat the cell for 48 h while cisplatin was used to treated for 24 h. There were no significant differences for both treatments. The bars represent mean ± SEM of three independent experiments.

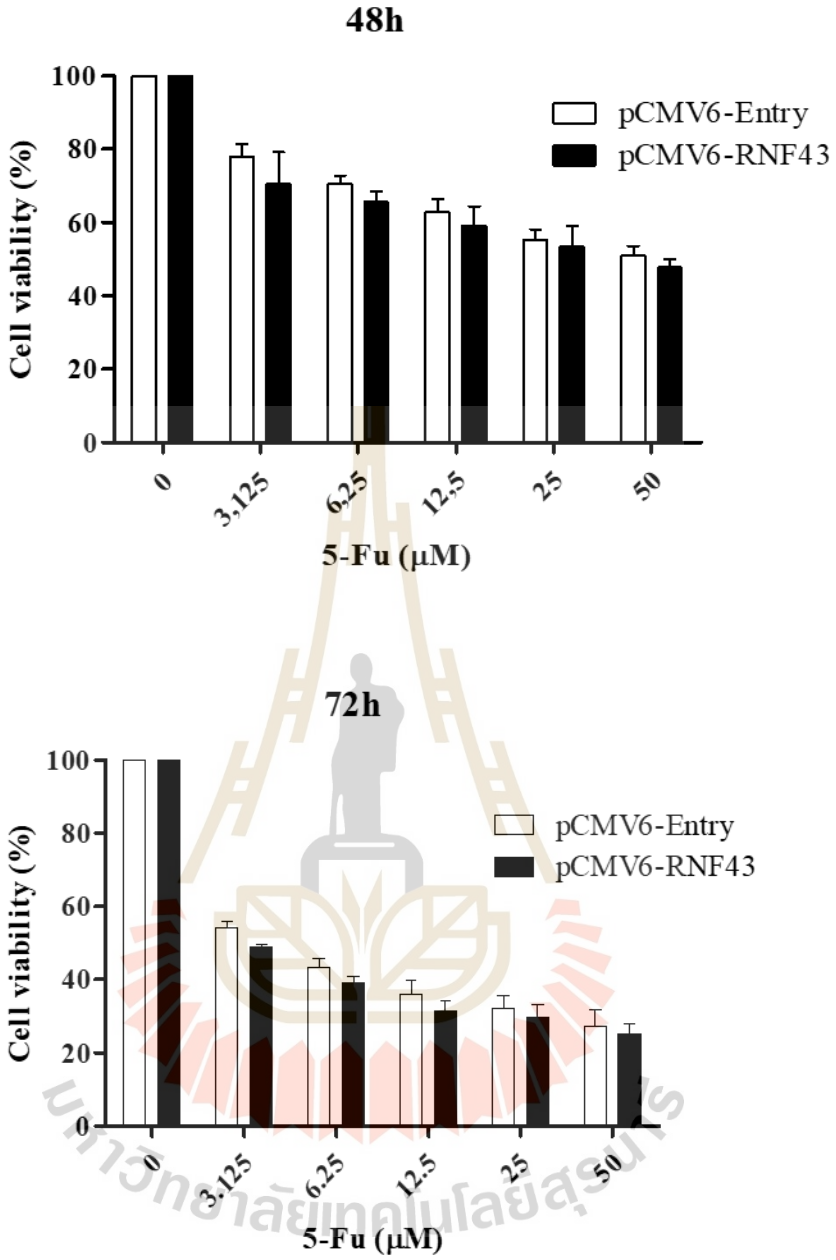

Figure 3.9 RNF43 overexpression increased 5-FU drug responses of KKU-213B. Cell viability was measured after 5-FU treatment with various gradient concentrations *(*0, 3.215, 6.25, 12.5, 25, and 50 µM*)*. The 5-FU drug was treated for 48 h and 72 h.

There were no significant differences for both treatments. The bars represent mean ± SEM of three independent experiments.

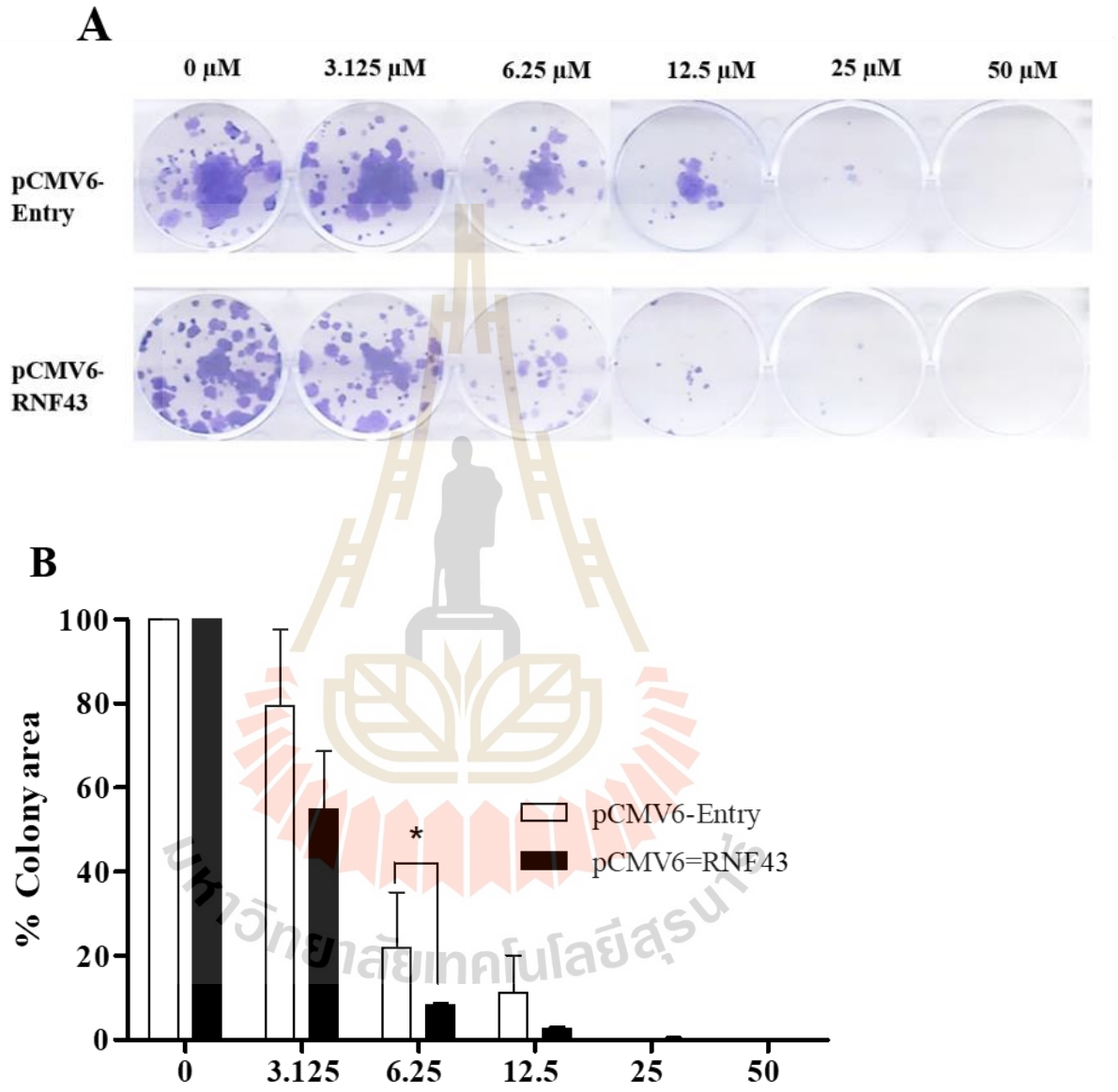

Figure 3.10 Colony formation assay in KKU-213B cell line treated with 5-FU for 48 h. $2x10^3$ cell/well transfected cells were plated and incubated for 14 days at 37°C. The cell were further fixed by methanol and stained with crystal violet solution. The bar graph represents mean ± SEM of three independent experiments. *$P<0.05$.

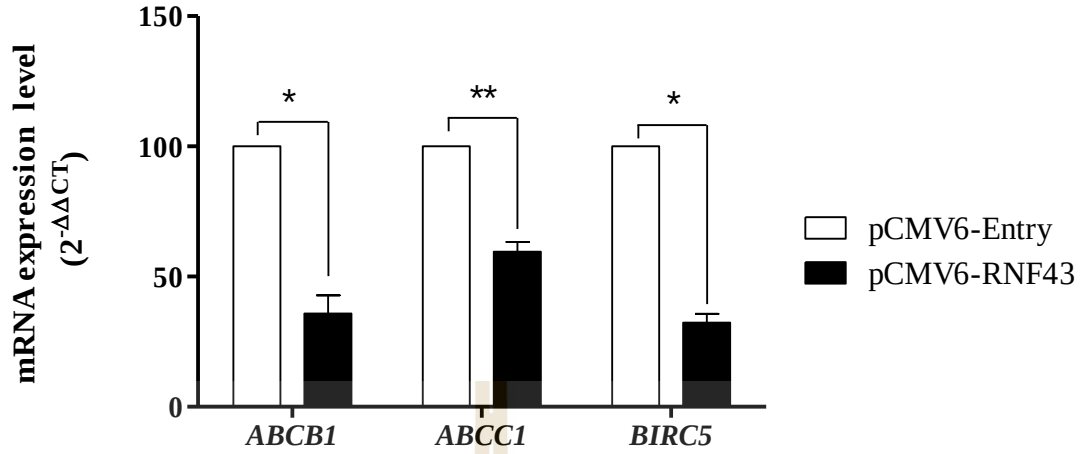

Figure 3.11 qRT-PCR was used to determine MDR genes and anti-apoptotic gene. *ABCB1* and *ABCC1* are ABC transporter genes which regulates drug efflux mechanism, whereas *BIRC5* is an anti-apoptotic marker gene encodes survivin protein. mRNA expression level was determined using $2^{-\Delta\Delta CT}$. Bar graph represents mean ± SEM of three independent experiments. **P*<0.05, ***P*<0.01.

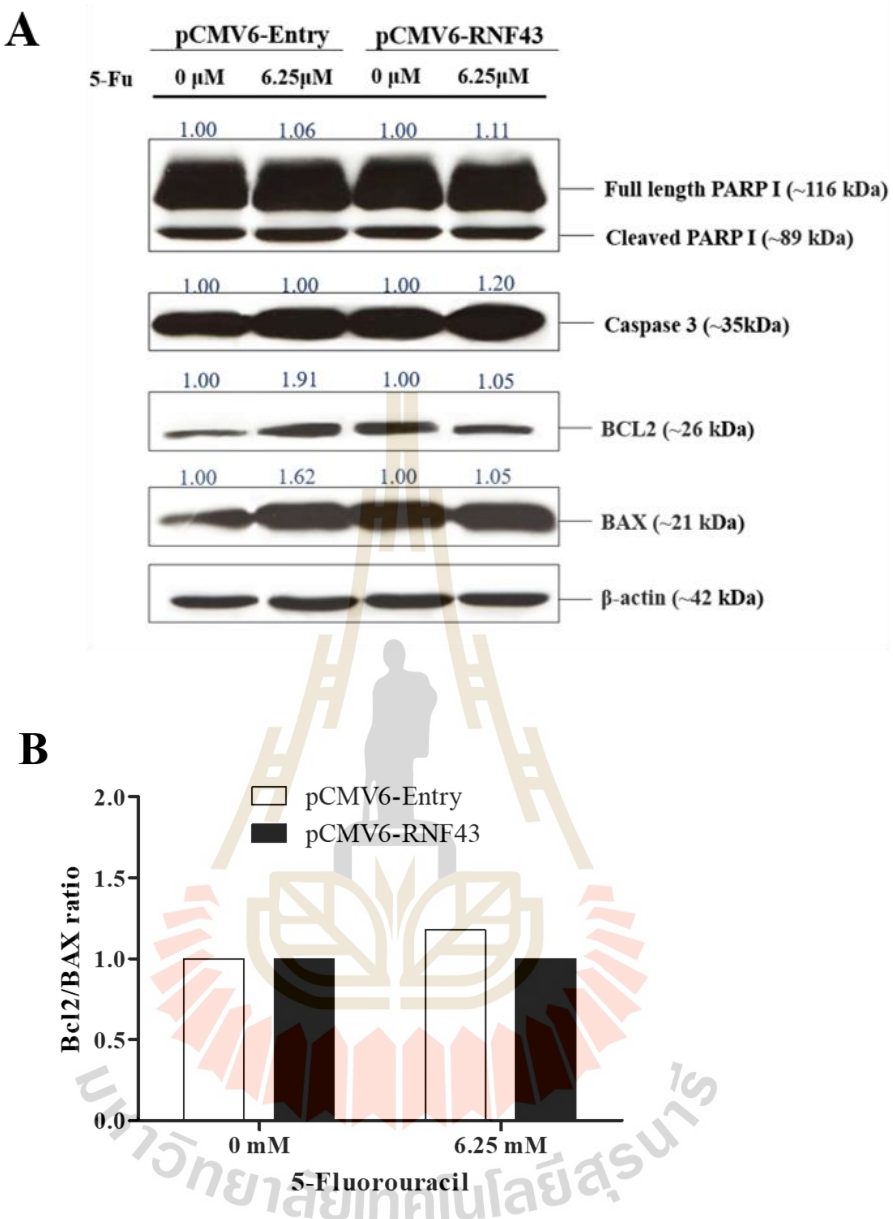

Figure 3. 12 *(*A*)*Various apoptotic markers were detected by the western blot analysis. *(*B*)* Bcl2/BAX ratio was calculated to determine cell susceptibility to apoptotic. The lower level of this ratio indicates higher apoptotic activity.

3.5 *RNF43* overexpression delays CCA cell migration

Wnt/β-catenin signaling activation also contributes to the progression of tumor growth and metastasis, which is initiated by local tumor migration (Uthaisar et al., 2016; Fares et al., 2020). In this study, we hypothesized the inactivation of Wnt/β-catenin signaling by *RNF43* overexpression would inhibit cell migration and subsequently hamper tumor progression in CCA cell lines. In order to test this hypothesis, we observed CCA cell motility by performing wound healing and transwell cell migration assays. The wound healing assay was used to observe the wound closure of cell monolayer on the surface of the culture plate. The result demonstrated that overexpression of *RNF43* in KKU-213B cell line delayed wound closure over time. As shown in Figure 3.13, the percentage of wound closure, which reflects migration rate, was observed to be higher in the control compared to the cell with *RNF43* overexpression (90.8% vs 48.1% at 16 h (***P*<0.001); 98.3% vs 69.5% at 20 h (***P*<0.001); and 99.6% vs 85.3% at 24 h, respectively).

The transwell cell migration assay was performed to measure the capacity of cell motility towards a chemo-attractant gradient. We use a complete media contained FBS as the chemo-attractant and incubate for 16 h and 24 h. Based on our observation, cells with *RNF43* overexpression less migrated compared to the control in both 16 and 24h incubation condition. The measurement of the OD value also shown there was a substantial cell migration (*P<0.05*) in the control compared to the cell with *RNF43* overexpression (Figure 3.14).

Likewise, mRNA expression level of the various genes which contribute to the uncontrolled cell migration were downregulated in the cell with RNF43 overexpression, including *MMP-2, MMP-7, MMP-9* and *CDH-2.* On the other hand,

an epithelial marker gene, *CDH-1,* which maintains cell-cell adhesion, was increased (Figure 3.17). These observations not only support a relevant cooperative function of RNF43 in inhibiting Wnt/β-catenin signaling, but also underline the importance of RNF43 in restraining the progression of CCA tumor by prevent initiation of metastasis.

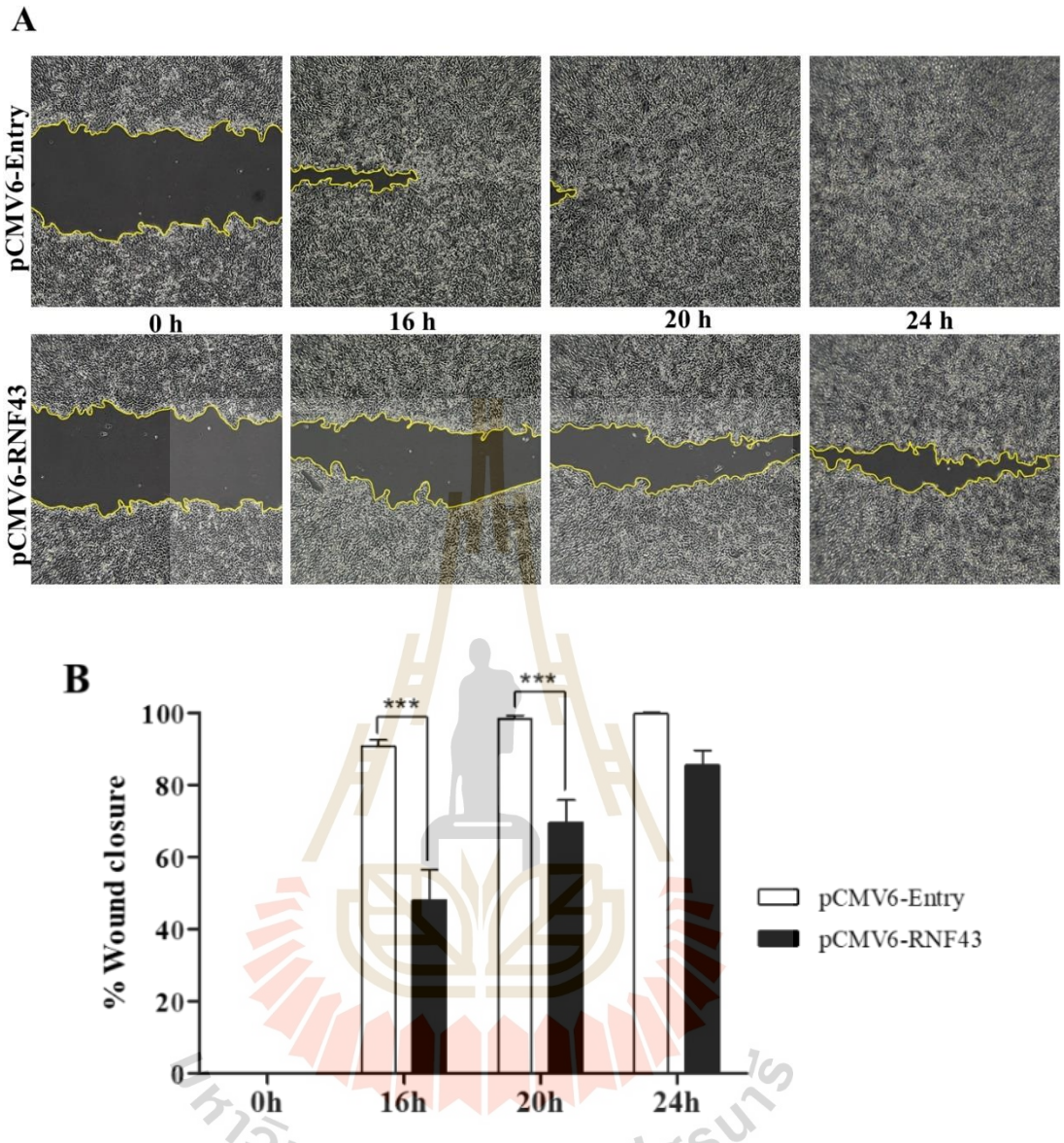

Figure 3.13 Wound closure was substantially observed to be slower in *RNF43*-overexpressing cell compared to the control (A) Representative figure of wound healing assay over time. The wound was measured at 0, 16, 20 and 24 hours followed by analysis by ImageJ (B) Statistical calculation of wound healing assay. The wound closure area was measured by ImageJ by compare % area. Values are expressed as means ± standard error of the mean (SEM) of three independent experiments. ***P<0.001.

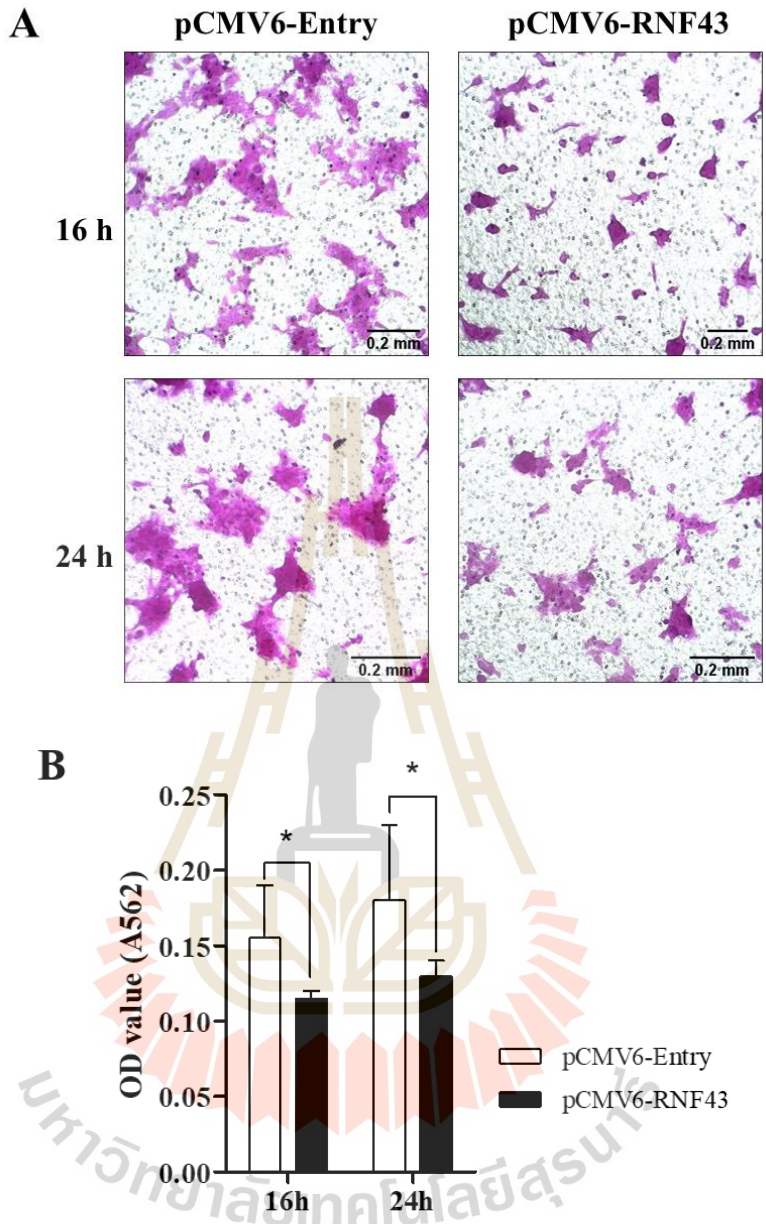

Figure 3.14 Transwell cell migration assay depicts the correlation of RNF43 overexpression and inhibition of cell migration towards chemoattractant. (A) SRB staining of migrated cell (x10 magnification) after RNF43 overexpression and incubation at16 h and 24 h. (B) OD measurement of migrated cell (A562). Values are expressed as means ± standard error of the mean (SEM) of three independent experiments. *P<0.05.

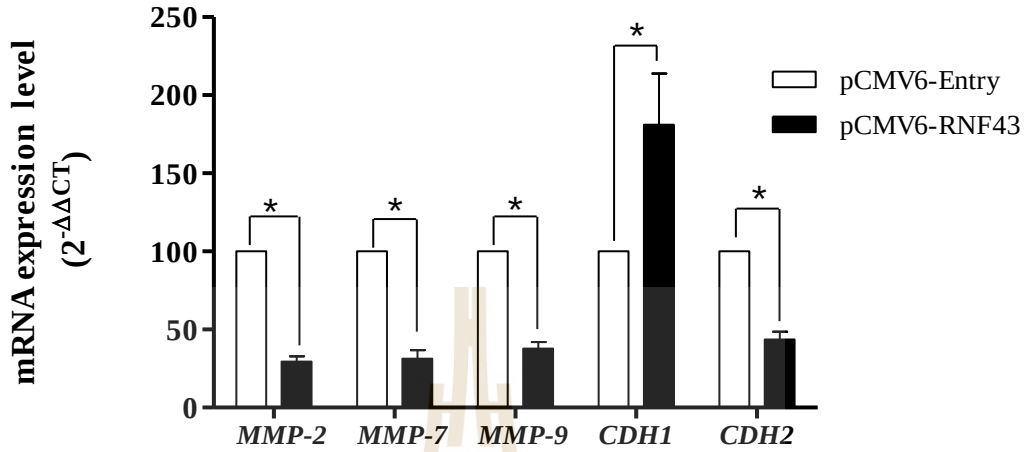

Figure 3.15 qRT-PCR was used to determine various genes that are contribute to the metastatic processes including several MMPs and EMT marker genes. mRNA expression level was determine using $2^{-\Delta\Delta CT}$. Values are expressed as means ± standard error of the mean (SEM) of three independent experiments. *P<0.05.

CHAPTER IV

DISCUSSION AND CONCLUSSION

RNF43 is a novel gene that frequently found to be mutated in various cancers, including pancreatic distal adenocarcinoma (Jiang et al., 2013), colorectal cancer (Yang and Tang 2000), gastric cancer (Gao et al., 2017) and *Opisthorchis viverrini*-related CCA (Ong et al., 2012; Talabnin et al., 2016). The inactivating mutation of RNF43 accounted for 9.3%, suggesting that potentially contributes to the development of CCA. Although the mechanism remains unclear, *RNF43* loss-of-function mutation was associated with poor prognosis of the CCA patients (Ong et al., 2012). In this study, low expression of RNF43 was observed in all of the CCA cell lines used. This result was consistent with previous study that found a remarkably low mRNA and protein expression level of RNF43 in CCA tissue (Talabnin et al., 2016). In light of this study, downregulation of RNF43 was highly correlated with the short survival of CCA patients (Talabnin et al., 2016). Given that inactivating mutation and altered gene expression of RNF43 have been reported in CCA and are associated with poor prognosis, understanding the functional significance of RNF43 in CCA is crucial and important for the development of effective cancer therapies.

The involvement of the Wnt/β-catenin signaling pathway has been reported in the progression of CCA, in which the vast majority of Wnt-associated genes are mutated or upregulated (Boulter et al., 2015; Zheng et al., 2018). Interestingly, Wnt/β-catenin signaling is tightly controlled by a ubiquitin ligase, RNF43 (Loregger et al., 2015; Ryland et al., 2013; Tsukiyama et al., 2015).

There are various possible Wnt/β-catenin signaling inactivation mechanisms by RNF43. At the membrane level, interaction of RNF43 with the Fzd receptor eventually stabilizes the destruction complex and prevents β-catenin translocation into the nucleus (Serra and Chetty, 2018; Zebisch and Jones, 2015). Subsequently, at the nucleus, interaction of β-catenin with TCF4 transcription factor initiates the transcription of Wnt/ β-catenin target genes. However, in the presence of RNF43, TCF4 is sequestered into the nuclear membrane, which prevents interaction between TCF4 and β-catenin and suppress the transcription of Wnt target genes (Loregger et al., 2015). In accordance with previous findings, we observed overexpression of RNF43 in KKU-213B resulted in β-catenin protein accumulated in the cytoplasm, whilst lower amount of β-catenin was found in nuclear fraction. Additionally, at the nuclear fraction, we also found that overexpression of RNF43 suppressed TCF4 in the nucleus. This finding supports the idea that a sufficient amount of RNF43 blocks β-catenin translocation from cytoplasm into the nuclear fraction, as well as decreases TCF4 expression at the nucleus, and ultimately inhibit Wnt/β-catenin signaling pathway.

In support of the idea that RNF43 suppresses Wnt/β-catenin signaling pathway, downregulation of known Wnt/β-catenin target genes, such *BIRC5*, *CCND1*, *c-MYC*, *MMP-7*, and *ABCB1*, was shown following RNF43 overexpression. However, it is noteworthy to mention that some of those genes are not specifically regulated by Wnt signaling only. There are various other signaling pathways which may contribute to the activation of these genes, such as the MEK/REK pathway, which regulates *CCND1* (Modi et al., 2012) and *c-MYC* (Marampon, Ciccarelli, and Zani, 2006), or the Notch signaling pathway, which regulates *MMP-7* (Sawey and Crawford, 2008). Therefore, in order to clarify whether RNF43 specifically inhibits the Wnt signaling cascade, we

further confirmed by measuring the mRNA expression level of the essential Wnt/β-catenin signaling target gene *AXIN2*. Here, we found that *AXIN2* was significantly downregulated following RNF43 overexpression. Thus, this finding further corroborates the idea of the involvement of RNF43 in specific inhibition of Wnt/β-catenin signaling.

Notably, downstream molecular marker genes which mediate the progression of cell growth and proliferation, such as *CCND1* (Wang et al., 2012; Fusté et al., 2016) and *c-MYC* (Melnik et al., 2019) were found to be downregulated following RNF43 overexpression. This finding supports cell proliferation analysis, in which a significant decreased of cell viability at 48 and 72 h was detected in RNF43-overexpressing KKU-213B compared to the control. These findings implicate an indispensable requirement of RNF43 to inhibit the activity of the Wnt/β-catenin signaling pathway, which is followed by inhibition of cell growth.

Unresectable CCA due to late diagnosis has made chemotherapeutic regiments as the best option to treat the patients. However, poor response to available chemotherapeutic drugs in advanced CCA patients remains a challenge (Marin et al., 2018). A synergistic powerful chemotherapeutic resistance mechanism is reported in CCA and characterized by multi drug resistance (MDR) phenotype (Marin et al., 2018; Shen et al., 2013). Some of the genes involved in the MDR phenotype are known to be regulated under the Wnt/β-catenin signaling pathway (Marin et al., 2018). Here we evaluated the role of RNF43 in mediating chemotherapeutic drug susceptibility. The result showed that RNF43-overexpressing cells were likely to be more prone to the treatment of 5-FU marked by lower percent cell viability and colony formation when compared to the control. Furthermore, two MDR-related genes which mediate

chemoresistance (*ABCB1* and *ABCC1*) and an anti-apoptotic gene (*BIRC5*) were downregulated upon RNF43 overexpression. As inhibitor of apoptotic (IAP) gene family *BIRC5*, is one of the genes which is activated under the WNT/ β-catenin signaling pathway (Zhang et al., 2013). In the present study, downregulation of *BIRC5* provides a possible mechanism for how cell survival inactivation is regulated by RNF43 via WNT/ β-catenin signaling pathway inhibition. Recently, involvement of RNF43 in mediating inhibition of chemotherapeutic resistance via DNA damage repair (DDR) mechanisms has been documented in gastric cancer, in which RNF43 ubiquitinates various DDR key players that eventually increase drug response (Neumeyer et al., 2021). Accordingly, the role of RNF43 in chemotherapeutic resistance via DNA damage repair in CCA is required in further study.

CCA is commonly known as high invasive tumor (Banales et al., 2020). The Wnt/β-catenin signaling pathway is reported to be one of the mechanisms underlying the aggressiveness CCA progression (Boulter et al., 2015; Noll et al., 2016). Local tumor migration is an initial step of metastasis commonly observed in tumor growth (Uthaisar et al., 2016; Fares et al., 2020). Downregulation of RNF43 in colorectal cancer increased cell migration and invasion, (Wang et al., 2016). Likewise, our cell migration analysis results suggested that RNF43 inhibits the migration of the CCA cell line via inactivation of Wnt/β-catenin signaling pathway. This discovery was further supported by RNF43 overexpression, which downregulated Wnt/β-catenin target gene, *MMP-7*, genes encode extra cellular matrix for angiogenesis and metastasis *MMP-2* and *MMP-9*, and mesenchymal marker gene *CDH-2*. Whereas an epithelial marker gene *CDH-1* was increased. Thus, the sufficient expression of RNF43 hinders CCA metastasis.

In conclusion, our study has elucidated the functional role of RNF43 in mediating the inhibition of the Wnt/β-catenin signaling pathway. The unique properties of RNF43 block β-catenin translocation and subsequentially followed by downregulation of Wnt/β-catenin target genes that are involved in the progression of CCA. Sufficient expression of RNF43 is essential to prevent further aberrant activation of Wnt/β-catenin signaling, which contribute to the aggressive CCA phenotypes. This discovery underscores the functional role of RNF43 as a Wnt/β-catenin signaling inhibitor (Figure 4.1)

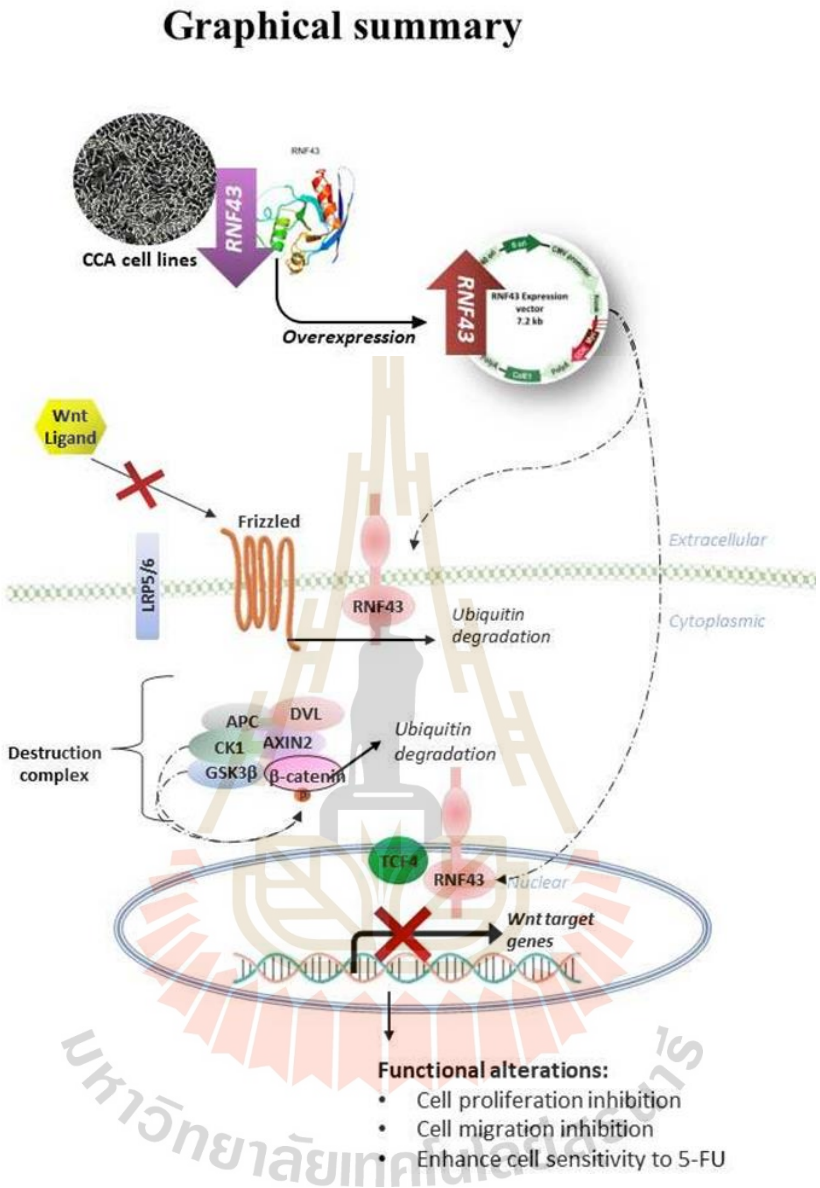

Figure 4.1 Graphical summary Wnt/β-catenin signaling inhibiton by RNF43 modulates various genotype and phenotype changes, including downregulation of Wnnt target genes and functional alteration which involved in the inhibition of cancer progression.

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