ROLE OF CORE 1 β1-3 GALACTOSYLTRANSFERASE 1 IN CHOLANGIOCARCINOMA

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Translational Medicine

Suranaree University of Technology

Academic Year 2020

บทบาทของเอมไซม์ คอร์วันเบต้าวันทรีกาแลคโตซิลทรานสเฟอเรสวัน ในมะเร็งท่อน้ำดี

นางสาวจุฑามาสฆ์ เขียววิจิตร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเวชศาสตร์ปริวรรต
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2563

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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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โอไกลโคซิเลชันเป็นกระบวนการเติมแต่งโปรตีนหลังจากกระบวนการแปลรหัสโปรตีนที่พบได้ในโปรตีนส่วนใหญ่ โดยกระบวนการนี้นิยมเรียกว่า มิวซินไกลโคซิเลชัน เนื่องจากเป็นกระบวนการหลักที่เกิดในโปรตีนที่หลั่งออกนอกเซลล์โดยเฉพาะอย่างยิ่งเช่น มิวซิน ซึ่งความผิดปกติของกระบวนการมิวซินไกลโคซิเลชันนี้มักถูกพบบ่อยในมะเร็งและมีความสัมพันธ์กับความผิดปกติของการแสดงออกของยีนกลูโคซิลทรานสเฟอเรส โดยเอมไซม์คอร์วันเบต้าวันทรีกาแลคโตซิลทรานสเฟอเรสวันหรือ C1GALT1 เป็นเอนไซม์หลักที่มีความสำคัญในการสร้างโครงสร้างมิวซินแบบที่ 1 โดย C1GALT1 จะทำงานร่วมกับโปรตีนผู้ช่วยที่มีความจำเพาะเจาะจงกัน โดยจะทำหน้าที่เติมหมู่น้ำตาลกาแลคโตสบนโครงสร้าง Tn-antigen เพื่อเกิดเป็นโครงสร้าง T-antigen หรือเรียกว่าโครงสร้างมิวซินแบบที่ 1 โดยโครงสร้าง T-antigen นี้จะสามารถถูกเติมแต่งต่อไปด้วยการเพิ่มน้ำตาลชนิดต่างๆผ่านการทำงานของเอนไซม์กลูโคลซิลทรานสเฟอเรสชนิดอื่นๆ มีรายงานความผิดปกติของการแสดงออกของยีน C1GALT1 ในมะเร็งหลากหลายชนิดและสัมพันธ์กับความรุนแรงของมะเร็ง ได้แก่ การแบ่งตัวเติบโต การรุกรานและการกระจายของมะเร็ง และยังรวมไปถึงการตอบสนองต่อยาเคมีบำบัดด้วย แต่อย่างไรก็ตามการแสดงออกของยีน C1GALT1 และบทบาทหน้าที่ยังไม่เคยมีการศึกษาในมะเร็งท่อน้ำดี ดังนั้นในการศึกษานี้ผู้วิจัยได้แสดงให้เห็นว่าการแสดงออกของยีน C1GALT1 มีอยู่ในระดับต่ำในมะเร็งท่อน้ำดีแต่การแสดงออกดังกล่าวไม่พบความสัมพันธ์ทางสถิติกับลักษณะอาการทางพยาธิคลินิกหรืออัตราการอยู่รอดของผู้ป่วยมะเร็งท่อน้ำดี เมื่อทำการศึกษาบทบาทหน้าที่พบการยับยั้งการแสดงออกของยีน C1GALT1 มีผลเพิ่มการเจริญเติบโตของเซลล์มะเร็งและการดื้อต่อยาเคมีบำบัดชนิดฟลูโอโรยูราซิลในรูปแบบตามความเข้มข้น และการยับยั้งการแสดงออกของยีน C1GALT1 ยังมีผลกระตุ้นกระบวนการส่งสัญญาณวิถี AKT และ ERK เพิ่มความสามารถการต้านการตาย ซึ่งถูกแสดงด้วยอัตราส่วนที่สูงขึ้นระหว่าง BCL2 ต่อ BAX และยังพบการแสดงออกที่เพิ่มขึ้นของยีนที่เกี่ยวข้องการเจริญเติบโตได้แก่ c-Myc และ CCND1 ยิ่งไปกว่านั้น ยังพบการยับยั้งการแสดงออกของยีน C1GALT1 สัมพันธ์กับการแสดงออกของสารไกลแคนชนิด VVL (VVL-binding glycans) ซึ่งผลการศึกษานี้แสดงให้เห็นว่าระดับการแสดงออกที่ลดลงของยีน C1GALT1 มีผลกระทบที่สำคัญในกระบวนการสร้างโครงสร้างของมิวซินแบบที่ 1 ให้สมบูรณ์ของโปรตีนที่เกี่ยวข้องกับการเจริญเติบโตและการดื้อต่อยาเคมี

บำบัดชนิดฟลูโอโรยูราซิลในมะเร็งท่อน้ำดี และด้วยเอนไซม์คอร์ทรีทรานสเฟอเรส (B3GNT6) และเซียลิวทรานสเฟอเรส (ST6GALNAC1) สามารถนำน้ำตาลไปเติมบนโครงสร้างของ Tn antigen ได้เช่นกัน ดังนั้นในการศึกษานี้พบการยับยั้งการแสดงออกของยีน C1GALT1 ไม่มีผลกระตุ้นการทำงานของเอนไซม์ทั้งสอง เนื่องจากการแสดงออกของยีนทั้งสองนี้มีระดับที่ลดลงเช่นกัน ซึ่งผลการศึกษานี้มีความสอดคล้องกับการเพิ่มขึ้นของการแสดงออกของสารไกลแคนชนิด VVL (VVL-binding glycans) ดังนั้นจากผลการศึกษาทั้งหมดที่กล่าวมาบ่งชี้ให้เห็นว่าการแสดงออกของยีน C1GALT1 ที่ลดลงในมะเร็งท่อน้ำดีมีความสัมพันธ์กับการแสดงออกของโครงสร้างมิวซินแบบที่ 1 ที่ไม่สมบูรณ์ และการแสดงออกที่ลดลงของยีน C1GALT1 นี้มีผลส่งเสริมการเจริญเติบโตของเซลล์มะเร็งท่อน้ำดีและทนทานต่อยาเคมีบำบัดชนิดฟลูโอโรยูราซิลที่อาศัยกระบวนการส่งสัญญาณวิถี AKT และ ERK

สาขาวิชาเวชศาสตร์ปริวรรต ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2563 ลายมือชื่ออาจารย์ที่ปรึกษา_____

JUTHAMAS KHIAOWICHIT : ROLE OF CORE 1 β1-3 GALACTOSYLTRANSFERASE 1 IN CHOLANGIOCARCINOMA. THESIS ADVISOR : ASSOC. PROF. KRAJANG TALABNIN, Ph.D., 75 PP.

CHOLANGIOCARCINOMA/ CORE 1 β1-3 GALACTOSYLTRANSFERASE 1 (C1GALT1)/ CANCER AGGRESSIVENESS

O-Glycosylation is one of the most common protein post-translational modification that often-called mucin-type O-glycosylation because of its present on many extracellular secreted glycoproteins especially mucins. Aberrant mucin-type O-glycosylation is often observed in cancers and associated with the alteration of glycosyltransferase expression. Core 1 β1-3 Galactosyltransferase (C1GALT1) is a key glycosyltransferase for the formation of mucin-type Core 1 structure. C1GALT1, with the help of its C1GALT1-specific chaperone (COSMC), transfers galactose to Tn antigen to form T antigen (core 1 structure). T antigen is further modified by adding other sugar via other glycosyltransferases to form the complex mucin-type O-glycans. Aberrant C1GALT1 expression has been documented in several types of cancers and is associated with cancer aggressiveness, including cell proliferation, invasion and metastasis as well as chemotherapeutic sensitivity. However, the C1GALT1 expression and its role in cholangiocarcinoma (CCA) have never been studied. In this study, we demonstrated that low expression of C1GALT1 was detected in CCA tissues at both mRNA and protein levels. However, there was no statistically significant association between C1GALT1 protein expression with any clinicopathological features and survival of CCA patients. The functional studies revealed that suppression of

C1GALT1 increased cell proliferation and 5-fluorouracil resistance in a dose-dependent manner. The activation of phosphorylation of AKT and ERK was significantly increased in silencing C1GALT1 CCA cells. Suppression of C1GALT1 enhanced anti-apoptotic properties in CCA cell lines via a high ratio of BCL2/BAX and increased of cell growth-related genes including *c-Myc* and *CCND1*. Moreover, inhibition of C1GALT1 was associated with high expression of VVL-binding glycans. These observations indicated that low expression of C1GALT1 has a critical effect on mucin-type O-glycans truncation of the protein involving in cell growth and 5-fluorouracil response in CCA. Addition to C1GALT1, Core 3 GlcNAc-transferase (*B3GNT6*) and sialyl-transferase (*ST6GALNAC1*) can transfer sugar molecules to Tn-antigen. Our results showed that suppression of C1GALT1 lead to decreased expression of *B3GNT6* and *ST6GALNAC1*. This observation was consistent with increase expression of VVL-binding glycans. Therefore, our findings indicate that downregulation of C1GALT1 expression in CCA associates with the expression of immature core 1 O-glycan and that subsequently enhances the tumor growth and 5-FU resistance in CCA via altering AKT/ERK signaling pathway.

School of Translational Medicine Student's Signature_____

Academic Year 2020 Advisor's Signature_____

ACKNOWLEDGEMENTS

First, I would like to express sincere appreciation to my co-advisors, Asst. Prof. Dr. Chutima Talabnin, who patiently educated, encouraged, guided, and strengthened my knowledge in order for me to complete my master's degree in Translational medicine. In addition, her kindness was the most important factor in assisting me to pass through every problem. For the above reasons, it is a great pleasure to be one of her students. Moreover, I wish to express my deepest appreciation to my advisor, Asst. Prof. Dr. Krajang Talabnin, who gave me the opportunity to do the challenging and interesting study and always add valuable suggestion and guidance in throughout.

I would like to acknowledge to Asst.Prof.Dr. Chavaboon Dechsukhum, Dr. Sanong Suksaweang and my external examiner, Assistant Professor Dr. Atit Silsirivanit, who provided valuable suggestions.

I would also like to thank to Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, for providing the specimens and clinical data in this study. I would like to acknowledge to the Thailand Research Fund and Kittibandit scholarship. Moreover, sincere thank to the Translational medicine and Biochemistry Research Unit, Suranaree University of Technology for supporting the equipment and laboratory facilities.

Grateful is expressed to all my friends in the Translational Medicine and Biochemistry, for helping me to get through the difficult times, and for all the emotional support, friendship, and entertainment.

Last, but not least, I would like to express my deepest appreciation to my family, especially my beloved mother and sister for their endless love, encouragement and support.

Juthamas Khiaowichit

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LIST OF ABBREVIATIONS

AKT	Protein kinase B
ERK	Extracellular-signal-regulated kinase
C-Myc	MYC Proto-Oncogene, BHLH Transcription Factor
CCND1	B-cell leukemia/lymphoma 1
BIRC5	Baculoviral IAP repeat containing 5
BCL2	B-Cell Leukemia/Lymphoma 2
BAX	Bcl-2-associated X protein
OV	Opisthorchis viverrini
GalNAc	N-Acetylgalactosamine
Cosmc	C1GALT1-specific chaperone 1
MUC	Mucin
EGFR	Epidermal Growth Factor Receptor
HER2	Human epidermal growth factor receptor 2
SBA	Soybean Agglutinins Lectin
WFA	Wisteria Floribunda Lectin
SJA	Styphnolobium Japonicum Lectin
IHC	Immunohistochemistry
Kb or bp	Kilobase or base pair
kD	Kilodalton
PBS	Phosphate-buffered saline
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamind gel electrophoresis

CHAPTER 1

INTRODUCTION

1.1 Rationale for the study

Cholangiocarcinoma (CCA) is an aggressive tumor arising from bile duct epithelial cells (Alsaleh et al., 2019). CCA is quite rare around the globe but, the high incidence was documented in the northeastern part of Thailand. Liver fluke (*Opisthorchis viverrini*, OV) infection is identified to be a major risk factor (Sripa et al., 2008). The clinical progression of CCA is unnoticed, resulting in diagnosis of the advanced stages of cancer in most patients, leading to limited surgical treatment of CCA. Moreover, CCA with advanced stage of the disease responds poorly to current chemo- and radiotherapy (Tushar Patel, 2021). Therefore, identifying targets for developing new therapeutic treatment are extreamly wanted.

The major post-translational modification of many cellular proteins is glycosylation. N-glycosylation and O-glycosylation are the two major types of glycosylation in human (Franca et al., 2014). O-glycosylation especially mucin-type O-glycosylation is mostly found in mammals, and is regulated by a series of glycosyltransferases. Aberrant Mucin-type O-glycosylation including extended or truncated glycosylation has been documented as hallmarks of cancer such as tumor-promoting inflammation, sustain proliferative signaling and resisting cell death (Munkley et al., 2016). Core 1 β1,3 galactosyltransferase I

(C1GALT1) is a key glycosyltransferase in biosynthetic pathways of mucin-type O-glycosylation. Dysregulation of C1GALT1 has been found in various cancers and was associated with malignant phenotypes such as cell proliferation and chemotherapeutic drug sensitivity, and poor prognosis (Lin et al., 2018, Chou et al., 2015, Lee et al., 2020 and Dong et al., 2018). In CCA, the aberrant mucin-type O-glycosylation e.g., mucin MUC1 and MUC5AC have shown to be related with the progression of CCA (Atit et al., 2021). Truncation of mucin-type O-glycans was also observed in CCA and its expression was increasing during carcinogenesis (Marutpong et al., 2020; Krajang et al., 2016 and 2021). However, the underlying mechanism of aberrant mucin-type O-glycosylation in CCA is still unclear. C1GALT1 has been shown to play crucial roles in mucin-type O-glycosylation of multiple types of cancer. The present study hypothesized that low expression of C1GALT1 may promote truncation of mucin-type O-glycosylation and lead to CCA progression. Therefore, the expression of C1GALT1 in CCA and its functional role on cell growth and drug response were explored in this study.

1.2 Thesis objectives

The objectives of this thesis focused on expression of C1GALT1 and its function in CCA.

1. To examine the expression profiles of glycosyltransferases in CCA tissues and nontumorous tissue of the same patients.

2. To determine the protein expression of C1GALT1 in paraffin-embedded CCA tissues

3. To determine the association of C1GALT1 protein expression and clinicopathological features and survival of CCA patients.

4. To examine the biological functions and molecular meachinsm of C1GALT1 in CCA cell lines.

CHAPTER 2

LITERATURE REVIEW

2.1 Cholangiocarcinoma

2.1.1 General information and epidemiology of CCA

Cholangiocarcinoma originates in the biliary epithelium with features of cholangiocyte differentiation (Groen et al., 1999), which is classified by anatomical location as intrahepatic CCA (iCCA), which are located proximal to 66 between the secondary branches of the right and left hepatic ducts, and extrahepatic CCA (eCCA), which include perihilar CCA (pCCA) and distal cholangiocarcinomas (dCCA). pCCA is located between the secondary branches of the right and left hepatic ducts to the cystic duct origin and dCCA is located at the common bile duct (CBD) as shown in Figure 1.1 (Boris Blechacz, 2017).

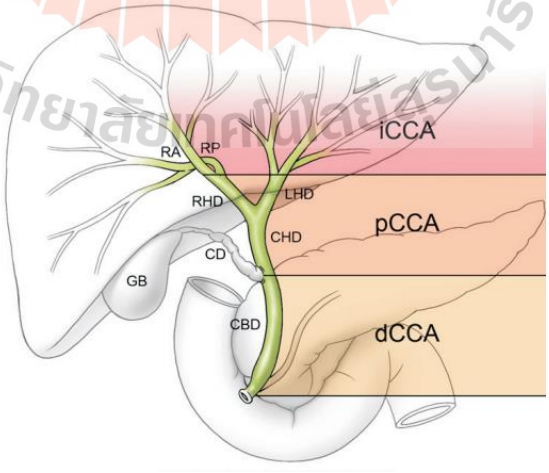


Figure 2.1 Classification of cholangiocarcinoma based on its anatomic location within the biliary tree (Boris Blechacz, 2017).

Among them, the incidence of iCCA is increasing in both Western and Eastern countries, while that of eCCA has remained stable (Jesus et al., 2020). In the case of iCCA, it is further divided into categories based on its growth characteristics or macroscopic into three subtypes including mass-forming (MF), periductal infiltrating (PI) and intraductal growth (IG) types as shown in Figure1.2. Mass-forming (MF) type is occurred in the peripheral bile duct that generally demonstrates as a large mass, and high metastasis. Periductal infiltrating (PI) type is occurred in the central intrahepatic large bile duct, not formed as a mass, but it can spread along the Glisson's sheath where around the bile duct. Intraductal growth (IG) type is occurred within the bile duct, the least common of iCCA. This type can slowly spread to the lymphatic system and has a prognosis than MF or PI (Masanori et al., 2012).

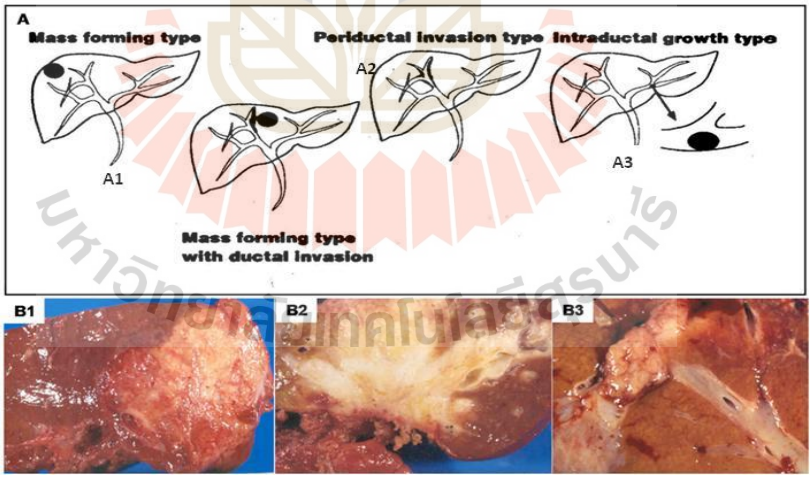


Figure 2.2 Intrahepatic or peripheral cholangiocarcinoma can be presented as mass-forming (A1, B1), periductal infiltrating (A2, B2), and intraductal growth (A3, B3) (Bhudhisawasdi et al., 2012).

Southeast Asia, especially in Northeast Thailand has high incidences of CCA (Parkin et al., 1993); however, its incidence rates vary substantially in different countries, including 5% in Japan, 20% in Korea (Jung et al., 1993) and up to 90% in Thailand especially Khon Kaen province (Parkin et al., 1993).

2.1.2 Pathogenesis of Opisthorchis viverrini-related cholangiocarcinoma

The causes of CCA are still unclear, although chronic inflammation along with partial bile duct blockage (Sirica et al., 2002) is a predisposed factor for CCA development. Caroli's disease, Hepatolithiasis, and congenital choledochal cysts are well-known risk factors for CCA development in Western countries (Rizvi and Gores, 2013). Liver fluke infections by *Opisthorchis viverrini* (OV) is endemic mainly in Thailand, Laos PDR, and Malaysia, while *Clonorchis sinesis* infection is endemic mainly in Japan, Korea, and Vietnam (Sithithaworn et al., 1994). It was predominantly showed the association with CCA development in this geographical area (Sithithaworn et al., 1994). Furthermore, both type of liver flukes are classified by the International Agency for Research on Cancer (IARC) to be type I human carcinogen in 1994 and 2007, respectively.

Chronic inflammation is the major risk factors for CCA development. One of the mechanisms linked between cancer and inflammation is free radicals which are generated via immune cells that are infiltrated to inflammatory sites. The live fluke infection-induced chronic inflammation is involved with the generating of nitric oxide (NO) and other reactive oxygen species or nitrogene species (ROS & RNS). These oxidative/nitrosative stress can potentially cause damage to various cellular biomolecules including protein, lipid or DNA. (Ohshima, Tatemichi, & Sawa, 2003). The inducible

nitric oxide synthase (iNOS) is mainly produced by inflammatory cells, especially macrophages that induced by inflammatory cytokines (Mayer & Hemmens, 1997). There is evidence demonstrated that the activation of iNOS can occur through response to inflammatory cytokines, causing the over-production of NO, resulting in DNA damage and inactivating the enzyme involving in DNA repair process (Jaiswal, LaRusso, Burgart, & Gores, 2000). In addition, the study in hamster model of Pinlaor et al. demonstrated that OV infection can cause oxidative and nitrative DNA damage. As evident by the present of 8-nitroguanine (8-oxodG) and 8-oxo-7, 8-dihydro-2'-deoxyguanosine which are biomarkers for the damage of DNA in the liver of hamsters infected with OV. The nitrative and oxidative DNA damage and the expression of iNOS which are induced via the infection of Ov may participate in CCA carcinogenesis (Pinlaor et al., 2004). Moreover, high of 8-oxodG levels was observed in liver tissues of CCA patients and the level of 8-oxodG was also observed in urine and leukocytes of OV-infected patients comparing to healthy subjects (Thanan et al., 2008). Normally, genotoxic events caused by DNA damage can lead to DNA repair mechanism which is DNA mismatched repair or, if the damage is beyond repair, or cell death through apoptosis, then these mutated cells are permitted to survive and can transform into malignant cells. All of these events can be described in Figure 1.3.

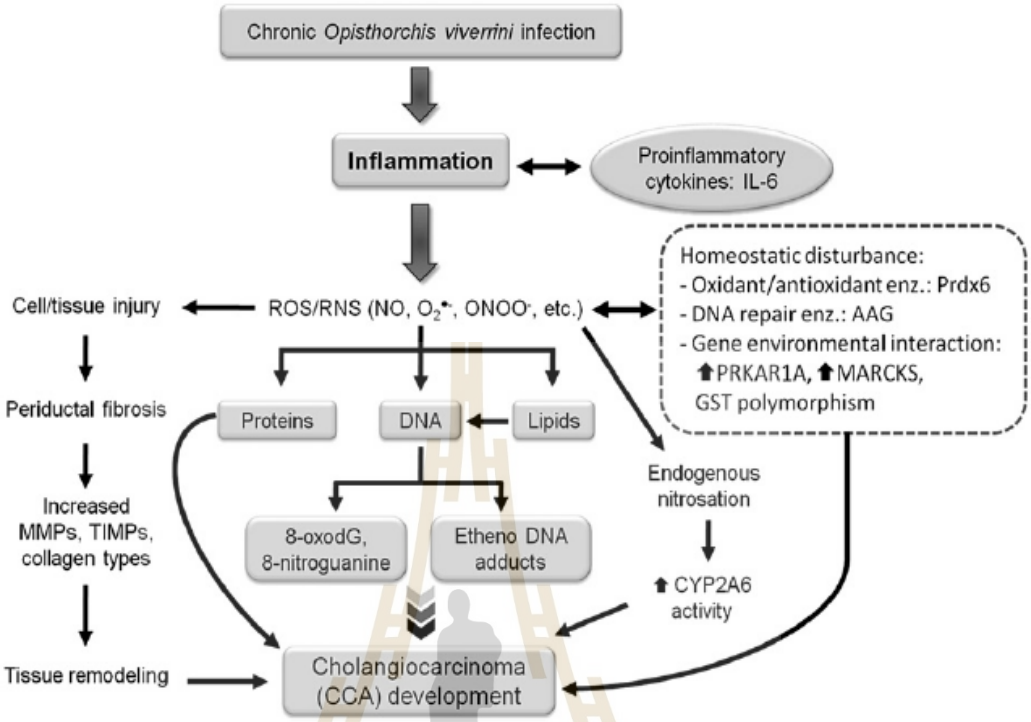


Figure 2.3 A possible mechanism of liver fluke-induced inflammation in association with cholangiocarcinogenesis (Yongvanit, Pinlaor, & Bartsch, 2012).

2.1.3 Diagnosis, treatment and chemotherapeutic drugs in CCA

Because of its silent clinical nature, the low specificity of most diagnostic methods, and the lack of precise diagnostic criteria, CCA is difficult to diagnose. The majority of patients with cholangiocarcinoma do not have symptoms until the cancer has progressed to an advanced stage (Boris et al., 2011). Magnetic resonance imaging (MRI) with magnetic resonance cholangiopancreatography (MRCP) is the best diagnostic tool for CCA diagnsosis (Park et al., 2013). There are no specific tumor markers for CCA now. But serum levels of matrix metalloproteinase-7 (MMP-7) (Leelawat et al., 2009) and tumor M2-PK (Li, Y.G., & Zhang, N., 2009 were showed to related with clinical features of CCA. A novel CCA-associated carbohydrate antigen may have potential as a

marker for the early diagnosis in Ov associated CCA in the hamster model (Kittisak et al., 2012). Tissue-based biomarkers may also give information on the diagnosis and prognosis of a disease. The biomarkers fascin, EGFR, MUC1, MUC4, and p27 have been reported to be associated with survival in patients with resected CCA, according to a recent meta-analysis of tissue markers for the prognosis of CCA, as evaluated by immunohistochemistry results (Ruys et al., 2014). Treatment choices for CCA are limited. Surgical excision is the only possibly curative method now available (Mitsugi Shimoda & Keiichi Kubota, 2007). Chemotherapy has been used in patients with unresectable, or metastatic CCA to try to overcome the disease and improve survival of the patients. The general chemotherapeutic drugs are used to treat the patients with unresected tumors, as a palliative treatment includes cisplatin, gemcitabine, folinic acid, oxaliplatin and *5-fluorouracil* (5-FU) (Jesus et al., 2020).

Fluorouracil (FU or 5-FU) is thought to be an antimetabolite drug which is the most commonly used for cancer treatment in many types of cancer, including colorectal cancer, breast cancer and aerodigestive tract cancers. The first-line chemotherapeutic treatment for CCA is 5-FU, even though efficacy has been limited by resistance (Kitti et al., 2018). The 5-FU structure is a uracil analogue having a fluorine atom at the C-5 position instead of a hydrogen atom. The mechanisms of 5-FU include misincorporation of fluoronucleotides into RNA and DNA and inhibition of the nucleotide synthetic enzyme thymidylate synthase (TS). Mechanically (as shown in Figure 1.4), 5-FU enters the cell by the same transport mechanism as uracil. (Wohlhueter et al., 1980). It can be changed intracellularly into three major active metabolites: fluorodeoxyuridine

monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridinetriphosphate (FUTP). The main mechanism of 5-FU activation is the conversion of orotate phosphoribosyltransferase (OPRT) with its cofactor, phosphoribosyl pyrophosphate (PRPP), to fluorouridine monophosphate (FUMP) or indirectly by fluorouridine (FUR) through the sequential action of uridine phosphorylase (UP) and uridine kinase (UK). FUMP is subsequently phosphorylated to fluorouridine diphosphate (FUDP), which can be further phosphorylated to fluorouridine triphosphate (FUTP) or transformed to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reducase (RR). In turn, FdUDP can subsequently be phosphorylated or dephosphorylated to create active metabolites FdUTP and FdUMP. Another activation pathway involves thymidine phosphorylase (TP), which can expedite the conversion of 5-FU to fluorodeoxyuridine (FUDR), which is subsequently phosphorylated to FdUMP by thymidine kinase (TK). The conversion of 5-FU to dihydrofluorouracil (DHFU) through dihydropyrimidine dehydrogenase (DPD) is the rate-limiting step of 5-FU catabolism in normal cells and cancer cells. DPD in the liver destroys up to 80% of the 5-FU given (Daniel et al., 2003).

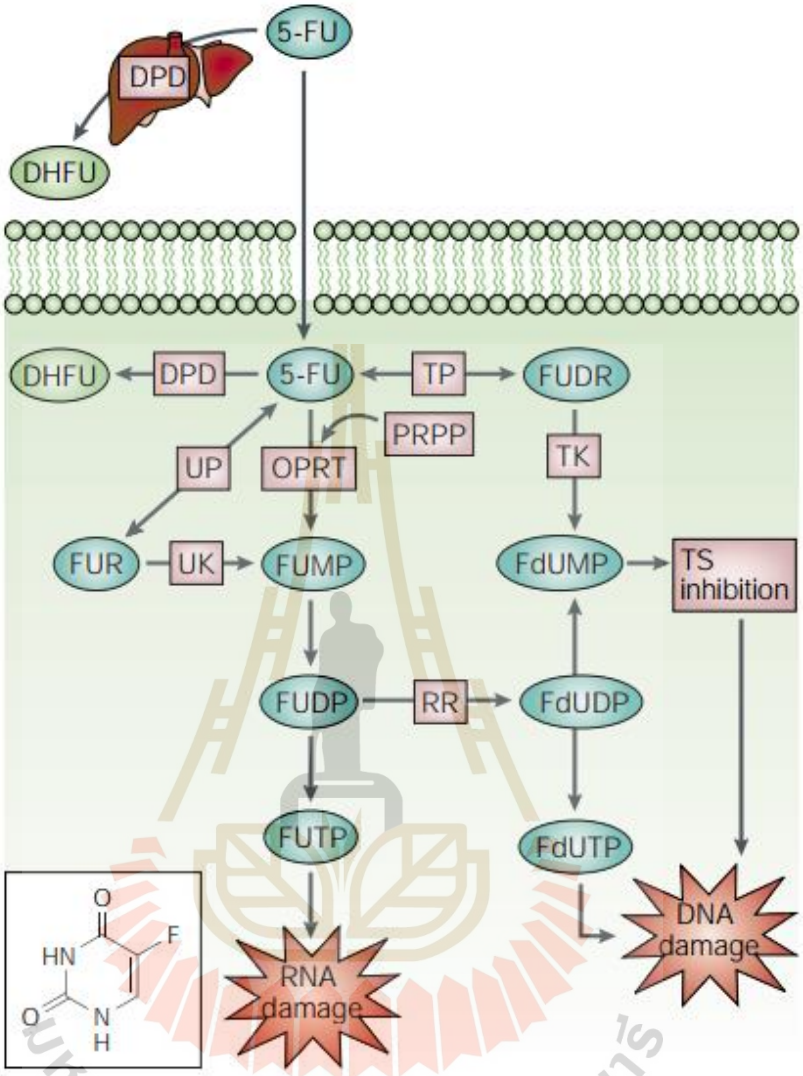


Figure 2.4 5-Fluorouracil (5-FU) metabolisms (Daniel et al., 2003)

Inhibition of TS is considered to be a key factor in 5-FU metabolism. The TS protein can normally function as a dimer and has two subunits that contain a nucleotide-binding site and a binding site for the reduced FOLATE 5,10-methylenetetrahydrofolate (CH2THF) which is the methyl donor. Active TS protein can catalyze the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). This

reaction is the synthesis of thymidylate, which are essential for DNA replication and repair. 5-FU can metabolite FdUMP binds to the nucleotide-binding site of TS, forming a stable TERNARY COMPLEX with the enzyme and CH2THF. Therefore, the normal substrate dUMP can be blocked by replacing FdUMP, leading to inhibiting dTMP synthesis (Sommer et al., 1974). Depletion of dTMP leads to depletion of deoxythymidine triphosphate (dTTP), which causes deoxynucleotide pool imbalances (especially the dATP/dTTP ratio) and leads to DNA damage by disrupting DNA replication and repair (Houghton et al., 1995). Furthermore, TS inhibition can increase the levels of dUMP, which might cause an increase in deoxyuridine triphosphate (dUTP) levels, causing misincorporation into DNA (Aherne et al., 1996). Uracil-DNA glycosylase (UDG) is a critical DNA repair enzyme. The repair of 5-FU-containing DNA is futile in the existence of a high amount of dUTP and leads to further incorrect nucleotide incorporation and, eventually, DNA strand breakage and cell death. In addition, dUTP pyrophosphatase (dUTPase) is an enzyme that limits dUTP accumulation. Therefore, DNA damage due to dUTP misincorporation depends on the level of dUTPase. Moreover, TS deficiency effects can also be relieved by thymidine via activation of the thymidine kinase (TK) as shown in Figure1.5. Therefore, this pathway is considered to be a mechanism of resistance to 5-FU. Based on the 5-FU mechanism, potential strategies for modifying the antitumor effects of 5-FU should include decreasing 5-FU degradation, increasing 5-FU activation, and increasing TS-FdUMP binding activity (Daniel et al., 2003).

Moreover, the tumor suppressor p53 can promote apoptosis and the removal of damaged cells by inducing FAS (CD95/APO1) and BAX expression and decreasing anti-apoptotic BCL2. (Petak et al., 2000). In vitro studies have reported that loss of p53 activity decreases cellular response to 5-FU (Bunz et al., 2008). As a result, studying the effectiveness of 5-FU is still required to completely overcome cancer treatment.

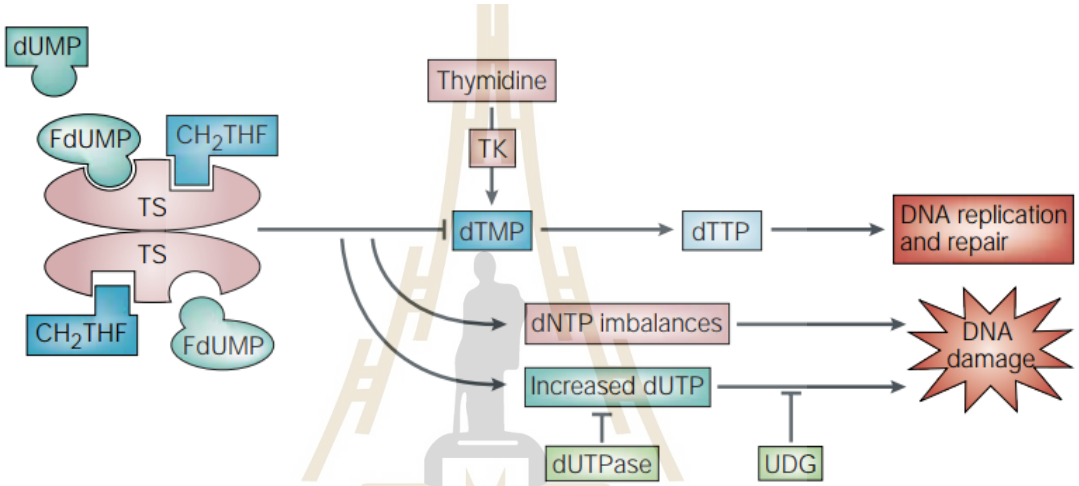


Figure 2.5 Mechanism of thymidylate synthase inhibition by 5-fluorouracil (5-FU) (Daniel et al., 2003).

2.2 Post-translational modification

Post-translational modification (PTMs) is the covalent and typically enzymatic modification of proteins that occurs after protein biosynthesis. Proteins are synthesized by mRNA into polypeptide chains, which may subsequently undergo PTMs to generate the matured protein product. PTMs can arise on the side chains of amino acids or on the C-or N-termini of proteins (Khoury et al., 2011). It regulates numerous biological processes, including protein localisation in the cell, protein stability, and enzymatic

activity control (Audagnotto et al., 2017). There are various types of PTMs as presented in Table 1.1 and Figure 1.6.

Table 2.1 Types of post-translational modification (PTMs)

Type of PTMS	Amino acid residues	Molecule (donor)	Molecular function
1. Glycosylation	Asparagine/Arginine/Serine/ Threonine/Tyrosine/Hydroxylysine/Hydroxyproline/Tryptophan	Carbohydrate	- Cell adhesion - Ligand–receptor interaction - Etc.
2. Methylation	Arginine/Lysine/Histidine/ Proline	Methyl groups	- Transcriptional regulation - RNA processing - Metabolism - Signal transduction - Etc.
3. Acetylation	Tyrosine/Serine/Threonine	Acetyl groups	- Protein stability - Enzymatic activity - subcellular localization - Etc.
4. Phosphorylation	Serine/Threonine/Tyrosine	Phosphate groups	- Signaling pathways - Metabolism - Etc.
5. SUMOylation	Lysine	Small Ubiquitin-like Modifier (or SUMO) proteins	- Protein stability - Nuclear-cytosolic transport - Transcriptional regulation - Etc.
6. Lipidation	Cysteine/Serine/Lysine	Lipids	- Protein localization - Signaling processes
7. Ubiquitination	Lysine/Cysteine/ Serine/ Threonine	Ubiquitin	- Apoptosis - Cell cycle and division - Etc.
8. Hydroxylation	Proline/Lysine/Asparagine/ Aspartate/Histidine	Hydroxyl group	- Enhance the stability of the triple helix - Influence on collagen fibril formation - Etc.
9. Disulfide bond	Cysteine	Sulfur atoms	- Protein folding - Protein function - Etc.

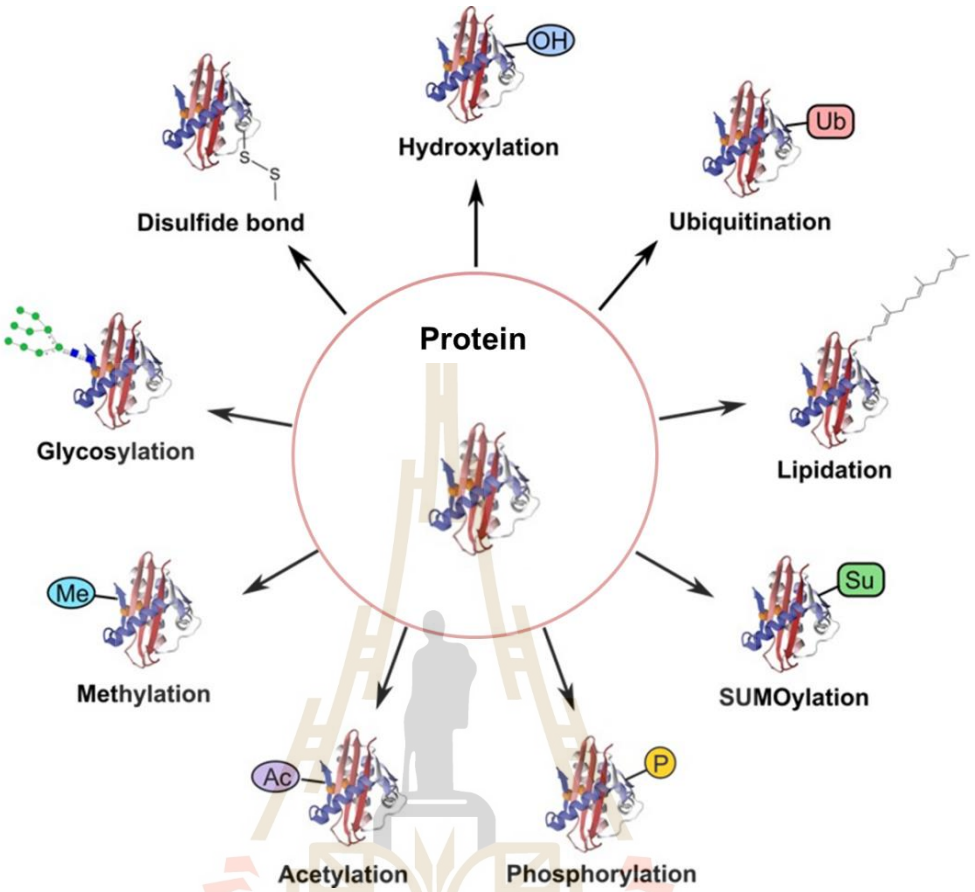


Figure 2.6 PTMs refers to the modification that occurs on a protein after translation catalyzed by enzyme (https://www.creative-proteomics.com)

2.2.1 Protein glycosylation

Glycosylation is the major post-translational modification of all protein that mainly occurs at endoplasmic reticulum (ER), and Golgi where all glycosyltransferases are located (Jung et al., 2011). Glycosyltransferases catalyze reactions that arise via a bi-bi substrate mechanism, in which a sugar-nucleotide donor and a carbohydrate acceptor collaborate to create a modified glycan and a nucleoside as the products. (Figure 1.7,

Gupta et al., 2016 & Katrine et al., 2020). In human, five classes of glycans are produced as shown in Figure 1.8 and Table 1.2 (Jung et al., 2011 & Katrine et al., 2020).

Table 2.2 Five classes of glycans in glycosylation

Classes of glycans	**Linkage**	**Amino acid residues**	**An example of a glycan-containing protein**
1. Phosphoglycans	Phosphate	Phosphoserine	Acid phosphatase (SAP)
2. C-linked glycans	Carbon	Tryptophan	Thrombospondins
3. Glypiation	Glycosylphosphatidylinositol (GPI) anchor	C-terminus (the end of an amino acid chain)	Alkaline phosphatase (ALP)
4. N-linked glycans	Nitrogen	Asparagine/ Arginine	Transferrin, Ceruloplasmin
5. O-linked glycans	Hydroxyl oxygen	Serine/Threonine	Mucins

Glycosylation is most commonly attached to the peptide chain that are frequently occurs in mammalian cells in regulating many biological functions (Reily et al., 2019). Additionally, both types of glycosylation have been demonstrated to participate in several step of cancer development and progression.

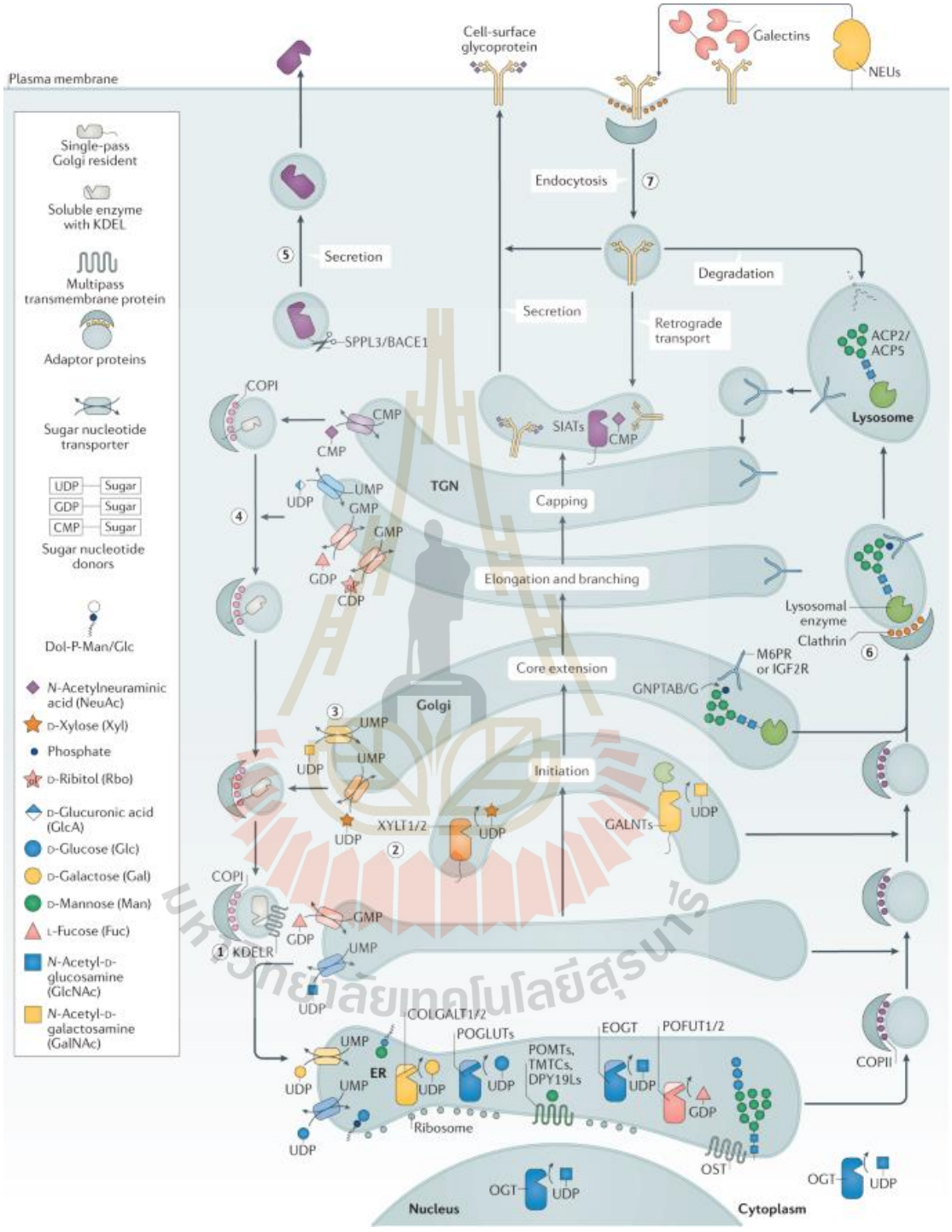


Figure 2.7 Subcellular organization of protein glycosylation (Katrine et al., 2020).

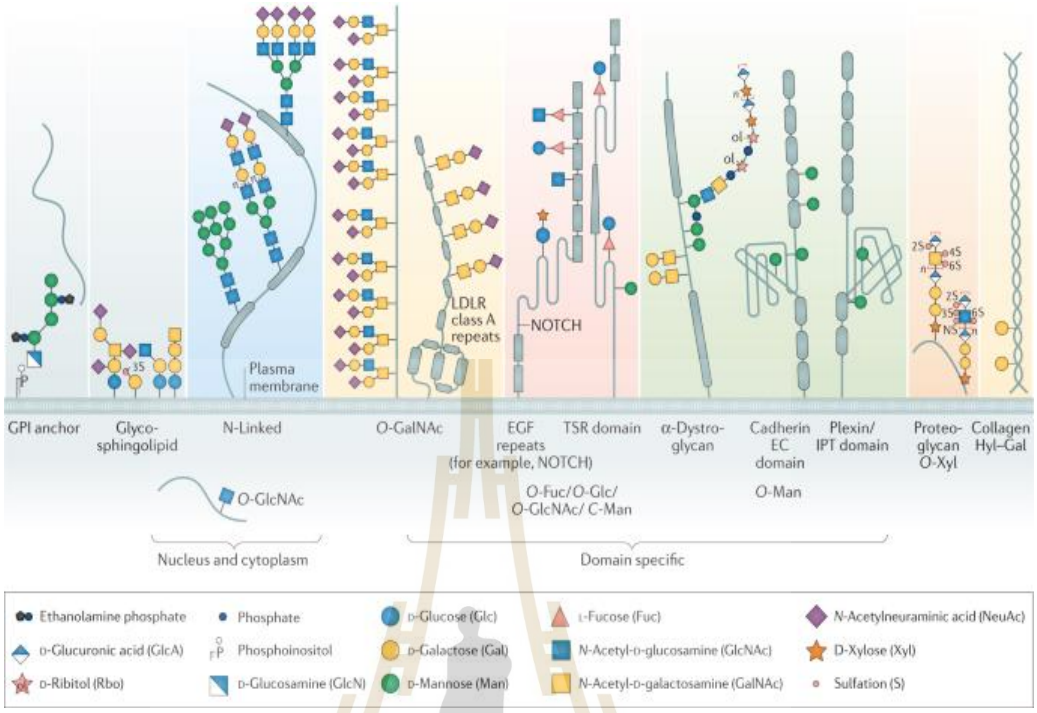


Figure 2.8 Key classes of glycoconjugates of the human cellular glycome (Katrine et al., 2020).

2.2.2 O-Glycosylation (mucin-type O-glycosylation)

O-glycosylation can occur on amino acid with functional hydroxy group, which are most often Ser and Thr. In human, the most O-glycosylation particularly GalNacylation, often called mucin-type-O-glycans, are found on many extracellular secreted glycoproteins especially mucins. Mucins are bulky secreted glycoproteins expressed by epithelial membranes for protecting the cellular surface from external stress (Guzman et al., 2010). Biosynthetic pathways of mucin-type O-glycosylation takes place in the Golgi apparatus. The first step is the transfer of GalNAc from UDP-GalNAc to Ser/Thr residues of a polypeptide by GALNTs (Polypeptide N-acetylgalactosaminyltransferase), which results in the formation of GalNAcα-Ser/Thr,

(Tn antigen). Following the addition of GalNac, either galactose (Gal) or N-acetylglucosamine (GlcNac) can be added by the action of C1GAlT1 (Core 1 Synthase, Beta-1-3 Galactosyltransferase) or B3GNT6 (Beta-1,3-N-Acetylglucosaminyltransferase) to promotes the formation of core 1 (Gal β1,3 GalNAcα-) or core 3 (GlcNAc β1,3 GalNAcα-) O-glycan structure respectively. C1GALT1 play a major role on the formation of core 1 O-glycan structure, often known Thomsen Friedenreich (TF or T) antigen, by the aid of its C1GALT1-specific chaperone (Cosmc) transfers UDP-galactose to Tn antigen to form core 1 structure. However, Tn antigen can receive a Neu5Ac unit (from CMP-Neu5Ac) to terminates the sugar chain and form to be sialyl Tn antigen when the expression of ST6GalNAc-I (ST6 N-Acetylgalactosaminide Alpha 2,6 Sialyltransferase 1) is increased, (sTn) (Chao et al., 2016).

However, core 1 and core 3 O-glycan structure can be further modified by other glycosyltransferase in a stepwise fashion to yield up 8 core complex glycosylation structures (Hounsell et al., 1996; Chia et al., 2016, Figure 1.9).

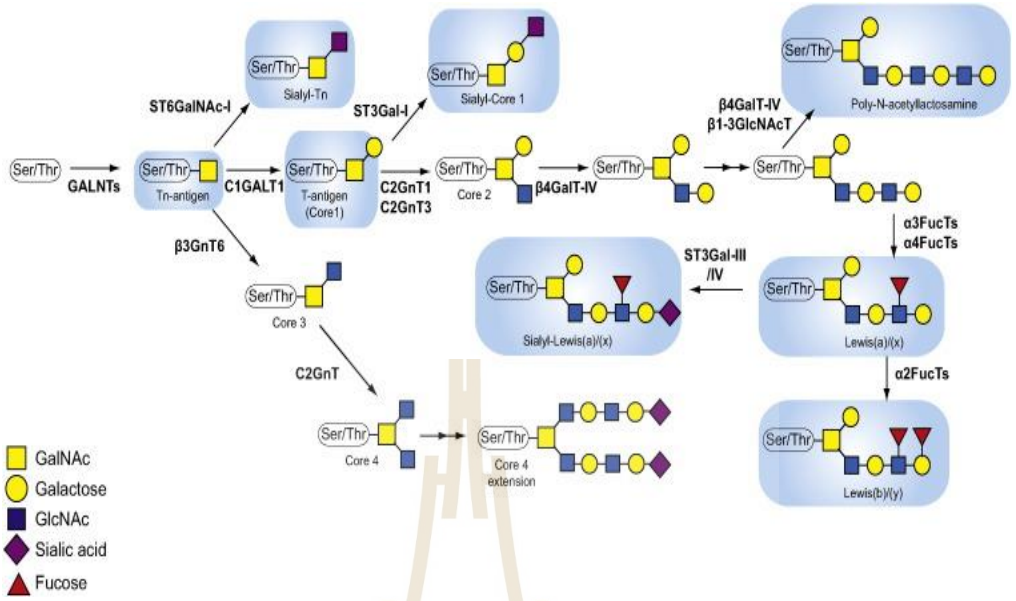


Figure 2.9 The O-GalNAc glycosylation biosynthetic pathway (Chia et al., 2016)

2.3 Truncation of mucin-type O-Glycosylation in cancers

Aberrant glycosylation has demonstrated in all key pathological steps of tumor development and progression (Jennifer et al., 2016). Overproduction of nucleotide-sugar donors and/ or altered expression of enzymes such as glycosyltransferase have been demonstrated in promoting the elongation or inducing truncation of O-glycan. However, the most frequently observed aberrant mucin type O-glycosylation in cancer is the immature truncated core 1 O-glycan which designed Tn and sialyl -Tn (sTn) antigens. High expressions of Tn and sTn antigens are observed virtually all epithelial cancer cells including colon cancer (Itzkowitz et al., 1989), breast cancer (Atsuo et al., 1999), ovarian cancer (Inoue et al., 1991) and cervical cancer (Kensuke et al., 1996), and many early epithelial premalignant lesions that precede the development of adenocarcinoma (Prakash

et al,.2014). Expression of truncated O-glycans is strongly associated with metastasis and poor prognosis in various cancer types such pancreatic ductal adenocarcinoma (Divya et al., 2019), and gastric cancer (Daniela et al., 2019). Furthermore, truncation of mucin-type O-glycans such as MUC-Tn was demonstrated in multiple cancer types and overexpression of MUC-Tn is correlated with short overall survival of lung cancer patients (Terufumi et al., 2021). Several factors have reported to contribute to the formation of Tn and sTn antigens on glycoprotein in cancer such as dysregulation of C1GALT1 expression, somatic mutations, or hypermethylation of COSMC (Figure 1.10). Mutation in COSMC have reported in cervical cancers (Woong et al., 2018). However, the major reason for the mucin O-glycan truncation in colorectal cancer and pancreatic ductal adenocarcinoma (PDAC) are the hypermethylation of COSMC (Xiwei et al., 2018 & Prakash et al., 2014). In pancreatic ductal adenocarcinoma (PDAC), hypermethylation of COSMC is the causes for the expression of truncated mucin type O-glycan leading to enhance the tumor aggressiveness through the induction of epithelial to mesenchymal transition (EMT). Moreover, truncation of mucin-type O-glycans show to impact gastric cancer cell-matrix adhesion and mobility through activation of receptor tyrosine kinases including EGFR and HER2 (Figure 1.11) (Daniela et al., 2019).

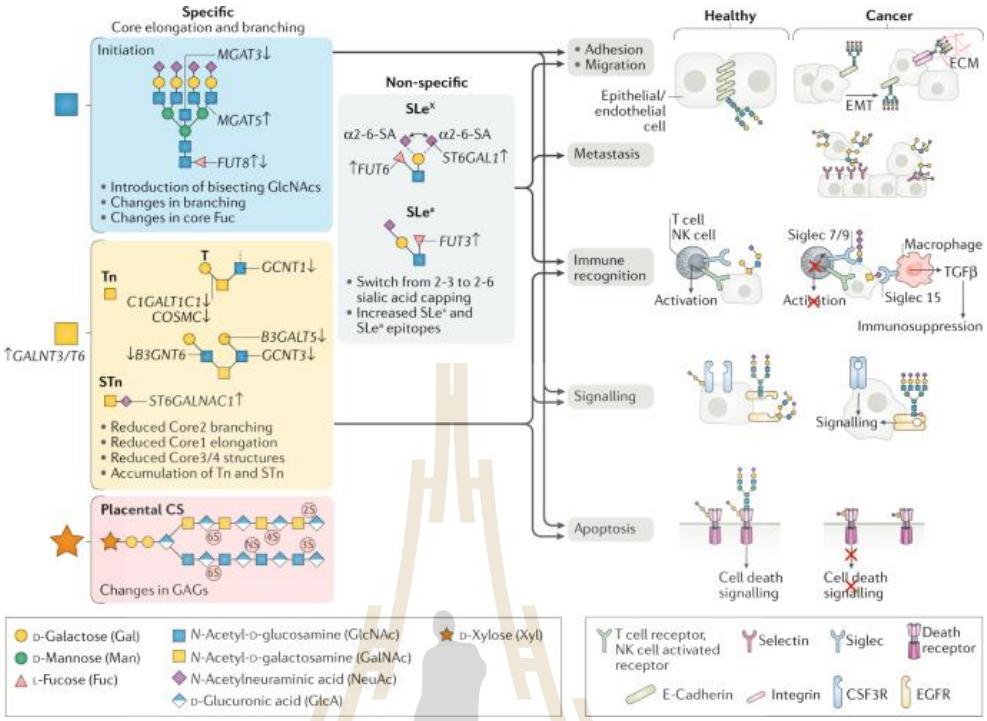


Figure 2.10 Common dysregulated glycosyltransferase genes in cancer (Katrine et al., 2020).

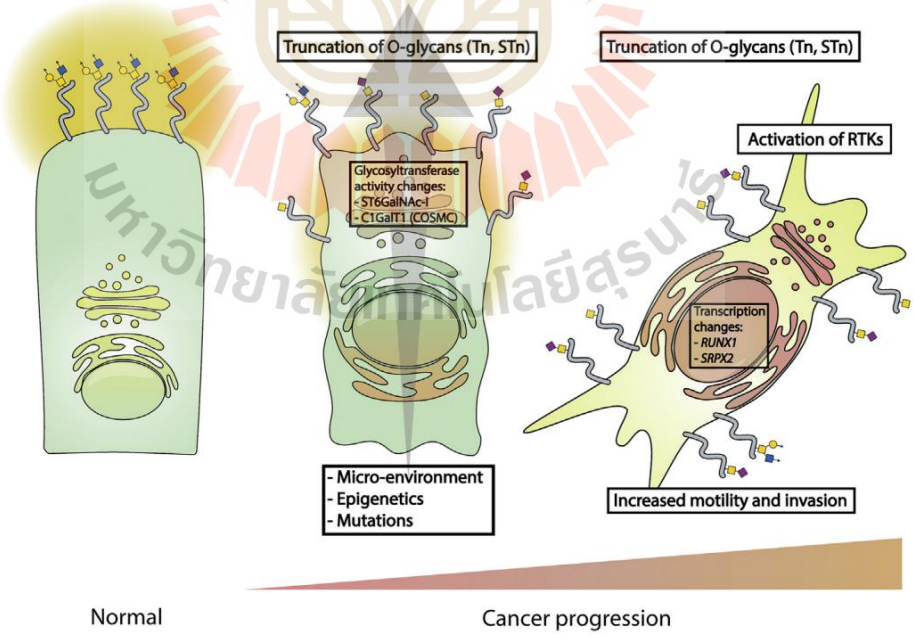


Figure 2.11 O-glycans truncation in gastric cancer cell modulates invasive features via the activation of receptor tyrosine kinase (Daniela et al., 2019).

2.4 Implication of mucin-type O-glycosylation in cholangiocarcinoma

The number of studies using lectin and direct structure analyses of glycan features have demonstrated diverse changes of glycosylation in CCA. The aberrant glycosylation in CCA e.g., mucin MUC5AC, MUC1, CCA-CA, carbohydrate antigen 19-9 (CA19-9), and CA-S27 have been applied for CCA diagnosis and its altered expression have shown to be correlated with the progression of CCA (Artit et al., 2021). It has been demonstrated that mucin MUC1 and MUC5AC strongly express in CCA and relates to aggressive phenotypes (Chanchai et al., 2005 & Anwar et al., 2010). However, there is no direct evidence and underlying mechanism on aberrant expression of mucin-type O-glycan on these two mucins. Using N-acetylgalactosamine (GalNAc)- specific lectin (Table 1.3), High expression of GalNAcylated glycans by Sophora japonica agglutinin (SJA) was significantly observed in precancerous bile duct epithelia and CCA (Waraporn et al., 2017). Similarly, Wisteria floribunda agglutinin (WFA) staining was detected with high frequency in CCA but much less frequency for normal bile duct and cancerous lesion. Additionally, WFA staining in CCA was shown to be closely related with that of MY.1E12 established previously against sialylated mucin 1 (MUC1) by double-staining experiments (Atsushi et al., 2010). Although SJA and WFA staining revealed the increased GalNAcylated glycans in CCA but the specificity of these two lectins was not exact to demonstrate the single O-GalNAc modification on the glycan structure (Tn antigen). The terminal GalNAc residue on Tn antigen has been identified by VVL, a lectin derived from Vicia villosa seed. Marutpong et al., 2020 demonstrated that the staining of VVL binding glycans is increasing during CCA carcinogenesis. Moreover,

mucin-type O glycans with having di- to hexa-saccharides with terminal galactose and sialic acids by direct structure analysis were also observed in CCA (Krajang et al., 2016 and 2021). Gather all information suggest that truncations of mucin-type O-glycans are observed in CCA and its expression is increasing during carcinogenesis.

Table 2.3 CCA-associated glycans and specific lectins in CCA (Artit et al., 2021).

lectins	**Glucan structure**	**CCA Application**
SBA WFA SJA VVL	O-GalNAc	Diagnostic marker
sWGA	O-GlcNAc	Prognostic marker
EUA-I	Fucose	Prognostic marker
MAL-II	Sialic acid	Prognostic marker

2.5 Significance of C1GALT1 in Cancer

C1galt1 gene encodes β1-3 galactosyltransferase 1 (T synthase) which is important enzyme in mucin-type O-glycosylation. C1GALT cooperates with COSMC to catalyze the second step of mucin-type O-glycosylation by the addition of galactose to N-acetylgalactosamine (Tn antigen) that form a core 1 carbohydrate structure (T antigen). Then, T antigen is further modified by other glycosyltransferases to generate a variety of complex O-glycans (Engang et al., 2009). Aberrant O-glycans such Tn and and sTn antigen have been found predominately in MUC protein and reported in multiple cancer types including gastric cancer (Tianwen et al., 2018), colon cancer (Konno et al., 2002 & Byrd et al., 2004), breast cancer (Welinder et al., 2013) and lung cancer (Rafael et al., 2012 & Takafumi et al., 2020). The truncation of O-glycan has been associated with the genetic and epigenetic alterations of both C1GALT1 and their specific chaperone,

COSMC (Wang et al., 2010; Radhakrishnan et al., 2014; Ju et al., 2014; Cheng et al., 2016). Loss of C1GAlT1 in Kras and p53 mutant mice demonstrates development of aggressive pancreatic ductal adenocarcinomas (PDACs) and increase metastasis. Additionally, knockout of C1GALT1 increases the truncation of O-glycosylation on MUC16, which leads to increase tumorigenicity and aggressiveness in PDAC (Chugh et al., 2018). Similar observation was reported in gastric cells, in which loss of C1GALT1 activity is cause of gastritis and gastric cancer by caspase-1/caspase-11 (Casp1/11)–dependent inflammasomes (Liu et al., 2019). Moreover, Sagar et al., 2021 have demonstrated that disruption of COSMC contributes increase truncated O-glycan on MUC4 that enhances gemcitabine resistance in PDAC tumors via altering ErbB/AKT signaling. Whereas C1GALT1 is also overexpressed in various cancer types such as ovarian cancer (Chou et al., 2017), head and neck cancer (Lin et al., 2018), hepatocellular carcinoma (Wu et al., 2013), breast cancer (Liu et al., 2020) colorectal cancer (Gao et al., 2020) and gastric cancer (Lee et al., 2020) and its overexpression was associated with tumor growth, metastasis and poor prognosis. It has been demonstrated that silencing of C1GALT1 expression inhibits cell growth, migration, and cancer stemness properties as well as increase apoptosis and chemotherapeutic response to various agents through blocking O-glycan elongation on several growth receptors such as EGFR (Lin et al., 2018), MET receptor (Wu et al., 2013), FGFR2 (Hung et al., 2014), β1-integrin (Chiung-Hui Liu et al., 2014 & Zhang et al., 2018) and EPHA2 (Lee et al., 2020). Moreover, C1GALT1 modulates O-glycan structures on mucin (MUC) 1 and promotes MUC1-C/β-catenin signaling in breast cancer cells (Chou et al., 2015). Taken together, dysregulation

of C1GALT1 is involved in cancer development and progression through either promoting O-glycan truncation or elongation. These evidences motivated us to explore the significance of C1GALT1 in CCA

CHAPTER 3

MATERAILS AND METHODS

3.1 Materials

3.1.1 Specimens

3.1.1.1 CCA tissues and clinical data

The Liver Fluke and Cholangiocarcinoma Research Center of Khon Kaen University provided paraffin-embedded tissues from 26 CCA patients, as well as 29 frozen CCA tissues and matched adjacent tissues. Each subject agreed to sign an informed consent form. The procedure for sample collection was authorized by the Khon Kaen University Ethics Committee for Human Research (HE521209) and Suranaree University of Technology (EC-57-25).

3.1.1.2 Cholangiocarcinoma cell lines and cell culture condition

KKU-100, KKU-213A, KKU-213B, and KKU-055, were established by Professor Banchob Sripa. All CCA cell lines along with MMNK-1 (normal human immortalized cholangiocyte) were acquired from the Japanese Collection of Research Bioresources Cell Bank in Osaka, Japan.

Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin was used to culture all cell

lines. The cells were incubated at 37°C with 5% CO2. 80% of confluent cells were trypsinized with 0.25 % trypsin-EDTA (Gibco) for subsequent use.

Chemicals and reagents

Chemicals, antibodies and lectins are obtained from various companies as listed below.

Table 3.1 Chemicals, antibodies and lectin used

Chemical	Company
RNA isolation and Gene expression	
Isopropanol	Carlo Erba
Agarose powder	Vivantis
SYBR™ Safe DNA Gel Stain	Invitrogen
SensiFAST cDNA Synthesis Kit	Bioline
LightCycler® 480 SYBR Green	Roche
Gene knockdown	
Control siRNA-A: sc-37007	Santa Cruz
C1GALT1 siRNA (h): sc-72690	Santa Cruz
DharmaFECT® Transfection Reagents	Cytiva
Opti-MEM® (Reduced Serum Medium)	hermo Fisher Scientific
Coomassie brilliant blue	PanReac AppliChem
Glycine	PanReac AppliChem
Tetramethylethylenediamine (TEMED)	PanReac AppliChem
ECL kit	Cytiva
Methanol	VWR Chemicals BDH
Protein ladder	Invitrogen
Antibodies	
Mouse monoclonal C1GALT1 antibody	Santa Cruz
Mouse monoclonal β-Actin antibody	Santa Cruz
Phospho-Akt Antibody	Cell Signaling Technology
Total Akt Antibody	Cell Signaling Technology
Phospho-Erk Antibody	Cell Signaling Technology
Total Erk Antibody	Cell Signaling Technology
Bax Antibody	Proteintech
BCL2 Antibody	Proteintech
HRP labelled polymer anti rabbit IgG	Dako
HRP labelled polymer anti mouse IgG	Dako
Lectins	
Vicia villosa lectin (VVL, VVA)	Vector Laboratories
Wheat germ agglutinin (WGA)	Vector Laboratories
Concanavalin A lectin (ConA)	Vector Laboratories

Table 3.1 Chemicals, antibodies and lectin used (Continued)

Chemical	Company
Immunohistochemistry (IHC)/Cytochemistry	
Absolute Ethanol	Carlo Erba
Sodium Citrate dihydrate **(**mw**:** 294**.**1 g/mol**)**	Carlo Erba
Citric Acid **(**mw**:** 192**.**1 g/mol**)**	Carlo Erba
hydrogen peroxide **(**H_2O_2**)**	Carlo Erba
Immunohistochemistry (IHC)/Cytochemistry	
Sodium chloride **(**$Nacl_2$**)**	Merck
Disodium Hydrogenphosphate **(**Na2HPO4**)**	Merck
Potassium dihydrogen phosphate **(**KH_2PO_4**)**	Merck
Triton X-100	Merck
Polyoxyethylene-20 (Tween-20)	Merck
Normal Horse Serum **(**NHS**)**	Vector Laboratories
DAB-Peroxidase Substrate Solution	Cell Signaling Technology
Haematoxylin	Biomall
Bovine Serum Albumin Standard	Thermo Fisher Scientific
RNA isolation and Gene expression	
TRIzol™ Reagent	Thermo Fisher Scientific
Chloroform	Carlo Erba

3.1.1.3 Oligonucleotide primers

Table 3.2 Primer sequences used

Primers	Forward primer sequence(5'- 3')	Reverse primer sequence(5'-3')	Product size (bp)
C1GALT1	5'-GGG AAT CTG GGC GGC A-3'	5'-GGG ACT GGT GAC CTT TGC TT-3'	89
COSMC	5'-AAC GTG AGA GGA AAC CCG TG-3'	5'-AAA GCA TTT TTC CCG CGT CT-3'	73
B3GNT6	5'-TCA ACC TCA CGC TCA AGC AC-3'	5'-CAG GAA GCG GAC TAC GTT GG-3'	125
ST6GALNAC1	5'-CAG AGG CAC AAT CAT GGA AG-3'	5'-GCT GAC TTT TGG GAA TGA GC-3'	150
C-Myc	5'-CTG CTG TGG ACC CTA CTG-3'	5'-AAC TGC GTC TCT GCC AGG AC-3'	122
CCND1	5'-CCA CTT GAG CTT GTT CAC CA-3'	5'-AAC TAC CTG GAC CGC TTC CT-3'	204
BIRC5	5'-TGA GGA GAC ACC GCC CAC-3'	5'-CAA CAT CGA TTT CTT CCT CAT CTT C-3'	71

3.2 Methodology

3.2.1 Determination of C1GALT1 mRNA and protein expression

3.2.1.1 Gene expression analysis from database

CCA gene expression data was obtained from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov) using GEO Series GSE76297. GEO Series GSE76297 comprised expression data from 91 CCA tumors and 92 paired non-tumors. Tumors and paired non-tumor tissues were analyzed individually utilizing the Affymetrix Human Transcriptome Array 2.0 for gene expression profiling. All expression data were log2 transformed.

3.2.1.2 Total RNA extraction

TRIzol reagent (Invitrogen) was used to extract total RNA from tumor tissues and adjacent nontumorous tissues from the same patient. In short, frozen tissues were extracted with 1 ml of TRIzol reagent and 200 µl of chloroform, and centrifuged at 12,000 xg for 15 min. After centrifuge, the upper phase (500ul) was precipitated with isopropanol 500 µl of for 10 min. After centrifuging the RNA pellet at 12,000 xg for 15 min, it was washed with 75% ethanol and left to dry. Finally, the acquired RNA was resuspended in 30 µl of RNAse free water. The NanoDrop (NanoDrop Technologies, Delaware, USA) was used to check the purity and concentration of the extracted RNA.

3.2.1.3 The first strand complementary DNA (cDNA) synthesis

Total RNA was used to synthesis 1st strand cDNA using the SensiFAST cDNA Synthesis Kit (Bioline). The reaction was carried out in a 20 µl containing 1 µg of

template RNA, 5x TransAmp buffer, Reverse transcriptase, and DNase/RNase free-water. The 10 ng/ul of cDNA was prepared for gene expression analysis.

3.2.1.4 Quantitative polymerase chain reaction (qPCR) analysis

C1GAT1 and other glycosyltransferases were determined by qPCR with SYBR-Green in the Light Cycler 480 II equipment (Roche). The PCR condition is concise in (Table2.3) The expression of C1GAT1 and other glycosyltransferases was normalized using β-Actin as an internal control. Melting curve analysis was performed to demonstrate the specificity of PCR reaction. (Figure2.1) The mRNA expression was analyzed by $2^{\wedge-\Delta\Delta Ct}$, 1.5 used as a fold change cut-off.

Table 3.3 The qPCR thermal cycling profiles

Reagent and condition	Volume
Reaction volume	20 µl
2X Light cycler SYBR Green mix	10 µl
Forward and Reverse primer (10 µM)	1 µl
cDNA(10 ng/µl)	5 µl
RNase free water	4 µl
PCR cycle	1 cycle at 95°C 5 min 40 cycle at 95°C 10 sec 55*, 58**, 60***°C 10 sec 72°C 10 sec

* Annealing temperature at 55 ºC for amplification of *ST6GALNACI, COSMC*

** Annealing temperature at 58 ºC for amplification of *C1GALT1, B3GNT6, C-Myc* and *BIRC5*

***Annealing temperature at 60 ºC for amplification of *CCND1*

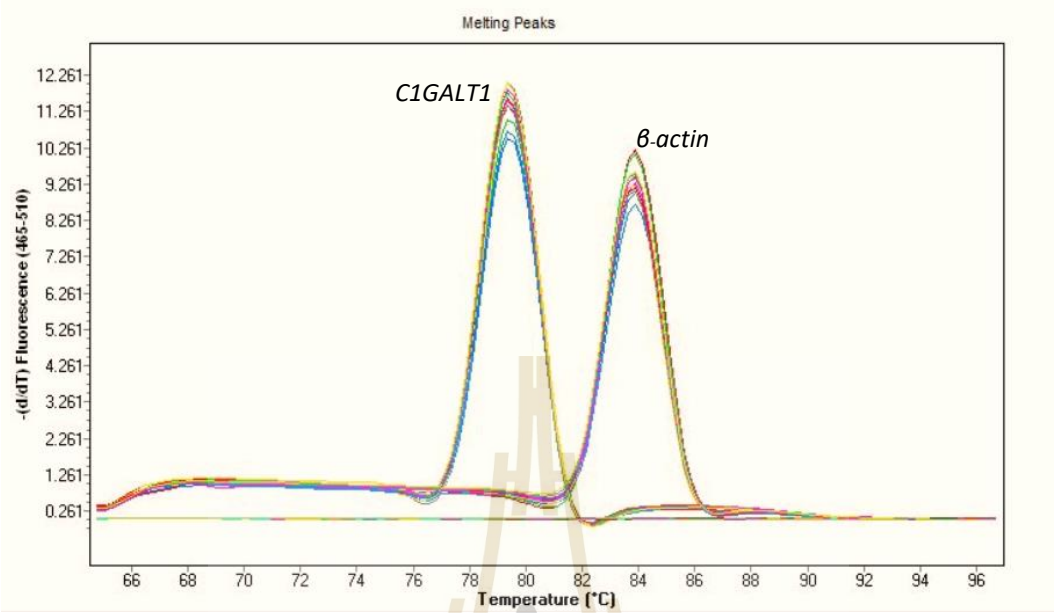


Figure 3.1 The melting temperature of the C1GALT1 amplicon is 79°C, while that of the (β-actin) internal control is 84°C.

3.2.1.5 Immunohistochemical analysis

Paraffin-embedded tissues were cut into 5 μm sections. For immunohistochemistry of C1GALT1, In brief, each section was deparafinized and rehydrated by xylene and ethanol (respectively) with decreasing concentration. Deparaffinized sections were boiled for 5 min. (in 0.01 mol/L citrate buffer at pH 6.0) in pressure cooker for epitope retrieval. After inactivating the endogenous peroxidase (3% hydrogen peroxide in methanol for 30 min. Normal horse serum was used for blocking nonspecific binding (20%), for another 30 min. Sections were incubated with primary mouse anti-human C1GALT1 monoclonal antibody (dilution 1:500, Santa Cruz) at room temperature for overnight. After that, sections were incubated with biotinylated secondary antibody in PBS for 1 hr at room temperature, and incubated with ABC-

Peroxidase Solution (Vector Laboratories) for 1 hr at room temperature, respectively. The visualization with Liquid DAB+ (Dako), and counterstained with hematoxylin were then performed. In the statistical analysis, the scores 0 was categorized as C1GALT1-negative cells; and the scores 1 was categorized as C1GALT1-positive cells, respectively. Immunohistochemical results were further analyzed the correlation with clinical data.

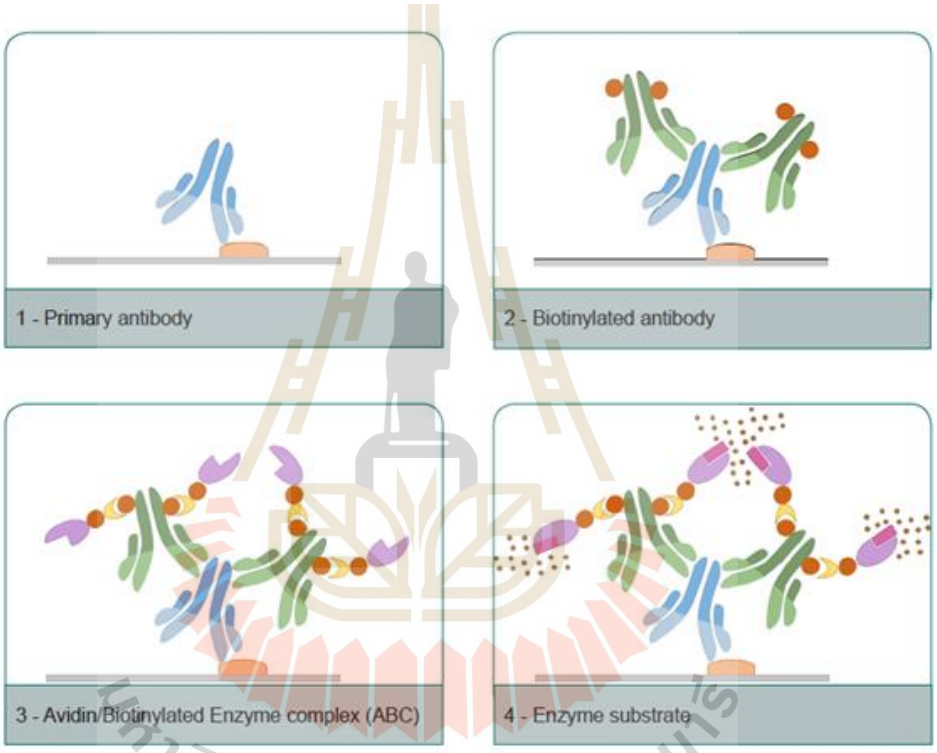


Figure 3.2 Principle of Avidin–Biotin Complex (ABC) staining method for IHC Detection (https://www.clinisciences.com).

3.2.2 Identifying the biological role of C1GALT1 in CCA cell lines

3.2.2.1 Cell culture and transfections

To knockdown the gene expression of C1GALT1 using small interfering RNA (siRNA), KKU-055 and KKU-100 cells were seed at the density of 2.5 x 10^5 cells/well into 6-well plate. After 24 hr seeding, the cells were transfected with si-

Control and si-C1GALT1 (Santa Cruz) using DharmaFECT® transfection reagents (Cytiva) according to the manufacturer's protocol. After transfection for 48 hr, transfected CCA cell lines were used in C1GALT1 expression and subsequent experiments.

3.2.2.2 Assessment of cell proliferation

Transfected cells and control cells were seeded at density 1 x 10^3 cells/well in to 96-well plate and then incubated at 37^{o}C for 0, 2, 3, 4 and 5 days. The effects of C1GALT1 knockdown on CCA viab measured using cell counting kit 8 (CCK-8) (Abcam). Cellular dehydrogenases convert WST-8 tetrazolium salts to an orange formazan product which is soluble in culture media. The amount of formazan generated is directly related to the number of live cells. In brief, 10 µl/well WST-8 reagent was applied to each well, which was then incubated for 1 h. The color development in this experiment was evaluated using a microplate spectrophotometer (Bio-Rad) at 460 nm.

3.2.2.3 Assessment of 5-fluorouracil (5-FU) chemotherapeutic sensitivity

Transfected cells and control cells were seeded (5 x 10^3cells, 100 ul/well) into 96-well plate. The cells were treated with 5-FU at 150 and 300 µM for 48 h. Cell viability was detected using CCK-8 kit

3.2.2.4 Protein collection and BCA assay

For protein collection, the cells were collected by washing twice with 1X PBS and added 80 µL lysis buffer with protease inhibitor followed by 30 min incubation on ice. Subsequently, the cells were scraped from the plate with cell scrapers, transferred the mixture into the tube and centrifuged at 16,000xg for 20 min. The

supernatant was transferred into new tube and stored at -80ºC until used. Pierce™ BCA Protein Assay Kit (Thermo Scientific) was used to measure the protein concentration in 96-well plate. Briefly, bovine serum albumin (BSA) standard curve was performed at 0, 5, 25, 50, 125, and 250 µg/mL by diluted with 1X PBS. The samples were diluted as 1:20 with 1X PBS. Then, 25 µL of the diluted samples and each concentrations of standard will be pipetted into a 96-well plate in duplicate. BCA working reagent was prepared with 196 µL/reaction of BCA solution (Reagent A) mixed with 4 µL/reaction of 4% Cupric sulfate and then added to each well followed by gently mixed and incubated at 60ºC for 15 min. Subsequently, microplate reader was required for measuring protein concentration at A_{562} nm.

3.2.2.5 SDS-PAGE and Western blot analysis

Protein samples were separated by SDS-polyacrylamide gel electrophoresis on a 10% separating gel before being transferred to nitrocellulose membranes using wet/tank electroblotting techniques and followed by incubation with primary antibodies including anti-C1GAL1 (1:1000, Santa Cruz Biotechnology), pAKT (1:1000, Cell Signaling Technology), totalAKT (1:1000, Cell Signaling Technology), pERK (1:1000, Cell Signaling Technology), totalERK (1:1000, Cell Signaling Technology), BAX (1:1000, Cell Signaling Technology) and BCL2 (1:1000, Cell Signaling Technology) overnight at 4°C. Secondary antibodies goat anti-rabbit IgG (GenScript) or rabbit anti-mouse IgG (Dako) linked with horseradish peroxidase were used to visualize the signal with the Luminata Forte Western HRP substrate (Merck). The

signal intensities were measured using ImageJ software (NIH) and the internal loading control was used to standardize the intensities.

3.2.2.6 Immunocytochemistry

Transfected cells and control cells were seeded were seed (6 x 10^4 cells/well) into the 24-well plate. Cells were then fixed by 4% paraformaldehyde in PBS (pH 7.4 for 15 minutes at room temperature and then permeabilized by 0.2% Triton X-100 in PBST for 10 min. Non-specific binding was blocked with 0.3% of FBS (Fetal Bovine Serum) for 30 min. After blocking, cells were incubated with lectins PNA (1:100), WGA (1:1000) and VVL (1:1000) for overnight at 4°C. ABC-Peroxidase Solution (Vector Laboratories) was used to develop the signal for 1 hr at room temperature. The visualization with 3,3 -diaminobenzidine-tetrahydrochloride, Liquid DAB+ (Dako), and counterstained with hematoxylin were then performed.

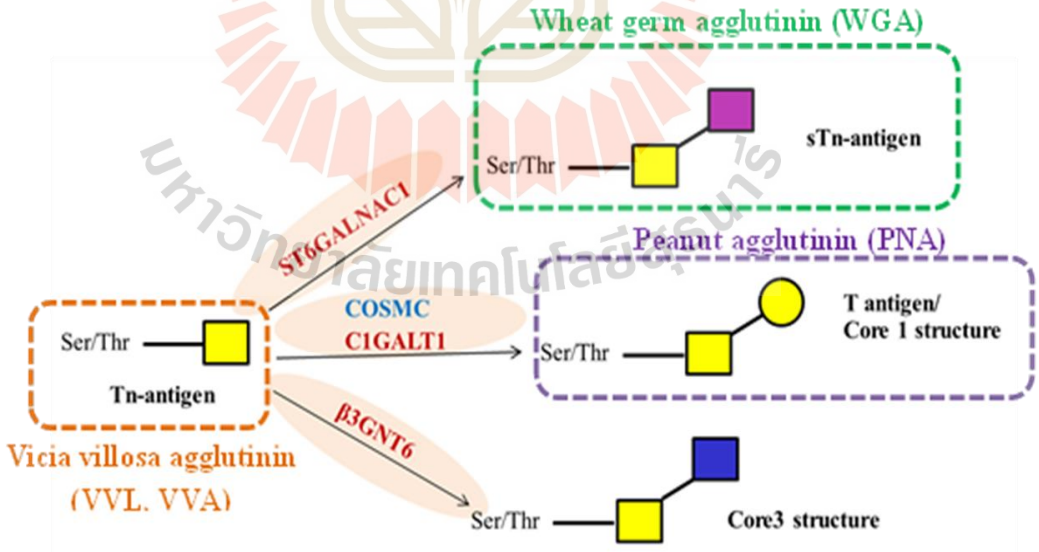


Figure 3.3 O-glycans-binding lectins are used in lectin-histrochemistry.

Table 3.4 List of lectins used in lectin-histrochemistry

Lectin	Origin	Abbreviation	Binding specificity	Possible glycan structure
Peanut agglutinin	*Arachis hypogea*	PNA	Gal-β-(1,3)-GalNAc-α-Thr/Ser	T antigen
wheat germ agglutinin	*Triticum vulgaris*	WGA	GlcNAc; Neu5Ac	Sialyl Tn antigen (sTn)
Vicia villosa agglutinin	*Vicia villosa*	VVL, VVA	Terminal GalNAc especially GalNAc-α Thr/Ser	Tn antigen

3.2.2.7 Statistical analysis

SPSS statistics software version 16.0.1 was used for all statistical analysis (SPSS, Illinois, USA). The correlations between C1GALT1 protein expression and clinicopathological characteristics of CCA patients were analyzed using Pearson's chi-squared test. The total survival curve was estimated using the Kaplan-Meier survival analysis, and the log-rank test was performed to compare groups. The results of functional investigations of C1GALT1, which included cell proliferation and 5-fluorouracil (5-FU) chemotherapeutic sensitivity testing, were obtained in three separate experiments and were expressed as the mean ± SD. The Student's t-test was used to determine the difference between the two groups. If the P value was less than 0.05, the statistical significance was accepted.

CHAPTER 4

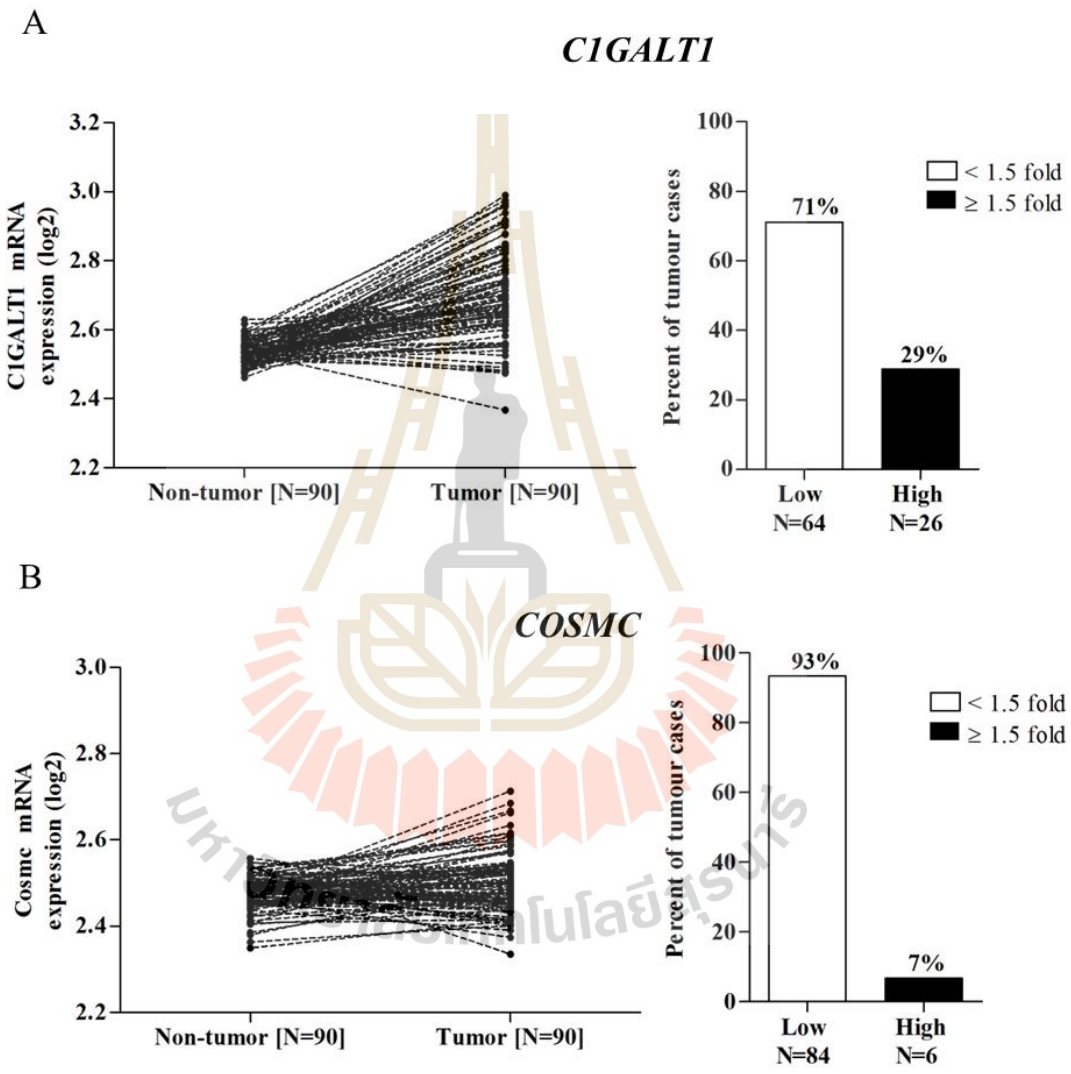
RESULTS

4.1 C1GALT1 mRNA expression in cholangiocarcinoma

The initiation of mucin type O-glycosylation occurs via the formation of Tn antigen. Then, either galactose (Gal) or N-acetylglucosamine (GlcNAc) is added onto Tn antigen via the action of C1GALT1 (Core 1 Synthase) or B3GNT6 (Core-3 synthase) to promote the formation of core 1 (T antigen) or core 3 O-glycans. Whereas high expression of ST6GalNAc is high, N-acetylneuraminic acid (sialic acid) acts on Tn antigen, resulting in the truncation of O-glycan by forming Sialyl-Tn antigen (Gupta R et., 2020). To address whether aberrant mucin-type O glycosylation in CCA is triggered by altered expression of enzymes such as glycosyltransferase and sialyltransferases. Firstly, the differential expression of *C1GALT1*, *COSMC*, *B3GNT6* and *ST6GALNAC1* were investigated through GEO Series GSE76297. Using cut off at 1.5-fold change, expression levels of all four genes were downregulated in CCA tissues when compared with non-tumor tissues (Figure 3.1). Percent of tumor cases showing high expression was *C1GALT1*: 29 % (26/90), *COSMC*: 7 % (6/90) *B3GNT6*: 7 % (15/90) and *ST6GALNAC1*: 24 % (22/90).

As the synthesis of Core-1 glycans is a precursors for the complex formation of mucin-type O-glycans. We further verified the differential expression of *C1GALT1* in 30 paired frozen CCA tissues using qPCR. Using cut off value at 1.5-fold change, there were

23 % (7/30) of tumor cases showing a high expression whereas 77% (23/30) showing a low expression compared with the adjacent normal tissue (Figure 3.2). This finding suggests us that *C1GALT1* was downregulated in CCA.



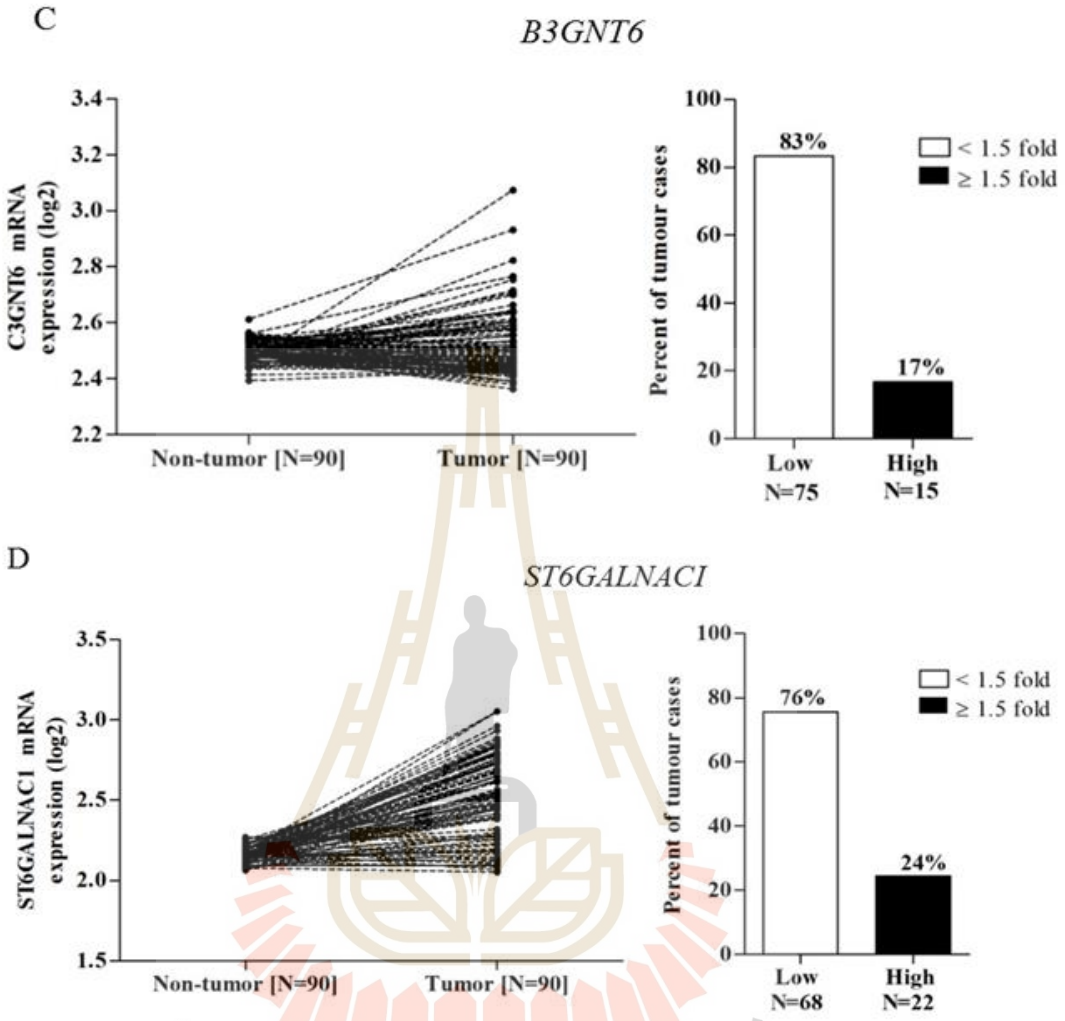


Figure 4.1 Expression levels of *C1GALT1, COSMC, B3GNT6 and, ST6GALNAC1* in 90 paired CCA tissues from GEO Series GSE76297.

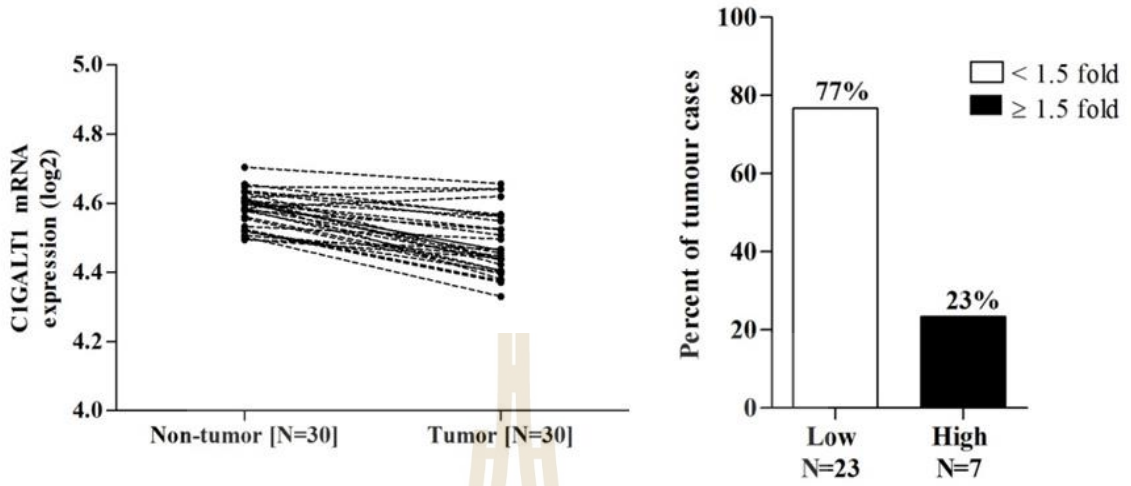


Figure 4.2 Downregulation of C1GALT1 mRNA expression in CCA. The C1GALT1 expression was determined by qPCR. Relative mRNA expression level was determined using $2^{-\Delta\Delta CT}$ normalized to non-tumor. Bar graph represents mean ± SEM of three independent experiments.

4.2 C1GALT1 protein expression in cholangiocarcinoma tissues

To evaluate the correlation of C1GALT1 expression and clinicopathological features of the CCA patients. We further determined the protein expression of C1GALT1 in 26 CCA tissues using immunohistochemistry. The result demonstrated that C1GALT1 protein expression was located at cytoplasmic area. There were 38% (10/26) with C1GALT1 positive staining whereas remaining was 62% (16/26) showing a negative staining of C1GALT1 (Figure 3.3). Due to there was no differences in intensity of C1GALT1 positive cells, CCA patients were dichotomized into two groups; C1GALT1 positive and C1GALT1 Negative. We next used Pearson's chi-squared test to evaluate

the correlation between C1GALT1 protein expression and clinicopathological features. There was no statistically significant association between C1GALT1 protein expression and sex, age, metastasis and invasion (Table 3.1). Kaplan-Meier analysis and the log-rank test demonstrated that there was no statistically significant in overall survival between patients with C1GALT1 positive VS C1GALT1 negative (log rank, P-value=0.342) (Figure3.4). However, the mean survival time of the CCA patients with C1GALT1 negative (19 weeks) was lesser than that of the patients with C1GALT1 positive (37 weeks). These findings suggest that downregulation of C1GALT1 may associate with poor prognosis of the CCA patients.

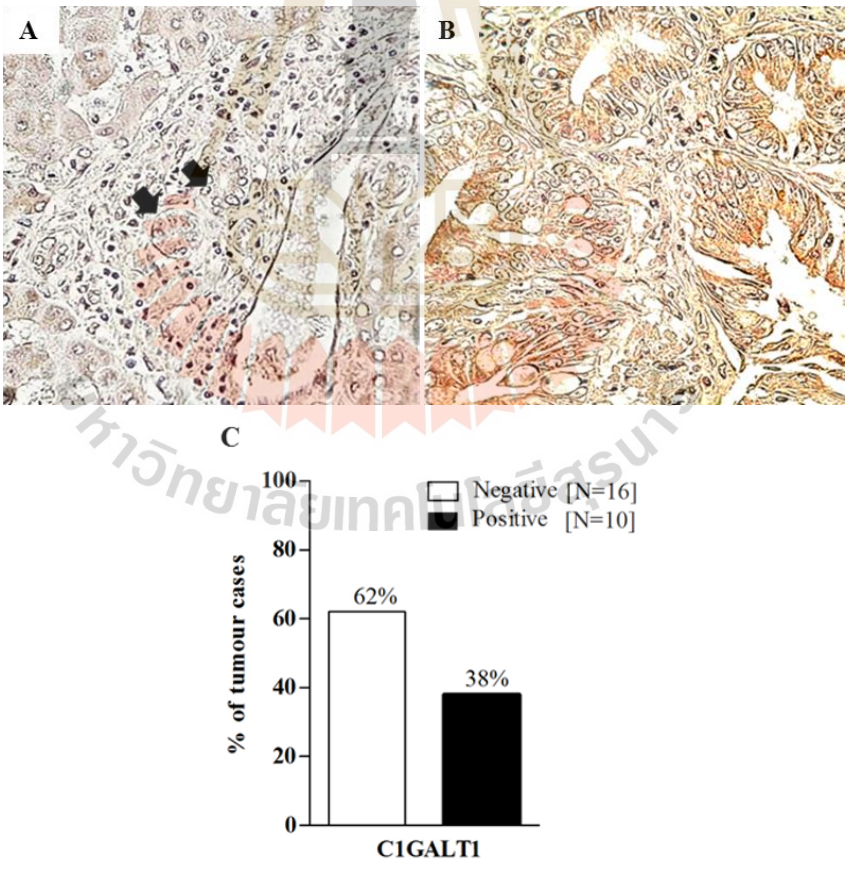


Figure 4.3 Immunohistological analysis of C1GALT1 protein expression in CCA

tissues. (A) C1GALT1 negative, (B) C1GALT1 positive, (C) % of tumor cases for C1GALT1 expression in CCA tissues.

Table 4.1 Correlation of C1GALT1 protein expression with clinicopathologic features (n = 26)

Factors		**Negative (N=16)**	**Positive (N=10)**	***p***
Sex	Male	14	4	0.300
	Female	2	6	
Age, y	<55	8	6	0.635
	≥55	8	4	
Metastasis	0	10	5	0.549
	1	6	5	
Invasion	0	1	3	0.111
	1	15	7	

The correlation was analyzed by using the Pearson's chi-squared test.

P < 0.05 is considered statistically significant.

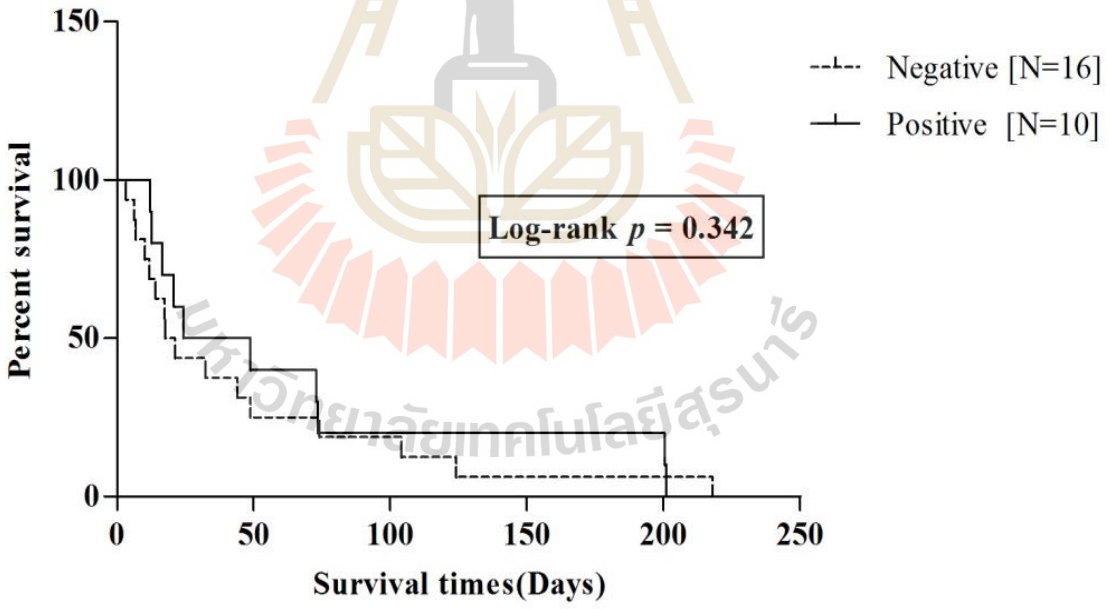


Figure 4.4 Kaplan-Meier analysis demonstrates cumulative overall survival determined of CCA patients with C1GALT1 negative VS C1GALT1 positive.

4.3 The functional analysis of C1GALT1 in CCA cell lines

4.3.1 Basal C1GALT1 expression in CCA cell lines and knockdown of the expression by small interfering RNA

To explore the biological functions of C1GALT1 in CCA, endogenous C1GALT1 expression was investigated in KKU-055, KKU-100, KKU-213A and KKU-213B and MMNK-1 using western blot analysis. High expression of C1GALT1 was demonstrated in KKU-055, KKU-100 and MMNK-1 whereas KKU-213A and KKU-213B had a relatively low endogenous expression of C1GALT1 (Figure3.5). KKU-055 and KKU-100 cells were used in gene knockdown model. KKU-055 and KKU-100 cells were transfected with specific siRNA to C1GALT1 (si-C1GALT1) and si-control. The expression levels (mRNA and protein) were significantly decreased following transfection with C1GALT1 siRNA at 48 hr (Figure 3.6 and Figure 3.7).

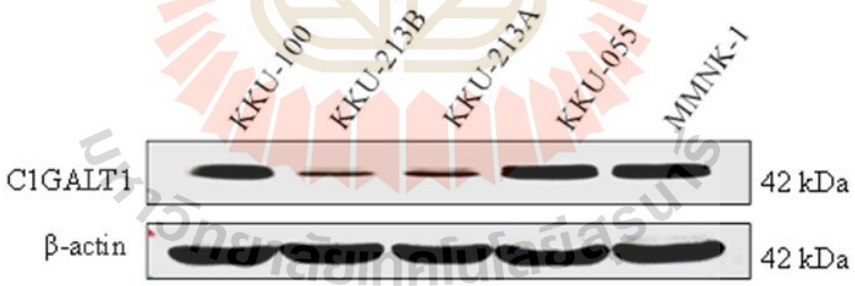


Figure 4.5 Basal expression of C1GALT1 in 4 different CCA cell lines (KKU-100, KKU-213B, KKU-213A and KKU-055) and a highly differentiated immortalized human cholangiocyte cell line (MMNK-1). Protein expression was detected by western blot analysis. β-actin was used as loading control.

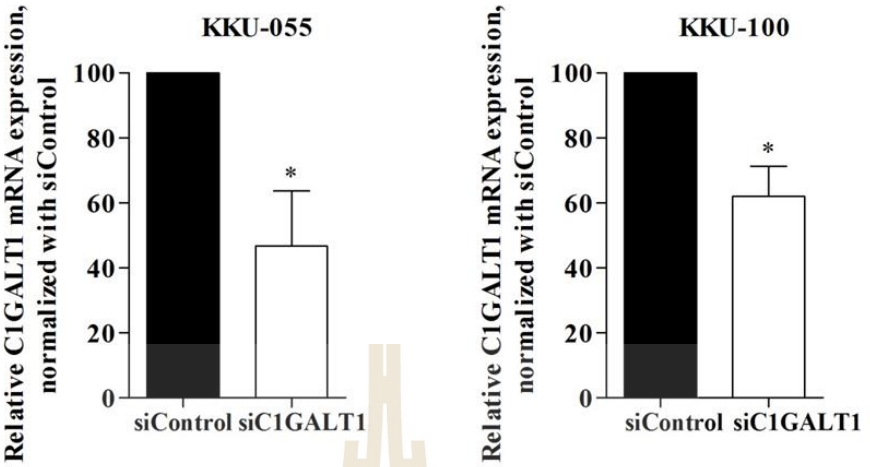


Figure 4.6 Knockdown of the C1GALT1 expression in KKU-055 and KKU-100 cells. The mRNA expression was performed using qRT-PCR. Relative mRNA expression level was determined using $2^{-\Delta\Delta CT}$ normalized to siControl. Expression data come from three independent experiments that are calculated as mean ± SD from (*P < 0.05 VS siControl).

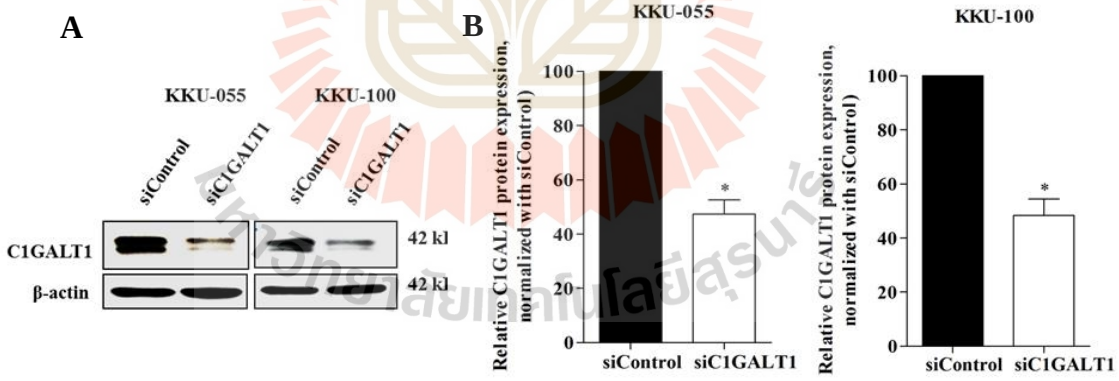


Figure 4.7 Knockdown of the C1GALT1 expression in KKU-055 and KKU-100 cells. (A) The protein expression was performed using western blot analysis. (B) Quantitative analysis was assessed by ImageJ. Expression data come from three independent experiments that are calculated as mean ± SD (*P < 0.05 VS siControl).

4.3.2 Silencing C1GALT1 enhances cell proliferation and 5-fluorouracil drug resistance

Dysregulation of C1GALT1 is involved in all steps of tumor development and progression as well as drug resistance (Gupta et al., 2020). We investigated whether suppression of C1GALT1 influences CCA cell growth and 5-fluorouracil (5-FU) chemotherapeutic sensitivity.Cell proliferation assay demonstrated that silencing C1GALT1 significantly increased cell growth at day 2 to day 5 in both KKU-055 and KKU-100 cells (Figure 3.8). To further explore the role of C1GALT1, both KKU-055 and KKU-100 were transfected with siC1GALT1 or siControl for 48 hr and then treated with various concentrations of 5-FU at 150 and 300 µM for 48 hr. Suppression of C1GALT1induced 5-FU resistance at both 150 and 300 µM in KKU-055 and KKU-100 (Figure 3.9). There findings suggest that C1GALT1 has a role in the regulation of CCA cell growth and drug response.

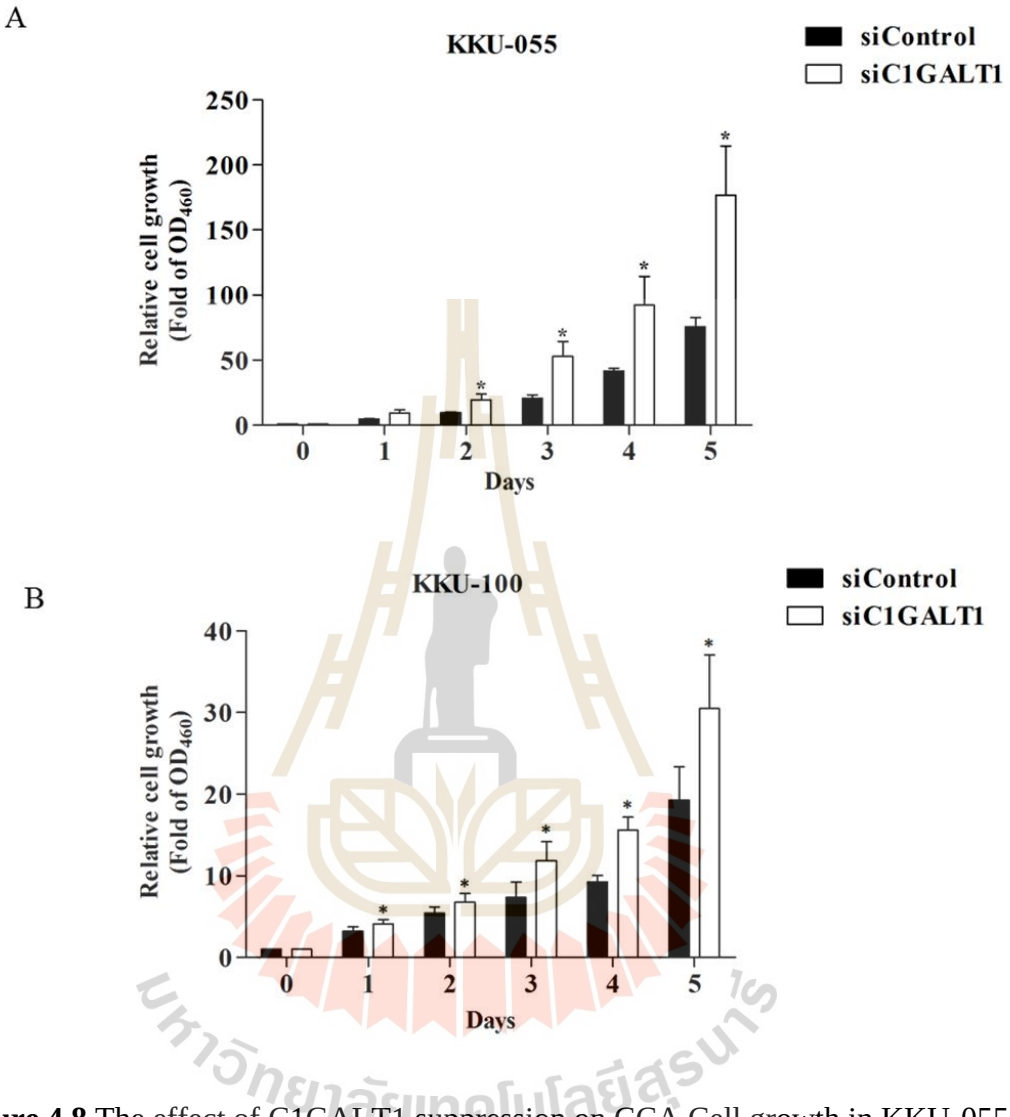


Figure 4.8 The effect of C1GALT1 suppression on CCA Cell growth in KKU-055 (A) and KKU-100 (B). Cell viability was performed using CCK-8. Relative cell growth come from three independent experiments that are presented as mean ± SD (*P < 0.05 VS day 0).

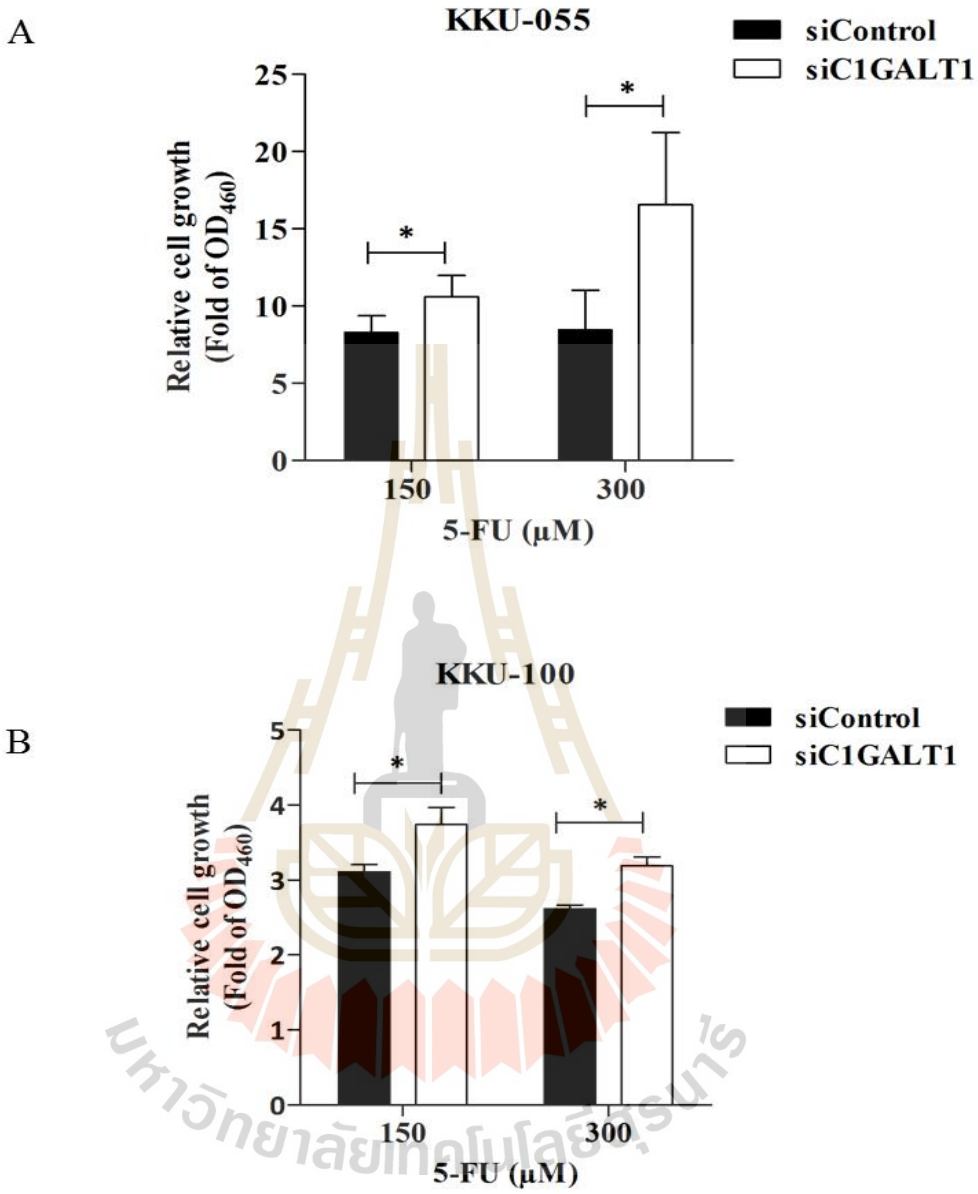


Figure 4.9 The effect of C1GALT1 suppression on 5-FU response in KKU-055 (A) and KKU-100 (B). After 48 hr of transfection, the cells were treated with 5-FU at 150 and 300 µM for another 48 hr. Cell viability was performed using CCK-8 kit). Relative cell growth come from three independent experiments that are presented as mean ± SD (*P < 0.05 VS siControl).

4.3.3 Silencing C1GALT1 promotes CCA cell growth and 5-FU resistance via the activation of AKT/ERK signaling

Activation of the PI3K/AKT and MAPK signaling pathway is a major determinant of tumor cell growth and survival in multiple solid tumors (Cervello et al., 2012 & Ostad et al., 2011). To evaluate the underlying mechanism of C1GALT1-mediated CCA progression, C1GALT1 expressions were suppressed using siC1GALT1 or siControl in KKU-055 and KKU-100 cells and survival markers including AKT and ERK were determined. Western blot analysis showed that the activation/phosphorylation of AKT and ERK was significantly increased in siC1GALT1 treated cells (Figure 3.10). Upregulation of anti-apoptotic protein (BCL2) and downregulation of apoptotic protein (BAX) were also observed after C1GALT1 suppression. Then, the effect of C1GALT1-mediated CCA survival was clearly demonstrated by a high ratio of BCL-2/BAX (Figure 3.11). Moreover, transcription levels of growth activating genes (*c-Myc*, *CCND1*) were increased in siC1GALT1 treated cells (Figure 3.12). These findings indicate that inhibition of C1GALT1 induced AKT/ERK activation, leading to CCA cell growth and survival.

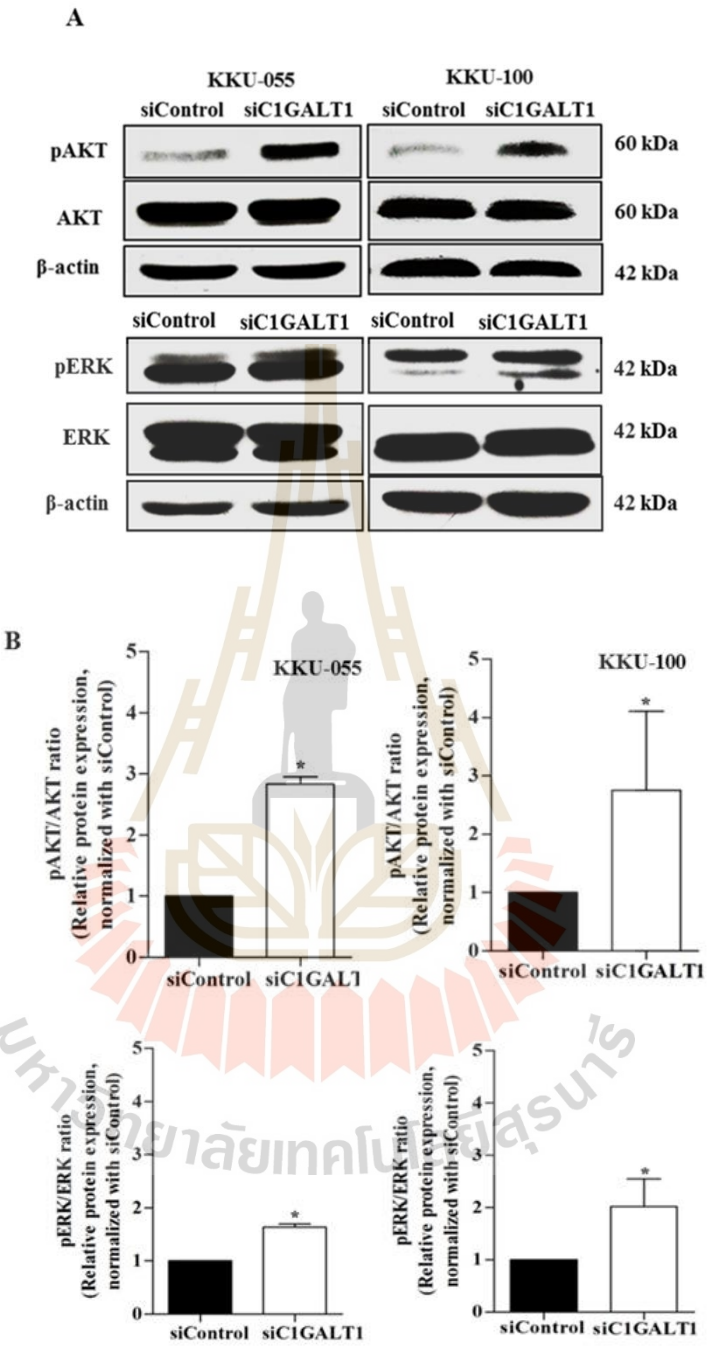


Figure 4.10 AKT/ERK signaling in siC1GALT1 treated KKU-055 and KKU-100. (A) The phosphorylation of AKT and ERK upon C1GALT1 suppression. (B) Quantitative analysis was assessed as phosphorylated form/total AKT and

ERK form by ImageJ. Expression data come from three independent experiments that are calculated as mean ± SD (*P < 0.05 VS siControl).

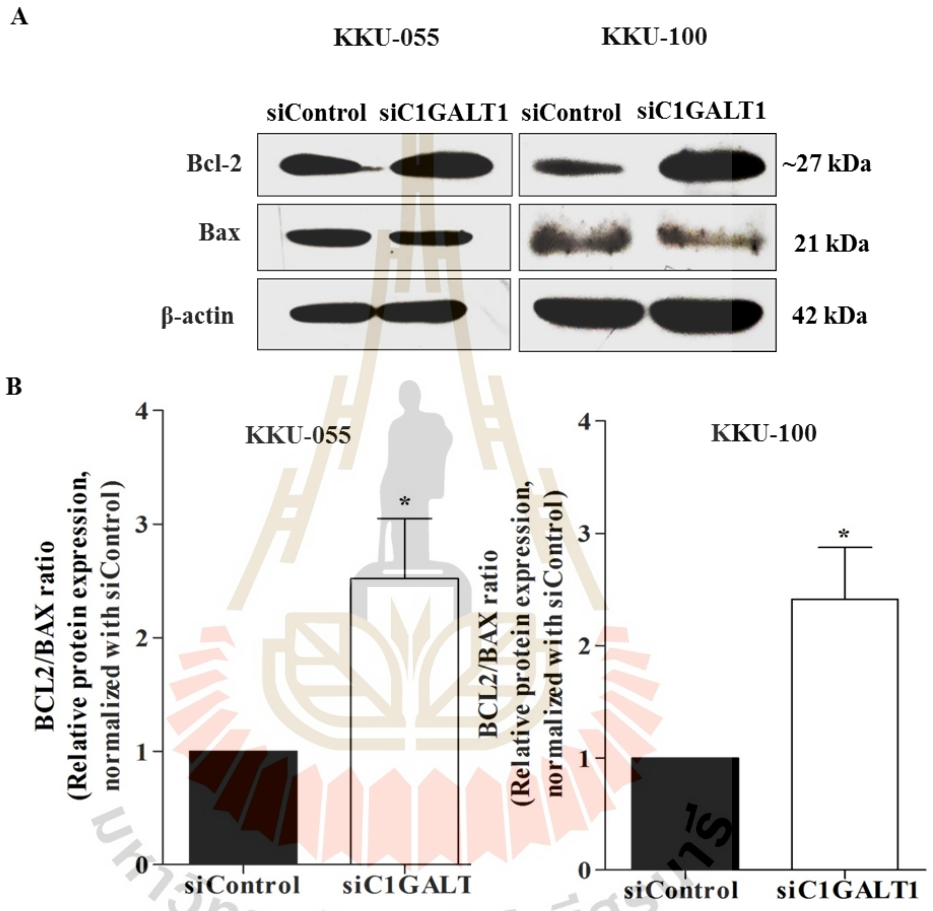


Figure 4.11 BCL-2 and BAX proteins expression in C1GALT1 knockdown KKU-055 and KKU-100. (A) Suppression of C1GALT1 increased expression of BCL-2 and decreased expression of BAX. (B) Quantitative analysis was assessed as BCL2 form/BAX form by ImageJ. Expression data come from three independent experiments that are calculated as mean ± SD (*P < 0.05 VS siControl).

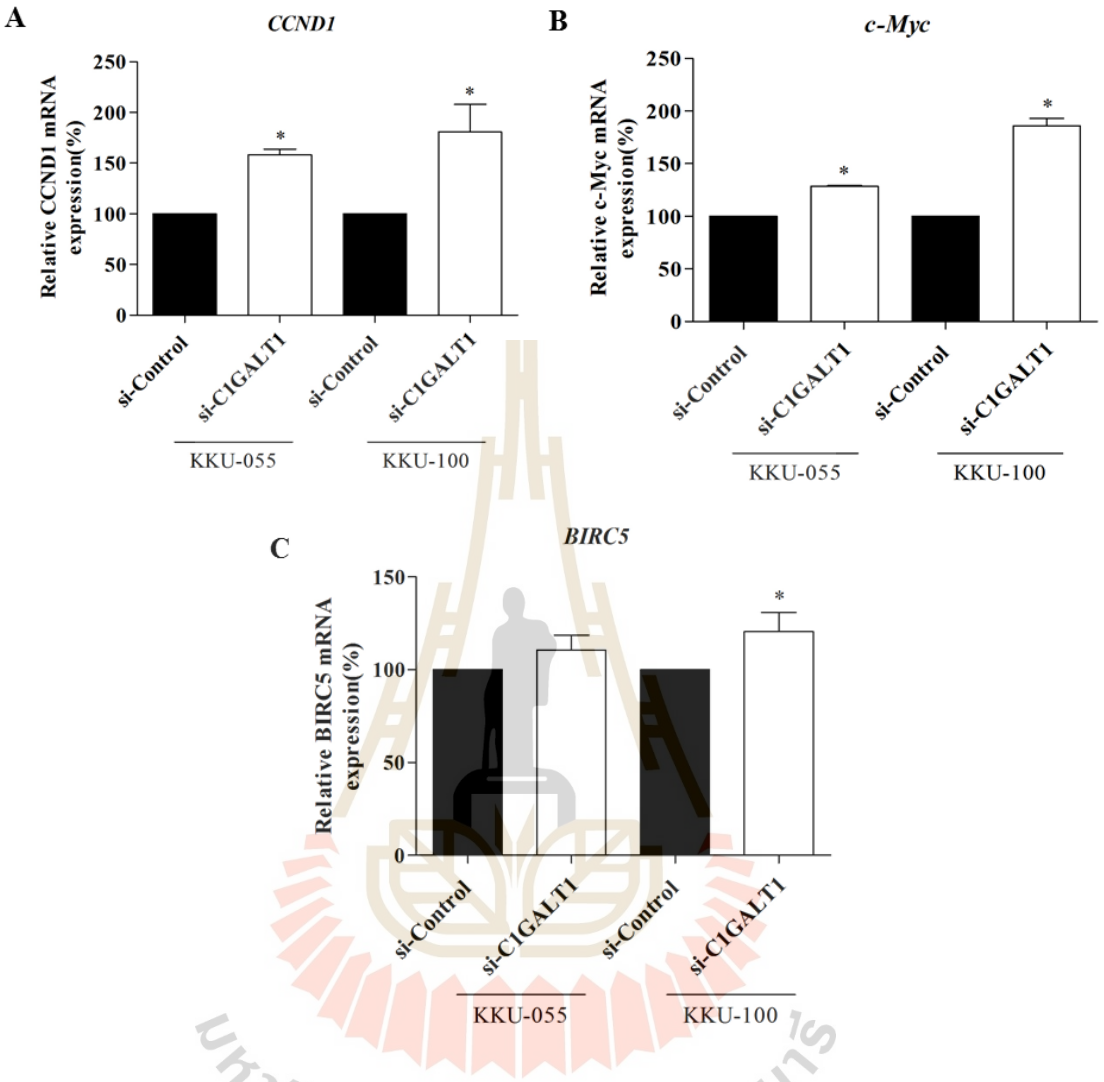


Figure 4.12 mRNA expression levels of *CCND1*, *C-Myc* and *BIRC5* in C1GAlT1 knockdown cells (KKU-055 and KKU-100). The mRNA expression was performed using qRT-PCR. Relative mRNA expression level was determine using $2^{-\Delta\Delta CT}$ normalized to siControl. Expression data come from three independent experiments that are calculated as mean ± SD (*P < 0.05 VS siControl).

4.3.4 Silencing C1GALT1 associates with truncated mucin-type O-glycosylation

The relative expression of *C1GALT1*, *B3GNT6*, and *ST6GalNAc-I* enzymes towards the O-GalNAc substrate (Tn antigen) determines variable synthesis of mucin-type O-glycan extension or truncation (Gupta et al., 2020). To understand the significance of *C1GALT1* knockdown on mucin-type O-glycosylation in CCA, the expression of *C1GALT1*, *COSMC*, *B3GNT6*, and *ST6GalNAc-I* were determined after C1GALT1 knocked-down KKU-055 and KKU-100. Decrease of *B3GNT6* and *ST6GalNAc-I* was detected in siC1GALT1 treated cells when compared with those of control cells. Whereas there was no transcription level change of a private C1GALT1 chaperone, *COSMC*, after C1GALT1 suppression (Figure 3.13). The aberrant mucin-type O-glycosylation upon C1GALT1 suppression was further investigated using lectin immunocytochemistry. Binding specificity of GalNAc-α Thr/Ser, Gal-β-(1,3)-GalNAc-α-Thr/Ser and GlcNAc; Neu5Ac was determined by Vicia villosa lectin (VVL), peanut agglutinin (PNA), and wheat germ agglutinin (WGA) respectively. The result showed that high expression of VVL-binding glycans but low expression of WGA-binding glycans were observed in siC1GAT cells when compared with those of sicontrol cells. However, there was no difference on the expression of PNA-binding glycans in between siC1GALT1 treated cells and control cells (Figure3.14-3.15). Therefore, inhibition of C1GALT1 diminished the activation of *B3GNT6*, and *ST6GalNAc-I* expression, leading to mucin-type O-glycans truncation in CCA.

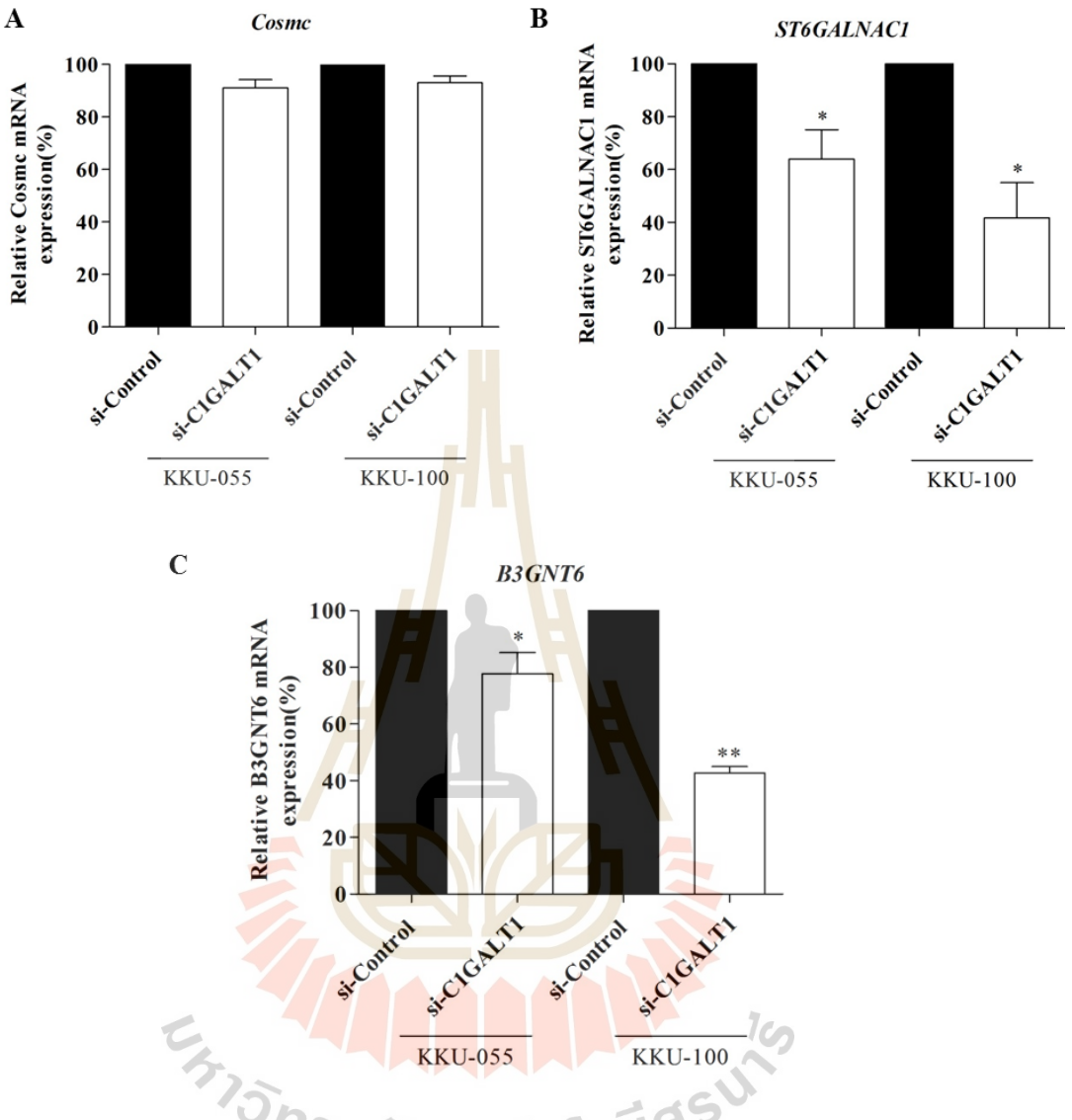


Figure 4.13 mRNA expression levels of *COSMC, ST6GALNAC1* and *B3GNT6* in C1GALT1 knocked down KKU-055 and KKU-100 cells. The mRNA expression was performed using qRT-PCR. β-actin was used as the reference gene. Relative mRNA expression level was determine using $2^{-\Delta\Delta CT}$ normalized to siControl. Expression data come from three independent experiments that are calculated as mean ± SD (*P < 0.05 VS siControl).

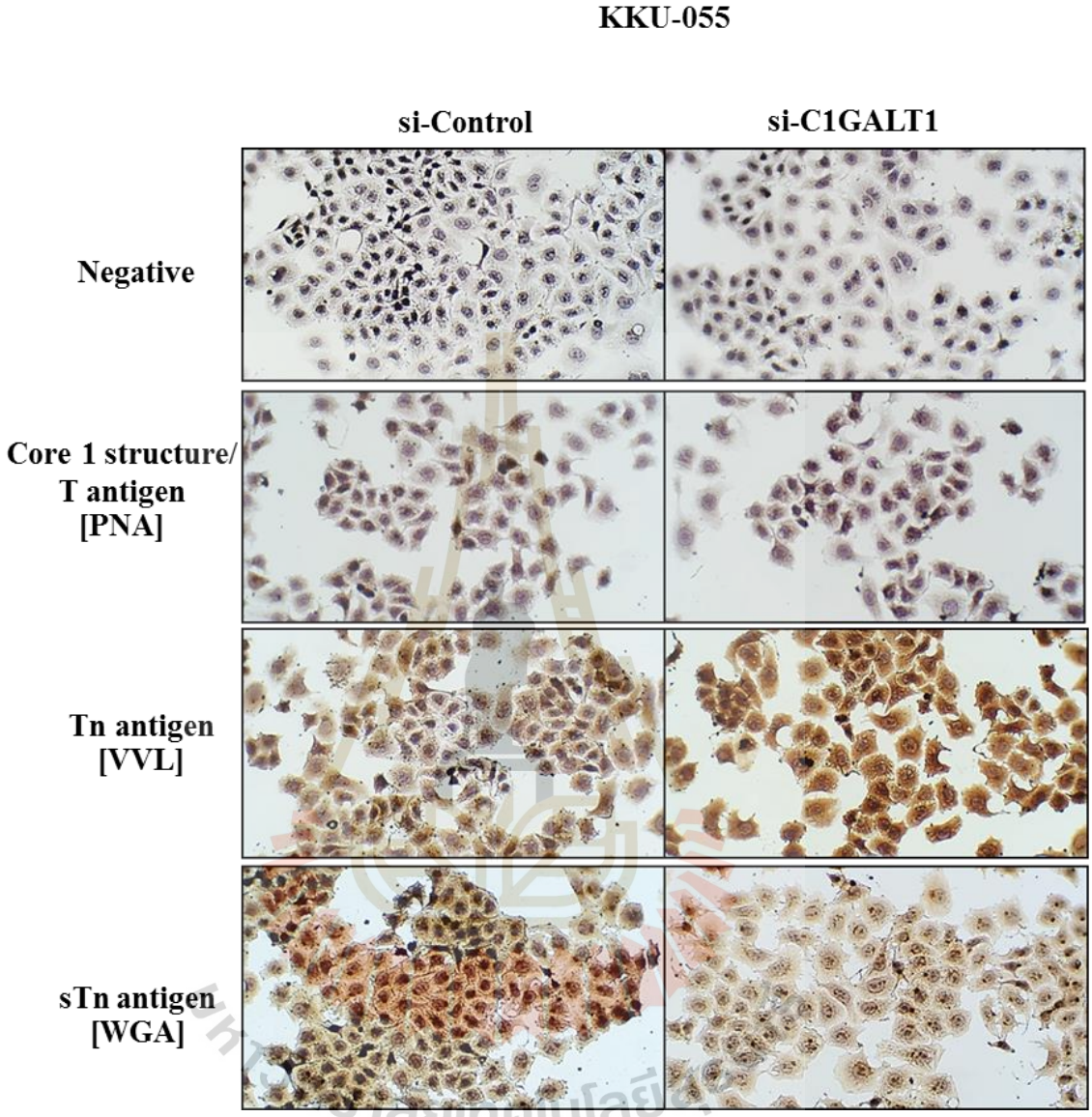


Figure 4.14 Immunocytochemical staining of PNA, VVL and WGA in KKU-055.

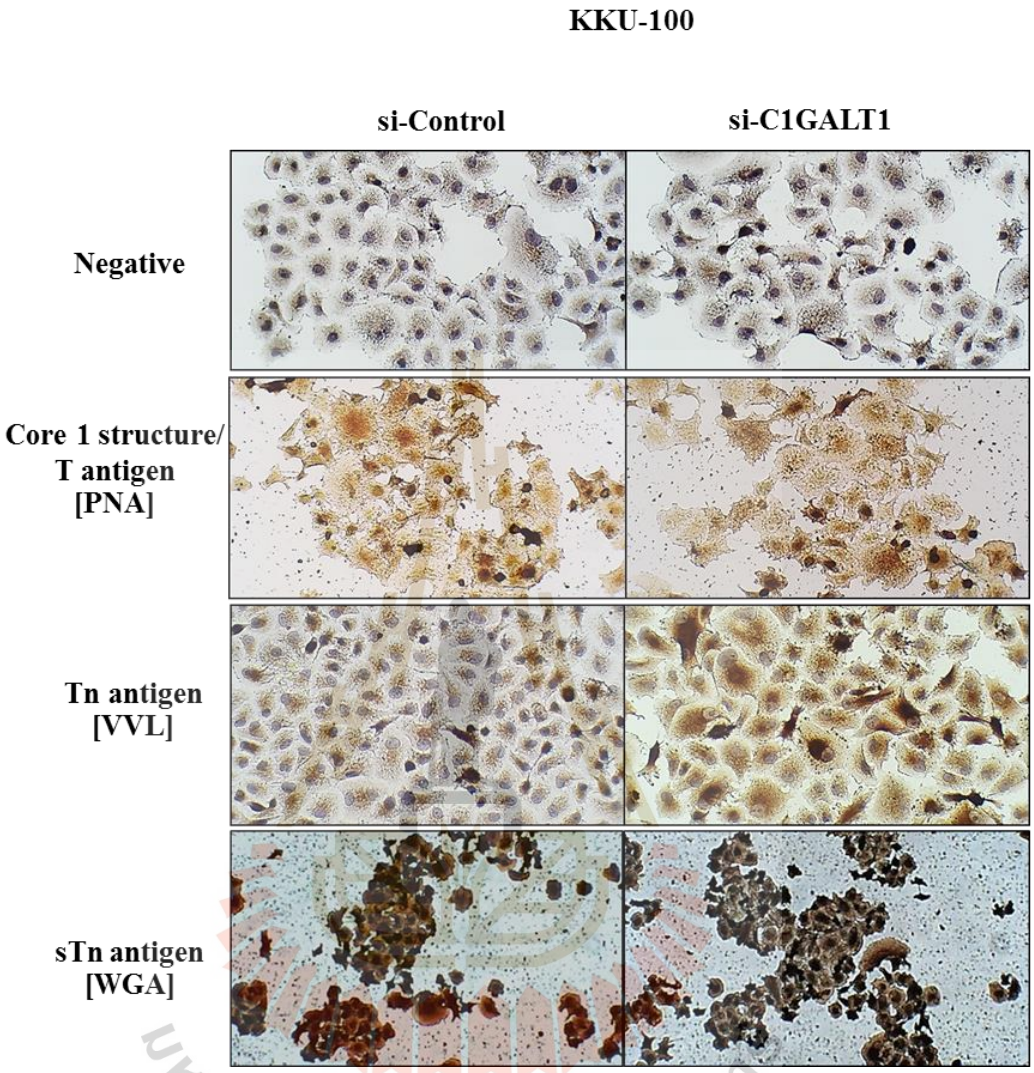


Figure 4.15 Immunocytochemical staining of PNA, VVL and WGA in KKU-100.

CHAPTER 5

DISCUSSION AND CONCLUSION

The alteration of the mucin type O-glycosylation is the most common characteristics in cancers and is involved in all key pathological step of tumor development and progression (Munkley et al., 2016). The most frequently observed aberrant mucin type O-glycosylation in cancer is the immature truncated core 1 O-glycan which designed Tn and sialyl -Tn (sTn) antigens (Itzkowitz et al., 1989; Tsuchiya et al., 1999; Inoue et al., 1991; Terasawa et al., 1996). Currently, numerous studies on O-glycosylation using lectin and direct structure analyses of glycan features have demonstrated diverse changes of glycosylation in CCA. However, the immature truncated core 1 glycans especially Tn is the common feature of CCA, in which increased expression of Tn antigen is observed during cholangiocarcinogenesis (Sasaki et al., 1999 & Marutpong et al., 2020). According to the relative expression of *C1GALT1*, *B3GNT6*, and *ST6GalNAc-I* determines the complexity of glycosylation on Tn antigen. In present study, we found that low mRNA expressions of *C1GALT1*, *B3GNT6*, and *ST6GalNAc-I* were observed in CCA tissues. This result indicates that aberrant mucin-type O-glycosylation in CCA may result from the altered expression of theses glycosyltransferases. As C1GALT is major glycosyltransferase that transfers UDP-galactose to Tn antigen to form core 1 structure (T antigen). Loss of C1GALT1 and their specific chaperone, COSMC contribute increase truncate O-glycans on several mucin proteins including MUC4 and MUC16 (Satish et al.,

2021 & Chugh et al., 2018). Moreover, the truncation of mucin-type O-glycan has been associated with the genetic and epigenetic alterations of both C1GALT1 and their specific chaperone, COSMC (Wang et al., 2010; Radhakrishnan et al., 2014; Ju et al., 2014; Fu et al., 2016). Furthermore, somatic mutation and hypermethylation of COSMC contribute the loss of C1GALT1 function resulting in development of colorectal cancer through induction of oncogenic properties such as proliferation, migration and apoptotic-resistant ability (Jiang et al., 2018). Given that low expression of C1GALT1 was also observed in CCA tissues at both mRNA and protein levels. Additionally, suppression of C1GALT1 contributed various aggressive malignant phenotypes including cell growth and 5-FU resistance via the activation of AKT and ERK signaling pathways. Moreover, increase transcription levels of growth activating genes (*c-Myc* and *CCND1*) and upregulation of anti-apoptotic protein, BCL-2, were detected in CCA cell line having C1GALT1 suppression. These observations are consistent with the studies in colorectal cancer, gastric cancer and pancreatic ductal adenocarcinoma, in which forced knockout of C1GALT1 in colorectal cancer cells significantly induced Tn antigen expression and subsequently enhanced cell proliferation, adhesion, as well as migration and invasiveness (Xichen et al., 2018). Similar observation was reported in gastric cells, in which loss of C1GALT1 activity is cause of gastritis and gastric cancer by caspase-1/caspase-11 (Casp1/11)–dependent inflammasomes (Liu et al., 2019). Loss of C1GAlT1 in Kras and p53 mutant mice demonstrates development of aggressive pancreatic ductal adenocarcinomas (PDACs) and increase metastasis. Additionally, knockout of C1GALT1 increases the truncation of O-glycosylation on MUC16, which leads to increase

tumorigenicity and aggressiveness in PDAC (Chugh et al., 2018). Moreover, knockout of C1GALT1-specific chaperone (COSMC) also increases truncated O-glycan on MUC4 that enhance the malignant phenotypes and gemcitabine resistance in PDAC tumors via altering ErbB/AKT signaling cascades and expression of nucleoside transporters, respectively (Satish et al., 2021).

As the formation of Tn antigen is mediated via the action of polypeptide N-Acetylgalactosaminyl-transferase (GALNTs). Marutpong et al., 2019 have demonstrated that the expression level of GALNT5 in CCA corresponds to the expression level of Tn antigens as detected by Tn-specific Vicia villosa lectin (VVL binding glycans). Additionally, overexpression of GALNT5 in CCA cells promotes the expression level of VVL binding glycans resulting in contribution the cell invasion and metastasis through the activation of AKT/ERK signaling pathway in CCA. However, Tn antigen can be further modified by the action of C1GALT1 to promote the formation of core 1 O-glycans (T antigen). The result of present study demonstrated that suppression of C1GALT1 by specific siRNA in CCA cell lines increased the expression level of VVL binding glycans. Moreover, decrease of *B3GNT6* and *ST6GalNAc-I* was also detected in C1GALT1 knockdown. These observations suggest that high expression of immature truncated core 1 O-glycan (Tn antigen) in CCA could be the result of downregulation of C1GALT1. Furthermore, lack of C1GALT1 in CCA may sustain the expression level of Tn antigen by diminishing the formation of sTn and core 3 O-glycans.

In conclusion, our study demonstrated that C1GALT1 was down-regulated in CCA. Silencing C1GALT1 enhanced cell proliferation and 5-fluorouracil resistance in CCA

cell lines. The activation of AKT/ERK signaling was detected in silencing C1GALT1 expression. Moreover, suppression of C1GALT1 seem to sustained the expression level of VVL binding glycans in CCA cell line by reducing the expression of Core-3 synthase (*B3GNT6*) and sialyl-transferase (*ST6GalNAc-I*) expression. These observations emphasize the essential role of C1GALT1 on aberrant expression of the immature truncated core 1 O-glycan (Tn antigen) and its associated CCA progression (Figure 4.1).

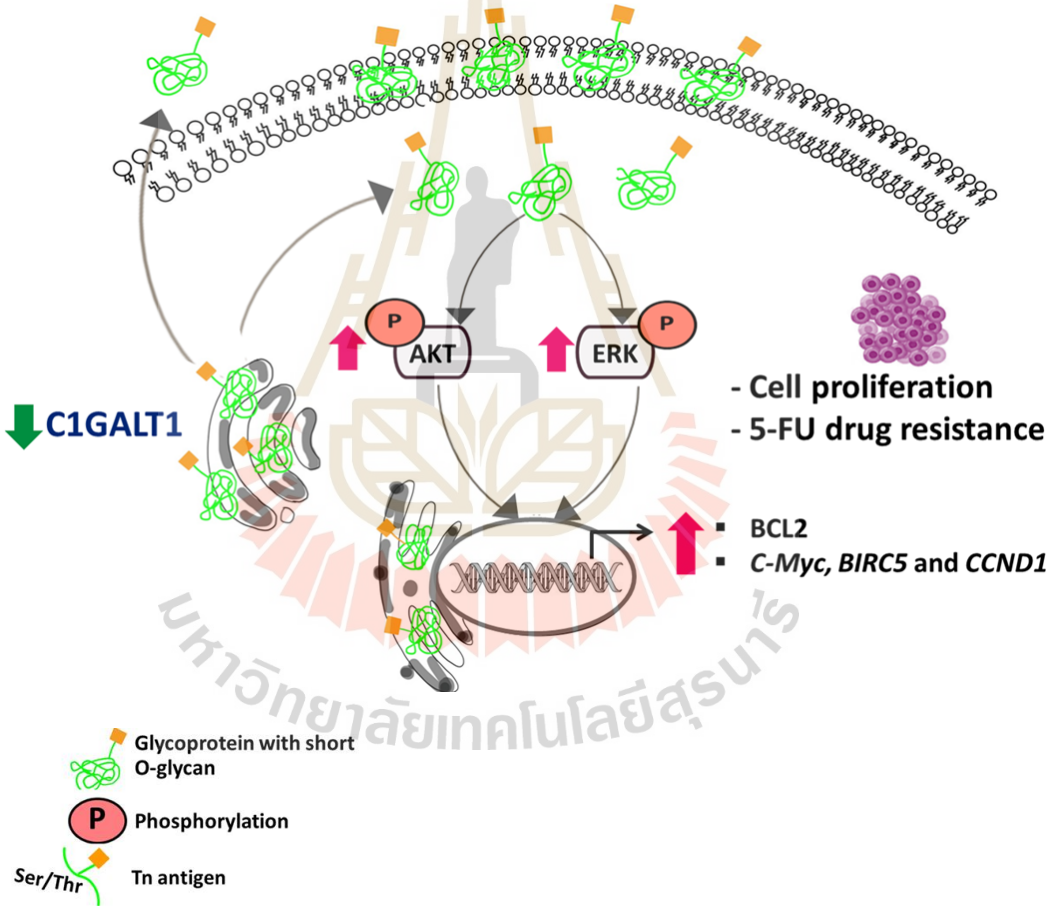


Figure 5.1 The schematic diagram depicts that the mechanism of C1GALT1 promotes cell proliferation and 5-FU resistance of CCA cells. Downregulation of C1GALT1 promotes truncation of O-glycan leading to CCA progression through activation of The AKT/ERK signalling pathway.

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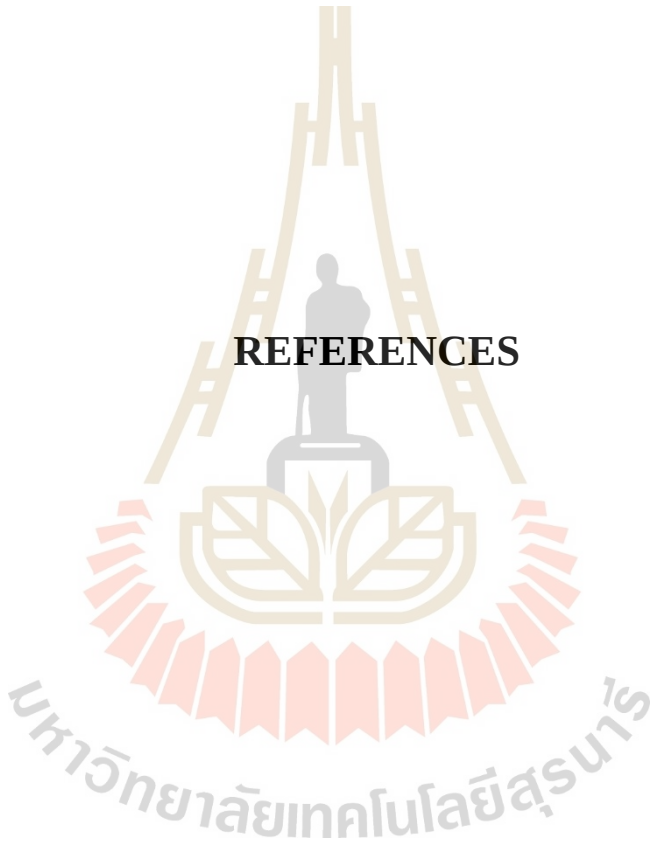

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Grants

- Thailand Research Fund under Grant no. RSA6180017

- Kittibandit scholarship, Suranaree University of Technology