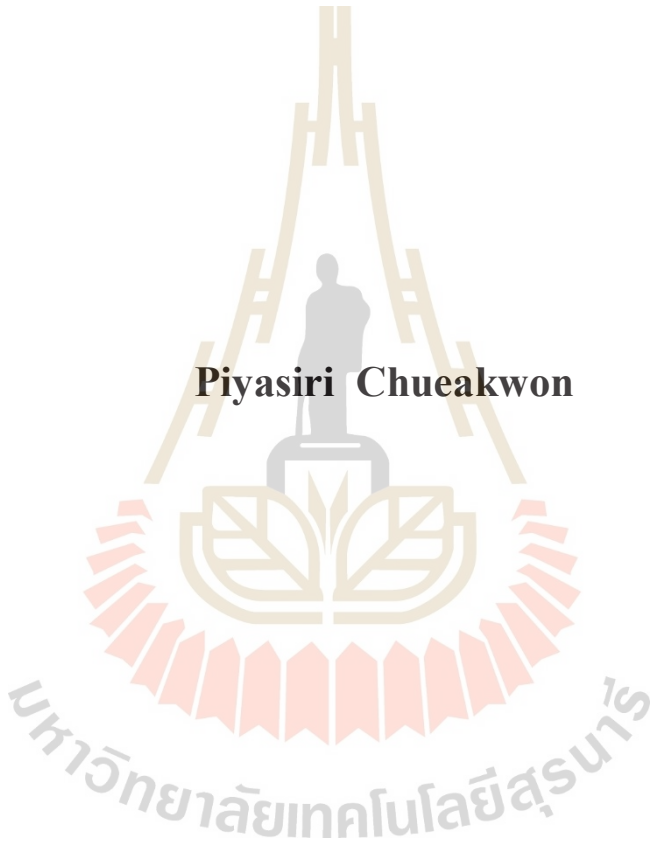
ROLE OF GLUCOSYLCERAMIDE SYNTHASE IN CHOLANGIOCARCINOMA

Piyasiri Chueakwon



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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ROLE OF GLUCOSYLCERAMIDE SYNTHASE IN CHOLANGIOCARCINOMA

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

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อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.ชุติมา ตลับนิล, 102 หน้า.

เอนไซม์กลูโคซิลเซอรามายด์ซินเทส หรือ GCS เป็นเอนไซม์ที่สำคัญในกระบวนการเติมสารคาร์โบไฮเดรตให้กับเซอรามายด์ (ceramide) โดยเอนไซม์นี้ทำหน้าที่จำเพาะเจาะจงในการย้ายน้ำตาลกลูโคสจาก UDP-glucose ไปยังเซอรามายด์เพื่อสังเคราะห์เป็นกลูโคซิลเซอรามายด์ (glucosylceramide; GlcCer) ซึ่งเป็นสารตั้งต้นสำหรับการผลิตไกลโคสฟิงโกลิพิดชนิดต่าง ๆ โดยกระบวนการดังกล่าวสามารถเกิดปฏิกิริยาย้อนกลับได้ด้วยบทบาทของเอนไซม์กลูโคซิลเซอรามิเดส 1 (GBA1) ที่อยู่บริเวณไลโซโซม และกลูโคซิลเซอรามิเดส 2 (GBA2) ที่มักจะอยู่ด้านบนผิวของเอนโดพลาสมิก เรติกคูลัม และกอลไจ คอมเพล็กซ์ โดยเอนไซม์นี้จะทำหน้าที่ในการสลายกลูโคซิลเซอรามายด์ให้เป็นน้ำตาลกลูโคสและเซอรามายด์ โดยในหลายการศึกษาแสดงให้เห็นว่า การสังเคราะห์กลูโคซิลเซอรามายด์ที่เพิ่มขึ้นที่เกิดจากการทำงานของเอนไซม์ GCS ที่เพิ่มขึ้น สามารถส่งเสริมให้เซลล์มะเร็งสามารถหลบหนีจากกระบวนการตายที่ถูกชักนำด้วยเซอรามายด์ และนำมาสู่การกระตุ้นการเพิ่มจำนวนของเซลล์ และการดื้อต่อยาเคมีบำบัด โดยการแสดงออกที่เพิ่มมากขึ้นของ GCS ถูกพบในเซลล์มะเร็งหลายชนิด และมีความเกี่ยวข้องกับการดื้อต่อยาเคมีบำบัด ในกรณีของมะเร็งท่อน้ำดีมีรายงานพบระดับการแสดงออกที่ผิดปกติของไกลโคสฟิงโกลิพิดเช่นกัน ได้แก่ เฮกโซซิลเซอรามายด์ (hexosylceramides; HexCer) และแลคโตซิลเซอรามายด์ (lactosylceramides; LacCer) โดยการแสดงออกที่ผิดปกติดังกล่าวมีความสัมพันธ์กับการเจริญพัฒนาของเซลล์มะเร็งท่อน้ำดี อย่างไรก็ตามยังไม่มีการศึกษาใดแสดงถึงกลไกความผิดปกตินี้ ดังนั้นในการศึกษาครั้งนี้ ผู้วิจัยมีวัตถุประสงค์ในการศึกษาบทบาทของกระบวนการเติมสารคาร์โบไฮเดรตให้กับเซอรามายด์ในมะเร็งท่อน้ำดี ด้วยการวิเคราะห์การแสดงออกของยีน GCS และบทบาทหน้าที่ของยีนนี้ โดยผู้วิจัยทำการวิเคราะห์การแสดงออกของยีน GCS GBA1 และ GBA2 โดยใช้ข้อมูลการแสดงออกของยีนจากฐานข้อมูลการแสดงออกของยีนสาธารณะที่เรียกว่า gene expression omnibus (GEO) พบการแสดงออกของยีน GCS และ GBA2 เพิ่มสูงขึ้นในเนื้อเยื่อมะเร็งท่อน้ำดีอย่างมีนัยสำคัญทางสถิติ ในขณะที่ GBA1 มีการแสดงออกที่ลดต่ำลงในเนื้อเยื่อมะเร็งท่อน้ำดีเมื่อเทียบกับเนื้อเยื่อปกติ และ

ผู้วิจัยได้ศึกษาการแสดงออกของยีน GCS ในเนื้อเยื่อมะเร็งท่อน้ำดีและเนื้อเยื่อปกติจำนวน 29 คู่ตัวอย่าง ด้วยปฏิกิริยาโพลีเมอร์เรสแบบเชิงปริมาณ เมื่อใช้เกณฑ์ค่าการแสดงออกของยีนที่เปลี่ยนแปลงไปในเนื้อเยื่อมะเร็งเทียบกับค่าการแสดงออกของยีนในเนื้อเยื่อปกติ ที่ค่ามัธยฐานของจำนวนเท่าของการแสดงออกของยีน พบจำนวนตัวอย่างที่ให้ผลการแสดงออกของยีน GCS ที่เพิ่มสูงเฉลี่ยที่ 1.79 เท่า คือ 14 ตัวอย่างจากตัวอย่างทั้งหมด 29 ตัวอย่าง แต่การแสดงออกของยีน GCS ที่เพิ่มสูงนี้ไม่พบความสัมพันธ์อย่างมีนัยสำคัญกับการรอดชีวิตของผู้ป่วย และอาการทางพยาธิคลินิกต่าง ๆ และเมื่อทำการศึกษาบทบาทของ GCS ในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี พบมีการแสดงออกของยีน GCS และ GBA2 เพิ่มสูงขึ้นเมื่อทดสอบเซลล์ด้วยซิสพลาตินและยังพบอัตราส่วนการแสดงออกของยีน GCS/GBA2 ที่ลดลงแสดงให้เห็นว่า มีความเปลี่ยนแปลงเกิดขึ้นในกระบวนการเติมสารคาร์โบไฮเดรตให้กับเซอราไมด์เมื่อเซลล์ได้รับซิสพลาติน เมื่อยับยั้งการแสดงออกของยีน GCS โดยการยับยั้งการแสดงออกด้วย siRNA หรือยับยั้งการทำงานของ GCS ด้วยสารเคมียับยั้ง (PPMP) หรือยับยั้งทั้งสองระบบร่วมกัน พบการเจริญเติบโตของเซลล์ลดลง และมีผลเพิ่มฤทธิ์ของซิสพลาตินให้มีการชักนำการตายของเซลล์แบบอะพอพโทซิสมากขึ้น จากผลการศึกษาวิจัยทั้งหมดแสดงให้เห็นว่า GCS มีบทบาทต่อการเจริญเติบโตของเซลล์มะเร็งท่อน้ำดี และความไวในการตอบสนองต่อซิสพลาติน ดังนั้นการยับยั้งการแสดงออกของยีน GCS อาจเป็นกลยุทธ์หนึ่งที่น่าจะช่วยเพิ่มประสิทธิภาพการรักษามะเร็งท่อน้ำดีได้

สาขาวิชาเคมี ลายมือชื่อนักศึกษา Piyasiri Chueakwon

ปีการศึกษา 2563 ลายมือชื่ออาจารย์ที่ปรึกษา

PIYASIRI CHUEAKWON : ROLE OF GLUCOSYLCERAMIDE SYNTHASE IN CHOLANGIOCARCINOMA. THESIS ADVISOR : ASST. PROF. CHUTIMA TALABNIN, Ph.D. 102 PP.

GLUCOSYLCERAMIDE SYNTHASE/PPMP/CHOLANGIOCARCINOMA/ CISPLATIN/GLYCOSPHINGOLIPID

Glucosylceramide synthase (GCS) is an essential enzyme for ceramide glycosylation that transfers a glucose residue from UDP-glucose to ceramide there by generating glucosylceramide (GlcCer), a precursor of glycosphingolipids (GSLs) synthesis. This process can be reverted by either glucosylceramidase 1 (GBA1) in the lysosome or glucosylceramidase 2 (GBA2) on the cytoplasmic surface of the ER and Golgi to hydrolyze GlcCer and generate ceramide (Cer) and glucose. Several experiments demonstrated that increasing of GlcCer synthesis via upregulation of GCS allows tumor cells to escape from ceramide-induced programed cell death, and promotes cell proliferation and multidrug resistance. Overexpression of GCS was found in various cancers and associated with chemotherapy resistance. In CCA, aberrant glycosphingolipids (GSLs) levels, such as those of hexosylceramides (HexCer) and lactosylceramides (LacCer) have been reported and are associated with CCA progression. However, the underlying mechanism of this alteration is still unknown. In the present study, we aimed to study on the role of ceramide glycosylation in CCA. GCS expression and its roles were explored. The expression of genes involved in ceramide glycosylation including GCS, GBA1, and GBA2, were retrieved from a publicly available human CCA database (GEO). The results showed that GCS and

GBA2 were significantly upregulated in tumor tissues, whereas GBA1 was downregulated when compared with normal tissues. We further verified GCS expression in 29 paired frozen tissues by qPCR. With median expression as the cut-off value, there were 14/29 cases of the cancer specimens showing highly expressed GCS as 1.79-fold, compared with the adjacent normal tissues. There was no statistical correlation between GCS expression and overall survival as well as clinicopathological parameters in CCA patients. High GCS and GBA2 expression were observed in cisplatin-treated CCA cells and low ratio of GCS/GBA2 expressions was clearly demonstrated the alteration of ceramide glycosylation upon cisplatin treatment. Reduction of CCA cell growth was shown in the presence of specific siRNA to GCS, PPMP (a chemical GCS inhibitor), and co-suppression of both. Subsequently, both of genetic and chemical inhibition of GCS were able to enhance cisplatin-induced CCA cell death through the apoptosis signaling pathway. Our findings suggest that GCS has a role in CCA cell growth and cisplatin sensitivity. Thus, targeting GCS may be a potential strategy for improving CCA treatment.

School of Chemistry

Academic Year 2020

Student's signature Piyasiri Chueakwon

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LIST OF ABBREVIATIONS

asGCS	A full-length of GCS antisense
BCSCs	Breast cancer stem cells
BMSCs	Bone marrow stem cells
CCA	Cholangiocarcinoma
C_4DGJ	N-butyl-deoxygalactonojirimycin
C_9DGJ	N-nonyl-deoxygalactonojirimycin
CDase	Ceramidase
Cer	Ceramide
CerS	Ceramide synthase
CERT	Ceramide transfer protein
Cis	Cisplatin
CLN3p	Neuronal ceroid-lipofuscinosis-3 protein
cDNA	Complementary deoxynucleic acid
CST	Cerebroside sulfotransferase
dCCA	Distal cholangiocarcinoma
DES	Dihydroceramide desaturase
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
ER	Endoplasmic reticulum
FBS	Fetal bovine serum

LIST OF ABBREVIATIONS (Continued)

GALC	Galactocerebrosidase (β-galactosidase)
GalCer	Galactosylceramide
GalCerS	UDP-galactose:ceramide galactosyltransferase
GEMs	GSL-enriched microdomains
GBA1	Glucocerebrosidase 1
GBA2	Glucocerebrosidase 2
GCS	Glucosylceramide synthase
GEO	Gene Expression Omnibus
GLA	α-Galactosidase A
GlcCer	Glucosylceramide
GSLs	Glycosphingolipids
HCC	Hepatocellular carcinoma
HexCer	Hexosylceramides
HPCs	Hepatic progenitor cells
HRP	Horse-radish-peroxidase
iCCA	Intrahepatic cholangiocarcinoma
IFNγ	Interferon-gamma
kDa	Kilo Dalton
LacCer	Lactosylceramides
LacCerS	Lactosylceramide synthase
MBO-asGCS	Mixed-backbone oligonucleotide of GCS antisense

LIST OF ABBREVIATIONS (Continued)

MDR1	Multidrug resistance 1
Min	Minute
NB-DNJ	N-butyl-deoxynojirimycin
pCCA	Perihilar cholangiocarcinoma
PenStrep	Penicillin and Streptomycin
PDMP	D-threo-1-phenyl-2-decanoylamino-3-morpholino-propanol
PPMP	D,L-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol
PPPP	1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol
PSC	Primary sclerosing cholangitis
qPCR	Quantitative Real-time PCR
S1P	Sphingosine-1-phosphate
SAT	Sialyltransferase
SDS	Sodium Dodecyl Sulfate
SEM	Standard error of mean
siRNA	Small interfering RNA
SK	Sphingosine kinase
SK1	Sphingosine kinase 1
SM	Sphingomyelin

LIST OF ABBREVIATIONS (Continued)

SMase	Acid sphingomyelinase
SMS	Sphingomyelin synthase
Sph	Sphingosine
SPT	Serine palmitoyltransferase
RNA	Ribonucleic acid
rpm	Revolutions per minute
ROS	Reactive oxygen species
TCA	Tricarboxylic acid
TLR-4	Toll-like receptor 4
TNFα	Tumor necrosis factor-alpha
(n/μ/m)g	(nano, micro, milli) Gram
(μ/m)L	(micro, milli) Liter
v/v	Volume per volume
w/v	Weight per volume

CHAPTER I

INTRODUCTION

1.1 Significance of research

Cholangiocarcinoma (CCA) is a malignant neoplasm of the bile duct that remains a serious health problem, particularly in South-East Asia and Eastern Europe (Kamsa-ard et al., 2018; Shaib et al., 2004; Sripa et al., 2007). CCA arises from the epithelial lining of the biliary tree either within the liver (intrahepatic cholangiocarcinoma or ICC) or outside the liver (extrahepatic cholangiocarcinoma or ECC) excluding the ampulla of Vater (Bergquist and von Seth, 2015; Blechacz, 2017). CCA is the second most common form of liver cancer, comprising approximately 10-15% of all hepatocellular carcinoma cases (B. Blechacz and G. J. Gores, 2008). CCA diagnosis is difficult at the early stage which leads to the patients often being found at advanced stages when cure and survival rates are low, despite treatment with aggressive therapy (Anderson et al., 2004). *Opisthorchis viverrini* is a major risk factor of CCA in Thailand (Ong et al., 2012; Sripa and Pairojkul, 2008). Several lines of evidence have indicated the possible mechanisms of liver fluke associated CCA, including chronic infection and inflammation, nitric oxide formation, intrinsic nitrosation and activation of drug-metabolizing enzymes that lead to oxidative DNA damage and CCA development (Sithithaworn et al., 2014; Sriplung et al., 2006; Watanapa and Watanapa, 2002).

Sphingolipids are bioactive molecules, a class of lipids with 18 carbon amino-alcohol backbones which are synthesized in the ER from serine and palmitic acid precursors (Gault et al., 2010). Ceramide (Cer) is a central molecule in sphingolipid metabolism. Cer can be converted to various glycosphingolipids (GSLs) that has been involved in many biological processes such as fluidity regulation, cell growth, and cell differentiation. However, unmodified ceramide is thought to induce death, growth inhibition, and senescence in cancer cells. Whereas glucosylceramide (GlcCer) shows opposite functions through inducing cell proliferation, oncogenic transformation, differentiation, tumor metastasis, and drug resistance (Gouazé et al., 2005; Ogretmen, 2018).

Glucosylceramide synthase (GCS) is a glucosyltransferase that transfers glucose residue from UDP-glucose to ceramide for promoting ceramide glycosylation, glucosylceramide, and the function is reversed by hydrolysis of glucocerebrosidase 1 (GBA1) and glucocerebrosidase 2 (GBA2). Various studies have been demonstrated that ceramide glycosylation allows tumor cells to escape from ceramide-induced programmed cell death and enhances cancer cell resistance to anticancer drugs (Y.-y. Liu et al., 2001; Liu et al., 2008; Modrak et al., 2006). Recently, high expression of GCS has been reported in various cancer types, including breast, cervix, colon, non-small cell lung cancer, and papillary carcinoma. GCS has been shown to promote cancer progression by inducing Bcl-xL-mediated anti-apoptosis and upregulation of MDR1 gene expression through c-Src and β-catenin signaling to promote multidrug resistance in cancer. Targeting of GCS was considered to overcome multidrug resistance and cancer progression (Chiu et al., 2015; Jennemann et al., 2017). In CCA, alteration of sphingolipid metabolism and GSLs has been documented by increasing

the levels of hexosylceramides (HexCer), and lactosylceramides (LacCer) in CCA tissues. Increase of LacCer (d18:1-h23:0) expression in tumors was associated with poor survival in CCA patients (Silsirivanit et al., 2019). However, the underlying mechanism of this aberrant expression has never been reported yet. Moreover, GCS is the rate-limiting enzyme that controls the biosynthesis of both hexosylceramides (HexCer), and lactosylceramides (LacCer). Therefore, we aim to 1) investigate the expression of GCS in CCA tissues and its association with clinicopathological features and survival of CCA patients 2) to demonstrate the functional roles of GCS in CCA development and progression.

1.2 Research objectives

1.2.1 To investigate the expression level of the enzymes in glucosylceramide synthesis and degradation, including GCS, GBA1, and GBA2, in CCA cell lines and CCA tissues.

1.2.2 To analyze the correlation between GCS expression with clinicopathological features and survival of CCA patients.

1.2.3 To investigate the effect of GCS inhibition on malignant phenotypes and drug sensitivity and its molecular mechanism.

CHAPTER II

LITERATURE REVIEW

2.1 Cholangiocarcinoma (CCA)

Cholangiocarcinoma (CCA) is the primary cancer of bile ducts that arises from the ductular epithelium of the biliary tract (Khan et al., 2005; Lazaridis and Gores, 2005). It can be divided into 3 subtypes base on the tumor location on the biliary tree (Figure. 2.1), including 1) Intrahepatic cholangiocarcinoma (ICC), located nearby the secondary branches of left and right hepatic ducts, 2) Perihilar cholangiocarcinoma (pCCA) located at the secondary branches of left and right the hepatic ducts and common hepatic ducts until the origin of cystic duct, and 3) Distal cholangiocarcinoma (dCCA) tumors - located along hepatic ducts excluding the ampulla Vateri (Blechacz, 2017).

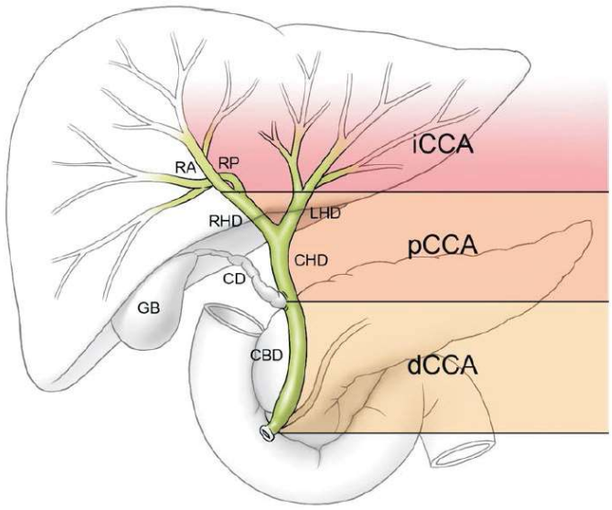

Figure 2.1 Classification of CCA. CCA can be divided into 3 subtypes, including Intrahepatic cholangiocarcinoma (iCCA), Perihilar cholangiocarcinoma (pCCA), and Distal cholangiocarcinoma (dCCA) (Blechacz, 2017).

ICC is the most common type of CCA. It has been considered to develop from biliary epithelial cells or hepatic progenitor cells, which can be subdivided into the conventional, bile ductular, or intraductal neoplasm type (Nakanuma et al., 2010). A marker of hepatic progenitor cells (HPCs), neural cell adhesion molecule was found in bile ductular and combined hepatocellular CCA types. Thus, this investigation was expected that ICC originates from hepatic progenitor cell. The other subtypes, dCCA, and pCCA have been considered to arise from the biliary epithelium and peribiliary glands. Transdifferentiation, followed by neoplastic conversion of normal hepatocytes into malignant cholangiocytes as a model of ICC tumors arising has also been considered (Rizvi and Gores, 2013) (Figure. 2.2). Surgical resection is the only chance for curing CCA. Inoperable tumors curing is inefficient when treated with chemotherapy and radiotherapy. Liver transplantation combines with neoadjuvant

chemoradiotherapy (administration of chemotherapy drugs before surgery to reduce the size of tumors) is suggested for long-term survival for patients (Khan et al., 2005).

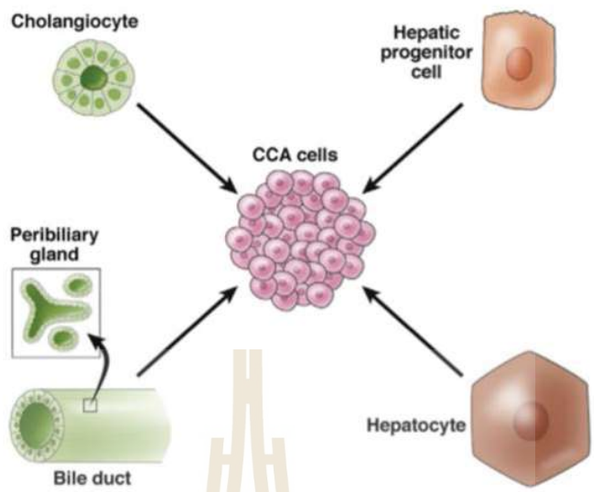

Figure 2.2 Cells of origin in CCA (Rizvi and Gores, 2013).

2.2 Epidemiology and risk factors for CCA

Globally, CCA has the incidence rate of almost 3% of all gastrointestinal cancers, which has different distribution among the different areas of the world. The CCA subtypes, ICC and ECC have different epidemiological features. Recent data has shown that the incidence and mortality rates of ICC have been increasing worldwide. Conversely, incidence and mortality rates of ECC have been decreasing (Figure. 2.3) (Shaib and El-Serag, 2004). The incidence has been reported in the Cancer Incidence in Five Continents initiative (Curado et al., 2007; Parkin et al., 2002). The data has shown approximately 20% of cases related to CCA. The majority occurs in patients older than 65 years. The marked occurrence of primary sclerosing cholangitis (PSC) in men causes CCA is more common in males. The high incidence rates of CCA occurs

in Asia have been reported as 113 per 100,000 in men and 50 per 100,000 in females (Tyson and El-Serag, 2011), when compared with the prognosis in Europe that only 0.5-2 per 100,000 in a population (Figure. 2.4) (Bragazzi et al., 2011).

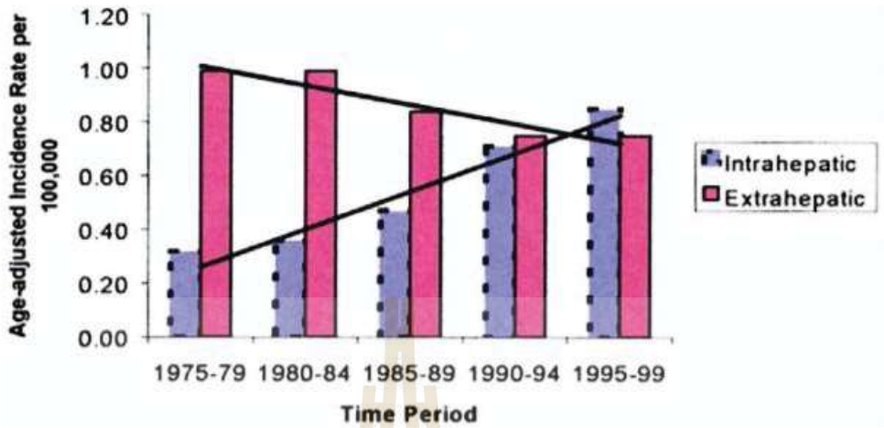

Figure 2.3 The incidence trends of ICC and ECC in the United States. Data showed for 5-year between 1975 and 1999, n = 2864 for ICC and n = 4317 for ECC (Lazaridis and Gores, 2005; Shaib and El-Serag, 2004).

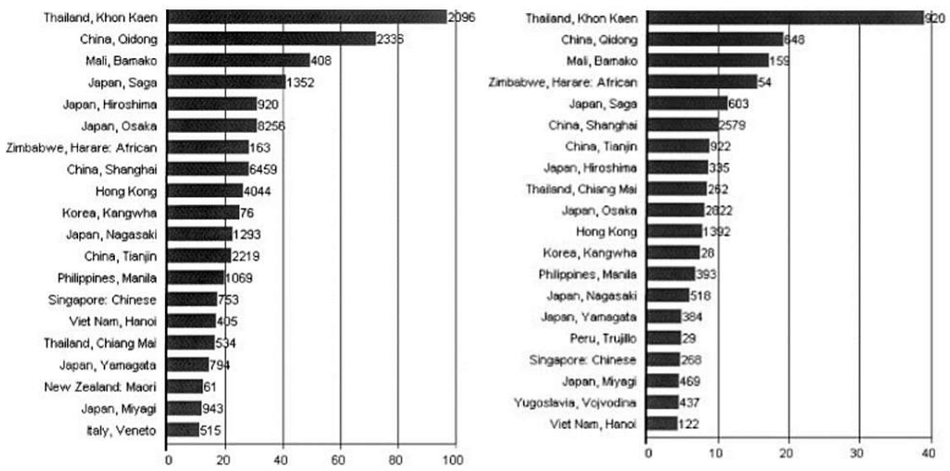

Figure 2.4 (Left) The age-adjusted incidence rates of CCA in men in 20 different geographic regions. CCA has been calculated as primary liver cancer that is not HCC. The frequency of cases is shown to the right of each bar. **(Right)** The age-adjusted incidence rates of CCA in women in 20 different geographic regions (Shaib and El-Serag, 2004).

Currently, several risk factors have been reported as the factors for CCA occurrence (Table 2.1) (Patel, 2006; Tyson and El-Serag, 2011). Geographic variability of different regions leads to variations of regional environmental risk factors. In the United States and Europe, the main causes of CCA are primary sclerosing cholangitis (PSC) and fibropolycystic liver disease, such as choledochal cyst. On the other hand, Asia especially South-East Asia has parasite infections as the major risk factors including liver fluke infection (Cai et al., 2011; Chapman, 1999; Suarez-Munoz et al., 2013). The survival rate of less than 5% generally dies within 5-years. 18-30 months are found for the median survival times for ICC. The shorter survival has been reported for 12–24 months in perihilar cholangiocarcinoma (Mosconi et al., 2009).

In Thailand, there are notably risk factors associated with CCA including, age, *Opisthorchis viverrini* infection, eating raw cyprinoid fish, family history of cancer, liquor consumption, and taking praziquantel (Kamsa-ard et al., 2018). Highly prevalent of CCA has been found in northeastern Thailand, especially Khon Kaen province, which has an incidence of CCA up to 36.3 in females and 87.7 in males per 100,000 population (Sriplung et al., 2006). The epidemiological and experimental evidence support that the major endemic risk factor of CCA in this region is liver fluke, *Opisthorchis viverrini*. Existing evidence has suggested possible mechanisms of liver fluke associated CCA, including chronic irritation, nitric oxide formation, intrinsic nitrosation, and activation of drug-metabolizing enzymes (Figure 2.5). The liver fluke can induce chronic inflammation that leads to oxidative DNA damage of the infected biliary epithelium and malignant transformation (Sriplung et al., 2006; Watanapa and Watanapa, 2002). If the damaged DNA involves cell cycle control genes, neoplastic changes may occur. Moreover, the data exhibited an increase in endogenous nitrosamine, which has cytotoxic and genotoxic in liver fluke infection patients (Watanapa and Watanapa, 2002).

Table 2.1 Overview of possible risk factors for CCA (Bergquist and von Seth, 2015).

Risk factor	Type of study	Types of CCA	Risk estimate (95% CI)
Liver flukes			
O. viverrini or *C. sinensis*	Meta-analysis	CCA	4.7 (2.2-9.8)
Biliary tract conditions			
Choledochal cysts	Case-control	ECC	47.1 (30.4-73.2)
	Case-control	ICC	36.9 (22.7-59.7)
Hepatolithiasis	Case-control	ECC	16.5 (1.9-146.3)
	Case-control	ICC	6.7 (1.3-33.4)
Choledocholithiasis	Case-control	ICC	8.8 (4.9-16.0)
	Case-control	ICC	22.5 (16.9-30.0)
	Case-control	ECC	34.0 (26.6-43.6)
Hepatic disorders			
Hepatitis B	Meta-analysis	ICC	5.10 (2.91-8.95)
	Meta-analysis	ICC	3.17 (1.88-5.34)
	Meta-analysis	ICC	3.42 (2.46-43.74)
Hepatitis C	Meta-analysis	ICC	4.84 (2.41-9.71)
	Meta-analysis	ICC	3.42 (1.96-5.99)
Cirrhosis	Meta-analysis	ICC	22.92 (18.24-26.79)
Other conditions			
Diabetes mellitus type II[a]	Meta-analysis	ICC	1.89 (1.74-2.07)
Obesity[a]	Meta-analysis	ICC	1.56 (1.26-1.94)
Alcohol use[b]	Meta-analysis	ICC	2.81 (1.52-5.21)

Abbreviations: CI, confidence interval; ICC, intrahepatic cholangiocarcinoma; ECC, extrahepatic cholangiocarcinoma.

[a] Less established risk factor.

[b] Study included alcoholic liver disease.

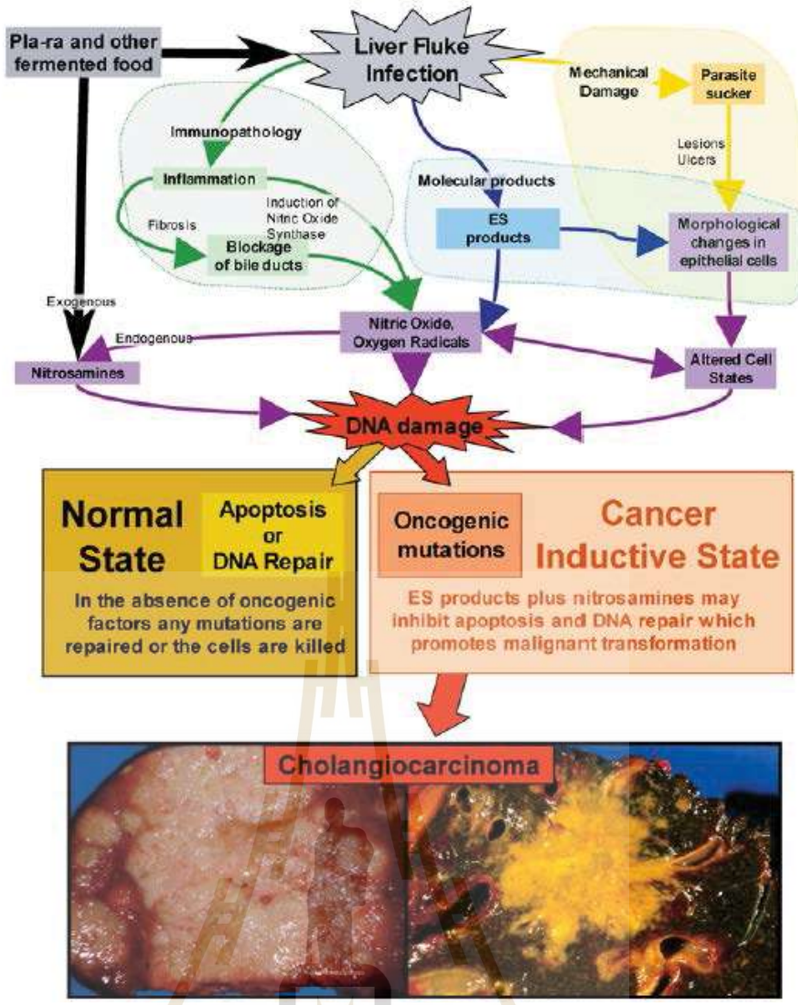

Figure 2.5 Mechanisms by which *Opisthorchis viverrini* infection leads to CCA initiation (Sripa et al., 2007).

2.3 The role of chemotherapy in CCA

Most of CCA patients are presented in advanced stage and unresectable with poor survival (Adeva et al., 2019; Alqahtani and Colombo, 2020). Surgical resection is the sole potentially treatment for long-term survival in all types of CCA (Sungkasubun et al., 2016). Although the patients have surgery, it is still disappointed with high rate recurrence approximately 60% (Aljiffry et al., 2009; Thongprasert, 2005). Chemotherapy has current used in postoperative treatment to increase the chances of a cure or unresectable tumor. The existing clinical data was collected from small case studies due to low incidence worldwide and uncontrolled. Therefore, different various chemotherapeutic agents have been tested. Currently, chemotherapeutic agents have been used either single or in combination with other agents to improve CCA therapy (B. R. Blechacz and G. J. Gores, 2008; Thongprasert, 2005). The most studied drugs were gemcitabine and 5-Fluouracil (5-FU) that were approved for treating CCA by U.S. Food and Drug Administration (FDA) in 2002 (B. R. Blechacz and G. J. Gores, 2008). In addition, both drugs also combined with other drugs, including cisplatin, oxaliplatin, leucovorin, streptozotocin, doxorubicin, epirubicin, mitomycin C, and etoposide (B. Blechacz and G. J. Gores, 2008; Choi et al., 2000; Ducreux et al., 1998; Lee et al., 2004; Patt et al., 2001; Takada et al., 1994). Either alone or co-administration of 5-Fluouracil (5-FU), cisplatin, and gemcitabine have improved survival in CCA patients (Asgar et al., 2015). The standard regimen of CCA treatment with advanced stage is combination of gemcitabine and cisplatin (Adeva et al., 2019; Alqahtani and Colombo, 2020; Vogel et al., 2014). Valle et al. reported the follow-up of CCA patients that received a different treatment between either gemcitabine alone or the combination of gemcitabine and cisplatin. The median

overall survival in gemcitabine/cisplatin group was significantly longer than gemcitabine alone (11.7 months VS 8.1 months, respectively (Valle et al., 2010). Double drugs combination showed significantly increased response rate and overall survival more than single drug. Triple or more drugs combination also were tested but there was no different in overall survival (Aiewtrakun et al., 2012). The additive or synergistic effect of cisplatin also found in other tumor types such as head and neck cancer, lung, and bladder. However, adverse effects was presented from cisplatin treatment results in drug resistant developed by tumor (Asgar et al., 2015; Valle et al., 2010).

2.4 Sphingolipids metabolism

Sphingolipids are a family of membrane lipids that play a key role in fluidity regulation. Many molecules are involved in sphingolipid metabolism, including sphingolipids ceramide, ceramide-1-phosphate, glucosylceramide, lactosylceramide, galactosylceramide, sphingosine, sphingosylphosphocholine, psychosine, and sphingosine-1-phosphate (S1P). Sphingolipids have an important role in cell growth, differentiation, senescence, and apoptosis (Ogretmen and Hannun, 2004). De novo synthesis of sphingolipids occurs in the endoplasmic reticulum (ER), and other ER-associated membranes, such as perinuclear and mitochondria-associated membranes (Figure 2.6).

Sphingolipids are firstly synthesized in the ER from serine and palmitic acid to proceed ceramide formation which is a key precursor for glycosphingolipids synthesis (Gault et al., 2010). Synthesized ceramides (Cer) at the ER membrane are transported to Golgi which is the site for sphingomyelin (SM) and glucosylceramide synthesis, via

the transfer protein CERT (which delivers Cer for SM synthesis) or vesicular transport (which delivers Cer for GlcCer synthesis) respectively. Next, the transport protein FAPP2 is required to deliver GlcCer for glycosphingolipid (GSL) synthesis. To synthesize complex GSLs, GlcCer is synthesized at the cytosolic side of the Golgi requires flipping to the inside of Golgi by ABC transporter, P-glycoprotein (MDR1). Consequently, vesicular transport appears to deliver SM and complex GSLs to the plasma membrane. Acid sphingomyelinase (SMase) and neutral SMase present in the outer and inner leaflet of the membrane bilayer, which are able to metabolize SM to Cer and followed by other bioactive lipids. Moreover, this metabolism network is also influential to the circulatory system especially in association with lipoproteins that are rich in SM and Cer and several enzymes can be found in the circulatory system, such as acid SMase, neutral ceramidase (CDase), and sphingosine kinase (SK). Internalization of membrane sphingolipids proceeds via the endosomal pathway, so SM and GlcCer are sent to the lysosomal compartment, and are subsequently degraded by SMase and glucocerebrosidase (GBA) to Cer. After that, Cer is hydrolyzed by ceramidase (CDase), producing sphingosine (Sph) that may get out from the lysosome even though its ionizable positive charge favors partitioning in lysosomes. So, sphingosine kinase 1 (Sk1) that is present near the lysosome is assumed to act as a 'trap' to trap Sph via phosphorylation. To recycle Sph to Cer, a salvage pathway (showed by dashed arrows in figure 2.6) is required by the action of sphingosine-1-phosphate (S1P) phosphatases in the ER and Sph from this salvage pathway is soluble in the cytosol and move across membranes (Figure 2.6) (D'Angelo et al., 2007; Hannun and Obeid, 2008).

Taken together, ceramides are key lipids in sphingolipid metabolism pathways and signaling cascades regulating critical physiological functions in cells (García-González et al., 2018; Ogretmen, 2018).

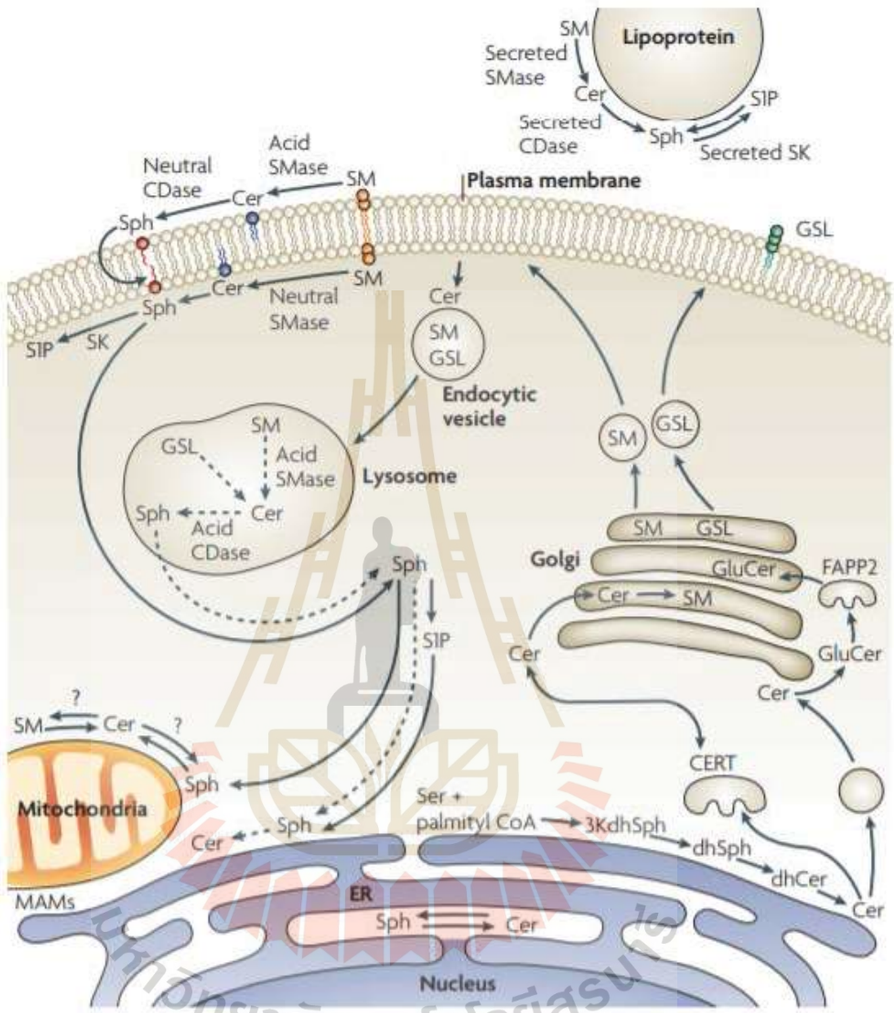

Figure 2.6 Compartmentalization of sphingolipid metabolism pathways (Hannun and Obeid, 2008).

2.5 Ceramide metabolism and cancer

Ceramide is considered as the center of sphingolipid metabolism that accomplished by hydrolysis of sphingomyelin, salvage and recycling pathway and de novo synthesized from palmitate and serine (Figure 2.7). Ceramides compose of sphingosine long-chain base (LBC) containing 18 carbons (d18), amide-linked to fatty acyl chain that has variable numbers of carbons (14 to 26). Ceramide synthesis occurs in the ER, but proteins that are associated with ceramide metabolism are located on the mitochondria membrane (García-González et al., 2018; Ogretmen, 2018). Synthesis of ceramide can be induced by different stimuli, including increased intracellular concentration of postprandial palmitate, hypoxia, intracellular stress, activation of the neutral sphingomyelinase, and inflammatory stimuli mediated by Toll-like receptor 4 (TLR-4). TLR-4 is involved in response to tumor necrosis factor-alpha (TNFα) and interferon-gamma (IFNγ) signaling, which can induce the expression of enzymes such as serine palmitoyltransferase (SPT).

De novo synthesis is activated by increasing saturated fatty acid accumulation. Then, palmitoyl-CoA and serine are conjugated by SPT to form an unstable molecule, 3-ketosphinganine that is rapidly reduced to dihydro-sphingosine, followed by N-acylation by ceramide synthase (CerS) located in the ER and nuclear envelope leading to dihydroceramides production. After that, dihydroceramides can be converted into ceramides by dihydroceramide desaturase (DES 1 and 2). From here, ceramides are delivered by vesicular transport to the Golgi apparatus or via a carrier protein, such as ceramide transfer protein (CERT) (Garcia-Gonzalez et al., 2018; Hannun and Obeid, 2008). Ceramide is a substrate for GlcCer synthesis (delivered by vesicular transport) that GlcCer is synthesized at the cytosolic surface of the ER by glucosylceramide

synthase (GCS). Synthesized GlcCer is then translocated across the Golgi membrane to trans-Golgi for GSLs synthesis (Boslem et al., 2012; Hannun and Luberto, 2004).

To synthesize complex GSLs, flipping activity from flippase and MDR1 is required for flipping GlcCer to the inside of the Golgi (Antje et al., 2002; Chalat et al., 2012; Higgins and Gottesman, 1992; Y.-Y. Liu et al., 2013). FAPP2 is a lipid transfer protein that transfers GlcCer to synthesize lactosylceramide (LacCer) by lactosylceramide synthase (LacCerS). Several lines of evidences showed that FAPP2 is associated with apical transport from the Golgi complex in epithelial of Madin–Darby canine kidney (MDCK) cells. The in vitro study revealed FAPP2 is involved in tubule formation of flat membrane sheets that depends on the phosphoinositide-binding activity of its PH domain and also involved ciliogenesis in MDCK cells (Cao et al., 2009; Vieira et al., 2006; Yamaji et al., 2008). Downregulation of the FAPP2 gene by siRNA transfection was reported to increase the apoptosis rate in glioma and breast cancer when incubated with Fas ligand (FasL), but was not correlated with Fas expression (Tritz et al., 2009).

For production of LacCer in the Golgi, lactosylceramide synthase (LacCerS), also known as UDP-galactose:glucosylceramide β-1→4-galactosyltransferase, and encoded by β4GalT-5 or β4GalT-6, plays a role to transfer galactose from UDP-galactose to GlcCer to synthesize LacCer. LacCerS was reported that synthesized by β4GalT-6 in rat brain but LacCerS is mainly produced from β4GalT-5 during early mouse embryogenesis (Hosain et al., 2013; Nishie et al., 2010; Takizawa et al., 1999). LacCer is a precursor for ganglioside series, including the isoglobo series, globo series, lacto series, neolacto series, and ganglio series (Merrill Jr, 2011).

Mostly of gangliosides are synthesized from LacCer, excluding GM4 that is synthesized from galactosylceramide (GalCer). GM3 is sialylated LacCer synthesized, by CMP-N-acetyl-neuraminate:lactosylceramide α2→3-sialyltransferase (ST-I or known as GM3 synthase). Subsequently, addition of sialic acids to GM3 can synthesize GT3 by activity of CMP-sialic acid:GD3 α2→8-sialyltransferase (ST-III, also known as GT3 synthase) and GD3 by CMP-sialic acid:GM3 α2→8-sialyltransferase (ST-II or known as GD3 synthase) (Robert K et al., 2011). β1,4-N-acetylgalactosaminyltransferase (GM2/GD2 synthase) is responsible for converting GM3→GM2 and GD3→GD2 and LacCer→asialo-GM2 (GA2) (Furukawa et al., 2002).

For globo-series GSLs synthesis, α1→4-galactosyltransferase, α1→4GalT (Gb3 synthase) is required to synthesized globotriaosylceramide Gb3 (Labilloy and Weisz, 2019; Russo et al., 2018), followed by synthesis of Gb4 and Gb5 by β3GalNAcT (Gb4 synthase) and β1,3-galactosyltransferase-V (β3GalT-V), respectively. In addition, Gb5 is also known as SSEA-3 (stage-specific embryonic antigen-3) that using as a marker for embryonic stem cells (Suila et al., 2010).

For synthesis of lacto-/neolacto-series gangliosides, β1,3-N-acetylglucosaminyltransferase-V (β3Gn-T5) is an enzyme that transfers GlcNAc in β1,3-linkage to lactosylceramide producing GlcNAcβ1,3Galβ1,4Glc-ceramide to initiate the formation of the gangliosides (He et al., 2018). The clusters of GSLs and other membrane components which are found within the cell membrane can be formed as GSL-enriched microdomains (GEMs), glycosynapses, and lipid rafts that play an important role in membrane trafficking, regulating signal transduction and associated with cell-cell adhesion. Thus, it can modulate cells responses to stress and play a role

in development of drug resistance in cancer. It was reported that stem cell properties are modulated by GEMs through the c-Src/β-catenin and extracellular-signal-regulated kinase 1/2 (ERK1/2) signaling pathways that are associated with tumorigenesis and cancer progression (Hosain et al., 2013).

Moreover, ceramide can be converted to galactosylceramide (GalCer) by the ER transmembrane protein, galactosylceramide synthase or UDP-galactose:ceramide galactosyltransferase (GalCerS or CGT) which transfers galactose from UDP-galactose to ceramide at the 1-hydroxyl moiety (Shah, 1971; Sprong et al., 1998; Stahl et al., 1994). GalCer is a major component of myelin found in many epithelial cells, which serves as one of the first specific markers that is synthesized during differentiation of Schwann cells and oligodendrocytes (Schaeren-Wiemers et al., 1995; Sprong et al., 1998). GalCer is transported from the ER to the Golgi and serves as a precursor of sulfatide and (3-sulfogalactosylceramide) and GM4 that is synthesized in the the Golgi. Cerebroside sulfotransferase (CST) is responsible for adding a sulfate to galactose at 3'-OH group for sulfatide synthesis. For GM4 synthesis, sialyltransferase (SAT) is the key enzyme that catalyzes GalCer conversion to GM4 (Jackman et al., 2009). Transporting GalCer from the ER-Golgi to lipid rafts through recycling endosomes involves the aid of CLN3p (neuronal ceroid-lipofuscinosis-3 protein), GalCer transporter (an Haack et al., 2011; Persaud-Sawin et al., 2007; Rusyn et al., 2008).

Ceramide is also delivered to the Golgi which converted to sphingomyelin (SM) by sphingomyelin synthase (SMS) (Olsen and Færgeman, 2017). SMS has three homologs including, SMS1, SMS2 and SMS3, but only SMS1 and SMS2 promote SM synthesis. SM can be degraded by sphingomyelinase (Olsen and Færgeman, 2017; Zheng et al., 2019a). Moreover, ceramide can be deacetylated to form sphingosine by

action of ceramidases (CDase) and sphingosine can be converted back to ceramide by ceramide synthase (CerS) (Espaillat et al., 2015).

In cancer, ceramide can function as a potent tumor suppressor lipid that can limit cancer cell proliferation by inducing apoptosis and cell growth arrest. Several key players such as protein phosphatase 2A, p38, JUN N-terminal kinase (JNK), AKT, protein kinaseCζ (PKCζ) and surviving are well established to communicate in ceramide signaling (Morad et al., 2012). As ceramide is a second messenger, it could activate at the post-receptor action of a variety of cytokines, hormones, and growth factors, including members of the tumor necrosis factor superfamily (Liu et al., 2004; Zheng et al., 2019a). Ceramide can be produced in cancer cells in order to respond to treatment with anticancer drugs that are commonly used in the clinic, such as doxorubicin, paclitaxel, vinblastine, etoposide, and actinomycin D. However, defect in ceramide production and metabolism can cause cellular resistance to apoptosis in response to radiotherapy and chemotherapy (Liu et al., 2004).

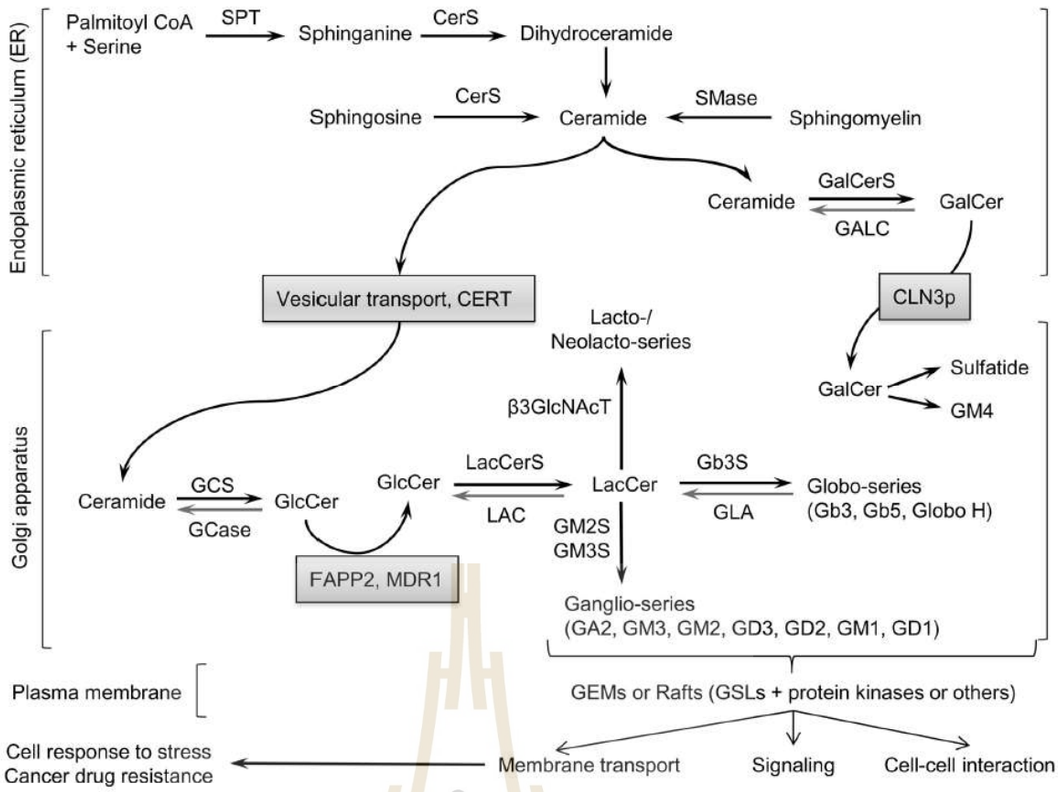

Figure 2.7 Glycosphingolipid biosynthesis and its cellular functions. SPT, serine-palmitoyl transferase; CerS, ceramide synthase; CERT, ceramide transporter; GCS, glucosylceramide synthase; GalCerS, galactosylceramide synthase; GALC, galactocerebrosidase (β-galactosidase); LacCer, lactosylceramide; LacCerS, lactosylceramide synthase; Gb3S, globotriaosylceramide synthase; GCase, glucocerebrosidase (β-glucosidase); GLA, α-galactosidase A; GM2S, GM2 synthase; GM3S, GM3 synthase; GEMs, GSL-enriched microdomains (Y.-Y. Liu et al., 2013).

2.6 Glucosylceramide synthase and cancers

Glucosylceramide synthase (GCS) is a transmembrane protein, EC2.4.1.80, 44.9 kDa, known as UDP-glucose: ceramide glucosyltransferase encoded by human

UGCG (1730 bp) that present on the cis-Golgi and catalytic site is located at cytoplasm. GCS is the rate-limiting enzyme that controls the first glycosylation step in the biosynthesis of glycosphingolipids by transfers glucose from UDP-glucose to ceramide and produces glucosylceramide (GlcCer) (Liu et al., 2004; Y.-Y. Liu et al., 2013; Roh et al., 2015). Many studied reported that GlcCer involved in several cellular processes including oncogenic transformation, cell proliferation, metastasis, and cell differentiation. High expression of GCS has been reported in various cancer types, including breast, cervix, colon, non-small cell lung cancer, and papillary carcinoma. GCS has been showed to promote cancer progression through generation of glucosylceramide to promote glycosphingolipid (GSL) synthesis for enhancing cell growth and cell survival. Therefore, increase apoptosis was demonstrated after inhibition of GCS expression (Bleicher and Cabot, 2002). Overexpression of GCS was found in multidrug-resistant cells in several types of cancer and contributed to poor chemotherapy response (Gupta and Liu, 2013; Liu et al., 2004; Liu, Patwardhan, Xie, et al., 2011). Upregulation of GCS expression was highly correlated with ER-positive and HER2-positive in metastatic breast cancer that (Liu et al., 2014).

Overexpression of GCS enhanced cell proliferation and abnormal nuclear morphology in breast cancer cells (MCF-7 cells) by alteration of GEMs and accumulation of Gb3 and GlcCer through the activation of Akt and ERK1/2 (Wegner et al., 2018). Moreover, Ceramide glycosylation by GCS also contributed to breast cancer stem cell properties by increase of globo-series GSLs (particularly Gb3). Gb3 then activated c-Src/β-catenin signaling pathway followed by upregulation the essential factors for stem cells involved in tumorigenesis (Gupta et al., 2012). BCL-2, anti-apoptotic protein and BAX, apoptotic protein are mediated cell death through apoptosis

pathway (Naseri et al., 2015). The study in human liver cells line HL-7702 revealed that GCS associated with cell survival and apoptosis via BCL-2/Bax signaling pathway (Li et al., 2017). Tumor necrosis factor- α (TNF-α) is a mediator of inflammation that producing reactive oxygen species (ROS) and leading cell death (Kim et al., 2010). Overexpression of GCS influenced TNF-α-induced apoptosis which caused cellular resistance to TNF-α (Liu, Han, Giuliano, Ichikawa, et al., 1999). Inhibition of GCS can restore p53 dependent apoptosis to sensitize doxorubicin in mutant p53 in ovarian cancer, however there is not affect in wild-type p53. Additionally, GCS suppression was reported to increase the levels of phosphorylated p53 and p53-responsive genes, including $p21^{Waf1/Cip1}$, Bax, and Puma (Liu, Patwardhan, Bhinge, et al., 2011; Ogretmen, 2018). Delay cytokinesis which enhanced cell growth arrest by induced bi-nucleation of atypical hepatocytes was also found after GCS silencing (Jennemann et al., 2017). Glutamine, non-essential amino acid was known involved in cell metabolism in tumor. In tricarboxylic acid (TCA) cycle, glutamine serves as a fuels anaplerosis producing fatty acid and nucleotide as well as important for cellular energy metabolism. Schömel et al revealed that overexpression of GCS in breast cancer cells influenced glutamine uptake subsequently increased glutamine oxidation and cell proliferation (Schömel et al., 2019).

Taken together, GCS has the potential roles in several aspects for promoting cancer progression as summarized in Table 2.2. However, there is no evidence of GCS in CCA. Thus, the GCS expression and its role need to be elucidated in CCA.

Table 2.2 Lists of GCS-associated cancer progression.

Type of cancer	GCS-associated cancer progression	Refs
Breast	GCS upregulated in metastatic breast cancer that highly associated with ER-positive and HER2-positive	(Gupta and Liu, 2013; Liu et al., 2004; Liu, Patwardhan, Xie, et al., 2011)
Breast	GCS overexpression enhanced cell proliferation and multidrug resistance through alteration of GEMs and accumulation of Gb3 and GlcCer, which subsequently activated Akt and ERK1/2 signaling pathway.	(Wegner et al., 2018)
Breast	GCS overexpression influenced cytotoxicity resistance to TNF-α-induced apoptosis which caused cellular resistance to TNF-α	(Liu, Han, Giuliano, Ichikawa, et al., 1999)
Breast	GCS overexpression increasing of breast cancer stem cell numbers. Gb3 was significantly higher and Gb3 subsequently activated c-Src/β-catenin signaling.	(Gupta et al., 2012)

Table 2.2 Lists of GCS-associated cancer progression (Continued).

Types of cancer	GCS-associated cancer progression	Refs
Breast	GCS overexpression influenced glutamine uptake subsequently increased glutamine oxidation and cell proliferation.	(Schömel et al., 2019)
Lung	Silencing of GCS decreased metastasis.	(Inokuchi et al., 1990)
Ovarian	GCS suppression increased p53-responsive genes, including $p21^{Waf1/Cip1}$, Bax, and Puma	(Liu, Patwardhan, Bhinge, et al., 2011; Ogretmen, 2018)
Liver	Silencing of GCS has significantly inhibited cell proliferation and increased apoptosis via Bcl-2/Bax signaling pathway.	(Li et al., 2017)
Liver	Impaired cytokinesis was found when GCS silencing	(Jennemann et al., 2017)
Colon	GCS overexpression increased numbers of CSCs numbers through Zeb1 and β-catenin transcription factors which essential for EMT	(Hosain et al., 2016)
Melanoma	GCS silencing suppressed melanoma tumor growth in vivo	(Weiss et al., 2003)

2.7 Glucosylceramide synthase and drug resistance in cancers

Overexpression of GCS is a cause of acquired drug resistance in multiple cancer cell lines. Liu et al., shown that overexpression of GCS significantly increased expression of globo series GSLs (globotriaosylceramide Gb3, globotetraosylceramide Gb4) on GSL-enriched microdomain (GEM). Then, activated cSrc kinase, decreased β-catenin phosphorylation, and increased nuclear β-catenin. Changing of various GSLs levels can alter lipid-lipid interactions or lipid-protein interactions and also affect the action of protein kinases (cSrc kinases) in GEMs of the plasma membrane (Y.-Y. Liu et al., 2013). Next, they also demonstrated that GCS can upregulate MDR1 expression by activation of cSrc signaling and TCF4/β-catenin recruitment on the MDR1 gene promoter (Figure 2.8) (Liu et al., 2010).

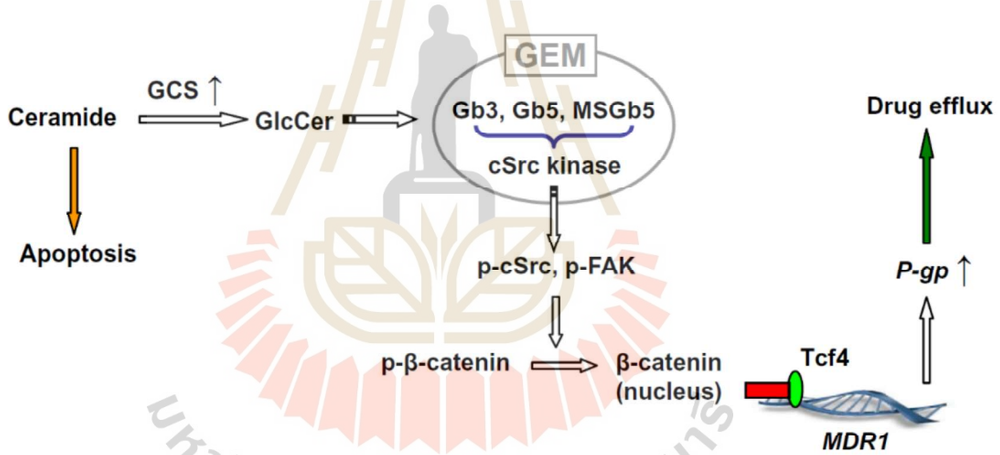

Figure 2.8 GSLs synthesis and MDR1 expression. GCS, glucosylceramide synthase; GlcCer, glucosylceramide; Tcf4, T-cell factor 4; FAK, focal adhesion kinase; cSrc, proto-oncogene (Schmidt-Ruppin A-2); Gb3, globotriaosylceramide; Gb5, globopentaosylceramide; MSGb5, monosyl-Gb5 (Liu et al., 2010).

It is known that anticancer agents can cause multidrug-resistant by promoting malignant pluripotency (Fairchild et al., 1987; Gupta and Liu, 2013). Many studied showed that overexpression of GCS increased MDR in several cancer cell lines (Liu et al., 2016). Several drugs including tamoxifen, verapamil, and cyclosporin A demonstrated the ability to inhibit MDR1. Tamoxifen decreased glucosylceramide synthesis and sensitized MCF-7AdrR cells to adriamycin (Lavie et al., 1997). Co-suppression of GCS and MDR1 in MCF-7/AdrR breast cancer cells could enhanced adriamycin sensitivity comparing with single suppression (Zhang et al., 2009). High expression of GCS has been reported to associated with Vinca alkaloid vinorelbine (VNR) resistance in A549 and CL1-5 human lung adenocarcinoma by increasing Bcl-xL expression (Chiu et al., 2015). Moreover, GCS overexpression in MCF-7 breast cancer cells (MCF-7/GCS) was associated with ceramide hyperglycosylation and lead to low response to adriamycin (Liu, Han, Giuliano and Cabot, 1999). Unable to accumulate ceramide was showed in paclitaxel-resistant T98G (PCL-R-T98G) and temozolomide-resistant T98G (TMZ-R-T98G) glioblastoma cells when GCS activity was presence. Suppression of GCS demonstrated sensitized these cells to paclitaxel and temozolomide (Giussani et al., 2012). GCS overexpression also contributed to sorafenib resistance in hepatocellular carcinoma (HCC). GCS was upregulated when exposed with sorafenib and markedly increased in sorafenib-resistant cells. Inhibition of GCS could resensitize sorafenib resistant cells to sorafenib. Furthermore, co-exposure of sorafenib and GCS inhibition enhanced cytochrome c releasing and ATP depletion leading cell death (Stefanovic et al., 2016). Cisplatin is a compound that widely used in several types of human solid neoplasms by interfering DNA repair mechanism leading to DNA damage and enhancing apoptosis. GCS and P-gp

expression were also reported significantly correlated with cisplatin resistance in head and neck cancer (HN9-cisR). Downregulation of GCS resensitized cisplatin resistant cells to cisplatin (Roh et al., 2015). Furthermore, overexpression of GCS was also found in other multidrug-resistant cancer cells such as melanoma, epidermoid carcinoma and leukemia (Gouazé et al., 2004; Xie et al., 2008). High expression of GlcCer was associated in multidrug-resistant cancer cells. GlcCer was accumulated in vinblastine-resistant epidermoid cancer cells, breast cancer cells, and ovarian adenocarcinoma cells that contributed to drug resistance and alterations in GlcCer metabolism (Lavie et al., 1996; Lavie et al., 1997). Doxorubicin (Dox) can increase ceramide-mediated apoptosis production by the *de novo* synthesis pathway in both cancer cells and noncancerous cells (Y.-Y. Liu et al., 2013; Liu et al., 2008; Lucci et al., 1999). In breast cancer, GCS have a crucial the opposite effects of Dox treatment between breast cancer stem cells (BCSCs) and bone marrow stem cells (BMSCs) in vivo, in which GCS suppressed BMSCs whereas GCS enhanced BCSCs progression (Figure. 2.9) (Bhinge et al., 2012; Gupta and Liu, 2013).

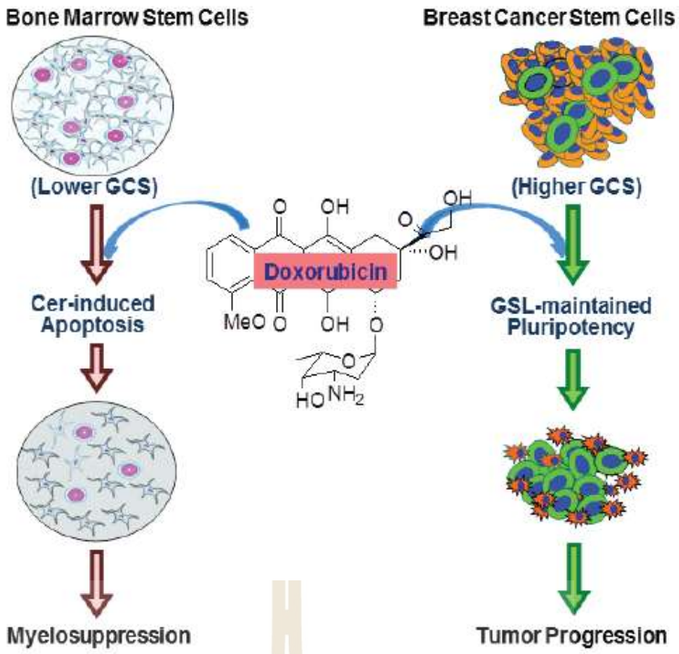

Figure 2.9 The opposite effects of doxorubicin in breast cancer stem cells versus bone marrow stem cells (Bhinge et al., 2012).

Upregulation of GCS expression in ovary cell lines, leukemia cell lines and invasive ductal breast cancer was also showed in Dox treatment. It was showed that DOX could induced GCS expression (Zhang et al., 2012). Gather all information suggested that inhibition of GCS expression alone was not able to reverse multidrug resistance in all cell types. However, to improve drug sensitivity in CCA by inhibiting GCS expression need to be explored. Taken together, GCS associated in multi-drug resistant as summarized in Table 2.3.

Table 2.3 List of GCS-associated multidrug-resistance in cancer cell lines (Huang et al., 2011).

GCS expression	Cancer cell lines	Drugs	Resistance	Refs
GCS ↑	Vinblastine-resistant epidermoid cancer cells (KB-V.01, KB-V.1, and KB-V1)	Vinblastine	↑	(Gouazé et al., 2004; Lavie et al., 1996)
	Adriamycin-resistant epidermoid carcinoma (KB-A.05 and KB-A1)	Adriamycin	↑	(Gouazé et al., 2004)
	Adriamycin-resistant breast cancer cells (MCF-7-AdrR)	Adriamycin	↑	(Lavie et al., 1997; Zhang et al., 2009)
	Developed breast cancer cells (MCF-7-GCS)	Adriamycin	↑	(Liu, Han, Giuliano and Cabot, 1999)
	VNR-resistant lung adenocarcinoma (A549 and CL1-5)	Vinca alkaloid vinorelbine	↑	(Chiu et al., 2015)
	Adriamycin-selected colon cancer (SW620Ad1000)	Adriamycin	↑	(Gouazé et al., 2004)
	Vincristine-resistant leukemia cells (HL-60/VCR)	Vincristine	↑	(Gouazé et al., 2004)

Table 2.3 Lists of GCS-associated multidrug-resistance in cancer cell lines (Huang et al., 2011) (Continued).

GCS expression	Cancer cell lines	Drugs	Resistance	Refs
GCS ↑	Adriamycin-resistant leukemia cells (K562/A02)	Adriamycin	↑	(Xie et al., 2008)
	Etoposide selected resistant subline melanoma cells (MeWoEto1)	Etoposide	↑	(Gouazé et al., 2004)
	Sorafenib-resistant liver cancer (HepG2-R and Hep3B-R)	Sorafenib	↑	(Stefanovic et al., 2016)
	Head and neck cancer (HN9-cisR)	Cisplatin	↑	(Roh et al., 2015)
GCS ↓	Drug-resistant breast cancer (NCI2ADR-RES and EMT/AR1)	Doxorubicin	↓	(Liu et al., 2010; Patwardhan et al., 2009)
	Adriamycin-resistant breast cancer cells (MCF-7-AdrR)	Adriamycin	↓	(Lavie et al., 1997; Zhang et al., 2009)

Table 2.3 Lists of GCS associated multidrug-resistance in cancer cell lines (Huang et al., 2011) (Continued).

GCS expression	Cancer cell lines	Drugs	Resistance	Refs
	Doxorubicin-resistant breast cancer cells (MCF-7-AdrR)	Paclitaxel Vinblastine	↓	(Gouazé et al., 2005)
	VNR-resistant lung adenocarcinoma (A549 and CL1-5)	Vinca alkaloid vinorelbine	↓	(Chiu et al., 2015)
GCS↓	Paclitaxel-resistant glioblastoma cells (PCL-R-T98G)	Paclitaxel	↓	(Giussani et al., 2012)
	Temozolomide-resistant glioblastoma cells (TMZ-R-T98G)	Temozolomide	↓	(Giussani et al., 2012)
	Doxorubicin-resistant ovarian carcinoma cells (A2780-AD)	Doxorubicin	↓	(Liu et al., 2010)

Table 2.3 Lists of GCS-associated multidrug-resistance in cancer cell lines (Huang et al., 2011) (Continued).

GCS expression	Cancer cell lines	Drugs	Resistance	Refs
GCS↓	Doxorubicin-selected cervical cancer cells (KB-A1)	Doxorubicin	↓	(Liu et al., 2010)
	Drug-resistant colon cancer cells (SW620/Ad)	Doxorubicin	↓	(Liu et al., 2010)
	Adriamycin-resistant leukemia cells (K562/A02)	Adriamycin	↓	(Xie et al., 2008)
	Sorafenib-resistant liver cancer (HepG2-R and Hep3B-R)	Sorafenib	↓	(Stefanovic et al., 2016)

2.8 Targeting glucosylceramide synthase for restraining drug resistance

2.8.1 Antisense Oligonucleotide

Overexpression of GCS could upregulate MDR1 expression and contributed to poor response to chemotherapy (Liu et al., 2010). Directly inhibited the GCS gene using genetic approach is an option to sensitize multi-drug resistant in tumors. A full-length of GCS antisense (asGCS) transfection in adriamycin-resistant breast cancer showed that GCS was suppressed and lead to increased ceramide and apoptotic effector level resulting in restored drugs sensitivity to taxanes, anthracyclines, Vinca alkaloids, and actinomycin D (Liu et al., 2000; Y. Y. LIU et al., 2001). Although, several of asGCS oligonucleotide were designed (Table 2.4), but especially ODN-7 provided high efficiency to increase doxorubicin sensitivity on doxorubicin-resistant breast and ovarian cancer cells by enhancing drug uptake (Liu et al., 2004). A new mixed-backbone oligonucleotide (MBO-asGCS), second generation of asGCS was designed for more stability, efficiency, and selectivity. It was showed that MBO-asGCS could increase doxorubicin sensitivity by 83-fold in human NCI/ADR-RES, and 43-fold in murine EMT6/AR1 breast cancer cells (Patwardhan et al., 2009). Furthermore, GCS suppression by small interfering RNA of GCS (siGCS) or GCS-shRNA transfection revealed that silencing GCS can restore the response of various drugs such as paclitaxel, adriamycin, and vinblastine in breast cancer (Gouazé et al., 2005; Sun et al., 2010; Zhang et al., 2009), sorafenib in liver cancer (Stefanovic et al., 2016), and cisplatin in head and neck cancer (Roh et al., 2015).

2.8.2 A chemical GCS inhibitors

Chemical inhibitors of GCS have been widely used for studying glycosphingolipids. There were two classes of GCS inhibitors, including 1) a group analogs of D-threo-1-phenyl-2-decanoylamino-3-morpholino-propanol (PDMP) also called 'P' drugs, including DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP), and Genz-123346 and 2) a group of imino sugars such as N-butyl-deoxynojirimycin (NB-DNJ), N-butyl-deoxygalactonojirimycin (C_4DGJ) and N-nonyl-deoxygalactonojirimycin (C_9DGJ) as showed in Figure 2.10 (Y.-Y. Liu et al., 2013; Payne, 2014). P drugs are ceramide analogue that can mimic and target ceramide effectors at downstream (Herzer et al., 2016; J. Liu et al., 2013). The difference structures of PDMP and PPMP are length of fatty acyl chains and amino acid substitution with shorter (PDMP: C_{10}: decanoyl) and longer (PPMP: C_{16}: palmitoyl). Longer fatty acyl chains showed more potent to inhibit GlcCer than shorter in mammals (Abe et al., 1992; Kovács et al., 2000). PPMP is a stronger GCS inhibitor than PDMP by disruption of Golgi and abrogated trafficking ER for Golgi rebuilding (Nakamura et al., 2001). PPPP is synthesized based on PDMP structure to increase specificity and efficacy by replacing morpholine group with pyrrolidine group. The accumulation of ceramide and the reduction of GlcCer were reported in PDMP and PPMP whereas PPPP only effect GlcCer (Nicholson et al., 1999). However, PDMP could inhibit other enzymes in GSLs metabolism with non-specific action. Furthermore, it has reported that PDMP affected calcium homeostasis and membrane fluidity (Chai et al., 2011). Genz-123345 is a novel GCS inhibitor that is improved for increasing specificity (Chai et al., 2011; McEachern et al., 2007). PDMP and PPMP were a widely used GCS

inhibitor for understanding lipid metabolism. PPMP generally exhibited using lower concentration for treatment than PDMP (Alam et al., 2015). Both inhibitors could enhance the response of human glioblastoma and neuroblastoma to paclitaxel and temozolomide (Dijkhuis et al., 2006; Giussani et al., 2012). Missense mutation of p53-R273H in colon cancer cells exhibited that PDMP sensitized the mutant to doxorubicin and restored the p-53 mutant and eliminated pluripotency property (Hosain et al., 2016). GCS inhibition by iminosugar such as N-butyl-deoxynojirimycin (NB-DNJ) also known as miglustat or OGT-18 was accepted for Gaucher disease type 1 treatment. It has reported that NB-DNJ not only inhibited GCS, but also inhibited glucosidases and intestinal glycosidases, which caused side-effect of life-long therapy in patients (Andersson et al., 2000; Butters et al., 2005; McEachern et al., 2007; Norris, 2012). N-butyl-deoxygalactonojirimycin (C_4DGJ) and N-nonyl-deoxygalactonojirimycin (C_9DGJ), are more selective to GCS in this class (Andersson et al., 2000; Y.-Y. Liu et al., 2013). C_9DGJ restored paclitaxel and temozolomide to drug resistant human glioblastoma cells similar with PDMP and PPMP (Giussani et al., 2012). Similar observation was demonstrated in the study of chronic lymphocytic leukemia (CLL), in which C_4DGJ and C_9DGJ sensitized CLL cells to cytotoxic agents (Gerrard et al., 2009). However, utilization of chemical GCS inhibitors still showed low specificity to GCS and limited with micromolar level inhibitory activity (Larsen et al., 2012).

Table 2.4 Characteristics of antisense oligonucleotides against GCS gene (Liu et al., 2004).

Oligomer	Sequence	Target	Hybridization Strength Parameter			
			-dG	Hairpin	Dimer	Percentage GC
					Kcal/mol	
ODN-1	GCCAGGTCCAGCAGCGCCAT	Start code (1-20)	29.1	2.3	-6.2	70
ODN-2	CCATAATAT CCCATCTGA AC	ORF (929-938)	21.1	3.4	-1.4	40
ODN-3	GCAGAGATA TAGTATCTT GG	ORF (579-598)	20.6	2.2	-3.2	40
ODN-4	GATTAAGTT AGGATCTAC CC	ORF (181-200)	21.1	2.6	-3.0	40
ODN-5	GCTGTAGTT ATACATCTA GG	ORF (1172-1191)	20.4	2.9	-3.0	40
ODN-6	CCACCTATA AACAATCTA GC	ORF (327-346)	21.4	3.0	-2.3	40
ODN-7	ACGGCCATT CCCTCCAAG GC	ORF (18-37)	28	0.95	-5.5	65
ODN-8	CTGCTGTAC CCCCACAGC GT	ORF (1146-1166)	27.2	-1.5	-5.8	65
ODN-9	TATCTTGGA TGTGAAGTT CC	His^{193} (568-585)	22.5	1.3	-3.5	45
ODN-10	GACATTGCA AACCTCCAA CC	Exon-7 (739-756)	25.2	2.2	-6.8	50
ODN-11	ATTCCTGTC ACACAAAAG AA	Cyc^{207} (613-632)	22.9	2.0	-4.2	35

ODN, oligodeoxyribonucleotide; ORF, open reading frame, Oligonucleotides were analyzed by HYBsimulator program.

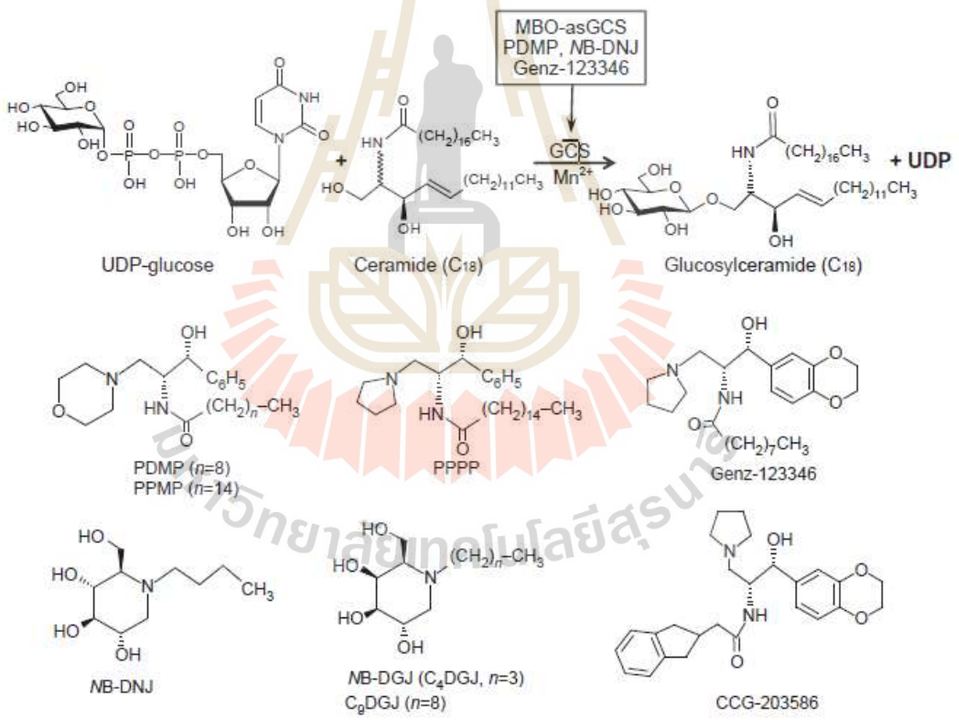

Figure 2.10 Structure of GCS inhibitors (Y.-Y. Liu et al., 2013).

CHAPTER III

MATERAILS AND METHODS

3.1 Biological materials

3.1.1 CCA tissues

The twenty-nine frozen CCA tissues and paired adjacent tissues were obtained from the specimen Bank of the Liver Fluke and Cholangiocarcinoma Research Institute, Faculty of Medicine, Khon Kaen University. Written informed consent was obtained from each subject. The Khon Kaen University Ethics Committee for Human Research and the Ethics Committee for Human Research (HE521209), Suranaree University of Technology (EC-57-25), approved the protocol for sample collection.

3.1.2 CCA cell lines

Three CCA cell lines, KKU-055, KKU-100, and KKU-213A were established by Dr. Banchob Sripa, the Liver fluke and Cholangiocarcinoma Research Institute, Khon Kaen University (Sripa et al., 2005; Sripa et al., 2020). All three cell lines were obtained from the Japanese Collection of Research Bioresources Cell Bank.

3.1.3 Chemicals and reagents

All chemicals are obtained from various companies as listed below.

Table 3.1 The lists of chemicals and their suppliers.

Chemicals	Suppliers
Dulbecco's Modified Eagle Medium (DMEM)	Gibco
Opti-MEM I	Gibco
Sodium hydrogen carbonate	CARLO ERBA
Fetal bovine serum (FBS)	Gibco
Pennicillin Streptomycin (PenStrep)	Gibco
0.25% Trypsin-EDTA	Gibco
Immobilon® Fort Western HRP Substrate	Millipore
Amersham™ ECL™ Prime Western Blotting Detection Reagent	GE healthcare
Nitrocellulose blotting membrane (0.45 µm)	GE healthcare
Developer and replenisher	Carestream
Fixer and replenisher	Carestream
Amersham Hyperfilm™ ECL	GE healthcare
SYBR safe DNA gel stain (10,000X)	Invitrogen
6X loading dye	Vivantis
VC 1 kb DNA ladder	Vivantis
VC 100 bp DNA ladder	Vivantis
Chromatin Prestained Protein Ladder	Vivantis
Protease inhibitor cocktail tablets	Roche
Skim milk	TMMEDIA
Sodium Dodecyl Sulfate (SDS)	Vivantis
Tris	Vivantis

Table 3.1 The lists of chemicals and their suppliers (Continued).

Chemicals	Suppliers
Tween-20	VWR
Methanol	RCI Labscan
30% Acrylamide solution (Mix 37.5:1)	ITW reagents
TEMED	ITW reagents
Agarose	Vivantis
Dimethylsulfozide (DMSO)	CARLO ERBA
Trichloroacetic acid solution (TCA)	Sigma
Sulforhodamine B sodium salt	Sigma
Control-siRNA-A sc-37007	Santa Cruz biotechnology
UGCG siRNA (h) sc-45404	Santa Cruz biotechnology
D,L-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol hydrochloride (PPMP) inhibitor	Santa Cruz biotechnology
UGCG and β-actin primers	Integrated DNA technologies
GBA1 and GBA2 primers	Bio Basic Inc.
TRIzol® Reagent	Invitrogen
Lipofectamine 3000	Invitrogen
DharmaFECT™ Transfection Reagents	Horizon
Pierce™ BCA Protein Assay Kit	Thermo Scientific
LightCycler® 480 SYBR Green I Master	Roche
SuperScript™ VILO™ cDNA synthesis Kit	Invitrogen

Table 3.2 The lists of antibodies and their suppliers.

Antibodies and dilution	Supplier
Rabbit polyclonal anti-Ceramide glucosyltransferase antibody (ab124296) (1:1000)	Abcam
Rabbit polyclonal anti-human GBA2 antibody (1:30)	GenScript
Mouse monoclonal anti-PARP1 antibody (1:5000)	Proteintech
Rabbit monoclonal anti Caspase-3 antibody (1:1000)	Cell signalling
Mouse monoclonal anti-BCL-2 antibody (1:500)	Proteintech
Rabbit anti-Cleaved caspase-3 (D175) antibody (1:1000)	Cell signalling
Rabbit polyclonal anti-BAX antibody (1:1000)	Proteintech
Mouse monoclonal anti-β-actin (C4) antibody (1:1000)	Santa Cruz biotechnology
Mouse monoclonal anti-flag-tag antibody (1:1000)	Cell signaling technology
ECL™ Peroxidase labelled anti-rabbit secondary antibody NA934VS (1:2000)	GE healthcare
HRP-conjugated sheep anti-mouse IgG secondary antibody NXA931V (1:2000)	GE healthcare
HRP-conjugated donkey anti-rabbit IgG secondary antibody NA934V (1:2000)	GE healthcare

3.2 Methods

3.2.1 Gene expression analysis

Gene expression data of CCA was retrieved through GEO Series GSE76297 at Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). GEO Series GSE76297 contained the expression data from 91 CCA tumors and 92 paired non-tumors. For gene expression profiling, tumors and paired non-tumor tissues were profiled separately using Affymetrix Human Transcriptome Array 2.0. All expression data were log2 transformed.

3.2.2 Cells culture

Three CCA cell lines, namely KKU-055, KKU-100, and KKU-213A were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS). The cells were maintained in humidified atmosphere 5% CO_2 at 37ºC. Approximately 80-90% confluence, the cells were washed once with 1X PBS, then trypsin/EDTA (0.25% w/v) was used to detach the cells by incubation at 37ºC for 3 minutes. After that, the cells were centrifuged at 1,200 rpm, 3 minutes. The supernatant was discarded and the cell pellets were resuspended with the media and processed according to the experiments.

3.2.3 RNA extraction

Total RNA from CCA cell lines was extracted by TRIzol® (Invitrogen). Briefly, the cell lysate in 1 mL TRIzol® was added with 200 µL of chloroform/1 mL TRIzol® and vigorous shaking for 15 seconds and incubates at room temperature for 2-3 minutes. Next, the sample tube was centrifuged at 12,000 xg for 15 minutes at 4ºC. The upper phase (400-500 µL) was transferred into new tube followed by adding 500 µL isopropanol/1 mL TRIzol®. Gently mixed by inverting and incubated at room

temperature for 10 minutes then centrifuged at 12,000 xg for 20 minutes, 4ºC. The supernatant was discarded and added 1 mL of 75% EtOH for washing the RNA. After centrifugation, the supernatant was discarded and RNA pellet was air dry for 5-10 minutes at room temperature. Finally, RNase free water was used to dissolve the RNA pellet and stored at 80ºC until used. Measurement of RNA concentration was performed using NanoDrop spectrophotometer (Thermo Scientific). The RNA purity should be in $2.0 \geq A260/280 \geq 1.8$ range. To check RNA quality, RNA concentration at least 1 µg was required. Gel electrophoresis with 0.8% agarose concentration was performed at 100 V for 30-45 minutes. The bands of 28s and 18s rRNA should be detected on the gel when visualized by gel documentation.

3.2.4 cDNA synthesis

cDNA synthesis was performed using SuperScript™ VILO™ cDNA synthesis kit (Invitrogen). The amount of 2 µg (up to 2.5 µg) of total RNA was required for cDNA synthesis. The reaction was prepared according to the manufacturer's instructions showed in Table 3.3. Subsequently, the sample tubes were gently mixed and loaded into thermocycler for cDNA synthesis at 25ºC for 10 minutes, 42ºC for 60 minutes and terminated the reaction at 85ºC for 5 minutes. The synthesized cDNA was diluted with nuclease-free water to be 10 ng/µL for further experiments.

Table 3.3 Reaction components of cDNA synthesis.

Components	Volume (µL)
5X VILO™ Reaction Mix	4
10X Superscript™ Enzyme Mix	2
RNA 2 µg	x
Nuclease-free water	Up to 20

3.2.5 Quantitative Real-time PCR (qPCR)

mRNA expression of target genes was quantified by qPCR using LightCycler® 480 SYBR Green I Master (Roche). The primer sequences were custom designed from NCBI (https://www.ncbi.nlm.nih.gov/) showed in Table 3.4. The amount of cDNA 50 ng per reaction was used for quantification. The reaction mixture and gene amplification conditions were performed as summarized in Table 3.5. The reaction mixes were prepared in 96-well plate on ice. After finished, sealed the plate with clear film and centrifuged at 1,500 rpm for 3 min. Roche LightCycler 480 Instrument II was used to proceed qPCR. The cycling program was set up following in Table 3.6. Amplification temperature for all of primers including, GCS, GBA1, GBA2, and β-actin was used at 58ºC. The gene expression data was collected that presented as cycle threshold values (CT). The CT values of target genes were analyzed by normalized against the house- keeping gene, β-actin. The relative gene expressions were determined by $2^{-\Delta\Delta CT}$ and $2^{-\Delta CT}$ equation.

Table 3.4 Oligonucleotide primers sequence for qPCR.

Oligonucleotide primers	Forward primer sequence (5' → 3')	Reverse primer sequence (5' → 3')
GBA1	GTT CCA GAA AGT GAA GGG AT	TTC TCT GAA GAA GGA ATC GG
GBA2	CCA CTA CAG GCG GTA TAC AA	GAT CTG TCA TCC AAT ACC GG
Ugcg	TGC TCA GTA CAT TGC CGA AGA	TGG ACA TTG CAA ACC TCC AA
β-actin	GAT CAG CAA GCA GGA GTA TGA CG	AAG GGT GTA ACG CAA CTA AGT CAT AG

Table 3.5 Reaction mixture for qPCR.

Components	Volume (µL)
2X SYBR Green Master Mix	10
10 µM Forward primer + Reverse primer	1
cDNA template (10 ng/µl)	5
Sterile distilled water	4
Total volume/well	**20**

Table 3.6 The thermo cycle program for LightCycler 480 PCR instrument II.

Target temperature (°C)	Time
Pre-incubation (1 cycle)	
95	5 min
Amplification (35 cycle)	
95	10 sec
58 (depends on primers)	10 sec
72	20 sec
Melting curve (1 cycle)	
95	5 sec
65	1 min
Cooling	
2	Until we took it out

3.2.6 Gene silencing by RNA interference transfection (siRNA)

KKU-213A was seeded at $3.5x10^5$ cells into 6-well plate for overnight. The cells were transfected with 10 µM siGCS or siCTRL in OptiMEM mixing with DharmaFECT™ Transfection Reagents (Horizon) for 24 h or replaced with new media for another 24 h. After 24 h or 48 h transfection, the transfected cells were harvested and counted for subsequent experiments.

3.2.7 Protein collection and BCA assay

For protein collection, the cells were harvested by washing twice with 1X PBS and added 80 µL lysis buffer with protease inhibitor followed by 30 minutes incubation on ice. Subsequently, the cells were scraped from the plate with cell scrapers,

transferred the mixture into the tube and centrifuged at 16,000 xg for 20 minutes. The supernatant was transferred into new tube and stored at -80°C 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 60°C for 15 min. Subsequently, microplate reader was required for measuring protein concentration at A_{562} nm.

3.2.8 SDS-PAGE and Western blot analysis

The SDS-PAGE consists of 10% separating gel and 4% stacking gel was used for protein separation. Twenty micrograms of protein lysate were required for mixed with 7 µL sample buffer and 1X PBS up to 28 µL. The protein samples were heated at 95°C for 5 minutes followed by loading 28 µL of samples into the wells. The electrophoresis was performed at 80 V for 15 minutes for proteins stacking running and followed at 110 V for 90 minutes for proteins separating. Next, the protein was transferred onto nitrocellulose membrane by electroblotting with a cold and wet blotting system. After blotting process, blocking of non-specific binding proteins on the membrane was performed by 5% skimmed milk in PBST for 1 h. Washed twice with PBST for 15 minutes/time and incubated with primary antibodies in 1% skimmed milk diluted with PBST at 4°C for overnight. Next day, the membrane was washed 3 times with PBST for 10 minutes/ time. Probed with horse-radish-peroxidase (HRP)-

conjugated secondary antibodies in 1% skimmed milk diluted with PBST for 1 h and washed 3 times with PBST for 10 minutes/time. Subsequently, performed the detection by using chemiluminescent HRP substrate, incubated in a dark place for 5 minutes and exposed in the dark room. The primary antibodies that used in this study, including GCS (Abcam), GBA2 (GenScript Corporation), caspase-3 and cleavage-caspase 3 (Cell signaling), PARP1, BAX, and BCL2 (Proteintech). β-actin (C4) (Santa Cruz) was used as internal control. All antibodies dilution was mentioned above in Table 3.2.

3.2.9 Sulforhodamine B (SRB) assay

The SRB assay was assessed to measure cell density based on cellular protein component of adherent cells. To measure the cell density, media was discarded and replaced by 100 µL/well 10% (W/V) tricarboxylic acid (TCA) to fix all cellular proteins, stored at 4°C overnight. Discarded the TCA and five times washed with 200 µL of deionizing water to remove residual solution 100 µL of SRB dye was added in 96-well plate then stained for 30 minutes at room temperature. After that, the SRB dye was discarded. Excess dye was removed by washing five times with 400 µL of 1% (V/V) acetic acid. Subsequently, the protein-bound dye was solubilized with 100 µL of 10 mM Tris base solution for measuring optical density value at A_{564} using a microplate reader.

3.2.10 Cell proliferation assay

The transfected KKU-213A or control cells were seeded at $5x10^3$ into 96-well plate, and then incubated at 37°C for 24 and 48 h. SRB assay was assessed to measure percent of cell viability. The wavelength at 564 nm was used to determine the optical density value by using microplate reader.

3.2.11 Drug treatment

The transfected KKU-213A or control cells were seeded at $7x10^3$ cells into 96-well plate and incubated at 37°C for 24 h. The cell was then exposed with cisplatin alone (10 or 20 µM), PPMP alone (10 µM) (Santa Cruz) or in combination of both agents for 24 and 48 h. Cell viability was evaluated by SRB assay

3.2.12 Statistical analysis

All experiments were performed in three independent experiments. All results expressed as a mean ± SEM (standard error of mean). The correlation of GCS mRNA expression and patient's clinicopathological data was analyzed by Chi square test. Survival analysis was plotted by Kaplan-Meier curve plotting between the percentages of survival cases (Y-axis) versus the specific time point (X-axis) and a comparison between two group was determined using log-rank test. A two-tailed t-test analysis was used to compare the statistical significance different results between two groups. Mann-Whitney t-test was used to analyze GEO database data compared between non-tumorous and tumor tissues as well as paired data. Two-way ANOVA for multiple comparison tests was performed to compare between two groups or more. Statistical analysis was evaluated by GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) and IBM SPSS 22.0 software (IBM Corporation, Armonk, NY, USA). All statistical analysis was two-sided and $P<0.05$ was defined as statistically significant.

CHAPTER IV

RESULTS

4.1 Glucosylceramide synthase (GCS) expression in cholangiocarcinoma tissues.

Ceramide (Cer) has been described to mediate cell death in cancer by inducing apoptosis. But, alteration of ceramide-metabolizing enzymes is observed in many types of cancer and has been involved in the ceramide elimination for promoting tumor growth and metastasis (Gouazé et al., 2005; Ogretmen, 2018). To understand the biological characteristic of ceramide metabolism in CCA, we examined the expression of ceramide-metabolizing enzymes including GCS, GBA1, and GBA2 in CCA tissues. Firstly, the differential expression of these three genes were investigated through GEO Series GSE76297. It was found that expression levels of GCS and GBA1 were significantly upregulated (P<0.001) in CCA tissues when compared with non-tumor tissues (Figure 4.1A, B), whereas the significant downregulation of GBA2 was observed in CCA tissues (P=0.006) (Figure 4.1C), because GCS is a key enzyme that catalyze ceramide glycosylation for regulating tumor progression. Next, GCS mRNA expression was further verified in 29 paired frozen CCA tissues using qPCR (Figure 4.2). With cut-off value at median fold-change was 1.00, there were 14/29 cases showing a high GCS expression as 1.79-fold, whereas 15/29 cases showing a low expression as 0.66-fold, compared with the adjacent normal tissues (Figure 4.3). This

finding suggests us that alteration of ceramide-metabolizing enzymes is occurred in CCA.

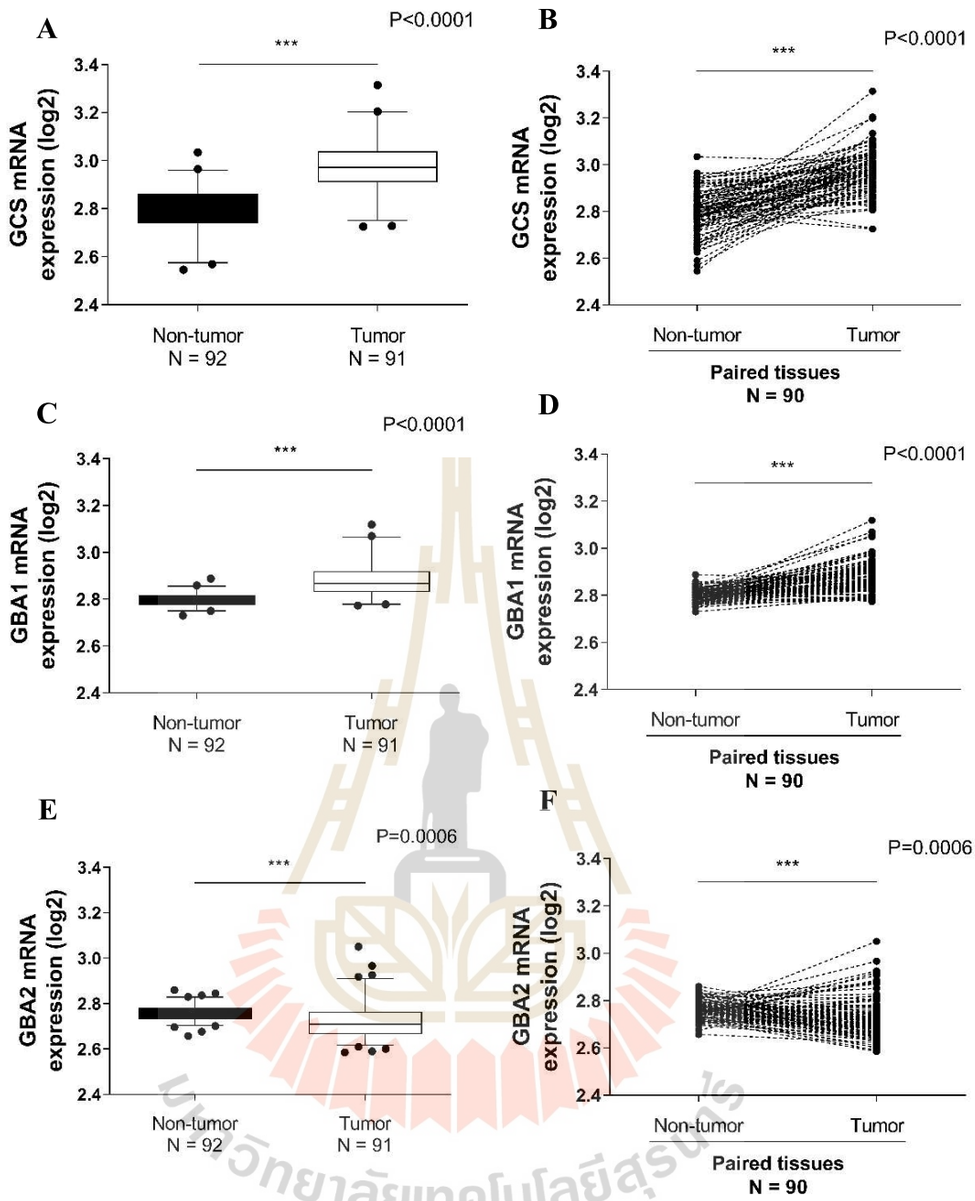

Figure 4.1 Expression of ceramide-metabolizing enzymes in GEO database. The Expression of (A) GCS, (B) GBA1, and (C) GBA2 in Gene Expression Omnibus (GEO) database (GSE76297 dataset) in non-paired (left) and paired (right) CCA tissues. ***, $P<0.001$ versus control. $P<0.05$ was defined as statistically significant.

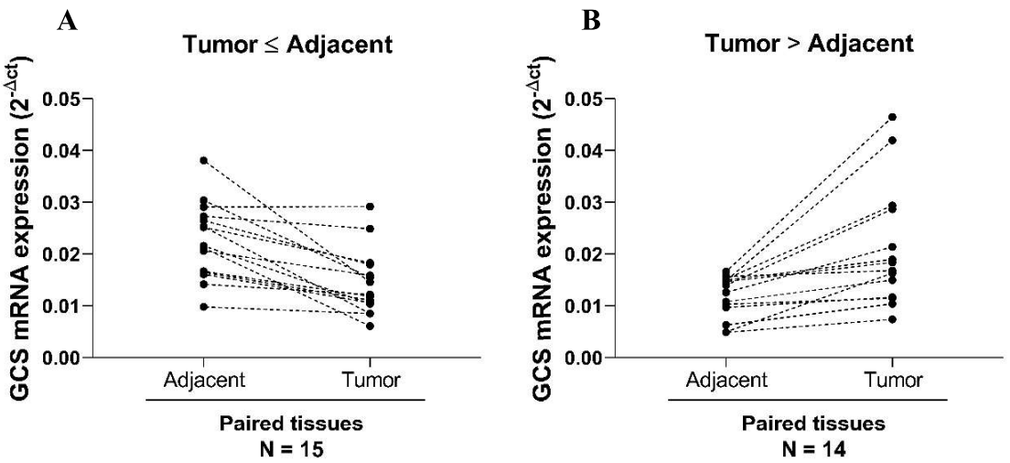

Figure 4.2 GCS expression in 29 paired frozen CCA tissues. Real-time PCR analysis was performed. The data were presented with GCS mRNA expression of (A) tumor ≤ adjacent normal tissues and (B) tumor > adjacent normal tissues. Data are expressed as mean ± SD of two dependent experiments.

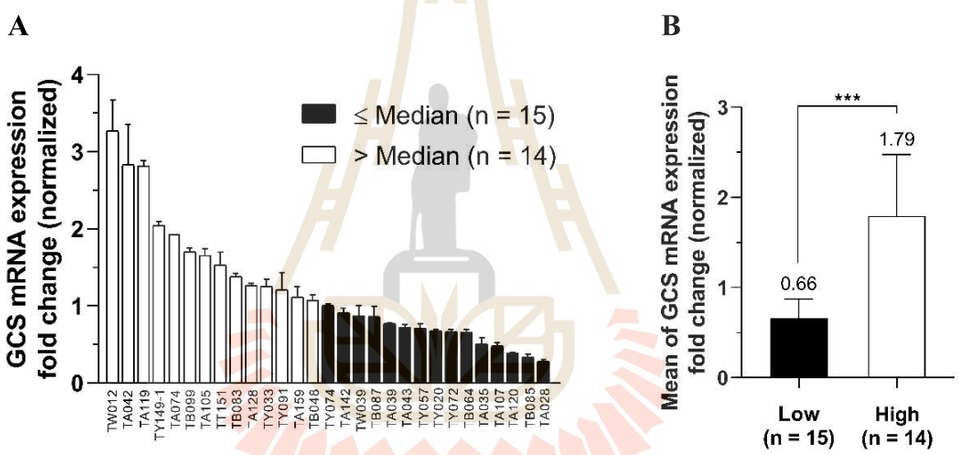

Figure 4.3 GCS mRNA expression fold-change in 29 paired frozen CCA tissues. Tumor was normalized by adjacent normal tissues. (A) Two groups were dichotomized with cut-off at median fold-change (1.00 fold). Cut-off value at ≤ median fold-change denoted as low expression and > median fold-change denoted as high expression. (B) mean of GCS mRNA expression fold-change in both groups. ***, $P<0.001$ versus dissected group. $P<0.05$ was defined as statistically significant.

4.2 High expression of GCS is not associated with clinicopathological features and survival in CCA patients.

To address the significance of GCS mRNA expression with clinical characteristic of the CCA patients. We further quantitatively analyzed the correlation between GCS mRNA expression in CCA and the clinicopathological features, using a univariate analysis. There was no statistically significant association between GCS expression and age, sex, tumor stages, lymphatic invasion, and histologic types (Table 4.1). Then, the correlation between GCS mRNA expression and overall survival of CCA patients was also observed. Kaplan-Meier analysis demonstrated that there was no statistically significant in overall survival between patients with high versus low GCS mRNA expression (log rank test provides $P = 0.67$) (Figure 4.4).

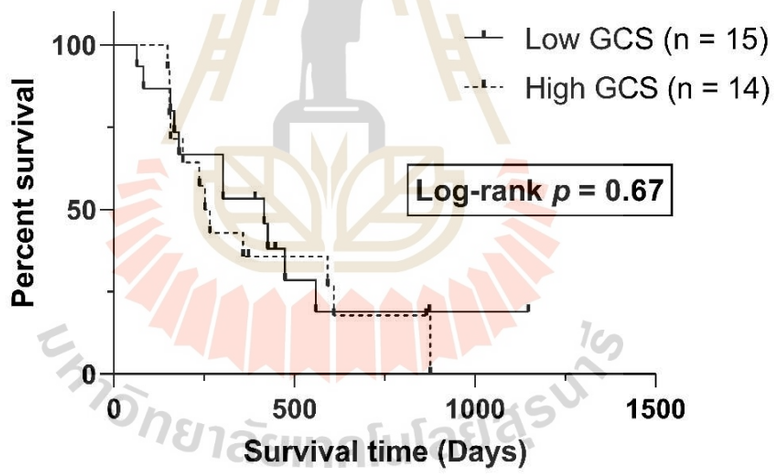

Figure 4.4 Cumulative overall survival was determined using Kaplan-Meier analysis for CCA patients (n = 29). Log Rank test was used for analysis.

Table 4.1 The association of clinicopathological features of 29 patients and GCS expression.

Oncopathologic variable	Cases (n = 29)	GCS mRNA expression levels*		*P*-value**
		Low	High	
Sex				
Male	15	10	5	0.362
Female	14	7	7	
Age (years old)				
≤ 50	5	4	1	0.286
>50	24	13	11	
Tumor stage				
I	1	1	0	0.067
II	4	3	1	
III	20	13	7	
IV	4	0	4	
Normal stage (Lymph node)				
0	17	9	8	0.460
I	12	8	4	
Histological				
Papillary carcinoma	10	8	2	0.090
Tubular adenocarcinoma	19	9	10	

* Median fold-change at 1.00-fold was used as cut-off value.

** *P*-value < 0.05 is a statistically significance from chi-square test.

4.3 Basal expression of ceramide-metabolizing enzymes and inducible expression by cisplatin treatment.

The balance between glucosylceramide and ceramide level is a regulator factor of cell survival or death (Mashhadi Akbar Boojar et al., 2018). To evaluate the regulation of glycosylation in CCA. First, we determined the basal expression of three ceramide-metabolizing enzymes including GCS, GBA1 and GBA2 in CCA cell lines, KKU-055, KKU-100, and KKU-213A. High expression level of GCS was found in all CCA cell lines whereas all CCA cell lines showed low expression of both GBA1 and GBA2 (Figure 4.5). Then, KKU-213A was treated with various concentrations of cisplatin (0, 10, 20, 40, 60 μM) for 24 and 48 h to see how it affected cell viability. When the concentration of cisplatin was increased, the viable cell continued to decline, especially at 48 h (Figure 4.6). The IC_{50} values of cisplatin was 20.29 and 18.27 μM for cisplatin treated in KKU-213A at 24 and 48 h, respectively. Next, to examine the alteration of these three ceramide-metabolizing enzymes upon cisplatin treatment. KKU-213A cell was treated with the same concentrations of cisplatin as before for 24 h. The results revealed that both of GCS and GBA2 at both mRNA and protein levels were significantly induced at cisplatin 10 and 20 μM (Figure 4.7 and 4.8). However, the inducible expression of these two ceramide-metabolizing enzymes tended to decline at 40 and 60 μM cisplatin. Interestingly, the ratio of GCS/GBA2 was decreased in a dose-dependent manner (Figure 4.9). These results implied that the GCS and GBA2 expression were altered upon cisplatin treatment for promoting ceramide-induced CCA cell death.

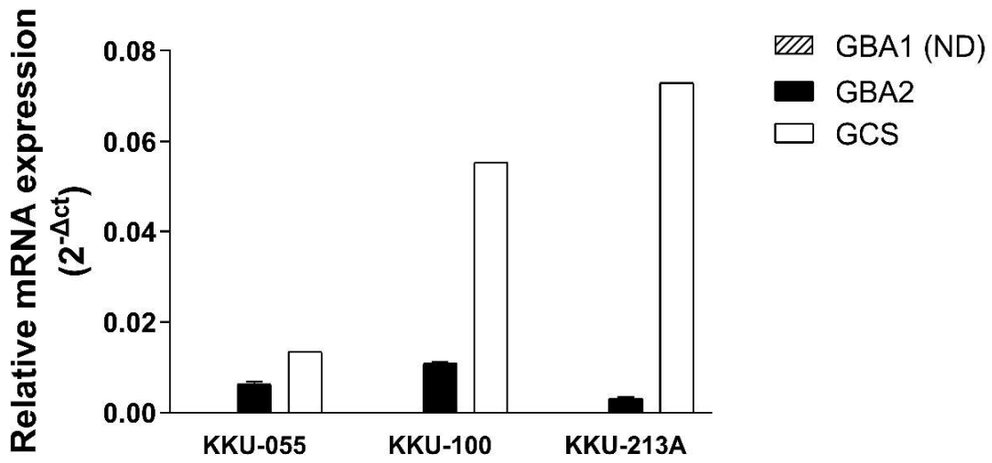

Figure 4.5 The endogenous mRNA expression levels of ceramide-metabolizing enzymes in three human CCA cell lines. Real-time PCR analysis was analyzed and normalized with β-actin. Data are expressed as mean ± SD of two dependent experiments. ND, not determined.

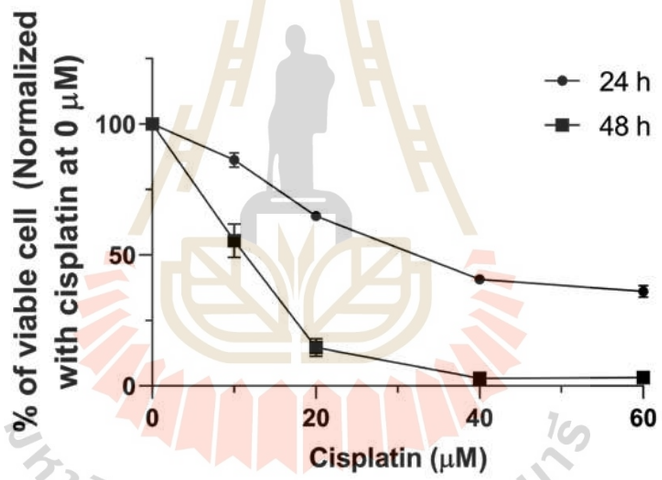

Figure 4.6 Cell viability of KKU-213A against cisplatin treatment. Various concentrations of cisplatin (0, 20, 40, and 60 μM) were exposed in KKU-213A for 24 (left) and 48 h (right). Data are expressed as mean ± SD of three independent experiments.

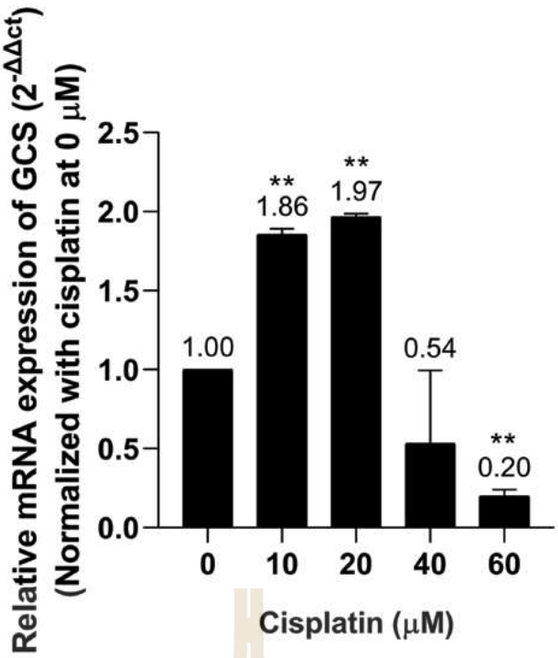

Figure 4.7 GCS mRNA expression levels against cisplatin exposure. KKU-213A was exposed with cisplatin for 24 h. GCS mRNA expression was validated by qPCR. Relative mRNA expression levels were presented by normalized with non-treatment. Data are expressed as mean ± SD of three independent experiments. **, $P<0.01$ versus control. $P<0.05$ was defined as statistically significant.

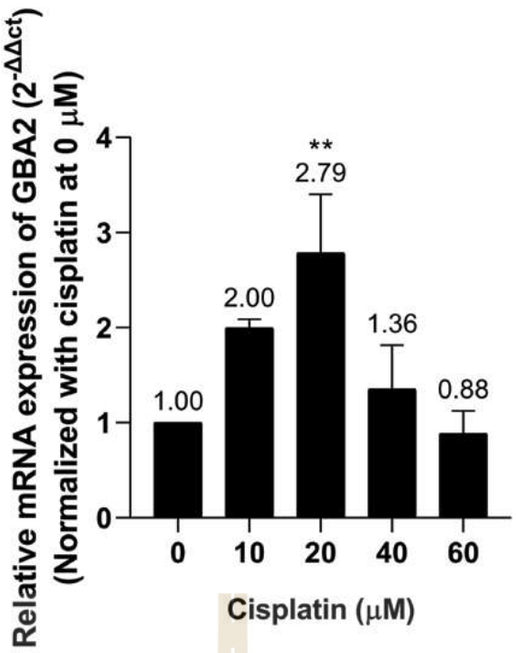

Figure 4.8 GBA2 mRNA expression levels against cisplatin exposure. KKU-213A was exposed with cisplatin for 24 h. GBA2 mRNA expression was validated by qPCR. Relative mRNA expression levels were presented by normalized with non-treatment. Data are expressed as mean ± SD of three independent experiments. **, $P<0.01$ versus control. $P<0.05$ was defined as statistically significant.

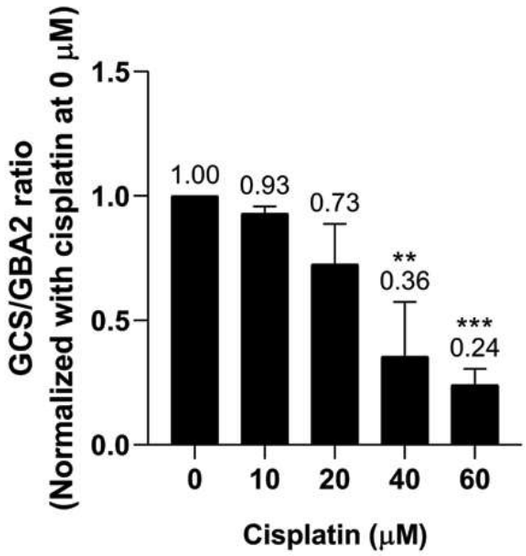

Figure 4.9 GCS/GBA2 mRNA expression ratio against cisplatin exposure. KKU-213A was exposed with cisplatin for 24 h. GCS mRNA expression was validated by qPCR. Data are expressed as mean ± SD of three independent experiments. **, $P<0.01$; ***, $P<0.001$ versus control. $P<0.05$ was defined as statistically significant.

4.4 Suppression of GCS reduces CCA cell growth.

To evaluate the role of GCS in CCA, KKU213A was selected as representative cells due to express the highest GCS expression among others cell lines (Figure 4.5). KKU213A cells were transfected with siGCS or siCTRL for 24 and 48 h. Both of mRNA and protein expression levels were significantly decreased following transfection with GCS siRNA at the time-points of 24 and 48 h (Figure 4.10 and 4.11). Then, we found that suppression of GCS significantly decreased cell growth at 24 and 48 h (Figure 4.12). PPMP, a chemical GCS inhibitor, was also used to inhibit GCS activity in KKU-213A. The effect of GCS inhibition by PPMP at 10 μM was consistency with the effect of GCS suppression by specific siRNA to GCS (Figure 4.13). Moreover, double suppression of GCS by genetic and chemical inhibition was demonstrated. Significantly reduction of cell growth was observed on day 2 ($P<0.001$) and this effect was greatly reduced more than either genetic inhibition or chemical inhibitor exposure alone (Figure 4.14). These findings suggest that GCS has a role on CCA cell growth.

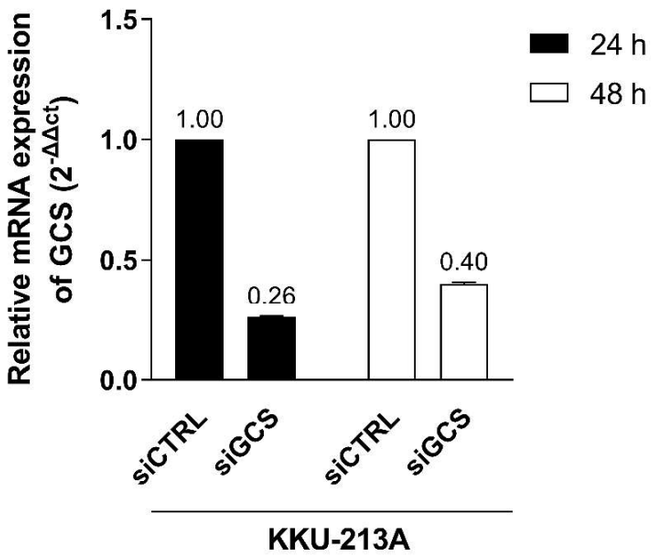

Figure 4.10 mRNA expression of GCS in KKU-213A after transfected with siRNAs. KKU-213A was transfected with siGCS or siCTRL for 24 and 48 h and analyzed by qPCR. Data are expressed as mean ± SD of two dependent experiments.

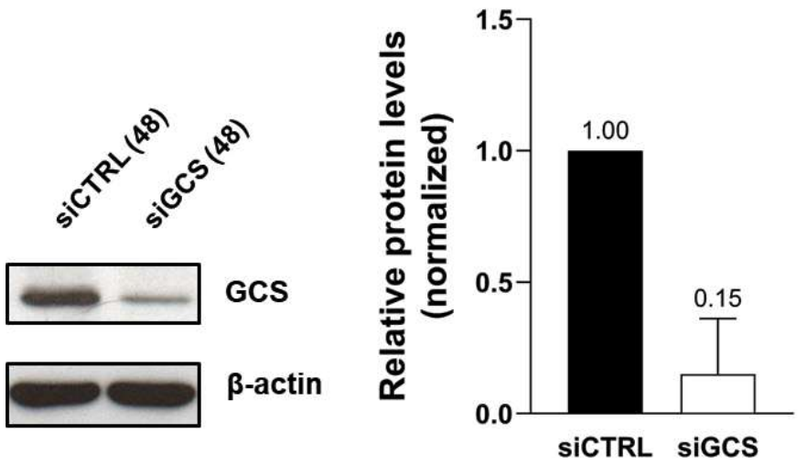

Figure 4.11 Protein expression of GCS in KKU-213A after transfected with siRNAs. KKU-213A was transfected with siGCS or siCTRL for 48 h. The whole cell lysate was used and Western blot analysis was performed. Relative protein levels were presented by normalized with β-actin. Data are expressed as mean ± SD of three independent experiments.

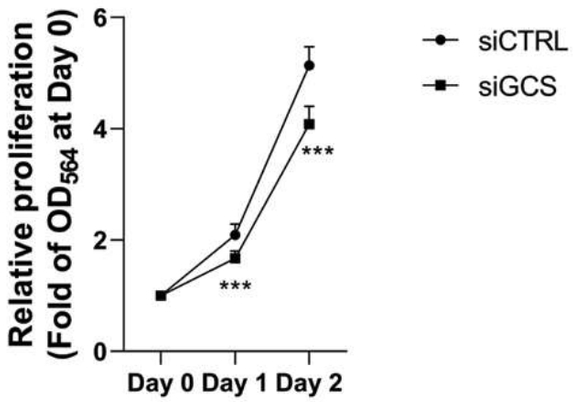

Figure 4.12 Effect of GCS on cell proliferation after genetic inhibition. KKU-213A was transfected against siGCS or siCTRL for 24 h. Cell viability was observed at 24 and 48 h and evaluated by SRB assay. Day 0 was counted after 24 h transfection. Data are expressed as mean ± SD of three independent experiments. ***, P<0.001 versus siCTRL. P<0.05 was defined as statistically significant.

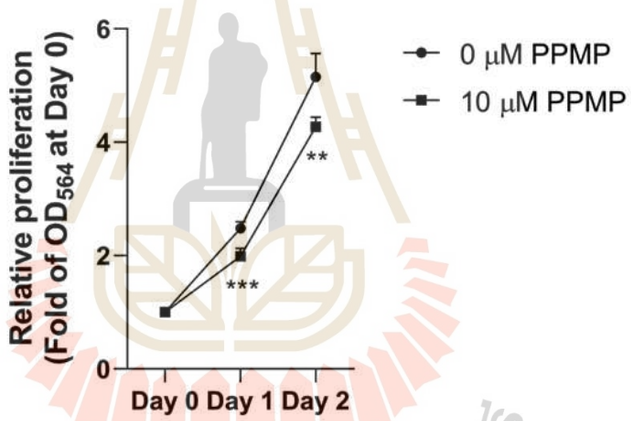

Figure 4.13 Effect of GCS on cell proliferation after pharmacological inhibition. KKU-213A was treated with PPMP for 24 h. Cell proliferation was observed for 48 h and visualized by SRB assay. Day 0 was counted after cells plating for 24 h. Data are expressed as mean ± SD of three independent experiments. **, P<0.01; ***, P<0.001 versus 0 μM PPMP. P<0.05 was defined as statistically significant.

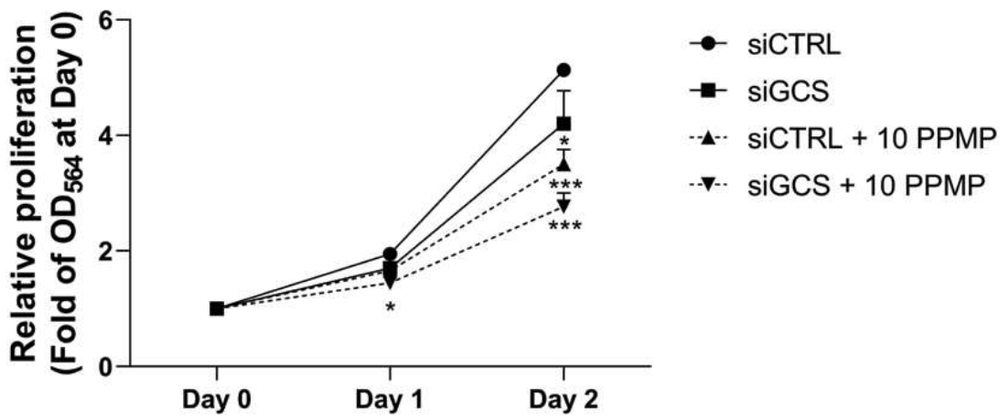

Figure 4.14 Effect of GCS on cell proliferation after genetic inhibition with PPMP treatment. Double suppression by siGCS or siCTRL plus PPMP was performed. KKU-213A was transfected with siGCS or siCTRL for 24 h followed by exposed with PPMP (10 μM) for another 48 h. Day 0 was counted after 24 h transfection. Cell viability was observed after PPMP exposure and evaluated by SRB assay. Data are expressed as mean ± SD of three independent experiments. *, $P<0.05$; ***, $P<0.001$ versus siCTRL. $P<0.05$ was defined as statistically significant.

4.5 Suppression of GCS enhances cisplatin sensitivity.

Ceramide glycosylation by GCS is a key step in regulating ceramide levels and glycosphingolipid synthesis for supporting cell growth and survival. Overexpression of GCS has been found in diver drug-resistant cancer cells and in several type of cancers (Y.-Y. Liu et al., 2013). To address the possible role of GCS on chemotherapeutic response in CCA, GCS expression or activity in KKU-213A were suppressed by siGCS or PPMP at 10 μM and then co-treatment with cisplatin at 10 and 20 μM. The result demonstrated that inhibition of GCS expression by GCS siRNA enhanced cisplatin-induced CCA cell death in a dose-dependent manner (Figure 4.15). Moreover, cisplatin-induced cell death was also increased in combination treatment with 10 μM PPMP especially at 48 h of incubation. However, there were no further increase on cisplatin sensitivity in KKU-213A having double inhibition of GCS by GCS siRNA and PPMP (Figure 4.16 and 4.17).

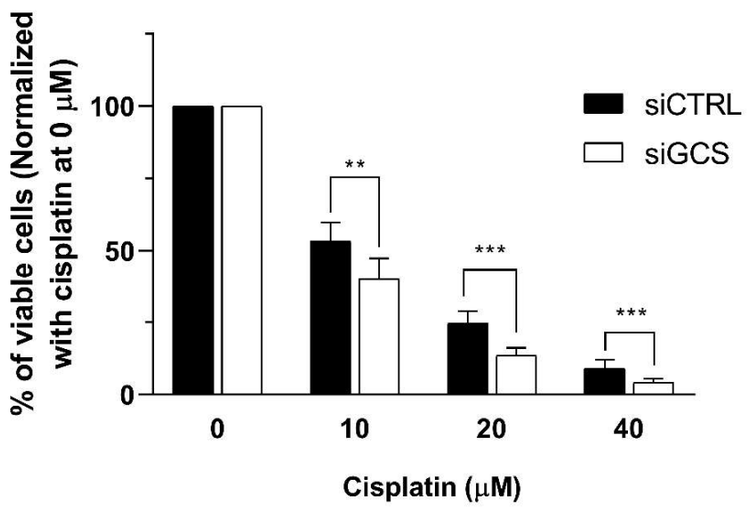

Figure 4.15 Effect of GCS on cisplatin sensitivity. KKU-213A was transfected with siGCS or siCTRL for 24 h. Cell viability was determined for 48 h after cisplatin treatment and assessed by SRB assay. Data are expressed as mean ± SD of three independent experiments. **, $P<0.01$; ***, $P<0.001$ versus siCTRL. $P<0.05$ was defined as statistically significant.

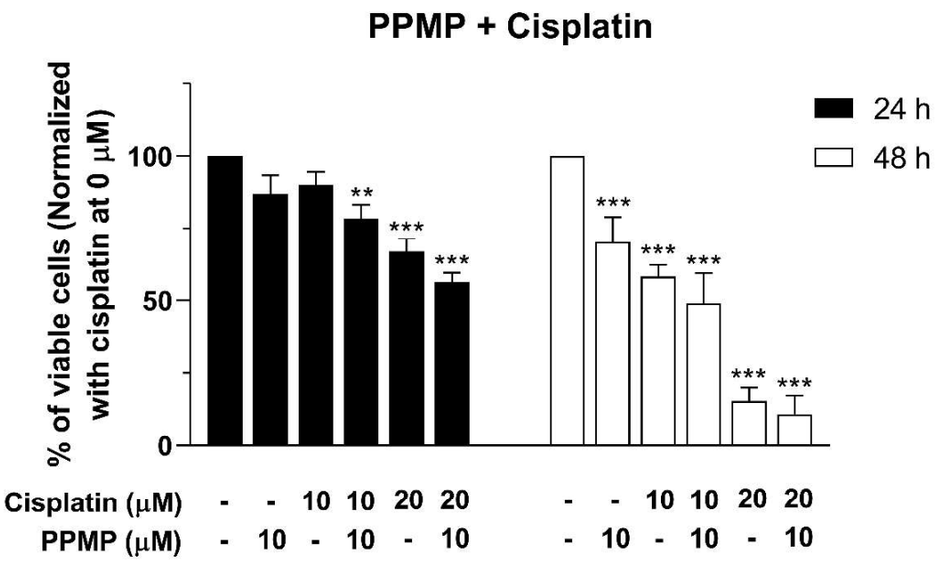

Figure 4.16 Cell viability of cisplatin and PPMP co-treatment. The viable cells were determined for 24 and 48 h after treated with cisplatin, PPMP, or their combination. SRB was performed to evaluate. Data are expressed as mean ± SD of three independent experiments. **, $P<0.01$; ***, $P<0.001$ versus control. $P<0.05$ was defined as statistically significant.

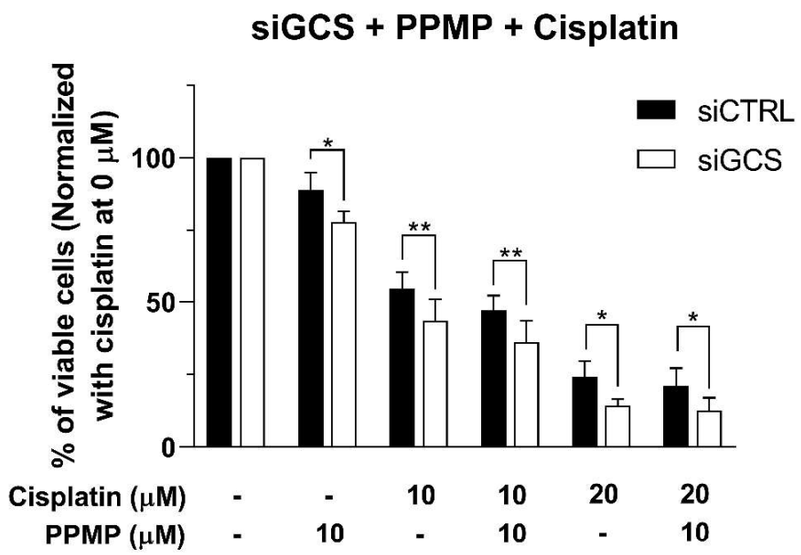

Figure 4.17 Cell viability after genetic suppression of cisplatin and PPMP co-treatment. GCS was suppressed in KKU-213A by siRNAs transfection for 24 h. The viable cells were investigated for 48 h after treated with cisplatin, PPMP, or their combination. SRB was performed to evaluate. Data are expressed as mean ± SD of three independent experiments. *, $P<0.05$; **, $P<0.01$ versus siCTRL. $P<0.05$ was defined as statistically significant.

4.6 Suppression of GCS promotes cisplatin-induced cell death through apoptosis pathway.

Ceramide can enhance cell cycle arrest and cell death via inducing mitochondria dysfunction and apoptosis cascade (Y.-Y. Liu et al., 2013). Whereas, ceramide glycosylation is the process of reducing cellular ceramide by preventing ceramide-induced apoptosis in cancer cells (Gupta et al., 2012). To verify the underlying mechanism of GCS inhibition on enhancing cisplatin-induced cell death. The suppression of GCS with PPMP and co-treatment with cisplatin was performed in KKU-213A. The cells were treated with either PPMP alone (10 μM), cisplatin alone (10 or 20 μM), or the combination of both agents for 24 h. The expression of apoptosis-related proteins, including PARP1, cleaved PARP1, caspase-3, cleaved caspase-3, BCL-2, and BAX were then examined. The results demonstrated that the expression of cleaved PARP1 and cleaved caspase-3 was significantly increased in KKU-213A treated with cisplatin alone at 10 and 20 μM and combination treatment with PPMP at 10 μM in a dose-dependent manner (Figure 4.18, 4.19, and 4.20). Moreover, the expression of BCL-2, an anti-apoptotic protein, was decreased especially cisplatin treatment at 20 μM with and without PPMP treatment. However, none of these proteins was significantly altered upon GCS inhibition by PPMP alone (Figure 4.18). Furthermore, the effect of PPMP on cisplatin sensitivity was clearly demonstrated by a low ratio of BCL-2/BAX (Figure 4.21). Therefore, inhibition of GCS enhanced cisplatin-induced CCA cell apoptosis.

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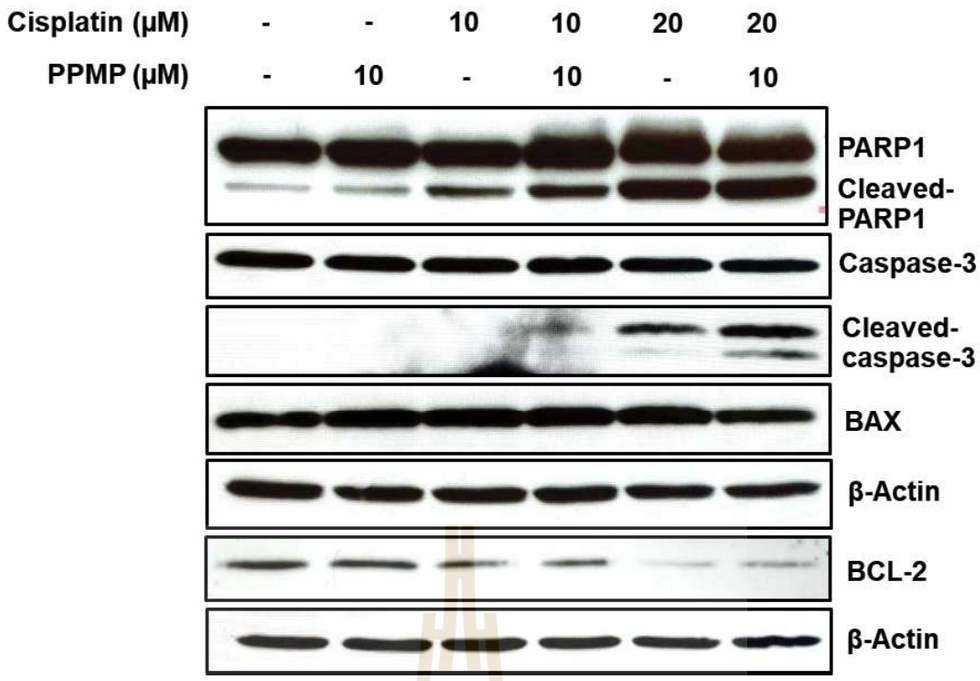
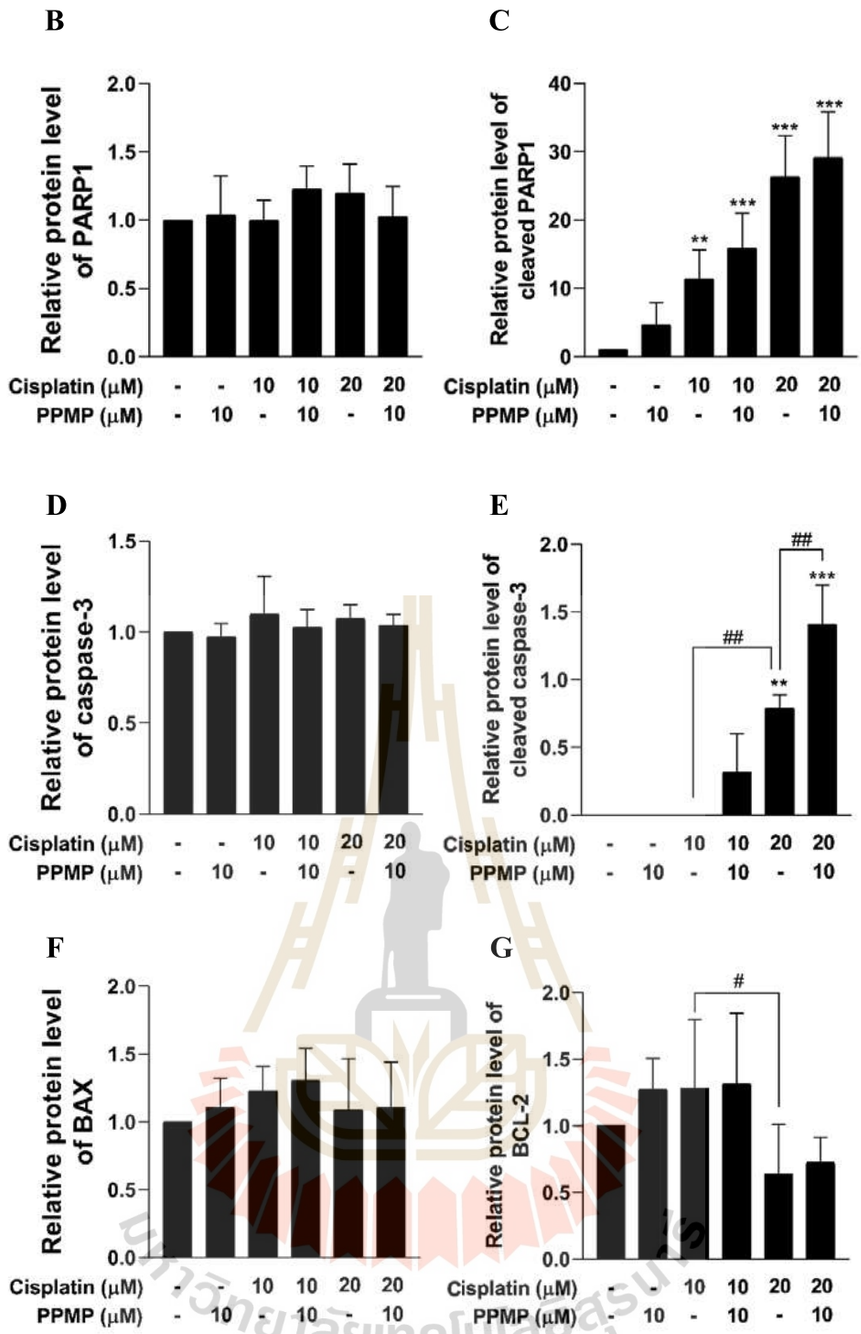


Figure 4.18 Alteration of apoptotic markers upon GCS inhibition combined with cisplatin. KKU-213A was exposed with the combination of PPMP (10 μM) and cisplatin (10 or 20 μM) for 24 h. The whole cell lysate was utilized. (A) The proteins involved with apoptosis pathway were determined by Western Blot assay. Relative protein levels of all proteins, including (B) PARP1, (C) Cleaved PARP1, (D) Caspase-3, (E) Cleaved caspase 3, (F) BAX, and (G) BCL-2 were normalized using β-actin. Data are expressed as mean ± SD of three independent experiments. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ versus control; $^{\#}$, $P<0.05$; $^{\#\#}$, $P<0.01$; $^{\#\#\#}$, $P<0.001$ versus treatment group. $P<0.05$ was defined as statistically significant.



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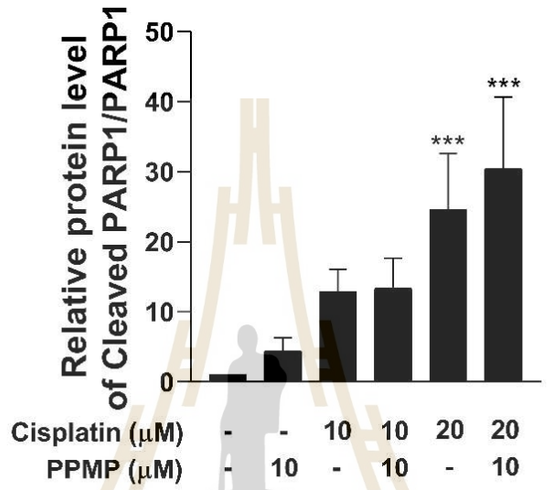

Figure 4.19 The ratio of cleaved PARP1/PARP1 protein expression upon GCS inhibition combined with cisplatin. Data are expressed as mean ± SD of three independent experiments. ***, $P<0.001$ versus control. $P<0.05$ was defined as statistically significant.

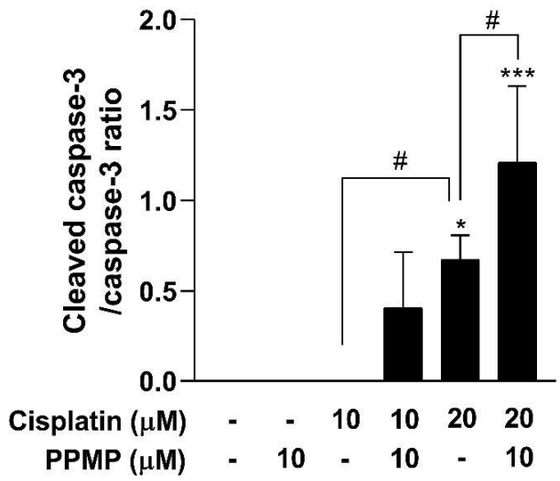

Figure 4.20 The ratio of cleaved caspase-3/caspase-3 protein expression upon GCS inhibition combined with cisplatin. Data are expressed as mean ± SD of three independent experiments. *, $P<0.05$; ***, $P<0.001$ versus control; #, $P<0.05$ versus treatment group. $P<0.05$ was defined as statistically significant.

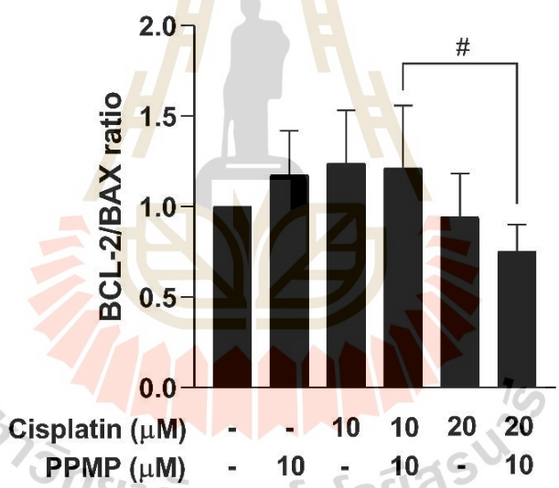

Figure 4.21 The ratio of BCL-2/BAX protein expression upon GCS inhibition combined with cisplatin. Data are expressed as mean ± SD of three independent experiments. #, $P<0.05$ versus treatment group. $P<0.05$ was defined as statistically significant.

CHAPTER V

DISCUSSION AND CONCLUSION

Ceramide has been clearly recognized as a key player in the induction of cell apoptosis and cell growth arrest from a variety of assaults, including chemotherapy in various cancer types, such as hepatocellular carcinoma, retinopathy, and renal cell carcinoma (Zheng et al., 2019a). However, the tumor suppressor effect of ceramide has been obviously neutralized by glucosylceramide synthase (GCS) that is a key enzyme for the conversion of ceramide to glucosylceramide (GlcCer) in promoting glycosphingolipid synthesis to enhance cell growth and survival. High GCS expression has been reported in multiple types of cancer, but not CCA (Abdul-Hammed et al., 2017; Gupta and Liu, 2013; Liu et al., 2010; Liu et al., 2004; Patwardhan et al., 2009; Roh et al., 2015). Increasing of glycosphingolipid synthesis, a downstream process of ceramide glycosylation by GCS, and its hydroxylation has been demonstrated to associate with short survival of the CCA patients (Silsirivanit et al., 2019). However, the underlying mechanism is still unclear. According to GEO databases, GCS and GBA2 expression were significantly upregulated, whereas GBA1 was downregulated in CCA tumor tissues, when compared with non-tumor tissues. Subsequently, GCS expression was further verified in 29 paired frozen CCA tissues. High expression of GCS was found that upregulated as 1.79-fold of these CCA cases and there was no statistical significance in the correlation between GCS expression and overall survival, as well as clinicopathological features, including age, gender, tumor stage, lymph node

stage and histological types. These observations are consistent with the study of breast cancer, in which the levels of GCS expression may not correlate with the clinicopathological features of the cancer patients. Nevertheless, high GCS protein expression was associated with estrogen receptor (ER) positivity and human epidermal growth factor-2 (HER-2) negativity in breast cancer (Liu et al., 2014). Additionally, it has been demonstrated that GCS has an oncogenic role to enhance drug resistance. However, as in our study verification of GCS expression was performed only at the mRNA levels and has a limited number of validated samples, further studies with larger sample size and measurement of GCS protein expression are needed.

GCS plays a key role in producing GlcCer for promoting glycosphingolipid synthesis to encourage several cellular processes, such as cell proliferation, metastasis, and multi-drug resistance (Bleicher and Cabot, 2002). In this study, GCS was most highly expressed among all ceramide-metabolizing enzyme genes, while GBA1 could not be detected in all CCA cell lines. Additionally, inducible expression of GCS and GBA2 was found upon cisplatin treatment at 10 and 20 μM, but this induction was strongly declined at high concentrations of cisplatin (40 and 60 μM). Moreover, the GCS/GBA2 ratio was significantly decreased in a dose-dependent manner. These results indicated that induction of both GCS and GBA2 expression serves a vital role in the response to cisplatin treatment and the low ratio of GCS/GBA2 may imply cisplatin-induced ceramide production leading to CCA cell death. These results of the present study are consistent with a previous study, in which lower doses of exposure to anticancer drugs, such as cisplatin and doxorubicin, were capable of stimulating ceramide glycosylation by upregulating GCS expression in cancer cells resulting in low chemotherapy response (Roy et al., 2020). It has been demonstrated that

overexpression of GCS enhanced cell proliferation and stem cell properties by alteration of GEMs and accumulation of Gb3 and GlcCer through the activation of Akt and ERK1/2 (Wegner et al., 2018) and c-Src/β-catenin signaling pathways (Gupta et al., 2012). In contrast, GCS silencing suppressed tumor growth and increased apoptosis via the BCL-2/BAX signaling pathway (Li et al., 2017). In ovarian cancer, suppression of GCS reduced cell proliferation via induction of p53 (Liu, Patwardhan, Bhinge, et al., 2011; Ogretmen, 2018). In the present study, suppression of GCS by both genetic inhibition and chemical inhibitor has been observed to reduce the CCA cell growth. Our findings suggest that GCS has a role on cell growth and drug response in CCA.

It is well established that GCS is overexpressed in several drug-resistant cell lines and overexpression of GCS was associated with upregulation of multi-drug resistance genes (MDR) (Liu et al., 2016). Thus, inhibition of GCS resulted in increased chemotherapeutic response to several anticancer drugs, such as cisplatin, doxorubicin, adriamycin, and sorafenib (Liu et al., 2000; Liu et al., 2004; Roh et al., 2015; Song et al., 2012; Stefanovic et al., 2016; Yandım et al., 2013). Our results demonstrated that suppression of GCS by either specific siRNA to GCS or PPMP increased cisplatin-induced CCA cell death in a dose- and time-dependent manner. However, there is no addition effect of cisplatin-induced CCA cell death when suppression of GCS was done by both genetic inhibition and a chemical inhibitor. Ceramide holds promise as an anticancer agent. Ceramide can function as a messenger to drive apoptosis, autophagy response and cell cycle arrest via p53 activation and inactivation of AKT (Morad et al., 2012). BCL-2 is an anti-apoptotic protein that binds to inactivate pro-apoptotic proteins. Upregulation of BCL-2 is associated with cell survival and cisplatin resistance in many cancer types by inhibiting pro-apoptotic BAX

translocation (Cho et al., 2006; Kang and Reynolds, 2009; Leisching et al., 2015). However, the ceramide treatment promoted p53 activation that is followed by an increase in BAX level and a decrease in Bcl-2 level and lead to apoptosis (Kim et al., 2002; Li et al., 2017; Ogretmen, 2018; Zheng et al., 2019b). In the present study, GCS inhibition by PPMP-combined with cisplatin markedly increased cleaved caspase-3/caspase-3 cleaved PARP1/PARP1 ratio but diminished BCL-2/BAX ratio. These observations suggested that GCS inhibition may enhance the conversion of GlcCer to ceramide to increase the response to cisplatin in CCA. Similar to previously studies, GCS suppression resulted in increased levels of apoptotic markers, including BAX, cleaved PARP, and cleaved caspase-3, while decreased BCL-2 expression was seen in liver cells, head and neck cancer, lung endothelial cells, and colon cancer (Koike et al., 2019; Li et al., 2017; Roh et al., 2015; Song et al., 2012). Cleaved PARP1 has been reported to mediate both apoptosis and necrosis pathway by inducing DNA damage (Shin et al., 2015). However, there was no additional effect on the expression of cleaved PARP1 upon the combination of PPMP with high concentration of cisplatin.

In conclusion, our study demonstrated that GCS expression is upregulated in CCA. GCS has a role on CCA cell growth and drug response. Suppression of GCS is essential to enhance cisplatin sensitivity in CCA. Thus, targeting GCS with chemotherapy would be a potential strategy for improving CCA treatment.

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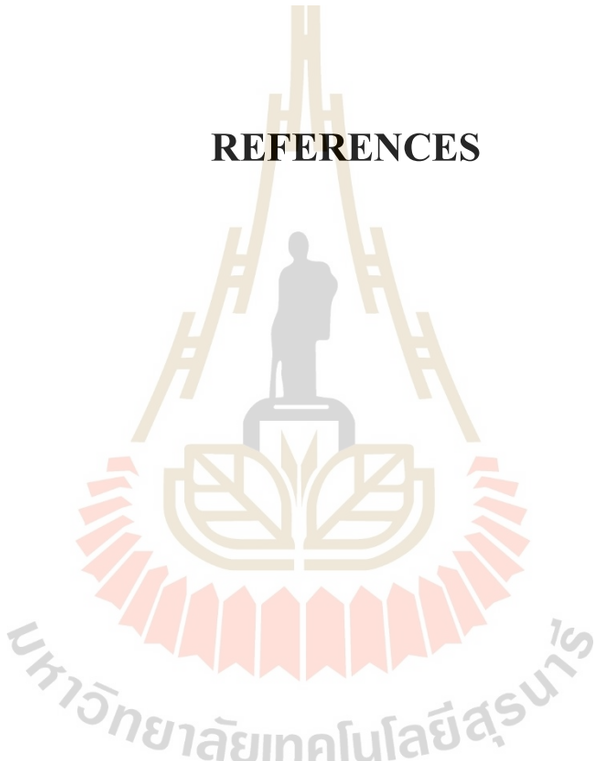

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