DETECTION OF GLYCOSPHINGOLIPID GM2 IN CHOLANGIOCARCINOMA

NUCHANARD SUTATUM

A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Master of Science in Translational Medicine
Suranaree University of Technology
Academic Year 2021

การตรวจวิเคราะห์ไกลโคสฟิงโกลิพิด GM2 ในมะเร็งท่อน้ำดี

นางสาวนุชนาถ สุตธรรม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเวชศาสตร์ปริวรรต
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ปีการศึกษา 2564

DETECTION OF GLYCOSPHINGOLIPID GM2 IN CHOLANGIOCARCINOMA

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นุชนาถ สุตธรรม: การตรวจวิเคราะห์ไกลโคสฟิงโกลิพิด GM2 ในมะเร็งท่อน้ำดี (DETECTION OF GLYCOSPHINGOLIPID GM2 IN CHOLANGIOCARCINOMA) อาจารย์ที่ปรึกษา: รองศาสตราจารย์ ดร.กระจ่าง ตลับนิล, 34 หน้า.

คำสำคัญ: มะเร็งท่อน้ำดี ไกลโคสฟิงโกลิปิด GM2 B4GALNT1 HEXA HEXB GM2A

องค์ความรู้ด้านการแสดงออกและกลไกทางชีวโมเลกุลของสาร ganglioside GM2 ในโรคมะเร็งท่อน้ำดียังมีข้อมูลขำกัด ดังนั้นผู้วิจัยจึงได้ศึกษาการแสดงออกของ ganglioside GM2 ในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีและเนื้อเยื่อของผู้ป่วยมะเร็งท่อน้ำดี ด้วยวิธีการทางอิมมูโนไซโตเคมี และอิมมูโนฮิสโตเคมี รวมถึงการแสดงออกของกลุ่มเอนไซม์ที่ใช้ในการสร้างและทำลายสาร ganglioside GM2 ด้วยวิธี quantitative real-time polymerase chain reaction (qRT-PCR) จากการศึกษาพบว่า สาร ganglioside GM2 มีการแสดงออกเพิ่มมากขึ้นในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีชนิด KKU055, KKU100 และ KKU213A เมื่อเปรียบเทียบกับเซลล์เยื่อบุผิวท่อน้ำดีมาตรฐาน (MMNK1) การศึกษาการแสดงออกของเอนไซม์ที่เกี่ยวข้องกับการสร้างและการทำลายสาร ganglioside GM2 พบว่ามีการแสดงออกที่เพิ่มขึ้นของเอนไซม์ beta-1,4-N-Acetyl-Galactosaminyltransferase 4 (B4GALNT1) ที่ทำหน้าที่สังเคราะห์ GM2 และตรวจพบการแสดงออกที่ลดลงของเอนไซม์ Hexosaminidase A (HEXA) และ Hexosaminidase B (HEXB) ซึ่งทำหน้าที่ในการสลาย GM2 ในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี เมื่อศึกษาความสัมพันธ์ของการแสดงออกของสาร ganglioside GM2 กับลักษณะพยาธิวิทยาคลินิกของผู้ป่วยมะเร็งท่อน้ำดีพบว่าการแสดงออกที่เพิ่มขึ้นของสาร ganglioside GM2 มีความสัมพันธ์กับการรุกรานหลอดเลือด (vascular invasion) ของผู้ป่วยมะเร็งท่อน้ำดีอย่างมีนัยสำคัญทางสถิติ (P=0.024) จากการศึกษาที่กล่าวมาทั้งหมดแสดงให้เห็นว่าการแสดงออกที่เพิ่มขึ้นของ ganglioside GM2 เป็นผลเนื่องมาจากการเพิ่มขึ้นของเอนไซม์ B4GALNT1 และการลดลงของเอนไซม์ HEXA และ HEXB และการแสดงออกที่เพิ่มขึ้นของสาร ganglioside GM2 มีความสัมพันธ์กับการรุกรานหลอดเลือดของผู้ป่วยมะเร็งท่อน้ำดี

สาขาวิชาเวชศาสตร์ปริวรรต ลายมือชื่อนักศึกษา.....นุช.....

ปีการศึกษา 2564 ลายมือชื่ออาจารย์ที่ปรึกษา.....

NUCHANARD SUTATUM: DETECTION OF GLYCOSPHINGOLIPID GM2 IN CHOLANGIOCARCINOMA. THESIS ADVISOR: ASSOC. PROF. KRAJANG TALABNIN, Ph.D., 34 PP.

Keyword: Cholangiocarcinoma GM2 B4GALNT1 HEXA HEXB GM2A

Elevated expression of ganglioside GM2 has been demonstrated in cholangiocarcinoma (CCA), but their molecular mechanisms are not well understood. In this study, we demonstrated the expression of GM2 in CCA cell lines and tissues by immunocyto- /histo- chemistry and further investigated the expression of GM2 metabolizing enzymes by quantitative real-time polymerase chain reaction (qRT-PCR). High expression of GM2 was detected in CCA cell lines, KKU055, KKU100, and KKU213A compared with an immortalized human cholangiocytes, MMNK1, with plasma membrane and cytoplasmic staining. High expression of GM2 metabolizing enzymes, Bata-1,4-N-Acetyl-Galactosaminyltransferase 4 (B4GALNT1) and low expression of Hexosaminidase A (HEXA) and Hexosaminidase B (HEXB) in CCA cell lines were demonstrated. The associations of high expression of GM2 with vascular invasion (P=0.024) were also demonstrated in tissue of patients with CCA (n=60). These data suggested that, high expression of ganglioside GM2 in CCA were contributed by an up regulation of GM2 synthesizing enzyme, B4GALNT1 and down regulation of GM2 degradation enzymes, HEXA and HEXB

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Academic Year 2021

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my thesis advisor, Assoc. Prof. Dr. Krajang Talabnin, thesis co-advisor, Asst. Prof. Dr. Chutima Talabnin who's patiently in instruction, encouragement, guidance, and strengthen the knowledge in order for me to complete my master's degree in Translational medicine. I wish to express my deepest appreciation to Dr. Pundit Asavaritikrai, M.D. and Dr. Sanong Suksaweang who gave valuable suggestion and guidance in my thesis. I am very thankful to my external examiner, Assoc. Prof. Dr. Atit Silsirivanit, who provided valuable suggestion.

I would like to express my sincere thanks to Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, for providing the specimens and clinical data in this study. I would like to acknowledge the One Research One Graduate (OROG) scholarship of Suranaree University of Technology and the National Research Council of Thailand for financial support. Moreover, sincere thanks to the Translational medicine and Biochemistry Research Unit, Suranaree University of Technology for supporting the equipment and laboratory facilities.

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

Nuchanard Sutatum

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LIST OF ABBREVIATIONS

CCA	Cholangiocarcinoma
cfDNA	cell – free DNA
cfRNA	cell – free RNA
DMEM	Dulbecco's Modified Eagle Medium
EVs	Extracellular vesicles
HCC	Hepatocellular carcinoma
lncRNAs	long non-coding RNAs
miRs	microRNAs
MMNK1	immortalized human cholangiocytes
GalCer	galactosylceramide
GSLs	Glycosphingolipids
GlcCer	glucosylceramide
GlcNAc	N-Acetylglucosamine
IHC	Immunohistochemistry
ICC	Immunocytochemistry
O-GlcNAc	O-linked-Acetylglucosamine
OV	*Opisthorchis viverrine*
qRT-PCR	Quantitative real-time polymerase chain reaction
sLea	Sialyl Lewis A

CHAPTER 1
INTRODUCTION

1.1 Thesis title

DETECTION OF GLYCOSPHINGOLIPID GM2 IN CHOLANGIOCARCINOMA

1.2 Background and Problem

Cholangiocarcinoma (CCA), a cancer of an epithelium lining of the bile duct, is a concern public health problem in Thailand, particularly in the northeast area where *Opisthorchis viverrini* (OV) infection is endemic. The incidence of CCA increasing remains a highly fatal malignancy and mortality is still gradually increasing because early diagnosis is difficult (Izquierdo-Sanchez et al. 2022) (Shin, Moon, and Kim 2023). The malignancy of CCA is normally difficult to diagnose until the disease becomes advanced, at which the prognosis is poor. At present, there is no diagnosis tool for early detection of CCA. Therefore, the candidate biomarkers for early diagnosis or prognosis are still needed (Blechacz and Gores., 2018).

Alteration of glycolipid expression has been reported in many types of cancer, so several glycosphingolipids (GSLs) have been evaluated for diagnosis, prognosis, and cancer therapy (Daniotti et al., 2013). GSLs are implicated in several physiological and pathological processes, including cell growth and differentiation, regulation of cell signaling, and association with acute and chronic diseases (Allende., 2014). Characterization of cancer related GSLs expression may facilitate discovery of candidate biomarkers or novel therapeutic targets (Kim et al., 2011). The glycosyltransferases and glycosidase involved in the synthesis and catabolism of GSLs maybe also be used as cancer biomarkers (Meany et al., 2011).

Biomarker studies for CCA are growing both in laboratory and in clinical settings. The expression of Sialyl Lewis A (sLea) in tissue of patients with CCA have been found to be related to a poor prognosis (Juntavee et al., 2015). In serum of patient with CCA,

the association of carbohydrate marker S121 and prognosis were demonstrated (Silsirivanit et al., 2011). S121 epitope was found in biliary cells of an animal model and the expression was increased with tumor progression (Sawanyawisuth et al., 2012). Overexpression of GlcNAc (Indramanee et al., 2012) and *O*-GlcNAc transferase (Phoomak et al., 2012) have been demonstrated by immunohistochemistry in CCA tissues. A study in CCA sera by ELISA technique showed the diagnostic value of carbohydrate epitope (CA-S27) in CCA (Silsirivanit et al., 2013). The lectin micro array-based biomarker detection for CCA has also been demonstrated (Matsuda et al.,2015). In CCA cell lines, differential expression of *O*-linked glycans in each pathological origin has been demonstrated (Talabnin et al., 2016). Our study in CCA sera revealed the increased expression of *N*-linked glycoprotein glycans compared to healthy controls (Talabnin et al., 2017). A recent study in CCA tissue demonstrated the high expression of GSLs is associated with shorter survival of the patients with CCA (Silsirivanit et al., 2019). Although several GSLs of CCA have been reported, the glycosyltransferase and glycosidase involved in the synthesis and catabolism of the GSLs are never been investigated.

In this study, we determined the expression of glycosphingolipid GM2 in cell lines and tissue of patients with CCA. The associations of GM2 expression and clinicopathological features were demonstrated. The contribution of GSLs metabolizing enzymes in synthesis and catabolism of GM2 were characterized. Practical investigation of GSLs metabolizing enzymes may help in understanding molecular mechanism of GSLs in CCA and can be applied for clinical use.

CHAPTER 2
LITERATURE REVIEWS

2.1 Cholangiocarcinoma

Cholangiocarcinoma (CCA) is a malignancy that arises from the biliary tract. This CCA can be classified into intrahepatic (iCCA) and extrahepatic (eCCA) (Qurashi M et al., 2023). Based on their macroscopic growth patterns, Intrahepatic cholangiocarcinoma is classified into mass-forming, periductal infiltrating, and intraductal growth types. Mass-forming lesions are the predominant type, accounting for 60-80% of Intrahepatic cholangiocarcinoma whereas periductal infiltrating type was considered 15%-35% of Intrahepatic cholangiocarcinoma and expanded the portal tracts presenting as bile duct strictures with luminal narrowing (Vij et al. 2022). There is also intraductal growth, they are the least common variant and account for 8-29 % of Intrahepatic cholangiocarcinoma. There characterized by a papillary or polypoid lesion within a dilated bile duct and most often represents a malignant progression of intraductal papillary mucinous neoplasm (IPNB). Several studies found Intrahepatic cholangiocarcinoma can have mixed growth patterns as shown in Figure 2.1 (Vij et al. 2022). The incidence of intrahepatic cholangiocarcinoma is expected to increase worldwide by up to 10-fold during the next two to three decades whereas treatment strategies are still limited. Most patients are typically diagnosed at advanced stages when the available systemic therapies are of limited effectiveness (Dong et al. 2022). Moreover, the prognosis for cholangiocarcinoma remains dismal, this leads to the search for new treatments. Some researchers have proposed using aspirin as a potential preventive and adjuvant agent for cholangiocarcinoma (Shen and Shen 2021).

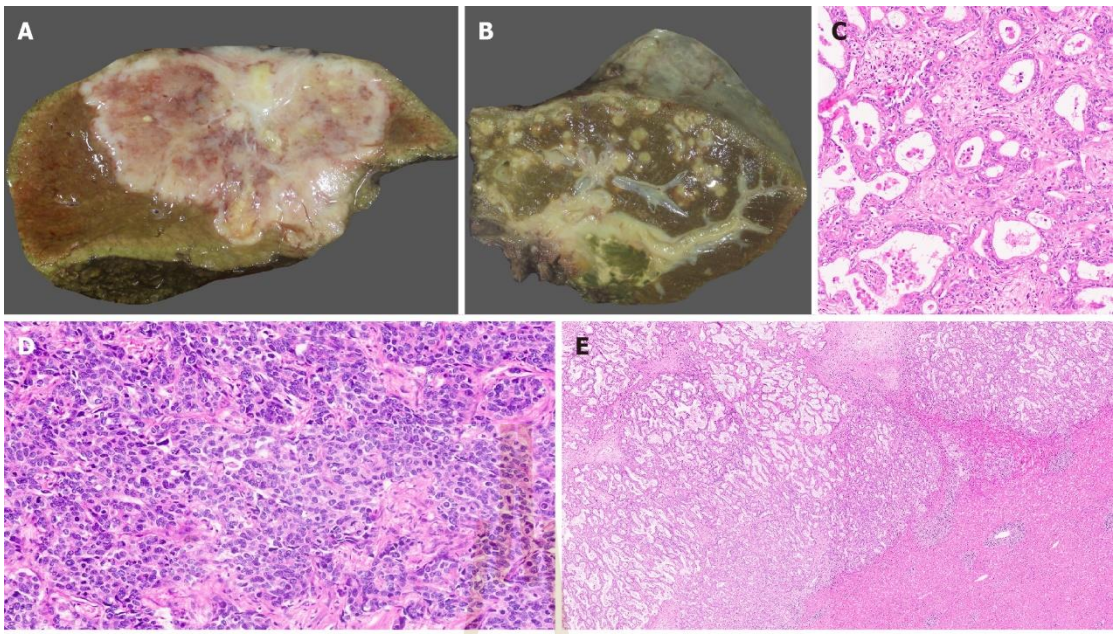

Figure 2.1 Gross features and morphology of cholangiocarcinoma.

A: Mass forming intrahepatic cholangiocarcinoma (IHCC); **B:** Extrahepatic cholangiocarcinoma with periductal infiltrating growth (arrow) and markedly greenish liver; **C:** Well differentiated cholangiocarcinoma [hematoxylin and eosin (H&E, × 25)]; D: Poorly differentiated cholangiocarcinoma (H&E, × 25); E: Large duct variant of IHCC (H&E, × 8). (Vij et al. 2022)

2.1.1 Epidemiology and Etiology of CCA

Cholangiocarcinoma (CCA) is anatomically classified into 3 groups: (1) intrahepatic CCA, (2) perihilar CCA, and (3) distal CCA (Rizvi et al., 2018). Intrahepatic CCA arises above the second-order bile ducts. The anatomical point of distinction between perihilar cholangiocarcinoma and distal cholangiocarcinoma is the cystic duct (Rizvi et al., 2013). perihilar CCA accounts for ~50%-60% of all CCA, distal CCA 20%-30%; and intrahepatic CCA. Intrahepatic CCA is the second most common primary hepatic malignancy after hepatocellular carcinoma (HCC). The incidence of CCA is geographical variation. It is showing higher incidence and mortality rates in Asia compare to Western country, with significant difference between regions of the same country too (Table 2.1) (Khan et al., 2019)

Table 2.1 Global prevalence of CCA, per 100 000 population (Khan et al., 2019)

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

Chronic inflammation and the obstruction of bile duct are the high-risk conditions for CCA development, but the real etiologies of CCA are still unknown (Gores, 2003; Sirica et al., 2002; Blechacz et al., 2008). The risk factors, which well characterized of CCA are primary sclerosing cholangitis, hepatolithiasis, and choledochal cysts, while inflammatory bowel disease, cirrhosis hepatitis B and C virus infection, diabetes, obesity, alcohol consumption and smoking are less-developed risk factors (Rizvi and Gores, 2013). In Eastern, the major risk factor of CCA in Thailand, Laos PDR, and Malaysia is the infection of liver fluke, *Opisthorchis viverrini*, whereas *Chlonorchis sinesis* infection is a prominent risk factor of CCA in Japan, South Korea, and Vietnam (Kullavanijaya et al., 1999; Sithitthaworn et al., 1994). Epidemiology and experimentally (both in laboratory and animal models) has been established the association of *Opisthorchis viverrini* infection and CCA. In 1994, The International Agency for Research on Cancer of the WHO (IARC) lists *Opisthorchis viverrini* as a group 1 carcinogen (IARC, 1994).

2.1.2 Clinical symptoms of CCA

The symptoms of patients with CCA are different between extra-hepatic and intra-hepatic CCA depends on the degree of bile duct obstruction (Table 2.2). Symptoms of biliary obstruction, including painless jaundice, pale stools, dark urine, and pruritus were mostly presented in extra-hepatic CCA patients, while acute cholangitis and paraneoplastic syndromes including, hypoglycemia, hypercalcemia, diabetes, porphyria, cutanea tarda, and migratory thrombophlebitis were rarely presented (less than 10% of cases). For intra-hepatic CCA, the symptoms of hepatic mass, including abdominal pain, malaise, night sweats, and cachexia were mostly presented (Gatto, M., & Alvaro, D., 2010).

Unfortunately, most of the patients with CCA were clinically diagnosed at advanced stages when the treatment with surgery, radiology and chemical therapy was not effective. The lack of effective diagnosis markers for early detection and the nature of CCA that clinically silent of their symptoms in an early state is the major problem for late diagnosis of CCA.

Table 2.2 CCA clinical presentation.

Intra-Hepatic CCA	Extra-Hepatic CCA
Abdominal pain	90% Painless, jaundice
Diminished appetite	10% Cholangitis
Weight loss	Rare symptoms:
Malaise	Diabetes, Hypoglycemia
Night sweats	Hypercalcemia
Cholestasis	Porphyria cutanea tarda
Incidental mass	Migratory thrombophlebitis

(Gatto, M., & Alvaro, D., 2010)

2.1.3 Diagnosis of CCA

The combination of clinical, biochemical, radiological and histological information was used for diagnosis of CCA. For CCA subtype, the different imaging techniques were used: ultrasound (US), computed tomography (CT), magnetic resonance imaging/magnetic resonance cholangiopancreatography (MRI/MRCP) and positron emission tomography (PET) (Khan et al., 2005). Histological analysis has been used to confirm the diagnosis for CCA (Eloubeidi et al., 2004). The new biomarkers for early diagnosis and prognostic values for CCA are being investigated.

Non-invasive biomarkers for diagnosis CCA such as circulating nucleic acids; (cell-free DNA (cfDNA), RNA (cfRNA), microRNAs (miRs), and long non-coding RNAs (lncRNAs) can be found in most biofluids. The circulating nucleic acids are less resistant to degradation and/or modification and can be easily detected and amplified when compared with the others potential biomarkers such as proteins or metabolites. In the recent years, a few studies have been investigated the circulating nucleic acids profiles in patients with CCA (Figure 2.2) (Macias et al., 2018)

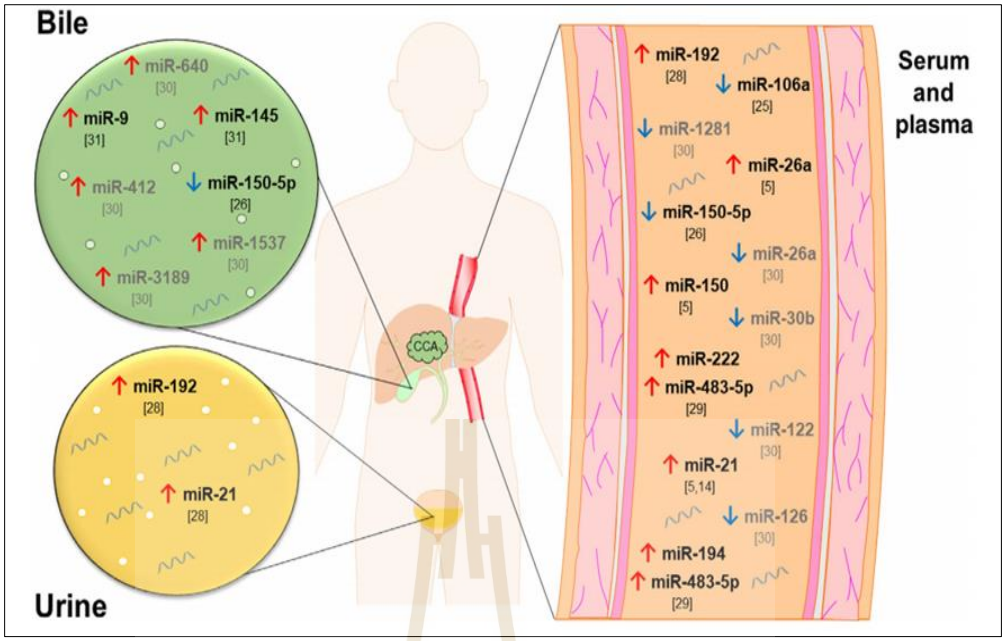

Figure 2.2 Circulating miRs up - or down- regulated in the serum, plasma, bile and urine of patients with CCA

Up regulation (red arrows) and down regulation (blue arrows) of circulating microRNAs (miRs) in the serum, plasma, bile and urine of patients with CCA, compared with sclerosing cholangitis (PSC) (in grey) or with healthy individuals (in black). (Macias et al., 2018).

Proteins and cytokines are the other promising biomarkers for diagnosis and prognosis of CCA, including cytokeratin-19 fragment (CYFRA 21-1), matrix metalloproteinase-7 (MMP-7) and osteopontin (Ost) (Table 2.3).

Table 2.3 Proteins/cytokines as circulating biomarkers for CCA. (Macias et al., 2018)

Protein/Cytokine	Source	Levels	Comparison	SEN (%)	SPE (%)	AUC
MMP7	Serum	Up	CCA (n = 44) vs benign biliary tract disease (n = 36)	75	78	0.730
Osteopontion	Serum	Up	CCA (n = 80) vs healthy controls (n = 42)	88	100	0.964
IL-6	Serum	Up	CCA (n = 26) vs healthy controls (n = 23)	73	92	0.875
S100A6	Serum	Up	CCA (n = 29) vs healthy controls (n = 22)	86	91	0.909
DKK1	Serum	Up	iCCA (n = 37) vs healthy controls (n = 50)	76	100	0.872
SSP411	Serum	Up	CCA (n = 35) vs "cholangitis (n = 13) and healthy controls (n = 23)"	90	83	0.913

SEN, sensitivity; SPE, specificity.

The candidate biomarkers that have been investigated and have potential for diagnosis of CCA include CA19-9, CCA, S100A6, 50 DKK1, 51 KL-6-Mucin52 and SSP411.53. However, more sample sizes and multiple geographic distribution of CCA need to be evaluated.

The biomarker of extracellular vesicles (EVs) such as FCN2, ITIH4, FIBG, EpCAM, ASGPR1, CD133 can be found in body fluids of patients with CCA. During CCA progression, EVs have been shown to help generate tumor environment by inducing the differentiation of mesenchymal stem cells into fibroblasts (Table 2.4).

Table 2.4 The extracellular vesicles (EVs) biomarkers for diagnosis of CCA. (Macias et al., 2018)

Biomarker	Source	Method	Controls	SEN	SPE	AUC
FCN2, ITIH4, FIBG	Serum Evs	Proteomics	PSC	92-100	81	0.88-0.96
EpCAM ASGPR1 CD133	Serum Evs	FACS	Healthy	90	50	0.82
Total amount	Serum Evs	NTA	Non-malignant bile duct stenoses	47	80	0.81
Total amount	Bile Evs	NTA	Non-malignant bile duct stenoses	100	100	0.1
miR-191 miR-486-3p miR-1274b miR-16 miR-484	Serum Evs	MmiR arrays	Non-malignant bile duct stenoses	67	96	-

The good prognosis biomarkers for resected CCA are in tumor tissues. In the resent years, specific genomic and transcriptomic profiles for prognosis of CCA have been investigated. The genetic alteration of CCA have been reported; TP53 for DNA repair, KRAS, BRAF, SMAD4, FGFR2, and PTPN3 for growth pathways, KMT2C, ARID1A, PBRM1 and BAP1 for chromatin remodeling and NOTCH1, NICD, WNT7B and WNT10A for signaling pathways (Table 2.5).

Table 2.5 Tumor tissue prognostic biomarkers for CCA. (Macias et al., 2018)

Gene	Description	Expression (high/low)	Method	Overall survival	Recurrence-free survival
KRAS	Kristen rat sarcoma viral oncogene homolog	High	TES/WES	Decreased	Decreased
TP53	Tumour protein 53	Low	TES/WES	Decreased	Decreased
PROM 1	Prominin-1/CD133	High	IHC	Decreased	-
CTGF	Connective tissue growth factor	High	IHC	Increased	-
VIM	Vimentin	High	IHC	Decreased	-
DKK1	Dickkopf WNT signalling pathway inhibitor 1	High	IHC/PCR	Decreased	-
SOX 2	SRY-box 2	High	IHC	Decreased	-
SOX17	SRY-box 17	Low	PCR	Decreased	-
MUC1	Mucin 1, cell surface associated	High	PCR	Decreased	-
PTEN	Phosphatase and tensin homologue	Low	ISH	Decreased	-
PTPN14	Protein tyrosine phosphatase, non-receptor type 14	Low	ISH	Decreased	-
inc RNA AFAP1-A51	AFAP1 antisense RNA1	High	PCR	Decreased	-
inc RNA PANDAR	Promotor of CDKN1A antisense DNA damage activated RNA	High	PCR	Decreased	-
CEACAM 6	Carcinoembryonic antigen-related cell adhesion molecule 6	High	PCR/ IHC	-	Decreased
CD151	Cluster of differentiation 151	High	PCR/ IHC/ WB	Decreased	Decreased
C-met	MET proto-oncogene, receptor tyrosine kinase	Low	PCR/ IHC	Increased	Increased
BECN1	Beclin 1	High	PCR	Increased	Increased
STAT3	Signal transducer and activator of transcription 3	High	PCR/ IHC	Decreased	Decreased
CAPN4/CAPNS1	Calpain small subunit 1	High	PCR/ IHC/ WB	Decreased	Decreased
SOX9	SRY-box 9	High	IHC	Decreased	-
CDH1	E-cadherin	Low	IHC	Decreased	-

Table 2.5 Tumor tissue prognostic biomarkers for CCA. (Macias et al., 2018) (continued)

Gene	Description	Expression (high/low)	Method	Overall survival	Recurrence-free survival
FASCIN/FSCN1	Fascin action-bunding protein 1	High	IHC	Decreased	-
S100A4	S100 calciun-binding protein A4	High	IHC	Decreased	-
EGFR	Epidermal growth factor receptor	High	IHC	Decreased	-
VEGF	Vascular endothelial growth factor	High	IHC	Decreased	-
MUC4	Mucin 4, cell surface associated	High	IHC	Decreased	-
MUC16/CEA 125	Mucin 16, cell surface associated	High	IHC	Decreased	-
CD44	Cluster of differentiation 44	High	IHC	Decreased	-
FBXW7	F-box and WD repeat domain containing 7	Low	IHC	Decreased	Decreased
CDKN1B/p27	Cyclin-dependent kinase inhibitor 1B	Low	IHC	Decreased	Decreased
CCND1	Cyclin D1	High	IHC	Decreased	Decreased
HDGF	Heparin-binding growth factor	High	IHC	Decreased	-
KRT103	Keratin103	Low	IHC	Increased	-
HDAC1	Histone deacetylase 1	High	IHC	Decreased	Decreased
NOTCH4	Notch4	High	IHC	Decreased	-
PTP4A3/PRL3	Protein tyrosine phosphatase type IVA, member 3	High	IHC	Decreased	-
AKT1	AKT serine/threonine kinase 1	High	IHC	Increased	-
MTOR	Mechanistis target of rapamycin kinase	High	IHC	Increased	-
SMAD7	SMAD family member 7	High	IHC	Decreased	Decreased
FOXC2	Forkhead box C2	High	IHC	Decreased	Decreased
SKP2	S-phase kinase-associated protein 2	High	IHC	Decreased	-
CTL4	Cytotoxic T-lymphovyte antigen 4	High	mRNA microarray	-	Decreased
IL-33	Interleukin 33	High	PCR/ ISH	-	Increased
MIR21	MicroRNA21	High	ISH/ PCR	Decreased	Decreased

2.2 Glycosphingolipid GM2

Glycosphingolipids (GSLs) are subclass of glycolipids found in cell membranes of organisms from bacteria - humans. GSLs are the major glycolipids of animals. Galactosylceramide (GalCer) was the first glycosphingolipid that characterized in 1884. It is also one of the most abundant GSLs molecules in the brain of vertebrate. GalCer consists of ceramide and a galactose residue linked by glycosidic interaction at ceramide (C-1) hydroxyl group. In the past, "Sphingosine" means its structure was difficult to determine and analyze (Varki et al., 2015-2017). GSLs were identified because they accumulate to pathological levels in tissues of the patients. For example, lysosomal storage diseases, a genetic disorders in which the enzymes that degrade glycans are defect. Tay–Sachs disease, in which it accumulates "ganglioside" GM2 in nerve clusters or "ganglion" of the brain. GlcCer (Glucosylceramide) was first isolated from the spleen of a patient with Gaucher disease. Recently, many unique GSL structures have been found and identified in all vertebrate tissues. (D'Angelo et al., 2013) The pathways of the biosynthesis of ganglioside are shown in Figure 2.4 (Konrad Sandhoff et al., 2013).

In plasma membrane, GSLs are expressed in the outer part of the cells with glycans as the terminal structures. The function of GSLs are classified into two major categories: (1) trans recognition (modulating activities of cells via binding to molecules on other plasma membranes and (2) cis regulation (mediating interaction of proteins in the same plasma membrane). In experimental environment using chemical inhibitor or genetic modification, the cells without GSLs can still survive and proliferate. (Hakomori et al., 1981)

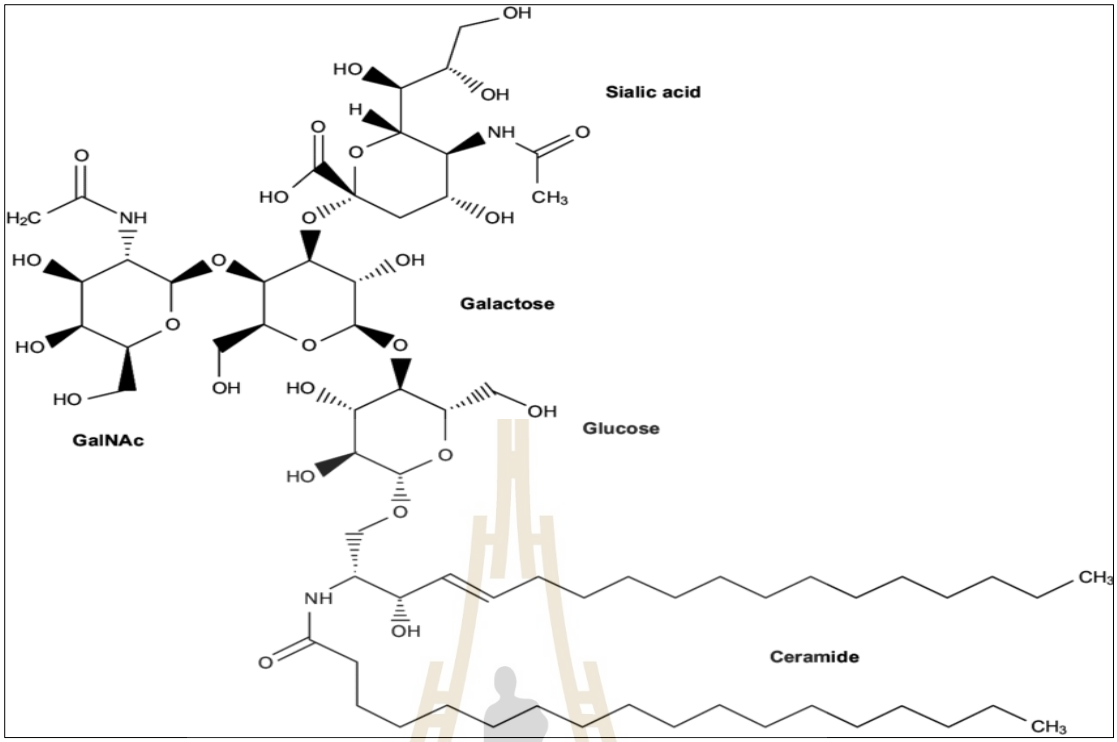

Figure 2.3 Structure of Ganglioside GM2. (Lawson et al., 2016)

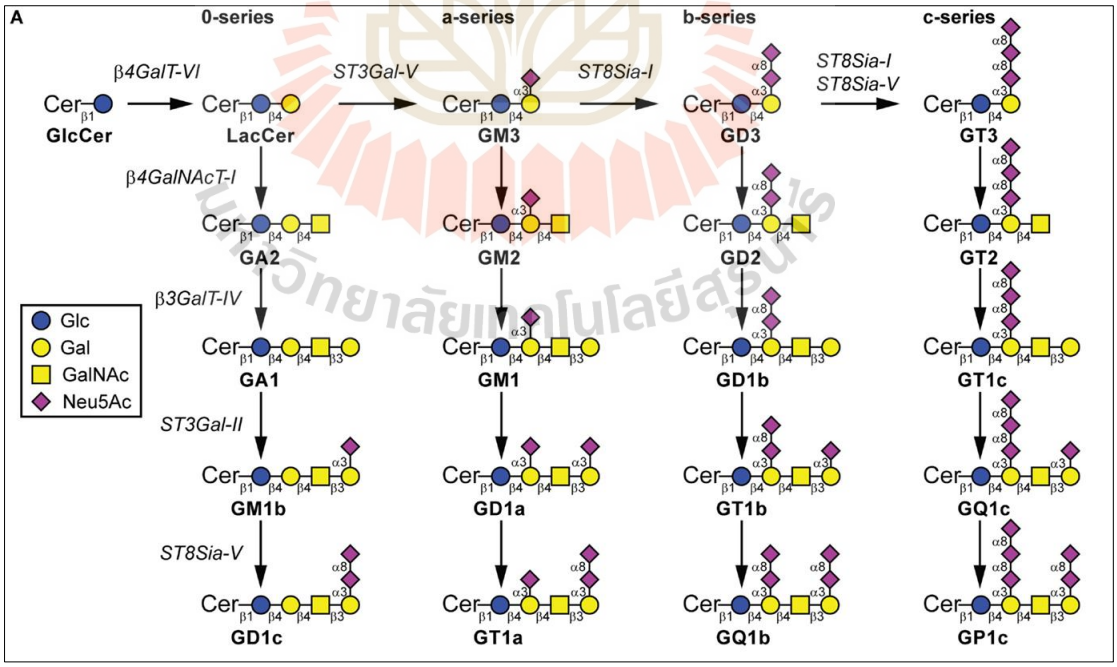

Figure 2.4 Biosynthesis of gangliosides. (Daniotti et al., 2016)

2.2.1 Role of GM2 in Cancers

Genetic mutations of glycosphingolipid biosynthetic enzymes in humans are extremely rare. Mutations of ganglioside GM3 synthesis enzyme, ST3GAL5, result in severe infantile seizures, profound motor and intellectual deficits. Mutations in B4GALNT1, a ganglioside-specific biosynthetic gene is less severe. It is responsible for biosynthesis of GM2 and GD2, resulting in hereditary spastic paraplegia accompanied by intellectual disability. Mutations in genes of GSL degradation enzymes lead to the accumulation of GSLs in lysosomes, which cause GSL storage diseases (Todeschini et al., 2008). Gaucher disease is the most common GSL storage disease, which is caused by mutations in the enzyme β-glucocerebrosidas, resulting in the accumulation of GlcCer in the liver and spleen. Another example, Tay–Sachs disease, is caused by mutations in a β-hexosaminidase and results in the build-up of GM2, culminating in irreversible fatal deterioration of brain function (Hakomori et al., 1981). Change in glycosylation in glycoprotein and glycolipid is often associated with Malignancy and tumor progression. The altered levels of glycosyltransferase activities are involved. For example, the increase of GD3 or GM2 in melanoma, and sialyl-Lewis in gastrointestinal cancers were demonstrated (Ichikawa et al., 1994).

The roles of ganglioside GM2 in tumor progression were investigated in molecular level using cell line models. The interaction of GM2 with integrin and integrin receptor leading to downstream signaling and phosphorylation of Erk–MAPK, which leading to increased tumors migration (Figure 2.6) (Kundu et al., 2016). In pancreatic cancer, GM2 may be involved in control of cell growth, cell proliferation, cell differentiation, and apoptosis through regulation of growth factor, TGF-β1 (Sasaki et al., 2019). Regulation of GM2 expression was demonstrated by downregulation of Neuraminidase 3 (NEU3), a plasma membrane-associated sialidase (removing sialic acid) (Tringali C et al., 2012). The inhibition of GM2 and TGF receptor interaction by AMP-dNM treatment could inhibit TGF- β1 signaling and invasion. In melanoma, increase expression of GM2/GD2 could enhance tumorigenesis by angiogenesis induction (Yoshida et al., 2020).

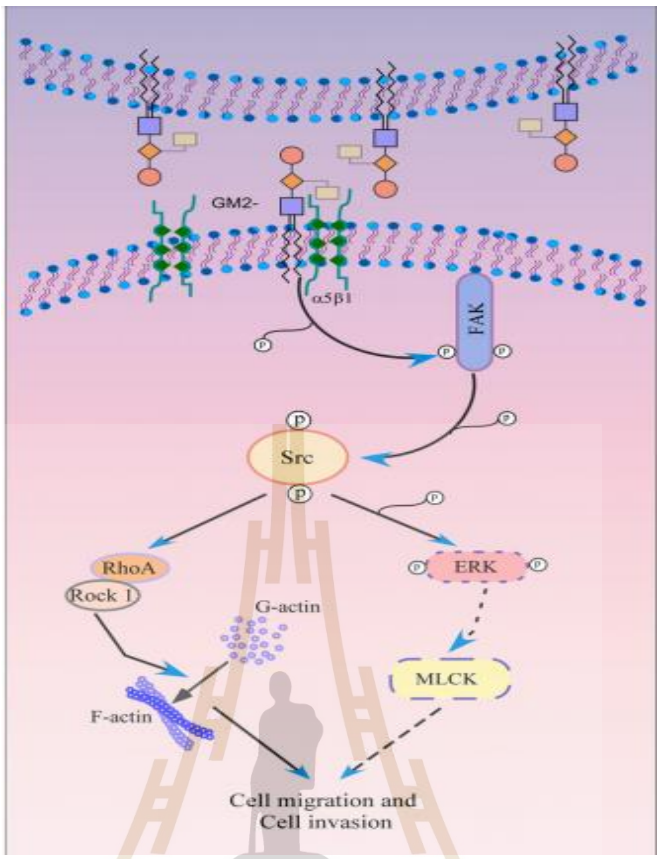

Figure 2.5 GM2 mediates tumor cell migration and invasion, a hypothetical model. (Kundu et al.,2016)

The expression of GM2 also demonstrated in pancreatic ductal adenocarcinoma cell line (Sasaki et al., 2019), Irradiation-tolerant Lung Cancer Cells (Ishihara et al., 2018), melanoma (Lo, Agnes SY, et al., 2010), small-cell lung cancer (SCLC) (Cheresh DA., 1986), sarcoma, and neuroblastoma (Zhang et al., 1997) (Table 2.6). It has been showed that overexpression of GM2 in pancreatic ductal adenocarcinoma cell line is associated with positive growth, invasion, and advanced tumor stage (Sasaki et al., 2019). These evidences indicate the significance of GM2 in cancer which may have an important role in tumorigenesis and tumor progression.

Table 2.6 GM2 expression in cancers

Sample	GM2 expression	References
Pancreatic ductal adenocarcinoma cell line	High	Sasaki et al., 2019
Irradiation-tolerant Lung Cancer Cells	High	Ishihara et al., 2018
Melanoma	High	Lo, Agnes SY, et al., 2010
Small-cell lung cancer (SCLC)	High	Cheresh DA., 1986
Sarcoma, and neuroblastoma	High	Zhang et al., 1997

Nevertheless, the previous studies show characteristic, biosynthesis, role and malignant transformation in melanoma, gastrointestinal cancers of GSLs. But the observation on characteristic and malignant transformation of GSLs in cancerous bile duct tissue is unexplored. The aims of this study are: (1) to examine the expression of GSLs GM2 in CCA tissue and CCA cell lines (2) to Investigated the correlation of GM2 metabolizing enzymes, B4GALNT1, B3GALT4, GM2A, HEXA, and HEXB.

CHAPTER 3
RESERCH METHODOLOGY

3.1 CCA cell lines

CCA cell lines (KKU055, KKU100, and KKU213A) and an immortalized human cholangiocytes (MMNK1) were obtained from Cholangiocarcinoma Research Institute, Khon Kaen University, Thailand. CCA cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 100 U/mL penicillin and 100 μg/mL streptomycin with 10% FBS (Gibco, Grand Island, NY, USA). Cell growth were performed at 37 °C under 5% CO_2 and 95% humidified air.

3.2 CCA tissues

Tissue samples were obtained from the specimen bank of the Cholangiocarcinoma Research Institute, Khon Kaen University, Thailand. Written informed consent was obtained from each subject. The Ethics Committee of Khon Kaen University approved the study protocol (registration number: HE521209). We obtained 60 intrahepatic CCA tissue samples from patients with CCA and their adjacent normal tissues (mean age, 54.78±9.00; 17 females and 43 males).

3.3 Immunocytochemistry

Cell lines were seeded (1×10^4 and 5×10^4 cells/well) into the 24-well plates and fixed by 4% paraformaldehyde in PBS, pH 7.4 for 15 minutes at room temperature. Fixed cells were then permeabilized by 0.2% Triton X-100 in PBST for 10 min at room temperature. Non-specific binding was blocked by 0.3% of FBS (Fetal Bovine Serum) for 30 min at room temperature. After washing with PBS, cell lines were (a) incubated with anti-ganglioside GM2 rabbit polyclonal antibody (dilution 1:500) for 18 hours at 4°C, then (b) incubated with Envision/HRP, Rabbit (Dako, Carpinteria, CA, USA). DAB+ solution (3,3'-diaminobezidine-tetrahydrochloride) was used for visualization. The cell

lines were then counterstained with hematoxylin. GM2-positive cells were evaluated compared with negative control.

3.4 Quantitative real-time PCR analysis (qRT-PCR)

RNA was extracted from CCA cell lines (KKU055, KKU100, and KKU213) and an immortalized human cholangiocytes (MMNK1) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized using superscript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). The primer sequences used for GM2 metabolizing enzyme genes, B4GALNT1, HEXA, HEXB, GM2A, and *β-actin* were listed in Table 3.1. Expression levels of GM2 metabolizing enzyme genes were evaluated by SYBR-Green-based real-time PCR in Light Cycler® 480 II machine (Roche Applied Sciences, Indianapolis, IN, USA). The amount of starting cDNA was adjusted using *β-actin* as an internal control. GM2 metabolizing enzyme genes expression was calculated by the $2^{-\Delta_{CT}}$ equation.

Table 3.1 The primer sequences used for reverse transcription-quantitative PCR (qRT-PCR).

Gene	Forward (5'-3')	Reverse (5'-3')
B4GALNT1	5'-GACACAGTCCGGTTCTCCAC 3'	5'-TCGTGACTAGAGCGCTGATG 3'
HEXA	5'-ACCAGCGCTACGTCCTTTAC-3'	5'-TATGCCGTTTCCCTGTGAGG-3'
HEXB	5'-GAGTGTGATGCTTTCCCCAAC-3'	5'-CCTCGTAATGCTCCCCAAAC-3'
GM2A	5'TCAAGATCCCATGCACAGAC 3'	5' GCTCTTGGGCAGTGAGTAGG 3'

B4GALNT1, β-1,4-N-Acetyl-Galactosaminyltransferase 1; HEXA, hexosaminidase A ; HEXB, hexosaminidase B; GM2A, GM2 activator protein

3.5 Immunohistochemistry

Anti-ganglioside GM2 (Sigma-Aldrich, Merck, Darnstadt, Germany) was used for GM2 detection. Paraffin sections of CCA tissues were deparafinnized in xylene, hydrated in downgraded ethanol and distilled water, respectively. The antigens were unmasked by hearting each section (in 0.1 mol/L citrate buffer, pH 6.0) in a pressure cooker.

Endogenous peroxidase was removed by treating the sections with absolute methanol containing 3% hydrogen peroxide for 30 minutes at room temperature. The non-specific binding were blocked by 20% normal horse serum for 30 minutes at room temperature. The sections were then incubated with (1) anti-ganglioside GM2 rabbit polyclonal antibody (dilution 1:500) for 2 hours at room temperature, (2) incubated with Envision/HRP, Rabbit (Dako, Carpinteria, CA, USA), for 1 hours at room temperature. The sections were visualized with 3,3'-diaminobezidine-tetrahydrochloride, Liquid DAB+ (Dako, Carpinteria, CA, USA), and counterstained with hematoxylin. The staining results were evaluated as a frequency of GM2-positive cells at the tumor area—classified into 4 scoring categories (0, negative; 1+, 1%-10%; 2+, 11%-50%; and 3+, >50%). Two researchers without any knowledge of the clinicopathological variables evaluated the specimens. The score 0 and 1+ were categorized as "low expression"; and the score 2+ and 3+, as "high expression" for statistical analysis.

3.6 Statistical analysis

The expression of GM2 metabolizing enzyme genes in CCA cell lines (KKU055, KKU100, and KKU213) and a non-tumor cell line (MMNK1) were reported as means ± SD. The X^2-test was used for determination of the association between ganglioside GM2 expression and the clinicopathological features of CCA. All analyses were done using SPSS statistical software (version 22.0; SPSS, Inc., Chicago, IL, USA). A $P< 0.05$ indicated a statistically significant difference.

CHAPTER 4

RESULTS

4.1 High expression of ganglioside GM2 in CCA cell lines

The expression of ganglioside GM2 in CCA cell lines (KKU055, KKU100, and KKU213A) and an immortalized human cholangiocytes (MMNK1) was investigated by immunohistochemistry. GM2 was highly expressed in plasma membrane and cytoplasm of CCA cells. Three of CCA cell lines, KKU055 (poorly differentiated cholangiocarcinoma cell line), KKU100 (poorly differentiated adenocarcinoma), and KKU213A (poorly differentiated squamous cell carcinomas) were evaluated as high expression of GM2 with specific staining of plasma membrane and cytoplasm of the cells (score 2+ and 3+) while an immortalized human cholangiocytes (MMNK1) were evaluated as low expression with negatively or partially staining (score 0 and 1+, Figure 4.1.).

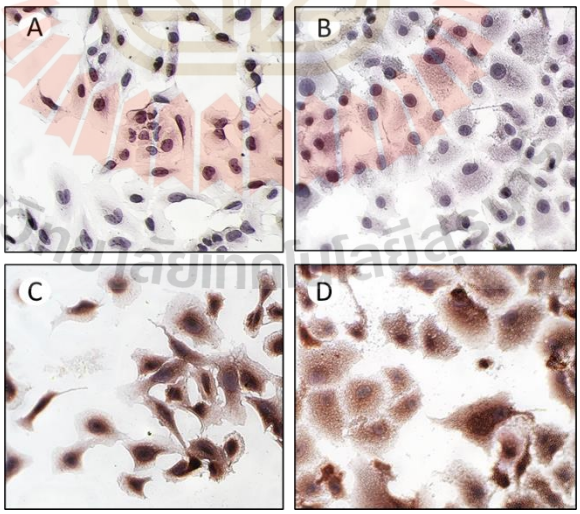

Figure 4.1 Grading of Immunocytochemistry of ganglioside GM2 in CCA cell line: Negative (A), 1+; 1%-10% of GM2 staining (brown color) (B); 2+, 11%-50% of GM2 staining (brown color) (C); and 3+, >50% of GM2 staining (brown color) Original magnification, x200.

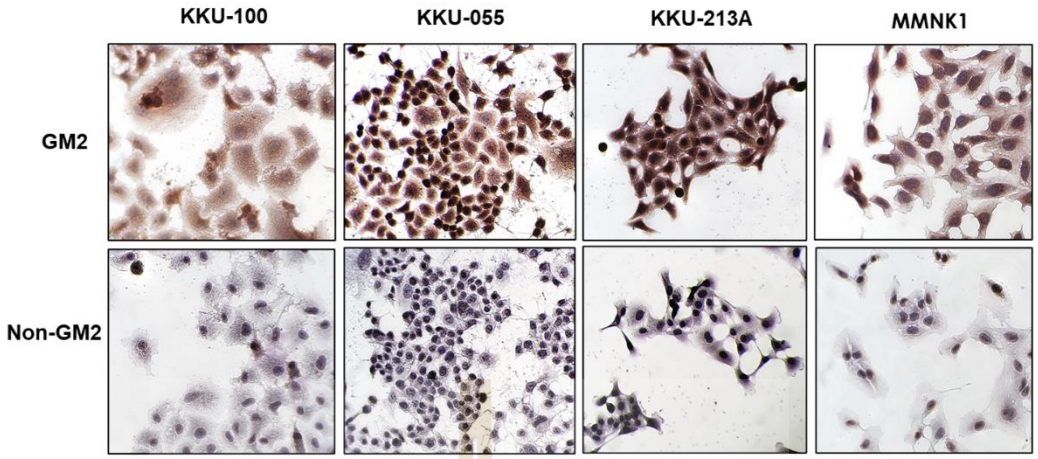

Figure 4.2 Immunocytochemistry of ganglioside GM2 in CCA cell lines (KKU055, KKU100, and KKU213A) compared with an immortalized human cholangiocytes (MMNK1). High expression of GM2 was demonstrated in CCA cell lines with cell membrane and cytoplasmic staining. Original magnification, x200.

4.2 Altered expression of GM2 metabolizing enzymes in CCA cell lines

To investigate the enzymes that contribute to GM2 expression, we further determined the expression of GM2 metabolizing enzymes in CCA cell lines (KKU055, KKU100, and KKU213A) compared with an immortalized human cholangiocytes (MMNK1) by RT-PCR. B4GALNT1, HEXA, HEXB, and GM2A expression level were demonstrated. Scheme of GM2 metabolizing enzymes was shown in Figure 4.3. B4GALNT1 and GM2A were up regulated in CCA cell lines, while HEXA and HEXB were down regulated (Figure 4.4A.). The comparison between GM2 synthesizing enzymes (B4GALNT1) and GM2 catabolism enzymes (HEXA, HEXB, and GM2A) were shown in Figure 4.4B. These data demonstrated that high expression of GM2 in CCA cell lines were contributed by up regulation of GM2 synthesizing enzymes (B4GALNT1) and down regulation of GM2 catabolism enzyme (HEXA and HEXB). However, an up regulation of GM2A (GM2 catabolism protein) in CCA cell lines may represent a compensation process for their cofactors (HEXA and HEXB) low expression.

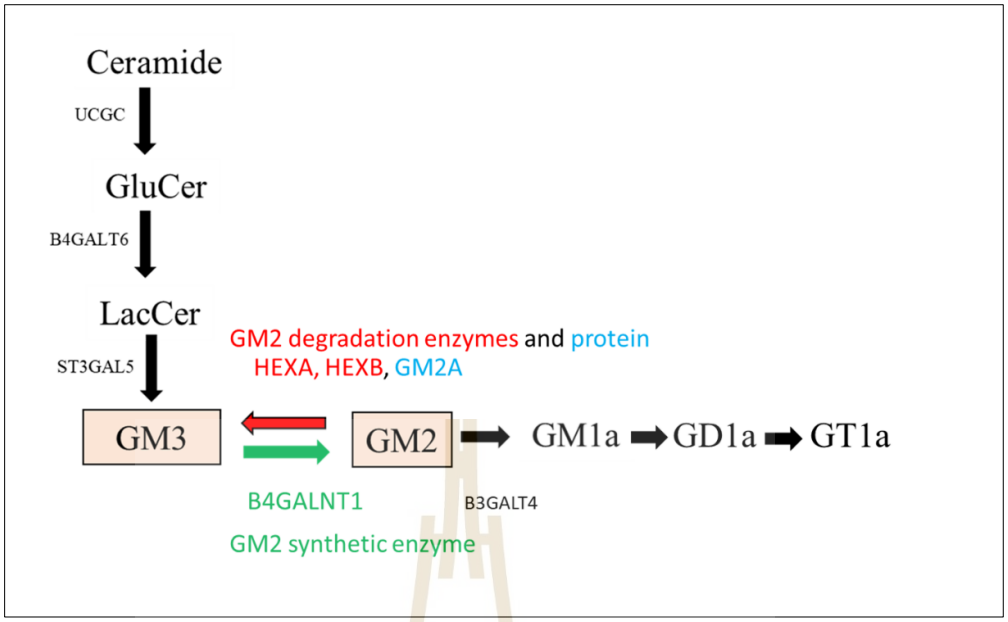

Figure 4.3 Scheme of biosynthesis and degradation of ganglioside GM2. (Modified from Okuda., 2019)

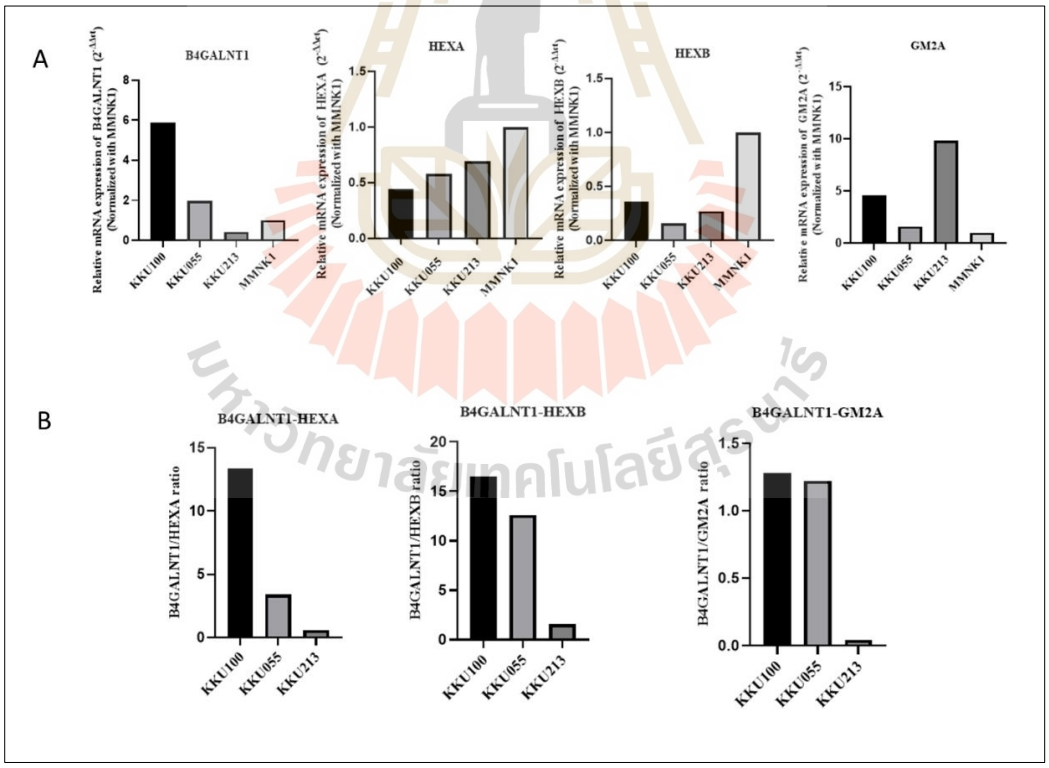

Figure 4.4. Altered expression of GM2 metabolizing enzymes. Expression level of B4GALNT1, HEXA, HEXB, and GM2A (A); comparison between GM2 synthesizing enzymes (B4GALNT1) and GM2 catabolism enzyme (HEXA, HEXB, and GM2A) (B).

4.3 High expression of ganglioside GM2 is associated with vascular invasion of CCA

We then further investigated the expression of ganglioside GM2 in 60 intrahepatic CCA tissues by immunohistochemistry. Ganglioside GM2 was highly expressed in the cell membrane, cytoplasm of tumor cells and in the epithelium lining of the bile ducts (Figure 4.6.). Fifty-seven of CCA tissues were classified as high expression of GM2 (95.00%) with specific membrane and cytoplasm staining (score 2+ and 3+), while the remaining 3 CCA tissues (5.00%) with negative or partially staining were classified as low expression (score 0 and 1+) (Figure 4.5. Grading of immunohistochemistry). The associations between GM2 expression and clinicopathological features were further analyzed, using a univariate analysis. High expression of ganglioside GM2 was associated with vascular invasion (P=0.024). However, there were no significant associations between GM2 expression and patient age, sex, histological type, tumor stage, and lymphatic invasion (Table 4.1).

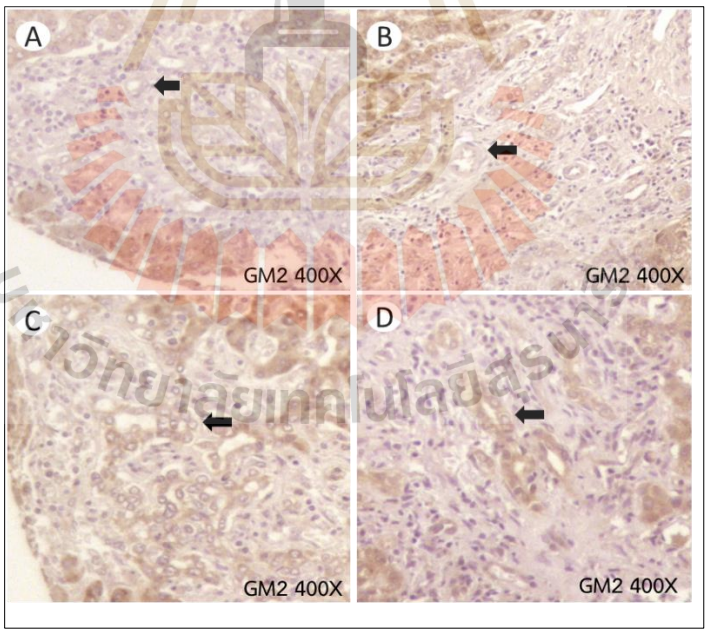

Figure 4.5 Grading of Immunohistochemistry of ganglioside GM2 in tissue of patients with CCA: Negative (A), 1+; 1%-10% of GM2 staining (brown color) (B); 2+, 11%-50% of GM2 staining (brown color) (C); and 3+, >50% of GM2 staining (brown color) (D). Original magnification, x400.

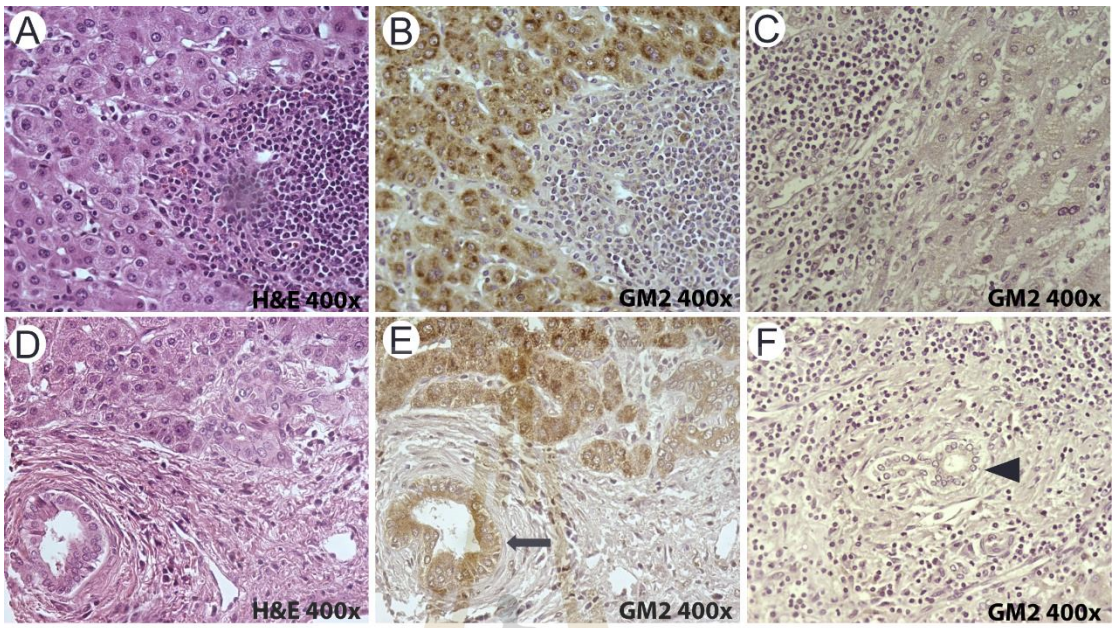

Figure 4.6 Immunohistochemistry indicates high expression of ganglioside GM2 in tissue of patients with CCA: hematoxylin and eosin (H&E) staining of CCA tissue (A), high expression of GM2 in CCA tissue (B), low expression of GM2 in CCA tissue (C), H&E staining of bile duct epithelium in CCA tissue (D), high expression of GM2 in bile duct epithelium of CCA tissue (E, arrow), low expression of GM2 in bile duct epithelium of CCA tissue (F, arrow head). Original magnification, x400.

Table 4.1 Demographic data of patient with CCA and ganglioside GM2 expression in clinicopathologic features.

Variables	GM2 expression		P-value
	Low (n=3)	High (n=57)	
Age (y)			
< 55	1	29	0.554
≥ 55	2	28	
Sex			
Male	2	41	0.844
Female	1	16	

n=60; *P < 0.05 vs. low expression. CCA, cholangiocarcinoma.

Table 4.2 Association between ganglioside GM2 expression and clinicopathologic features of patient with CCA.

Variables	GM2 expression		P-value
	Low (n=3)	High (n=57)	
Histologic type			
Papillary	2	34	0.809
Nonpapillary	1	23	
Stage			
I	0	12	0.374
II-IV	3	45	
Lymphatic invasion			
Absent	1	20	0.95
Present	2	37	
Vascular invasion			
Absent	3	20	*0.024
Present	0	37	

n=60; *P < 0.05 vs. low expression. CCA, cholangiocarcinoma.

CHAPTER 5
DISCUSSION AND CONCLUSION

In the current study using immunocytochemistry, we demonstrated the high expression of ganglioside GM2 in CCA cell lines compared with a non-tumor cell line and further investigated the expression of GM2 metabolizing enzymes by qRT-PCR. The correlations between GM2 expression and clinicopathological features in tissue of patients with CCA were then investigated by immunohistochemistry.

GM2 expression has been reported in various cancers, such as colon cancer, pancreatic cancer, melanoma, and neuroblastoma (Zhang et al., 1997). GM2 has also been reported to play a role in tumor cell migration/invasion (Kundu et al., 2016). In this study, we demonstrated the high expression of GM2 in CCA cell lines compared with a non-tumor cell line and the association between GM2 expression and vascular invasion in tissue of patients with CCA suggesting that GM2 plays an important role in the progression and metastasis of CCA.

B4GALNT1 was up regulated in CCA cell lines (KKU055 and KKU100) compared with a non-tumor cell line (MMNK1). High expression of B4GALNT1 has been reported with poor prognosis in renal cell carcinoma, neuroblastoma (Cheung et al., 2003), and melanoma (Tringali et al., 2014). B4GALNT1 has been shown to induce angiogenesis and promotes tumorigenesis in melanoma by induction of ganglioside GM2/GD2 (Yoshida et al., 2020). Up regulation of B4GALNT1 in CCA may represent a biosynthesis change in GSLs during tumor progression.

HEXA, HEXB, and GM2A are GM2 degradation protein and enzymes. Defect of HEXA and HEXB enzymes were found in neurodegenerative disorders (GM2 gangliosidosis) (Breiden et al., 2020). In CCA cell lines, up regulation of GM2A, but down regulation of

HEXA and HEXB were demonstrated. Down regulation of HEXA and HEXB in CCA cell lines represent a schematic change in degradation of GM2 and reflect GM2 expression in CCA.

In summary, elevated expression of ganglioside GM2 in CCA cell lines are contributed by an up regulation of B4GALNT1 enzyme and down regulation of HEXA and HEXB.

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University	Burapha University	Medical Science	Bachelor of Science (B.S.)	2017	2.87

Grants

- One Research One Graduate scholarship (OROG) of Suranaree University of Technology, Thailand