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Ganglioside GM2: a potential biomarker for cholangiocarcinoma

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Abstract

Objective: To investigate the expression of glycosphingolipids in serum and tissue from patients with cholangiocarcinoma compared with healthy controls.

Methods: Nanospray ionization-linear ion trap mass spectrometry (NSI-MS^n) was used to demonstrate the comparative structural glycomics of glycosphingolipids in serum from patients with cholangiocarcinoma (n=15), compared with healthy controls (n = 15). GM2 expression in cholangiocarcinoma tissues (n = 60) was evaluated by immunohistochemistry.

Results: Eleven glycosphingolipids were detected by NSI-MS^n: CMH (ceramide monohexose), Lac-Cer (galactose (Gal)β1-4 glucose (Glc)β1-1'-ceramide), Gb3 (Galα1-4Galβ1-4Glcβ1-1'-ceramide), Gb4/Lc4 (N-acetylgalactosamine (GalNAc)β1-3Galα1-4Galβ1-4Glcβ1-1'-ceramide/ Galβ1-4 N-acetylglucosamine (GlcNAc)β1-3Galβ1-4Glcβ1-1'-ceramide), GM3 (N-acetylneuraminic acid (NeuAc)2-3Galβ1-4Glcβ1-1'-ceramide), GM2 (GalNAcβ1-4[NeuAc2-3]Galβ1-4Glcβ1-1'-ceramide), GM1 (Galβ1-3GalNAcβ1-4[NeuAc2-3]Galβ1-4Glcβ1-1'-ceramide), hFA (hydroxylated fatty acid)-CMH, hFA-Lac-Cer, hFA-Gb3, and hFA-GM3. Lac-Cer was the most abundant structure among the lactosides and globosides (normal, 24.40% ± 0.11%; tumor, 24.61% ± 2.10%), while GM3 predominated among the gangliosides (normal, 29.14% ± 1.31%;

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tumor, 30.53% ± 4.04%). The two glycosphingolipids that significantly differed between healthy controls and patients with cholangiocarcinoma were Gb3 and GM2. High expression of GM2 was associated with vascular invasion in tissue from patients with cholangiocarcinoma.

Conclusions: Altered expression of glycosphingolipids in tissue and serum from patients with cholangiocarcinoma may contribute to tumor growth and progression. The ganglioside GM2, which significantly increased in the serum of patients with cholangiocarcinoma, represents a promising target as a biomarker for cholangiocarcinoma.

Keywords

Cholangiocarcinoma, glycosphingolipids, GM2, Gb3, vascular invasion, gangliosides, globosides, lactosides, glycomics, biomarkers

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Introduction

A malignancy of the bile duct epithelium, cholangiocarcinoma is a major health problem in northeastern Thailand, particularly where infection with the liver fluke *Opisthorchis viverrini* is endemic.[1] Although it is rare worldwide, the incidence of cholangiocarcinoma is high in East and Southeast Asia; the incidences of cholangiocarcinoma have also been increasing in England, the United States, and Australia.[2,3] Diagnosis of cholangiocarcinoma most often occurs when the disease is advanced or disseminated, such that surgery and other therapies are ineffective.[4] Current clinical serum markers for cholangiocarcinoma are carcinoembryonic antigen and CA19-9; these have low sensitivity/specificity and are inadequate for early detection. Thus, there is an urgent need for novel target biomarkers to facilitate early detection of cholangiocarcinoma.

Aberrant expression of glycolipids has been observed in different types of cancer cells; accordingly, several glycosphingolipids and gangliosides have been tested for use in cancer therapy.[5] Glycosphingolipids have been associated with acute and chronic diseases.[6] There have been multiple in vitro and in vivo biomarker studies of glycolipids in cholangiocarcinoma. These studies have shown that tissue expression levels of sialyl Lewis A[7] and serum levels of the carbohydrate marker S121 are related to cholangiocarcinoma prognosis;[8] in an animal model, S121 was expressed in the cytoplasm and at the apical surface of biliary cells at the early stage of tumor development, then increased with tumor progression.[9] Furthermore, the studies have shown that tissue levels of GlcNAc[10] and *O*-GlcNAc transferase,[11] as well as serum levels of the glycan epitope CA-S27, are related to cholangiocarcinoma prognosis.[12] Notably, a lectin microarray-based sero-biomarker has been reported for the detection of *O*-linked glycosylation in patients with cholangiocarcinoma;[13] cholangiocarcinoma cell lines exhibit differential expression of *O*-glycans, based on the histological type of cholangiocarcinoma.[14] Our prior research revealed elevated expression of *N*-linked glycoprotein glycans in serum from patients with cholangiocarcinoma, compared with healthy controls;[15] elevated glycosphingolipid levels have also

been associated with shorter survival in patients with cholangiocarcinoma.[16]

Because the above studies of glycolipids have not yielded sufficiently compelling biomarkers, the present study investigated the structural detail and the quantities of glycosphingolipids in serum samples from patients with cholangiocarcinoma, compared with healthy controls. The candidate glycosphingolipid biomarkers identified in this study may aid in development of cholangiocarcinoma biomarkers and their use in clinical applications.

Materials and methods

Materials

Glycolipid standards were purchased from Matreya (State College, PA, USA) and fine chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium hydroxide (KOH) and sodium hydroxide (NaOH) were obtained from Sigma-Aldrich.

Participants and samples

Serum and tissue samples were obtained from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Institute, Khon Kaen University, Thailand. Written informed consent was obtained from each participant for inclusion of their serum samples in this study. The Ethics Committee of Khon Kaen University approved the study protocol (registration number: HE521209).

Preparation of glycosphingolipids

Twenty microliters of each serum sample—from patients with cholangiocarcinoma and healthy controls—were homogenized by vortexing in ice-cold 50% methanol. To extract the lipid components, the homogenate was extracted for 2 hours at room temperature in a 4:8:3 ratio of chloroform to methanol to water. The extracts were then centrifuged at $2500 \times g$ for 15 minutes at room temperature. The resulting supernatants were dried using a rotary evaporator. To remove the glycerolipids, the dry lipid extracts were saponified using 0.5 M KOH at 37°C for 18 hours. Ether phospholipids were removed using ice-cold concentrated HCl at pH 2 for 30 minutes. The extracts were dialyzed against tap water at 4°C for 18 hours, then dried using a rotary evaporator. Three microliters of each respective glycosphingolipid extract were spotted onto a thin-layer chromatography plate and developed for 9 minutes using solvent in a 6:4:1 ratio of chloroform to methanol to water. The developed glycosphingolipids were visualized using an orcinol-sulfuric acid reaction; the amounts of glycosphingolipids for permethylation were adjusted with respect to the intensity of Lac-Cer on the thin-layer chromatography plate.

Permethylation of glycosphingolipids

To facilitate analysis of glycosphingolipids by mass spectrometry, glycosphingolipid mixtures were permethylated in accordance with the method described by Anumula and Taylor.[17] Briefly, glycosphingolipid mixtures were permethylated under water-free conditions using 500 μL DMSO, 10 μg NaOH, and 200 μL methyl iodide (all from Sigma-Aldrich) for 30 minutes at room temperature.

Nanospray ionization-linear ion trap mass spectrometry

Permethylated glycosphingolipids were dissolved in methanol: 1-propanol: 2-propanol: 13 mM aqueous ammonium acetate (16: 3: 3: 2 by volume) and infused into a linear ion trap mass spectrometer (LTQ Orbitrap Discovery; Thermo Fisher Scientific, Inc., Waltham, MA, USA), using a nanospray source at a syringe flow rate of 0.5 μL/min. The capillary temperature was set to 210°C, and mass

spectrometry analysis was performed in positive ion mode with 45% collision energy. Glycosphingolipids with 16 to 24 fatty acids were analyzed. The prevalence of each glycosphingolipid in each profile was quantified by comparing its signal intensity with the sum of signal intensities for all identified glycosphingolipids GSLs in the profile; this comparison yielded "% Total profile." Permethylated maltotri- and maltotetra-saccharides (Dp3 and Dp4) were used as external glycan standards.

Immunohistochemistry

Anti-ganglioside GM2 (Sigma-Aldrich) was used for GM2 detection. Paraffin sections of cholangiocarcinoma tissues were deparaffinized in xylene, then hydrated in a series of graded ethanol and distilled water mixtures. The antigens were unmasked by heating each section in a pressure cooker, while sections were immersed in 0.1 mol/L citrate buffer (pH 6.0). The sections were then treated with absolute methanol containing 5% hydrogen peroxide for 30 minutes at room temperature. Subsequently, sections were washed with phosphate-buffered saline and nonspecific binding was blocked by incubation in 20% normal horse serum for 30 minutes at room temperature. Sections were then incubated with anti-ganglioside GM2 rabbit polyclonal antibody (dilution 1:500) for 2 hours at room temperature, followed by incubation with Envision/HRP, Rabbit (Dako, Glostrup, Denmark), in accordance with the manufacturer's instructions. The sections were visualized with 3,3'-diaminobenzidine-tetrahydrochloride (Liquid DAB+; Dako), and counterstained with hematoxylin. The staining results were evaluated as the frequency of GM2-positive cells in the tumor area—classified into four scoring categories (0, negative; 1+, 1% to 10%; 2+, 11% to 50%; and 3+, >50%). The specimens were evaluated by two researchers blinded to the clinicopathological variables. In statistical analysis, scores 0 and 1+ were categorized as "low expression"; scores 2+ and 3+ were categorized as "high expression".

Statistical analysis

The respective prevalences of glycosphingolipids (% total profile) in serum samples from patients with cholangiocarcinoma and healthy controls are reported as means ± standard deviations. Differences in expression between groups were analyzed using independent *t*-tests. The chi-squared test was used to measure associations between ganglioside GM2 expression and clinicopathological features of cholangiocarcinoma. All analyses were performed using SPSS Statistics, version 22.0 (IBM Corp., Armonk, NY, USA). Differences with $P < 0.05$ were considered statistically significant.

Results

Participant characteristics

We obtained 15 serum samples from patients with cholangiocarcinoma (mean age, 55.73 ± 9.68 years; five women and 10 men) and 15 samples from healthy controls (mean age, 55.46 ± 9.58 years; 4 women and 11 men). The serum samples were maintained at –80°C until analysis. We also obtained 60 intrahepatic cholangiocarcinoma tissue samples and adjacent normal tissues from patients with cholangiocarcinoma (mean age, 54.78 ± 9.00 years; 17 women and 43 men).

Altered expression of glycosphingolipids in serum from patients with cholangiocarcinoma and healthy controls

Mass analysis of glycosphingolipids in serum samples from patients with cholangiocarcinoma and healthy controls is

shown in Figure 1. The glycosphingolipids assigned were lactosides, globosides, gangliosides, and their hydroxylated fatty acid forms (hFA). The lactosides detected included: Lac-Cer (structure 2; galactose (Gal)β1-4 glucose (Glc)β1-1'-ceramide), Lc4 (structure 4; Galβ1-4 N-acetylglucosamine (GlcNAc)β 1-3Galβ1-4Glcβ1-1'-ceramide), and hFA-Lac-Cer (structure 9). The globosides detected were Gb3 (structure 3; Galα1-4Galβ1-4Glcβ1-1'-ceramide), Gb4 (structure 4; N-acetylgalactosamine (GalNAc)β 1-3Galα1-4Galβ1-4Glcβ1-1'-ceramide), and hFA-Gb3 (structure 10). The gangliosides detected were GM3 (structure 5; N-acetylneuraminic acid (NeuAc)2-3Galβ1-4Glcβ1-1'-ceramide), GM2 (structure 6; GalNAcβ1-4[NeuAc2-3]Galβ1-4Glc β1-1'-ceramide), GM1 (structure 7; Galβ1-3GalNAcβ1-4 [NeuAc2-3]Galβ1-4Glcβ1-1'-ceramide), and hFA-GM3 (structure 11). CMH (structure 1; ceramide monohexose) was assigned to the isomeric structures GlcCer or GalCer, while hFA-CMH (structure 8) was the corresponding hydroxylated form. Gb4/Lc4 (structure 4) was the corresponding isomeric structure of globoside Gb4 and lactoside Lc4. Lac-Cer (structure 2) was most abundant among the lactosides and globosides (normal, $24.40 \pm 0.11\%$; tumor, $24.61 \pm 2.10\%$), while GM3 (structure 5) was most abundant among the gangliosides (normal, $29.14 \pm 1.31\%$; tumor, $30.53 \pm 4.04\%$). The abundance of ganglioside GM2 (structure 6, $P = 0.042$) was significantly elevated in serum samples from patients with cholangiocarcinoma, compared with serum samples from healthy controls. In contrast, the abundance of globoside Gb3 (structure 3, $P = 0.041$) was significantly

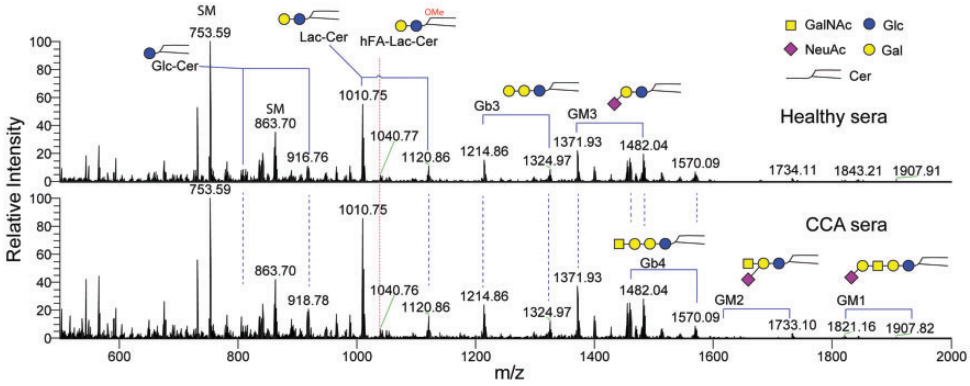

Figure 1. Mass spectrometry spectra of permethylated glycosphingolipids in serum samples from patients with cholangiocarcinoma, compared with healthy controls, as determined by nanospray ionization-linear ion trap mass spectrometry. Glycosphingolipid extracts from the cholangiocarcinoma sera (CCA sera) and healthy sera were permethylated and analyzed. The respective mass spectrometry spectra demonstrate the predominance of glycosphingolipids (i.e., lactosides, globosides, and gangliosides) in cholangiocarcinoma sera, compared with healthy control sera. GM2 was significantly elevated in serum samples from patients with cholangiocarcinoma, while Gb3 was significantly reduced. The prevalences of glycosphingolipid profiles in serum samples from patients with cholangiocarcinoma, compared with healthy controls, are shown in Table 1. Graphical representations of monosaccharide residues are shown in the legend, consistent with the suggested nomenclature of the Consortium for Functional Glycomics (http://glycomics.scripps.edu/CFGnomenclature.pdf).
Abbreviations: SM, sphingomyelin; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; NeuAc, N-acetylneuraminic acid; Cer, ceramide; Lac-Cer (Galβ1-4 glucose (Glc)β1-1'-ceramide), Gb3 (Galα1-4Galβ1-4Glcβ1-1'-ceramide), Gb4 (GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1'-ceramide), GM3 (NeuAc2-3Galβ1-4Glcβ1-1'-ceramide), GM2 (GalNAcβ1-4[NeuAc2-3]Galβ1-4Glcβ1-1'-ceramide), GM1 (Galβ1-3GalNAcβ1-4[NeuAc2-3]Galβ1-4Glcβ1-1'-ceramide), hFA (hydroxylated fatty acid).

reduced in serum samples from patients with cholangiocarcinoma (Table 1).

Association of high expression of ganglioside GM2 with vascular invasion of cholangiocarcinoma

We used immunohistochemistry to investigate the expression of ganglioside GM2 in intrahepatic cholangiocarcinoma tissues from 60 patients with cholangiocarcinoma. Ganglioside GM2 was highly expressed in the cell membrane and cytoplasm of tumor cells, as well as in the epithelial lining of bile ducts (Figure 2). Fifty-seven cholangiocarcinoma tissues exhibited high expression of GM2 (95.00%) with specific membrane and cytoplasm staining (scores 2+ and 3+); the remaining three cholangiocarcinoma tissues (5.00%) exhibited low expression of GM2 with negative or partial staining (scores 0 and 1+). The associations between GM2 expression and clinicopathological features were then examined by univariate analysis.

Table 1. Characteristics and prevalences of glycosphingolipids in serum samples from patients with cholangiocarcinoma and healthy controls.

Structures	Group[$]	n	Relative abundance (%) Mean ± standard deviation	*P* values
1 CMH	N	15	14.09 ± 1.95	0.931
	T	15	14.25 ± 2.10	
2 Lac-Cer	N	15	24.40 ± 0.11	0.846
	T	15	24.61 ± 2.29	
3 Gb3	N	15	11.42 ± 0.19	0.041*
	T	15	8.02 ± 2.57	
4 Gb4/Lc4	N	15	10.18 ± 0.76	0.868
	T	15	10.00 ± 1.98	
5 GM3	N	15	29.14 ± 1.31	0.523
	T	15	30.53 ± 4.04	
6 GM2	N	15	1.40 ± 0.18	0.042*
	T	15	2.45 ± 0.80	
7 GM1	N	15	0.44 ± 0.29	0.973
	T	15	0.43 ± 0.25	
8 hFA-CMH	N	15	3.66 ± 0.77	0.858
	T	15	3.54 ± 0.40	
9 hFA-Lac-Cer	N	15	1.49 ± 0.03	0.798
	T	15	1.45 ± 0.31	
10 hFA-Gb3	N	15	0.38 ± 0.24	0.323
	T	15	0.67 ± 0.40	
11 hFA-GM3	N	15	3.39 ± 0.76	0.431
	T	15	4.04 ± 1.01	

Note: The respective prevalence of each indicated glycosphingolipid is expressed as a percentage of the total pool of detected glycosphingolipids (i.e., % total profile, mean ± standard deviation). The same numerical designations, assigned to each structure, are used in the figures, tables, and text.

[$]N, Healthy sera; T, cholangiocarcinoma sera; *$P < 0.05$, significant difference.

Abbreviations: CMH (ceramide monohexose), Lac-Cer (galactose (Gal)β1-4 glucose (Glc)β1-1'-ceramide), Gb3 (Galα1-4Galβ1-4Glcβ1-1'-ceramide), Gb4/Lc4 (N-acetylgalactosamine (GalNAc)β1-3Galα1-4Galβ1-4Glcβ1-1'-ceramide/Galβ1-4 N-acetylglucosamine (GlcNAc)β1-3Galβ1-4Glcβ1-1'-ceramide), GM3 (N-acetylneuraminic acid (NeuAc)2-3Galβ1-4Glcβ1-1'-ceramide), GM2 (GalNAcβ1-4[NeuAc2-3]Galβ1-4Glcβ1-1'-ceramide), GM1 (Galβ1-3GalNAcβ1-4[NeuAc2-3] Galβ1-4Glcβ1-1'-ceramide), hFA (hydroxylated fatty acid).

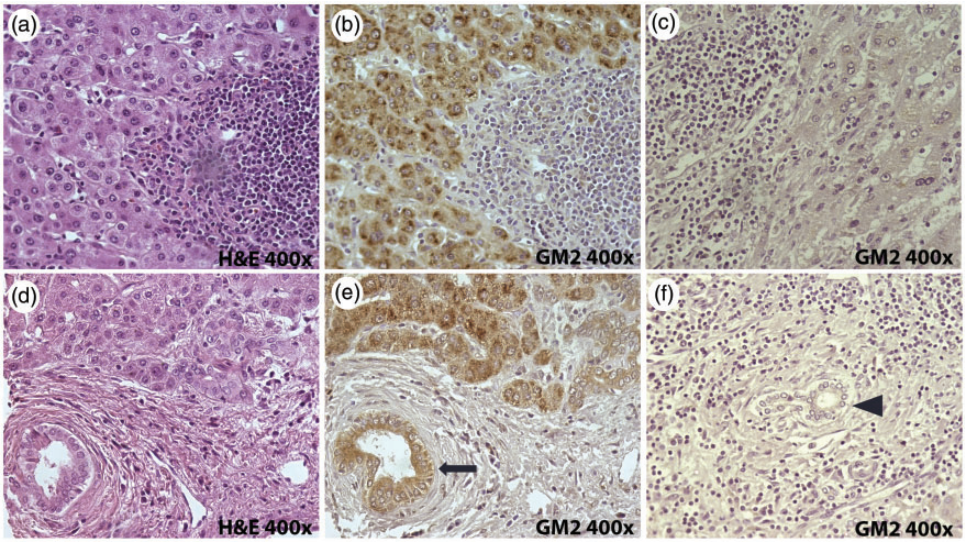

Figure 2. Immunohistochemistry analysis demonstrated high expression levels of ganglioside GM2 (N-acetylgalactosamine (GalNAc)β1-3 galactose (Gal)α1-4Galβ1-4 glucose (Glc)β1-1'-ceramide) in tissue from patients with cholangiocarcinoma. Hematoxylin and eosin (H&E) staining of cholangiocarcinoma tissue (a); high expression of GM2 in cholangiocarcinoma tissue (b); low expression of GM2 in cholangiocarcinoma tissue (c); H&E staining of bile duct epithelium in cholangiocarcinoma tissue (d); high expression of GM2 in bile duct epithelium of cholangiocarcinoma tissue (e, arrow); low expression of GM2 in bile duct epithelium of cholangiocarcinoma tissue (f, arrow head). Original magnification, $\times$400.

High expression of ganglioside GM2 was associated with vascular invasion ($P=$ 0.024). However, there were no significant associations between GM2 expression and any of the following factors: patient age, sex, histological type, tumor stage, or lymphatic invasion (Table 2).

Discussion

The current study used mass spectrometry to demonstrate the differential expression of glycosphingolipids in serum samples from patients with cholangiocarcinoma, compared with healthy controls. It also investigated the expression of ganglioside GM2 in the tissues of patients with cholangiocarcinoma.

Notably, this study showed that ganglioside GM3 (structure 5) was the most abundant glycosphingolipid in serum samples from patients with cholangiocarcinoma and healthy controls. High expression levels of GM3 have been observed in various tumor tissues;[18] moreover, serum GM3 was proposed as a biomarker for kidney cancer[19] and a risk factor for metabolic syndrome.[20] In the current study, however, serum GM3 levels did not significantly differ between patients with cholangiocarcinoma and healthy controls.

This study revealed that the two glycosphingolipids with significantly different expression levels between patients with cholangiocarcinoma and healthy controls were GM2 (structure 6, $p=0.042$) and Gb3 (structure 3, $p=0.041$). GM2 was significantly elevated in serum samples from patients with cholangiocarcinoma, while Gb3 was significantly reduced in those samples. Elevated expression of GM2 has been observed in various cancer tissues, such as

Table 2. Associations between expression of ganglioside GM2 and clinicopathologic features of patients with cholangiocarcinoma.

Variables	GM2 expression		*P* value
	Low (n=3)	High (n=57)	
Age (years)			
<55	1	29	0.554
≥55	2	28	
Sex			
Male	2	41	0.844
Female	1	16	
Histologic type			
Papillary	2	34	0.809
Nonpapillary	1	23	
Stage			
I	0	12	0.374
II–IV	3	45	
Lymphatic invasion			
Present	2	37	0.95
Absent	1	20	
Vascular invasion			
Present	0	37	0.024*
Absent	3	20	

n=60; *$P<0.05$ vs. low expression.

melanoma, neuroblastoma, breast cancer, colon cancer, pancreatic cancer, ovarian, and endometrial cancer;[18] however, reports of elevated expression of GM2 in serum samples from patients with cancer have been limited.[21] GM2 reportedly plays a role in tumor cell migration/invasion.[22] Thus, high expression of the ganglioside GM2 may serve as a prognostic marker for cholangiocarcinoma. Furthermore, the present study demonstrated an association between expression of GM2 and vascular invasion of tissue in patients with cholangiocarcinoma, suggesting that GM2 is important in the progression of cholangiocarcinoma.

In contrast, expression of Gb3—which is highly expressed by various types of cancers, including pancreatic and colon cancers[23,24]—was significantly reduced in serum samples from patients with cholangiocarcinoma. Reduced expression of Gb3 has been observed in breast cancer cell cultures and in cancer stem cells.[25] Gb3 has been shown to increase the expression of human multidrug resistance gene (*MDR1*) through recruitment of c-Src kinases and inhibition of apoptosis.[26] The reduction of Gb3 in cholangiocarcinoma may represent a change in the glycosphingolipid biosynthesis pathway during tumor progression. The same precursor (Lac-Cer) is used by Gb3 and gangliosides (i.e., GM3 and GM2); thus, the significant increase in GM2 expression observed in the present study may reflect changes in the relative amount of Gb3.

Hydroxylated forms of glycosphingolipids—hFA-CMH, hFA-Lac-Cer, hFA-Gb3, and hFA-GM3—were detected in serum samples from patients with cholangiocarcinoma and healthy controls. hFA-glycosphingolipids are present in various tissues including the nervous system, epidermis, kidney, and tumors.[27] Elevated expression of hFA-glycosphingolipids has been observed in drug-resistant human ovarian carcinoma cell lines.[28,29] There is recent evidence that hFA-glycosphingolipids exhibit specific roles in membrane homeostasis and cell signaling.[30] However, the present study did not demonstrate significant differences in hFA-glycosphingolipid expression in serum samples from patients with cholangiocarcinoma, compared with healthy controls. Importantly, the sample size was limited in the present study, which may have affected the statistical power of the findings.

In summary, the altered expression of glycosphingolipids in serum samples from patients with cholangiocarcinoma appears to contribute to tumor growth and progression. The ganglioside GM2, which exhibited significantly greater expression in serum samples from patients with cholangiocarcinoma,

represents a promising target as a biomarker for cholangiocarcinoma.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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