RESEARCH ARTICLE

Aberrant Glycosylation in Cholangiocarcinoma Demonstrated by Lectin-histochemistry

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Abstract

Cholangiocarcinoma (CCA) is an aggressive malignant tumor which is difficult to diagnose at an early stage. Because no reliable CCA specific markers are available at present, most patients are diagnosed after late clinical presentation. In many tumors, aberrant glycans participate in various steps of pathogenesis and progression. In this study, we investigated aberrant glycosylation in CCA tissues using lectin histochemistry to allow associations of specific glycans with clinicopathological features of the patients to be investigated. For this purpose, 14 lectins specific to 5 main glycan structures were used for screening. Nine lectins showed positive staining in hepatocyte sand stromal cells in liver tissues whereas three lectins, sWGA, SJA and UEA-I, had negative lectin binding to hepatocytes and normal bile duct epithelia but exhibited positive staining with CCA. sWGA was selected for further evaluation of (β-D-GlcNAc)$_n$-glycoconjugate expressions in 44 CCA tissues. We found that sWGA-specific glycans were aberrantly expressed along with CCA development and the level of expression varied with histological types. It was highly expressed in papillary and well-differentiated types but was significantly reduced in poorly-differentiated lesions. Specific associations of sWGA-specific glycoconjugate expression with clinicopathological features or overall survival of patients were not apparent in this cohort study.

Keywords: Bile duct - GlcNAc - lectin - sWGA - cholangiocarcinoma

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Introduction

Cholangiocarcinoma (CCA) is an aggressive malignant tumor, arising from bile duct epithelia at any part of biliary tree. It counts for around 10-25% of primary liver cancers but current epidemiological reports show a globally increasing incidence and mortality rate of CCA over the past decades (Sripa and Pairojkul, 2008). Although the underlining causes remain unclear, patients who have gotten liver fluke infection (*Opisthorchis viverrini*) are associated with a high incidence in northeast Thailand (Zhou et al., 2008; Shin et al., 2010). Patients often show late stages of clinical manifestation and most of them become fatal. The prognosis of CCA is poor with a short survival even in the patients who underwent surgical resection (Khan et al., 2005).

As available, serological tumor markers, carbohydrate antigen (CA19-9) and carcinoembryonic antigen (CEA) are widely used to diagnose CCA (Qin et al., 2004). However, they are not the potential biomarkers for CCA with unsatisfied sensitivity and specificity. On the other hand, imaging techniques such as ultrasonography, computed tomography and magnetic resonance cholangiography as well as pathological diagnoses such as brush cytology and forceps biopsy are performed to detect CCA (Weber et al., 2008). Nevertheless, these tools are difficult to detect CCA in an early stage because of its lesion, size and localization (Qin et al., 2004). A new CCA-related diagnostic biomarker is required to identify the lesion as an early stage as possible.

Aberrant glycosylation is frequently associated with carcinogenesis and tumor progression (Fuster and Esko, 2005). There is a body of evidence showing that cancer cells often display aberrant glycans on the membrane-bounded surface in the aspect of structure and expression level. Hence, tumor-related glycoconjugates may act as potential biomarkers that draw attention of investigators. For example, sialyl Lewis^a (sLe^a) which was expressed highly on CCA cells played an important role in tumor invasion (Juntavee et al., 2005). MUC5AC, a highly glycosylated mucin, was elevated in CCA patient tissues (Wongkham et al., 2003; Silsirivanit et al., 2011) and serum (Silsirivanit et al., 2011) with high sensitivity and specificity.

Lectins are proteins or glycoproteins that can recognize specific glycan structures. Lectins with different glycan-specific bindings can demonstrate a pattern of signals related to glycosylation during cancer-

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associated pathological conditions, such as cancers of prostate, ovary and colon (Jass and Smith, 1992; Arenas et al., 1999; Mitchell and Schumacher, 1999). At present, lectin-based strategies are the popular techniques to observe glyco-alteration in various cancers. In this study, we determined the glycan profile of CCA tissues using histochemistry with 14 lectins. Succinylated wheat germ agglutinin (sWGA) which binds specifically to bile duct epithelia of CCA was investigated in association with clinicopathological features and survival of CCA patients.

Materials and Methods

Human CCA specimens

The 59 paraffin-embedded liver tissues from the primary tumors of intrahepatic CCA patients were obtained from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center (LFCRC), Faculty of Medicine, Khon Kaen University, Thailand. Our study design was approved by the Human Research Ethics Committee, Khon Kaen University and informed consent was obtained from each subject.

All human CCA specimens were histologically proven. Tumor classifications and staging were done according to the American Joint Committee on Cancer (AJCC, 6th edition) (Greene and American Joint Committee on Cancer, 2006). Patient characteristics including age, sex, tumor location, tumor size, histological grading and staging were evaluated from the medical charts and pathology records.

Four different histological types of CCA specimens were used for screening with 14 different lectins. A lectin which specifically binds to CCA was selected as the candidate for lectin-histochemistry determination in a larger sample size (n=44).

Lectin-histochemistry

All biotinylated lectins were products of Vector laboratories (Berlingame, CA). Lectin-histochemistry was done in serial sections of CCA tissues. All paraffin-embedded liver tissues were deparaffinized in xylene and rehydrated in ethanol according to routine histochemistry. The sections were boiled under pressure 3 min in 0.01 M citrate buffer, pH 6.0, and cooled down in phosphate-buffered saline (PBS), pH 7.2 at room temperature. Endogenous peroxidase activity was eliminated using 0.3% (v/v) H_2O_2 in methanol for 30 min. The non-specific glycoconjugate binding was blocked using 0.5% (v/v) periodate treated BSA in PBS, for 30 min in a humidified chamber at room temperature. Sections were incubated with biotinylated lectins 20 μg/ml for all lectins (except RCA and Con A which were 50 μg/ml and 2 μg/ml, respectively) for 2 h and with 12.5μg/ml streptavidin-conjugated horseradish peroxidase (Invitrogen^{TM}; Federick, MD) for 40 min. The peroxidase activity was developed with 25 mg/ml diaminobenzidine (DAB; Dako; Glostrup, Denmark) as the substrate at room temperature in the dark. The sections were counterstained with Mayer's hematoxylin (Bio-optica; Milan, Italy). A positive control and a negative control (without lectin) were included in each run.

Each section was scored under a light microscope according to frequency of presence in cells as<25%=1; 25-50%=2; 50-75%=3; >75%=4 and intensity of the stain as 0=low intensity and 1=high intensity. The lectin-histochemistry score was calculated as frequency x intensity.

Statistical analysis

Statistical analysis was performed using the SPSS software package (version 13.0; SPSS, Chicago, Ill). The chi-square test was used to compare the differences between lectin histochemistry score and clinicopathological features of CCA patients. Results obtained from the determination of sWGA reactivity in different histotypes of CCA tissues were presented as mean±SD and the significance of differences was analyzed by Student's t-test. Patients' survival times were calculated from the date of surgical resection until death. Survival analyses were performed using the Kaplan-Meier method and the differences were compared using the log-rank test. P-value<0.05 was considered as a statistical significance.

Results

Expression profile of glycoconjugates in intrahepatic CCA patients

Glycoconjugates expressed on CCA bile duct epithelium were demonstrated by lectin-histochemistry using biotin-streptavidin system. Fourteen lectins used in this study were grouped according to the glycan specific preference into 5 groups, including glucose/mannose, *N*-acetylglucosamine, oligosaccharide, fucose and *N*-acetylgalactosamine/galactose groups (Table 1).

Different expression profiles of glycoconjugates among normal bile ducts in the non-tumorous adjacent areas, hyperplastic/dysplastic biliary cells and CCA tissues were screened in 4 histologically proven CCA cases (Figure 1). The patterns of lectin bindings to hepatocytes, endothelial cells connective tissues, and CCA, were compared (Table 2). As an overview, almost all lectins bound to liver and CCA tissues; only DBA which binds to α-D-GalNAc, showed negative staining to all cell types examined. Lectins of the glucose/mannose group (Con A, LCA, PSA) and those of *N*-acetylglucosamine group (WGA), *N*-linked oligosaccharide group (PHA-E, PHA-L) and *N*-acetylgalactosamine/galactose group (GSL-I, PNA, RCA_{120} and SBA) gave positive signals in all cell types determined. In contrast, differential staining of lectins, namely sWGA (N-acetylglucosamine), UEA-I (fucose), and SJA (N-acetylgalactosamine/galactose) were observed with varied staining patterns between hepatocytes, normal bile ducts and CCA tissues. These lectins gave positive lectin signals to hyperplasia/dysplasia bile duct epithelia and CCA but not other cell types found in the liver tissues. sWGA which had a specific signal to CCA tissue and gave higher positive frequency than other lectins was selected as the candidate for a larger sample size.

Staining pattern of sWGA-histochemistry in intrahepatic CCA tissues

Lectin histochemical analysis with sWGA of 44

intrahepatic CCA showed strongly intense signals at the apical site with focal or diffuse cytoplasmic staining while all normal biliary cells showed negative or slightly positive signals (Figure 2Aa). A majority of hyperplastic/ dysplastic bile ducts (39/44; 88.6%) gave positive signal for sWGA. Moreover, differential expression of sWGA-binding glycoconjugates in different histological types of CCA was observed (Figures 2Ab-d). Secreted materials in the luminal bile ducts of CCA tissues also gave positive signals of sWGA (Figure 2Ac). The sWGA-histochemical scores of every histological type of CCA were higher than those of normal bile duct epithelia (P<0.001) and those of papillary type and well-differentiated type CCA were significantly higher than those of moderately- and poorly-differentiated types (P=0.026 and P=0.002) (Figures 2A, B).

sWGA-specific glycoconjugates and clinicopathological features of CCA patients

Clinicopathological features of CCA patients included in this study are described in Table 3. Of the 44 intrahepatic CCA patients, 55% were male, with a male to female ratio 1.2: 1. Most of patients (84%) were dead at the end of the follow-up period. The common histotype was the non-papillary type CCA (64%) and 57% of patients were stage IVA and IVB. The univariate analysis between CCA

Table 1. List of Lectins Used in Lectin-histochemistry

Lectin	Abbreviation	Major sugar specific
Glucose/ Mannose group		
Conavalin ensiformis agglutinin	Concanavalin A (Con A)	α-D-Man, α-D-Glc
Lens culinaris agglutinin	LCA	α-D-Man
Pisum sativum agglutinin	PSA	α-D-Man
N-acetylglucosamine group		
Triticum vulgaris agglutinin	WGA	$(\beta\text{-D-GlcNAc})_n$, NeuNAc
Succinylated WGA	sWGA	$(\beta\text{-D-GlcNAc})_n$
Oligosaccharide group		
Phaseolus vulgaris Erythroagglutinin	PHA-E	Bisected, triantennary *N*-glycan
Phaseolus vulgaris Leucoagglutinin	PHA-L	Bisected, triantennary *N*-glycan
Fucose group		
Ulex europaeus agglutinin-I	UEA-I	α-L-Fuc
N-acetylgalactosamine/ Galactose group		
Dolichos biflorus agglutinin	DBA	α-D-GalNAc
Griffonia simplicifolia agglutinin	GSL-I	α-D-Gal, α-GalNAc
Arachis hypogaea agglutinin	Peanut agglutinin (PNA)	β-D-Gal(1-3)D-GalNAc
Ricinus communis agglutinin	RCA_{120}	β-D-Gal, α-D-Gal
Glycine maxi agglutinin	Soybean agglutinin (SBA)	α,β-D-Gal, α,β-D-GalNAc
Sophora japonica agglutinin	SJA	β-D-GalNAc

*Fuc: fucose, Gal: galactose, Glc: glucose, GalNAc: *N*-acetylgalactosamine, GlcNAc: *N*-acetylglucosamine, Man: mannose, NeuNAc: *N*-acetylneuraminic acid

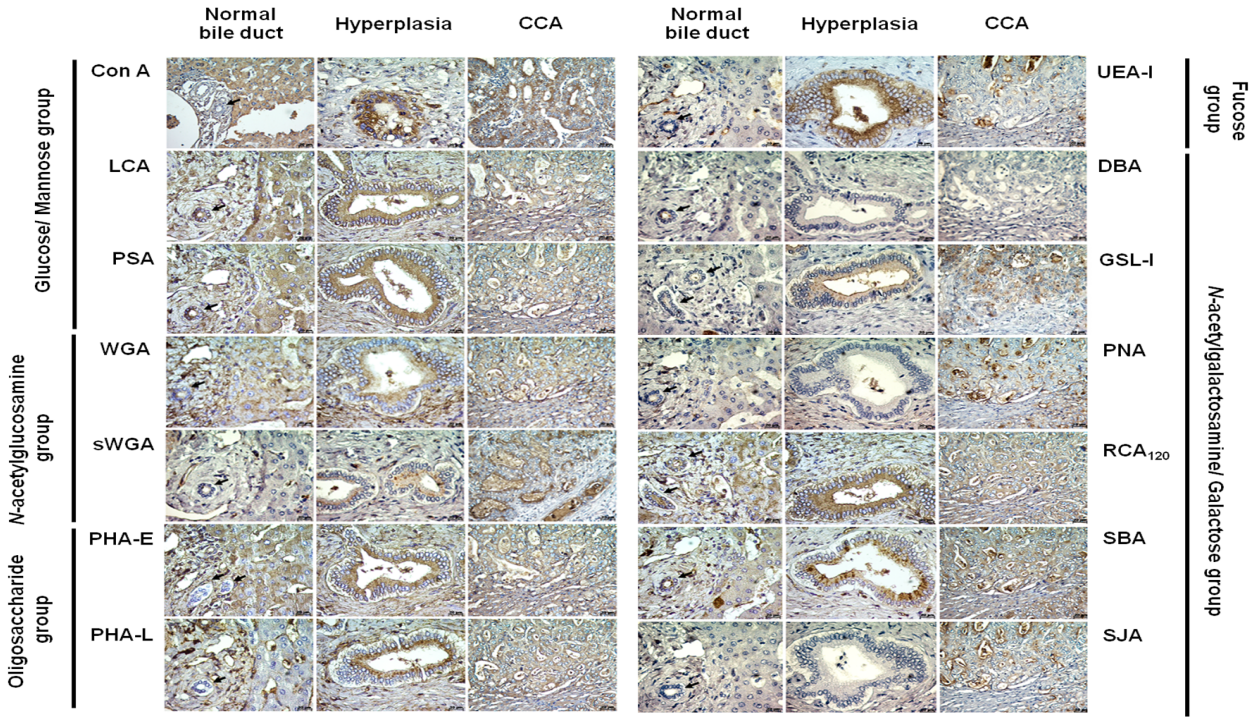

Figure 1. Lectin-histochemistry Profiles of CCA. Different glycoconjugates of serial sections of CCA tissues were determined using 14 different lectins. Normal bile ducts in the adjacent non-tumorous areas are shown by arrow (magnification, x 400)

Table 2. Intensity of Lectin Binding in Patients with Intrahepatic CCA

Lectin	Hepatocytes	Endothelia	CNT	NBD	Hyperplasia	CCA
Glucose/Mannose group						
Con A	+	+	+	+	+	+
LCA	+	+	+	+	+	+
PSA	+	+	+	+	+	+
N-acetylglucosamine group						
WGA	+	+	+	+	+	+
sWGA	-	+	-	-	+	+
Oligosaccharide group						
PHA-E	+	+	+	+	+	+
PHA-L	+	+	+	-	+	+
Fucose group						
UEA-I	-	-	-	-	+	±
N-acetylgalactosamine/Galactose group						
DBA	-	-	-	-	-	-
GSL-I	-	+	+	-	+	+
PNA	+	+	±	±	-	±
RCA_{120}	+	+	+	+	+	+
SBA	+	+	+	+	+	+
SJA	-	-	-	-	±	±

*CNT: connective tissue; NBD: normal bile duct epithelia; +, Positive glycoconjugate expression; -, Negative glycoconjugate expression; ±, slightly positive

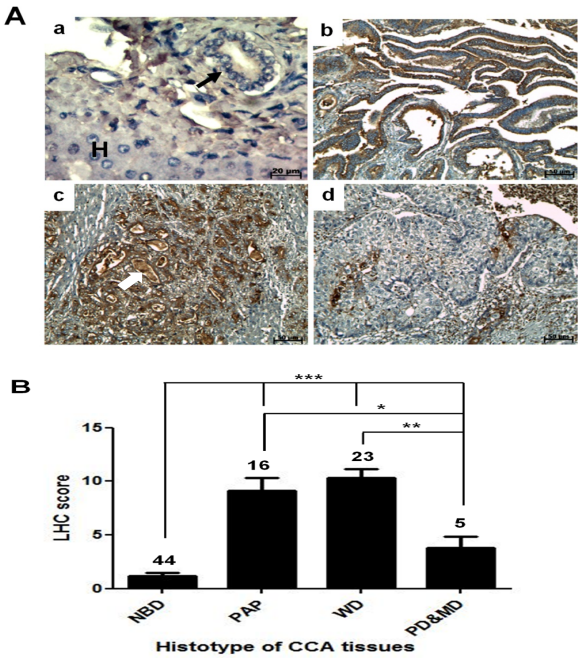

Figure 2. Patterns of sWGA-histochemistry in CCA. (A) $(D\text{-}GlcNAc)_n$, sWGA binding glycan, was differentially expressed in CCA; a) normal bile duct (black arrow) and hepatocytes (H) are negative for sWGA (magnification, x 400); b) papillary type; c) well-differentiated CCA; d) poorly-differentiated CCA (magnification, x 100). The luminal materials (white arrow) are positive for sWGA bindings; (B) The reactivity of sWGA-staining of all histological types of CCA was significantly higher than that of normal bile duct. In addition, level of sWGA-specific glycoconjugates of well-differentiated and papillary type CCA was significantly higher than those of moderately- and poorly-differentiated CCA. *P=0.026; **P=0.002; ***P<0.001

patients with low (sWGA-histochemical score ≤9) scores and high expression of sWGA-specific glycoconjugates (sWGA-histochemical score >9) were not associated

Table 3. Clinicopathological Features of Patients with Intrahepatic CCA

			sWGA-specific glycoconjugates expression		
		n (%)	Low	High	P
Age (years)	≤56	24 (55)	10	14	0.824
	>56	20 (45)	9	11	
Sex	Male	25 (57)	9	16	0.270
	Female	19 (43)	10	9	
Histopathology	Papillary type	16 (36)	8	8	0.490
	Non-papillary type	28 (64)	11	17	
Staging	I-III	19 (43)	6	13	0.176
	IVA-IVB	25 (57)	13	12	
Survival time	1 year-survival				
	<1 year	26 (59)	13	13	0.272
	≥1 year	18 (41)	6	12	
	2 year-survival				
	<2 years	35 (80)	16	19	0.710
	≥2 years	9 (20)	3	6	

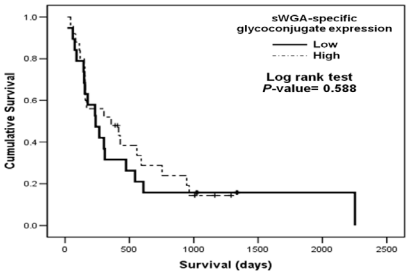

Figure 3. Survival Curves of Intrahepatic CCA Patients According to sWGA-glycoconjugates. Survival curves of intrahepatic CCA patients according to sWGA-glycoconjugates. Survival curves were analyzed using Kaplan-Meier method. No correlation of sWGA-specific glycan expression and patients' survival was observed (log rank test, P=0.588)

with clinicopathological features of patients. Cumulative survival was compared among CCA patients with low and high expression of sWGA-specific glycoconjugates (Figure 3). The mean survival time was 558 days (95%confidence interval; CI, 210-905 days) for CCA patients with low sWGA expression and 490 days (95%CI, 319-661 days) for those with high expression. However, we did not find a significant difference in the survival times of CCA patients with low vs. high sWGA-specific glycoconjugate expression (log rank, P-value=0.588) (Figure 3).

Discussion

Early detection and accurate diagnosis of CCA can help to obtain early treatment and improve survival rate of CCA patients. Most of the existing CCA-associated serological markers such as CA19-9, CEA, MUC5AC, MUC1 and MAC2-binding proteins are not sensitive or specific enough to be used for CCA screening and early detection (Wongkham et al., 2003; Koopmann et al., 2004; Qin et al., 2004). Since aberrant glycosylation frequently occurs during the pathological development as well as in tumors, this directed us to find the CCA-associated glyco-alteration. Advanced technologies of glycoproteomics are the important tools to develop a strategy to discover high

performance glycoprotein biomarkers (Matsuda et al., 2010; Adamczyk et al., 2011). Of these, a lectin-based approach is one of the efficient tools for screening of glyco-alteration in biological specimens. In the present study, we used lectin-histochemistry to characterize glycan profiles in CCA.

Aberrant glycosylations in intrahepatic CCA patient's tissues were observed using histochemistry with 14 different lectins. Liver sections, hepatocytes, endothelial cells, connective tissues and bile duct epithelia had strong expressions of glycans which were preferentially bound by Con-A, LCA, PSA, WGA, PNA, RCA, SBA, PHA-E and PHA-L, indicating similar glycan profiles of these cell types for α-D-Man, $(\beta\text{-D-GlcNAc})_n$, NeuNAc, β-D-Gal(1-3) D-GalNAc, β-D-Gal, α-D-Gal, and Bisected/triantennary *N*-glycan. In contrast, DBA, a lectin, recognizes *N*-acetylgalactosamine/galactose and gave a negative staining with all cell types in liver tissues, signifying the lack of DBA binding glycans in the liver tissues. In contrast, DBA showed different staining in different cell types, for example, malignant cells of prostatic carcinoma showed positive staining with DBA (Khabaz et al., 2011) whereas less staining intensity was observed in rectal tissues from patients at risk for hereditary nonpolyposis colorectal cancer (Sams et al., 1990). It is of interest that glycans which are specifically recognized by, sWGA, UEA-I, and SJA were not detected in hepatocytes, connective tissues and normal bile duct epithelia but were expressed in the hyperplastic/dysplastic and malignant biliary cells. This indicates that $(\beta\text{-D-GlcNAc})_n$, α-L-Fuc and β-D-GalNAc recognized by sWGA, UEA-I, and SJA are necessary for CCA development. Since this set of glycans was detected in the precancerous hyperplastic/dysplastic bile duct, it is possible that these glycoconjugates may be used as markers for the early stage of CCA. In addition, this set of lectins can also be used to differentiate CCA bile duct epithelia from normal bile duct and other cell types in the liver tissues.

As demonstrated in this study, sWGA-histochemistry can differentiate normal bile duct epithelia from CCA. sWGA-specific glycoconjugates were expressed significantly higher in CCA bile duct epithelia than normal bile duct epithelia. The staining pattern of β-D-GlcNAc/$(\beta\text{-D-GlcNAc})_n$, the sWGA-binding glycan, in CCA tumor cells tended to intensify at the membranous/apical site and varied from weak to intense intensity in cytoplasm. sWGA-specific glycoconjugates were differentially expressed in CCA. The expression of β-D-GlcNAc/$(\beta\text{-D-GlcNAc})_n$-glycoconjugates was increased when CCA developed, however, it was expressed in moderately and poorly differentiated CCA to a lower extent when comparing with those in well-differentiated and papillary type CCA. Since determination of glycoconjugates using lectin approach is targeted to glycans but not the core proteins or lipids, therefore, the differential expression of glycans detected by lectin can be either the alteration of core protein/lipid synthesis or of glycosylation process. It is of interest to identify the sWGA-specific glycoconjugates detected in different types of CCA. It is well recognized that poorly differentiated CCA is associated with tumor progression and poor prognosis (Shirabe et al., 2010), the sWGA-specific glycoconjugates specificity to poorly-differentiated CCA may reveal the glycoproteins which are associated with tumor progression and poor prognosis.

$(\beta\text{-D-GlcNAc})_n$, the glycan specifically recognized by sWGA was increased in many cancers such as breast, lung and colorectal cancers (Gu et al., 2010; Mi et al., 2011; Slawson and Hart, 2011). Low expression of *N*-acetylglucosaminyltransferase V (GnT-V), a β-D-GlcNAc transferring enzyme which generates a sWGA recognizing glycan, was related with poor prognosis and shorter survival time of non-small lung cancer patients (Dosaka-Akita et al., 2004). Even though sWGA-containing glycoconjugates were differentially expressed in CCA, the correlation of sWGA-specific glycoconjugates expression and clinicopathological features or patient's survival could not be demonstrated. This is probably due to a too small number of samples with low expression of sWGA-specific glycoconjugates in this study.

In summary, aberrant glycosylation was demonstrated in intrahepatic CCA. $(\text{D-GlcNAc})_n$, α-L-Fuc and β-D-GalNAc recognized by sWGA, UEA-I, and SJA were aberrantly expressed in precancerous and CCA tissues. Lectins have been applied in CCA for the detection of aberrant glycans in many clinical samples such as tissue and serum (Bamrungphon et al., 2007; Matsuda et al., 2010; Silsirivanit et al., 2011). The CCA associated glycoconjugates revealed by specific lectins raise the potential of using these glycoconjugates as markers for early detection and diagnosis of CCA.

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