

RESEARCH ARTICLE

Differential Expression of O-glycoprotein Glycans in Cholangiocarcinoma Cell Lines

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Abstract

Protein glycosylation is the most common posttranslational modification in mammalian cells. Aberrant protein glycosylation has been reported in various diseases, including cancer. We identified and quantified the glycan structures of O-linked glycoprotein from cholangiocarcinoma (CCA) cell lines from different histological types and compared their profiles by nanospray ionization-linear ion trap mass spectrometry (NSI-MSⁿ). Five human CCA cell lines, K100, M055, M139, M213 and M214 were characterized. The results showed that the O-linked glycans of the CCA cell lines comprised tri- to hexa-saccharides with terminal galactose and sialic acids: NeuAc1Gal1GalNAc1, Gal2GlcNAc1GalNAc1, NeuAc2Gal1GalNAc1 NeuAc1Gal2GlcNAc1GalNAc1 and NeuAc2Gal2GlcNAc1GalNAc1. All five CCA cell lines showed a similar glycan pattern, but with differences in their quantities. NeuAc1Gal1GalNAc1 proved to be the most abundant structure in poorly differentiated adenocarcinoma (K100; 57.1%), moderately differentiated adenocarcinoma (M055; 42.6%) and squamous cell carcinoma (M139; 43.0%), while moderately to poorly differentiated adenocarcinoma (M214; 40.1%) and adenosquamous cell carcinoma (M213; 34.7%) appeared dominated by NeuAc2Gal1GalNAc1. These results demonstrate differential expression of the O-linked glycans in the different histological types of CCA. All five CCA cell lines have abundant terminal sialic acid (NeuAc) O-linked glycans, suggesting an important role for sialic acid in cancer cells. Our structural analyses of glycans may provide important information regarding physiology of disease-related glycoproteins in CCA.

Keywords: Cholangiocarcinoma - glycoprotein - O-linked glycans - sialic acid

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Introduction

Cholangiocarcinoma (CCA) is a malignancy of the bile duct epithelium. The incidence of CCA is rare worldwide, but it is high in East and Southeast Asia. There is, however, an increasing incidence of CCA reported in England, the USA and Australia (Patel, 2001; Shaib et al., 2004). CCA is a major health problem in northeastern Thailand, particularly in areas where infection with the liver fluke *Opisthorchis viverrini* (OV) is endemic. Typically, diagnosis of CCA occurs when the disease is advanced or disseminated and prognosis is consequently poor. At present, there is no standard protocol for successful treatment of disseminated CCA. There is, consequently, an urgent need for novel target biomarkers for early detection of the disease or as biomarkers of therapeutic efficacy for treatments of CCA.

Glycosylation is one of the most common posttranslational modifications and is found on over

50% of human proteins (Apweiler et al., 1999; Wong, 2005). The modifications of glycans are important in host-pathogen interactions, inflammation, development, and malignancy. Aberrant glycosylation may result in abnormal changes in biological function/activity, protein folding, and ultimately assist with molecular recognition of disease. Thus, analysis of altered cancer-related glycoprotein expression may facilitate discovery of potential biomarkers, as well as discovery of novel targets for therapeutics (Kim and Misek, 2011).

The glycobiology of CCA is growing. The immunohistochemistry of sialyl Lewis (a) (sLea) and in vitro assays on adhesion and transmigration of CCA sLea cells revealed that the expression of sLea relates to poor prognosis in CCA (Juntavee et al., 2005). The carbohydrate marker for serum glycoprotein mucin 5AC from CCA patients was studied using monoclonal antibody (Silsirivanit et al., 2011). The study revealed that the level of serum glycan epitope (S121) was related

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to prognosis and was specific to CCA. Association of the glycan epitope (S121) to CCA was further studied in an animal model; glycan epitope (S121) was found expressed in the cytoplasm and apical surface of biliary cells at the early stage (1 month) of tumor development and increased with tumor progression (Sawanyawisuth et al., 2012). Further immunohistochemistry studies demonstrated overexpression of GlcNAc (Indramanee et al., 2012) and *O*-GlcNAc transferase in CCA patient tissues (Phoomak et al., 2012). Recent studies in serum of CCA patients by sandwich ELISA showed the glycan epitope CA-S27 was related to prognosis and specific to CCA and may have immunodiagnostic value (Silsirivanit et al., 2013). Although several glycan epitopes of CCA have been reported, glycan characterization was limited to detection by antibody or lectin staining. To gain a better understanding of cancer biology and the association of glycan structures to clinico-pathological feature, the glycans expressed on CCA glycoproteins need greater characterization.

In the current study, the structural detail and the quantities of *O*-linked glycans from the 5 different histological types of CCA cell lines were demonstrated. Knowledge of these specific *O*-glycans may help in understanding the mechanisms of tumorigenesis, progression and metastasis of CCA, and may be applied for clinical diagnosis or effective treatment.

Materials and Methods

Materials

Sodium hydroxide (NaOH) and Sodium borohydride (NaBH₄) were obtained from Sigma. Other glycoprotein standards and fine chemicals were from standard sources.

CCA cell lines

CCA cell lines-K100, M055, M139, M213 and M214-were obtained from the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand. CCA cell lines were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA), containing 100 U/mL penicillin and 100 µg/mL streptomycin with 10% FBS (Hyclone Laboratories). Cell growth was performed at 37 °C under 5% CO₂ and 95% humidified air.

Preparation of CCA cell lines protein powder

Five hundred microliters of packed cells of each of the CCA cell lines-K100, M055, M139, M213 and M214-were homogenized on ice in cold 50% methanol. The homogenate was then adjusted to a 4:8:3 ratio of chloroform to methanol to water and extracted for 2 h at room temperature. The extracts were centrifuged at 3000 rpm for 15 min and the resulting pellets dried under nitrogen to yield a protein powder, which was stored (desiccated) at -20 °C until used.

Preparation of *O*-glycans by Reductive β- Elimination

The protein powder of the CCA cell lines (1 mg each) was subjected to reductive β-elimination in 100 mM NaOH, containing 1.0 M NaBH₄ at 45 °C for 18 h. The

reaction mixture was neutralized with 10% acetic acid and desalted on a column of AG50W-X8 (H⁺) (Aoki et al., 2008). The material was eluted with 5 % acetic acid and lyophilized. The boric acid was removed by evaporation with methanol. Released *O*-glycans were purified by Sep-Pak C18 cartridge column (Aoki et al., 2007).

Permethylolation of Glycans

To facilitate analysis by mass spectrometry (MS), portions of released oligosaccharide mixtures were permethylated as per Anumula and Taylor (Anumula and Taylor, 1992).

Nanospray Ionization-Linear Ion Trap Mass Spectrometry

Mass analysis by NSI-MSⁿ was performed as per (Aoki et al., 2007). Briefly, permethylated glycans were dissolved in 1 mM NaOH in 50% methanol and infused directly into a linear ion trap mass spectrometer (LTQ; Thermo Scientific) using a nanospray source (at a syringe flow rate of 0.40-0.60 µL/min). The capillary temperature was set to 210 °C, and MS analysis performed in positive ion mode. MS and MS/MS spectra (at 28 % collision energy) were obtained using the total ion mapping (TIM) function of the Xcalibur (software version 2.0). The nomenclature used by Domon and Costello (Domon and Costello, 1988) was used to describe the fragmentation derived from the MS/MS spectra.

Results

Structural characterization of *O*-Glycans from CCA cell lines

The *O*-glycomes of the CCA cell lines (K100, M055, M139, M213 and M214) were qualitatively and quantitatively compared. The *O*-glycans from these cell

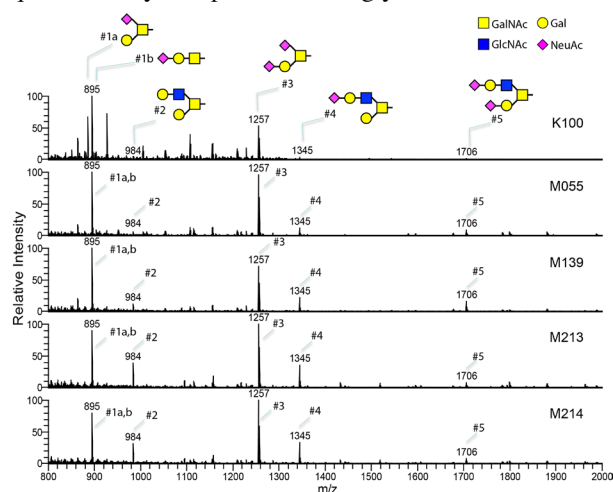


Figure 1. MS Spectra of Permethylated *O*-linked Oligosaccharides of CCA Cell Lines by NSI-MS.

Glycans released from the CCA cell lines (K100, M055, M139, M213 and M214) glycopeptides were permethylated and analyzed. MS spectra demonstrate the predominance of mono sialic acid ($m/z = 895$) and disialic acid ($m/z = 1257$) oligosaccharides in CCA cell lines. The total *O*-linked glycan profiles of CCA cell lines are shown in Table 1. Graphical representations of monosaccharide residues are shown in the legend and are consistent with the suggested nomenclature of the Consortium for Functional Glycomics

Table 1. Characteristics and Prevalence of O-linked Glycans of 5 CCA Cell Lines

Structures	Observed m/z	Glycan Prevalence (% Total Profile)				
		K100	M055	M139	M213	M214
NeuAc-[Hex]-HexNAc-ol (1a) and NeuAc-Hex-HexNAc-ol (1b)	895	57.1±3.8	42.6±2.1	43.0±2.0	32.3±1.0	30.9±1.2
Hex-HexNAc-[Hex]-HexNAc-ol	984	5.3±4.6	4.8±4.2	7.2±2.8	16.9±3.5	13.0±1.1
NeuAc-[NeuAc-Hex]-HexNAc-ol	1257	33.3±4.6	42.5±3.9	31.1±3.9	34.7±4.4	40.1±3.5
NeuAc-Hex-HexNAc-[Hex]-HexNAc-ol	1345	3.0±1.8	5.9±1.2	10.4±0.3	12.9±0.2	12.6±0.7
NeuAc-Hex-HexNAc-[NeuAc-Hex]-HexNAc-ol	1706	1.6±0.9	4.4±0.5	8.2±0.7	3.2±0.7	3.3±0.9

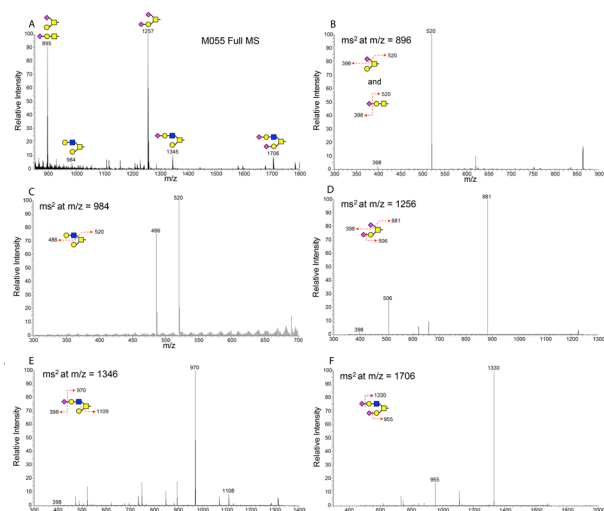


Figure 2. Representative MS² Spectra of Permethylated O-linked Oligosaccharides of CCA Cell Lines. Shown are the full MS spectra (A) and fragmentation of parent ion at m/z = 895 (B), m/z = 984 (C), m/z = 1257 (D), m/z = 1345 (E), and m/z = 1706 (F). Parent ion at m/z = 895 (Structure 1) fragments in MS² to give m/z = 520 (loss of terminal NeuAc, $\Delta m/z = 375$), and m/z = 398 (terminal NeuAc with Na⁺). Parent ion at m/z = 984 (Structure 2) fragments in MS² to give m/z = 520 (loss of terminal Hex-HexNAc, $\Delta m/z = 464$), and m/z = 486 (terminal Hex-HexNAc with Na⁺). Parent ion at m/z = 1257 (Structure 3) fragments in MS² to give m/z = 881 (loss of terminal NeuAc (1st), $\Delta m/z = 375$), m/z = 506 (loss of terminal NeuAc (2nd), $\Delta m/z = 375$), and m/z = 398 (terminal NeuAc with Na⁺). Parent ion at m/z = 1345 (Structure 4) fragments in MS² to give m/z = 1108 (loss of terminal Hex with O, $\Delta m/z = 237$), m/z = 970 (loss of terminal NeuAc, $\Delta m/z = 375$), and m/z = 398 (terminal NeuAc with Na⁺). Parent ion at m/z = 1706 (Structure 5) fragments in MS² to give m/z = 1330 (loss of terminal NeuAc (1st), $\Delta m/z = 375$), and m/z = 955 (loss of terminal NeuAc (2nd), $\Delta m/z = 375$)

lines were chemically released, purified, and analyzed in their free reduced forms, using positive ion NSI-MS/MS. Identification of the glycan structures was based on (i) parent ion mass determined by NSI-MS; (ii) fragmentation of permethylated glycans by automated TIM (NSI-MS/MS) and further manual fragmentation (NSI-MSⁿ); and, (iii) similarity to known structures of characterized glycans and known biosynthetic limitations. The prevalence of each individual glycan in each profile was quantified by comparing its signal intensity to the sum of the signal intensities for all identified glycans in the profile yielding “% Total Profile” for each glycan.

Figure 1 presents the mass profiles for the O-glycans

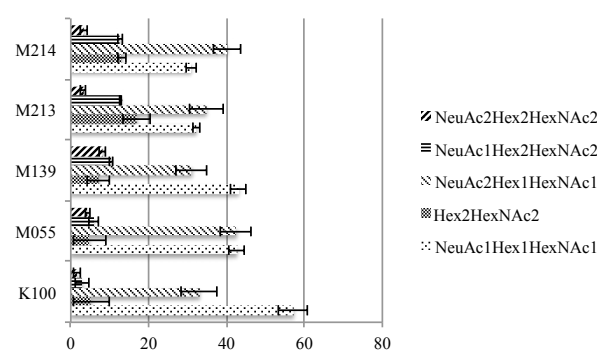


Figure 3. O-linked Oligosaccharides of 5 CCA Cell Lines. Prevalence of each glycan is expressed as a percentage of total pool of detected glycans (% Total Profile). O-linked glycans of CCA cell line consisted of tri- to hexa-saccharides with terminal galactose and sialic acid. NeuAc1Gal1GalNAc1 and NeuAc2Gal1GalNAc1 are the two most abundant structures in all 5 CCA cell lines

of the CCA cell lines. The profiles of each cell line showed a similar glycan pattern, but a more detailed analysis revealed some unique glycan features. In total, 5 monosaccharide compositions yielding 6 glycan structures were identified from each cell line. The tri- to hexa-saccharides with the terminal galactose and/or sialic acid were detected-viz., NeuAc1Gal1GalNAc1 (Structure 1a and 1b), Gal2GlcNAc1GalNAc1 (Structure 2), NeuAc2Gal1GalNAc1 (Structure 3), NeuAc1Gal2GalNAc2 (Structure 4), and NeuAc2Gal2GalNAc2 (Structure 5). Fragmentation revealed that the MS signals (at m/z=896) arise from isobaric mixtures of two structures (Table 1, Figure 2). A summary of the O-glycan structures for each cell line and their relative abundance are presented in Table 1. The fragmentation of each oligosaccharide is presented in Figure 2.

Differential expression of O-Glycan structures in CCA cell lines

All five CCA cell lines (K100, M055, M139, M213 and M214) showed similar glycan profiles, albeit differences in their quantities. The two most abundant structures among the 5 CCA cell lines were NeuAc1Gal1GalNAc1 (Structure 1a and 1b) and NeuAc2Gal1GalNAc1 (Structure 3). The NeuAc1Gal1GalNAc1 (Structure 1a and 1b) was the most abundant in poorly differentiated adenocarcinomas (K100; 57.1%), moderately differentiated adenocarcinomas (M055; 42.6%), and squamous cell carcinomas

(M139; 43.0%). NeuAc2Gal1GalNAc1 (Structure 3) dominated moderately to poorly differentiated adenocarcinomas (M214; 40.1%) and adenosquamous cell carcinomas (M213; 34.7%). The non-sialic *O*-glycan-Gal2GlcNAc1GalNAc1 (Structure 2)-accounted for 16.9%, 13.0%, 7.2%, 5.3%, and 4.8% of M213, M214, M139, K100, and M055, respectively (Table 1).

Discussion

Alterations in *O*-glycan structures have been reported in various diseases including cancer. Many reports suggests that changes in protein glycosylation may play an important role in promoting tumors and may, therefore, provide biomarkers for tumor progression. The data presented in this report represents comparative structural glycomics of the *O*-linked glycans of human CCA cell lines. The diversity of the carbohydrate part of the glycoproteins of the cell lines was identified and quantified using mass spectrometry.

We demonstrated differential expression of *O*-glycans among the different histological types of CCA cell lines that may contribute to the progression and nature of the cancer. Our results demonstrated that complex types of *O*-glycans dominated *O*-glycosylation of the 5 CCA cell lines (viz., NeuAc1Gal1GalNAc1 (Structure 1a and 1b) and NeuAc2Gal1GalNAc1 (Structure 3).

These 3 major structures may contribute to the differentiation of each cell line. NeuAc1Gal1GalNAc1 was the most abundant structure in the poorly differentiated adenocarcinoma cell line (K100), the moderately differentiated adenocarcinoma cell line (M055), and the squamous cell carcinoma cell line (M139). Meanwhile, NeuAc2Gal1GalNAc2 dominated the moderately to poorly differentiated adenocarcinoma cell line (M214) and the adenosquamous cell carcinoma cell line (M213). This divergence in *O*-glycan processing indicates specific expression of glycosyltransferases that act on the same core glycan acceptor substrates. The well known, previously reported, pan-carcinoma glycan markers in CCA tissues-sialyl-Tn (STn) antigen and sLea (Juntavee et al., 2005) -were not detected in the 5 CCA cell lines. The differential expression of *O*-glycans structures might have been regulated by many factors, including: mRNA turnover, the availability of substrate, nucleotide sugar transporter, and/or relevant glycosyltransferase (Chik et al., 2014).

Interestingly, all 5 CCA cell lines highly expressed *O*-glycans with a terminal sialic acid. These data suggest an important role for sialic acid in the cancer cell. The aberrant expression of sialoglycans impairs apoptotic signaling, allowing tissue evasion and metastasis (Bull et al., 2014). We detected non-sialic *O*-glycan in small amounts compared with the sialic acid. This differential expression in *O*-glycan processing indicates specific expression of glycosyltransferases that play the role on glycosylation of CCA cell lines.

Compared to other cancers, the *O*-glycans from the CCA cell lines are at once similar and distinct. As in cultured breast cancer cells (Brockhausen et al., 1995; Burchell et al., 1999), CCA cell lines express a sialylated

core 1 (NeuAc1Gal1GalNAc1), which may play an important role in the *O*-linked glycoprotein of these 5 cancer cell lines. Unlike other cancers, CCA cell lines express a complex *O*-glycans with a terminal sialic acid, and yet the common tumor-specific antigen STn, T and Tn antigens were not detected. This result may be due to the high expression of the *O*-glycan and the sialic acid could be suppressing the signal from the minor core and short chain oligosaccharide structures.

Comparing the glycan profiles of each of the 5 CCA cell lines, differences in glycan prevalence most likely reflects specific changes in glycosyltransferase expression. The abundance of NeuAc1Gal1GalNAc1 and NeuAc2Gal1GalNAc1 in the CCA cell lines could be attributed to the dominant activity of ST3Gal and ST6GalNAc, which competes for substrate with C2GnT. The aberrant expression of sialic acid in CCA cells (NeuAc) may affect tumor growth and progression (Bull et al., 2014).

In conclusion, this study yielded baseline information on the *O*-glycan profiles of the 5 different CCA cell lines. Differences in the amounts of specific glycan structures may reflect their role in tumor development. The high expression of complex *O*-glycans with the terminal sialic acid suggests that it has a role in cell immortalization. Further glycomic investigation into the specific glycoproteins in the CCA cell lines is essential for understanding the glycobiology of this cancer and may lead to the discovery of biomarkers.

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