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Accumulation of free Neu5Ac-containing complex-type *N*-glycans in human pancreatic cancers

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Abstract We have analyzed the structures of glycosphingolipids and intracellular free glycans in human cancers. In our previous study, trace amounts of free *N*-acetylneuraminic acid (Neu5Ac)-containing complex-type *N*-glycans with a single GlcNAc at each reducing terminus (Gn1 type) was found to accumulate intracellularly in colorectal cancers, but were undetectable in most normal colorectal epithelial cells. Here, we used cancer glycomic analyses to reveal that substantial amounts of free Neu5Ac-containing complex-type *N*-glycans, almost all of which were α2,6-Neu5Ac-linked, accumulated in the pancreatic cancer cells from three out of five patients, but were undetectable in normal pancreatic cells from all five cases. These molecular species were mostly composed of five kinds of glycans having a sequence Neu5Ac-Gal-GlcNAc-Man-Man-GlcNAc and one with the following sequence Neu5Ac-Gal-GlcNAc-Man-(Man-)Man-GlcNAc. The most abundant glycan was Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAc, followed by Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6Manβ1-4GlcNAc. This is the first study to show unequivocal evidence for the occurrence of free Neu5Ac-linked *N*-glycans in human cancer tissues. Our findings suggest that free Neu5Ac-linked glycans may serve as a useful tumor marker.

Keywords Free oligosaccharide · *N*-glycans · Pancreatic cancer · *N*-acetylneuraminic acid

Abbreviations

Neu5Ac	*N*-acetylneuraminic acid
SGP	Sialylglycopeptide
Cer	Ceramide
GM3	Neu5Acα3Galβ4GlcCer
LST-c	Neu5Acα6Galβ4GlcNAcβ3Galβ4GlcCer
GM1	Galβ3GalNAcβ4(Neu5Acα3)Galβ4GlcCer
LST-a	Neu5Acα3Galβ3GlcNAcβ3Galβ4GlcCer
Disialyl Lc_4	Neu5Acα3Galβ3(Neu5Acα6)GlcNAcβ3Galβ4GlcCer
SSEA-4	Neu5Acα3Galβ3GalNAcβ3Galα4Galβ4GlcCer
Disialyl SSEA-3	Neu5Acα3Galβ3(Neu5Acα6)GalNAcβ3Galα4Galβ4GlcCer

Introduction

Extensive studies on the oligosaccharide structures of cancers have revealed that aberrant glycosylation occurs in essentially all types of human cancers [1–4]. Altered carbohydrate determinants, including tumor associated carbohydrate antigens such as SLe^a and SLe^x have been utilized as useful tumor

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markers for the diagnosis of cancer [5–7]. Thus, the application of glycomic analyses to cancer research can highlight changes in the profiling of glycan structures during tumor development, leading to the identification of novel carbohydrate tumor markers. Hence, we comprehensively and precisely analyzed the structures of glycosphingolipids (GSLs) using highly purified colorectal cancer cells and normal colorectal epithelial cells from more than 60 patients and identified two kinds of novel tumor-associated carbohydrate antigens, Neu5Acα2-6(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ1-4Glc (α2-6-sialylated type 2H) and Neu5Acα2-6(Fucα1-2)Galβ1-3GlcNAcβ1-3Galβ1-4Glc (α2-6-sialylated type 1H), both of which are isomers of SLe^a and SLe^x [8–11]. The α2-6-sialylated type 2H was found in colorectal cancer cells from half of the cases, whilst α2-6-sialylated type 1H was specifically found in colorectal cancer cells from Lewis-negative patients. The suitability of these two carbohydrate antigens as tumor markers is currently being evaluated in our group.

In the present study, extensive structural analyses of oligosaccharides derived from pancreatic cancers have been undertaken. These analyses revealed the occurrence of substantial amounts of intracellular free Neu5Ac (*N*-acetylneuraminic acid)-containing complex-type *N*-glycans with a single GlcNAc at each reducing terminus in human pancreatic cancers. We observed both free glycans and GSLs in our assay. These molecules are extractable in chloroform-methanol solution, which was used to homogenize the cancer cells and tissues [12].

The occurrence of free high-mannose type *N*-glycans is well demonstrated in mammalian cells [13–15]. However, with the exception of mouse liver and two kinds of human stomach cancer derived cell lines (MKN7 and MKN45) [12, 16], free complex-type *N*-glycans, especially sialylated species, are not normally observed.

In our previous study, free Neu5Ac-containing complex-type *N*-glycans were found in colorectal cancers, but only at trace amounts [11]. However, a large amount of free Neu5Ac-containing complex-type *N*-glycans were found to accumulate in some pancreatic cancers. In this paper, the detailed structures of these free sialylated complex-type *N*-glycans that accumulate in human cancers are presented.

Materials and methods

The majority of the experimental procedures including purification of cancer cells and normal epithelial cells, isolation of GSLs and free oligosaccharides, preparation and separation of pyridylaminated (PA)-oligosaccharides, and mass spectrometry analyses have been reported previously [11]. In brief, pancreatic cancer cells and normal pancreatic epithelial cells were highly purified from primary lesions of pancreatic cancers and their surrounding normal pancreatic lesions, respectively, using the epithelial cell marker, CD326, and magnetic beads. CD326 positive cells were extracted with 1,200 μl of chloroform/methanol (2:1, v/v), followed by 800 μl of chloroform/methanol/water (1:2:0.8, v/v/v). This methodology extracts both GSLs and free oligosaccharides. The extracts were loaded onto a DEAE-Sephadex A25 column and flow-through fractions were collected as neutral oligosaccharides. Acidic oligosaccharides were subsequently eluted with 200 mM ammonium acetate in methanol. The neutral and acidic fractions were digested with endoglycoceramidase II from *Rhodococcus* Sp. (Takara Bio) to release the oligosaccharide portion from GSLs. Liberated oligosaccharides from GSLs and free oligosaccharides were then labeled with 2-aminopyridine (2-AP) [17].

PA-oligosaccharides were separated on a Shimadzu LC-20A HPLC system equipped with fluorescence detector. Normal phase HPLC was performed on a TSK gel Amide-80 column (0.2×25 cm, Tosoh). The molecular size of each PA-oligosaccharide is given in glucose units (Glc) based on the elution times of PA-isomaltooligosaccharides. Reversed phase HPLC was performed on a TSK gel ODS-80Ts column (0.2× 15 cm, Tosoh). The retention time of each PA-oligosaccharide is given in glucose units based on the elution times of PA-isomaltooligosaccharides. Thus, a given compound on these two columns provides a unique set of Glc (amide) and Gu (ODS) values, which correspond to coordinates of the 2-D map. PA-oligosaccharides were analyzed by LC/ESI MS/MS according to our previously established procedures [9].

Glycosidase digestions

Linkage position of Neu5Ac to the terminal galactose was determined as described previously [9]. Briefly, Neu5Ac-linked PA-oligosaccharides were digested with 2 U/ml of α2,3-sialidase from *Salmonella typhimurium* (Takara Bio) in 100 mM sodium acetate buffer, pH 5.5, for 2 h at 37 °C. Under these conditions, α2,3-sialidase specifically digests α2-3-Neu5Ac linked to the terminal residue, but not Neu5Ac with an α2-6-linkage. However, under conditions using 10 U/ml for 16 h, even so-called α2,3-sialidase can hydrolyze α2-6-Neu5Ac linked to the terminal residue, but not Neu5Ac linked to a non-terminal residue.

Preparation of free Neu5Ac-containing complex-type *N*-glycans

Authentic PA-oligosaccharides required for the analysis of GSLs have been already obtained in our laboratory and are listed in Table 1. However, we had no authentic PA-oligosaccharides of free oligosaccharides, and they were not commercially available. Hence, authentic PA-oligosaccharides of free oligosaccharides required for this study were prepared enzymatically. The procedure is outlined in Fig. 1. PA-oligosaccharides were prepared from sialylglycopeptide (SGP) (Tokyo Chemical Industry) (Fig. 1) and the results are

Table 1 Structures and elution positions in HPLC of standard PA-oligosaccharides. Peak numbers shown in Fig. 2 are given in parentheses

Abbreviation	Structure	Elution position in HPLC	
		Size (Gu)	RP (Gu)
GM3 (G1)	Neu5Acα2-3Galβ1-4Glc-PA	2.46	3.00
LST-c (G2)	Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA	4.40	3.76
GM1 (G3)	Galβ1-3GalNAcβ1-4Galβ1-4Glc-PA 3 | Neu5Acα2	3.85	2.92
LST-a (G4)	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA	4.01	4.69
Disialyl Lc_4 (G5)	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA 6 | Neu5Acα2	4.52	5.54
SSEA-4 (G6)	Neu5Acα2-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glc-PA	4.98	4.81
Disialyl SSEA-3 (G7)	Neu5Acα2-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glc-PA 6 | Neu5Acα2	5.26	7.47
S1-4G-Hex	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1 ＼ 6 Manβ1-4GlcNAc-PA 3 ／ Manα1	6.11	6.55
S2-4G-Hex (F6)	Manα1 ＼ 6 Manβ1-4GlcNAc-PA 3 ／ Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1	6.01	5.15
S1-4G-Hex-Man (F4)	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1 ＼ 6 Manβ1-4GlcNAc-PA	5.21	5.93
S2-4G-Hex-Man (F3-2)	Manβ1-4GlcNAc-PA 3 ／ Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1	5.00	6.84

summarized in Table 1. Two hundred micrograms of SGP was digested with 0.5 U/ml of recombinant endoglycosidase F2 from *Elizabethkingia meningosepticum* (Merck) in 100 mM sodium acetate buffer, pH 4.5, for 16 h at 37 °C. The released Gn1-type oligosaccharide (designated as SGP-F2, Fig. 1) was labeled with 2-aminopyridine (PA-SGP-F2). To cleave either side of the mannose arm, PA-SGP-F2 was partially digested with 1 U/ml of α2,3-sialidase from *S. typhimurium* in 100 mM sodium acetate buffer pH 5.5, for 16 h at 37 °C. Under these conditions, even so-called α2,3-sialidase can partially hydrolyze α2-6-Neu5Ac linked to the terminal residue. Two kinds of single digested oligosaccharides (S1 and S2) were separated and collected by reversed phase HPLC. S1 and S2 were sequentially digested with 0.4 U/ml β1,4-galactosidase from *Streptococcus pneumonia* (Prozyme) in 100 mM sodium citrate buffer, pH 6.0, for 2 h at 37 °C, followed by 10 U/ml of β-*N*-acetylhexosaminidase from jack bean (Seikagaku Kogyo) in 100 mM sodium citrate buffer pH 5.0, for 16 h at 37 °C (S1-4G-Hex, and S2-4G-Hex). Linkage position of the non-reducing terminal mannose of the two oligosaccharides (S1-4G-Hex and S2-4G-Hex) was determined by utilizing the specificity of jack bean α-mannosidase. S1-4G-Hex and S2-4G-Hex were digested with jack bean α-mannosidase (Seikagaku Kogyo) at either 10 U/ml or 25 U/ml in 100 mM sodium acetate buffer pH 5.0 containing 2 mM $ZnCl_2$, for 16 h at 37 °C. At the lower concentration, R-Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc but not R-Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNA is susceptible, where R is not H or Man. The substrate specificity of this enzyme was described previously [18]. Digestion with a low concentration

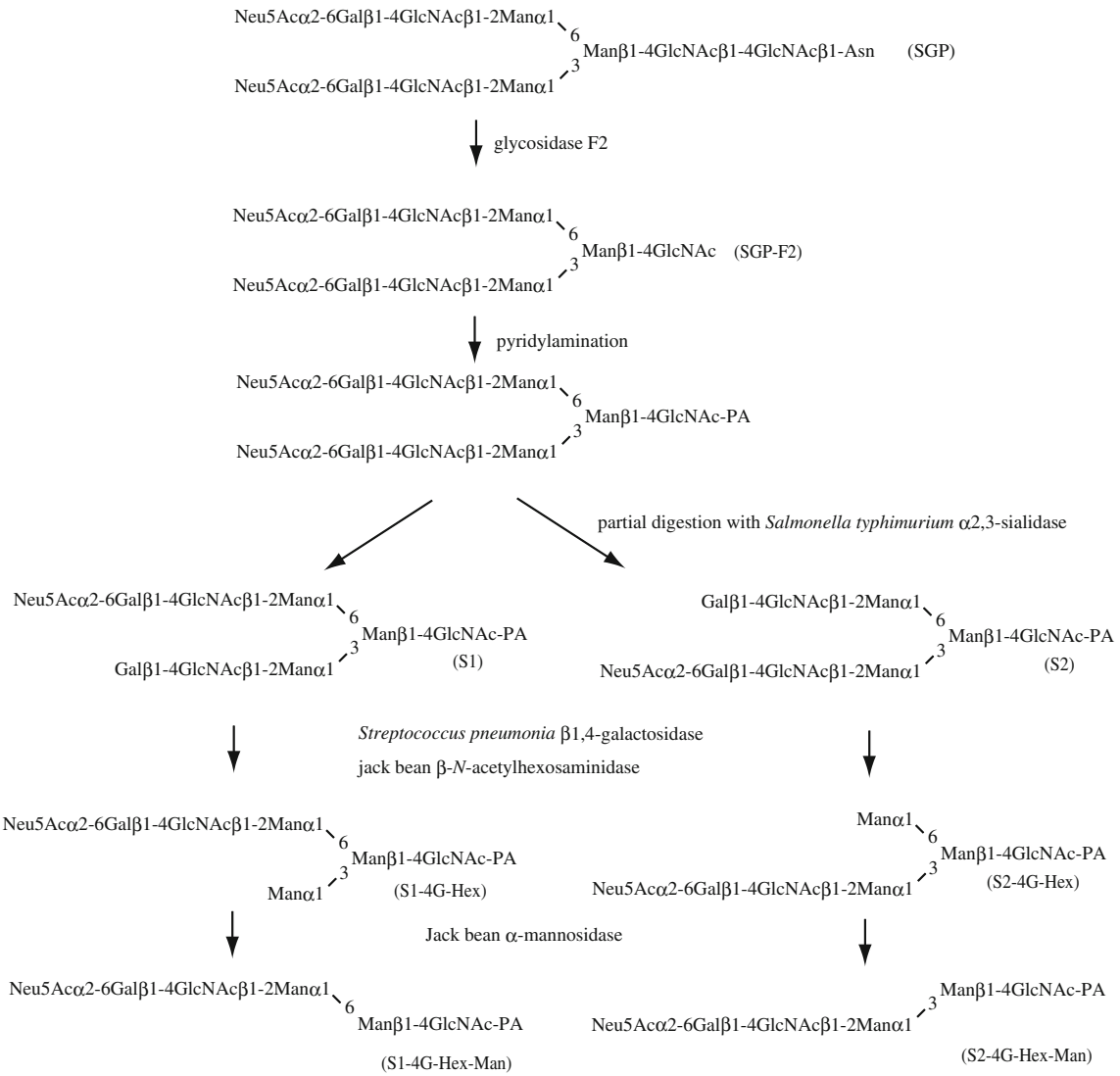

Fig. 1 The strategy used to synthesize authentic PA-free complex-type *N*-glycans

of α-mannosidase completely liberated the mannose residue from S1-4G-Hex (S1-4G-Hex-Man), indicating that the cleaved mannose was on the α1-3 side and the digested product (S1-4G-Hex-Man) was Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6Manβ1-4GlcNAc-PA. S2-4G-Hex was partially cleaved by a high concentration of α-mannosidase, indicating that the cleaved mannose was on the α1-6 side and the digested product (S2-4G-Hex-Man) was Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAc-PA.

Results

Preparation of PA-oligosaccharides from colon and pancreatic adenocarcinoma

Colon and pancreatic cancer cells and their normal epithelial cells were isolated with high purity from the cancer tissues and surrounding normal epithelial tissues using magnetic beads labeled with antibody against the epithelial cell marker, CD326. We analyzed the structures of GSLs and free oligosaccharides of pancreatic cancer cells and normal pancreatic cells derived from five patients. The clinicopathological features of the five pancreatic cancer patients are described in Supplementary Table 1.

It is well known that both free oligosaccharides and GSLs, but not glycoproteins, are recovered in lipid fractions from biological samples [12]. In this study, both free oligosaccharides and GSLs were extracted from the isolated cells using organic solvent, chloroform-methanol. The oligosaccharide portions of GSLs were released by endoglycoceramidase II, and the reducing ends of the released oligosaccharides of GSLs and free oligosaccharides were tagged with the fluorophore, 2-aminopyridine (see Materials and Methods).

Major acidic free oligosaccharides accumulated in human cancer cells

The acidic PA-oligosaccharides from cancer cells and normal epithelial cells were analyzed by size-fractionation HPLC (Fig. 2a–h). Each of the peaks separated by the size-fractionation HPLC was further separated by reversed phase HPLC. Additionally, the separated PA-oligosaccharides were subjected to LC/ESI MS^2 analysis. Some of the PA-oligosaccharides were also analyzed after digestion with glycosidase to help ascertain their structures. By means of these combinational analyses, the structures of all the major GSLs could be estimated and the occurrence of a large amount of free sialylated complex-type *N*-glycans in cancer cells was unequivocally demonstrated. In total, 7 distinct Neu5Ac-containing free oligosaccharides (F1, F2, F3-1, F3-2, F4, F5, and F6) were detected as major components in human pancreatic cancers (Fig. 2, Tables 2, 3). All these glycans were easily predicted to be Neu5Ac-containing free complex-type *N*-glycans having a single HexNAc (probably GlcNAc) at their reducing termini (Gn1 glycans) by mass analyses. Elution positions, mass data and estimated composition of the

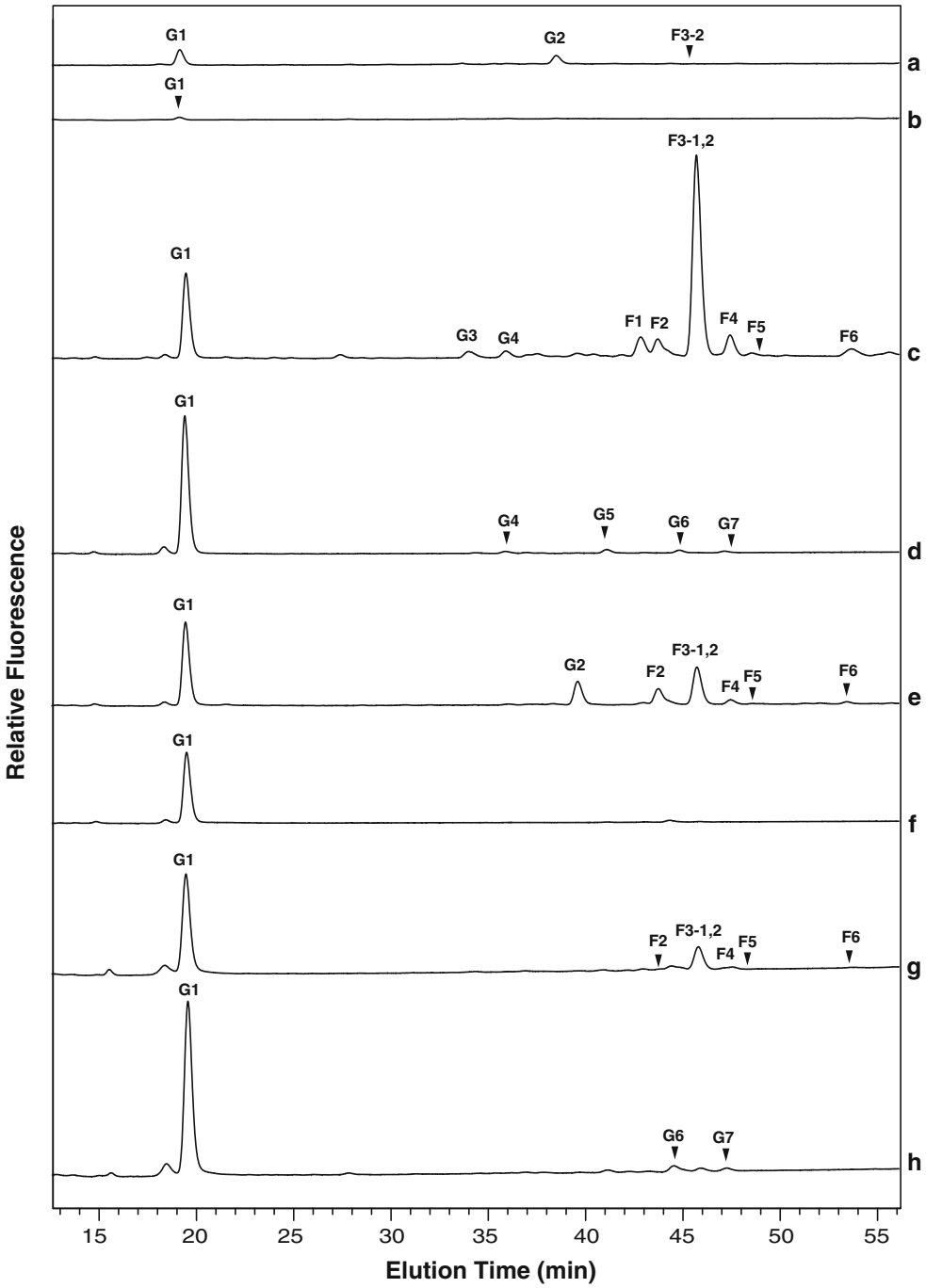

Fig. 2 Size fractionation HPLC of acidic PA-oligosaccharide mixtures obtained from human cancer and normal epithelial cells. **a** and **b** colon cancer cells (**a**) and normal colon epithelial cells (**b**) from the representative case (1×10^6 cells each), **c**–**h** pancreatic cancer cells (**c**, **e** and **g**) and normal pancreatic epithelial cells (**d**, **f** and **h**) (1×10^6 cells each). **c** and **d**, **e** and **f**, and **g** and **h** are from the same case (case 1, case 2 and case 3, respectively, Supplementary Table 1). Six major peaks derived from free *N*-glycans found in pancreatic cancer (**c**) are represented as F1–F6 with fraction numbers as per Tables 1, 2 and 3. Free *N*-glycan peaks found in other cells are numbered as per the peak numbers of C. G1–G7 are the peaks derived from GSLs. The structures of G1–G7 are listed in Table 1. The positions of minor peaks that were barely detectable are highlighted by an arrowhead

Table 2 Elution positions in HPLC and mass analysis of sialylated PA- free oligosaccharides obtained from human pancreatic cancer cells.

Group	Fraction	Elution position in HPLC		Mass (observed)	Mass (calculated)	Estimated composition
		Size (Gu)	RP (Gu)			
1	F3-1	5.03	5.55	1280.5	1280.5 $[M+H]^+$	$NeuAc_1Hex_3HexNAc_2$-PA
	F3-2	5.02	6.92	1280.4	1280.5 $[M+H]^+$	$NeuAc_1Hex_3HexNAc_2$-PA
	F4	5.26	6.00	1280.5	1280.5 $[M+H]^+$	$NeuAc_1Hex_3HexNAc_2$-PA
	F5	5.38	6.03	1280.5	1280.5 $[M+H]^+$	$NeuAc_1Hex_3HexNAc_2$-PA
	F1	4.76	7.91	1280.5	1280.5 $[M+H]^+$	$NeuAc_1Hex_3HexNAc_2$-PA
2	F6	6.07	5.20	1442.4	1442.5 $[M+H]^+$	$NeuAc_1Hex_4HexNAc_2$-PA
3	F2	4.84	7.80	1321.5	1321.5 $[M+H]^+$	$NeuAc_1Hex_2HexNAc_3$-PA

oligosaccharides are presented in Table 2. Based on the composition of monosaccharide, we were able to classify the 7 free *N*-glycans into three groups as follows; group 1 (F1, F3-1, F3-2, F4 and F5), group 2 (F6), and group 3 (F2) (Tables 2, 3). In the following results sections, the structures of these PA-oligosaccharides are explained in detail.

Structural analyses of free Neu5Ac-containing complex-type N-glycans

Group 1

The $MS^{1,2}$ spectra of the five group 1 glycans (F1, F3-1, F3-2, F4 and F5) were essentially the same. A representative $MS^{1,2}$ spectrum of F3-2 is shown in Fig. 3a, b. The structures of group 1*N*-glycans were judged to be Neu5Ac-Hex-HexNAc-Hex-Hex-HexNAc-PA or branched Neu5Ac-HexNAc-Hex-(Hex-)Hex-HexNAc-PA by MS^2 analysis (Fig. 3a, b, Table 3), and they were anticipated to be Neu5Ac-Gal-GlcNAc-Man-Man-GlcNAc-PA or Neu5Ac-GlcNAc-Man-(Man-)Man-GlcNAc-PA, respectively. However, the latter candidate could be excluded because these oligosaccharides were resistant to jack bean α-mannosidase (data not shown). Neu5Ac is linked *via* α2-6 to the non-reducing terminal residue of F3-1, F3-2, F4 and F5, and α2-3 to that of F1 as determined by the specificity of α2-3-siallidase digestion as described in Material and Methods (Fig. 4, Table 3). These data suggested that two of the four oligosaccharides (F3-1, F3-2, F4 and F5) are

Table 3 Neu5Ac-containing free complex-type *N*-glycans accumulated in human cancers. Estimated structures and the amount of glycans in colon cancer cells (case of Fig. 2a), pancreatic cancer cells (cases of Fig. 2c, 2e, and 2g) are presented. The amount of glycan is expressed as pmol/1×10^6 cells. (−) indicates that this glycan was not detected in the cells

Group	Fraction	Structure	Amount of glycan (pmol/1 X 10^6 cells)			
			colon (A)	pancreas (C)	pancreas (E)	pancreas (G)
1	F3-1	Neu5Acα2-6Galβ1-4GlcNAcβ-Manα–Manβ1-4GlcNAc-PA	(-)	10.3	1.7	0.7
	F3-2	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1 ⟋3 Manβ1-4GlcNAc-PA	0.1	121.1	22.3	17.6
	F4	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1 ⟍6 Manβ1-4GlcNAc-PA	(-)	16.6	2.9	1.9
	F5	Neu5Acα2-6Galβ1-4GlcNAcβ-Manα–Manβ1-4GlcNAc-PA	(-)	0.9	0.9	0.3
	F1	Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1 ⟋3 Manβ1-4GlcNAc-PA	(-)	6.9	(-)	(-)
2	F6	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1 ⟋3 Manα1 ⟍6 Manβ1-4GlcNAc-PA	(-)	2.8	1.0	0.5
3	F2	Neu5Acα2-6HexNAc-GlcNAcβ-Manα–Manβ1-4GlcNAc-PA	(-)	8.9	8.4	0.5

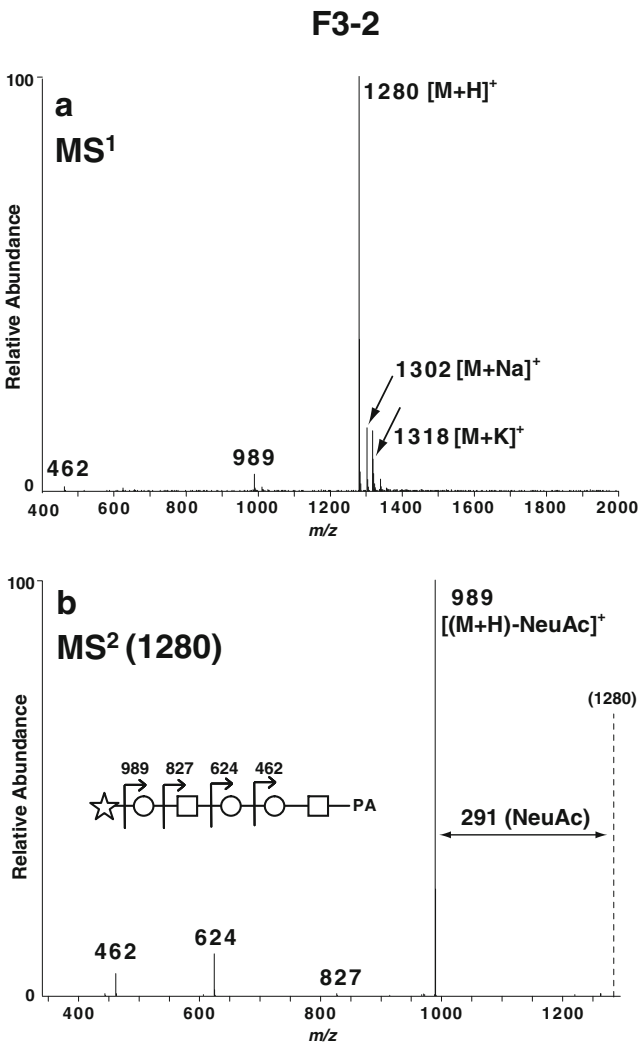

Fig. 3 $MS^{1,2}$ spectra of F3-2. **a** MS^1 spectra of F3-2; **b** MS^2 spectra of $[M+H]^+$ precursor ion at *m/z* 1280 detected in MS^1 of A. The MS/MS fragment ions were assigned as shown schematically. Symbol representations of glycans are as follows: Hex, open circle; HexNAc, open square; Neu5Ac, open star

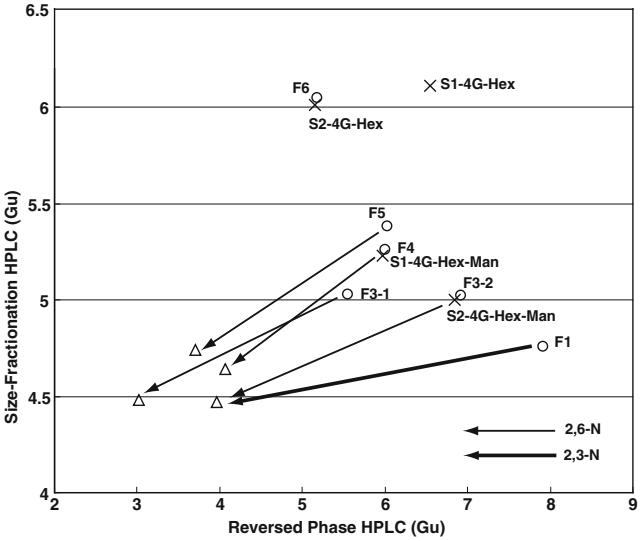

Fig. 4 Digestion of PA-oligosaccharides with sialidase. The elution profiles of PA-*N*-glycans are summarized in the form of a 2-D map. Xs indicate the positions of the authentic PA-*N*-glycans (Table 1). Open circles indicate the positions of free Neu5Ac-containing *N*-glycans, F1, F3-1, F3-2, F4, F5 and F6. Triangles indicate the positions of the digested products with sialidase. Thin lines indicate the direction of the change after α2,3-sialidase digestions of F3-1, F3-2, F4 and F5 under the conditions where the enzyme digests Neu5Ac in both α2-3- and α2-6-linkages (abbreviated as 2,6-N). Thick lines indicate the direction of the change after α2,3-sialidase digestions of F1 under the conditions where the enzyme specifically digests α2-3-linked Neu5Ac (abbreviated as 2,3-N)

likely to be digestion products of a disialylated complex biantennary *N*-glycan; specifically, cleavage of the *N*,*N*'-diacetylchitobiose core linkage (GlcNAcβ1-4GlcNAc) and removal of either the Manα1-3 or Manα1-6 arm from the biantennary form (Fig. 1). Hence, the authentic free complex-type *N*-glycans were synthesized from SGP (Fig. 1, see Material and Methods). F4 and F3-2 coincided with the position of the standard S1-4G-Hex-Man and S2-4G-Hex-Man, respectively, on the 2-D map (Fig. 4). These results were also confirmed by MS analyses. Thus, free oligosaccharides F4 and F3-2 were estimated to be Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6Manβ1-4GlcNAc and Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAc, respectively (Table 3). The structures of the other two kinds of free α2,6-Neu5Ac-linked oligosaccharides, F3-1 and F5, were thought to be either Neu5Acα2-6Galβ1-4GlcNAcβ1-6Manα1-6Manβ1-4GlcNAc or Neu5Acα2-6Galβ1-4GlcNAcβ1-4Manα1-3Manβ1-4GlcNAc, based on the branching pattern of typical tetraantennary *N*-glycans. However, we were unable to unambiguously confirm this structure. Hence, the structures of F3-1 and F5 were indicated to be Neu5Acα2-6Galβ1-4GlcNAcβ-Manα-Manβ1-4GlcNAc, without the linkage position of the fourth GlcNAc and third Man residues from the reducing terminus (Table 3). Neu5Ac is linked to F1 *via* α2-3 linkage. The desialylated product of F1 is identical to desialylated F3-2, indicating that the structure of F1 is Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAc (Table 3, Fig. 4).

Group 2 and Group 3

On the 2D map, F6 coincided with the position of S2-4G-Hex, but not with S1-4G-Hex (Figs. 1 and 4). The structure of F6 was predicted to be Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Manα1-6)Manβ1-4GlcNAc (Table 3). MS analysis revealed the compositional sequence of F2 (Group 3 glycan) to be Neu5Ac-HexNAc-HexNAc-Hex-Hex-HexNAc (data not shown). Neu5Ac was linked *via* α2-6 to the non-reducing terminus. Previous reports showed the presence of a (sialyl) LacdiNAc structure (GalNAcβ1-

4GlcNAc) [19], so we reasoned that F2 is Neu5Acα2-6GalNAc-GlcNAcβ-Manα-Manβ1-4GlcNAc. However, the subterminal residue could not be confirmed as GalNAc in this study, F2 is indicated to be Neu5Acα2-6HexNAc-GlcNAcβ-Manα-Manβ1-4GlcNAc (Table 3).

The structure of GSLs and free oligosaccharides of colorectal cancers

The profiling of acidic GSLs and free oligosaccharides of colorectal cancer cells and normal colorectal epithelial cells from representative cases are shown in Fig. 2a and b, respectively. Two major peaks, G1 (GM3) and G2 (LST-c), were obtained in acidic GSLs of colorectal cancer cells (Fig. 2a). The free oligosaccharide, corresponding to F3-2, was observed as a very minor peak, which is much lower than the GSLs, G1 (GM3) and G2 (LST-c). In the profiling of normal epithelial cells, free oligosaccharides could not be detected (Fig. 2b).

Accumulation of large amounts of free Neu5Ac-containing complex-type N-glycans in pancreatic cancer cells

The structures of GSLs and free oligosaccharides of pancreatic cancer cells and their normal pancreatic cells from five patients have been analyzed. Free oligosaccharides were obtained as major components in the pancreatic cancer cells from three out of the five cases (cases 1, 2 and 3, Supplementary Table 1). Pancreatic cancer cells derived from the two other cases contained free oligosaccharides as very minor components (cases 4 and 5, Supplementary Table 1). However, these free oligosaccharides were undetectable in normal pancreatic cells from all five cases. Furthermore, the amount of the major free *N*-glycans of pancreatic cancer cells was much higher than those of colorectal cancers cells. The profile of oligosaccharides from pancreatic cancer cells (Fig. 2c, e, g) and the corresponding normal pancreatic epithelial cells (Fig. 2d, f, h) from three cases, all of which showed an accumulation of free oligosaccharides as major components in the pancreatic cancer cells, are shown in Fig. 2c–h.

Figure 2c shows the profile of PA-glycans of case 1 (Supplementary Table 1) i.e., where free *N*-glycans were most abundantly accumulated in pancreatic cancer cells among the five cases. The corresponding profile for normal pancreatic cells from the same case is shown in Fig. 2d. In the GSLs, G1 (GM3) was expressed as the dominant component in both pancreatic cancer cells and normal pancreatic cells, and the expression levels of GM3 of these cells were much higher than those of colorectal cancer cells and normal colorectal cells (Figure 2a–d). In addition to GSLs (G1, G3 and G4), a variety of free oligosaccharides were also detected in cancer cells, but not normal cells. The abundance of the free *N*-glycans exceeded about 2-fold that of GSLs (Fig. 2c, d). Six peaks, F1, F2, F3, F4, F5 and F6, corresponding to free *N*-glycans, were observed. Reversed phase HPLC of each peak revealed that they were composed of 7 oligosaccharides, F1, F2, F3-1, F3-2, F4, F5 and F6. The abundance of each *N*-glycan is summarized in Table 3. All but 1 (F1) of the 7 glycans were α2-6-Neu5Ac-linked *N*-glycans, which represented more than 95 % of the total amount of free *N*-glycans in these samples (Table 3). F3-2 was most abundant, and the amount of other 6 glycans was much lower than that of F3-2. Among the four kinds of α2-6-sialylated oligosaccharides in group 1, the relative amount of each species was as follows: F3-2 (72 %) >>F4 (10 %)>F3-1 (6 %)>F5 (1 %) (numbers in parentheses indicate the percentage of each glycan relative to total free *N*-glycan). It is noteworthy that this order is common to all the cases of pancreatic cancers.

The profiling of GSLs and free *N*-glycans of pancreatic cancer and normal pancreatic cells from the other two cases are shown in Fig. 2e and f (case 2, Supplementary Table 1) and in Fig. 2g and h (case 3, Supplementary Table 1). The profiles are similar to that of case 1 described above, although the amounts of free *N*-glycans were considerably lower for cases 2 and 3. Nonetheless, the amount of free *N*-glycans was still comparable to that of GSLs (Fig. 2c, e, g). Akin to the previous case, the relative amount of group 1 oligosaccharides was as follows: F3-2 >>F4>F3-1>F5 (Table 3).

Discussion

Structural analyses of oligosaccharides associated with pancreatic cancers revealed, unlike colorectal cancer cells, the presence of a variety of free Neu5Ac-containing complex-type *N*-glycans. The relative amounts of these free Neu5Ac-containing complex-type *N*-glycans were comparable to or much higher than those of GSLs in most, but not all, pancreatic cancers. High levels of free Neu5Ac-containing N-glycans were observed in the pancreatic cancer cells from three out of the five cases, but correlation between clinicopathological features and the amounts of free oligosaccharides were not found.

The free *N*-glycans accumulated in cancer cells derived from the three human pancreatic cancer cases described in this study (cases 1, 2 and 3) and the previously studied colorectal cancer cases [8–11] were found to possess several common characteristic features. Specifically, (i) almost all (>95 %) of the free *N*-glycans were composed of α2-6-Neu5Ac-linked glycans, with α2-3-sialylated glycans making up a very minor part, and (ii) the proportion of each free *N*-glycan relative to total free glycans was to some extent dependent on its pentasaccharide backbone. Namely, free α2-6-

Neu5Ac-linked *N*-glycans having a Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAc backbone were the most abundant species (i.e., 48–72 % of total free *N*-glycan content). The second most abundant glycans had a Galβ1-4GlcNAcβ1-2Manα1-6Manβ1-4GlcNAc backbone (8–24 %), followed by glycans with either a Galβ1-4GlcNAcβ1-6Manα1-6Manβ1-4GlcNAc or Galβ1-4GlcNAcβ1-4Manα1-3Manβ1-4GlcNAc backbone. These results indicated that the branch on the α6-Man arm of biantennary *N*-glycans was preferentially removed. Thus, F3-2 (Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAc) was most abundant among the free Neu5Ac-containing *N*-glycans in the cancers examined.

The occurrence of free complex-type *N*-glycans in mammals has been reported in mouse liver and in human stomach cancer derived cell lines, MKN7 and MKN45 cells [12, 16]. In mouse liver, only free complex-type biantennary, but not monoantennary, *N*-glycans were observed. This observation differs from the findings reported in this study. Free monoantennary *N*-glycan was found in MKN7 and MKN45 cells. Similar to our findings, F3-2 (Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAc) was most abundant in MKN7 and MKN45 cells. Furthermore, the amounts of F3-2 in MKN7 and MKN45 were about 600 and 380 pmol/10^6 cells, respectively [12]. However, significant differences in the composition of accumulated free oligosaccharides were found between the two kinds of cell lines and human pancreatic cancers. Almost all the free *N*-glycans in human cancers examined in this study were composed of monosialylated hexasaccharide with a singly trimmed core structure (group 1, and 3) along with F6 (group 2), possessing an intact trimannosyl core structure (Table 3). By contrast, in MKN7 and MKN45 cells, F3-2 was the only monosialylated hexasaccharide with a singly trimmed core structure among the various free *N*-glycans, and F6 as the major component. Furthermore, free multiply sialylated *N*-glycans, which could not be detected in human cancers, were abundantly present in MKN7 and MKN45 cells.

In the same report of MKN7 and MKN45 cells, a mechanism responsible for the accumulation of free Neu5Ac-containing *N*-glycans was suggested based on biochemical analyses to be due to insufficient lysosomal function, inefficient degradation of the free *N*-glycans in the lysosomes, as well as the leakage of lysosomal components including free *N*-glycans into the cytosol [12]. This hypothesis is further supported by the findings that F3-2, F1 and F6 are contained in excessive urinary excretion from patients with the lysosomal disease, sialidosis [20]. A similar mechanism might be responsible for the accumulation of free complex-type *N*-glycans in human pancreatic cancer cells, though experimental evidence needs to be provided by further studies.

In this study, we focused on the analysis of the structures of GSLs and fee glycans. The other kinds of glycoconjugates, such as *N*-linked glycans attached as glycoproteins, are also very interesting and important targets to investigate the difference between pancreatic and normal pancreatic cells.

In summary, we have unequivocally demonstrated that substantial amounts of free Neu5Ac-complex-type *N*-glycans accumulate in human pancreatic cancers. By contrast, in most normal epithelial cells these glycan species were undetectable. It is possible that these glycans might be developed as novel tumor markers for cancers. However, further validation studies will be needed prior to clinical application of these candidate markers.

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