Glycoconjugate Journal 15, 1033–1037 (1998)

Alteration of *N*-acetylglucosaminyltransferases in pancreatic carcinoma

Bi-cheng NAN[1], Dong-min SHAO[3], Hui-li CHEN[1]*, Yong HUANG[1], Jian-xin GU[3], Yi-bin ZHANG[2], Zhao-guang WU[2]

[1]*Key Laboratory of Glycoconjugate Research, Ministry of Public Health, Shanghai Medical University, Shanghai, 200032, China*
[2]*Department of General Surgery, Zhongshan Hospital, Shanghai Medical University, Shanghai, 200032, China*
[3]*Department of Biochemistry, Shanghai Medical University, Shanghai, 200032, China*

The activities of three N-acetylglucosaminyltransferases (GnT III, GnT IV and GnT V) were determined in 10 samples of pancreatic carcinoma (PCa) and compared with those in 9 samples of normal pancreatic tissue (NP). It was found that the specific activities of GnT III , GnT IV and GnT V increased in all of the PCa samples. GnT III increased most significantly, up to 22.3 fold of normal, GnT IV was elevated 12.3 fold, while GnT V increased only 2.4 fold. The elevation of GnTs in pancreatic carcinoma was consistent with the increase in the number of antenna and bisecting GlcNAc structures in N-glycans of pancreatic ribonuclease (RNase) as assessed by Con A affinity chromatography. Polycytidylate specific RNase from the serum of PCa patients showed the same structural changes as that found in in N-glycans of the RNase from PCa tissue.

Keywords: **Pancreatic carcinoma, N-acetylglucosaminyltransferase, Glycan, Ribonuclease, ConA affinity chromatography, WGA affinity chromatography.**

Abbreviations: **PCa, pancreatic carcinoma; NP, normal pancreas; RNase, ribonuclease; GnT, N-acetylglucosaminyltransferase; GlcNAc or Gn, N-acetylglucosamine; Man or M, mannose; UDP-, uridine diphospho-; HPLC, high-performance liquid chromatography; N-glycan, asparagine linked glycan, EDTA, ethylenediamine tetraacetic acid; ConA, concanavalin A; WGA, wheat germ agglutinin; α-MeMan, Methyl-α-D-mannopyranoside; polyC, polycytidylate; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol, EGTA, ethyleneglycol-bis (β-aminoethyl) ether-N,N,N',N'-tetraacetic acid; C_2C_2, GlcNAcβ1- 2Manα1-6(GlcNAcβ1-2Manα1-3)ManβR; $C_{2,4}C_2$, GlcNAcβ1-2Manα1-6(GlcNAcβ1-4[GlcNAcβ1-2]Manα1-3)ManβR; $C_2C_{2,6}$, GlcNAcβ1-6 [GlcNAcβ1-2]Manα1-6(GlcNAcβ1-2Manα1-3)ManβR;$C_{2,4}C_{2,6}$,GlcNAcβ1-6[GlcNAcβ1-2]Manα1-6(GlcNAcβ1-4[GlcNAcβ1-2]Manα1 -3)Man βR where R = 1-4GlcNAcβ1-4GlcNAcAsnX; $Gn_2M_3Gn_2$-PA, C_2C_2 where R = 1-4GlcNAcβ1-4GlcNAc-PA.**

Introduction

Changes in glycan structures of glycoproteins from cancer cells are closely related to malignant behaviors such as rapid growth, invasion, and metastasis [1-3]. The enzymatic mechanisms of the structural changes in glycans include alterations of enzymatic activities of some glycan processing enzymes[4, 5]. *N*-acetylglucosaminyltransferases play a critical role in the structure of asparagine linked [N-] glycans such as antennary (outer chain) number, linkage of sugars, and bisecting structure of glycans. GnT III is involved in the synthesis of a bisecting GlcNAc residue, which attaches to the core β-mannose with a β-1,4 linkage in *N*-glycans. GnT IV participates in the synthesis of a β-1,4 branch to the mannose of the α 1.3 arm in complex type N-glycans, and GnT V catalyzes the attachment of the GlcNAc residue to the mannose of the α 1.6 arm with a β1,6 linkage. Using a biantennary N-glycan (C_2C_2) as the substrate, the product of GnT IV or GnT V is triantennary ($C_{2,4}C_2$ or $C_2C_{2,6}$ respectively) N-glycan [6]. The changes in GnT activities in human malignancies have been reported by many authors. GnT-V increased in human breast cacinoma [4] ,and GnT III was elevated in patients with chronic myelogenous leukemia in blast crisis [7]. In our laboratory, it was found that the activities of GnT III and IV decreased, but GnT V remained unchanged in renal cell carcinoma [8]. The activity of GnT V also significantly increased in most samples from human primary liver cancer [9]. However, the question whether the changes in GnTs always show different patterns in different malignancies needs to be further investigated. In the present study, we report that all of the activities of GnT III, GnT IV, and GnT V increased in human pancreatic carcinoma (Pca). To know whether the activity changes in GnTs may affect some structures of *N*-glycans on the glycoproteins in cancerous pancreatic tis-

*To whom correspondence should be addressed. Fax: 86 21 64039987; E-mail: hlchen@shmu.edu.cn

sue, polycytidylate specific ribonuclease (RNase), one of the marker enzymes of pancreatic tissue was selected, because it was reported that human pancreatic RNase is a *N*-glycan containing enzyme [10]. The results showed that some structural changes in the glycans of RNase from cancer tissues and sera of PCa patients were consistent with the activity changes of GnTs in PCa tissues.

Materials and methods

Samples

Tissues of PCa and normal region (at least 5 cm apart from the PCa tissue) of pancreas were resected from patients (8 males and 2 females, aged 34–62) during operation and stored at −80°C. All carcinoma samples were pathologically confirmed. These were 2 with stage I, 2 with stage II, 4 with stage III, and 2 with stage IV lesions, according to the American Committee for Staging of Cancer. Serum samples were obtained from PCa patients and normal healthy blood donors.

Preparation of cell extracts for GnT assays

After thawing, the pancreatic samples were cut into small pieces and homogenized in an adequate volume of buffer A (50 mM sodium maleate, pH6.5, 0.30 M sucrose, 5mM $MnCl_2$, 1 mM PMSF, 1mM DTT and 1 mM EGTA) for 5 min at 0°C and centrifuged with 900×g for 20 min at 0°C. The supernatants were recentrifuged with 105,000×g for 60 min and the precipitates were dissolved in an adequate amount of buffer A and used as the enzyme preparations for GnT assays.

Preparation of glycan substrate

Fluorescence-labeled substrate of GnTs was prepared from human transferrin (Sigma, St. Louis, MO, USA) by using pronase digestion, hydrazinolysis, *N*-acetylation, desialylation, and fluorescence labeling with 2 aminopyridine (PA, Sigma, St. Louis, MO, USA) at the reducing end of the glycanaccording to the method of Hase et al. [11]. The labeled C_2C_2 biantennary glycans were treated with β-galactosidase (Sigma, St. Louis, MO, USA), and the final product ($Gn_2M_3Gn_2$-PA heptasaccharide) was purified with HPLC by the method of Nishikawa et al. [12].

Assay of GnTs

GnT III, GnT IV, and GnT V activities were determined according to the method of Nishikawa et al. [12] and modified by Ju et al [13]. The concentration of the fluorescence labeled glycan (GlcNAc acceptor) was increased to 400 μM, and the GlcNAc donor, UDP- GlcNAc, was 50mM. Ten mM $MnCl_2$ was added to the reaction mixture. The products of the GnTs were separated from the substrate by reverse phase HPLC with a TSK-Gel ODS-A C18 column (6 × 150 mm) and identified with the standards provided by Professor Noriko Takahashi of Nagoya City University Medical School. The GnT activity was calculated according to the ratio of the peak areas of the product and the standard, and expressed as pmol of GlcNAc transferred per h per milligram of protein.

ConA-affinity chromatography

The enzyme preparation for GnT assays and corresponding serum sample were subjected to Con A-Sepharose 4B (Sigma) affinity-column chromatography as described previously [14]. The ConA-sepharose 4B column (60 × 10 mm) was preequilibrated with buffer T (50mM Tris-HCl, pH7.4, containing 0.15M NaCl, 1mM $MgCl_2$, $CaCl_2$, $MnCl_2$ for each). After the enzyme preparation or serum was applied to the column, the unbound fraction ($ConA^-$) was eluted with 10 ml buffer T, the weakly bound fraction (ConA+) with 10 ml buffer T containing 10mM α-MeMan, and the strongly bound fraction ($ConA^{++}$) with 10 ml buffer T containing 0.2M α-MeMan. Fractions of 1 ml were collected at a flow rate of 0.2 ml/min Three subfractions (ConA−, ConA+, ConA++) were combind seperately, and RNase activities in each fration were measured. The percentage of RNase activity in each subfraction was calculated.

WGA-affinity chromatography of serum ribonuclease

Serum was treated with neuraminidase from Clostridium perfringeus (Sigma, St. Louis, MO, USA) to remove the terminal sialic acids in glycans of glycoproteins, according to the method described previously [15], then subjected to a WGA-Sepharose 4B (Sigma) affinity column. The WGA-sepharose 4B column (6 × 10 cm) was preequilibrated with buffer T. After enzyme preparation was applied to the column,the unbound fraction was eluted with 10 ml buffer T and the bound fraction with 10 ml buffer T containing 0.2M GlcNAc. The calculation of the percentage of RNase activity in two WGA subfractions was the same as in ConA chromatography.

Measurement of ribonuclease activity

The RNase activities in the chromatographic subfractions and saline-diluted sera were determined by Reddi's spectrophotometric method using polycytidylate (poly C, Sigma) as the relative specific substrate for pancreatic RNase [16].

Protein determination

Protein content was measured by the method of Lowry et al. [17].

Results

Activities of GnTs in normal and cancerous pancreatic tissues

As indicated in Table 1, all of the specific activities of GnT III, GnT IV, and GnT V increased in samples of PCa as compared with the values from the normal counterparts. GnT III increased most significantly, up to 22.3 fold of normal values. GnT IV activity was elevated up to 12.3 fold of normal values, but GnT V increased only to 2.4 fold of normal values. The *P* values of GnT III to GnT V were $<.001$, $<.001$, and $<.05$, respectively, when calculated statistically with Student's-T test. The specific activities of GnT III and GnT IV were higher than the upper limits of normal range in all (100%) of the PCa samples, and GnT V activities were higher in 60% of samples.

ConA-affinity chromatography of ribonuclease in cell extracts of pancreatic tissues

The activity changes in GnT III, GnT IV, and GnT V may cause the structural alterations of *N*-glycans in some glycoproteins from PCa tissue, such as RNase, a marker enzyme of pancreas. Therefore, *N*-glycans of RNase in the cell extracts (enzyme preparation for GnT assays) from normal and cancerous pancreatic tissues were analyzed by means of Con A lectin affinity-column chromatography in six pairs of samples. As shown in Table 2, the unbound fraction ($ConA^-$) of poly C specific RNase increased significantly in PCa samples ($P < .005$). In contrast, the weakly ($ConA^+$) and strongly ($ConA^{++}$) bound fractions decreased markedly, and the *P* values were $<.01$ and $<.05$ respectively.

ConA-affinity chromatography of ribonuclease in serum samples

The poly C-specific RNase activities in sera from PCa patients were about 3.7 times as compared with the mean normal value ($P < .01$, Table 3). When sera from normal persons or PCa patients were subjected to ConA column, the serum RNase was also separated into unbound, weakly bound, and strongly bound fraction as in the normal pancreatic or PCa tissue. The weakly and strongly bound fractions were combined as bound fraction, since the strongly bound fraction was only less than 5% of the total activity. The RNase activities in ConA bound fractions were also decreased significantly in the serum samples from PCa patients as compared with the normal values ($P < .01$).

WGA-affinity chromatography of ribonuclease in serum samples

RNase *N*-glycans was also analyzed with WGA chromatography. Sera from PCa patients and normal persons were treated with neuraminidase to remove the sialic acids at the terminal of *N*-glycans in RNase and then subjected to WGA column. It was shown in Table 4 that the RNase activity in the WGA bound fraction of neuraminidase-treated serum from PCa patients was markedly higher than that in serum from normal persons ($P < .01$).

Table 1. Comparison of GnT activities between normal pancreas and pancreatic cancer.

			GnT activities (pmol GlcNAc transferred/hr mg protein)					
			GnT III		*GnT IV*		*GnT V*	
Sample No.	*Age*	*Sex*	*Normal*	*PCa*	*Normal*	*PCa*	*Normal*	*PCa*
1	62	M	24.45	630.15	15.30	197.34	87.88	252.26
2	61	M	57.59	886.84	29.96	281.19	28.12	94.58
3	43	M	34.15	833.23	4.25	64.90	31.87	98.65
4	44	M	18.38	433.76	13.28	125.98	67.59	118.34
5	60	F	10.58	383.38	3.43	61.32	19.30	84.46
6	51	M	9.96	238.62	3.97	60.00	17.73	51.53
7	55	M	29.50	831.63	18.15	242.30	40.03	98.75
8	55	M	—	740.80	—	229.58	—	79.20
9	56	M	55.93	838.43	11.95	89.71	85.70	128.43
10	34	F	10.65	389.88	5.41	84.12	18.95	70.56
Mean±			27.91±	621.67±	11.70±	143.64±	44.13±	107.68±
SD			18.47	237.88	8.67	85.41	28.64	55.44
p value			<0.001		<0.001		<0.05	

Cancer stage No. 1, 2 stage I; No. 3, 4 stage II; No. 5–8 stage III; No. 9, 10 stage IV, according to the American Committee for Stafging of Cancers. Cancer size (cm) No. 1–10, $3 \times 3 \times 3$, $5 \times 5 \times 5$, $5 \times 5 \times 6$, $3 \times 2 \times 3$, $6 \times 6 \times 12$, $6 \times 7 \times 4$, $7 \times 9 \times 6$, $5 \times 3 \times 4$, $5 \times 5 \times 5$, $10 \times 4 \times 5$, respectively.

Table 2. Percentages of RNase activity found in the ConA subfractions of normal pancreas and pancreatic carcinoma.

Group	*No.*	*Unbound (ConA⁻)%*	*Weakly bound (ConA⁺)%*	*Strongly bound (ConA⁺⁺)%*
Normal	6	64.93 ± 6.67	29.59 ± 6.21	5.48 ± 1.61
Pancreatic cancer	6	82.33 ± 3.45	14.65 ± 2.42	3.02 ± 1.23
P value		<.005	<.01	<.05

Data are expressed as mean % ± SD.
The method of ConA-Sepharose 4B column chromotography was described in Materials and methods.

Discussion

In the present study, it was found that the activities of GnT III, GnT IV, and GnT V, especially GnT III, increased significantly in PCa (Table 1). These results were different from those of human hepatocarcinoma and breast carcinoma [4] in which GnT V was the mainly increased GnT. It also differed from renal cell carcinoma which showed reduced activities of GnT III and IV [8].

RNase is one of the marker pancreatic enzymes, since it is most abundant in pancreas. It was reported that human pancreatic RNase is *N*-glycosylated [10]. The increased GnTs in PCa may influence the glycan structures of glycoproteins synthesized in PCa. For example, the elevation of GnT III in PCa may cause the increase of bisecting GlcNAc in the *N*- glycans of RNase, and elevation of GnT IV and V will stimulate the synthesis of tri- and tetra-antennary structures from biantennary complex type *N*-glycans [6], resulting in the increase of antennae of RNase *N*-glycans. These structural changes in RNase glycans lead to the reduction of binding affinity of RNase to Con A column, since Con A does not bind to the glycan products of GnT IV and GnT V, and very weakly bind to biantennary glycans with a bisecting GlcNAc [18, 19]. In general, the increase of RNase activity in unbound (Con A$^-$) fraction and the decrease of that in weakly bound (Con A$^+$) fractions on Con A column were consistent with and may have resulted from the elevated activities of GnT III, GnT IV, and GnT V in PCa tissue. The enzymatic mechanism of the decreased RNase activity in the Con A strongly bound fraction (Con A^{++}) is not well understood now and needs to be further investigated.

Pancreatic RNase is an important source of serum poly C specific RNase, but serum poly C specific RNase may also originate from other tissues. As shown in Table 2, the RNase activity found in the ConA bound fraction (ConA$^+$ plus ConA^{++}) is about 35% of the total RNase from normal pancreas, while the enzyme activity in this bound fraction increases to 74.7% of the total RNase from normal serum (Table 3). Therefore, a significant amount of ConA-bound RNase in normal serum may come from extra-pancreatic sources. The ConA unbound fraction of poly C specific RNase increased in PCa sera as in PCa tissues, indicating that the serum RNase with abnormal glycans containing more bisecting GlcNAc and antenna comes from the cancerous pancreatic tissues. The increased RNase activity in WGA-bound fraction of neuraminidase-treated serum from PCa patients (Table 4) provides another evidence for the increase of bisecting GlcNAc in *N*-glycans of RNase, since WGA binds to desialo *N*-glycans containing a bisecting GlcNAc [18]. The increase of bisect-

Table 3. Total activities and percentage of RNase activity found in the ConA subfractions of serum samples from normal persons and patients with pancreatic cancer.

		Total activities		*RNase activity % in Con A subfraction*	
Group	*No.*	*units/ml*	*No.*	*Unbound(ConA⁻)*	*Bound(ConA⁺)*
Normal	15	19.98 ± 7.24	6	22.29 ± 12.25	74.71 ± 12.25
Pancreatic cancer	15	74.21 ± 55.06	8	44.71 ± 6.16	55.29 ± 6.16
P value		<.01		<.01	<.01

Data are expressed as mean ± SD.
Unit of enzyme was expressed as nmol of cytidylic acid produced per min in the expermimental condition.
The method of ConA-Sepharose 4B column chromatography was described in Materials and methods. The activities and percentage of RNase activity in two Con A subfractions of serum samples were determined and calculated.

Table 4. Percentage of RNase activity found in the WGA subfractions of serum samples from normal persons and patients with pancreatic cancer

Group	*No.*	*Unbound %*	*Bound %*
Normal	6	91.55 ± 2.11	8.45 ± 2.11
Pancreatic cancer	8	86.32 ± 2.33	13.68 ± 2.33
P value			<0.01

Data are expressed as mean ± SD.
The method of WGA-Sepharose 4B column chromatography was described as in Materials and methods.

ing GlcNAc in serum RNase from PCa patients is also in accordance with the increase of GnT III in PCa tissues (Table 1).

The total activity of serum poly C specific RNase is not a specific index for PCa diagnosis, owing to the multiple origins of this enzyme. In our experiments, it was found that serum poly C specific RNase also increased in patients with some extra-pancreatic diseases. Structural analysis of *N*-glycans in serum RNase using lectin affinity chromatography may become an available method for the clinical diagnosis of PCa. This is now under investigation in our laboratory with more cases of PCa, as well as other chronic pancreatic diseases and extra-pancreatic diseases.

Acknowledgment

This work was supported by the grants from National Natural Science Foundation of China (No. 39570784), Ministry of Public Health and Education Committee of Shanghai Municipal Government for Key Academic Department.

References

1 Kobata A (1988) *Biochimie* 70: 1575–85.
2 Dennis JW (1988) *Cancer Surv* 7: 573–95.
3 Yamamura K, Takasaki S, Ichihashi M, Kobata A (1991) *J Invest Demerol* 97: 176–82.
4 Dennis JW, Laferte S (1989) *Cancer Res* 49: 945–50.
5 Nakao H, Nishikawa A, Karasuno T, Nishiura T, Iida H, Kanayama Y, Yonezawa T, Tarui S, Taniguchi N (1990) *Biochem Biophys Res Commun* 172: 1260–6.
6 Brockhausan I, Narasimhan S, Schachter H (1988) *Biochimie* 70: 1521–33.
7 Yoshimura M, Ihara Y, Taniguchi N (1995) *Glycoconjugate J* 12: 234–40.
8 Zhu TY, Chen HL, Gu JX, Zhang YF, Zhang RA (1997) *J Cancer Res Clin Oncol* 123: 296–9.
9 Yao M, Zhou DP, Jiang SM, Huang QH, Zhou XD, Tang ZY, Gu JX (1998) *J Cancer Res Clin Oncol* 124: 27–30.
10 Yamashita K, Hitoi A, Irie M, Kobata A (1986) *Arch Biochem Biophys* 250: 263–66.
11 Hase S, Ibuki T, Ikenaka T (1984) *J Biochem (Tokyo)* 85: 197–203.
12 Nishikawa A, Gu J, Fujii S, Taniguchi N (1990) *Biochem Biophys Acta* 1035: 313–8.
13 Ju TZ, Chen HL, Gu JX, Qin H (1995) *Glycoconjugate J* 12: 767–72.
14 Chen HL, Dong SC, Ju TZ, Yang XP (1995) *J Cancer Res Clin Oncol* 121: 397–401.
15 Zha XL, Ai ZW, Chen HL (1989) *Acta Biochem Biophys Sinica* 21: 431–6.
16 Reddi KK, Holland JF (1976) *Proc Natl Acad Sci U S A* 73: 2308–10.
17 Lowry OH, Rosebrough NJ, Farr AL, Randall RZ (1951) *J Biol Chem* 193: 265–75.
18 Osawa T, Tsuji T (1987) *Ann Rev Biochem* 56: 21–42.
19 Brewer CF, Bhattacharyya L (1986) *J Biol Chem* 261: 7306–10.

Received 4 March 1998, revised 24 July 1998 and accepted 6 August 1998