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Alteration in *N*-acetylglucosaminyltransferase activities and glycan structure in tissue and bile glycoproteins from extrahepatic bile duct carcinoma

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The activities of three *N*-acetylglucosaminyltransferases (GnT)-III, IV and V, as well as the structural alterations of N-glycans on the glycoproteins in cancer tissues and bile specimens from 28 cases of extrahepatic bile duct carcinoma (EBDC) were compared with those from 18 cases of benign biliary duct diseases (BBDD). GnT activities were determined with fluorescence-labeled substrate using a HPLC method. It was found that GnT-III and GnT-V activities in EBDC were increased to 3.14 and 15.96 times respectively of the mean BBDD values, but GnT-IV remained unchanged. The activity of GnT-V was correlated with the grade of differentiation and TMN stage of EBDC. The up-regulation of GnT-III resulted in the increased bisecting-GlcNAc on the N-glycans of glycoproteins in cancer tissues and a 201 kDa bile glycoprotein when analyzed with HRP-labeled E_4-PHA. The increased GnT-V activity led to the elevation of the β1,6GlcNAc branch (or antennary number) on the N-glycans in cancer tissue glycoproteins and 201, 163, 122 kDa proteins in the bile as probed with HRP-labeled DSA. These findings suggest that the alteration in GnT activities may be involved in the malignant transformation and development of EBDC, resulting in the aberrant glycosylation of some tissue and bile proteins. The latter was expected to be used in the clinical diagnosis and prognosis evaluation in EBDC patients.
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Keywords: **extrahepatic bile duct carcinoma (EBDC), *N*-acetylglucosaminyltransferase (GnT), asparagine-linked glycan (*N*-glycan), bile glycoprotein, lectin**

Abbreviations: **EBDC, Extrahepatic bile duct carcinoma; BBDD, Benign bile duct diseases; SA, Sialic acid or *N*-acetylneuraminic acid; Gal, Galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Man, Mannose; UDP, Uridine diphosphate; GnT, *N*-acetylglucosaminyl transferase; MES, 2-(*N*-morpholino) ethanesulfonic acid; E-PHA, Phaseolus vulgaris Erythroagglutinin, DSA, Datura stramonium agglutinin; HRP, Horse radish peroxidase; ECL, Enhanced chemiluminescence; BSA, Bovine serum albumin; TBS, Tris buffered saline.**

Introduction

It has been well documented that alterations in the glycan structure on cancer cell surface are closely related to the malignant behaviors, such as uncontrolled growth, invasion and metastasis. The enzymatic mechanism of the structural changes in glycans is the altered activities of some glycosyltransferases [1,2]. For instance, *N*-acetylglucosaminyltransferase (GnT) is a family of enzymes catalyzed the transference of the *N*-acetylglucosamine (GlcNAc) group from UDP-GlcNAc to an acceptor sugar residue (Man, Gal, GalNAc) in asparagine-linked (N-) glycans or serine/threonine-linked (O-) glycans. At least five GnTs were cloned from mammalian or human tissues [2,3] participating in the processing of N-glycan, transforming N-glycan from high mannose-type to hybrid type and complex-type. Among them, GnT-I and GnT-II transfer GlcNAc group to the trimannoside core of N-glycans. GnT-III transfers GlcNAc group to the β1,4 mannoside in the core portion of N-glycans to form a bisecting β1,4 GlcNAc structure (bis-GlcNAc). GnT-IV or GnT-V transfers GlcNAc to the α1,3 mannoside or α1,6 mannoside of biantennary and triantennary N-glycans, producing a β1,4 or β1,6 GlcNAc branching structure, respectively, in the product tri- or tetra-antennary N-glycan [3,4]. It was reported that the activities of GnT-III, IV and V were increased in many human malignancies, such

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as breast cancer [5], primary liver cancer [6], pancreatic cancer [7], trophoblastic cell tumor [8] and bladder cancer [9], but remained unchanged in renal cancer [10].

Furthermore, numerous reports showed that the glycoproteins produced from cancer cells frequently carried aberrant sugar chains when compared with the same proteins from corresponding normal cells. For example, the γ-glutamyltransferase (γ-GT) from human hepatocellular carcinoma contains more β1,6 GlcNAc branching structure on its N-glycans than the γ-GT from normal human liver [11]. The N-glycans in serum transferrin (Tf) from hepatocarcinoma patients are multiantennary containing bis-GlcNAc and outer chain fucose, while these structures are almost absent in the serum Tf from normal persons [12]. The urine hCG (human chorionic gonadotropin) from patients with invasive mole and choriocarcinoma showed an increase in the antennary number and abnormal attachment of the antennae to the core portion of N-glycans [13]. The binding affinity to lectin concanavalin A (Con A) of serum ribonuclease from patients with pancreatic carcinoma and the urine fibronectin from patients with bladder cancer was reduced due to the increase in β1,6 GlcNAc branch and bis-GlcNAc on the N-glycans of ribonuclease and fibronectin [7,9].

The incidence of extrahepatic bile duct cancer (EBDC) has been apparently increasing in recent years; the increasing rate in Shanghai China was reported to be approximately 5% per year, especially in the aged persons [14]. Some imaging diagnostic methods were commonly used in the diagnosis of EBDC, but all of these methods need expensive instruments or the procedures are harmful and difficult in the discrimination from benign diseases of the bile duct. In addition, some tumor markers, such as CEA, CA-19-9 and CA-50 were not specific for EBDC, and often increased in other cancers or benign biliary disorders [15,16]. Therefore, a simple biochemical method was required for the specific and accurate diagnosis of this cancer. In the present investigation, the alterations of the expressions of GnT-III, IV and V were studied in EBDC tissue with different pathological conditions, such as the grade of differentiation, the TNM (tumor-node-metastasis) stage of cancer development. The aberrant structures of the *N*-glycans in the tissue and bile glycoproteins from EBDC were also analyzed in order to find a biochemical method for the clinical diagnosis and prognosis evaluation of EBDC.

Materials and methods

Samples and materials

EBDC tissues from 28 patients (age 36–83, mean 54.0 $\pm$ 12, 7 male 17, female 11) were resected from the upper (23 cases), middle (3 cases) and lower (2 cases) segments of bile duct, anatomically classified according to Longmire *et al.* [17]. The diagnosis of histological types, the grade of differentiation (G1–G3) and the TNM stage (I–IV) of the EBDC samples were determined by the professors in the Department of Pathology of our Medical College according to the standard recommended by UICC (Union of International Cancer Committee). Table 1 shows the pathological data of 28 EBDC cases. Control samples from 18 patients (age 42–73, mean 59.0 $\pm$ 8.7, male 9, female 9) with benign biliary duct diseases (BBDD), including 12 cases of hepatolithiasis, 6 of extrahepatic bile duct stenosis, were obtained from the remnant ends of bile duct during cholangiojejunostomy. All samples were immediately frozen in liquid nitrogen after resection and stored at -70°C. A piece of each tissue was embedded in paraffin for preparing the tissue slice. The bile specimens from patients with EBDC and BBDD were collected with puncture during the surgical operation and stored at -20°C until use.

Lectins, including E_4-PHA (homotetramer) and DSA, HRP, neuraminidase (type V from Clostridium perfringens), GlcNAc, UDP-GlcNAc, Tf, 2-aminopyridine and Sephadex G-150 were purchased from Sigma. Enhanced chemiluminescence (ECL) plus Western blot system and nitrocellulose membranes were obtained from Amersham Corp. Other reagents were commercially available in China.

Preparation of acceptor substrate and assay of GnTs

The acceptor substrate, pyridylamino (PA)-flurorescence-labled biantennary N-glycan, ($GlcNAc_2Man_3GlcNAc_2$-PA), was prepared from normal Tf and purified with high performance liquid chromatography (HPLC) by Hase method [18] as described in our published papers [7,9]. The tissue samples from EBDC and BBDD were homogenized with ultra-sonification, and centrifuged as described previously [9]. The second supernatant was used as the enzyme preparation after protein determination with Lowry's methods [19]. GnTs were assayed according to our modified method [7,9] of Nishikawa *et al.* [4] (1990). Briefly, reaction mixture (50 μl) contained 80–100 μg enzyme, 0.1 M pH 6.25 MES buffer, 10 mM $MnCl_2$, 0.8 mM acceptor substrate, 50 mM UDP-GlcNAc, 0.2 M GlcNAc and 2% Triton-X100. After incubation at 37°C for 5 h, the reaction was stopped by boiling. Then the samples were centrifuged and 20 μl of each supernatant was applied to reverse-phase HPLC with ODS C_{18} column to separate the products of GnTs from the acceptor substrate. GnT-III and GnT-IV were determined simultaneously in one test tube, and GnT-V was assayed in a separate tube without the addition of $MnCl_2$. All the samples were assayed in duplicate. The enzyme activities were calculated according to the peak areas of the products and the standard with known concentration.

Histochemical staining using HRP-E_4-PHA and HRP-DSA as probes

Paraffin sections (5 μm thick) of the tissues were de-waxed with xylene and re-hydrated using ethanol with gradually decreasing concentrations. Then the sections were washed twice with TBS containing 3% H_2O_2 and followed by 30 min treatment of TBS containing 2% BSA (buffer 1). The sialyl residues at the terminal of glycans in the tissue glycoproteins were removed with the treatment of neuraminidase at a concentration of 1.8 U/ml

Table 1. Pathological data of 28 EBDC cases

Case	*Sex*	*Age*	*Histological type*	*Location*	*Grade*	*TNM stage*
1	M	63	Adeno*	Upper	G3	IV
2	M	67	Adeno*	Upper	G3	IV
3	F	65	Adeno*	Upper	G3	IV
4	M	44	Adeno*	Lower	G3	IV
5	M	66	Adeno*	Middle	G3	IV
6	M	65	Adeno*	Upper	G3	III
7	M	38	Adeno*	Upper	G3	III
8	M	69	Adeno*	Upper	G3	IV
9	F	53	Adeno*	Upper	G3	IV
10	M	47	Adeno*	Upper	G3	III
11	F	40	Adeno*	Upper	G3	IV
12	M	36	Adeno*	Upper	G3	IV
13	M	55	Pap Adeno	Middle	G1	II
14	M	56	Pap Adeno	Upper	G1	I
15	F	36	Pap Adeno	Upper	G1	II
16	M	63	Adeno*	Upper	G3	IV
17	F	40	Muc Adeno	Lower	G1	II
18	F	53	Muc Adeno	Upper	G1	II
19	F	46	Tub Adeno	Upper	G1, G2	II
20	M	51	Tub Adeno	Upper	G1, G2	IV
21	F	62	Tub Adeno	Upper	G1, G2	II
22	F	68	Tub Adeno	Upper	G1, G2	II
23	F	73	Tubular Adeno	Upper	G1, G2	III
24	M	36	Adeno*	Upper	G3	IV
25	M	50	Tubular Adeno	Upper	G1, G2	IV
26	F	42	Pap & Tub Adeno	Upper	G1, G2	II
27	M	83	Pap & Tub Adeno	Upper	G1, G2	II
28	M	49	Pap & Tub Adeno	Middle	G1, G2	II

EBDC: extrahepatic bile duct carcinoma; M: Male; F: Female; Adeno: Adenocarcinoma; Pap: Papillary; Muc: Mucinous; Tub: Tubular; *Adenocarcinoma could not be divided into papillar, mucinous or tubular type owing to the poor differentiation (G3) of this type.

in 0.1 M sodium acetate/0.04 M $CaCl_2$ (pH 5.0). After that, the slices were incubated over night at 4°C with HRP-E_4-PHA or HRP-DSA (1:100 diluted in buffer 1) and followed by 3 washes with TBS. The HRP-lectin conjugates were prepared according to the method of Wilson and Nakane [20]. Finally, the slices were stained with diaminobezene (DAB)/H_2O_2, and counterstained with hematoxylin.

Western blot of bile proteins and detection of glycoproteins with HRP-labeled lectins

After centrifugation of the thawed bile specimens, an equal volume of saturated ammonium sulfate was added to each supernatant. Then the specimens were stood overnight and centrifuged again. The lipids in the precipitate were removed with methanol-chloroform extraction, and the protein in the water phase was collected and dialyzed to eliminate the salts. The sialyl residues of glycans in the bile glycoproteins were removed with neuraminidase as described before. After protein determination, the bile proteins (total 30 μg) were separated with 7% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 4% BSA in 0.05 M Tris buffered saline (TBS), and treated with 1:200 HRP-labeled lectin (E_4-PHA or DSA), then stored overnight at 4°C. After being washed with TBS containing 1% Tween-20, the membrane was treated with ECL reagent according to the manual and the protein bands were visualized by exposure of the membrane to X-ray film. The semi-quantitative data were obtained using ImageMaster System and the screened photos were analyzed with NIH Image software.

Statistics analysis

Values were expressed as mean $\pm$ SD. Statistical significance was determined by unpaired two-tailed Student's t-test. $P < 0.05$ and $p < 0.01$ were considered statistically significant and very significant respectively.

Results

Activities of GnTs in tissue samples from EBDC and BBDD

The average activity of GnT-III was the highest one among the three assayed GnTs in the BBDD group, and increased to 3.14 times in the EBDC group ($p < 0.05$) as shown in Table 2.

Table 2. The activities of GnT-III, GnT-IV and GnT-V in EBDC and BBDD tissues

		Enzyme activity (p mol GlcNAc transferred/ h · mg)		
Group	*No. of cases*	*GnT-III*	*GnT-IV*	*GnT-V*
EBDC	28	$28.9 \pm 15.2^{\#}$	3.5 ± 2.2	$76.6 \pm 64.2^{*}$
		12.0–76.0	1.0–13.0	13.0–298.0
BBDD	18	9.2 ± 5.5	3.4 ± 0.8	4.8 ± 2.4
		3.9–21.0	1.7–4.3	1.8–13.0

EBDC: extrahepatic bile duct carcinoma; BBDD: benign biliary duct diseases; GnT: *N*-acetylglucosaminyltransferase. Data were expressed as mean ± SD and range from three independent experiments. $^{\#}p < 0.05$ compared to BBDD group; $^{*}p < 0.01$ compared to BBDD group. The experimental procedure for GnT assay and the method for statistic analysis were described in the "Methods".

There were 6 cases in the BBDD group showed high GnT-III activity and overlapped with the EBDC samples. The average activity of GnT-IV in EBDC group was much lower than that of GnT-III, and was almost equal to the mean GnT-IV level of the BBDD group. In contrast, the elevation of GnT-V activity in the EBDC group was very significant ($p < 0.01$), its mean value was 15.96 times of that in BBDD group, and only one case overlapped with the BBDD group. If 20.0 and 13.0 p mol GlcNAc transferred/h · mg were taken as the upper limits of GnT-III and GnT-V activities in BBDD group respectively, the sensitivity and specificity of GnT-III were 67.9% (19/28) and 88.9% (16/18), while those of GnT-V were 96.4% (27/28) and 100% (18/18) respectively.

Relations of the GnT activities to different pathological conditions of EBDC

The EBDC group can be divided into various pathological subgroups according to their histological type, differentiation grade and TNM stage. Table 3 indicated that the average activities of GnT-V in the subgroups with poor differentiation (G3) and advanced stage (TNM III, IV) were significantly higher than those in the corresponding subgroups with well/medium differentiation (G1, G2) and earlier stage (TNM I, II). The ratio of GnT-V activity of G3 to G1/G2 subgroup was 2.82 ($p < 0.05$), and TNM III/IV to TNM I/II was 3.48 ($p < 0.01$). Therefore, GnT-V activity was supposed to be related with the differentiation and development of EBDC. In TNM stage III and IV, the cancer cells invaded into extra-gallbladder tissues and adjacent organs. It indicated that GnT-V was highly associated with the invasion and metastasis of EBDC. In contrast, the activities of GnT-III and GnT-IV in each subgroup were not different from their corresponding counterparts ($p > 0.05$).

Histochemical studies on GnT products with HRP-lectins

The above experiments revealed that the expression of GnT-III was elevated and GnT-V was very significantly increased in the samples from EBDC. These findings indicate that the products of GnT-III and GnT-V, bisecting GlcNAc and β1,6 GlcNAc branch, respectively, on the N-glycans of glycoproteins may be increased in the cancer cells. This was evidenced by the histochemical studies with HRP-lectins as shown in Figure 1. E_4-PHA recognizes *N*-glycans with bisecting GlcNAc (the product of GnT-III) [21,22]. DSA strongly binds to multiantennary N-glycans containing the β1,6GlcNAc branch (the products of GnT-V) or repeat sequence of *N*-acetyllactosamine [23], but the latter sequence is frequently located at the outside of the β1,6GlcNAc branch [24]. In the slices of hepatolithiasis (an example of BBDD), the tissue was slightly stained by both HRP-E_4-PHA and HRP-DSA, suggesting that the N-glycans of glycoproteins in the benign epithelium of bile duct contained only little amount of bisecting-GlcNAc and β1,6GlcNAc branch. In contrast, the cancer cells of tubular adenocarcinoma (an example of EBDC) were very strongly stained by

Table 3. Relation between GnT activities with different pathologiacal conditions of EBDC

		Enzyme activity (p mol GlcNAc transferred/ h · mg)		
Group	*No. of cases*	*GnT-III*	*GnT-IV*	*GnT-V*
Histological grade				
G1, G2	14	26.5 ± 15.6	3.1 ± 1.6	40.1 ± 30.8
		12.0–76.0	1.0–8.0	13.0–143.0
G3	14	31.3 ± 14.4	3.9 ± 2.6	$113.1 \pm 68.0^{\#}$
		13.5–70.2	1.3–13.0	49.0–298.0
TNM stage				
I, II	11	26.3 ± 16.9	2.6 ± 1.0	30.6 ± 10.0
		12.0–76.0	1.0–4.0	13.0–48.0
III, IV	17	30.6 ± 13.8	4.1 ± 2.6	$106.4 \pm 66.8^{*}$
		13.5–70.2	1.3–13.0	24.0–298.0

EBDC: extrahepatic bile duct carcinoma; GnT: *N*-acetylglucosaminyltransferase. Data were expressed as mean ± SD and range from three independent experiments. $^{\#}p < 0.05$ (G3 vs G1/G2); $^{*}p < 0.01$ (III/IV vs I/II). The experimental procedure for GnT assay and the method for statistic analysis were described in the "Methods".

HRP-E_4-PHA and HRP-DSA, revealing that bisecting-GlcNAc and β1,6GlcNAc branch were greatly increased in the N-glycans of glycoproteins in EBDC cells. The positive granules were located in the cytoplasm and plasma membranes of the cancer cells, as well as the secreting substance in the bile duct.

Western blot and HRP-lectin analysis of bile glycoproteins

If the glycoproteins with aberrant structure of N-glycans in cancer tissue are secreted into the bile duct, they can be found in the bile of the patients. In order to verify this speculation, the HRP-E_4-PHA and HRP-DSA were also used to analyze the N-glycan structures of bile glycoproteins after Western blot. It was found that only a few glycoproteins were detected using HRP labeled lectins as the probes, though many proteins including non-glycosylated proteins were appeared when silver staining was used instead of HRP-lectins (photos not shown). A 201 kDa glycoprotein was obviously increased in the samples from EBDC when it was stained with HRP-E_4-PHA (Figure 2A), indicating that bisecting-GlcNAc was increased in the 201 kDa glycoprotein from EBDC bile. After this protein was semi-quantified by ImageMaster scanning System followed by the analysis of the screened photos with NIH Image software, it was found that the average intensity of this protein in EBDC bile samples was 13.2 times higher than that in BBDD samples.

On the other hand, three glycoproteins (201, 163 and 122 kDa) were apparently appeared in EBDC samples when probed with HRP-DSA (Figure 2B). The rates of appearance of these three glycoproteins in EBDC bile were 73.3%, 66.7% and 80.0% respectively. Among them, the 122 and 163 kDa glycoproteins were absent in all of the benign specimens, and the 201 kDa glycoprotein was appeared in 10% of benign samples with low intensity. If the 201 kDa protein was semi-quantified, its average intensity in EBDC bile specimens was 17.6 times higher than that in BBDD ones. The higher intensity in HRP-DSA staining of 201, 163 and 122 kDa glycoproteins from EBDC bile demonstrated that the β1,6GlcNAc branch or antennary number of N-glycans was increased in EBDC bile glycoproteins.

Discussion

In the present investigation, it was found that GnT-V was increased most significantly in EBDC as compared with GnT-III and GnT-IV, and became the most abundant GnT in EBDC. The activity of GnT-III, but not GnT-IV, was also increased to a lesser extent in EBDC. This GnT pattern of EBDC is unique, which is different from those of other cancers studied in our laboratory, including hepatocellular [6], pancreatic [7] and bladder carcinoma [9].

As the indexes of EBDC, both sensitivity and specificity of tissue GnT-V were higher than those of GnT-III (refer to the part 1 of the 'Results'). We have also determined two tumor markers in the bile specimens of 31 cases of EBDC and 13 cases of BBDD patients. It was found that the sensitivity and the specificity of carcino-embryonic antigen (CEA) were 80.6% (25/31) and 100% (13/13) respectively (the cut off value of CEA was 7.0 ng /ml bile), and those of antigen CA19-9 were only 19.3% (6/31) and 76.9% (10/13) (the cut off value of CA19-9 was 400 U/ml bile). Therefore, the sensitivity of tissue GnT-V was much better than that of bile CEA and CA19-9, and the specificity of GnT-V was equal to CEA and much higher than that of CA-19-9. However, it must be noted that both GnTs and other tumor markers are not specific for EBDC and are frequently increased in other malignancies.

In addition, the elevation of GnT-V activity was related to the differentiation and invasion (or metastasis) of EBDC, as GnT-V was elevated more significantly in G3 grade and TNM III/IV stages than in G1/G2 grade and TNM I/II stages. This was compatible with a number of reports that GnT-V was associated with the metastasis of some cancers, such as mammary and primary liver cancer [5,25]. In our laboratory, it was demonstrated that the *in vitro* metastatic potential (cell adhesion to laminin, cell migration and invasion through artificial membrane) of a human hepatocarcinoma cell line, H7721, was promoted after the transfection of the cDNA of GnT-V, and reduced after transfection of antisense cDNA of GnT-V [26]. Moreover, transfection of oncogene (*H-ras* or *v-sis*) or anti-metastasis gene (*nm23*-H1) into H7721 cells resulted in the concomitant increase or decrease, respectively, in both GnT-V expression and metastatic potential [27,28]. The up-regulation of GnT-V expression is always accompanied by the increase in its product, β1,6GlcNAc branch, on surface *N*-glycans, indicating that the enhancement of metastatic potential by GnT-V was probably mediated by this branching structure [26–28]. The molecular mechanism of the metastasis of EBDC is not well established; the increased expression of GnT-V and its products might be one of the reasons. In Taniguchi's lab, it has been reported that the transcriptional regulation of GnT-V is mediated by transcription factor Ets-1 in a human bile duct carcinoma cell line, HuCC-T1 [29]. However, not all of the cancer metastasis is related to GnT-V, for instance, GnT-V may be not involved in the metastasis of bladder and renal cancer, since its activity was only slightly increased or unchanged in these two cancers [9,10]. Moreover, GnT-III is more important than GnT-V in the development of bladder cancer [9]. This suggests that probably different glycosyltransferases participate in the malignant transformation and development of different cancers.

In the early stages of EBDC (TNM stage I, II), GnT-III and GnT-V were increased to 2.87 and 6.38 times of the BBDD level respectively when the data shown in Tables 2 and 3 were compared, suggesting that both GnT-III and V are the indexes of malignant transformation of bile duct epithelial cells. However, in the advanced stages of EBDC (TNM stage III and IV), GnT-V was further elevated to more than three times of the level in early stages, but GnT-III increased very slightly with no statistic significance, indicating that only GnT-V might be a parameter of EBDC progression. We have not evaluated the relation between the activity of GnT-V and the survival rate, since

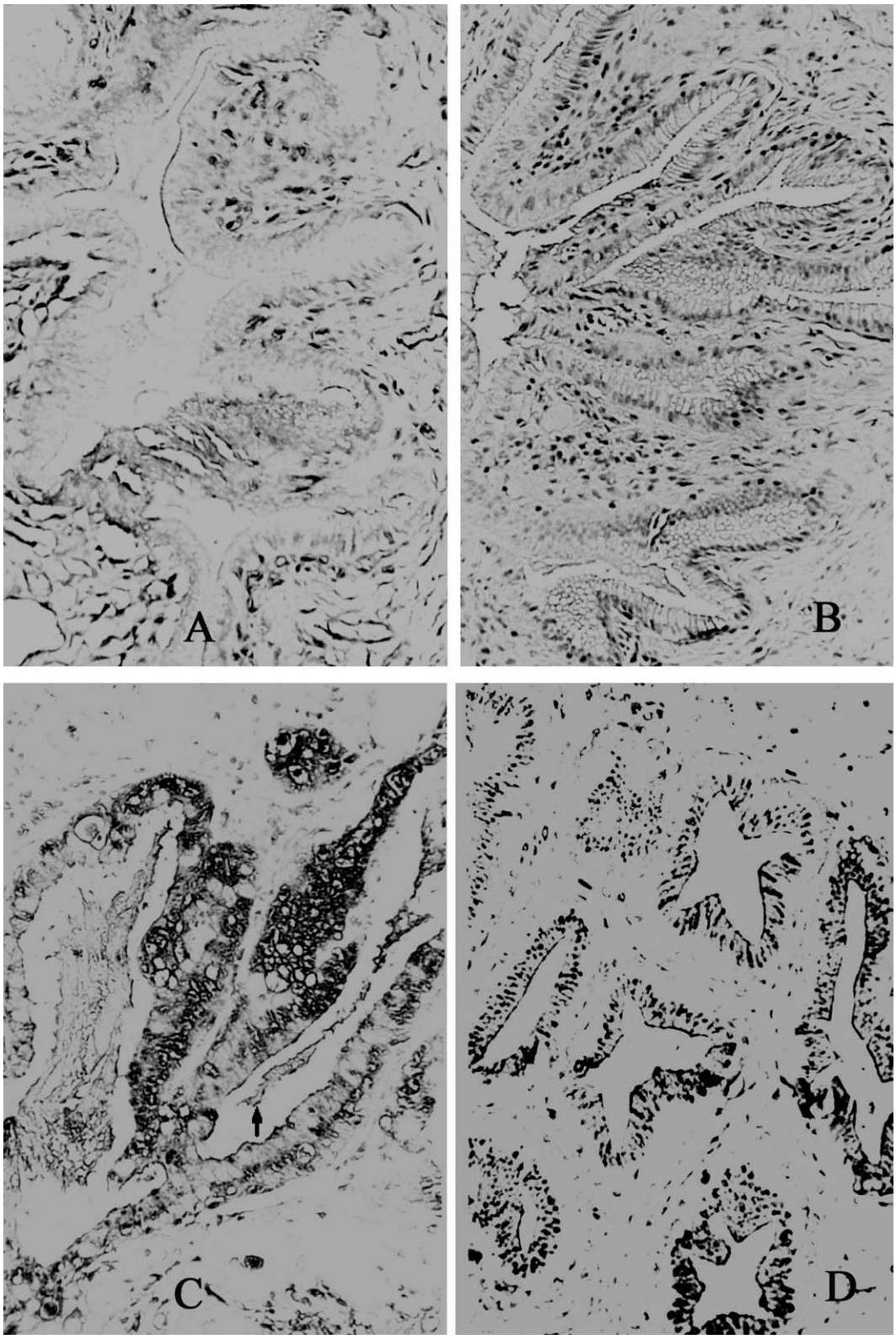

Figure 1. Histochemical staining using HRP-E_4-PHA and HRP-DSA as probes. The slices were pre-treated with neuraminidase. The procedures for the treatment of slices with HRP-labeled lectin, staining with DAB/H_2O_2 and counterstained with hematoxylin were described in the "Methods". (A) Hepatolithiasis (BBDD), HRP-E4-PHA as probe; (B) Hepatolithiasis (BBDD), HRP-DSA as probe; (C) Tubular adenocarcinoma (EBDC), HRP-E_4-PHA as probe; (D) Tubular adenocarcinoma (EBDC), HRP-DSA as probe. The arrows in (C) indicate the secreted substances in the duct of tubular adenocarcinoma.

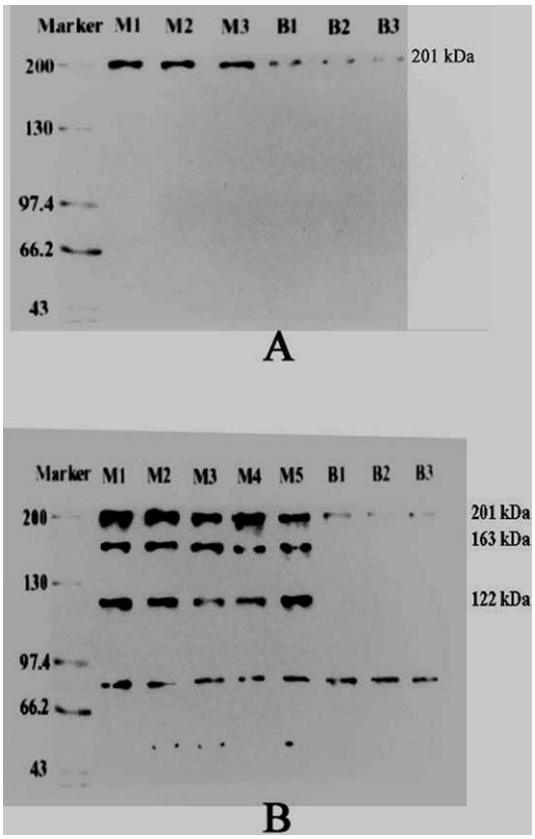

Figure 2. Western blot of bile glycoproteins with HRP-lectins. The bile samples were treated with neuraminidase before SDS-PAGE as described in the "Methods". The kDa of the marker proteins was indicated at the left side of the photos. (A) HRP-E_4-PHA as probe. M1–M3: Three examples of bile specimens from EBDC; B1–B3: Three examples of bile specimens from BBDD. 201 kDa is the molecular mass of the increased glycoprotein in EBDC bile specimens. (B) HRP-DSA as probe. M1–M5: Five examples of bile specimens from EBDC; B1–B3: Three examples of bile specimens from BBDD. 201, 163 and 122 kDa are the molecular mass of the increased or appeared glycoproteins in EBDC bile specimens.

the survival rate of EBDC was influenced by many factors, not only decided by GnT-V activity.

The findings using a histochemical method as shown in Figure 1 demonstrated that the up-regulated GnT-III and -V in the cancer cells can produce their products in the presence of endogenous substrates. The N-glycans of glycoproteins with high affinity to E_4-PHA (products of GnT-III) and DSA (products of GnT-V) were much higher in the cancer cells than in the normal ones, suggesting that GnTs were mainly distributed in the cancer cells of EBDC. These results were in agreement with the observation of the elevated expressions of GnT-III and GnT-V in EBDC as shown in Table 2.

The findings with Western blot of bile proteins (Figure 2) revealed that some bile glycoproteins with higher affinity to E_4-PHA and DSA in EBDC tissue were secreted into the bile. These results were compatible with the findings shown in Figure 1 that the glycoproteins carrying the products of GnT-III and GnT-V were increased in EBDC tissue. The staining intensity of 201 kDa protein was increased to 13.2 times and 17.6 times when HRP-E_4-PHA and HRP-DSA were used as the probes, respectively, which was generally in agreement with the 15.96 times increase of GnT-V activity in EBDC tissues. These aberrantly glycosylated proteins, especially 201 kDa protein are probably specific for EBDC, and were expected to be used in the clinical diagnosis and prognosis evaluation of EBDC after extensive studies.

Collectively, the present studies demonstrate that the glycoproteins with abnormal glycan structures are secreted into bile by EBDC cells, and the aberrant glycosylation is attributed to the alterations in the expressions of some corresponding glycosyltransferases. As a consequence, the invasion or metastatic potential of the EBDC cells were enhanced.

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