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# A Glycomic Approach to Hepatic Tumors in *N*-acetylglucosaminyltransferase III (GnT-III) Transgenic Mice Induced by Diethylnitrosamine (DEN): Identification of Haptoglobin as a Target Molecule of GnT-III

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A glycomic approach to the identification of target molecules in glycosyltransferase gene targeting mice is a promising strategy to understand the biological significance of glycosyltransferase genes *in vivo*. In order to understand the biological effects of *N*-acetylglucosaminyltransferase III (GnT-III) on tumor formation in the liver, diethylnitrosamine (DEN) induced tumor formation in the GnT-III transgenic mice was examined. Our findings show that the incidence of hepatic tumor could be dramatically suppressed.

A glycomic approach using two-dimensional gel electrophoresis followed by lectin blot analysis and sequence analysis revealed that haptoglobin, a radical scavenger molecule in serum was heavily glycosylated in hepatic tumor-bearing GnT-III transgenic mice that had been treated with DEN. Immunoprecipitation followed by $E_4$-PHA lectin blot analysis also confirmed that the bisecting GlcNAc, a product of GnT-III was added to haptoglobin molecules. Since the use of DEN is known to lead to the production of lipid peroxidation products which facilitate this reaction and haptoglobin is an acute phase reactant, acting as a radical scavenger against hemoglobin or iron stimulated lipid peroxidation, a relationship between the glycosylation of haptoglobin and the suppression of hepatoma development can not be ruled out.

This paper is the first report that shows a relationship between the sugar chains of glycoproteins with radical scavenger activity and hepatocarcinogenesis.

*Keywords*: GnT-III; DEN; Hepatoma; Haptoglobin; Glycomics; Mice

*Abbreviations*: GnT-III, *N*-acetylglucosaminyltransferase III; $E_4$-PHA, erythroagglutinating phytohematoagglutinin; DEN, diethylnitrosamine; MMP, matrix metalloproteinase

## INTRODUCTION

*N*-Acetylglucosaminyltransferase III (GnT-III) catalyzes the transfer of *N*-acetylglucosamine (GlcNAc) to the core mannose of *N*-glycans via a β1–4 linkage and forms a bisecting GlcNAc structure[1] as shown in Fig. 1. While a high level of expression of GnT-III is observed in rat brain and kidney, the expression in the normal liver is quite low.[2] However, dramatic increases in GnT-III expression in the liver are observed during hepatocarcinogenesis in the case of a rodent model[3] as well as at the stage of liver cirrhosis of human liver diseases.[4]

In order to better understand the biological meaning of the expression of GnT-III during hepatocarcinogenesis, we established GnT-III transgenic mice using a liver specific promoter.[5] Our previous study indicated an abnormal accumulation of lipid in hepatocytes of GnT-III transgenic mice at 12 weeks of age. However, the formation of hepatic tumors is rare in these mice at 1–2 years after birth. Therefore, it is possible that GnT-III may play a

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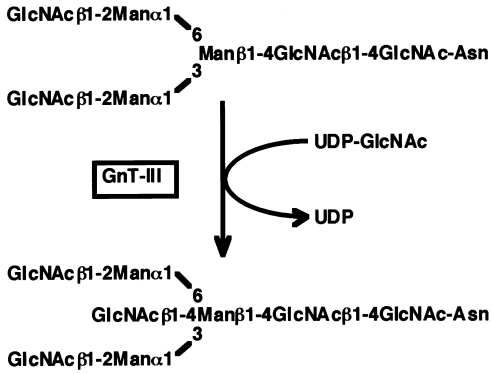


FIGURE 1 Reactions catalyzed by GnT-III. GlcNAc, *N*-acetylglucosamine; Man, mannose; Asn, asparagine; UDP, uridine 5′-diphosphate.



protective role against hepatocarcinogenesis. On the other hand, Bhaumik *et al.* reported the importance of extrahepatic GnT-III in hepatic tumor formation using GnT-III deficient mice.[6] However, the biological significance of GnT-III in hepatocytes themselves remains unknown in terms of hepatocarcinogenesis.

In this study, we report on an investigation of the effect of DEN treatment on the formation of hepatic tumors in GnT-III transgenic mice and report on a glycomic approach[7] to the identification of target molecules of GnT-III. The findings indicate that an overexpression of GnT-III in the liver suppresses tumor formation induced via treatment with low dose levels of DEN. Furthermore, a glycomic approach identified haptoglobin as one of the target proteins of GnT-III during hepatocarcinogenesis, as evidenced by two-dimensional gel electrophoresis followed by lectin blot analysis and sequence analysis. Possible implications of the mechanisms underlying the oligosaccharide modification of GnT-III are discussed.

## MATERIALS AND METHODS

### Animals

The experimental animals were maintained in the Institute of Experimental Animal Sciences Osaka University Medical School (IEXAS). All experiments were performed according to the Osaka University Medical School Guideline for the Care and Use of Laboratory Animals. The establishment of GnT-III transgenic mice has been described previously[5] and BDF1 mice (Japan SLC, Shizuoka, Japan) were used as controls. GnT-III transgenic mice (hetero-type) and control BDF1 mice were mated. The hetero-type of GnT-III transgenic mice and the other mice were used as GnT-III positive mice and GnT-III negative mice, respectively. Selection was performed by Southern blot analysis using a specific probe for human GnT-III cDNA.

### Induction of Hepatoma

To induce hepatic tumors, the mice were treated with diethylnitrosamine (DEN) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to previous methods[8,9] with slight modifications. Briefly, a single intraperitoneal injection (5 μg/g of body weight) in saline was administered to both the GnT-III transgenic mice and the control mice 14 days after birth. These mice were maintained in temperature-controlled conditions with free access to standard mouse chow and water. After sacrificing the mice at 26 weeks of age under comfortable euthanasia, their livers and serum were collected, and stored at −80°C until used. For histological analysis, the liver tissues, which were not frozen, were fixed in 10% buffered formalin and paraffin-embedded blocks were produced for each case. Four-micrometer slices of tissue sections were deparaffinized and stained with hematoxylin–eosin using standard procedures. The degree of histological liver damage was evaluated by microscopic observation.

### Two-dimensional Gel Electrophoresis Followed by Lectin Blot Analysis and Sequence Analysis

Isoelectric focusing (IEF) was carried out at room temperature using an ATTO SJ-1060D instrument (ATTO Corp., Tokyo, Japan). Aliquots containing 5 or 15 μl of mouse serum were diluted with 95 or 85 μl of urea buffer (9.5 M urea, 2% Triton X-100, 2% ampholine, and 5% 2-mercaptoethanol) for lectin blots or for Coomassie Brilliant Blue (CBB) staining, respectively. Samples were separated on gels by running at 400 V for 20 h. The resulting gels were equilibrated in 125 mM Tris–HCI (pH 6.8) buffer containing 1.25% SDS, 5% 2-mercaptoethanol and 0.0125% bromophenol blue. The second dimension was run on 8% SDS-PAGE with the PROTEAN II xi 2-D Cell (Bio-Rad Laboratories, CA, USA). The separated proteins were transferred to an Immobilon-P Transfer Membrane (MILLIPORE Corp., Massachusetts, USA) with 1.8 mA for 1 h. One of the membranes was used for lectin blot analysis using $E_4$-PHA. Detailed procedures have been described previously.[10] Proteins that strongly bind to $E_4$-PHA were identified from the membrane stained with CBB, and then sequenced from the N-terminal amino acid.

### Immunoprecipitation

A 5-μl aliquot of mouse serum diluted in 0.5 ml PBS was incubated with 10 μl of rProtein A Sepharose®

TABLE I Incidence of tumors in GnT-III transgenic mice livers as the result of DEN treatment

| DEN (μg/g) | Mice | Numbers of DEN treated mice | Numbers of mice with tumor developed ($p < 0.01$*) | Numbers of nodules observed per liver (mean ± SD) |
|---|---|---|---|---|
| 5 | GnT-III tg | 19 | 1 | 0.05 ± 0.23 |
| 5 | Control | 16 | 11 | 1.38 ± 1.78† |

All mice were males. The numbers of nodules on the surface of the liver were visually counted. GnT-III tg, GnT-III transgenic mice. * By $\chi^2$ test. † $p < 0.01$ vs. GnT-III transgenic mice treated with DEN by the Student's *t*-test.

Fast Flow (Amersham Pharmacia Biotech, Buckinghamshire, England) pre-conjugated with normal rabbit serum at 4°C overnight. After centrifugation at 300*g* for 5 min, 4.5 μg of anti human haptoglobin rabbit polyclonal antibody was added to the supernatant followed by incubation for 4°C for 6 h. An additional overnight incubation at 4°C was performed in the presence of 10 μl of rProtein A Sepharose®. This was performed at room temperature as follows. After washing four times with PBS containing 0.05% Tween 20 (PBS-T), the immune complexes were subjected to 10% SDS-PAGE and transferred to an Immobilon-P Transfer Membrane. The membranes were incubated with 5% skim-milk overnight and then incubated further with 1/2000 diluted anti human haptoglobin antibody for 2 h. After washing three times with PBS-T for 10 min each, the membrane was incubated with peroxidase-conjugated goat affinity purified antibody to rabbit IgG (ICN Biomedicals, Inc., CA, USA) for 1 h. The membrane was washed three times with PBS-T for 10 min each, and then developed using an ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, England) following standard protocols. After dehybridizing the membrane, a lectin blot analysis using $E_4$-PHA was performed as described above.

## RESULTS

### GnT-III Transgenic Mice Escape from Chemical Carcinogenesis

The administration of DEN to normal control mice caused nodules at six months after the peritoneal injection. When DEN was administered, the number of tumors that developed and the number of visible nodules in the liver was significantly lower in the case of the GnT-III transgenic mice than in the control mice (Table I). No significant difference was observed for female mice because the number of visible nodules was quite low both in GnT-III transgenic and the control mice (data not shown). Histological examination revealed that visible nodules in the liver were similar in both groups of mice, i.e. adenoma nodules (Fig. 2).

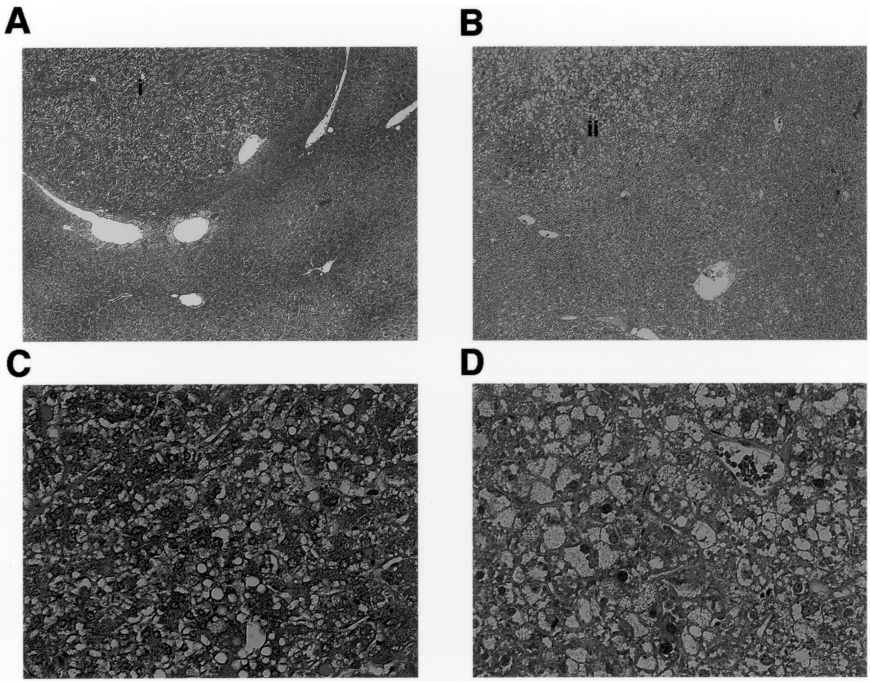


FIGURE 2 Histological analysis of hepatic tumors of DEN-treated mice. Livers from GnT-III transgenic mice (A, C) and control mice (B, D) were removed 26 weeks after DEN treatment and stained with hematoxylin and eosin. C and D correspond to areas i and ii, respectively. Magnification, ×40, A and B; ×200, C and D. Detailed procedures are described in "Materials and Methods".

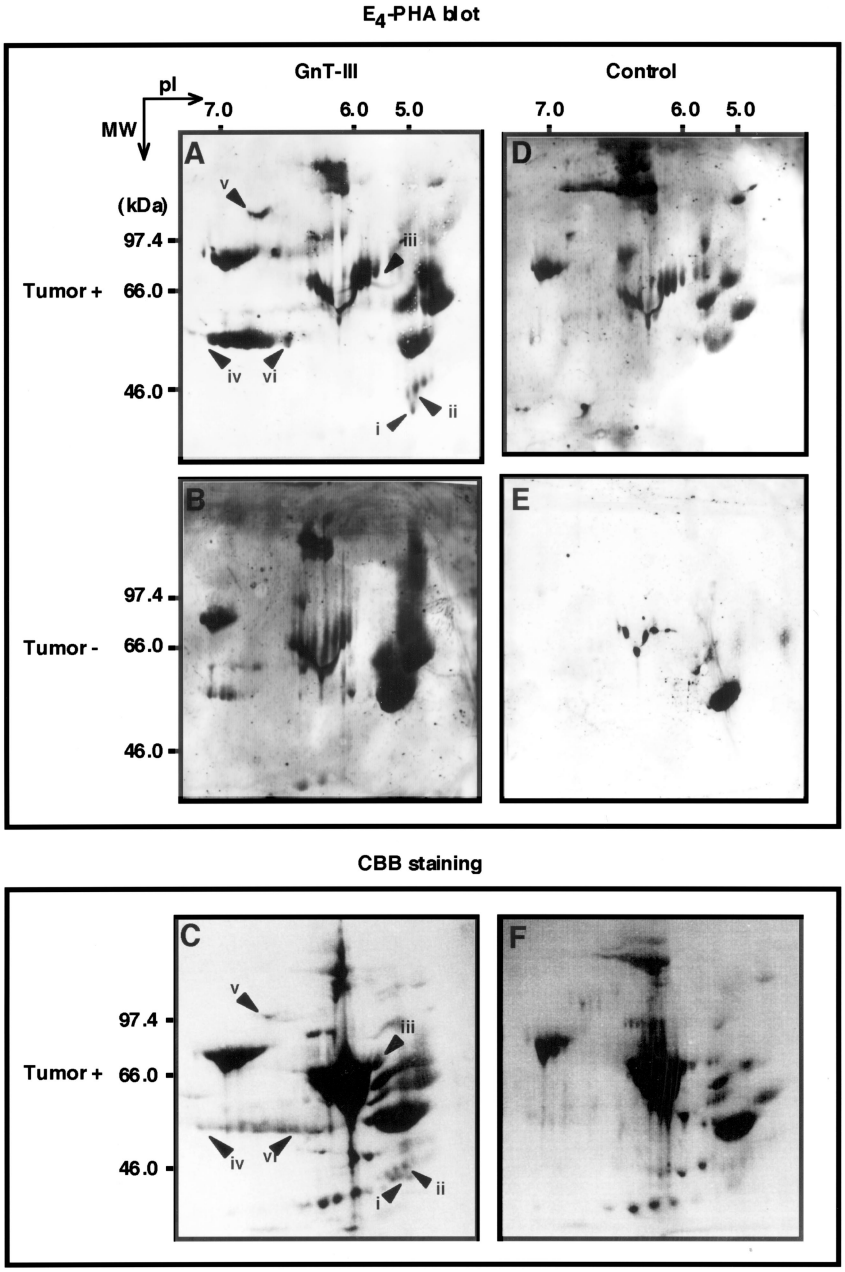


FIGURE 3 Two-dimensional electrophoresis followed by $E_4$-PHA lectin blot analysis. Serum from GnT-III transgenic mice (A, B, C) and control mice (D, E, F) were used. Panels A and D represent the $E_4$-PHA lectin blotting pattern of the serum (5 μl) from mice which had visible liver nodules. Panels B and E represent the $E_4$-PHA lectin blot pattern where no visible nodules were observed. In panels C and F, 15 μl of serum from the GnT-III transgenic and control mice were run, respectively, and stained with Coomassie Brilliant Blue. Detailed procedures are described in "Materials and Methods". Each arrowhead indicates glycoprotein(s) with high levels of bisecting GlcNAc.

TABLE II Identification of the target protein of GnT-III

| Sample No.* | Amino acid sequence | Protein |
|---|---|---|
| i | LVPTD | T-cell receptor |
| ii | IIGGSMDA | Mouse haptoglobin β chain |
| iii | EAHKS | Mouse serum albumin |
| iv | DVDLQESG | Mouse IgG heavy chain |
| v | DXLDGYIS | Plasminogen? |
| vi | QVQLQES | Mouse IgG heavy chain |

*These numbers are indicated in Fig. 3.

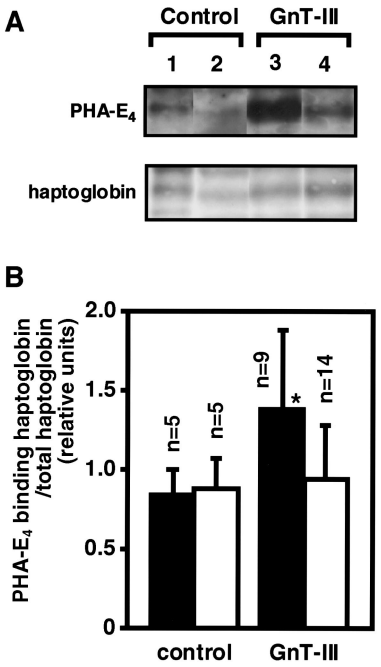


FIGURE 4 $E_4$-PHA binding to immunoprecipitated haptoglobin. 5 μl of serum was immunoprecipitated using anti-haptoglobin IgG, and a lectin blot analysis using $E_4$-PHA was then performed. After dehybridization of the membrane, a Western blot analysis of haptoglobin was performed. Representative data are shown in panel A. Lane 1 indicates control mice bearing hepatic tumors. Lane 2 indicates control mice with no tumors. Lane 3 indicates GnT-III transgenic mice bearing hepatic tumors. Lane 4 indicates GnT-III transgenic mice with no tumors. In panel B, the relative intensity of $E_4$-PHA binding to haptoglobin to total haptoglobin was calculated by densitometry and plotted. All data represent the mean ± SD. :$*p < 0.05$ vs. the non-tumor group (lane 4) by Student's *t*-test. Numbers at the top indicate the number of samples investigated.

### Identification of Target Glycoproteins of GnT-III in Hepatocarcinogenesis

While the development of hepatic tumors in GnT-III transgenic mice that had been treated with DEN was suppressed, certain hepatoma tissues in a rodent model showed high expression of GnT-III (3). To investigate the molecule responsible for stimulating the hepatomas to express high levels of GnT-III, GnT-III transgenic mice were treated with a high dose of DEN to induce hepatic tumors. A glycomic approach such as two-dimensional gel electrophoresis followed by an $E_4$-PHA blot was performed to identify changes in the oligosaccharides of serum glycoproteins of the GnT-III transgenic mice livers, (Fig. 3). The total binding of serum proteins to $E_4$-PHA was increased in the GnT-III transgenic mice, irrespective of whether or not they had hepatic tumors. In these proteins, six spots were dramatically increased in the hepatic tumor-bearing GnT-III transgenic mice. The N-terminal amino acid sequences were determined on the proteins corresponding to these spots (Table II). The sequence of one of these proteins was IIGGSMDA and was identified as the mouse haptoglobin β-chain. Increases in $E_4$-PHA binding to immunogloblin were not observed in other tumor bearing GnT-III transgenic mice and albumin binds non-specifically with $E_4$-PHA because of its high levels in serum. Although one spot was identified as fragments of a T-cell receptor, it was not investigated further because of the difference in molecular weight. The last spot was very similar to the partial sequence of plasminogen, but one amino acid was different.

### Increases of $E_4$-PHA Binding to Haptoglobin

To confirm whether the oligosaccharide structure of haptoglobin is modified in the progression of hepatic tumor in the GnT-III transgenic mice, immunoprecipitation followed by $E_4$-PHA binding of serum haptoglobin was performed (Fig. 4). No differences in $E_4$-PHA binding to haptoglobin were observed among control mice with/without hepatic tumors, and the GnT-III transgenic mice without tumors. However, significant increases in $E_4$-PHA binding to haptoglobin were observed in the serum of hepatic tumor-bearing GnT-III transgenic mice. The levels of haptoglobin in the serum were almost the same among these four groups.

## DISCUSSION

The biological functions of GnT-III are controversial, because glycoproteins that are modified by GnT-III are different for each type of cancer cells. For example, the overexpression of GnT-III suppresses lung metastasis of melanoma cells, suggesting that GnT-III alters cancer cells with mild characteristics.[11] In contrast, the expression of GnT-III is dramatically enhanced in the blast crisis of chronic myelogenous leukemia[12] and an overexpression of GnT-III in K562 human leukemic cells inhibits NK cell-mediated cell lysis,[12] suggesting that GnT-III plays a role in malignant transformation, at least in the case of leukemia.

While a striking retardation of the progression of hepatic neoplasms was observed in GnT-III deficient mice,[6] the author hypothesized that a glycoprotein growth factor, which is synthesized outside the liver is important in facilitating the progression of DEN-induced hepatic tumors. When a glycoprotein growth factor lacks the bisecting GlcNAc, it might not function well. More recently, this group has reported on the importance of the extra hepatic role of GnT-III in the progression of DEN-induced liver tumors.[13] When GnT-III deficient mice were mated with GnT-III transgenic mice, in which GnT-III expression was regulated under a liver-specific promoter, treatment with DEN and phenobarbital had no effect on the progression of tumors. This

indicates the importance of GnT-III in extra-hepatic organs during hepatocarcinogenesis.

The present study, in contrast to a previous study by Bhaumik *et al.*, clearly demonstrated that the addition of bisecting GlcNAc structures is of biological importance in hepatocytes during hepatocarcinogenesis. A marked increase in bisecting GlcNAc structures in the liver was found to suppress the progression of hepatic tumor formation, as induced by a low dose of DEN. This indicates that the bisecting GlcNAc inhibited the malignant transformation of hepatocytes. While treatment with a low dose of DEN did not induce hepatitis, a deformity in the hepatocytes was observed both in GnT-III transgenic and control mice (data not shown), suggesting that the increased GnT-III activity in hepatocytes suppressed DEN-induced hepatocarcinogenesis at a later stage. In contrast, the study by Yang *et al.* demonstrated the importance of bisecting GlcNAc derived from extra hepatic cells during DEN-induced hepatocarcinogenesis.[13] The reason for the discrepancy between our data and Yang's data may be explained by differences in the method used for the induction of the hepatomas. While we used a low dose of DEN, they used a high dose of DEN and phenobarbital.

Recent findings indicate that *N*-acetylglucosaminyltransferase V (GnT-V) is essential for the progression of cancer as well as metastasis.[15] The action of GnT-V is inhibited by GnT-III via substrate competition of each enzyme.[13] A variety of glycoproteins were glycosylated by GnT-III and -V during hepatocarcinogenesis. Some of these reactions might be directly linked to malignant transformation through the addition of a β1–6 GlcNAc branching by GnT-V. An overexpression of GnT-III in hepatocytes might inhibit β1–6 branching formation in such proteins, which, in turn, would lead to the suppression of tumor formation in the liver. Increased levels of GnT-III and GnT-V are higher in non-parenchymal liver cells than in heptocytes.[16] A circulating glycoprotein, which is a key factor for hepatocarcinogenesis, if it exists, might be secreted from these non-parenchymal cells.

In contrast, high levels of expression of GnT-III were observed in hepatomas of LEC rats,[3] suggesting that certain proteins with bisecting GlcNAc might promote hepatocarcinogenesis. Therefore, in order to identify target molecules of GnT-III, a glycomic approach of serum proteins was performed. We identified haptoglobin as one of the target molecules of GnT-III during hepatocarcinogenesis of GnT-III transgenic mice. When we investigated glycosylation levels of other five spot proteins between serum of GnT-III transgenic mice with tumors and those without tumors, there was no statistical significance (data not shown). Increases in addition of bisecting GlcNAc were observed only in haptoglobin. Haptoglobin is a serum protein, which is produced in the liver. It binds to free hemoglobin, released from erythrocytes preventing iron from being made available as an acute reactant and acts as a radical scavenger.[17] In fact, increases in the fucosylation of the haptoglobin have been reported in breast cancer and ovarian cancer.[18] The biological significance of this glycosylation of haptoglobin is not fully understood at present. Haptoglobin acts as a natural inhibitor for matrix metalloproteinases 2 (MMP-2),[19] which plays a pivotal role in hepatoma progression.[20] Oligosaccharides associated with this protein might easily lead to conformational changes of the protein. Our recent findings suggest that the glycosylation of TIMP-1 (tissue inhibitory metalloproteinase-1) by GnT-III enhances its affinity for MMP-9,[21] resulting in a decrease in MMP-9 activity. Similarly, haptoglobin, when bearing bisecting GlcNAc might suppress the activity of radical scavengers as well as the inhibitory activity of MMP-2. Haptoglobin β-chain (GenBank accession number L10353) has four potential sites of *N*-linked oligosaccharides and two of them exist near the binding sites to free heme and the site for inhibition of lipid peroxidation.[22] Aberrant glycosylation of haptoglobin β-chain might decrease the capacity of radical scavenger, leading to accumulation of free radicals. Recently, Roemer *et al.* reported that changes in glycosylation of mouse haptoglobin β-chain were observed in the serum of nude mice whose mammary carcinoma cells were xenotransplantated.[23] The biological meaning of bisecting GlcNAc modified haptoglobin needs further clarification at the molecular levels in terms of hepatocarcinogenesis.

Collectively, the overexpression of GnT-III in the liver was found to suppress the progression of DEN-induced hepatic tumors and haptoglobin represents one of the target molecules of GnT-III during hepatocarcinogenesis.



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