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Determination of UDP-*N*-acetylglucosamine:β-D-mannoside-1,4-*N*-acetylglucosaminyltransferase-III in patients sera with chronic hepatitis and liver cirrhosis using a monoclonal antibody

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The glycoprotein UDP-*N*-acetylglucosamine:β-D-mannoside-1,4-*N*-acetylglucosaminyltransferase-III (GnT-III) catalyzes the addition of *N*-acetylglucosamine via a β-1, 4-linkage to the β-linked mannose of the trimannosyl core of *N*-linked glycans. It has been reported that the expression of GnT-III increases in many oncogenically transformed cells and human hepatocellular carcinoma (HCC) tissues, and GnT-III enzyme activity in serum can be used for the detection and monitoring of primary hepatomas and hepatocellular carcinomas. A solid-phase enzyme-linked immunosorbent sandwich assay in which a polyclonal antibody (PAb) to aglycosylrecombinant GnT-III (AGR-GnT-III) and a monoclonal antibody (mAb) are employed as a capture protein and probe protein, respectively, is described. The sensitivity of the PAb-mAb sandwich assay, as determined by the dose-response effect for AGR-GnT-III, was 10 ng/ml. This assay was specific for GnT-III and did not detect β-1, 6-*N*-acetylglucosaminyltrasferase-V (GnT-V). AGR-GnT-III concentrations in 377 serum specimens were determined by the PAb-mAb sandwich assay and the results were analyzed based on the disease category, using 1.99 μg/mL (AGR-GnT-III) as a cut-off value. The AGR-GnT-III level of 61 normal serum samples was 0.57 $\pm$ 0.71 μg/ml (mean $\pm$ SD). The results revealed an elevation in serum AGR-GnT-III levels in 60 of 86 patients (3.03 $\pm$ 2.04 μg/ml) with liver cirrhosis (LC) and 86 of 91 patients (2.73 $\pm$ 0.59 μg/ml) with chronic hepatitis (CH). By contrast, 3 of 61 normal subjects, 9 of 34 patients (1.02 $\pm$ 1.03 μg/ml) with acute hepatitis and 8 of 38 patients (1.79 $\pm$ 0.56 μg/ml) with a variety of non-hepatic diseases exhibited a slight increase above the cut-off value. These results indicate that serum AGR-GnT-III levels are elevated predominantly in LC or CH cases. Serum AGR-GnT-III concentration, as measured by the developed PAb-mAb sandwich assay, may be a useful differential marker as a diagnostic aid for CH and/or LC and warrants further investigations with expanded serum panels. ***Published in 2003.***

Keywords: **human *N*-acetylglucosaminyltransferase-III, enzyme linked immunosorbent assay, monoclonal antibody, liver disease**

Abbreviations: **GnT-III, UDP-*N*-acetylglucosamine:β-D-mannoside-1,4-*N*-acetylglucosaminyl-transferase-III; GnT-V,β-1, 6-*N*-acetylglucosaminyltrasferase-V; AGR-GnT-III, aglycosyl recombinant GnT-III; PA, pyridylamine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; PBS, phosphated buffered saline; mAb, monoclonal antibody; PAb, polyclonal antibody; LC, liver cirrhosis; HCC, hepatocellular carcinoma; BSA, bovine serum albumin; DAB, dimethylaminobenzidine; OPD, orthophenylenediamine; γ-GTP, γ-glutamyltranspeptidase; ALT, alanine aminotransferase; AST, aspatrate aminotransferase; ALP, alanine phosphatase; PT, prothrombin time.**

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Introduction

Oligosaccharides in glycoproteins have various biological functions including cell adhesion, sorting, differentiation, and carcinogenesis [1]. UDP-*N*-acetylglucosamine:β-D-mannoside-1,4-*N*-acetylglucosaminyltransferase-III (GnT-III) catalyzes the attachment of an *N*-acetylglucosamine (GlcNAc) residue to mannose in a β1–4 configuration in the region of *N*-glycans and forms a bisecting GlcNAc. GnT-III activity was first discovered in the hen oviduct [2], and its expression in normal rats has been reported to be high in the kidney, stomach, and brain, but barely detectable in the liver [3,4]. High GnT-III activities were observed in hepatic nodules of rat livers during hepatocarcinogenesis [5], as well as in various cells including Novikoff ascites tumor cells, human B lymphocytes, CaCO-2 cell, HuH-6 cell, HL-60 cells, Hep3B and HepG2 [6–11]. GnT-III enzyme activity has been reported to be significantly elevated in the serum of human subjects with hepatomas, liver cirrhosis, as well as in hepatocellular carcinoma (HCC) tissues [11–13]. Therefore, it would appear that an elevation in GnT-III activity might cause the uncontrolled expression of GnT-III by altering the glycosylation pattern of proteins and lipids.

A cDNA clone of human GnT-III was expressed and partially characterizated by Ihara et al. [14] and also by Kim et al. [15]. The cDNA sequence of GnT-III was determined to have an open reading frame that encoded a protein with 531 amino acids and was expressed in *E. coli* [16]. Human GnT-III was purified from HCC tissues by a simplified procedure based on QAE and a immunoaffinity chromatography technique [16].

GnT-III enzyme activity is currently assessed by measuring the reaction product by HPLC. However, this method is time-consuming laborious. In this study, we report on the development of an ELISA utlizing polyclonal Ab and monoclonal Ab to AGR-GnT-III and an investigation of the relationship between serum GnT-III like protein (AGR-GnT-III) levels and the onset of hepatic disease including hepatitis, liver cirrhosis and hepatocellular carcinomas.

Materials and methods

Serum specimens

Serum specimens were obtained from 61 normal healthy subjects, 278 patients with various hepatic diseases, and 38 patients with a variety of non-hepatic diseases. Consent from each patient was obtained for the use of the serum specimen in these studies, and the protocols were consistent with the guidelines approved by the committee at the Catholic University, St. Mary's Hospital (Daejeon, Korea).

Production AGR-GnT-III and antibodies to the protein

Aglycosylrecombinant *N*-acetylglucosaminyltransferase-III (AGR-GnT-III) protein, deficient in the first 23 amino acids was expressed in *E. coli* and purified by DEAE-Sephacel chromatography (Pharmacia, NJ, USA), followed by Sephacryl S-200 gel (Pharmacia, NJ, USA) filtration and, finally, preparative gel electrophoresis [16].

The procedure employed for the production of monoclonal antibodies was based on the protocol described by Kohler and Milestein [17]. Balb/c mice were immunized by an intraperitoneal injection of AGR-GnT-III and mixed with Freund's complete adjuvant. Spleen cells from the mice were fused with the murine myeloma cell line SP2/0-Ag14. The mAb-producing hybridomas were cloned by the limiting dilution technique, and the selected hybridoma clones were propagated by injecting them into mice. Subsequently, ascitic fluids were harvested and processed by Protein G-Sepharose 4B chromatography to obtain purified mAb. AGR-GnT-III-specific mAb, designated as GT273, was selected based on its ability to bind AGR-GnT-III and was coupled to HRP (Sigma, St. Louis, USA) by the Periodate method [18].

Rabbits were immunized with purified AGR-GnT-III in Freund's complete adjuvant (FCA, Sigma, St. Louis, USA) and boosted with 50–200 μg of protein prepared in Freund's incomplete adjuvant (Sigma, St. Louis, USA). The antibodies in the antiserum were purified by Protein G-Sepharose 4B chromatography (Pharmacia, NJ, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

SDS-PAGE experiments were performed using a Phast PAGE System (Pharmacia, NJ, USA), following the manufacturer's instructions. A ten percent gel under reducing conditions (5% w/v mercaptoethanol) was employed in the SDS-PAGE experiments, and the protein bands in the gel were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA, USA). The proteins, after being resolved by SDS-PAGE were transferred electrophoretically onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). The membrane was then treated with non-fat dried milk for 2 h at room temperature, and probed overnight at 4°C using mAb or PAb which is specific to AGR-GnT-III. After rinsing with PBS, the membrane was incubated for 1 h with goat anti-mouse antibody conjugated with horse raddish peroxidase (HRP). The specific binding of mAb to AGR-GnT-III was visualized by adding a dimetylaminobenzidine solution (DAB, Sigma, St. Louis, USA) as a chromogenic substrate.

mAb-PAb sandwich assay for AGR-GnT-III

PAb and 6 mAbs were identified as having specificity and sensitivity for human AGR-GnT-III. These antibodies were evaluated as candidates for capture and/or probe proteins in the sandwich assay for AGR-GnT-III. Briefly, a 96 well microtiter plate was coated, with 100 μL per well of 2 μg/mL PAb to AGR-GnT-III, overnight at 4°C and then washed with PBS solution. The wells

were then overcoated with a 1% BSA solution. 100 μL of appropriately diluted AGR-GnT-III samples were applied per well in duplicate and allowed to react with the PAb for 2 h, at room temperature. After washing with PBS solution, appropriately diluted HRP-conjugated antibodies were applied at a levels of 100 μL per well for 1 h. Orthophenylenediamine (OPD) solution was subsequently added to the wells and, after stopping the reaction by the addition of 1 N H_2SO_4 solution, the absorbance was measured at 490 nm on a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Among the 6 antibodies, GT-273 was selected as the most promising probe protein for the sandwich assay.

The dose-response effect and sensitivity of the PAb-mAb sandwich assay was determined using serially diluted AGR-GnT-III samples. Serum specimens were diluted with Dilution Buffer (PBS, 1% w/v BSA, 0.05% w/v Tween 20) and assayed for AGR-GnT-III, following the protocol described earlier. AGR-GnT-III concentrations of serum specimens, obtained from normal subjects or from clinically diagnosed patients, were assayed after diluting the specimens 10-fold with Dilution Buffer. A recovery test was performed with normal serum samples to which predetermined amounts of AGR-GnT-III were added.

Determination of the serum parameters included in the liver function test

Each serum specimen was analyzed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alanine phosphatase (ALP), prothrombin time (PT), total bilirubin, total protein, direct bilirubin, γ-glutamyltranspeptidase (γ-GTP), and albumin concentration, using an automated analyzer (Hitachi 747, Japan).

GnT-III enzymatic activity

GnT-III enzymatic activity was assayed according to the procedure described previously by Song et al. [11] using a pyridylamino-sugar oligosaccharide chain (PA-sugar, Takara, Shiga, Japan) as the substrate. Typically, the reaction was carried out using 20 mM UDP-GlcNAc and 20 mM pyridylaminated agalacto biantennary oligosaccharide (GlcNAcβ-1,2Manα1-6(GlcNAcβ-1,2Manα1-3)Manβ-1,4-GlcNAcβ-1,4GlcNAc-PA) in the presence of 60 mM MES, 10 mM$MnCl_2$, 200 mM GlcNAc, and 0.5% Triton X-100, pH 6.5. The assay mixtures were incubated at 37°C for 2 h, and the reactions were then terminated by heating in a boiling water bath for 5 min, followed by centrifugation at 15,000 $\times$ g for 5 min. The enzymatic reaction was monitored using an HPLC system, equipped with an Ultrasphere ODS-C18 column (4.6 $\times$ 150 mm, Beckman, Fullerton, CA) by eluting with 0.1 M ammonium acetate, 0.3% v/v n-butanol (pH 4.0). The HPLC profile was analyzed by the fluorescence signal generated by the products (excitation at 320 nm and emission at 400 nm).

Results

Production and characterization of antibodies to AGR-GnT-III

A 1,524 bp portion of human the GnT-III DNA sequence, which encodes the amino acid sequence of the human GnT-III protein, a mutant protein deficient in the first 23 amino acids, were constructed and expressed in *E. coli.* The recombinant AGR-GnT-III protein was purified by DEAE-Sephacel chromatography, followed by Sephacryl S-200 gel filtration and, finally, preparative gel electrophoresis [11].

Mice were then immunized with the purified AGR-GnT-III. Among the hybridomas generated against AGR-GnT-III, six clones produced mAbs that strongly bound AGR-GnT-III. The specificity of the monoclonal antibodies was determined by a Western blot method (Figure 1). Immunoglobulin iso-typing showed that 5 clones (GT122, 125, 94, 10 and 6) were IgM class and 1 clone (GT 273) was IgG2b. Particularly, mAb GT273 for which the isotype was determined to be IgG_{2b} was selected for this investigation because of its high affinity for AGR-GnT-III. As a capture protein, purified AGR-GnT-III was immunized to rabbits, and the resulting polyclonal antibodies obtained.

Performance of PAb-mAb sandwich assay

The performance of the PAb-mAb sandwich assay was evaluated using a PAb to AGR-GnT-III as a capture protein, AGR-GnT-III as an analyte and mAb-HRP as a probe protein. Purified

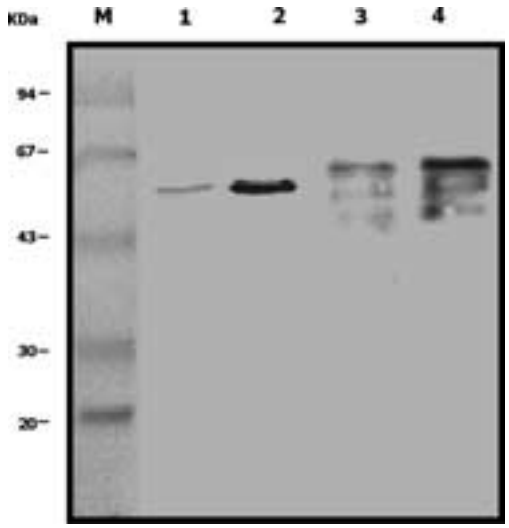

Figure 1. *Western blot probed with the monoclonal antibody and polyclonal antibody.* SDS-PAGE was performed under reducing conditions (10% gels, 0.5% mercaptoethanol). Proteins were transferred onto a membrane and probed with mAb and PAb for human AGR-GnT-III. Lane 1, Mw. standards (prestained); Lane 2, monoclonal antibody against AGR-GnT-III (GT 273); Lane 3, polyclonal antibody against AGR-GnT-III; Lane 3, monoclonal antibody against a patient serum sample (GT 273); Lane 4, polyclonal antibody against a patient serum sample.

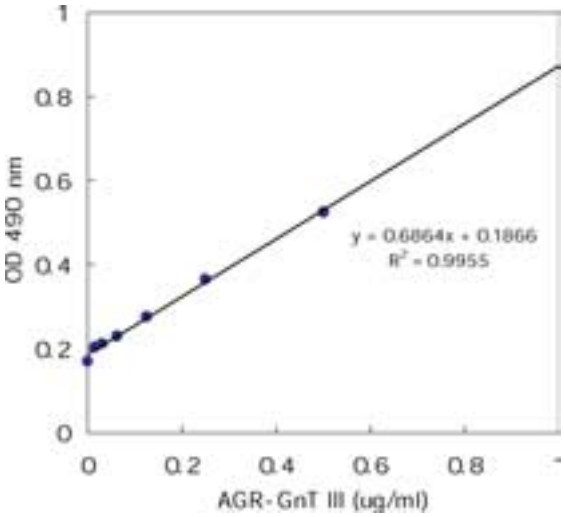

Figure 2. *Dose-response relationship between AGR-GnT-III and OD 492 in the ELISA.* A microtiter plate was coated with polyclonal antibody and overcoated with 1% BSA. One hundred μl of serially diluted GnT-III solution was added per well, followed by incubation for 2 h at R.T. Following the incubation, 1:500 dilution of HRP conjugated monoclonal antibody (IgG of GT273) was reacted for l h, and the color development was measured spectrophotometrically at 492 nm.

GT-273 (IgG) was conjugated with HRP by the periodate oxidation method. The optimum concentration of HRP-conjugated antibody for use in ELISA was determined to be a 1:500 fold dilution in a buffer solution (1% BSA-PBST).

A typical dose-response curve generated by the PAb-mAb sandwich assay, for AGR-GnT-III at various concentrations, is shown in Figure 2. The sensitivity of the mAb-RCA sandwich assay was determined to be 10 ng/mL (AGR-GnT-III). High levels of serum AGR-GnT-III were serially diluted and the concentration of AGR-GnT-III measured by ELISA and a GnT-III enzymatic activity by HPLC. AGR-GnT-III and GnT-III enzymatic activities were correlated well (Figure 3).

Serum dilution test by the mAb-RCA sandwich assay

The serum dilution effect in the PAb-mAb sandwich assay was examined using serum specimens obtained from the pool of normal serum and the pool obtained from liver disease patients who were diagnosed with acute hepatic, chronic hepatitis, liver cirrhosis or a hepatocellular carcinoma. The results presented in Figure 4 clearly show a significant elevation in serum AGR-GnT-III concentration in patients with hepatic disease at various serum dilutions, compared with those of normal subjects.

Determination of serum AGR-GnT-III concentration by the PAb-mAb sandwich assay

A dose-response curve was generated, using known amounts of AGR-GnT-III samples, with each set of the assay and served as a standard curve. A recovery test with normal specimens, to

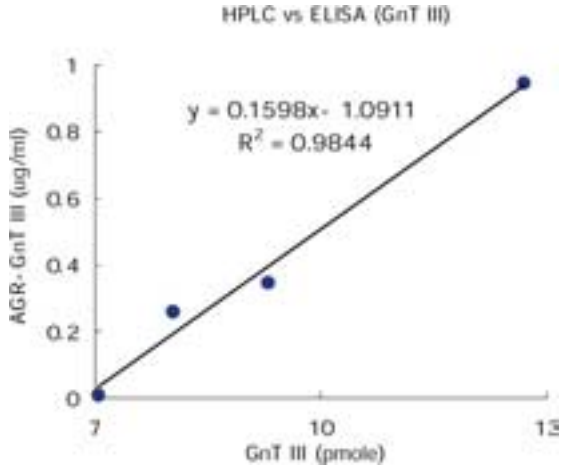

Figure 3. *Correlation of the concentration of AGR-GnT-III and GnT-III enzymatic activity.* High level of AGR-GnT-III serum was serially diluted and measured concentration of AGR-GnT-III by ELISA and GnT-III enzymatic activity by HPLC.

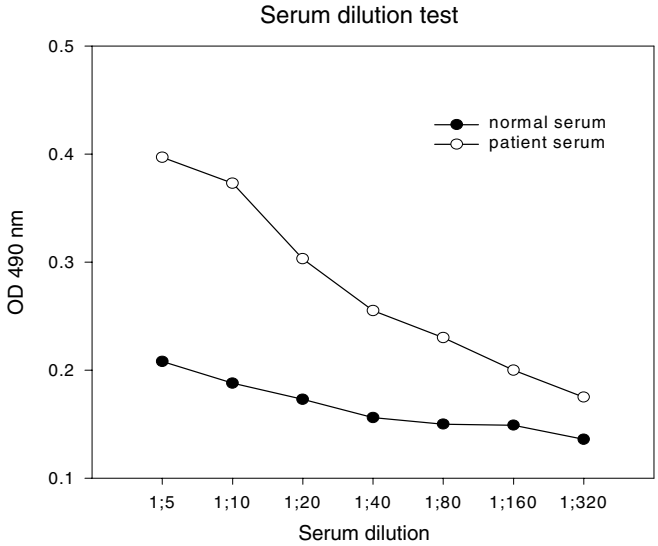

Figure 4. *Dilution test for AGR-GnT-III ELISA with a human serum specimen.* The PAb-mAb sandwich assay protocol was the same as in Figure 2. A sample with a high serum content of AGR-GnT-III and normal serum were serially diluted with dilution solution (PBST- 1% BSA).

which a known amount of AGR-GnT-III was spiked, yielded a recovery in excess of 94% recovery of the added AGR-GnT-III at 5–20-fold dilutions of serum specimens (Table 1).

Serum specimens were obtained 278 patients with hepatocellular carcinoma, liver cirrhosis, chronic hepatitis or acute hepatitis and 34 patients with non-hepatic disease as well as healthy individuals. Serum AGR-GnT-III concentrations were determined, after a 10-fold dilution and in duplicate, by the PAb-mAb sandwich assay.

The results were statistically analyzed based on the disease category, using 1.99 ug/mL (AGR-GnT-III) as a cut-off value,

Table 1. Recovery test of serum AGR-GnT-III by the mAb-PAb sandwich assay[a]

Serum dilution (-fold)	*AGR-GnT-III (μg/mL)*	*AGR-GnT-III measured (μg/mL)*	*Recovery (%)*
5	1.00	0.96	96
10	1.00	1.02	102
20	1.00	0.94	94

[a]Normal human serum was diluted with Dilution Buffer and used in this test. The specimen was assayed in triplicate.

and are summarized in Table 2. The serum AGR-GnT-III concentration of patients with CH or LC were significantly elevated, compared with that of normal subjects. However, patients with acute hepatitis, as well as those with non-hepatic diseases, showed no significant change in serum AGR-GnT-III concentration.

Correlation of serum AGR-GnT-III level with the parameters included in the liver function test

ALT, AST, ALP, total bilirubin, total protein, direct bilirubin, γ-GTP, PT and albumin concentrations in each serum specimen were determined. Serum AGR-GnT-III concentrations were not significantly correlated with any of the parameters included in the typical liver function serum test (Table 3). These results indicate that serum AGR-GnT-III concentration may be an independent parameter that could be used to identify some clinical condition(s), in which the typical liver function test cannot. Furthermore, GnT-III activities of 221 individual sera were also assayed by HPLC for the correlation between GnT-III activity levels and GnT-III protein levels, but serum AGR-GnT-III concentration did not exhibit any significance ($r^2 = 0.0109$).

Discussion

Cell surface carbohydrates are major components of the outer surface of mammalian cells and these carbohydrates often represent characteristic and function of cell types. Carbohydrate structures change dramatically during fertilization, development, differentiation, and malignant transformation [19]. In mature organisms, it is assumed that cell type-specific carbohydrates are probably involved in cell-cell interactions, and protein targeting. In particular, alterations in various glycans occur during the tumorigenesis, for example, an increase in the degree of branching of trimannosyl core [20], the formation of a polylactosaminoglycan chain [21,22] and an increase in sialylation [23]. Such alterations in glycan structure are caused by the action of glycosyltransferases and glycosidases that affect the branching and terminal structure of glycan side chains [24].

It has been reported that, in human liver disease, GnT-III activity in serum and liver tissues is significantly elevated with the progression of the disease [12,13]. Currently, biological function studies of GnT-III in hepatoma cell lines or human liver disease are carried out by means of an activity test using HPLC. In this study, we attempted to develop an immunoassay system capable of detecting GnT-III levels more rapidly and accurately. To obtain the glycosylated form of human GnT-III, we attempted to express human GnT-III gene DNAs in COS cells, following the procedure of Ihara et al. [14], but were unable to produce a significant level of human GnT-III protein. On the other hand, we were successful in expressing human GnT-III gene DNAs in *E.coli* and generating a relatively large amount of aglycosyl recombinant human GnT-III [16]. As a result, we proceeded to use the AGR-GnT-III to generated murine monoclonal antibodies. Six monoclonal hybridomas were identified, in which AGR-GnT-III-binding mAbs were observed. However, immunoglobulin iso-typing showed that 5 clones (GT122, 125, 94, 10 and 6) to be IgM class and only 1 clone (GT 273) was IgG2b. To establish a sandwich assay, as a capture protein, purified AGR-GnT-III was immunized to rabbits, and the resulting polyclonal antibodies were obtained.

Although we immunized with aglycosyl GnT-III, the polyclonal antibody and monoclonal hybridomas were capable of binding AGR-GnT-III to the same degree as the endogenous GnT-III-like proteins in the extract of the HCC tissues. The availability of specific mAb and PAb would allow the development of an immunoassay to quantitatively assess the human

Table 2. Serum AGR-GnT-III levels of the patients with liver disease

Clinical diagnosis	*Total number*	*AGR-GnT-III $>$ 1.99 μg/mL[a] number (%)*	*AGR-GnT-III $<$ 1.99 μg/mL[a] number (%)*	*Mean $\pm$ SD[b] (μg/mL)*
Normal	61	3 (5)	58 (95)	0.57 $\pm$ 0.71
Acute hepatitis	34	9 (26)	25 (74)	1.02 $\pm$ 1.03
Chronic hepatitis	91	86 (95)	5 (5)	2.73 $\pm$ 0.59
Liver cirrhosis	86	60 (70)	26 (30)	3.03 $\pm$ 2.04
Hepatocellular carcinoma	67	7 (10)	60 (90)	0.70 $\pm$ 0.92
Non-hepatic disease	38	8 (21)	30 (79)	0.79 $\pm$ 0.56

[a]A cut-off value of 1.99 μg/mL(AGR-GnT-III) was employed in the analysis.
[b]Mean $\pm$ SD: Arithmetic mean value of AGR-GnT-III(μg/mL) $\pm$ standard deviation.

Table 3. Correlation between serum AGP-GnT-III concentration and parameters included in the liver function test

Parameter	*Number of samples*[a]	*Correlation coefficient (r^2)*[b,c]
Total protein	339	0.000947
Total bilirubin	343	0.00231
Direct bilirubin	339	0.00232
GnT-III	221	0.0109
ALT	326	0.00188
AST	325	0.0106
ALP	328	0.0000026
γ-GTP	319	0.000103
PT	237	0.000159
Albumin	341	0.000537

[a]Serum specimens were obtained from normal healthy subjects, patients with various hepatic diseases, as well as patients with a variety of non-hepatic diseases.
[b]Correlation between AsAGP concentration and the concentration of each parameter was analyzed based on all the serum specimens included in (a).
[c]None of the parameters examined showed a significant correlation with AsAGP concentration ($r^2 \sim 0.0$).

GnT-III levels in serum and other body fluids. It is also desirable to investigate the mechanism of the liver diseases as well as the relationships between human GnT-III and liver diseases. Although the activity of GnT-III in hepatocellular carcinoma patient serum were elevated compare to normal subjects, as shown in a previous study [16], elevated levels of GnT-III were not found in hepatocellular carcinoma patient sera in this study. Therefore, it seems likely that enzyme is not correlated with the amount of protein.

The present study was undertaken to develop an assay system, which is capable of measuring serum AGR-GnT-III with a sensitivity, specificity and reproducibility that is acceptable for clinical testing. In addition, the new assay system must be stable and amenable to the production of a large quantity of the test kits required for a clinical study.

The newly developed PAb-mAb sandwich assay is capable of measuring serum AGR-GnT-III concentration as low as 10 ng/mL and is specific in detecting only GnT-III. It was determined, based on the serum dilution test (Figure 3), that a 10-fold dilution of patient serum reflected the best discrimination between normal subjects and patients with hepatic disease. Therefore, AGR-GnT-III concentrations were determined using a panel of serum specimens from normal subjects, patients with various hepatic diseases, or patients with a variety of non-hepatic diseases, including non-hepatic carcinomas, cardiac disorders, pneumatic diseases, digestive organ disorders and strokes. Serum AGR-GnT-III concentrations were elevated in 60 of 86 (70%) patients with LC and 86 of 91 (95%) patients with CH. In contrast, only 3 of 61 (5%) of the normal subjects, 9 of 34 (26%) patients with acute hepatitis and 8 of 38 (21%) patients with non-hepatic disease showed a slight elevation above the cut-off value (Table 2). Although only AGR-GnT-III concentrations were examined in this study, the results shown in Table 2 illustrate a unique characteristic of serum GnT-III, and suggest that AGR-GnT-III may be used as an adjunct serum marker for CH and LC.

A clinical study with a large number of diverse serum specimens will be necessary to validate serum AGR-GnT-III as a marker for diagnosing CH or LC. Such a study should include patients diagnosed with CH or LC, whose disease status can be monitored along with the serum AGR-GnT-III determination. The outcome of these studies would determine whether serum AGR-GnT-III concentration might serve as a useful parameter, not only as a diagnostic aid, but also, as a tool to assess the disease status of patients with CH or LC during clinical therapy.

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