

Identification of Soluble NH₂-Terminal Fragment of Glypican-3 as a Serological Marker for Early-Stage Hepatocellular Carcinoma

Yoshitaka Hippo,¹ Kiyotaka Watanabe,⁴ Akira Watanabe,¹ Yutaka Midorikawa,⁵ Shogo Yamamoto,² Sigeo Ihara,² Susumu Tokita,⁷ Hiroko Iwanari,⁷ Yukio Ito,⁷ Kiyotaka Nakano,⁶ Jun-ichi Nezu,⁶ Hiroyuki Tsunoda,⁶ Takeshi Yoshino,⁶ Iwao Ohizumi,⁶ Masayuki Tsuchiya,⁶ Shin Ohnishi,⁴ Masatoshi Makuuchi,⁵ Takao Hamakubo,³ Tatsuhiko Kodama,³ and Hiroyuki Aburatani¹

¹Genome Science Division, ²Division of Dynamical Bioinformatics, and ³Division of Molecular Biology and Medicine, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan; Departments of ⁴Gastroenterology and ⁵Hepato-Biliary-Pancreatic Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; ⁶Chugai Pharmaceutical Co., Ltd., Shizuoka, Japan; and ⁷Perseus Proteomics, Inc., Tokyo, Japan

ABSTRACT

For detection of hepatocellular carcinoma (HCC) in patients with liver cirrhosis, serum α -fetoprotein has been widely used, but its sensitivity has not been satisfactory, especially in small, well-differentiated HCC, and complementary serum marker has been clinically required. Glypican-3 (GPC3), a heparan sulfate proteoglycan anchored to the plasma membrane, is a good candidate marker of HCC because it is an oncofetal protein overexpressed in HCC at both the mRNA and protein levels. In this study, we demonstrated that its NH₂-terminal portion [soluble GPC3 (sGPC3)] is cleaved between Arg³⁵⁸ and Ser³⁵⁹ of GPC3 and that sGPC3 can be specifically detected in the sera of patients with HCC. Serum levels of sGPC3 were 4.84 ± 8.91 ng/ml in HCC, significantly higher than the levels seen in liver cirrhosis (1.09 ± 0.74 ng/ml; $P < 0.01$) and healthy controls (0.65 ± 0.32 ng/ml; $P < 0.001$). In well- or moderately-differentiated HCC, sGPC3 was superior to α -fetoprotein in sensitivity, and a combination measurement of both markers improved overall sensitivity from 50% to 72%. These results indicate that sGPC3 is a novel serological marker essential for the early detection of HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers worldwide, and its incidence is still increasing (1). Because HCC develops from cirrhotic liver after chronic infection with hepatitis virus B or C, patients with liver cirrhosis (LC) are advised to undergo periodical screening of serum α -fetoprotein (AFP) levels and liver ultrasound for the purpose of early detection of cancer (2). AFP is a glycoprotein expressed abundantly in fetal liver but not in normal adult liver and is re-expressed by HCC as it dedifferentiates from a premalignant lesion in the cirrhotic liver through well-differentiated (WD) and moderately differentiated (MD) HCC to poorly differentiated HCC (3). AFP has been used as a serum marker of HCC for more than 40 years. However, ultrasound imaging has been more effective lately in early detection of small WD HCC, in which AFP has yet to be elevated (4), highlighting the clinical need for novel sensitive serum markers for WD HCC.

Many previous studies have identified genes up-regulated in HCC

compared with surrounding noncancerous lesions using differential display or cDNA subtraction (5–8). Recently, microarray studies on HCC presented gene lists containing a number of overexpressed genes (9–14). However, to determine whether a gene is a good candidate as a serological marker of WD HCC, it is crucial to determine the following: (a) whether it is overexpressed in WD HCC; (b) whether it is not expressed abundantly in other normal organs; and (c) whether it is detectable in the serum.

Overexpression of *GPC3* mRNA in HCC has been reported by ourselves and several other groups (15–18). Moreover, frequency of *GPC3* mRNA overexpression was significantly higher than that of elevated serum level and mRNA level of AFP in small HCC (16). We also observed frequent overexpression of *GPC3* in WD HCC compared with AFP with microarray analysis.⁸ Together with minimal expression in normal organs (16, 19), GPC3 has, undoubtedly, previously existed as an attractive candidate marker of HCC. We showed previously using a monoclonal antibody (mAb) that GPC3 protein is also highly expressed in HCC (15). In this study, we further characterized GPC3 protein using a panel of newly generated mAbs and investigated whether it could be detected specifically in the sera of the patients with HCC. Finally, we successfully established a detection system for the soluble fragment of GPC3 (sGPC3) and confirmed its usefulness as a novel biomarker for HCC.

MATERIALS AND METHODS

Serum Samples. Serum samples were collected at Tokyo University Hospital with informed consent from 69 patients with HCC and 38 patients with LC, defined according to the following criteria: patients with a pathological diagnosis of HCC after surgery or with evidence of tumor stain on computed tomography or angiography were diagnosed with HCC; and patients diagnosed with LC were limited to those who had no history of HCC and no ultrasound evidence of tumor for more than 6 months from the day of serum collection.

Purification of Recombinant GPC3 Proteins. For protein expression, we used modified pCXN vector that contained dihydrofolate reductase expression unit as a selection marker. Original pCXN vector (20) was generously provided by J. Miyazaki (Osaka University Medical School, Osaka, Japan). An expression vector for GPC3 that lacks the COOH-terminal hydrophobic glycosylated phosphatidylinositol (GPI)-anchoring domain, GPC3 Δ GPI, was constructed by introducing cDNA corresponding to amino acid residues 1–563 of GPC3 into modified pCXN with a FLAG tag added at the COOH terminus. An expression vector for GPC3 Δ GPI without heparan sulfate, GPC3 Δ GPI Δ HS, was constructed by changing Ser⁴⁹⁵ and Ser⁵⁰⁹ to Ala to abolish the heparan sulfate attachment site. These constructs were stably transfected into Chinese hamster ovary cells deficient in the dihydrofolate reductase gene. Culture media containing GPC3 Δ GPI-FLAG or GPC3 Δ GPI Δ HS-FLAG recombinant proteins were collected and loaded to DEAE ion-exchange chromatography DEAE Sepharose FF (Amersham Bioscience, Tokyo, Japan). After washing, eluted protein solutions were applied to anti-FLAG M2 antibody beads (Sigma, St.

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Requests for reprints: Hiroyuki Aburatani, Genome Science Division, Research Center for Advanced Science and Technology, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan. Phone: 81-3-5452-5235; Fax: 81-3-5452-5355; E-mail: haburata-ky@umin.ac.jp.

⁸ Y. Midorikawa, S. Tsutsumi, K. Nishimura, N. Kamimura, M. Kano, H. Sakamoto, M. Makuuchi, and H. Aburatani. Transcriptional Signature in Progression of Hepatocellular Carcinoma, manuscript in preparation.

Louis, MO). Proteins eluted with solution containing 200 $\mu\text{g/ml}$ FLAG peptide (Sigma) were subjected to gel filtration chromatography with HiLoad 26/60 Superdex200pg (Amersham Bioscience). Finally, recombinant protein was concentrated using DEAE Sepharose FF.

Generation of Anti-GPC3 mAbs. We used recombinant GPC3 Δ GPI as an immunogen. Spleen cells were isolated and fused with mouse myeloma P3-X63Ag8U1 cells (American Type Culture Collection, Manassas, VA). Hybridomas were selected by ELISA against the purified recombinant GPC3 Δ GPI Δ HS-FLAG, followed by cloning with limited dilution. Three mouse mAbs (A1836A, M18D04, and M19B11) were used in this study. For epitope mapping of these mAbs, a pGEX-5X (Amersham Biosciences) construct for the NH₂-terminal portion of GPC3 (amino acids 25–358) was expressed in *Escherichia coli* BL21 Codon Plus (DE3) pLys (Stratagene, La Jolla, CA) as a glutathione *S*-transferase-fusion protein and subject to immunoblotting analysis.

Immunoblotting. Total cell lysates were obtained after lysis in 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1.0% Triton X-100, 1.0% sodium deoxycholate, and 0.1% SDS with protease inhibitor mixture (Sigma). Culture supernatant was obtained from serum-free medium used for culture of hepatoma cells. Proteins were separated with 12% SDS-PAGE and transferred to polyvinylidene difluoride Hybond P membrane (Amersham Biosciences). The membrane was treated with 2% nonfat milk in TBS containing 0.05% Tween 20 (TBST) followed by incubation with anti-GPC3 mAb in TBST and subsequent incubation with horseradish peroxidase-conjugated secondary antibody (dilution, 1:5000; Amersham Biosciences) in TBST. The protein was visualized using the enhanced chemiluminescence plus detection system (Amersham Biosciences).

Immunoprecipitation. We first prepared antibody beads by covalently linking 25 μg of protein G-Sepharose (Amersham Biosciences) and 50 μg of anti-GPC3 mAb M18D04 or M19B11 with 20 mM dimethyl pimelimidate (ICN Aurora, Aurora, OH). We then added 50 μl of sera from the patients or culture media of HuH7 cells diluted in 250 μl with PBS to 25 μl of antibody beads and incubated them for 2 h at 4°C. After extensive washing with PBS, antibody beads were boiled for 5 min in 50 μl of SDS-PAGE loading buffer containing 10% 2-mercaptoethanol, and subsequently, immunoblotting was performed.

Sandwich ELISA. One μg of anti-GPC3 mAb A1836A per well was immobilized to 96-well plate Maxisorp (Nalge Nunc International, Roskilde, Denmark) and stabilized with Immunoassay Stabilizer (Advanced Biotechnologies Inc., Columbia, MD). Twenty-five μl of sera or standard were diluted with 100 μl of buffer containing 20% normal rabbit serum (Pel-Freez Biologicals, Rogers, AR), 1% BSA (Oriental Yeast Co., Ltd., Osaka, Japan), and 2% mouse ascites Hyb-3423 (Institute of Immunology, Tokyo, Japan) in 50 mM Tris-Cl (pH 8.0), 0.15 M NaCl, and 1 mM EDTA and incubated at room temperature for 2 h. After washing, 25 μl of biotinylated antibody solution containing anti-GPC3 mAbs M18D04 (1.88 $\mu\text{g/ml}$) and M19B11 (3.75 $\mu\text{g/ml}$) and 100 μl of horseradish peroxidase-labeled streptavidin (Vector Laboratories Inc., Burlingame, CA) were added to the plate and incubated twice at room temperature for 30 min. TMB Soluble Reagent and Stop Buffer (Scy Tek Laboratories, Inc., Logan, UT) were added as substrate, and absorbance at 450 nm was read with EIA Reader (Corona Electric Co., Ltd., Ibaraki, Japan). Recombinant GPC3 Δ GPI was used as a standard sample in each assay.

Amino Acid Sequence Analysis. Recombinant GPC3 Δ GPI and GPC3 Δ GPI Δ HS were purified and separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane ProBlott (Applied Biosystems, Foster City, CA). The membrane was stained with CBB R-250, and sections containing bands of M_r 40,000 and M_r 30,000 were cut out separately. These polyvinylidene difluoride membrane sections were washed with a solution including 50% acetonitrile and 0.1% trifluoroacetic acid and applied to an ABI 492 Protein Sequencer (Applied Biosystems) to sequence the NH₂ terminus of the protein. Because the NH₂ terminus of the M_r 40,000 protein was blocked, the membrane was further incubated in acetate with 0.6 mg/ml 3-bromo-3-methyl-2-nitrophenyl-mecapto-3*H*-indole (ICN Biomedicals Inc., Irvine, CA) at 80°C for 1 h in the dark to chemically cleave the protein at the COOH terminus of tryptophan residues. After washing twice with 80% acetate and once with 10% methanol, the peptide was analyzed using an ABI 492 Protein

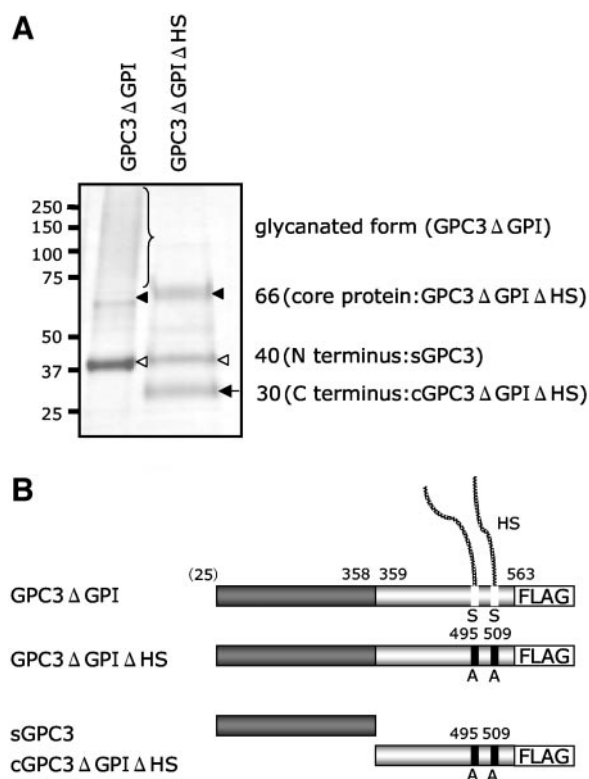


Fig. 1. Characterization of recombinant glypican-3 (GPC3) proteins. A, CBB R-250-stained SDS-PAGE of purified recombinant GPC3. *Brace*, glycanated GPC3 (smearing), GPC3 Δ GPI; *closed arrowhead*, core protein of GPC3 (M_r 66,000) that lacks heparan sulfate glycosaminoglycan, GPC3 Δ GPI Δ HS; *open arrowhead*, sGPC3 (M_r 40,000); *arrow*, cGPC3 Δ GPI Δ HS (M_r 30,000). B, schematic diagram of recombinant proteins. Numbers above the boxes indicate amino acid residue number. Note that NH₂-terminal residue 25 is putative and indicated in parentheses. HS, heparan sulfate glycosaminoglycan.

Sequencer. The detected sequence was aligned using FASTS software available online,⁹ and the protein was identified.

RESULTS

The NH₂-Terminal Portion of GPC3 Is Cleaved between Arg³⁵⁸ and Ser³⁵⁹ *in Vitro*. We have previously generated mAb K6534 raised against a peptide corresponding to amino acids 355–371 of GPC3 protein, and we demonstrated, for the first time, overexpression of its core protein in HCC with immunoblotting using this antibody (15). Another antibody is required to construct a sandwich ELISA system for serum examination of GPC3, so we started generating high-affinity mAbs using recombinant GPC3 Δ GPI as an immunogen. While purifying the immunogen from the culture supernatant of Chinese hamster ovary cells, we observed a M_r 40,000 band (Fig. 1A) in addition to the M_r 66,000 band that corresponds to core protein of GPC3 as observed with K6534 (15).

Because the NH₂ terminus of this M_r 40,000 band was modified, as revealed by initial amino acid sequencing, we performed sequencing of internal amino acids of the band after cleavage at the COOH terminus of tryptophan residues to verify its origin. We detected six cycles of three amino acid residues VRY, EPX, YES, ITY, LPX, and QSV, each cycle corresponding to the first to sixth residue following tryptophan (W), respectively. After alignment with FASTF algorithm, these sequences matched with the (W)VPETPV (amino acid 51–57), (W)YCSYCQ (amino acid 261–267), and (W)REYILS (amino acid

⁹ <http://fasta.bioch.virginia.edu/>.

296–302) partial sequences of GPC3, respectively, indicating that this band is derived from an NH₂-terminal portion of GPC3. We designated this soluble cleaved fragment of GPC3 as sGPC3.

To further characterize sGPC3, we next tried to precisely identify the undetermined cleavage site by sequencing the residual COOH-terminal portion of GPC3 (designated cGPC3). However, the corresponding band was not visible by SDS-PAGE, presumably due to attachment of heparan sulfate glycosaminoglycan, leading to smearing (Fig. 1A). After substituting the two heparan sulfate attachment sites of the expression construct and purifying the resultant GPC3ΔGPIΔHS, we could observe a band of M_r 30,000, as expected (Fig. 1A). The NH₂-terminal sequence of this band was identified as SAYYPEDLF, identical to amino acids 359–367 of GPC3. Thus, the cleavage site was identified as being between Arg³⁵⁸ and Ser³⁵⁹ (Fig. 1B). We do not have precise information on the NH₂-terminal sequence of sGPC3 due to modification, but considering that amino acid 1–24 is a putative signal sequence, sGPC3 is likely to consist of amino acids 25–358 with an estimated molecular weight of 38,100, consistent with the M_r 40,000 band observed in SDS-PAGE (Fig. 1A).

Soluble GPC3 Is a Major Form of GPC3 Specifically Detected in the Sera of Patients with HCC. We succeeded in generating a number of high-affinity mAbs specific for GPC3 and classified these antibodies into two groups, N-mAbs and C-mAbs, according to their epitopes within amino acids 25–358 or 359–563, respectively (data not shown). These antibodies could also recognize endogenous GPC3 protein in immunoblotting: core protein (M_r 66,000) and glycanated form (smearing) of GPC3 were detected by both N-mAbs and C-mAbs; whereas sGPC3 (M_r 40,000) was detected only by N-mAbs (Fig. 2A). An additional M_r 50,000 band was detected strongly in the cell lysate of HepG2 with both N-mAbs and C-mAbs (Fig. 2A). This band was only weakly detectable in HuH6 cells and was undetectable in five other hepatoma cell lines (Fig. 2, A and C; data not shown), suggesting cell-specific variations in the processing of the protein. In the culture supernatant, sGPC3, rather than a core protein or a glycanated form of GPC3, was the major form of GPC3 detected (Fig. 2A).

Based on the above *in vitro* finding, we speculated that sGPC3, instead of core protein of GPC3, might be the major form of GPC3 in the sera of HCC patients. To avoid possible interference on immunoblotting by significant migration of albumin or immunoglobulin in the serum, we performed immunoprecipitation before immunoblotting using three N-mAbs (Fig. 2B). sGPC3 alone was successfully detected by immunoprecipitation with M18D04 (Fig. 2C) or M19B11 (data not shown) followed by immunoblotting with A1836A in the sera of patients with HCC, but not in sera from normal liver (NL). These results clearly demonstrate that sGPC3 is the major diagnostic target specifically detectable in the sera of HCC patients.

Soluble GPC3 Is Useful as a Serological Marker of WD HCC and MD HCC. We next constructed a sandwich ELISA system with these three antibodies to measure the serum level of sGPC3 (Fig. 3A). To verify the specificity of the assay, we performed immunoblotting of 10 sera samples from HCC with sGPC3 levels ranging from 4.0 to 55.0 ng/ml and 3 samples from NL with sGPC3 levels of <0.1 mg/ml. We detected only sGPC3 in all 10 HCC samples, whereas no band was detected in 3 samples from NL, indicating high sensitivity and specificity of the assay (Fig. 3B). When we examined sera from 69 cases with HCC, 38 cases with LC, and 96 cases with NL, the level of sGPC3 (mean ± SD) was 4.84 ± 8.91 ng/ml for HCC, 1.09 ± 0.74 ng/ml for LC, and 0.65 ± 0.32 ng/ml for NL and was significantly higher in HCC than in NL ($P < 0.001$, Student's *t* test) or in LC ($P < 0.01$; Fig. 3C).

We then evaluated sGPC3 as a general marker for HCC in com-

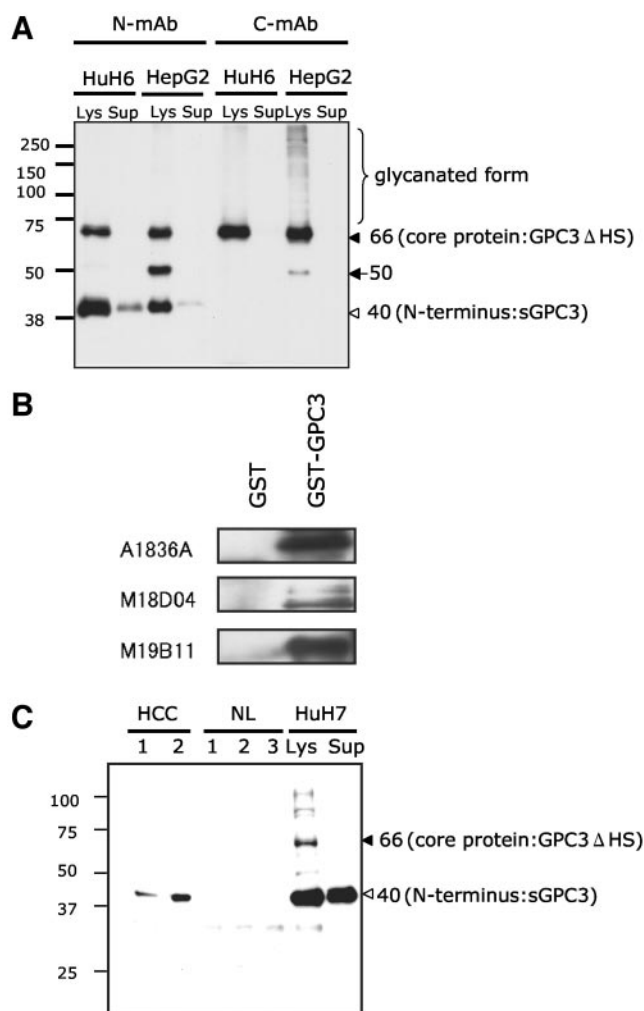


Fig. 2. Characterization of endogenous glypican-3 (GPC3) proteins with monoclonal antibodies (mAbs). A, representative immunoblotting of endogenous GPC3 in the cell lysate and culture supernatant with N-mAb and C-mAb. HepG2 and HuH6 were analyzed. Note that soluble GPC3 (sGPC3) alone is detected in the culture supernatant. *Brace*, glycanated GPC3 (smearing); *closed arrowhead*, core protein of GPC3 (M_r 66,000); *open arrowhead*, sGPC3 (M_r 40,000); *arrow*, uncharacterized processed fragment of GPC3 (M_r 50,000). *Lys*, lysate; *Sup*, supernatant of culture media. B, immunoblotting analysis with anti-GPC3 antibodies A1836A, M18D04, and M19B11 recognized glutathione *S*-transferase-sGPC3 but did not recognize glutathione *S*-transferase. C, detection of sGPC3 alone in the sera of the patients with hepatocellular carcinoma. Sera from two patients with hepatocellular carcinoma and three healthy adults (NL) were analyzed by immunoprecipitation with M18D04 followed by immunoblotting with A1836A. HuH7 cells were analyzed as a reference. *Closed arrowhead*, core protein of GPC3 (M_r 66,000); *open arrowhead*, sGPC3 (M_r 40,000).

parison with AFP. Initial analysis of the receiver-operating characteristic curve using the data from 69 cases with HCC and 38 cases with LC suggested that, used in isolation, sGPC3 is not as good as AFP: the calculated area under the receiver-operating characteristic curve was 0.729 for sGPC3 and 0.799 for AFP (Fig. 3D). The sensitivity and specificity of sGPC3 for the diagnosis of HCC (cutoff value, 2.0 ng/ml) were 51% and 90%, respectively, whereas those of AFP measured in parallel (cutoff value, 20 ng/ml) were 55% and 90%, respectively. AFP and sGPC3 were not correlated ($r = 0.13$), and combination measurement of both markers markedly improved sensitivity to 72%.

HCC may be divided into two subgroups correlating to the extent of disease: (a) one first treated by surgery, mainly with a solitary tumor or few tumors; and (b) the second treated with transcatheter arterial chemoembolization, mostly with multiple and advanced tumors. The serum level of sGPC3 was 2.61 ± 2.69 ng/ml for the former group,

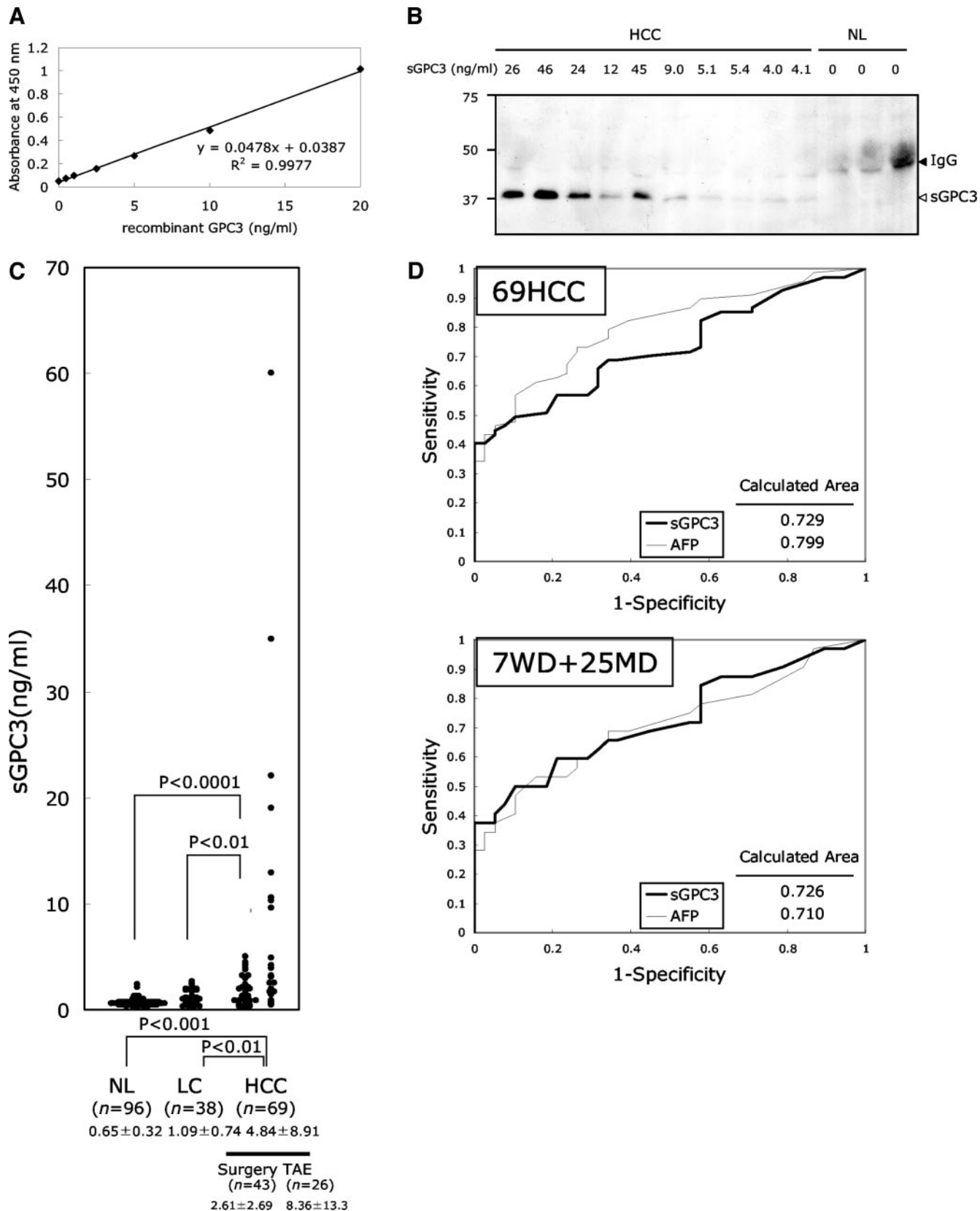


Fig. 3. Evaluation of soluble glypican-3 (sGPC3) as a serological marker of hepatocellular carcinoma (HCC). **A**, standard curve of sandwich ELISA. **B**, high specificity of sandwich ELISA. Specific detection of sGPC3 alone solely in the sera with elevated sGPC3 level measured with sandwich ELISA. Sera from 10 patients with HCC and 3 healthy adults (NL) were analyzed by immunoprecipitation with M18D04 followed by immunoblotting with A1836A. Serum sGPC3 level is indicated for each sample. *Open arrowhead*, sGPC3 (M_r 40,000); *closed arrowhead*, IgG. **C**, distribution of sGPC3 in the sera of patients with normal liver, liver cirrhosis (LC), and HCC (surgery and transcatheter arterial chemoembolization subgroup). Mean ± SD (ng/ml) of serum sGPC3 is indicated. Number of samples is indicated as *n*. **D**, receiver-operating characteristic curve analysis of sGPC3 (*thick line*) and α -fetoprotein (*thin line*). *Top panel*, all of the 69 HCCs and 38 cases of LC were included in the analysis. *Bottom panel*, 32 HCCs (including 7 well-differentiated and 25 moderately differentiated HCCs) and 38 cases of LC were analyzed. Area under the receiver-operating characteristic curve is indicated.

significantly higher than that for NL ($P < 0.0001$, Student's t test) or LC ($P < 0.01$), and 8.36 ± 13.3 ng/ml for the latter group (Fig. 3C), suggesting that the serum level of sGPC3 is elevated in an earlier stage and rises as HCC progresses. We then evaluated sGPC3 as a marker for HCC in relatively early-stage disease. When 43 cases treated by surgery were confined to 32 cases with relatively early-stage HCC (7 cases with WD HCC and 25 cases with MD HCC), calculated areas under the receiver-operating characteristic curve for sGPC3 and AFP were 0.726 and 0.710, respectively, indicating that sGPC3 is superior to AFP (Fig. 3D). The sensitivity of sGPC3 and AFP for the diagnosis of WD HCC and MD HCC was 50% and 47%, respectively. Moreover, combination measurement of both markers in WD HCC and MD HCC also markedly improved sensitivity to 72%. These results clearly demonstrate the utility of sGPC3 as a serological marker for HCC, especially for relatively early-stage HCC, and its complementarity to AFP.

DISCUSSION

GPC3 (alternatively called OCI-5 or MXR-7) is a heparan sulfate proteoglycan. The structural characteristics of the glypican family are (a) a core protein of approximately M_r 60,000, (b) binding to the membrane through GPI anchor, (c) heparan sulfate glycosaminoglycan attachment at Ser-Gly sequence within the COOH-terminal portion, and (d) a highly conserved pattern of 14 Cys residues (19). *GPC3* was originally isolated as a gene that is developmentally expressed in fetal rat intestine (21, 22). Mutation of *GPC3* is found in Simpson-Golabi-Behmel syndrome characterized by an overgrowth phenotype, hence its putative function was associated with an apoptotic effect (23). Silencing of *GPC3* in some types of cancer (24–26) is in line with this notion.

Overexpression of *GPC3* mRNA in HCC has been reported by ourselves and several other groups (15–18), although the role of GPC3 in carcinogenesis or progression of HCC has yet to be determined. In general, transcription level and protein level do not necessarily correlate. We have succeeded previously in generating an anti-GPC3 mAb against a peptide within the COOH-terminal portion, and we demonstrated using the antibody that the expression level of GPC3 core protein correlated well with its transcription level and that GPC3 was also overexpressed at protein level for the first time (15). Difficulties in making high-affinity antibodies against GPC3 (27), presumably due to its complex structure derived from disulfide bonds between 14 Cys residues, prohibited further analysis. We tried to generate high-affinity mAbs again by using recombinant GPC3 protein expressed in mammalian cells as an immunogen, and we finally succeeded in generating numerous high-affinity mAbs; to our knowledge, this is the first establishment of mAbs that can react with sGPC3. We did not recognize sGPC3 in a previous study (15) because we used a mAb against a relatively COOH-terminal portion (amino acids 355–371).

In the present work, we have precisely characterized GPC3 and demonstrated that the M_r 40,000 protein, sGPC3, derives from the NH₂-terminal portion of GPC3 and is cleaved between Arg³⁵⁸ and Ser³⁵⁹. The M_r 40,000 protein was previously described by Mast *et al.* (19), who were searching for the binding protein on the plasma membrane of HepG2 cells for tissue factor pathway inhibitor. They purified a M_r 40,000 protein from culture supernatant of HepG2 cells and showed that it was derived from the NH₂-terminal portion of GPC3. They did not identify a cleavage site for the protein, unlike our study, but it is highly likely that the soluble protein they observed is sGPC3. They described purification of a M_r 40,000 protein only when protease inhibitors were used throughout the procedure, strongly suggesting that GPC3 cleavage is mediated by a protease (19). In

addition, they found that washing the cells with dextran sulfate or heparin released significantly higher amounts of GPC3 than seen before treatment, strongly suggesting that most GPC3 is noncovalently attached to the cell surface after cleavage of the GPI anchor, but not in the culture supernatant (19). Our finding that sGPC3 alone is the major form of GPC3 in the culture supernatant of hepatoma cells and the serum of patients with HCC is consistent with these findings.

Very recently, two other groups reported elevated levels of GPC3 in the serum of HCC patients. The results still seem preliminary, although they are quite similar to ours. Here, we have made significant improvements in the reliability of the assay. Nakatsura *et al.* (28) used a polyclonal antibody raised against 303–464 amino acids of GPC3 in their analysis. The specificity of their ELISA is to be confirmed because it is not sandwich ELISA, despite the many non-specific bands the antibody detected in their immunoblotting. Moreover, the standard used in the assay was not recombinant GPC3 but a supernatant of HepG2 cells that is a mixture of many heterogeneous proteins. It is possible that they are measuring a mixture of non-specific but HCC-related proteins. Capurro *et al.* (29) used a polyclonal antibody and a mAb, both raised against the last 70 amino acids of the COOH-terminal portion of GPC3, to detect glycanated GPC3 in serum with their sandwich ELISA. However, the major detectable form of GPC3 in serum is sGPC3, which cannot be detected with these antibodies against the COOH-terminal portion, as shown clearly in the present study. In fact, we examined many combinations of mAbs in our sandwich ELISA, but we could detect signal only when we used a combination of two N-mAbs (data not shown). Furthermore, the only evidence reported previously for the extracellular localization of glycanated GPC3 is immunoblotting of HepG2 cell culture supernatant, rather than serum from HCC patients. Here, we demonstrated that sGPC3 is in the culture supernatant and serum of the HCC patients using both immunoblotting and sandwich ELISA with the same combination of mAbs. One possible interpretation of the result, obtained by Capurro *et al.*, is that they are detecting some short fragments derived from a COOH-terminal portion but not the glycanated form of GPC3, and this issue should be further investigated.

We have delineated the usefulness of sGPC3 as highly sensitive to early-stage HCC. In addition, there were several cases with elevated serum sGPC3 among LC patients, although not included in this study, where HCC developed within 6 months after serum examination or some tumor was already detected by ultrasound without final diagnosis of HCC by computed tomography or angiography. We have also demonstrated the complementarity of sGPC3 to another HCC marker, AFP. These findings promise future bedside use of sGPC3 as a serological marker of HCC. Another attractive aspect of GPC3 is that the membrane-anchored portion is a potential target for antibody therapy. In this context, diagnosis with serum sGPC3 is useful not only in early detection of HCC but also for future identification of patients with high sGPC3 levels for tailor-made HCC therapy. Thus, further investigation into the clinical aspects of GPC3 in HCC is warranted.

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