

Identification of β 2-microglobulin as a candidate for early diagnosis of imaging-invisible hepatocellular carcinoma in patient with liver cirrhosis

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Abstract. Glypican-3 (GPC3) is overexpressed in hepatocellular carcinoma (HCC) but not in chronic hepatitis (CH) and liver cirrhosis (LC). We have reported the possibility of GPC3-specific cytotoxic T lymphocytes (CTLs) serving as a marker for the early diagnosis of imaging invisible HCC. In this study, to identify new early diagnostic biomarker of imaging invisible HCC, we analyzed plasma of healthy donors and patients with CH, LC and HCC using surface-enhanced laser desorption-ionization time-of-flight mass spectrometry (SELDI-TOF-MS). The intensities of four peaks were significantly increased in HCC patients compared with healthy donors. Two of these four peaks were significantly higher in CH and LC patients with GPC3-specific CTLs than in those without. One peak (11.7 kDa) was predicted to be β 2-microglobulin (β 2-MG) by molecular mass. There was a correlation between concentration of β 2-MG by latex agglutination immunoassay in plasma and peak intensity using SELDI-TOF-MS. The 11.7 kDa protein was fractionated by gel filtration and was identified as β 2-MG by

Western blot analysis. These results suggest that the level of β 2-MG in plasma from patients with CH and LC could be a useful marker for the early diagnosis of imaging invisible HCC, however further investigation is needed.

Introduction

Glypican-3 (GPC3) belongs to glypican family that is a group of heparan sulfate proteoglycans linked to the outer surface of cell membrane through a glycosylphosphatidylinositol anchor (1). GPC3 is overexpressed in hepatocellular carcinoma (HCC) (2) and was a useful diagnostic marker for a component HCC (3). Also, GPC3 is a useful tumor marker in early HCC (2,4,5). We have reported that GPC3 was correlated with poor prognosis in HCC (6). Furthermore, we showed the usefulness of GPC3 as a target for cancer immunotherapy (7-9). We are undertaking a phase I clinical trial of GPC3 peptide vaccine for patients with advanced HCC.

HCC is one of the most common malignant tumors worldwide (10). Patients with liver cirrhosis (LC) are at higher risk for the development of HCC (11). To date diagnostic imaging such as Computer Tomography (CT) or Magnetic Resonance Imaging (MRI) is taken as the gold standard for definitive diagnosis of HCC. Several serum markers developed for the diagnosis of HCC, including evaluation of α -fetoprotein (AFP) and protein induced by vitamin K absence (PIVKA-II) (12,13). AFP and PIVKA-II is most widely used as a diagnostic serum marker for HCC, however its early diagnostic value is poor (14,15). Thus there are no available tumor markers or means for detecting invisible HCC by CT or MRI.

We have previously reported that anti-GPC3 IgG was detected in the serum of patients not only with HCC but also with chronic hepatitis (CH) and LC (16). In addition, in the same study, we reported that GPC3-specific cytotoxic T lymphocytes (CTLs) were present in the peripheral blood mononuclear cells (PBMCs) of patients not only with HCC but also with CH or LC using *ex vivo* IFN- γ enzyme-linked immunospot (ELISPOT) assay. GPC3-specific CTLs should react to the GPC3 expressing HCC cells. The detection of GPC3-specific CTLs shows the existence of GPC3 expressing

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Abbreviations: β 2-MG, β 2-microglobulin; HCC, hepatocellular carcinoma; GPC3, glypican-3; CH, chronic hepatitis; LC, liver cirrhosis; CTLs, cytotoxic T lymphocytes; AFP, α -fetoprotein; SELDI-TOF-MS, surface-enhanced laser desorption-ionization time-of-flight mass spectrometry; LAIA, latex agglutination immunoassay; PIVKA-II, protein induced by vitamin K absence

Key words: surface-enhanced laser desorption-ionization time-of-flight mass spectrometry, glypican-3, cytotoxic T lymphocyte, chronic hepatitis, liver cirrhosis, hepatocellular carcinoma

HCC cells in these CH and LC patients. This suggested that GPC3-specific CTLs could serve as a marker for the early diagnosis of imaging invisible HCC. Therefore, in this study, we tried to identify the HCC producing protein in the serum of CH and LC patients who were positive for GPC3-specific CTLs.

ProteinChip, based on surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) has recently been shown to be useful in discovering biomarkers for the diagnosis of breast, liver and various other cancers (17-21). In order to establish the possibility of early diagnosis of imaging invisible HCC, we analyzed plasma from patients with CH and LC patients with or without GPC3-specific CTLs and HCC patients using ProteinChip Arrays.

Materials and methods

Plasma samples. Plasma samples were obtained from 6 patients with HCC at National Cancer Center Hospital East, 16 patients with CH or LC at Tokyo Rosai Hospital and 8 healthy volunteers after obtaining their written consent. CH and LC patients who were confirmed to be HCV-RNA (+) or HBs antigen (+) within six months prior to registration were eligible for the study. The diagnosis of CH or LC was made clinically by imaging and laboratory data. The patients had no medical history of HCC and no evidence of HCC on ultrasonography, CT or MRI conducted prior to registration. All plasma samples were stored at -80°C until analysis.

SELDI-TOF-MS analysis. For SELDI-TOF-MS analysis, we used CM10 ProteinChip (weak cation-exchange) with anionic surface chemistry. The chips were washed twice with shaking for 5 min in 150 μ l binding buffer (50 mM sodium acetate, pH 4.0) per well. Plasma samples diluted 1:10 with PBS buffer and then diluted 1:10 with pH adjusted buffer. One hundred microliters of all diluted plasma samples were applied on each ProteinChips. The samples were applied in duplicate. Binding was allowed to proceed for 1 h with shaking at room temperature. The chips were then washed twice using 150 μ l of binding buffer (5 min with shaking), rinsed, dried and then added 0.5 μ l of a matrix solution (50% acetonitrile, 0.5% trifluoroacetic acid) to each spot. Matrix solution was repeatedly put to each spot. These ProteinChip Arrays were analyzed using a ProteinChip reader (ProteinChip Biology Systems II; Bio-Rad Laboratories, Inc., Tokyo, Japan).

High performance liquid chromatography (HPLC) analysis. Plasma samples were analyzed by the HPLC (Shimadzu, Kyoto, Japan). All samples were fractionated by size-exclusion HPLC chromatography equipped on a shodex protein KW-802.5 column (Showa Denko, Tokyo, Japan). One hundred microliters of the sample was loaded into the column: a mobile phase composed of A solution (20 mM NaH₂PO₄·2H₂O 300 mM NaCl) and B solution (20 mM NaH₂PO₄·2H₂O); a flow rate of 1.0 ml/min; UV detection at 280 nm. Appropriate fractions were concentrated in Vivaspin 2 column (3 kDa cut off) (GE Healthcare, UK Ltd.). The search for retention time of β 2-MG of purified standard protein (Biocode Hycl, Liege, Belgium), fractionated using the same method for all plasma samples.

Table I. Characteristics of patients involved in this study.

	Male/ Female	Average age	HBV/HCV
GPC3-specific CTL			
Negative patients	5/1	60.3 (51-75)	1/5
GPC3-specific CTL			
Negative patients	3/3	67.7 (59-74)	2/4
HCC patients	2/4	66.1 (48-77)	2/4

Detection of β 2-microglobulin (β 2-MG) in plasma by latex agglutination immunoassay (LAIA). The concentration of β 2-MG in plasma samples was measured using a latex agglutination immunoassay (LAIA) at SRL, Tokyo, Japan. The normal range of healthy donor plasma levels of β 2-MG was 0.9-2.0 mg/l.

Western blot analysis. Plasma samples were measured by Bradford protein assay and adjusted to equal concentration for Tris-tricine SDS-polyacrylamide gel electrophoresis (PAGE). The samples in each were separated on 16.5% gels and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked by 5% milk powder in TBS-0.5% Tween-20 buffer (TBS-T) for 1 h at room temperature and then incubated with mouse anti- β 2-MG antibody (1:1000, Hokudo, Sapporo, Japan) overnight at 4°C, followed by reaction with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (1:20000, Jackson ImmunoResearch, USA). The signal was developed using ELC plus Western Blotting Detection Reagent (GE Healthcare, UK).

Results

Selection of the candidate protein for detecting invisible HCC using SELDI-TOF-MS. To search for novel markers for detecting invisible HCC by CT or MRI, we performed proteomic analysis using ProteinChip Array. At first, we compared the protein profiling in the plasma between 6 HCC patients and 6 healthy donors (Table I). We found four peaks (2.7, 11.7, 51.7 and 118.6 kDa) to be significantly higher in HCC patients than in healthy donors (Fig. 1). Intensity of the peaks discriminated significantly between HCC patients and healthy donors, P=0.01 (2.7 kDa), P=0.006 (11.7 kDa), P=0.04 (51.7 kDa), P=0.001 (118.6 kDa).

For further analyses, the protein expression profiles in the plasma between 6 CH, LC patients with GPC3-specific CTLs and 6 CH, LC patients without GPC3-specific CTLs were compared (Table I, Fig. 2A and B). The intensity of two peaks (11.7 and 51.7 kDa) were significantly higher than in the plasma of GPC3-specific CTLs positive patients as compared with GPC3-specific CTLs negative patients. A protein peak of 11.7 or 51.7 kDa discriminated significantly between GPC-specific CTLs negative patients and HCC patients (P=0.002 or P=0.007), as well as between GPC3-

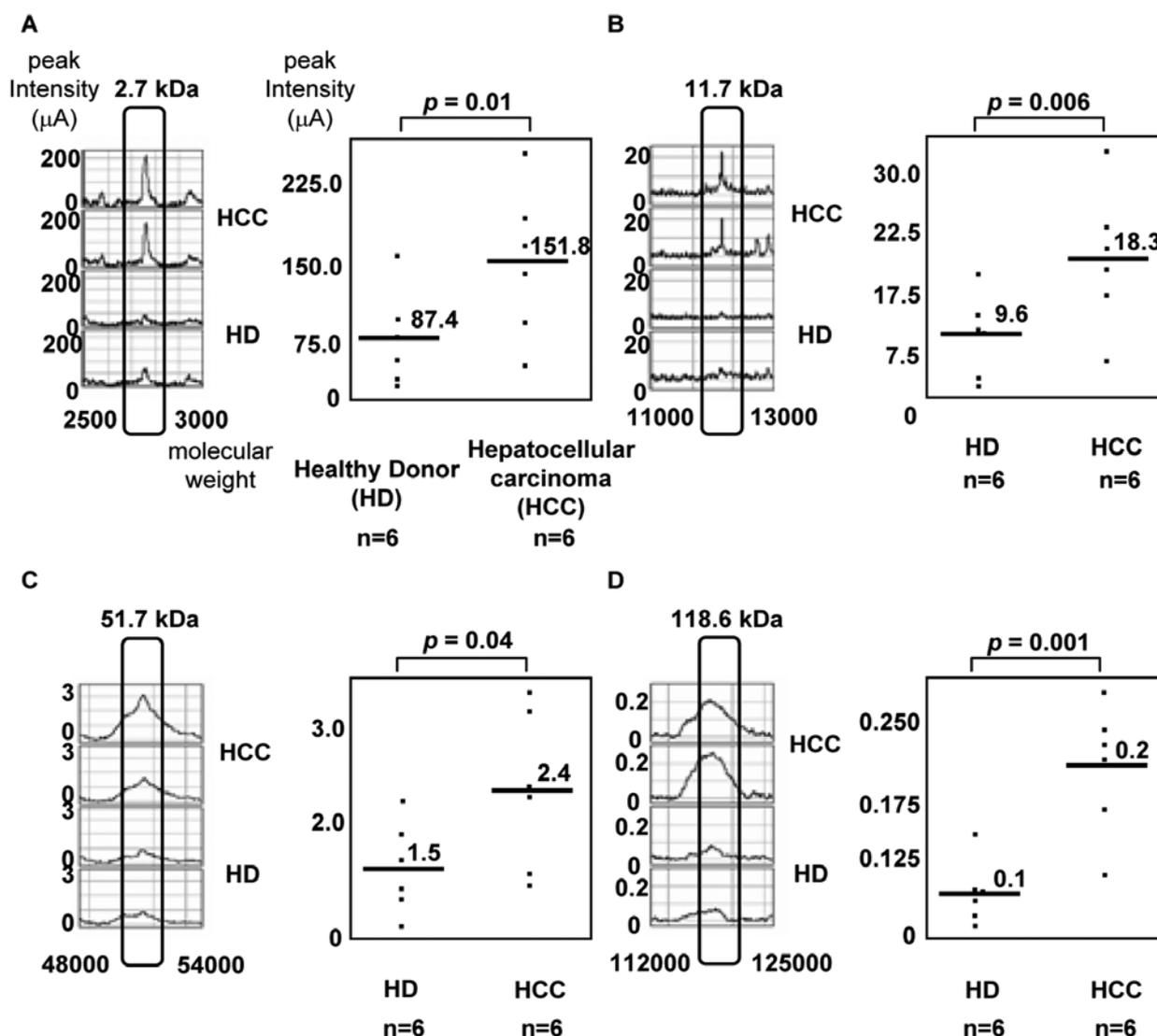


Figure 1. Comparison of the intensity of different expressed peaks in hepatocellular carcinoma (HCC) patients and healthy donors (HD). The spectra were obtained using CM10 ProteinChip (weak cation-exchange) arrays. Distribution of the intensity of different expressed peaks in plasma samples. Distribution of signal intensities for the 2.7 kDa protein (A), the 11.7 kDa protein (B), the 51.7 kDa protein (C) and the 118.6 kDa protein (D) were shown. Black bars indicate mean intensity.

Table II. Search results from SELDI-TOF-MS analysis data using ExpASY TagIdent.

Molecular weight	Entry name	Protein name	Accession no.
11731	B2-MG_HUMAN	β 2-microglobulin	P61769
11684	NRTN_HUMAN	Neurturin	Q99748
11710	KCNE3_HUMAN	Potassium voltage-gated channel subfamily E member 3	Q9Y6H6

specific CTLs positive patients and HCC ($P=0.04$ or $P=0.02$).

Prediction of β 2-microglobulin (β 2-MG) as a 11740 kDa protein using ExpASY server. To identify the 11740 kDa protein, we used the TagIdent tool from the ExpASY proteomic server (<http://www.expasy.ch/tools/tagident.html>). By entering the molecular mass unknown protein, this tool will search in

the TrEMBL and Swiss-Plot protein database for proteins that will match with the requested molecular mass. From the results of the search from ExpASY TagIdent, we focused on β 2-MG. Peak of 11.7 kDa was predicted to be β 2-MG by molecular mass (Table II).

Identification of the β 2-microglobulin (β 2-MG). We examined the plasma level of β 2-MG in six novel donors (HD-1, 2, CH-

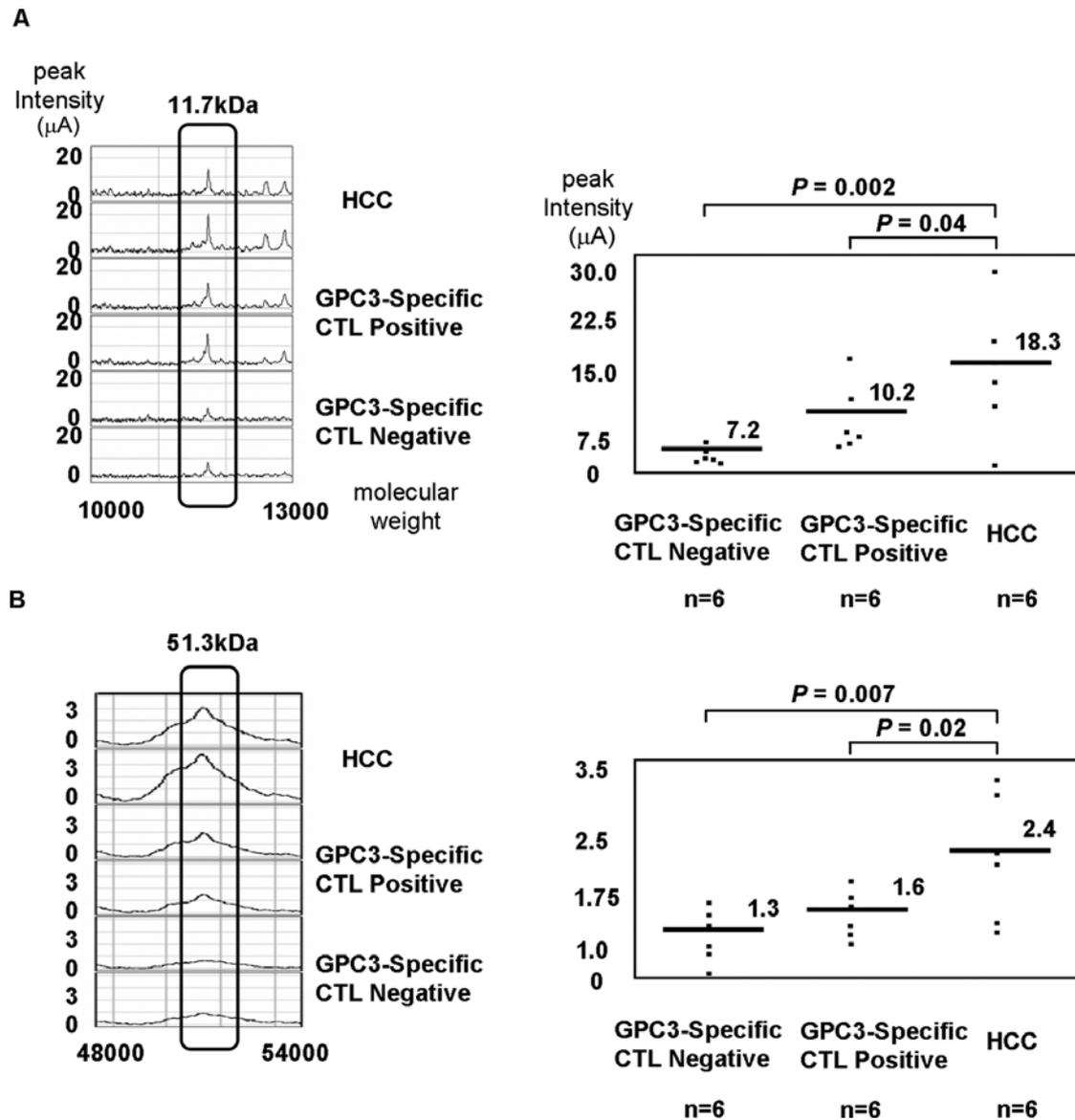


Figure 2. Comparison of the intensity of different expressed peaks in CH/LC patients who are positive for Glypican-3 (GPC3)-specific CTLs. CH/LC patients who are negative and HCC. Data were obtained using CM10 arrays. Distribution of signal intensities for the 11.7 kDa protein (A) and the 51.7 kDa protein (B) are shown.

1, 2 and LC-1, 2) by LAIA. To see whether relative peak intensity of 11.7 kDa protein in SELDI-TOF-MS analysis was correlated with plasma β 2-MG levels determined by an LAIA or not (Fig. 3). As shown in Fig. 3A and B, there was a positive correlation between the peak intensity and the concentration of β 2-MG. To further confirm our results, Western blot analysis with mouse anti- β 2-MG antibody was performed on the plasma (HD-1, 2, CH-1, 2, LC-1, 2, in Fig. 3A). As expected, a specific band at 11.7 kDa was clearly detected in plasma samples (Fig. 3C). These results suggest that 11.7 kDa band should be β 2-MG.

Fractionation of the 11.7 kDa protein peak, β 2-MG. To confirm 11.7 kDa protein as β 2-MG, the plasma was fractionated by gel filtration and HPLC. We fractionated β 2-

MG standard using size-exclusion chromatography. The peak eluted for the β 2-MG standard at retention time 20.049 min (Fig. 4A). Fractions were collected at retention times from 15 to 18 min (Fr. 1), 18 to 24 min (Fr. 2: the same retention time as the fraction of β 2-MG standard), 24 to 31 min (Fr. 3), 31 to 34 min (Fr. 4) and β 2-MG standard (Fig. 4B). Western blot analysis with mouse anti- β 2-MG antibody was performed to a set of collected fractions. As expected, only a single specific band at \sim 12 kDa was detected with unpurified plasma, Fr. 2 and β 2-MG standard (Fig. 4C).

Discussion

SELDI-TOF-MS has been successfully applied in biomarker detection and identification in ovarian, lung, colon and

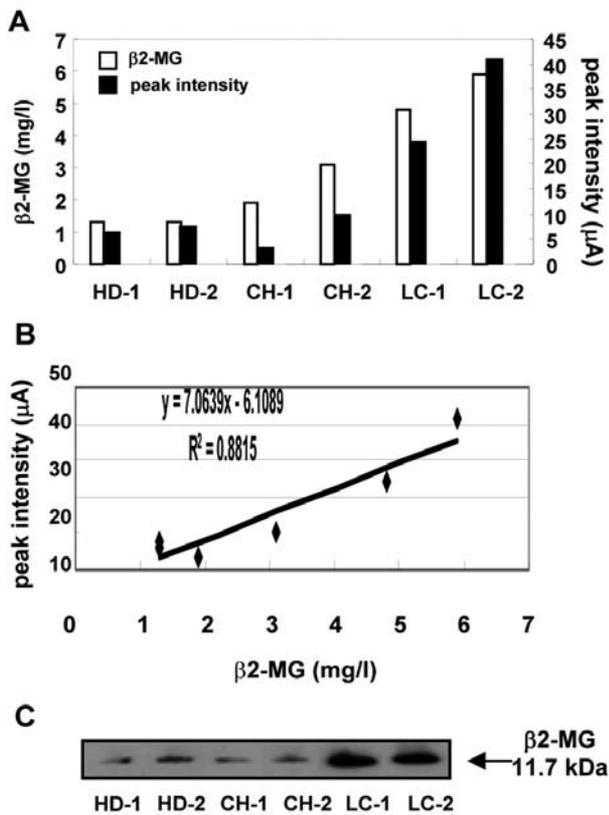


Figure 3. Prediction of 11.7 kDa protein peak as β 2-microglobulin (β 2-MG). (A) Correlation between peak intensity of 11.7 kDa in healthy donors (HD)-1, 2, patients with chronic hepatitis (CH)-1, 2, liver cirrhosis (LC)-1, 2 by SELDI-TOF-MS and concentration of β 2-MG by latex agglutination immunoassay (LAIA). (B) Correlation between 11.7 kDa proteins intensity by SELDI-TOF-MS and concentration of β 2-MG by LAIA are shown. The values are well correlated. (C) Western blot analysis of β 2-MG in two pairs of healthy donors (HD) and patients (CH, LC) plasma. The band of β 2-MG is shown with an arrow.

various cancers (22-25). In this study, in order to search for new biomarkers of CH and LC, we analyzed plasma using ProteinChip Array. We have identified β 2-MG as a new biomarker in CH and LC patients who have GPC3-specific CTLs.

β 2-MG is a non-glycosylated polypeptide composed of 99 amino acids (26). It is one of the components of major histocompatibility complex HLA class I molecules on the cell surface of all nucleated cells (27). Increased serum levels of β 2-MG also occur in a variety of multiple myeloma, lymphoma, Sjögren's syndrome and amyloid fibrils in patients receiving hemodialysis for long periods (28-30). It has been reported that the level of serum β 2-MG was elevated in patients with chronic hepatitis C, HCV-related HCC when compared to HCV-negative patients or healthy donors (31,32). However, it has not yet been reported that the detection of β 2-MG in plasma may show the invisible HCC by CT or MRI and that high β 2-MG in the plasma is a risk factor for developing HCC.

The mechanism of the increase of β 2-MG in amyloid fibrils in patients receiving hemodialysis for long periods has been elucidated, but previously the relationship between mechanism in β 2-MG and risk for the developing of HCC has not been reported.

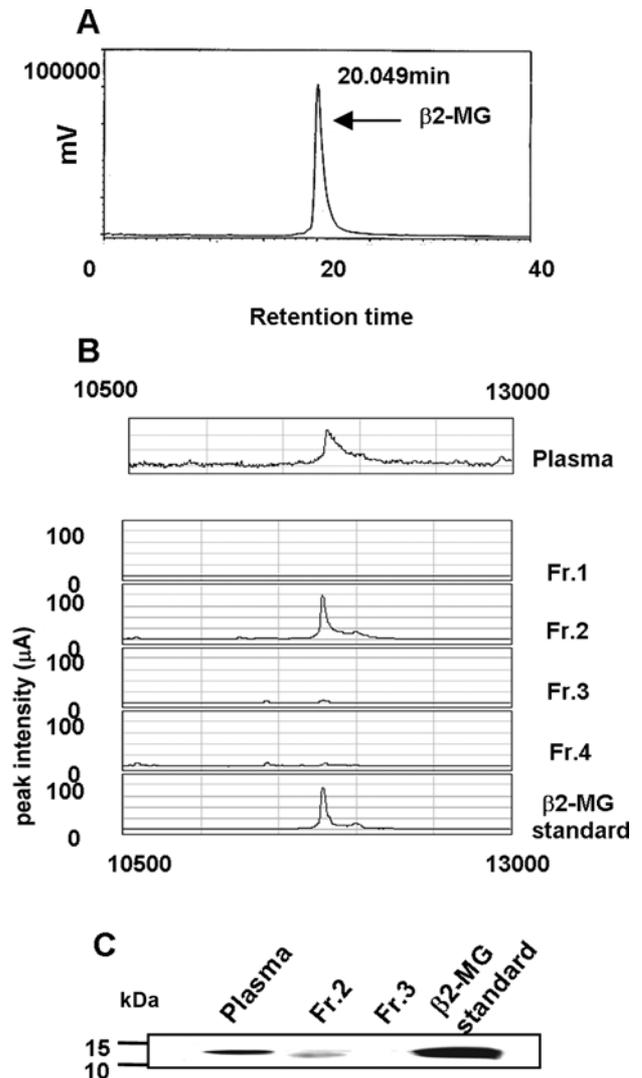


Figure 4. Fractionation of 11.7 kDa protein peak as β 2-MG. (A) High performance liquid chromatography (HPLC) of β 2-MG standard, absorbance at 280 nm. (B) Fractionation of HPLC by SELDI-TOF-MS. (C) Western blot analysis of each fractionation of HPLC and β 2-MG standard.

In this study, we showed that β 2-MG in plasma increased in CH and LC patients with GPC3-specific CTLs, and suggested that the β 2-MG in plasma could be a marker to detect imaging-invisible HCC. To confirm these results, we will evaluate the correlation between level of β 2-MG and risk for developing HCC in a large-scale analysis using many plasma samples of CH and LC patients. In addition, we aim at identifying a good diagnostic marker for imaging-invisible HCC.

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