Association of glypican-3 expression with growth signaling molecules in hepatocellular carcinoma

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Abstract

AIM: To clarify the association of glypican-3 (GPC3) expression with Wnt and other growth signaling molecules in hepatocellular carcinoma (HCC).

METHODS: Expression of GPC3, Wnt, matrix metalloproteinases (MMPs), sulfatase (SULF)1, SULF2, and other growth signaling molecules was analyzed in HCC cell lines and tissue samples by real-time reverse transcription-polymerase chain reaction, immunoblotting, and/or immunostaining. Expression of various genes in GPC3 siRNA-transfected HCC cells was analyzed.

RESULTS: GPC3 was overexpressed in most HCCs at mRNA and protein levels and its serum levels were significantly higher in patients with HCC than in non-HCC subjects (P < 0.05). Altered expressions of various MMPs and growth signaling molecules, some of which were correlated with GPC3 expression, were observed in HCCs. Down-regulation of GPC3 expression by siRNA in GPC3-overexpressing HCC cell lines resulted in a significant decrease in expressions of MMP2, MMP14, fibroblast growth factor receptor 1, insulin-like growth factor 1 receptor. GPC3 expression was significantly correlated with nuclear/cytoplasmic localization of β-catenin.

CONCLUSION: These results suggest that GPC3, in conjunction with MMPs and growth signaling molecules, might play an important role in the progression of HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most com-
mon cancers in the world[1]. HCC is associated with well-defined viral and non-viral etiological factors. Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), chemical carcinogens (i.e. aflatoxins), and other environmental and host factors causing liver injury have etiologically been linked to HCC[2-3]. Various genetic and epigenetic abnormalities have been identified in HCC, suggesting a multi-step nature of hepatocarcinogenesis[3-4].

The pathogenesis of HCC has been known to involve p53[4], β-catenin[3], TGFβ[1] and the retinoblastoma gene[6]. p53 gene mutation occurs in one-third of HCC[5]. Activating mutations in β-catenin have been reported in 18% of HCC patients and axin mutations in 6%[6,7]. HCCs are also known to express various Wnt family members[8] and the activation of the canonical Wnt signaling pathway occurs in 18% of HCC[9].

Glypican-3 (GPC3) is a member of the glypican family of glycosylphosphatidylinositol-anchored cell-surface heparan sulfate proteoglycans. GPC3 is highly expressed in HCC cells and tissues[10-12]. It is thought that GPC3 stimulates the growth of HCC cells by upregulating autocrine/paracrine canonical Wnt signaling[13]. GPC3 was shown to bind to fibroblast growth factor (FGF)2 and may function as a coreceptor for FGF2[14]. Two recently identified human heparin-degrading endosulfatases, named sulfatase 1 (SULF1) and SULF2, have been found to be involved in liver carcinogenesis[15,16]. Interestingly, SULF2 reportedly regulates migration, adhesion, and actin cytoskeleton organization in mammary tumor cells through Wnt signaling modulation[17].

Matrix metalloproteinases (MMPs) also play an important role in HCC[12,13]. It has been reported that GPC3 may regulate MMP activity in breast cancer[18]. The following genes were analyzed: MMP2, MMP3, MMP7, MMP9, MMP14, SULF1, SULF2, FGFR1, IGF1R, FGFR4, WNT2b, WNT3, WNT5a, and WNT7b, GPC3, MMP2, MMP3, MMP7, MMP9, and MMP14, SULF1, SULF2, FGFR, FGFR receptor (FGFR) 1, FGFR2, FGFR3, FGFR4, epidermal growth factor receptor (EGFR), erb-b2 (ERBB2), IGF2 and IGF1R. A comparative threshold cycle was used to determine gene expression relative to the no-tissue control (calibrator).

**Materials and Methods**

**Cell lines**

HCC cell lines, HepG2, Hep3B, JHH-4, HuH-7, HLE, HLF, PLC/PRF/5, Li-7, huH-1, HT17, CHC4, and CHC32, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan), Riken Cell Bank (Tokyo), or the American Type Culture Collection (Rockville, MD, USA), and cultured as recommended. Cells were maintained at 37°C in an atmosphere of humidified air with 5% CO2.

**Tissue samples**

All tissue and serum samples were obtained from Japanese patients. Informed consent was obtained from each patient. Tissue microarray of HCC tissues was purchased from SuperBioChips Laboratories (Seoul, Korea). Each tissue specimen was divided into two pieces. One sample was used for total RNA extraction. The other sample was processed for pathological examination using hematoxylin and eosin staining for the evaluation of the tumor cell content. Only specimens containing more than 80% tumor cells were used for analysis. The tumor-node-metastasis (TNM) system of the American Joint Committee on Cancer and the International Union against Cancer was used for the pathologic diagnosis and classification of variables.

**Semiquantitative RT-PCR and real-time RT-PCR**

Semiquantitative RT-PCR was performed as described previously[19]. The primer sequences used were 5′-AGGGTAGCTGCGAGGAAC-3′ and 5′-AGGTCAGTGCTTGGCTCTTC-3′ for GPC3 and 5′-TGGACATCAATGAGTG-7TTGCCTC-3′ and 5′-CAATTTGCTGGTACGACATTG-3′ for GAPDH. Real-time RT-PCR was performed by using TaqMan real-time PCR system as described previously[20]. The following genes were analyzed: GPC3, WNT (WNT1, WNT2, WNT2b, WNT3a, WNT4, WNT5a, and WNT7b), MMP (MMP2, MMP3, MMP7, MMP9, and MMP14), SULF1, SULF2, FGFR, FGFR receptor (FGFR) 1, FGFR2, FGFR3, FGFR4, epidermal growth factor receptor (EGFR), erb-b2 (ERBB2), IGF2 and IGF1R. A comparative threshold cycle was used to determine gene expression relative to the no-tissue control (calibrator).

**Immunoblotting**

Immunoblotting using total cell lysates was performed as previously described[20]. The antibodies used were MMP2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), MMP14 (Abcam, Cambridge, UK), FGFR1 (Abcam), and IGF1R (Santa Cruz Biotechnology). The protein was visualized using the enhanced chemiluminescence plus detection system (Amersham Biosciences, Piscataway, NJ, USA), and the membranes were stripped and reprobed with mouse anti-actin monoclonal antibody (Chemicon, Temecula, CA, USA).

**Gelatin zymography**

Gelatinase activity of conditioned media of siRNA-transfected HCC cells was analyzed.
Expression of GPC3 mRNA in HCC cell lines

GPC3 expression was detected in 7 (58%) of 12 HCC cell lines (Figure 1). GPC3 mRNA was detected in HT17, PLC/PRF/5, HuH-1, HepG2, Hep3B, and HuH-7 at a high level and HLE at a low level. GPC3 mRNA was positive in well differentiated HuH7 and poorly differentiated HT17 cell lines. There was no correlation between GPC3 positivity and histopathology. Concordant results were obtained by real-time RT-PCR (Figure 2). There was no correlation between quantitative GPC3 mRNA levels and histopathology.

mRNA expression of WNT, MMPs, SULF1, SULF2, and other growth signaling molecules in HCC cell lines

WNT1, WNT2, WNT9b, WNT3a, WNT4, WNT5a, WNT7b expression was detected in 0 (0%), 7 (58%), 0 (0%), 0 (0%), 5 (41%), 9 (75%), 4 (33%) of 12 HCC cell lines. Thus, WNT2 and WNT5a were frequently detected compared with other WNTs. MMP2, MMP3, MMP7, MMP9, MMP14 expression was detected in 7 (58%), 2 (16%), 8 (67%), 2 (16%), 11 (92%) of 12 HCC cell lines. Thus, the positivity was considerably different among MMPs. SULF1 and SULF2 expression was detected in all of the 12 HCC cell lines. EGF-R, ERBB3, FGFR2, FGFR3, FGFR4 expression was detected in 12 (100%), 12 (100%), 12 (100%), 12 (100%), 6 (50%), 2 (20%) of 12 HCC cell lines.

Expression of GPC3 mRNA in HCC cell lines

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RESULTS

Expression of GPC3 mRNA in HCC cell lines

GPC3 expression was detected in 7 (58%) of 12 HCC cell lines (Figure 1). GPC3 mRNA was detected in HT17, PLC/PRF/5, HuH-1, HepG2, Hep3B, and HuH-7 at a high level and HLE at a low level. GPC3 mRNA was positive in well differentiated HuH7 and poorly differentiated HT17 cell lines. There was no correlation between GPC3 positivity and histopathology. Concordant results were obtained by real-time RT-PCR (Figure 2). There was no correlation between quantitative GPC3 mRNA levels and histopathology.
expression of GPC3 was not related to HBV or to HCV infection. Overexpression of GPC3 was not significantly correlated with other clinicopathological characteristics (data not shown).

**mRNA expression of WNT, MMPs, SULF1, SULF2, and other growth signaling molecules in HCC tissue**

mRNA expression of SULF1 was downregulated in 17 (74%) and SULF2 was upregulated in 7 (30%) of 23 HCC tissue samples compared with non-tumor tissue. Overexpression of GPC3 was significantly correlated with MMP2 (P = 0.0460), FGF2 (P = 0.0001), FGFR1 (P = 0.0417), FGFR2 (P = 0.0023), SULF1 (P = 0.0202), and SULF2 (P = 0.0081).

**Knockdown effect of GPC3 on expression of various molecules in HCC cells**

To assess the effect of GPC3 expression on gene expression in HCC, mRNA expression of WNT, MMPs, SULF1, SULF2, and other growth signaling molecules was analyzed by real-time PCR after treatment with specific siRNA for the GPC3 gene. Transfection with siRNA resulted in over 80% inhibition of mRNA and protein expression of GPC3 (Figure 3). Among the genes analyzed, mRNA expression of MMP2, MMP14, FGFR1, and IGF1R was downregulated in GPC3 siRNA-transfected cells compared with control siRNA-transfected counterparts (Figure 3A). Down-expression of MMP2, MMP14, FGFR1, and IGF1R was confirmed at protein levels analyzed by immunoblotting (Figure 3B). Down-regulation of MMP2 activity was further confirmed by zymography (Figure 3C).

**Immunohistochemical expression of GPC3 and β-catenin in HCC tissue**

Figure 4 shows representative results of immunohistochemistry for GPC3 in HCC tissue samples. GPC3 protein was strongly expressed in the cytoplasm and/or membrane of carcinoma cells, when compared with adjacent non-tumor cells. GPC3 expression was positive in 54 (75%) of the 72 cases. GPC3 positivity was not significantly correlated with clinicopathological characteristics. Figure 5 shows representative results of immunohistochemistry for β-catenin in HCC tissues. Membranous, weak, cytoplasmic, and accumulated pattern was observed in 18 (64%), 0 (0%), 6 (22%), and 4 (14%), respectively. Nuclear/cytoplasmic localization of β-catenin was observed in a significantly higher percentage of carcinomas with GPC3 expression (9 of 18, 50%) than in those without (1 of 10, 10%, P = 0.040).

**Serum GPC3 levels**

Serum GPC3 levels were under cut-off levels in healthy volunteers, HBV carriers and chronic hepatitis patients (Figure 6). One patient with liver cirrhosis and 32 (50%) of the 64 patients with HCC showed elevated serum GPC3 levels. Serum GPC3 levels in patients with HCC were significantly higher than in non-HCC subjects (P < 0.05). There was no correlation in positivity between...
GPC3, α-fetoprotein (AFP) levels and vitamin K absence or antagonist-Ⅱ (PIVKA-Ⅱ) levels (data not shown).

**DISCUSSION**

In this study, we found overexpression of *GPC3* mRNA in HCC cell lines and tissue samples. The overexpression of GPC3 in HCC was also observed at protein level analyzed by immunohistochemistry. These results further support the notion that GPC3 plays an important role in hepatocarcinogenesis.

We analyzed the association of GPC3 with WNT, MMPs, SULF1, SULF2, and other growth signaling molecules, in HCC cell lines and tissue samples. *GPC3* expression was correlated with expression of *MMP14, ERBB2, FGFR3*, and *FGFR4* in HCC cell lines. Overexpression of *GPC3* was significantly correlated with *MMP2, FGF2, FGFR1, FGFR2, SULF1*, and *SULF2* in HCC tissue. To assess the effect of GPC3 expression on gene expression in HCC cells, expression of WNT, MMPs, SULF1, SULF2, and other growth signaling molecules was analyzed in HCC cell lines after treatment with specific siRNA for the *GPC3* gene. Down-expression of MMP2, MMP14, FGFR1, and IGF1R was observed at mRNA and

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**Figure 4** Immunohistochemistry for glypican-3 in liver tissue. A: Normal liver negative for glypican-3 (GPC3); B: Hepatocellular carcinoma positive for GPC3. Original magnification, × 200. Expression of GPC3 was immunohistochemically analyzed with an anti-human GPC3 mouse monoclonal antibody. GPC3 protein was expressed in the cytoplasm and/or membrane of carcinoma cells.

**Figure 5** Immunohistochemistry for β-catenin in hepatocellular carcinoma tissue. A: Membrane staining, cytoplasmic staining, nuclear staining of β-catenin. Expression of β-catenin was immunohistochemically analyzed with an anti-human β-catenin monoclonal antibody. Original magnification, × 200; B: Association of nuclear/cytoplasmic localization of β-catenin with glypican-3 (GPC3) expression.

- Nuclear: 14%
- Cytoplasmic: 22%
- Membranous: 64%

![Bar graph showing comparison between GPC3 positive and negative groups (P = 0.04)](image)

A: GPC3 (+); B: GPC3 (-)

Nuclear/cytoplasmic (%)

GPC3 (+) GPC3 (-)

P = 0.04
protein levels, and that of MMP2 was also observed at gelatinolytic activity levels.

MMP2 and its activator MMP14 play an important role in HCC progression. Therefore, down-regulation of both MMP2 and MMP14 by GPC3 suppression is interesting. It has been reported that GPC3 may regulate MMP activity. GFG-FGFR signaling plays an important role in hepatocarcinogenesis. GPC3 has been shown to bind to FGF2 and may function as a coreceptor for FGF2. SULF2 reportedly up-regulates GPC3, and SULF2 gene expression is important. Considering the expression pattern of GPC3 in HCC, GPC3 may play a role in nuclear/cytoplasmic localization of β-catenin in HCC. These results may explain, in part, the association between GPC3 and growth signaling molecules in HCC. Further studies are necessary to clarify the direct and/or indirect interactions between GPC3 and growth signaling molecules in HCC.

The implication of a part played by GPC3 in HCC was further substantiated by the fact that serum GPC3 levels were significantly higher in patients with HCC than non-HCC subjects. However, there was a discrepancy between GPC3 overexpression in HCC tissues (75%) and GPC secretion (50%). The discrepancy was also reported in previous studies. The sandwich ELISA kit used in this study used a polyclonal antibody and a monoclonal antibody, both raised against the last 70 amino acids of the COOH-terminal portion of GPC3, to detect glycated GPC3. This may be one of the reasons why serum GPC3 positivity was lower than GPC3 positivity in HCC tissue. Comparison of our results with those analyzed by a kit using antibody against the NH2-terminal portion of GPC3 will further strengthen the notion that GPC3 is a useful serum marker for HCC. There was no correlation between GPC and AFP levels. Therefore, detection of both glycated and NH2-terminal truncated GPC3, as well as AFP, may provide additional useful markers for HCC.

Taken together, our results suggest that GPC3 overexpression plays an important role in HCC. As a target gene for molecular therapy, its expression in normal adult tissues is important. Considering the expression pattern of GPC3 together with its oncogenic function, GPC3 could be an attractive target for molecular therapy. Antitumor effects of the anti-GPC3 antibody have been reported. Interestingly, we have recently reported the tumor suppressive effect of tyrosine kinase inhibitor of IGF1R, NVP-AEW541, on GPC-3-expressing HCC cell line PLC/PRF/5. Combination of the anti-GPC3 antibody and molecular therapy targeting GPC3-related molecules, such as FGFR, found in this study will be a promising new cancer therapy in the future.

**Figure 6** Serum glypican-3 (GPC3) levels were measured using a commercially available sandwich ELISA kit. Bar: Standard error. Serum GPC3 levels were significantly higher in patients with hepatocellular carcinoma (HCC) than in non-HCC subjects (P < 0.05). HBV: Hepatitis B virus.

**Figure 7** Associations of glypican-3 expression with matrix metalloproteinases and growth signaling molecules in hepatocellular carcinoma. A summary diagram is shown. MMPs: Matrix metalloproteinases; SULF2: Sulfatase 2; GPC3: Glypican-3; FGF: Fibroblast growth factor; IGF: Insulin-like growth factor.
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S- Editor Wang JL  L- Editor Logan S  E- Editor Zheng XM