Evaluation of glypican-3 in patients with hepatocellular carcinoma

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Received June 6, 2024; Accepted October 3, 2024

DOI: 10.3892/mco.2024.2796

Abstract. Hepatocellular carcinoma (HCC) is one of the most common cancers occurring worldwide, including Mongolia. Although alpha-fetoprotein (AFP) is a widely used marker for HCC, conflicting studies have been published regarding its specificity and sensitivity towards HCC. Glypican-3 (GPC3) is a different promising biomarker for HCC, and there is some evidence to suggest that this protein may be a more specific marker compared with AFP. GPC3 has been shown to fulfill important roles in cell proliferation and division during embryogenesis, and is rarely found in the tissues of healthy adults. The aim of the present study was to investigate the levels of serum GPC3 (sGPC3) and tissue GPC3 in Mongolian patients with HCC. Serum samples from a total of 270 individuals [HCC group, 90 patients; risk group (RG), 90 subjects; and control group, 90 subjects] were evaluated using enzyme-linked immunosorbent assay to identify the sGPC3 levels. In addition, immunohistochemical analysis of the GPC3 was performed on tissue samples from 50 patients with HCC to evaluate the expression of GPC3. sGPC3 level was found to be significantly increased in the HCC group compared with the RG and the control group, with the area under the curve=0.85 (P<0.001). sGPC3 was found to be significantly associated with hepatitis C virus status and cirrhosis (P<0.05). In addition, the tissue expression of GPC3 was associated with the serum AFP (sAFP) level. Finally, positive staining of GPC3 was observed when the sAFP level of the patient was >20 ng/ml. In conclusion, the results from the present study have supported that GPC3 may be a promising marker for HCC, and can be used as a diagnostic marker alongside AFP.

Introduction

As of 2022, liver cancer ranked as the 8th most common cancer worldwide. According to Global Cancer Statistics 2022, there were 865,269 new cases of liver cancer diagnosed and 757,948 deaths were reported. Mongolia demonstrates the highest occurrence of hepatocellular carcinoma (HCC) globally, with a rate of 86 cases per 100,000 people. This prevalence greatly surpasses that of neighboring nations, being 4 times higher compared with China, and >20 times higher compared with Russia, as well as surpassing the incidence rates observed in any other country worldwide (1).

In the diagnosis of HCC, alpha-fetoprotein (AFP) is widely used as a biomarker. However, problems associated with its poor specificity and sensitivity have been reported in certain studies (2,3). According to international standards, AFP test results are typically confirmed with the assistance of computerized tomography scans and magnetic resonance imaging analyses (4-6). However, in Mongolia, there are fewer opportunities to perform these scans at present. A recent study has shown that the use of biomarkers such as glypican-3 (GPC3), Golgi protein 73 and des-gamma carboxyprothrombin, in

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Abbreviations: HCC, hepatocellular carcinoma; AFP, alpha fetoprotein; GPC3, glypican 3; RG, risk group; sGPC3, serum GPC3; ELISA, enzyme-linked immunosorbent assay

Key words: glypican-3, hepatocellular carcinoma, early detection, biomarker

addition to AFP, has practical benefits for the early diagnosis of liver cancer (7). Among these markers, GPC3 has attracted significant attention, primarily due to the fact that it is specifically expressed in tumors. Moreover, it is potentially useful as a marker for cases of HCC where AFP levels are either low or absent, and in combination with AFP (8-11).

GPC3 is a heparan sulfate proteoglycan consisting of 580 amino acids, with a molecular weight of 70 kDa, which is attached to the glycosyl-phosphatidylinositol region of cell membranes (12,13). The GPC3 gene is located on the human X chromosome (in the Xq26 chromosomal region) and has important roles in cell proliferation and division during embryogenesis. The expression of GPC3 has been identified in fetal placenta, liver, lung and kidney tissues, although it is rarely found in the tissues of healthy adults (14). During embryonic development, GPC3 interacts with signaling pathways and proteins, including the Wnt, fibroblast growth factor and bone morphogenetic proteins signaling pathways, to regulate cell division, proliferation and apoptosis (15-18). In addition, studies have shown that activated GPC3 protein increases the rates of cell proliferation and growth by increasing the synthesis of heparan sulfate growth factors from tumor cells through the sulfatase-2 enzyme (19,20).

A number of studies have also shown that the levels of GPC3 are increased in the serum and tissues of patients with HCC, but not in cases of liver injury, cirrhosis or viral hepatitis (21,22). Moreover, several studies have performed immunohistochemical (IHC) evaluations of GPC3 protein expression in HCC tissue samples, wherein it was noted that GPC3 may be useful as a marker for tumor diagnosis, staging, treatment outcome, disease progression and recurrence (23-26).

To the best of the authors' knowledge, the tissue expression of GPC3 protein has not been reported in Mongolian patients with HCC. Therefore, the aim of the present study was to assess the association between GPC3 protein and the clinical characteristics of Mongolian patients with HCC.

Materials and methods

Study subjects and samples. Laboratory experiments were performed in collaboration with the Central Scientific Research Laboratory of the Institute of Medical Sciences (Ulaanbaatar, Mongolia) and the Hepato-Biliary-Pancreatic Surgical Department of the National Cancer Center of Mongolia (Ulaanbaatar, Mongolia). Liver tissue and serum samples from patients with HCC were collected between October 2022 and March 2023 at the National Cancer Center in Ulaanbaatar, Mongolia. Serum samples from the control and risk groups were collected during the same period at The Third Central Hospital in Ulaanbaatar, Mongolia.

Serum samples were collected from a total of 270 participants, comprising the HCC group (n=90), the risk group (RG) (n=90) and the healthy control group (n=90). The RG included patients with chronic hepatitis, toxic hepatitis, alcohol-associated liver disease and other liver disorders. The average age of the participants was 61.0±9.5 years. A total of 107 (39.7%) of the subjects were males and 163 (60.3%) were females.

A total of 64 people who were diagnosed with HCC and underwent surgical treatment were included in the tissue analysis. Following surgery, 14 participants were excluded from the study, as pathological examination of the cancer tissues extracted during surgery revealed that their diagnoses were other than HCC. GPC3 protein expression was evaluated in both the cancerous tissue and the surrounding tissue of 50 patients with HCC using an IHC staining method. The average age of the participants was 64.06±9.1 years. A total of 21 (42%) of the subjects were males and 29 (58%) were females.

Tumor staging was conducted based on the 8th tumor-node-metastasis (TNM) classification system for liver cancer [The National Comprehensive Cancer Network® (NCCN®), version 5.2020 (27): AJCC, 8th edn, 2017 (28)]. Tumors were classified according to the predominant histological subtype proposed by the 2017 American Joint Committee on Cancer classification system.

The present study was approved by the Ethics Committee of Mongolian National University of Medical Sciences (approval nos. 2022/3-05 and 2022.05.20; Ulaanbaatar, Mongolia), and the various experimental procedures were performed according to the Declaration of Helsinki (2013). All patients provided written informed consent.

Detection of the serum GPC3 (sGPC3) level. GPC3 in serum of patients was detected using a Human Glypican 3 Quantikine® QuicKit™ enzyme-linked immunosorbent assay (ELISA) Kit (cat. no. DGLY30) from R&D Systems, Inc. ELISA analysis was performed according to the manufacturer's protocol using a BIOBASE-EL10A ELISA microplate reader. The optical density was determined at 450 nm. All procedures were performed at room temperature and all samples were measured in triplicate.

IHC analysis. The protein expression of GPC3 was assessed on extracted HCC tissues through IHC staining using an anti-GPC3 antibody (200:1; cat. no. SC-65443; Santa Cruz Biotechnology, Inc. https://www.scbt.com/home). Liver tissues were fixed in 10% neutral buffered formalin for 24 h at room temperature. Paraffin embedded tissue blocks were cut into 4-μm sections for IHC staining. The prepared tissues were deparaffinized in xylene at room temperature (18-22˚C) for 10 min, followed by rehydration in a 100, 100, 95, 80 and 70% ethanol series at room temperature (2 min each dilution of ethanol). Subsequently, the tissue slices were subjected to antigen retrieval at 120˚C for 10 min in 10 mmol/l citrate buffer (pH 6.0).

Subsequently, endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide dissolved in methanol, followed by treatment with the anti-GPC3 primary antibody. The antibody was incubated with the tissues at room temperature for 1 h. Subsequently, a secondary antibody [broad-spectrum secondary antibody solution; cat. no. D01-110; Golden Bridge International (GBI) (Labs) Ltd. https://www.gbiinc.com/] was added, and incubated with the tissue at room temperature for 15 min according to the manufacturer's protocols. Streptavidin-HRP was added, and incubated at room temperature for a further 15 min. Finally, a drop of aminoethyl carbazole solution [cat. no. C01-12; GBI (Labs) Ltd. https://www.gbiinc.com/] was applied to the tissue, which was observed under a

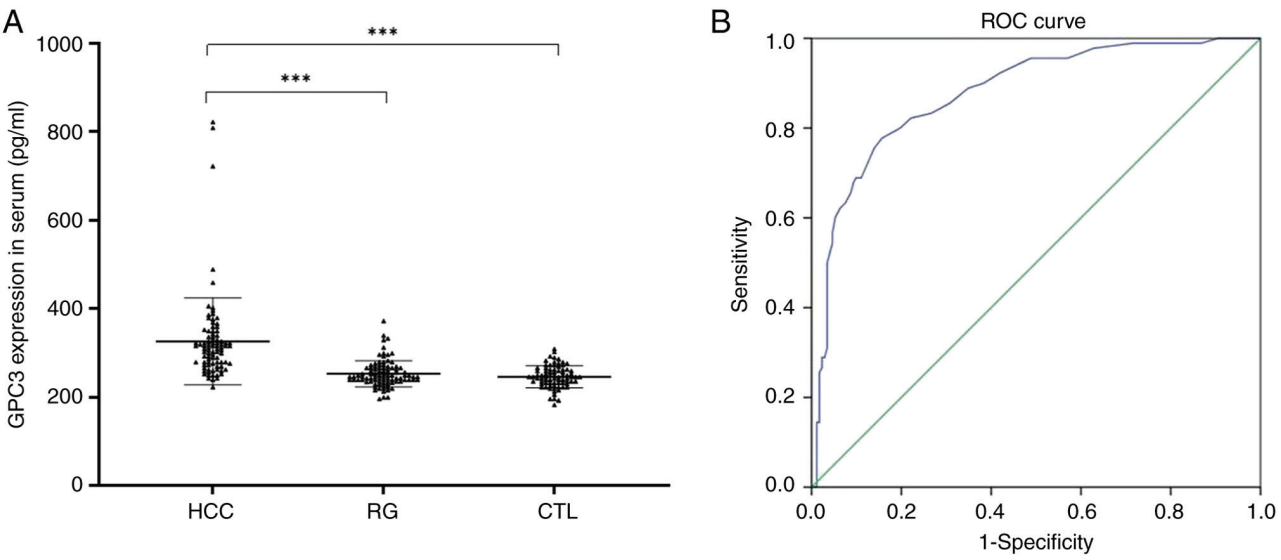

Figure 1. (A) The boxplot of sGPC3 levels is shown. ***P<0.001. (B) ROC curves of sGPC3 for the diagnosis between HCC and precancerous lesions of HCC. ROC, receiver operating characteristic; sGPC3, serum glypican 3; HCC, hepatocellular carcinoma; RG, risk group; CTL, normal control.

microscope (Olympus BX41; Olympus Corporation) until a red color developed.

Tissue microarray analysis (TMA). The paraffin-embedded liver cancer tissue was observed under a light microscope (Olympus CHA; Olympus Corporation), and a suitable area was marked out. A 2-mm diameter punch was inserted into the cancer tissue to a depth of 5 mm, which was then placed into a newly prepared paraffin block. After inserting all the tissues, 1-μm thick tissue sections were cut from the prepared tissue microarray block using a microtome prior to IHC analysis as aforementioned.

Evaluation of GPC3 in tissue samples. When evaluating the results, the expression of GPC3 protein was categorized as negative, weakly positive or strongly positive based on the percentage of cells exhibiting red-brown staining, and the intensity of the staining within the cell membrane, cytoplasm and nucleus. The following criteria were used to assign scores, which were determined according to the average scores calculated by histopathologists and researchers: Score, 0: Percentage of stained cells <5%; score, 1: Percentage of stained cells 5-25%; score, 2: Percentage of stained cells 26-50%; and score, 3: Percentage of stained cells >50%. In terms of the color intensity, the following values were assigned: Score, 0: No staining; score, 1: Weak staining; score, 2: Moderate; and score, 3: Strong staining. To calculate the final score, the percentage of stained cells score was multiplied by the intensity of staining score (range 0-9) as follows: Negative, 0-1; weakly positive, 2-4; moderate staining, 5-7; strongly positive, 8-9.

Statistical analysis. Statistical analysis for the tables was performed using Chi-square (χ^2) test or Fisher's exact test [to test the association between the sGPC3 level and the patient's clinical and histopathological characteristics] with SPSS software (version 24.0; IBM Corp.). One way ANOVA test was applied and followed by Tukey's test to analyze the differences in sGPC3 levels among the groups. P<0.05 was considered to indicate a statistically significant difference. Receiver operating characteristic (ROC) curves were performed to define the optimal cut-off values, and to assess sensitivity, specificity and respective areas under the curve (AUCs). Data are expressed as the mean ± SD, or n (%). All experiments were conducted in triplicate.

Results

sGPC3 is a diagnostic marker for HCC. The sGPC3 levels in the 3 different experimental groups (HCC group, RG and control group) are shown in Fig. 1. The median sGPC3 level was found to be 327.25±98.22 pg/ml in the HCC group (n=90), whereas the median values ± range were 253.21±29.53 pg/ml in the RG (n=90) and 245.31±23.38 pg/ml in the control group (n=90).

Significant differences in the sGPC3 level were noted among these 3 groups (P=0.001); however, the sGPC3 levels in the RG were not significantly different from those in the control group (P>0.05; Fig. 1A).

The ROC curve of sGPC3 is presented in Fig. 1B. Based on the ROC curve, the cut-off value was set to 270 pg/ml. The sensitivity and specificity percentage values were 83.3 and 84.4% respectively, with AUC=0.892. The positive predictive value was 83%, whereas the negative predictive value was 91.02%.

The χ^2 test and Fisher's exact test were performed on the HCC group (n=90) to test the association between the sGPC3 level and the patients' clinical and histopathological characteristics (Table I). Patients were grouped into two groups with respect to the sGPC3 level, namely >270 pg/ml (n=17) or <270 pg/ml (n=73). A statistically significant association was observed between sGPC3 levels and cirrhosis, as well as between sGPC3 levels and hepatitis C virus (HCV) infection. However, no significant associations were observed between sGPC3 and the other clinical characteristics of the patients. Statistically significant values are shown in bold (Table I).

Table I. Serum GPC3 level and baseline characteristics of the patients with hepatocellular carcinoma.

No.	Characteristics		GPC3 (pg/ml) ≥270 (n=73)	GPC3 (pg/ml) <270 (n=17)	P-value
1	Age	≤60	29	10	0.152
		>60	44	7	
2	Sex	Male	36	9	0.788
		Female	37	8	
3	HBV	Yes	34	11	0.178
		No	39	6	
4	HCV	Yes	38	4	0.034[a]
		No	35	13	
5	Cirrhosis	Yes	58	8	0.013[a]
		No	15	9	
6	TNM stage	T1	12	3	0.541
		T2	26	6	
		T3	30	4	
		T4	5	4	
7	Tumor number	Single	43	13	0.338
		Multiple	30	4	
8	Tumor size	≤5 cm	46	12	0.711
		>5 cm	27	5	
9	AFP	≤20 ng/ml	31	7	0.923
		>20 ng/ml	42	10	

[a], indicates statistically significant difference. GPC3, glypican 3; HBV, hepatitis B virus; HCV, hepatitis C virus; TNM, tumor-node-metastasis; AFP, alpha-fetoprotein.

A total of 50 participants from the HCC group (21 men and 29 women aged 33-79 years, with an average age of 64.06±9.1 years) were evaluated for GPC3 expression with IHC analysis. Through IHC staining, GPC3 protein was found to be positively stained in the cytoplasm, membrane and canaliculi of cells in 38 out of 50 (76%) participants. Representative images of IHC staining are demonstrated in Fig. 2. Among these, 16 of 38 (42.1%) participants exhibited weak positive staining, whereas the remaining 22 (57.9%) displayed strong positive staining. TMA construction and representative images in different magnifications are shown in Fig. 3.

In early-stage cancers, GPC3 protein expression was found to be absent in 8 out of 32 cases (25%), weakly positive in 10 out of 32 cases (31.3%), and strongly positive in 14 out of 32 cases (43.8%). Similarly, the expression of GPC3 was absent in 4 out of 18 cases (22.2%), weakly positive in 6/18 cases (33.3%), and strongly positive in 8/18 cases (44.4%) (P>0.05) in late-stage cancers.

In Table II, the patients are grouped according to the tissue expression of GPC3. After having performed the χ^2 test and Fisher's exact test, clinical characteristics such as age, sex, hepatitis B virus (HBV) infection, HCV infection, cirrhosis, tumor number and tumor size did not show statistically significant association with GPC3 protein expression (P>0.05).

However, a statistically significant association was observed between GPC3 protein expression and serum AFP (sAFP) levels: In particular, when the sAFP level was either normal or <20 ng/ml, the GPC3 protein was positively stained in 13/21 (61.9%) of cases. On the other hand, when the sAFP level was >20 ng/ml, the GPC3 protein was positively stained in 25/29 (86.2%) of cases (P=0.047).

In addition, histopathological characteristics, including fibrosis, steatosis, histological grade, histological cell type, differentiation, TNM stage and vascular invasion, did not show statistically significant association with GPC3 protein expression (P>0.05) (Table II).

Discussion

In the present study, the GPC3 levels in the serum of 270 patients were initially evaluated to assess differences between the HCC, the RG and the control group. The HCC group was found to have significantly higher levels of sGPC3 compared with the RG and the control group. By contrast, no significant differences were noted between the RG and the control group. Similarly, other research groups have shown that the sGPC3 level was significantly elevated in patients with HCC, but not in the healthy control group (29). In the aforementioned study, the average sGPC3 level was found to be 99.94±267.2 ng/ml, whereas the HCC group in the present study showed an sGPC3 level of 327.25±98.22 pg/ml (30). This difference may have been due to differences in the assay kit sensitivity or population genetic characteristics. On the other hand, Baatarkhuu *et al* (31) investigated the difference in sGPC3 expression levels between Mongoloids and Caucasians. They reported no significant differences in sGPC3 between these ethnic groups, and an increased sGPC3 level was detected in 50-55% of the patients with HCC.

Moreover, the ROC curve of sGPC3 showed AUC=0.892, with 83.3% sensitivity and 84.07% specificity, to distinguish HCC from the control group. These results indicated that the sGPC3 level may effectively be used to detect HCC. Qiao *et al* (32) found that the sGPC3 level was the best marker, with an AUC of 0.892 and a cut-off value of 26.8 ng/ml, and a sensitivity of 51.5% and specificity of 92.8% compared with AFP and human cervical cancer oncogene. In addition, the study by Liu *et al* (10) revealed that sGPC3 levels were >300 ng/l in 50% of patients with HCC with sAFP levels <100 mcg/l.

The χ^2 and Fisher's exact test results in the present study did not show any significant association between sGPC3 and the sAFP; however, the sGPC3 level was found to be associated with HCV infection and cirrhosis.

In several previous studies (33-42), the range of percentages of positive GPC3 expression in the tissues of HCC was found to be 52.5-85%, and GPC3 was not detected in healthy liver tissue, liver injury, cirrhosis or viral hepatitis, suggesting the possibility of this marker being used as a diagnostic biomarker for HCC. In the present study, GPC3 protein was shown to be positive in 76% (38/50) of the participants diagnosed with HCC, which was similar to that reported in previous studies. In addition, no direct associations were identified between GPC3 protein expression and other clinical and histopathological characteristics, including age, sex, tumor size, tumor number,

Table II. Tissue expression of GPC3 protein and clinical characteristics of the patients.

No.	Characteristics	Classification	GPC3		P-value
			Positive	Negative	
1	Age (years)	≤60	12	5	0.728
		>61	26	7	
2	Sex	Male	17	4	0.485
		Female	21	8	
3	HBV	Yes	16	5	0.979
		No	22	7	
4	HCV	Yes	18	8	0.243
		No	20	4	
5	Cirrhosis	Yes	24	11	0.079
		No	14	1	
6	Fibrosis (Ishak score)	Stage 2	6	2	0.989
		Stage 3	9	2	
		Stage 4	15	5	
		Stage 5	5	2	
		Stage 6	3	1	
7	Steatosis	Grade 0	26	4	0.058
		Grade 1	11	7	
		Grade 2	1	0	
		Grade 3	0	1	
8	Histological grade	Low grade	18	5	0.730
		High grade	20	7	
9	Histological cell type	Classic	32	10	1.000
		Clear cell	6	2	
10	Differentiation	Poorly differentiated	14	2	0.622
		Moderately differentiated	20	10	
		Well differentiated	4	0	
11	TNM stage (pT)	T1	6	0	0.974
		T2	18	8	
		T3	9	4	
		T4	5	0	
12	Tumor number	Single	28	10	0.705
		Multiple	10	2	
13	Tumor size	≤5 cm	27	10	0.480
		>5 cm	11	2	
14	Vascular invasion	Yes	32	12	0.314
		No	6	0	
15	AFP	≤20 ng/ml	13	8	0.047
		>20 ng/ml	25	4	

GPC3, glypican 3; HBV, hepatitis B virus; HCV, hepatitis C virus; TNM, tumor-node-metastasis; AFP, alpha-fetoprotein, pT, pathological stage.

TNM stage, differentiation, histological grade, histological cell type and vascular invasion, in the present study (P>0.05).

However, GPC3 protein was found to be positive in 61.9% (13/21) of all participants with normal sAFP levels (≤20 ng/ml), which was consistent with the results of other studies. Therefore, GPC3 may have more diagnostic value compared with AFP, the traditional biomarker for the diagnosis of liver cancer (43). Similar to this finding, Liu *et al* (11) reported that, when GPC3 was combined with AFP, the AUC and sensitivity values were increased from 0.879 and 79.52% to 0.925 and 88.10%, respectively. In addition, 43/68 AFP-negative patients had elevated sGPC3 levels. These findings indicated that GPC3 may serve as a valuable marker alongside AFP in HCC diagnosis.

The present study did, however, had certain limitations. First, the number of samples was small and, it was not possible to collect survival and recurrence data due to the limited source.

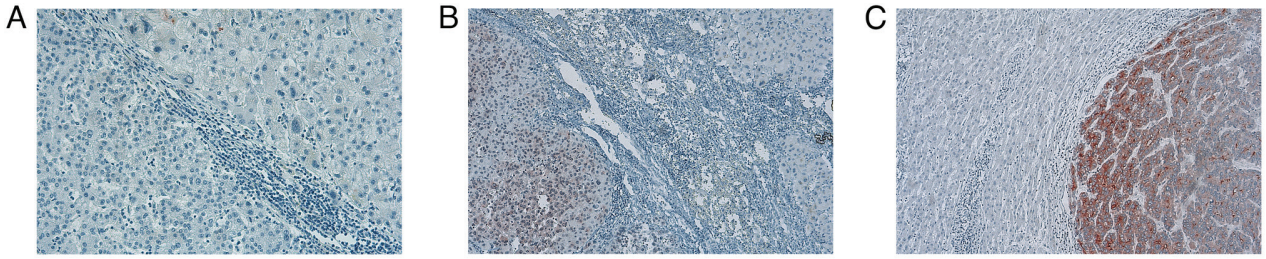

Figure 2. IHC staining of GPC3 in HCC tissue samples. Representative images of IHC slides including both tumor and tumor adjacent normal tissue are shown. (A) A liver tissue stained negatively for GPC3 with poorly differentiated hepatocellular carcinoma, marked by disorganized and bizarre cells. The tissue section shows irregular and distorted tumor cells against the adjacent liver tissue. The cancerous regions exhibit highly abnormal cell shapes and sizes, contrasting with the surrounding normal hepatocytes. (B) A liver tissue stained moderately for GPC3 with moderately differentiated hepatocellular carcinoma, characterized by a more irregular cellular arrangement. The GPC3 staining is patchy, highlighting areas of tumor cells against the adjacent liver tissue. The cancerous regions exhibit less organized trabeculae, contrasting with the surrounding normal hepatocyte. (C) A liver tissue stained strongly for GPC3 with a micro-trabecular pattern of well-differentiated hepatocellular carcinoma. The cancerous cells, marked by intense GPC3 staining, contrast with the adjacent normal liver tissue, highlighting the irregular trabeculae of the carcinoma against the orderly structure of the healthy hepatocytes. Magnification, x100. IHC, immunohistochemical; GPC3, glypican 3; HCC, hepatocellular carcinoma.

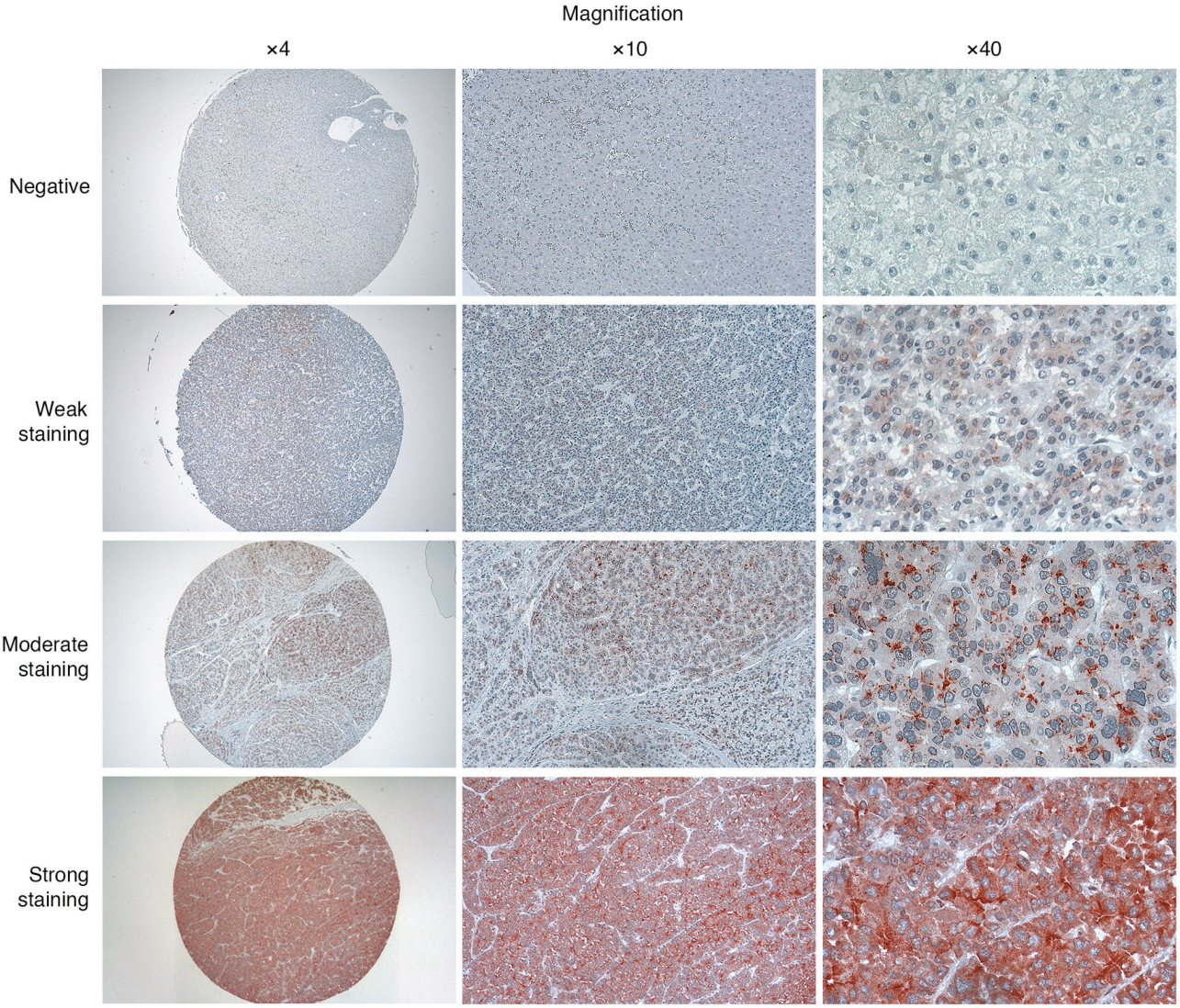

Figure 3. GPC3 staining intensities according to the tissue microarray analysis. GPC3 staining images with the indicated magnifications are shown. Hepatocellular carcinoma samples (n=50); negative (n=15), weakly positive (n=13), moderately positive (n=11), strong positive (n=11). GPC3, glypican 3.

Therefore, larger samples are required in future studies with survival analysis. Furthermore, benign liver diseases could not be included for making comparisons with the HCC tissue samples due to the small sample size. Lastly, the experiments

were conducted in a limited amount of time. In a future study, the authors plan to employ *in vitro* assays using an expression vector to further evaluate the potential of the marker.

In conclusion, in the present study, a high level of sGPC3 was observed in the HCC group, and this was found to be associated with the HCV status and cirrhosis. In the tissue analysis, GPC3 protein was specifically expressed in the cytoplasm, membrane and canaliculi of HCC. The expression of GPC3 was not found to be associated with other clinical features, such as age, sex, viral hepatitis, cirrhosis, tumor, size, histological cell type and vascular invasion. However, the tissue expression of GPC3 was directly correlated with the AFP level in the serum. Hence, it is considered that the potential importance of GPC3 in HCC diagnosis should be further studied by determining the amount of GPC3 protein in the serum of participants, and comparing this with the results of the IHC analysis.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Mongolian National University of Medical Sciences, Supporting Foundation for Science and Technology (grant no. 2022/24).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

BBat, MS, OT, NN and BK analyzed and interpreted patient data on ELISA, IHC and TMA. MS, BBat, OT and BM designed and conducted the present study. BBat, OT and NEN collected serum samples from the subjects. UG, GK, EB, MR, DEO, MBo, MBy, YA, MC, NG, AB, TD, LNO and BBay collected tissue samples and performed histological examinations of hepatocellular carcinoma and combined the clinical data of patients. BBat, BM and MS were major contributors to the writing of the manuscript. SJ, MBat and TL designed the present study, confirm the authenticity of all the raw data, revised the manuscript and gave the final approval for publishing the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Mongolian National University of Medical Sciences (approval nos. 2022/3-05 and 2022.05.20; Ulaanbaatar, Mongolia), and all procedures were conducted according to the Declaration of Helsinki. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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