Serum anti-osteopontin autoantibody as a novel diagnostic and prognostic biomarker in patients with hepatocellular carcinoma

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Abstract. Osteopontin (OPN) is a secreted phosphorylated and glycosylated protein, which plays an important role in carcinogenesis and metastasis. In hepatocellular carcinoma (HCC), OPN is being investigated either as a therapeutic target gene or as a biomarker for diagnosis. Yet, the role of the anti-OPN autoantibody in HCC remains unclear. In the present study, the level of serum anti-OPN autoantibody in HCC was analyzed by enzyme-linked immunosorbent assay. Immunohistochemistry (IHC) was also performed to analyze protein expression profiles and the prognostic significance of OPN in HCC. In this study, the prevalence and titer of anti-OPN autoantibodies in HCC were significantly higher than these values in normal human serum (NHS) (P=0.001, P=0.000, respectively). When both α-fetoprotein and the autoantibody against OPN were used simultaneously as diagnostic biomarkers, the sensitivity was up to 65%. In IHC, 59 of the 83 (65.6%) HCC specimens expressed OPN with cytoplasmic positive staining. The overall survival (OS) of HCC patients with OPN-positive tumors was 28.81 months compared to 39.37 months for HCC patients with OPN-negative tumors (P<0.01). Furthermore, multivariate analysis showed that OPN overexpression was the strongest independent adverse prognostic factor for OS (P=0.02). Taken together, our data indicate that the anti-OPN autoantibody may be a supplementary serological biomarker for HCC, and is correlated with poor prognosis in HCC patients.

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Abbreviations: HCC, hepatocellular carcinoma; OPN, osteopontin; CH, chronic hepatitis; NHS, normal human serum; AFP, α-fetoprotein; SPP1, secreted phosphoprotein 1; ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemistry; IIFA, indirect immunofluorescence assay; OS, overall survival; TAAs, tumor-associated antigens; PVDF, polyvinylidene fluoride; TBS, Tris-buffered saline; RGD, arginine-glycine-aspartate; SIBLING, small integrin-binding ligand N-linked glycoprotein; MAPK, mitogen-activated protein kinase pathway

Key words: osteopontin, autoantibody, hepatocellular carcinoma, biomarker, diagnosis

Introduction

Hepatocellular carcinoma (HCC), the fifth most common cancer worldwide, is not only a highly aggressive carcinoma of the liver but is also the third leading cause of cancer-related mortality (1). Hepatitis B virus, hepatitis C virus, and chronic heavy alcohol consumption leading to liver cirrhosis remain the most important causes (2). Few target molecules have been identified that enable the diagnosis of HCC with a high sensitivity and specificity, particularly in the early clinical stages of HCC. Currently α-fetoprotein (AFP) is widely used as a surveillance and detection test for HCC, despite its limited performance, particularly in early-stage HCC (3). Other molecular markers, for example, lectin-bound AFP (4), glypican-3 (5) and des-γ carboxyprothrombin (6) have been proposed for HCC detection. However, an ideal marker for HCC diagnosis and prognosis has not yet been identified. Development of novel biomarkers for the early diagnosis and prognosis of HCC is vital.

Our previous studies demonstrated that cancer serum contains antibodies that react with a unique group of autologous cellular antigens called tumor-associated antigens (TAAs), whose abnormal regulation or excessive expression are closely related to carcinogenesis (7,8). The TAAs in blood circulation rapidly degrade, and their use in early cancer diagnosis has been hampered by false-positive rates in the normal population. In contrast, autoantibodies can identify aberrant cellular antigens in carcinogenesis and serve as markers for cancer detection and surveillance since anti-TAA autoantibodies are more stable (9). Furthermore, the absence of these autoantibodies in normal individuals and non-cancer conditions makes them potential markers for different types of cancer (7). For example, p62, a fetal protein absent in adult tissues, is recognized as a TAA. The anti-p62 autoantibodies were found to be positive in 21% of HCC patients but negative in controls (10).

Osteopontin (OPN) is a secreted phosphorylated and glycosylated protein, which is expressed widely and has multiple functions in cell adhesion and migration (11), antiapoptosis (12),

immune and inflammatory responses (13,14), calcification (15) and suppression of nitric oxide synthase (16). In addition, it is also known as secreted phosphoprotein 1 (SPP1). In normal liver tissue, OPN is expressed in bile duct epithelium, stellate cells, and Kupffer cells but not in normal hepatocytes (17). Recent studies have confirmed that OPN plays an important role in carcinogenesis and metastasis (18,19). In many types of cancer, including breast (20), lung (21), colon (22) and kidney cancer (23) elevated expression of OPN was found to be associated with carcinogenesis, progression, metastasis and poor prognosis. Other studies further found elevated expression levels of serum OPN in HCC compared to liver cirrhosis (LC), chronic hepatitis (CH) and normal human serum (NHS) groups (24,25). However, little is known concerning the role of the autoantibody against OPN in HCC and its relationship with the prognosis of HCC patients.

In the present study, the autoantibody response to OPN in HCC and control groups was analyzed by enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay. Immunohistochemistry (IHC) with an HCC tissue array was also investigated to understand OPN protein expression profiles. In addition, the relationship between OPN expression and overall survival (OS) of HCC patients was analyzed to investigated the role of OPN in the prognosis of HCC patients.

Materials and methods

Serum samples from 148 HCC patients, 32 patients with CH, 32 patients with LC, and 75 normal controls were collected at The First Affiliated Hospital of Xi'an Jiaotong University (China) from 2011 to 2013. This study was approved by the Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University. All HCC serum samples (n=148) had been collected before patients were treated with chemotherapy, radiotherapy or surgery, and were pathologically confirmed. The normal controls were negative for the hepatitis virus and had no evidence of malignancy. All blood samples were collected in vacuum tubes, clotted at room temperature for 30 min, and then centrifuged at 3,000 rpm for 5 min. The suspension was distributed into 300-μl aliquots each and stored at -80˚C until analysis. The general information for these patients was available (Table I), and AFP levels of 140 of the 148 HCC serum samples were assessed by ELISA assay.

Indirect enzyme-linked immunosorbent assay (ELISA). Microtiter plates (96-well; Corning, NY, USA) were coated overnight at 4˚C with 100 μl/well recombinant osteopontin protein (R&D, Minneapolis, MN, USA) at a final concentration of 1.0 μg/ml in phosphate-buffered saline (PBS). Then the plates were blocked with 1% bovine serum albumin (BSA) in PBS, 300 μl/well, for 2 h at room temperature with gentle shaking. Following 4-time washes with PBST (0.1% Tween-20 in PBS), 100 μl/well diluted human serum (1:50) in 1% BSA/PBS was incubated for 2 h at room temperature in the antigen-coated wells with gentle shaking. Plates were washed 4 times again followed by 100 μl/well of HRP-conjugated goat anti-human IgG (1:4000 diluted) for 1 h. After 4 washes, 100 μl/well of tetramethylbenzidine (TMB) was added and the reaction was stopped by adding 50 μl 2 M sulphuric acid (H_2SO_4). The optical density (OD) of each well was read at a wavelength of 405 nm, and the mean OD of 75 NHS+3SD was designated as a cutoff value for a positive reaction.

Cell lines and cell extracts. Ten different cancer cell lines, human hepatocellular carcinoma cell lines (HepG2 and Hep3B), human breast cancer cell lines (MCF-7 and MDA-MB-453), human cervical cancer cell lines (HeLa and SiHa), human colorectal cancer cell line (HCT116), non-small cell lung cancer cell line (H1299), prostate cancer cell line (C42), and human epidermal carcinoma cell line (A431), were obtained from the Department of Transformation Medical Center of Xi'an Jiaotong University. Cancer cells were solubilized in extraction buffer supplemented with the protease inhibitor cocktail.

Western blotting. Cancer cell lysates were electrophoresed on 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) at 37˚C for 3 h and then incubated overnight at 4˚C with a 1:1,000 dilution of the rabbit monoclonal anti-SPP1 antibody (Epitomics, Burlingame, CA, USA) and a 1:5,000 dilution of the mouse monoclonal anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA). HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG (both at 1:2,000; Abcam Inc, Cambridge, MA, USA) were used as secondary antibodies. An enhanced chemiluminescence kit (Millipore Corporation, Billerica, MA, USA) was used to detect the immunoreactive bands.

Absorption of antibodies with recombinant protein. The serum was incubated with recombinant OPN protein (the final concentration of the protein diluted with HCC serum was 0.01 μg/μl) overnight at 4˚C, and then centrifuged at 10,000 x *g* for 15 min. The supernatant was the pre-absorbed serum used for the immunofluorescence assay.

Indirect immunofluorescence assay (IIFA). HeLa cells ($2x10^4$ cells/ml) were cultured in 12-well plates, after 24 h, serum (1:1) and pre-absorbed serum (1:1) were incubated with these HeLa antigen substrates overnight at 4˚C. FITC-conjugated goat anti-human IgG (Protein Tech Group, Inc., Chicago, IL, USA) was used as the secondary antibody at a 1:20 dilution. Fluorescence microscope (Leica DM1000, Germany) was used for examination.

Immunohistochemical (IHC) analysis of the tissue assay slides. HCC tissue array slides (including 90 HCC tissue specimens) with information regarding clinicopathological characteristics comprising age, gender, clinical stage, pathology grade, and prognosis information were commercially purchased (Outdo Biotech Co., Ltd., Shanghai, China). This tissue assay was used to detect the expression of OPN in HCC specimens and identify the relevant prognostic factors. The protocol for IHC was carried out as previously described (26). The primary antibody was rabbit monoclonal anti-SPP1 antibody (1:120 dilution; Epitomics).

Three independent pathologists evaluated and scored the IHC staining. Scoring of cytoplasmic OPN staining was

Table I. Characteristics of the patients for ELISA assay.

Group	Male/Female	Age range (years)	Mean age (years)	Viral infection			
				HBV	HCV	HBV+HCV	No virus
NHS	52/23	16-82	51.41	0	0	0	75
LC	28/4	27-65	45.91	27	3	0	2
CH	17/15	34-73	49.09	21	10	1	0
HCC	123/25	14-78	52.95	127	8	1	12

HCC, hepatocellular carcinoma; LC, liver cirrhosis; CH, chronic hepatitis; NHS, normal human serum; HBV, hepatitis B virus; HCV, hepatitis C virus.

Table II. Frequency of the autoantibody against OPN in human serum samples by ELISA.

Type of serum	No. tested	Autoantibody to OPN, n (%)
HCC	148	19 (12.8)[b]
LC	32	5 (15.6)[a]
CH	32	1 (3.1)
NHS	75	0 (0.0)

Cutoff value, mean + 3SD of NHS samples; [b]P=0.001, [a]P=0.002 relative to NHS; OPN, osteopontin; HCC, hepatocellular carcinoma; LC, liver cirrhosis; CH, chronic hepatitis; NHS, normal human serum.

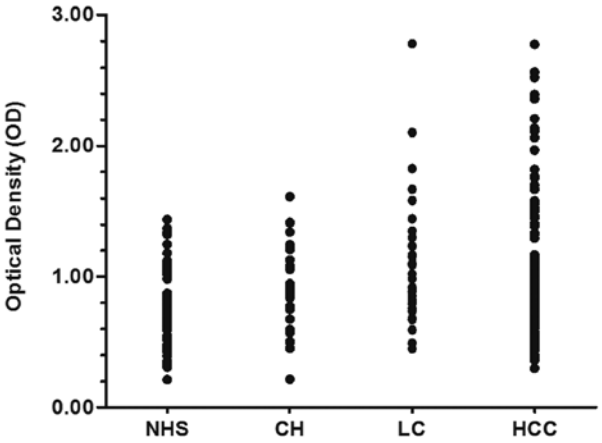

Figure 1. Titer of anti-OPN autoantibodies in human serum samples by ELISA. The range of antibody titers to OPN was expressed as optical density (OD) obtained from ELISA. The mean titer of NHS, CH, LC and HCC was 0.768, 0.902, 1.087 and 1.02, respectively. The mean + 3SD of the NHS samples was used as a cutoff value. Titer of anti-OPN in LC and HCC was much higher than that in the NHS group (P<0.001). OPN, osteopontin; NHS, normal human serum; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma.

based on both the staining intensity and extent according to a previous report (27). Microscopically, each section was observed randomly in 10 high-power fields (x40 magnification), each of which included 100 cells. First, the staining extent was scored according to the proportion of positive tumor cells: 0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%. Second, the staining intensity was scored: 0, no cell staining; 1, weak staining; 2, moderate staining; 3, strong staining. Finally, scores were calculated by multiplying the intensity and extent scores and the results were divided as follows: negative ('I', score: 0-1), weak positive ('II', score: 2-4), moderate positive ('III', score: 5-8) and strong positive ('IV', score: 9-12). Staining results were also divided into negative expression and positive expression for further study.

Statistical analysis. We adopted SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) for Windows to analyze the data. The mean OD value of each group was compared using the Mann-Whitney U test and the frequency of the autoantibody to OPN in each group of patient serum was compared by means of the χ^2 test with Fisher's exact test. Comparison of the clinicopathological parameters with OPN expression was conducted by the two-tailed Mann-Whitney U test. The survival curve was estimated using the Kaplan-Meier method, and the prognostic significance of these markers was analyzed by log-rank test. Univariate and multivariate Cox regression analyses were performed to analyze the relevant prognostic factors. Two-tailed P-values <0.05 were considered to indicate statistically significant differences.

Results

Frequency and titer of the autoantibodies against OPN in HCC, LC, CH and NHS groups. Recombinant human OPN protein was used in ELISA as a coated antigen with which to detect autoantibodies against OPN in serum samples from 148 HCC patients, 32 CH patients, 32 LC patients and 75 healthy individuals. The mean titer of the autoantibodies against serum OPN in the NHS group was 0.768±0.266, the mean + 3SD of the NHS samples (1.566) was used as a cutoff value. Table II shows that the prevalence of autoantibodies against OPN was 12.8% (19/148) in HCC, 15.6% (5/32) in LC, 3.1% (1/32) in CH and 0% (0/75) in the NHS group. Compared with these 4 groups, the prevalence of anti-OPN autoantibodies in the HCC (P=0.001) and LC (P=0.002) group was significantly higher than that in the NHS groups. As shown in Fig. 1, the titer of the autoantibodies against OPN in the 4 groups was different (P=0.001). The titer in the HCC and LC groups was higher than that in the NHS group (both P=0.000). The average titer of the anti-OPN autoantibodies was 1.015±0.502, 1.087±0.498, 0.902±0.328 and 0.768±0.266 in the HCC, LC,

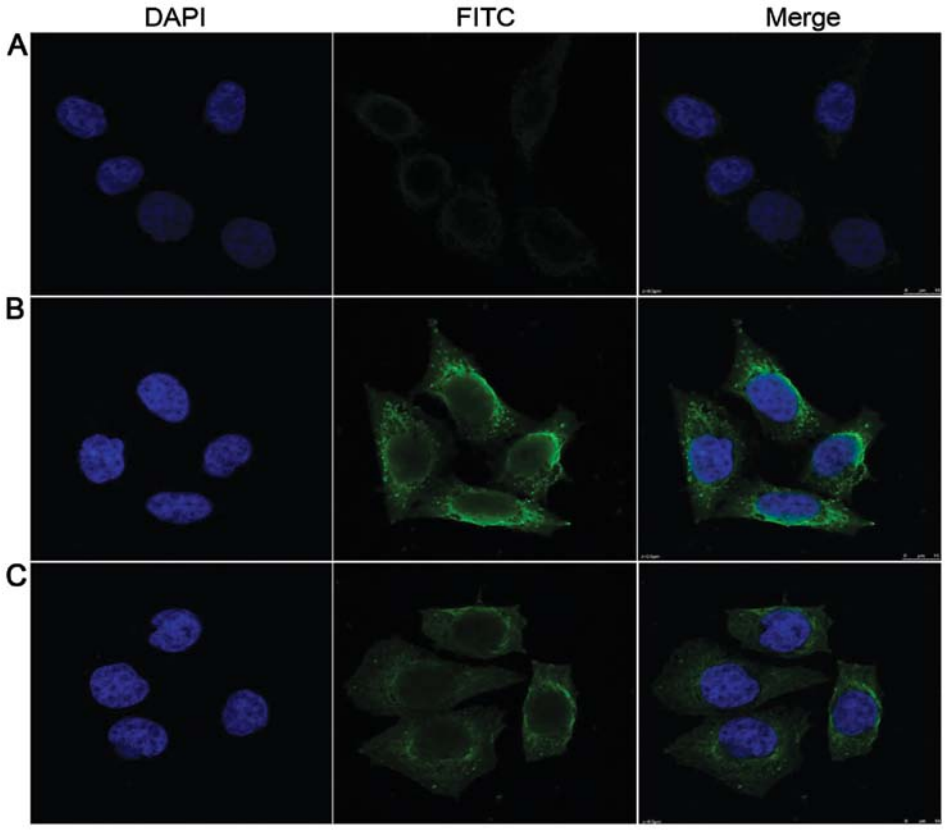

Figure 2. Representative immunofluorescence staining pattern of anti-OPN antibody-positive HCC serum samples. (A) A normal human serum sample. (B) A representative anti-OPN antibody-positive HCC serum sample. (C) The same HCC serum sample in B was pre-absorbed with recombinant OPN protein. OPN, osteopontin; HCC, hepatocellular carcinoma.

CH, and NHS group, respectively. In fact, of the 148 HCC patients, 140 patients were tested for both AFP and anti-OPN autoantibody levels by ELISA. The AFP level in 84 (60%) HCC patients was >100 ng/ml, whereas in 75 (53.6%) HCC patients the level was >200 ng/ml. When both AFP and anti-OPN autoantibody were used simultaneously as biomarkers, 91 (65%) HCC patients (AFP >100 ng/ml) were positive, and 83 (59.3%) HCC patients (AFP >200 ng/ml) were positive, respectively.

Perinuclear intense staining pattern detected in HeLa cells by indirect immunofluorescence assay with representative positive HCC serum. Indirect immunofluorescence was used to confirm the reactivity of OPN autoantibodies in HCC serum samples and the intracellular location of OPN. In this assay, HeLa cell slides and HCC serum samples with anti-OPN-positive expression in ELISA were selected. As shown in Fig. 2, HCC serum samples with anti-OPN-positive expression had a perinuclear staining pattern. When the same HCC serum samples were pre-absorbed by recombinant OPN protein, the fluorescent staining was significantly reduced.

OPN expression in the different cancer cell lines. OPN contributes to carcinogenesis and metastasis. To determine the expression levels of OPN protein in different tumor cell lines, 10 tumor cell lines (MCF-7, MDA-MB-453, SiHa, HeLa, H1299, HCT116, C42, A431, HepG2, Hep3B) were cultured, and analyzed by western blotting. As shown in Fig. 3, MCF-7,

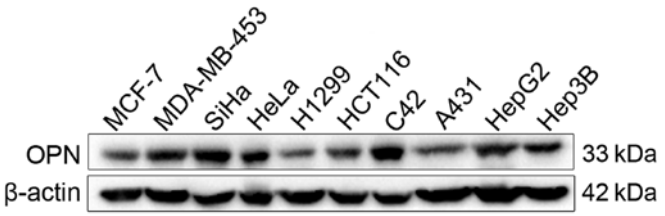

Figure 3. Ten tumor cell lines analyzed by western blotting. The monoclonal anti-OPN antibody was used as a probe. MDA-MB-453, SiHa, HeLa, C42, HepG2 and Hep3B cells exhibited strong reactivity. MCF-7, H1299, HCT116 and A431 cells exhibited weak reactivity compared to the cell lines which had clear reactive bands.

H1299, HCT116 and A431 cancer cell lines showed relatively weaker reactive bands compared to those cell lines that had strong reactivity such as MDA-MB-453, SiHa, HeLa, C42, HepG2 and Hep3B.

Expression of OPN in hepatocellular carcinoma tissues. Expression of OPN protein in the HCC tissues was examined by immunohistochemistry with tissue array slides. The monoclonal anti-OPN antibody was used as a primary antibody to detect the expression of OPN. The expression of OPN showed no correlation with clinical and pathological characteristics such as age, gender, tumor size, histological grade, capsular infiltration and portal vein invasion ($P<0.05$). The patients included 77 males and 13 females, with a mean age of 54.51±9.49 years (range 18-73 years). The expression of OPN

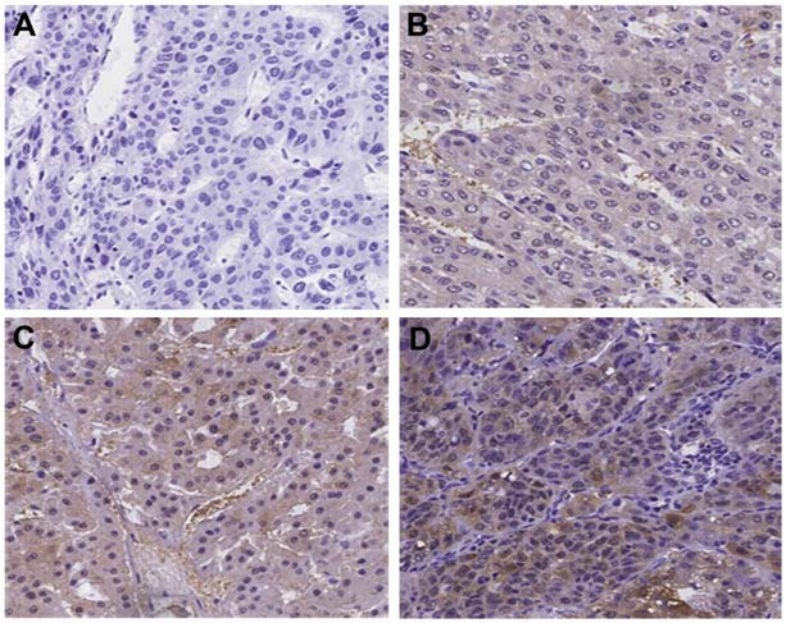

Figure 4. Expression of osteopontin (OPN) in the HCC tissue array. (A) Negative OPN immunohistochemical staining. (B) Weak positive OPN immunohistochemical staining. (C) Moderate positive OPN immunohistochemical staining. (D) Strong positive OPN immunohistochemical staining (A-D: magnification, x200).

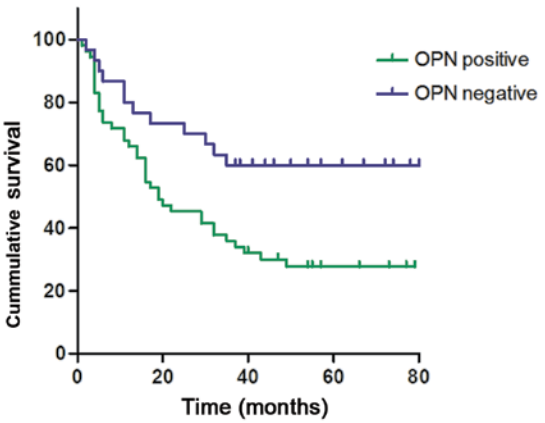

Figure 5. Kaplan-Meier curves for cumulative overall survival of the HCC patients. The HCC patients with OPN-positive expression had a worse survival rate than those with OPN-negative expression. (P<0.01).

protein in the HCC tissues is shown in Fig. 4. Of the total 90 HCC specimens, 59 HCC samples (65.6%) were positive for OPN staining and OPN-positive staining was observed in the cytoplasm of the cancer cells. However, there was no significant correlation between patient age, gender, tumor size, tumor differentiation grade and OPN expression.

Furthermore, the HCC patients of the tissue array slides were followed up over the course of 3-7 years (7 patients were lost to follow-up). At the end of the follow-up period, 33 HCC patients were alive and 50 HCC patients were deceased. We further analyzed the relationship between OPN expression and overall survival (OS) of the HCC patients. Mean OS was 39.37±23.48 months in the HCC patients with negative OPN expression. In contrast, mean OS in the patients with positive OPN expression was 28.81±24.22 months. OPN expression was significantly correlated with OS of the HCC patients (Table III). Kaplan-Meier curve and log-rank test

Table III. Univariate analysis of the associations between prognostic variables and overall survival in the HCC patients.

Markers	n	P-value (OS)
Age (years)		0.893
≤55	45	
>55	38	
Gender		0.367
Male	70	
Female	13	
Tumor size (cm)		0.012
≤5	35	
>5	47	
Histological grade		0.432
I-II	52	
III	31	
Capsular infiltration		0.035
With	2	
Without	81	
Portal vein invasion		0.016
With	6	
Without	77	
Lymph node metastasis		0.257
With	1	
Without	82	
OPN		0.009
Positive	53	
Negative	30	

HCC, hepatocellular carcinoma; OPN, osteopontin; OS, overall survival.

Table IV. Multivariate analysis of overall survival according to the Cox model.

				95% CI for Exp(B)			
Factors	B	S.E.	Exp(B)	Lower	Upper	Wald	P-value
OPN	0.742	0.336	2.099	1.086	4.059	4.859	0.020
Tumor size	0.049	0.020	1.050	1.009	1.093	5.675	0.028

B, Cox regression coefficient; Exp(B), risk ratio; 95% CI, 95% confidence interval; OPN, osteopontin.

showed that HCC patients with positive OPN expression had shorter OS than those with negative OPN expression (P<0.01) (Fig. 5). Overexpression of OPN in HCC is an independent prognostic factor for OS of these HCC patients. Cox multivariate analysis showed that OPN-positive expression and tumor size were prognostic factors (Table IV), and the hazard ratio of OPN expression was 2.099 (95% CI 1.086-4.059; P=0.02). Collectively, these data indicate that HCC patients with OPN-positive expression have a poor prognosis.

Discussion

OPN is an arginine-glycine-aspartate (RGD)-containing acidic member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family of proteins. Various tissues including brain, liver, lung, bone, kidney can produce this protein, and it can be detected in different fluids, for example, blood, urine, milk and seminal fluid (28). Previous studies found that OPN can be produced by activated lymphocytes and macrophages as an early T-cell activation factor and plays a role as a cytokine (29,30). OPN is also a protein with diverse functions due to its different functional domains, such as calcium-binding domain, aspartate-rich domain, heparin-binding domain, thrombin cleavage site, α9β1/α4β1 domain as well as an integrin-binding RGD motif (31). The multifunction OPN has been revealed in promoting tumor formation and prognosis. It exerts these functions through direct binding to integrin and/or CD44, and the subsequent activation of various pathways leads to an increased malignant phenotype (32). Other studies have also reported the related signaling pathways and mechanisms of OPN. For example, OPN protein can trigger the mitogen-activated protein kinase pathway (MAPK) to promote tumor growth and matastasis, while the effect can be reversed through the knockdown of OPN expression (33). OPN can also induce NF-κB activity through phosphorylation and degradation of IκBα by activating IKK that ultimately triggers the activation of pro-MMP-2 (34).

OPN is an attractive potential tumor marker, since it functions not only as an immobilized extracellular matrix molecule but also as a secreted form in body fluids including serum. Previous studies have demonstrated that OPN is overexpressed with invasion and metastasis in a wide variety of human malignancies including HCC (20,21,24). The role of OPN in cancer has recently attracted attention. Our study showed that OPN was expressed at a higher level in MDA-MB-453, SiHa, HeLa, C42, HepG2 and Hep3B cell lines, while expressed relatively weaker in MCF-7, H1299, HCT116 and A431 cell lines. In addition, an immunohistochemistry assay was further used to detect the OPN protein expression profiles. Most importantly, the data showed that the OS of HCC patients with OPN-positive expression was 28.81±24.22 months which was significantly shorter than 39.37±23.48 months of HCC patients with OPN-negative expression. Furthermore, multivariate analysis showed that OPN overexpression was the strongest independent adverse prognostic factor for OS. Taken together, these results suggest that overexpression of OPN in HCC is related to poor prognosis of HCC patients, which is consistent with the findings of previous studies. Collectively, OPN may be considered as an independent prognostic marker of HCC. Further studies have found that OPN is related to capsular infiltration and portal vein invasion (25,27). Yet, our study did not find a relationship due to the limited pathological information or the small sample size.

However, the role of anti-OPN autoantibody levels in HCC patients is not clearly known although elevated anti-OPN autoantibodies have been detected in prostate cancer (35) and rheumatoid arthritis (36). In the present study, we demonstrated that 12.8% of HCC patients expressed autoantibodies against OPN, and the frequency and the mean titer of the anti-OPN antibodies in HCC serum samples were higher than these values in normal human serum (NHS). However, there was no statistical differences between the HCC group and the LC group or the chronic hepatitis (CH) group, which may be because of the limited patient population of the two groups. Notably, when both the anti-OPN antibody and α-fetoprotein (AFP) were used as diagnostic biomarkers simultaneously, the sensitivity of HCC diagnosis reached 65%, which was higher than that using the single anti-OPN autoantibody or AFP as a biomarker. Moreover, indirect immunofluorescence assay was also used to confirm the immune response of the anti-OPN autoantibody in HCC serum samples to recombinant OPN protein. Collectively, these data indicate that the anti-OPN autoantibody may be a potential diagnostic marker for HCC, particularly in conjunction with AFP.

In conclusion, we identified a high level of anti-OPN autoantibody in HCC serum samples and the poor prognosis of HCC patients with OPN-positive expression. It is plausible that OPN and the anti-OPN autoantibody are closely correlated to HCC; both may play an important role in the diagnosis and prognosis of HCC. Further studies with large sample size and detailed pathological information are warranted, and research aimed to investigate the mechanisms of OPN and the anti-OPN autoantibody in carcinogenesis, progression and metastasis will also be proposed.

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