

Osteopontin promotes hepatocellular carcinoma progression via the PI3K/AKT/Twist signaling pathway

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Abstract. The epithelial-mesenchymal transition (EMT) serves critical roles in the migration, invasion and metastasis of human cancer cells. This process is initiated by regulation of E-cadherin expression by the major inducers of EMT. Previous studies reported that osteopontin (OPN) is essential for hepatocellular carcinoma (HCC) metastasis as it facilitates the EMT in HCC. However, the role and clinical significance of OPN as an EMT regulator in HCC remains unknown. The present study revealed that OPN regulated the expression of Twist by activating RAC serine/threonine-protein kinase (Akt), a critical EMT regulator. Interfering with the phosphoinositide 3-kinase (PI3K)/Akt pathway may suppress the expression of Twist enhanced by OPN. Increased Twist levels in HCC were associated with poor survival and tumor recurrence in patients with HCC following surgery. A significant association was observed between OPN expression and Twist levels in HCC, and a combination of these two parameters was revealed to be a more powerful predictor of poor patient prognosis. The findings of the present study indicate that Twist serves an notable role in OPN-mediated metastasis of HCC through activation of the PI3K/Akt pathway. Twist may be a potential therapeutic target for the prevention of HCC metastasis in patients exhibiting high OPN expression.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of cancer and primarily occurs in South Africa and Asian countries (1,2). According to statistical analyses, the incidence of liver cancer has increased in the majority of countries over the past 5 years (1). China alone accounted for ~50% of cases of newly diagnosed liver cancer and mortality in 2012 (2). Although curative resection is beneficial for the long-term survival of patients with HCC, the prognosis of these patients remains poor owing to the high rate of metastasis and recurrence (3,4). Therefore, identifying more accurate prognostic biomarkers of HCC is of great clinical value for the understanding HCC and to develop novel therapeutic strategies.

Osteopontin (OPN), a secreted glycosylated phosphoprotein encoded by the secreted phosphoprotein 1 gene, has been implicated as being a major mediator and potential therapeutic target of cancer metastasis (5,6). A previous study has demonstrated that OPN is a ligand that binds to $\alpha\beta$ integrins or receptors of the cluster of differentiation 44 family to promote cell adhesion, extracellular matrix degradation, and the prevention of apoptosis, angiogenesis and indolent tumor growth (7). Furthermore, OPN has been identified as a key inducer of tumor invasion and metastasis (8,9).

The epithelial-mesenchymal transition (EMT) serves a major role in tumor metastasis, a developmental process whereby E-cadherin, an epithelial marker, is downregulated and vimentin and N-cadherin, which are mesenchymal markers, are upregulated (10-12). Additionally, the EMT results in reduced epithelial cell intercellular adhesion and causes these cells to acquire fibroblastoid properties, thereby improving the ability of cells to migrate. Therefore, the EMT is important for the development, invasion and metastasis potential of cancer.

Twist, a major EMT regulator, is essential for tumor metastasis (13,14). In the process of metastasis, Twist serves a crucial role in the EMT by downregulating E-cadherin and β -catenin, and by regulating cell motility, invasiveness and metastasis (14-17). Furthermore, Twist expression was observed to be increased in different types of tumor, including prostate cancer (17), melanoma (18), pediatric osteosarcoma (19), T-cell lymphoma (20), gastric cancer (21), breast carcinoma (22) and

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HCC (23,24). Previous studies have proven that Twist and Snail, but not Slug, are major EMT inducers in HCC, and that Twist serves a major role in hepatitis C-associated HCC, unlike Snail (25).

A recent study demonstrated that OPN promotes EMT of HCC (8). The present study examined the role and clinical significance of OPN on the EMT regulator, Twist, in HCC cell lines and tumor tissues. OPN was revealed to regulate the expression of Twist, a major regulator of HCC metastasis. Furthermore, interfering with the phosphoinositide 3-kinase (PI3K)/RAC serine/threonine-protein kinase (Akt) pathway may suppress the expression of Twist enhanced by OPN. Therefore, we hypothesize that OPN and Twist may serve as synergistic prognostic biomarkers and therapeutic targets for HCC.

Materials and methods

Patients and follow-up. A total of 374 patients, including 306 males and 68 females (age range, 30-70 years, median age 55 years) with HCC, underwent a hepatectomy at the Liver Cancer Institute, Zhongshan Hospital, Fudan University (Shanghai, China) by the same surgical team between March 2004 and December 2006. In addition, these patients had not received any neo-adjuvant or adjuvant treatments, but did undergo a pathological examination and complete follow-up. Formalin-fixed, paraffin-embedded tissues, which included 374 HCC patient tissues and 192 matched adjacent non-tumor tissues were used to organize a tissue microarray (TMA) for immunohistochemistry (IHC) studies. The clinicopathological characteristics of patients whose tissues were used for the TMA are summarized in Table I. The present study was approved by the Research Ethics Committee of Zhongshan Hospital, Fudan University (Shanghai, China) and written informed consent was obtained from each patient. Follow-up was completed in May 2013. The follow-up procedures and treatment modalities following relapse are described in previous studies (26-28). To diagnose recurrence, α -fetoprotein (AFP) levels were analyzed and computed tomography (CT) and/or magnetic resonance imaging (MRI) scans were performed. Patient mortality and disease recurrence were used as endpoints and the endpoints included the overall survival (OS) time and the time to recurrence (TTR). The OS time was defined as the interval between the dates of surgery and mortality. The TTR was defined as the time between surgery and the first report of intrahepatic or distant recurrence or the last follow-up for patients who had not experienced recurrence at the time of mortality (patients who had succumbed to other causes were not included) (29). The TTR was recorded at the date of mortality or the last follow-up (30,31).

TMA and IHC. The resected specimens (2-3 mm) were fixed in 10% formalin for four days at room temperature, and send to Pathology department of Zhongshan hospital. The construction of the TMA (in collaboration with Shanghai Biochip Co., Ltd., Shanghai, China) and IHC were performed as described previously (32). Immunostaining was performed on TMA slides using a two-step process according to the manufacturer's protocols. Following deparaffinization, 4 μ m sections were rehydrated in a descending alcohol series and subjected to

antigen retrieval by microwaving in 0.01 mol/l sodium citrate (pH 6) for 10 min. When microwaving, sodium citrate was boiled at a \sim 100°C and sections placed in it, followed by the temperature \sim 30-40°C for 10 min, followed by the sections being allowed to cool naturally to room temperature. Then, sections were washed using phosphate buffered saline (PBS).

Sections were incubated at 4°C overnight with monoclonal antibodies against OPN (dilution, 1:100; cat no. ab8448; Abcam, Cambridge, UK) and Twist (dilution, 1:100; cat no. ab50581; Abcam). Immunostaining was performed using ChemMate DAKO EnVision Detection kit, Peroxidase/DAB, Rabbit/Mouse (cat no. GK500705; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA), according to the manufacturer's protocol. Subsequently, the sections were counterstained with hematoxylin at room temperature until the microscopic observation of sections were discoloration, used PBS to rinse and soaked it for 2 min, then mounted in dimethyl benzene. Negative controls were included in all assays and were treated identically but with the primary antibodies omitted. An optical microscope was used at a magnification x200.

The intensity of staining was scored manually (0, no staining; 1, weak staining; 2, moderate; and 3, strong staining) by two independent experienced pathologists. Tumor cells in 5 randomly selected fields were scored based on the proportion of positively stained cells (0-100%). The final IHC scores were determined by multiplying the intensity scores and the proportion scores of the positive cells. Expression levels of OPN and Twist in all 374 samples were quantified. 'High' vs. 'low' OPN and Twist expression was defined according to the cut-off values of OPN and Twist level, which were defined as the median of the cohort. To evaluate the combined influence of OPN and Twist on the prognosis of patients, the 374 patients with HCC were separated into four groups: Group I, patients with low OPN and low Twist expression (n=117); Group II, patients with high OPN and low Twist expression (n=65); Group III, patients with low OPN and high Twist expression (n=87); and Group IV, patients with high OPN and high Twist expression (n=105).

Cell lines and plasmids. Three human HCC cell lines with various metastatic potentials, MHCC97-L, MHCC97-H and HCC-LM3, and the human non-transformed hepatic L-02 cell line, were used in the present study. MHCC97-L, MHCC97-H and HCC-LM3 with stepwise increasing metastatic potential were established from the same parent human HCC cell line at the Liver Cancer Institute, Fudan University (Shanghai, China). They have a genetically identical background (33,34). The L-02 cells were obtained from American Type Culture Collection (Manassas, VA, USA). These cell lines were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS; both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified incubator containing 5% CO₂.

Expression vectors for OPN and short hairpin RNA targeted at OPN (shOPN), as well as methods of cell transfection, were constructed as previously described (8). Twist small interfering RNA (siRNA) constructs were obtained from Sigma-Aldrich; Merck KGaA. The sequences of the primers used were as follows: Twist-siRNA forward, 5-gatccGCTG

Table I. Association between Twist expression levels and clinicopathological characteristics in HCC patients.

Variable	Twist expression, n		P-value
	Low (n=204)	High (n=170)	
Sex			0.687
Female	39	29	
Male	165	141	
Age, years			0.467
≤50	98	89	
>50	106	81	
HBsAg			0.543
No	16	10	
Yes	188	160	
ALT, U/l			0.498
≤75	185	150	
>75	19	20	
Liver cirrhosis			0.745
No	24	18	
Yes	180	152	
AFP, ng/ml			0.830
≤20	74	64	
>20	130	106	
Tumor size, cm			0.613
≤5	158	136	
>5	46	34	
Tumor number			0.052 ^a
Single	190	166	
Multiple	14	4	
Tumor capsule			0.677
Complete	112	89	
None	92	81	
Vascular invasion			0.013
No	153	107	
Yes	51	63	
Tumor differentiation			0.633
I/II	155	125	
III/IV	49	45	
BCLC stage			0.157
0 and A	59	38	
B and C	145	132	

HBsAg, hepatitis B surface antigen; AFP, α -fetoprotein; ALT, alanine amino transferase; BCLC, Barcelona Clinic Liver Cancer. ^aFisher's exact tests, and χ^2 tests for all other analyses.

AGCAAGATTCAGACCTtcaagagaGGTCTGAATCTTGCTCAGCttttta-3 and reverse, 3-gCGACTCGTTCTAAGTCTGgaagttctctCCAGACTTAGAACGAGTCGaaaaattcga-5 and Twist-siRNA-scramble forward, 5-gatccCGGTAACACAGACTGCAGTtcaagagaACTGCAGTCTGTGTTACCGttttta-3 and

reverse, 3-gGCCATTGTGTCTGACGTCAaagttctctGCCATTGTGTCTGACGTCAaaaaattcga-5. Recombinant plasmids were prepared as described previously (34). Then, 10 μ g plasmids were transfected into the MHCC-97L cells using lipofectamine 2000 (cat no. 11668019; Thermo Fisher Scientific, Inc.). Subsequently, the cells were collected after 24 h and cells were cleaved to extract protein for western blot analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from HCLM3, MHCC97H, MHCC97L and L02 cell lines (that had not undergone transfection) and frozen tumor specimens using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (1 μ g) was reverse transcribed using PrimeScript[®] Reverse Transcriptase Master mix (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocols. The sequences of the primers used were as follows: Twist forward, 5-GTCCGCAGTCTTACGAGGAG-3 and reverse, 5-GCTTGAGGGTCTGAATCTTGCT-3; and β -actin forward, 5-CATGTACGTTGCTATCCAGGC-3 and reverse, 5-CTCCTTAATGTCACGCACGAT-3. An AceQ[®] qPCR SYBR Green Master mix kit (Vazyme, Piscataway, NJ, USA) was used for qPCR. β -actin was used as the reference gene. Amplification and detection were performed using the ABI PRISM[®] 7900HT Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Thermocycling conditions were as follows: 50°C for 2 min (required for optimal AmpErase UNG activity Applied Biosystems; Thermo Fisher Scientific, Inc.), template denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 sec, and combined primer annealing/elongation at 60°C for 1 min. The Twist level was normalized to β -actin to yield a $2^{-\Delta\Delta C_q}$ value for relative expression of Twist (35).

Detection of protein by western blot analysis. Cells lysates were prepared as described previously (8). RIPA lysis buffer (cat no. P0013E; Beyotime Institute of Biotechnology, Haimen, China) was used for lysis. Protein concentrations were measured using a Bicinchoninic Acid Assay kit (Pierce; Thermo Fisher Scientific, Inc.). A total of 40 μ g/ μ l of protein was separated using SDS-PAGE (5% concentration gel and 10% separation gel), according to protein mass and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). 5% skimmed milk was used to block polyvinylidene fluoride membranes at room temperature for one h. Proteins were then incubated with primary antibodies against OPN (cat no. sc-21742; dilution, 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and Twist (cat no. ab50581; dilution, 1:1,000; Abcam) at 4°C overnight. A mouse anti-human monoclonal antibody against GAPDH (cat no. 8884; dilution, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) was used as an internal control. In addition, primary antibodies against Akt (cat no. ab8805; dilution, 1:1,000; Abcam), p-Akt (cat no. ab38449; dilution, 1:1,000; Abcam), matrix metalloproteinase 2 (MMP2) (cat no. ab37150; dilution, 1:1,000; Abcam) and urokinase (uPA) (cat no. ab82220; dilution, 1:1,000; Abcam) were used to analyze the mechanism of EMT. Secondary antibodies were goat anti-rabbit IgG (dilution, 1:5,000; cat no. ab6721; Abcam) and goat anti-mouse IgG

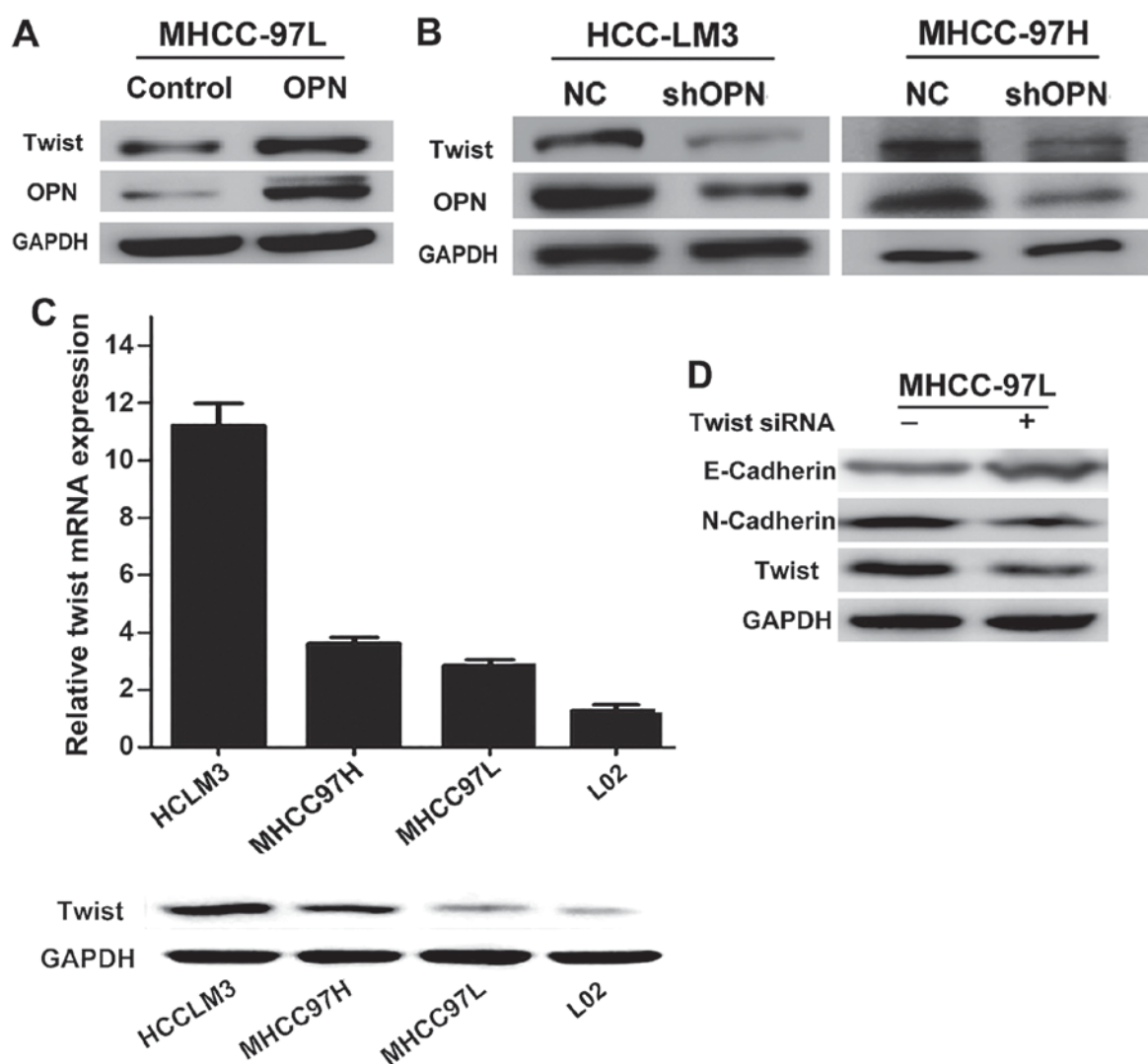


Figure 1. OPN enhances Twist expression in HCC cell lines. (A) Western blot analysis demonstrates the effect of OPN overexpression on the expression of Twist in HCC cell lines. (B) Western blot analysis demonstrates the effect of knockdown of OPN on the expression of Twist in HCC cell lines. (C) Top panel represents the mRNA expression of Twist by RT-qPCR, and the bottom panel is the protein expression of Twist by western blot analysis. The mRNA levels of Twist in HCC cell lines with different metastatic potentials, as determined by reverse transcription-quantitative polymerase chain reaction. Western blot analysis also demonstrated that Twist expression was increased in human HCC cell lines with increasing metastatic potentials. (D) EMT-associated markers were detected in the MHCC-97L-OPN cell line transfected with siTwist or scrambled siRNA. OPN, osteopontin; HCC, hepatocellular carcinoma; EMT, epithelial-mesenchymal transition; siRNA, small interfering RNA; siTwist, siRNA targeted at Twist; NC, negative control; shOPN, short hairpin RNA targeted at OPN.

(dilution, 1:5,000; cat no. ab6789; Abcam). We used the ECL Western Blotting Detection Kit (Thermo Fisher Scientific, Inc) to detect immobilized specific antigens in chemiluminescent Western blots through horseradish peroxidase (HRP) labeled antibodies. The bands were quantified using ImageJ v.2.0 software (National Institutes of Health, Bethesda, MD, USA). In order to improve the accuracy of the present study, each experiment was repeated ≥ 3 times.

Chemicals. LY294002 (cat no. s1105; Selleck Chemicals, Houston, TX, USA), which is able to inhibit Akt activation to assess whether PI3K/Akt signaling was involved in OPN-mediated metastasis. MHCC 97L cells with OPN overexpression were harvested after 1 h incubation with 50 $\mu\text{mol/l}$ PI3K/Akt inhibitor LY294002 (Selleck Chemicals) to suppress Akt activation, and collected cells to extract protein for western blot analysis.

Statistical analysis. Statistical analyses were performed using SPSS 15.0 (SPSS, Inc., Chicago, IL, USA). The Kaplan-Meier method was used to create survival and recurrence curves and to estimate OS and TTR. The significance of OS and TTR was determined using the log-rank test. Fisher's exact and χ^2 tests were used to demonstrate clinicopathological association. Univariate and multivariate analyses were performed using Cox's proportional hazards model. Values are expressed as the mean \pm standard deviation. All statistical tests were two-sided and $P < 0.05$ was considered to indicate a statistically significant difference. For OPN or Twist density, the cut-off for the definition of subgroups was the median value. Samples were separated into two groups for each analysis. The first group was comprised of HCC with OPN and/or Twist levels exceeding the median value, and the second group comprised the rest. Each data set was analyzed separately.

Results

OPN enhances Twist expression in HCC cell lines. Recent studies have demonstrated that OPN may induce EMT in HCC (8,36). To determine the effects of OPN on the EMT regulator Twist in HCC metastasis, the expression of Twist was analyzed in HCC cell lines with downregulated or upregulated expression of OPN. Twist expression was elevated when OPN was overexpressed in the MHCC-97L cell line, which is usually not highly metastatic and exhibits decreased levels of OPN (Fig. 1A). However, downregulation of OPN in highly metastatic the HCC MHCC97-H and HCC-LM3 cell lines markedly reduced Twist expression (Fig. 1B). Therefore, we hypothesized that OPN may serve an important role in Twist expression and therefore, may affect the metastasis of liver cancer.

To evaluate the association between OPN, Twist and HCC metastasis, Twist levels were detected in a panel of human HCC cell lines with different metastatic potentials. Expression levels of Twist protein and mRNA were substantially increased in three established HCC cell lines compared with the non-transformed hepatic L-02 cell line (Fig. 1C). Additionally, the expression levels of Twist in the highly metastatic HCC MHCC97-H and HCC-LM3 cell lines, which exhibit increased levels of OPN, were much higher than that in the HCC MHCC97-L cell line, which is not highly metastatic and exhibits a low expression of OPN. These data indicated that expression of Twist is upregulated in HCC cell lines and that this increased expression is positively associated with the malignant phenotype of HCC cells.

The present study also aimed to determine whether or not Twist is involved in OPN-induced EMT. EMT markers were detected in the lowly metastatic HCC MHCC97-L cell line, which exhibits stable overexpression of OPN when transfected with a siRNA targeted at Twist (siTwist) or scrambled siRNA. Knockdown of Twist significantly reduced the expression of N-cadherin and increased the expression of E-cadherin (Fig. 1D). These results demonstrated that Twist is required for OPN-driven EMT.

OPN increases the expression of Twist via the PI3K/Akt signaling pathway. Previous studies have elucidated the mechanisms of metastasis induced by OPN, including the role of the mitogen-activated protein kinase, nuclear factor- κ B and the PI3K/Akt pathways (9,37). The PI3K/Akt pathway is crucial in promoting invasion and metastasis and has been documented to be involved in the EMT of several types of human cancer (38-40). To assess whether PI3K/Akt signaling was involved in OPN-mediated metastasis, the present study examined the effect of OPN on the activation of PI3K/Akt. Phosphorylation of Akt was notably enhanced by OPN overexpression, whereas knockdown of OPN significantly decreased the phosphorylation of Akt (Fig. 2A and B). Consistently, suppression of Akt activation by the specific PI3K/Akt inhibitor LY294002 markedly attenuated the expression of OPN-induced Twist (Fig. 2C). These results indicated that the PI3K/Akt pathway is critical in the increased expression of Twist induced by OPN.

To investigate whether OPN mediated metastasis via the PI3K/Akt/Twist pathway, genes associated with metastasis, including MMP2 and uPA, were further investigated. As

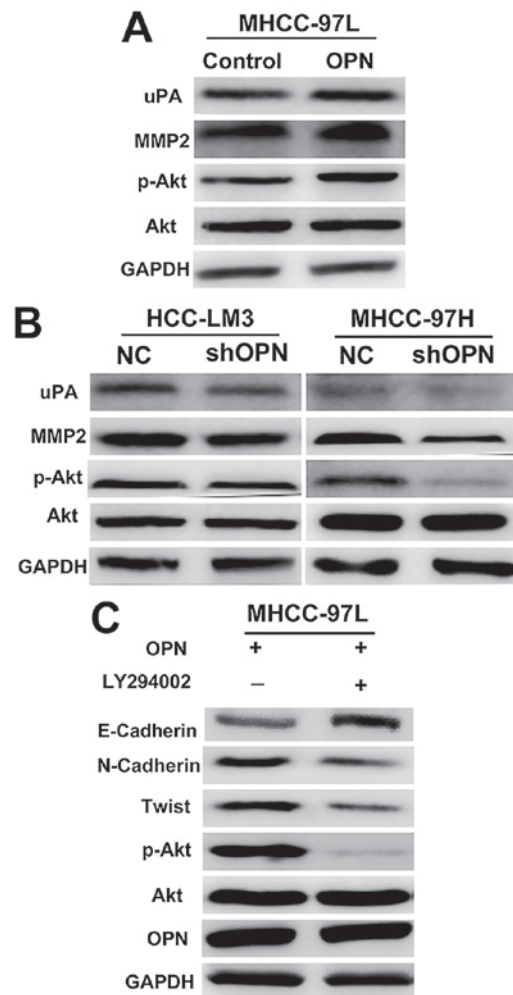


Figure 2. OPN induced the expression of Twist through the PI3K/Akt signaling pathway. (A) Western blot analysis demonstrates the effect of overexpression of OPN on the protein levels PI3K and Akt in HCC cells. (B) Western blot analysis depicting the effect of knockdown of OPN on the protein levels of PI3K and Akt in HCC cells. (C) Suppression of Akt activation by the specific PI3K/Akt inhibitor LY294002 markedly attenuated OPN-elicited EMT. OPN, osteopontin; PI3K, phosphoinositide 3-kinase; Akt, RAC serine/threonine-protein kinase; HCC, hepatocellular carcinoma; EMT, epithelial-mesenchymal transition; NC, negative control; uPA, urokinase; MMP2, matrix metalloproteinase 2; p-Akt, phosphorylated Akt; shOPN, short hairpin RNA targeted at OPN.

demonstrated in Fig. 2A, MMP2 and uPA were upregulated in MHCC-97L cells stably overexpressing OPN, but were markedly decreased in cells transfected with shOPN (Fig. 2B). Taken together, these results suggested that OPN activates PI3K/Akt signaling, which increases the expression of Twist and the metastasis of HCC cells.

OPN and Twist expression detected by TMA and IHC staining. To evaluate the potential role of Twist in HCC, the expression levels of Twist in 192 human HCC tissues were identified by IHC analyses. Higher Twist levels were detected in tumor tissues than in their paired non-cancerous liver tissues (Fig. 3A and B). The clinical significance of Twist was further investigated using TMAs containing HCC tissues from 374 patients. IHC staining revealed that high expression of Twist was significantly associated with the vascular invasion of HCC ($P=0.013$; Table I), whereas no significant association

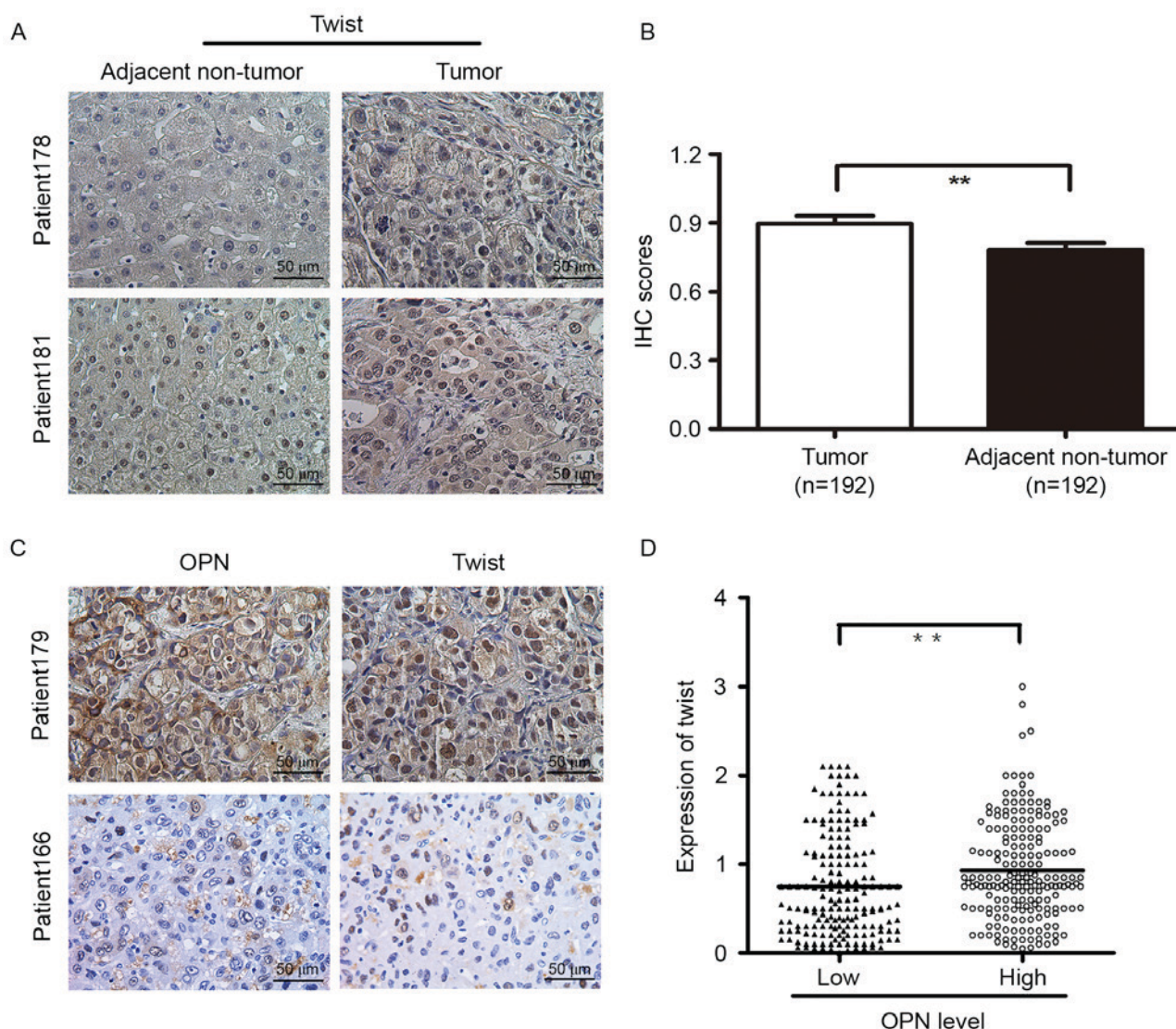


Figure 3. Twist expression detected by TMA and immunohistochemical staining. (A) Different expression statuses of Twist in tumor tissues and their paired non-cancerous liver tissues. (B) Immunohistochemical analyses demonstrated markedly increased Twist levels in tumor tissues compared with their paired non-cancerous liver tissues. * $P<0.05$, ** $P<0.01$. (C) OPN and Twist, detected by immunohistochemical staining in consecutive sections of HCC tissues from the same patient. (D) OPN is significantly associated with Twist expression, as determined by immunohistochemistry in a TMA constructed from HCC tumor samples. ** $P<0.01$. TMA, tissue microarray; OPN, osteopontin; HCC, hepatocellular carcinoma; IHC, immunohistochemistry.

was observed between Twist density and other clinicopathological characteristics of HCC patients. Next, the association between OPN and Twist expression in human HCC tissues were analyzed. On the basis of the IHC results, TMA analysis of HCC specimens revealed that OPN expression was positively associated with Twist expression in the HCC samples ($P<0.001$; Fig. 3C and D).

Prognostic value of Twist in HCC patients. Using the integrated optical density median value as the cut-off value, the 374 HCC patients were divided into two groups. The OS time of HCC patients with high Twist expression was significantly lower than that of the patients with low Twist expression ($P=0.041$; Fig. 4A), whereas the TTR of the high-Twist-expression group was significantly higher than that of the low-Twist-expression group ($P=0.017$; Fig. 4B). The OS and TTR times of the patients in group I were markedly longer than those of the patients in group IV (Fig. 4C and D).

Univariate and multivariate analyses of the prognostic value of Twist in HCC patients. To determine the prognostic value of Twist for HCC patients, univariate and multivariate analyses were performed on the clinicopathological characteristics and Twist expression levels of patients (Table II). Univariate analysis revealed that OPN expression, Twist expression, serum AFP level, hepatitis B surface antigen, tumor size, tumor capsulation, vascular invasion, Barcelona Clinic Liver Cancer stage (41) and tumor differentiation were significantly associated with the OS and TTR times of patients with HCC (Table II). However, no prognostic significance for OS or TTR was observed in association with the other characteristics, including sex, age, liver cirrhosis, ALT and tumor number. Individual characteristics that exhibited significance by univariate analysis were adopted as covariates in a multivariate Cox's proportional hazards model and combined variables were further analyzed. When OPN was combined with Twist, the combination of the two was a more potent independent

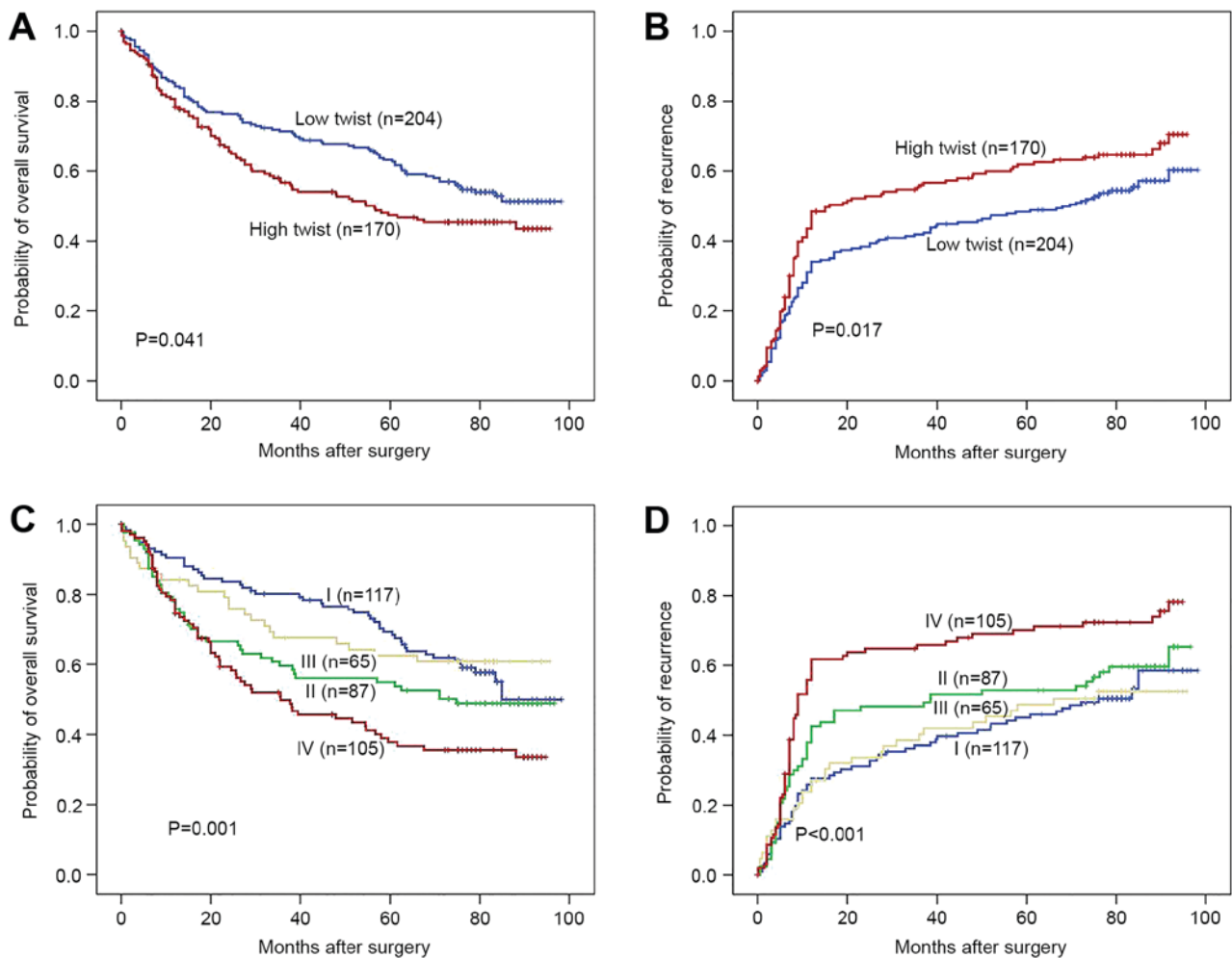


Figure 4. Prognostic value of Twist in HCC patients. Kaplan-Meier curves of (A) OS and (B) TTR in HCC patients expressing different levels of Twist. The patients with higher Twist levels exhibited significantly shorter OS and TTR times than patients with lower Twist levels. The patients of subgroup I had (C) the longest OS and (D) the lowest possibility of tumor recurrence, among the four subgroups, which were divided according to combinations of OPN and Twist expression (I, low OPN+low Twist; II, low OPN and high Twist; III, high OPN and low Twist; IV, high OPN and high Twist). HCC, hepatocellular carcinoma; OS, overall survival; TTR, time to recurrence; OPN, osteopontin.

prognostic indicator for OS ($P=0.001$) and TTR ($P<0.001$) than each factor alone.

Discussion

It is well-known that liver cancer is associated with a high mortality and primarily occurs in less developed countries (1,2). Although patients with HCC exhibit significantly improved survival times following curative resection, the prognosis of these patients remains poor owing to tumor invasiveness and metastasis (42). Therefore, identifying more accurate prognostic biomarkers is of great clinical value, allowing for further understanding of HCC and the development of novel therapeutic strategies.

EMT is a major step in tumor metastasis (10,43). This process is regulated by major EMT regulators, including Twist, Snail and Slug, and is initiated by suppression of E-cadherin expression (10). Twist serves an important role through its regulation of E-cadherin expression in human cancer. The role of Twist in cancer metastasis was first reported in study on a breast cancer model, the results of which indicating that Twist induced EMT, resulting in the promotion of tumor invasion (13). Twist has been

revealed to be associated with metastasis in various types of cancer, including HCC, through the induction of EMT changes and cancer invasiveness (23). The present study detected an association between Twist expression and OPN expression in HCC cell lines and in patients with HCC. The results of the present study demonstrated that Twist expression was induced by OPN in HCC. This finding was further confirmed using TMA, which revealed that OPN overexpression in HCC tumor tissues was associated with Twist expression. Additionally, Twist expression was observed in various HCC cell lines with different metastatic potentials. Twist was overexpressed in metastatic cell lines compared with non-metastatic primary cell lines. The TMA immunohistochemical assay performed in the present study also supported the hypothesis that Twist expression was positively associated with metastasis in HCC tumor tissues. Therefore, these data indicated that OPN serves a crucial role in the induction of HCC progression through the regulation of Twist expression.

A further important finding from the present study is that the hyper-activation of the PI3K/Akt signaling pathway is responsible for the progression of HCC cells induced by OPN. Twist is the most important transcription factor in the

Table II. Univariate and multivariate analyses of factors associated with OS and TTR of HCC (n=374).

Variable	OS			TTR		
	HR	95% CI	P-value	HR	95% CI	P-value
Univariate analysis						
OPN (high vs. low)	1.52	1.13-2.04	0.005	1.42	1.09-1.86	0.009
Twist (high vs. low)	1.35	1.01-1.81	0.043	1.38	1.06-1.79	0.018
Sex (female vs. male)	1.06	0.72-1.55	0.779	1.13	0.76-1.70	0.548
Age, years (>50 vs. ≤50)	1.11	0.83-1.48	0.478	1.09	0.81-1.47	0.555
ALT, U/l (≥75 vs. <75)	1.09	0.68-1.73	0.722	1.04	0.65-1.68	0.874
AFP, ng/ml (>20 vs. ≤20)	1.66	1.21-2.28	0.002	1.47	1.08-2.02	0.016
Liver cirrhosis (yes vs. no)	1.42	0.85-2.38	0.179	1.58	0.93-2.68	0.092
HBsAg (positive vs. negative)	1.91	0.94-3.87	0.075	2.50	1.23-5.06	0.011
Tumor size, cm (>5 vs. ≤5)	1.64	1.18-2.29	0.003	1.55	1.09-2.21	0.015
Tumor number (multiple vs. single)	1.59	0.84-3.00	0.157	1.18	0.58-2.41	0.641
Tumor capsule (none vs. complete)	1.53	1.15-2.05	0.004	1.39	1.03-1.87	0.031
Vascular invasion (yes vs. no)	2.03	1.50-2.73	<0.001	1.23	1.05-1.45	0.010
BCLC stage (B and C vs. 0 and A)	1.79	1.24-2.58	0.002	1.53	1.07-2.18	0.020
Tumor differentiation (III-IV vs. I-II)	1.57	1.14-2.16	0.006	1.43	1.02-2.00	0.037
Combination of OPN and Twist			0.006			0.004
II vs. I	1.40	0.93-2.10	0.193	1.28	0.88-1.86	0.193
III vs. I	1.15	0.73-1.81	0.543	1.16	0.78-1.74	0.459
IV vs. I	1.92	1.31-2.82	0.001	1.87	1.32-2.65	<0.001
Multivariate analysis						
AFP, ng/ml (>20 vs. ≤20)	1.47	1.06-2.04	0.023	1.37	1.02-1.84	0.035
Tumor size, cm (>5 vs. ≤5)	1.43	1.00-2.04	0.051	1.47	1.02-2.10	0.088
Tumor capsule (none vs. complete)	1.45	1.08-1.95	0.013	1.38	1.06-1.80	0.018
Vascular invasion (yes vs. no)	1.43	1.01-2.02	0.042	1.30	0.95-1.79	0.104
BCLC stage (B and C vs. 0 and A)	1.38	0.90-2.09	0.138	1.46	1.00-2.11	0.048
Tumor differentiation (III-IV vs. I-II)	1.19	0.85-1.67	0.320	1.14	0.83-1.56	0.419
Combination OPN and Twist			0.038			0.019
II vs. I	1.40	0.93-2.13	0.111	1.27	0.87-1.86	0.223
III vs. I	1.16	0.74-1.83	0.525	1.18	0.79-1.76	0.430
IV vs. I	1.77	1.19-2.65	0.005	1.77	1.23-2.54	0.002

Univariate and multivariate analyses were performed using Cox's proportional hazards regression model. Variables were adopted in multivariate analysis based on their prognostic significance by univariate analysis. CI, confidence interval; HR, hazard ratio; OS, overall survival; TTR, time to recurrence; OPN, osteopontin; ALT, alanine amino transferase; AFP, α -fetoprotein; HBsAg, hepatitis B surface antigen; BCLC, Barcelona Clinic Liver Cancer. I, low OPN and Twist; II, low OPN and high Twist; III, high OPN and low Twist; IV, high OPN and Twist.

negative regulation of E-cadherin expression and the EMT of epithelial cells. Evidence indicates that Twist phosphorylation is predominantly regulated by the PI3K/Akt pathway (44). An increase in Akt signaling is a key tumor survival mechanism that promotes tumor metastatic processes. A previous study (45) have demonstrated that activated Akt serves a critical role in hematogenous intrahepatic metastasis in an orthotopic implantation model of HCC. The present study revealed that, through the activation of PI3K/Akt signaling induced by OPN, the level of Twist increased correspondingly,

which led to the downregulation of E-cadherin. This concept was further supported by the observation that knockdown of OPN markedly reduced OPN-induced Akt activation. When HCC cell lines stably overexpressing OPN were incubated with LY294002, a PI3K inhibitor, the expression of Twist and N-cadherin was markedly reduced. Taken together, these results indicated that OPN activates PI3K/Akt/Twist signaling, which promotes the metastasis of HCC cells. To the best of our knowledge, the present study is the first to document a link among OPN, PI3K/Akt and Twist in human HCC.

Previous studies identified that OPN was associated with aggressive and metastatic HCC phenotypes, and with poor patient prognosis (46-48). The results of the present study indicated that patients with high Twist expression exhibit significantly shorter OS and TTR times than patients with low Twist expression. Additionally, according to univariate and multivariate Cox proportional hazards regression analyses, the predictive range of OPN expression levels combined with those of Twist was more sensitive than that of OPN alone for OS and TTR. Taken together, the results of the present study clearly demonstrate that a combination of OPN and Twist expression levels may serve as a powerful prognostic indicator of HCC.

In conclusion, the data presented in the current study revealed that Twist was markedly upregulated in HCC patients and that high expression of Twist was associated with poor patient prognosis. Additionally, the expression of Twist, as a major regulator of EMT, was regulated by OPN through the PI3K/Akt signaling pathway. These findings indicate that OPN and Twist may serve as synergistic indicators for HCC patients following curative resection. Therefore, Twist may be a potential therapeutic target to inhibit HCC metastasis in patients with high OPN expression.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XXY, YZ, XCZ and XMG designed and performed the experiments, analyzed data. CQW, YYS and WC participated in collecting the samples and correcting patient sample's following-up investigation data. XXY and YZ performed bioinformatics analyses. NR and LXQ participated in data interpretation and provided valuable discussions with regard to clinical correlates. QZD and HLJ designed and supervised the entire project, designed the experiments, and prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Zhongshan Hospital, Fudan University (Shanghai, China). Clinical samples were collected from these patients after obtaining informed consent according to an

established protocol approved by committee's regulations. The data did not contain any information that could lead to patient identification.

Patient consent for publication

Written informed consent for publication was obtained from all participants.

Competing interests

The authors have declared that no competing interests exist.

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