Osteopontin as a Key Mediator for Vasculogenic Mimicry in Hepatocellular Carcinoma

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Osteopontin (OPN) is overexpressed in a variety of cancers including hepatocellular carcinoma (HCC), and is likely involved in the process of vasculogenic mimicry (VM) in some tumor cells. In this study, we explored whether OPN plays a role for VM in HCC. Metastatic MHCC97-H human HCC cells and non-metastatic Hep3B human HCC cells were compared for their abilities to establish VM. Three-dimensional-culture assays showed that MHCC97-H cells but not Hep3B cells were able to form the chord-like structure that represents VM. Real-time RT-PCR arrays were used to detect gene expression profiles of the two HCC cell lines in three-dimensional culture. PCR array analyses revealed the increased expression of OPN in MHCC97-H cells forming VM compared with Hep3B cells. Small interfering RNA was employed to investigate whether OPN knockdown could influence VM, and the expression of matrix metalloproteinase (MMP)-2, MMP-9 and urokinase-type plasminogen activator (uPA) in MHCC97-H cells. OPN knockdown resulted in a significant decrease in the ability of MHCC97-H cells to form VM, which was accompanied by the down-regulation of MMP-2 and uPA expression. Furthermore, human HCC tissue samples were studied by immunohistochemistry to analyze the correlations between VM and the expression of OPN, MMP-2 and uPA. There existed significant positive correlations between VM and the expression of OPN, MMP-2 and uPA in HCC tissue samples. In conclusion, OPN is required for VM in HCC cells, and its action may be associated with activation of MMP-2 and uPA. OPN-targeted therapeutics may be useful for patients with advanced HCC.

Keywords: osteopontin; vasculogenic mimicry; hepatocellular carcinoma; matrix metalloproteinase; plasminogen activator


Growth, proliferation, and metastasis of most tumors have been thought to be angiogenesis-dependent processes (Folkman and Klagsbrun 1987). Antiangiogenic targeting of the neovasculature within tumors was once considered one of the most promising strategies in the search for novel antineoplastic therapies (Folkman 1996). However, the clinical success of current antiangiogenesis therapy is still very limited (Jain 2005).

It is now clear that tumor angiogenesis does not necessarily depend on endothelial cell proliferation and sprouting of new capillaries. In addition to the well-studied angiogenesis, recent reports have revealed several new patterns by which tumor tissues nourish themselves, including the pattern of mosaic vessels from both endothelium and tumor cells (Chang et al. 2000; Semela and Dufour 2004), and the pattern of vessels lined exclusively with tumor cells. This last process is termed vasculogenic mimicry (VM). VM describes the unique ability of highly aggressive tumor cells, but not poorly aggressive tumor cells, to form matrix-rich networks de novo when cultured on a three-dimensional matrix, thus mimicking embryonic vasculogenesis (Maniotis et al. 1999). VM, advanced by Maniotis et al. (1999), has been described in several other aggressive tumor types, including gallbladder carcinoma (Fan et al. 2007), breast cancer (Basu et al. 2006), prostate cancer (Sood et al. 2001), ovarian carcinoma (Heyman et al. 2010), colorectal cancer (Baeten et al. 2009), clear cell renal cell carcinoma (Vartanian et al. 2009), and acute leukemic bone marrow stromal cells (Mirshahi et al. 2009). In recent years, we and others have described the presence of VM in human HCC samples and initiated an investigation of its clinical significance (Sun et al. 2006; Sun et al. 2010; Liu et al. 2011).
The precise molecular events underlying the process of VM displayed by highly aggressive tumor cells remain poorly understood. In a recent report, epithelial-mesenchymal transition regulator Twist1 was identified as an important mediator of VM in HCC cells (Sun et al. 2010). Our previous report demonstrated that hypoxic inducible factor-1α, matrix metalloproteinase (MMP)-2, and MMP-9 were important for HCC cells to form VM (Liu et al. 2010). A smattering of plot studies have shown that osteopontin (OPN), an oncoenic protein that has been clinically correlated with increased tumor burden and adverse prognosis in patients with tumor metastasis, might be associated with VM in melanoma and breast cancer (Kadkol et al. 2010). However, it is not known whether OPN plays an important role in regulating the ability of HCC cells to display VM. It has been reported that MMP-2 and urokinase-type plasminogen activator (uPA) were related to progression, metastasis (Theret et al. 2001; Chan et al. 2004), and OPN-induced migration and invasion in vitro (Chen R.X. et al. 2010) in HCC. Whether MMP-2 and uPA are involved in the regulation of OPN and VM is also under confirmation.

In the present study, we compared the ability of two different metastatic potentials human HCC cell lines to form VM in vitro. Then, real-time reverse transcription-polymerase chain reaction (RT-PCR) arrays were used to screen differentially expressed genes in the two HCC cell lines in three-dimensional culture. To determine whether OPN, an up-regulated gene detected by RT-PCR arrays in metastatic human HCC cell lines to display VM. It has been reported that MMP-2 and uPA are involved in the regulation of OPN and VM is also under confirmation.

In the present study, we compared the ability of two different metastatic potentials human HCC cell lines to form VM in vitro. Then, real-time reverse transcription-polymerase chain reaction (RT-PCR) arrays were used to screen differentially expressed genes in the two HCC cell lines in three-dimensional culture. To determine whether OPN, an up-regulated gene detected by RT-PCR arrays in highly metastatic HCC cells, regulates the process of VM, we assessed whether silencing OPN expression with small interfering RNA (siRNA) inhibited VM. Moreover, we investigated the relationship between VM and OPN expression in HCC tissue samples. In addition, the role of MMP-2, MMP-9 and uPA in the regulation of OPN was also explored.

Materials and Methods

Three-dimensional Matrigel Culture

The highly metastatic human HCC cell line MHCC97-H was purchased from Liver Cancer Institute of Fudan University in Shanghai. The non-metastatic human HCC cell line Hep3B was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The two cell lines were cultured in Dulbecco’s modified Eagle medium (DMEM; Hyclone) high-glucose medium with 10% fetal bovine serum (FBS; Hyclone) at humidified 37°C incubator supplied with 5% CO₂. Primary human umbilical vein endothelial cells (HUVECs) were obtained from umbilical cords at the Anhui Provincial Hospital. The cells were isolated by collagenase treatment and were also grown in DMEM. HUVECs between passages 2 and 5 were used as described below. The following experiments were performed with 70% to 80% confluent cultures. A 24-well tissue culture plate was evenly coated with 25 μL/well growth factor-reduced Matrigel (BD Biosciences, Bedford, MA), which was allowed to solidify at 37°C for 60 min, before cells were plated. The cell suspension was added (2 × 10⁵ cells/well) onto the surface of the matrigel and incubated at 37°C for varying times.

Real-time RT-PCR array Analysis

Human HCC cells cultured on Matrigel for 24 h were collected by trypsin, from which total RNA was extracted using Trizol (Invitrogen life technologies, Carlsbad, CA). Contaminant genomic DNA was removed from total RNA by Dnase treatment using Qiagen RNaseasy kit (Qiagen, Valencia, CA). First strand cDNA synthesis was performed with RT² PCR array first strand kit (SuperArray Bioscience, Frederick, MD). The cDNAs were then added to a 96-well plate from the RT² Profiler PCR array system (human extracellular matrix and adhesion molecules PCR array; SuperArray Bioscience), in which 84 wells contain individual primer sets specific to different genes important for cell-cell and cell-matrix interactions and the remaining wells serve as genomic DNA contamination controls, reverse transcription controls, and positive PCR controls. Quantitative RT-PCR was performed using ABI PRISM 7,900 system (Applied Biosystems, Foster City, CA). Data analysis was performed using the ΔΔCt method, with the housekeeping gene β-2-microglobulin as the normalization factor. Changes in gene expression were determined by comparing gene transcription levels in MHCC97-H cells to those in Hep3B cells. According to the manufacturer’s instructions, a significant threshold is defined as a 2-fold change in gene expression.

RNA Interference Against OPN

RNA interference technique was used to knock down the expression of OPN in the MHCC97-H cells. A small interfering RNA of OPN (sc-36129) and control siRNA (sc-37007) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). MHCC97-H cells were plated in six-well tissue culture plate at 2 × 10⁵ cells per well 24 h before transfection. Before transfection, the medium was replaced with 2 ml of antibiotic-free medium, and then cells were transfected with siRNA using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. Eight hours after transfection, cells were replaced with fresh medium. To confirm the effect of siRNA on the expression of OPN, sixty hours after siRNA transfection, some of the cells were subjected to western blot, immunofluorescence and real-time RT-PCR analysis. In the next studies, some transfected cells were harvested and used for proliferation, migration and invasion assay, as well as three-dimensional culture assay for VM.

Real-time RT-PCR Analysis

The DNA Engine Opticon® 2 System (MJ Research, Waltham, MA) was used for 2-step reverse transcription-polymerase chain reaction. cDNA was amplified using SYBR® Premix Ex Taq™ II (Perfect Real Time) kit (TaKaRa, Japan) with specific oligonucleotide primers for target sequences of OPN, MMP-2, MMP-9 and uPA in a total of 25 μL reaction mixture (2 μL of cDNA, 12.5 μL of 2 × SYBR® Premix Ex Taq™ II, 2 μL of each 10 μM forward and reverse primers and 8.5 μL of H₂O). The real-time quantitative PCR thermal cycling conditions were 95°C for 30 s, followed by 95°C for 5 s and 60°C for 30 s for 40 cycles. The primer sequences were as follows: sense 5'-AGGAGGAGGCGAGGCAACA-3’ and anti-sense 5'-CTGGTATGCGCAGGTAGATG-3’ for OPN; sense 5'-GCCCCAGACAGGATGATC-3’ and anti-sense 5'-GCTGTTGCGAGGAAAGAGT-3’ for MMP-2; sense 5'-TTCGAGCCAGCAGAACTAGT-3’ for MMP-9; sense 5'-TGGTCTGTGAGGGTGGGG-3’ and anti-sense 5'-TCCAGGCGAGCATAGAAGT-3’ for MMP-2; sense 5'-CCGGCTTTCGCTTTGTCGTC-3’ and anti-sense 5'-TAT...
TGTCGTTGCGCCTGTGAG-3′ for uPA; sense 5′-GGACCTGACCTGGCTACTAAG-3′ anti-sense 5′-TAGCCGAGATGCCCCTTGAG-3′ for GAPDH. All primers were synthesized by TaKaRa Biotechnology (Japan). Data were analyzed according to the comparative Ct method and were normalized by GAPDH expression in each sample. Relative mRNA levels were calculated based on the Ct values, corrected for GAPDH expression, according to the equation: 2ΔΔCt [ΔCt = Ct (OPN, MMP-2, MMP-9 or uPA) - Ct (GAPDH)]. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product.

**Western Blot Analysis**

MHCC97-H cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% sodium dodecyl sulphate). Fifty micromolars of protein extract was electrophoresed on a sodium dodecyl sulphate–polyacrylamide (SDS-PAGE) gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). After incubation with 5% non-fat milk to block non-specific binding, the membranes were exposed to specific anti-OPN or control anti-β-actin (all 1:1,000, Santa Cruz) monoclonal antibodies overnight at 4°C. Membranes were then washed and exposed to peroxidase-conjugated anti-IgG secondary antibody (1:5,000), and the antigen-antibody complex was visualized using an enhanced chemiluminescence detection system according to the manufacturer’s instructions (GE Healthcare, Chalfont St Giles, UK).

**Immunofluorescence Staining**

The transfected MHCC97-H cells were plated on cover slips in each well of an 8-well chamber for 48 h. The cells were then fixed with paraformaldehyde for 15 min, rinsed with PBS, and incubated with 5% goat serum for 30 min. Next the cells were incubated with anti-OPN antibody (Santa Cruz) for 2 h. After washing with PBS, the cells were incubated with FITC-conjugated secondary antibody (Zhongshang Goldenbridge Biotechnology CO Ltd, Beijing, China) for 45 min. Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical, St. Louis, MO). Cells were visualized with an Olympus FSX100 fluorescence microscope.

**Cell Proliferation Assay**

Cell proliferation assays were performed by 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma) colorimetry. The MHCC97-H cells transfected with control siRNA or OPN siRNA were harvested and plated in 96-well plates at 1 × 10⁴ cells per well in 100 µL cell culture medium and maintained at 37°C in a humidified incubator containing 5% CO₂. After every 24 h, 10 μL of the MTI solution was added into the triplicate wells and incubated at 37°C for another 4 h, 100 μL of DMSO was added and surged for 10 min to dissolve the crystal completely. Then absorbance at 540 nm was measured to calculate the numbers of vital cells in each well by micro-enzyme-linked immunosorbent assay plate reader.

**In Vitro Transwell Migration and Invasion Assay**

The transwell migration and invasive ability of MHCC97-H cells was determined using 24-well transwell chambers with upper and lower culture compartments separated by polycarbonate membranes with 8-µm pore (Corning Incorporated, Corning, NY). For the invasion assay, 100 μL of serum-free DMEM-diluted Matrigel (0.4 mg/ml) (BD Biosciences, Bedford, MA) was added to the membranes and incubated at 37°C for 2 h to form matrix gels. However, Matrigel was not used in the migration assay. The transfectected cells (1 × 10⁵) were suspended in 100 μL serum-free DMEM and seeded on the top chamber. 750 μL DMEM with 10% FBS was added to the lower chamber. After 24 h of incubation at 37°C, the upper surface of the filters was carefully wiped with a cotton-tipped applicator. Cells that had invaded across the matrigel and passed through the Transwell filter pores toward the lower surface of the filters were stained with crystal violet solution, and counted in five no overlapping 200× fields under a light microscope.

**Enzyme-linked Immunosorbent Assay (ELISA)**

Serum-free supernatants from 4 × 10⁵ cells were measured for MMP-2, MMP-9 and uPA by ELISA according to manufacturer’s directions (MMP-2 and MMP-9 ELISA kit from R&D Systems, Minneapolis, MN; uPA ELISA kit from American Diagnostica Inc, Greenwich, CT). Absorbance was measured at 450 and 570 nm using a microplate spectrophotometer.

**Immunohistochemistry**

Tumor tissues from 151 patients from Anhui Provincial Hospital were studied, who underwent curative resection by the same surgical team for pathologically confirmed HCC between January 2003 and December 2008. The use of these tissues in this study was approved by the ethics committee at Anhui Provincial Hospital. Written informed consent was obtained from all patients.

Formalin-fixed paraffin-embedded samples were sectioned at 4 μm and stained with hematoxylin and eosin for identification. Sections adjacent to the hematoxylin and eosin-stained sections were used for immunohistochemical staining. All slides were dewaxed in xylene, rinsed in graded ethanol, and finally rehydrated in double-distilled water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide methanol at ambient temperature for 20 min. Antigen retrieval was carried out with 20-min microwave treatment in 10 mmol/L citrate buffer (pH 6.0) for CD34, hepatocyte, OPN, MMP-2, MMP-9 and uPA, and with 6-min 0.3 mg/ml proteinase K (Sigma-Aldrich, St. Louis, MO) for laminin. After washing in 0.1 M phosphate-buffered saline (PBS), pH 7.4, and exposure to 3% normal goat serum for 20 min to reduce non-specific binding, the slides were incubated for 2 h at 37°C with a 1:100 dilution of anti-laminin monoclonal antibody (Millipore), or a 1:100 dilution of anti-CD34 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or a working solution of anti-hepatocyte antibody (Zhongshan Goldenbridge Biotechnology CO Ltd, Beijing, China) for 45 min. Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical, St. Louis, MO). Cells were visualized with an Olympus FSX100 fluorescence microscope.

Serum-free supernatants from 4 × 10⁵ cells were added to the membranes and incubated at 37°C for 2 h to form matrix gels. However, Matrigel was not used in the migration assay. The transectectected cells (1 × 10⁵) were suspended in 100 μL serum-free DMEM and seeded on the top chamber. 750 μL DMEM with 10% FBS was added to the lower chamber. After 24 h of incubation at 37°C, the upper surface of the filters was carefully wiped with a cotton-tipped applicator. Cells that had invaded across the matrigel and passed through the Transwell filter pores toward the lower surface of the filters were stained with crystal violet solution, and counted in five no overlapping 200× fields under a light microscope.

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nohistochemical scoring of less than 1 were defined as negative, otherwise they were defined as positive.

**Statistical Analysis**

Statistical analyses were performed using SPSS 13.0 for Microsoft Windows (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as means ± s.d., and analyzed using the two-tailed Student’s t-test or Two-way ANOVA. The correlations between VM and the expression of OPN, MMP-2, MMP-9 and uPA were analyzed by the χ² test and Pearson correlation test. The level of significance was set at P < 0.05.

**Results**

**Differences in VM in MHCC97-H and Hep3B Cells**

Highly metastatic MHCC97-H cell line and non-metastatic Hep3B cell line were assayed for their ability to form VM. Three-dimensional Matrigel culture was utilized as a well-established *in vitro* model for investigating the process of VM. As shown in Fig. 1, MHCC97-H cells exhibited a chord-like phenotype with the formation of an interlacing arborizing pattern on Matrigel, which was called VM. The formation of the chord-like structures was initiated within 8 h after seeding MHCC97-H cells onto Matrigel, with obvious structure formation achieved by 24 h. HUVECs were used as a positive control and formed similar structure under identical culture conditions. In contrast, Hep3B cells failed to form chord-like structures *in vitro* under identical culture conditions on Matrigel (Fig. 1).

**The Results of Real Time RT-PCR Array Analysis**

To find out molecular mechanisms underlying the process of VM in HCC cells with different metastatic potentials, we compared MHCC97-H and Hep3B cells under three-dimensional culture conditions using real-time PCR arrays to screen differentially expressed genes. Our results indicated that there were 36 differentially expressed genes, in which 23 genes including OPN were up-regulated and 13 genes were down-regulated in expression by two fold or more in MHCC97-H cells compared with Hep3B cells (Table 1).

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**Table 1. Partial genes of MHCC97-H and Hep3B cells expressing over 5-fold differences.**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>GeneBank</th>
<th>Description</th>
<th>Average ratio (MHCC97-H / Hep3B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>NM_000610</td>
<td>CD44 molecule (Indian blood group)</td>
<td>102.78 ± 30.13</td>
</tr>
<tr>
<td>COL6A1</td>
<td>NM_001848</td>
<td>Collagen, type VI, alpha 1</td>
<td>11.62 ± 3.35</td>
</tr>
<tr>
<td>COL6A2</td>
<td>NM_001849</td>
<td>Collagen, type VI, alpha 2</td>
<td>5.15 ± 1.01</td>
</tr>
<tr>
<td>ITGA1</td>
<td>NM_181501</td>
<td>Integrin, alpha 1</td>
<td>6.18 ± 1.51</td>
</tr>
<tr>
<td>MMP1</td>
<td>NM_002421</td>
<td>Matrix metalloproteinase 1 (interstitial collagenase)</td>
<td>10.83 ± 2.93</td>
</tr>
<tr>
<td>MMP2</td>
<td>NM_004530</td>
<td>Matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)</td>
<td>12.09 ± 3.46</td>
</tr>
<tr>
<td>SPP1</td>
<td>NM_000582</td>
<td>Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)</td>
<td>5.17 ± 1.46</td>
</tr>
</tbody>
</table>
Inhibition of OPN Expression in MHCC97-H Cells by siRNA

The effect of siRNA for knocking down OPN mRNA and protein levels was confirmed by real-time RT-PCR, western blotting and immunofluorescence analyses. As shown in Fig. 2A and B, OPN at mRNA and protein levels was very low in OPN siRNA-transfected MHCC97-H cells, compared with control siRNA-transfected MHCC97-H cells. Further experiment using immunofluorescence indicated that there was a dramatic decrease in the amount of OPN in the cytoplasm of OPN siRNA-transfected MHCC97-H cells (Fig. 2C and D). These results suggested that the expression of OPN mRNA and protein was significantly suppressed in MHCC97-H cells using siRNA techniques.

Effect of OPN Knockdown on MHCC97-H Cells Proliferation

To determine the effect of OPN on cell proliferation, MHCC97-H cells were transfected with OPN siRNA or control siRNA and assessed for cell proliferation by MTT assay. OPN siRNA resulted in a slight, but not significant, inhibition of proliferation in MHCC97-H cells when compared to control siRNA (P = 0.7304; Fig. 3).

Effect of OPN Knockdown on MHCC97-H Cells Invasion and Migration

Having observed the effect of OPN siRNA on cell proliferation, we wished to determine whether OPN might contribute to invasion and migration. In the in vitro invasion assays, the number of cells invaded through the transwell membrane in OPN siRNA-transfected group was significantly lower than those in the control group (5.25 ± 1.50 vs. 13.75 ± 3.59, P = 0.005). In the in vitro transwell migration assays, we also found that a significant difference in
the number of cells invaded through the membrane between the OPN siRNA-transfected group and control group (8.50 ± 2.08 vs. 21.75 ± 5.37, \(P = 0.004\)) (Fig. 4A and B).

**Effect of OPN Knockdown on VM in MHCC97-H Cells**

To address whether OPN was important to VM in HCC cells, the cells treated by OPN siRNA or control siRNA were used to carry out VM assay in three-dimensional Matrigel culture. As shown in Fig. 5, The control siRNA-transfected cells efficiently formed the chord-like structures with the formation of an interlacing arborizing pattern when cultured on Matrigel for 3 d. Likewise, HUVECs which served as a positive control also exhibited the chord-like structures on Matrigel under identical culture conditions. However, the chord-like structures were not observed obviously when OPN siRNA-transfected cells were cultured on Matrigel for 1 d or 3 d.

**Effect of OPN Knockdown on MMP-2, MMP-9 and uPA Expression**

To detect the effect of OPN knockdown on the expression of MMP-2, MMP-9 and uPA, real-time RT–PCR and ELISA were used to assess gene and protein expression levels in MHCC97-H cells. The results showed that the expression of MMP-2 and uPA mRNA was lower in the HCC cells transfected with OPN siRNA than in the HCC cells transfected with control siRNA (Fig. 6A). The decreased expression of MMP-2 and uPA protein was also observed in the HCC cells transfected with OPN siRNA. However, the levels of MMP-9 mRNA and protein did not show significant difference between the HCC cells transfected with OPN siRNA and the HCC cells transfected with control siRNA (Fig. 6B).

**VM Expression in Human HCC Samples Detected by Immunohistochemistry**

VM in HCC samples was identified by the detection of laminin-positive loops surrounding clusters of 3 to 15 tumor

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**Fig. 4.** Effect of OPN knockdown on invasion and migration of MHCC97-H cells. (A) Invasion (upper) and migration assay (low) showed that invasion and migration ability was significantly decreased in MHCC97-H cells transfected with OPN siRNA as compared with those transfected with control siRNA. (B) Results were plotted as the number of cells per field migrated through the membrane or invaded Matrigel and growing on the under side of the membrane. All data are expressed as means ± s.d. *\(P < 0.05\), compared with control siRNA-transfected cells.
cells (Fig. 7A) (Folberg and Maniotis 2004; Guzman et al. 2007). The same HCC lesion in Fig. 7A showing VM expression by laminin immunostain did not express the endothelial cell marker CD34, indicating that the laminin-positive loops were not hepatic sinusoids. Hepatic sinusoids were CD34 positive (Fig. 7B). The cells external to the lumen of the patterned channels were positive for hepatocyte, which indicated the channels were formed by HCC cells (Fig. 7C). In comparing Fig. 7A to Fig. 7B, the laminin-positive loops seemed to connect to hepatic sinusoids partly. VM was observed in 31 of the 151 HCC samples.
OPN, MMP-2 and uPA Expression in Human HCC Samples is Correlated With VM

We further examined OPN, MMP-2 and uPA expression in 151 HCC samples by immunohistochemistry. The results showed that OPN, MMP-2 and uPA expression was located in the cytoplasm of the HCC cells (Fig. 8A and B). The expression of these protein in VM-positive and VM-negative samples was compared using the \( \chi^2 \) test. The positive rate of these protein expression in the VM-positive samples was higher compared with the VM-negative samples (\( P = 0.009, P = 0.024, P = 0.017 \), respectively; Fig. 8C, D and E). Correlations analysis between VM and these protein expression was also tested in VM-positive samples by way of Pearson correlation test. The results showed that VM had positive correlations with OPN, MMP-2 and uPA expression in HCC samples (\( P = 0.004, r = 0.728; P = 0.022, r = 0.409; P = 0.008, r = 0.469 \), respectively; Fig. 8F, G and H).

Discussion

OPN, an integrin-binding glycoposphoprotein, is normally expressed in and secreted by bone, teeth, kidney, epithelial, and activated immune cells (Kim et al. 2009). It has been demonstrated that OPN is overexpressed in a variety of human tumors, including carcinomas of the thyroid (Briese et al. 2010), stomach (Imano et al. 2009), colon-rectum (Likui et al. 2010), breast (Macri et al. 2009), pancreas (Chen R. et al. 2010), prostate (Caruso et al. 2008), lung (Zhang et al. 2010), ovary (Kato and Motoyama 2008), uterus (Cho et al. 2008), and liver (Huang et al. 2010; Xue et al. 2010). OPN is involved in tumor proliferation, invasion and metastasis, and high levels of OPN expression are associated with a poor prognosis in cancer patients (Wai and Kuo 2004; Kato et al. 2008; Caruso et al. 2008; Cho et al. 2008; Imano et al. 2009; Macri et al. 2009; Chen R. et al. 2010; Briese et al. 2010; Huang et al. 2010; Likui et al. 2010; Zhang et al. 2010). The potentially important role of OPN in HCC proliferation, invasion, and metastasis has also been suggested by a growing body of in vitro evidence (Huang et al. 2010; Xue et al. 2010).

There are already quite a few reports involved in the relationship between OPN and VM. A recent study showed that tissue sections of primary uveal melanomas lacking looping VM patterns either did not stain for OPN or exhibited weak, diffuse staining. In primary melanomas containing looping VM patterns, strong OPN staining was detected in the tumor periphery where patterns were located (Kadkol et al. 2006). Another study suggested that increased expression of OPN by decreased levels of hsa-mir-299-5p (a miRNA) played a critical role in enhancing proliferation, tumorigenicity and the ability to display VM of the spheroid-forming cells of breast cancer cells (Shevde et al.
2010). However, there is no report about the relationship between OPN and VM in HCC.

In the current study, we firstly compared the ability of highly metastatic MHCC97-H cell line with that of non-metastatic Hep3B cell line to display VM in three-dimensional Matrigel culture, and found that the MHCC97-H cells efficiently formed VM, whereas Hep3B cells formed hardly any chord-like structures at all. This finding is consistent with other results showing that highly aggressive tumor cells, but not poorly aggressive tumor cells, form VM in vitro when cultured on a three-dimensional matrix (Maniotis et al. 1999; Sood et al. 2001; van der Schaft et al. 2005).

The molecular mechanisms underlying differential VM in the two HCC cell lines with different metastatic potentials remain uncertain. We speculated that some genes were likely to contribute to the differential formation. Therefore, we used real-time RT-PCR arrays to detect gene expression profiles of the two HCC cell lines in three-dimensional culture for 24 h to screen differentially expressed genes. Our results demonstrated that OPN, which was among 36 differentially expressed genes, was obviously up-regulated in MHCC97-H cells. Consistent with our results, OPN was previously observed to be expressed differentially in the original profiling analysis of metastatic melanomas with increased invasiveness (Bittner et al. 2000). Taken together, we conjectured that OPN could partly contribute to VM in MHCC97-H cells.

Next, we used siRNA knockdown approach to investigate whether OPN would play an important role in VM in HCC cells. We found that the transfection of MHCC97-H cells with OPN siRNA could markedly knock down OPN gene and protein expression. Furthermore, we investigated the effect of OPN knockdown on proliferation, migration,
invasion and VM of MHCC97-H cells \textit{in vitro}. On the basis of high transfection efficiency, our studies showed that OPN knockdown could obviously inhibit the migration and invasion ability, and more importantly, significantly inhibit VM \textit{in vitro}. These \textit{in vitro} data show that OPN not only plays an important role in tumor cell migration and invasion but is also closely associated with tumor cell plasticity to VM. VM is associated with cell migration and invasion, and it is a similar mechanism among endothelial cells (Hendrix et al., 2001, 2003; Sun et al., 2010). It is also possible that OPN knockdown inhibits VM via the inhibition of migration and invasion ability.

In our previous work (Liu et al., 2010), we found that VM existed in 31 of 151 HCC patients who underwent radical hepatectomy and had an adverse influence on the HCC patient’s prognosis. In this study, we examined OPN protein expression in these HCC tissues by immunohistochemistry. We found that OPN expression located in the cytoplasm of the HCC cells could be detected in 68 of the 151 HCC samples and there existed a significant positive correlation between VM and OPN expression. This result was further confirmed by evaluating the correlation between OPN expression and the number of VM loops in 31 VM-positive samples.

It is still unknown about precise mechanism how OPN regulates VM in HCC cells. MMP-2 and uPA have been previously reported to be involved in HCC progression and metastasis (Theret et al., 2001; Chan et al., 2004). Recent report indicated that MMP-2 and uPA might have a role in OPN-induced migration and invasion \textit{in vitro} (Chen R.X., et al., 2010). Our \textit{in vitro} experiments revealed that OPN knockdown was able to decrease MMP-2 and uPA expression at both gene and protein levels. Further research on clinical specimen demonstrated that VM also had positive correlations with MMP-2 and uPA expression. Together, these results show that OPN knockdown may inhibit VM through inhibiting MMP-2 and uPA expression. Nevertheless, OPN knockdown is also likely to impair VM via its effect on invasion and migration by regulating MMP-2 and uPA expression.

In summary, we have provided evidence that OPN has a crucial role in VM in HCC cells by regulating MMP-2 and uPA expression. Further representing a possible choice for targeted drug therapy for patients with advanced HCC. Although these \textit{in vitro} and clinical sample studies suggest that OPN is associated with VM, animal experiments \textit{in vivo} need to be done in order to further confirm OPN function in VM in HCC cells.

Acknowledgments

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Conflict of Interest

We have no conflict of interest.

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