Expression and Functional Significance of Twist1 in Hepatocellular Carcinoma: Its Role in Vasculogenic Mimicry

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The up-regulation and nuclear relocation of epithelial-mesenchymal transition (EMT) regulator Twist1 have been implicated in the tumor invasion and metastasis of human hepatocellular carcinoma (HCC). The term vasculogenic mimicry (VM) refers to the unique capability of aggressive tumor cells to mimic the pattern of embryonic vasculogenic networks. However, the relationship between Twist1 and VM formation is not clear. In this study, we explored HCC as a VM and EMT model in order to investigate the role of Twist1 in VM formation. We first examined the expression of Twist1 in human HCC samples and cell lines and found that Twist1 was frequently overexpressed in the nuclear relocation occurring in VM-positive HCCs (13/18 [72%]). Twist1 nuclear expression was likewise significantly associated with VM formation. Clinicopathological analysis revealed that both VM and Twist1 nuclear expressions present shorter survival durations than those without expression. We consistently demonstrated that an overexpression of Twist1 significantly enhanced cell motility, invasiveness, and VM formation in an HepG2 cell. Conversely, a knockdown of Twist1 by the short hairpin RNA approach remarkably reduced Bel7402 cell migration, invasion, and VM formation. Using chromatin immunoprecipitation, we also showed that Twist1 binds to the vascular endothelial (VE)-cadherin promoter and enhances its activity in a transactivation assay. Conclusion: The results of this study indicate that Twist1 induces HCC cell plasticity in VM cells more through the suppression of E-cadherin expression and the induction of VE-cadherin up-regulation than through the VM pattern in vivo and in a three-dimensional in vitro system. Our findings also demonstrate a novel cogitation in cancer stem-like cell differentiation and that related molecular pathways may be used as novel therapeutic targets for the inhibition of HCC angiogenesis and metastasis. (HEPATOLOGY 2010;51:545-556.)

Hepatocellular carcinoma (HCC), as a prevalent malignancy, is the most common malignant tumor of the liver. The malignant tumors require adequate blood supply to support their growth. It was believed that only endothelial cells could form blood vessels. However, a blood vessel's rigid cellular identity has been challenged with the observation that tumor cells also form extravascular networks in melanoma.1-3 The process

Abbreviations: 3D, three-dimensional; cDNA, complementary DNA; ChIP, chromatin immunoprecipitation; EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma; MMP, matrix metalloproteinase; PAS, periodic acid-Schiff; PCR, polymerase chain reaction; RT-PCR, reverse-transcription polymerase chain reaction; shRNA, short hairpin RNA; siRNA, small interfering RNA; VE, vascular endothelial; VM, vasculogenic mimicry.

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by which a vessel is formed from tumor cells is called vasculogenic mimicry (VM). We and others have proposed that tumors, including HCC, may develop vascularization through angiogenesis, as well as develop alternative pathways such as VM and mosaic vessels.1-8

The term vasculogenic mimicry refers to the unique ability of aggressive tumor cells to form periodic acid-Schiff-positive and CD31-negative cells that line VM networks in vivo and form tubular structures and patterned networks in three-dimensional (3D) culture in vitro; this mimics the pattern of embryonic vasculogenic networks, referred to as a tumor aggressive pattern.9,10 The VM networks do not result from the hemorrhage and necrosis of tumors, because no necrosis or inflammatory cells are found around them. In addition, we assume that when the growth of endothelium-dependent vessels is insufficient for the rapid proliferation of tumor tissues, some tumor cells alter their gene expression program and the cell’s fate becomes similar to that of endothelial cells. Furthermore, tumor cells lining the VM networks secrete matrix metalloproteinases (MMPs) and express vascular endothelial (VE)-cadherin and laminin to induce extracellular matrix remodeling, promoting the formation of VM.11,12

Epithelial cells can convert into mesenchymal cells through a process known as epithelial-mesenchymal transition (EMT). EMT describes a series of events during which epithelial cells lose many of their epithelial characteristics and take on properties typical of mesenchymal cells, which require complex changes in cell architecture and behavior.13,14 Twist1 has been identified as an EMT inducer; it binds DNA using similar E-box sequence motifs, repressing E-cadherin, and up-regulating mesenchymal markers expression.13 The VM formation involving tumor cells mimics endothelial cells consisting of a type of mesenchymal cell, similar to the EMT process. However, it is still unclear whether the EMT mechanism is associated with VM formation or if EMT causes VM.

Given that Twist has been found to be a crucial inducer contributing to tumor EMT, it is of great interest to examine the role of Twist in VM formation in HCC. Thus far, there have been no data on the role of EMT in VM. This study aims to investigate the expression and possible role of Twist in VM using HCC tissue samples and cell lines. Moreover, the correlation between Twist and a VM-associated marker was examined by performing immunostaining in the tissue samples. We likewise performed an ectopic up-regulation or short hairpin RNA (shRNA) knockdown of Twist to the HCC cell lines. Our evidence suggests that Twist1, not Twist2, is correlated with VM formation in HCC and that it is associated with invasion and short survival in HCC patients.

Materials and Methods

Patient Samples. Through the Tumor Tissue Bank of Tianjin Cancer Hospital, tissue specimens were obtained from 97 patients who underwent hepatectomy for HCC between 2001 and 2005. The diagnoses of these HCC samples were verified by pathologists. Detailed pathologic and clinical data were collected for all samples including Edmondson tumor grade, metastasis, and survival duration. We collected paraffin-embedded tumor tissue samples from patients who had not undergone therapy prior to tumor surgical operation. The use of these tissue samples in this study was approved by the institutional research committee.

Immunohistochemical and Histochemical Double-Staining Methods. The sections were pretreated with microwave, blocked, and incubated using a series of antibodies (Supporting Table 1). The staining systems used in this study were PicTure PV6000 (Zhongshan Chemical Co., Beijing, China) and Elivision Plus (Zhongshan Chemical Co., Beijing, China). Finally, the sections were counterstained with hematoxylin or periodic acid-Schiff (PAS). Phosphate-buffered saline was used in place of the primary antibodies for the negative control. The results were quantified according to the method described by Bittner et al.15

Cell Culture and Transfection. The HCC cell lines used in this study were HepG2, Bel7402, PLC, SMMC7221, and Huh-7 (from the American Type Culture Collection, Rockville, MD). These cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen). The vectors were transfected into cells by way of percutaneous ethanol injection (Polysciences, Inc., Cat#23966).

Expression Plasmids. Full-length Twist complementary DNA (cDNA) was generated by normal human embryo total cDNA, and digested with XhoI/EcoRI and subcloned into pcDNA3.1 vectors. The resulting constructs were confirmed by DNA sequencing.

Twist1 Gene Silencing. The small interfering RNA (siRNA) kit (pGP-Twist1-shRNA) was purchased from GenePharm (Shanghai, China). The target sequence (AAGCTGAGCAAGATTCAGACC [siTwist1 nucleotides 505-525]) was used to down-regulate Twist1 in vitro.16 A nonsilencing siRNA sequence (target sequence 5’-AATTCTCCGAACGTGTCACGT-3’), was used as a negative control.

Invasion and Wound Healing Assay. Cell migration assay was performed using Transwell cell culture inserts (Invitrogen). The transfected cells were maintained for 48 hours and allowed to migrate for another 24 hours. The passed cells were stained with crystal violet solution and determined its absorbance at 595 nm. In wound healing
assays, cell motility was assessed by measuring the movement of cells into a scarped. The speed of wound closure was monitored after 12 and 24 hours by measuring the ratio of the distance of the wound at 0 hours. Each experiment was also performed in triplicate.

**3D Cultures.** Tumor cells were transfected for 24 hours, then mixture-seeded with Matrigel (Collaborative Biomedical) to allow them to polymerize. The addition of conditioned media-soluble added with 10% fetal bovine serum (Hyclone) were performed by pretreatment and continuous treatment regimes during the 10-day incubation period in 3D cultures. Cells were collected from Matrigel by trypsin, to which Trizol or RIPA buffer was isolated to isolate cells of total RNA or protein.

**Reverse-Transcription Polymerase Chain Reaction.** Total cellular RNA was isolated using Trizol reagent (Invitrogen Life Technologies), reverse transcript (TaKaRa Biotechnology Co., Ltd., Japan), and polymerase chain reaction (PCR) analysis. The forward primer for human Twist1 was 5'-GCAAGAAGTGCAGCGAAGAT-3', and the reverse primer was 5'-GCTCTGCACTCTCGAA-3'. The forward primer for human E-cadherin was 5'-GTCAGTGAACACGATAT-3', and the reverse primer was 5'-TTTCAGTTGATTACGACGT-3'.

**Western Blot Analysis.** The whole cell lysates were resolved by way of sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore). Blots were blocked and incubated with the monoclonal antibody (1:200: Santa Cruz Biotechnology) beta-actin antibody (1:200; Santa Cruz Biotechnology) catabway, NJ). For protein loading analyses, a monoclonal conjugated mouse and rabbit immunoglobulin G antibody was diluted and pre-cleared with protein-A/G beads blocked with salmon sperm DNA and preimmune serum. The pre-cleared chromatin solution was divided and used in immunoprecipitation assays with an anti-Twist1 antibody. Following the wash, the antibody-protein-DNA complex was eluted from the beads and reversed cross-link incubation. After removed protein and RNA, purified DNA was subjected to PCR with primers specific for human VE-cadherin promoter (AC012325). The sequence of the PCR primers used was 5'-AGCCAGCCCATGCCCTCAC-3' and that for the reverse primer was 5'-CCTGTCAGCGACGTCTTTG-3'.

**Reporter Gene Assays.** The VE-cadherin promoter was PCR-amplified from human genomic DNA and cloned into the pGL4.3 Luciferase Reporter Vector (Promega) (Supporting Fig. 1). Transactivation assays were performed by way of percutaneous ethanol injection and cultured in a 3D Matrigel system for 72 hours. Luciferase activities were measured with the use of a Synergy 2 microplate reader system (Gene).

**Zymography Assays.** All media were collected and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 0.01% wt/vol gelatin containing 10% polyacrylamide gel. After electrophoresis, gels were equilibrated in 2.5% Triton X-100 and incubated in 50 mM Tris-HCl (pH 7.5), 10 mM CaCl2, 150 mM NaCl, 1 mM ZnCl2, and 0.02% NaN3 for 40 hours at 37°C. These were stained with Coomassie R250 and destained until the wash became clear with apparent cleared zones associated with MMP activity.

**Statistical Analysis.** All data in the study were evaluated with SPSS version 11.5 software (SPSS Inc.). Differences were considered significant at values of $P < 0.05$. 

**Results**

**VM Present in HCC Associated with Twist1 Nuclear Expression.** In the definition of VM, tumor cells mimic endothelial cells to form cell extracellular matrix–rich channels (PAS-positive), the sinusoidal structures surrounding clusters of tumor cells. The VM was identified by the presence of red blood cells in vessels lined by tumor cells (not by endothelial cells) and by the absence of necrosis and inflammatory cells infiltrating around the channels; it was found in 18 out of 97 HCC samples (19%). Succeeding clinical data are shown in Table 1.
The cells in the slide were negative for CD31 and positive for PAS, indicating that they were not endothelial cells. Rather, the cells stained positive for hepatocytes, indicating that they were HCC cells (Fig. 1A). In the 97 HCC samples, Twist expression could be detected in 13 of the 18 (72%) samples in the VM-positive group and in 38 of the 79 (48%) samples in the VM-negative group. Twist nuclear expression could be detected in 13 of the 18 (72%) samples in the VM-positive group and in 20 of the 79 (25%) samples in the VM-negative group. Twist1 protein expression in the VM-positive and VM-negative group was not significant, but Twist1 nuclear expression was significant between the VM-positive and the VM-negative groups (P value not significant) (Fig. 1B,C). To further confirm the correlation of Twist1 with VM, we compared the number of VM-PAS–positive pattern and Twist expression or nuclear expression positive rate and found a significant value between Twist nuclear expression cells and PAS-positive pattern (Pearson correlation test; P = 0.000, r = 0.762) (Fig. 1D).

**Twist1 Expression Level in HCC Cell Lines, Ectopic Introduction of Twist1 with Up-regulation in HepG2 Cells, and Knockdown in Bel-7402 Cells.** To further screen the expression level of Twist1, we compared the level of mRNA and protein of Twist1 expression in various HCC cell lines using reverse-transcription PCR (RT-PCR) and western blotting. We found that HepG2 had a low-level Twist1 expression in contrast with the Bel7402, which presented a high level (Fig. 2A). HepG2 cells were observed in the up-regulation Twist1 expression when transfected with Twist1 cDNA. Immunofluorescence staining revealed increased nuclear Twist1 protein expression in the HepG2-Twist1 transfectants compared with the negative vector controls. Twist1 overexpression resulted in morphologic changes from tightly packed colonies to scattered growth structure. To investigate the molecular changes in EMT in the HepG2-Twist1 transfectant, we detected the expression of epithelial marker E-cadherin (Fig. 2B) in up-regulated Twist1 expression. Meanwhile, E-cadherin expression was repressed as detected with RT-PCR, western blotting, and immunofluorescence compared with the negative vector controls. For Bel7402 cells, knockdown by siRNA decreased Twist1 expression detected with RT-PCR, western blotting, and immunofluorescence. The results revealed a high transfection (about 60%) and gene knockdown (about 70%) efficiency. When Twist1 expression was knocked down, the morphology of Bel7402 cells changed from a scattered growth structure to tightly packed colonies, but E-cadherin expression was not detected.

**Twist1 Up-regulation Leading to Increased HCC Cell Invasion, Migration, and VM Formation In Vitro.** Given the up-regulated cell model in HepG2 and knockdown cell model in Bel7402, as well as the detection of down-regulated E-cadherin following the ectopic introduction of Twist1 nuclear relocation, we then investigated the effect of Twist1 activation on cell invasion, migration, and VM formation in the 3D culture system. E-cadherin is one of the most frequently reported characteristics of metastatic HCC and VM formation.18 VM formation is associated with cell migration and invasion, and it is a similar mechanism among endothelial cells.12,18 We studied the invasion and migration ability of HepG2 and Bel7402 after performing Twist1 ectopic transfection and Twist1 knockdown, respectively. As shown in Fig. 3A, following a wound healing assay, the quantitative analysis suggests a significant difference in the speed of wound healing between the transfection groups and the control group. In the invasion assay presented in Fig. 3B, following Matrigel invasion assay, about twice an increase in cell invasion was observed in the Twist1-transfected HepG2 cell line compared with the negative vector control (P < 0.01). We also observed a two-fold to three-fold decrease in Twist1-shRNA–transfected Bel7402 cells compared with the negative vector control (P < 0.01).

We then used a well-established in vitro model of 3D culture for investigating vasculogenic mimicry formation in order to elucidate whether Twist1 mediates morphological alterations of the HCC cells. Our results demonstrated that both HepG2 and Bel7402 cells, when up-regulated or down-regulated by Twist1, formed typical pipe-like structures within the 3D Matrigel medium. There was no significant difference for the HepG2 in the efficiency of network

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### Table 1. Correlation Between VM and Clinicopathologic Characteristics of Patients with HCC

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<td>0</td>
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<tr>
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<td>36</td>
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<td>0.014*</td>
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<tr>
<td></td>
<td>≤5</td>
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<td>43</td>
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<tr>
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<td>50</td>
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<td></td>
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<tr>
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<td>III IV</td>
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<td>36</td>
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<tr>
<td>AbSENT</td>
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<td>43</td>
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*Significantly different.
Fig. 1. The correlation of Twist1 nuclear expression with VM formation in clinical samples. (A) Evidence of VM in HCC. Morphologic observation with hematoxylin-eosin staining. The channels (yellow arrow) lined with tumor cells containing red blood cells and shuttle-like endothelium cells do not appear in the VM wall (a). The cells lining VM were positive for hepatocytes (b). Tumor cells were negative for CD31 and PAS double-staining (yellow arrow), and the base membrane-like structure between red blood cells and tumor cells was positive for PAS. The endothelial-dependent and mosaic vessels were both positive for CD31 and PAS (white arrows) (c). VM was identified by the presence of red blood cells in the vessels lined by tumor cells, not by endothelial cells, and by the absence of necrosis and inflammatory cells infiltrating the channels, which was found in 18 HCC samples (19%). The PAS-positive networks were shown by a green filter in the photomicroscope; VM was marked by yellow arrow (d). (B) Twist1 expression in HCC. Overview of the tissue section containing 51 samples showing Twist1 expression and 33 samples showing Twist1 nuclear expression. Immunostaining of Twist1 cytoplasm expression in VM-negative and VM-positive HCC samples (a and b, respectively); Twist1 nuclear expression in VM-negative and VM-positive HCC samples (c and d, respectively). (C) Twist1 expression in the VM(+) and VM(−) groups was not significant (P = 0.064), but Twist1 nuclear expression was significantly associated with VM formation (P = 0.000). (D) Quantum analysis of the correlation of Twist1 with VM. VM was analyzed by the VM-PAS pattern, and it was revealed that the Twist1 cytoplasm and nuclear expression positive rate both have significant values (cytoplasm: P = 0.000, r = 0.741; nucleus: P = 0.000, r = 0.762).
formation, but Bel7402 had the ability to form a network. In the control groups of HepG2, there was a lack of tube formation, providing further support for the possible role of Twist1 in promoting VM formation. However, cells accumulating in clumps could suggest that the lack of Twist1 expression could only lead to cell-clonal proliferation. We also observed the emergence of pipe-like structures and cellular plasticity in the Bel7402 control group, but this was not observed in the Twist knockdown group (Fig. 3C). These results indicate that Twist1 activation might lead to increased invasiveness and migration of HCC cells and that it could also play an important role in VM formation in vitro.

Twist1 Leading to VM Formation Through VE-cadherin Transcription and MMP Activation. In the process of VM formation by tumor cells, VE-cadherin plays an important role as an intercellular adhesion and cascade signal transduction molecule.12,19 Our data indicate an up-regulation of VE-cadherin expression in the 3D culture system based on the Twist1 transfection of HepG2 cells compared with the control group. We used an siRNA-based technique to silence specifically the Twist1 expression of the Bel7402 cells, the results of which showed a down-regulation of Twist1 as well as a significant decrease of VM formation in the 3D cultures compared with the control group. There was also a significant change in VE-cadherin as shown by these two groups (Fig. 4A). The MMPs act as effector molecules, and they are critical in cell plasticity and VM-formation.3,10,11 Based on zymographic assays, the activities of MMP2 and MMP9 were significantly higher in the Twist1 up-regulated group in HepG2 compared with the control group. In particular, the activity of MMP9 with Twist1 in HepG2 cells was five times higher than that in the control group (Fig. 4B). When we knocked down the expression of Twist1 in Bel7402 cells, there was a down-regulation of MMP9 and MMP2 activities. Through this process, tumor cells enhanced their expression of VE-cadherin and active MMPs through the up-regulated Twist1 expression, which is ultimately important to VM formation. We performed ChIP assays to determine if the transcription activation of Twist1 on the VE-cadherin promoter had been a result of the direct recruitment of Twist1 to the promoter. Transient transfection of HCC HepG2 cells with a Twist1 expression vector and Bel7402 cells with a Twist1 knockdown vector were used. Here, 5- and 10-day cultures were performed with a Twist1 antibody in 3D Matrigel system after conducting transfection using a ChIP assay. We found that Twist was present in VE-cadherin transcription in the 3D culture niche but not in the monolayer culture (Fig. 4C). Further results showed that Twist1 overexpression could enhance VE-cadherin promoter activity. HepG2 cells were cotransfected with a VE-cadherin promoter-luciferase construct and Twist1-cDNA in an expression vector. Twist1 overexpression resulted in an approximate two-fold transactivation of VE-cadherin promoter activity in a 3D Matrigel system (Fig. 4D). These results indicate that VE-cadherin promoter is also the target of Twist binding in a 3D culture system.

Relationship Between Twist1, VM-Associated Marker, and Clinical Data. Immunohistochemical analysis was performed to assess the expression of Twist1, Twist1 (nuclear), Twist2, E-cadherin, VE-cadherin, MMP-2, and MMP-9 in the HCC samples. The results
Fig. 3. Effect of Twist1 overexpression and knockdown on cell motility, invasion abilities, and VM formation in the Matrigel of HCC cells. (A) Following the wound healing assay, quantitative analysis showed a significant difference in the speed of wound healing between the Twist1 overexpression and knockdown group compared with the negative control. The arrows signify the different cell motilities between the cells with and without low Twist1 expression (n = 3). (B) For the invasion assay, the Twist1 overexpression group presents a twofold increase in cell invasion as compared to the control group (P < 0.01) in HepG2. The Twist1 knockdown group presents a threefold decrease as compared to the control group (P < 0.01) in Bel7402 (n = 3). (C) Matrigel 3D culture was utilized as a well-established in vitro model for investigating VM formation. Bel7402 cells can form typical pipe-like structures within the Matrigel medium, but HepG2 cannot. When up-regulated by Twist1, HepG2 gains the capability to carry out VM formation in vitro. To knockdown Twist1 expression by shRNA, Bel7402 cells lose the capability to carry out VM formation in vitro as compared with the control group. The arrow shows the pipe-like structures and hollow tubular networks in the 3D culture.
showed that Twist1, Twist2, E-cadherin, VE-cadherin, MMP-2, and MMP-9 expressions were located in the cytoplasm, nuclei, or membranes of the HCC cells (Fig. 5, Supporting Fig. 2). The expression of these proteins in the cells with and without VM was compared using the \( \chi^2 \) test (Table 2). The positive rates of Twist1 (nuclear), VE-cadherin, and MMP-9 expression with VM were higher compared with cancers without VM. The differences in Twist1 (nuclear), VE-cadherin, and MMP-9 were statistically significant (\( P < 0.01 \)). The correlation between
Twist1, Twist1 (nuclear), Twist2, E-cadherin, VE-cadherin, MMP-2, and MMP-9 was tested by way of association analysis. The results are shown in Supporting Table 2. Numeration data were counted by positive cell rate, and analysis was conducted using the Pearson correlation test. We found that Twist (nuclear) is correlated to VE-cadherin, Twist1, and MMP9 (P < 0.05). Results showed that the numeration of the VM-PAS pattern was correlated to Twist (nuclear), VE-cadherin, Twist1, and MMP9 (P < 0.01) (Fig. 6). A Kaplan-Meier survival analysis revealed that patients with VM and expression of Twist1, Twist1 (nuclear), VE-cadherin, and MMP9 had a shorter survival period than those without expression. At the same time, E-cadherin expression had a longer survival compared with those without expression (Supporting Fig. 3). Clinical data for Twist1 expression and relocation into the nucleus showed that both had a significant effect on VM-positive patients. The expression of Twist1, VE-cadherin, E-cadherin, and MMP9 may play an important role in the process of VM formation.

**Discussion**

To our knowledge, this is the first report discussing the relationship between EMT-associated molecular Twist and VM. In this study, we explored HCC and presented a well-established VM as the model. The EMT has an important role in the development of tissues during embryogenesis, but similar changes are recapitulated during pathological processes, as in the case of cancer.\(^{20,21}\) Most of the research conducted regarding EMT focuses on the invasion and metastasis of cancer cells.\(^{22,23}\)

The existence of VM shows that tumor cells have more than one “trick” to ensure that they receive their food supply. Under conditions that are being extensively investigated (as in the case of hypoxia niche), it has been found that cancer cells have tremendous plasticity and can alter their cell marker and function in order to adapt to a specific microenvironment.\(^{5,9,10,11,15}\) This phenomenon is similar to the pattern of embryonic vasculogenic network. Interestingly, EMT is an integral part of the tissue remodeling that occurs during embryogenesis, and it is considered extremely critical because it often allows the formation of a three-layered embryo through gastrulation. During embryogenesis, several extracellular signals can convert epithelial cells into mesenchymal cells by triggering EMT.\(^{13,21}\) The mechanism between cancer cell plasticity in the endothelial cells and the EMT process may be the same and may thus have a similar relevance.

Attempts to elucidate the mechanism of VM formation have presented a significant challenge using traditional views.\(^{1,10}\) A new perspective for VM highlights the answer to this question, which can be better addressed by first considering the embryonic footprint of cancer cells comprising specific tumors, especially in poorly aggressive tumors.\(^{10,15}\) There is a view pointing out that the tumor cell plasticity to a VM network is present in a cancer stem cell–like phenotype or a cancer stem-like cell phenotype.\(^{24}\) This process is referred to as EMT, which is characterized by the down-regulation of the molecular markers of epithelial cells together with the loss of intercellular junction that can result in the reduction of intercellular adhesion and increased mesenchymal molecules.\(^{21-23,25}\) In VM, HCC cells mimic endothelial-like cells and function as vessels. Whether these cells are derived from true HCC cells or cancer stem cells through the EMT present in the tumor remains to be investigated. It is conceivable that a population of cancer stem cells or stem-like cells can exist in high-grade HCC and that this stem cell population is responsible for VM formation.\(^{26,27}\)

In this study, we found that the transfection of HepG2 cells with ectopic-induced Twist1 expression could change in cellular morphology consistent with EMT. We also found that this could promote invasion and migration and presents an aggressive pattern of tubular structures and patterned networks in 3D culture system. Moreover, based on the results from the high-expression Twist1 cell line Bel7402, we found that the transfection of Bel7402 cells with Twist1 shRNA plasmid could knock down the expression of Twist1 and inhibit cell invasion, migration, and VM formation in vitro. These in vitro data show that Twist1 not only plays an important role in tumor cell invasion and migration but is also closely associated with tumor cell plasticity to VM pattern. From human HCC tissue samples, we found a significant positive correlation between Twist1 nuclear expression and the number of VM pattern (counts by PAS-positive and CD31-negative pattern \(P = 0.000; r = 0.762)\). This result was further confirmed by evaluating the Twist1 nuclear expression by Western blotting.

### Table 2. Relationship Between VM and VE-cadherin, Twist-2, E-cadherin, MMP-2, and MMP-9 Expression

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<tr>
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<td>9.431</td>
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<tr>
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<td>Positive</td>
<td>44</td>
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*Significantly different.
expression, leading some HCC cell plasticity to VM cells by way of a specific molecular pathway.

A biologically significant marker is necessary to detect VM-associated tumor cells. VE-cadherin is exclusively expressed by highly aggressive tumor cells and is critical in tumor VM. It is not only considered an adhesive transmembrane protein exclusively expressed at the interendothelial junctions in differentiated cells, but it can also lead to the induction of vascular signaling cascades. In the VM formation of tumor cells, a highly expressed VE-cadherin is usually present. We also used this marker to detect HCC cell plasticity or EMT into endothelial cells. Previous studies have examined the VE-cadherin expression with respect to Twist1 up-regulation or knockdown in HepG2 and Bel7402 cells. Data show that VE-cadherin expression is up-regulated following Twist1 nuclear expression up-regulation in the 3D culture system; down-regulated Twist1 also results in decreased VE-cadherin. Data on ChIP-PCR provide the basis for the conclusion that Twist1, when up-regulated in the nucleus, can promote VE-cadherin transcription. This

Fig. 6. Correlation analysis between VM-PAS pattern and Twist1, Twist1 nuclear, Twist2, VE-cadherin, E-cadherin, MMP2, and MMP9 expression was tested by way of a Pearson correlation test. The results show that the VM-PAS pattern has a correlation with Twist1 (nuclear), Twist1, VE-cadherin, and MMP9 ($P < 0.01$).
means that Twist1 can also regulate endothelial cell marker transcription not only for the fibroblast marker. These contrasting data suggest that Twist1 can promote endothelial cell–associated molecule expression such as VE-cadherin in a certain niche.

We have reported that VM is associated with metastasis in HCC and shows a shorter survival period. In this study, we provide evidence that VM is associated with Twist1 expression in the nucleus and that Twist1 nuclear expression is associated with metastasis. Clinical data present a logical relationship between Twist1 nuclear expression, VM, metastasis, and poor prognosis. Immuno-staining showed the up-regulation and relocation of Twist1 in the nuclear compartment found in VM-positive tissue samples. Concomitantly, Twist1 overexpression in the nucleus revealed down-expression of E-cadherin and up-expression of VE-cadherin, all of which are consistent with common characteristics between EMT and VM.

By further detecting HCC tissue samples and in vitro cells, our data showed that MMP expression and activity up-regulated after Twist1 nuclear up-regulation. The MMP9 activity involved invasion and metastasis, which might be useful in preventing tumors from developing in the vessels by way of VM. In this study, Twist1 overexpression in HepG2 and knockdown in Bel7402 cells correlated with MMP activity, especially with MMP9. In tissue samples, MMP9 also showed a significant expression in the VM-positive group as well as a correlation with Twist1 nuclear expression.

In mammals, two Twist-like proteins, Twist1 and Twist2, share high structural homology. Gene deletion experiments have shown that Twist1 and Twist2 have some functional redundancy. In this report, Twist1 was found to be significant in VM formation, the same cannot be said for Twist2. The HCC tissue samples analysis show that Twist2 is usually expressed in the cytoplasm, not in the nucleus, and has no correlation with VM, MMPs, and survival time. This is in contrast with the roles that Twist1 and Twist2 play in VM formation. Therefore, further investigation is required to determine the function differences between Twist1 and Twist2 in tumors.

In conclusion, we showed for the first time that Twist1 was correlated with HCC VM formation and that the EMT mechanism potentially includes epithelial–endothelial transition. Twist1 induced HCC cell plasticity to VM cells through the suppression of E-cadherin expression and the induction of VE-cadherin up-regulation, rather than through a VM pattern in vivo and in a 3D in vitro system. Our findings not only provide a molecular basis for the role of EMT mechanism in HCC VM formation but also suggest a novel cognition in cancer stem-like cell differentiation. More importantly, the study showed that Twist1 and related molecular pathways might be used as novel therapeutic targets for the inhibition of HCC angiogenesis and metastasis.

References


