Twist Overexpression Correlates with Hepatocellular Carcinoma Metastasis through Induction of Epithelial-Mesenchymal Transition

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Abstract

Purpose: Hepatocellular carcinoma (HCC) is a rapidly growing tumor associated with a high propensity for vascular invasion and metastasis. Epithelial-mesenchymal transition (EMT) is a key event in the tumor invasion process. Recently, Twist has been identified to play an important role in EMT-mediated metastasis through the regulation of E-cadherin expression. However, the actual role of Twist in tumor invasiveness remains unclear. The purpose of this study is to investigate the expression and possible role of Twist in HCC.

Experimental Design: We evaluated Twist and E-cadherin expression in HCC tissue microarray of paired primary and metastatic HCC by immunohistochemical staining. The role of Twist in EMT-mediated invasiveness was also evaluated in vitro in HCC cell lines.

Results: We first showed that overexpression of Twist was correlated with HCC metastasis (P = 0.001) and its expression was negatively correlated with E-cadherin expression (P = 0.001, r = -0.443) by tissue microarray. A significant increase of Twist at the mRNA level was also found in metastatic HCC cell lines MHCC-97H, MHCC-97L, and H2M when compared with nonmetastatic Huh-7, H2P, and PLC cell lines. The MHCC-97H cell line, which has a higher metastatic ability, was found to have a higher level of Twist than MHCC-97L. Accompanied with increased Twist expression in the metastatic HCC cell lines when compared with the nonmetastatic primary ones, we found decreased E-cadherin mRNA expression in the metastatic HCC cell lines. By ectopic transfection of Twist into PLC cells, Twist was able to suppress E-cadherin expression and induce EMT changes, which was correlated with increased HCC cell invasiveness.

Conclusion: This study shows that Twist overexpression was correlated with HCC metastasis through induction of EMT changes and HCC cell invasiveness.

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and is the second leading cause of cancer death in Hong Kong (1, 2). HCC is associated with a high potential for vascular invasion, metastasis, and recurrence even after surgical resection, leading to poor prognosis (3). Intrahepatic and extrahepatic metastases occur in >50% of patients after resection of HCC in most reports in the literature, with intrahepatic metastases occurring more frequently (3). The common sites of extrahepatic metastasis include lung, bone, peritoneum, spleen, and lymph nodes (3). HCC invasiveness is related to the ability of tumor cell to invade the capsule and portal vein (4, 5). Epithelial-mesenchymal transition (EMT) is a key event in the tumor invasion process whereby epithelial cell layers lose polarity and cell-cell contacts and undergo a dramatic remodeling of the cytoskeleton (6). A hallmark of EMT is the loss of E-cadherin expression (6). E-cadherin is a central component of cell-cell adhesion junctions in the maintenance of cell polarity and environment (7, 8). In HCC, loss of E-cadherin expression is associated with tumor invasiveness, metastasis, and prognosis (7). The expression of E-cadherin is regulated at the genetic level through gene mutation, loss of heterozygosity, and hypermethylation of its promoter in various cancers (9–13). However, E-cadherin promoter methylation is not closely related to the loss of E-cadherin expression (13), and hence, other repression mechanisms have been suggested recently. The regulatory mechanism of E-cadherin is controversial and consensus has not been reached.

Several EMT-inducing regulators repress E-cadherin transcription via interaction with specific E-boxes of the proximal E-cadherin promoter (6). Snail-related zinc finger transcription factors (Snail and Slug) are the most prominent ones (14, 15). Recently, the basic helix-loop-helix transcription factor Twist, a known protein identified in Drosophila melanogaster (16, 17) as an organizer of the EMT during gastrulation and regulator of mesoderm differentiation, has been added to the list of developmental genes with a key role in E-cadherin repression and EMT induction (6). Consistent with its function in EMT, Twist has been found to be correlated with metastasis in various malignancies. Recently, Twist has been found to be correlated with metastasis in various malignancies. This study shows that Twist overexpression was correlated with HCC metastasis through induction of EMT changes and HCC cell invasiveness.

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cancers including breast and prostate cancers (6, 18). Twist knockdown by RNAi approach in metastatic mammary tumor prevented metastasis, presumably by blocking intravasation (6). In addition, overexpression of Twist in two human epithelial cell lines caused both complete EMT and E-cadherin repression (6). However, it is still unclear whether Twist interacts with these E-boxes directly via E2A protein or through indirect mechanisms.

Because EMT has been found to be a crucial step contributing to tumor invasiveness, it is of great interest to examine the role of Twist in HCC. Thus far, there has been no data on the role of Twist in HCC. The aim of this study was to investigate the expression and possible role of Twist in HCC metastasis by tissue microarray (TMA) of paired primary and metastatic HCC. In addition, the correlation between Twist and E-cadherin was examined by immunostaining in the TMA specimens and ectopic transfection of Twist cDNA into the HCC cell line. Our evidence suggests that Twist is correlated with HCC metastasis through repression of E-cadherin.

**Materials and Methods**

**Patient samples.** Tissue specimens from TMA were obtained from 60 patients who underwent hepatectomy for HCC between 1995 and 1999 in the Eastern Hepatobiliary Surgery Hospital, Shanghai, China, and subsequently developed intrahepatic or extrahepatic metastases. Matched pairs of primary and metastatic HCC samples were obtained for the TMA.

**Construction of TMA.** The HCC TMA was constructed as previously described (19). Briefly, all tissue samples embedded in paraffin for array studies were freshly sectioned and stained with H&E. The representative regions of lesion were reviewed carefully and defined by two pathologists. Based on the clinicopathologic information, specimens were grouped in tissue cylinders and a diameter of 0.6 mm was taken from the selected regions of the donor block and then punched precisely into a recipient paraffin block using a tissue array instrument (Beecher Instruments, Silver Spring, MD). Consecutive 5 μm sections of the microarray blocks were made with a microtome. Finally, a TMA section with 60 pairs of primary and matched metastatic HCC samples, including 31 intrahepatic and 29 extrahepatic (19 peritoneal, 6 lymph nodes, 1 intestine, 2 spleen, and 1 lung) metastases were constructed.

**Immunostaining.** Formalin-fixed and paraffin-embedded sections with a thickness of 4 μm were dewaxed in xylene and graded alcohols, hydrated, and washed in PBS. After pretreatment in a microwave oven [12 minutes in sodium citrate buffer (pH 6)], the endogenous peroxidase was inhibited by 0.3% H2O2 for 30 minutes, and the sections were incubated with 10% normal goat serum for 30 minutes. Primary antibodies [rabbit polyclonal anti-Twist, H-81 (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-E-cadherin, clone 4A2C7 (Zymed Laboratories Inc., South San Francisco, CA)] were applied overnight in a moist chamber at 4°C. A standard avidin-biotin peroxidase technique (DAKO, Carpinteria, CA) was applied. Briefly, biotinylated goat anti-rabbit immunoglobulin, goat anti-mouse, and avidin-biotin peroxidase complex were applied for 30 minutes each, with 15-minute washes in PBS. The reaction was finally developed by Dako Liquid DAB+ substrate chromogen system (DAKO).

**Plasmids.** pcDNA-Twist was a gift from Dr. Glackin, Beckman Research Institute of the City of Hope, Duarte, CA (20) and E-cadherin promoter was a gift from Prof. S.W. Tsao, Department of Anatomy, the University of Hong Kong, Hong Kong.

**Cell lines.** Two metastatic HCC cell lines, MHCC-97L and MHCC-97-H (low and high metastatic potential, respectively, from Liver Cancer Institute, Fudan University, Shanghai, China; ref. 21), non-

**Fig. 1.** A, correlation of Twist expression with metastasis in clinical samples. Overview of a tissue array section containing 60 pairs of primary HCC and their matched metastatic tumors showing Twist expression (a). Immunostaining of Twist in a pair of HCC (b and c). Higher magnification of one pair of a primary and its matched metastatic HCC (case 22) is depicted from the boxed area (b and c). B, using quantitative PCR, a significant increase of Twist mRNA level was found in MHCC-97H, MHCC-97L, and H2M when compared with nonmetastatic Huh-7, H2P, and PLC cell line. In addition, elevated Twist mRNA was found in MHCC-97H when compared with MHCC-97L.

metastatic primary HCC cell lines Huh-7 (a gift from Dr. H. Nakabayashi, Hokkaido University School of Medicine, Sapporo, Japan; ref. 22), PLC (Japanese Cancer Research Bank, Tokyo, Japan), and H2P and H2M (a pair of primary and its matched metastatic
HCC cell lines; ref. 19) were maintained in DMEM with high glucose (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 100 mg/mL penicillin G, and 50 μg/mL streptomycin (Life Technologies) at 37°C in a humidified atmosphere containing 5% CO2. All cell lines used in this study correspond to malignant hepatocytes.

Quantitative reverse transcription-PCR. Total RNA was isolated using Trizol reagent according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). cDNA was synthesized using the SuperScript first strand synthesis system (Invitrogen). Portions of double-stranded cDNA were subjected to PCR with a SYBR Green PCR kit (Applied Biosystems, Foster City, CA). The amplification protocol comprised incubations at 94°C for 15 seconds, 63°C for 30 seconds, and 72°C for 60 seconds. Incorporation of the SYBR Green dye into PCR products was monitored in real time with an ABI PRISM 7700 sequence detection system (PE Applied Biosystems), thereby allowing determination of the threshold cycle (Ct) at which exponential amplification of products begins. The amount of target cDNAs relative to that of the 18S cDNA

### Table 1. Twist and E-cadherin expression in HCC TMA

<table>
<thead>
<tr>
<th>Twist</th>
<th>E-cadherin</th>
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<tr>
<td></td>
<td>Negative (-ve)</td>
</tr>
<tr>
<td>Primary HCC</td>
<td>40 of 54 (74%)</td>
</tr>
<tr>
<td>Metastatic HCC</td>
<td>24 of 57 (42%)</td>
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<tr>
<td></td>
<td>6 of 54 (11%)</td>
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<td></td>
<td>21 of 57 (37%)</td>
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<td>39 of 54 (72%)</td>
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Fig. 2. A, correlation of Twist with E-cadherin in HCC. The correlation between Twist with E-cadherin was shown in consecutive sections of HCCs. Case 5 showed strong immunostaining of Twist but was negative for E-cadherin (a and b). In case 36, it showed negative staining for Twist but strong membrane staining of E-cadherin (c and d). B, correlation of Twist with E-cadherin in various HCC cell lines with different metastatic potential. Using quantitative PCR, we found decreased E-cadherin mRNA expression in metastatic HCC cell lines, MHCC-97L and MHCC-97H, when compared with nonmetastatic primary ones (PLC and Huh-7). In addition, H2M showed decreased E-cadherin expression when compared with its matched primary HCC cell line-H2P. The E-cadherin expression was negatively correlated with Twist expression in vitro.
was calculated from the C_{T} values with the use of Sequence Detector ver. 1.6.3 software (PE Applied Biosystems), and was then amplified by PCR with Twist and E-cadherin primers: Twist-f, 5'-GGGAAGGCTGACAGAATT-3' and Twist-r, 5'-CTCTGCTGAAACAATGAC-3'; E-cadherin-f, 5'-GAAACGATGAAATGAC-3' and E-cadherin-r, 5'-TTGTCAGCAACAGGAT-3'.

**Twist and E-cadherin immunofluorescence staining.** Cells were plated onto chamber slides in DMEM medium at approximately 70% confluence for 24 hours. They were fixed in ice-cold acetone and methanol (1:1), washed with PBS, and then stained with mouse anti-E-cadherin and anti-rabbit Twist overnight at 4°C. After washing, the cells were applied with goat anti-mouse FITC or anti-rabbit TRITC conjugated secondary antibody for 30 minutes at room temperature and then counterstained with 4',6-diamidino-2-phenylindole for 1 minute at room temperature. The cells were examined under a fluorescent microscope.

**Cell transfection.** pcDNA and pCDNA-Twist were transfected into PLC cells using Lipofectin (Life Technologies Inc., Carlsbad, CA) and a pool of transfectants (~150-200 colonies) was selected using G418 at a dose of 800 μg/mL.

**Luciferase promoter assay.** PLC cells (5 × 10^4 cells per well) were plated into 24-well culture plates and allowed to grow for 24 hours. E-cadherin promoter (provided by Prof. S.W. Tsao) and pRL-CMV-Luc were cotransfected with either pCDNA-Twist or pcDNA into the cells using Fugene 6 reagent (Roche Diagnostics, Indianapolis, IN). The cells were lysed 48 hours after transfection and were assayed for luciferase activity.

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**Fig. 3.** Effect of ectopic Twist expression on EMT changes. The effect of Twist activation on EMT by ectopic transfection of Twist cDNA into PLC cell. A, using immunofluorescence staining, we found increased nuclear Twist protein expression (white arrows) in PLC-Twist transfectants when compared with PLC transfected with empty vector control. The cells were counterstained with 4',6-diamidino-2-phenylindole. B, Twist induced morphologic changes from tightly packed colonies to scattered growth structure. C, we examined the expression of Twist, E-cadherin, β-catenin, α-catenin protein, fibronectin, and Sm-actin in PLC-Twist transfectant and PLC-pcDNA by Western blot. Decreased E-cadherin was noted in PLC-Twist transfectant.
activity using the dual luciferase reporter assay system (Promega, Madison, WI). Firefly luciferase activity was measured at 48 hours after transfection and the reading was then normalized with the Renilla luciferase activity, which served as an internal control for transfection efficiency. Luciferase assay was also done in stable PLC-pcDNA and PLC-Twist transfectants by transfection of E-cadherin promoter and pRL-CMV-Luc. Each experiment was done at least thrice in duplicate wells and each data point represented the mean and SD. The percentage decrease in luciferase activity of E-cadherin promoter was calculated relative to that of the vector controls. The mean percentage decrease in luciferase activity was presented as the final results and the SD of the means was used as error bars.

**Wound healing assay and invasion assay.** Cell motility was assessed by measuring the movement of cells into a scarped, acellular area created by a 200 μL pipette tube (time 0), and the speed of wound closure was monitored after 24 hours. Invasion assays were done with 24-well BioCoat Matrigel Invasion Chambers (Becton Dickinson and Company, Franklin Lakes, NJ) using 5 × 10^4 cells in serum-free DME and plated onto either control or Matrigel-coated filters. Conditioned medium from PLC or PLC-Twist cells were placed in the lower chambers as chemoattractants. After 22 hours in culture, the cells were removed from the upper surface of the filter by scraping with a cotton swab. The cells that invaded through the Matrigel and were adherent to the bottom of the membrane were stained with crystal violet solution. The cell-associated dye was eluted with 10% acetic acid and its absorbance at 595 nm determined. Each experiment was done in triplicate and the mean values ± SE were presented.

**Western blotting.** The cells were lysed and protein extraction was done. The samples were separated in 10% SDS acrylamide gel and electrophoretically transferred to polyvinylidene fluoride membrane (Amersham, Buckinghamshire, United Kingdom). The membrane was blotted with 10% nonfat milk, washed and then probed with Twist, actin, fibronectin, and Sm-actin (Santa Cruz Biotechnology), E-cadherin, β-catenin, and α-catenin (Zymed Laboratories). After

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**Fig. 3** Continued. D, consistent with decreased E-cadherin expression in PLC-Twist, E-cadherin was mainly detected in the cytoplasm of PLC-Twist transfectant, indicating function inactivation. **Red arrows,** the membrane staining of E-cadherin in PLC-pcDNA. E-cadherin promoter activity was decreased in PLC-Twist transfectant when compared with empty vector control. **E,** to further examine whether Twist inactivated E-cadherin transcriptionally, we did a promoter assay. **F,** increased promoter activity was found in PLC-Twist transfectant when compared with the vector control.
washing, the membrane was incubated with horseradish peroxidase–conjugated anti-mouse, anti-rabbit, or anti-goat antibody (Amersham) and then visualized by Enhanced Chemiluminescence Plus according to the manufacturer’s protocol.

**Statistical analysis.** Continuous data were expressed as median and range and compared between groups using the Mann-Whitney U test. Categorical variables were compared using the χ² test (or Fisher’s exact test where appropriate). All statistical analyses were done using a statistical software (SPSS 9.0 for Windows; SPSS, Inc., Chicago, IL). *P* < 0.05 was considered statistically significant.

**Results**

**Twist overexpression correlated significantly with HCC metastasis.** To determine the possible role of Twist in HCC metastasis, we evaluated Twist expression in TMA by immunostaining. In 60 pairs of primary HCC and their matched metastatic tumors, nuclear Twist expression could be detected in 14 of 54 (26%) and 33 of 57 (58%) tumors, respectively (Fig. 1A). Twist expression was significantly associated with HCC metastasis (*P* = 0.001). Twist protein expression in primary and their matched metastatic HCC was summarized in Table 1. To further confirm the correlation of Twist with metastasis, we compared Twist expression in various HCC cell lines with different metastatic potentials by reverse transcription-PCR. A significant increase of Twist at the mRNA level was found in MHCC-97H, MHCC-97L, and H2M when compared with nonmetastatic Huh-7, H2P, and PLC cell lines (Fig. 1B). The MHCC-97H cell line, which has a higher metastatic ability, was found to have a higher level of Twist than MHCC-97L (Fig. 1B).

**Twist overexpression negatively correlated with E-cadherin expression in HCC.** To further assess the possible correlation of Twist expression with E-cadherin, we evaluated the protein expression of E-cadherin in another set of TMA specimens. It was found that Twist was negatively correlated with E-cadherin expression (*P* = 0.001, *r* = −0.443; Fig. 2A). The E-cadherin expression in TMA was summarized in Table 1. The correlation of Twist with E-cadherin in HCC was also investigated by examination of E-cadherin mRNA expression in the above HCC cell lines with different metastatic potentials by reverse transcription-PCR. Accompanied with increased Twist expression in the metastatic HCC cell lines when compared with the nonmetastatic primary ones in Fig. 1B, we found decreased E-cadherin mRNA expression in the metastatic HCC cell lines (Fig. 2B).

**Ectopic introduction of Twist led to EMT activation through E-cadherin repression.** Given the negative correlation between Twist and E-cadherin in HCC samples and cell lines, we then investigated the effect of Twist activation on EMT by ectopic transfection of Twist cDNA into the PLC cell. After transfection, a pool of clones (150-200 clones) was isolated after stable selection by G418. There are many reports showing gene silencing by CMV promoter methylation in hepatocytes (23, 24). In this experiment, Twist expression was sustained after long-term cultivation of PLC-Twist transfectants. Therefore, promoter methylation in the hepaticocyte might be cell line–specific. By immunofluorescence staining, we found increased nuclear Twist protein expression in the PLC-Twist transfectants when compared with empty vector controls (Fig. 3A). Twist overexpression resulted in morphologic changes from tightly packed colonies to scattered growth structure (Fig. 3B). To further examine the molecular changes in EMT in the PLC-Twist transfectant, we examined the expression of epithelial and fibroblast markers. As shown in Fig. 3C, decreased epithelial markers, E-cadherin, β-catenin, and α-catenin protein, in the PLC-Twist transfectant and increased fibroblast markers, fibronectin and Sm-actin, were observed. As the hallmark of EMT is the loss of E-cadherin expression, and the membrane localization indicates its biological function, we did immunofluorescence staining to examine the localization of E-cadherin in the PLC-Twist transfectant. E-cadherin was mainly localized at the cell membrane of PLC cells transfected with empty vector, whereas E-cadherin was mainly detected in the cytoplasm of PLC-Twist transfectants (Fig. 3D). Consistently, E-cadherin promoter activity was decreased in PLC-Twist transfectant when compared with the empty vector control (Fig. 3D). To further examine whether Twist inactivated E-cadherin transcriptionally, we then cotransfected pcDNA3.1 or pcDNA3.1-Twist together with a luciferase reporter harboring E-cadherin promoter and generated transient transfectants. We found that E-cadherin promoter activity was decreased by ~7.8-fold in Twist transfectants when compared with the vector control (Fig. 3E).

**Twist up-regulation led to increased invasion ability.** The aforementioned results suggested that Twist induced EMT changes in the PLC cell line. Given that the down-regulation of E-cadherin is one of the most frequently reported characteristics in metastatic HCC and decreased membrane E-cadherin expression was found in the PLC-Twist transfectant, we hypothesized that Twist regulation may lead to an increase in the invasion ability of the nonmetastatic HCC cell line. To test this hypothesis, we studied the invasion and migration ability of PLC after ectopic Twist transfection. As shown in Fig. 4A, following Matrigel invasion assay, an ~81% increase in cell invasion was observed in the Twist-transfected PLC cell line when compared with empty vector control. This result showed that Twist increased the invasive ability in this HCC cell line. Cell motility was investigated by a wound-healing assay. PLC-Twist showed higher cell motility than the vector control (Fig. 4B). These results indicated that Twist activation might lead to the increased invasiveness of HCC cell lines through regulation of EMT changes.

**Discussion**

HCC invasiveness is a key step that leads to metastasis, resulting in poor prognosis (3). Therefore, it is of great value to study the molecular mechanism of HCC invasiveness. Recently, increasing evidence has shown that EMT, a process first identified in embryogenesis (25), mediates tumor progression, including local invasion, spreading through the circulation and metastasis. Several developmental genes that induce EMT have been shown to act as E-cadherin repressors. The first of these is the zinc finger protein, Snail, a DNA-binding factor that recognizes E-box motifs in target promoters such as E-cadherin (15). The recent findings of Yang et al. added Twist to the class of EMT inducers (6). Twist was first suggested to be an oncogene (26, 27). For example, ectopic Twist introduction promotes colony formation of mouse embryonic fibroblasts in soft agar (27). Recently, Twist overexpression has been found to correlate with malignant transformation of melanoma (28). The role of Twist in cancer metastasis was first reported in a...
breast cancer model, which suggested that Twist induced EMT resulting in the promotion of tumor invasion (6). However, the role of EMT-mediated tumor metastasis is controversial. Some studies have shown that Twist overexpression was correlated with EMT-mediated metastasis in prostate and breast cancers (6, 18). In contrast, other studies reported that there was no correlation between gastric and colon cancers (29, 30). In this study, we first reported that Twist overexpression in HCC tumor tissues correlated with metastasis by TMA. This result was further confirmed by comparing Twist expression in various HCC cell lines with different metastatic potentials. As shown in Fig. 1B, we found that Twist was overexpressed in metastatic cell lines when compared with nonmetastatic primary ones. The above results strongly suggested the role of Twist in HCC metastasis.

Twist, like other EMT-inducing transcription factors such as Snail, Slug, and SIP, bind DNA using similar E-box sequence motifs, repressing E-cadherin (15). Therefore, we hypothesized that Twist induced EMT-mediated metastasis through E-cadherin repression. To confirm this, we evaluated the correlation between Twist and E-cadherin in HCC clinical samples by performing E-cadherin immunostaining on another TMA slide. From TMA, we found significant negative correlation between Twist and E-cadherin protein expression ($P = 0.001, r = -0.443$). This result was further confirmed by evaluating E-cadherin mRNA expression in the cell lines with different metastatic potentials. As shown in Fig. 2B, we found underexpression of E-cadherin mRNA expression in metastatic cell lines which showed strong Twist expression. Taken together, our results suggested that Twist might be correlated with HCC metastasis by repression of E-cadherin expression.

As loss of E-cadherin expression is the hallmark of EMT, we examined whether Twist directly induced EMT changes by stable ectopic transfection of Twist cDNA into PLC cells. After Twist transfection, ectopic Twist was mainly located in the nucleus of the cell. The staining pattern agrees with our immunostaining result on HCC clinical samples as well as with a previous report on rhabdomyosarcoma showing nuclear Twist expression (27). Ectopic Twist conferred morphologic changes from epithelial to fibroblastic appearance, which was accompanied by a gain of mesenchymal markers such as fibronectin and sm-actin, and loss of epithelial markers such as E-cadherin, $\beta$-catenin, and $\alpha$-catenin protein. In addition, these changes were accompanied with increased translocation of E-cadherin from the membrane to the cytoplasm, indicating function inactivation. To examine whether Twist represses transcription from E-cadherin promoter via E-box that are also targeted by snail, we did a promoter assay. The results show that Twist repressed E-cadherin expression transcriptionally by the suppression of E-cadherin promoter (Fig. 3E). However, it remains to be determined if the interaction of Twist with these E-boxes is direct or through other mediators. Although...
promoter methylation has been widely studied to be one of the major mechanisms for control of E-cadherin expression (11). E-cadherin promoter methylation is not closely related to loss of E-cadherin expression (13). In this study, we found that Twist-mediated transcriptional repression provides a novel mechanism for loss of E-cadherin in HCC rather than genetic control. The EMT changes were correlated with increased invasion after ectopic Twist introduction into PLC cell (Fig. 4A and B). Together with the immunostaining result in the TMA specimens, we showed that Twist increased the metastatic potential of HCC by the promotion of cell invasiveness. Although increasing evidence has shown the importance of Twist in the development and progression of human cancers, the underlying function is controversial. Recently, promoter methylation of Twist gene has been reported in breast cancer, especially in the metastatic lesions (31). These contrasting data suggested that the role of Twist in tumor progression might be cell type-specific. The role of Twist in cancer progression deserves further investigation.

In conclusion, we showed for the first time that Twist was correlated with HCC metastasis and that Twist induced HCC invasiveness through the suppression of E-cadherin expression and the induction of EMT. Our findings not only provide a molecular basis for the role of Twist in HCC metastasis, but also suggest a novel therapeutic target for the inhibition of HCC metastasis.

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