T-cell immunoglobulin mucin-3 as a potential inducer of the epithelial-mesenchymal transition in hepatocellular carcinoma

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Abstract. T-cell immunoglobulin mucin (TIM)-3 is an important member of the TIM gene family, which was thought to contribute to the progression of numerous types of cancer, including hepatocellular carcinoma (HCC); however, the mechanism underlying TIM-3 functions in HCC progression has not yet been extensively investigated. The present study aimed to investigate the function of TIM-3 in the metastasis of HCC and to determine whether the alteration of TIM-3 expression levels regulated the epithelial-mesenchymal transition (EMT) occurrence of HCC, using epithelial (E)-cadherin, neuronal (N)-cadherin, matrix metallopeptidase-9 (MMP-9), Twist 1, Slug, Snail, and Smad as EMT biomarkers. The results demonstrated that upregulation of TIM-3 using TIM-3 lentiviral activation particles (5 μl) increased cell migration and invasion, which was decreased in TIM-3 short interfering RNA-infected cells (10 μM, 3 μl) correspondingly. SMMC-7721 HCC cells were used as the control. EMT was aggravated in TIM-3 upregulated SMMC-7721 cells, which was attenuated in the TIM-3 interference group, accompanied by an alteration of E-cadherin, N-cadherin, MMP-9, Twist 1, Slug, Snail and Smad expression levels. The data presented suggests that TIM-3 serves an essential role in the metastasis of HCC, the mechanism of which was associated with EMT occurrence. Interference of TIM-3 is expected to be an effective means to prevent and control EMT, and further the metastasis of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of malignancy worldwide and has been a serious worldwide public health problem (1). In China, the mortality rate induced by HCC ranks highest, particularly in economically underdeveloped areas (2-4). Metastasis remains the primary cause of HCC-associated mortality. It was reported that >60% of HCC cases had encountered metastasis at the time of diagnosis (5-7). The five-year survival rate of patients with HCC and metastasis is markedly higher compared with patients with HCC without metastasis (8). Investigations into the molecular mechanism underlying metastasis are important for understanding HCC.

Epithelial-mesenchymal transition (EMT), a biological process during whereby epithelial cells transform into mesenchymal cells under a specific program, serves an important role in numerous physiological and pathological processes, including embryogenesis, organ development, tissue repair, organ fibrosis, and tumor metastasis (9,10). In epithelial malignancies, tumor cells acquire potent migratory and invasive abilities, transfer to a different site via the blood, and form further tumor metastasis through mesenchymal-epithelial transition (MET) (11-13). The mechanism underlying EMT in a number of solid tumors has been investigated extensively, including HCC (14,15). However, the prevention and control of HCC metastasis in clinics requires further study. Further investigations targeting the regulation of the mechanism underlying EMT progression of HCC are required.

T-cell immunoglobulin and mucin domain-containing molecule 3 (TIM-3), a novel participant in HCC progression, has recently been reported to regulate the biological behaviors of HCC. Tumor-derived TIM-3$^+$ cluster of differentiation (CD)4 T cells were revealed to suppress the proliferation of autologous CD8$^+$ T cells *in vitro* significantly, compared with tumor-derived TIM-3$^-$ CD4 T cells, suggesting the regulatory role of TIM-3 for T cells in human hepatocellular, cervical, colorectal and ovarian carcinomas (16). Furthermore, the impact of TIM-3 on hepatitis B virus (HBV) infection progression has been assessed, and genetic variants of TIM-3 were demonstrated to serve important roles in the disease progression of HBV infection (17,18). TIM-3 is also involved in the pathogenesis of human osteosarcoma, and TIM-3-triggered tumor cells have been observed to acquire the characteristics of aggressive EMT, indicating the possible role of TIM-3 in EMT occurrence (19). However, to the best of our knowledge, no study has been performed regarding the function of TIM-3 in the EMT progression of HCC.

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The present study focused on the role of TIM-3 in EMT progression of HCC based on cultured SMMC-7721 cells. TIM-3 short interfering (si) RNA and TIM-3 lentiviral activation particles were applied to alter the TIM-3 expression level, and EMT biomarkers, including epithelial (E)-cadherin, neuronal (N)-cadherin, matrix metallopeptidase-9 (MMP-9), Twist 1, Slug, Snail and Smad were analyzed. The results of the present study revealed that the migratory and invasive ability of SMMC-7721 cells was positively associated with the expression level of TIM-3. The EMT biomarkers all changed accordingly with the TIM-3 expression level, trending to EMT occurrence in TIM-3 upregulated cells. It was concluded that TIM-3 serves an important role in the metastasis of HCC and the underlying mechanism is associated with EMT occurrence. The results of the present study suggest that TIM-3 may be a potential inducer of EMT and promote the metastasis of HCC.

Materials and methods

Materials. The human HCC SMMC-7721 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cell culture medium Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and 0.02% EDTA-trypsin digestion were all obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The primary antibodies anti-TIM-3 and anti-β-actin supplied by OriGene Technologies, Inc. (Rockville, MD, USA); TIM-3 siRNA (human; h), TIM-3 lentiviral activation particles (h), primary antibodies anti-E-cadherin, anti-N-cadherin, anti-MMP-9, anti-Twist 1, anti-Slug, anti-Snail and anti-Smad were all purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-β-actin antibody was obtained from OriGene Technologies, Inc. All primers were designed by the PrimerBank (http://pga.mgh.harvard.edu/primerbank/) and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Quantitative polymerase chain reaction (qPCR) reagents were supplied by Thermo Fisher Scientific, Inc. All others reagents, mainly including Chemiluminescence Western Blotting kit, crystal violet dye, QuantiPro™ BCA Assay Kit and SYBR-Green, were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Cell culture. The SMMC-7721 human HCC cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 mg streptomycin. The conditions of the incubator were 37˚C and saturated humidity with 5% CO_2. Cells passage was performed in 0.02% EDTA-trypsin digestion when fully integrated.

Interference or activation of TIM-3 expression. Interference or activation of TIM-3 expression were performed according to the manufacturer's instructions (Santa Cruz Biotechnology, Inc.). In brief, for TIM-3 interference, TIM-3 siRNA (10 μM, 3 μl) was added to confluent cells for 6-8 h at 37˚C. For activation of TIM-3, 5 μl TIM-3 lentiviral activation particles (h) was added to the medium of confluent cells for 6-8 h at 37˚C. Subsequently, cells were further cultured in updated growth medium (10% FBS-DMEM) for 48 h for further detections at 37˚C.

Migration assay. Transwell™ chambers were used to analyze the migration of SMMC-7721 cells. In brief, 600-700 μl DMEM without FBS was added to the lower chamber of each well at room temperature and then co-cultured with inoculated cells at 37˚C for 24 h. Subsequently, cells were seeded in the upper chamber at an appropriate density ($5.0x10^4$/ml) in 100 μl 10% FBS-DMEM. Following 24 h incubation at 37˚C, the chamber was removed and fixed with 95% alcohol for 15 min at room temperature following cleaning cells of the upper chamber using a cotton swab. Subsequently, the chamber was immersed in crystal violet dye for staining for 15 min at room temperature. After being thoroughly washed in PBS, migrated cells were observed under a phase contrast microscope. Analyses of each group were repeated in three replicates and all experiments were repeated three times. Cell numbers from five random fields was determined to represent the migration ability of each group.

The invasion ability detection. To assess invasion, each well was first coated with 80 μg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) overnight at 4˚C. Subsequently, protocols similar to the migration assay were performed under the same conditions. The cell density was $5.0x10^3$ cells/well. A total of five random fields were selected and cell numbers was determined to represent the invasion ability of each group. All experiments were repeated three times.

qPCR and western blotting. Proteins were extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) in ice for 30-45 min, during which, tubes were agitated on an oscillator for 1 min every 5-10 min. The proteins were quantified using a BCA kit (Sigma-Aldrich; Merck KGaA). Total mRNA was extracted from the control, TIM-3 activation and TIM-3 siRNA groups respectively, using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR and western blotting were performed as common (20,21). For qPCR detection, cDNA was firstly obtained based on reverse transcription from total mRNA. SYBR Green (1X; Sigma-Aldrich; Merck KGaA) was used as fluorophore. The qPCR reaction was performed under the following conditions: 95˚C denaturing for 5 min, followed by 36 cycles each of 95˚C 40 sec, 58˚C 30 sec and 72˚C 60 sec. For Western blotting, 40 μg of total protein was loaded in each lane, 12.5% SDS-PAGE gels were used to detect each protein, and 5% skimmed milk was used as blocking reagent to block the unspecific proteins in each membrane for 1 h at room temperature. Polyvinylidene difluoride (PVDF) membranes (0.45 μm) were applied. The dilutions of the primary antibodies for TIM-3 (TA-807034; OriGene Technologies, Inc.; 1:1,000) and β-actin (TA-09; OriGene Technologies, Inc.; 1:2,500) were used. Primary antibodies from Santa Cruz Biotechnology, Inc. for E-cadherin (sc-71008), N-cadherin (sc-59987), MMP-9 (sc-21733), Twist 1 (sc-81417), Slug (sc-166476), Snail (sc-271977), Smad (sc-7965) were all 1:200. They were incubated with PVDF membrane for 30-60 min at room temperature and then incubated overnight at 4˚C. The secondary goat-anti-mouse horseradish peroxidase (HRP)-conjugated antibody and goat-anti-rat HRP-conjugated antibody were both from OriGene Technologies, Inc., incubated with the PVDF membrane at room temperature for 1 h at 1:5,000 dilutions. Enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA) was used as visualization reagent, by

Table I. Sequence information of the primers used for quantitative polymerase chain reaction.

Genes	Sequences	Product size (bp)	Gene Bank
E-cadherin	Forward: 5'-CGAGAGCTACACGTTCACGG-3'	119	NM 004360.3
	Reverse: 5'-GGGTGTCGAGGGAAAAATAGG-3'		
N-cadherin	Forward: 5'-AGCCAACCTTAACTGAGGAGT-3'	136	NM_001792.3
	Reverse: 5'-GGCAAGTTGATTGGAGGGATG-3'		
MMP-9	Forward: 5'-GGGACGCAGACATCGTCATC-3'	139	NM_004994.2
	Reverse: 5'-TCGTCATCGTCGAAATGGGC-3'		
Twist 1	Forward: 5'-GAGACTCTGGAGCTGGATAACT-3'	100	NM_000474.3
	Reverse: 5'-CGTCTGGGAATCACTGTCCA-3'		
Slug	Forward: 5'-TGTGACAAGGAATATGTGAGCC-3'	203	NM_003068.4
	Reverse: 5'-TGAGCCCTCAGATTTGACCTG-3		
Snail	Forward: 5'-TCGGAAGCCTAACTACAGCGA-3'	140	NM_005985.3
	Reverse: 5'-AGATGAGCATTGGCAGCGAG-3'		
Smad	Forward: 5'-AGAGACTTCTTGGGTGGAAACA-3'	157	NM_001003688.1 and
	Reverse: 5'-ATGGTGACACAGTTACTCGGT-3'		NM_005900.2

MMP-9, matrix metallopeptidase-9; bp, base pair; E, epithelial; N, neuronal.

mixing by equal solution A and B and incubating for 2-3 min in the dark. The target bands of TIM-3, E-cadherin, N-cadherin, MMP-9, Twist 1, Slug, Snail, Smad and β-actin were visualized at apparent molecular weights of 33, 120, 130, 92, 28, 30, 29, 52-56 and 43 kDa, respectively. Semi-quantitative analysis was performed by ImageJ software 1.41o (National Institutes of Health, Bethesda, MD, USA). β-actin was used as the control. For qPCR, Ct value was compared to represent the transcription level of each molecule, using the $2^{-\Delta\Delta Cq}$ method (22). Each experiment was repeated three times. The primers are presented in Table I.

Statistical analysis. Data are presented as the mean ± standard deviation and were analyzed using SPSS software package, version 16.0 (SPSS, Inc., Chicago, IL, USA). Comparisons between two groups were analyzed using a t-test. Comparisons of datasets containing multiple groups (three or more) were performed using analysis of variance and Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

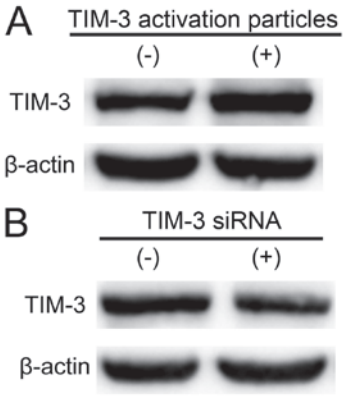

Figure 1. TIM-3 expression level was evaluated in SMMC-7721 cells based on TIM-3 activation particles and TIM-3 siRNA applications. (A) TIM-3 activation particles (5 μl) were transfected into SMMC-7721 cells and upregulated TIM-3 expression level significantly. (B) The expression of TIM-3 was downregulated markedly in SMMC-7721 cells transfected with TIM-3 siRNA (10 μM, 3 μl). TIM-3, T-cell immunoglobulin mucin-3; siRNA, short interfering RNA.

Results

Monitoring of TIM-3 expression level under TIM-3 siRNA and TIM-3 lentiviral activation particles in SMMC-7721 cells. To investigate the role of TIM-3 in the metastasis of HCC, TIM-3 siRNA and TIM-3 lentiviral activation particles were applied in cultured SMMC-7721 cells to regulate the expression level of TIM-3. As presented in Fig. 1, TIM-3 was upregulated markedly by TIM-3 lentiviral activation particles (5 μl; Fig. 1A) and downregulated markedly in SMMC-7721 cells transfected with TIM-3 siRNA (10 μM, 3 μl; Fig. 1B). This result suggested that TIM-3 lentiviral activation particles (5 μl) and TIM-3 siRNA (10 μM, 3 μl) upregulated, and downregulated TIM-3 expression levels, respectively.

Alteration of TIM-3 expression level affects the migration and invasion of SMMC-7721 cells. As presented in Fig. 2A, in the migration and invasion assays, more TIM-3 overexpressed cells and fewer cells transfected with TIM-3 siRNA passed through the filter compared with the control group. A total of five random fields were selected and the number of cells in each group was determined. Taking the relative number of migrated and invaded cells in control group as 100, the migrated and invaded number of cells in the TIM-3 upregulated group was 157±10 and 179±11, and the number in the TIM-3 interference group was 61±8 and 34±7, respectively (Fig. 2B). Significant differences were revealed in the migration and invasion abilities of the cells compared with the control ($P<0.05$).

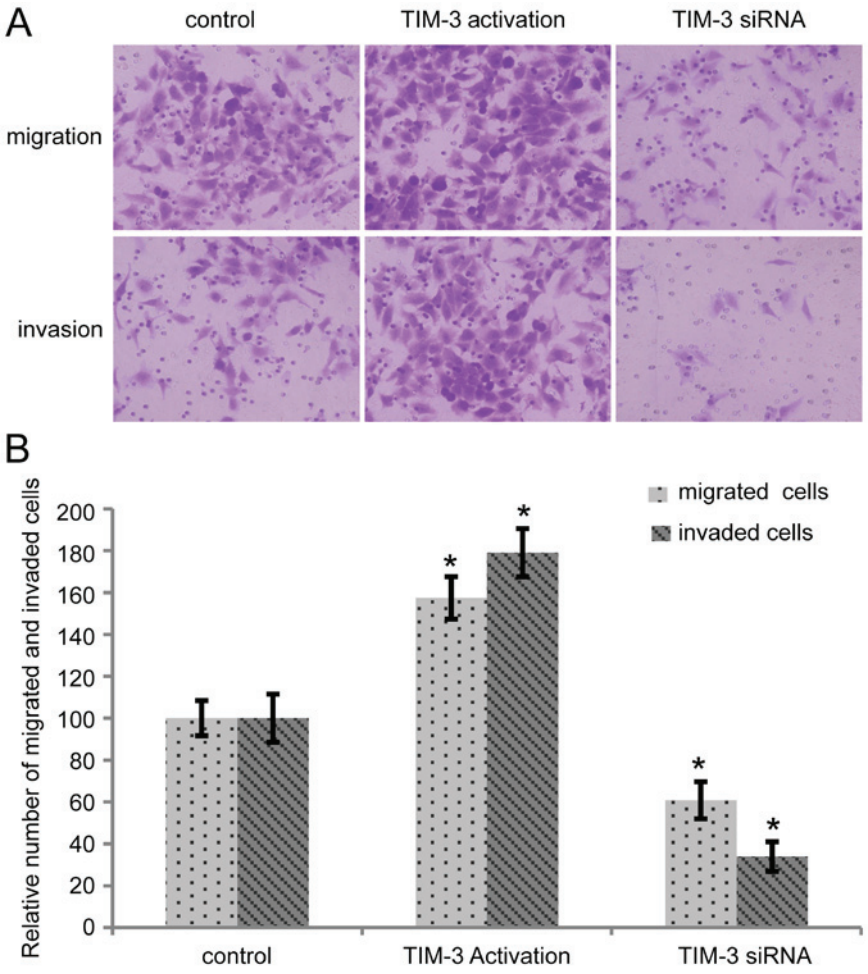

Figure 2. Alteration in TIM-3 expression level affects migration and invasion of SMMC-7721 cells (magnification, x200). (A) Migration and invasion abilities were detected by Transwell and Matrigel assays in control cells, cells transfected with TIM-3 activation particles (5 μl), and cells transfected with TIM-3 siRNA (10 μM, 3 μl). (B) Five random fields were selected, and the number of cells in each group was evaluated. Compared with the control, TIM-3-overexpressed cells revealed significantly higher migration and invasion abilities, which were reversed in TIM-3 downregulated cells (*P<0.05). TIM-3, T-cell immunoglobulin mucin-3; siRNA, short interfering RNA; MMP-9, matrix metallopeptidase-9.

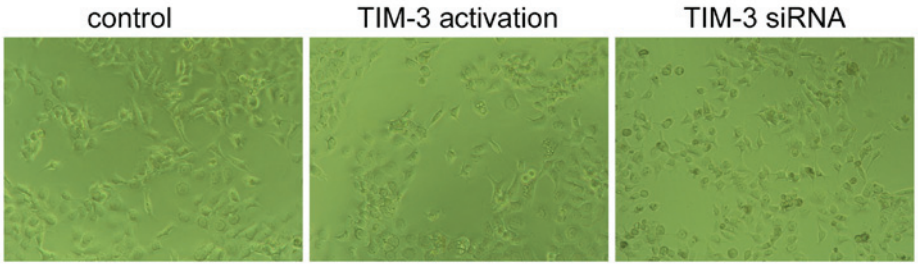

Figure 3. Alteration in TIM-3 expression level affects cell morphology of SMMC-7721 cells (magnification, x200). Morphological changes were observed using an inverted microscope in control cells, cells transfected with TIM-3 activation particles (5 μl) and cells transfected with TIM-3 siRNA (10 μM, 3 μl). TIM-3, T-cell immunoglobulin mucin-3; siRNA, short interfering RNA.

Alteration of TIM-3 expression level influences cell morphology of SMMC-7721 cells. As presented in Fig. 3, cells transfected with TIM-3 activation particles (5 μl) a more spindle-like morphology and connections between cells were fewer, which was previously revealed to be more conducive of migration and invasion (23). Simultaneously, SMMC-7721 cells transfected with TIM-3 siRNA (10 μM, 3 μl) demonstrated epithelial and the adhesion showed stronger, with more antennas, indicating a lower aggressive type of cancer.

Alteration in TIM-3 expression levels affects the transcription of EMT biomarkers in SMMC-7721 cells. EMT occurrence was indicated by the reduced expression level of epithelial cell markers, with E-cadherin being the most apparent, and increased expression levels of mesenchymal cell markers, including N-cadherin, MMP, Twist 1, Snail, Slug and Smad. In order to investigate the effect of TIM-3 in EMT occurrence of HCC, the present study designed numerous specific primers targeted to E-cadherin, N-cadherin, MMP, Twist 1, Snail, Slug

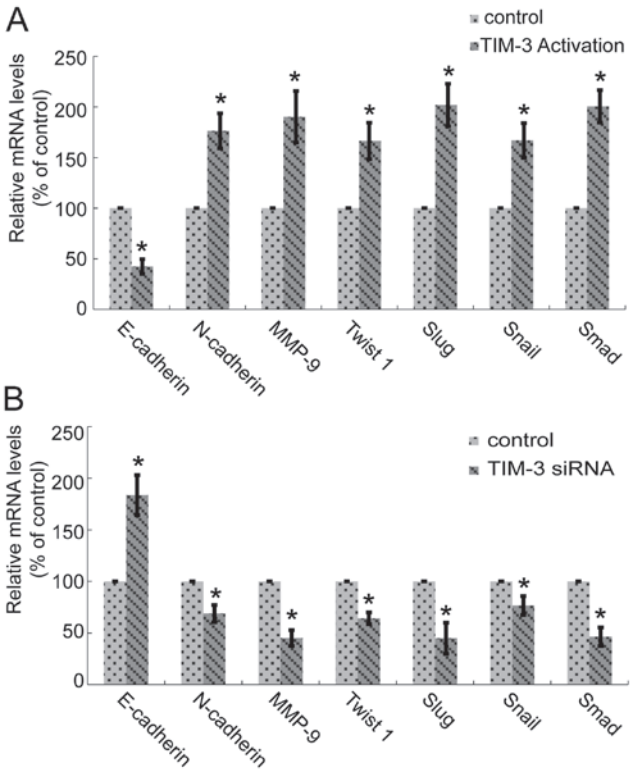

Figure 4. Alteration in TIM-3 expression level affects the transcription of epithelial-mesenchymal transition biomarkers of SMMC-7721 cells. (A) In TIM-3-overexpressed cells, the expression levels of E-cadherin were reduced, and N-cadherin, MMP-9, Twist 1, Snail, Slug and Smad were increased (*P<0.05). (B) Downregulation of TIM-3 increased the mRNA expression levels of E-cadherin, and decreased the N-cadherin, MMP-9, Twist 1, Snail, Slug and Smad mRNA expression levels (*P<0.05). TIM-3, T-cell immunoglobulin mucin-3; MMP-9, matrix metallopeptidase-9; E, epithelial; N, neuronal.

and Smad. qPCR was performed. Fig. 4 presents the results, with reduced E-cadherin expression levels and increased expression levels of N-cadherin, MMP, Twist 1, Snail, Slug and Smad in SMMC-7721 cells transfected with TIM-3 activation particles (5 μl; Fig. 4A). Downregulation of TIM-3 based on TIM-3 siRNA (10 μM, 3 μl) revealed the opposite changes for all the aforementioned molecules (Fig. 4B). Each molecular was analyzed more than three times. The differences between control and TIM-3 activation (Fig. 4A) or between control and TIM-3 siRNA (Fig. 4B) were all significant for the expression of E-cadherin, N-cadherin, MMP, Twist 1, Snail, Slug and Smad, respectively (*P<0.05).

Expressions of EMT biomarkers are regulated by TIM-3 in SMMC-7721 cells. As presented in Fig. 5, the results demonstrated were consistent with the results of mRNA detections in Fig. 4. Overexpression of TIM-3 reduced E-cadherin expression levels, and increased the expression levels of N-cadherin, MMP, Twist 1, Snail, Slug and Smad (Fig. 5A). Downregulation of TIM-3 revealed overexpression of E-cadherin and downregulation of N-cadherin, MMP, Twist 1, Snail, Slug and Smad (Fig. 5B). Each experiment was repeated three times. Semi-quantitative analysis using ImageJ software revealed significance in the TIM-3-upregulated (Fig. 5C) and TIM-3-interference groups, compared with the control (Fig. 5D; P<0.05).

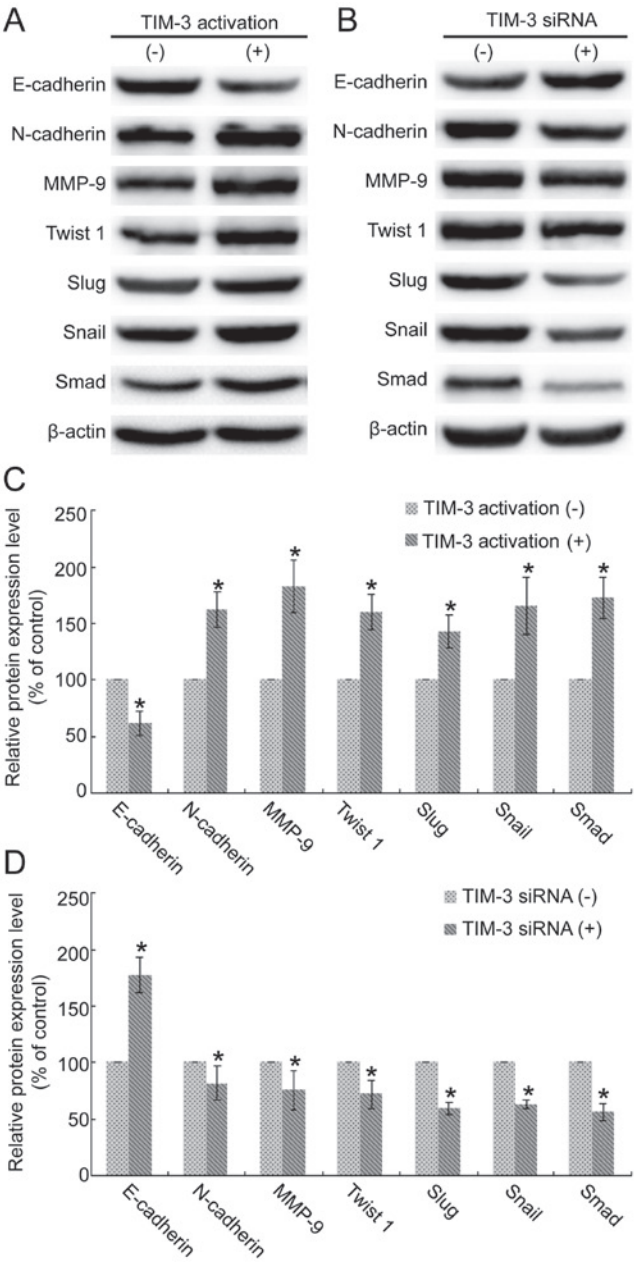

Figure 5. Alteration in TIM-3 expression level affects the translation of epithelial-mesenchymal transition biomarkers in SMMC-7721 cells. (A) Overexpression of TIM-3 reduced E-cadherin expression level and downregulated expression levels of N-cadherin, MMP-9, Twist 1, Snail, Slug and Smad. (B) Downregulation of TIM-3 increased E-cadherin and reduced N-cadherin, MMP-9, Twist 1, Snail, Slug and Smad expression levels. (C) Quantitative analysis based on ImageJ software revealed the significant downregulation of E-cadherin and upregulations of N-cadherin, MMP-9, Twist 1, Snail, Slug and Smad under TIM-3 overexpression (*P<0.05). (D) Quantitative analysis based on ImageJ software demonstrated the significant upregulation of E-cadherin and downregulations of N-cadherin, MMP-9, Twist 1, Snail, Slug and Smad under TIM-3 siRNA (*P<0.05). TIM-3, T-cell immunoglobulin mucin-3; MMP-9, matrix metallopeptidase-9; siRNA, short interfering RNA; E, epithelial; N, neuronal.

Discussion

Although certain previous studies have investigated the regulation of HCC metastasis (24-26), the prevention and control of HCC metastasis in clinics requires further study. Further studies investigating the regulation of HCC metastasis are

required. The present study explored the role of TIM-3 in EMT the occurrence of HCC and aimed to provide specific signs for HCC metastasis. The results demonstrated that alteration of TIM-3 expression levels correlated positively with EMT occurrence, and the migration and invasion ability of SMMC-7721 cells, suggesting that TIM-3 may be a potential inducer of EMT and further promote the metastasis of HCC.

Certain regulating factors in HCC metastasis have been previously reported, including microRNA, oncogenes and tumor suppressor genes (27). The inactivation of tumor suppressor genes is considered to be essential in HCC progression. TIM-3 is a type of suppressive molecule located at the T cell surface, and can induce T cell depletion in cancer and chronic viral infections. TIM-3 has been studied mostly as an immunotherapeutic target in various types of cancer, including HCC. Cooperation or interaction of TIM-3 and programmed cell death-1 has been suggested to be more relevant than either molecule alone to immune dysfunction in HCC in chronic HBV infections (28). A previous study investigating the role of TIM-3 in patients with HCC with chronic hepatitis B virus infection also supports the role of TIM-3 in persistent HBV infections and HCC development (18). The present study demonstrated the essential role of TIM-3 in the migration and invasion of HCC by performing a Transwell assay. Overexpression of TIM-3 promoted the migration and invasion of SMMC-7721 cells, which was reduced in TIM-3 interference cells. Previous studies and the present study indicated the potential function of TIM-3 in HCC metastasis (29,30); however, the underling molecular mechanism remains unclear.

EMT is an iconic phenomenon in cancer metastasis, particularly in epithelial cell-derived cancer, including HCC. A number of studies have investigated the EMT regulation of HCC (31,32). For example, transforming growth factor-β was revealed to be involved in regulating EMT of HCC, and is the primary inducer of EMT (31). However, a large number of other factors limit the prevention and control of EMT, and further metastasis of HCC in the clinic, such as small nucleolar RNA ACA11 (ACA11) (33), vitamin C (34), free fatty acid (35) and membrane-associated heparan sulfate proteoglycan Glypican-3 (GPC3) (36). The present study focused on the possible role of TIM-3 in EMT occurrence of HCC, by monitoring alterations of cell morphology and biomarkers of EMT. From the phase contrast microscope, it was observed that normal HCC cells showed regular polygon and were connected closely and arranged regularly. When cells encountered EMT, they were arranged in a loose fashion and intercellular adhesion among cells was weakened (32). The present study observed the morphological changes under TIM-3 alteration. Cells with TIM-3 overexpression changed to a spindle-like morphology and connections between cells were fewer, which contrasted with the effects in TIM-3 downregulated cells. This morphological change indicated that alteration of TIM-3 influenced the morphology, and upregulation of TIM-3 promoted EMT occurrence of SMMC-7721 cells.

A variety of biomarkers have been used to represent the EMT progress, primarily cell surface markers, cytoskeletal markers, extracellular matrix proteins and transcriptional factors (37). The present study investigated the changes of specific EMT biomarkers, including E-cadherin, N-cadherin, MMP-9, Twist 1, Slug, Snail and Smad. E-cadherin, a classic marker of epithelial cells is expressed at low levels in EMT of numerous types of cancer (38,39). N-cadherin, a mesenchymal cell marker, has an increased expression level in EMT. The conversion of E-cadherin to N-cadherin was previously used to monitor EMT process (40). In the present study, overexpression of TIM-3 reduced E-cadherin and upregulated N-cadherin, which was in contrast to the TIM-3 interference group at the transcription, and translation levels. This result revealed the function of TIM-3 in HCC EMT.

Snail, Slug, Twist 1 and MMP-9 are also primary regulators of EMT. Snail is the most well-known E-cadherin suppressor gene, and a significant expression level of Snail was reported in HCC, and was associated with occurrence and metastasis (41). Slug belongs to the same gene family as Snail and is expressed in the membrane and cytoplasm of HCC cells. Twist 1, a basic helix-loop-helix protein, may inhibit the expression of E-cadherin independent of Snail, and enhanced the expression levels of fibronectin and N-cadherin (42,43). In the present study, in the TIM-3 upregulated group of SMMC-7721, Snail, Slug, Twist 1 and MMP-9 had high expression levels, suggesting TIM-3 may be an inducer of EMT in HCC and serve an essential role in the induction of EMT in HCC.

Taken together, the results of the present study provided evidence suggesting the role of TIM-3 expression in regulating EMT occurrence and further metastasis of HCC. To substantiate our findings, samples from patients with HCC samples have been collected. Our future experiments will focus on the association between TIM-3 expression level and HCC clinical stages. This will further the knowledge of the mechanisms underlying TIM-3 in HCC metastasis.

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