TC21 promotes cell motility and metastasis by regulating the expression of E-cadherin and N-cadherin in hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) is an aggressive type of cancer, and it may be at an advanced stage when it is detected. It has been shown that TC21, a member of the Ras superfamily, is associated with the proliferation, migration and transformation of tumor cells. Previous studies have shown that TC21 is overexpressed in breast, esophageal and oral carcinomas, and that it is closely associated with the early stages of tumorigenesis. In this study, we demonstrate that TC21 overexpression promotes the motility of HCC cells *in vitro* and intrahepatic metastasis *in vivo*. Furthermore, experiments examining the effects of both the ectopic expression of TC21 and siRNA treatment in HCC cells showed that TC21 alters the expression of the adhesive molecules E-cadherin and N-cadherin. Our data suggest that TC21 is associated with tumor progression and poor prognosis in HCC.

Introduction

The Ras superfamily of proteins has been shown to play an important role in the tumorigenesis of >30% of human cancers. TC21, also known as R-Ras2, is a member of the R-Ras subfamily of the RAS superfamily and shares ~60% nucleotide homology and 55% amino acid homology with the H-, K- and N-Ras genes. TC21 was initially cloned from a human teratocarcinoma cDNA library during the search for Ras-related genes (1-3). TC21 was also the first Ras-related protein that was found to exhibit a transforming activity that was equivalently potent to that of Ras (2,4). Mutation and overexpression of TC21 have been detected in many human cancers including breast tumors, ovarian and uterine sarcomas, oral squamous cell carcinomas and esophageal cancers (5-10). It has been proposed that the activation and upregulation of TC21 promote malignant transformation and proliferation of a wide variety of normal cells, such as murine NIH/3T3 cells. TC21 also enhances the migration of breast tumor cell lines and neurofibromatosis type 1 (NF1) gene-deficient Schwann cells (7,11,12). A recent study demonstrated that TC21 induces cell transformation through the PI3K-AKT, MAPK and NF-κB signaling pathways (11,13-15). However, the underlying mechanism of TC21-mediated tumor metastasis remains unclear.

Hepatocellular carcinoma (HCC) is one of the most common tumors worldwide. Individuals living in Asian countries account for ~8% of the ~600,000 new cases of HCC that are globally reported each year (16). HCC is an aggressive tumor that may be at an advanced stage when it is detected. Only a small proportion of hepatocellular carcinomas can be completely removed by surgery. Several members of the Ras superfamily, including Rac, Ra1 and RhoA, have been found to play an important role in the pathogenesis of HCC (17,18). So far, there have been no reports on the role of TC21 in HCC.

A hallmark of epithelial-mesenchymal transition (EMT) is the disruption of adherence junctions, of which the transmembrane glycoprotein E-cadherin is a major component. Loss of E-cadherin is associated with invasion, metastasis and a poor prognosis in breast, colon and other cancers (19,20). A recent study indicated that the downregulation of E-cadherin expression in HCC was also associated with both a large tumor size and multi-nodular tumors (21,22). Previous studies showed that two members of the Snail family of zinc finger transcriptional repressors (i.e., Snail and Slug) lead to a dysregulated E-cadherin expression in breast cancer. It has also been reported that H-Ras and oncogenic

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Ras cause downregulation and mislocalization of E-cadherin in rat intestinal epithelial cells (23-25). We report herein that the overexpression of TC21 directly decreases E-cadherin expression and increases N-cadherin expression, thereby enhancing both the motility of HCC cells *in vitro* and their ability to metastasize to intrahepatic locations *in vivo*.

Materials and methods

Immunohistochemical analyses of TC21, E-cadherin and N-cadherin expression in human primary HCC tissues. Paraffin-embedded HCC tissue microarray sections (5 μm thick) containing 57 pairs of HCC tissue samples and their corresponding adjacent non-cancerous liver tissue samples (2 mm each) were first routinely dewaxed and rehydrated. The endogenous horseradish peroxidase (HRP) activity was then quenched using H_2O_2 and antigen retrieval was performed by heating the sample in sodium-citrate buffer (10 mM, pH 6.0). Sections were incubated with rabbit anti-E-cadherin (1:25, Santa Cruz), rabbit anti-N-cadherin (1:25, Santa Cruz), and rabbit anti-R-Ras2 (TC21) (1:50, Abcam) primary antibodies; labeled using EnVision HRP/DAB (rabbit) kits (Dako); and counterstained with Mayer's hematoxylin (Dako). The sections were finally examined and photographed using an Axioskop 2 microscope (Carl Zeiss, Germany) with a DP70 CCD system (Olympus, Japan).

Cell lines and cell culture. The HEK293T cell line was obtained from Invitrogen (Carlsbad, CA, USA). The HCC cell lines MHCC-97L and MHCC-LM3 were obtained from Zhongshan Hospital, Fudan University (Shanghai, China) and the HepG2, Huh7, Hep3B, SMMC-7721, Focus, PLC/PRF/5, SK-HEP-1 and HUVEC cell lines were obtained from the cell bank of the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai). All cells were cultured in Dulbecco's modification of Eagle's medium Dulbecco (DMEM) (Sigma, St. Louis, MO) supplemented with fetal bovine serum (FBS; Hyclone, Logan, UT) to a final concentration of 10% and antibiotics (100 U/ml penicillin-G and 100 μg/ml streptomycin) at 37˚C in a humidified atmosphere containing 5% CO_2.

Lentiviral vector construction and cell transduction. The open reading frame (ORF) of the TC21 gene was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from the genomic DNA of normal liver tissues and cloned into the Pme1 sites of the pWPI vector (Addgene). Viral packaging was performed in HEK293T cells via co-transfection of the pWPI-TC21 vector or pWPI vector with the envelope plasmid pMD2.G and the packaging plasmid psPAX2 by using Lipofectamine 2000 (Invitrogen). Viral particles were harvested 48 h after transfection and concentrated by ultracentrifugation. Target cells ($1x10^6$), including MHCC-97L, MHCC-LM3 and PLC/PRF/5 cells, were infected with $1x10^7$ recombinant lentivirus-transducing units in the presence of 10 μg/ml polybrene (Sigma).

TC21 siRNA sequences and transient transfection. Small-interfering RNA (siRNA) oligonucleotides for TC21 and a non-targeted siRNA-negative control were synthesized by GenePharma (Shanghai, China). The sequences of the three TC21 siRNA fragments and the negative control that were used are listed below: i) Human (TC21) 1: 5'-CACCAGAAC CAACACGGAATT-3' (sense) and 5'-UUCCGUGUUGGUU CUGGUGAA-3' (antisense); ii) Human (TC21) 2: 5'-GGC UGCCAUUGUGUCAUUUTT-3' (sense) and 5'-AAAUGA CACAAUGGCAGCCTT-3' (antisense); iii) Human (TC21) 3: 5'-AGACAGAUUCUCAGAGUAATT-3' (sense) and 5'-UUACUCUGAGAAUCUGUCUTT-3' (antisense); and iv) Negative control: 5'-UUCUCCGAACGUGUCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (antisense). Gene silencing studies were performed using the reverse transfection method. Briefly, prior to cell plating, siRNA oligonucleotides (40 pmol) and Lipofectamine 2000 (2 μl; Invitrogen) were added to Opti-MEM (Invitrogen) and mixed. The resulting mixture was then incubated in 12-well plates. The cells were trypsinized into suspension at a concentration of $1x10^5$ cells per ml, plated into the mixture medium, cultured at 37˚C for 48 h, and harvested for further analysis.

Real-time reverse transcription-polymerase chain reaction. Total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA). Reverse-transcribed cDNA was synthesized using the PrimeScript RT reagent Kit (Takara, Dalian, China). The expressions of E-cadherin and TC21 mRNA were measured by real-time PCR. The reaction was performed using the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) with the SYBR Premix Ex Taq II solution (Takara Biotechnology). The primers that were used for the target genes were: i) E-cadherin forward: 5'-TCC ATTTCTTGGTCTACGCC-3' and reverse: 5'-CACCTTCA GCCAACCTGTTT-3'; and ii) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (control).

Western blot analysis. The total protein content of the cells was obtained using RIPA lysis buffer (Santa Cruz) containing a cocktail of proteinase inhibitors (Roche Applied Science) and phosphatase inhibitors (Roche Applied Science). Protein levels were quantified using a BCA reagent (Sigma). Equal amounts of protein (30-60 μg) were denatured and loaded onto SDS-polyacrylamide gels (SDS-PAGE). Proteins were then transferred to nitrocellulose membranes and probed with the following primary antibodies: rabbit anti-E-cadherin and rabbit anti-N-cadherin (Santa Cruz) at a dilution of 1:100, mouse anti-TC21 (Abnova) at a dilution of 1:200, mouse anti-ß-actin (Sigma) at a dilution of 1:10000. In all cases, goat anti-mouse IgG-HRP (Santa Cruz) was used as the secondary antibody at a dilution of 1:4000. The membranes were developed using the SuperSignal West Femto Maximum Sensitivity Substrate Kit (Pierce, Rockford, IL) and exposed to X-ray film (Kodak, Rochester, NY).

Fluorescent immunocytochemical staining. Cells were grown on glass slides overnight. Adherent growing cells were then washed twice with PBS, fixed in 4% paraformaldehyde at 4˚C for 20 min, and used for fluorescent immunocytochemical staining. The cells were incubated with a primary antibody (rabbit anti-N-cadherin and E-cadherin) overnight at 4˚C. After washing with PBST, the cells were incubated

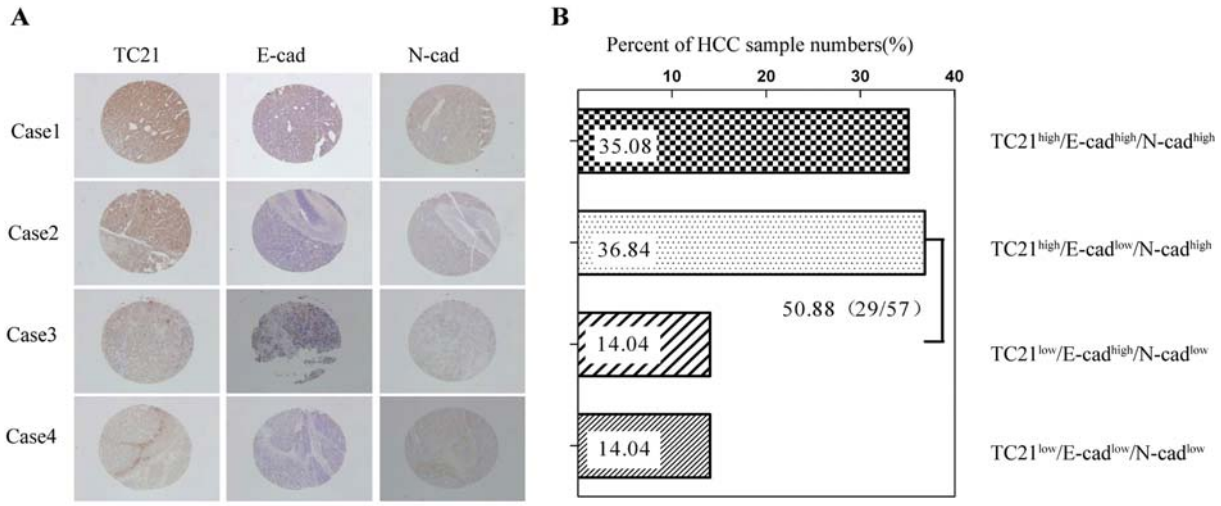

Figure 1. Immunohistochemical analysis of the expression of TC21, E-cadherin and N-cadherin in human HCC tissues. (A) Representative images of immunohistochemical staining for TC21, E-cadherin (E-cad) and N-cadherin (N-cad) proteins in 4 HCC patients from an HCC tissue array containing 57 tissue samples. (B) Distribution of immunohistochemical staining patterns: the TC21^{high}/E-cad^{high}/N-cad^{high} pattern was present in 20 cases (35.08%); the TC21^{high}/E-cad^{low}/N-cad^{high} pattern was present in 21 cases (36.845%); the TC21^{low}/E-cad^{high}/N-cad^{low} pattern was present in 8 cases (14.04%); and the TC21^{low}/E-cad^{low}/N-cad^{low} pattern was present in 8 cases (14.04%). Original magnification of all images, x50.

with a fluorescence-conjugated secondary antibody (Alexa 594-conjugated goat anti-rabbit IgG, Molecular Probes). The nuclei were counterstained using DAPI for 30 min at room temperature. Finally, imaging was performed using a laser confocal microscope (FV1000, Olympus, Japan).

In vitro cell migration assay. For the *in vitro* transwell migration assay, 1x10^5 cells were placed on the top chamber of each insert (BD Biosciences, NJ, USA) on the non-coated membrane. Cells were trypsinized and resuspended in DMEM and 0.6 ml of medium supplemented with 10% FBS was added to the lower chambers. After several hours (i.e., 20 h for the MHCC-97L and PLC/PRF/5 cells, and 30 h for the MHCC-LM3 cells) of incubation at 37˚C, the cells that remained in the top chambers or on the upper membrane of the inserts were carefully removed using medical cotton. After fixation in 10% neutral phosphate-buffered formalin and staining with Giemsa solution (Sigma), the cells that had migrated across the membrane and the cells that had adhered to the back side of the inserts were examined, counted and photography-taken under an Axioskop 2 microscope (Carl Zeiss, Germany).

Trans-endothelial cell migration assay. Human umbilical vein endothelial cells (HUVEC) were seeded into gelatin-coated FluoroBlok transwell polycarbonate membrane inserts with 8.0-μm pores (BD Biosciences) at a concentration of 1x10^5 cells per 24-well insert and allowed to grow to confluence for 20 h. Thereafter, tumor cells were trypsinized and recounted. The thousands of tumor cells that were seeded into the transwell inserts that had grown to a confluent endothelial monolayer were fixed in 10% PB-buffered formalin after 16 h of incubation. Cells on the top side of each insert were scraped off. Cells that had migrated to the back side of the membrane were observed and photographed using an Axioskop 2 microscope. Three random fields were used for statistical analysis.

Intrahepatic metastasis assay in a mouse model. Six-week-old male nude mouse (BALB/c-nu/nu) were divided into 4 groups (6 mice each), in which two groups were inoculated orthotopically the 2x10^6 MHCC-97L or PLC/PRF/5 cells infected with TC21-pWPI lentivirus in the left hepatic lobe, and the other two groups were inoculated orthotopically the 2x10^6 MHCC-97L or PLC/PRF/5 cells infected with pWPI-empty vector lentivirus as controls. Cells were suspended in 40 μl serum-free DMEM/Matrigel (1:1) for each mouse. Four weeks later, the mice were sacrificed, their livers were dissected and fixed in 10% phosphate-buffered formalin to standard histological examination. The number of intrahepatic metastatic nodules in mouse models between TC21-pWPI and pWPI-empty vector treatments were counted and compared. During the period of animal experiment, the mice were housed and cared for according to the guidline of Shanghai Medical Exprimental Animal Care Commission.

Statistical analysis. Statistical analysis was performed using the SPSS 13.0 software (SPSS, Chicago, IL). Continuous data were presented as mean ± SDs and compared using Student's t-test. All P-values at <0.05 were considered to be statistically significant.

Results

Immunohistochemical analysis of TC21, E-cadherin and N-cadherin expression in primary HCC tissues. To characterize the differential expression patterns of the TC21, E-cadherin and N-cadherin proteins, immunohistological analysis was performed using a tissue microarray that included samples from 57 tumors from HCC patients who had not undergone neoadjuvant chemotherapy prior to surgical treatment. Of the 57 HCC samples, four common expression patterns were identified: i) TC21^{high}/E-cadherin^{high}/N-cadherin^{high} (35.08%, 20/57); ii) TC21^{high}/E-cadherin^{low}/N-cadherin^{high} (36.84%, 21/57), iii) TC21l^{low}/E-cadherin^{high}/N-cadherin^{low} (14.04%,

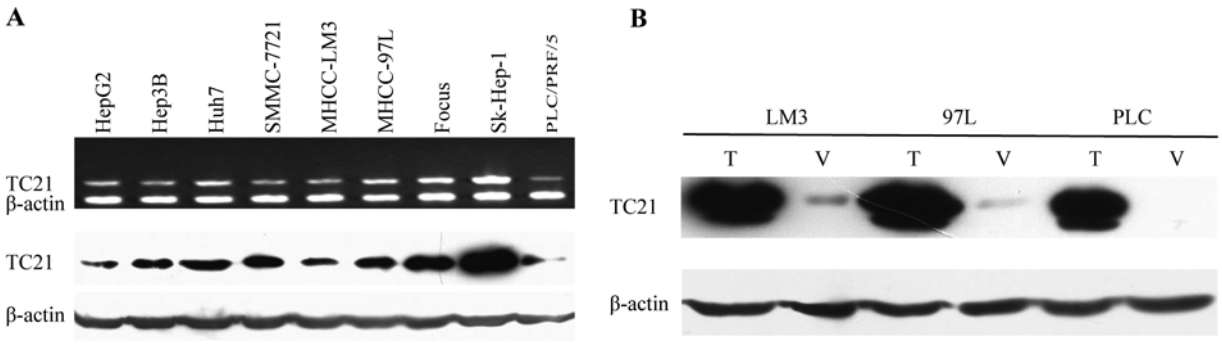

Figure 2. The expression level of TC21 in HCC cell lines and validation of stable TC21-overexpressing HCC cell lines. (A) TC21 mRNA and protein expressions were determined in nine HCC cell lines by RT-PCR (top panel) and Western blot analysis (bottom panel), respectively. High levels of expression of both TC21 mRNA and protein were detected in Sk-Hep-1, Focus and Huh7 cells; low expression levels were detected in PLC/PRF/5 cells; and moderate expression levels were observed in the other five HCC cell lines that were tested. ß-actin was used as the loading control. (B) TC21 protein expression in the stable MHCC-LM3 (LM3), MHCC-97L (97L) and PLC/PRF/5 (PLC) cell lines that had been infected with TC21-pWPI (T) and the pWPI-empty vector (V) lentivirus was confirmed by Western blot analysis. ß-actin was used as the loading control.

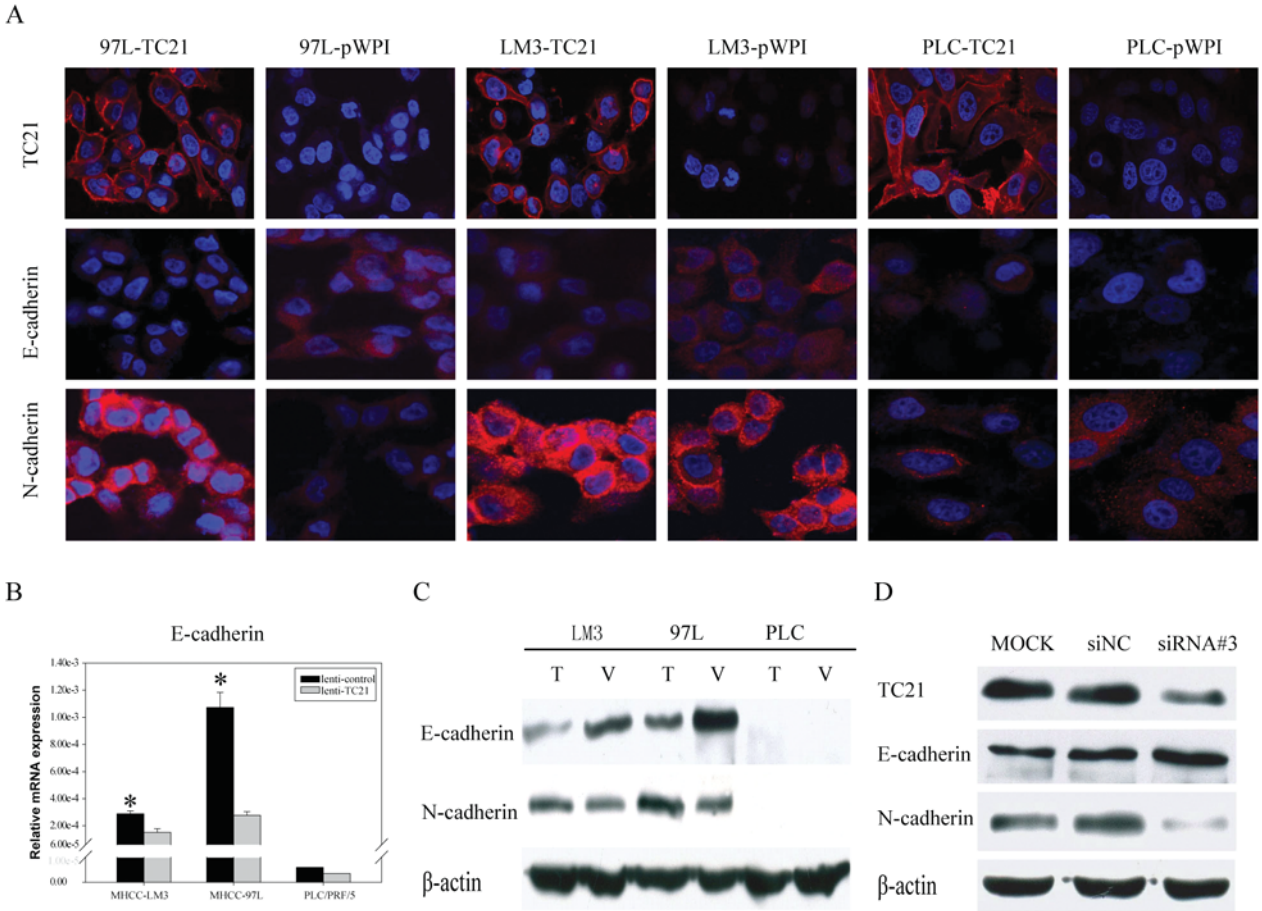

Figure 3. TC21 overexpression downregulates E-cadherin expression and upregulates N-cadherin expression. (A) Fluorescent immunocytochemical stainings of E-cadherin (red) and N-cadherin (red) were performed in the stable MHCC-LM3 (LM3), MHCC-97L (97L) and PLC/PRF/5 (PLC) cell lines infected with TC21-pWPI (T) and the pWPI-empty vector (V) lentivirus. The cells were observed under a laser confocal microscope. DAPI was used for nuclear counterstaining (blue). Original magnification, x400. (B) The expression of E-cadherin mRNA was measured in the stable MHCC-LM3 (LM3), MHCC-97L (97L), PLC/PRF/5 (PLC) cell lines infected with TC21-pWPI (T) and the pWPI empty vector (V) lentivirus by qRT-PCR. (C) Western blot analysis indicated downregulation of E-cadherin expression and upregulation of N-cadherin expression in the stable TC21-overexpressing LM3-TC21 and 97L-TC21 cell lines. ß-actin was used as the loading control. (D) In the TC21-overexpressing Huh7 cells, upregulation of E-cadherin and downregulation of N-cadherin were observed at the protein level after they were transfected with a siRNA targeted against TC21 or a negative control siRNA.

8/57), and iv) TC21^{low}/E-cadherin^{low}/N-cadherin^{low} (14.04%, 8/57) (Fig. 1). Patterns ii and iii suggest that the expression of the E-cadherin protein is downregulated by TC21.

Establishment of stable TC21 HCC cell lines that overexpress TC21. To select HCC cell lines with the lowest underlying expression of TC21, the TC21 expression levels of nine HCC

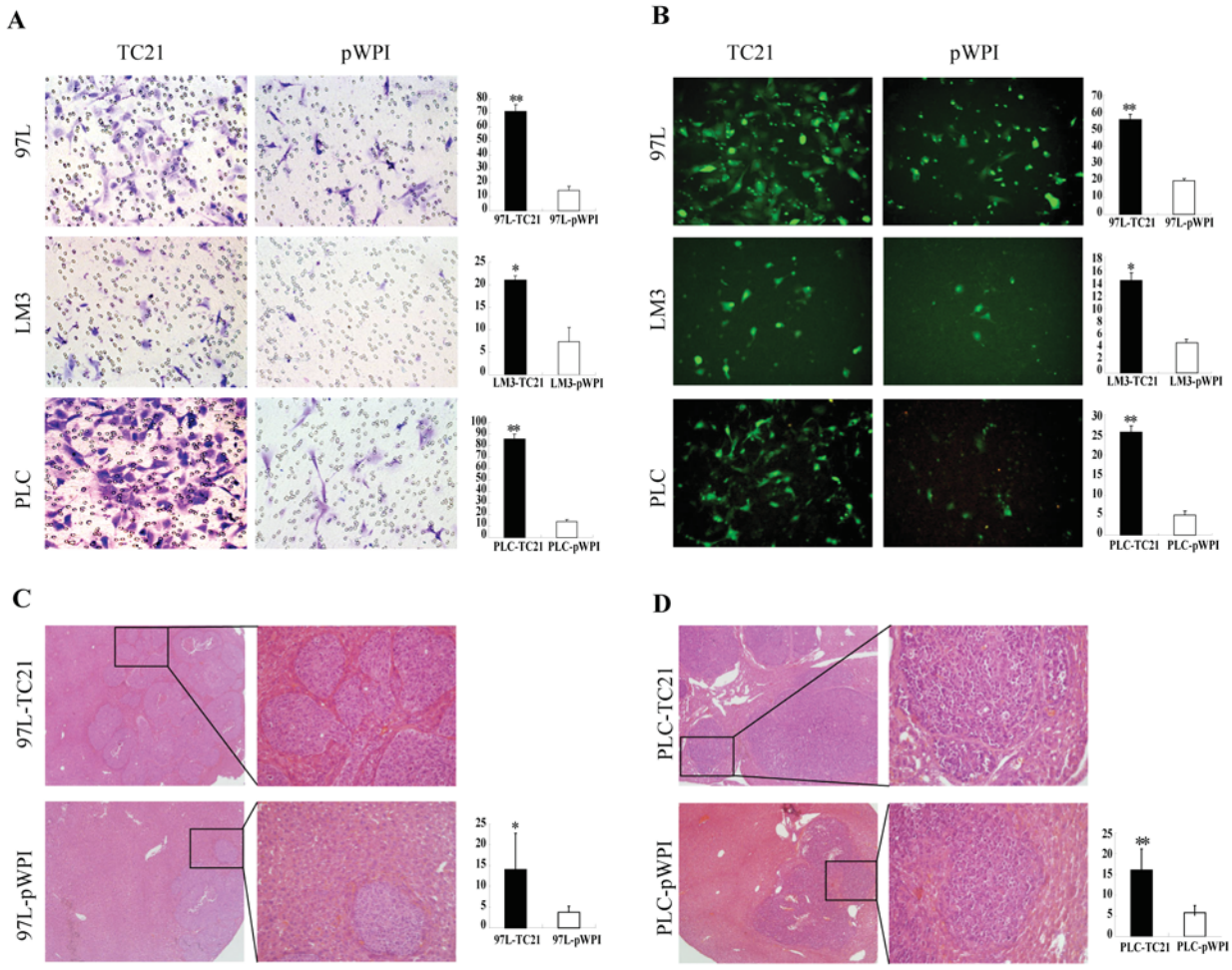

Figure 4. The overexpression of TC21 induced the migration and intrahepatic metastasis of HCC cells. (A) Transwell migration assay of the stable MHCC-LM3 (LM3), MHCC-97L (97L) and PLC/PRF/5 (PLC) cell lines infected with TC21-pWPI (TC21) and the pWPI empty vector (Vector) lentivirus. Representative images are shown (left pictures). The number of HCC cells that had migrated between TC21 and pWPI-empty vector treatments were counted and analyzed using Student's t-test. Data are presented as mean ± SDs (from five random low-magnification fields) (right curves), *P<0.05, **P<0.01. (B) Trans-endothelial migration assay of the stable MHCC-LM3 (LM3), MHCC-97L (97L) and PLC/PRF/5 (PLC) cell lines that had been infected with TC21-pWPI (TC21) and the pWPI-empty vector (pWPI) lentivirus. The number of HCC cells that had undergone trans-endothelial migration (green) were counted under a fluorescent inverted microscope and analyzed using Student's t-test. Data are presented as mean ± SDs (from five random low-magnification fields) (right curves), *P<0.05, **P<0.01. (C and D) Metastatic potential of the stable MHCC-97L (97L) and PLC/PRF/5 (PLC) cells infected with TC21-pWPI (TC21) and the pWPI-empty vector (pWPI) lentivirus. The number of intrahepatic metastatic nodules in mouse models between TC21 and pWPI-empty vector treatments were counted and analyzed using Student's t-test. Data are presented as mean ± SDs (from five liver lobes) (right curves), *P<0.05, **P<0.01.

cell lines were analyzed using RT-PCR and Western blot analysis. It was found that the highest expression of TC21 occurred in the SK-Hep-1 and Focus cell lines, while the lowest expression occurred in the HepG2, Hep3B, MHCC-97L, SMMC-7721 and Huh7 cell lines (Fig. 2A). Based on their intrinsic TC21 expression levels, three HCC cell lines, i.e., MHCC-LM3, MHCC-97L and PLC/PRF/5, were chosen to undergo transfection with TC21 using a lentiviral vector system in order to obtain stable HCC cell lines. The stable HCC cell lines obtained were confirmed by Western blot analysis (Fig. 2B).

Overexpression of TC21 may downregulate the expression of E-cadherin and upregulate the expression of N-cadherin in HCC cell lines. Based on the findings of our immunohistochemical analysis of the intrinsic TC21, E-cadherin and N-cadherin expression levels in human HCC tissues, the expression levels of metastasis-associated adhesive molecules were investigated at the mRNA and protein levels in stable TC21-overexpressing HCC cell lines. We found that ectopic expression of TC21 decreased the expression of E-cadherin and increased the expression of N-cadherin in MHCC-LM3 and MHCC-97L cells (Fig. 3A, B and C).

Using different siRNA oligonucleotides specific for human TC21, we attempted to knock down endogenous TC21 expression in Huh7 cells and evaluated its effect on E-cadherin expression. By transiently transfecting Huh7 cells with three different TC21 siRNA fragments, it was found that siRNA 3 significantly decreased the expression of TC21 in Huh7 cells. Treatment with this TC21 siRNA not only resulted in the overexpression of E-cadherin, but also in a decreased expression of N-cadherin (as compared to the levels observed in NC and MOCK cells) (Fig. 3D). These results suggest that E-cadherin is directly regulated by TC21 and is a downstream target gene of TC21 in HCC cells.

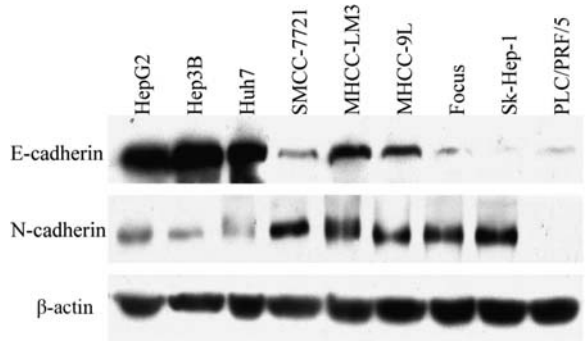

Figure 5. E-cadherin and N-cadherin protein expressions were determined in 9 HCC cell lines by Western blot analysis. Five cell lines (i.e., SMMC-7721, MHCC-LM3, MHCC-97L, Focus and Sk-Hep-1) displayed low N-cadherin expression levels and E-cadherin overexpression, three cell lines (i.e., HepG2, Hep3B and Huh7) exhibited E-cadherin overexpression and low N-cadherin expression levels, and one cell line (i.e., PLC/PRF/5) exhibited low N-cadherin and E-cadherin expression levels. ß-actin was used as the loading control.

Ectopic expression of TC21 promoted cell migration, trans-endothelial cell migration and intrahepatic metastasis in HCC cells in vivo. The transwell migration assay demonstrated that TC21 overexpression could significantly promote the migration of MHCC-97L, MHCC-LM3 and PLC/PRF/5 cells, as compared to the migratory activity observed in vector controls ($P<0.05$) (Fig. 4A). This result is consistent with the finding that the overexpression of activated TC21 enhances the migration of Schwann cells derived from Nf1-null mice (12). Moreover, in our trans-endothelial cell migration assays, we observed that the overexpression of TC21 promoted the migration of HCC cells across the endothelial layer (Fig. 4B). The results obtained by orthotopic inoculation of the stable TC21-overexpressing SMMC-7721 and PLC/PRF/5 cells showed that TC21 enhances the intrahepatic metastatic capability of these cells (Fig. 4C).

Discussion

The pathogenesis of HCC is a multistage process that is accompanied by accumulated genetic alterations, including an overexpression of oncogenic genes and tumor suppressor gene mutations. The Rb and Ras/MAPK pathways have been shown to play a role in the development of HCC (17). In this study, we demonstrated that the overexpression of oncogenic TC21, the fourth member of the Ras supergene family, enhances the migration and invasion capabilities of HCC cells both *in vitro* and *in vivo*. We also found that the overexpression of TC21 in MHCC-LM3 and MHCC-97L cells leads to a downregulation of E-cadherin expression and an upregulation of N-cadherin expression. On the other hand, the silencing of the intrinsic TC21 expression significantly decreases N-cadherin protein levels and increases E-cadherin protein levels in Huh7 cells. However, because the expression of endogenous E-cadherin in PLC/PRF/5 cells is very low (both at mRNA and protein levels), the changes in the expression level of E-cadherin in response to ectopic overexpression of TC21 were undetectable, which is easy to understand given the co-expression profile of endogenous E-cadherin and N-cadherin. Among the nine HCC cell lines that were used in this study, five cell lines (i.e., SMMC-7721, MHCC-LM3, MHCC-97L, Focus and Sk-Hep-1) displayed low N-cadherin expression levels and E-cadherin overexpression, three cell lines (i.e., HepG2, Hep3B and Huh7) exhibited E-cadherin overexpression and low N-cadherin expression levels, and one cell line (i.e., PLC/PRF/5) exhibited low N-cadherin and E-cadherin expression levels (Fig. 5).

It has been demonstrated that the loss of E-cadherin is involved in tumor invasion and metastasis, and that it leads to a poor prognosis in breast, colon and other cancers (19,20). Previous studies have also shown that E-cadherin is associated with recurrence and/or metastasis in HCC (21,22). The altered expression of E-cadherin in HCC was found to be associated with the expressions of twist, Snail and ß-catenin (25). Furthermore, studies have shown that activated H-Ras and Ras can lead to downregulation and mislocalization of E-cadherin in rat epithelial cells (23,24). Conclusively, our findings demonstrate that the overexpression TC21 promotes the motility of HCC cells both *in vitro* and *in vivo* through regulation of the expression of E-cadherin and N-cadherin.

Acknowledgements

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