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The EMT transcription factor, Twist1, as a novel therapeutic target for pulmonary sarcomatoid carcinomas

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Abstract. Pulmonary sarcomatoid carcinomas (PSCs) are a rare subtype of non-small-cell lung cancer and are typically biphasic neoplasms. No effective treatment for PSCs is currently available in clinical practice. The expression of the epithelial-mesenchymal transition (EMT) transcription factors, Twist1, Slug and Snail, as well as the EMT phenotype and vasculogenic mimicry (VM) were analysed in 41 PSC and 79 pulmonary squamous carcinoma (PSCC) samples. Compared with the PSCCs, the PSCs exhibited an EMT phenotype and VM, and they also exhibited an increased expression of the Twist1, Slug, Snail and VM markers. Twist1 expression was associated with metastasis and TNM stage. Twist1-positive patients exhibited a poorer prognosis for overall survival (OS) than those with Twist1-negative PSCs. Transforming growth factor β1 (TGFβ1) was used to induce an EMT transition in a PSCC cell line. SK-MES-1 cells treated with TGFβ1 exhibited an increased expression of Twist1. The EMT phenotype, VM and increased migratory and invasive abilities were induced following TGFβ1 treatment. Importantly, in cells treated with TGFβ1, the EMT phenotype was reversed, VM marker expression was decreased, and the migratory and invasive ability of the PSCC cell line was decreased following Twist1 knockdown. Collectively, this study provides a new perspective of Twist1 in the aggressiveness of PSCs. The identification of Twist1 as an independent marker of poor prognoses may lead to the development of novel strategies for improving the treatment of patients with PSC.

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Introduction

Pulmonary sarcomatoid carcinomas (PSCs) are rare tumours, accounting for <1% of all lung cancers. PSCs include spindle cell carcinoma, giant cell carcinoma, pleomorphic carcinoma, carcinosarcoma and pulmonary blastoma, with pleomorphic carcinoma being the most frequent subtype. No major changes in the terminology or diagnostic criteria have been made since the 2004 World Health Organization (WHO) classification (1). These tumours have a strong association with tobacco exposure. The clinical behaviour of PSCs is extremely aggressive, and patient prognosis is extremely poor (2,3), since these tumours have a high rate of distant metastasis. The effect of conventional chemotherapy for PSCs remains controversial due to their low incidence (4); thus, progress on tumour treatments is moderate.

PSCs are typically biphasic neoplasms, including both epithelial and fusiform components (5). The fusiform components of PSCs may originate from the epithelial-mesenchymal transition (EMT) of epithelial cancer cells (6,7). EMT plays a pivotal role in cancer aggressiveness, metastasis and resistance to therapy (7-10). Manzotti *et al* (5) provided a formal validation of EMT in the development of PSCs and functional insights into the mechanisms through which EMT occurs during PSC evolution. Previous studies (11,12) have demonstrated that non-small cell lung cancer (NSCLC) cell lines treated with transforming growth factor β1 (TGFβ1) underwent significant EMT-related morphological changes. The cells exhibited mesenchymal features, including morphology and increased Vimentin and reduced E-Cadherin protein expression following treatment with TGFβ1. TGFβ1 induces EMT to promote lung cancer cell proliferation, invasion and metastasis (11,12).

Vasculogenic mimicry (VM) represents a specific tumour blood supply pattern (13). VM provides an advantage for rapidly growing tumours that require a blood supply. Previous studies have demonstrated that VM occurs in highly invasive tumours and is associated with a high histological grade, invasion, metastasis and a short survival in patients with malignant tumours (14,15). Previous studies by the authors of this study and others (16-19) have demonstrated that VM channel formation in highly aggressive human tumour cells has a close association with the EMT process. Therefore, both EMT and

VM are synonymous with tumour plasticity, the transdifferentiation of epithelial cells to a mesenchymal phenotype, tumour aggressiveness and metastasis.

EMT transcription factors contribute to the development of resistance against cancer therapy, and they may be targeted as novel therapeutic approaches for the treatment of cancer (9). Twist1, a transcription factor of the basic helix-loop-helix class, was originally reported as a master regulator of embryonic morphogenesis. Twist1 is known to induce EMT in a variety of tumours (20-22). Previous studies by the authors have revealed that Twist1-induced EMT enhances the invasive, metastatic and VM formation abilities of hepatocellular carcinoma cells (23,24). Yochum *et al* (25) demonstrated that Twist1 functions to suppress oncogene-induced senescence and apoptosis in multiple oncogene-driver dependent settings, including tumours with EGFR mutations. The genetic or pharmacologic inhibition of Twist1 induces EGFR-mutant NSCLC cell growth inhibition and apoptosis, and it contributes to restoring tumour cell sensitivity to the chemotherapeutic agent, erlotinib.

In this study, the EMT phenotype, EMT transcription factor expression and VM were examined in 41 PSC and 79 pulmonary squamous carcinoma (PSCC) samples. The association of Twist1 expression with clinicopathologic parameters was explored. The prognostic role of Twist1 in PSCs was evaluated using Cox regression and Kaplan-Meier analysis. Furthermore, PSCC cells were treated with TGFβ1 *in vitro* to mimic PSC cells and to demonstrate the biological behaviour of PSCs and the function of Twist1 in PSCs.

Materials and methods

Patient samples. Human lung cancer tissue collection and analysis in this study were consented to by the patients and were approved by the Ethical Committee of Tianjin Medical University. Specimens from 41 cases of PSC and 79 cases of PSCC that were fixed with formalin and paraffin-embedded from 1995 to 2010 were selected. The specimens of patients who had not undergone chemotherapy or radiotherapy prior to surgery were exclusively employed. The pathological diagnosis was reviewed by two senior pathologists based on haematoxylin and eosin-stained sections according to the 2015 WHO classification of lung tumours. All the clinicopathological parameters, including sex, age, metastasis status, histological grade, tumour size and TNM stage, were obtained from the records.

All of the patients were followed-up by a clinical interview or phone call. The overall survival (OS) time was calculated as the duration from the date of surgery to the date of death.

Immunohistochemical and histochemical double-staining methods. Tissue sections (4-5-μm-thick) were deparaffinized and hydrated utilizing standard procedures. Immunostaining was performed using a super-sensitivity S-P IHC kit. Following immersion in 3% H_2O_2 for 10 min to eliminate endogenous peroxidase, the sections were microwaved for antigen retrieval in Tris/EDTA pH 9.0 or sodium citrate pH 6.0 for 15 min. After blocking in 10% goat serum for 30 min and incubation with primary antibodies (Twist1: 1:100, sc-15393, Santa Cruz Biotechnology; Slug: 1:150, LS-C175161, LifeSpan Biosciences; Snail: 1:100, NBP1-19529, Novus Biologicals; p63: ready to use, ZM-0406, Zhongshan Goldenbridge Biotechnology; CK5/6: ready to use, ZM-0313, Zhongshan Goldenbridge Biotechnology; CD31: ready to use, ZA-0568, Zhongshan Goldenbridge Biotechnology; Vimentin: 1:400, 2707-1, Epitomics; EPH receptor A2 (EphA2): 1:100, sc-924, Santa Cruz Biotechnology; VE-cadherin: 1:100, ab33168, Abcam; MMP2: 1:100, ab37150, Abcam; E-cadherin: 1:200, sc-8426, Santa Cruz Biotechnology) in commercialized antibody diluent at 4˚C overnight, the tissue sections were incubated with appropriate secondary antibodies (ready to use, PV-6001 and PV-6002, Zhongshan Goldenbridge Biotechnology) for 1 h at room temperature, and positive signals were developed in 3,3-diaminobenzidine tetrahydrochloride (DAB) solution. After counterstaining with haematoxylin for 5 min or Periodic acid-Schiff (PAS) (Zhongshan Goldenbridge Biotechnology) at room temperature, the slides were ready for microscopic examination. All sections with PAS staining were oxidized in 0.5% periodic acid solution for 5 min, rinsed in distilled water, placed in Schiff reagent for 15 min and washed in tap water for 5 min.

VM channel quantification was assessed by light microscopy (CX23, Olympus) analysis of the tumour areas. A total of 50 non-overlapping high-power fields (x400 magnification) were randomly selected per case. VM quantification was scored as follows: 0 (no VM channels were found), 1 [1 VM channel/50 high-power field (HPF)], 2 (2-4 VM channels/50 HPF), 3 (5-7 VM channels/50 HPF) and 4 (>8 VM channels/50 HPF). The cases with a score ≥1 were considered VM-positive.

Staining was defined as positive for significant nuclear and cytoplasmic (Twist1, Slug Snail and p63), cytoplasmic [CK5/6, Vimentin, CD31, EphA2, VE-cadherin and matrix metalloproteinase (MMP)2] or membranous (E-cadherin) immunoreactivity in neoplastic cells. Protein expression levels were quantified according to a previous standard (26) with minor modifications. The percentage of stained cells ('P') was scored as follows: 0 (staining of <5% of cells), 1 (5-10% of cells), 2 (10-30%), 3 (30-50%) and 4 (>50%). Staining intensity ('I') was graded as follows: 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (intense staining). Samples were evaluated for both factors, i.e., 'P' multiplied by 'I'. Ten high-power fields were randomly selected per case. The scoring of each case was a mean value of selected high-power fields. Cases with a score >3 were considered positive.

Western blot analysis. Cells (please see below) were lysed by using lysis buffer for western blotting (P0013, Beyotime) and loaded onto 10% sodium dodecyl sulphate-polyacrylamide gels and were then transferred onto polyvinylidene difluoride membranes (Millipore). The quantification of total protein was performed using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific), and equal amounts of protein (30 μg) were used for analysis. Blots were blocked with 5% milk/TBST and incubated with primary antibodies (Twist1: 1:500, sc-15393, Santa Cruz Biotechnology; Slug: 1:1,000, 6591, Cell Signaling Technology; Snail: 1:1,000, ab53519, Abcam; Vimentin: 1:1,000, 2707-1, Epitomis; EphA2: 1:500, sc-924, Santa Cruz Biotechnology; VE-cadherin: 1:500, ab33168, Abcam; MMP2: 1:500, ab37150, Abcam; E-cadherin: 1:200, SC-8426, Santa Cruz Biotechnology; p-smad2/3: 1:500, sc-11769, Santa

Cruz Biotechnology) at 4˚C overnight, followed by incubation with secondary antibodies (goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP, 1:2,000; sc-2005 and sc-2030; Santa Cruz Biotechnology) for 2 h at room temperature. Blots were developed using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). For protein loading analyses, β-actin antibody (1:1,000, P30002, Abmart) or GAPDH (1:2,000, ab9485, Abcam) was used.

Immunofluorescence. Cells cultured on glass slides were washed with PBS after discarding the medium, and they were then fixed with cold methanol at -20˚C for 15 min. The cells were permeabilized with 0.1% Triton X-100 in PBS for 20 min and blocked with 5% FBS in PBS at room temperature for 30 min. The cells were then incubated with primary antibodies (E-cadherin: 1:100, sc-8426, Santa Cruz Biotechnology; Vimentin: 1:100, 2707-1, Epitomics) for 1 h at 37˚C. The cells were then incubated for 1 h with secondary antibodies conjugated to Alexa 488 (A32723, Invitrogen; Thermo Fisher Scientific) or Alexa 568 (A-11011, Invitrogen; Thermo Fisher Scientific), and then they were washed with PBS. Nuclear staining with 4',6-diamidino-2-phenylindole (DAPI) (Sigma) for 10 min at room temperature was then performed. Slides were viewed under a fluorescence microscope (Nikon).

Cell culture, treatment and plasmid transfection. The SK-MES-1 cells (American Type Culture Collection) were cultured in minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS) (HyClone). The H1299 and H460 cells (Cell Resource Center, Institute of Basic Medical Sciences, Peking Union Medical College) were cultured in RPMI-1640 with 10% FBS. The cells were incubated in media with 10 ng/ml human recombinant TGFβ1 (R&D Systems) in a humidified 5% CO_2 incubator at 37˚C for 15 days to induce EMT.

The pGP-Twist1-shRNA plasmid was purchased from GenePharma. The target sequence [AAGCTGAGCAAG ATTCAGACC (siTwist1 nucleotides 505-525)] was used to downregulate Twist1. A non-silencing siRNA sequence (target sequence 5'-AATTCTCCGAACGTGTCACGT-3'), was used as a negative control. Plasmid vectors were transfected into the cells with polyethylenimine (PEI) (Cat. no. 23966, PolyScience, Inc.).

Wound-healing assay. The cells were seeded in 6-well plates. When the cells reached 100% confluency, a wound was created using a 100-μl sterile pipette tip. The wound was then photographed by using inverted phase contrast microscope (TS2, Nikon) (0 h). The rate of gap closure was measured at 24 h. To prevent apoptosis and cell detachment, 1% FBS medium was used for 24 h. The presence of serum in the culture medium may permit cell proliferation, influencing the results of the assay; however, the low percentage of FBS should sufficiently inhibit proliferation such that the gap closure was mainly due to cell migration. Each experiment was performed in triplicate.

Cell migration assay and invasion assay. Transwell inserts were used in 24-well plates, and cells ($1x10^5$) were added to the upper chamber with serum-free medium; and MEM with 10% FBS was added to the bottom chamber. Following 24 h of incubation at 37˚C and 5% CO_2, cells that remained on the upper side of the insert were removed with a cotton swab. The migratory and invasive cells were fixed with methanol and stained with crystal violet (Sigma) for 20 min at room temperature. Invasion assays were performed as with the migration assays, with the exception that the Transwell chambers were coated with Matrigel before the cells were seeded in the upper chamber. These cells were counted using an inverted light microscope (Nikon). Each experiment was performed in triplicate.

3D Matrigel culture. Tumour cells were mixed with Matrigel (BD Biosciences) and were seeded to allow for polymerization. The addition of regular medium was performed during the incubation at 37˚C, 5% CO_2 for 1 week. Cells were incubated until tubular structures were formed and were photographed using a phase contrast microscope.

Statistical analysis. All data in this study were evaluated using SPSS17.0 software (SPSS, Inc.). The correlation between E-cadherin and Vimentin expression and VM formation was analysed using the Spearman's rank test. E-cadherin, Vimentin, VM, EphA2, VE-cadherin, MMP2, Twist1, Slug and Snail immunohistochemical staining score data were transformed into categorical data by setting up a score of >3 as positive and a score of ≤3 as negative. Therefore, the comparison of the number of patients with positive or negative expression of these factors between PSCs and PSCCs [i.e., the comparison of positive rate (the number of patients with positive expression divided by the total number of patients with PSC or PSCC)] was performed using Chi-square test. Twist1, Slug and Snail expression in groups with different clinicopathologic parameters was analysed using the Chi-square test. Survival curves were estimated using the Kaplan-Meier method and were compared by the log rank test. Multivariate analysis of prognostic factors was tested for using Cox regression analysis. The data analysis of wound closure, invasive and migratory cell numbers was performed an using independent-samples t-test. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

PSCs display an EMT phenotype and VM formation. In this study, 41 PSC and 79 PSCC specimens were collected. Positive CK5/6 or p63 staining was found in all 79 PSCCs (data not shown). For the 41 sections of PSCs, there were 30 pleomorphic carcinomas, 7 carcinosarcomas, and 4 spindle cell carcinomas. The 30 pleomorphic carcinomas were composed of squamous cell carcinoma with positive CK5/6 or p63 expression (Fig. 1A), and they contained at least 10% spindle and/or giant cells (data not shown). This finding suggested that these pleomorphic carcinomas may originate from monoclonal malignant transformed squamous epithelium that they partially differentiate and exhibit mesenchymal characteristics. In the 7 carcinosarcomas, positive CK5/6 or p63 expression was found in 5 cases, and CK8/18 was found in 2 cases. Positive panCK staining was found in 4 spindle cell carcinomas, which demonstrated their epithelial origin (data not shown).

Compared with the PSCCs, the PSCs exhibited an EMT phenotype. E-cadherin expression was higher in the PSCC

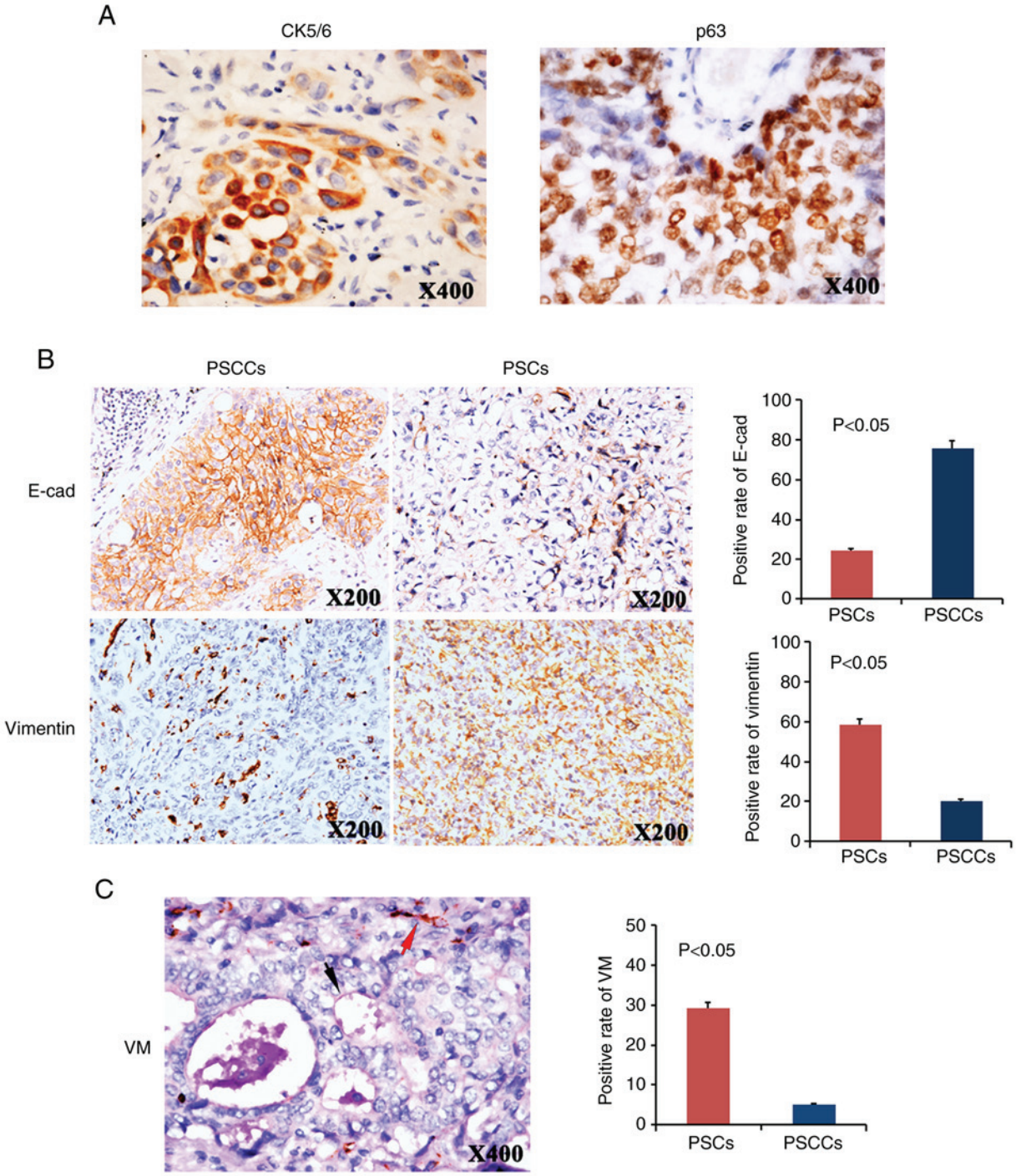

Figure 1. PSCs displayed an EMT phenotype and VM formation. (A) CK5/6 and p63 positive staining in PSCs. (B) The decreased expression of E-cadherin was more frequently detected in the PSCs group than it was in the PSCCs group. Vimentin expression was found to be higher in the PSCs group than it was in the PSCCs group. (C) VM channels (black arrow indicates VM, red arrow indicates typical CD31-positive blood vessel). The percentage rate of VM was higher in PSCs than it was in PSCCs. PSC, pulmonary sarcomatoid carcinoma; EMT, epithelial-mesenchymal transition; PSCC, pulmonary squamous carcinoma; VM, vasculogenic mimicry.

group (positive rate, 75.9%) than it was in the PSC group (positive rate, 24.4%). By contrast, Vimentin expression was higher in the PSC group (58.5%) than it was in the PSCC group (20.3%) (Fig. 1B).

Since the EMT phenotype represents tumour cell plasticity, which can contribute to VM formation, the VM channels were analysed in the PSCs and PSCCs. Using CD31/PAS double-staining (Fig. 1C), the tubular channels lined with tumour cells were considered VM formation. These tumour cells were negative for CD31 staining, demonstrating that they were not endothelial cells. The membrane-like matrix around the VM structure was positive for PAS. Necrosis and infiltration of inflammatory cells in the periphery of the channels were not observed, and red blood cells were found inside the channels. The positive VM rate differed markedly between the PSCs and PSCCs. VM was found in 12 out of 41 PSC samples (29.3%) and in 4 out of 79 PSCC samples (5.1%, P<0.05, Fig. 1C).

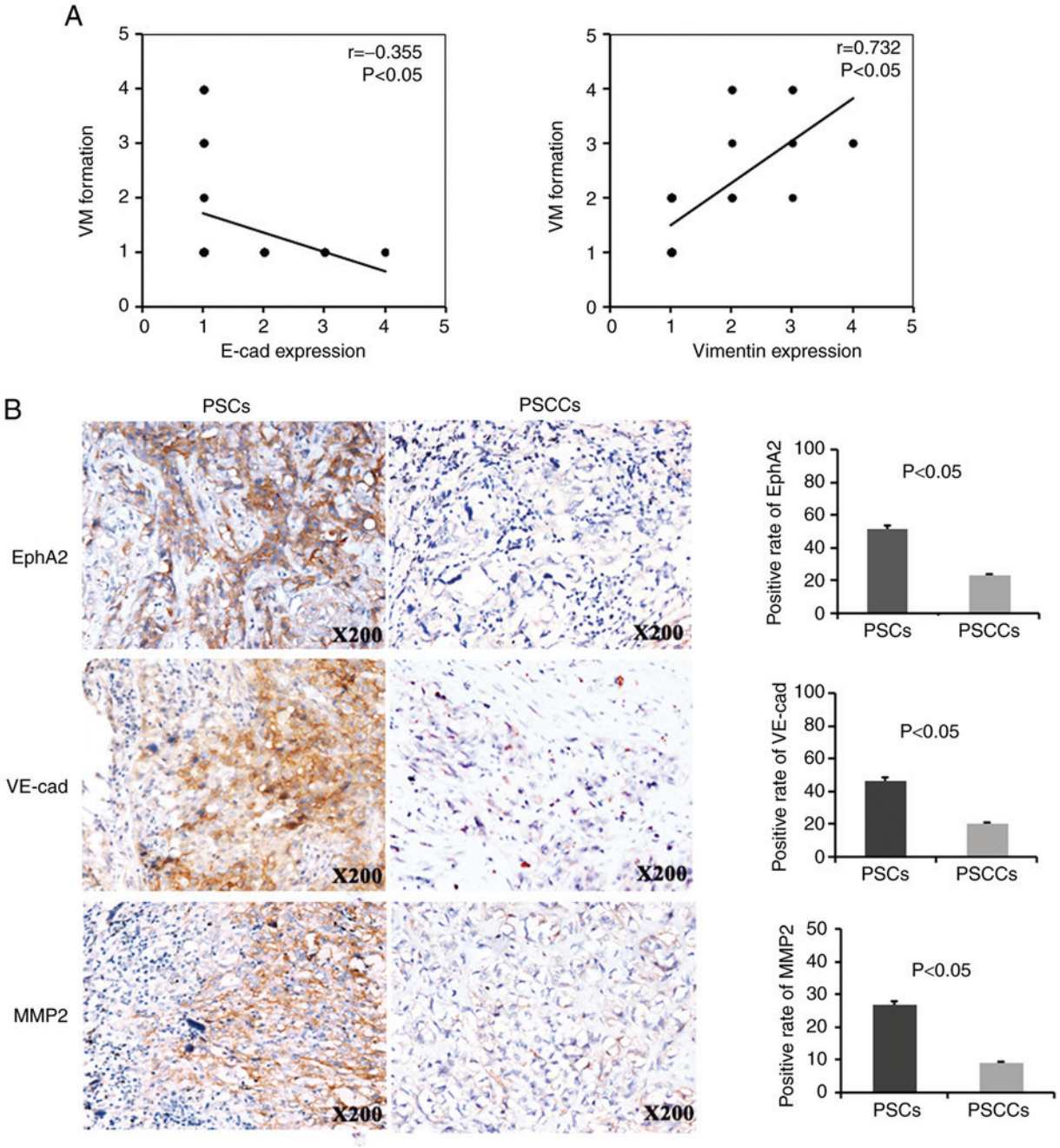

Figure 2. Expression of the VM markers, EphA2, VE-cadherin and MMP2, was increased in PSCs compared with PSCCs. (A) VM formation exhibited a negative correlation with E-cadherin expression and a positive correlation with Vimentin expression in PSCs. (B) EphA2, VE-cadherin and MMP2 positive expression in PSCs and negative expression in PSCCs. VM, vasculogenic mimicry; EphA2, EPH receptor A2; MMP2, matrix metalloproteinase 2; PSC, pulmonary sarcomatoid carcinoma; PSCC, pulmonary squamous carcinoma.

In the PSCs, VM formation exhibited a negative correlation with E-cadherin expression and a positive correlation with Vimentin expression (r=-0.355, P<0.05; r=0.732, P<0.05, respectively) (Fig. 2A). Of the 41 PSCs analysed, 21 (21/41, 51.2%), 19 (19/41, 46.3%) and 11 (11/41, 26.8%) were positive for EphA2, VE-cadherin and MMP2, respectively. Of the 79 PSCCs analysed, 18 (18/79, 22.8%), 16 (16/79, 20.3%) and 7 (7/79, 8.9%) were positive for EphA2, VE-cadherin and MMP2, respectively. Therefore, the expression of the VM markers, EphA2, VE-cadherin and MMP2, was higher in the PSCs compared with the PSCCs (Fig. 2B).

Twist1 expression indicates a poor survival of patients with PSC. In previous studies, the EMT regulators Twist1, Slug and Snail were found to play a role in EMT and VM in hepatocellular carcinoma and breast cancer (19,24,27). Therefore, this study examined Twist1, Slug and Snail expression in PSCs and PSCCs (Fig. 3A). Correspondingly, Twist1, Slug and Snail exhibited a significantly increased expression in the PSCs (61.0, 39.0 and 34.1%, respectively) over what was observed in the PSCCs (26.6, 21.5 and 17.7%, respectively) (Fig. 3A).

The association of Twist1, Slug and Snail expression with the patient clinicopathological variables was analysed in the PSCs and PSCCs. A positive Twist1 expression, but not that

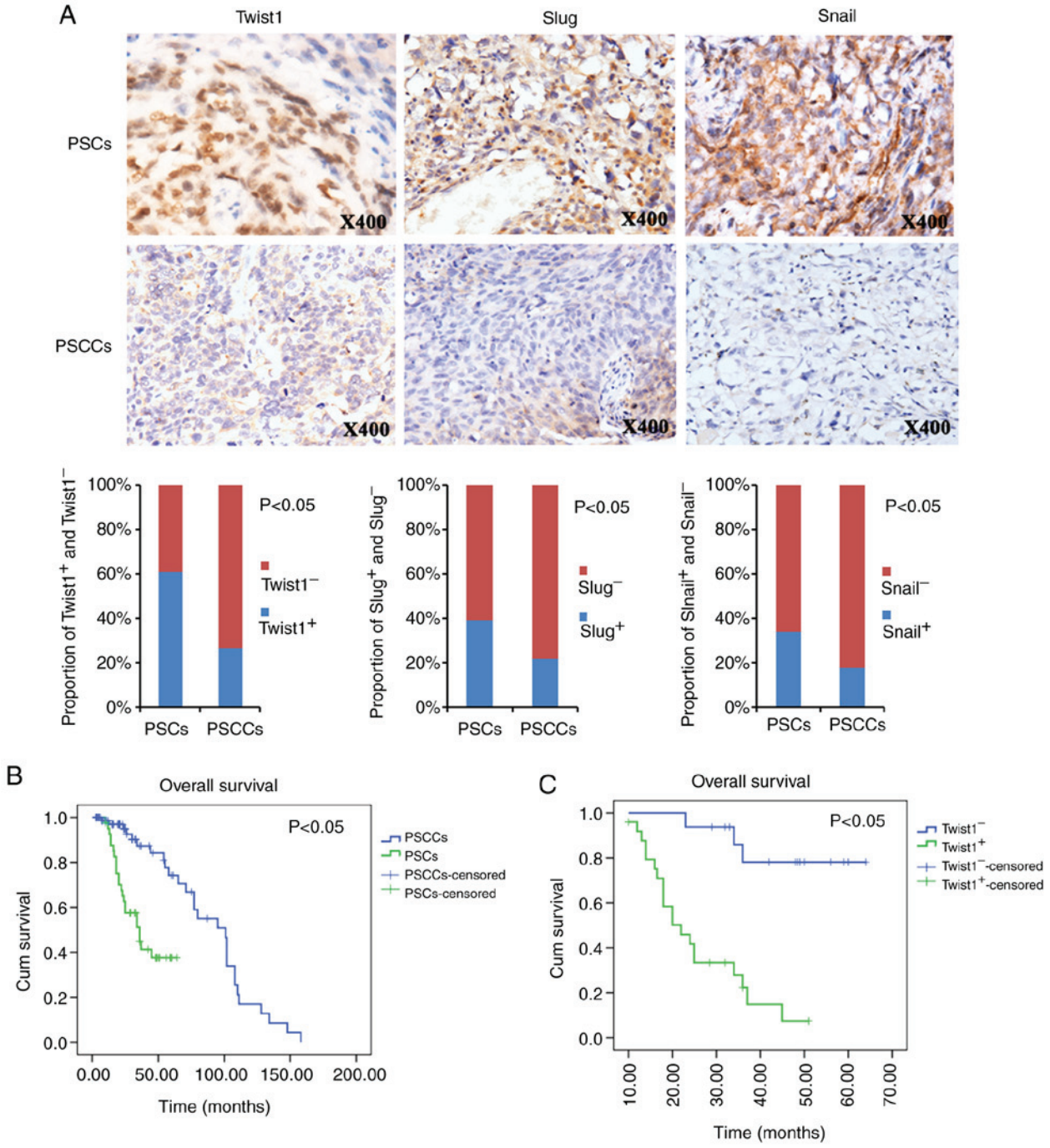

Figure 3. Twist1 positivity was an independent marker of a poor prognosis for OS in patients with PSC. (A) Twist1, Slug and Snail showed increased expression in PSCs than they did in PSCCs. (B) Patients with PSC had a worse OS than patients with PSCC. (C) Twist1-positive patients with PSC exhibited a poorer prognosis for OS than those with Twist1-negative expression. OS, overall survival; PSC, pulmonary sarcomatoid carcinoma; PSCC, pulmonary squamous carcinoma.

of Slug or Snail, was not only associated with metastasis (including lymph node metastasis, haematogenous metastasis and pleural implantation metastasis) (P<0.05), but also with TNM stage (P<0.05) in the PSCs (Table I). In the PSCCs, Snail expression, but not that of Twist1 or Slug, was associated with the histological grade (P<0.05) (Table II).

Kaplan-Meier survival analysis revealed that patients with PSC had a worse OS than the patients with PSCC (Fig. 3B). The mean (95% CI) OS times were 39.2 (32.5-45.8) months for the patients with PSC and 88.0 (75.3-100.8) months for patients with PSCC (P<0.05).

Additional survival analysis revealed that the Twist1-positive patients with PSC exhibited a poorer prognosis for OS than patients with Twist1-negative expression (Fig. 3C). The mean (95% CI) OS times were 25.7 (20.7-30.7) months for Twist1-positive patients, and 56.9 (49.7-64.1) months for Twist1-negative patients (P<0.05). However, there was no difference in survival time between the Slug-positive and -negative patients with PSC or between the Snail-positive and -negative patients with PSC (Fig. S1). Twist1, Slug and Snail expression did not exhibit any prognostic significance for OS in the patients with PSCC (Fig. S2).

Table I. Twist1, Slug and Snail expression in patients with PSC with different clinicopathologic parameters.

	Twist1			Slug			Snail		
Characteristic	+	-	P-value	+	-	P-value	+	-	P-value
Sex			0.072			0.160			0.153
Male	15	5		10	10		9	11	
Female	10	11		6	15		5	16	
Age, years			0.087			0.288			0.228
≤60	13	4		5	12		4	13	
>60	12	12		11	13		10	14	
Metastasis			0.005[a]			0.960			1.000
Yes	12	1		5	8		4	9	
No	13	15		11	17		10	18	
Tumour size			0.444			0.248			0.062
≥5 cm	11	9		6	14		4	16	
<5 cm	14	7		10	11		10	11	
Stage			0.002[a]			0.848			0.706
I	4	11		5	10		4	11	
II	9	3		5	7		5	7	
III	12	2		6	8		5	9	

[a]Statistical significance (P<0.05). PSC, pulmonary sarcomatoid carcinoma.

Table II. Twist1, Slug and Snail expression in groups with different clinicopathologic parameters in PSCCs.

	Twist1			Slug			Snail		
Characteristic	+	-	P-value	+	-	P-value	+	-	P-value
Sex			0.300			0.729			0.186
Male	16	37		12	41		12	41	
Female	5	21		5	21		2	24	
Age, years			0.793			0.621			0.425
≤60	13	34		11	36		7	40	
>60	8	24		6	26		7	25	
Metastasis			0.591			0.759			0.160
Yes	9	21		7	23		3	27	
No	12	37		10	39		11	38	
Tumour size			0.670			0.264			0.149
≥5 cm	9	28		10	27		9	28	
<5 cm	12	30		7	35		5	37	
Grade			0.714			0.506			<0.001[a]
I	3	13		4	12		2	14	
II	13	33		11	35		1	45	
III	5	12		2	15		11	6	
Stage			0.556			0.278			0.851
I	6	23		6	23		6	23	
II	9	24		5	28		5	28	
III	6	11		6	11		3	14	

[a]Statistical significance (P<0.05). PSCC, pulmonary squamous carcinoma.

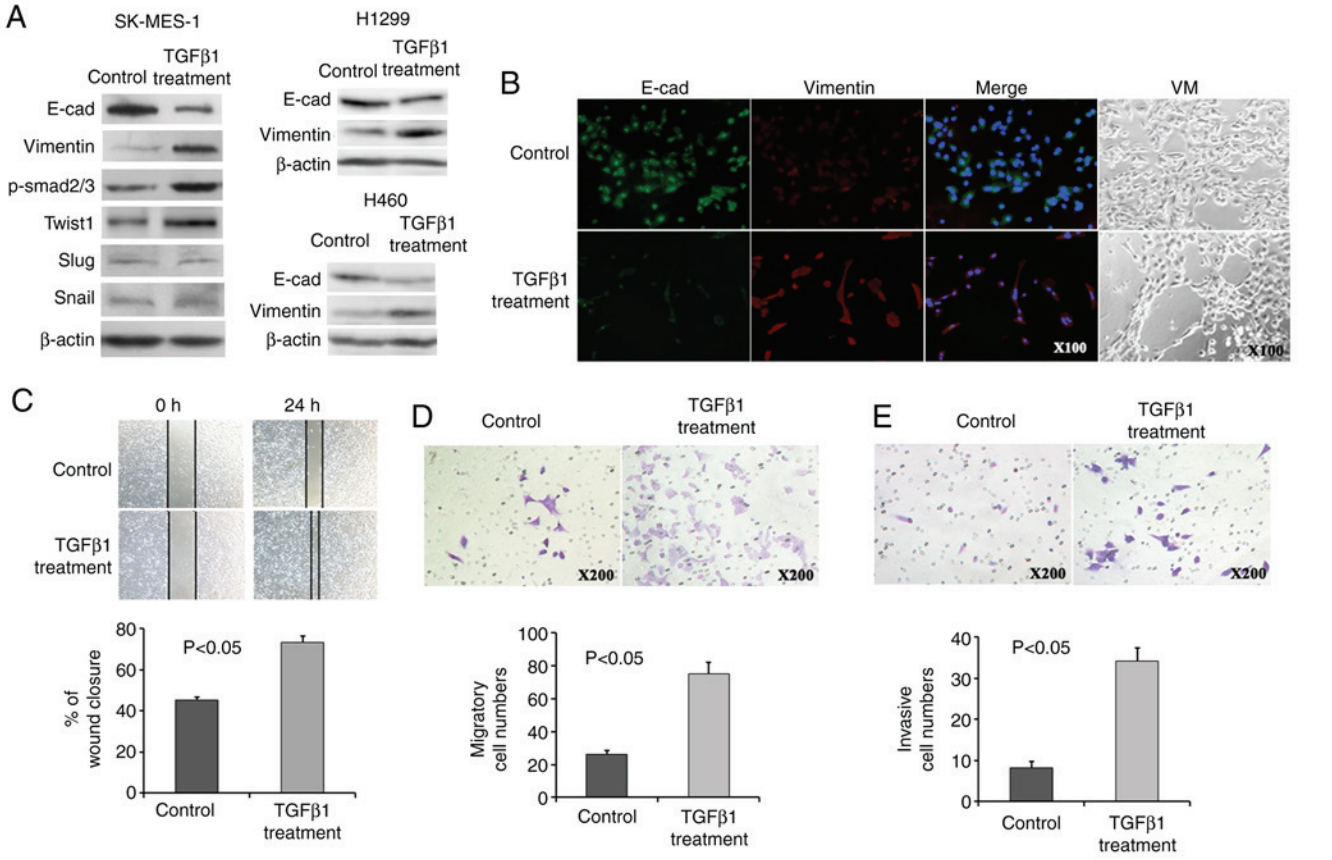

Figure 4. The PSC cell line analogue exhibited an increased Twist1 expression, EMT phenotype, VM formation and migratory and invasive ability. (A) NSCLC cells treated with TGFβ1 exhibited a decreased E-cadherin and increased Vimentin expression. Twist1 and p-Smad2/3 expression was induced, while that of Slug and Snail expression was not altered following TGFβ1 treatment in SK-MES-1 cells. (B) Immunofluorescence assays revealed a decreased E-cadherin expression and an increased Vimentin expression, and 3D Matrigel culture revealed VM formation following TGFβ1 treatment. (C-E) Increased wound closure (C), migratory (D), and invasive (E) ability was observed following TGFβ1 treatment. PSC, pulmonary sarcomatoid carcinoma; EMT, epithelial-mesenchymal transition; PSCC, pulmonary squamous carcinoma; VM, vasculogenic mimicry.

In addition, multivariate Cox regression analysis was performed (Table SI), and Twist1 positivity and metastasis were identified as independent markers of a poor prognosis for OS in patients with PSC. However, TNM stage was identified as an independent marker of a poor prognosis for OS in patients with PSCC (Table SI).

EMT transition of lung cancer cell lines by TGFβ1 addition. To the best of our knowledge, no PSC-derived cell lines are currently available. Thus, TGFβ1 was used to induce the EMT transition of the NSCLC cell lines, SK-MES-1 (28), H1299 and H460, in order to obtain an *in vitro* PSC cell line analogue.

Consistently, all cells exhibited a decreased E-cadherin and increased Vimentin expression following the addition of TGFβ1, as assessed by immunofluorescence and western blot analysis (Fig. 4A and B). TGFβ1 proteins bind to receptors on the cell surface, initiating a signalling cascade that leads to phosphorylation of Smad2 and Smad3. Phosphorylated Smad then translocates to the nucleus and regulates the transcription of its target genes. Therefore, phosphorylated Smad2/3 (p-Smad2/3) was detected as a surrogate marker for TGFβ1 signalling activity, and p-Smad2/3 expression exhibited a marked increase in the SK-MES-1 cells following treatment with TGFβ1 (Fig. 4A). Importantly, the expression of Twist1 exhibited a tendency towards an increased expression, although the expression of Slug and Snail did not exhibit a similar trend (Fig. 4A). These results suggest that the downstream targets of TGFβ1 signalling, as well as Twist1, may be useful markers for the evaluation of the occurrence of EMT, which converts epithelial PSCCs into PSCs with biphasic differentiation containing epithelial and mesenchymal features. In addition, the PSC cell line analogue exhibited VM formation in 3D Matrigel culture following the occurrence of EMT (Fig. 4B).

Twist1 knockdown (KD) inhibits the migration and invasion of the PSC cell line analogue. Quantitative analyses of the wound-healing assay suggested a significant difference in the speed of wound healing between the PSC cell line analogue and control SK-MES-1 cells. The PSC cell line analogue displayed a faster speed of wound healing (Fig. 4C). An increased migratory and invasive ability following treatment with TGFβ1 was observed in the PSC cell line analogue by Transwell assays (Fig. 4D and E). Twist1 downregulation in SK-MES-1 cells under standard conditions or TGFβ1 treatment was observed following transfection with shTwist1 vector (Figs. 5A and S3). Importantly, the EMT phenotype was reversed (Fig. 5A), VM marker expression was decreased (Fig. 5A), the migratory and invasive abilities were decreased (Fig. 5B), and wound closure (Fig. 5C) and VM formation (Fig. 5D) were also decreased in the PSC cell line analogue following Twist1 knockdown.

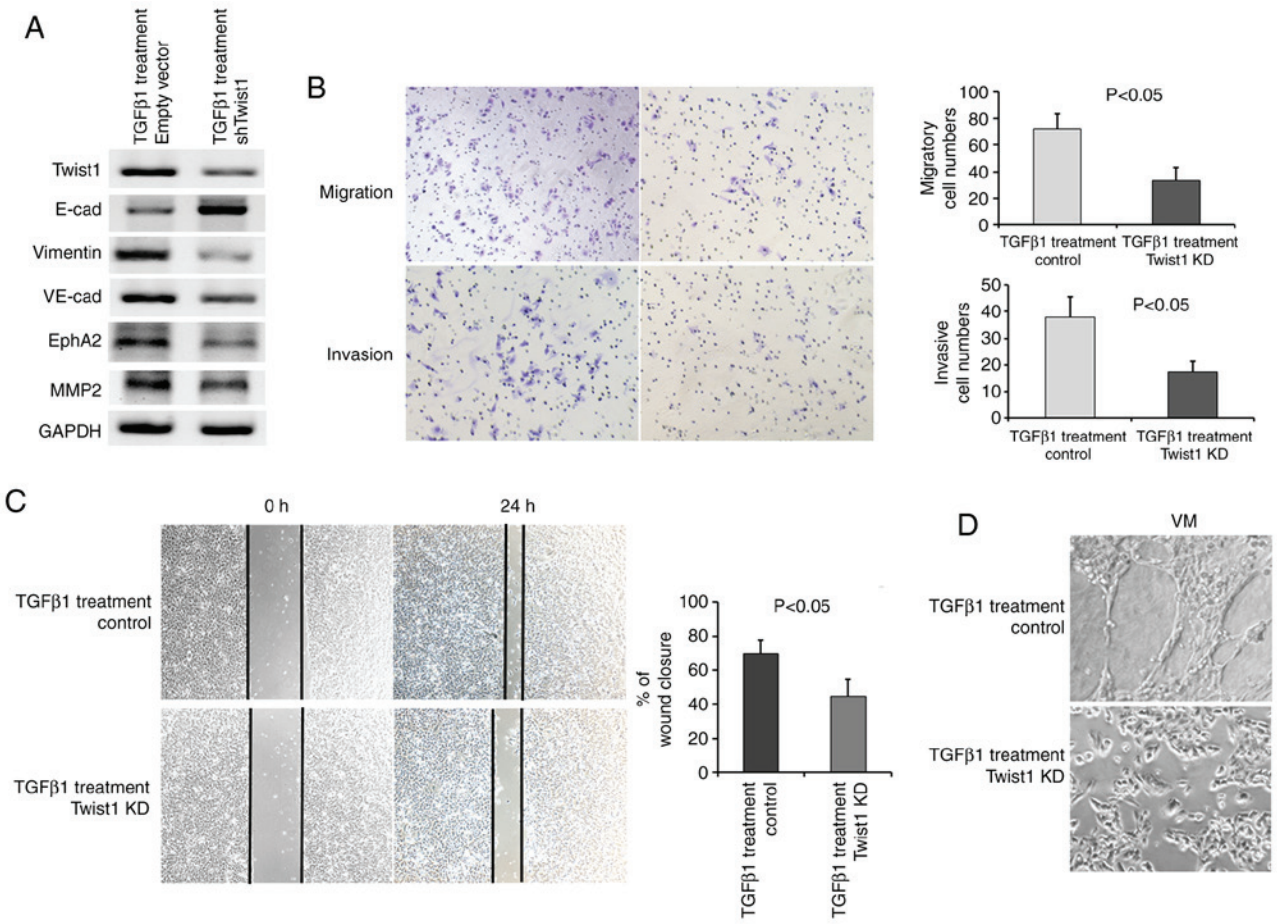

Figure 5. Twist1 knockdown (KD) inhibits the migration and invasion of the PSC cell line analogue. (A) The EMT phenotype was reversed, and VM marker expression was decreased following Twist1 KD. (B) The migratory and invasive ability of the PSC cell line analogue was decreased following Twist1 KD. Decreased wound closure (C) and VM formation (D) ability was observed following Twist1 KD. EMT, epithelial-mesenchymal transition; VM, vasculogenic mimicry.

Discussion

NSCLC with spindle and/or giant cells or sarcomas that contain heterologous elements (rhabdomyosarcoma, chondrosarcoma, and osteosarcoma) are unified under the umbrella term sarcomatoid carcinoma. Molecular evidence indicates that these tumours are essentially carcinomas with varying degrees of divergent differentiation (supporting tumour metaplasia, rather than the collision 'polyclone hypothesis') (29-31). In pulmonary sarcomatoid carcinoma, the sarcomatous-like and carcinomatous components have the same molecular pedigree and are composed of the same pattern of acquired allelic absence (30), p53 mutations and X chromosome inactivation (31). In this study, the expression of CK5/6 or p63 was observed in both carcinomatous and spindled components of the sarcomatoid carcinoma, indicating that sarcomatoid carcinoma may be histogenetically converted from lung squamous cell carcinoma. Demonstrating the gene expression differences existing between PSCs and PSCCs should aid in the understanding of the mechanisms through which this conversion occurs, providing new insight into the biology of PSCs and paving the way to the definition of novel therapies.

As a transcription factor, Twist1 can exert multiple biological effects through various downstream pathways by regulating the expression of target genes. The most critical pathological function of Twist1 in cancer is facilitating tumour invasion and metastasis by promoting EMT. EMT is a cellular plasticity process (32), which involves the loss of epithelial characteristics by epithelial cells, such as decreased cell-cell contact and the downregulation of E-cadherin, with the simultaneous acquisition of mesenchymal properties, including a spindle-like shape, increased cell motility, and the upregulation of mesenchymal markers such, as vimentin. Twist1 interacts with several components of the Mi2/nucleosome remodelling and deacetylase (Mi2/NuRD) complex (MTA2, RbAp46, Mi2, HDAC2) and recruits the factors to the proximal regions of the E-cadherin promoter to downregulate promoter activity and repress E-cadherin gene expression (33). As the downstream target of Twist1, in this study, the reduced expression of E-cadherin and the increased expression of vimentin were more frequently detected in the PSC group than the PSCC group, and they were more frequent in SK-MES-1 cells treated with TGFβ1 than in the control cells, suggesting that Twist1-induced E-cadherin repression and the EMT phenotype may be characteristics of PSCs.

The authors previously demonstrated that tumour cells with EMT characteristics can form VM, and the occurrence of both EMT and VM indicates that tumour cells harbour high plasticity (19,24). In this study, there was a significant

difference between the positive rate of VM in PSCs and PSCCs, which is consistent with the occurrence of EMT in PSCs. The tumour cells of the VM channel wall can fall off, flow into the blood and arrive at other organs to grow as a metastatic tumour. This may explain the shorter survival period observed in patients with PSC with a higher VM formation ability compared to that of patients with PSCC. In the present study, it was also demonstrated that the expression of the VM markers, EphA2, VE-cadherin and MMP2, was increased in PSCs. Remodelling of the extracellular matrix is one of the factors that governs VM channel formation, and MMP2, which can degrade various extracellular matrix proteins and facilitate tumour invasion and metastasis, is positively correlated with VM formation (34). VE-cadherin and EphA2 were also found to be co-localized in cell-cell adhesion junctions both *in vitro* and *in vivo*. It has been previously demonstrated that EphA2 and VE-cadherin interact directly and/or indirectly during VM (35,36). Previous research has shown that EphA2 is a factor upstream of phosphatidylinositol 3-kinase (PI3K) and that phosphorylated EphA2 can activate PI3K and then regulate the expression of MMP-2 (37). An increased EphA2, VE-cadherin and MMP2 expression are hallmarks of VM that were more commonly present in PSCs than they were in PSCCs in this study, indicating that PSCs develop a more malignant phenotype.

To demonstrate the role of EMT in the occurrence of PSC *in vitro*, NSCLC cells were used and were treated with TGFβ1 to mimic PSC cells, since TGFβ1 is a well-known EMT inducer. All NSCLC cells exhibited a decreased E-cadherin and an increased vimentin expression following the addition of TGFβ1. The cells undergoing EMT cells exhibited an increased VE-cadherin, MMP2 and EphA2 expression and VM formation, suggesting that these cells may possess the biological characteristics of PSCs. The migratory and invasive ability was elevated in these cells, suggesting aggressive biological behaviour in the PSC cell line analogue. Importantly, these cells exhibited an increased Twist1 expression, and Twist1 knockdown reversed the EMT phenotype, reduced the migratory and invasive ability and the expression of VM markers. In addition, in this study, the expression of Twist1, Slug and Snail was found to be higher in the PSC group than in the PSCC group. It has been reported that Twist1, Slug and Snail can stimulate carcinoma metastasis by mediating E-cadherin repression (19,38,39). Twist1 had been found not only to function in EMT, but also to function in VM in previous studies by our group and others (24,38). VE-cadherin expression is upregulated following the upregulation of Twist1 expression in the 3D Matrigel culture system (24). Of note, in this study, only Twist1 expression, not Slug or Snail, was associated with metastasis and TNM stage in patients with PSC, suggesting an important role for Twist1 in PSC metastasis by promoting and maintaining the EMT phenotype and VM formation.

Consistently, it was found that patients with PSC with Twist1-positive expression exhibited a poorer OS than Twist1-negative patients, as indicated by Kaplan-Meier analyses. There was no difference in survival time between Slug-positive and -negative or between Snail-positive and -negative PSCs. Cox multivariate analysis demonstrated that a Twist1-positive expression was an independent prognostic factor for OS in patients with PSC. These results indicated that Twist1-positive expression predicted a worse survival and may serve as the key molecular prognostic indicator for PSCs. Mechanistically, Twist1 expression in PSCs promoted tumour cell plasticity, which contributed to the EMT phenotype through the suppression of E-cadherin and the upregulation of Vimentin, and it contributed to VM formation by the upregulation of VE-cadherin; thus, Twist1 plays a role in the aggressive behaviour of PSCs. The association between Twist1-positive expression and poor survival suggests a feasible therapeutic strategy for targeting Twist1 in PSCs.

To the best of our knowledge, this study is the first report on the expression of Twist1, which is closely associated with tumour cell plasticity, EMT and VM, and may regulate the molecular mechanisms of aggressiveness in PSCs. Therefore, the findings of this study may prove to be useful in the identification of novel therapeutic targets for the inhibition of PSC angiogenesis and metastasis.

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Availability of data and materials

The datasets and data used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BS, TL, XiZ, XuZ and YZ conceived and carried out experiments. TL and XuZ conceived the experiments and analysed the data. XuZ, XiZ, YZ, XD, NZ and SL carried out immunochemistry and cell culture experiments. All authors were involved in the writing of the manuscript and gave the final approval of the submitted and published versions.

Ethics approval and consent to participate

Tissue collection and analysis in this study were approved by the Ethics Committee of Tianjin Medical University, China. Written consent had been signed by all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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