Expression of DPC4/Smad4 in non-small-cell lung carcinoma and its relationship with angiogenesis

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The DPC4 influences tumourigenesis and tumor progression by various mechanisms, including angiogenesis. The aim of this study was to determine whether the expression of DPC4 is related to the angiogenesis in lung cancer and whether it could be involved in its clinical behaviour. Immunohistochemistry revealed that DPC4 was expressed at high level in normal broncho-tracheal epithelium, but at low level in tumor tissues, and closely correlated with tumor lymph node metastasis. This result was further confirmed by Western blot analysis. Furthermore, carcinomas with high DPC4 expression demonstrated low VEGF expression and low MVD (microvessel density) labelled with CD34. In addition, DPC4 siRNA in A549 cells also showed that DPC4 could decrease VEGF protein and mRNA expression, and increase TSP1 protein and mRNA expression. Our findings indicated that DPC4 might be an important biomarker for malignant transformation and be involved in preventing the tumor metastasis by inhibiting tumor angiogenesis.

Key words: DPC4, Lung cancer, VEGF, TSP-1, Angiogenesis

DPC4(deleted in pancreatic carcinoma locus 4,DPC4) is a new tumor suppressor gene frequently inactivated in carcinomas [1]. It is involved in many different and even contradictory activities, such as cell-cycle regulation, differentiation, and angiogenesis [2–5]. Loss of heterozygosity (LOH) at the DPC4 locus occurred in >50% of the pancreatic carcinomas and has been found to extent in carcinomas of the colon, breast, ovary, lung, and head and neck [6,7]. Devereux [8] also reported previously that DPC4 remained at high loss in lung cancers.

Already in the late 1960s, there was preliminary evidence indicating that tumor angiogenesis was mediated by diffusible factors produced by tumor cells [9,10]. DPC4 inhibits also tumor progression by decreasing neovascularization under certain circumstances [11–12]. Previous studies have suggested that Smad4 decreased the expression of vascular endothelial growth factor (VEGF) causing the cells to switch from angiogenic to antiangiogenic capacity in vitro and in vivo [12]. However,little is known about DPC4 expression in NSCLC, especially its relationship with tumor angiogenesis. The aim of the work is to determine the possible association between DPC4 expression and tumor angiogenesis in NSCLC.

Materials and methods

Case selection. In this study, 52 cases coded as "lung cancer" were selected consecutively from the surgical pathology archives of the Department of Pathology of Hubei Tumor Hospital between 2000 and 2003 .The previous histologic diagnosis was confirmed by a experienced pathologist. The age of these selected patients ranged from 20 to 70 years at the time of surgery (mean age, 52.3 years), and the male/female ratio was 2.6:1. According to the criteria of the World Health Organization(WHO, 2004), 52 cases were divided into 25 squamous cell carcinomas and 27 adencarcinomas. With regards to differentiation, 14 were well-differentiated, 18 were moderately differentiated, and 20 were poorly differentiated. Among 52 cases lymph node metastasis were in 17 cases. In addition, among 52 cases with lung cancer, 19 cases containing primary carcinoma and corresponding normal lung tissue

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was chosen for labeling. All cases were selected for study on the basis of availability of a paraffin-embedded, formalin-fixed tissue blocks. An approval for this study was given by the Medical Ethics Committee of Sun Yat-sen University.

Immunohistochemistry for DPC4,VEGF and CD34. Unstained 4μm sections were cut from the selected paraffin blocks and deparaffinized by routine techniques. The slides were steamed for 20 minutes in sodium citrate buffer (diluted to 1×from 10×heat-induced epitope retrieval buffer). After cooling for 5 minutes, the slides were labeled for 60 minutes at room temperature with either a 1:100 dilution of monoclonal antibody to DPC4 (Maxim-Bio, China) (Clone No: 4C6F8), a 1:200 dilution of a monoclonal antibody to VEGF(Maxim-Bio, China)(Clone No: VG-1) or 1:200 dilution of a monoclonal antibody to CD34 (Maxim-Bio, China) (Clone No: QBEnd/10) using the Bio Tek 1000 automated stainer. Labeling was detected by adding biotinylated secondary antibodies, avidin-biotin complex, and 3, 3'-diaminobenzidine. Sections were then counterstained with hematoxylin. DPC4, VEGF and CD34 immunolabeling were evaluated jointly by two authors using a multi-headed microscope, with agreement on all cases. In negative controls, we used PBS to replace DPC4, VEGF and CD34.We used the known positive slice in the SP Kit(Maxim-Bio, China) as positive control. In all positive controls, the known positive slices were from the tissues of breast carcinoma.

Evaluation of immunohistochemistry. We determined the scoring criteria during a preliminary evaluation using a multi-headed microscope in order to reach a consensus. Staining results for each antibody were then interpreted by two of the authors independently, without prior knowledge of the clinicopathological parameters. Discordant cases were reviewed and agreed upon before data were statistically analyzed. For each sample, at least five fields (×400) and more than 500 cells were analyzed. Under microscope, we observed the distribution, positive intensity and positive ratio of DPC4, and VEGF protein. The number of immunopositive cells was semi-quantitatively estimated as follows: Firstly scoring according to staining intensity: colorless(0); buff(1); brownly yellow(2); darkly brown(3);Then scoring according to the percentage of positive cells: no positive cells (0); <10 % of the cells staining positive (1); 11%-50% of cells staining positive (2); 51%-75% of cells staining positive (3); and >75% of cells staining positive (4).If the product of multiplication between staining intensity and the percentage of positive cells is ≥ 2,it is thought as immunoreaction positive(+).A known positive control was included with each run of staining to monitor batch-to-batch consistency. In all positive controls, the known positive slices were from the tissues of breast carcinoma.

MVD counting: The reference pathologist also performed the MVD scoring. MVD was determined by light microscopy in regions of invasive tumor containing the highest numbers of capillaries and small venules (microvessels) per area (i.e., areas with the most intense neovascularization). Tumor sections were scanned first at a low power (40×and 100×) to identify areas of invasive carcinoma having the greatest numbers of distinct CD34 staining microvessels per area (brown staining), usually at the margins of the carcinoma. Individual microvessel counts were then made on a 200×field within the area of most intense tumor neovascularization. Any endothelial cell or endothelial cell cluster positive for CD34 and clearly separate from an adjacent cluster was considered to be a single countable microvessel. Results were expressed as the highest number of microvessels identified within any single 200× field. The review was performed without any knowledge of the clinical outcome.

Cell culture and RNA Interference. The non-small-cell lung cancer A549 cell line was purchased from the Wuhan Cell Bank. The cells were cultured in RPMI medium supplemented with 10% heat-inactivated fatal calf serum at 37? under 5% CO_2 in a humidified incubator. Lipofectamine 2000 was used for siRNA transfections.A549 cells in the exponential phase of growth were grown for 24 h and plated in antibiotic-free RPMI at 2×10^4 cells/ml, then transfected with siRNA(DPC4 siRNA,>97% pure). The ion-exchange high-performance liquid chromatography-purified siRNA (DPC4 siRNA) was purchased from(Ruibo, China). For the selection of the DPC4 siRNA, Homo sapiens DPC4 mRNA sequence (GenBank Accession No. NM_005359) was subjected to NCBI Blast search against Bos taurus EST cDNA library. The following base pair duplexes of siRNA were used for DPC4: (sense) 5'-GUAUGAUGGUGAAGGAUGA dTdT-3' and (antisense) 3'-dTdT CAUACUACCACUUCCUACU-5'.In addition to medium control, cells were transfected with negative control siRNA. Twenty-four, forty-eight and seventy-two hours after transfection, cells were harvested and used for experiments.

Western blotting. Protein extracts were prepared using a lysis buffer(10 mM Tris–HCl (pH 7.5), 1% Triton X-100, 20% glycerol, 1 mM EDTA,50 mM NaCl, and 1 mM PMSF). Ninety micrograms of protein was loaded onto SDS–polyacrylamide gels and subjected to electrophoresis, and transferred onto nitrocellulose membrane . Blotted membranes were then incubated with a 1:2000 dilution of the anti-DPC4, VEGF and TSP-1 antibody(Sigma) in 5% milk/TBST for 24 h. Following three 10-min washes in TBST, the membranes were incubated with a 1:1000 dilution of the goat horseradish peroxidase-conjugated secondary antibody(Sigma) in 5% milk/PBST for 3 h. Finally, membranes were subjected to three 10-min washes in PBST, and then immunocomplexes were visualized using an enhanced chemiluminescence system (ECL; Amersham). The same membrane was reprobed with β-actin-specific antibody to ensure equal control.

Real-time reverse transcription-polymerase chain reaction(RT-PCR). For real-time RT-PCR, the total RNA was extracted using the RNAeasy kit (Qiagen, USA) from A549 cells 48h after transfection . Briefly, total RNA (1μg) of each cell sample was reversely transcribed in 20μL using 0.5 μg of oligo dT and 200 U Superscript II RT (Invitrogen, USA). The amplification was carried out in a total volume of 20μL containing 0.5μM of each primer, 4 mM MgCl2, 2μL LightCyclerTM FastStart DNA Master SYBR green I (Roche, USA), and 2μL of 1:10 diluted cDNA. Ct Value

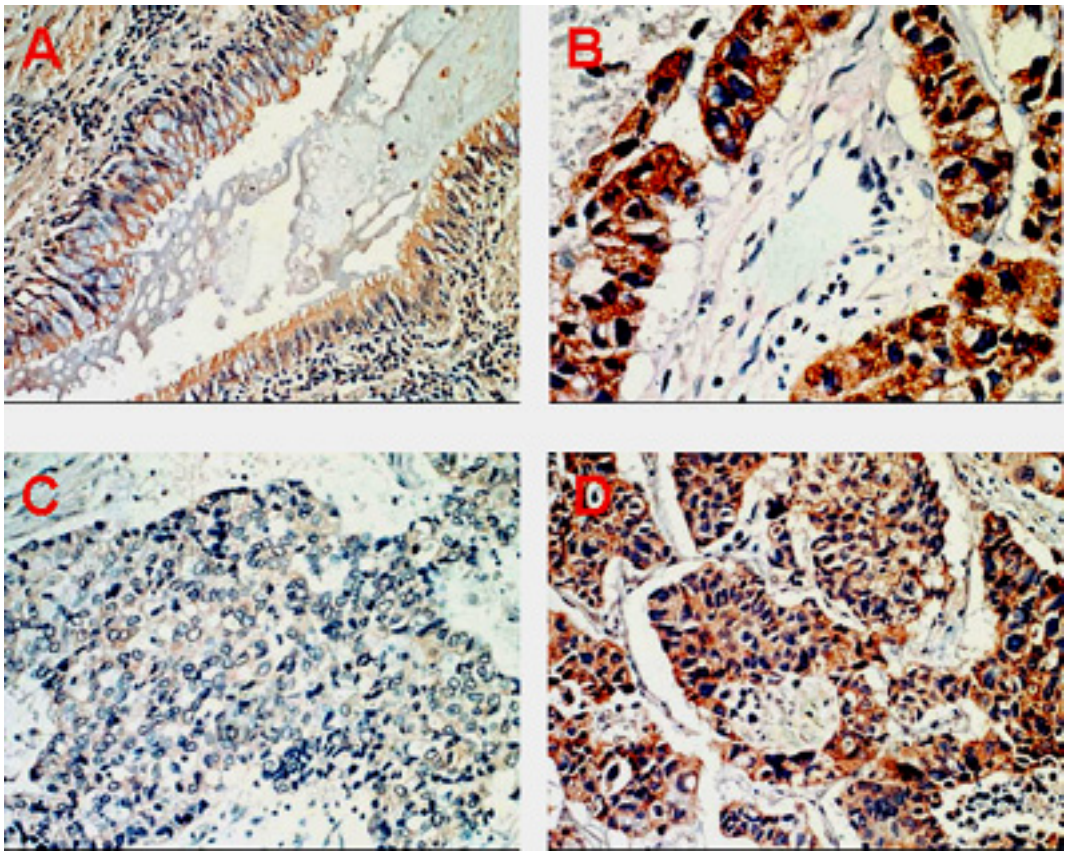

Fig 1 Immunohistochemical staining for DPC4 is mainly observed in the cytoplasm of the lung cancer cells.DPC4 staining was also occasionally found in interstitial cells.(A).Strongly positive expression of DPC4 in normal bronch-tracheal epithelium(SP×200).(B).Strongly positive expression of DPC4 in moderately differentiated adenocarcinoma(SP×200).(C). Negative expression of DPC4 in poorly differentiated squamous carcinoma(SP×400).(D).Positive expression of DPC4 in well differentiated squamous carcinoma(SP×200).

(initial amplification cycle) of each standard dilution was plotted against standard cDNA copy numbers. On the basis of the standard curves for each gene,the sample cDNA copy number was calculated according to the sample Ct value. Standard curves and PCR results were analyzed using ABI7000 software (Appliedbiosystem, USA).The target gene primers were VEGF(f):5'-CAAGGCCAGCACATAGGAGA-3';VEGF(r): 5'-ACGCGAGTCTG TGTTTTTGC-3' TSP-1(f):5'-CAGGTCGATGAGTGCAAAGA-3';TSP-1(r):5'-GTCTGCTTGGTCAGGGTTGT -3';β-actin (f):5'-GATCATTGCTCCTCCTGAGC-3'; β-actin (r),:5'-TGTGGA CTTGGGAGAGGACT-3'.All PCR reactions were performed in duplicate.

Statistical analyses

Statistical analysis from SPSS(11.5) was performed by using the one-way or two-way ANOVA test between DPC4 expression and clinical parameters, followed by Tukey's test or Student's t-test for protein semiquantity and RNA quantity, and Spearman correlation analysis between DPC4 expression and VEGF expression. P value of less than 0.05 was considered significant.

Results

DPC4 expression in NSCLC. By immunostaining of DPC4, positive granules were mainly located in the cytoplasm of normal bronch-tracheal epithelium and tumor cells (Fig 1). DPC4 was also occasionally found in fibroblast cells, smooth muscle cells and inflammatory cells in the stroma of the surrounding tissue. The preserved expression of DPC4 in normal lung tissues was much higher than that in lung carcinoma tissues($P<0.05$)(89.5%and 63.5%, respectively).But the expression of DPC4 was not associated with histological types and differentiation degrees($P>0.05$). There was statistically significant difference in DPC4 expression in lung carcinomas with lymph node metastasis (5 of 17,29.4%) versus those without lymph node metastasis (28 of 35, 80.0%) ($p<0.001$)(See Table 1).

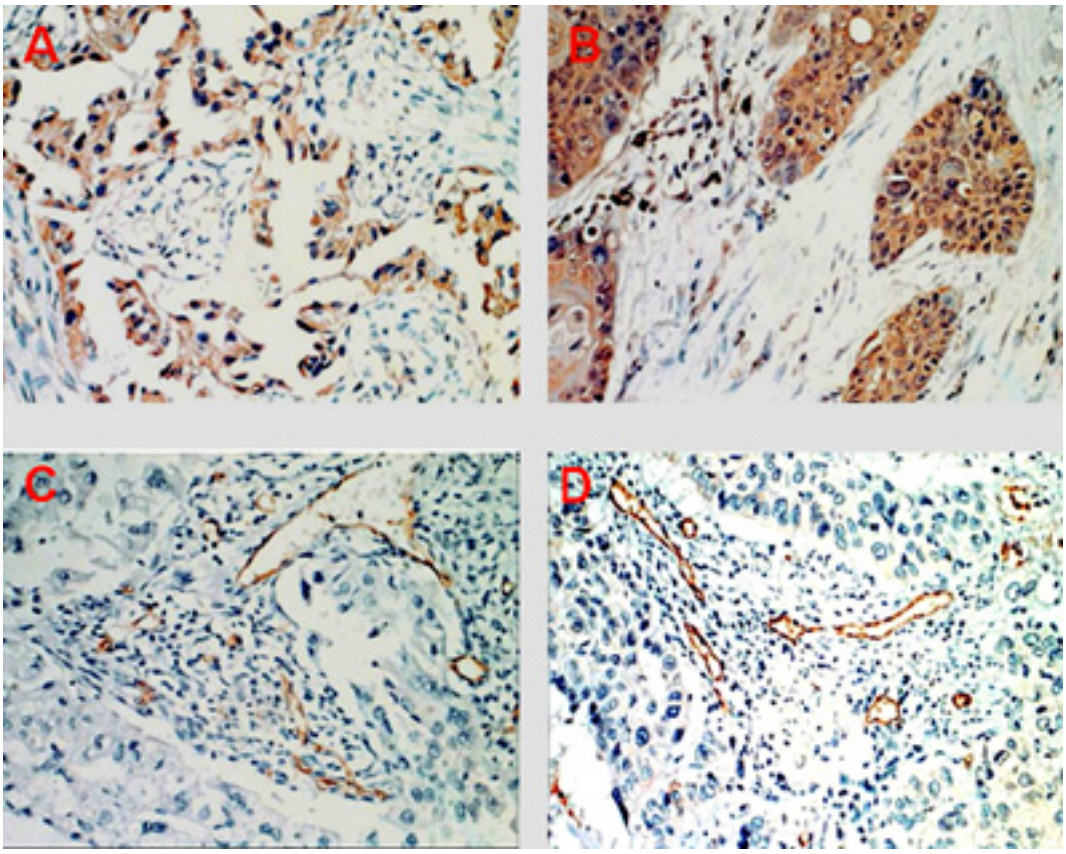

Fig 2. Immunohistochemical staining for VEGF is mainly observed in the cytoplasm of the lung cancer cells.(A).Strongly positive expression of VEGF in moderately differentiately adenocarcinoma(SP×200).(B). Positive expression of VEGF in moderately differentiated squamous carcinoma(SP×200).CD34 staining was mainly located in the surrounding micro-vessels of lung carcinoma.(C). Positive expression of CD34 in moderately differentiately adenocarcinoma(SP×200).(D). Positive expression of CD34 in moderately differentiated squamous carcinoma(SP×200).

Interrelationship between DPC4 and VEGF, MVD. VEGF was expressed in the tumor cells cytoplasm with a homogenous or granular pattern (Fig 2.A,B). In all lung carcinomas, 13 of 33 cases with preserved DPC4 expression showed VEGF positivity (13/33, 39.4%). Among 19 cases with reduced DPC4, there were also 14 cases displaying VEGF positivity (14/19, 73.7%). Significant correlationship was found between DPC4 and VEGF(P=0.020).CD34 positive granules were located in the vascular endothelial cells(Fig 2.C,D). MVD in DPC4 positive group was 43.74±7.25,which was much lower than that in DPC4 negative group (61.77±7.09). DPC4 staining was inversely associated with MVD(P<0.001)(See Table 2).

Immunoblot confirmation of the expression of DPC4 in cancerous tissues. To confirm the immunohistochemical

Table 1 Correlation of expression of DPC4 in NSCLC with clinical data

Clinical data	n	DPC4		P
		+	–	
Normal lung tissues	19	17	2	0.033
NSCLC	52	33	19	
Tissue type				0.282
Squamous cancer	25	14	11	
Adenocarcinoma	27	19	8	
Differentiation level				0.583
Well	14	10	4	
Moderately	18	12	6	
Poorly	20	11	9	
Lymph node metastasis				<0.001
NO	35	28	7	
Yes	17	5	12	

Table2 Relationship between DPC4 and VEGF, MVD

DPC4	n	VEGF[a]		MVD[b](χ±s)
		+	–	
+	33	13	20	43.74±7.25
–	19	14	5	61.77±7.09

a: P =0.020
b: P<0.001

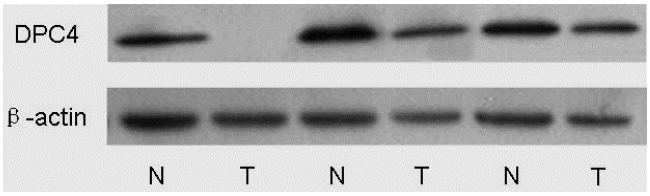

Fig. 3. Western blot analysis revealed markedly down-regulated DPC4 expression in NSCLC samples (T) when compared with that in normal (N) bronch-trachea tissues. Depicted are 3 individual-matched pairs of normal and neoplastic tissue samples.

results, DPC4 expression in 12 additional NSCLC tissue specimens was compared with that in individual-matched normal bronch-tracheal tissue samples by Western blot analysis. To confirm equal protein loading, parallel actin-immunoblotting was performed and signal quantification was performed by densitometric scanning. The results showed that DPC4 was down-regulated in all (12 / 12) of the tested NSCLC samples as compared with individual-matched normal bronch-tracheal tissues (Fig. 3).

DPC4 siRNA inhibits the expression of DPC4 in A549 cells. To investigate the role of DPC4 on VEGF and TSP1 expression in A549 cells, we used siRNA to specifically knock down DPC4 expression. A549 cells were transfected with DPC4 siRNA. The efficacy of DPC4 siRNA on DPC4 protein was confirmed by Western blot analysis at 24h, 48h and 72h after siRNA transfection. As shown in Fig 4, treatment with DPC4 siRNA significantly decreased DPC4 protein by about 50% in A549 cells at 48h and 72h as compared to siRNA control(P<0.01), suggesting that the DPC4 siRNA achieved a successful knockdown.

Role of DPC4 in VEGF and TSP1 mRNA and protein expression in A549 cells. In response to DPC4 siRNA, VEGF protein increased by 201.13% after DPC4 siRNA transfection, compared with siRNA-transfected control(P<0.01). However,TSP-1 protein decreased by 39.32%, compared with siRNA-transfected control (P<0.01) (Fig 5 A). These were associated with a significant increase in VEGF mRNA, and decrease in TSP-1 mRNA expression in DPC4 siRNA-transfected A549 cells(Fig 5 B). These datum suggest that DPC4 signalling can induce TSP1 mRNA and protein expression, and inhibit VEGF mRNA and protein expression in A549 cells.

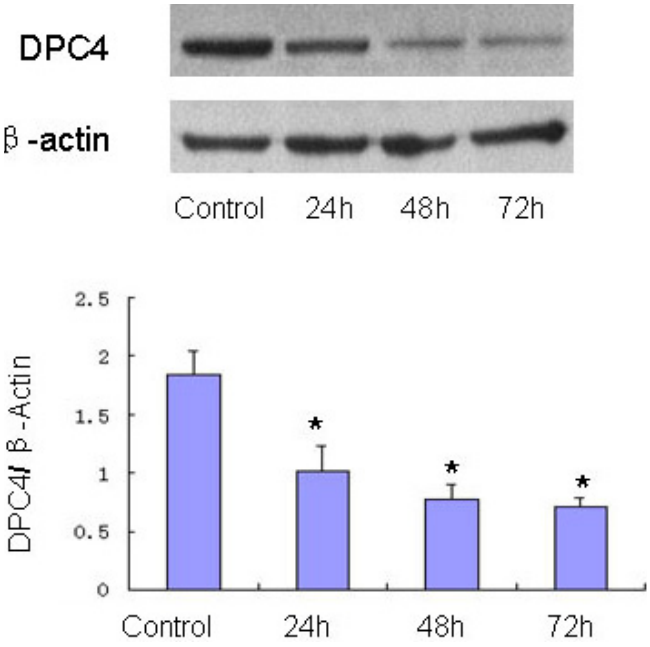

Fig 4. A representative Western blot for DPC4 protein expression showed down-regulation of DPC4 expression by siRNA. Datum are means±SD of 3 independent experiments.* P<0.05 versus Con-siRNA

Discussion

In vitro and in vivo studies have shown that the loss of DPC4 played a significant role in tumorigenesis and progression of pancreatic , colorectal and lung cancer [6, 13]. In present study, the positive rate of DPC4 expression in NSCLC tissues was 63.5%, decreasing significantly compared to that (89.5%) in normal lung epithelial cells (P<0.05). Western blot analysis also confirmed that DPC4 was down-regulated in all (12 / 12) of the tested NSCLC tissues as compared with individual-matched normal broncho-tracheal tissues. Gregory et al reported that Smad4 protein expression was correlated with the grade and stage in prostatic adenocarcinomas [14]. By the immunohistochemical staining of Smad4 protein in varying stages of colorectal cancers, Maitra et al [15]confirmed the loss of Smad4 function occured at later stages of malignancy, playing a role in the acquisition of advanced phenotypes. However, we found that DPC4 expression did not relate to differentiation of NSCLC. This phenomenon may be caused by the tissue type difference. These data indicated that inactivation of DPC4 might be an early event in NSCLC carcinogenesis. Previous studies reported that the loss of DPC4 increased from 0% in adenoma to 10% in carcinoma, and up to 35% in invasive carcinomas with lymph node metastasis [16]. In our study, there was statistically significant difference in DPC4 expression in lung carcinomas that present with lymph node metastasis (29.4%) versus those without lymph node metastasis (80.0%) (p<0.001). Thus, reduced expression of DPC4 in NSCLC may indicate that patients have a poor prognosis.

This ‘angiogenic switch’ as it is called, is necessary for tumors to obtain the necessary nutrients and oxygen to grow. However, the integrity of these new neoplastic capillaries is compromised due to the lack of a smooth muscle wall, and an irregular leaky basement membrane, which may also facilitate tumor cell leakage into the circulation and development of metastasis [17, 18]. Therefore, to study the tumor angiogenesis seems to be important to elucidate the tumor metastasis mechanism. Our results showed that MVD in DPC4 positive

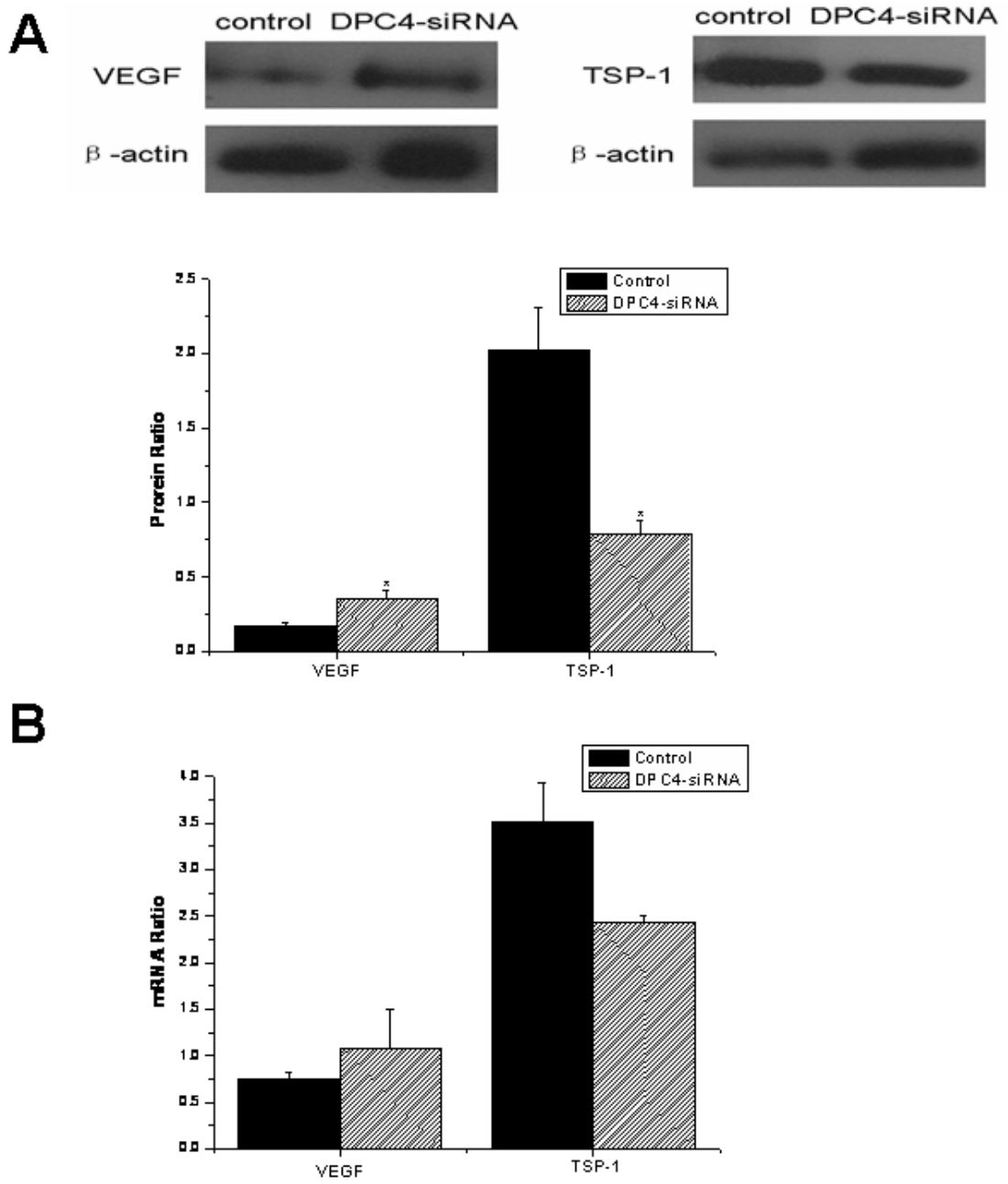

Fig 5. Effect of DPC4 siRNA on VEGF and TSP1 expression. A549 cells were transfected with DPC4 siRNA and a scramble siRNA(Con-siRNA). (A) VEGF and TSP1 were determined by Western blot analysis. Upper panel showed a representative blot for VEGF and TSP1 protein. Lower panel was quantification of VEGF and TSP1 protein levels. (B) VEGF and TSP1 mRNA was determined by real-time RT-PCR in A549 cells, and levels of VEGF and TSP1 mRNA were expressed as (VEGF and TSP1)/β-actin mRNA ratio). Datum are means±SD of 3 independent experiments.* P<0.05 versus Con-siRNA

group was 43.74±7.25,which was much lower than that in DPC4 negative group(61.77±7.09)(P<0.001). This indicated that DPC4 might inhibit tumor angiogenesis and play an important role in tumor angiogenesis. VEGF is known to be the most important proangiogenic factor, critical to the process of angiogenesis. It has been shown to increase vascular permeability, which may facilitate tumor dissemination via the circulation[19,20]. In our immunohistochemistry experiment, DPC4 staining was inversely associated with VEGF expression(P<0.05). This showed that VEGF might be involved in the process of DPC4 angiogenesis inhibition. In addition, it was reported that the TSP-1 is an anti-angiogenic

factor related to the tumor development, metastasis and invasion in vitro [21–23]. Reduced expression of TSP-1 mRNA was demonstrated in melanoma, lung cancer and breast cancer cell lines with a high metastatic potential[24]. However, the study on the relationship between DPC4 and VEGF, TSP1 was rarely reported. Thus, to assess the role of DPC4 on VEGF and thrombospondin-1 (TSP-1), DPC4 siRNA was used to treat A549 cells. Western blot showed that DPC4 protein expression is remarkably down-regulated by DPC4 siRNA, which demonstrated that siRNA technology was extremely successful. However, VEGF protein and mRNA level was up-regulated after the blockade of DPC4 signaling. This fact is concordant with a previous study showing that SMAD4 restoration in pancreatic cancer cells can reduce angiogenesis rates through the down-regulation of VEGF expression [12]. Inversely, Tsp1 protein and mRNA level was down-regulated. This further demonstrated that VEGF and TSP1 might be the down-stream gene of DPC4 and played an important role in the process of DPC4 angiogenesis inhibition.

Together, our findings revealed that DPC4 might be involved in early carcinogenesis of NSCLC and was thought as an important clinical prognostic index. Our data suggested also that DPC4 participated in the complex regulatory mechanism of angiogenesis, possibly by down-regulating VEGF expression and up-regulaing TSP1 expression..

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