The expression and underlying angiogenesis effect of DPC4 and VEGF on the progression of cervical carcinoma

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Abstract. The present study aimed to investigate the expression and roles of deleted in pancreatic carcinoma locus 4 (DPC4) and vascular endothelial growth factor (VEGF) in the development of cervical carcinoma. A total of 115 patients aged between 25 and 60 years were involved, including 19 cervical inflammation, 35 cervical intraepithelial neoplasia (CIN), and 61 cervical squamous-cell carcinoma (CSCC). The protein expression rates of DPC4 and VEGF in all samples were detected using immunohistochemistry. The protein levels of DPC4 and VEGF in CSCC samples were measured using ELISA. Microvessel density (MVD) of each CSCC sample was measured according to the Winder method. Association analysis between DPC4, VEGF and thrombospondin-1 (TSP-1) was conducted using Spearman's correlations. The negative expression rate of DPC4 [DPC4 (-)] and positive expression rate of VEGF [VEGF (+)] of the CSCC group were significantly higher compared with that in the cervical inflammation and CIN groups ($P<0.05$). In the CSCC group, the protein level of DPC4 decreased, while the VEGF level increased significantly compared with the healthy control group ($P<0.05$). The MVD in the DPC4 (-), VEGF (+) and TSP-1 (-) groups was significantly increased compared with that of the DPC4 (+), VEGF (-), and TSP-1 (+) groups ($P<0.05$). The expression of DPC4 was negatively associated with VEGF and TSP-1 ($P<0.01$). These results suggest that DPC4, VEGF and TSP-1 are involved in the carcinogenesis of cervical carcinoma by inducing angiogenesis. In addition, the loss of DPC4 induces angiogenesis through increasing VEGF. Thus, VEGF may be a target gene regulated by DPC4.

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Introduction

Cervical carcinoma, also known as invasive carcinoma of cervix uteri, is a cancer arising from the cervix. It is ranked as the second most common type of cancer among women worldwide (1,2). Furthermore, cervical cancer was associated with 275,000 mortalities in 2008, ~88% of which occurred in developing countries (3). Therefore, the identification of molecular genes involved in the regulation of cervical carcinoma progression is warranted in order to develop novel therapeutic approaches for this cancer.

Several molecular genes have been reported to be associated with tumor progression, including deleted in pancreatic carcinoma locus 4 (DPC4/Smad4), vascular endothelial growth factor (VEGF) and thrombospondin-1 (TSP-1) (4). Loss of DPC4 contributes to the switch of transforming growth factor (TGF)-β from a tumor-suppressive to a tumor-promoting pathway in cancer (5). Previous research demonstrated that a decrease in DPC4 mRNA expression is associated with lack of growth inhibition in human cervical carcinoma (6). VEGF and TSP-1 are regulatory molecules of angiogenesis (7), which is essential for the progression of cervical carcinoma (8). High expression of VEGF is associated with the degree of vascularization (9). In addition, the positive expression rate of VEGF in cervical carcinoma is higher compared with that in normal, inflammatory and cervical intraepithelial neoplasia (CIN) cervix (10). It has also been revealed that decreased TSP-1 expression correlates inversely with microvessel count in cervical carcinoma (11) and alters tumor growth by modulating angiogenesis (12). Furthermore, recent research suggests that VEGF and TSP-1 are key target genes for DPC4 (4), and the stable expression of DPC4 downregulates the expression of VEGF in cervical carcinoma (13). Although DPC4 and VEGF have been identified to be associated with cervical carcinoma, the involvement of the two with tumorigenesis, and the correlation between DPC4 and VEGF/TSP-1 in cervical carcinoma remain unclear.

In the present study, the expression levels of DPC4 and VEGF in cervical carcinoma were assessed in order to investigate the association between DPC4, VEGF, and clinicopathological characteristics of patients with cervical carcinoma. Furthermore, the microvessel density (MVD) of each cervical squamous-cell carcinoma (CSCC) sample was detected to investigate the effect of DPC4, VEGF and TSP-1

on angiogenesis. In addition, the correlation between DPC4, VEGF and TSP-1 was investigated in order to elucidate the roles of DPC4 and VEGF in the progression of tumorigenesis, and their possible associations in carcinogenesis.

Materials and methods

Patients. The field study took place at the Second Clinical Hospital of Jilin University (Changchun, China) between August 2012 and August 2013. A total of 115 patients with invasive cervical carcinoma aged between 25 and 60 years (mean age, 46 years) were surgically treated to remove a tumor. All the patients were treated without radiotherapy, chemotherapy or other adjuvant therapy prior to surgery, and did not recently use drugs that affected the metabolism of prostaglandin and thromboxane. The 115 samples were identified by three pathologists independently, and the final diagnosis was confirmed when ≥2 of the same results were obtained from the three pathologists. Lymph node metastasis and classification of tumors were regarded as the criteria. The samples included 19 cervical inflammation, 10 CIN I, 11 CIN II, 14 CIN III and 61 CSCC. Local pathologists were asked to provide complete clinical data of the patients with CSCC. The 61 CSCC samples, which were staged according to the International Federation of Gynecology and Obstetrics (14), were divided into stage I (n=20), stage II (n=25) and stage III (n=16). Pathological grade assessment was also performed, and the CSCC samples were graded into G1 (n=18), G2 (n=26) and G3 (n=17) according to the proportion of differentiated cells (15). A total of 21 lymph node metastasis samples were involved in these CSCC samples. Finally, 12 fresh normal cervix tissue samples, which were acquired from individuals confirmed via necropsy to have succumbed to natural causes (and therefore unassociated with cervical complications) at the Second Clinical Hospital of Jilin University between August 2012 and August 2013, were used as normal controls.

Written informed consent was obtained from all the subjects or their parents. The present study was approved by the Ethics Committee of the Second Clinical Hospital of Jilin University and all study procedures were performed according to ethical standards.

Immunohistochemical analysis. Excess tissue and moisture were removed from the freshly obtained tissue samples. The samples were then dehydrated routinely and embedded in paraffin. Embedded tissues were sliced into 5-μm thick sections, deparaffinized in xylene followed by treatment with 95, 70 and 50% ethanol, and rehydration with PBS (pH 7.4). Paraffin-embedded tissues were used for hematoxylin and eosin staining, and immunohistochemical analysis. Pathological diagnosis and pathological grade were established by two pathologists, respectively. Sections analyzed for protein expression (DPC4, VEGF and TSP-1) were microwaved for 5 min at 98˚C in citrate buffer (pH 6.0) for antigen retrieval. Following these pretreatment procedures, all samples were incubated with a solution of 1% hydrogen peroxide in methanol for 15 min at room temperature to block endogenous peroxidase. Sections were washed three times with PBS (pH 7.5) and then incubated for 10 min at room temperature in 50 μl normal non-immune goat serum (YeSen Biotechnology Co., Ltd., Shanghai, China). The rabbit anti-human primary monoclonal antibodies for DPC4, TSP-1 or VEGF (all at a dilution of 1:100; Shanghai Westang Bio-Tech Co., Ltd., Shanghai, China) were applied to the sections overnight at 4˚C. The next day the sections were rinsed three times in PBS and incubated for 10 min with the addition of goat anti-rabbit peroxidase-conjugated secondary antibody (1:50; Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 1 h at room temperature. Following rinsing three times in PBS, the samples were incubated with 50 μl streptavidin-peroxidase for another 10 min at room temperature. Then, the samples were washed with PBS and visualized with 100 μl stable 3,3'-diaminobenzidine (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 10 min at room temperature. The reaction was stopped by washing with water and the sections were counterstained with Gill No. 3 hematoxylin solution (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Following rinsing in PBS, the slides were dehydrated with 50, 70, 95% and absolute ethanol, and mounted with neutral balsam. The slides were observed using an optical microscope (CH20BIMF2000; Olympus Corporation, Tokyo, Japan). Negative controls involved the same procedure; however, the primary antibody was replaced with PBS. As a positive control, a specimen of CSCC with strong expression of DPC4, VEGF or TSP-1 was used.

Protein expression was determined by two independent observers who semi-quantitatively assessed the staining intensity and the percentage of stained tumor cells. The staining intensity was rated as follows: 0 points, negative; 1 point, weak intensity; 2 points, moderate intensity; and 3 points, strong intensity. The percentage of positive cells was rated as follows: 0 points, 0% positive tumor cells; 1 point, <30% positive cells; 2 points, 30-60% positive cells; and 3 points, >60% positive cells. Points of staining intensity and percentage of positive cells were added, positive expression was regarded as ≥3 points.

ELISA. The protein expression levels of DPC4 and VEGF in different pathological periods of CCSC were detected using an ELISA kit (Wuhan Boster Biological Technology, Ltd.) according to the manufacturer's protocol. The serum samples (100 μl) were obtained by drawing peripheral blood and centrifuging for 10 min (1,760 x g at 4˚C). The substrate used as the enzyme conjugate was tetramethylbenzidine. Optical density values were read at 450 nm. Normal cervix samples were used as controls.

Quantification of MVD. The MVD of CSCC samples were determined according to the Winder method (16). Microvessels were highlighted by staining endothelial cells for Factor VIII (FVIII) with immunohistochemistry. The most vascularized areas (hot spots) with a high density of FVIII-positive cells were picked up using a low power field magnification (x40). The MVD was quantified using a x200 magnification high-power field (x10 ocular and x20 objective; field area; 0.25 mm^2). All positively stained discrete cells or cell clusters with, or without visible lumina were counted as a microvessel. The branch structure of discrete cells, which did not connect with the primary structure, was also counted as a microvessel. However, vessels with a diameter greater than the sum of eight red blood cells, which had an evident muscular layer, were not

counted. A total of three fields of view in each sample were counted and the average was represented as the MVD.

Statistical analysis. Statistical analysis was performed using SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA). All data are expressed as the mean ± standard deviation. Statistical significance of protein expression (DPC4, TSP-1 or VEGF) in the different groups was conducted using the χ^2 test. In addition, paired t-tests were performed to estimate the statistical significance of MVD. Correlation analysis between DPC4 and VEGF/ TSP-1 was conducted by Spearman's correlations. P<0.05 was considered to indicate a statistically significant difference.

Results

Protein expression rates of DPC4 and VEGF detected using immunohistochemistry. The negative expression rates of DPC4 in the normal control and chronic cervicitis groups were 0% (Fig. 1). The rates in the CIN I, CIN II and CIN III groups were 10, 9 and 14.3% respectively, which were all significantly lower compared with that in the CSCC group (40.9%; P<0.05; Table I; Fig. 1). Regarding the expression of VEGF, the positive expression rates in the CIN I (10%), CIN II (18.2%) and CIN III (35.7%) groups were significantly lower compared with that in the CSCC group (73.8%; P<0.05). Furthermore, no VEGF expression was detected in the normal control and chronic cervicitis groups.

Association analysis between DPC4/VEGF and the clinical pathology of CSCC. As presented in Fig. 1A, DPC4 protein was located in the cytoplasm and karyon of normal cervical tissue, and irregularly distributed; however, the protein was located primarily in the cytoplasm of CSCC tissue (Fig. 1B) and was granulated. DPC4 protein staining exhibited claybank spot distribution. The negative expression rate of DPC4 in the stage I group was 20%, significantly lower compared with that in the stage II group (56.3%; P<0.05; Table II). However, no statistical difference was observed in pathological grade. In addition, the DPC4 protein expression was associated with lymph node metastasis (Table II), the negative rate in the lymph node metastasis group (71.4%) was significantly higher compared with that of the lymph node metastasis-negative group (25.0%; P<0.05; Table II).

For VEGF, no expression was detected in normal cervical tissues (Fig. 1E), but the protein was located in the cytoplasm in CSCC (Fig. 1F) with granulated and dispersedly distributed claybank spot staining. The positive expression rate of VEGF in stage I was 60%, which was decreased significantly compared with that in stage II (84%; P<0.05; Table II). However, no statistical difference was observed in pathological grade. Furthermore, the positive rate of VEGF protein expression in the lymph node metastasis group (80.9%) was significantly higher compared with that of the lymph node metastasis-negative group (47.5%; P<0.05; Table II).

Expression levels of DPC4 and VEGF in different clinical stages. As presented in Table III, the protein expression level of DPC4 in stage I, II and III were 39±2.01, 34±2.2, and 50±2.83, respectively, which were significantly lower compared with that in the normal control group (86±4.21; P<0.05). However, for VEGF, the expression level in stage I (90±4.40), stage II (106±4.89) and stage III (70±3.89) increased significantly compared with the normal control group (26±1.83; P<0.05).

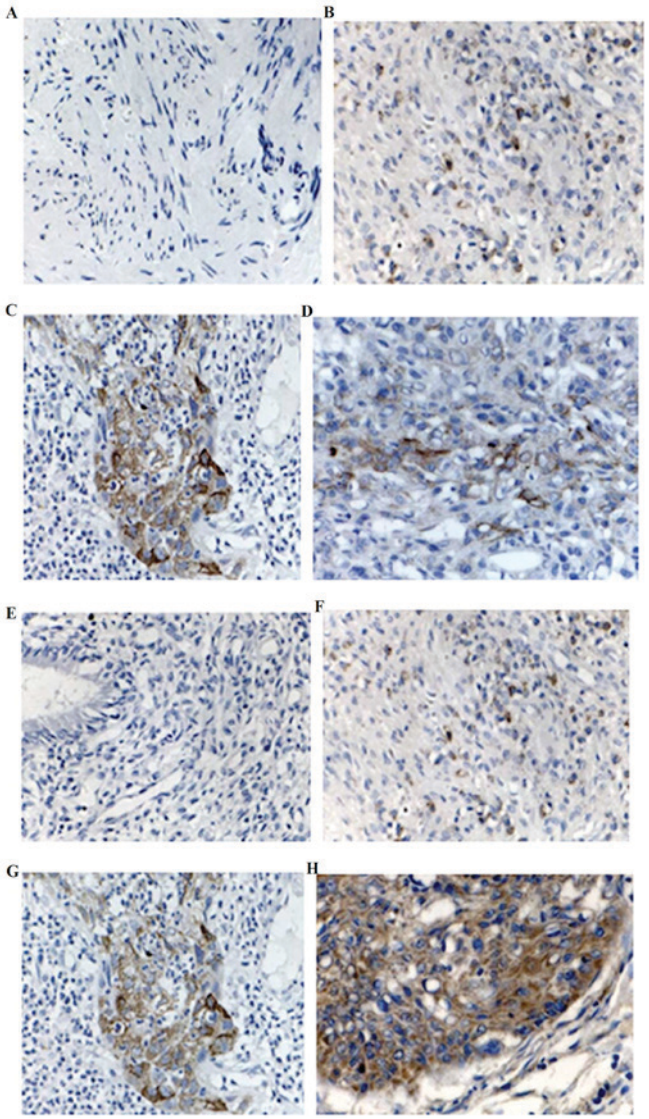

Figure 1. Immunohistochemistry results of DPC4 and VEGF expression levels. The expression of DPC4 in (A) normal cervix (magnification, x200), (B) stage I CSCC, (C) stage II CSCC and (D) stage III CSCC. The expression of VEGF in (E) normal cervix, (F) stage I CSCC, (G) stage II CSCC and (H) stage III CSCC. (B-H) Magnification, x400. CSCC, cervical squamous-cell carcinoma; DPC4, deleted in pancreatic carcinoma locus 4; VEGF, vascular endothelial growth factor.

Association between DPC4, VEGF, TSP-1 and tumor angiogenesis. As presented in Table IV, in CSCC samples, the MVD of the DPC4 (-) group (8.38±3.15) was significantly higher compared with that of the DPC4 (+) group (11.28±3.55; P<0.05). The MVD of the VEGF (-) group (10.51±3.90) was significantly lower compared with the VEGF (+) group (15.37±4.59; P<0.01). In addition, the MVD of the TSP-1 (-) group (15.37±3.10) was significantly higher compared with the TSP-1 (+) group (9.31±2.4; P<0.01).

Statistical analysis demonstrated that the MVD in the DPC4 (-) TSP-1 (+) group (12.74±3.59) was significantly

Table I. Expression of DPC4 and VEGF in cervical tissues of differential pathological types.

		Negative expression of DPC4		Positive expression of VEGF	
Group	No. of cases	No. of cases	Negative rate, %	No. of cases	Positive rate, %
Normal cervix	12	0	0.0[a]	0	0.0[a]
Cervical inflammation	19	0	0.0[a]	0	0.0[a]
CIN I	10	1	10.0	1	10.0[a]
CIN II	11	1	9.0[a]	2	18.2[a]
CIN III	14	2	14.3	5	35.7[a]
CCSC	61	21	40.9	45	73.8[a]

[a]P<0.05, compared with the CSCC. CSCC, cervical squamous-cell carcinoma; DPC4, deleted in pancreatic carcinoma locus 4; VEGF, vascular endothelial growth factor; CIN, cervical intraepithelial neoplasia.

Table II. Expression of DPC4 and VEGF in CSCC samples classified according to different clinicopathological factors.

		Negative expression of DPC4		Positive expression of VEGF	
Clinicopathological factor	No. of cases	No. of cases	Negative rate, %	No. of cases	Positive rate, %
Stage					
I	20	4	20.0	11	60.0[b]
II	25	7	48.0	22	84.0
III	16	8	56.3[a]	12	75.0
Grade					
G1	18	5	22.2	13	72.2
G2	26	10	38.5	19	73.1
G3	17	10	64.7	13	76.5
Lymph node metastasis					
R	21	15	71.4[c]	17	80.9[c]
F	40	10	25.0	19	47.5

[a]P<0.05, compared with stage I; [b]P<0.05, compared with stage II; [c]P<0.05, compared with F. R, CSCC samples with lymph node metastasis; F, CSCC without lymph node metastasis; CSCC, cervical squamous-cell carcinoma; DPC4, deleted in pancreatic carcinoma locus 4; VEGF, vascular endothelial growth factor.

Table III. Expression of DPC4 and VEGF in different stage of cervical squamous-cell carcinoma.

Expression	Normal cervix	Stage I	Stage II	Stage III
DPC4	86±4.21	39±2.01[a,b]	34±2.20[a]	50±2.83[a]
VEGF	26±1.83	90±4.40[a,b]	106±4.89[a]	70±3.89[a]

[a]P<0.05, compared with the normal cervix; [b]P<0.05, compared with the stage III. DPC4, deleted in pancreatic carcinoma locus 4; VEGF, vascular endothelial growth factor. All data are presented as the mean ± standard deviation.

increased compared with the DPC4 (+) TSP-1 (-) group (11.49±3.89), and significantly decreased compared with the DPC4 (+) TSP-1 (+) group (19.38±3.35; P<0.01). The MVD of DPC4 (-) VEGF (+) group (18.38±3.25) was significantly higher compared with the DPC4 (+) VEGF (+) group (11.74±3.89) and the DPC4 (+) VEGF (-) group (10.39±3.89; P<0.01).

A negative correlation was identified between the expression of DPC4 (r=-0.762) and TSP-1 (r=-0.578), and tumor angiogenesis (both P<0.01; Table IV). However, a positive correlation between VEGF expression and tumor angiogenesis was reported (r=0.478; P<0.01; Table IV).

Correlation between DPC4, VEGF and TSP-1. As presented in Table V, the expression of DPC4 was identified to be negatively correlated with VEGF (r=-0.486; P<0.01) and TSP-1 (r=-0.480; P<0.01).

Discussion

Cervical carcinoma is a common type of gynecologic cancer caused by various factors. However, the mechanism underlying tumorigenesis remains to be elucidated. Loss of DPC4 and

Table IV. Mean MVD of cervical squamous-cell carcinoma samples with positive/negative expression of DPC4, VEGF or TSP-1.

Expression	No. of cases	MVD (mean ± SD)	[d]R
DPC4			-0.76
Negative	22	18.38±3.15	
Positive	39	11.28±3.55[a]	
VEGF			0.48
Negative	18	10.51±3.90	
Positive	43	15.37±4.59[a]	
TSP-1			-0.58
Negative	16	15.37±3.10	
Positive	45	9.31±2.40[a]	
DPC4 and TSP-1			
DPC4 (+) TSP-1 (-)	16	11.49±3.89[b]	
DPC4 (-) TSP-1 (+)	24	12.74±3.59	
DPC4 (+) TSP-1 (+)	21	19.34±3.55[b]	
DPC4 and VEGF			
DPC4 (+) VEGF (-)	18	10.4±3.89[c]	
DPC4 (-) VEGF (+)	22	18.4±3.25	
DPC4 (+) VEGF (+)	21	11.74±3.89[c]	

[a]P<0.001, compared with the negative expression group; [b]P<0.001, compared with the DPC4 (-) TSP-1 (+) group; [c]P<0.001, compared with the DPC4 (-) VEGF (+) group; [d]R values were only calculated for the correlations between DPC4, VEGF and TSP-1 with MVD. DPC4, deleted in pancreatic carcinoma locus 4; VEGF, vascular endothelial growth factor; MVD, microvessel density; TSP-1, thrombospondin-1; SD, standard deviation.

Table V. Correlation between DPC4 and VEGF or TSP-1.

	VEGF				TSP-1			
DPC4	+	-	R	P-value	+	-	R	P-value
+	21	18	-0.49	<0.01	21	16	0.48	<0.01
-	22	0			24	0		

DPC4, deleted in pancreatic carcinoma locus 4; VEGF, vascular endothelial growth factor; TSP-1, thrombospondin-1.

overexpression of VEGF have been reported to be associated with cervical carcinoma carcinogenesis (17,18). Thus, DPC4 and VEGF may be candidate molecules that contribute to the progression of cervical carcinoma. In the present study, it was demonstrated that the negative expression rate of DPC4 and positive expression rate of VEGF in the CSCC group were increased significantly compared with that in the normal, inflammatory, and CIN groups (P<0.05). In addition, DPC4 (-) and VEGF (+) were associated with clinical stages, and lymph node metastasis. Furthermore, the expression levels of DPC4 and TSP-1 were identified to be negatively associated with angiogenesis. However, the expression of VEGF and angiogenesis were positively associated (P<0.01). The expression of DPC4 was negatively associated with VEGF and with TSP-1 (P<0.01).

DPC4 is a tumor suppressor gene frequently inactivated in various types of carcinoma, including cervical carcinoma (19-22). Reduced or lost expression of DPC4 is frequently observed during cancer progression (13). In the present study, it was revealed that DPC4 was negatively expressed in CIN and CSCC tissues, and the negative expression rate increased gradually along with the progression of cervical carcinoma, corresponding with a study by Baldus *et al* (17). The results of the present study suggest that DPC4 is associated with the tumorigenesis of cervical carcinoma. Furthermore, previous evidence has reported that DPC4 alterations are associated with the specific loss of TGF-β-induced growth resulting in increased angiogenesis (23,24). In the present study, the results demonstrated that the negative expression of DPC4 was associated with angiogenesis. In addition, it has been reported that DPC4 restoration can inhibit angiogenesis in different carcinoma types, including colon and pancreatic (25), lung (26) and pancreatic (4) cancer. These results indicate that the loss of DPC4 may contribute to cervical carcinoma progression via angiogenesis.

VEGF is one of the most important angiogenesis factors in regulating angiogenesis. Its expression is associated with MVD (27) and a high expression level has been detected in numerous human tumor types, including lung (28), colon (29), and gastric (30) cancer. In the present study, a high positive expression rate and high level of VEGF were detected in cervical carcinoma. Furthermore, these results revealed that the expression of VEGF was positively associated with angiogenesis. As angiogenesis serves an important role in the progression of tumorigenesis, VEGF may participate in the tumorigenesis of cervical carcinoma by inducing angiogenesis.

As a potent inhibitor of neovascularization, TSP-1 also serves a key role in regulating angiogenesis. Reduced expression of TSP-1 can switch normal cells to an angiogenic phenotype, thus progressing cells towards malignancy (31). Overexpression of TSP-1 can reduce MVD and inhibit the growth of prostate cancer cells (32). The results of the present study reported that the MVD in the TSP-1 (-) group was significantly higher compared with that of the TSP-1 (+) group, indicating that angiogenesis of cervical carcinoma may be suppressed by TSP-1. Consistent with these results, a previous study demonstrated that microvessel counts are significantly higher when decreased TSP-1 mRNA expression is evident in cervical carcinoma (11). Therefore, TSP-1 may inhibit the angiogenesis of cervical carcinoma.

Due to the observations of decreased DPC4 expression and VEGF overexpression in the progression of angiogenesis, the correlation between DPC4, and VEGF was analyzed. The results demonstrated that they were associated. Correspondingly, it has been reported that carcinomas with high DPC4 expression are accompanied with low VEGF expression (26). Increased expression of DPC4 results in decreased expression levels of VEGF, shifting cells from an angiogenic to antiangiogenic phenotype in various cancer types, including pancreatic (4), gastrointestinal (19) and lung carcinoma (10). Furthermore, the loss of DPC4 has been demonstrated to enhance VEGF protein expression in human ovarian cancer cells (13). DPC4 expression is negatively associated with VEGF-C expression in colon cancer (33). The results of the aforementioned studies are all consistent with the results of the present study. Therefore, the loss of DPC4 may induce VEGF expression, increasing angiogenesis and consequently promoting the progression of cervical carcinoma. Although it is not yet clear how DPC4 modulates VEGF expression, DPC4 may be a candidate target for inhibiting cancer progression.

The association between DPC4 and TSP-1 was also investigated as TSP-1 is involved in angiogenesis, and may be a target gene of DPC4. The results demonstrated that the expression of DPC4 and TSP-1 was negatively associated, which suggested that the loss of DPC4 may induce the expression of TSP-1. Although reduced DPC4 expression and high TSP-1 expression are common in various cancer types (34-37), few studies have investigated the association between DPC4 and TSP-1 in tumorous tissue. Furthermore, contrary to the results of the present study, Schwarte-Waldhoff *et al* (4) indicated that DPC4 restoration may increase the expression of TSP-1 in human pancreatic adenocarcinoma cells. The correlation between DPC4 and TSP-1 in cervical carcinoma remains to be elucidated with further investigations required.

In conclusion, the loss of DPC4 and overexpression of VEGF may play important roles in the cervical carcinoma progression. The conceivable mechanism may involve the loss of DPC4, which induces angiogenesis by increasing VEGF expression, subsequently promoting the progression of cervical carcinoma. The results of the present study suggest that VEGF is a target gene regulated by DPC4. Furthermore, the negative expression of TSP-1 contributes to angiogenesis, and the correlation between DPC4 and TSP-1 requires further investigation.

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