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P-cadherin promotes cervical cancer growth and invasion through affecting the expression of E-cadherin and p120 catenin

Baohua Li, Danzhu Gong, Fenfen Wang, Xiaoyun Wan

Department of Gynecologic Oncology, Women's Hospital, School of Medicine, Zhejiang University, Hangzhou (China)

Summary

Objective: The aim of the present study was to investigate the role of P-cadherin in cervical carcinogenesis and cancer progression. *Materials and Methods:* The expression of placental cadherin (P-cadherin) protein was examined by Western blot in different cervical samples and cervical cell lines. Through regulating P-cadherin expression in Siha or 293T cells, cell growth, migration, and invasion were examined separately using a cell-counting kit-8 (CCK-8) or Transwell assays, and meanwhile the expression of cadherin/catenin complex was tested using Western blot. *Results:* Western blot showed that P-cadherin was overexpressed in cervical cancer tissues and cancer-derived cell lines compared with normal cervical tissues. Silencing P-cadherin expression resulted in increase of epithelial cadherin (E-cadherin) and decrease of p120 catenin (p120ctn) expression, which was accompanied by inhibiting cell growth and reducing cell migration and invasion. Conversely, overexpression of P-cadherin led to downregulation of E-cadherin and upregulation of p120ctn expression, which was accompanied by promoting cell growth and increasing cell migration and invasion. *Conclusions:* P-cadherin could promote cervical cancer growth and invasion, at least partly through affecting the expression of E-cadherin and p120 catenin, consequently contributing to cervical carcinogenesis and cancer progression.

Key words: P-cadherin; E-cadherin; p120ctn; Metastasis; Cervical cancer.

Introduction

Cervical cancer is one of the most common female malignant tumors in the world, and is still one of the leading causes of cancer-related deaths in less developed countries including China [1]. Persistent infection of high-risk human papillomavirus (HR-HPV) has been regard as key factor in cervical carcinogenesis [2]. In recent years, due to the widespread application for cervical screening programs, the majority of cervical cancer patient was detected at an early-stage and usually had a relative favorable prognosis. However, those patients with local invasion and/or distant lymph node metastasis indeed presented a poorer survival [3, 4]. Hence, understanding the molecular details of cervical carcinogenesis and cancer progression and identifying the signaling pathways involved in them would likely contribute to new therapeutic interventions.

Placental cadherin (P-cadherin) is a member of the classic cadherins family and its encoding gene CDH3 is located in the human chromosome 16q22.1. P-cadherin expression was firstly observed in the placenta tissues, both in the embryonic and maternal regions, hence named as the denomination of placental-cadherin. In normal physiological conditions, P-cadherin is involved in the formation of the specific areas of epithelia and maintenance of the tissues homeostasis [5]. Moreover, the role and function of P-cadherin in normal development is also well-determined by gene knockouts in mice. Radice *et al.* reported that loss of function of P-cadherin in mouse embryogenesis is not lethal, but is associated to development defects of the breast [6]. Recently, the relationship between P-cadherin expression and human malignancies has been extensively studied. Mounting evidence has demonstrated that the changes in the expression or function of P-cadherin could cause abnormal adherens junctions, consequently contributing to initiation and progression of tumor cells [7]. Furthermore, aberrant expression of P-cadherin has been reported to be associated with a poorer clinical outcome and prognosis in several carcinomas, such as bladder carcinoma [8] and invasive breast carcinoma [9]. However, the role of P-cadherin in cervical cancer has not been explored.

In the present study, the authors examined the level of P-cadherin expression in 30 cervical cancer samples and in 30 normal cervical tissues, as well as five cervical cell lines. Furthermore, they modulated separately the expression of P-cadherin in human renal epithelial cell line 293T and human cervical cancer cell line Siha, and subsequently investigated the effect of P-cadherin change on cell growth, migration, and invasion, as well as expression of cadherin/catenin complex. The aim of this study was to explore the role of P-cadherin expression in cervical carcinogenesis and cancer progression.

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Materials and Methods

The use of all tissues was approved by the hospital research ethics committee. An informed consent was obtained from all the patients. A total of 30 cervical cancer tissues were collected, which were derived from the patients undergoing radical hysterectomy with pelvic lymph node dissection and not receiving any anti-cancer therapy prior to their surgery in cervical lesion clinic of Women's Hospital, School of Medicine, Zhejiang University from January 2013 to December 2013. Additionally, 30 normal cervical tissues were used as controls, which were derived from the patients undergoing hysterectomy because of benign gynecologic diseases. All collected tissues were immediately snap frozen in liquid nitrogen after surgical removal and stored at -70°C until used.

The human normal epithelial cell line Hacat and the human renal epithelial cell line 293T were cultured separately in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum. Three cervical cancer cell lines Siha, Caski, and Hela were cultured separately in RPMI 1640 medium with 10% fetal bovine serum. All cells were grown at 37°C in a humidified incubator with 5% CO2.

SiRNA against P-cadherin was designed depending on literature [10], and was chemically synthesized. Furthermore, siRNA was transfected into the cervical cancer cell lines Siha using lipofectamine according to the manufacturer's instruction. The cells transfected with a scrambled oligonucleotides siRNA were used as negative control. The inhibition rate of P-cadherin was identified separately by qRT-PCR and Western blot analyses.

Recombinant expressed vector containing P-cadherin gene was constructed, and was transfected into the human renal epithelial cell line 293T using lipofectamine according to the manufacturer's instruction. The cells transfected with empty vector served as negative control. The exogenous expression of P-cadherin was confirmed by qRT-PCR and Western blot analyses.

The cDNAs were synthesized using an RNA reverse transcription amplification kit. Then, qRT-PCR for mRNA was performed using SYBER green detection. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. All primers for qRT-PCR were listed: for P-cadherin, forward 5'-AGTGGAGGACCCCATGAACA-3', antisense5'-TTGGGCTTGTGGTCATTCTG-3'; for GAPDH, forward 5'-GACAGTCAGCCGCATCTTCT-3', antisense 5'-TTAAAAGCA GCCCTGGTGAC-3'. Relative quantification of the mRNA expression was calculated with the 2-ΔΔCT method.

The proteins from cervical samples, cervical cell lines or transfected Siha and 293T cells, were harvested. Then, Western blot was performed as described previously [11]. Briefly, the membranes were incubated with primary antibodies against P-cadherin (diluted 1:500, epithelial cadherin (E-cadherin) (diluted 1:1000), beta-catenin (β-catenin) (diluted 1:500) and p120 catenin (diluted 1:1000). GAPDH (diluted 1:1000) was used as an internal reference for western blot analysis.

Siha cells (6×10^3cells/well) transfected with P-cadherin siRNA or scrambled oligonucleotides siRNA, and 293T cells (1×10^4cells/well) transfected with P-cadherin expressed vector or empty vector, were cultured separately in 96-well plates overnight. At 24, 48, 72, and 96 hours post-transfection, CCK-8 solution was added. After one hour of incubation at 37°C in a humidified incubator with 5% CO2, the absorbance of samples was recorded at 450nm with a multifunction reader. The data were summarized based on three independent experiments.

Siha cells (1×10^5cells/well) transfected with P-cadherin siRNA or scrambled oligonucleotides siRNA, and 293T cells (2×10^5cells/well) transfected with P-cadherin expressed vector or empty vector, were suspended in 200 ul serum-free medium and seeded into the upper chamber of a Transwell insert. The lower chamber was filled with the optimal medium with 10% fetal bovine serum. After 24 hours, the cells on the upper surface of membrane were wiped off, while the cells on the lower surface of membrane were fixed and stained with crystal violet, and counted in four randomly selected microscopic fields using an inverted microscope. Then, to assess the invasive capability of transfected cells, the same protocol was used except that inserts were pre-coated with Matrigel. The data were summarized based on three independent experiments.

All statistical analysis was performed using SPSS 16.0 software. Data were presented as the mean ± standard deviation (SD) from at least three independent experiments. All statistical tests were two-sided, and p values less than 0.05 were considered to be statistically significant (* represents < 0.05; ** represents < 0.01).

Results

To evaluate the level of P-cadherin expression in vivo and vitro, the authors firstly collected a series of cervical samples, including 30 cervical cancer tissues and 30 normal cervical tissues, and subsequently examined the level of P-cadherin protein using Western blot analysis. As shown in Figure 1, the results showed that P-cadherin expression was significantly elevated in cervical cancer tissues with average value of 3.8 in protein level, compared with that of the normal cervical tissues as 1.0 ($p = 4.69E^{-9}$). Moreover, among 30 cervical cancer cases, lymph node metastasis (LNM) was found in 15 of 30 cases. Furthermore, the authors compared to the expression of P-cadherin in cervical cancer tissues with (n=15) or without (n=15) LNM and found that P-cadherin expression exhibited a statistical increase in those with lymph node metastasis ($p = 0.028$).

The authors also separately examined the expression of P-cadherin mRNA and protein using qRT-PCR and Western blot assays in human normal cell lines 293T and Hacat, and three cervical cancer-derived cell lines, including Siha, Caski, and Hela. As shown in Figure 2, both qRT-PCR and Western blot analyses showed that P-cadherin expression was significantly higher in all cervical cancer-derived cell lines than that in normal cell lines. These results altogether suggest that P-cadherin is significantly overexpressed in cervical cancer tissues and cancer-derived cell lines, implying P-cadherin might contribute to cervical carcinogenesis and cancer progression as a tumor oncogene.

To identify the effect of aberrantly expressed P-cadherin on cell growth, migration and invasion in cervical cell lines, the authors firstly reduced P-cadherin expression using a specific P-cadherin siRNA in Siha cell that exhibits originally high expression of P-cadherin. As shown in Figure 3A, the siRNA resulted in the significant reduction of P-cadherin expression of Siha cells in mRNA and protein levels at 48 hours post-transfection. Furthermore, the authors examined cellular proliferation rate using CCK-8 assay in Siha cells that were transferred with P-cadherin siRNA or

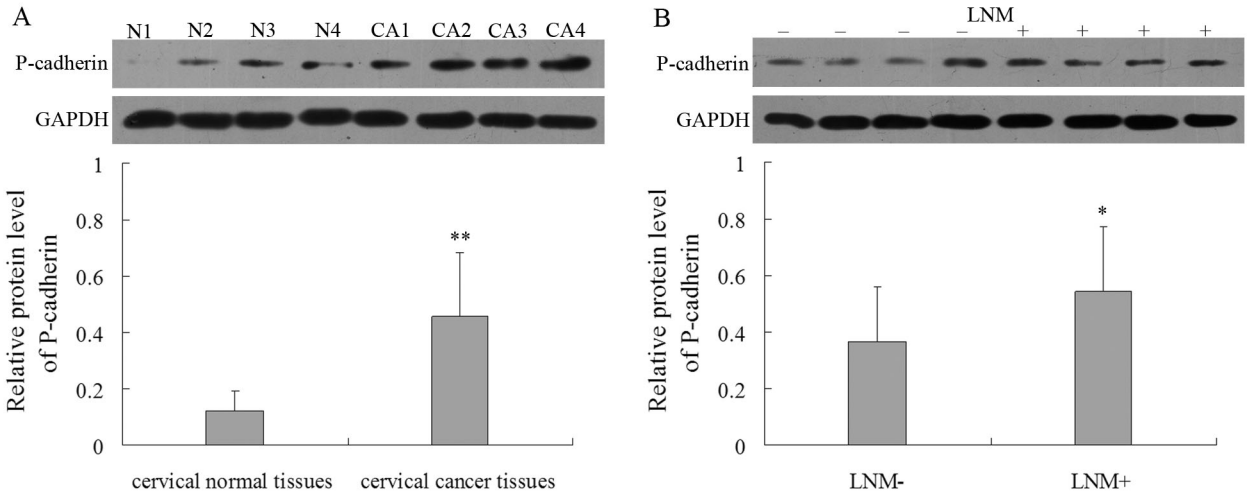

Figure 1. — The relative expression of P-cadherin protein in cervical cancer tissues. (A) Western blot analysis shows that the protein level of P-cadherin is significantly elevated in cervical cancer tissues (CA) compared with that of normal cervical tissues (N). (B) The protein level of P-cadherin is significantly increased in cervical cancer tissues with lymph node metastasis (LNM) compared with that without LNM using Western blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as an internal reference. Each lane is a representative sample of each group.

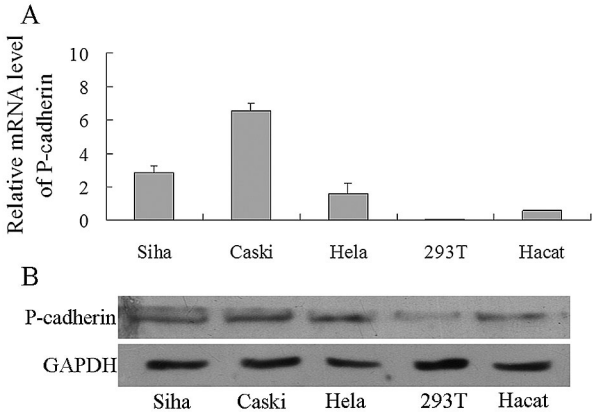

Figure 2. — The relative expressions of P-cadherin mRNA and protein in different cervical cell lines. Both qRT-PCR (A) and Western blot (B) analyses show that P-cadherin expression is significantly higher in all cervical cancer-derived cell lines than that in normal cell lines.

negative control at 24, 48, 72, and 96 hours. The results showed that reduced expression of P-cadherin significantly suppressed cellular growth at 72 hours ($p = 0.024$) and 96 hours ($p = 0.004$), not at 24 and 48 hours post-transfection (Figure 4A). Furthermore, cellular migratory and invasive capability of P-cadherin siRNA transferred Siha was examined separately using Transwell assays. At 24 hours post-transfection, reduction of P-cadherin expression using P-cadherin siRNA dramatically impaired the migratory ($p = 0.01$) and invasive ($p = 0.002$) capability of Siha cells compared to negative control (Figure 5). Conversely, the authors also constructed a recombinant expressed vector containing P-cadherin gene, and subsequently transfected into 293T cells that exhibits originally low expression of P-cadherin. At 48 hours post-transfection, both qRT-PCR and Western blot analyses determined the overexpression of P-cadherin in 293T cells (Figure 3B). Furthermore, the results of CCK-8 assay showed that exogenous overexpression of P-cadherin promoted cellular proliferation ($p = 0.038$) at 96 hours post-transfection (Figure 4B). More importantly, exogenous P-cadherin overexpression statistically resulted in increased cell migration ($p = 0.001$) and invasion ($p = 0.004$) in P-cadherin expressed vector transferred 293T cells compared to negative control at 24 hours post-transfection (Figure 5). The present results suggest that alteration of P-cadherin expression could affect cellular proliferation, migration, and invasion in vitro cervical cell lines.

To identify the effect of P-cadherin alteration on expression of cadherin/catenin complex in cervical cell lines, the authors firstly reduced the expression of P-cadherin using P-cadherin siRNA and subsequently used Western blot assay to examine the level of cadherin/catenin complex that included epithelial cadherin (E-cadherin), beta-catenin (β-catenin), and p120 catenin (p120ctn) expression. Western blot analysis demonstrated that reduction of P-cadherin expression resulted in increased expression of E-cadherin ($p = 0.015$) and decreased expression of p120ctn ($p = 0.039$), and no change of β-catenin expression ($p = 0.717$) (Figure 6A). Conversely, the authors also tested the expression of E-cadherin, β-catenin, and p120ctn in P-cadherin overexpression 293T cells and control cells. The results showed that exogenous overexpression of P-cadherin significantly

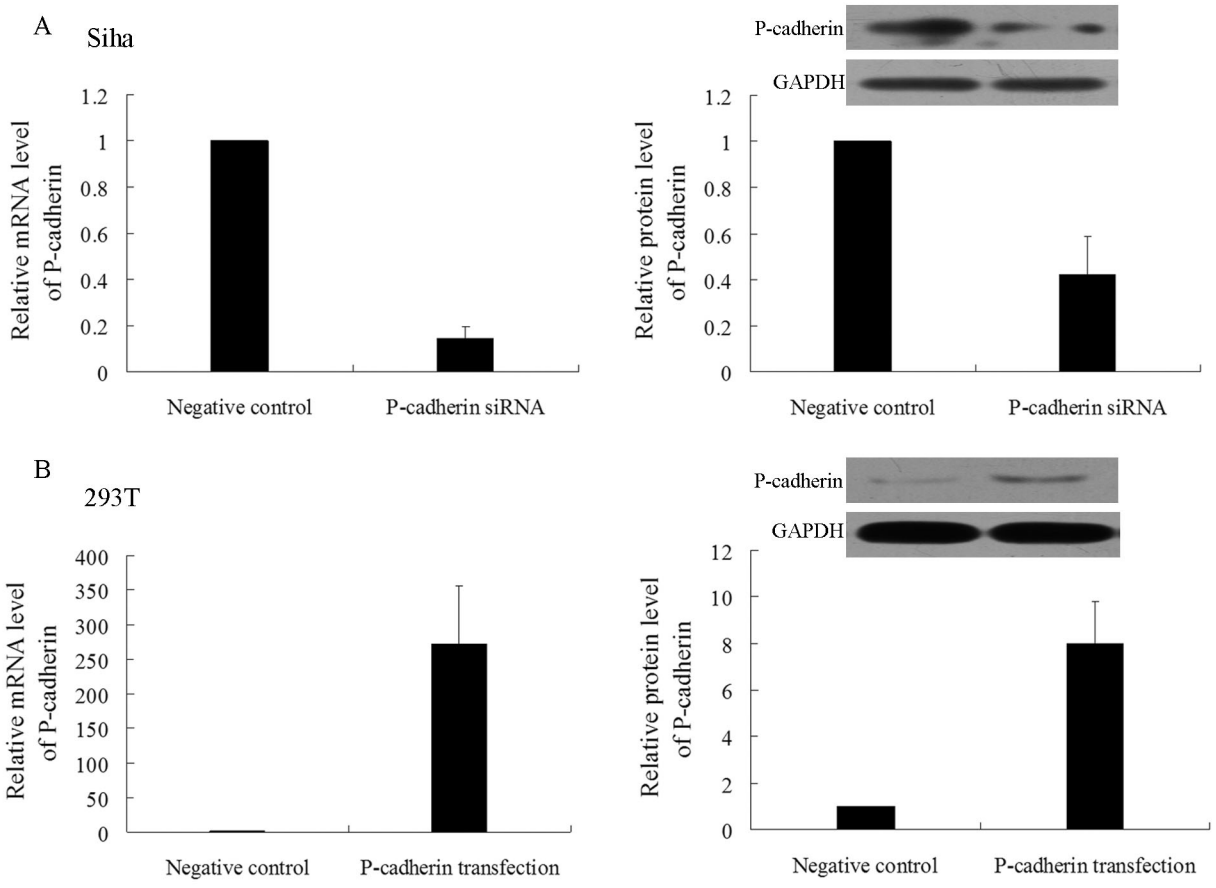

Figure 3. — The modulation of P-cadherin expression in cervical cell lines using P-cadherin siRNA or expressed vector. (A) The interference effects are determined by qRT-PCR and Western blot analyses in Siha cells transfected with P-cadherin siRNA. (B) The results from qRT-PCR and Western blot analyses demonstrate the exogenous overexpression of P-cadherin in 293T cells transfected with P-cadherin expressed vector. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as an internal reference. Data are presented as the mean ± SD from three independent experiments.

inhibited E-cadherin expression ($p = 1.82E^{-5}$) and increased p120ctn expression ($p = 0.011$), but had no effect on β-catenin expression ($p = 0.962$) (Figure 6B). The present finding suggests that the alteration of P-cadherin expression could affect the expression of E-cadherin and p120ctn proteins in vitro cervical cell lines.

Discussion

Local invasion and distant lymph node metastasis are the key processes of cervical cancer progression. The acquisition of this invasive and metastatic behavior requires different cellular events including cytoskeletal alteration, disruption of cell-cell adhesion, matrix protein proteolysis, and migration. Among them, disruption of cell-cell adhesion is the basis for migration, invasion, and metastasis of tumor cells.

P-cadherin is a calcium-dependent cell-cell adhesion molecule. Its protein is comprised of an extracellular N-terminal domain, a transmembrane domain and a highly conserved cytoplasmic carboxy-terminal domain. The previous studies demonstrated that cytoplasmic domain of P-cadherin could bind directly to the catenin family members (α-, β-, γ-, and p120-catenin), which link them with the actin cytoskeleton, eventually providing the molecular basis for adherens junctions. In general, P-cadherin plays a major role in cell-cell adhesion, and has an important effect on cell growth, differentiation, motility, and survival [12]. However, the change of P-cadherin expression or function has been reported to cause the disruption of cell-cell adhesion and contribute to tumor initiation and development.

Up to date, accumulated evidence has demonstrated that P-cadherin is aberrantly expressed in many types of tumors. However, it is contradictory that P-cadherin serves as a tumor suppressor gene or oncogene in different cancer cells. Several preclinical and clinical studies have shown that P-cadherin upregulation was frequently detected in

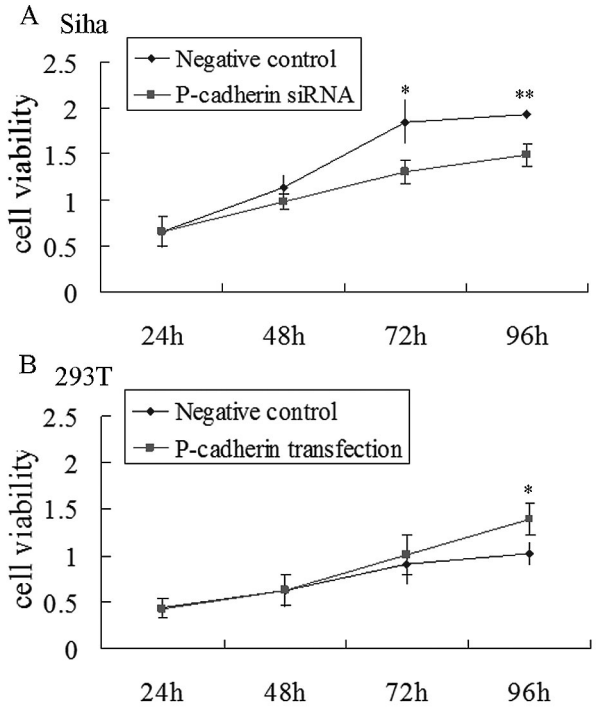

Figure 4. — The modulation of P-cadherin expression affects the growth of cervical cell lines. (A) Siha cells treated by P-cadherin siRNA result in a significantly decreased rate of cell growth compared to negative control at 72 and 96 hours post-transfection. (B) The exogenous overexpression of P-cadherin in 293T cells transfected with P-cadherin expressed vector promote cell growth compared to negative control at 96 hours post-transfection. Data are presented as the mean ± SD from three independent experiments.

some malignant tumors, including breast [13], gastric [14], colorectal [15], and pancreatic carcinomas [16], and contributes to more invasive phenotype of tumor and unfavorable patient prognosis. In contrast, reduced expression of P-cadherin was observed in progressive melanomas [17], oral squamous cell carcinoma [18], and Merkel cell carcinoma [19], and is correlated with increased cell invasion and poor patient survival. Thus, the role and mechanisms of P-cadherin in different tumors needs further investigation to confirm and identify P-cadherin function. In the current authors previous study, they reported that upregulated expression of P-cadherin was significantly associated with clinical parameters indicating poor prognosis and shorter patient survival in cervical squamous cell carcinoma. However, it is still unclear whether aberrant P-cadherin expression could contribute to cervical carcinogenesis and cancer progression [20]. Recently, Paredes *et al.* set up breast cancer cell lines with wild-type E-cadherin, and subsequently upregulated the expression of P-cadherin using P-cadherin expressed vector. The results showed that P-cadherin overexpression promoted tumor cell invasion, motility and migration by inducing the secretion of matrix metalloproteases (MMPs), specifically MMP-1 and MMP-2 [21]. Furthermore, the authors found that E- and P-cadherin co-expressed in breast cancer cell lines significantly promoted tumor cell migration and survival in vitro assay, and enhanced in vivo tumor growth, compared with those cells expressing only E- or only P-cadherin [22]. Hence, they provided a hypothesis that P-cadherin is able to induce invasion only in cell systems that already express an endogenous and functional E-cadherin by disrupting the interaction between E-cadherin and catenins. Contrarily, P-cadheirn is able to suppress invasion in the absence of E-cadherin expression by its strong interaction with catenins [12].

In normal cervical tissues, the expression of P-cadherin protein is mainly detected in the basal layer of uterine cervix, and plays a key role in the maintenance of the epithelial phenotype and the migration of basal cells to the intermediate and superficial layers of epidermis. The previous studies have demonstrated that functional expression of E-cadherin has been found in cervical cancer tissues and cervical cancer cell lines, and is associated with invasive and migratory potential of tumor [23, 24]. However, the role and function of P-cadherin in cervical carcinogenesis and cancer progression were unclear. According to Paredes *et al.*'s opinion [25], the present authors speculated that P-cadherin might be as a tumor oncogene in cervical cancer because of the existence of functional E-cadherin. To elucidate the hypothesis, they firstly examined the expression of P-cadherin in different cervical samples, as well as different cervical cell lines. The results showed that P-cadherin expression was significantly upregulated not only in cervical cancer tissues, but also in cervical cancer-derived cell lines, compared with normal cervical tissues and normal cell lines. More importantly, they found that expression of P-cadherin was remarkable increased in cervical cancer tissues with LNM compared with that without LNM. Furthermore, they downregulated expression of P-cadherin in Siha cell using P-cadherin siRNA or upregulated expression of P-cadherin in 293T cell using P-cadheirn expressed vector in vitro. The present findings showed that reduction of P-cadherin expression inhibited cell growth and decreased cell migration and invasion in Siha cell; in contrast, exogenous overexpression of P-cadherin in 293T cell indeed promoted cell proliferation and increased cell migratory and invasive capability. These data together suggest that P-cadherin could be involved in cervical carcinogenesis and cancer progression as a tumor oncogene.

Presently, there are several mechanisms by which P-cadherin could regulate initiation and development of tumor cells. Previous studies found that P-cadherin was correlated with destabilization of the normal cadherin/catenin complex and subsequently affected cell migration and invasion, depending on the aberrant activation of signaling pathways.

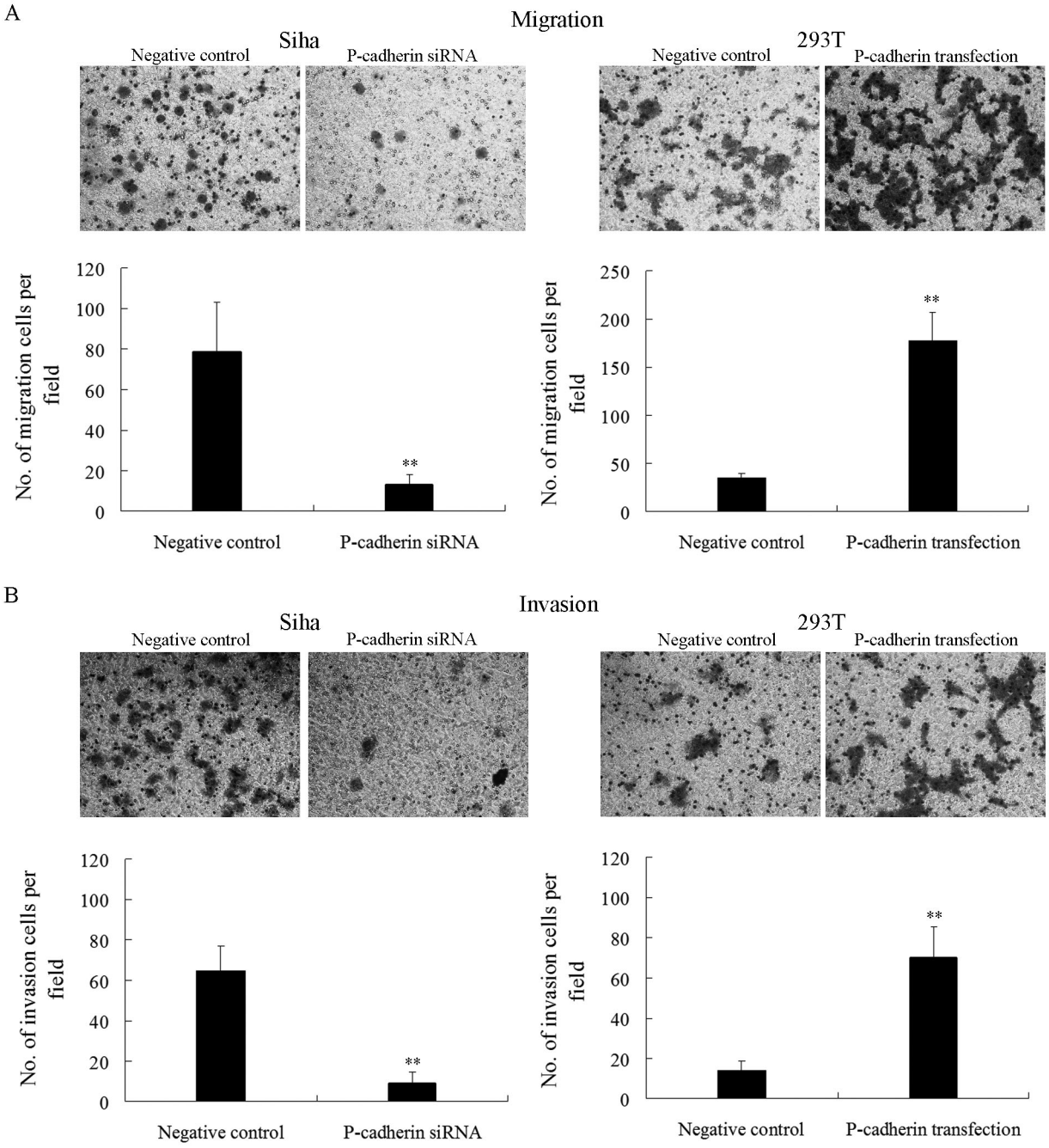

Figure 5. — The modulation of P-cadherin expression affects the migratory and invasive capability of cervical cell lines. According to Transwell migration and invasion assay, reduction of P-cadherin expression by P-cadherin siRNA results in a decreased cell migratory and invasive capability in Siha cells, whereas exogenous overexpression of P-cadherin by P-cadherin expressed vector presents an increased cell migratory and invasive capability in 293T cells, at 24 hours post-transfection compared to negative control. Data are presented as the mean ± SD from three independent experiments. Magnifications: ×200.

Sun *et al.* found that inhibition of P-cadherin expression induced the up-regulation of E-cadherin and the downregulation of β-catenin that promoted colon cancer metastasis to the liver [10]. Cheung *et al.* also reported that P-cadherin could cooperate with insulin-like growth factor-1 receptor to promote ovarian cancer metastasis via P120ctn [26]. The further study showed that breast carcinomas with co-express E- and P-cadherin were associated with p120ctn cytoplasmic localization and poor patient survival, probably because P-cadherin interferes with the normal binding of

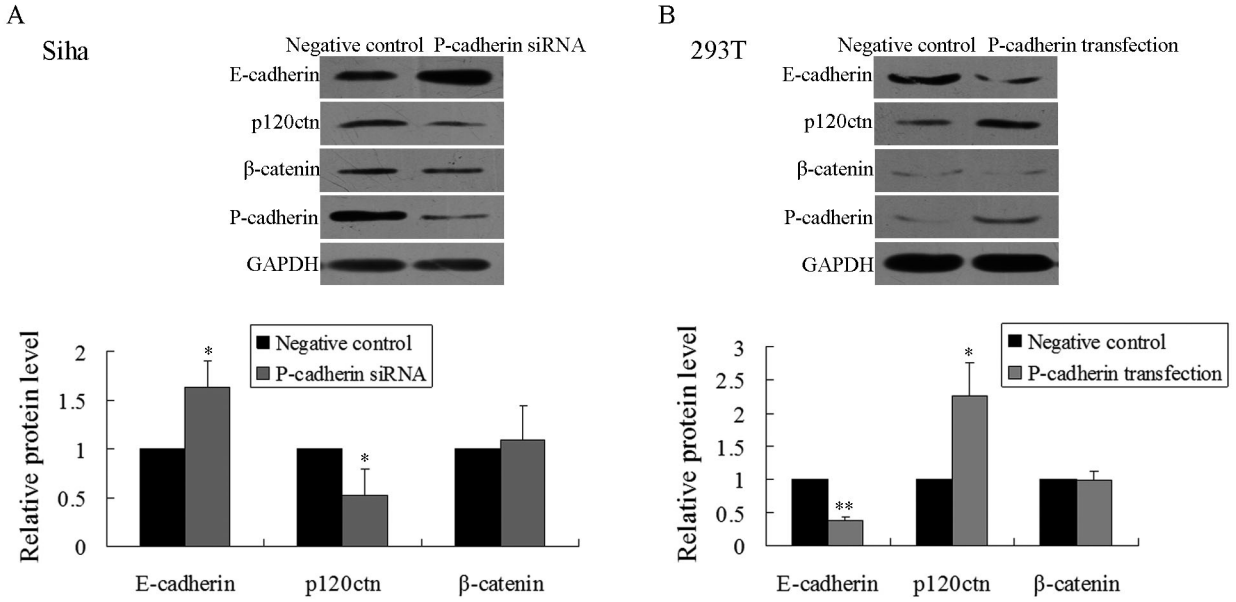

Figure 6. — The modulation of P-cadherin expression affects the expression of E-cadherin and p120ctn protein in cervical cell lines. Siha or 293T cells are transfected separately with P-cadherin siRNA, expressed vector and negative control (NC). At 48 hours post-transfection, the protein expressions of cadherin/catenin complex, including E-cadherin, β-catenin, and p120ctn, are examined by Western blot assay. The results show that downregulation of P-cadherin expression in Siha cells (A) result in an increased expression of E-cadherin and decreased expression of p120ctn; conversely, exogenous overexpression of P-cadherin in 293T cells (B) significantly inhibit E-cadherin expression and upregulated p120ctn expression, compared to negative control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as an internal reference. Data are presented as the mean ± SD from three independent experiments.

p120ctn to E-cadherin [26]. Additionally, it has been shown that the regulatory role of P-cadherin in cell migration is related with the organisation of the non-muscle myosin II-B isoform [27]. Up to date, little is known regarding the regulatory mechanisms of aberrantly expressed P-cadherin in cervical carcinogenesis and cancer progression. In the present study, the authors focused on the cadherin/catenin complex, including E-cadherin, β-catenin, and p120ctn, and determined whether these molecules could be affected by P-cadherin, so as to be involved in cervical carcinogenesis and cancer progression. The present authors firstly modulated P-cadherin expression in vitro cervical cell lines and further examined the expression of cadherin/catenin complex using Western blot assay. The present results showed that reduction of P-cadherin expression could upregulate the level of E-cadherin expression and downregulate the level of p120ctn, but not that of β-catenin expression; contrarily, exogenous overexpression of P-cadherin indeed could lead to the decrease of E-cadherin level and increase of p120ctn level, but no change of β-catenin expression. E-cadherin, as a tumor invasion suppressor gene, has been extensively reported to be involved in cervical cancer cell invasion and metastasis [28, 29]. Furthermore, some studies have demonstrated that in tumor cells that had decreased E-cadherin expression, p120ctn could promote cell invasion and anchorage-independent growth [30]. Thus, the present results and those of previous studies together suggest that P-cadherin could promote cervical cancer cell growth and invasion at least partly through affecting the expression of E-cadherin and p120 catenin.

Conclusion

In summary, the present results demonstrated that P-cadherin expression was significantly elevated in cervical cancer tissues and cancer-derived cell line compared with that of normal cervical tissues and normal cell lines. Furthermore, the authors found that modulation of P-cadherin expression in vitro cervical cell lines could affect cell growth, migration, and invasion, and result in the change of E-cadherin and p120ctn expression. The present data together suggest that P-cadherin, as a tumor oncogene, could participate in cervical carcinogenesis and cancer progression at least partly through affecting the expression of E-cadherin and p120 catenin.

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Corresponding Author:
XIAOYUN WAN, M.D.
Department of Gynecologic Oncology
Women's Hospital, School of Medicine
Zhejiang University
Xueshi Rd no.1, Hangzhou
Zhejiang 310006 (China)
e-mail: wanxy@zju.edu.cn