

RESEARCH COMMUNICATION

Platelet-derived Growth Factor-D Promotes Ovarian Cancer Invasion by Regulating Matrix Metalloproteinases 2 and 9

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Abstract

Objective: Platelet-derived growth factor-D (PDGF-D) can enhance invasion and metastasis in several human malignancies, though little is known about its functions in ovarian cancer. **Methods:** In this study, we detected expression of PDGF-D in ovarian cancer tissues and cell lines by qRT-PCR, immunohistochemistry and western blotting, investigating the influences on cellular proliferation, invasion and apoptosis by upregulating its expression. **Results:** 79.5% (62/78) of ovarian cancer samples proved to be PDGF-D positive, in contrast to just 38.5% (30/78) in their adjacent non-cancer tissues ($p < 0.001$). Moreover, we found high levels of PDGF-D were correlated with lymph node metastasis ($p = 0.025$) and positive cancer cells in abdominal washings/ascites ($p = 0.042$). In vitro, upregulation of PDGF-D enhanced the invasiveness of SKOV3 cells ($p < 0.01$), but had no impact on cellular proliferation or apoptosis. Furthermore, expression of matrix metalloproteinases 2/9 (MMP2 and MMP9) was positively related with PDGF-D, indicating their involvement in the invasion and metastasis of ovarian cancer. **Conclusions:** Our findings proved that PDGF-D could promote ovarian cancer invasion by upregulating MMPs, which might be a potential target for ovarian cancer treatment.

Keywords: Ovarian cancer - platelet-derived growth factor-D - matrix metalloproteinases - invasion

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Introduction

Up to date, ovarian cancer is still the largest challenge for gynecologists. Globally, the overall five-year survival rate for ovarian cancer is just about 40% (StatBite, 2011). According to a recent report, in 2010, there were about 21,880 new cases and 13,850 new deaths in the USA (Jemal et al., 2010). The mainly reason for its high mortality is tumor invasion, which could lead to spreadly metastases. However, our knowledge about tumor invasion and metastasis is very limited. Recently, several studies highlighted that platelet derived growth factor-D (PDGF-D) maybe a key factor in tumor invasion (Wang et al., 2010; Zhao et al., 2010).

PDGF-D belongs to the PDGFs (platelet derived growth factors) family (Bergsten et al., 2001; Li et al., 2003; Reigstad et al., 2005). Overexpression of PDGF-D was found in pancreatic cancer, prostate cancer, gastric cancer and renal carcinoma, implying the relationships between high PDGF-D levels and human malignant tumors (Ustach et al., 2005; Xu et al., 2005; Wang et al., 2007; Zhao et al., 2010). As previous researches reported, PDGF-D primarily interacts with PDGFR- β (PDGF receptor- β) and activates several downstream signaling cascades, such as β -catenin, notch-1, nuclear factor- κ B (NF- κ B), phosphatidylinositol 3-kinase (PI3K)/Akt signalings and so on, finally contributing to tumor development, progression and metastasis (Wang et al.,

2009; Wang et al., 2010).

As we now know little about the functions and mechanisms of PDGF-D in human ovarian cancer, in this study, we detected PDGF-D expression in ovarian cancer tissues and cell lines. By upregulating the expression level of PDGF-D, we further studied the detailed functions of PDGF-D.

Materials and Methods

Tissue samples

A total of 78 ovarian cancer tissues and their corresponding non-malignant tissues were collected from the International Peace Maternity and Child Health Hospital (Shanghai Jiaotong University School of Medicine, Shanghai, P.R.China). All patients provided consent and approval was obtained from the ethics committee. All cases were classified and graded according to the criteria of the International Federation of Obstetrics and Gynecology (FIGO 2009).

Immunohistochemistry

Protocols for immunohistochemical staining were described previously (Xu et al., 2005). Primary antibody against human PDGF-D was purchased from Invitrogen ((Invitrogen, USA). Sections stained without anti-PDGF-D were used as the negative control. For evaluation of PDGF-D's protein level, classification standards were

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as follows: negative: totally no staining or <10% tumor cells showed positive staining; positive: $\geq 10\%$ tumor cells were positively stained.

Cancer cell lines and culture

The ovarian cancer cell line SKOV3, HO-8910, 3AO, A2780, OVCAR-3 were routinely cultured in appropriate growth medium, supplemented with 10% FBS at 37°C, 5% CO₂.

RNA extraction and qRT-PCR assay

RNA was extracted with Trizol (Invitrogen, USA) according to the manufacturer's instructions. The qRT-PCR assay was performed on Stepone (Applied Biosystems, USA). The primers and conditions used in this study were same as previously studies (Ahmad et al., 2010). GAPDH was used as the endogenous control. PCR reactions were performed in triplicate.

Generation of SKOV3 cell lines stably overexpressing PDGF-D

PDGF-D overexpressed SKOV3 cell lines were generated following the steps in previously publications (Kong et al., 2008). Briefly, the plasmid pcDNA3-PDGF-D:His was constructed and verified by sequence analysis, then SKOV3 cells were stably transfected with pcDNA3-PDGF-D:His or the corresponding empty vector pcDNA3, qRT-PCR was performed to certify the upregulation of PDGF-D expression.

Proliferation assay

Proliferation of SKOV3 cells was determined by MTT assay. Briefly, SKOV3 cells were incubated with 20 μ l of MTT (5 mg/ml; Sigma, USA) for 4 hours, then in formazan crystals dissolved in 200 μ l of DMSO (Sigma, USA). The absorbance of the solution was measured at 490nm. All procedures were repeated triplicate.

Invasion assay

Cell invasion was investigated using transwell chamber system (Millipore, USA) according to the manufacturer's instructions. Briefly, 1×10^5 SKOV3 cells were seeded into the upper chamber. The bottom chamber was added with 600 μ l of condition medium. After 24 hours incubation, cells migrated to the membrane bottom were fixed, stained and counted by microscope. SKOV3 cells transfected with empty plasmid were used as control. The experiment was performed triplicate.

Western blotting

Equal amounts of protein were resolved by 10% SDS-PAGE, transferred to PVDF membrane, and probed with primary antibodies against Bcl-2 (DAKO, USA), PDGF-D (Invitrogen, USA), MMP-2 and MMP-9 (Santa Cruz, USA). Following incubation with the secondary antibody, the bands of specific protein on the membranes were developed with enhanced chemiluminescence (Beyotime, China). β -actin (Santa Cruz, USA) was used as the endogenous control.

Flow cytometry analysis

Table 1. PDGF-D Expression in Ovarian Cancers

	Tumor	Non-tumor	P
PDGF-D expression			
Positive	62	48	<0.001
Negative	16	30	

Table 2. Relationship Between PDGF-D Expression and Lymph Node Metastasis

	lymph node metastasis Positive	Negative	P
PDGF-D expression			
Positive	35	27	0.025
Negative	4	12	

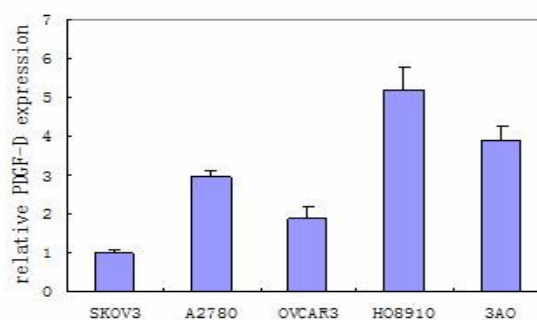


Figure 1. PDGF-D Expression in Ovarian Cancer Tissue (A and B) and Adjacent Non-cancer Tissues (C and D)

Apoptosis of two kinds of SKOV3 cells was analyzed by Annexin V-FITC/propidium iodide method as described previously. In brief, cells were resuspended in 100 μ l binding buffer and adjusted to about 1×10^6 /ml, 5 μ l Annexin V-FITC and 10 μ l PI (20 μ g/ml) were added, after a 15 minutes incubation, flow cytometry was performed on a FACS caliber system (BD Biosciences, USA).

Statistical analysis

The software SPSS 16.0 (SPSS Inc., USA) was used. χ^2 test and t-test were used appropriately. $P < 0.05$ were considered to be statistically significant.

Results

Overexpression of PDGF-D in human ovarian cancer. PDGF-D was overexpressed in 79.5% (62/78) ovarian cancer tissues, whereas, it was just 38.5% (30/78) in their adjacent non-cancer tissues ($p < 0.001$, Figure 1, Table 1); statistical analysis proved that high levels of PDGF-D were correlated with lymphatic node metastasis ($p = 0.025$, Table 2) and positive rate of cancer cell in abdominal washings/ascites ($p = 0.042$, Table 3). We did not detect any relationships between the PDGF-D expression and other clinico-pathological characteristics (data not shown). Upregulation of PDGF-D in SKOV3 cells inhibits cell invasion.

We upregulated PDGF-D expression in SKOV3 cells, in which the level of PDGF-D was relatively low (Figure 2). SKOV3 cells transfected with pcDNA3.1-PDGF-D had an approximately 4.2-fold rise of PDGF-D mRNA level (Figure 3). Next, we examined the effects of

Table 3. Relationship Between PDGF-D Expression and Positive Rate of Cancer Cell in Abdominal Washings or Ascites

PDGF-D expression	cancer cell in abdominal washings and ascites		P
	Positive	Negative	
Positive	29	33	0.042
Negative	3	13	

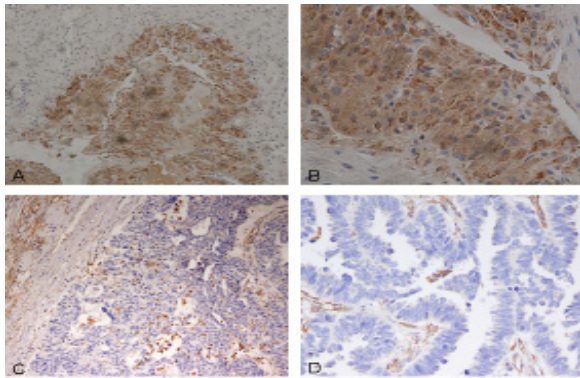


Figure 2. PDGF-D Expression in Ovarian Cancer Cell Lines

PDGF-D on cellular proliferation, invasion and apoptosis. As the data shown, upregulation of PDGF-D enhanced invasion of SKOV3 cells significantly ($p < 0.01$, Figure 4); concomitantly, the protein levels of MMP2 and MMP9 increased enormously (2.3-fold and 3.9-fold, respectively, Figure 3), taken together, these findings suggested that PDGF-D overexpression positively contributed to the cell invasion through upregulating MMP2 and MMP9. Inconsistent with previous publications, we detected that PDGF-D had almost no influence on cellular proliferation and apoptosis ($p = 0.083$ and 0.071 , respectively). No obviously change of anti-apoptotic factor Bcl-2 also support our conclusions.

Discussion

In the last decades, completely surgical resection and chemotherapy are still the mainly treatments for ovarian cancer, unfortunately, which seemed to be of limited efficacy (Schorge et al., 2010). The latest data showed much lower 5-year survival rates for ovarian cancer: 92% for stage I, 55.1% for stage II, 21.9% for stage III and just 5.6% for stage IV, underlining the huge damage to female of ovarian cancer in the worldwide (StatBite, 2011).

Several genes were proved to be involved in tumor invasion, PDGF-D is one of them. Overexpression of PDGF-D was detected in a great many human malignancies, such as pancreatic cancer, prostate cancer, gastric cancer, breast cancer and so on, but rare in the normal tissues, implying PDGF-D's roles in cancer development and progression; what is more, it was proved that PDGF-D could enhance cancer cell growth, migration, invasion and metastasis in different kinds of cancer cell lines (Kong et al., 2005; Xu et al., 2005; Wang et al., 2007; Kong et al., 2008; Liu et al., 2011). As there is little knowledge concerning the functions and mechanisms of this gene in ovarian cancer, we performed this research.

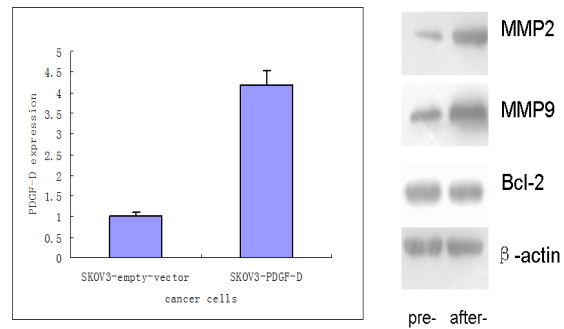


Figure 3. Upregulation of PDGF-D in SKOV3-PDGF-D Cells (left); Changes of MMP2, MMP9 and Bcl-2 pre and after the Upregulation of PDGF-D

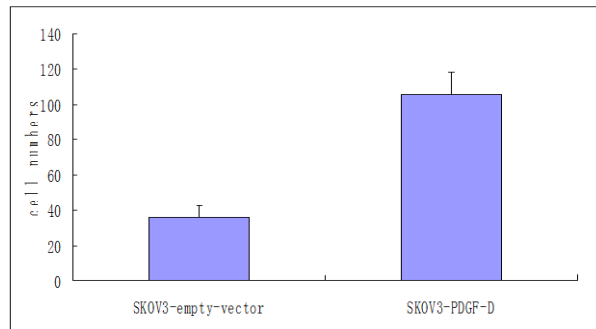


Figure 4. Invasion Assay. The data shown were in the form of mean±SD from three independent experiments (35.2 ± 5.8 VS 105.6 ± 12.1 , $p < 0.01$)

According to our results, 79.5% (62/78) ovarian cancer tissues showed upregulated PDGF-D, while just 38.5% (30/78) in their corresponding tissues, it is significantly different ($p < 0.001$); furthermore, by statistical analysis, we found that PDGF-D's upregulation was correlated with lymphatic node metastasis ($p = 0.025$) and positive rate of cancer cell in abdominal washings/ascites ($p = 0.042$), highlighting the potential roles of PDGF-D in ovarian cancer invasion.

In vitro, by generating SKOV3 cells which could stably overexpress PDGF-D, we further studied the exact functions of this gene on cellular proliferation, invasion and apoptosis. In the invasion assay, much more SKOV3-PDGF-D cells migrated into bottom chamber, in addition, increased levels of MMP2 and MMP9 (which could impair the basilar membrane) also evidenced that PDGF-D can promoter invasion.

According to previous reports, PDGF-D could initiate several downstream signaling pathways to regulate development and progression of cancer. In pancreatic cancer, overexpressed PDGF-D enhanced cell growth, invasion and anti-apoptosis through Notch and NF- κ B pathways (Wang et al., 2007); In prostate cancer cells, overexpressed PDGF-D led to higher invasiveness, which was proved to be associated with activation of mTOR pathway (by targeting S6K and 4E-BP1) and downregulation of Akt (Kong et al., 2008). This study also demonstrated B-DIM (a new chemopreventive agent) could impair cancer invasion and angiogenesis by inhibiting both Mtor/Akt pathway. Another study on human renal cell carcinoma demonstrated that high PDGF-D level promoted angiogenesis and metastasis of human renal cell carcinoma in an SCID mouse

model (Xu et al., 2005); concomitantly, in tumor tissue, angiopoietin-1 and matrix metalloproteinase-9 (MMP-9) were overexpressed. One latest publication proved that PDGF-D could promote lymphatic metastasis by activating CXCR4 in breast cancer (Liu et al., 2011). Similar to these results, we proved that PDGF-D could strengthen tumor invasion in ovarian cancer, and the clinico-pathological characteristics also evidenced our findings.

Although several studies reported that PDGF-D enhanced tumor growth and anti-apoptosis, we failed to detect any changes of cell proliferation and apoptosis by MTT assay and flowcytometry; expression Bcl-2 also showed no variation. We hypothesized that PDGF-D might have no or just very slightly fluence on proliferation and apoptosis in ovarian cancer, which need further evidences.

Summarily, expression of PDGF-D was upregulated in most of human ovarian cancers, enhanced tumor invasion and caused lymphatic node and abdominal metastasis. In vitro assay, we demonstrated that MMP2 and MMP9 (which could impair the basilar membrane) were upregulated by PDGF-D and invasiveness of cancer cells was significantly reinforced. In conclusion, inhibition of this gene maybe an advantaged therapeutic site for ovarian cancer.

Acknowledgements

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