Smad4 mediates malignant behaviors of human ovarian carcinoma cell through the effect on expressions of E-cadherin, plasminogen activator inhibitor-1 and VEGF

Chen Chen1,2,* Ming-Zhong Sun1,2, Suyaing Liu1, Dongmei Yeh1,2, Lijun Yu1, Yang Song1, Linlin Gong1, Lihong Hao1, Jun Hu1 & Shujuan Shao1

1Key Laboratory for Proteomics of Liaoning Province, Dalian Medical University, 2Department of Anatomy, Dalian University, Dalian 116044, China

INTRODUCTION

Ovarian cancer is the 4th leading cause of cancer death in women accounting for 6% of all cancers, and ranks 2nd among gynecologic cancers and increases dramatically (American Cancer Society, 2008 & 2009, http://www.cancer.org). It is an aggressive and fatal disease with a mortality rate of ~50%. Ovarian carcinoma is expected to develop in 1 out of 70 women in their life times, over one woman out of 100 will ultimately succumb to complications associated with this disease (1-3). It is scarcely detected until its recurrence in the advanced stages. The pathogenesis and malignance of ovarian cancer at molecular level are still poorly understood (1, 2, 4, 5).

Smad4, initially identified as DPC4 (deleted in pancreatic carcinoma, locus 4), belongs to Smad gene family encoding intracellular signaling mediators of the TGF-β superfamily of cytokines (6). It is a central molecule for TGF-β signaling pathway. The interaction of Smad4 with phosphorylated Smad2/Smad3 makes it possible for TGF-β executing its biological activity (7, 8). TGF-β/Smad4 signaling pathway is a potent regulator of cellular adhesion, motility and extracellular matrix. Reduced or lost expression of Smad4 is frequently observed during cancer progression. Smad4 over-expression in Smad4-deficient SW480 colon carcinoma cells suppressed their tumorigenicity in nude mice (9). Smad4 expression negatively correlated with liver metastasis in colorectal cancer patients (10). Loss of Smad4 contributes to the switch of TGF-β from a tumor-suppressive to a tumor-promoting pathway in pancreatic cancer through its interaction with vimentin, beta-catenin and E-cadherin (11). For non-small-cell lung carcinoma cells, Smad4 could prevent their metastasis by inhibiting tumor angiogenesis by decreasing VEGF and increasing TSP1 expressions at both protein and mRNA levels (12). Down-regulated Smad4 was observed in microdissected specimens from patients suffering with advanced-stage ovarian cancers, which suggested that Smad4 might enhance TGF-β signaling (13).

However, is Smad4 really associated with the tumor invasion and metastasis of ovarian cancer? Is Smad4 related with other genes, VEGF, E-cadherin and PAI-I, already known relevant to other cancer progressions?

In current study, to gain insights into the potential Smad4-regulated molecules that contribute to ovarian tumor malignancy, two ovarian cancer cell lines originated from the ascitic fluid of a patient with poorly differentiated ovarian papillary serous cyst-adenocarcinoma, HO-8910 with a low metastasis capacity and HO-8910PM with a high metastasis capacity (1, 2, 11, 12, 14-17), were utilized as our initial experimental subjects. Those two cell lines sharing same genetic back-
Smad4 expression mediates ovarian cancer cell malignance
Chen Chen, et al.

Fig. 1. (a) The expression assay of Smad4 in HO-8910 and HO-8910PM cell lines. Plot (up), immunocytochemistry result; Plots (middle and bottom), Western blot analysis. (b) Transient transfection of HO-8910PM with pCMV5-Smad4 vector and the re-expression of Smad4 in HO-8910PM cell. Protein expression of Smad4 was measured in the HO-8910PM cell after a 24-hour-transient-transfection.

RESULTS

Different expressions of Smad4 in HO-8910 and HO-8910PM cells, restoration of Smad4 in HO-8910PM cell
Smad4 showed higher expression in HO-8910 cell relative to HO-8910PM (Fig. 1a, up plot). Smad4 is distributed in the nuclear and cytoplasm of HO-8910 cell. Western blot analysis was consistent with IHC result. The protein level of Smad4 for HO-8910 cell was about 4-fold of that for HO-8910PM (Fig. 1A, middle and bottom plots).

Transient transfections of Smad4 were performed for HO-8910PM cells with the cell density of 4 × 10^5/ml in a 24-well plate inoculated with 5 μg pCMV5-Smad4 plasmid plus 5 μl lipofectamine 2000 in Opti-MEM. pCMV5-Smad4 plasmid contains the coding sequence of human Smad4. In 6 h, the transient HO-8910PM/Smad4 cells were continuously cultured in RPMI1640 media for another 24 h and 48 h. Western results indicated that the protein level of Smad4 was increased significantly in the transfected cell (Fig. 1b). The protein levels of Smad4 in HO-8910PM/Smad4 (pCMV5-Smad4-transfected HO-8910PM) were 8.8-fold and 2.6-fold of that in the HO-8910PM control cell at the time points of 24 h and 48 h, respectively. Finally, 24 h culture was performed for further experiments for HO-8910PM/Smad4 cell. The HO-8910PM/Smad4 cells were obtained by pulling of several transfectants.

Loss of Smad4 increases migration and invasion abilities for HO-8910 cell
Cell scratch-wound assay indicated that more HO-8910PM cells passed through the membrane. The relative cell migration capacity of HO-8910PM cell was ~5-fold of that of HO-8910 (Fig. 2a). A modified Boyden chamber assay was performed to examine the effect of Smad4 on the invasion capacities of HO-8910 and HO-8910PM cells. The HO-8910PM cell number in low chamber was 1.8-fold of that of HO-8910 cell (Fig. 2b). The invasive and migratory capacities of HO-8910PM cell were much higher than HO-8910 cell.

Smad4 restoration reduces migration and invasion capacities for HO-8910PM
HO-8910PM cell motility was reduced following the Smad4 restoration. The relative cell migration capacity of HO-8910PM/Smad4 was comparable with that of HO-8910 (Fig. 2a). The invasion ability of HO-8910PM/Smad4 cell was ~45% of that of HO-8910 and ~26% of that of HO-8910PM (Fig. 2b), respectively.
Smad4 expression mediates ovarian cancer cell malignance
Chen Chen, et al.

**Fig. 2.** (a) Migratory behaviors of HO-8910, HO-8910PM and HO-8910PM/Smad4 cells were determined by scratch-wound assay. Cells were seeded at very low density and allowed to scratch a wound. The wound were photographed after 24 h. Asterisks marked P values for HO-8910 and HO-8910PM/Smad4 groups are 0.01 and 0.03. (b) Invasion abilities of HO-8910, HO-8910PM and HO-8910PM/Smad4 cells determined by Transwell assay. 6 × 10^4 cells were seeded in the upper chamber. The cells migrating to the under surface were fixed and stained with crystal violet. Asterisks marked p-values for HO-8910 and HO-8910PM/Smad4 groups are 0.05 and 0.02. For P values for Fig. 2a and 2b, SPSS 12.0 software was used for all statistical analyses. The data were expressed as the mean ± standard deviation (S.D.). P values were obtained from t-test analysis.

**Fig. 3.** (a) The expressions of E-cadherin, PAI-1 and VEGF in the cell lines of HO-8910 and HO-8910PM; and the effect of the re-expressions of Smad4 on the protein expressions for E-cadherin, PAI-1 and VEGF in HO-8910PM/Smad4 cell. For PAI-1 group, P values for HO-8910 and HO-8910PM/Smad4 are 0.02 and 0.01; For E-cad group, P values for HO-8910 and HO-8910PM/Smad4 groups are 0.03 and 0.05 from t-test analysis; (b) Cell adhesion abilities comparative analysis of the HO-8910, HO-8910PM and HO-8910PM/Smad4 cells. Asterisks marked P values for HO-8910 and HO-8910PM/Smad4 groups are 0.01 and 0.02, respectively. One-way analysis of variance (ANOVA) was used to determine the significant differences in two comparisons.

**Smad4 up-regulates the expression of E-cadherin**
E-cadherin was undetectable in HO-8910PM cell by Western blot analysis. Smad4 restoration increased the E-cadherin level with a magnification of 2.8-fold (Fig. 3a). The loss of E-cadherin might increase the HO-8910PM cell metastasis capacity.

**Smad4 down-regulates PAI-1 expression**
PAI-1 was up-regulated in HO-8910PM cell. The protein level of PAI-1 was increased by 131% in HO-8910PM relative to HO-8910 cell (Fig. 3a). Smad4 restoration in HO-8910PM cell induced a ~30% reduction for PAI-1.

**Smad4 down-regulates the expression of VEGF**
VEGF protein level in HO-8910PM was ~2-fold relative to HO-8910 cell (Fig. 3a), which might facilitate HO-8910PM cell metastasis capacity. Smad4 re-expression reduced VEGF protein expression by 15% (Fig. 3a).

The loss of Smad4 reduces E-cadherin expression, increases PAI-1 expression and enhances VEGF protein expression, which might accelerate the metastatic progression of human ovarian cancer cells.

**Smad4 inhibits adhesion capability of ovarian cancer cell**
Smad4 was associated with the adhesion capacities of HO-8910 and HO-8910PM cells. The number of HO-8910 cell adhered to Matrigel was 1.5-fold relative to HO-8910PM (Fig. 3b). Smad4 re-expression made the cells adhering to Matrigel doubled. Loss of Smad4 might result in decreased adhesion ability of ovarian cancer cell.
E-cadherin, PAI-1 and VEGF expressions regulated by Smad4

Fig. 4 shows IHC results for the expressions of E-cadherin, PAI-1 and VEGF in HO-8910, HO-8910PM and HO-8910PM/Smad4 cells. IHC analysis indicated that E-cadherin was highly suppressed with undetectable (−) level in HO-8910PM cell. Moderate levels of E-cadherin expressions were found in HO-8910 cell (+/−) and in HO-8910PM/Smad4 cell (+ +). E-cadherin expression is regulated by the expression level of Smad4. Strong expression level (+ + +) of PAI-1 was detected in HO-8910PM cell comparing to moderate level (+ +) PAI-1 expressions in both the HO-8910 and HO-8910PM/Smad4 cells. Following Smad4 restoration in HO-8910PM cell, the protein level of PAI-1 is dramatically decreased to the comparable level of PAI-1 in HO-8910 cell. Herein, the above result implicates again that the Smad4 suppression or loss potentially enhance the metastatic ability of ovarian cancer cell through up-regulating the protein expression of PAI-1. VEGF was moderately expressed (++) in both HO-8910PM cell and HO-8910PM/Smad4 cell, which was much higher than the low (+) expression of VEGF in HO-8910 cell (Fig. 4). The protein expression level of VEGF in HO-8910PM/Smad4 seems slightly lower than that in HO-8910PM cell. And the IHC results were consistent with Western blot analysis.

DISCUSSION

Ovarian cancer is an aggressive and fatal disease with low mortality rate as it is scarcely detected until its recurrence in advanced stages. The pathogenesis and malignance of human ovarian cancer at molecular level are unclear (1, 2, 4, 5, 12). TGF-β/Smad4 signaling pathway is believed to play a critical role in the progression of a variety of cancers and potentially associated with ovarian cancer progression (1-5, 7-9, 12, 13, 18-20). Smad4 is regarded as a central molecule for the TGF-β signaling pathway (6-9). Suppressed and lost expressions of Smad4 are associated with the increase of tumorigenicity for nude mice colon carcinoma cells (9), enhancement of liver metastasis in colorectal cancer patients (10), promotion of tumor-promoting pathway in pancreatic cancer (11) and tumor metastasis capacity increase for NSCL cell (12). Down-regulation of Smad4 was discovered in advanced-stage ovarian cancers patients, which implicates that Smad4 might function through TGF-β signaling pathway (13).

Metastasis is a complex biological process which mainly relies on the invasion and migration abilities of malignant tumor cells (21). HO-8910 and HO-8910PM cells have same genetic background with low and high metastasis capacities, which have been used as the ideal cell models for studying the tumor metastasis progression for human ovarian cancer and excellent subjects for detecting the dynamic changes for genes and proteins following pharmaceutical treatment, gene silencing and protein restoration (1, 2, 11, 12, 14-17). HO-8910PM cell migrated much faster than HO-8910 cell (Fig. 2a). Moreover, HO-8910PM cell had much higher invasion ability than HO-8910 cell (Fig. 2b). Concordantly, the protein level of Smad4 in HO-8910PM was much lower relative to HO-8910 cell (Fig. 1). The loss of Smad4 in highly metastatic HO-8910PM cell might result in its higher migration and invasion capacities, which confirmed by Smad4 restoration in HO-8910PM cell. Following the stable Smad4 over-expression, the migration ability of HO-8910PM decreased by 400% (Fig. 2a); and the invasion ability of HO-8910PM reduced to ~45% of that of HO-8910 cell (Fig. 1). The loss of Smad4 in highly metastatic HO-8910PM cell might result in its higher migration and invasion capacities, which confirmed by Smad4 restoration in HO-8910PM cell. Following the stable Smad4 over-expression, the migration ability of HO-8910PM decreased by 400% (Fig. 2a); and the invasion ability of HO-8910PM reduced to ~45% of that of HO-8910 cell (Fig. 1). The re-expression of Smad4 strategy might be implicated in inhibiting and reversing ovarian cancer malignancy.

E-cadherin is a cell-surface glycoprotein acting as one of key molecules in the establishment and maintenance of cell adhesion (9). Reduced level of E-cadherin is associated with in-
increased invasion and migration capacities of epithelial tumor cells (22, 23). IHC and Western blot analysis indicated that protein level of E-cadherin was highly suppressed in HO-8910PM. E-cadherin protein level could be regulated by Smad4 protein level. Smad4 stable over-expression enhanced E-cadherin protein level with a magnification of 2.8-fold (Fig. 3a). Smad4 probably regulates the metastatic capacity of HO-8910/HO-8910PM cells through regulating the protein expression level of E-cadherin.

VEGF-induced pathological angiogenesis has been implicated in promoting tumor dissemination, invasion and metastasis by supplying the required oxygen and nutrient for tumor cells (24-26). Protein expression level of VEGF in HO-8910PM cell was 2 times of that in HO-8910 cell (Fig. 3a), which implicates VEGF over-expression of might increase the metastatic capacity of ovarian cancer cell. Smad4 re-expression in HO-8910PM reduced the protein expression level of VEGF by 15% (Fig. 3a). VEGF might be potentially involved in TGF-β/Smad4 pathway for regulating the malignancy behavior of HO-8910PM.

The initial step for cancer invasion and metastasis is to break the ECM barrier. Plasminogen activator inhibitor (PAI-1) is an ECM and TGF-β15% (Fig. 3a). VEGF might be potentially involved in TGF-

Materials and reagents

Materials and methods

E. coli DH5α alpha competent cells. Lipofactamine 2000 and RPMI1640 medium were purchased from Invitrogen (USA). The pCMV5 and pCMV5-Smad4 plasmids were kind gifts from Dr. Joan Massague from Cancer Biology and Genetics Program, Memorial Sloan-Kettering Cancer Center, USA. Transwells (8-μm pore size, 12 mm I.D.) were purchased from Costar (Cambridge, MA, USA). Matrigel was obtained from BD Biosciences company (USA). ECL for Western blot and gel electrophoresis device were from GE HealthCare (USA). The antibodies for Smad4, PAI-1, E-cadherin and β-actin were from Santa Cruz Biotechnology Inc (USA). Labwork gel imaging system was from Media Cybernetics (USA).

Cell line and culture conditions

HO-8910 and HO-8910PM cells were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (GIBCO, Rockville, USA) at 37°C in a humidified atmosphere containing 5% CO2. Cells were passaged with 0.25% trypsin. Cells in the logarithmic growth phase were used for experiments.

IHC for Smad4, E-cadherin, PAI-1 and VEGF

Slices of HO-8910 and HO-8910PM were fixed in 4% paraformaldehyde for 10 min and then incubated with the corresponding antibodies for 1 h and with goat anti-rabbit biotin IgG for 30 min at room temperature. Having been reacted with streptavidin-biotin-peroxidase complex for 30 min, the immunoreactivity was determined by 3,3′-diaminobenzidine-hydrochloride. To better appreciate negative nuclei, the slices were counterstained with hematoxylin. Selected sections were imaged at 100× magnification for visualizing the localization and distribution of proteins. -, +, ++ and +++ were used to represent the protein expressions at undetectable, low, moderate and strong levels in cells by IHC analysis.

SDS-PAGE and Western blot

The harvested HO-8910 and HO-8910PM cells were washed with cold phosphate-buffered saline (PBS) three times. Then the cells were sonicated in ice-cold RIPA buffer (Santa Cruz, CA) (20 min) on ice. The supernatants were collected by centrifugation at 12,000 rpm (15 min) at 4°C. Protein concentrations of the samples were determined by the Bradford Assay (Bio-Rad) for Western blots.

Some amount of proteins from each samples were resolved in 10% SDS-PAGE and transferred onto nitrocellulose membranes. β-actin was used as the internal standard. Membranes were washed three times for 10 min with PBS-T (20 mM, pH 7.4, 100 mM NaCl, 0.5% Tween-20) and blocked with 5% nonfat milk PBS-T (0.5% Tween-20) for 1 h at room temperature. Being washed extensively with PBS-T, the membranes were then incubated with the horseradish peroxidase-coupled secondary antibodies and were developed by ECL according to the kit instructions (GE HealthCare, USA). The antibodies used were as follows: mouse anti-human Smad4 monoclonal antibody (1:500, Santa Cruz); rabbit anti-PAI-1 monoclonal antibody (1:300, Santa Cruz); rabbit anti-human E-cadherin monoclonal antibody (1:500, Santa Cruz); mouse anti-human VEGF monoclonal antibody (1:500, Santa Cruz) and rabbit anti-human beta-actin monoclonal antibody (1:1,000, Santa Cruz).
Transwell assay (boyden chamber invasion assay)
24-well transwell units with 8 μm I.D. polyester membrane plates (Costar, USA) were used for cell invasion assay. The filter surfaces were coated with 40 μl ice-cold Matrigel (15 mg/ml; BD Biosciences). Conditioned media (200 μl RPMI1640 media with 0.1% bovine serum) were added into the lower compartment of the chamber. Cells (6 x 10^5 cells in 100 μl RPMI 1640 per well) suspended in serum-free RPMI1640 were seeded in the upper chamber. After 24 h of incubation, non-invasive cells on the upper surface of the filter were wiped out with a cotton swab. The cells that had invaded to the lower surface of the filter were fixed with methanol, stained with crystal violet and counted by using five random fields per well. Cells were counted under a light microscope at a magnification of 400×. Triplicate samples were acquired and the data were expressed as the average cell number of five fields.

Cell scratch-wound assay (migration ability analysis)
The cells HO-8910, HO-8910PM and HO-8910PM/Smad4 were grown in 10% fetal bovine serum until confluence. Monolayer cells seeded in 24-well plates were wounded by scratching with 0.1 ml pipette tips. The wounded cells were carefully washed with PBS to remove floating cells. After 8 h and 24 h incubation, the cells in the wounded monolayer were counted randomly at multiple fields. The relative cell migration capacity was compared by the relative area of cell migration defined by the rest of scratch area (at the time point of 0 h) divided by the initial scratch area (at the time point of 0 h).

Cell adhesion assay
Prepared cells (4 x 10^5) were seeded in a 96-well plates coated with Matrigel at 37°C in a CO₂ incubator for 2 h. Being fixed with 4% formaldehyde, the cells were then stained with 0.5% crystal violet in 20% methanol and viewed under a microscope. The cells adhered to the wells were released by equal amounts of 10% acetic acid. The absorbance at 490 nm was utilized to compare the relative numbers of different cells adhered to the Matrigel. Triplicate determinations were done at each data point.

Statistical analysis
SPSS 12.0 software was used for all statistical analyses. Each assay was performed three times. The data were expressed as the mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) was used to determine the significant differences in two comparisons. Statistical significance was set at P < 0.05.

Acknowledgements
This work was supported by grants from National Natural Science Foundation of China (30672392, 20935004) and from the Liaoning Provincial Department of Education, China (2006R17, 2007T025). We thank Dr. Joan Massague from Cancer Biology and Genetics Program, Memorial Sloan-Kettering Cancer Center, USA, for pCMV5-Smad4 vector. We appreciate it the great help from Dr. Frederick Greenaway from Clark University, USA reading the manuscript and revising the English extensively.

REFERENCES


