Low Expression of Protocadherin-8 Promotes the Progression of Ovarian Cancer

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Objective: Ovarian cancer (OC) is the second most lethal gynecological cancer among women throughout the world. Protocadherin-8 (PCDH8) could function as a candidate tumor suppressor. However, the link between PCDH8 and OC development is poorly understood.

Materials and Methods: A total of 68 OC patients were retrospectively enrolled. Clinical information was collected and cancer tissues were used for tissue microarray. The PCDH8 expression was determined on tissue microarray by immunohistochemical staining, and PCDH8 protein was detected in cancer tissues and adjacent tissue by western blotting. Human OC cell lines (SKOV-3 and OVCAR-3) were used to assess the effects of PCDH8 overexpression by western blot and real-time PCR analysis. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay, wound healing migration assay, colony formation assay and invasion assays were performed to assess the influence of PCDH8 on cell function. Cells with Luc-nonspecific Lentiviral or Luc-Lentiviral with PCDH8 gene were subcutaneously injected into nude mice to observe the effect of PCDH8 gene on tumor growth. Bioluminescence imaging was used to observe tumor volume.

Results: We found a low expression of PCDH8 in OC tissues versus the corresponding adjacent tissue. The PCDH8 expression, International Federation of Gynecology and Obstetrics stage, metastasis and recurrence were the independent prognostic factors for over-all survival by multivariate analyses. Furthermore, the patients with recurrence presented a low level of PCDH8 in OC tissues, and patients with advanced tumor stage also had a low PCDH8 expression. Importantly, the low expression of PCDH8 in OC tissues had a poor prognostic with a low overall survival rate. Overexpression of PCDH8 could inhibit OC cell growth/proliferation, migration, invasion, and colony formation in vitro. In vivo experiments also proved that overexpression of PCDH8 could inhibit OC cell growth/proliferation.

Conclusions: Protocadherin-8 might be considered as a candidate tumor suppressor and play an important role in the progression of OC.

Key Words: Ovarian cancer, PCDH8, Prognosis, Tissue microarray
Abbreviations: OC - ovarian cancer, PCDHs - protocadherins, PCDH8 - protocadherin-8, TMA - tissue microarray, IHC - immunohistochemical

Received May 25, 2017, and in revised form November 8, 2017. Accepted for publication November 16, 2017.

(Int J Gynecol Cancer 2018;28: 346–354)

Ovarian cancer (OC) is the second most lethal gynecological cancer among women throughout the world.1 The American Cancer Society estimates that in 2016, about 22,280 new cases of OC will be diagnosed and 14,240 women will die of OC in the United States.1 Currently, the standard care for OC includes surgical treatment and platinum/taxane-based chemotherapy. Despite the development of various new treatment strategies (targeted therapy, immune therapy, and so on), the relative 5-year survival rate for OC patients is 46.2% in 2012, improved slightly from 33.7% in 1975.2 Survival rates vary greatly depending on the stage of diagnosis. Because of the absence of specific symptoms in early stages and lack of early diagnosis markers, 60% to 70% of OC patients are often diagnosed in advanced stage (stage III–IV).3 Therefore, early detection to improve OC outcome and survival remains the cornerstone of OC control, and new diagnosis markers for the progress of OC and novel therapeutic strategies are urgently needed to improve the survival rate of this population.4 As a malignancy, the development of OC is a multistep process including complex molecular changes, but the underlying molecular mechanism has not been fully elucidated. Thus, a better understanding of the molecular biology of OC will lead to the development of early detection and new therapeutic strategies.

The invasion and metastasis is one of the most important hallmarks of tumor progression.5 Ovarian cancer patients diagnosed at an early stage (before the cancer has spread) have a much higher 5-year survival rate (92.1%) than those diagnosed at an advanced stage (28.8%).6 The local invasion and distant metastasis of carcinomas that were associated with cancer cells typically developed alterations in their shape as well as in their attachment to other cells and to the extracellular matrix.5 The abnormal expression and mutational inactivation of cell-to-cell and cell-to-extracellular matrix adhesion molecules were considered as key suppressors of this hallmark capability.6 A major class of cell-cell adhesion molecules is the cadherin superfamily, including E-cadherin, N-cadherin, P-cadherin, protocadherins (PCDHs), and so on.7,8 Protocadherin-8 (PCDH8) is a nonclustered protocadherin, which belongs to the protocadherin family, a subfamily of the cadherin superfamily, and plays multiple roles in cell adhesion, proliferation, differentiation, and migration.9–11 Previous studies have indicated that PCDH17 and 20 could act as tumor suppressors in gastric and colorectal cancers, nasopharynx carcinoma, breast cancer, or non–small-cell lung cancers.12–15

Recently, some studies proved that PCDH8 was inactivated in liver cancer, nasopharyngeal carcinoma, renal cell carcinoma, mantle cell lymphoma and breast cancer, and could function as a candidate tumor suppressor via inhibiting cell growth, colony formation, and migration.16–20 However, less definitive evidence has been reported in OC, and the link between PCDH8 and OC development is poorly understood. In this study, we compared the expression of PCDH8 in OC tissues and the corresponding adjacent tissue. Subsequently, we evaluated the functional role of PCDH8 in growth/proliferation, migration/invasion, and colony formation of OC cell lines. Furthermore, we analyzed the relationship of PCDH8 expression and the prognostic significance of OC patients. Our results suggested that the low expression of PCDH8 predicted a poor prognosis in clinical OC patients, and PCDH8 can be considered as a candidate tumor suppressor for OC patients.

MATERIALS AND METHODS

Construction of the OC Tissue Microarray

A retrospective study of 68 OC patients was identified for tissue microarray (TMA) immunohistochemical (IHC) staining. The study was approved by the human ethic committee of the first affiliated hospital of Zhengzhou University. All patients signed the approval letters that their clinical information would be kept in the databases of the first affiliated hospital of Zhengzhou University and used for research. Those patients with archival tissue blocks available through the Department of Pathology were selected. The data of age, sex, International Federation of Gynecology and Obstetrics (FIGO) stages, TNM stages, CA-125 levels, metastasis, and recurrence of patients were collected, as shown in Table 1. The TMA was constructed by the Tissue Microarray and Imaging Core at the First affiliated hospital of Zhengzhou University.

IHC Staining on TMA

Expression level of PCDH8 was determined based on the immunohistochemistry protocol (Paraffin) from Cell Signaling Technology (Beverly, MA) as previously described.21 Immunohistochemical analysis was performed using PCDH8 antibodies (1:100 dilution, Abcam, Cambridge, MA) and secondary antibody conjugated with HRP (SuperPicture Polymer Detection Kit, HRP, broad spectrum; Thermo Fisher Scientific, Inc., Waltham, MA).

Assessment of PCDH8 staining was conducted according to staining showing and positive cells percentage. Thereby, PCDH8 staining patterns were categorized into 5 groups: 1+, less than 10% of cells stained positive; 2+, 10% to 25% positive cells; 3+, 26% to 50% positive cells; 4+, 51% to 75% positive cells; 5+, greater than 75% positive cells. The PCDH8 staining images for IHC and hematoxylin-eosin staining were obtained using the NanoZoomer 2.0-RS system (Hamamatsu Photonics Inc.,
Germany), and the digital slides were analyzed by NDP. view 2.5.14 version.

### Western Blotting Assay

Total protein extracted from 8 pairs OC tissues (tumor tissue [T]) and the corresponding adjacent tissue (normal tissue [N]), and Western blot analysis was performed with the standard protocol as previous studies. The primary antibodies for PCDH8 (1:1000 dilution) and GAPDH (1:2000 dilution) were purchased from Abcam. Goat anti-mouse HRP antibodies were obtained from Zhongshan Jinqiao Company, Beijing. Quantification analysis of Western blot values was performed with Odyssey software 3.0 (Li-COR Bioscience, NE).

### Cell Lines and Cell Culture

Human OC cell lines (SKOV-3 and OVCAR-3) were purchased from American Type Culture Collection (Rockville, MD). The OC cell lines were incubated in RPMI-1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (Life Technologies, Carlsbad, CA). All cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. Light microscopic images were recorded by Zeiss microscope from Carl Zeiss, Inc. (Oberkochen, Germany) with an attached Nikon D40 digital camera (New York, NY).

### Lentiviral PCDH8 Transduction

The nonspecific lentiviral (as control) and lentiviral with PCDH8 gene (PCDH8-OE) were purchased from RiboBio, and the transfection was carried out using Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to the manufacturer’s instructions. At 72 hours after transfection, the effects of PCDH8 overexpression were measured by Western blot and real-time PCR (RT-PCR) analysis.

### Total RNA Isolation and Real-Time PCR

Total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA), and cDNA was generated by the SuperScript III First-Strand Synthesis System (Life Technologies) using the standard protocol. Real-time PCR was performed using a Quantstudio 6 system with powerup SYBR Green kit (ABI, Foster City, CA). The expression of the target genes were normalized to the expression of GAPDH. The data were performed using the \( 2^{-\Delta\Delta Ct} \) method.

### 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Cell Proliferation Assay

5 × 10⁵ cells in a volume of 100 μL were seeded into a 96-well plate. Each group included three repeated wells. At 1, 2, 3, 4, 5, 6, and 7 days after incubation, proliferation assays were performed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Promega, Madison, WI) using the standard protocol. The absorbance at a wavelength of 490 nm (A490) was measured on a SPECTRAMax Microplate Spectrophotometer from Molecular Devices (Sunnyvale, CA). All results were analyzed by GraphPad Prism 5 software (San Diego, CA).

### Table 1: The relationship between PCDH8 expression and clinicopathological features of OC patients

| Clinicopathological Features | PCDH8 Expression | Survival |
|-----------------------------|------------------|---------|
|                             | Low (n = 37)     | High (n = 31) | \( \chi^2 \) | \( P \) | Live (n = 33) | Dead (n = 35) | \( \chi^2 \) | \( P \) |
| Age, y                      | >Median          | 18 | 13 | 0.306 | 0.579 | 16 | 18 | 0.0588 | 0.808 |
|                             | ≤Median          | 19 | 18 |          |       | 17 | 17 |          |       |
| FIGO stage                  | Stage I and II   | 16 | 22 | 5.258 | 0.021 | 24 | 14 | 7.379 | 0.0065 |
|                             | Stage III and IV | 21 | 9  |          |       | 9  | 21 |          |       |
| TNM stage                   | Stage I and II   | 20 | 14 | 0.533 | 0.465 | 18 | 16 | 0.5298 | 0.466 |
|                             | Stage III and IV | 17 | 17 |          |       | 15 | 19 |          |       |
| Histologic type             | Serous           | 24 | 21 | 0.068 | 0.967 | 21 | 24 | 1.429 | 0.490 |
|                             | Mucinous         | 5  | 4  |          |       | 6  | 3  |          |       |
|                             | Other            | 8  | 6  |          |       | 6  | 8  |          |       |
| CA-125                      | Median (range), U/mL | 625 | 743 | 0.845 | | 569 | 695 | 0.541 | |
|                             | (17–10,682)      | (21–9,675) |          |       | | | | | |
| Metastasis                  | Absent           | 16 | 21 | 4.08  | 0.0430 | 22 | 15 | 3.881 | 0.048 |
|                             | Present          | 21 | 10 |          |       | 11 | 20 |          |       |
| Recurrence                  | Absent           | 17 | 23 | 5.556 | 0.0184 | 26 | 14 | 10.550 | 0.0011 |
|                             | Present          | 20 | 8  |          |       | 7  | 21 |          |       |
| PCDH8 expression            | Low              | —  | —  |          |       | 14 | 24 | 4.710 | 0.029 |
|                             | High             | —  | —  |          |       | 19 | 11 |          |       |
RESULTS

The Expression of PCDH8 Was Low in OC Tissue and Was Closely Related to the Prognosis of OC Patients

To determine the expression of PCDH8 in human OC, we constructed the OC TMA and analyzed the protein expression of PCDH8 by IHC. A total of 68 OC samples were enrolled. PCDH8 staining was assessed according to nuclear staining intensity and the percentage of positive cells among the entire spot. The PCDH8 staining score ranged from score 1+ to score 5+, according to the staining intensity distributed from poor to strong. The details were shown in Figure 1A.

To analyze the correlation between PCDH8 expression and clinicopathological features in 68 OC patients, we divided the evaluable specimens into two grades: low expression (scores 1+, 2+, and 3+) and high expression (scores 4+ and 5+) based on PCDH8 staining intensity. In 68 clinical patients with OC, there was no difference in age, TNM stage, and CA-125 expression between PCDH8 low-expression patients and PCDH8 high-expression patients (Table 1). However, according to FIGO stage (stages I and II, stages III and IV), PCDH8 expression was correlated with FIGO stage (stages III and IV) (P = 0.021) (Table 1). There also were significant relationships between high PCDH8 expression and metastasis, as well as recurrence (Table 1). Moreover, there was a low survival rate in patients with low-expression PCDH8 compared with patients with high-expression PCDH8 (P = 0.029) (Table 1). We also found that FIGO stage, metastasis, recurrence, and PCDH8 expression were the independent prognostic factors for overall survival by multivariate analyses in 68 OC patients (Table 2).

To further verify the relationship between the expression of PCDH8 and clinicopathological features of OC patients, PCDH8 expression in the TMA was analyzed. We firstly found that the expression of PCDH8 was a remarkably low expression in OC tissue compared with the corresponding adjacent tissue by PCDH8 staining score (P = 0.0008) (Fig. 1B). Meanwhile, we found that patients with recurrence possessed a low PCDH8 expression (Fig. 1C). The similar result was found in the patients with metastasis (Fig. 1D). In addition, PCDH8 expression in patients with different FIGO stages was analyzed. We found that PCDH8 expression reduced gradually along with the FIGO stages (Fig. 1E). More importantly, Kaplan-Meier analysis showed the survival time of patients with low PCDH8 was significantly poorer than patients with high PCDH8.

These data suggest that the expression of PCDH8 was low in OC tissue and is closely related to the prognosis of OC patients.

PCDH8 Is Crucial for OC Cell Growth and Proliferation

To further verify the expression of PCDH8 in OC tissue, we analyzed the expression of PCDH8 protein in OC tissue compared with the corresponding adjacent tissue by Western blotting. In normal tissue, the expressions of PCDH8 protein were high, but significantly decreased in OC tissue (Fig. 2A).
To illustrate the function of PCDH8 in OC cell, lentiviral with human PCDH8 gene was transfected in the SKOV-3 and OVCAR-3 cell lines, respectively. The nonspecific lentiviral served as the control of lentiviral with human PCDH8 gene. The normal SKOV-3 and OVCAR-3 cell lines served as blank controls. First, we identified the successful overexpression of PCDH8 in the OC cell line by Western blot and RT-PCR (Figs. 2B and E). Next, we observed cell growth in SKOV-3 and OVCAR-3 cell lines after PCDH8 overexpression. We found that PCDH8 overexpression could also inhibit the cell growth and proliferation in vitro by MTT assay (Fig. 2C and D).

These data further suggested that high PCDH8 expression inhibited cell growth and proliferation in OC cells.
PCDH8 Is Crucial for OC Cell Migration, Colony Formation, and Invasion

We further examined whether PCDH8 overexpression by lentiviral transfection could influence the migration ability of OC cells by the wound healing assay. We found that the wounds were almost recovered after 48 hours of migration in non-specific lentiviral treated cells. In contrast, the wound healing was remarkably inhibited after PCDH8 overexpression (Fig. 3A and B). Next, we observed cells growth in SKOV-3 and OVCAR-3 cell lines after PCDH8 overexpression. We found that colony formation was inhibited by PCDH8 overexpression in vitro (Fig. 3C and D). Meanwhile, the invasion ability of OC cells after PCDH8 overexpression were observed by trans-well assay, and we found that PCDH8 overexpression inhibited the invasion ability of OC cells.

These data further suggested that high PCDH8 expression inhibited cell migration, colony formation, and invasion of OC cells.

Overexpression of PCDH8 Could Inhibit the Cancer Cell Growth In Vivo

To further confirm the role of PCDH8 in OC, a tumor-burdened mouse model was established by injecting subcutaneously OC cells which expressed a luciferase reporter and Lentiviral with PCDH8 gene (or nonspecific Lentiviral) stably. First, we found that PCDH8 overexpression inhibited tumor growth by the comparison of tumor volume and the intensity of luminescence (Fig. 4A). Meanwhile, we detected the expression of PCDH8 in the tumor of Lenti-PCDH8 and Lenti-MOCK and identified the successful overexpression of PCDH8 in the OC tumor of Lenti-PCDH8 (Fig. 4B). Moreover, the tumor weight and the intensity of luminescence were determined, and we found that the weight and the intensity of luminescence of Lenti-PCDH8 tumor were decrease compared with Lenti-MOCK tumor (Fig. 4C and D). Meanwhile, we analyzed tumor growth curve and found that there was significant between Lenti-PCDH8 tumor and Lenti-MOCK tumor.

**TABLE 2.** Independent prognostic factors for OS by multivariate analyses in 68 OV OC patients

| Univariate Analysis | Relative Risk | (95% CI) | P     |
|---------------------|--------------|----------|-------|
| FIGO stage (III and IV vs I and II) | 3.165 | 2.746–3.417 | 0.039 |
| Metastasis (present vs absent) | 1.695 | 1.439–2.007 | 0.049 |
| Recurrence (present vs absent) | 2.314 | 1.965–2.548 | 0.027 |
| PCDH8 expression (low vs high) | 2.014 | 1.789–2.214 | 0.045 |

**FIGURE 2.** PCDH8 is crucial for OC cell growth and proliferation. A, Expressions of PCDH8 were present in 8 OC specimens (T) with paired normal tissue (N). B, Lentiviral with human PCDH8 gene was transfected in the SKOV-3 and OVCAR-3 cell lines and overexpression of PCDH8 were verified by western blot. Cell proliferation OVCAR-3 (C) and SKOV-3 (D) after PCDH8 overexpression was evaluated by MTT assay. E, Lentiviral with human PCDH8 gene was transfected in the SKOV-3 and OVCAR-3 cell lines and overexpression of PCDH8 were verified by RT-PCR. Control, blank control; NS, the nonspecific lentiviral; PCDH8-OE, PCDH8 overexpression.
These data further suggested that high PCDH8 expression inhibited OC cell growth in vivo.

DISCUSSION

Protocadherins are the largest mammalian subgroup of the cadherin superfamily. It has been proven that PCDH17 could suppress Wnt/β-catenin signaling and cell metastasis and was frequently downregulated or silenced in 78% of breast tumor cell lines, as well as 89% of primary breast tumors. It also could induce tumor cell apoptosis and autophagy in gastric and colorectal cancers. Moreover, PCDH17, combined with POU4F2, has yielded the highest sensitivity and specificity for detecting bladder cancer. Protocadherin-20, another number of PCDH family, was silenced in non–small-cell lung cancers, nasopharyngeal carcinoma and liver cancer, and suppress tumor through antagonizing the Wnt/β-catenin signaling pathway in liver cancer. These studies indicated that PCDH17 and 20 could act as tumor suppressors in a series of cancers.

Protocadherin-8 also belongs to the PCDH family and plays multiple roles in cell adhesion, proliferation, differentiation, and...
and migration. Recently, some studies proved that PCDH8 was inactivated in liver cancer, nasopharyngeal carcinoma, renal cell carcinoma, mantle cell lymphoma, and breast cancer, and could function as a candidate tumor suppressor via inhibiting cell growth, colony formation, and migration. However, less definitive evidence has been reported in OC, and the link between PCDH8 and OC development is poorly understood. In this study, we found that the low expression of PCDH8 was observed in OC tissues with the corresponding adjacent tissue. The PCDH8 expression, as well as FIGO stage, metastasis, and recurrence, were the independent prognostic factors for overall survival by multivariate analyses. Furthermore, we evaluated the relationship of PCDH8 expression with the prognostic significance of OC patients. We found that patients with recurrence possessed a low level of PCDH8 in OC tissues. Meanwhile, patients in advanced tumor stage (FIGO stages) also had a low PCDH8 expression. More importantly, the low expression of PCDH8 in OC tissues of patients had a poor prognosis with low overall survival. Overexpression of PCDH8 could inhibit OC cell growth/proliferation, migration, invasion, and colony formation in vitro. In in vivo experiments, we also proved that overexpression of PCDH8 could inhibit OC cell growth/proliferation. Our results suggest that PCDH8 can be considered as a candidate tumor suppressor and play a crucial role in cell growth/proliferation, migration, invasion, and colony formation of OC.

Recent studies have proved that the silenced expression of PCDHs, such as PCDH17 and PCDH20, was due to the promoter methylation of PCDHs genes in a series of cancers. Moreover, the promoter methylation also induced inactivated PCDH8 in liver cancer, nasopharyngeal carcinoma, renal cell carcinoma, mantle cell lymphoma, resulting in the inhibition of cancer cell growth, colony formation, and migration. Thus, we suspected that the low expression of PCDH8 might also attribute to the promoter methylation in OC. We will investigate this point in our future study.

**FIGURE 4.** Overexpression of PCDH8 could inhibit cancer cell growth in vivo. A, The tumor of Lenti-PCDH8 and Lenti-MOCK were isolated and presented after mice sacrificed (upper) and were presented in mice by bioluminescence imaging (under). B, Expression of PCDH8 in the tumor of Lenti-PCDH8 and Lenti-MOCK were detected to identify the successful overexpression of PCDH8 in OC tumor of Lenti-PCDH8. C, The tumor of Lenti-PCDH8 and Lenti-MOCK were isolated and the weight was evaluated. D, The tumor of Lenti-PCDH8 and Lenti-MOCK were evaluated by bioluminescence imaging. E, Tumor growth curve were analysis by bioluminescence imaging.
In conclusion, the present study demonstrates that PCDH8 is low in OC tissue. Moreover, the low expression of PCDH8 in OC tissues of patients had a poor prognosis. More importantly, improved expression of PCDH8 could inhibit OC cell growth/proliferation, migration/invasion, and colony formation in vitro and in vivo. These data suggested that the low expression of PCDH8 predicted a poor prognosis in clinical OC patients and PCDH8 can be considered as a candidate tumor suppressor for OC patients.

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