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Pigment Epithelium-Derived Factor Inhibits Invasion and Metastasis of Breast Cancer Cells by Regulating Epithelial-Mesenchymal Transition Post-print

Authors: Zhou Dan, Xu Pengcheng, Zhang Min, Yu Yang, Wu Aiguo

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Abstract

Objective: To investigate whether pigment epithelium-derived factor (PEDF) inhibits breast cancer invasion and metastasis by acting on epithelial-mesenchymal transition (EMT). Methods: Immunohistochemistry was employed to detect the expression of PEDF, vimentin, and E-cadherin in 119 cases of invasive ductal carcinoma tissues; a PEDF-siRNA-vector interference vector was constructed, RNA interference technology was utilized to block PEDF expression in breast cancer SK-BR-3 cells, and a recombinant adenovirus vector construct (Lentivirus-PEDF-vector) was constructed for transfection of SK-BR-3 cells. Cell scratch assay, cell invasion assay, and Western blot were applied to detect changes in PEDF expression in SK-BR-3 cells, and Western blot was used to detect alterations in the expression of the epithelial marker E-cadherin and mesenchymal marker vimentin, and to observe changes in the in vitro proliferation, invasion, and adhesion characteristics of breast cancer cells. Statistical analysis was performed using chi-square test, Fisher' s exact probability test, Spearman rank correlation test, and paired enumeration data test. Results: The positive expression rate of PEDF in invasive ductal carcinoma was significantly lower than that in normal breast tissue; PEDF positive expression was correlated with tumor size, positively correlated with E-cadherin expression ($r=0.473$, $P<0.001$), and negatively correlated with vimentin expression ($r=-0.412$, $P<0.001$). After culturing breast cancer SK-BR-3 cells in PEDF-conditioned medium: cellular morphology was altered; Transwell invasion assay indicated that cell invasion and metastatic capacity was diminished; PEDF-siRNA enhanced the migration and invasion of SK-BR-3 cells, while Lentivirus-PEDF-vector suppressed the invasion and migration capacity of SK-BR-3 cells. Western blot results demonstrated: cellular E-cadherin expression was significantly decreased ($P<0.05$), vimentin expression was significantly increased ($P<0.05$). Conclusion: The PEDF

gene is closely related to the invasion and metastasis process of breast cancer; PEDF can act on SK-BR-3 cells to undergo EMT thereby inhibiting breast cancer invasion and metastasis.

Full Text

Preamble

Dan Zhou[1], Pengcheng Xu[2], Min Zhang[2], Yang Yu[2], Aiguo Wu[3]
[1]Breast Oncology Division, Foshan Hospital Affiliated to Sun Yat-Sen University, Foshan 528000, China
[2]Department of Histology and Embryology, School of Basic Medical Sciences, Southern Medical University, Guangzhou 510515, China
[3]Department of General Surgery, Zhujiang Hospital, Southern Medical University, Guangzhou 510282, China

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Abstract

Objective: To investigate whether pigment epithelium-derived factor (PEDF) inhibits invasion and metastasis of breast cancer through regulation of epithelial-mesenchymal transition (EMT). **Methods:** The expression patterns of PEDF, vimentin, and E-cadherin were examined in 119 breast cancer tissues using immunohistochemistry. SK-BR-3 breast cancer cell models with PEDF knockdown and overexpression were established by transfecting cells with a PEDF-siRNA vector and a lentivirus-PEDF vector, respectively. Western blotting was employed to detect changes in PEDF, vimentin, and E-cadherin expression, while cell invasion and migration capabilities were assessed using scratch wound healing and Transwell assays. **Results:** The PEDF positivity rate was significantly lower in breast cancer tissues compared with adjacent normal tissues. PEDF expression positively correlated with tumor size and E-cadherin expression level ($r=0.473$, $P<0.001$), but negatively correlated with vimentin expression ($r=-0.412$, $P<0.001$). Transwell invasion experiments demonstrated that PEDF knockdown enhanced cell invasion and metastasis, whereas PEDF overexpression inhibited the invasion and migration of SK-BR-3 cells. Western blotting revealed that PEDF silencing significantly decreased E-cadherin expression ($P<0.05$) and increased vimentin expression in cells ($P<0.05$). **Conclusion:** PEDF is closely associated with breast cancer metastasis and functions by regulating epithelial-mesenchymal transition.

Keywords: breast cancer; pigment epithelium-derived factor; epithelial-mesenchymal transition; invasion; metastasis

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Introduction

Breast cancer has become one of the most common malignant tumors among women worldwide, ranking first in incidence among female malignancies in several major Chinese cities. Despite continuous improvements in treatment modalities, 20-30% of early-stage patients still progress to distant metastasis. Investigating the mechanisms underlying breast cancer invasion and metastasis is therefore crucial for identifying molecular therapeutic targets and improving patient survival rates and outcomes. Genome-wide transcriptional profiling of breast cancer cell lines has revealed that expression of EMT-related markers significantly correlates with invasive and metastatic potential. Recent studies indicate that breast cancer stem cells play a critical role in the EMT process through their self-renewal capacity, thereby influencing tumor metastasis and progression. Pigment epithelium-derived factor (PEDF) is a protein that effectively inhibits neovascularization and works in concert with vascular endothelial growth factor (VEGF) to regulate angiogenesis. Previous research has demonstrated that PEDF protein expression is significantly associated with microvessel density (MVD) and disease prognosis. Our preliminary experiments confirmed that PEDF protein participates in the regulation of EMT in invasive ductal carcinoma tissues, though the precise mechanisms by which PEDF regulates EMT, reduces distant metastasis, and affects patient prognosis remain unclear. Therefore, this study further investigates the specific mechanisms through which PEDF influences EMT characteristics and metastasis. By examining changes in tumor cell properties after co-culture with PEDF, we explore the potential role of PEDF in the tumor microenvironment during breast cancer metastasis, providing insights for targeted gene therapy in breast cancer.

Methods

1.1 Clinical Specimens

We collected 119 paraffin-embedded tissue samples from female patients with invasive ductal carcinoma who underwent surgical resection at the Department of Pathology, Foshan First People' s Hospital between January 2009 and October 2010. All patients had complete medical records and had not received preoperative radiotherapy, chemotherapy, or endocrine therapy. Additionally, 50 breast tissue samples obtained >5 cm from tumor masses were used as controls, with histological sections confirming normal tissue architecture.

1.2 Reagents and Materials

Mouse anti-human PEDF monoclonal antibody and rabbit anti-human E-cadherin monoclonal antibody were purchased from Millipore. Rabbit anti-human vimentin monoclonal antibody was from Cell Signaling Technology. SABC immunohistochemistry kits and DAB chromogen kits were obtained

from Wuhan Boster Biological Engineering. The human breast cancer cell line SK-BR-3 was maintained at the Central Laboratory of Southern Medical University. Lenti-Pac HIV Expression packaging kits were purchased from GeneCopoeia, and siRNA sequences were synthesized by Invitrogen. PEDF primers (product length 240 bp) were: forward 5' -GGA UUU CUA CUU GGA UGA ATT-3' , reverse 5' -UUC AUC CAA GUA GAA AUC CTC-3' . GAPDH primers (product length 227 bp) were: forward 5' -ACC TGA CCT GCC GTC TAG AA-3' , reverse 5' -TCC ACC ACC CTG TTG CTG TA-3' . All primers were synthesized by TaKaRa.

1.3 Experimental Procedures

1.3.1 Immunohistochemical Detection of PEDF, E-cadherin, and Vimentin SABC method was used to detect PEDF, E-cadherin, and vimentin expression in invasive ductal carcinoma and normal tissues. PBS substituted for primary antibody served as negative control. Procedures followed the SABC kit instructions. PEDF positivity was evaluated by randomly selecting 10 fields at 400× magnification and counting 200 cells; <10% positive cells was considered negative, $ $10% as positive. E-cadherin positivity was assessed using Tanaka' s modified quantitative scoring method. Vimentin positivity was defined as: $ $20% positive cells indicating low expression, >20% indicating high expression.

1.3.2 Cell Culture and Treatment SK-BR-3 cells were divided into two groups: blank control group (cultured in regular medium without treatment) and experimental group (cultured in PEDF-conditioned medium). Cells were incubated at 37°C in 5% CO_2 for 72 hours before subsequent experiments.

1.3.3 siRNA Transfection and Lentiviral Packaging Lipofectamine RNAiMAX and siRNA diluent were mixed at 1:1 ratio (siRNA added dropwise to Lipofectamine RNAiMAX). Culture medium was changed after 4-6 hours. Transfected cells were cultured for 48 hours (37°C, 5% CO_2 incubator) before green fluorescent protein expression was observed under fluorescence microscopy to assess transfection efficiency. For lentiviral packaging, 293T cells were transfected according to the liposome transfection reagent instructions. Fluorescence microscopy was performed after 2 days, and cells were collected after 7-10 days, subjected to 4 freeze-thaw cycles (37°C to -80°C), then centrifuged at 8000 rpm for 10 minutes at 4°C. The viral supernatant was used to infect SK-BR-3 cells again to amplify the lentiviral recombinants.

1.3.4 Scratch Wound Healing Assay SK-BR-3 cells in logarithmic growth phase were harvested after 48 hours to prepare single-cell suspensions. Cells were seeded into 6-well plates at 2$×10^{6}$ cells per well and incubated at 37°C in 5% CO_2. A 200 μL pipette tip was used to create scratches following pre-drawn horizontal lines on the plate bottom. Cells were washed twice with

D-Hank' s buffer to remove detached cells, then serum-free medium was added. Changes at the scratch site were observed and photographed at 0 hour. Plates were returned to 37°C, 5% CO_2 incubator for continued culture. Samples were taken and photographed at 6, 12, 36, 48, 60, and 72 hours to monitor cell migration.

1.3.5 Transwell Invasion Assay Matrigel was applied to Transwell chambers to form an artificial basement membrane layer. Cell suspensions (200 µL at 2$\times 10^{5}/mL) from control and experimental groups were added to the upper chamber, while 500 L of 10_{2}, cel
power fields (200\times$) were counted continuously and averaged.

1.3.6 Western Blot Analysis After 72-hour culture, experimental and blank control group cells were lysed and proteins extracted. Western blotting was performed to detect E-cadherin and vimentin expression.

1.4 Statistical Analysis

SPSS 18.0 statistical software was used. Descriptive statistics were expressed as mean ± standard deviation. Analysis of variance was used for multi-group cell migration width comparisons. Factorial design ANOVA was applied for overall comparison of scratch wound area across different time points and groups. Single-factor ANOVA was used for separate effect analysis of multi-group parameters.

Results

2.1 Expression of PEDF, Vimentin, and E-cadherin in Invasive Ductal Carcinoma and Correlation with Clinicopathological Features

PEDF protein expression in invasive breast cancer was associated with tumor size ($P<0.05$) but not with patient age, TNM stage, histological grade, ER status, PR status, or axillary lymph node metastasis ($P>0.05$). Vimentin protein expression correlated with TNM stage, tumor size, axillary lymph node metastasis, histological grade, and PR status ($P<0.05$), but not with patient age or ER status ($P>0.05$). E-cadherin protein expression was associated with TNM stage, tumor size, axillary lymph node metastasis, and PR status ($P<0.05$), but not with patient age, histological grade, or ER status ($P>0.05$) (Table 1).

2.2 Relationship Between PEDF Expression and Vimentin/E-cadherin

In 119 invasive ductal carcinoma cases, Spearman correlation analysis revealed that PEDF expression was significantly negatively correlated with vimentin

expression (r=-0.412, P<0.001) and significantly positively correlated with E-cadherin expression (r=0.473, P<0.001). Vimentin and E-cadherin expression showed a significant negative correlation (r=-0.628, P<0.001) (Table 2).

2.3 PEDF Inhibits Breast Cancer Cell Invasion and Migration

After validating PEDF expression in cell lines and breast cancer specimens, which suggested a close relationship between PEDF and breast cancer metastasis with EMT phenotype, we functionally tested this hypothesis. We downregulated PEDF in SK-BR-3 cells (transfected with PEDF-siRNA) and upregulated PEDF (transfected with Lentivirus-PEDF-vector), then examined PEDF effects on invasion and migration using Transwell assays. Results demonstrated that PEDF significantly inhibited breast cancer cell invasion and migration. Western blotting confirmed that both siRNA and lentivirus vector effectively altered PEDF expression levels in breast cancer cell lines (Figure 2 [Figure 2: see original paper]). Transwell experiments showed that PEDF-siRNA markedly increased SK-BR-3 cell migration and invasion potential, while Lentivirus-PEDF-vector significantly suppressed these capabilities (Figure 3 [Figure 3: see original paper]). These findings indicate that PEDF downregulation indeed enhances breast cancer invasive potential (Figure 4 [Figure 4: see original paper]).

2.4 PEDF Regulates Cytoskeletal Morphology and EMT Marker Expression in Breast Cancer Cells

PEDF overexpression in SK-BR-3 cells caused significant upregulation of the epithelial marker E-cadherin and downregulation of the mesenchymal marker vimentin. In contrast, compared with normal and negative control groups, PEDF-siRNA group showed significantly reduced E-cadherin expression (P<0.05) and elevated vimentin expression (P<0.05). No significant differences in E-cadherin and vimentin expression were observed between normal and negative control groups (P>0.05). These results demonstrate that mesenchymal-like SK-BR-3 cells underwent significant EMT phenotype reversal toward an epithelial-like phenotype under PEDF influence (Figure 5 [Figure 5: see original paper]).

Discussion

PEDF is a 50 kDa protein found in the extracellular matrix that belongs to the serine protease inhibitor family and contains heparin and collagen binding sites. It functions not only as a potent neurotrophic and angiogenic inhibitor but also correlates with tumor growth suppression, reduced metastasis, and favorable prognosis. PEDF inhibits breast cancer growth and metastasis by downregulating fibronectin through the laminin receptor AKT/ERK pathway. Previous studies have demonstrated that PEDF induces endothelial cell apoptosis via the Fas/FasL death pathway and reduces VEGF expression. VEGF derived from breast cancer cell line MDA-MB-231 significantly induced in vitro angiogenesis,

which was subsequently reduced by PEDF addition, establishing PEDF as a VEGF antagonist. Further research indicates PEDF inhibits VEGF-mediated angiogenesis through the VEGFR-1 pathway, and VEGFR-1 promotes breast cancer cell invasion and metastasis via EMT. Therefore, further investigation into PEDF regulation of EMT and its role in breast cancer invasion and metastasis is warranted.

Our results show that PEDF protein is significantly downregulated in the highly metastatic breast cancer SK-BR-3 cell line. Western blotting confirmed PEDF expression in SK-BR-3 cells, consistent with preliminary findings. At the molecular level, PEDF gene silencing enhanced proliferation and in vitro invasion of breast cancer SK-BR-3 cells. Cell-matrix adhesion experiments demonstrated that reduced PEDF expression decreased SK-BR-3 cell adhesion to the matrix.

A key characteristic of EMT is the reduction of epithelial properties and enhancement of mesenchymal features. Epithelial cells gradually lose polarity, cell-cell adhesion weakens, and cytoskeletal remodeling occurs. EMT induces changes not only in cell phenotype but also in marker expression: downregulation of E-cadherin and cytokeratins, and upregulation of mesenchymal markers such as vimentin and N-cadherin. Our study found that high vimentin expression in breast cancer significantly correlated with distant metastasis and pathological stage ($P<0.05$). E-cadherin expression gradually decreased with increasing pathological stage, while vimentin showed the opposite pattern, with both markers exhibiting negative correlation in breast cancer tissues ($r=-0.628$, $P<0.001$), confirming EMT occurrence in breast cancer and its potential involvement in invasion and metastasis. Additionally, Spearman analysis revealed significant correlations between PEDF expression and both E-cadherin ($r=0.473$, $P<0.001$) and vimentin ($r=-0.412$, $P<0.001$), suggesting PEDF may regulate breast cancer invasion and metastasis by modulating these markers. Western blotting further confirmed that SK-BR-3 cells transfected with PEDF-siRNA showed significantly reduced E-cadherin and increased vimentin expression. Conversely, Lentivirus-PEDF-vector transfection caused SK-BR-3 cytoskeletal morphology to shift from mesenchymal to epithelial, with E-cadherin upregulation and vimentin downregulation.

Current research has investigated EMT-promoting factors and transcription factors, particularly in breast cancer, with progress in understanding how matrix metalloproteinases and miRNAs regulate EMT. However, studies identifying a targeted gene for EMT regulation in breast cancer are limited. This study is the first to explore the relationship between PEDF expression and EMT in breast cancer and its role in invasion and metastasis. Our findings demonstrate that PEDF participates in and regulates the epithelial-to-mesenchymal transition in breast cancer cells, thereby inhibiting migration and invasion. These results provide a basis and potential therapeutic target for preventing tumor cell invasion and metastasis in clinical practice.

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