Original Article

Inhibitory effects of pigment epithelium-derived factor on epithelial-mesenchymal transition, migration and invasion of breast cancer

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Abstract: Objective: Pigment epithelium-derived factor (PEDF) is a ubiquitously expressed secreted protein that suppresses tumor growth and metastasis by targeting tumor cells and their microenvironment. However, the exact mechanism of PEDF on breast cancer metastasis including liver and lung metastasis remains unclear. Epithelial-mesenchymal transition (EMT) is a pivotal event in the progression of cancer towards metastasis. In the present report, we investigated whether PEDF inhibits breast cancer metastasis through epithelial-mesenchymal transition and elucidate the association of PEDF expression and EMT in vitro.

Methods: Our analyses were performed on 102 tissue samples of patients with primary BC and a set of 20 control samples of healthy women, respectively. Lentiviruses were used to stably express PEDF in SkBr3 breast cancer cell line to determine EMT factors changes of invasion ability following PEDF re-expression. PEDF and EMT factors protein levels were measured in SkBr3 breast cancer cell line using western blot analyses.

Results: We show that the important inhibitor of angiogenesis, pigment epithelium-derived factor expression positively correlated with lymph node-positive tumor status and tumor size, low expression level of vimentin, and high expression levels of membranous E-cadherin. In addition, we found that PEDF activation suppressed migration and invasion in SKBR3 (luminal) cells and led to morphologic and molecular changes of epithelial-mesenchymal transition (EMT). Loss of PEDF promotes mesenchymal phenotype, whereas PEDF was shown to effectively promote epithelial phenotype and inhibited the growth of endocrine-resistant SkBr3 breast cancer cells invitro. Finally, western blot examination of PEDF/siRNA-expressing tumor showed down-regulation of E-cadherin and up-regulation of vimentin.

Conclusions: These findings suggest that PEDF is directly linked to the mechanisms that suppress metastasis of breast cancer through regulating epithelial-mesenchymal transition. In the future, contribute to evaluate the efficacy of PEDF targeted therapy early during the course of the disease, may be beneficial in the treatment of breast cancer patients.

Keywords: Breast cancer, pigment epithelium-derived factor, metastasis, epithelial-mesenchymal transition

Introduction

Breast cancer is the first leading cause of cancer mortality in women worldwide. Every year, above 1.3 million women are diagnosed with breast cancer and nearly 450,000 women die from it [1]. Metastasis, a process that cancer cells invade surrounding tissues and migrate to distal organs including lung, liver, brain, bone, and lymph nodes, is a major cause of mortality in breast cancer patients [2]. Due to the breast cancer presents as a heterogeneous disease, displaying a variety of histopathological features, genetic markers and diverse prognostic outcomes [3]. So, exploring the mechanism of cancer metastasis is instrumental to develop molecule targets for clinical treatment and improve patients’ survival. Thus within each treatment modality, ongoing investigations to improve therapeutic benefits continue.

Epithelial-mesenchymal transition (EMT) is a complex molecular program that regulates changes in cell morphology and function during embryogenesis and tissue development. EMT also contributes to tumor progression and metastasis [4]. To date, the epithelial-mesenchymal transition phenomenon has been the
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The steps of EMT include a combination of the microenvironment molecules adjusting to accommodate cellular expansion to either promote tissue development in physiological or pathological contexts [6-10]. EMT is typically characterized as loss of epithelial cell adhesion protein E-cadherin and cytokeratins together with the gain of mesenchymal-associated molecules N-cadherin, Vimentin, and fibronectin [11]. The process is described as “cadherin switching”, i.e., down-regulation of E-cadherin and up-regulation of N-cadherin [12, 13]. EMT is associated with therapy resistance and tumor recurrence. Although the role of EMT in tumor invasion and metastasis becomes a topic of interest, the molecular mechanism by which EMT is regulated has not been fully understood.

Pigment epithelium-derived factor (PEDF) is a 50 kDa glycoprotein that belongs to the non-inhibitory serine protease inhibitor superfamily but it does not inhibit proteases [14]. It is a ubiquitously expressed secreted protein that suppresses tumor growth and metastasis by targeting tumor cells and their microenvironment. In breast cancer PEDF has been as a potentially useful prognostic marker, markedly identified reduced in breast tumors compared with normal tissue and this reduction is associated with disease progression and poor patient outcome [15]. Recently, it was also identified as a dual effector in limiting breast cancer growth and metastasis and highlighted a new avenue to block breast cancer progression [16]. It inhibited breast cancer metastasis by down-regulating fibronectin via laminin receptor/AKT/ERK pathway. In cells grown on a thick collagen bed, PEDF overexpression and exogenous PEDF blocked the rapid invasiveness, rounded morphology, and promoted an elongated, mesenchymal-like phenotype associated with reduced invasion [17]. However, the mechanism show PEDF signaling participates in the acquisition and suppression of invasive phenotype for breast cancer cells remain speculative.

In this report, we identified the specific role of PEDF stimulation in inhibition of EMT and weakened of invasive phenotype in breast cancer cell line SKBR3. Our studies cast new light on understanding the molecular mechanisms by which PEDF serves as a potential therapeutic target for cells that have undergone EMT.

Materials and methods

Breast cancer tissue immunohistochemistry

Tumor sections were processed for immunohistochemistry as described before [18]. The sections were incubated with primary antibodies specific for PEDF (1:100; Millipore, Billerica, MA, USA), E-cadherin (1:500; Millipore), vimentin (1:100; Cell Signaling Technology Inc., Danvers, MA, USA), diluted in phosphate-buffered saline containing 0.1% Tween-20 (PBST) and 5% bovine serum albumin overnight at 4°C. Subsequent to being washed three times with PBST, the sections were incubated with secondary antibodies, avidin-biotin-peroxidase complex and DAB reagent.

Cell culture

The SKBR3 cells used in this study were cloned from trastuzumab-insensitive cell line SKBR3 breast cancer cells originally obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in full serum medium composed of RPMI 1640 medium, penicillin at 100 U/ml, streptomycin at 100 µg/ml, 1 × nonessential amino acids (Invitrogen, Grand Island, NY, USA) and 10% fetal bovine serum (Invitrogen, Grand Island, NY, USA) at 37°C in a humidified atmosphere of 5% CO₂ for 72 h. SKBR3 cells stably expressing PEDF were grown in phenol red-free RPMI 1640 medium supplemented with 10% phenol red-free RPMI, 10% fetal bovine serum treated three times with dextran-coated charcoal and 4 µg/ml blasticidin (In vivo Gen, San Diego, CA, USA).

Adhesion, invasion and migration analysis

Adhesion assays were performed 48 hours after transfection with PEDF using a Cytoselect 48-well cell adhesion assay (Cells Bios, San Diego, CA) according to the manufacturer’s protocol. The invasion assays were performed in 24-well transwell chambers (Corning, Acton, MA, USA) containing polycarbonate filters with 8-µm pores coated with matrigel (BD Biosciences, Bedford, MA, USA). Initially, the chambers were rehydrated with DMEM (serum free) for 2 h at 37°C in 5% CO₂ atmosphere. Five hundred microlitres of balanced mixture of the conditional medium from NIH3T3 fibroblasts and the complete medium was added to the lower compartment as the chemotactic factor. Then 1 × 10⁵ cells in serum-free DMEM were
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added to the upper compartment of the chamber. Each cell group was plated in 3 duplicate wells. After incubation for 24 h, the noninvasive cells were removed with a cotton swab. Cells that had migrated through the membrane and stuck to the lower surface of the membrane were fixed with methanol and stained with crystal violet. Finally, the cells in the lower compartment of the chamber were counted under a light microscope in at least ten random visual fields. The migration assay was similar to the invasion assay described above, except that the upper side of the membranes was not coated with the matrigel.

**Lentiviral vector design, production, and transduction**

For PEDF overexpression, we generated a lentiviral construct encoding the full-length human PEDF cDNA inserted between XbaI and BamHI sites of the prrl. CMV. EGFP. wpre. SIN lentiviral vector. Lenti-Pac™ HIV Expression kit were purchased from Gene Copoeia. Cells were transfected with plasmids by using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) or Lipofectamine 2000 (Invitrogen Carlsbad, CA), according to the manufacturer’s instructions.

**Knockdown of PEDF with small interference RNA**

To assess the role of EMT factors in biological effects that are mediated by PEDF, we silenced PEDF in SKBR3 cells by siRNA, the sequence for PEDF was synthesized by primers: sense 5’-GGA UUU CUA CUU GGA UGA Att-3’, antisense 5’-UUC AUC CAA GUA GAA AUC Ctc-3’. Transfection was done by using Lipofectamine 2000 (Invitrogen). As a control, scrambled non-silencing siRNA was used (TaKaRa). The siRNA-scrambled sequence was as follows: GAPDH primers: sense 5’-ACC TGA CCT GCC GTC TAG AA-3’, antisense 5’-TCC ACC ACC CTG TTG CTG TA-3’.

**Western blot analyses**

Cells were washed with PBS and re-suspended in RIPA lysis buffer containing 1% Sodium Deoxycholate, 10% triton pH 7.4, 50 mM Heps, Int J Clin Exp Pathol 2017;10(10):10593-10602

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**Figure 1.** A. Immunostaining of pigment epithelium-derived factor (PEDF) mainly in the cytoplasm of certain epithelial cells. B. Immunostaining of PEDF in invasive ductal breast cancer samples. C. Immunostaining of E-cadherin in the normal breast tissues. D. Immunostaining of E-cadherin in invasive ductal breast cancer samples. E. Immunostaining of vimentin in the normal breast tissues. F. Immunostaining of vimentin in the cytoplasm of invasive ductal breast cancer samples. G-I. Immunostaining of PBS as control in normal and invasive ductal breast cancer samples.
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Table 1. Expression of PEDF, E-cadherin and Vimentin in Breast cancer and its relationship with clinical pathological characteristics

<table>
<thead>
<tr>
<th>Clinical pathological</th>
<th>PEDF</th>
<th>E-cadherin</th>
<th>Vimentin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Positive cases (%)</td>
<td>$\chi^2$</td>
</tr>
<tr>
<td>Age (year) &lt;50</td>
<td>50</td>
<td>21</td>
<td>0.420</td>
</tr>
<tr>
<td></td>
<td>≥50</td>
<td>52</td>
<td>25</td>
</tr>
<tr>
<td>Tumor diameter (cm) &lt;2</td>
<td>13</td>
<td>6</td>
<td>0.462</td>
</tr>
<tr>
<td></td>
<td>≥2</td>
<td>89</td>
<td>40</td>
</tr>
<tr>
<td>TNM stage I and II</td>
<td>63</td>
<td>31</td>
<td>0.492</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>39</td>
<td>15</td>
</tr>
<tr>
<td>Lymph-node metastasis</td>
<td>Yes</td>
<td>57</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>45</td>
<td>20</td>
</tr>
<tr>
<td>Histopathological grade</td>
<td>G1</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>G2/G3</td>
<td>91</td>
<td>44</td>
</tr>
<tr>
<td>ER status</td>
<td>Positive</td>
<td>62</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>40</td>
<td>18</td>
</tr>
<tr>
<td>PR status</td>
<td>Positive</td>
<td>57</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>45</td>
<td>15</td>
</tr>
</tbody>
</table>

Note: P<0.05 is statistically significant.

Statistical analysis

SPSS 18.0 (SPSS Inc., Chicago, IL) was used to analyze these data. Chi-square tests and Fisher’s exact tests (two-sided) were performed to assess the correlation between clinicopathological parameters and protein expression. A Spearman’s rho test was used to determine the relationship among expression levels of each protein. Analysis of variance was used to analyze cell migration width among multiple groups. Factor design was used to analyze scratch wound area at different time and overall comparison between groups. P<0.05 was considered statistically significant. The data are shown as the mean ± standard deviation. All data points represent the mean of triplicates.

Results

Immunohistochemical analysis PEDF, E-cadherin and vimentin expression and correlation with clinicopathological features

PEDF protein was not detectable in normal breast tissues (Figure 1A), but was predominantly expressed in the cytoplasm of the tumor cells (Figure 1B) in 44.5% of patients (Table 1).
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E-cadherin was normally present in the cell membranes of normal breast tissues, but failed to express in tumor tissues (Figure 1C and 1D). Approximately 26.5% (27/102) of the tumor sections showed loss or reduction of E-cadherin expression. Consistent with the previous studies, expression of PEDF and E-cadherin in IDC were only associated with a large tumor size, and were not related to age, tumor size, histological type, status of estrogen receptor (ER) and progesterone receptor (PR) and axillary nodal metastasis (P>0.05). Conversely, vimentin was absent in normal breast tissues (Figure 1E, 1G). However, vimentin was highly expressed in tumor tissues, and was detected in 85.3% of the tumor tissues analyzed (Figure 1F and Table 1). Consistent with the association in the low-expressed E-cadherin subgroup, high vimentin expression was strongly associated with high-grade and late-stage tumors (P<0.001) (Table 1). Positive membranous vimentin expression also correlated with the TNM stage, lymph-node positive group and a negative PR status (P=0.003 and P=0.016, respectively) (Table 1).

Association between expression levels of PEDF, E-cadherin and vimentin in invasive breast carcinoma samples

Next we analyzed the correlation between the expression levels of PEDF and EMT-related proteins in breast carcinoma tissues. In accordance with the protein changes found during EMT, high expression levels of vimentin was associated with the weak expression of membranous E-cadherin (P<0.001; Table 2). Furthermore, a high level of PEDF expression was strongly associated with a low level of E-cadherin expression (r=-0.473, P<0.001) and a high level of vimentin (r=-0.412, P<0.001).

Effects of PEDF deficiency in cell adhesion, migration, and invasion

To further assess the significance of PEDF downregulation in breast cancer, we compared the breast cancer cell line SKBR3 expressing control siRNA with that expressing PEDF-overexpression (Figure 3). First, the PEDF-siRNA-expressing SKBR3 cells were found to be significantly more invasive compared with the PEDF-overexpression and control cell line,
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as judged by Matrigel migration assay (Figure 3B). The invasiveness was confirmed also by scratch healing assay (Figure 3A; Table 3). Moreover, in soft agar colony formation assay, the PEDF-siRNA-expressing cells produced significantly more colonies than the PEDF-overexpression and control cells (Figure 3B).

PEDF inhibits EMT of the breast cancer cells

This morphological change suggested a reminiscent of the phenotypic change of EMT. Unlike the epithelial cuboid appearance of the parental cells, the PEDF-deficient SKBR3 cells exhibited elongated mesenchymal-like morphology, a change that is observed during EMT. Also, there was a clear loss of surface expression of E-cadherin and increase in expression of the mesenchymal markers vimentin in the PEDF-deficient cells (Figure 4A and 4B). By contrast, PEDF-overexpression SKBR3 cells highly expressed E-cadherin but lacked vimentin expression. Knockdown of PEDF in the SKBR3 cells caused a significant reduction (about

Table 3. A comparison of scratching wound width between groups

<table>
<thead>
<tr>
<th>Group</th>
<th>0 h (mm)</th>
<th>12 h (mm)</th>
<th>48 h (mm)</th>
<th>60 h (mm)</th>
<th>Sum (mm)</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.60 ± 0.03</td>
<td>0.54 ± 0.03</td>
<td>0.43 ± 0.01</td>
<td>0.31 ± 0.02</td>
<td>0.47 ± 0.02</td>
<td>85.217</td>
<td>0.000</td>
</tr>
<tr>
<td>SKBR3+Lentivirus</td>
<td>0.59 ± 0.04</td>
<td>0.55 ± 0.03</td>
<td>0.40 ± 0.02</td>
<td>0.35 ± 0.01</td>
<td>0.47 ± 0.025</td>
<td>53.433</td>
<td>0.000</td>
</tr>
<tr>
<td>SKBR3+SIRNA</td>
<td>0.60 ± 0.02</td>
<td>0.50 ± 0.01</td>
<td>0.23 ± 0.03</td>
<td>0.19 ± 0.09</td>
<td>0.38 ± 0.018</td>
<td>335.279</td>
<td>0.000</td>
</tr>
<tr>
<td>Sum</td>
<td>0.60 ± 0.03</td>
<td>0.53 ± 0.024</td>
<td>0.35 ± 0.020</td>
<td>0.28 ± 0.013</td>
<td>0.44 ± 0.022*</td>
<td>128.015*</td>
<td>0.000*</td>
</tr>
<tr>
<td>F</td>
<td>0.103</td>
<td>3.316</td>
<td>74.786</td>
<td>111.813</td>
<td>193.316*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>0.903</td>
<td>0.107</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000*</td>
<td>(F=56.263, P=0.000)#</td>
<td></td>
</tr>
</tbody>
</table>

Note: *P<0.05 is statistically significant.
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5-fold) in the level of E-cadherin, whereas overexpression of PEDF in the SKBR3 cells caused a significant increase in the epithelial phenotype, as measured by increased E-cadherin and reduced vimentin expression (Figure 4A and 4B). Together, these observations suggest that PEDF is a regulator of EMT in breast cancer cells.

Discussion

EMT, a critical physiological process during development and wound healing, has been implicated in tumor progression and metastasis, can lead to increased cellular adhesion, apical-basal polarity, cellular motility, increasing the potential for invasion/metastasis [19-21]. To date, the epithelial-mesenchymal transition phenomenon has been the favored explanation of distant metastases for epithelial cancers including breast cancer [22]. This phenomenon is characterized by the loss of cell-cell adhesion molecules, down-regulation of epithelial differentiation markers, and transcriptional induction of mesenchymal markers [23]. Loss of epithelial characteristics in favor of a mesenchymal phenotype a process referred to as epithelial-to-mesenchymal transition-bestows carcinoma cells at the tumor front with stem cell-like and more motile properties needed for their invasion into surrounding tissues [24].

PEDF is ubiquitously expressed in many tissues and possesses potent antiangiogenic activity, being more than twice as potent as angiostatin and more than seven times as potent as endostatin [25]. Recent studies indicate that PEDF expression is significantly reduced in a wide range of tumor types and that its re-expression in these tumors delays the onset of primary tumors and decreases metastases [26]. It has been reported that PEDF expression is significantly reduced in breast cancer, and associated with disease progression and poor patient outcome [15]. However, the exact mechanism of PEDF on breast cancer metastasis including liver and lung metastasis remains unclear. In this report, we identified the specific role of PEDF stimulation in induction of EMT and rein-
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First, we found that overexpression of PEDF was strongly associated with low vimentin expression, as well as high E-cadherin expression (Table 2) in invasive breast carcinoma tissues, which are particularly associated with tumor invasion, metastasis and aggressiveness. Among the 102 breast cancer samples in our study, 26.5% showed loss or reduction of E-cadherin, while 85.3% showed positive vimentin expression. The correlation analysis data showed that PEDF expression was associated with low vimentin expression and high E-cadherin expression, suggesting that PEDF may be involved in EMT. This finding suggests that overexpression of PEDF may serve as an indicator for cancers with high metastatic potential. To our best knowledge, it is the first time to report that PEDF is a possible mediator for EMT in invasive breast cancer. In our previous study [18], since PEDF expression was strongly associated with cadherin switching during EMT, we hypothesized that PEDF could regulate EMT of breast cancer cells, inhibiting breast cancer progression and metastasis. Cell metastasis not only requires cell movement, but also the ability to degrade the basement membrane and invade the extracellular matrix (ECM). Metastasis is a hallmark of cancer and the leading cause of mortality among cancer patients. The first step in metastasis is the migration of cancer cells away from the primary tumor, a process called tumor invasion [27]. Therefore, much research effort in recent years has been directed toward disruption of this step of the metastatic process [28, 29]. In this study, we chose SKBR3 cells with high metastatic potential to explore the effects of metastriestosterone on the metastatic activity of human breast cancer cells. Importantly, the decrease in expression of PEDF showed enhanced migratory and invasive ability by promoting EMT in cancer cell line SKBR3 (Figure 2), as evidenced by changes in cell morphology and the expression of EMT-related markers.

The present study further demonstrated the association of PEDF expression and EMT in vitro, and elucidated that PEDF is directly linked to the mechanisms that suppress metastasis of breast cancer through regulating epithelial-mesenchymal transition. Vimentin is a well-known metastasis marker and therapeutic target, as inhibiting vimentin function reduces the ability of cells to migrate [30]. E-cadherin plays a pivotal role in cadherin-catenin-cytoskeleton complexes, and it grants anti-invasive and anti-migratory properties to epithelial cells [31, 32]. Our study, for the first time, identifies an extracellular factor PEDF, which promotes the mesenchymal-like phenotype and restricts surface distribution and function of the mesenchymal marker vimentin expression and increased E-cadherin expression in invasive breast cancer cells (Figure 4A, 4B), a hallmark of EMT for the invasion of adherent tumor cells.

The epithelial-to-mesenchymal transition has been considered as the initiation process of cancer metastasis, when non-invasive and non-metastatic tumor cells lose their epithelial phenotype, acquire invasive properties, infiltrate surrounding tissues and metastasize to secondary sites [33, 34]. Turning an epithelial cell into mesenchymal cell requires loss of epithelial polarity, alteration in cellular architecture and acquisition of migration capacity [35]. Interestingly, we found that in addition to inhibiting migration and invasion, PEDF activation also led to morphologic and molecular changes related to MET. Furthermore, up-regulation of PEDF in post-EMT SKBR cells resulted in a partial MET morphologic change, suggesting a role of PEDF in regulating EMT-MET.

Trastuzumab treatment has improved the overall survival rate of patients with HER2-overexpressing breast cancer. Despite the success of trastuzumab as a monotherapy agent, a significant portion of HER2-positive breast cancers respond poorly to the treatment [36, 37]. HER2-enriched tumors are also more likely to develop metastatic disease. Current studies indicate that the development of trastuzumab resistance in HER2 overexpressing breast cancer cells is accompanied by partial EMT-like transition [38]. Due to the missing expression of HER-2 in interstitial tissue cells, one of the reasons for trastuzumab treatment resistance was HER-2 membrane with deletion leading by EMT, so more and more research aim to combine classic treatment with targeting EMT therapy to prevent the occurrence of EMT and the acquired drug resistance. Interestingly, we found that expression of PEDF inhibited the metastatic activity of the SKBR3 cells, which could reverse the mesenchymal phenotype to epithelial phenotype.
In conclusion, our data show that PEDF inhibits migration, adhesion, and invasion abilities of breast cancer cells. We also identified previously unknown function of PEDF, where it arises conversion to a mesenchymal-like morphology, and that induction of EMT occurs mainly after a loss of PEDF expression. This finding suggests that PEDF suppresses breast cancer metastasis by inhibiting the EMT process, and serve as an attractive therapeutic target for cancer therapy.

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Disclosure of conflict of interest

None.

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