Expression of pigment epithelium-derived factor is associated with a good prognosis and is correlated with epithelial-mesenchymal transition-related genes in infiltrating ductal breast carcinoma

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Abstract. Epithelial-mesenchymal transition (EMT) is a pivotal event in the progression of cancer towards metastasis. Given that pigment epithelium-derived factor (PEDF) inhibits angiogenesis, the present study analyzed whether PEDF expression is associated with EMT and prognosis in invasive ductal breast cancer (IDC). Immunohistochemical analysis was used to examine the expression levels of PEDF, E-cadherin, vimentin, Snail and nuclear factor-κB (NF-κB) in 119 cases of IDC. Correlations between PEDF expression and EMT-related genes, and clinicopathological features and clinical prognosis were analyzed. E-cadherin, vimentin, Snail and NF-κB expression was correlated with tumor size, lymph node metastasis and clinicopathological stage. PEDF expression was closely associated with tumor size. Spearman's rank correlation analysis revealed a positive correlation between PEDF and E-cadherin, vimentin, Snail and NF-κB expression (P<0.05). Additionally, Kaplan-Meier survival analysis demonstrated that the five-year survival rate was higher for patients with PEDF- and E-cadherin-positive tumors, but was lower for those with vimentin-, Snail- and NF-κB-positive tumors. Vimentin, E-cadherin and NF-κB levels were dependent prognostic factors of favorable outcomes in IDC, as determined by Cox multivariate analysis. PEDF expression in breast cancer was significantly associated with EMT-related genes, suggesting that it may be an EMT suppressor. However, its potential as a prognostic indicator in breast cancer warrants further investigation.

Introduction

As the most commonly diagnosed cancer and the main cause of cancer-related mortality among women, breast cancer accounts for 23% of all cancer cases and 14% of the cancer-related fatalities (1). Breast cancer is also one of the most common malignant tumors in Chinese women, with >100 new cases per 100,000 women aged 55-69 years estimated to occur by 2021 (2). Advances in the treatment of this disease using a multi-disciplinary approach with improved combinations of surgery, radiotherapy, chemotherapy and endocrine therapy have resulted in marked improvements in patient outcomes. However, more than half a million women will continue to succumb to breast cancer annually despite these advances (3).

Epithelial-mesenchymal transition (EMT) is an essential process during cancer progression to tumor metastasis (4,5). Mesenchymal cell marker expression, such as that of vimentin, Snail (6,7) and E-cadherin (8), is considered a hallmark for EMT. EMT can also be induced in mammary epithelial cells by expression of various factors, such as the Twist or Snail families (9,10). In addition, the upregulation of nuclear factor-κB (NF-κB) is found in human breast tumor cell lines, carcinogen-transformed mammary epithelial cells, and the majority of primary human and rodent breast tumor tissue samples (11). NF-κB has also been shown to be a central mediator of EMT in a breast cancer progression mouse model (12,13). Although activation of these processes in breast cancer cells has greatly increased their invasive and metastatic potential, the exact role of EMT in tumor metastasis remains unknown (14).

Pigment epithelium-derived factor (PEDF) is a potential independent prognostic marker for breast cancer, and reduction in its expression levels is associated with the progression of disease and a poor patient outcome (15,16). In addition to its anti-angiogenic functions, PEDF also inhibits tumor cell

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migration by promoting cell adhesion, inducing apoptosis and regulating tumor cell differentiation (17). Specifically, PEDF induces endothelial cell apoptosis via NF-κB activation and its downstream target Fas ligand (18,19), Furthermore, PEDF expression is significantly reduced in a wide range of tumor types, and its recovered expression in these tumors delays the onset of primary tumors and decreases metastasis (20). In the brain, PEDF acts as a metastatic suppressor and a neuroprotectant, highlighting its role in limiting brain metastasis and local consequences in primary breast tumors (21). Thus, PEDF may play a critical role in breast cancer development and progression. However, the exact molecular mechanisms by which PEDF elicits its antitumor effects remain unknown (22). The effects of PEDF on tumor suppression and endothelial cell apoptosis combined with its inhibition of tumor cell migration suggest that it may have therapeutic value in the context of EMT. The present study systemically investigated the association between PEDF levels and EMT-related proteins in 119 cases of primary invasive ductal breast cancer (IDC), and analyzed their correlation with clinicopathological factors and patient survival to determine the association between PEDF expression and EMT in breast cancer.

Materials and methods

Patients and tissue specimens. Paraffin-embedded surgical specimens were randomly obtained from 119 non-consecutive breast cancer patients that underwent modified radical masectomy at the Zhujiang Hospital Affiliated to Southern Medical University (Guangzhou, Guangdong, China) between 2006 and 2008. The 119 surgical specimens were selected in accordance with the following criteria: Female patients presenting with unilateral, primary IDC without a history of breast cancer. Patients who received neoadjuvant chemotherapy prior to surgery, presented with secondary breast cancer or exhibited peritumorous carcinoma *in situ* in the tumor sample were excluded. Tumor histology was determined according to the 2003 World Health Organization criteria (23), while disease stage was assessed according to the Union for International Cancer Control (24). Tumors were graded according to Bloom and Richardson, as modified by Elston and Ellis (25), and hormone receptor status was assessed according to the scoring system developed by Remmele and Stegner (26). Inclusion criteria for the study were as follows: Female patients presenting with unilateral, primary IDC, without a history of breast cancer. Patients who received neo-adjuvant chemotherapy prior to surgery, presented with secondary breast cancer or had peritumorous carcinoma *in situ* present in the tumor sample were excluded. Normal mammary parenchyma obtained from 30 women who underwent breast reduction was also analyzed. Ethical approval was obtained from the Medical Ethics Committee of Zhujiang Hospital Affiliated to Southern Medical University and written informed consent was obtained from all patients.

Immunohistochemical staining. Paraffin-embedded sections (5-μm thick) were deparaffinized by immersion in dimethylbenzene for 20 min and then rehydrated in graded concentrations of ethanol (100, 90, 80 and 70%; Beyotime

Biotechnology, Haimen, China). The sections were then subjected to immunohistochemical analysis, as previously described by Zhang *et al* (27). Subsequent to blocking endogenous peroxidase (3% hydrogen peroxidase; Beyotime Biotechnology), the sections were incubated with primary mouse anti-human monoclonal PEDF (1:100; Millipore, Billerica, MA, USA), rabbit anti-human monoclonal E-cadherin (1:500; Millipore), mouse anti-human monoclonal vimentin (1:100; Cell Signaling Technology Inc., Danvers, MA, USA), goat anti-human polyclonal Snail (1:50; Santa Cruz Biotechnology Inc., Dallas, TX, USA) and rabbit anti-human monoclonal NF-κB (1:600; Cell Signaling Technology Inc.) antibodies diluted in phosphate-buffered saline containing 0.1% Tween-20 (PBST) and 5% bovine serum albumin (Beyotime Biotechnology) overnight at 4˚C. Subsequent to being washed three times with PBST, the sections were incubated with secondary antibodies (goat anti-mouse IgG/biotin, rabbit anti-goat IgG/biotin or goat anti-rabbit IgG/biotin; 1:100), avidin-biotin-peroxidase complex and DAB reagent (Wuhan Boster Biological Technology, Ltd., Wuhan, China). Subsequently, all sections were counterstained with hematoxylin (Beyotime Biotechnology) and visualized by microscopy (DM40008; Leica, Solms, Germany). Images were captured by Leica Application Suite 3.7 (Leica), and 5-10 photomicrographs were randomly selected from each section.

Immunohistochemical evaluation. The expression levels of PEDF, E-cadherin, vimentin, Snail and NF-κB were independently reviewed and scored by two pathologists who were blinded to the clinical parameters. The expression of Snail and NF-κB was observed in the cytoplasm, nucleus or both; however, only nuclear expression was considered immunopositive for Snail. Expression of PEDF, E-cadherin and vimentin in the cytoplasm and/or plasma membrane were each considered positive.

The semi-quantitative analysis of the distribution of staining was scored according to the percentage of cells showing immunoreactivity: Negative immunoreactivity indicated the absence of staining or weak staining in 1% of the tumor cells; + indicated focal staining in 1-10% of the tumor cells; ++ indicated positive staining in 11-50% of the tumor cells; and +++ indicated positive staining in >50% of the tumor cells. Tumors were defined as immunopositive when >10% (++/+++) of tumor cells show immunoreactivity. Thus, (+) is defined as low expression, whereas (++/+++) is defined as high expression.

Statistical analysis. SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. The χ^2 test was used to analyze the correlation between PEDF, E-cadherin, vimentin, Snail and NF-κB expression, and the clinicopathological features of the IDC patients. Spearman's correlation coefficient analysis was used to evaluate the correlations between the variables. The Kaplan-Meier method and log-rank tests were used to evaluate the correlation between marker expression and overall survival (OS). The Cox proportional hazards model was used for the multivariate analysis to identify independent prognostic factors. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Correlation between PEDF, E-cadherin, vimentin, Snail and NF-κB expression, and clinicopathological features.

Feature	PEDF, n (+)	PEDF, n (-)	P-value	E-cadherin, n (+)	E-cadherin, n (-)	P-value	Vimentin, n (+)	Vimentin, n (-)	P-value	Snail, n (+)	Snail, n (-)	P-value	NF-κB, n (+)	NF-κB, n (-)	P-value
Age, years			0.809			0.929			0.407			0.775			0.246
<50	26	35		31	30		32	29		31	30		38	23	
≥50	26	32		29	29		26	32		31	27		30	28	
Menopausal status			0.918			0.936			0.621			0.738			0.791
Premenopausal	29	38		34	33		34	33		34	33		39	28	
Postmenopausal	23	29		26	26		24	28		28	24		29	23	
Lymph node metastasis			0.555			0.013			<0.001			<0.001			0.001
Negative	22	32		34	20		16	38		18	36		22	32	
Positive	30	35		26	39		42	23		44	21		46	19	
Tumor size, cm			0.039			0.004			0.017			<0.001			0.001
≤2.0	26	21		32	15		16	31		15	32		18	29	
>2.0	26	46		28	44		42	30		47	25		50	22	
Histopathological grade			0.113			0.126			0.005			0.672			0.001
G1	3	10		8	5		4	9		6	7		4	9	
G2/G3	49	57		52	54		54	52		56	50		64	42	
Pathological stage[a]			0.495			<0.001			<0.001			<0.001			<0.001
I/II	35	41		51	25		24	52		26	50		32	44	
III/IV	17	26		9	34		34	9		36	7		36	7	
ER status			0.862			0.314			0.248			0.348			0.419
Positive	31	41		39	33		32	40		35	37		39	33	
Negative	21	26		21	26		26	21		27	20		29	18	
PR status			0.137			0.014			0.008			0.111			0.186
Positive	32	32		39	25		24	40		29	35		33	31	
Negative	20	35		21	34		34	21		33	22		35	20	
Adjuvant treatment			0.304			0.216			0.173			0.388			0.910
None	14	24		16	22		22	16		22	16		22	16	
Therapy[b]	38	43		44	37		36	45		40	41		46	35	

[a]Tumor-node-metastasis staging. [b]Includes chemotherapy and endocrine therapy or chemotherapy, endocrine therapy and radiation therapy. PEDF, pigment epithelium-derived factor; ER, estrogen receptor; PR, progesterone receptor; NF-κB, nuclear factor κB.

Table II. Association between PEDF, E-cadherin, vimentin, Snail and NF-κB expression in invasive breast carcinoma samples.

Factor	E-cadherin, n (+)	(−)	r	P-value	Vimentin, n (+)	(−)	r	P-value	Snail, n (+)	(−)	r	P-value	NF-κB, n (+)	(−)	r	P-value
PEDF			0.496	<0.001			-0.337	<0.001			0.340	<0.001			0.383	<0.001
Positive	41	11			14	38			16	36			19	33		
Negative	19	48			45	22			46	21			50	17		
E-cadherin							-0.613	<0.001			-0.480	<0.001			-0.519	<0.001
Positive					11	49			17	43			19	41		
Negative					47	12			45	14			49	10		
Vimentin											0.428	<0.001			0.437	<0.001
Positive									42	16			46	12		
Negative									20	41			22	39		
Snail															0.291	0.002
Positive													44	18		
Negative													24	33		

PEDF, pigment epithelium-derived factor; NF-κB, nuclear factor κB.

Results

PEDF, E-cadherin, vimentin, Snail and NF-κB expression in IDC samples. The expression of PEDF, E-cadherin, vimentin, Snail and NF-κB was analyzed in 119 IDC tissues by immunohistochemistry (Fig. 1). As shown in Fig. 1A, PEDF was detected in the cytoplasm of the epithelial cells, and high levels of staining were observed in 44.5% of the breast carcinoma tissues analyzed, which were classified as PEDF-positive. Although E-cadherin was present in the cell membranes of normal breast tissues, it was absent in the tumor tissues (Fig. 1G). Approximately 49.6% (59/119) of the tumor sections exhibited an absence or reduction in E-cadherin expression (Table I). Reduced E-cadherin expression was observed in 20.9% (9/43) of the late-stage (III/IV) and 49.1% (52/106) of the high-grade tumors, which was significantly more than that observed for the early-stage (I/II; 67.1%) and low-grade (61.5%) tumors (both P=0.001) (Table I). Conversely, vimentin and Snail were absent in the normal breast tissues (Fig. 1C and E).

However, vimentin was highly expressed in the tumor tissues, and Snail was detected in 52.1% of the tumor tissues analyzed (Fig. 1H and J; Table I). Finally, NF-κB was detected in the cytoplasm of IDC cells in 57.1% of the tumors analyzed (Fig. 1I).

Correlation between PEDF, E-cadherin, vimentin, Snail and NF-κB expression, and clinicopathological features. The correlation analysis between the expression levels of PEDF, E-cadherin, vimentin, Snail, NF-κB and clinicopathological features is summarized in Table I. PEDF protein was detected in 40.7% of lymph node-negative tumors and 46.2% of lymph node-positive tumors (Table I). The cytoplasmic expression of PEDF was significantly correlated with the tumor size (P=0.039). Furthermore, a low level of E-cadherin expression was correlated with a large tumor size (P=0.004), positive lymph node metastasis status (P=0.013), early pathological stage (P<0.001) and positive progesterone receptor (PR) status (P=0.014; Table I). High vimentin expression was strongly associated with high pathological stage and large tumor size (P<0.001 and P=0.009, respectively); it was also correlated with positive lymph node status and negative PR status (P<0.001 and P=0.008, respectively) (Table I). High nuclear Snail staining was significantly associated with a large tumor size (P<0.001), positive lymph node metastasis status (P<0.001) and late pathological stage (P=0.001). For example, 83.7% of patients with late-stage tumors (III-IV) expressed high levels of nuclear Snail, compared with 26.3% of patients with early-stage (I-II) tumors. Positive nuclear expression of NF-κB was also associated with all adverse clinicopathological variables, namely tumor size, high tumor grade, late tumor stage and lymph node positivity (all P≤0.001) (Table I). No significant association existed between PEDF, E-cadherin, vimentin, Snail, NF-κB expression and gender, age, menopausal status, adjuvant treatment and estrogen receptor (ER) status.

Association between PEDF, E-cadherin, vimentin, Snail and NF-κB expression in IDC samples. Next, the correlation between low level PEDF expression levels and EMT-related

Table III. Correlation of PEDF, E-cadherin, vimentin, Snail and NF-κB expression with patient survival.

Factor	No. of cases	Positive, n	Negative, n	Score range	Median	P-value
PEDF						
NPI						0.701
1	24	9	15	2.2-4.4	3.3	
2	52	24	28	2.6-6.4	4.4	
3	43	19	24	3.5-7.2	5.8	
Survival status						0.014
Good	49	28	21			
Poor	70	24	46			
E-cadherin						
NPI						<0.001
1	24	20	4	2.2-4.4	3.3	
2	52	29	23	2.6-6.4	4.4	
3	43	11	32	3.5-7.2	5.8	
Survival status						<0.001
Good	49	39	10			
Poor	70	21	49			
Vimentin						
NPI						<0.001
1	24	5	19	2.2-4.4	3.3	
2	52	20	32	2.6-6.4	4.4	
3	43	33	10	3.5-7.2	5.8	
Survival status						<0.001
Good	49	9	40			
Poor	70	49	21			
Snail						
NPI						<0.001
1	24	6	18	2.2-4.4	3.3	
2	52	24	28	2.6-6.4	4.4	
3	43	32	11	3.5-7.2	5.8	
Survival status						0.005
Good	49	18	31			
Poor	70	44	26			
NF-κB						
NPI						<0.001
1	24	5	19	2.2-4.4	3.3	
2	52	24	28	2.6-6.4	4.4	
3	43	39	4	3.5-7.2	5.8	
Survival status						<0.001
Good	49	16	33			
Poor	70	52	18			

PEDF, pigment epithelium-derived factor; NPI, Nottingham prognostic index; NF-κB, nuclear factor κB.

proteins was analyzed in the breast carcinoma tissues. In accordance with the protein changes found during EMT, high expression levels of vimentin, Snail and NF-κB were associated with the weak expression of membranous E-cadherin (P<0.001; Table II). In addition, a high level of vimentin expression was significantly correlated with low level nuclear Snail expression (P<0.001, r=0.428); a high level of Snail expression was also significantly correlated with the low level expression of NF-κB (P=0.002, r=0.291). These data indicated the presence of EMT in the IDC tissues. Furthermore, a high level of PEDF expression
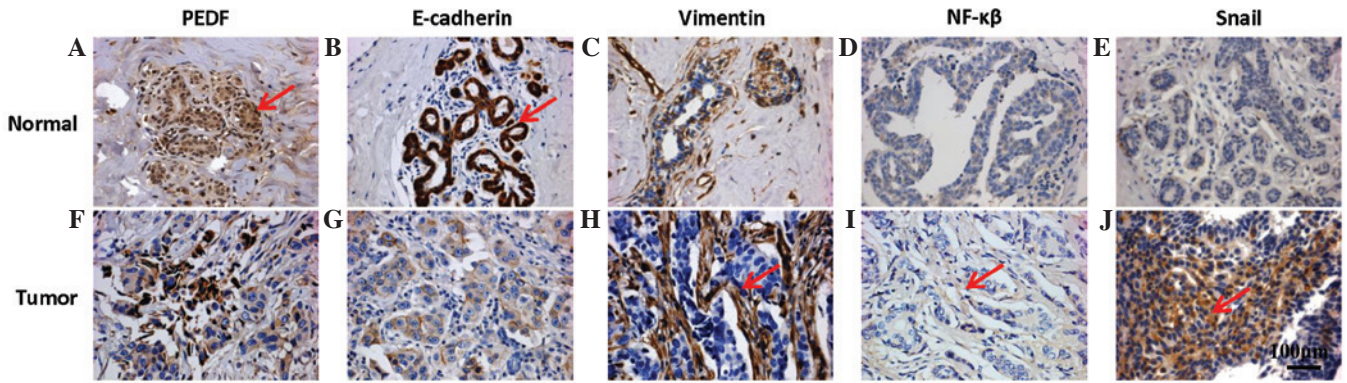
Figure 1. Immunohistochemical analysis of E-cadherin, vimentin, Snail and NF-κB in invasive ductal breast cancer (IDC) and normal breast samples (original magnification, x400). (A) Immunostaining of pigment epithelium-derived factor (PEDF) mainly in the cytoplasm of certain epithelial cells (yellow-brown granules indicated by the red arrow). (B) Immunostaining of E-cadherin in the cell membranes of normal breast tissues (yellow-brown granules indicated by the red arrow). (C) Immunostaining of vimentin in the normal breast tissues. (D) Immunostaining of nuclear factor κβ (NF-κβ) in the normal breast tissues. (E) Immunostaining of Snail in the normal breast tissues. (F) Immunostaining of PEDF in invasive ductal breast cancer samples. (G) Immunostaining of E-cadherin in invasive ductal breast cancer samples. (H) Immunostaining of vimentin in the cytoplasm of certain epithelial cells (yellow-brown granules indicated by the red arrow). (I) Immunostaining of NF-κβ in the nuclei and cytoplasm of cancer cells in a positive specimen (yellow-brown granules indicated by a red arrow). (J) Immunostaining of Snail in the cytoplasm of IDC cells in a positive specimen (yellow-brown granules indicated by a red arrow).
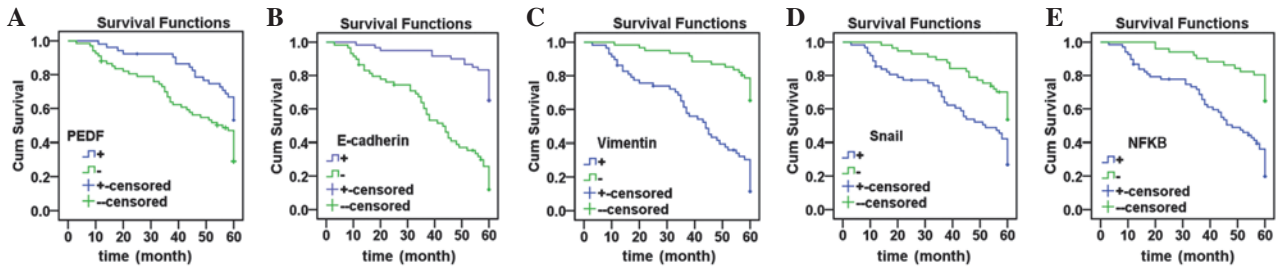

Figure 2. Kaplan-Meier analysis for overall survival curves of breast cancer patients with pigment epithelium-derived factor (PEDF), E-cadherin, vimentin, Snail and nuclear factor κB (NF-κB) expression. Survival curves are stratified by negative (-) and positive (+) (A) PEDF, (B) E-cadherin, (C) vimentin, (D) Snail and (E) NF-κB expression.
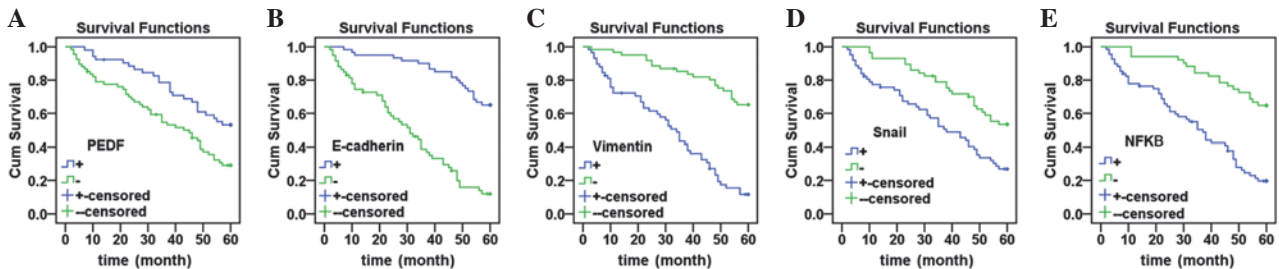

Figure 3. Kaplan-Meier analysis for disease-free survival curves of breast cancer patients with pigment epithelium-derived factor (PEDF), E-cadherin, vimentin, Snail and nuclear factor κB (NF-κB) expression. Survival curves are stratified by negative (-) and positive (+) (A) PEDF, (B) E-cadherin, (C) vimentin, (D) Snail and (E) NF-κB expression.

was strongly associated with a low level of E-cadherin expression ($P<0.001$, $r=0.496$) and a high level of vimentin ($P<0.001$, $r=-0.337$), Snail ($P<0.001$, $r=0.34$) and NF-κB ($P<0.001$, $r=0.383$) expression. These data suggest that PEDF may be involved in the regulation of EMT.

Association between PEDF, E-cadherin, vimentin, Snail and NF-κB expression, and patient survival. Next, the Nottingham prognostic index (NPI) was used as an indicator of patient prognosis, as previously described (28). As shown in Table III, the overexpression of E-cadherin, vimentin, Snail and NF-κB was significantly associated with NPI status, as determined by the Kruskal-Wallis test (all $P<0.001$); however, PEDF expression was not associated with NPI. Specifically, high NF-κB expression was more frequently observed in patients with a high NPI (≥5.4; 37.7%) compared with those with a low NPI (<5.4; 24.4%). The patients were then divided into two groups on the basis of their prognosis. Patients were considered to have a good prognosis (n=49) if they remained disease-free at the 5-year follow-up; those with a poor prognosis (n=70) included patients who developed recurrence, metastasis to a distant site, or those who had succumbed as a result of the breast cancer. As

Table IV. Multivariate analysis of overall survival of patients with invasive breast carcinoma.

Factor	Regression coefficient	Standard error	Wald	RR	95% CI	P-value
Age	-0.001	0.012	0.010	0.999	0.975-1.023	0.921
Histopathological grading	0.472	0.516	0.838	1.604	0.583-4.410	0.360
Lymph node metastasis	0.055	0.384	0.020	1.056	0.498-2.240	0.886
Pathological stage	0.735	0.505	2.120	1.329	0.775-5.614	0.145
Tumor size	0.285	0.109	6.853	2.086	1.074-1.645	0.009
ER status	0.399	0.263	2.298	1.49	0.890-2.497	0.130
PR status	0.368	0.270	1.844	1.445	0.849-2.459	0.174
PEDF	-0.232	0.351	0.435	0.793	0.398-1.579	0.510
Vimentin	-0.847	0.353	5.751	0.429	0.215-0.857	0.016
E-caherin	1.186	0.406	8.517	3.274	1.476-7.262	0.004
Snail	-0.001	0.328	0.000	0.999	0.525-1.900	0.997
NF-κB	-0.609	0.364	2.796	0.544	0.267-1.110	0.094

ER, estrogen receptor; PR, progesterone receptor; PEDF, pigment epithelium-derived factor; RR, relative risk; CI, confidence interval; NF-κB, nuclear factor κB.

Table V. Multivariate analysis of disease-free survival of patients with invasive breast carcinoma.

Factor	Regression coefficient	Standard error	Wald	RR	95% CI	P-value
Age	-0.001	0.013	0.003	0.999	0.975-1.025	0.959
Histopathological grading	0.309	0.510	0.368	1.362	0.501-3.702	0.544
Lymph node metastasis	-0.201	0.380	0.281	0.818	0.388-1.723	0.596
Pathological stage	0.528	0.505	1.095	1.696	0.631-4.563	0.295
Tumor size	0.219	0.108	4.134	1.245	1.008-1.537	0.042
ER status	0.404	0.268	2.270	1.497	0.886-2.531	0.132
PR status	0.257	0.270	0.907	1.294	0.762-2.197	0.341
PEDF	-0.324	0.354	0.840	0.723	0.361-1.446	0.034
Vimentin	-0.918	0.366	6.301	0.399	0.195-0.818	0.012
E-caherin	1.141	0.407	7.847	3.129	1.409-6.950	0.005
Snail	-0.102	0.319	0.101	0.903	0.483-1.690	0.751
NF-κB	-0.705	0.362	3.787	0.494	0.243-1.005	0.052

ER, estrogen receptor; PR, progesterone receptor; PEDF, pigment epithelium-derived factor; RR, relative risk; CI, confidence interval; NF-κB, nuclear factor κB.

shown in Table III, patients with poor prognoses had low levels of PEDF and E-cadherin expression (P=0.014 and P<0.001, respectively) and high levels of vimentin, Snail and NF-κB expression (P≤0.005).

Univariate and multivariate Cox regression analysis of PEDF, E-cadherin, vimentin, Snail and NF-κB expression, and clinicopathological variables. Univariate analyses of OS using Cox regression analysis identified PEDF, vimentin, E-cadherin, Snail and NF-κB expression (P=0.006, P<0.001, P<0.001, P=0.001 and P<0.001, respectively), lymph node metastasis (P=0.015), tumor size (P=0.012), pathological stage (P=0.012) and PR status (P=0.003) as significant prognostic

predicators (Table IV). To determine whether PEDF-positive expression was an independent predictor of patient survival, a multivariate analysis was performed using Cox proportional regression models, together with vimentin, E-cadherin, Snail and NF-κB expression, as well as basic patient and tumor characteristics, such as age, tumor clinical stage, lymph node metastasis, tumor size and ER/PR status. Cox multivariate analysis showed that the expression of vimentin and E-cadherin were independent prognostic factors associated with OS (P=0.016 and P=0.004, respectively) and disease-free survival (DFS; P=0.012 and P=0.005, respectively). Although PEDF expression was not correlated with OS (P=0.51), a significant correlation with DFS was noted (P=0.034). With the exception

of tumor size (P=0.009), no clinicopathological factors were independently predictive of patient survival (Table V).

Kaplan-Meier survival analysis. Kaplan-Meier survival curves are shown in Figs. 2 and 3. Among the 119 patients with IDC analyzed, the patients with PEDF- and E-cadherin-positive tumors exhibited higher OS rates compared with those with PEDF- and E-cadherin-negative tumors (P=0.004 and P<0.001, respectively; Fig. 2A and B). Patients with PEDF- and E-cadherin-positive tumors also exhibited significantly higher DFS rates (P=0.004 and P<0.001, respectively; Fig. 3A and B). By contrast, the patients with vimentin-, Snail- and NF-κB-positive tumors exhibited lower OS rates compared with the patients with vimentin-, Snail- and NF-κB-negative tumors (all P≤0.001; Fig. 2C-E). Patients with vimentin-, Snail- and NF-κB-positive tumors also exhibited significantly lower DFS rates (all P≤0.001; Fig. 3C-E).

Discussion

PEDF is a 50-kDa protein found in the extracellular matrix (ECM). It belongs to the serpin (serine protease inhibitor) family, and contains heparin and collagen binding sites (29-31). PEDF is a potent neurotrophic and angiogenesis-inhibiting factor with tumor suppressor properties (32). *In vitro* studies have demonstrated that the silencing of PEDF may be a novel mechanism for the development of endocrine resistance in breast cancer and that its expression may be a predictive marker of endocrine sensitivity (33). In addition, previous studies have shown that the expression of PEDF is significantly decreased in a number of tumor types, including pancreatic adenocarcinoma (34), glioblastoma (35) and ovarian carcinoma (36).

Vascular endothelial growth factor receptor-1 (VEGFR-1) regulates EMT for the promotion of breast cancer progression and metastasis (37). In various types of tumor cells, PEDF has been shown to decrease VEGF levels (38-40); suppression of VEGF signaling by PEDF may be a novel therapeutic target. In addition, Cai *et al* (41) also identified two novel pathways through which VEGF-induced angiogenesis is inhibited by PEDF: Regulated intramembrane proteolysis and phosphorylation inhibition. Therefore, we hypothesized that PEDF may represent a potential biomarker of EMT in breast cancer and analyzed its expression using immunohistochemistry analysis. In the present study, high PEDF expression was strongly associated with low E-cadherin expression, as well as with high vimentin, Snail and NF-κB expression. These data suggest that PEDF may be involved in the regulation of EMT. This is the first study to show that PEDF may serve as a novel EMT suppressor and to reveal its potential as a prognostic indicator in breast cancer.

EMT exhibits certain characteristic phenotypic changes that are a result of complex genetic changes, which to a certain degree are mediated by specific transcription factors that are able to modulate E-cadherin expression and the expression of numerous other EMT-associated genes *in vitro*. Vimentin is a widely recognized EMT-like phenotype marker, and its expression has been shown in a number of aggressive breast cancer cell lines (42). In the present study, low E-cadherin expression was associated with increased tumor size (P=0.004), lymph node metastasis status (P=0.013) and the pathological stage

(P<0.001). In addition, high vimentin expression was strongly associated with high-grade and late-stage tumors (P<0.001), which is consistent with the results reported by Lee *et al* (43). Notably, vimentin overexpression and reduced E-cadherin expression were significantly correlated with reduced survival and were independent predictors in multivariate analysis. Moreover, multivariate analysis showed that the expression of vimentin and E-cadherin were independent prognostic factors correlated with shorter OS and DFS times.

Several studies have demonstrated the expression of Snail at the tumor-stroma interface and in invasive breast cancers (44). As expected, high nuclear Snail staining was significantly associated with large tumor size (P<0.001), status of lymph node metastasis (P<0.001) and pathological stage (P=0.001) in the present study. Moreover, it was observed that positive nuclear NF-κB expression was associated with all adverse clinicopathological variables analyzed, including large tumor size, high tumor grade, late tumor stage and lymph node positivity, confirming its significance in the development and progression of cancer. Furthermore, low E-cadherin expression, and high vimentin, Snail and NF-κB expression levels were associated with shorter OS and DFS times, which is consistent with studies of other cancers (45,46). Taken together, these results suggest that the expression of certain transcription factors, including NF-κB and Snail, is associated with a poor prognosis in a range of different human cancer types (47).

Previous studies have reported that low PEDF expression is associated with angiogenesis in breast cancer (16). The present study also demonstrated that PEDF was a statistically significant prognostic factor in multivariate Cox regression analysis. Additionally, although the association between EMT and metastasis in patients has been indicated, the present study is the first to suggest a possible mechanism by which PEDF may be capable of reversing tumor growth and metastasis in breast cancer. To the best of our knowledge, studies have rarely been focused on the PEDF and EMT-related genes; Hirsch *et al* (48) concluded that PEDF upregulates PPARγ by binding to PEDF receptor, resulting in the suppression of NF-κB-mediated transcriptional activation in prostate cancer cells. Therefore, further studies are required to elucidate the mechanisms by which PEDF regulates EMT in breast cancer. Therapies targeting PEDF may provide a novel therapeutic approach for untreatable patients.

In summary, to the best of our knowledge, the present study is the first to demonstrate a link between the expression of PEDF in breast cancer and EMT-related genes. These findings suggest that PEDF may be capable of reversing tumor growth and metastasis in breast cancer; however, further studies are necessary to elucidate the association of PEDF expression and EMT *in vitro* and *in vitro*.

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