Original Paper

Silencing of Prrx2 Inhibits the Invasion and Metastasis of Breast Cancer both *In Vitro* and *In Vivo* by Reversing Epithelial-Mesenchymal Transition

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Key Words

Breast cancer • Epithelial-mesenchymal transition • Paired-related homeobox 2 • Invasion • Proliferation

Abstract

Background/Aims: Epithelial-mesenchymal transition (EMT) is recognized as a crucial mechanism in breast cancer progression and metastasis. Paired-related homeobox 2 (Prrx2) has been identified as a new EMT inducer in cancer, but the underlying mechanisms are still poorly understood. **Methods:** The expression of Prrx2 was assessed by immunohistochemistry in breast cancer tissues to evaluate the clinicopathological significance of Prrx2, as well as the correlation between Prrx2 and EMT. Short hairpin RNA knockdown of Prrx2 was used to examine cellular effects of Prrx2, detect the expression of Wnt/ β -catenin signaling and EMT-associated proteins, and observe cell proliferation, invasion and migration abilities *in vitro* and *in vivo*. **Results:** Clinical association studies showed that Prrx2 expression was related to tumor size, lymph node metastasis, tumor node metastasis stages, EMT and poor survival. Results also showed that knockdown of Prrx2 could alter cell morphology, suppressed the abilities of cell proliferation, invasion and migration in breast cancer. Moreover, silencing of Prrx2 induced the mesenchymal-epithelial transition and prevented nuclear translocation of β -catenin, inhibited wnt/ β -catenin signaling pathway. **Conclusion:** Our study indicated that Prrx2 may be an important activator of EMT in human breast cancer and it can serve as a molecular target of therapeutic interventions for breast cancer.

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Introduction

Breast cancer is the most prevalent cancer in women and the leading cause of cancer death in female worldwide [1, 2]. Distant metastasis and therapeutic failure have been the major challenges in the treatment of human breast cancer [3]. Thus, we need to unravel more markers to predict responsiveness of treatment, tumor progression, and use the knowledge to develop novel targets and therapies for breast cancer [4]. Epithelial-mesenchymal transition (EMT) has been demonstrated to promote cell invasion, leading to tumor cell metastasis in various cancers, including breast cancer [5, 6]. During the EMT, epithelial cells lose apical-basal polarity and their junctions, acquire the motility and the invasiveness properties of mesenchymal cells characterized by the down-regulation of cell-cell adhesion molecules, such as E-cadherin, claudins and occludin and the up-regulation of vimentin, fibronectin and N-cadherin [7, 8]. Conversely, the mesenchymal-epithelial transition (MET) describes the reverse process. Transcriptional factors such as Twist1, ZEB2, Snail and FOXC2 can directly or indirectly trigger the EMT [9]. However, the molecular mechanisms by which EMT and MET occur during cancer progression are still unclear [10].

One family of transcriptional factors implicated with regulation of EMT is the paired-related homeobox protein family, which includes Prrx1 and Prrx2 [11]. The Prrx1 gene is alternatively spliced to yield two proteins, Prrx1a and Prrx1b. These proteins differ at their C-termini, where Prrx1a and Prrx2 contain a regulatory otp, aristaless and rax domain, whereas Prrx1b contains a repressor domain. Prrx1 and Prrx2 are important factors for the development of mesenchymal tissues. They further involved in the organogenesis of many tissues during developmental processes. Prrx1/Prrx2 double-deficient mice particularly demonstrate the important roles performed by these factors in craniofacial and limb morphogenesis. We previously reported that silencing of Prrx1b repression of Wnt/ β -catenin signaling pathway and inhibited the proliferation, invasive and migration in triple-negative breast cancer by reversing the EMT phenotypes [12]. A recent study reported that Prrx2 as a TGF- β -induced factor that enhances invasion and migration in breast cancer [13]. Similarly, the role of Prrx2 has been implicated in acute myeloid leukaemia, in which translocation was mediated by the fusion of the nucleoporin gene Nup98, and Prrx2 has been identified as a new transcriptional factor in metastatic gastric cancer [14, 15]. However, the molecular mechanism of Prrx2 in breast cancer requires further investigation.

In this study, we demonstrated that the expression of Prrx2 is closely associated with poor disease-free survival and tumor progression in human breast cancer. In addition, we found that silencing of Prrx2 suppressed cell proliferation, invasion and migration, and induced a MET phenotype in breast cancer. Furthermore, we verified that silencing of Prrx2 prevented nuclear translocation of β -catenin and inhibited wnt/ β -catenin signaling pathway in breast cancer.

Materials and Methods

Tissue samples

133 breast cancer samples were obtained from the Affiliated Hospital of Qingdao University between 2010 and 2012. The matched non-cancerous adjacent tissue specimens were collected 5 cm away from the tumor site. No patients had received chemotherapy or radiotherapy before surgery. This study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University and each participant signed an informed consent document prior to participation in our study.

Cell lines and culture conditions

Human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from Shanghai Cancer Institute (China). Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 ug/ml streptomycin and incubated at 37°C with 5% CO₂.

RNA interference-based gene silencing experiment

Lentivirus plasmid containing short hairpin RNA of Prrx2 were designed by Genechem (Shanghai, China). The target sequences used in this study as follows: 5'-CTGGGTGGACAGCAATAGAAA-3', a scramble sequence (Scr-shRNA) 5'-TTCTCCGAACGTGTCACGT-3' was used for negative control. These viruses were used to transduce 5×10^5 cell/well in the presence of 8 μ g/ml polybrene in 6-well plates. Transduced cells were selected in DMEM containing 2 μ g/ml puromycin for 1 week.

MTT proliferation assay

Approximately 6×10^4 cells were seeded into 96-well culture plates with or without Prrx2 knockdown at 24, 48, 72 and 96 h, respectively. Then cells were incubated with 20 μ l MTT (10 mg/ml) for 4 h at 37°C and 150 μ l DMSO was pipetted to solubilize the formazan product for 30 min at room temperature. The optical density was determined using a spectrophotometer at a wavelength of 570 nm.

Immunohistochemistry (IHC)

Briefly, sections (5 μ m) were dewaxed, hydrated, and heated for 2 min for antigen retrieval in a conventional pressure cooker by using citrate buffer. Then sections were treated with 3% hydrogen peroxide in PBS for 25 min and incubated with 10% goat serum for 30 min. Next, the primary antibody was applied at 4°C overnight. After washing, biotin labeled secondary antibody against rabbit immunoglobulin was applied for 30 min at room temperature. The results were evaluated as described previously [12].

Western blot

Total proteins were extracted using cell lysis buffer and quantified using protein quantification reagents from Bio-Rad. Next, 80 μ g of the protein were electrophoresed on 8% SDS-PAGE gels and transferred to polyvinylidene difluoride membrane by electroblotting. Membranes were incubated with first antibody (i.e., Polyclonal rabbit antibodies against human Prrx2, E-cadherin, vimentin and α -SMA) for 20 h. After using the secondary antibodies, the blots were visualized using an ECL and exposed to X-ray.

Immunofluorescence cell staining

Cells were incubated with β -catenin (1: 250, Rabbit, Sigma) primary antibodies overnight at 4 °C, followed by incubation with a 1: 100 dilution of goat anti-rabbit TRITC-conjugated IgG at room temperature for 2 h and DAPI for 30 min. Images were acquired using confocal microscopy.

Transcriptional reporter gene assay

Prrx2 silenced cells and control cells were seeded on white bottomed 96-well plates at a concentration of 6000 cells per well. 180 ng TOPflash (TCF Reporter Plasmid) or FOPflash (mutant TCF binding sites) expression plasmids and 12 ng pRL-TK (Renilla-TK-luciferase vector, Promega) were co-transfected into the cells. After 20 h, luciferase activity was measured by using Dual-Glo Luciferase reporter Assay System according to the manufacturer's instructions. The ratio of TOPflash /FOPflash was determined, each normalized to the luciferase activities of the Renilla-TK-luciferase vector.

Migration and invasion assay

Breast cancer cells (MCF-7: 8×10^4 and MDA-MB-231: 6×10^4) in 150 μ l per well were plated in the top chamber in media without FBS. 15 % FBS-containing medium was used as a chemoattractant in the lower chamber. The cells on the lower surface of the membrane were stained with 4 % paraformaldehyde and 0.1% crystal violet. 5 random selected visual fields on average were counted.

Xenograft assays in nude mice

Six-week-old female athymic nude (nu/nu) mice (SLAC, Shanghai, China) were injected subcutaneously in the left flank with the stable single cell clones of Prrx2-shRNA and control cells at 4×10^6 cells in 100 μ l serum-free DMEM medium for each nude mouse (n=8). The situation of tumor growth was recorded every week since 7 days after implantation. After 42 days, the mice were killed, and the tumors were weighed. The tumor volumes were determined as described previously [12]. 2×10^6 cells were injected into the tail vein to establish a lung metastatic model of breast cancer (n=8). 60 days later, mice were killed and lung metastasis was quantified by counting the number of tumor foci.

Fig. 1. Prrx2 expression in breast cancer tissues and results in poor prognosis in breast cancer patients. (A) IHC analysis of Prrx2 protein in adjacent noncancerous (a) and breast cancer tissue samples. The serum contained normal saline was set as negative control (b). The IHC results were divided into two groups: the low expression level group (c) and the high expression level group (d). Scale bars, 20 μ m. (B) Statistical analysis the level of Prrx2 in breast cancer tissues from 133 patients by IHC. $*P < 0.05$. (C) Over-expression of Prrx2 in breast cancer with poor prognosis. Kaplan-Meier survival curves for 133 patients with breast cancer, grouped according to Prrx2 expression ($P = 0.005$).

Statistical analysis

Statistical analysis was carried out with the SPSS17.0 (San Rafael, CA, USA) software package for Windows. Comparison of categorical data was performed using the Chi-square test (χ^2 test) and comparison of continuous variables was performed using the Mann-Whitney U test. The relationship between Prrx2 and the differential expression of vimentin and E-cadherin was analyzed using χ^2 test and Pearson's correlation test. P values < 0.05 were considered statistically significant.

Results

Prrx2 expression were excessively elevated in breast cancer tissues

The IHC results showed that the stronger Prrx2 staining was detected in breast cancer tissues than that in corresponding adjacent tissues, with high expression in 48.12% (64/133) of breast cancer samples and only in 7.52% (10/133) of matched distal normal tissues (Fig. 1A, B). Statistical analysis revealed high expression of Prrx2 was associated with tumor size, lymph node metastasis and tumor node metastasis (TNM) stages (Table 1). After median follow up time was 63.2 months (rang 57.4-78.1 months), the calculation of the survival duration revealed that the disease-free survival rate of the lower-expression group ($n = 69$) was significantly better than that of the higher-expression group ($n = 64$) (Fig. 1C, $P = 0.005$).

Silencing of Prrx2 induces MET in breast cancer

Western blot showed that Prrx2 expression levels were significantly decreased by RNA interference ($P < 0.01$; Fig. 2). We further examined the expression of EMT-related proteins

Fig. 2. Effects of Prrx2-shRNA on Prrx2 expression in MDA-MB-231 and MCF-7 cells. The protein level of Prrx2 in MDA-MB-231 (A) and MCF-7 (B) was detected by western blot. (C) Data were expressed as the mean $\pm$ SD of three independent experiments relative to GAPDH. $*P < 0.05$.

in breast cancer and the results revealed that epithelium-specific E-cadherin was significantly elevated after silencing of Prrx2, whereas the intensity of mesenchyme-specific α -SMA and vimentin was decreased in Prrx2 silenced cells (Fig. 3A, B, C). Meanwhile, Prrx2 silenced cells lost their fibroblast-like appearance and acquired an epithelial morphology (Fig. 3D).

In order to confirm the relationship between Prrx2 and EMT in human breast cancer, we investigated the level of Prrx2, vimentin and E-cadherin in the tissue microarray that involved 118 breast cancer tissues. The results showed that majority of Prrx2 highly expressed tissues exhibited high vimentin (53/63) and low E-cadherin expression (46/63). Pearson correlation future analysis showed that Prrx2 expression positively correlated with the expression of vimentin ($P=0.012$) and negatively correlated with the expression of E-cadherin ($P=0.001$). Therefore, Prrx2 was closely associated with increased mesenchymal traits and decreased epithelial characteristics in breast cancer (Table 2).

Silencing of Prrx2 inhibits cell proliferation in vitro and tumor growth in vivo

MTT assay showed that the growth of Prrx2-shRNA group was slower than that of control group in MDA-MB-231 and MCF-7 *in vitro* ($P<0.05$; Fig. 4A, B). To investigate the effect of Prrx2 depletion on tumor growth *in vivo*, a nude mouse xenograft model was performed. The results showed that the tumor weight was smaller in Prrx2-shRNA cell xenografts than Scr-shRNA (160.2 ± 26.3 mg vs. 365.4 ± 19.7 mg, $P<0.01$, Fig. 4C, D).

Silencing of Prrx2 inhibits cell invasion and migration in breast cancer

To investigate the effect of Prrx2 depletion on cell migration and invasion in breast cancer, a transwell assay was performed in MDA-MB-231 and MCF-7. The results showed that silencing of Prrx2 significantly suppressed MDA-MB-231 and MCF-7 cell migration and invasion *in vitro* (Fig. 5A, B). In order to investigate the effect of Prrx2 on tumor metastasis *in vivo*, Prrx2 silenced cells were injected into the tail vein to establish a lung metastatic model of breast cancer. As shown in Fig. 5C, D, the number of macroscopic lung metastases found in the control group was much more than that infected with Prrx2-shRNA.

Table 1. Distribution of Prrx2 status in breast cancer according to clinicopathological characteristics (* $P < 0.05$)

Clinicopathological variables	Cases	Prrx2 expression level		P value
		No. of low expression	No. of high expression	
Age(years)				0.936
≤45	68	35	33	
>45	65	34	31	
Tumor size(cm)				0.000*
≤2	59	41	18	
> 2	74	28	46	
Differentiation grade				0.150
Well-moderate	60	27	33	
Poor-undifferentiation	73	42	31	
Lymph node status				0.010*
Negative	61	39	22	
Positive	72	30	42	
TNM stage				0.009*
I	46	31	15	
II -III	87	38	49	
ER				0.201
Negative	29	12	17	
Positive	104	57	47	
PR				0.506
Negative	32	18	14	
Positive	101	51	50	
Her-2				0.353
Negative	84	41	43	
Positive	49	28	21	

Table 2. Correlations between Prrx2 and E-cadherin, Vimentin in 118 cases of breast cancer (* $P < 0.05$)

Variable expression	Cases (n=118)	Prrx2 expression level		P value
		No. of high expression	No. of low expression	
E-cadherin				0.001*
Low	72	46	26	
High	46	17	29	
Vimentin				0.012*
Low	55	10	45	
High	63	53	10	

Fig. 3. Silencing of Prrx2 induces MET in MDA-MB-231. Silencing of Prrx2 increased the expression of E-cadherin (A), but decreased the expression of vimentin (B) and α -SMA (C) detected by western blot and analysed by normalizing to GAPDH expression. Results are presented as the mean $\pm$ SD of triplicate determinations from three independent experiments. (D) The morphological changes of Prrx2 silenced MDA-MB-231 cells observed by phase contrast microscopy. Scale bars, 20 μ m. * P < 0.05.

Fig. 4. Silencing of Prrx2 suppresses breast cancer cells proliferation *in vitro* and tumor growth *in vivo*. The cell proliferation of control and Prrx2-shRNA groups in MCF-7 (A) and MDA-MB-231 (B) was determined by MTT assay. Results are presented as the mean $\pm$ SD of triplicate determinations from three independent experiments. Prrx2-shRNA and control cells were injected subcutaneously into nude mice (n=8). The tumor volume was measured every 7 days. After 42 days, the mice were killed. The photograph of excised tumors were performed (C), and tumor weights of control and Prrx2-shRNA groups from C (D). * P < 0.05.

Silencing of Prrx2 inhibits Wnt/ β -catenin signaling pathway

To investigate the potential mechanisms of MET induced by silencing of Prrx2, we examined β -catenin distribution in the nucleus and cytoplasm by using cell fractionation method. We found that nuclear β -catenin was significantly decreased in Prrx2-shRNA group compared to that in control (Fig. 6A, B). Second, we detected the distribution of β -catenin with immunofluorescence staining. Compared with control cells, nuclear translocation of β -catenin was decreased in Prrx2 silenced cells (Fig. 6C). Furthermore, we determined the activation of the Wnt signaling pathway with a Wnt/ β -catenin reporter plasmid in the

Fig. 5. Silencing of Prrx2 suppresses breast cancer migration and invasion *in vitro* and *in vivo*. (A) Representative migration images of Prrx2 silenced and control cells. (B) Representative invasion images of Prrx2 silenced and control cells. Quantifications of cells on the lower surface of the membrane were performed with five randomly selected fields and shown on the right panels. Scale bars, 20 μ m. Prrx2-shRNA and control cells were injected into the tail vein to establish a lung metastatic model of breast cancer. After 60 days, the mice were killed. (C) The photograph of nude mice with lung dissemination from each group. (D) The number of macroscopic lung metastases were evaluated (n=8). * $P < 0.05$.

Fig. 6. Silencing of Prrx2 inhibits the Wnt/ β -catenin signaling pathway. Immunoblotting of β -catenin in cytosolic (A) and nuclear (B) extracts of MCF-7 and MDA-MB-231 cells was carried out, expression of GAPDH and Histon 3 was used as a loading control, respectively. Densitometric analysis of β -catenin from three independent experiments shown below. (C) Nuclear β -catenin was decreased in Prrx2-shRNA by immunofluorescence. Scale bars, 50 μ m. (D) Silencing of Prrx2 decreased TOP-flash activity by TOP/FOP flash report gene assay. (E) Silencing of Prrx2 induced a reduction in cyclin-D1 expression. Results are presented as the mean $\pm$ SD of triplicate determinations from three independent experiments and shown on the right panels. * $P < 0.05$.

breast cancer. After transfection with the plasmids, the luciferase activity of cancer cells was determined (Fig. 6D). In addition, the downstream target gene cyclin-D1 of the Wnt/ β -catenin signaling was down-regulated after Prrx2 was silenced (Fig. 6E).

Discussion

Invasion and metastasis remain the main obstacles in the effective treatment of breast cancer [16, 17]. Recent evidence indicates that paired-related homeobox protein family play a critical role in tumor progression and drug resistance by promoting cell growth, immune escape and angiogenesis [18]. Previous study reported that Prrx1 over-expresses was associated with more aggressive capability and poor prognosis of patients with thyroid cancer and colorectal cancer [19, 20]. In addition, we reported that silencing of Prrx1b inhibits cellular proliferation, invasion and migration in triple-negative breast cancer [12]. However, the role of another number of paired-related homeobox protein, Prrx2, in tumors are not yet clear. In this study, we found that the expression of Prrx2 was associated with tumor size, lymph node metastasis and TNM stages in breast cancer. We also found that the Prrx2 expression increased with the rising clinical stage, indicating participate in the development of breast cancer. These results indicated that Prrx2 might serve as a potential prognostic marker and tumor promoter in breast cancer.

EMT plays an important role in the differentiation of tissues and organs and in the formation of the body plan, as well as contributes to cancer progression and other diseases [21, 22]. In the current study, we found that Prrx2 may participate in the EMT process, which is supported by the following evidence. First, the breast cancer lost mesenchymal characteristics and acquired epithelial properties by silencing of Prrx2. Second, our result demonstrated that silencing of Prrx2 significantly decreased the mesenchymal markers such as α -SMA and vimentin, while increasing the epithelial marker E-cadherin expression, which lead to strengthening cell-cell junctions. Third, the expression of Prrx2 in patients' tissues positively correlated with vimentin expression and negatively correlated with E-cadherin expression. Based on these findings, our data revealed that silencing of Prrx2 induced MET in breast cancer. Additionally, our study also showed that the proliferative capacity of cancer cells significantly decreased *in vitro* and tumor growth was suppressed *in vivo* after Prrx2 was silenced. Our data further indicated that knockdown of Prrx2 in breast cells significantly decreased cell migration and invasion *in vitro*. Importantly, an animal model confirmed that Prrx2 facilitated tumor formation and metastasis *in vivo*. Taken together, these data indicated that silencing of Prrx2 resulted in not only morphological change but also decreased invasion and migration of breast cancer through inhibiting EMT.

The Wnt/ β -catenin signaling pathway has long been established as one of the most important intracellular signaling pathways in the EMT of human cancer. To our knowledge, activation of the Wnt/ β -catenin signaling pathway has an important impact on the EMT in gastric carcinoma and contributes to carcinogenesis in colorectal cancers [23, 24]. These findings from our study have identified that the role of Prrx2 in EMT in breast cancer might be related to the regulation of β -catenin nuclear translocation and the activation of the Wnt/ β -catenin signaling pathway. It was discovered through nuclear/cytoplasmic fractionation assay that the expression of β -catenin in the nucleus decreased after Prrx2 was silenced, and immunofluorescence staining also revealed that the expression of β -catenin in the nucleus was decreased compared with the cells in the control group after Prrx2 was silenced. Moreover, we revealed that the activity of the Wnt/ β -catenin signaling pathway was decreased after Prrx2 silenced by using the luciferase reporter assay system. Cyclin-D1 is one of the important target genes downstream of the Wnt/ β -catenin signaling pathway [25, 26], silencing of Prrx2 could significantly inhibit the expression of cyclin-D1. The above experiments revealed that Wnt/ β -catenin signaling pathway may participate in Prrx2-induced EMT of breast cancer.

In conclusion, our findings demonstrated that Prrx2 expression was associated with tumor size, lymph node metastasis, TNM stages and poor survival in breast cancer. Furthermore, silencing of Prrx2 may suppress the proliferation, invasive and migration capacities of breast cancer by reversing the EMT phenotypes through repression Wnt/ β -catenin signaling pathway. Thus, our data suggested that Prrx2 might be served as a target for new therapeutic strategies against breast cancer.

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Disclosure Statement

The authors declare that they have no competing interests exist.

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