Prrx1 enhanced the migration ability of breast cancer cell by initiating epithelial-mesenchymal transition

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Abstract: To study the effect of paired related homoeobox1 (Prrx1) on the epithelial-mesenchymal transition (EMT) and migration ability of breast cancer cells. Breast cancer cell lines (including BT-474, ZR-75-1 and Mccoy's 5A) were filtrated by Real-time PCR to obtain the cells with the lowest expression of Prrx1. Then the cells were infected with Prrx1 over-expression lentiviral vectors. The phenotype changes of the cells were observed after the infection was verified to be performed through Real-time PCR. The expression of Twist1 protein was tested by Western blot. At last, the effect of Prrx1 on the migration ability of the breast cancer cell was determined by Transwell assay. Mccoy's 5A cells were screened to be the lowest Prrx1 expression cells of the three kinds of breast cancer cells. Then the Mccoy's 5A cells were infected by lentiviral vectors to be Prrx1 over-expressing. We observed that the morphology of these cells changed from polygon to long spindle appearance. The real-time PCR results showed that the expression of Prrx1, E-adherin and Vimentin was 225.80, 0.55, and 4.20 times compared with groups without transfection respectively (p<0.05). Western blotting results showed that the expression of Twist1 protein in experimental group was higher than two control groups (p<0.05). Transwell assay showed that the number of the migration cells in Prrx1 over-expression group (72.64□ 5.32)was higher than both non-treated group (39.52□ 3.87)and lentiviral vectors group (42.35□ 4.11) (p<0.05), while the difference between the two control groups were not statistically significant (p□ 0.05).Prrx1 up-regulation can prompt EMT of breast cancer cells and enhance their migration abilities.

1. Introduction

Breast cancer is one of the common malignancy Worldwide [1]. Basic research shows that the abnormal expression of Prrx1 is closely related to the occurrence and development of pancreatic cancer [2], lung cancer [3], colon cancer [4]. And other studies have shown that Prrx1 gene up-regulation in gastric cancer and malignant glioma cells can promote EMT and closely related to invasion and metastasis [5-6]. In lung cancer, Prrx1 gene expression can reverse the EMT and prompt the distant metastasis of the tumor [3], however, in colorectal cancer, Prrx1 gene over-expression can promote distant colonization of tumor cells [4]. It is indicated that different expression of Prrx1 in different tumor cells on the occurrence of tumor EMT and distant colonization and metastasis are not the same. Furthermore, Prrx1 up-regulation often accompanying with Twist1 expression mediate the production of EMT [7]. At present, There was few research about Prrx1 up-regulation accompanying Twist1 up-regulation promoting the occurrence of EMT, invasion and metastasis in breast cancer cells, We constructed Prrx1 over-expressing breast cancer cell line by lentiviral infection technique to observe the effect of Prrx1 over-expression on Twist1 in EMT, invasion and migration ability in vitro.

2. Materials and Methods

2.1. Cell culture

Breast cancer cells BT-474, ZR-75-1 and Mccoy's 5A were purchased from Shanghai cell bank. The three kind of breast cancer cells were cultured in RPMI1640 mediem supplemented with 10% fetal bovine serum (Hyclone, US), 100mg/ml streptomycin and 100U/ml penicillin (Hyclone, US) in a saturated humidity, 37 ℃, 5% CO_2 incubator. All cells were cultured in a logarithmic growth phase for experimental use.

2.2. Real-time PCR

Total RNA was extracted from the three kind of cancer cells using Trizol (TaKaRa, Japan) and 500 ng total RNA was reverse to cDNA. Real-time PCR was used to screen out the lowest expression of Prrx1 from the above three kind of cells. The reaction system is 20ul and the reaction conditions were as follows: 95^oC, 30s pre-denaturation; 98^oC, 10s degeneration; 60^oC, 30s annealing; after 40 cycles, 95 ^oC extension, 40^oC cooling. Every group set three sub-holes and every experiment was repeated in 3 times. The results were analyzed by cycle threshold

(ΔCt). The Prrx1 primer sequence was:
    F:ACAGGCGGATGAGAAC
    R:CTGAGTTCAGCTGGTCAT

2.3. Lentivirus transduction

Prrx1 over-expression lentiviral vector was customized to Kyrgyzstan company in Shanghai. The order of the components is: Ubi-MCS-EGFP-IRES-Puromycin. The experiment was divided into 3 groups: Mccoy's 5A cells without treatment and infected with negative Prrx1were used for negative control (NC) and blank control group. Mccoy's 5A cells infected with Prrx1 over-expressing lentiviral vector were used as experimental groups. The Mccoy's 5A cells were seeded in 12-well plates at a rate of 5×10^4 cells / ml at about 1.5ml per well. The complex infection index (MOI) 30 was obtained according to pre-experiment and the adding to the lentiviral vector when the cells reached approximately 40% confluency. After 72 hours of infected with Lentiviral vector we observe the expression of green fluorescent protein.

2.4. Western blotting analysis

The protein concentration was determined by BCA kit (Kangwei century company). Mouse anti-human primary antibody (actin)(Santa Cruz industry, US, diluted 1:1000). Goat anti-human Twis1 primary antibody (Raybiotech industry, US, diluted 1:500). Horseradish enzyme labeled goat anti-mouse IgG secondary antibody (Zhongshan Jinqiao Company, China, diluted 1:10000). Horseradish enzyme labeled mouse anti-goat secondary antibody (Jackson Immuno Research Laboratories Inc., diluted 1:10000). Equal quantities of 20μl total protein was loaded into per well and then subjected to electrophoresis, Transfer membrane and closed at room temperature for 1h. The membrane was hatched in primary antibody hybrid bag in the 4°C refrigerator overnight. Then the membrane was washed 3 times using phosphate buffer (PBS, Hyclone, US), each lasting 15min, the membrane was incubated with the secondary antibody for 1h. The protein brands and the protein were detected by enhanced chemiluminescence kit (VILBER, France).

2.5. Prrx1,E-cadherin, Vimentin mRNA Expression

After infected with lentiviral vector 72 hours, three groups of cells were collected and the total RNA was extracted and reverse transcribed into cDNA. Real-time PCR procedure was carried out according detail description of TaKaRa. Every well set three parallel wells and the Ct value was read. The results of the experiment were analyzed using the value of $2^{-\Delta\Delta Ct}$. Protein primer was:

E-cadherin   F:ACACCATCCTCAGCCAAGA
R:CGTAGGGAAACTCTCTCGGT
Vimentin
F:GAGTCCACTGAGTACCGGAGAC
R:TAGGTGGCAATCTCAATGTC
GAPTH   F:GGGTGTGAACCATGAGAA
R:CATGGACTGTGGTCATGAG

2.6. Migration assay

Migration assays were performed using Transwell (Corning Life Science, Corning, NY, USA) 72 hours after infection, the cells of three groups were digested with trypsin and suspended with serum free medium at a density of 3×10^8 cells/L. 200ul cells were respectively added to the upper Transwell chamber and 600ul RPMI1640 supplement with FBS was added to the bottom chamber. After incubation at 37°C, 5% CO_2 incubator 24 hours, the cells were fixed 30 min with 5% pentanediol at 4°C and stained with crystal violet. Five fields were selected to observe with microscope and each assay was performed for three times.

2.7. Statistical analysis

SPSS19.0 statistical software analysis, multi-group average comparison using single factor analysis of variance, the two groups were compared using LSD-t test, $P<0.05$ for the difference was statistically significant.

3. Results

3.1. Screening Prrx1 lowly expressed cell lines by Real-time PCR

The expression of Prrx1 mRNA in breast cancer BT-474, ZR-75-1 and Mccoy's 5A cells were determined using real-time PCR. The results showed that the expression of Prrx1 mRNA in Mccoy's 5A cells was the lowest one in the three kind of cell lines. So we chose Mccoy's 5A cell for subsequent testing (Figure 1).
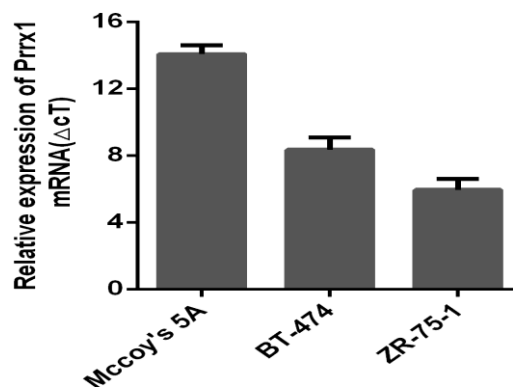

Figure 1. The value of ΔcT of the relative expression of Prrx1 mRNA(x̄±s).

3.2. Transduction Efficiency Observation

All the photos taken under 200 times of the microscope after the cells were infected with Lentiviral vector at 72h. We observe the same field of vision under ordinary light and fluorescence in the fluorescence microscope respectively. The number of fluorescent cells accounted for 80% -90% of the total cells was defined as successful of infection in the majority of experiments. The two control groups showed closely conjunction with each other, such as "cobblestone-like clusters" and the experimental group appear to long spindle appearance and became loose under ordinary light. The cells in the blank control and experimental groups showed green fluorescence and the infection efficiency was more than 80% under fluorescence, which proved successful infection of Lentiviral vector (Figure 2).
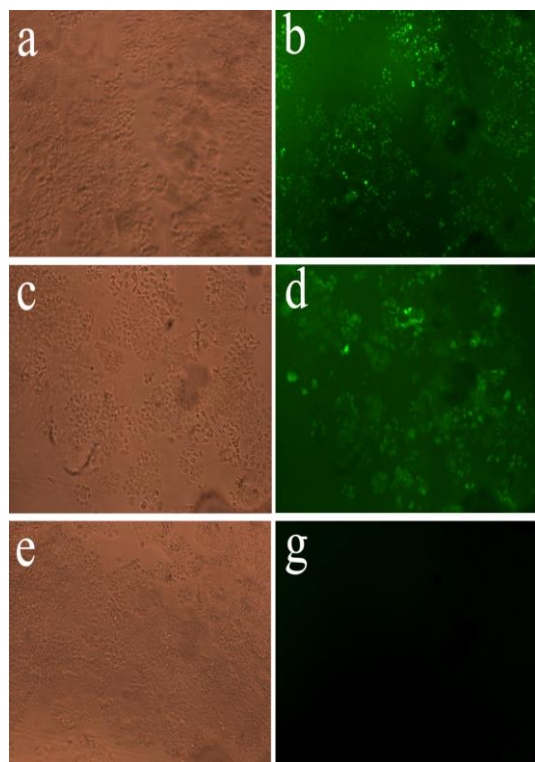

Figure 2. Image of Mccoy's 5A cells infected with lentiviral vector by fluorescence microscopy after 72 hours. Image of experimental group(a, b), Negative control group(c, d) and blank control group(e, f) under white and fluorescence microscope, respectively   (magnification×200).

3.3. Prrx1, E-cadherin, Vimentin mRNA Expression

After infected Lentiviral vector 72h, our results shows that there was no significant difference between the two control groups in the expression of Prrx1 Vimentin and E-cadherin (p>0.05). While the difference between the experimental group and the negative control group were statistically significant (p<0.001). The mRNA level of Prrx1 in experimental group was 225.80 times than negative control group .Vimentin was 4.20 times than negative control group and E-cadherin was 0.55 times than negative control group. The results showed that Successful infection of Prrx1 over-expression of lentiviral vector and the relative expression of E-cadherin of epithelial phenotype was decreased and the relative expression of Vimentin of phenotype interstitial was increased (Figure 3).

3.4. The expression of Twist1 protein

Western blot showed that the expression of Twist1 protein in the experimental group was significantly higher than that in the negative and blank control groups (Figure 4). Suggesting that Prrx1 up-regulation accompanying Twist1 up-regulation.

3.5. Prrx1 prompting the ability of migration of Mccoy's 5A cell by inducing EMT

We examined whether Prrx1 promotes Mccoy's 5A cell migration by inducing EMT. Our Transwell assay shows that Prrx1 over expression Mccoy's 5A cells had obviously higher migration capacities compared with Mccoy's 5A cells infected with negative Prrx1 (NC) and Mccoy's 5A parental cells(blank control) (p<0.05, Figure 5). Our data indicate that Prrx1 up-regulated Mccoy's 5A cells acquired higher migration behaviors compared with NC and blank control cells, while there was no significant difference between the two groups (p<0.001).

4. Discussion

The underlying mechanism of invasion and metastasis in malignant tumor is still not understood, the process is very complex, including: local cancer cell interstitial transformation, actin skeleton recombination, micro-environment formation and colonization formation [6]. In recent years, interstitial transformation has been paid attention to the research in cancer cell invasion and metastasis [7]. Transcription factors related to EMT include SNAI1, SNAI2, ZEB1, ZEB2, Twis1 [8]. However, Prrx1, a new EMT inducer, leads to complete EMT in cancer cells [2].After the occurrence of EMT, cell loss inherent epithelial phenotype and obtain interstitial phenotype, which conducive to invasion, migration and stretching of cells[9,10]. The results showed that after the breast cancer Mccoy's 5A cells was infected with lentiviral vector, the mRNA level of Prrx1 in experimental group was significantly increased than the two control groups, which proved

that Prrx1 gene has been successfully transfected into breast cancer Mccoy's 5A cells. After the transfection of Prrx1 we observed that the morphology of the cells changed from polygon to long spindle appearance under the microscope. Due to the lack of mutual restraint, the polarity of these type of phenotype cells is very high and form the trend of easy to distant metastasis. In general Prrx1 overexpression can induce EMT in Mccoy's 5A breast cancer cell lines and provide a histological basis for breast cancer metastasis.
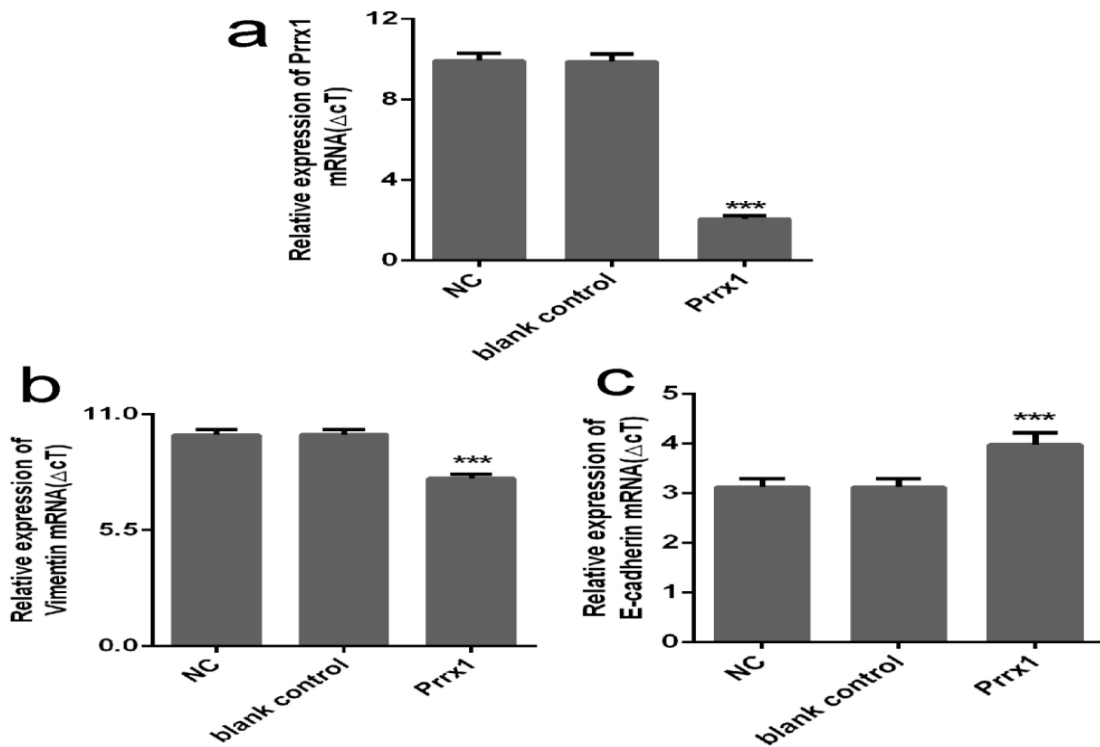

Figure 3. RT-PCR analysis of Prrx1,E-cadherin, Vimentin relative expression in three different groups (a,b,c) The value of ΔcT of the relative expression of mRNA(x̄±s). a Relative expression of Prrx1 mRNA between Prrx1 over expression group and NC, blank control cells (P<0.05). b Relative expression of Vimentin mRNA between Prrx1 over expression group and NC, blank control cells (P<0.05). b Relative expression of E-cadherin mRNA between Prrx1 over expression group and NC, blank control cells (P<0.05) the difference between NC and blank control group was not statistically significant (*p*>0.05).
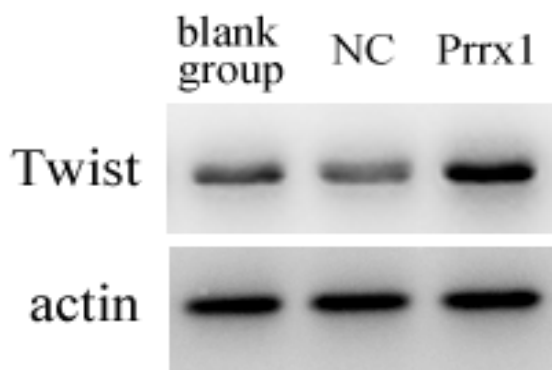

Figure 4. The expression of Twist1 protein between blank control group, negative control group and experimental group(p<0.05).
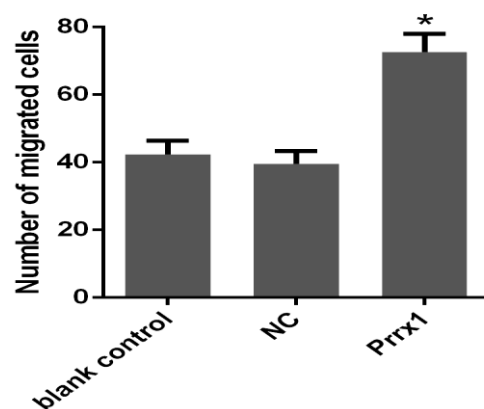

Figure 5. Measurement of migration properties in Mccoy's 5A cells transfected with Prrx1 over expression group, NC and blank control(P<0.05).

Epithelial phenotype E-cadherin is a tumor suppressor that plays an important role in the normal epithelial adhesion process. When it expresses down, tumor epithelial cells develop into EMT [11,12]. Furthermore, E-cadherin deficiency is related to the ability of invasion and metastasis in cancer cells[13], Interstitial phenotypic Vimentin, mainly expressed in mesenchymal, were abnormal expression in many epithelial tumors and closely related to differentiation, invasion and metastasis in cancer cells. During EMT process, interstitial markers such as Vimentin is up-regulation and epithelial markers such as E-cadherin is down-regulation [14,15]. Our study shows that the expression of E-cadherin is decreased and the expression of Vimentin is increased in Prrx1 over-expression Mccoy's 5A cells. The changes of protein expression make cells changing from stable epithelial cells phenotype into unstable stromal cells phenotype. Therefore, the cells were easily to distant metastasis and provide a molecular basis for breast cancer metastasis. After transfection with Prrx1, Mccoy's 5A cells not only occur EMT but also accompanying with Twist1 high expression comparing with other two control groups. This illustrates Prrx1 coordination with Twist1 trigger EMT in breast cancer cells. The results also showed that the ability of invasion and migration in Prrx1 overexpression of breast cancer Mccoy's 5A cells was significantly increased. In conclusion, the EMT was inducted by Prrx1 and/or Twist1 enhancing invasion and metastasis of cancer cells.

The study shows that Prrx1 overexpression after accompanying Twist1 up-regulation induce EMT in breast cancer cells and enhance the ability of cell migration. Which may be one of the important mechanisms of invasion and metastasis of breast cancer cells.Twist1 may be the downstream target gene of Prrx1, but its molecular mechanism needs to be further studied.

Acknowledgements

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