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Original Article

Overexpression of EPDR1 has an antitumorigenic effect on breast cancer in vitro

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Abstract: Background: EPDR1 is widely expressed in cancer, especially colorectal cancer. However, the biologic function of EPDR1 in breast cancer is uncertain. Methods: The expression profile of EPDR1 was assessed by Gene Expression Profiling Interactive Analysis (*GEPIA*; gepia.cancer-pku.cn). We constructed EPDR1-overexpressing (EPDR1-Ov) plasmids that were transfected into breast cancer cells (MCF-7 and MDA-MB-453) to examine the EPDR1 effect on their malignant behavior. The EPDR1 overexpression and the critical components of the P53 signaling pathway were determined by western blot or RT-PCR. Cell proliferation, colony formation, invasive capacity, and cell apoptotic proportions were examined after transfection. Results: mRNA expression of EPDR1 was significantly lessened in breast cancer tissues when compared to the adjacent normal tissues by data analysis from GEPIA. There was an impairment in proliferative ability, viability, invasion, and anti-apoptotic effect in EPDR1 overexpressed breast cancer cells. Mechanistic studies showed that EPDR1 overexpression increased the p53, p21 and Bcl-2 expression while inhibiting Bax expression. Conclusion: EPDR1 inhibited malignant behaviors and promoted apoptosis in breast cancer cells by activation of the p53 signaling pathway.

Keywords: Breast cancer, EPDR1, invasive capacity, proliferation, viability, P53

Introduction

Epithelial tumor transformation of breast tissue leading to breast cancer ranks as one of the four most prevalent cancers globally. In the USA alone, this disease contributed to 279,100 newly diagnosed cases and 42,690 deaths in 2019 [1]. Considerable advancement has been made in screening techniques and targeted therapies, causing improvement of the 5-year relative survival rate [2-4]. However, genetic variants and mutations that are known to be associated with the carcinogenesis and metastasis of breast cancer lead to rapid deterioration of the patient [5, 6]. Therefore, to improve its prognosis and decrease morbidity and mortality, it is necessary to understand the underlying etiological factors as well as pathological mechanisms of the cancer.

Ependymin related 1 (EPDR1), also known as EPDR, UCC1, MERP1; MERP-1 is a member of mammalian ependymin-related proteins (MERPs). The protein shares a conserved critical amino acid and primary structure with piscine pendymins, which is a type II transmembrane protein and opposes cell adhesion by intracellular and extracellular mechanisms [7, 8]. EPDR1 is differentially expressed in a wide range of tissues [9] including various malignant tissues and cell lines. This suggests EPDR1 may play a vital role in the occurrence or progression of certain cancers [10]. In human colorectal cancer overexpression of EPDR1 can facilitate the viability and invasion of cancer cells and serve as a reliable prognostic marker [5, 11, 12]. Data mined from the TCGA database revealed that EPDR1 expression may be down-regulated in certain types of cancer such as uterine carcinosarcoma [13]. Due to the varied expression of EPDR1 in different cancers, it is thought to possess a complex function in carcinogenesis and metastasis.

In the present work, the mRNA level of EPDR1 in breast cancer tissue is compared with nor-

mal tissues, and the data were analyzed using the GEPIA database. EPDR1 was further overexpressed in breast cancer cells, namely, MCF-7 and MDA-MB-45, aiming to reveal the role of EPDR1 in cell proliferation, viability, invasion, and apoptosis.

Materials and methods

Cell culture, maintenance, and antibodies

Two breast cancer cell lines, MCF-7, and MDA-MB-453 were obtained from the Wuhan University Type Culture Collection (Wuhan, China). Both breast cancer cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were maintained in a 37°C cell culture incubator. Anti-EPDR1 and anti-GAPDH antibodies were acquired from Proteintech Group, Inc., Wuhan, Hubei.

EPDR1 overexpression and transfection

For the overexpression of EPDR1, the primer sequence of the human EPDR1 gene was constructed as 5'-TAGGTTGGATGGAGTGTAGTGGTAT and 3'-ACTCCCATTCCCAATAAAAAATCTA. The EPDR1 cDNA was further cloned into a pAd-Easy/CMV plasmid vector (named EPDR1-Ov). For every well of 12-well plate $1*10^4$ MCF-7 and MDA-MB-45 cells at 70-80% confluence were transfected with 0.50 μg EPDR1-Ov or its corresponding empty vector (named NC) using Lipofectamine 2000 for 48 h. The overexpression of EPDR1 was finally validated by western blot.

Cell proliferation assay

The cell viability of MCF-7 and MDA-MB-453 cells was elucidated by the Cell Counting Kit-8 (CCK-8) following the manufacturer's protocol. Briefly, $1×10^3$ cells were incubated in a 96-well plate for 4 h. Further, 100 mL of culture medium containing 10% CCK-8 reagent was added to each well at a specific time interval (0th day, 1st day, 2nd day, and 3rd day) followed by continuous culture for 2 h. The absorbance was measured at 450 nm by a Multiskan.

Colony formation assay

The transfected MCF-7 and MDA-MB-453 cells were cultured, maintaining a density of 1000 cells/well in 10 cm plates for two weeks. Cell colonies over 0.5 mm diameter were taken into consideration and counted. Furthermore, the cells were fixed, stained with 0.5% crystal violet, and photographed.

Cell invasion assay

For quantification of the motility of transfected cells, a transwell insert was added to the Matrigel layer on the 24-well plate. The transfected cells ($1×10^5$) were seeded onto the upper compartment, which was placed in the lower compartment containing 2.6 mL of DMEM. The transwell plate was incubated for 2.5 h. The migrated cells were fixed by the addition of 5% glutaraldehyde. Crystal violet was subsequently added for staining the cells which were quantified under the microscope.

Flow cytometric analysis

The overexpressed-EPDR1 and the control of breast cancer cells were maintained in the 6-well plates for two days. The harvested cells were then trypsinized and washed. Acridine orange (AO) and propidium iodide (PI) was used for staining the cells for 15 min. Flow cytometry was employed to monitor the apoptotic cells.

Western blot assay

Cells were harvested, and to them, 30 μL ice-cold radioimmunoprecipitation assay (RIPA) buffer was added for cell lysis. The protein concentration of cell lysate was quantified following Bicinchoninic Acid (BCA) protein assay. Protein lysate of 25 μg was run on 12% SDS-PAGE. The proteins were transferred to blotting membrane by electroblotting technique. Blocking buffer was added onto the blot and incubated to prevent any unspecific binding. Subsequently, the primary antibody was added against EPDR1, P53, or GAPDH followed by treatment with the secondary antibody. The blots were developed following the instructions provided with the kit. GAPDH used as endogenous control.

Gene expression analysis

RNA from the cells was isolated with TRIzol reagent followed by RNase-free DNase digestion. iScript cDNA synthesis kit (Bio-Rad USA) was utilized for cDNA synthesis. TaqMan Gene Expression Assay protocol was followed for the quantification of mRNA expression.

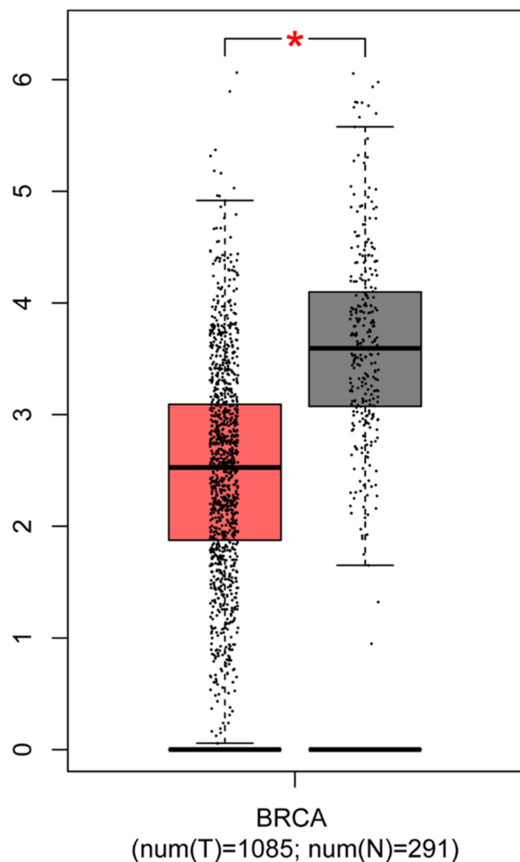


Figure 1. Expression of EPDR1 in breast cancer patients. Down-regulated expression of EPDR1 in breast cancer patients when compared with adjacent normal tissues by an open database, namely, GEPIA database.

Transcript level analysis of EPDR1, p53, p21, Bax, and Bcl2 was carried out by the $2^{-\Delta\Delta Ct}$ method.

Bioinformatic analysis and statistical analysis

The EPDR1 mRNA level in breast cancer was investigated by the GEPIA database (http://gepia.cancer-pku.cn). The unpaired Student's t-test was adopted to analyze the EPDR1 mRNA difference between 1081 invasive breast carcinoma (BRCA) tissues and 291 normal tissues from TCGA gene expression data. |Log2FC| Cutoff and *p*-value Cutoff were set as 1 and 0.01, respectively.

The data collected were compared between two groups with the help of a nonparametric test using Prism GraphPad 8. Differences were considered significant when $P < 0.05$.

Results

Reduced expression of EPDR1 mRNA in breast cancer tissues in TCGA

EPDR1 mRNA expression was investigated in invasive breast cancer (BRCA) compared with normal controls following the instruction of GEPIA website creator. The expression of EPDR1 was downregulated in breast cancer tissues (**Figure 1**).

Overexpression of EPDR1 inhibits proliferation and promotes apoptosis of breast cancer cells

To further investigate the EPDR1 in breast cancer, EPDR1-overexpressing plasmid was constructed and infected into MCF-7 and MDA-MB-453 cells. RT-PCR analysis and western blotting technique were used to validate the efficacy in the overexpressed cells. The exogenous EPDR1 expression was found to be significantly enhanced when compared with the cells infected with plasmids without constructs (**Figure 2A** and **2B**). The CCK8 and colony formation assays were carried out to assess whether the overexpression of EPDR1 influences the viability of breast cancer cells. As shown in **Figure 2C** and **2D**, the proliferative rate of cells overexpressing EPDR1 cells decreased in comparison to that of the blank control cells. Similarly, the colony formation capacity of EPDR1-overexpressing cells was attenuated when compared to the control group (**Figure 2E-H**). Apoptosis plot obtained from flow cytometry revealed that EPDR1 promoted apoptosis of breast cancer cells (**Figure 3**). Thus, ectopic expression of EPDR1 might suppress the proliferation and viability, and significantly promote apoptosis of breast cancer cells.

Overexpression of EPDR1 inhibits the migration of breast cancer cells

The transwell migration assay was performed to examine further the effect of EPDR1 on tumorigenicity of breast cancer cells. The results revealed that wild-type MCF-7 and MDA-MB-453 cells had higher migration ability compared with the EPDR1-overexpressing cells (**Figure 4**). This study suggests EPDR1 may suppress the migration of breast cancer cells.

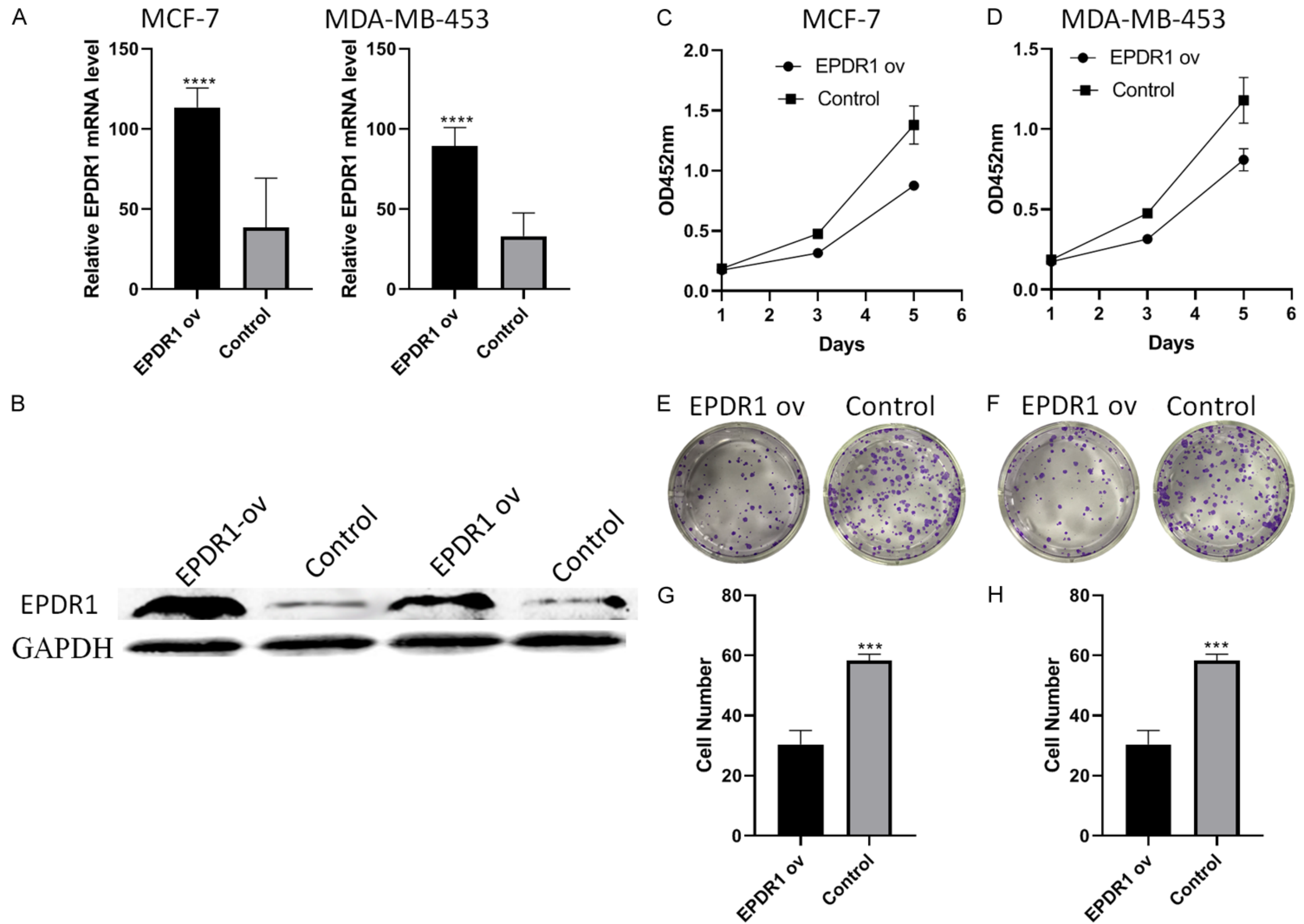
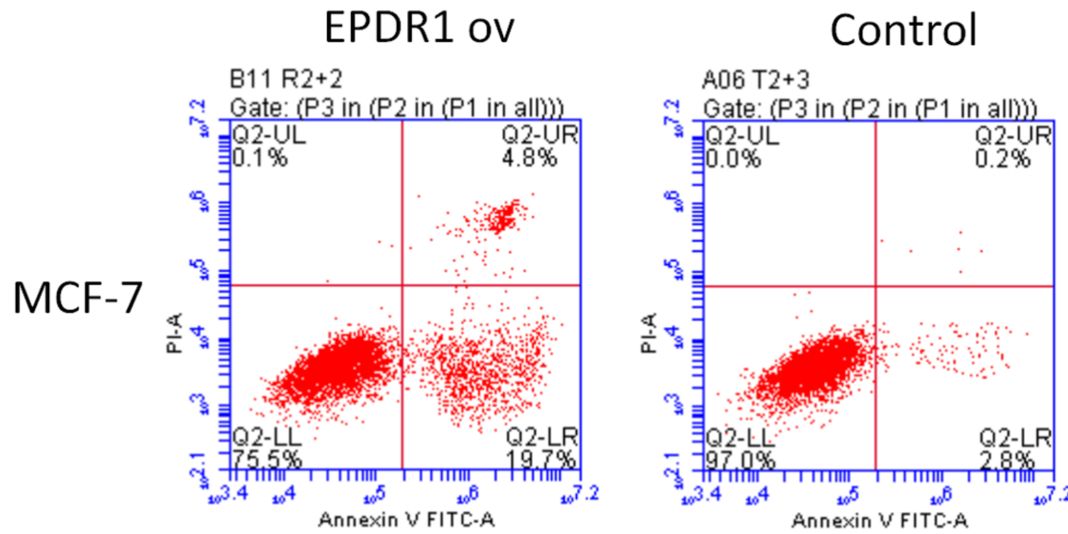
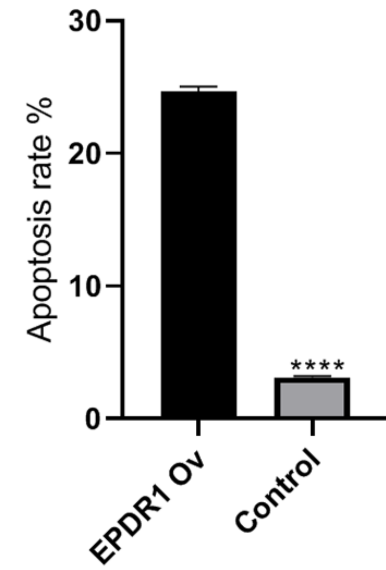
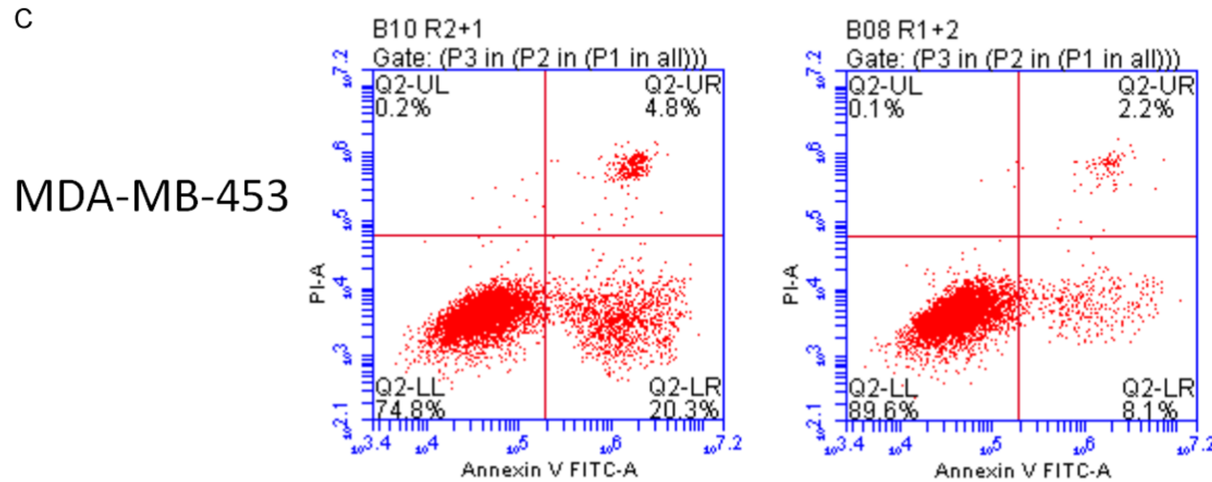


Figure 2. Overexpressing EPDR1 inhibited cell proliferation, and colony formation efficiency. (A and B) RT-PCR analysis to estimate the EPDR1 mRNA in both cell types. Western blot results corroborated the efficiency of EPDR1 overexpression in both cell types. (C and D) CCK8 assay indicating the proliferation of overexpressing-EPDR1 cells. (E-H) Demonstration of the colony formation capacity of overexpressing-EPDR1 cells by colony formation assay. ****$P < 0.0001$ vs. control; ***$P < 0.001$ vs. control.


A
EPDR1 ov
Control
MCF-7
B11 R2+2
Gate: (P3 in (P2 in (P1 in all)))
Q2-UL 0.1%
Q2-UR 4.8%
Q2-LL 75.5%
Q2-LR 19.7%
A06 T2+3
Gate: (P3 in (P2 in (P1 in all)))
Q2-UL 0.0%
Q2-UR 0.2%
Q2-LL 97.0%
Q2-LR 2.8%
PI-A
Annexin V FITC-A


B
Apoptosis rate %
****
EPDR1 Ov
Control


C
MDA-MB-453
B10 R2+1
Gate: (P3 in (P2 in (P1 in all)))
Q2-UL 0.2%
Q2-UR 4.8%
Q2-LL 74.8%
Q2-LR 20.3%
B08 R1+2
Gate: (P3 in (P2 in (P1 in all)))
Q2-UL 0.1%
Q2-UR 2.2%
Q2-LL 89.6%
Q2-LR 8.1%
PI-A
Annexin V FITC-A

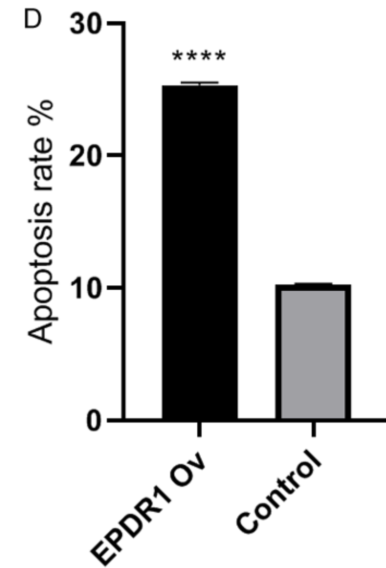

D
Apoptosis rate %
****
EPDR1 Ov
Control

Figure 3. Flow cytometry analysis was done to monitor the apoptotic cells after overexpressing-EPDR1 MCF-7 and MDA-MB-453 cells. A and C. An apoptosis assay revealed the overexpression of EPDR1 resulted in cell apoptosis. In the dot plots, top left quadrant: dead cells; bottom left quadrant: living cells; bottom right quadrant: cells in early apoptosis; and top right quadrant: cells in late apoptosis. B and D. Apoptosis rate was assessed in EPDR1-overexpressing cells and normal controls. ****$P < 0.0001$ vs. control.

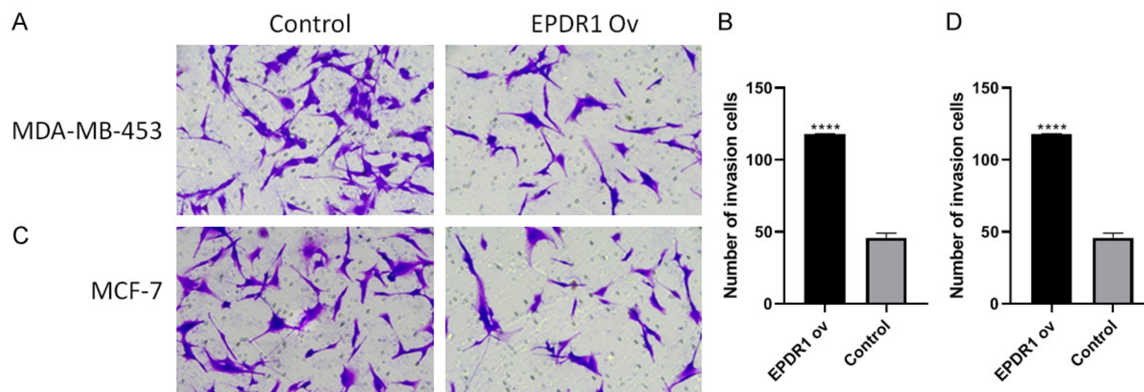


Figure 4. The invasive capacities after overexpressing-EPDR1 in MCF-7 and MDA-MB-453 cells. **$P < 0.01$ vs. control; ****$P < 0.0001$ vs. control.

Overexpression of EPDR1 activates P53 signaling pathway

Since P53 signaling pathway is transcriptionally or translationally alternated in cancer progression [14], we examined the P53 expression and its critical downstream effectors (P21, bcl-2, Bax) in EPDR1-Ov MCF-7 and MDA-MB-453 cells. The results showed that P53 was enhanced in EPDR1-ov breast cancer cells compared to the corresponding NC cells. Subsequent RT-PCR analysis also revealed that EPDR1 overexpression dysregulated the P53 mRNA level. In addition, we also identified increased expression level of P21 and Bax as well as reduced expression level of Bcl-2 in breast cancer cells (**Figure 5**).

Discussion

We have reported the first account of the biologic role of EPDR1 in malignant behavior of breast cancer cells and its underlying mechanisms. EPDR1 has a possible anti-tumor role and a pro-apoptotic effect in MCF-7 and MDA-MB-453 cells, at least partially by activating P53 signaling pathway.

The overexpression of EPDR1 was observed first in fish, and later it was found to be widely distributed in human tissues as well as in certain cancer cells [8, 12, 15]. Recently, a study done by Gimeno-Valiente et al. exhibited that expression of EPDR1 is elevated in human colorectal cancer tissues and it serves as an adverse diagnostic marker [13]. In the present study, downregulated EDPR1 was observed in breast cancer tissues from the GEPIA data. These data differed from the results exhibited in patients diagnosed with colorectal cancer (CRC) [13], which may be supportive of the notion that expression of EPDR1 behaves in a contrasting way in various tissues.

A series of *in vitro* assays was performed based on the above experimental outcomes to reveal the biological function of EPDR1 in breast cancer cells. By overexpression EPDR1 in MCF-7 and MDA-MB-45 cells, it was observed that EPDR1 inhibited cell viability and invasion, and stimulated cell apoptosis, suggesting that EPDR1 may inhibit tumorigenesis as well as prevent progression of breast cancer. However, recent research indicated that in SiEPDR1 colorectal cancer cells also showed a reduction of malignant behaviors of cancer when compared with the parental cells, indicating a protumorigenic role of EPDR1, which is contradictory to the results from the present study [12, 13]. Therefore, from the literature, it can be speculated that EPDR1 may function both ways in a context-dependent manner. This dual regulatory mode is common in tumor progression [16].

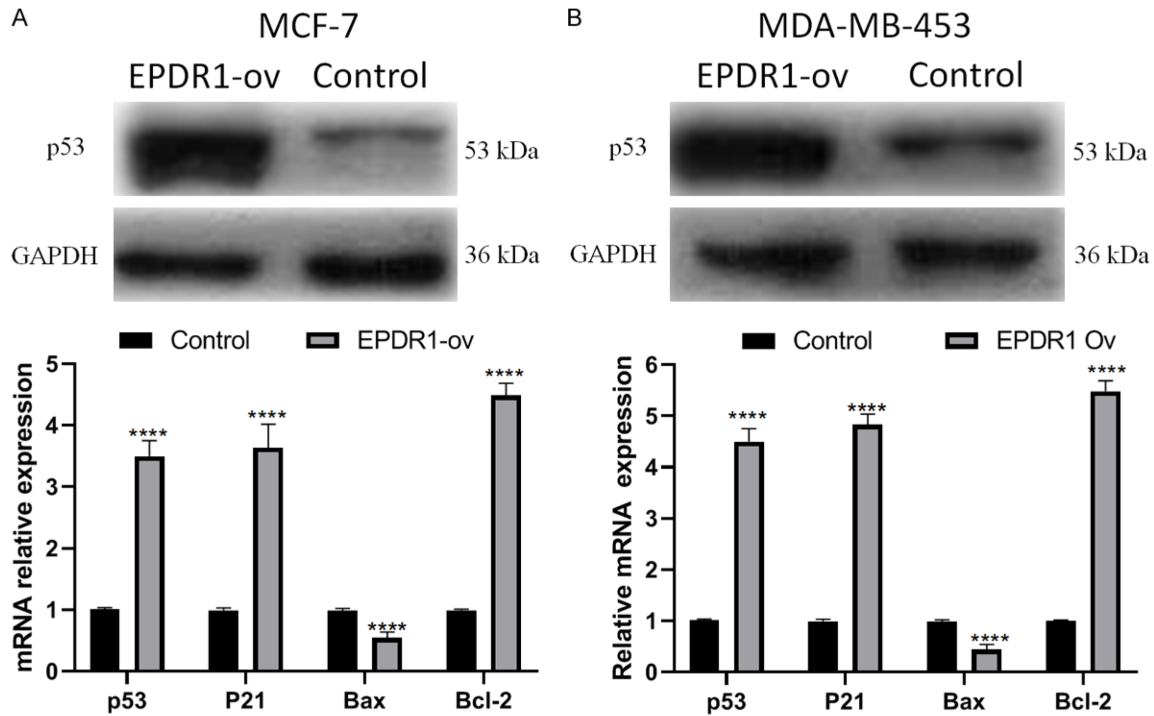


Figure 5. Enforced expression of EPDR1 activated p53 signaling pathway. The p53 and its crucial effectors p21 and Bax showed a considerably upregulated expression while bcl-2 was attenuated in MCF-7 (A) and MDA-MB-453 (B) cells. ****P* < 0.0001 vs. its NC.

p53, a multifunctional anti-tumor factor, mediates a potent tumor-suppressive effect by hyperactivation or crosstalking with various signaling circuits [17-19]. Functional defects of P53 were examined in diverse types of tumors which can disturb normal cell and tissue hemostasis, progressively resulting in tumor transformation [20]. Due to its clinical applicability, multiple lines of study demonstrated that P53 can be a target of therapy for cancer patients [14, 21, 22]. In our study, we found that ectopic expression of EPDR1 could significantly increase the p53 signaling pathway to modulate the breast cancer cell phenotype. Hence, EPDR1 might exert its anti-tumor activity by inactivation of the P53 signaling pathway. As for the underlying mechanism of EPDR1 in cancer progression, two simultaneous studies, done by Chu et al. and Gregorio-King et al. demonstrated that differential methylation of EPDR1 may be involved in the progression of CRC patients [12, 15]. Similar to ependymins, EPDR1 may be associated with cell-cell extracellular matrix interactions that facilitate EMT [23, 24]. The detailed molecular mechanistic pathway of EPDR1 and its role in *in vivo* models should be studied.

In conclusion, EPDR1 is downregulated in breast cancer tissues and functions a tumor suppressor in breast cancer cells by deactivating the p53 signaling pathway. EPDR1/p53 might be a new avenue for breast cancer diagnosis and therapy.

Disclosure of conflict of interest

None.

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