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Research

PWRN1 low expression facilitates cancer progression and is an unfavorable prognosis factor in breast cancer

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Abstract

Background Triple-negative breast cancer (TNBC) has a poor prognosis due to metastasis and recurrence. The LncRNA PWRN1 is lowly expressed in breast cancer, so we investigated the regulatory mechanism and prognostic value of PWRN1 in TNBC.

Methods 135 TNBC patients were included. PWRN1 levels and miR-21-5p level were analyzed by RT-qPCR. Independent prognostic factors for TNBC were analyzed using multifactorial Cox regression. CCK-8 was used to test the proliferative capacity of cells. The effects of PWRN1 and miR-21-5p on cell migration and invasive properties were analyzed by Transwell, and their target binding relationship was reported by DLR assay.

Results PWRN1 was lowly expressed in TNBC patients, while miR-21-5p was highly expressed. PWRN1 expressions were downregulated and miR-21-5p levels were upregulated in TNBC tissues. miR-21-5p is a downstream target gene of PWRN1 and their expression is negatively correlated. Overexpression of PWRN1 led to low expression of miR-21-5p, which reduced cell proliferation, migration, and invasion. In addition, PWRN1 was an independent prognostic factor. Patients in the PWRN1 low expression group had shorter overall survival.

Conclusions PWRN1 may be a prognostic marker for TNBC. Overexpression of PWRN1 leads to low expression of miR-21-5p, which affects cell proliferation, migration, and invasive behaviors, thereby preventing poor prognosis in TNBC patients.

Keywords PWRN1 · MiR-21-5p · Triple-negative breast cancer · Prognostic marker

1 Background

Breast cancer (BC) is frequently found in the female population, with the incidence rate increasing every year and the disease occurring at an increasingly younger age [1]. Among women in the United States, BC is reported to have the highest prevalence rate at 31% and the second-highest mortality rate at 15% [2]. BC has no obvious symptoms in the early stages, often presenting as a painless lump in the breast, which is difficult for most patients to detect in time [3]. It is only recognized when there are obvious symptoms such as breast skin indentation, redness, and nipple abnormalities [4]. As a result, the best time for treatment is often missed. In recent years, advances in cervical cancer screening have led to early detection and treatment of breast cancer. However, the prognosis for breast cancer remains poor due to drug resistance and recurrence [5]. BC can be divided into four subtypes. Among them, triple-negative breast cancer (TNBC) refers to the subtype that is based on the expression of human epidermal growth factor receptor 2 (HER2), progesterone

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receptor (PR), and estrogen receptor (ER) on the surface of the tumor cells are all negative [6]. It is the most aggressive subtype of BC [7]. Triple-negative breast cancer (TNBC) has the worst prognosis and is associated with a high rate of metastasis and low patient survival [8, 9]. The lack of effective biomarkers for the development of TNBC has led to limited and suboptimal treatment [10]. Therefore, the search for an effective prognostic biomarker for the development of TNBC is of great importance. Novel biomarkers such as lncRNA are often used as an effective biomarker to determine the prognostic outcome of the disease.

Long non-coding RNA (lncRNA) is a non-coding RNA molecule that is longer than 200 nucleotides and cannot code for proteins [11, 12]. LncRNA is associated with the development of malignant tumors and has the potential to become a new biomarker for the diagnosis and prognosis of tumors and even for targeted gene therapy [13]. LncRNAs can often contribute to tumor development by modulating epigenetic or regulating signaling pathways and protein interactions to promote tumorigenesis [14]. For example, the lncRNA CRNDE promotes glioma cell growth and invasion through the mammalian target of rapamycin (mTOR) signaling pathway [15]. They can make an important contribution to diagnosing TNBC status, identifying drug targets, and assessing the efficacy of therapeutic interventions. And PWRN1, as a lncRNA, has great potential as a BC biomarker.

Available studies have reported that the LncRNA PWRN1 is aberrantly expressed in a variety of cancers [16], with low expression in BC [17]. However, the expression of PWRN1 in TNBC is not yet known. The regulatory role of PWRN1 in TNBC has not yet been reported by researchers. In addition, hsa-miR-21-5p is a target miRNA of PWRN1 in glioblastoma [18], and miR-21-5p is also a tumor promoter in BC [19]. However, the clinical role of PWRN1 in TNBC and whether the underlying mechanism is related to miR-21-5p are unknown. We hypothesized that PWRN1 and miR-21-5p are similarly aberrantly expressed in TNBC and that miR-21-5p could act as a downstream target gene of PWRN1 to jointly participate in the regulation of TNBC progression. Therefore, by conducting clinical samples and in vitro cellular studies, this study aimed to investigate the expression of PWRN1 and miR-21-5p in TNBC and their effects on the physiological functions of breast cancer cells, to explore the potential regulatory mechanisms of both on TNBC, and to explore the new avenues that PWRN1 and miR-21-5p bring to the treatment of TNBC.

2 Materials and methods

2.1 Patients included

The research followed the tenets of the Declaration of Helsinki and was approved by the Ethics Committee of The Affiliated Hospital of Shaanxi University of Chinese Medicine. In addition, all participants gave informed consent.

135 TNBC patients who attended The Affiliated Hospital of Shaanxi University of Chinese Medicine from March 2017 to January 2019 were included. Inclusion criteria: a) all met the relevant diagnostic criteria of TNBC; b) all were patients with first-ever, primary TNBC and met the indications for surgery; c) no treatment such as radiotherapy before surgery. Exclusion criteria: a) those with coagulation disorders; b) those with hepatic and renal failure; c) patients with BC other than TNBC; d) those with other malignant tumors. All patients were treated with surgical resection and the patient's tumor tissues and paracancerous normal tissues (confirmed by pathological examination) were collected. The tissues were stored at −80℃ for preservation. All patients were followed up for 3–60 months after surgery. Recurrence, metastasis, and TNBC-related death were considered terminal events for the follow-up investigations. The specific clinical data of the patients are described in Table 1.

2.2 Cell culture and transfection

Four breast cancer cells (MDA-MB-231, MDA-MB-436, BT-549, HCC1806) and human normal mammary epithelial cells MCF-10 A were selected for the in vitro cell experiments and were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). MDA-MB-231, MDA-MB-436, BT-549, and HCC1806 were grown in DMEM medium (with 10% FBS, 10 mg/mL streptomycin, 100 U/mL penicillin; Gibco, USA) and MCF-10 A was grown in DMEM/F12 (1:1) medium (with 10% fetal bovine serum, 10 mg/mL streptomycin, 100 U/mL penicillin). The incubation temperature was 37 ℃ and the carbon dioxide concentration was 5%.

PWRN1 overexpression plasmid (oe PWRN1) and empty vector (oe NC), as well as miRNA blank control (miR NC) and miRNA mimic (miR mimic) were purchased from RiboBio (Guangzhou, China). Transfection was performed according to the steps of the Lipofectamine 3000 kit (Invitrogen, USA). HCC1806 and MDA-MB-231 cells were inoculated into 6-well

Table 1 Correlation between PWRN1 expression levels and clinical features in TNBC patients

Parameters	Total n = 135	PWRN1 expression levels		*P*
		Low (n = 69)	High (n = 66)	
Age				
< 50	72	36	36	0.782
≥ 50	63	33	30	
Menopause				
NO	66	37	29	0.303
YES	69	32	37	
Tumor size (cm)				
≤ 2	75	35	40	0.299
> 2	60	34	26	
Histological grade				
G1 + G2	76	33	43	0.056
G3	59	36	23	
Lymph node metastasis				
NO	87	39	48	0.072
YES	48	30	18	
TNM stage				
I + II	81	35	46	**0.035**
III	54	34	20	

P-values less than 0.05 in the table are highlighted by bolding

plates at a density of 3.0×10^5 cells/well. The oe PWRN1, empty vector (2 μg), as well as miR NC and miR mimic (100 nM), were added to HCC1806 and MDA-MB-231 cells, and the medium and transfection system was mixed well, placed in an incubator, and transfected for 6 h.

2.3 Real-time quantitative reverse transcription PCR (RT-qPCR)

PWRN1 and miR-21-5p were detected by RT-qPCR. Frozen tissue samples were thoroughly crushed and 1 mL of Trizol reagent was added to extract RNA from the tissues and cDNA by means of reverse Transcription kit (Takara, Dalian, China). The KAPA SYBR Rapid One-Step Kit (Roche, Switzerland) was used for PCR. The experimental procedure was performed according to the kit instructions, using GAPDH and U6 as the PWRN1 and miR-21-5p endogenous regulation. Cycle threshold (Ct) values were normalized using the $2^{-\Delta\Delta Ct}$ method. Differences in Ct values ((ΔDelta Ct) of PWRN1 and GAPDH or miR-21-5p and U6 were calculated to determine the relative RNA levels, as follows formula: ΔΔ Ct = (Δ Ct of the patient sample) – (Δ Ct of the control sample).

2.4 Cell proliferation assay

Proliferation of MDA-MB-231 and HCC1806 cells was measured within 72 h by the CCK-8 kit (Yeasen, Shanghai). Cells were inoculated into 96-well plates at 3000 cells/well and cultivated at 37 ℃ with 5% CO_2 for 24 h. Next, 10 μL of CCK-8 solution was added and the assay was incubated for 2 h. To prevent the presence of air bubbles in the 96-well plate from affecting the results, the absorbance value was read at 450 nm and the plate was shaken for 5 s before detection.

2.5 Cell migration and invasion assay

A 24-well Transwell chamber coated with 50 mg/L Matrigel (BD Biosciences) to evaluate cell migration and invasion ability. Cells transfected with 100 μL DMEM basal medium (without FBS) were put in the upper chamber of the transwell. The inoculation density was 2×10^4 cells/well. DMEM complete medium with FBS was added to the lower chamber. Migrated or invaded cells were fixed with methanol (10 min) and stained with crystal violet for 20 min after 24 h incubation. Excess dye was then washed off and five randomly selected fields of view were observed, photographed, and counted using a camera-equipped light microscope (Olympus BX53, Japanese).

2.6 Dual-luciferase Reporter (DLR) assay

Prediction of PWRN1 and miR-21-5p target binding sites using the ENCORI database. The gene plasmid was designed based on bioinformatic analysis of the binding site between the two. Wild-type (WT)-PWRN1 and mutant (MUT)-PWRN1 recombinant plasmids were constructed. The recombinant plasmids were co-transfected with miR mimic and miR inhibitor into HCC1806 and MDA-MB-231 cells. Luciferase activities were assayed by the DLR Kit (Shanghai Beo Tianmei Biotechnology Co., Ltd.).

2.7 Statistical analysis

At least three replicates of all experiments with parallel tests and results are presented as mean ± standard deviation. Cox and patients'clinical physiological data were analyzed and tabulated using SPSS, while the rest of the data were analyzed and plotted using GraphPad. The relationship between PWRN1 levels and clinicopathological features was investigated by chi-square test. Differences between groups were analyzed using a t-test. The prognosis of TNBC patients was analyzed using Kaplan–Meier plotter curves. A p-value of less than 0.05 denotes a difference that is statistically significant in both groups.

3 Results

3.1 Expression of lncRNA PWRN1

The database indicated that PWRN1 level was notably lower in cancer tissues (n = 115) from TNBC patients compared to normal tissues (n = 113) (Fig. 1A). The same result was detected by RT-qPCR, where the level of PWRN1 was significantly downregulated in cancer tissues ($P < 0.001$, Fig. 1B). In addition, PWRN1 low expression in TNBC cancer tissues was associated with the TNM stage ($P < 0.05$), regardless of other factors such as age, Menopause, and tumor size ($P > 0.05$, Table 1).

3.2 Prognostic value of PWRN1 in patients with TNBC

The horizontal coordinate is the follow-up time and the vertical coordinate is the overall survival rate in Fig. 1C. With increasing follow-up time, the overall survival of patients in the PWRN1 low expression group was lower than that in the PWRN1 high expression group. At around month 40, the survival rate of patients in the PWRN1 low expression group decreased significantly. Low expression of PWRN1 was linked to shorter overall survival in TNBC patients (Log-rank $P = 0.037$). This suggests that PWRN1 has potential as a clinical therapeutic target for TNBC. In addition, PWRN1 was an important predictor of TNBC by multifactorial Cox survival analysis ($P = 0.027$, HR = 3.194; Table 2). Furthermore,

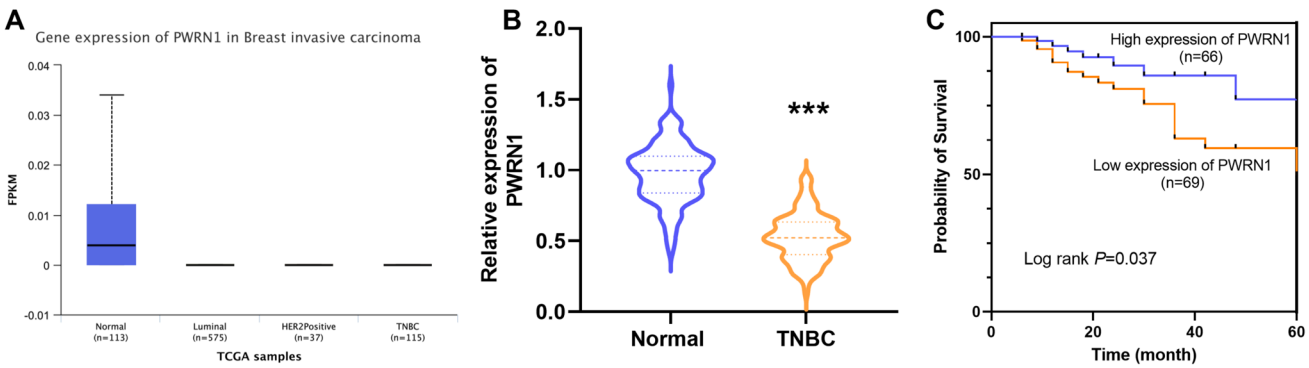

Fig. 1 PWRN1 expression and prognostic role in TNBC. **A** In the database, PWRN1 was expressed at low levels in TNBC tissues; **B** PWRN1 level in TNBC tissues was lower than in normal tissues (*** $P < 0.001$); **C** Patients in the low PWRN1 expression group had a shorter overall survival compared to the high expression group (Log-rank $P = 0.037$)

low expression of PWRN1 was 3.194 times more likely than high expression to have adverse prognostic events (95% CI 1.143–8.924). This suggests that high expression of PWRN1 contributes to improved poor prognosis in TNBC patients. Therefore, in a clinical sense, increasing PWRN1 expression improves the prognosis of patients.

3.3 PWRN1 expression level affects cell biological functions

The expression levels of PWRN1 in four TNBC cell lines (MDA-MB-231, MDA-MB-436, BT-549, HCC1806) and in MCF-10 A were further investigated. The results are shown in Fig. 2A, where the vertical coordinate is the expression level of PWRN1 and the horizontal coordinate is the five cells. Compared with MCF-10 A, PWRN1 was reduced in all four cell lines ($P < 0.001$), especially in HCC1806 and MDA-MB-231, and therefore HCC1806 and MDA-MB-231 were selected as cells for subsequent experiments. Transfection with oe PWRN1 significantly increased PWRN1 expression in both cell types and inhibited the abnormal proliferation, migration, and invasion ability of the cells ($P < 0.001$, Fig. 2B–I). This suggests that PWRN1 is present as a favourable factor in TNBC and that high expression of PWRN1 can inhibit the physiological activities of breast cancer cells.

3.4 Targeted binding of PWRN1 and miR-21-5p

The target gene of PWRN1, miR-21-5p, was identified by bioinformatics analysis, and the predicted target binding location of both is shown in Fig. 3A. StarBase database indicates that miR-21-5p has high levels in cancer tissues (Fig. 3B). Our assay showed the same trend, with miR-21-5p levels increasing in TNBC tissue ($P < 0.001$, Fig. 3C). DLR experiments demonstrated the target binding between PWRN1 and miR-21-5p. miR-21-5p mimic decreased PWRN1-WT luciferase activity, whereas miR-21-5p inhibitor increased activity ($P < 0.001$) but had no effect on PWRN1-MUT ($P > 0.05$, Fig. 3D–E). The expression of PWRN1 and miR-21-5p were negatively correlated, with decreased expression of PWRN1 leading to increased expression of miR-21-5p ($r = -0.7036$, $P < 0.0001$; Fig. 3F). In addition, transfection overexpressing PWRN1 decreased miR-21-5p level, whereas transfection overexpressing PWRN1 and miR mimics increased miR-21-5p level (Fig. 3G–H). The above results indicate that PWRN1 and miR-21-5p can target bind and that PWRN1 can regulate the expression level of miR-21-5p.

3.5 PWRN1 and miR-21-5p together affect the physiological function of cells

The co-regulation of physiological activities of cells by PWRN1 and miR-21-5p was verified by cell physiological activities. PWRN1 overexpression decreased the proliferation, migration, and invasion ability of HCC1806 and MDA-MB-231 cells ($P < 0.001$). In contrast, the addition of miR-21-5p mimic reversed this effect ($P < 0.001$, Fig. 4A–F). This suggests that increasing the level of PWRN1 inhibits the activity and function of breast cancer cells by decreasing the level of miR-21-5p. It provides a theoretical basis for the clinical treatment of TNBC through the PWRN1/miR-21-5p axis.

Table 2 Multivariate Cox analysis of PWRN1 and clinical parameters with overall survival

Characteristics	Multivariate analysis		
	P	HR	95%CI
PWRN1	**0.027**	3.194	1.143–8.924
Age	0.255	1.627	0.704–3.761
Menopause	0.128	0.486	0.192–1.230
Tumor size	0.089	0.458	0.186–1.127
Histological grade	0.205	0.554	0.222–1.380
Lymph node metastasis	**0.036**	0.381	0.155–0.940
TNM stage	**0.032**	0.358	0.140–0.914

P-values less than 0.05 in the table are highlighted by bolding

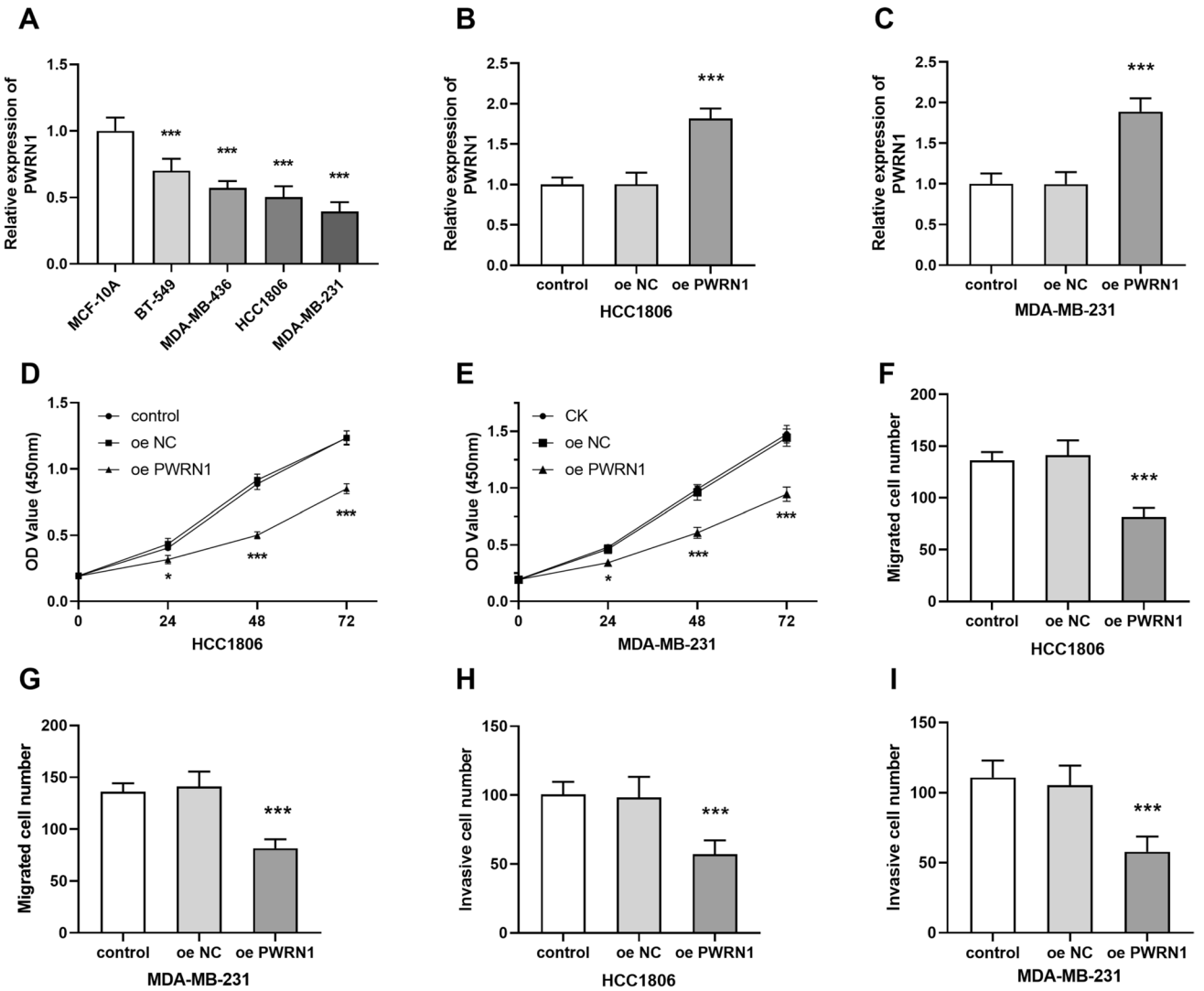

Fig. 2 Effect of PWRN1 expression on the physiological function of cells. All experiments were repeated three times. **A** PWRN1 expression was significantly reduced in four TNBC cell lines; **B** and **C** verified that transfection of overexpressed PWRN1 increased PWRN1 levels in cells; **D** and **E** oe PWRN1 reduced the proliferative ability of HCC1806 and MDA-MB-231 cells; **F** and **G** PWRN1 overexpression inhibited breast cancer cell migration ability; **H** and **I** Increasing the expression level of PWRN1 inhibited the invasive ability of breast cancer cells (* $P < 0.05$, *** $P < 0.001$ vs oe NC)

4 Discussion

TNBC has the worst prognosis of all BC subtypes [20]. Due to the higher degree of disease progression, larger tumor diameters, and greater aggressiveness, about 30–40% of TNBC patients will develop metastases and have a very poor prognosis [21]. Thus, an effective prognostic marker is needed. Long-stranded non-coding RNA (lncRNA) has been reported to have potential value in the prognosis and diagnosis of many cancers [22]. In addition, lncRNA is implicated in the pathogenesis of many cancers and even acts as a tumorigenic or tumor suppressor factor in cancer [23]. It is therefore important to investigate the potential function of lncRNA in the development of TNBC and its prognostic value.

The lncRNA PWRN1 plays a regulatory role and has diagnostic/prognostic potential in a variety of diseases[16]. A study by Shi et al. reported that PWRN1 expression is downregulated in osteosarcoma and may be a biomarker for predicting poor prognosis in cancer patients [24]. The same trend was observed in our findings. In TNBC cancer tissues, PWRN1 expression levels were significantly downregulated. This is in contrast to the trend of lncRNA HAGLR

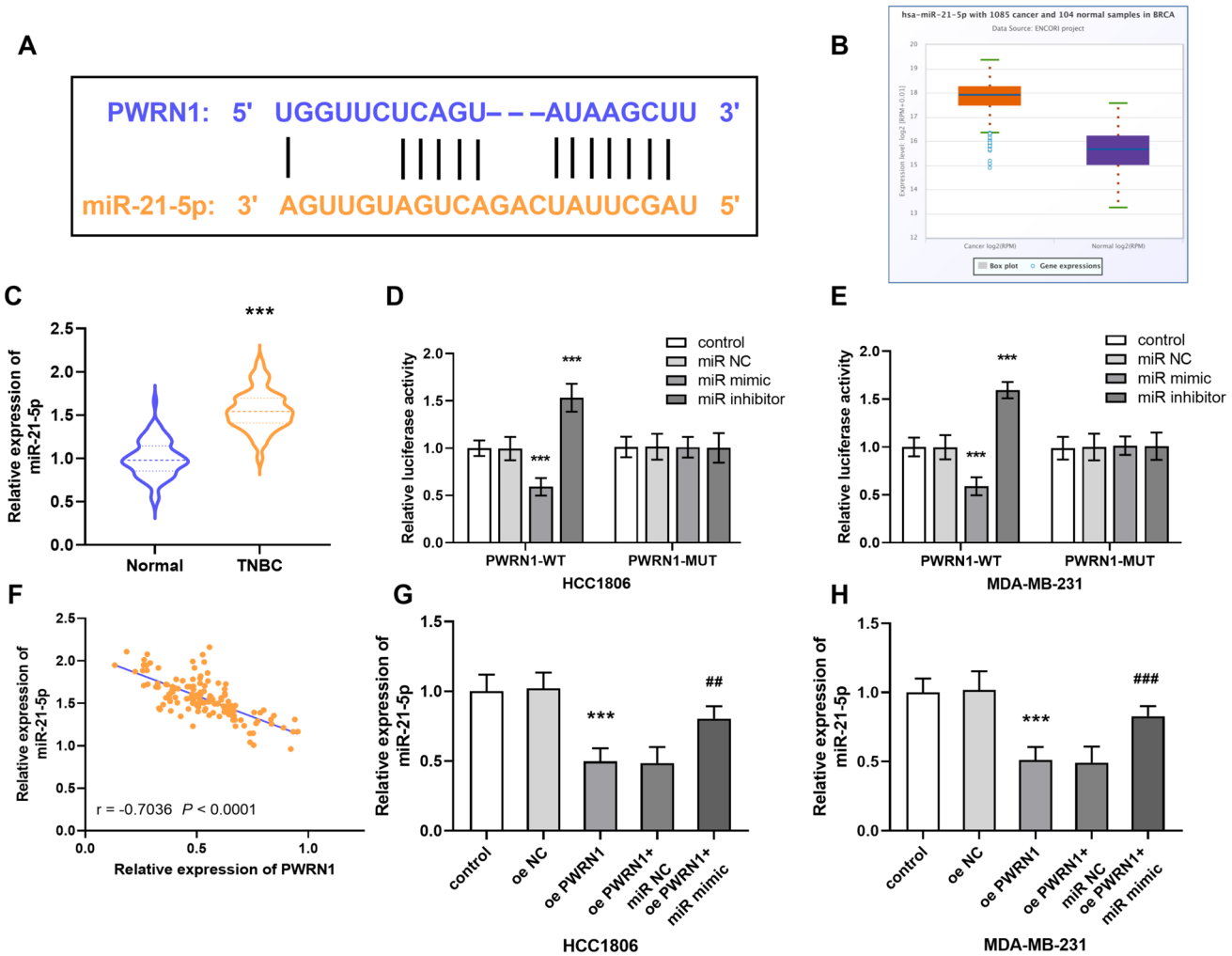

Fig. 3 Targeted binding of PWRN1 to miR-21-5p. **A** Predicted target binding location of PWRN1 to miR-21-5p; **B** The database indicated that miR-21-5p had relatively high levels in TNBC tissues; **C** PWRN1 expression was increased in TNBC tissues compared to normal tissues; **D** and **E** DLR assays showed that miR mimics caused a significant decrease in the luciferase activity of PWRN1-WT, and PWRN1 targeted binding to miR-21-5p; **F** miR-21-5p level was inversely correlated with PWRN1 (r = –0.7036, $P < 0.0001$); **G** and **H** PWRN1 overexpression inhibited miR-21-5p levels, whereas miR mimics increased miR-21-5p levels (*** $P < 0.001$ vs normal or control, ## $P < 0.01$, ### $P < 0.001$ vs oe PWRN1 + miR NC)

in TNBC, which was significantly upregulated in TNBC tissues and cells [25]. In addition, patients with low PWRN1 level had shorter overall survival (Log-rank P = 0.037). Such results suggest that, in a clinical sense, if low PWRN1 expression is detected in the pre-existing period of TNBC patients, early intervention can be made to manage the patients and reduce the possibility of a poor prognosis. Multifactorial Cox regression analysis showed that PWRN1 level was an independent prognostic factor. It is clear that PWRN1 levels significantly affect the overall survival of patients. TNBC Lower overall survival was associated with higher recurrence and metastasis rates [26]. LncRNA levels may affect the overall survival of patients by influencing the rates of recurrence and metastasis of tumours after surgery. As reported by LEE, higher lncRNA ANRIL expression was associated with increased metastasis and decreased overall survival in osteosarcoma [27]. survival was associated with a decreased rate of metastasis and a decreased rate of survival. And we need to further explore the effect of PWRN1 levels on the recurrence and metastasis rates of tumors in TNBC patients after surgery to more closely illustrate the effect of PWRN1 on the prognosis of TNBC patients.

It has been proposed that lncRNA can regulate the course of cancer development by affecting pathways such as cell division, growth, and migration [28]. For example, PWRN1 can affect glioblastoma progression by inhibiting cell proliferation and migration thereby affecting glioblastoma progression [18]. In this study, overexpression of PWRN1 reduced the proliferative ability and reduces the ability to migrate and invade of cells. It is suggested that PWRN1

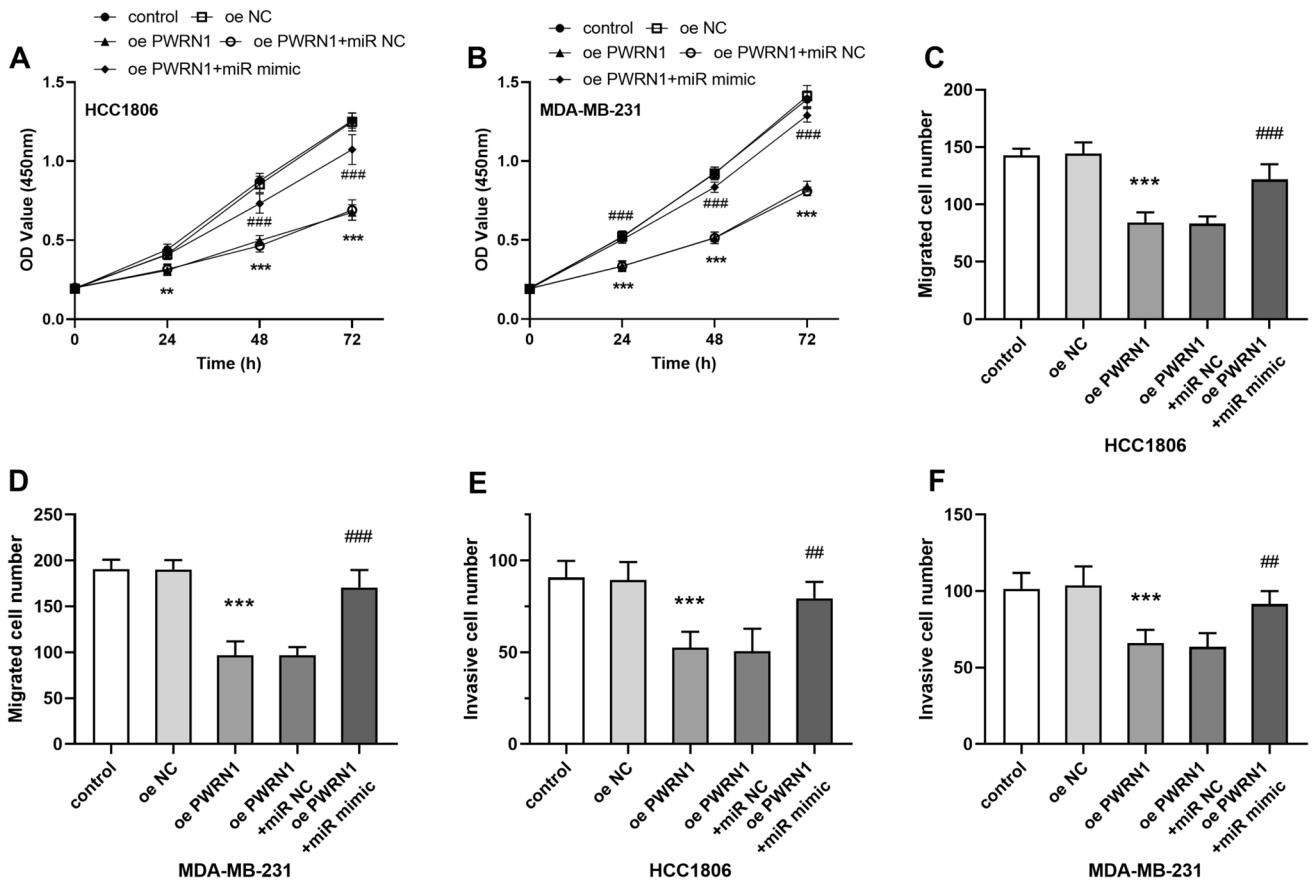

Fig. 4 PWRN1 and miR-21-5p co-regulate cell physiological activity. **A** and **B** Overexpression of PWRN1 markedly reduced cell proliferation, while the addition of miR mimics reversed this effect and restored cell proliferation; **C** and **D** PWRN1 overexpression significantly reduced the migratory capacity of the cells, whereas the addition of miR mimics reversed the effect, resulting in a mostly restored migration ability of the cancer cells; **E** and **F** PWRN1 overexpression reduced the cells invasive capacity, whereas the increase of levels of miR-21-5p reversed this effect, contributing to the recovery of cancer cell invasiveness (** $P < 0.01$, *** $P < 0.001$ vs control; ## $P < 0.01$, ### $P < 0.001$ vs oe PWRN1 + miR NC)

may be an oncogenic factor in TNBC. It is suggested that PWRN1 may be a cancer suppressor in TNBC. This is similar to the findings of Song et al. whose study reported that overexpression of LncRNA NEF inhibited TNBC cell migration and invasion [29].

In addition, lncRNAs can act as sponges for miRNAs and participate in cancer progression by regulating miRNAs [30]. Bioinformatics analysis showed that PWRN1 could target and bind miR-21-5p. miR-21-5p was expressed at elevated levels in TNBC tissues and in the database showed the same trend. PWRN1 was negatively correlated with the expression level of miR-21-5p. MiR-21-5p is reported to be involved in the regulation of a variety of diseases [31, 32]. miR-21-5p also affects the physiological functional activities of cells, such as proliferation, differentiation, and migration [33]. When PWRN1 was overexpressed in cells, a reduced level of miR-21-5p expression was observed and cell proliferation, migration, and invasive activities were inhibited, but this effect was reversed when miR-21-5p was highly expressed. Thus, PWRN1 may play a role in TNBC formation and progression by targeting miR-21-5p expression. This is similar to the findings of Gao et al. whose study reported that overexpression of lncRNA BRE-AS1 inhibited cancer cell proliferation, migration and invasion by down-regulating miR-21 [34]. The specific expression of PWRN1 in TNBC and its regulation of the physiological functions of breast cancer cells give it the potential to be a therapeutic target for TNBC, and poor prognosis of TNBC can be prevented clinically by transferring PWRN1 into the body.

However, we acknowledge some limitations of the present study: first, the signaling pathways involved in TNBC development by PWRN1 targeting miR-21-5p need to be further explored. Second, more experimental models are needed to validate our findings, especially in vivo models. Finally, prognostic data of patients over time as well as a discussion of the relationship between their expression levels and recurrence and metastasis are needed to detect and further explore the effects of PWRN1 levels on postoperative recurrence and metastasis rates in TNBC patients.

In conclusion, low expression of PWRN1 may predict a poor prognosis of TNBC. PWRN1 may be useful in predicting the occurrence of adverse events in TNBC prognosis. Overexpression of PWRN1 induces low level of miR-21-5p, which reduces cell proliferation, migration, and invasion, and slows down the development of TNBC.

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Data availability The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate The research followed the tenets of the Declaration of Helsinki and was approved by the Ethics Committee of The Affiliated Hospital of Shaanxi University of Chinese Medicine. In addition, all participants gave informed consent.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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