Original Paper

WBP2 Downregulation Inhibits Proliferation by Blocking YAP Transcription and the EGFR/PI3K/Akt Signaling Pathway in Triple Negative Breast Cancer

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Key Words

Ww domain-binding protein 2 (WBP2) • Triple Negative Breast Cancer (TNBC) • MiR-613 • Proliferation • Apoptosis

Abstract

Background/Aims: Dysregulated expression of Ww domain-binding protein 2 (WBP2) is associated with poor prognosis in ER+ breast cancer patients. However, its role in triple negative breast cancer (TNBC) has not been previously assessed. Therefore, we aimed to elucidate the functional mechanism of WBP2 in TNBC cells. **Methods:** qRT-PCR, western blotting, and immunohistochemical staining were used to evaluate WBP2 expression in TNBC patient tumors and cell lines. HCC1937 and MDA-MB-231 cells transiently transfected with WBP2 small interfering RNA (siRNA), miR-613 mimics, or miR-613 inhibitors were subject to assays for cell viability, apoptosis and cell cycle distribution. Co-immunoprecipitation, western blotting or qRT-PCR were employed to monitor changes in signaling pathway-related genes and proteins. Luciferase assays were performed to assess whether WBP2 is a direct target of miR-613. The effect of miR-613 on tumor growth was assessed *in vivo* using mouse xenograft models. **Results:** The expression of WBP2 was upregulated in TNBC tissues and cells. Expression of WBP2 was significantly correlated with Ki67 in TNBC patients. Knockdown of WBP2 inhibited cellular proliferation, promoted apoptosis, and induced cell cycle arrest of TNBC cells. miR-613 directly bound to the 3'-untranslated region (3'-UTR) of WBP2 and regulated the expression of WBP2. Moreover, miR-613 reduced the expression of WBP2 and suppressed tumor growth of TNBC cells *in vivo*. Knockdown of WBP2 inhibited YAP transcription and the EGFR/PI3K/Akt signaling pathway in TNBC cells, and these effects were reversed by inhibition of miR-613. **Conclusion:** WBP2 overexpression is associated with the poor prognosis of TNBC patients and the miR-613-WBP2 axis represses TNBC cell growth by inactivating YAP-mediated gene expression and the EGFR/PI3K/Akt signaling pathway.

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Introduction

Triple negative breast cancer (TNBC) accounts for 15%–20% of all breast cancers, and is a highly aggressive subtype of tumors that lack estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) [1]. Patients with TNBC have a higher rate of distant recurrence and a worse prognosis than women with other breast cancer subtypes [2, 3]. Surgical resection and chemotherapy are currently the mainstay of systemic treatment for TNBC patients as there are no clearly defined TNBC-specific therapeutic targets [4].

WW domain-binding protein 2 (WBP2) interacts with WW domains, which regulate protein-protein interactions containing proline rich motifs [5]. WW domain-containing proteins such as Yes-associated protein (YAP) in the Hippo pathway, play significant roles in human malignancies related to dysregulation of cell survival and proliferation, such as lung, breast, gastric and liver cancers [6]. WBP2-deficient mice exhibit a progressive high-frequency hearing loss, which may be due to the coactivation of WBP2 on the estrogen receptor α (ESR1) and the progesterone receptor (PGR) [7]. In addition, WBP2 behaves as an adaptor molecule of Pax8 [8], which is essential for the differentiation of thyroid cells [9]. It can also interact with YAP/TAZ as a bridge linking Hippo and Wnt pathways in breast cancer [10]. In ER+ breast cancer, WBP2 modulates tamoxifen sensitivity by facilitating G1/S transition [11]. However, the roles for WBP2 in TNBC, the hormone receptor absent subtype, remain unclear.

MicroRNAs (miRNAs) are a class of small non-coding RNAs (~ 22 nucleotides) that negatively regulate gene expression at the post-transcription level. miRNA-based therapy has gained traction as a therapeutic avenue for multiple diseases, including cancer. Several miRNAs have been found to play important roles in the tumorigenesis and progression of TNBC due to their aberrant expression in tumor tissues. For example, miR-9 and miR-200 regulate the vasculogenicity of TNBC [12], miR-212-5p and miR-143 are tumor suppressors in TNBC [13, 14], and high levels of miR-21 are associated with poor prognosis in TNBC patients [15]. However, the application of miRNAs as biomarkers or specific anti-cancer therapeutics for TNBC remains to be investigated.

In the present study, we found that WBP2 was up-regulated in TNBC tissues and cell lines compared with paired adjacent normal tissues and normal breast epithelial MCF-10A cells. The overexpression of WBP2 positively correlated with that of Ki67 (%) in TNBC patients. Knockdown of WBP2 inhibited cell proliferation, promoted apoptosis and caused cell cycle arrest in TNBC cells. We found that the endogenous interaction of YAP with WBP2 and downstream transcriptional targets of YAP were decreased when WBP2 was knocked down by small interfering RNA (siRNA). Knockdown of WBP2 also repressed the EGFR/PI3K/Akt signaling pathway in TNBC cells. Furthermore, we confirmed that WBP2 was a direct target of miR-613, which may act as a tumor suppressor in TNBC cells by targeting WBP2, thereby inactivating YAP-mediated gene expression and the EGFR/PI3K/Akt signaling pathway. In contrast, miRNA-613 inhibitors up-regulated WBP2 expression and activated YAP-driven gene expression and the EGFR/PI3K/Akt signaling pathway.

Materials and Methods

TNBC samples

Thirty cases of TNBC and their paired normal adjacent tissues were obtained from the Department of Breast and Thyroid Surgery of Shanghai Tenth People's Hospital, Shanghai, China. The samples were immediately snap frozen in liquid nitrogen and stored at -80 °C. None of the patients had received radiotherapy and chemotherapy before surgery. All patients participating in the study gave their informed consent and the protocols were approved by Institutional Ethics Committees of the hospital (the approval number: SHSY-IEC-KY-4.0/17-83/01).

Cell culture and transfection

Breast cancer cell lines BT-549, MDA-MB-231, MDA-MB-468, HCC 1937 and MCF-7 cell were obtained from the cell bank of the Chinese Academy of Science (Shanghai, China), where they were characterized by mycoplasma detection, DNA fingerprinting, isozyme detection, and determination of cell viability. Human normal breast cell line MCF-10A was purchased from Zhongqiaoxin Zhou Biotech (Shanghai, China). BT-549 cells were cultured in RPMI 1640 medium (Gibco BRL, Rockville, MD, USA), supplemented with 10% fetal bovine serum (FBS, Gibco BRL), 1% penicillin-streptomycin (PS, 100 µg/ml) (Enpromise, Hangzhou, China) at 37 °C in a humidified atmosphere containing 5% CO₂. MCF-10A cells were cultured in Mammary Epithelial Cell Medium (MEpiCM, ScienCell, Research Laboratories, Carlsbad, CA, USA). All other cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, Corning, Corning, NY, USA) medium containing 10% FBS, 1% PS in 5% CO₂ incubator at 37 °C.

miR-613 mimics (miR-613) and negative control (miR-NC), miR-613 inhibitors (anti-miR-613) and inhibitor negative control (anti-miR-NC) were synthesized and purified by RiboBio (Guangzhou, China). WBP2 siRNA, YAP siRNA and their negative controls (siRNA-NC) were synthesized by Sangon Biotech (Shanghai, China). MDA-MB-231 and HCC1937 cells were seeded in six-well plates with a concentration of 1.0×10^5 and 1.3×10^5 respectively. When the cells reached approximately 30%–50% confluence, transfections of miR-613, anti-miR-613, siRNAs and their respective NC (100 nmol/L) were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Lipofectamine 2000 alone treated cells served as a mock control. For the WBP2 siRNA study, siRNA-NC was termed as S1-NC, and the mock control as S2-NC. After 6 h incubation, the medium was changed with fresh medium containing 10% FBS and the cells were cultured for further experimentation.

Cell proliferation assay

MDA-MB-231 and HCC 1937 cells were seeded in 96-well plates at a density of 500 cells/well. Cell viability at different time points (24, 48, 72 and 96 h) was evaluated by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay. Briefly, 20 µl MTT solution (5 mg/mL; Sigma-Aldrich, St. Louis, Mo, USA) was added into each well, and cells were incubated at 37 °C for another 4 h. The reaction was terminated with 150 µL of dimethylsulfoxide (DMSO, Sigma-Aldrich) per well. Absorbance was measured at optical density (OD) of 490 nm using a microplate reader (BioTek, Winooski, VT, USA). All experiments were independently repeated three times in sextuplicate wells.

Colony formation assay

The cells were plated at 5×10^2 cells per well in the six-well plates and incubated at 37 °C for 7–10 days. When colonies were visible, the cells were gently washed twice with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min and stained with 0.1 % crystal violet solution for 20 min. The colonies were photographed using a digital camera, and the number of colonies containing at least 50 cells were counted. Each experiment was performed in triplicate.

Cell cycle and apoptosis assay

For analysis of cell cycle, cells were harvested 36 h post-transfection, and fixed in 70% ethanol overnight at 4 °C. The cells were washed twice with PBS and stained with propidium iodide (PI) for 30 min at room temperature. For apoptotic assay, 24h after transfection, cells were treated with 10 mmol/L 5-fluoro-2, 4(1H, 3H)-pyrimidinedione (5-FU) (Xudonghaipu, Shanghai) for 36 h. The cells were collected and washed with cold PBS. Subsequently, the cells were treated with FITC Annexin V Apoptosis Detection Kit (BD Biosciences, Bedford, MA, USA) following the manufacturer's protocols. Cell cycle distribution and the rate of apoptosis were both analyzed using flow cytometry (BD Biosciences, San Jose, CA, USA). All experiments were performed in triplicate.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from transfected cells or tissues was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions and served as template for synthesizing cDNA by reverse transcription PCR (RT-PCR) as per PrimeScript™ RT-PCR kit (Takara, Tokyo, Japan) instructions. qRT-PCR reaction was performed using the SYBR® FAST qRT-PCR Master Mix kit (Invitrogen, Burlington,

ON, Canada). The reactions were set up as follows: 95 °C for 3 min; 40 cycles of 95 °C for 3 s, 60 °C for 30 s; followed by 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s. Expression of mRNAs or miRNAs were assessed by evaluating threshold cycle (CT) values. β -actin mRNA was employed as an endogenous control for mRNAs. U6 RNA was used to normalize miRNAs. Relative expression was calculated using the relative quantification equation (RQ) = $2^{-\Delta\Delta Ct}$. Primer blasting and the melting curve were analyzed to ensure the specificity of amplification. Primer sequences are provided in Table 1.

Western blot analysis

Cells or tissues were lysed with RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). The protein concentrations were quantified using a BCA protein assay kit (Beyotime, Jiangsu, China). Equal amounts of protein samples were separated by 8% or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Beyotime) and then electro-transferred to 0.45 μ m nitrocellulose membrane (Beyotime). The membranes were incubated with PBST containing 5% bovine serum albumin (BSA) or 5% fat-free milk and 0.1 % tween20 at room temperature for 1 h. Membranes were incubated with the primary antibodies at 4 °C overnight, and then washed three times with PBST for 10 min each, followed by incubation with the corresponding secondary antibodies diluted in blocking buffer for 1 h at room temperature. After three washes in PBST, immunoreactive protein bands were detected with an odyssey scanning system (Li-Cor, Lincoln, NE, USA). Intensities of protein bands were calculated and normalized with respect to β -actin.

Immunohistochemical (IHC) staining

The formalin-fixed, paraffin-embedded specimens were cut into 5 μ m sections, placed on slides, and baked at 65 °C for 2 h. The sections were deparaffinized with xylenes and rehydrated by graded washes with ethanol. For antigen retrieval, the slides were placed in a water bath with antigen retrieval buffer at 90 °C for 10 min, followed by cooling for 20 min at room temperature. The endogenous peroxidase activity were blocked with 3% H₂O₂ for 15 min at 37 °C. Non-specific binding was blocked with 5% BSA (Beyotime) for 30 minutes at room temperature. Then the sections were incubated with primary antibody at 4 °C overnight. The next day secondary antibody was added to the sections for 2 h at room temperature. Finally, the antibody-antigen binding was visualized using a diaminobenzidine (DAB) kit (ZSGB-BIO, Beijing, China) under a light microscope. Five views of each slice were randomly selected for evaluation. The ratio of brown areas to the total areas was analyzed with Image-Pro Plus 6.0 Software (Media Cybernetics, Silver Spring, MD, USA).

Immunoprecipitation assay

Cells were lysed in RIPA buffer (Beyotime) and cell lysates (0.5mg) were mixed with 5 μ g primary antibody. 25 μ l of protein A/G plus-agarose (Santa Cruz, Dallas, TX, USA) was added and the mixture was incubated at 4 °C overnight with rotation. Then beads were washed three times with high salt buffer (1 M Tris-HCl, pH 7.4, 0.50 M NaCl, and 1% Nonidet P-40) and subsequently eluted with loading buffer for western blot analysis.

Animal experiments

All animal experiments were carried out according to protocols approved by the Institution Animal Feed and Use Committee. The five-week-old female BALB/c athymic nude mice were purchased from the laboratory animal center (Shanghai, China). Recombinant lentiviruses containing miR-613 precursor or miR-NC sequences were purchased from Biolink Biotechnology (Shanghai, China). Stable cell lines were produced by infecting MDA-

Table 1. Nucleotide sequences of primers used for PCR

Gene	Primer	Sequence (5'–3')
WBP2	Forward	GCGGAGTGATCGTCAATAACA
	Reverse	GACCCGGTAAGGGGTAAGGT
YAP	Forward	TAGCCCTGCGTAGCCAGTTA
	Reverse	TCATGCTTAGTCCACTGTCTGT
CTGF	Forward	ACCGACTGGAAGACACGTTTG
	Reverse	CCAGGTCAGCTTCGCAAGG
CYR61	Forward	CAGGACTGTGAAGATGCGGT
	Reverse	AGCCTGTAGAAGGAAACGC
ANKRD1	Forward	TACGGAACCTGTGGATGTGC
	Reverse	GGTTCAGTCTCACCCATCA
EDN1	Forward	GTTTGTGGCTTGCAAGGAG
	Reverse	CACGGTCTGTTCCTTTGTG
PTGS2	Forward	GTTCCACCCGAGTACAGAA
	Reverse	AGGGCTTCAGCATAAAGCGT
INHBA	Forward	TCATCACGTTTGCCGAGTCA
	Reverse	TGTTGGCTTGGGACTTTT
β -actin	Forward	CAGAGCTCGCCTTTGGC
	Reverse	GTCCGCCACATAGGAATC
U6	RT	GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAAATATGG
	Forward	TGCGGGTCTCGCTTCGGCAGC
miR-613	Reverse	CCAGTGCAGGTCCGAGG
	RT	GTCGTATCCAGTGCAGTGTGCGAGTCCGCAATTGCACTGGATACGACGGCAAA
miR-613	Forward	AGGAATGTTCTTCTTTGCC
	Reverse	CAGTGCCTGCTGGAGT

MB-231 cells with lentiviruses and subsequently selected in puromycin (2 µg/mL). MDA-MB-231 cells (3×10^6 per mice) stably expressing miR-613 or miR-NC were implanted into the second mammary fat pad on the right side of the nude mice (n = 5, each group), respectively. Tumor size was measured every three days, 5 weeks later, the mice were sacrificed, and the tumors were harvested, weighed, and photographed.

Dual-luciferase reporter assay

MDA-MB-231 cells were seeded into 48-well plates and cultured until the confluence reached to 80%. The psiCHECK-2/WBP2 3'-UTR wild type (WBP2 3'-UTR-WT) and psiCHECK-2/WBP2 3'-UTR mutant type (WBP2 3'-UTR-MT) reporter plasmids were obtained from IBSBIO (Shanghai, China). Cells were transfected with psiCHECK-2/WBP2 3'-UTR-WT (40 ng), psiCHECK-2/WBP2 3'-UTR-MT reporter plasmids (40 ng) and miR-613 or miR-NC (100 nmol/L) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. 24 h after transfection, cells were harvested and assayed with dual-luciferase reporter assay kit (Promega, Madison, WI, USA). The firefly luciferase (FL) values were normalized to the renilla luciferase (RL) values, with the results presented as the ratio of FL to RL activity (FL/RL).

Statistical analysis

Statistical analyses were performed using SPSS 20.0 (IBM, Somers, NY, USA). Each experiment was repeated at least three times independently. Data are represented as the mean $\pm$ standard error of the mean (SEM). The Student's t test was used for the comparison of parameters between two groups. A value of $p < 0.05$ was considered to indicate a statistically significant difference. The correlation between disease parameter and protein or mRNA expression was analyzed using the Pearson's correlation method.

Results

WBP2 expression is up-regulated in TNBC

In the present study, we included 30 cases of TNBC with para-cancer normal breast tissues. qRT-PCR analysis was performed to determine mRNA expression of WBP2 in TNBC (Fig. 1A). WBP2 mRNA levels were significantly higher in TNBC tissues than in para-cancer normal tissues. As the proliferation biomarker Ki-67 is considered to be a prognostic factor for breast cancer, we determined the correlation between WBP2 mRNA and Ki67 (%) expression in TNBC. Association analysis showed that the expression level of WBP2 mRNA was correlated with Ki67 (%) expression (Fig. 1B). Western blot analysis of WBP2 protein expression in 21-paired TNBC samples (C) and para-cancer normal breast tissues (N) (Fig. 1C), showed that WBP2 protein levels were significantly higher in the majority of TNBC samples (16/21) (Fig. 1D), and IHC staining showed that the mean density of WBP2 staining was significantly higher in TNBC tissues than in para-cancer normal breast tissues (Fig. 1E). WBP2 protein levels were also significantly higher in four human TNBC cell lines (BT-549, MDA-MB-231, HCC1937, MDA-MB-468) than in the human breast epithelial cell line (MCF-10A). However, WBP2 protein levels were lower in ER+ breast cancer cell lines (MCF-7) than in MCF-10A cells (Fig. 1F).

WBP2 downregulation suppresses proliferation of MDA-MB-231 and HCC1937 cells

To determine the function of WBP2 in TNBC, we used two different siRNAs to knock down the expression of WBP2 (S1-WBP2, S2-WBP2) in the human TNBC cell lines MDA-MB-231 and HCC1937. qRT-PCR and western blot analysis were performed to examine the knockdown efficiency and confirmed that the expression of both mRNA and protein levels of WBP2 was significantly decreased after transfection with WBP2 siRNAs (Fig. 2A and 2B). In the colony formation assays (Fig. 2C), knockdown of WBP2 expression significantly decreased colony formation compared to cells transfected with control siRNA treatment in both MDA-MB-231 and HCC1937 cells. Similarly, in MTT assays (Fig. 2D), a marked decrease in cell proliferation was detected at 96 h after transfection with WBP2 siRNA, compared to the cells transfected with control siRNA in MDA-MB-231 and HCC1937 cells. In addition,

Fig. 1. The expression of WBP2 is up-regulated in TNBC samples and cell lines. A. The relative expression of WBP2 mRNA in 30 paired TNBC samples (Cancer tissues) and matched normal breast tissues (Normal tissues), the graph represents the $2^{-\Delta\Delta Ct}$ values $\pm$ SEM. B. The correlation between the expression of Ki67(%) and WBP2 mRNA in 30 paired TNBC samples. C. Western blot analysis of WBP2 expression in 21, randomly picked, paired TNBC samples (C) and adjacent normal breast tissues (N), β -actin was used as a loading control. D. The relative expression level of WBP2 (Cancer/Normal) in each patient. E. Top panel: representative IHC staining ($\times 200$), bottom panel: percentage of WBP2 staining positive cells in TNBC and adjacent normal tissues (n=10). F. The relative expression of WBP2 mRNA in breast cancer cell lines BT-549 MDA-MB-231, HCC1937, MDA-MB-468 and MCF-7 compared to MCF-10A. Data represent the $2^{-\Delta\Delta Ct}$ values $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 2. WBP2 knockdown inhibits the growth of MDA-MB-231 and HCC1937 cells. A-B. The expression of WBP2 both at mRNA (A) and protein (B) levels after transfection with WBP2 siRNA (100 nmol/L). C. Colony formation assays, the WBP2 siRNA group exhibited fewer colonies than the NC group in MDA-MB-231 (S1-WBP2 vs. S1-NC) and HCC1937 (S2-WBP2 vs. S2-NC) cells. D. MTT assays, WBP2 siRNA inhibited the proliferation of MDA-MB-231 (S1-WBP2 vs. S1-NC) and HCC1937 (S2-WBP2 vs. S2-NC) cells. E. The relative protein expression of PCNA in MDA-MB-231 and HCC1937 cells (S1-WBP2 and S2-WBP2 vs. S1-NC or S2-NC) after transfection with WBP2 siRNA. β -actin was used as a loading control. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

we also demonstrated that knock down of WBP2 expression reduced the expression level of proliferating cell nuclear antigen (PCNA) in MDA-MB-231 and HCC1937 cells (Fig. 2E). These results indicated that suppression of WBP2 expression inhibits the proliferation of both MDA-MB-231 and HCC1937 cells.

Fig. 3. The effect of WBP2 knockdown on the cell cycle distribution and apoptosis in MDA-MB-231 and HCC1937 cells. A-B. The cell cycle distribution in MDA-MB-231(S1-WBP2 vs. S1-NC) and HCC1937 (S2-WBP2 vs. S2-NC) cells after treatment with WBP2 siRNA. C-D. The proportion of apoptotic cells in MDA-MB-231 (S1-WBP2 vs. S1-NC) and HCC1937(S2-WBP2 vs. S2-NC) cells after treatment with WBP2 siRNA. Notes: Cell apoptosis was induced by 5-FU (10 mmol/L) for 36 h, then measured by flow cytometry. Q2+Q4 represents apoptotic cells (%). * $p < 0.05$; ** $p < 0.01$; NS, no significance.

WBP2 downregulation promotes apoptosis and arrests the cell cycle in MDA-MB-231 and HCC1937 cells

The effect of WBP2 on apoptosis and cell cycle progression was analyzed with flow cytometry after transfection with WBP2 siRNA. The results showed that the percentage of cells in S phase was increased but decreased in G2/M phase in the WBP2-siRNA group compared to the siRNA-NC group in MDA-MB-231 and HCC1937 cells (Fig. 3A and 3B). Cell apoptosis was induced by 5-FU (10 mmol/L) for 36 h after transfection with WBP2-siRNA, and then measured with flow cytometry. The results showed that apoptotic cells were increased in the siRNA group compared with the NC group in MDA-MB-231 and HCC1937 cells (Fig. 3C and 3D). These results indicated that downregulation of WBP2 could impact cell cycle progression and apoptosis in TNBC cells.

WBP2 downregulation inhibits YAP transcription and the EGFR/PI3K/Akt Signaling Pathway

YAP is a transcriptional coactivator and is the major effector of the Hippo pathway. To investigate whether there was an interaction between YAP and WBP2 protein, we performed reciprocal co-immunoprecipitation (Co-IP) experiments and found that WBP2 and YAP were readily pulled down by each other. Downregulation of WBP2 decreased the endogenous WBP2-YAP interaction in MDA-MB-231 cells. We also observed decreased interaction between YAP-TEAD4 after silencing WBP2. However, YAP and TEAD4 protein expression was not significantly altered by silencing WBP2 (Fig. 4A and B). To determine the effect of silencing WBP2 expression on YAP transcription, MDA-MB-231 cells were transfected with WBP2 or YAP siRNA (Fig. 4C-A). The mRNA expression of YAP downstream effectors was lower in the WBP2 and YAP siRNA co-transfection group than that in the WBP2 siRNA group. This suggested that knockdown of WBP2 could decrease the transcription-promoting ability of YAP (Fig. 4C-b). Furthermore, WBP2 siRNA treatment resulted in decreases in EGFR, Phospho-mTOR (Ser2448, p-mTOR), Phospho-Akt (Ser473, p-Akt), p85, and Phospho-p70 S6 Kinase (Ser371, p-p70S6K) expression, but no change in total mTOR, Akt and p70S6K expression in MDA-MB-231 and HCC1937 cells (Fig. 4D and E). These results indicated that down-regulation of WBP2 inhibited the EGFR/ PI3K/Akt signaling pathway.

WBP2 is a direct target of miR-613 in MDA-MB-231 cells, and miR-613 can inhibit the proliferation of TNBC cells in vitro

Increasing evidence has shown that miRNAs play important roles in various biological processes in different types of cancer, including tumor proliferation, drug resistance,

Fig. 4. WBP2 downregulation inhibits YAP transcription and the EGFR/PI3K/Akt signaling pathway A-B. Endogenous WBP2-YAP interaction was validated in MDA-MB-231 cells by reciprocal Co-IP. Representative western blot bands (A) and quantification of protein expression levels (B), β -actin was used as a loading control for the input group. C. The expression of the downstream targets of YAP in MDA-MB-231 cells after transfection with WBP2 siRNA or YAP siRNA. D-E. Representative western blot analysis showing that transfection with WBP2 siRNA suppressed the EGFR/PI3K/Akt signaling pathway in MDA-MB-231 and HCC1937 cells (S1-WBP2 and S2-WBP2 vs. S1-NC or S2-NC). β -actin was used as a loading control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; NS, no significance.

Fig. 5. WBP2 is a direct target of miR-613 in MDA-MB-231 cells, and miR-613 can inhibit the proliferation of breast cancer cells in vitro. A-B. The expression of miR-613 was down-regulated in TNBC samples (A, $n = 30$) and cell lines (B). C. The colony formation ability of MDA-MB-231 cells after transfection with miR-613 or anti-miR-613. D. MTT assays, the proliferation of MDA-MB-231 cells after transfection with miR-613 or anti-miR-613. E. The relative protein expression of WBP2 and PCNA in MDA-MB-231 cells (miR-613 vs. miR-NC, anti-miR-613 vs. anti-miR-NC). β -actin was used as a loading control. F. F-a. The design of luciferase reporters with the wild type WBP2 mRNA 3'-UTR (WBP2 3'-UTR-WT) or the sitesdirected mutant WBP2 mRNA 3'-UTR (WBP2 3'-UTR-MT); F-b. The relative luciferase activity (FL/RL) of miR-613 and miR-NC in MDA-MB-231 cells transfected with either the WBP2 3'-UTR-WT or the WBP2 3'-UTR-MT. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; NS, no significance.

apoptosis and metastasis. To assess the possibility of functional miRNAs as an upstream regulator of WBP2, we performed a search of several online databases, including TargetScan, miRanda, and miRBase. According to a computational prediction, we found that WBP2 may be reduced by miR-613. We then measured the expression of miR-613 in TNBC samples ($n = 30$) and cell lines (Fig. 5A and 5B). The results indicated that the expression of miR-613

was lower in TNBC tissues compared with adjacent normal tissues. Similarly, the expression of miR-613 was significantly lower in the four human breast cancer cell lines (BT-549, MDA-MB-231, HCC1937 and MCF-7) than in the human breast epithelial cell line (MCF-10A). However, there was no statistically significant difference between MDA-MB-468 and MCF-10A cells.

The effect of miR-613 on the colony forming ability of MDA-MB-231 cells was evaluated by colony formation assays. The colony formation rate in the miR-613 groups was significantly lower than that in the miR-NC groups (Fig. 5C). However, the colony formation rate in the anti-miR-613 groups was significantly higher compared with that in the anti-miR-NC groups. The effect of miR-613 on the proliferation of MDA-MB-231 cells was analyzed by the MTT assay. The proliferation of transfected cells was measured and compared with their respective negative control at 24, 48, 72 and 96 h post-transfection. miR-613 overexpression significantly inhibited the proliferation of MDA-MB-231 cells. Conversely, suppression of miR-613 promoted cell proliferation (Fig. 5D). Western blot was carried out to determine the effect of miR-613 on the expression levels of WBP2 and indicated that the protein expression of WBP2 in MDA-MB-231 cells was down-regulated by transfection with miR-613. A loss of function study using anti-miR-613 found that the protein expression of WBP2 was upregulated in the anti-miR-613 group compared with that in the anti-miR-NC group. In addition, PCNA protein expression is a well-accepted marker of proliferation. The expression of PCNA protein was analyzed by western blot, and the results showed that PCNA protein was down-regulated in the miR-613 group compared with that in the miR-NC group. Conversely, PCNA protein expression in MDA-MB-231 cells was substantially upregulated by anti-miR-613 (Fig. 5E). These findings indicated that miR-613 may play a role in suppressing TNBC cell proliferation.

To confirm the possibility of WBP2 as the direct target of miR-613, luciferase reporter assays were performed to measure whether these sites could directly mediate the suppression of WBP2 expression. The luciferase activity was analyzed after miR-613 and miR-NC in MDA-MB-231 cells were transfected with either psiCHECK-2/WBP2 3'-UTR-WT or psiCHECK-2/WBP2 3'-UTR-MT in which the potential binding site of miR-613 is mutated (Fig. 5F-a). Luciferase activity was significantly decreased in the miR-613 group (co-transfection of psiCHECK-2/WBP2 3'-UTR-WT with miR-613) compared with the NC group (co-transfection of psiCHECK-2/WBP2 3'-UTR-WT with miR-NC) (Fig. 5F-b). However, the effect of miR-613 was abolished in the miR-613 group/mutant sites (co-transfection of psiCHECK-2/WBP2 3'-UTR-MT sites with miR-613). Taken together, these findings showed that WBP2 may be the direct target of miR-613.

Fig. 6. The effect of miR-613 on apoptosis and cell cycle regulation in MDA-MB-231 cells. A-B. The cell cycle distribution in MDA-MB-231 cells (miR-613 vs. miR-NC, anti-miR-613 vs. anti-miR-NC). C. Top panel, representative western blots. Bottom panel, bar graphs show the relative protein expression levels of CDK4, CDK6 and CyclinD1 in MDA-MB-231 cells (miR-613 vs. miR-NC, anti-miR-613 vs. anti-miR-NC). β -actin

was used as a loading control. D-E. Cell apoptosis in MDA-MB-231 cells (miR-613 vs. miR-NC, anti-miR-613 vs. anti-miR-NC). F. Left panel, representative western blots; Right panel, bar graphs show the relative protein expression levels of Cleaved-Caspase-3 and Bcl-2 in MDA-MB-231 cells (miR-613 vs. miR-NC, anti-miR-613 vs. anti-miR-NC). β -actin was used as a loading control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, no significance.

The effect of miR-613 on cell cycle and apoptosis

miR-613 overexpression resulted in a significant increase in MDA-MB-231 cells in the S phase, but a decrease in those in the G0/G1 and G2/M phase (Fig. 6A and 6B). Conversely, anti-miR-613 increased the cell population in the G2/M phase and decreased that in S phase; however, there was no significant difference in G0/G1 phase. At 72 h after transfection with miR-613 mimics or inhibitors (100 nmol/L), we assessed the cell cycle-related proteins in MDA-MB-231 cells. We found that the protein levels of CDK4, CDK6 and CyclinD1 were decreased after overexpression of miR-613, whereas these protein levels were enhanced by transfection with anti-miR-613, when compared with their respective controls (Fig. 6C). In addition, we confirmed the regulation of miR-613 in cell apoptosis. The percentage of apoptotic cells was markedly increased in cells with miR-613 overexpression and silencing of miR-613 reversed this effect (Fig. 6D and 6E). Furthermore, we analyzed the protein expression levels of the apoptosis-related marker, Cleaved-Caspase-3, and anti-apoptosis-related marker, Bcl-2, and found that overexpression of miR-613 down-regulated the expression of Bcl-2 and up-regulated the expression of Cleaved-Caspase-3. Inhibition of miR-613 consistently resulted in the opposite results (Fig. 6F). In summary, these observations suggest that miR-613 may function as a tumor suppressor in TNBC cells by promoting cell cycle arrest at the S checkpoint and increasing cell apoptosis.

miR-613 suppresses YAP-driven gene expression and the EGFR/PI3K/Akt signaling pathway by targeting WBP2 in MDA-MB-231 cells

As WBP2 depletion can suppress YAP transcription, and inactivate the EGFR/PI3K/Akt signaling pathway, we further determined the connection between miR-613 and YAP transcription, as well as the EGFR/PI3K/Akt signaling pathway. miR-613 overexpression decreased the endogenous interaction between WBP2-YAP and YAP-TEAD4. Notably, western blot was performed to analyze the cell lysates from the input group, and the results showed that the protein expression of WBP2 was suppressed by miR-613, but the protein expression of YAP and TEAD4 was not changed by miR-613 (Fig. 7A). Using qRT-PCR, we confirmed that

Fig. 7. miR-613 suppresses YAP-driven gene expression and the EGFR/PI3K/Akt signaling pathway by targeting WBP2 in MDA-MB-231 cells. Top panel, representative western blots. Bottom panel, bar graphs show the effects of miR-613 on the interaction of endogenous WBP2 with YAP, β -actin was used as a loading control for the input group. B. B-a. The mRNA expression levels of WBP2 and YAP in MDA-MB-231 cells after transfection with miR-613; B-b. The mRNA expression levels of PTGS2, CYR61, CTGF, ENO1, INHBA and ANKRD1 in MDA-MB-231 cells after transfection with miR-613. C. Representative western blots bands. D. Bar graphs show the corresponding band intensity, normalized to β -actin, of WBP2 protein, of EGFR protein, of p85 protein, of Akt protein, of p-Akt protein, of mTOR protein, of p-mTOR protein, of p70S6K and of p-p70S6K protein. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; NS, no significance.

Fig. 8. miR-613 inhibits the proliferation of breast cancer cells *in vivo*. Tumor formation at week 5 after inoculation of MDA-MB-231 cells infected with lentivirus carrying miR-613 or miR-NC. B. Weights of the xenografts are shown in the histogram (n=10). C. Tumor volumes were evaluated periodically at the indicated days post-inoculation in MDA-MB-231 cells. D. Representative IHC staining of WBP2, p-Akt, Bcl-2 and CDK4 stained cells from the indicated tumors (n=6). **p<0.01; ****p<0.0001.

WBP2 mRNA expression was downregulated, but YAP mRNA expression was not changed after transfection with miR-613 (Fig. 7B-a). In addition, we found that overexpression of miR-613 in MDA-MB-231 cells could reduce the mRNA expression levels of several YAP-regulated genes, such as PTGS2, CYA61, CTGF, ANKRD1, INHBA and END1 (Fig. 7B-b), which indicated that overexpression of miR-613 could suppress YAP-driven gene expression in TNBC cells. On the other hand, western blot showed that miR-613 overexpression significantly inhibited the expression of EGFR, p85, p-Akt, p-mTOR, and p-p70S6K, the key proteins involved in the EGFR/PI3K/Akt signaling pathway. Conversely, inhibition of miR-613 promoted these key proteins of the EGFR/PI3K/Akt signaling pathway. However, the expression of total mTOR, Akt and p70S6K proteins could not be altered by overexpression or inhibition of miR-613 (Fig. 7C and 7D). These data showed that miR-613 may suppress TNBC progression via inhibition of YAP transcription and the EGFR/PI3K/Akt signaling pathway by targeting WBP2.

miR-613 inhibits the proliferation of breast cancer cells in vivo

To further assess the effects of miR-613 on anti-tumorigenesis *in vivo*, MDA-MB-231 cells were infected with a lentivirus carrying the human miR-613 or miR-NC precursor sequence, and the cells were expanded and subcutaneously injected into nude mice. The results showed that the growth of the xenograft tumors derived from miR-613 cells was significantly lower than that derived from miR-NC cells (Fig. 8A). In addition, the weight and volume of the tumors in miR-613 group were lower than those in miR-NC group (Fig. 8B and 8C). IHC staining suggested that WBP2 expression in tumor sections from the miR-613 group was lower than that in tumor sections from the miR-NC group. Furthermore, we detected changes in p-Akt, a downstream signal of WBP2, in CDK4, a cell cycle-related marker, and in Bcl-2, an apoptosis-related marker in tumor sections by IHC staining. These results showed that p-Akt, CDK4 and Bcl-2 expression in the miR-613 group was lower than that in the miR-NC group (Fig. 8D). In brief, the *in vivo* data supported the conclusion that miR-613 served as a tumor suppressor in TNBC.

Discussion

WBP2 expression is significantly elevated in invasive ductal carcinoma and metastatic lesions [10]. High WBP2 expression correlates with poor prognosis in ER+ breast cancer patients [11]. However, the expression and molecular mechanism of WBP2 in TNBC have not been fully elucidated. Here, we show that mRNA and protein expression of WBP2 were significantly higher in TNBC tissues than in para-cancer normal tissues. Similarly, WBP2 protein expression was significantly higher in the TNBC cell lines, BT-549, MDA-MB-231, HCC1937 and MDA-MB-468 than in the human breast epithelial cell line, MCF-10A, but WBP2

protein levels were lower in MCF-7 than in MCF-10A cells. MCF-7 is a breast cancer cell line characterized by overexpression of ER, while MCF 10A is a non-tumorigenic epithelial breast cell line. We reviewed the literature and found no reports comparing the expression of WBP2 in MCF7 cells and MCF-10A cells, and this finding is worth further study. Genomic profiles have revealed that WBP2 is located on chromosome 17q25, a region frequently rearranged in many breast cancer cell lines and tumors [16]. Therefore, upregulation of WBP2 in TNBC may be due to gene arrangements during the amplification process. Interestingly, a positive correlation was observed between WBP2 and Ki67 (%) expression in TNBC samples. Ki67 is a marker widely used to assess tumor proliferation [17]. This indicated that WBP2 may be associated with cell proliferation in TNBC.

To investigate the functional role of WBP2 in TNBC, WBP2 siRNA was transfected and confirmed in MDA-MB-231 and HCC1937 cells. WBP2 interference induced apoptosis in MDA-MB-231 and HCC1937 cell lines and blocked proliferation, accompanied by cell cycle arrest in the S phase. We also found that downregulation of WBP2 inhibited the expression of PCNA, which is a protein bio-marker of cancer cell proliferation in MDA-MB-231 and HCC1937 cells. These results provide direct evidence that silencing of WBP2 inhibits cell proliferation in TNBC and may be the result of increased apoptosis and cell cycle arrest.

The transcriptional co-activator YAP is a downstream target of the Hippo tumor suppressor pathway. YAP possesses WW domains, which are important protein-protein interaction modules that mediate interaction with proline-rich motifs. We found that WBP2 was a YAP-interacting factor in MDA-MB-231 cells and knockdown of endogenous WBP2 decreased the interaction between YAP and WBP2. These findings were similar to those in recent reports which showed that WBP2 can directly interact with or affect YAP through WW domains [18-20]. The TEA domain (TEAD) family of transcription factors (TEAD1-4) play an important role in tumorigenesis by regulating various cellular processes, including cell growth, proliferation, differentiation, and survival [21]. YAP in conjunction with TEAD1-4 mediate major physiological functions of the Hippo pathway [22, 23]. Here, we found that WBP2 downregulation can disrupt the protein-protein interaction between TEAD4 and YAP. Moreover, knockdown of endogenous WBP2 could suppress YAP-driven gene expression in MDA-MB-231 cells.

Positive expression of EGFR is correlated with poor prognosis in several tumors, including TNBC [24, 25]. EGFR overexpression has been detected in TNBC cell lines such as MDA-MB-231, BT-20, and HCC1937 [26]. We found that knockdown of WBP2 decreased the expression of EGFR, and key proteins involved in the EGFR/PI3K/Akt signaling pathway in TNBC cell lines. It is known that the EGFR/PI3K/Akt signaling pathway is the major cell survival pathway and plays an important role in cell growth, apoptosis, cell cycle progression, drug resistance, migration and invasion [27-31]. The EGFR signaling pathway has been reported to play an important role in the occurrence of TNBC, and the key proteins involved in this pathway may be effective therapeutic targets in TNBC [32-35]. Therefore, WBP2 inhibition may be an emerging therapeutic target in the treatment of TNBC.

The aberrant expression of miRNAs has emerged as novel biomarkers and potential therapeutic targets in cancer. miRNAs can regulate gene expression at the post-transcriptional level by binding to the 3'-UTR of target mRNAs [36]. WBP2 is the putative target gene of miR-613 as shown by three microRNA prediction databases, and we found that miR-613 expression was downregulated in TNBC samples. At present, miR-613 is rarely reported in breast cancer. Our results showed that miR-613 suppressed TNBC cell proliferation, promoted cell apoptosis, and blocked the cell cycle *in vitro*. miR-613 inhibition markedly increased the viability of TNBC cells, and significantly suppressed cell apoptosis *in vitro*. Further studies showed that miR-613 suppressed the protein expression of WBP2 in TNBC cells. Conversely, the protein expression of WBP2 in TNBC cells was upregulated by anti-miR-613. More importantly, we identified WBP2 as the direct target of miR-613 through the dual-luciferase reporter system. miR-613 upregulation in TNBC cells significantly decreased the expression of key proteins involved in the EGFR/PI3K/Akt signaling pathway when compared with the miR-NC, and the results were reversed when cells were transfected

with anti-miR-613. Moreover, the interaction between endogenous YAP and WBP2 was significantly decreased by miR-613.

Previous studies suggested that WBP2 was a positive regulator of YAP [37]. WBP2 was identified as a target gene of miR-613 in MDA-MB-231 cells. The downstream target genes of YAP, such as PTGS2, CYA61, CTGF, ANKRD1, INHBA and END1, were down-regulated in MDA-MB-231 cells by up-regulation of miR-613. Notably, YAP expression, at both mRNA and protein levels, was not changed by miR-613, therefore, the effect of miR-613 on the EGFR/PI3K/Akt signaling pathway may not be blocked upon YAP inhibition. However, the mRNA and protein levels of WBP2 were down-regulated by miR-613. miR-613 may inhibit the YAP-driven gene expression and the EGFR/PI3K/Akt pathway by targeting WBP2.

In previous studies, miR-613 acted as a tumor suppressor in various tumors, including ovarian cancer [38], papillary thyroid carcinoma [39], gastric cancer [40] and hepatocellular carcinoma [41]. Inhibition of 6-phosphogluconate dehydrogenase by miR-613 could reverse cisplatin resistance in lung and ovarian cancer [38]. Our results suggested that miR-613 inhibited the proliferation of TNBC cells *in vitro* and *in vivo*, and suppressed the YAP transcription and the EGFR/PI3K/Akt signaling pathway by targeting WBP2. Therefore, the miR-613/WBP2 axis may act as an important regulator in TNBC.

In summary, we demonstrated that knockdown of WBP2 inhibited cell proliferation, increased apoptosis and caused cell cycle arrest in S phase in TNBC cells. Knockdown of endogenous WBP2 decreased the interaction of YAP and WBP2, suppressed the expression of transcriptional targets of YAP and the activity of the EGFR signaling pathway in TNBC cells. In addition, our results revealed that miR-613 acts as a novel regulator of WBP2 and indicated that the miR-613/WBP2 axis may serve as a novel potential therapeutic target in the treatment of TNBC.

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Disclosure Statement

The authors declare that there is no conflict of interests.

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