Gonadotropin-releasing hormone receptor inhibits triple-negative breast cancer proliferation and metastasis

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Abstract

Background: Gonadotropin-releasing hormone receptor (GnRHR) is expressed in several malignant tumors and inhibits the proliferation and metastasis of cancer cells, but its role in triple-negative breast cancers (TNBCs) is unclear. This study investigated the biological effects of GnRHR and their influence on TNBC prognosis.

Methods: The GSE21653 database was used to obtain information about GnRHR expression and clinicopathological factors in patients with TNBC. GnRHR was activated in cultured MDA-MB-231 and MDA-MB-468 cells by leuprolide acetate and antagonized by elagolix sodium. Cell proliferation was assessed by the cell counting kit-8 and colony formation assays. Cell metastasis was detected by the wound healing assay and Transwell assay. Apoptosis and the cell cycle were investigated by flow cytometry. GnRHR protein expression was determined by western blotting.

Results: GnRHR mRNA expression was significantly higher in patients with TNBC than in hormone receptor+/human epidermal growth factor receptor (HER)2– and HER2+ patients with breast cancer. Patients with high GnRHR expression had significantly better disease-free survival than those with lower expression. Activated GnRHR significantly inhibited cell proliferation and metastasis, increased apoptosis, and enhanced GnRHR protein expression levels.

Conclusion: GnRHR inhibits TNBC proliferation and metastasis, suggesting it could be targeted for TNBC treatment.

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Background

Triple-negative breast cancers (TNBCs) have a relatively high degree of malignancy. They do not express estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor-2 (HER2), so are not sensitive to endocrine therapy or HER2-targeted therapy.[1] A study involving more than 140,000 patients with TNBC reported a 5-year breast cancer-specific survival rate of 78.78%, which was lower than that of other breast cancer subtypes.[2] Identifying new therapeutic targets for TNBC will improve treatment and patient survival.[3]

Gonadotropin-releasing hormone receptor (GnRHR) is a transmembrane G protein-coupled receptor that is mainly expressed in the pituitary gland, but also in a variety of tumor tissues including hormone-dependent tumors (ER/PR-positive breast cancer, prostate cancer, and ovarian cancer) and hormone-independent tumors (ER/PR-negative breast cancer, glioma, and liver cancer). Tumor cells activate the GnRHR pathway through autocrine and paracrine mechanisms to regulate biological behavior.[4–9] The POEMS study found that the addition of gonadotropin-releasing hormone (GnRH) analogs to postoperative chemotherapy for ER/PR-negative breast cancer significantly improved disease-free survival (DFS) and overall survival (OS) ($P = 0.09$ and 0.06, respectively).[10] In neoadjuvant chemotherapy, it was also observed that GnRH analogs improved the complete tumor remission rate of ER/PR-negative breast cancers.[11] These results indicate that the GnRHR pathway may be involved in TNBC therapeutic effects.

Preclinical studies showed that GnRHR inhibits tumor cell proliferation, migration, and angiogenesis.[12–16] However, the biological effects of GnRHR on MDA-MB-231 and MDA-MB-468 TNBC cells are not clear. This study investigated the effect of GnRHR on the prognosis of TNBC and its biological effects on MDA-MB-231 and MDA-MB-468 cells *in vitro*.

Materials and methods

Bioinformatics analysis

This study was approved by the medical ethics committee of the Affiliated Hospital of Jiaxing University. mRNA expression data were obtained from the GEO database (no. GSE21653), which included the age, pathological type, histological grade, pT, pN, ER, PR, HER2, and DFS status of 266 patients with breast cancer.[17,18] Forty-two patients were excluded because of incomplete data; missing details included age (n = 1), pathological information (n = 3), pT or pN status (n = 11), histological grade (n = 3), HER2 status (n = 15), and DFS status (n = −9). A total of 224 cases with complete data were used for analysis (Figure 1). Because the GSE21653 dataset does not include immunohistochemical findings of Ki67 expression, we used ER, PR, and HER2 values to group patients into three categories, TNBC (ER-, PR-, and HER2-negative), HR+/HER2− (ER- or PR-positive and HER2-negative), and HER2+ (HER2-positive), which was previously shown to be accurate for identifying patients with TNBC.[19,20]

The platform of this dataset was GPL570, and the probes corresponding to GnRHR and GnRH were 211523_at and 207987_s_at, respectively.

Cell culture and grouping

MDA-MB-231 and MDA-MB-468 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). They were cultured in Leibovitz's L-15 medium containing 10% fetal bovine serum (FBS; Gibco, Shanghai, China) and
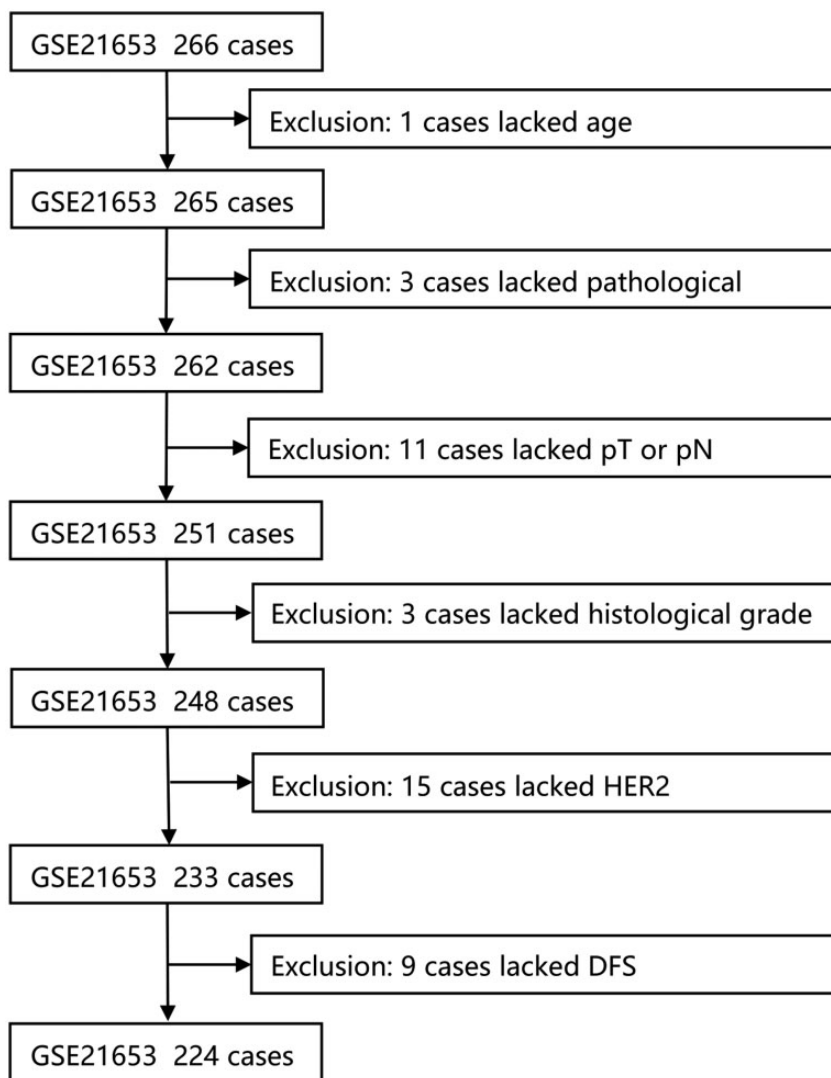
Figure 1. Study flow chart. Forty-two patients were excluded because of incomplete data, including age (n = 1), pathological information (n = 3), pT or pN stage (n = 11), histological grade (n = 3), HER2 status (n = 15), and DFS time (n = 9). A total of 224 cases with complete data were used for analysis.

1% (10,000 U/mL) penicillin + (10,000 μg/mL) streptomycin at 37°C with 5% CO_2. Leuprolide acetate or elagolix sodium (Selleck, Shanghai, China) was added to the cells for 72 hours at a final concentration of 10 μmol/L, which was confirmed by the manufacturer to have no significant effect on ion channels, enzymes, or transporters.[21] Untreated cells were used as controls.

Cell counting kit-8 (CCK-8) assay

Cell viability was assessed using the CCK-8 assay. Briefly, cells were seeded in 96-well plates at 4×10^3/well, with multiple pores

added to each well. Cells were incubated for 24, 48, or 72 hours, then $10\,\mu L$ of CCK-8 was added to each well for 3 hours. The absorbance at 450 nm was analyzed in each well using a BIOBASE-EL10A microplate reader (BIOBASE, Shandong, China).

Colony formation assay

Cells were plated in 12-well plates at 400 cells per well, then incubated for 7 days. They were fixed in methanol and stained with crystal violet, then images of cell colonies were photographed under a Leica DM500 microscope with $\times 10$ magnification (Leica, Heidelberg, Germany). The experimental procedure was carried out at least three times. The colony formation rate was calculated as: (number of cell colonies/ $400) \times 100\%$.

Apoptosis assay

Cell apoptosis was determined by flow cytometry using an Annexin V-FITC/PI Kit (4A Biotech, Beijing, China) according to the manufacturer's instructions. Cells (2×10^5) were collected and washed with phosphate-buffered saline (PBS), then resuspended in $100\,\mu L$ of $1\times$ binding buffer and incubated with $5\,\mu L$ Annexin V/FITC at room temperature for 5 minutes. They were centrifuged at $179 \times g$ for 5 minutes at $4°C$, resuspended in $400\,\mu L$ PBS, and $10\,\mu L$ $20\,\mu g/mL$ propidium iodide solution was added, then the cells were subjected to flow cytometry at 488 nm.

Cell cycle assay

The cell cycle distribution was determined using a Cell Cycle Analysis Kit (4A Biotech, Beijing, China) following the manufacturer's instructions. Cells (2×10^5) were collected and washed twice with ice-cold PBS. Then, 1 mL of cell suspension was added to 4 mL of ice-cold 95% ethanol and fixed at

$4°C$ for 12 to 24 hours. The cells were centrifuged at $179 \times g$ for 5 minutes at $4°C$, then washed once with ice-cold PBS. After treatment with 0.4 ml propidium iodide for 30 minutes, the cells were subjected to fluorescence-activated cell sorting analysis.

Transwell assay

Cell invasion was measured in Transwell chambers (Millipore, Billerica, MA, USA) with Matrigel coating. Cells (5×10^4) were seeded in the top chamber, and $600\,\mu L$ of 10% FBS medium was added to the lower chamber. After incubation for 48 hours, cells that had invaded the lower chamber were collected and stained with 0.1% crystal violet. Images of the invading cells were photographed under a microscope.

Wound healing assay

Cells were plated in 24-well plates at 3×10^5/well, with two multiple pores in each well. When they were almost 100% confluent, a 10-µl pipette tip was used to create an artificial wound. Culture was continued, and images of the wound at 0 and 24 hours were visualized under a microscope. The migration rate at three locations was measured as: Migration rate = (0 hour scratch width – 24 hour scratch width)/0 hour scratch width.

Western blotting

Total protein was extracted from cells by washing them with pre-cooled PBS and lysing with $200\,\mu L$ $1\times$ loading buffer. Proteins were then run on sodium dodecyl sulfate–pulsed field gel electrophoresis, and electroblotted onto polyvinylidene fluoride membranes. Membranes were incubated with a primary anti-GnRHR antibody (Immunoway, Suzhou, China) at $4°C$ overnight, then incubated with a goat anti-rabbit IgG secondary antibody (Proteintech Group, Wuhan, China) for 1 hour at room

temperature. for 1 hour at room temperature. Visualization was carried out using a chemiluminescence system (Leica).

Statistical analysis

Experimental data were analyzed by SPSS 22.0 statistical software (IBM Corp., Armonk, NY, USA). Data with a non-normal distribution were tested by the non-parametric Mann–Whitney U test, and the Kendall rank correlation coefficient method was used for correlation analysis. Pearson's chi-square test was used to analyze categorical data. The Kaplan–Meier method was used for survival analysis, and the log-rank test was used to test the difference in survival curves between groups. Multivariate survival analysis was performed with the Cox regression model. Cell data were averaged from three independent experiments. All statistical tests were bilateral tests, and differences were considered to be statistically significant at $P < 0.05$.

RESULTS

High expression of GnRHR is associated with increased DFS

Of the 224 patients with complete data obtained from GSE21653, 81 had TNBC, 120 had HR+/HER2−, and 23 had HER2+ breast cancer (Table 1). GnRHR ($P < 0.05$) and GnRH ($P < 0.001$) mRNA expression was significantly higher in patients with TNBC than in other patients (Figure 2a, b). In the TNBC group, there was a positive correlation between GnRHR and GnRH (correlation coefficient = 0.247, $P = 0.001$, Figure 2c). In the TNBC group, patients were divided into low, medium, and high expression groups according to GnRHR mRNA expression, with 27 cases in each group. The age difference among the three groups was statistically significant,

**Table 1.** Patients and sample characteristics.

	Patient groups		
	TNBC (n = 81)	HR+/HER2− (n = 120)	HER2+ (n = 23)
Age (years)			
≤55	40	60	14
>55	41	60	9
Pathology			
IDC	68	95	22
others	13	25	1
pT			
1	16	29	5
2	45	58	10
3	20	33	8
pN			
0	50	46	8
≥1	31	74	15
Tumor grade			
1	7	32	0
2	16	52	7
3	58	36	16

TNBC, triple-negative breast cancer; HR, hormone receptor; HER2, human epidermal growth factor receptor-2; IDC, invasive ductal carcinoma; pT, tumor size; pN, number of positive lymph nodes.

with GnRHR expression in patients ≤55 years old in the low expression group being significantly lower than in medium and high expression groups ($P = 0.023$, Table 2). Survival analysis showed that DFS was significantly longer in patients with TNBC with high GnRHR expression than in those with low or medium GnRHR expression ($P = 0.038$; Figure 2d). Multivariate survival analysis suggested that GnRHR was an independent influencing factor of DFS ($P = 0.004$; odds ratio 0.428, 95% confidence interval: 0.242–0.757); Table 3).

Activated GnRHR inhibits TNBC cell proliferation

To study the effect of activated GnRHR on TNBC cell proliferation, we used leuprolide
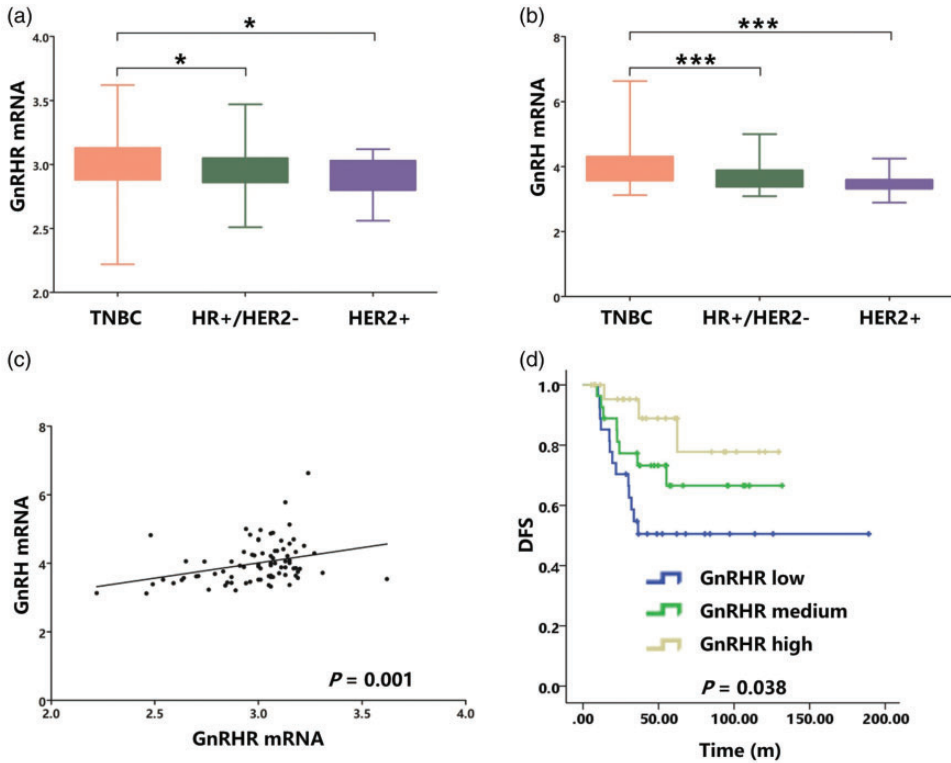
Figure 2. Expression of GnRHR and GnRH mRNA. (a) GnRHR mRNA expression in the TNBC group was significantly higher than in the other two groups. (b) GnRH mRNA expression in the TNBC group was significantly higher than in the other two groups. (c) Positive correlation between GnRHR and GnRH in TNBC. (d) DFS was significantly longer in TNBC patients with high GnRHR expression. *P < 0.05, **P < 0.01, ***P < 0.001.

TNBC, triple-negative breast cancer; GnRHR, gonadotropin-releasing hormone receptor; GnRH, gonado-tropin-releasing hormone; DFS, disease-free survival.

acetate to simulate GnRHR activation and elagolix sodium to simulate GnRHR antagonism in MDA-MB-231 and MDA-MB-468 cells *in vitro*.[22–24] After adding leuprolide acetate for 72 hours, the OD values of both cell types at 450 nm were significantly lower than those of the control group in the CCK8 assay ($P < 0.01$). Similarly, the OD values of both cell types were significantly higher than those of the control group after adding elagolix sodium for 72 hours ($P < 0.05$; Figure 3a). This suggested that activated GnRHR inhibited cell viability, while GnRHR antagonism promoted cell viability. The clone formation rate of MDA-MB-231 and MDA-MB-468 cells was significantly lower than that of the control group after 7 days of adding leuprolide acetate ($P < 0.01$), but did not increase significantly compared with the control group after the addition of elagolix sodium (Figure 3b). This indicated that activated GnRHR inhibited colony formation. Western blotting showed that leuprolide increased GnRHR protein expression in MDA-MB-231 and MDA-MB-468 cells, while elagolix decreased it (Figure 3c).

Table 2. Relationship between GnRHR expression and clinicopathological characteristics in TNBC.

	n	GnRHR expression low (n = 27)	medium (n = 27)	high (n = 27)	χ^2	P
Age (years)					7.507	**0.023**
≤55	40	18	14	8		
>55	41	9	13	19		
Pathology					2.382	0.304
IDC	68	21	25	22		
others	13	6	2	5		
pT					2.133	0.711
1	16	6	6	4		
2	45	14	13	18		
3	20	7	8	5		
pN					1.986	0.370
0	50	17	19	14		
≥1	31	10	8	13		
Grade					2.673	0.614
1	7	3	1	3		
2	16	5	4	7		
3	58	19	22	17		

TNBC, triple-negative breast cancer; GnRHR, gonadotropin-releasing hormone receptor; IDC, invasive ductal carcinoma; pT, tumor size; pN, number of positive lymph nodes.

Table 3. Multivariate analysis of DFS in TNBC.

	Groups	β	P	OR (95% CI)
GnRHR	low, medium, high	−0.848	0.004	0.428 (0.242–0.757)
Age (years)	≤55, >55	0.12	0.483	1.012 (0.979–1.046)
Pathology	IDC, others	0.318	0.538	1.374 (0.500–3.779)
Grade	1, 2, 3	0.042	0.906	1.043 (0.520–2.092)
pT	1, 2, 3	−0.107	0.763	0.899 (0.450–1.797)
pN	0, ≥1	1.388	0.004	4.005 (1.539–10.426)

OR, odds ratio; CI, confidence interval; GnRHR, gonadotropin-releasing hormone receptor; IDC, invasive ductal carcinoma.
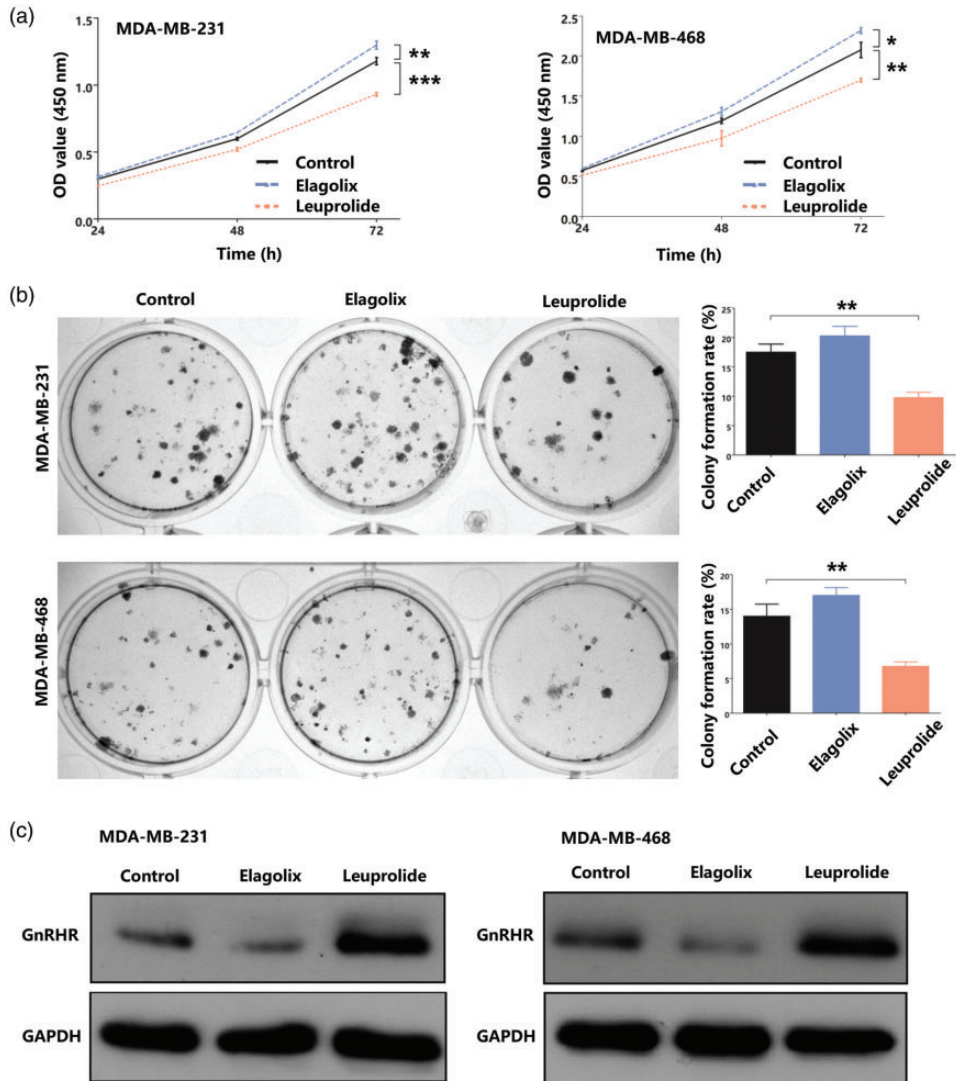
Activated GnRHR inhibits TNBC cell metastasis

To explore the effect of activated GnRHR on the migration and invasion of TNBC cells, we used the wound healing assay and Transwell assay. After adding leuprolide acetate for 24 hours, both the migration rate ($P < 0.001$; Figure 4a) and invasion ($P < 0.01$; Figure 4b) of MDA-MB-231 and MDA-MB-468 cells were significantly lower than that of the control group. This suggested that activated GnRHR inhibited migration and invasion capabilities of TNBC cells.

## Activated GnRHR promotes apoptosis in TNBC cells

Next, we used flow cytometry to determine the effects of activated GnRHR on TNBC

Figure 3. Activated GnRHR inhibits TNBC cell proliferation. (a) CCK8 assay analysis of MDA-MB-231 and MDA-MB-468 cells. OD values at 450 nm were significantly lower after adding leuprolide acetate than controls. OD values were significantly higher after adding elagolix sodium than controls. (b) The clone formation rate of MDA-MB-231 and MDA-MB-468 cells was significantly lower after adding leuprolide acetate than controls, but was unchanged after adding elagolix sodium. (c) Activated GnRHR increased the expression of GnRHR in MDA-MB-231 and MDA-MB-468 cells, and antagonized GnRHR decreased the expression of GnRHR. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.
TNBC, triple-negative breast cancer; CCK, cell counting kit; OD, optical density; GnRHR, gonadotropin-releasing hormone receptor.
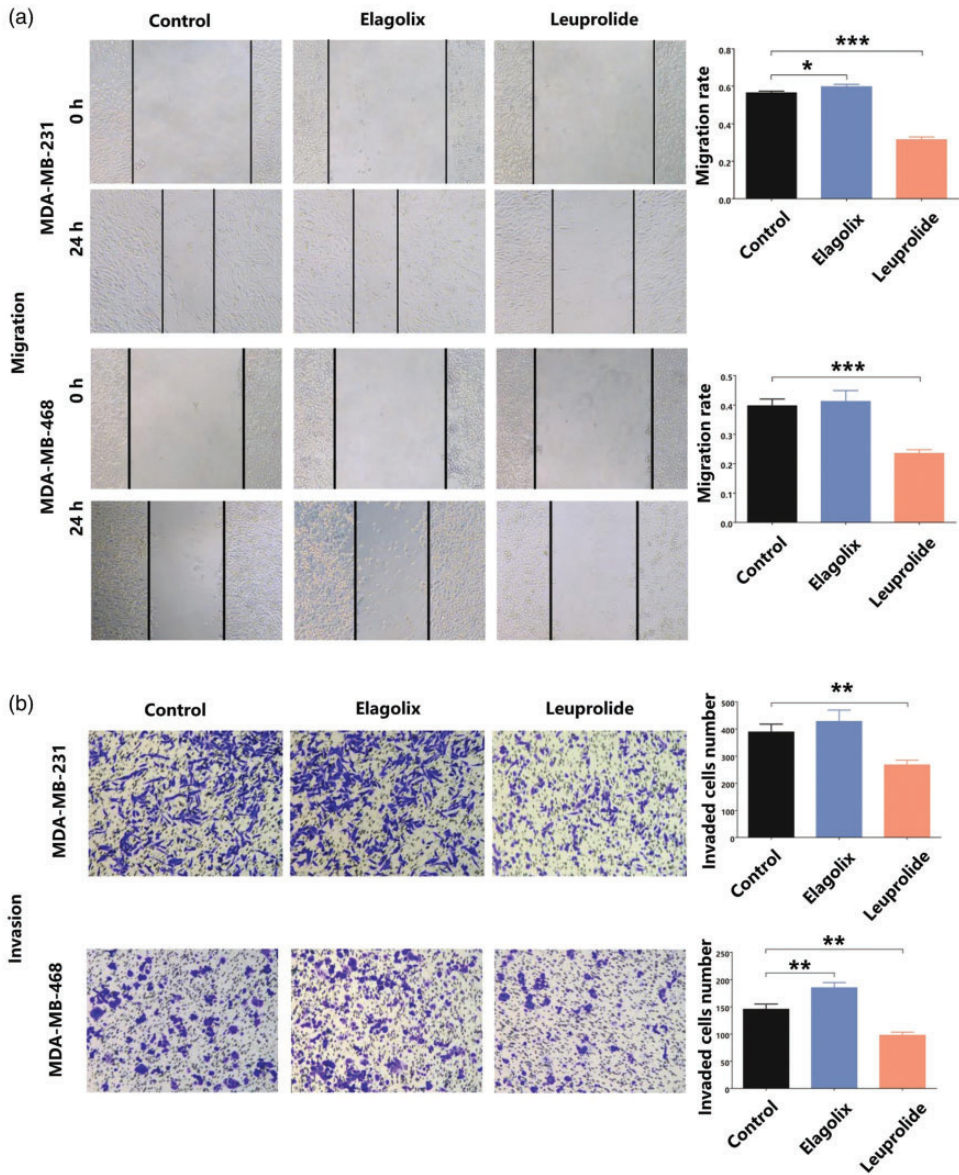
Figure 4. Activated GnRHR inhibits TNBC cell metastasis. (a) MDA-MB-231 and MDA-MB-468 cell migration was significantly lower after adding leuprolide acetate than controls. (b) MDA-MB-231 and MDA-MB-468 cell invasion was significantly lower after adding leuprolide acetate than controls. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

GnRHR, gonadotropin-releasing hormone receptor; TNBC, triple-negative breast cancer.
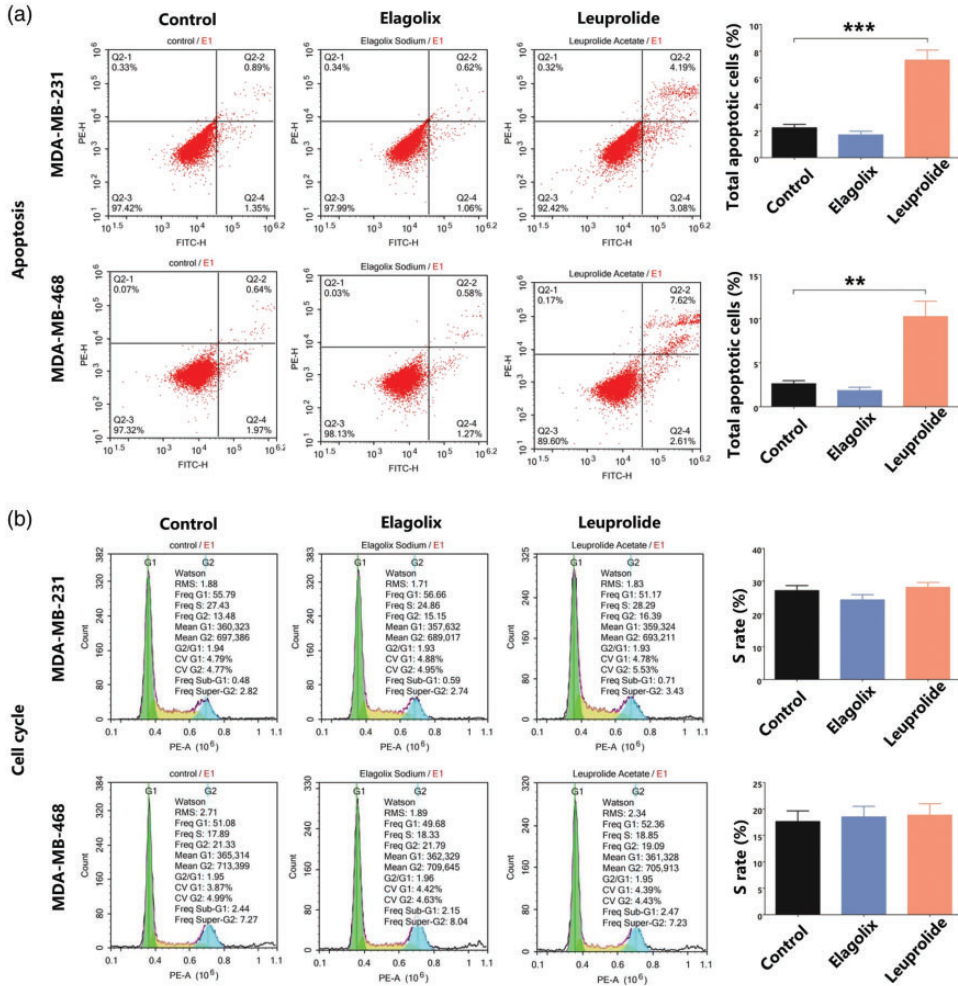
Figure 5. GnRHR effects on TNBC cell apoptosis and the cell cycle. (a) Significantly more MDA-MB-231 and MDA-MB-468 cells showed apoptosis after adding leuprolide acetate than controls. (b) The MDA-MB-231 and MDA-MB-468 cell S-phase rate was unchanged after adding leuprolide acetate or elagolix sodium compared with controls. *P < 0.05, **P < 0.01, ***P < 0.001.
GnRHR, gonadotropin-releasing hormone receptor; TNBC, triple-negative breast cancer.

cell apoptosis and the cell cycle. After adding leuprolide acetate for 72 hours, significantly higher numbers of apoptotic MDA-MB-231 and MDA-MB-468 cells were detected compared with controls, suggesting that activated GnRHR promoted apoptosis (*P* < 0.01; Figure 5a). However, there was no significant effect of either leuprolide acetate or elagolix sodium on the S-phase rate of MDA-MB-231 and MDA-MB-468 cells compared with controls, suggesting that the activation or antagonism of GnRHR had no effect on the cell cycle (Figure 5b).

Discussion

This study showed that the expression of GnRHR and GnRH mRNA in patients with TNBC was significantly higher than in those with HR+/HER2– or HER2+ breast cancer, suggesting that GnRHR and GnRH play an important role in the occurrence and development of TNBC. GnRHR mRNA expression in older patients with TNBC was also higher than in younger patients, which could be because many were menopausal and low levels of estrogen upregulate GnRHR expression through a feedback mechanism.[25] However, this finding differed from previous studies; for example, Paradiso et al. detected a higher proportion of GnRHR protein-positive cases in premenopausal breast cancer patients.[26] The present study did not analyze GnRHR protein expression. Given that mRNA expression does not directly correlate with protein levels because translation is affected by multiple factors, we plan to evaluate the effects of GnRHR protein in a future study.

We used MDA-MB-231 and MDA-MB-468 cells as *in vitro* models of TNBC, which are both confirmed TNBC cell lines.[27,28] The addition of leuprolide to these cells activated GnRHR, resulting in decreased proliferation and mobility and increased apoptosis, while the addition of elagolix antagonized GnRHR, resulting in increased cell proliferation and mobility. These results suggest that activated GnRHR inhibits TNBC proliferation and metastasis, which is consistent with the improved DFS observed in patients with high GnRHR expression, and is similar to the role of GnRHR in other malignant tumors.[12–16]

Conclusions

We showed that GnRHR inhibits proliferation and metastasis and promotes apoptosis in MDA-MB-231 and MDA-MB-468 TNBC cells. Once the molecular mechanism of this effect is understood, it might be possible to use GnRHR as a therapeutic target to improve the treatment of TNBC.

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