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GGNBP2 suppresses triple-negative breast cancer progression through inhibition of IL-6/STAT3 signaling activation

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Abstract

Triple negative breast cancer (TNBC) is the most aggressive subtype of breast cancer, lacking effective targeted therapies, and whose underlying mechanisms are still unclear. The gene coding for Gametogenetin-binding Protein (GGNBP2), also known as Zinc Finger Protein 403 (ZNF403), is located on chromosome 17q12-q23, a region known as a breast cancer susceptibility locus. We have previously reported that GGNBP2 functions as a tumor suppressor in estrogen receptor-positive breast cancer. In this study we find that GGNBP2 expression decreases in TNBC tissues and is associated with the outcome of breast cancer patients. Furthermore, experimental overexpression of GGNBP2 in MDA-MB-231 and Cal51 cells suppresses cell proliferation, migration and invasion, reduces the cancer stem cell subpopulation and promotes cell apoptosis *in vitro* as well as inhibits tumor growth *in vivo*. In these cell models, overexpression of GGNBP2 decreases the activation of IL-6/STAT3 signaling. Thus, GGNBP2 suppresses cancer progression by inhibition of IL-6/STAT3 activation in TNBC.

Keywords: triple negative breast cancer, progression, STAT3, GGNBP2,

Introduction

Breast cancer is the most frequently diagnosed cancer and the second leading cause of deaths among females worldwide [1]. Clinically, breast cancer patients are categorized into four subtypes based on the status of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and the expression of Ki-67: Luminal A, Luminal B, HER2-enriched and Triple negative breast cancer (TNBC). TNBC exhibits the highest aggressiveness and recurrence rates and chemotherapy remains the standard systemic treatment for TNBC due to lack of targeted therapies [2]. Therefore, the identification of new therapeutic targets for TNBC is urgently needed.

Gametogenetin binding protein 2 gene (*GGNBP2*), also known as zinc finger protein 403 (*ZNF403*), or laryngeal carcinoma-related protein 1 (*LCRG1*), is highly conserved, as *Drosophila*, mouse, rat and human share 87% homology in their nucleotide sequence [3]. *GGNBP2* is located on human chromosome 17q12-21, which is one of the most common loss of heterozygosity regions associated with many types of cancers, including breast cancer [4] and harbors several tumor suppressors or oncogenes such as *BRCA1*, *HER2* and *TP53*. The full-length transcript is 2869 nucleotides, including 14 exons, and encodes a protein of 698 amino acids containing a single C2H2 (Cystine-Cystine-Histidine-Histidine) zinc finger motif at the N-terminus as well as a consensus nuclear receptor binding box LxxLL (Leucine-X-X-Leucine-Leucine) [5,6]. *GGNBP2* was first identified in 2001 as a mouse gene dioxin inducible factor 3 (*DIF3*). It was induced by tetrachlorodibenzo-p-dioxin, which could lead to immunosuppression, organ toxicity, and tumorigenesis. The abnormal expression of *DIF3* was also detected in the testis and is associated with spermatogenesis and male germ cell development [3,7,8]. In addition, *GGNBP2* is expressed in the placenta during pregnancy and is essential for pregnancy maintenance through regulation of mouse trophoblast stem cell differentiation and proliferation [9].

In humans, *LCRG1*, a shorter form of *GGNBP2* containing 288 amino acids, is down-regulated in primary laryngeal carcinoma and experimental overexpression of *LCRG1* suppresses cell proliferation in laryngeal carcinoma [10]. Furthermore,

overexpression of GGNBP2 suppresses tumor proliferation and metastasis and its down-regulation is associated with drug resistance in ovarian cancer [11,12]. In glioma, GGNBP2 also functions as a tumor suppressor [13]. However, some studies indicate that depletion of GGNBP2 leads to cell cycle arrest at G2/M phase and inhibition of cell proliferation, suggesting that GGNBP2 may also function as an oncogene [14]. Our previous study indicated that GGNBP2 acts as a tumor suppressor by inhibiting ER activity in ER+ breast cancer cells [15].

In the present study, we investigate the role of GGNBP2 in TNBC. We observed a decreased GGNBP2 expression in TNBC and experimental overexpression of GGNBP2 suppresses cancer progression and decreases the stemness in TNBC. The expression of GGNBP2 is a potential prognostic predictor in patients with TNBC.

Materials and methods

Cell culture

Human breast cancer MCF7, T47D, SKBR3, MDA-MB-231, BT549, MDA-MB-468 and non-transformed breast cell line MCF10A were purchased from ATCC (Manassas, VA, USA). Cal51 cells were obtained from the German Collection of Microorganisms and Cell Cultures (Leibniz Institute DSMZ, Braunschweig, Germany). All the cells were cultured as previously described [15] and supplemented with 1% penicillin/streptomycin (Life Technologies, Grand Island, NY, USA), in a 5% CO₂ and humidified atmosphere at 37 °C. IL-6 (PeproTech, Rocky Hill, NJ, USA) was added to all cultures at final concentrations of 50 ng/mL.

Clinical samples and immunohistochemistry (IHC)

Two types of human breast cancer tissue microarrays used in this study were purchased from Shanghai Biochip Company Ltd. (Shanghai, China). One tissue microarray included 11 ductal carcinoma in situ (DCIS) and 59 invasive breast cancer (IBC) tissue specimens. The second tissue microarray contained 138 breast cancer samples with survival times, which were collected from 2001 to 2004. This study was approved by the Institutional Review Board of the Tianjin Medical University Cancer Institute and Hospital. IHC staining of GGNBP2 was performed as described

previously [16]. Briefly, slides were deparaffinized in xylene and rehydrated through a graded alcohol series to water. The slides were incubated with an anti-GGNBP2 primary antibody (Santa Cruz Biotechnology) for 3 h at room temperature. Reaction products were visualized following incubation with 3, 3'-diaminobenzidine. The negative control samples were treated identically, but without primary antibody. Slides were scanned using the Panoramic SCAN scanner (3DHISTECH Ltd.) for analysis. The staining score for each tumor sample was recorded separately. The percentage of immunostaining and the staining intensity (0, negative; 1+, weak; 2+, moderate; and 3+, strong) were recorded. An H-score was calculated using the following formula, $H - score = (\% \text{ of cells of weak intensity} \times 1) + (\% \text{ of cells of moderate intensity} \times 2) + (\% \text{ of cells of strong intensity} \times 3)$ [17].

RNA interference (RNAi)

For RNAi-mediated knockdown of *GGNBP2*, cells were transfected with 100 nM of either siGGNBP2 or siControl (Ribobio, Shanghai, China) using Lipofectamine 3000 (Life Technologies) according to the manufacturer's instructions for 48 h. The target sequence of GGNBP2 siRNA is GGGUAAUUAGCAGAUUGGAATT. The scrambled sequence has no significant homology to any known human gene sequence in the GenBank database.

RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from frozen tissues or cultured cells using TRIZOL reagent (Life Technologies) and reverse transcription was performed using First-strand cDNA Synthesis System (Life Technologies) according to the manufacturer's instructions. qPCR was performed to detect mRNA expression using GoTaq qPCR Master Mix according to the manufacturer's protocol (Promega, Madison, WI, USA). The real-time quantitative PCR was performed on a Bio-Rad iQ5 Optical System (Bio-Rad, Hercules, CA, USA). PCR was carried out after incubation at 50 °C for 2 min and predenaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 30 s and 62 °C for 1 min. *RPS14* was used as an internal mRNA quantity control. The

1 sequences of PCR primers were as follow: *GGNBP2*
2 (forward:5'-ATCACGCGAAGTCCTGAGTG-3'; reverse:
3 5'-GGGAAAAGAGACGCTCCACA-3'), *OCT4* (forward:
4 5'-AGCAAAACCCGGAGGAGT-3'; reverse:
5 5'-CCACATCGGCCTGTGTATATC-3'), *MYC* (forward:
6 5'-GGCACTTTGCACTGGAACCTT-3'; reverse:
7 5'-AGGCTGCTGGTTTTCCACTA-3'), *NOTCH1* (forward:
8 5'-ACTGTGAGGACCTGGTGGAC-3; reverse: 5;-
9 TTGTAGGTGTTGGGGAGGTC-3'), *RPS14* (forward:
10 5'-TCACCGCCCTACACATCAAAC-3'; reverse:
11 5'-CTGCGAGTGCTGTCAGAGG-3')

22 *Western blot*

23 Cells were lysed in Triton-X 100 lysis buffer containing 1% (v/v) Triton-X 100, 150
24 mM NaCl, 50 mM Tris-HCl with protease and phosphatase inhibitors. The cell lysates
25 were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and
26 then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA).
27 The membranes were then blocked with 5 % nonfat milk and incubated overnight
28 with anti-STAT3 (Cell signaling, Beverly, MA, USA), anti-mTOR (Cell Signaling),
29 anti-GGNBP2 (Santa Cruz Biotechnology), anti-pAKT (Cell Signaling),
30 anti-p-MAPK (Cell Signaling), anti-p-Jak1 (Cell Signaling), anti-Bax (Cell Signaling),
31 anti-Mcl-1 (Cell Signaling) and anti-pSTAT3 (Cell Signaling), respectively.
32 Peroxidase-conjugated anti-mouse or anti-rabbit IgG (Vector Laboratories,
33 Burlingame, CA, USA) was used as the secondary antibody. Immunoblotting signals
34 were detected using the ECL reagent (Millipore). All membranes were reprobed with
35 anti- β -actin antibody (Cell Signaling), which served as a loading control.

52 *Cell proliferation assays*

53 Cell counting and EdU assays were used to evaluate cell proliferation ability. For cell
54 counting assay, cells were seeded into 24-well plates at a density of 2.5×10^4 cells/per
55 well and cultured for 2, 4, and 6 days. Medium was changed with fresh media every

other day. Cells were harvested by trypsinization and counted using a hemocytometer under an Olympus light microscope. The EdU assay was performed using the Cell-Light EdU Apollo488 kit according to the manufacturer's protocol (Ribobio). EdU-positive cells were calculated as (EdU add-in cells/Hoechst stained cells) × 100% under a fluorescence microscope.

Fluorescence-activated cell sorting analyses (FACS)

For cell cycle distribution assay, cells were harvested and fixed with 70% ethanol, washed with ice cold PBS and resuspended in 0.1% (v/v) Triton-X 100/PBS and concomitantly treated with RNase A (200 µg/ml) and stained with 50 µg/ml propidium iodide (PI) for 15 min at room temperature as previously described [18].

Cell apoptosis was assessed using an Annexin V Staining Kit (BD Biosciences, San Diego, CA, USA) essentially as described [19]. Briefly, cells were harvested, washed with cold PBS and resuspended in binding buffer and incubated with Annexin V and PI for 15 min at room temperature. Sorting was performed and were analyzed using BD FACSCanto II (BD Biosciences).

For stem cell marker analysis, APC-conjugated CD44 and phycoerythrin (PE)-conjugated CD24 monoclonal antibodies (BD Biosciences) were used essentially as described [16]. Cells were resuspended in PBS at 1×10^6 cells/ml and both APC-CD44 and PE-CD24 antibodies, or their respective isotype controls APC-IgG and PE-IgG, added and incubated for 40 min at 4 °C in the dark. Labeled cells were washed in PBS and then analyzed in a flow cytometer. Gating was set to relevant isotype control (APC-IgG and PE-IgG)-labeled cells for each cell line. An Aldefluor assay kit (StemCell Technologies, Vancouver, BC, Canada) was used for the determination of ALDH activity with flow cytometry essentially as described [16].

Xenografts

To generate stable cells, the lentiviruses (RiboBio) were used to infect MDA-MB-231 cells according the manufacturer's recommendations. MDA-MB-231-GGNBP2 and control cells (1×10^6 cells/injection) were suspended in 100 µl PBS containing 20% matrigel (BD Biosciences) and injected into the mammary fat pad of 5-week-old

female nude mice (Vital River Company, Beijing, China). Tumor size was recorded once a week with a caliper. Tumor volume was calculated according to the formula $\text{volume} = 0.5 \times \text{width}^2 \times \text{length}$. Twenty-eight days after inoculation, mice were sacrificed and the final volume and weight of tumor tissues were determined. The use of animals in this study has been approved by the animal care and use committee of Tianjin Medical University Cancer Institute and Hospital.

Statistical analysis

Statistical evaluations were performed by Student's t-test for paired data, and data were considered significant at a p-value <0.05 . Kaplan-Meier survival curves and the log-rank test were used to evaluate patients of breast cancer with different GGNBP2 expression. All calculations were performed with the SPSS for Windows statistical software package (SPSS Inc., Chicago, IL, USA).

Results

GGNBP2 is associated with breast cancer progression and prognosis

We have previously reported that the expression of GGNBP2 is decreased in breast cancer tissues compared with the normal breast tissues [15]. In this study, we further determined the expression of GGNBP2 in DCIS (n=11) and IDC (n=59) by IHC. As shown in Figure 1A and 1B, the expression of GGNBP2 was significantly down-regulated in IDC compared with the DCIS ($P<0.0001$). Next, the expression of GGNBP2 was determined in a tissue array containing 138 cases of breast cancer specimens by IHC (Figure 1C). A receiver operating characteristic curve (ROC) was made based on the expression of GGNBP2 and overall survival status of breast cancer patients and the optimal cut-off value (staining score=43.46) was determined to group all patients into two groups, GGNBP2_{high} (n=86) and GGNBP2_{low} (n=52) (Figure 1D). Kaplan-Meier analysis indicated that the overall survival time of patients with low expression of GGNBP2 was shorter than those with high expression ($P=0.029$, Figure 1E). Together, these results indicate that decreased expression of GGNBP2 is associated with cancer progression and poor prognosis in patients with breast cancer.

GGNBP2 is down-regulated in triple-negative breast cancer

To further investigate the role of GGNBP2 in breast cancer progression, we next determined the expression of GGNBP2 in breast cancer cell lines by RT-qPCR and western blot. As shown in Figure 2A and 2B, the expression of GGNBP2 was down-regulated in breast cancer cell lines compared with the non-transformed breast cell line MCF10A. Furthermore, we observed a decreased GGNBP2 expression in TNBC cell lines compared with the other two subtypes (Figure 2A and 2B). To verify whether these cell line data were also reproduced in clinical samples, we analyzed GGNBP2 scores in luminal (n=87), Her2-positive (n=24) and TNBC (n=25). Importantly, TNBC patients showed down-regulation of GGNBP2 with respect to luminal ($P = 0.0328$, Figure 2C). Thus, GGNBP2 is down-regulated in TNBC.

Overexpression of GGNBP2 suppresses cancer progression in TNBC in vitro

To investigate the role of GGNBP2 in TNBC progression, we generated stable Cal51 and MDA-MB-231 GGNBP2 overexpressing cells through lentiviral transfection (Figure 3A and 3B). Cell growth curves indicated that Cal51-GGNBP2 and MDA-GGNBP2 cells grew more slowly than their respective controls transfected with empty vector (Cal51-control and MDA-control, respectively; Figure 3C). This was confirmed with another proliferation assay determining incorporation of EdU, as the population of EdU-positive cells significantly decreased in Cal51-GGNBP2 and MDA-GGNBP2 cells compared with their control cells (Figure 3D). Flow cytometry analysis indicated that there was also an accumulation of cells at the G0/G1 cell cycle stage in Cal51-GGNBP2 (65.4%) and MDA-GGNBP2 (46.8%) cells (Figure 3E). Furthermore, the migration and invasion capabilities of MDA-MB-231 were reversed by GGNBP2 overexpression (Figure 3F). Overexpression of GGNBP2 also led to an increased proportion of apoptotic, Annexin-V positive, cells (28% increase in Cal51-GGNBP2 cells and 23% increase in MDA-GGNBP2 cells; Figure 3G). Together, these results indicate that GGNBP2 inhibits cell proliferation, migration and invasion, and promotes apoptosis in TNBC.

Cancer stem cells (CSCs), a subpopulation of tumor cells possessing the extensive self-renewal capability necessary to successfully colonize distant organs,

relates to highly aggressive TNBC [20]. To determine whether GGNBP2 has the capacity to modulate stem cell phenotypes, we determined the CD44^{high}/CD24^{low} population by flow cytometry. Indeed, MDA-GGNBP2 cells exhibited a significant decrease (11.5%) in CD44^{high}/CD24^{low} stem cell population compared with control cells (Figure 4A) and this was accompanied by down-regulation of stem cell markers Oct4, c-Myc and Notch1, not only in MDA-GGNBP2 cells (Figure 4B) but also in Cal51-GGNBP2 cells (Figure 4D). As ALDH has also been identified as a CSC marker in different type of cancers, and represents the CSC subpopulation better than CD44^{high}/CD24^{low} [21] in Cal51 cells, we also tested ALDH activity by ALDEFLUOR assay in Cal51-GGNBP2 and control cells. Cal51-GGNBP2 cells had reduced the percentage of ALDH-positive cells to ~15% from ~50% in control cells; Figure 4C) These data indicate that GGNBP2 decreases the stemness of TNBC cells.

Overexpression of GGNBP2 suppresses tumor growth of TNBC in vivo

To further demonstrate the role of GGNBP2 *in vivo*, MDA-GGNBP2 and control cells were implanted into the mammary fat pads of nude mice and tumor growth monitored. As expected, GGNBP2 overexpression significantly reduced tumor growth. The tumor size of MDA-GGNBP2 xenografts was significantly smaller compared with the control xenografts (tumor volume at 28 days of ~450 mm³ in the control and ~100 mm³ in MDA-GGNBP2 xenografts; Figure 5A and 5B). RT-qPCR analysis indicated that the expression of *GGNBP2* was significantly elevated (approximately 2-fold) in MDA-GGNBP2 xenografts compared with the controls (Figure 5C). Together, these results indicate that ectopic overexpression of GGNBP2 suppresses tumor growth of TNBC *in vivo*.

GGNBP2 inhibits the IL-6/STAT3 signaling pathway in TNBC

GGNBP2 has been proved being significant in maintaining the balance of trophoblast stem cells proliferation and differentiation by regulating c-Met-STAT3 signaling [9]. Over phosphorylation of AKT, MAPK, STAT3 and JAK1, as well as overexpression of mTOR has been implicated in the development of TNBC and is associated with poor prognosis in patients with breast cancer, reported by our group and others

[16,22-25]. Hence, to further investigate the GGNBP2-regulated signaling in TNBC progression, we determined the expression of STAT3, pSTAT3, pAKT, pMAPK, mTOR, pJak1 in Cal51-GGNBP2 and MDA-GGNBP2, as well as the vector control cells by western blot. As shown in Figure 6A, STAT3 phosphorylation was markedly decreased in GGNBP2-overexpressed cells. Akt, MAPK, mTOR and Jak pathways were not affected by ectopic expression of GGNBP2. Activation of IL-6 has been identified to induce phosphorylation and activation of STAT3 transcription factor [26]. To further verify whether the apoptosis increase observed after GGNBP2 overexpression was due to the down-regulation of STAT3 activation, a rescue methodology was adopted. As shown in Figure 6B, the addition of IL-6 partially restored STAT3 phosphorylation in MDA-MB-231 and Cal51 cells after GGNBP2 overexpression. Additionally, the addition of IL-6 to GGNBP2 overexpressing cells decreased the expression of pro-apoptotic Bax, which was promoted after GGNBP2 overexpression. Similarly, the addition of IL-6 partially restored anti-apoptotic Mcl-1 expression which was inhibited in the cells with GGNBP2 overexpression. Furthermore, depletion of GGNBP2 by RNA interference enhanced STAT3 phosphorylation in MDA-MB-468 cells, a TNBC cell line with moderate GGNBP2 expression (Fig 2B), resulting in an activation of STAT3 signaling. Thus, these results indicate that GGNBP2 inhibits the IL-6/STAT3 signaling pathway in TNBC.

Discussion

In this study, we identify GGNBP2 as a tumor suppressor in TNBC and show evidence that GGNBP2 is decreased in TNBC, correlating with survival of breast cancer patients. Overexpression of GGNBP2 suppresses TNBC progression *in vitro* and tumor growth *in vivo*. Furthermore, overexpression of GGNBP2 decreases the stemness of TNBC. Thus, our results demonstrated that GGNBP2 functions as a tumor suppressor in TNBC and indicate that this is probably due in part to inhibition of the IL-6/STAT3 signaling pathway. This offers the possibility of its use as a potential biomarker for predicting the outcome in patients with TNBC.

GGNBP2 was first identified in male germ cells during spermatogenesis [3,27,28]. LCRG1, a shorter form of GGNBP2, was previously reported to function as

1 a tumor suppressor in cancer development and progression, including laryngeal
2 carcinoma [10], ovarian cancer [11] and glioma [13]. However, one study reported
3 that knockdown of GGNBP2 inhibits cell proliferation in cervical cancer [14]. Our
4 previous study indicated that overexpression of GGNBP2 inhibits breast cancer
5 proliferation by inhibition of ER α activity in ER positive breast cancer [15]. In this
6 study, we further investigate the role of GGNBP2 in TNBC development and
7 progression. The unrestrained cell proliferation and resistance to apoptosis have been
8 demonstrated as the fundamental traits of malignant cancer. The activation of
9 proliferative signals guides cancer cells into cell cycle progression, during which G1
10 to S transition is the most important step [29]. In the present study, overexpression of
11 GGNBP2 in MDA-MB-231 and Cal51 cells was found to enhance cell cycle arrest at
12 G0/G1 phase and apoptosis, resulting inhibition of cell proliferation *in vitro*.
13 Furthermore, ectopic expression of GGNBP2 in MDA-MB-231 suppresses tumor
14 growth *in vivo*. Taken together, GGNBP2 can reduce cell proliferation by induction of
15 G0/G1 phase arrest in TNBC. Therefore, GGNBP2 can likely exert both oncogenic
16 and anti-oncogenic functions depending on the cancer type. This pleiotropic nature is
17 not surprising because several genes had been identified to play diverse roles in
18 different types of cancer. For example, CBX4 has been proposed to function as an
19 oncogene in hepatocellular carcinoma by positively regulating proliferation,
20 angiogenesis and metastasis. However, CBX4 may be a tumor suppressor in
21 colorectal carcinoma [30]. EP300, a transcriptional co-activator, functions as histone
22 acetyltransferase that regulates transcription *via* chromatin remodeling and acetylates
23 all four core histones in nucleosomes. Consistent with our previous study, EP300
24 exert tumor suppressive effects through promoting the function of other tumor
25 suppressors, such as p53, RB1, E-cadherin [31,21]. Despite the tumor suppressive
26 roles of EP300, several lines of evidence suggest that EP300 can also participate in
27 promoting cancer and inhibition of EP300 can interfere with oncogene-driven
28 transcriptional programs in cancer cells [32,33].

29 Breast CSCs characterized by CD44^{high}/CD24^{low} or ALDH^{high} are responsible for
30 breast cancer formation, progression and metastasis [34,35]. In this study, we have
31 shown that overexpression of GGNBP2 in MDA-MB-231 and Cal51 cells decreases
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the CD44^{high}/CD24^{low} or ALDH^{high} subpopulation, a property of stem cells. Furthermore, overexpression of GGNBP2 down-regulates the expression of CSC marker OCT4, c-Myc and Notch1 in MDA-MB-231 and Cal51 cells. Metastasis is another hallmark of cancer and the main cause of many tumor deaths, which is a complex process, including migration, invasion, angiogenesis, extracellular matrix degradation and basement membrane breakdown [11,36]. Importantly, overexpression of GGNBP2 impairs the ability of migration and invasion in MDA-MB-231 cells, suggesting that GGNBP2 is involved in breast cancer metastasis. Thus, GGNBP2 suppresses TNBC progression by inhibition of metastasis and reduction of stemness.

IL-6, a key mediator of the inflammatory response, is involved in a variety types of cancer development and progression, including breast cancer [37]. IL-6 has been implicated as a potential regulator of normal and CSC self-renewal [38]. The signal transduction of IL-6 involves the activation of JAK family members, resulting in the activation of STAT3 [39]. The IL-6/STAT3 has also been identified to regulate CSCs in several types of cancers, including breast cancer [40-42]. Our previous study indicated that sabutoclax overcomes the drug-resistance phenotype of breast cancer and reduces the population of CSCs by abolition of the IL-6/STAT3 signaling [16]. STAT3 is an attractive target for anti-cancer drug discovery and many approaches have been used to discover drugs targeting STAT3 over the last twenty years. However, no STAT3-targeting therapeutics have been approved despite the fact that several drugs have entered clinical trials, including OPB-31121, OPB-516-2 and C188-9 [43]. GGNBP2 is a critical factor for pregnancy success acting through the maintenance of a balance of trophoblast stem cells proliferation and differentiation by regulating c-Met-STAT3 signaling [9]. Our current study indicates that GGNBP2 inhibits the activation of IL-6/STAT3 pathway in TNBC, suggesting that GGNBP2 suppresses TNBC progression through inhibition of IL-6/STAT3 pathway activation.

TNBC is the most aggressive form of breast cancer with high histologic grade, aggressive clinical behavior, high incidence of brain and lung metastases, and the lack of an effective therapeutic target [44]. Our results show that the expression of GGNBP2 is significantly decreased in TNBC and associated with the prognosis in patients with breast cancer, suggesting that GGNBP2 is a potential biomarker for

diagnosis and prognosis of TNBC.

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Compliance with Ethical Standards

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Conflict of interest: The authors declare no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Review Board of the Tianjin Medical University Cancer Institute and Hospital and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Figure legends

Figure 1 GGNBP2 is decreased in breast cancer and is associated with prognosis. A, Expression of GGNBP2 in DCIS (n = 11) and IDC (n = 59) by IHC. Two cases of each type are shown for illustration purposes. B, Statistical analysis of scoring values for GGNBP2 expression in DCIS and IDC. Median expression is marked. C, Overview of GGNBP2 expression by IHC in a tissue array containing 138 cases of breast cancer specimens. D, ROC based on the expression of GGNBP2 and overall

1 survival status of breast cancer patients. E) Kaplan-Meier analysis of overall survival
2 in patients with different GGNBP2 expression levels.
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6 Figure 2 GGNBP2 expression is decreased in TNBC. A, The expression of *GGNBP2*
7 mRNA levels in breast cancer cell lines and a non-transformed (*normal*) breast cell
8 line determined by RT-qPCR and normalized to that of *RPS14* mRNA. Data indicates
9 average $\pm$ SD of three independent experiments. B, The expression of GGNBP2
10 protein levels in breast cancer cell lines and normal breast cell line determined by
11 western blot. β -actin was used as a loading control. One representative blot is shown
12 out of three independent experiments. C, The expression of GGNBP2 in different
13 subtypes of breast cancer by IHC. Median expression is marked. * $P < 0.05$.
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24 Figure 3 Overexpression of GGNBP2 suppresses TNBC proliferation, migration,
25 invasion, and promotes cell apoptosis *in vitro*. A and B, The expression of GGNBP2
26 in stably transfected Cal51 and MDA-MB-231 cells with a GGNBP2 expression
27 vector or empty vector, as determined by RT-qPCR (A) and western blot (B). C and D,
28 Cell counting (C) and EdU incorporation (D) analyses of cell proliferation in
29 MDA-MB-231 and Cal51 cells expressing GGNBP2 or empty vector. Incorporation
30 of EdU is shown in green (magnification: $\times 200$) by fluorescence microscopy (upper
31 panels). Quantification of EdU incorporated cells after counting the cells (lower
32 panels). E, Cell cycle distribution of MDA-MB-231 and Cal51 cells expressing
33 GGNBP2 or empty vector by flow cytometry. Histograms represent the percentage of
34 cells in the G0/G1, S and G2/M phases. F, Transwell analysis of migration and
35 invasion in MDA-MB-231 cells expressing GGNBP2 or empty vector and stained
36 with crystal violet (magnification: $\times 200$) (upper panels). Quantification of migration
37 and invasion after counting the cells (lower panels). G, Apoptosis analysis by Annexin
38 V and PI staining of MDA-MB-231 and Cal51 cells expressing GGNBP2 or empty
39 vector by flow cytometry. Early apoptotic cells were defined as Annexin V-positive
40 and PI-negative. Numerical data indicate mean $\pm$ SD of three independent
41 experiments. Pictorial data show a representative of three independent experiments.
42 Microscopy data show a representative field out of 10 acquired. * $P < 0.05$.
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Figure 4 Overexpression of GGNBP2 decreases the cancer stem cell population in TNBC. A, Flow cytometry analysis of cell-surface markers CD44 and CD24 in MDA-MB-231 cells expressing GGNBP2 or empty vector. B, Expression of Oct4, c-Myc and Notch1 in stably transfected MDA-MB-231 cells overexpressing GGNBP2, or empty vector, determined by RT-qPCR and normalized to vector control. C, Flow cytometry analysis of ALDH in Cal51 cells expressing GGNBP2 or empty vector treatment with or without ALDH inhibitor DEAB. D, Expression of Oct4, c-Myc and Notch1 in stably transfected Cal51 cells overexpressing GGNBP2, or empty vector, determined by RT-qPCR and normalized to vector control. Numerical data indicate mean $\pm$ SD of three independent experiments. Pictorial data show a representative of three independent experiments. * $P < 0.05$.

Figure 5 Overexpression of GGNBP2 inhibits tumor growth *in vivo*. A, Representative photos of five tumors formed by MDA-MB-231 cells expressing GGNBP2 or empty vector after 28 days. B, Tumor volume of xenografts in mice injected with MDA-MB-231 cells expressing GGNBP2 or empty vector at the indicated times. C, Expression of GGNBP2 in tumors formed by MDA-MB-231 cells expressing GGNBP2 or empty vector by RT-qPCR. * $P < 0.05$.

Figure 6 GGNBP2 inhibits the IL-6/STAT3 signaling pathway in TNBC. A, Expression of STAT3, pSTAT3, pAKT, pMAPK, mTOR, pJak1 in GGNBP2-overexpressed Cal51 or MDA-MB-231 and the vector control cells by western blot. B, Expression of STAT3, pSTAT3, Mcl-1 and Bax in GGNBP2-overexpressed Cal51 or MDA-MB-231 and the vector control cells treatment with or without IL-6 by western blot. IL-6 was treated at final concentrations of 50 ng/mL for 24 h. C, Expression of GGNBP2, STAT3 and pSTAT3 by in siRNA GGNBP2-depleted MDA-MB-468 and scrambled siRNA control cells by western blot. Representative blots from three different experiments are shown.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

MDA-MB-231

MDA-MB-231-
GGNBP2

B

C

Figure 6

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