G protein subunit gamma 5 promotes the proliferation, metastasis and glycolysis of breast cancer cells through the Wnt/β-catenin pathway
Zuguo Yuan, Ruiping Ren and Zhengyang Xu

GNG5 is suggested to exert a critical effect on tumor development in human beings; however, its function and related mechanism within breast cancer (BC) are still unclear. In this regard, the present work focused on identifying and evaluating GNG5’s function and revealing its possible molecular mechanism. Subcutaneous tumorigenesis model of nude mice and in-vitro cell model was established. The relationship between GNG5 expression and BC was studied through knockdown and overexpression experiments. The proliferation, migration, invasion and epithelial–mesenchymal transition (EMT) of liver cancer cell lines overexpressing or silencing GNG5 were detected. Furthermore, the pathway mechanism of GNG5 was evaluated at the molecular level and was performed to further verify the possible targets and mechanisms of action. In comparison with that in normal tissue, GNG5 level within BC tissue was higher. In addition, GNG5 overexpression stimulated BC cell proliferation, invasion, migration and EMT. BC cells with reduced GNG5 expression exhibited significant decreases in glucose uptake, lactate levels, and ATP concentrations. In addition, GNG5 knockdown inhibited Wnt/β-catenin signaling. This study indicates that GNG5 may generate a vital function in BC. The results of the current work demonstrated GNG5’s effect on BC pathological process, also providing a reference for developing new targeted therapies for BC. Anti-Cancer Drugs 33: 1004–1011
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Keywords: breast cancer, glycolysis, GNG5, metastasis, proliferation, Wnt signaling pathway

Introduction
Breast cancer (BC), known as a frequently occurring cancer globally due to its high degree of malignancy and high lethality, has seriously threatened human health [1–3]. The mechanism underlying the invasion and metastasis of BC, however, remains to be elucidated. Therefore, it is necessary to discover new biomarkers that are more specific, stable and easier to detect for BC to assist in early diagnosis, personalized medical classification of patients and improve low survival results.

G protein subunit gamma 5 (GNG5) gene encodes G-γ5 subtype with oncogenes activity, which exerts an essential function in regulating cell adhesion, growth and migration [4]. Current research has shown that GNG5 is closely related to most types of cancer, such as gliomas [5], lymphoma [6] and metastatic thymic adenocarcinoma [7]. Nevertheless, GNG5’s effect on BC still needs further investigation.

Usually, detachment of cancer stem cells (CSCs) from the normal somatic system is thought to be the beginning of cancer [8]. CSC characteristics are modulated via pathways with evolutionary conservation (like Notch, Wnt/β-catenin 5 and Hedgehog), similar to healthy somatic stem cells. Among these pathways, Wnt pathway relates to numerous fundamental procedures in adulthood homeostasis and embryogenesis. It is related to diverse cell functions required for physiological organ growth, such as cell growth, survival, differentiation and self-renewal [9,10]. In addition, it also relates to regulating pathways within diverse disorders, especially cancer, such as liver cancer [11,12], colon cancer [13] and leukemia [14].

It has been reported that GNG5 serves as an oncogene in gliomas and is in association with the poor prognosis of the patients. However, its mechanism of action in BC is still unknown. This work detected and evaluated GNG5’s possible function and revealed the potential molecular mechanism of its role, in order to provide information for diagnosing and treating BC.

Materials and methods
Sample collection
Totally 88 BC patients from the Affiliated People’s Hospital of Ningbo University were recruited for this study. All patients were rigorously screened for the...
clinical characteristics of BC cases and excluded other causes. The cancer tissues were taken and washed three times with saline-treated with diethyl pyrocarbonate to rinse the blood clean. The tissues were then quickly frozen in liquid nitrogen. Tissue extraction should be performed within 15 min after cesarean section. Each subject provided informed consent. The present work gained approval from Ethics Committee of the Affiliated People’s Hospital of Ningbo University (Ethical Application Ref: the Affiliated People’s Hospital of Ningbo University).

Cell lines
We acquired human healthy breast epithelial MCF10A cells and BC cells (BT549, MDA-MB-231, MCF7, MDA-MB-468) in Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Afterward, we cultivated cells within DMEM (HyClone; Cytiva) that contained 10% fetal bovine serum (FBS, BI, USA) under 5% CO₂ and 37 °C conditions. Meanwhile, we obtained short hairpin (sh) RNA GNG5 lentiviral vector plasmid and the associated shControl in Shanghai GenePharma Co., Ltd. GNG5 expression was silenced in BT549 and MDA-MB-231 cells.

Quantitative real-time PCR analysis
We adopted quantitative real-time PCR (qRT-PCR) for analyzing expression of GNG5 in BC cells line. A TRIzol kit was adopted for extracting total RNA, cDNA synthesis reverse transcription kit for reverse transcription, SYBR-Green I Master kit for qRT-PCR, and finally detection on LightCycler 480 system. The above kits are from Takara and follow the manufacturer’s protocol. The 2−ΔΔCt approach was utilized to determine amplification products as previously depicted [15].

Western-blot assay
We conducted Western-blot (WB) assay to determine GNG5 level. In brief, we separated 25-µg proteins through 10% SDS-PAGE and transferred them onto PVDF membranes (Millipore, Billerica, Massachusetts, USA). Later, we blocked membranes using five defatted milk that contained Tris-buffered saline with 0.1% Tween 20 Detergent and incubated them using the primary antibody under 4 °C (against GNG5, 1:5000, Abcam, Shanghai, China; HK2, 1:5000, Abcam, Shanghai, China; and lactate dehydrogenase A (LDHA), 1:5000, Abcam, Shanghai, China; anti-beta-catenin, Proteintech, China; anti-cyclin D1, Proteintech, China; anti-c-myc, Proteintech, China; anti-GAPDH, Proteintech, China), and subsequent incubation using the horseradish peroxidase-labeled secondary antibody (1:10000, Solarbio, Beijing, China). Later, we visualized results using the ECL system (Millipore, Billerica, Massachusetts, USA) and quantified protein blots by adopting Quantity One software (Bio-Rad, Hercules, California, USA).

Transwell assays
We determined BC cell invasion and migration by Transwell assays. Briefly, we seeded 1 × 10⁵ cells/chamber from diverse groups into the Transwell chamber (Corning costar, Corning, New York, USA) to achieve 72-h culture. Then, medium was discarded, and fluorescein isothiocyanate-dextran (200 µl, 1 mg/ml, molecular weight 4000; Sigma; Cat No. 46944) was supplemented into upper chamber for 15-min incubation under 37 °C. Thereafter, we detected dye intensity within the bottom chamber by the fluorescence microplate reader (m200 pro, TECAN, Switzerland) at 485 nm. Each assay was carried out thrice.

Colony formation
We seeded cells (600/well) in the six-well plates, followed by 10-day culture within DMEM. After PBS washing, cells were subject to 10% formalin fixation as well as 0.1% crystal violet staining. Afterward, we dried the dish and took photographs. Finally, we counted the number of clones containing over 50 cells for subsequent analysis.

Assay of glucose, lactate and ATP
The 2-NBDG Glucose Uptake Detection Kits (BioVision, K682-50) were adopted to determine cell glucose uptake in line with specific instructions. We adopted lactate detection kits (Nanjing Jiancheng Bioengineering Institute, A019-2) for determining lactate content within supernatants from cell culture. ATP concentrations were extracted and assayed using, ATP assay kits (Nanjing Jiancheng Bioengineering Institute, A095) were adopted for measuring ATP content in cells. Three biological replicates were performed. Detailed operations followed the previous description SLC1A3 regulates PI3K/AKT pathway to enhance gastric cancer development.

Xenograft tumor models
We classified the BALB/c female nude mice (4 weeks old) as two groups, with six each. The model was established by subcutaneous injection of different groups of cells into mice. Subcutaneous tumor size was computed every 7 days. After 28 days, the rats were sacrificed. The obtained results were observed by naked eye and H&E staining. Each animal experiment gained approval from XXX and was carried out following related regulations and guidelines.

Immunohistochemistry
The slices were soaked in xylene for 15 min before ethanol-gradient water rehydration. Later, we soaked sections within citric acid (pH 6.0 DAKO), followed by another 10-min incubation. When cooling till ambient temperature, we rinsed each section by water and PBS for a 15-min period, followed by 10-min incubation using 3% H₂O₂. Each section was later subject to 30-min blocking using 5% BSA, probed using primary antibody under 4 °C overnight, and developed with DAB color-rendering kit.
Cell Counting Kit-8
We measured cell proliferation by Cell Counting Kit-8 (CCK-8) assay (Beyotime, C0037). The different transfection of the cells was inoculated in the 96-well plate to culture for 0, 12, 24 and 48 h, and 100 μl 10% CCK-8 solution were added respectively. We measured absorbance values of different groups using microplate reader (Biotek, Winooski, Vermont, USA) after 2-h incubation under 37 °C and 5% CO₂ conditions.

Statistical analysis
This study obtained the GNG5 datasets for BC in The Cancer Genome Atlas (TCGA) database. Results were displayed in a form of means ± SD. Both groups were compared through carrying out Student’s t-test. We performed one-way analysis of variance for comparison across diverse groups. P < 0.05 stood for statistical significance. We utilized GraphPad Prim 5 (GraphPad Software, La Jolla, California, USA) for carrying out statistical analysis.

Results
GNG5 expression elevated within breast cancer tissues and cells
As revealed by TCGA database analysis, GNG5 showed high expression within BC samples relative to healthy counterparts (P < 0.05; Fig. 1a). GNG5 levels within BC samples were markedly upregulated relative to matched noncanceroma samples using qRT-PCR (P < 0.01; Fig. 1b) and Western blot (P < 0.01; Fig. 1c). As revealed by Kaplan–Meier curve analysis, GNG5 upregulation predicted dismal overall survival (OS) (Fig. 1c); besides, BC cases showing GNG5 upregulation displayed poor relapse-free survival (Fig. 1d). Besides, GNG5 upregulation was detected within BC cells relative to MCF10A cells using qRT-PCR (P < 0.01; Fig. 1f) and Western blot (P < 0.01; Fig 1g). We chose BT549 and MDA-MB-231 cells in subsequent experiments because they expressed the top 2 in GNG5.

GNG5 knockdown inhibited breast cancer cell growth and migration
To explore GNG5’s function, we transfected BT5491 and MDA-MB-231 cells using sh-GNG5 or negative control (NC). As revealed by WB assay, sh-GNG5 #1, #2 and #3 could effectively knock down more than 50% of GNG5 within BT549 and MDA-MB-231 cells (P < 0.01; Fig. 2a). GNG5 knockdown markedly inhibited the above two cell lines as suggested through CCK-8 assay (P < 0.01; Fig. 2b). The clone formation capacity of GNG5 was assessed, which indicated that GNG5 silencing attenuated clone formation capacity of these two cell lines (P < 0.01; Fig. 2c). Migration and invasion are two important biological characteristics affecting tumor progression. Transwell migration assay showed that knockdown GNG5 markedly suppressed the migration of the above two cell lines. Besides, as revealed by Transwell invasion assay, sh-GNG5 group showed markedly decreased cell invasion of the above two cell lines in comparison with NC group (P < 0.01; Fig. 2d and e). WB assay was performed to detect epithelial–mesenchymal transition (EMT)-related markers. As a result, GNG5 knockdown upregulated E-cadherin but downregulated N-cadherin (P < 0.01; Fig. 2f). For investigating Wnt/β-catenin pathway’s effect on regulating GNG5 within BC, we conducted WB assay for detecting associated protein levels, containing c-myc, cyclin D1 and β-catenin. The results showed that GNG5 knockdown dramatically suppressed c-myc, cyclin D1 and β-catenin levels within the above two cell lines (P < 0.01; Fig. 2g).

Knocking down GNG5 hindered glucose metabolism of breast cancer cells
Glucose consumption, cell ATP level and lactate production were measured to evaluate how GNG5 affected cellular metabolism. According to Fig. 3a–c, GNG5 silencing reduced glucose consumption, cell ATP level and lactate production in BT549 and MDA-MB-231 cells. Additionally, knocking down GNG5 decreased the cell levels of HK2 and LDHA (P < 0.01; Fig. 3d).

GNG5-enhanced breast cancer cell malignant behaviors through modulating Wnt/β-catenin pathway
To better investigate, the role of GNG5 in promoting BC cell malignant progression through Wnt/β-catenin pathway activation, we exposed BT549 and MDA-MB-231 cells to LiCl, the Wnt/β-catenin pathway agonist. As revealed by WB assay, GNG5 silencing reduced c-myc, cyclinD1 and active-β-Catenin, levels. Adding LiCl simultaneously upregulated the above proteins to some extent (Fig. 4a). As revealed by clone forming and CCK-8 assays, LiCl exposure abolished the inhibition of GNG5 knockdown on the growth of these two cell lines (Fig. 4b and c). Additionally, according to Transwell assays, the GNG5 silencing-induced inhibited cell invasion and migration were restored via LiCl in BC cells (Fig. 4d and e). And the decreased lactate secretion, glucose consumption and ATP content within the above two cell lines showed recovery after GNG5 downregulation (P < 0.01; Fig. 4f–h).

Knocking down GNG5 suppressed breast cancer cell growth in vivo
For the purpose of investigating whether the GNG5 affected the BC proliferation, BC Xenograft tumor models of mouse were established, and the results demonstrated that knockdown expression of GNG5 can slow down the growth of BC tumor (P < 0.05; Fig. 5a and b). In addition, immunohistochemical results revealed that the expression levels of Ki-67 and GNG5 were lowered after GNG5 knockdown (P < 0.05; Fig. 5c).
Discussion

A growing body of data showed that GNG5 played a role in various biological processes, particularly in the initiation, progression and metastasis of gliomas. So far, the characteristics, biogenesis, function and progression of GNG5 in gliomas have been reported, and it has been considered as a diagnostic biomarker and therapeutic molecule of GNG5 [5,16]. However, the understanding of GNG5 within BC and GNG5 regulatory axis’s role in BC are still largely unknown. In addition, BC remains a frequently occurring malignancy worldwide, and its treatment and prognosis are not ideal. Therefore, it is necessary to more understand the molecular regulatory mechanism of occurrence and development of BC and to find its biomarkers for clinical treatment and diagnosis.

The current work concluded that GNG5 expression increased within BC tissues and cells, whereas GNG5 silencing reduced cell invasion ability and tumor volumes.
GNG5 knockdown inhibited BC cell invasion and proliferation. (a) GNG5 expression within BT549 and MDA-MB-231 cells was measured through WB assay. (b) Absorbance of BT549 and MDA-MB-231 cells was measured at 450 nm wavelength at 0, 24, 48 and 72 h in different groups by CCK-8 assay. (c) Clone forming assay was conducted to measure colonies formed in BT549 and MDA-MB-231 cells. (d) Transwell detects the ability of different groups of cells to migrate and invade. (f) WB assay was conducted to determine different groups of EMT of BT549 and MDA-MB-231 cells. (g) WB assay was conducted to measure cyclinD1, active-β-Catenin and c-myc levels within BT549 and MDA-MB-231 cells from diverse groups. *P < 0.05; **P < 0.01; ***P < 0.001. BC, breast cancer; CCK-8, Cell Counting Kit-8; GNG5, G protein subunit gamma 5; WB, Western blot.
This is the first report on GNG5 expression within BC. According to our results, alterations in GNG5 level modulate BC development. Consequently, the present data can indicate that GNG5 may contribute to BC progression. Furthermore, as revealed by survival analysis, BC showing upregulated GNG5 expression was associated with poor OS and disease free survival. These results indicate that GNG5 can independently predict OS of BC, indicating its critical effect on BC progression, which may serve as the diagnostic, therapeutic and prognostic marker for BC cases.

In this study, GNG5 was found to promote aerobic glycolysis, cancer cell growth and development. Increased GNG5 expression levels promote aerobic glycolysis, BC cell growth, invasion and migration. As reported in another study, GNG5 mRNA level markedly increased within glioma patients [16]. EMT has the molecular characteristics of epithelial phenotype loss (downregulated of E-cadherin, the epithelial marker) and mesenchymal phenotype acquisition (upregulation of N-cadherin, the mesenchymal marker). According to our results, GNG5 knockdown upregulated E-cadherin while downregulating N-cadherin, suggesting that GNG5 promoted the EMT process and, thus, promoted BC cell migration and invasion. Our present study showed that GNG5 protein and mRNA expression markedly increased within BC cells and tissues. Additionally, GNG5 protein level was positively related to cell proliferation marker protein Ki67 expression in BC. Energy metabolic alteration has a critical function in tumor occurrence [17,18]. The ability to accelerate glucose uptake and oxidation is characteristic of most malignancies. Glycolysis accounts for a major pathway that supplies energy for fast cancer cell proliferation, which tumor cells to hypoxia and increases their malignant potential [19]. We found that decreased GNG5 expression resulted in decreased glucose concentration, lactate level and ATP concentration in cell culture supernatant. This finding highlights the fact that phosphofructokinase P inhibits glycolysis and thus tumor growth.

Wnt/β-catenin pathway relates to cellular growth and differentiation, which may induce disease occurrence like cancer when it is abnormally activated. After activation, the Wnt signal triggers the nuclear import of β-catenin, leading to downstream target transcription (cyclinD1 and c-myC), which, thereby, promotes cancer growth and migration. Studies have shown that knocking down TRIM44 can suppress BC cell migration and proliferation via inhibiting Wnt/β-catenin pathway [20], whereas downregulation of SOX9 can inhibit EMT through Wnt/β-catenin pathway inactivation within PTC [21]. In this study, we found that GNG5 silencing also damaged β-catenin activity while inactivating c-myC and cyclin D1, and adding LiCl agonist restored GNG5 knockdown’s role in malignant progression.
GNG5 promoted BC cell malignant behaviors through modulating Wnt/β-catenin pathway. (a) cyclinD1, c-myC and active-β-catenin levels within BT549 and MDA-MB-231 cells from diverse groups were measured through WB assay. (b) Absorbance of BT549 and mda-mb-231 cells was identified at 450 nm by CKK-8 assay at 0, 24, 48 and 72 h in different groups. (c) The Colonies in MDA-MB-231 and BT549 cells were determined through Colony Formation. (d and e) Transwell detects the ability of different groups of cells to migrate and invade. (f) WB assay was conducted to detect EMT within BT549 and MDA-MB-231 cells from diverse groups. (g) c-myC, cyclinD1 and active-β-catenin levels within BT549 and MDA-MB-231 cells from diverse groups were measured through WB assay. *P < 0.05; **P < 0.01; ***P < 0.001. BC, breast cancer; CKK-8, Cell Counting Kit-8; GNG5, G protein subunit gamma 5; WB, Western blot.

Based on the above findings, GNG5 results in Wnt/β-catenin pathway activation, thus accelerating BC development.

In conclusion, this study found that GNG5 achieves BC cell growth and invasion through Wnt/β-catenin pathway activation, and preliminarily confirmed that GNG5 is an oncogene during BC occurrence. This new finding suggests that GNG5 may become a possible target and biomarker to treat BC.

Acknowledgments
Data availability statement: the data is available upon reasonable request.
GNG5 silencing suppressed BC cell growth in vivo. (a) Subcutaneous tumorigenesis experiment in nude mice. (b) Measurement of subcutaneous tumor quality of MDA-MB-231 cells from diverse groups. (c) The expression levels of Ki-67 and GNG5 in subcutaneous tumor tissues of MDA-MB-231 cells were identified through IHC. *P < 0.05; **P < 0.01; ***P < 0.001. BC, breast cancer; GNG5, G protein subunit gamma 5; IHC, immunohistochemistry.

Conflicts of interest
There are no conflicts of interest.

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