Knockdown of GALNT7 promotes cell apoptosis and autophagy of breast cancer cells by inactivation of STAT3

Jiao Xue1, Qiang Yu2,*

1Department of Breast Surgery, The Affiliated Changzhou No.2 People’s Hospital of Nanjing Medical University, 213000 Changzhou, Jiangsu Province, China
2Department of General Surgery, The Affiliated Changzhou No.2 People’s Hospital of Nanjing Medical University, 213000 Changzhou, Jiangsu, China

*Correspondence qiang_yu165@163.com (Qiang Yu)

Abstract
Breast cancer, a most common malignancy among women with high lethality. Polypeptide-N-acetyl-galactosaminyltransferase 7 (GALNT7) contributes to carcinogenesis and development of breast cancer. Activation of signal transducer and activator of transcription 3 (STAT3) is vital in the initiation and development of cancers. In this work, GALNT7 was upregulated in breast cancer cells, and promoted cell proliferation. GALNT7 knockdown suppressed proliferation of breast cancer cells. GALNT7 overexpression upregulated B-cell lymphoma protein 2 (BCL2) expression and suppressed the expression levels of Bcl-2-associated X protein (BAX), cleaved caspase 3, LC3-II/LC3-I ratio and Atg5, which was dramatically suppressed by knockdown of GALNT7 in breast cancer cells. GALNT7 overexpression favored the phosphorylation of STAT3, which was significantly prevented by knockdown of GALNT7 in breast cancer cells. Inactivation of STAT3 by Stat6ic increased the expressions levels of LC3-II/LC3-I ratio and Atg5, which was prevented by GALNT7 overexpression in breast cancer cells. Knockdown of GALNT7 promoted cell apoptosis and autophagy of breast cancer cells via inactivation of STAT3.

Keywords
GALNT7; STAT3; Breast cancer; Apoptosis; Autophagy

1. Introduction
Breast cancer is a most prevalent malignancy for women. Early diagnosis and substantial improvements in the therapies contributes to the decline of mortality caused by breast cancer [1, 2]. However, distant metastasis remains the main reason of breast cancer mortality [1, 2]. Many studies focus on exploring the molecular mechanism of carcinogenesis and metastasis. Autophagy is also involved in the development of breast cancer, and autophagy inhibits the invasion and migration of breast cancer [3]. Nevertheless, the mechanism through which autophagy suppresses the development of breast cancer remains unclear.

Polypeptide N-acetyl-galactosaminyltransferase (GalNAc-transferase) 7, named GALNT7, is a member of GalNAc-transferase family [4]. GALNT7 seems to be a follow-up enzyme that regulates the onset of O-glycosylation, and transfers N-acetylgalactosamine to threonine and serine [4]. GALNT7 is involved in cell proliferation and survival in various cancers, such as cervical cancer [5, 6], esophageal squamous cell cancer [7], hepatocellular carcinoma [8] and renal cell carcinoma [9]. The Cancer Genome Atlas database analysis demonstrates that GALNT7 is upregulated in glioblastoma [10]. GALNT7 facilitates cell proliferation and migration of glioma cells via multiple signaling pathways, for instance, the janus kinase-signal transducer and activator of transcription (STAT) signaling pathway [10]. STAT3 is not only a transcriptional activator but also an oncogene [11]. STAT3 is activated in cancer cells, which is critical in the onset and progression of tumor [11]. Activation of STAT3 was able to inhibit apoptosis in osteosarcoma [12].

However, the functional role of GALNT7 and its molecular mechanism in breast cancer progression remains to be elucidated. In this study, GALNT7 expression was assessed in different breast cancer cell lines. And then the effect of GALNT7 expression level on breast cancer cell proliferation, apoptosis and autophagy was studied.

2. Materials and Methods
2.1 Cell lines and antibodies
Human Mammary Epithelial Cells (HMEC), MDA-MB-231, MCF-7, T47D cell lines were supplied by The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Antibodies against GALNT7 (ab97645), BCL2 (ab182858), BAX (ab32503), cleaved caspase 3 (ab2302), p-STAT3 (ab76315), STAT3 (ab68153) and β-actin (ab8226) were purchased from Abcam (Shanghai, China). The primary antibodies against LC3-I/II (ABC929) and Atg5 (A0731) were purchased from Sigma-Aldrich (St. Louis, MO, USA).
2.2 Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

TRIzol (Invitrogen, Shanghai, China) was used for extraction of the total RNA. qRT-PCR was conducted with SYBR Green mix (Takara, Shiga, Japan). ABI PRISM 7500 RT PCR instrument (Thermo Fisher, Waltham, MA, USA) was used for the qRT-PCR reaction and detection. The transcript level of GALNT7 was measured with 2\(^{-\Delta\Delta Ct}\) method. \(\beta\)-actin served as an internal control. The primers were as following: GALNT7-forward: 5\('\)′-GGTTCATCTTACGCAGTTTGC-3\('\)′ and GALNT7-reverse: 5\('\)′-GGGCATGGGGTCATTGACA-3\('\)′; \(\beta\)-actin-forward: 5\('\)′-CATGTACGTGTCTATCCAGGC-3\('\)′ and \(\beta\)-actin-reverse: 5\('\)′-CTCCTTAAATGTCACGCAGAT-3\('\)′.

2.3 Generation of GALNT7 knockdown cell line

Two short hairpin RNA (shRNA) interference sequences targeting GALNT7 were designed as following: shGALNT7-1#: AACCCAGCATTTAGGTTTAGATAGCAGAAATGAGATTTGCACTT; shGALNT7-2#: GAAGGATGTCACCGCTAAGATGGAAGGTAAACTTCCCGTCAGGGAA. The synthesized oligonucleotides were inserted into the pLKO.1-Puro vector at AgeI-EcoRI site. Cells were infected with lentiviruses containing GALNT7 shRNA (shGALNT7-1# or shGALNT7-2#) or shRNA negative control (shNC). Western blotting assay showed the efficiency of GALNT7 knockdown.

2.4 Cell viability assay

Cell Counting Kit-8 (CCK8) kit (ab228554, Abcam) was used to assess the cell viability. Seeded cells at 1 × 10^4/well in a 96-well plate (100 \(\mu\)L/well) and cultured for indicated time. Each well was added 10 \(\mu\)L CCK-8 solution. After 1–4 h, the absorbance at 450 nm was measured with a plate reader.

2.5 Cell proliferation assay

EdU Staining Proliferation Kit (ab219801, Abcam) was used for cell proliferation assay. Seeded cells were loaded with 20 \(\mu\)M EdU solution and incubated for 2–4 h. Then replaced with fixative solution for 15 min, and rinsed with wash buffer. Permeabilized cells with the permeabilization buffer for 20 min. Rinsed cells twice with wash buffer. Incubated with the reaction mix and for 30 min. Rinsed cells once with wash buffer. Then incubated with Hoechst working solution for 30 min. Washed cells twice with wash buffer and kept the cells in the final wash. Images were photographed under a fluorescence microscope at Ex/Em = 491/521 nm.

2.6 Western blotting

Extracted cellular protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoreses. Immunoblotting was performed to blot the bands. Then the blots were visualized by enhanced chemiluminescence.

2.7 Bioinformatics analysis

Gene Expression Profiling Interactive Analysis (GEPIA) and The Cancer Genome Atlas (TCGA) databases were used for the prediction of the expression of GALNT7 in breast cancer.

2.8 Statistical analysis

GraphPad Prism 8 (Graph Pad Software Inc., San Diego, CA, USA) was used for data analysis. Data were presented as mean ± standard error of the mean (SEM). Comparisons between groups were conducted with one-way analysis of variance. \(p < 0.05\) was considered as statistically significant.

3. Results

3.1 GALNT7 was upregulated in breast cancer

Based on GEPIA and UALCAN database, GALNT7 was upregulated in breast cancer (Fig. 1A). TCGA showed that GALNT7 was upregulated in breast cancer (Fig. 1B). QRT-PCR analysis revealed that the relative mRNA level of GALNT7 is upregulated in breast cancer cells, in comparison with normal HMEC cells (Fig. 1C). The protein expression level of GALNT7 is upexpressed breast cancer cells, compared to HMEC cells (Fig. 1D). These data demonstrated that GALNT7 was upregulated in breast cancer.

3.2 Knockdown of GALNT7 suppressed proliferation of breast cancer cells

Next the effects of GALNT7 overexpression and knockdown on cell proliferation in breast cancer cells were investigated. Transfection with a plasmid carrying GALNT7 gene enhanced the expression levels of GALNT7 (Fig. 2A). Knockdown of GALNT7 using shRNA technique resulted in decreased expression level of GALNT7 in MCF-7 (HER2−/ER+) and MDA-MB-231 (HER2−/ER−) cells (Fig. 2A). shGALNT7-2# with higher efficiency for GALNT7 knockdown was used for the following experiments. Overexpression of GALNT7 promoted cell viability and proliferation, and knockdown of GALNT7 inhibited proliferation and cell viability in MCF-7 and MDA-MB-231 cells (Fig. 2B–D). These observations demonstrated that GALNT7 favored cell growth, and knockdown of GALNT7 suppressed cell proliferation in breast cancer cells.

3.3 Knockdown of GALNT7 promoted cell apoptosis and autophagy of breast cancer cells

Overexpression of GALNT7 suppressed cell apoptosis and knockdown of GALNT7 significantly promoted breast cancer cell apoptosis (Fig. 3A). Overexpression of GALNT7 enhanced the expression of BCL2, and inhibited the expressions of BAX and cleaved caspase 3 (Fig. 3B). Knockdown of GALNT7 significantly suppressed the expression of BCL2, and dramatically increased the expression levels of BAX and cleaved caspase 3 (Fig. 3B). Overexpression of GALNT7 inhibited the expression of LC3-II/LC3-I ratio and Atg5, which was strikingly enhanced by GALNT7 knockdown (Fig. 3C). These
FIGURE 1. GALNT7 (Polypeptide-N-acetyl-galactosaminlytransferase 7) was upregulated in breast cancer. (A) Gene Expression Profiling Interactive Analysis (GEPIA) revealed that GALNT7 was upregulated in breast cancer. (B) The Cancer Genome Atlas (TCGA) showed that GALNT7 was upregulated in breast cancer. (C) Real-Time Quantitative Reverse Transcription PCR (QRT-PCR) analysis assessed the relative mRNA level of GALNT7 in HMEC, MCF-7, MDA-MB-231 and T47D cell lines. (D) Left, representative blots of GALNT7 in HMEC, MCF-7, MDA-MB-231 and T47D cell lines. Right, the relative protein level of GALNT7 normalized to β-actin. **p < 0.01 vs. HMEC, ***p < 0.001 vs. HMEC. Data are mean ± standard error of the mean (SEM) of three independent experiments.

results indicated that GALNT7 had anti-apoptotic effect on breast cancer cells, and knockdown of GALNT7 promoted cell apoptosis and autophagy of breast cancer cells.

3.4 GALNT7 regulates STAT3 activation

Overexpression of GALNT7 favored the phosphorylation of STAT3 and knockdown of GALNT7 suppressed the phosphorylation of STAT3 (Fig. 4A). Inhibition of STAT3 activation by Statc significantly inhibited cell viability, which could be prevented by overexpression of GALNT7 (Fig. 4B). Statc treatment enhanced the expression ratio of LC3-II/LC3-I and the expression level of Atg5, which was dramatically prevented by GALNT7 overexpression (Fig. 4C). These data suggested that GALNT7 was able to regulate the activation of STAT3.

4. Discussion

Breast cancer is one of the most common malignancies among women, and distant metastasis is the main cause of high lethality by breast cancer [1, 2]. Autophagy is involved in the carcinogenesis and metastasis of breast cancer [3]. However, the molecular mechanism of autophagy in breast cancer remains to be elucidated. GALNT7 belongs to GalNAc-transferase family, and may acts as a follow-up enzyme of the starting step of O-glycosylation [4]. GALNT7 is upregulated in some cancers and regulates carcinogenesis and development [5–10]. The transcriptional activator STAT3 can also function as an oncogene [11]. STAT3 activation is vital in the onset and progression of tumor and has the ability to inhibits cancer cell autophagy [11, 12]. In this study, GALNT7 was shown to be upregulated in breast cancer cells, favoring cell viability and proliferation. Knockdown of GALNT7 promoted breast cancer cell apoptosis and autophagy probably via inactivation of STAT3.

STAT3 is ready to be activated in a variety of cancers including breast cancer [13]. STAT3 plays a critical role in cell proliferation, migration and immune response in breast cancer. STAT3 is phosphorylated and activated by upstream regulators’ signaling [13]. STAT3 is also can be activated by secretion of cytokines, G-protein signaling, serine kinases, receptor tyrosine kinases, nonreceptor tyrosine kinases [13]. STAT3 signaling is a central linking of multiple signaling processes via regulation of downstream genes involved in carcinogenesis and progression [13–15]. STAT3 is hyperactivated in cancer cells and immune cells, which drives tumor immunosuppression [14]. Therefore, targeting the activation of STAT3 is a promising therapeutic intervention, and further investigation of its role in cancers is necessary. GANLT7 expression level is associated tumor grade and survival time.
FIGURE 2. Knockdown of GALNT7 suppressed proliferation of breast cancer cells. (A) Plasmid DNA transfection and Short hairpin RNA (shRNA) technique were used to overexpress and knock down GALNT7. Left, representative blots of GALNT7 after transfections with NC and GALNT7 plasmids, or infection with lentiviruses containing GALNT7 shRNA (shGALNT7-1# and shGALNT7-2#). Right, the relative expression level of GALNT7 normalized to β-actin. (B) Cell Counting Kit 8 (CCK8) assay was used to detect the cell viability after transfections with NC and GALNT7 plasmids, or infection with lentiviruses containing shNC and shGALNT7-2# in MCF-7 and MDA-MB-231 cells. (C) Representative images of EdU and DAPI staining in MCF-7 and MDA-MB-231 cells after transfections with NC and GALNT7 plasmids, or infection with lentiviruses containing shNC and shGALNT7-2#. (D) Cell proliferation was analyzed by the fluorescence level of EDU normalized to that of DAPI. **p < 0.01 vs. NC, ***p < 0.001 vs. NC, ###p < 0.01 vs. shNC, ####p < 0.001 vs. shNC. Data are mean ± SEM of three independent experiments.

GALNT7 is likely to influence cancer cell proliferation via multiple signaling pathways including STAT signaling pathway [10]. STAT3 is one of the target genes of GALNT7 [10].

GALNT7 was upregulated in breast cancer cells. High expression levels of GALNT7 promoted cell viability and proliferation, and knockdown of GALNT7 suppressed cell proliferation in breast cancer cells. Overexpression of GALNT7 suppressed cell apoptosis via upregulation of BCL2 expression and inhibition of BAX, cleaved caspase 3, LC3-II/LC3-I ratio and Atg5 expressions in breast cancer cells. Knockdown of GALNT7 promoted cell apoptosis and autophagy via suppression of BCL2 expression and increase of BAX, cleaved caspase 3, LC3-II/LC3-I ratio and Atg5 expressions in breast cancer cells. GALNT7 favored the phosphorylation of STAT3, which could be significantly prevented by knockdown of GALNT7 in breast cancer cells. Inhibition of STAT3 activation by Static significantly inhibited cell viability, which could be prevented by overexpression of GALNT7 in breast cancer cells. Inhibition of STAT3 activation by Static promoted LC3-II/LC3-I
FIGURE 3. Knockdown of GALNT7 promoted cell apoptosis and autophagy of breast cancer cells. (A) Propidium iodide staining followed by flow cytometry analysis was used to assess the effect of GALNT7 expression level on cell apoptosis in MCF-7 and MDA-MB-231 cells. (B) Left, representative blots of BCL2, BAX and cleaved caspase 3 after transfections with NC and GALNT7 plasmids, or infection with lentiviruses containing shNC and shGALNT7-2#. Right, the relative expression levels of BCL2, BAX and cleaved caspase 3 normalized to β-actin. (C) Left, representative blots of LC3-I, LC3-II and Atg5 after transfections with NC and GALNT7 plasmids, or infection with lentiviruses containing shNC and shGALNT7-2#. Right, the relative expression levels of LC3-II/LC3-I and Atg5 normalized to β-actin. *p < 0.05 vs. NC, **p < 0.01 vs. NC, ***p < 0.001 vs. NC, ##p < 0.01 vs. shNC, ###p < 0.001 vs. shNC. Data are mean ± SEM of three independent experiments.
FIGURE 4. GALNT7 regulates STAT3 signaling pathway. (A) Left, representative blots of p-STAT3 and STAT3 after transfections with NC and GALNT7 plasmids, or infection with lentiviruses containing shNC and shGALNT7-2#. Right, the relative expression levels of p-STAT3 and STAT3 normalized to β-actin. (B) Cell Counting Kit 8 (CCK8) assay was used to detect the cell viability at indicated time points after transfections with NC and GALNT7 plasmids with or without Stattic treatment in MCF-7 and MDA-MB-231 cells. (C) Left, representative blots of LC3-I, LC3-II and Atg5 after transfections with NC and GALNT7 plasmids with or without Stattic treatment in MCF-7 and MDA-MB-231 cells. Right, the relative expression levels of LC3-II/LC3-I and Atg5 normalized to β-actin. ∗p < 0.05 vs. NC, ∗∗p < 0.01 vs. NC, ∗∗∗p < 0.001 vs. NC, #p < 0.05 vs. shNC, ##p < 0.01 vs. shNC, ###p < 0.001 vs. GALNT7. Data are mean ± SEM of three independent experiments.

ratio and Atg5 expressions, which was prevented by GALNT7 overexpression in breast cancer cells, indicating that GALNT7 could regulate the activation of STAT3. However, in this work, the role of GALNT7 in breast cancer and its molecular mechanism are limited to in vitro study, the further in vivo study is needed to be done.

5. Conclusions

Taken together, the present study demonstrated that knock-down of GALNT7 promoted cell apoptosis and autophagy in breast cancer cells by inactivation of STAT3. GALNT7 appeared to be an upstream regulator of STAT3, thus targeting GALNT7 to modulate the activation of STAT3 could be a promising therapeutic strategy for breast cancer.

AUTHOR CONTRIBUTIONS

JX—designed the study and carried them out; QY—supervised the data collection, analyzed the data, interpreted the data; JX and QY—prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.
ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

ACKNOWLEDGMENT

Not applicable.

FUNDING

This research received no external funding.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES


