Abstract. Role of long-chain non-coding ribonucleic acid (lncRNA) GACAT1 in the development of breast cancer and its possible mechanism were investigated. The levels of GACAT1, microRNA-875-3p and Stonin2 (STON2) in breast cancer tissues and adjacent normal tissues were detected by quantitative real-time polymerase chain reaction (qRT-PCR). The level of GACAT1 in breast cancer cell lines was further explored. The effects of GACAT1 and microRNA-875-3p on cell proliferation and cycle were detected by cell counting kit-8 (CCK-8) and flow cytometry. The binding relationship between microRNA-875-3p and STON2, microRNA-875-3p and GACAT1 was examined by a dual luciferase reporting assay. It was confirmed by rescue experiments whether GACAT1 can regulate the level of STON2 by binding to microRNA-875-3p. GACAT1 level was clearly enhanced in breast cancer tissues compared to that of the adjacent tissues. Similar result was observed in breast cancer cell lines. Upregulation of GACAT1 promoted the proliferation and cycle of breast cancer cells including MCF-7 and BCap-37. The dual luciferase reporting assay results indicated that GACAT1 had a binding relationship with microRNA-875-3p. Further experiments confirmed that microRNA-875-3p was conspicuously downregulated in breast cancer tissues, and upregulation of microRNA-875-3p could inhibit the proliferation ability of MCF-7 and BCap-37 cells, and partially reversed the promoting effect of GACAT1 on cell cycle. Through bioinformatics prediction and dual luciferase reporter gene experiments, we found that STON2 might be a target gene of microRNA-875-3p. Overexpression of STON2 could partially abolish the effect of microRNA-875-3p on cell proliferation and cycle of MCF-7 and BCap-37 cells. GACAT1 can participate in the progression of breast cancer by promoting the proliferation and cycle of breast cancer cells. The mechanism may be through the regulation of the level of STON2 by adsorbing microRNA-875-3p.

Introduction

Breast cancer is a highly heterogeneous malignant tumor, and its morbidity and mortality are second only to lung cancer, which poses a great threat to human health (1). In recent years, the mortality rate of breast cancer has been increasing year by year (2). Although the prognosis of breast cancer patients is greatly improved under comprehensive treatment, 20-25% of patients will eventually have local recurrence or even distant metastasis, eventually leading to death. Studies have shown that the development, early diagnosis, comprehensive treatment and prognosis of breast cancer are closely related to the abnormal level of non-coding ribonucleic acid (RNA) (3), so it is necessary to explore the pathogenesis of breast cancer from the perspective of non-coding RNA.

Long-chain non-coding RNA (lncRNA) is a RNA molecule that is more than 200 nt in length and has been proved to be involved in various biological processes in cells (4,5). lncRNA can actively participate in the regulation of various biological functions and plays an important role in a variety of tumors mainly through epigenetic changes, cell cycle regulation, regulation of miRNAs and participation in signaling pathways (6,7). For instance, lncRNA plays an important role in the development of breast cancer (8). By gene chip high-throughput analysis of differential level of lncRNA in breast cancer and normal breast tissue, studies have found that 220 lncRNAs are abnormally expressed in breast cancer tissues, of which 129 lncRNAs are specifically expressed in breast cancer and can be used as breast cancer biomarkers (9). Another study found that IncRNA H19 is overexpressed in estrogen receptor positive breast cancer and promotes breast cancer growth and proliferation (10). IncRNA GACAT1 is highly expressed in gastric cancer and closely related to the prognosis of gastric cancer, which can be used as a tumor marker for gastric cancer (11,12). Through previous studies, we found that GACAT1 is also highly expressed in breast cancer, but the study on GACAT1 in breast cancer has not been reported.

MicroRNAs (miRNAs) are a class of small-molecule non-coding RNAs found in eukaryotes. They are composed of ~22 nucleotides and bind to the corresponding target mRNA, resulting in degradation of target mRNA or inhibited
post-transcriptional translation. At present, scientists have identified 474 miRNAs in the human genome, which could regulate ~30% of the encoded protein genes, and each miRNA regulates ~100 target mRNAs (13). The relationship between miRNAs and human life activities and diseases is receiving increasing attention. In particular, recent studies have shown that they are closely related to the occurrence and development of human diseases. For example, in patients with lung cancer, the level of miRNA-let-7 is conspicuously reduced. Meanwhile, let-7a transfection into A549 cell line could inhibit lung cancer cell proliferation (14). In the study on chronic lymphocytic leukemia, it was found that the level of miR-15 and miR-16 in leukemia cells of patients with chronic lymphocytic leukemia is downregulated, while the level of B-cell lymphoma-2 (Bcl-2) is increased. There is a significant negative correlation between miR-15 and miR-16 expression and Bcl-2 protein levels, and miR-15 and miR-16 functions by targeting degradation of Bcl-2 (15).

In the present, through bioinformatics analysis, it was found that microRNA-875-3p might be a potential target gene for long-chain non-coding RNA GACAT1, moreover, its expression was low in breast cancer tissues and cells. This study investigated the role of GACAT1 in breast cancer to verify whether it could function by degrading microRNA-875-3p.

**Patients and methods**

**General information.** Fresh breast cancer tissue and adjacent normal tissues were collected from 24 patients with breast cancer who underwent surgery. The enrollment conditions included: i) exclusion of other malignant tumor history; ii) first surgery, no radiotherapy before surgery; iii) the pathology diagnosis was performed by the pathologist's reading film; iv) the clinical medical record data and the postoperative follow-up data were complete. This study was approved by the Ethics Committee of Linyi Cancer Hospital (Linyi, China). Signed informed consents were obtained from all participants before the study. The specimens were taken from the tumors removed during the operation, and stored in a liquid nitrogen tank within 15 min.

**Cell culture.** Normal Hs578Bst cells as well as breast cancer cell lines MDA-MB-231, BCap-37 and MCF-7 were purchased from American Type Culture Collection (ATCC). The cells were cultured in Dulbecco’s modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc.) (containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin) at 37°C, in a 5% CO₂ incubator.

**Cell transfection.** The cells were inoculated one day before the transfection. The next day, plasmid, miRNA mimics and their respective negative control sequences (50-100 nM) were transfected by Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). RNase-free diethyl pyrocarbonate (DEPC) water (both from Beyotime) and Eppendorf (EP) tubes were used for transfection.

**Tissue and cell total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR).** Extraction was carried out by the TRIzol method (Invitrogen; Thermo Fisher Scientific, Inc.). Chloroform (200 µl) was added to each 1 ml of TRIzol-dissolved EP tube, mixed by inversion, placed in an ice box for 5 min, and centrifuged at 4°C, 12,000 x g for 15 min carefully pipeting. The extracted RNA was applied to the NanoDrop 2000c assay to determine its concentration and the 260/280 absorbance was used to evaluate the quality. The measurement was repeated twice, and the 260/280 absorbance ratio was between 1.8 and 2.0. The total RNA was then reverse-transcribed into complementary deoxyribonucleic acid (cDNA) which was used for further real-time PCR. Detection of the level of the target gene was performed using an ABI StepOnePlus real-time quantitative fluorescent PCR instrument by the SYBR real-time qPCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each well contained 10 µl SYBR-Green Master Mix, 1 µl upstream primer, 1 µl downstream primer, 0.4 µl 50X Rox Dye, 2 µl cDNA and 5.6 µl enzyme-free water. The reaction conditions were 95°C for 10 min, 95°C for 15 sec and 60°C for 60 sec, for 40 cycles. The data were statistically analyzed using the StepOne Software version v2.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.) software and the primer sequences were derived as follows: microRNA-875-3p, forward, 5’-ACACTCCACGTTGGUAUA CUCAGUUUAU-3’ and reverse, 5’-CTCAACTGTTGTC GTGGAGTCCGCAATTCAGTTGAGCACCUGA-3’; U6, forward, 5’-TCGCCTTGGCCAGCACACATATA-3’ and reverse, 5’-AAATATGGAACGCTTCACGA-3’; GACAT1, forward, 5’-ACCCGAGAAGAAATTCCTCAGC-3’ and reverse, 5’-CCATAAAGGGGGCGCGTGT-3’; GAPDH, forward, 5’-GAAGAGAGAGACCCTCACGCTG-3’ and reverse, 5’-ACTGTGAGGAGGGGAGATTCAGT-3’; Stonin2 (STON2), forward, 5’-ACCATGTTGATGGCCACCCAC-3’ and reverse, 5’-AGCTCTCGGAGTTGCTGG-3’.

**Luciferase reporting assay.** Reporter plasmid was constructed and termed pmirGL0-STON2-7 UTR-wild type (Wt) or pmirGL0-STON2-37 UTR-mutant (Mut), which were inserted into the dual-fluorescence of wild-type and mutant STON2-3’UTR, respectively. Breast cancer cells were seeded into 48-well plates, and the plasmid was co-transfected with miRNA-875-5p mimics, then the luciferase activity was detected 48 h later.

**Detection of cell cycle.** Twenty-four hours after transfection, cells were collected, washed twice with phosphate-buffered saline (PBS) and then 1 ml of DNA Staining solution (Takara) was added, vortexed and mixed, and incubated at room temperature in the dark. After 30 min, the samples were detected by MACS flow cytometry (Beckman Coulter).

**Cell proliferation.** Transfected cells were seeded into 96-well plates (Corning, Inc.) at a density of 3-5x10³ cells per well, and 10 replicate wells were set for each group. 100 µl of a mixture of cell counting kit-8 (CCK-8) reagent (Dojindo) premixed in a ratio of 1:10 was added and then the cells were incubated in a 37°C incubator for 1 h. Then the enzyme was added. The optical density (OD) value of each replicate well was measured by microplate reader at 450 nm wavelength.

**Statistical analysis.** The measurement data were expressed as mean ± standard deviation, and t-test was used to examine the
mean difference between two groups. Single factor variance analysis was used when multiple sets of data meet normality and the homogeneity of the variance. Pairwise comparison was used if there was a difference between the groups. Nonparametric test was used when the data did not meet normality and the homogeneity of the variance. \( P<0.05 \) was considered statistically significant.

**Results**

**GACAT1 is clearly expressed in breast cancer.** First, we examined the level of GACAT1 in 24 breast cancer patients. The results showed that microRNA-875-3p was conspicuously expressed in breast cancer tissues compared to that of adjacent tissues (Fig. 1A). Furthermore, we found that in patients with cancer metastasis, the level of GACAT1 was conspicuously higher than that of patients without metastasis (Fig. 1B). We then examined the level of GACAT1 in breast cancer at different stages, and the results showed that GACAT1 level was conspicuously higher in patients with grade III to IV breast cancer than in patients with stage I to II (Fig. 1C). By analyzing the clinical information of breast cancer patients, the survival curve was plotted and the results showed that the area under the curve was 0.8583 and the cutoff value was 1.273 (Fig. 1D), which indicated that GACAT1 can be used as a biomarker for breast cancer.

**GACAT1 promotes proliferation and cycle of breast cancer cells.** In breast cancer cell lines, we also examined the GACAT1 level. The results showed that it was generally highly expressed in breast cancer cell lines compared to normal breast cells (Fig. 2A). After transfection of the GACAT1 overexpression plasmid in MCF-7 and BCap-37 cells, we examined the efficiency of transfection, and the results showed that GACAT1 was high and stably expressed (Fig. 2B). Subsequently, we applied CCK-8 assay and flow cytometry to examine the effect of GACAT1 on proliferation and cell cycle of breast cancer cells. The results showed that the upregulation of GACAT1 conspicuously increased the proliferation of BCap-37, and also clearly promoted the cell cycle of BCap-37 (Fig. 2C and D), which was consistent with MCF-7 cells (Fig. 2E and F). These results demonstrated that GACAT1 promoted proliferation and cell cycle of breast cancer cells.

**GACAT1 works by adsorbing microRNA-875-3p.** Through bioinformatics analysis, we found that GACAT1 might bind to microRNA-875-3p, and confirmed their binding by dual luciferase reporter gene experiments (Fig. 3A). Further studies found that microRNA-875-3p was conspicuously under expressed in breast cancer tissues (Fig. 3B). After upregulating the level of GACAT1 in BCap-37 and MCF-7 cells, the level of microRNA-875-3p was clearly decreased (Fig. 3C). The above results indicated that GACAT1 can inhibit the level of microRNA-875-3p. To verify whether GACAT1 functioned by targeting microRNA-875-3p, we first upregulated GACAT1 and then overexpressed microRNA-875-3p as well, and found that the effect of GACAT1 on cell proliferation in BCap-379...
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Figure 2. GACAT1 promotes proliferation and cycle of breast cancer cells. (A) GACAT1 expression is generally increased in breast cancer cells. (B) GACAT1 can be stably expressed after transfection of GACAT1 overexpression plasmid in MCF-7 and BCap-37 cells. (C and D) Cell proliferation and cycle can be promoted after GACAT1 is upregulated in BCap-37 cells. (E and F) In MCF-7 cells, upregulation of GACAT1 can significantly promote cell cycle and cell proliferation. *P<0.05, **P<0.01 and ***P<0.001.

Figure 3. GACAT1 works by adsorbing miR-875-5p. (A) Dual luciferase reporting assay results show that GACAT1 has a binding relationship with miR-875-5p. (B) miR-875-5p is significantly underexpressed in breast cancer cells. (C) After GACAT1 is upregulated in MCF-7 and BCap-37 cells, the expression of miR-875-5p was significantly decreased. (D and E) Upregulation of miR-875-5p in MCF-7 and BCap-37 cells partially reversed the promoting effect of GACAT1 on cell proliferation and cell cycle. *P<0.05, **P<0.01 and ***P<0.001.
and MCF-7 cells was partially inhibited by microRNA-875-3p overexpression (Fig. 3D). Moreover, we found that upregulation of microRNA-875-3p in BCap-37 and MCF-7 cells partially inhibited the promoting effect of GACAT1 on the cell cycle (Fig. 3E). Based on the above results, we concluded that GACAT1 may play a role by adsorbing microRNA-875-3p and then regulate its downstream target genes.

**Discussion**

The competitive endogenous RNA (ceRNAs) hypothesis reveals a novel mode of regulation of RNA-interacting interactions, including messenger RNA (mRNA), pseudogene, long-chain non-coding RNA (lncRNA) and circular RNA (circRNA). By stimulating the same miRNA through microRNA response elements (MREs), the level of miRNA is reduced, thereby reducing or reducing the effect of miRNA on target genes. ceRNA is widely involved in a series of physiological and pathological processes including cell differentiation, proliferation, apoptosis, and growth and invasion of tumor cells (16,17). There have been many studies on the role of ceRNA. Studies have found that lncRNA HOTAIR is conspicuously upregulated in renal cell tumors and acts as an endogenous RNA, which could promote tumor progression by downregulating the level of miR-217. Other studies have shown that lncRNA RMRP also plays a role as a miRNA sponge in lung cancer. By adsorbing miR-206, it upregulates the level of miRNA downstream target genes and plays a role in the development of lung cancer.
In this study, we found that GACAT1 was highly expressed in breast cancer by qPCR. After staging breast cancer patients and dividing them into metastasis group and non-metastasis group, we further analyzed that GACAT1 level was closely related to breast cancer staging and metastasis, suggesting that GACAT1 could indicate poor prognosis. In vitro, we overexpressed GACAT1 in breast cancer cell lines and found that GACAT1 conspicuously promoted the proliferation and cell cycle of breast cancer cells, suggesting that it may play a role in the development of breast cancer.

To explore whether GACAT1 also functioned as a miRNA sponge, we performed prediction analysis and found that microRNA-875-3p may be a potential target for GACAT1. Previous studies have shown that microRNA-875-3p plays a role as a tumor suppressor gene in a variety of tumors, and its upregulation can often inhibit the proliferation and invasion of tumor cells (18,19). Therefore, we also examined the level of microRNA-875-3p in breast cancer patients, and the results showed that microRNA-875-3p was conspicuously under-expressed in breast cancer. We confirmed the binding relationship between GACAT1 and microRNA-875-3p by luciferase reporter gene assay. Besides, the level of microRNA-875-3p was conspicuously decreased after upregulating GACAT1, indicating that GACAT1 can play the role of ceRNA to downregulate microRNA-875-3p level. To further confirm that GACAT1 functioned through microRNA-875-3p, we simultaneously upregulated GACAT1 and microRNA-875-3p in breast cancer cell lines, and found that microRNA-875-3p partially inhibited the inhibition effect of GACAT1 on cell proliferation and cycle.

In addition, since miRNAs play a role in the targeted degradation of their target genes, we further explored their potential target genes through site prediction and analysis. We found that STON2 may be a potential target gene for microRNA-875-3p. The STON2 gene is a clathrin-related protein that is closely related to the regulation of intracellular complexes (20,21). In ovarian cancer, studies have shown that high level of STON2 can promote tumor invasion and predict a poor prognosis (22). In this study, we verified that microRNA-875-3p could bind to STON2 by dual luciferase reporter gene assay and that STON2 was highly expressed in breast cancer. Through a series of recovery experiments, we found that overexpression of STON2 partially abolished the inhibitory effect of microRNA-875-3p on proliferation and cell cycle of breast cancer cells, indicating that microRNA-875-3p may exert its role through regulating STON2.

Based on the above results, we hypothesized that IncRNA GACAT1 can adsorb microRNA-875-3p to upregulate the level of STON2, thereby promoting the progression of breast cancer.

In conclusion, in this study, we conducted an in-depth study of the role and mechanism of GACAT1 in breast cancer, and demonstrated that GACAT1 could play a vital role in the progression of breast cancer, which also provides theoretical basis for new prevention and treatment for breast cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

QW and XQ designed the study and performed the experiments, QW and JX collected the data, QR and XL analyzed the data, QW and XQ prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Linyi Cancer Hospital (Linyi, China). Signed informed consents were obtained from the patients and/or guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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