circAGFG1 Facilitates the Progression of Non-Triple-Negative Breast Cancer Through miR-143-5p/USP14 Axis

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Abstract

Circular RNAs (circRNAs) emerged as a new class of regulators in various human diseases, including breast cancer (BC). Our previous study identified that circAGFG1 could promote triple-negative breast cancer (TNBC) progression. However, the clinical values and functions of circAGFG1 in non-TNBC remain elusive. Here, we applied qRT-PCR in 30 pairs of non-TNBC tissues and para-cancerous tissues and *in situ* hybridization (ISH) in 346 paraffin-embedded non-TNBC tissues to evaluate the expression pattern and clinical significance of circAGFG1 in non-TNBC. Gain- and loss-of-function studies were carried out to determine the biological function of circAGFG1 in non-TNBC *in vitro* and *in vivo*. Mechanistically, RNA pull-down, RNA immunoprecipitation, dual luciferase reporter, fluorescent *in situ* hybridization assays were employed to validate the interaction between circAGFG1 and miR-143-5p as well as miR-143-5p and USP14. We found that circAGFG1 was markedly increased in non-TNBC tissues as well. Moreover, patient suffering from non-TNBC with a higher expression level of circAGFG1 presents a worse clinical outcome. Multivariate analysis uncovered that high level of circAGFG1 might serve as a standalone risk factor for non-TNBC patients. Functional studies substantiated that enforced expression of circAGFG1 increased the malignance of non-TNBC cells, whereas silencing of circAGFG1 produced the opposite. Mechanistic investigations proved that circAGFG1 sponged miR-143-5p to regulate the level of oncogene USP14, leading to the progression of non-TNBC. Taken together, our data revealed that circAGFG1 could also accelerate the progression of non-TNBC via miR-143-5p/USP14 axis and might provide an alternative target for the diagnosis and treatment of non-TNBC.

Introduction

Breast cancer (BC), as the most common malignancy diagnosed among women, has become the main cause of cancer-related death among women more than 100 countries [1, 2]. It has been reported that BC accounts for approximately 30% of all cancer cases and 15% of all female cancer deaths [2, 3]. More than 2 million people were newly diagnosed with BC and more than 0.6 million people died of this disease in 2018 [2]. To our knowledge, BC is not a single disease, but a heterogeneous group categorized into three major subtypes in accordance with the expression status of human epidermal growth factor 2 (HER2), estrogen receptor (ER) and progesterone receptor (PR), namely, triple-negative, HER2 negative and HER2 positive subtype [4]. Each subtype of BC has its own biological and clinical characteristics. Despite the outstanding advances of early diagnosis, surgery, chemotherapy, radiotherapy, endocrine therapy and targeted therapy have been achieved over the past several decades, the morbidity and mortality of BC are still high. Novel biomarkers are urgent to be discovered to meliorate the diagnosis and therapy of BC.

Circular RNAs (circRNAs) are recognized as a novel type of single-stranded RNA transcripts with no 5'-end cap or 3'-end poly (A) tail, but a closed loop structure formed by non-canonical back-splicing [5]. CircRNA was firstly observed by Sanger in an RNA virus with electron microscopy as early as 1976 and later was found to be an endogenous circular RNA splicing product in the cytoplasm of eukaryotic cells in 1979 [6, 7]. After that, they were considered as junk products of mis-splicing for decades [8]. To date, this idea has been changed since plenty of circRNAs have been proved to exist in cells and serve as vital regulators in

various of biological processes [9]. Increasing evidence suggests that circRNAs participate in various kinds of human diseases, especially cancers [10, 11]. For instance, circLONP2 directly interacts with DDX1 to enhance the metastasis of colorectal cancer [12]. In lung squamous cell carcinoma, circTP63 sponges miR-873-3p to upregulate the FOXM1 level [13]. Additionally, circRHOT1 recruits TIP60 to initiate the expression of NR2F6 to promote the progression of hepatocellular carcinoma [14]. In BC, some circRNAs have been identified and characterized to be tumor suppressors or oncogenes, such as circTFF1, circANKS1B, circAHNAK1 and circKDM4C [15–18]. However, the function of circRNAs in BC need to be further investigated.

In our previous work, we identified a circRNA generated from the 2-5 exons of *AGFG1* gene, termed as circAGFG1, which promoted the progression of TNBC and served as a predictor for poor prognosis of TNBC patients [19]. However, the clinical values and biological roles of circAGFG1 in non-TNBC remain largely unclear. In the current work, we uncovered that circAGFG1 was also upregulated in non-TNBC samples, and high level of circAGFG1 predicted unfavorable clinical outcomes of patients suffering from non-TNBC. Functional investigations validated the pro-tumor effect of circAGFG1 in non-TNBC cell lines as well. Mechanistically, we reported that circAGFG1 could sponge miR-143-5p and thus to an increase the level of oncogene USP14 in non-TNBC. Our findings provide insights into how circAGFG1 facilitates to the progression of non-TNBC, raising the possibility of circAGFG1 to be an alternative therapeutic target for non-TNBC.

Materials And Methods

Tissue specimens

Totally, 30 cases of non-TNBC tissues and matched non-tumor tissues were acquired from patients who undergone mastectomy with informed consent at the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). None of these patients involved in the current study received preoperative chemoradiotherapy. All the samples were stored in liquid nitrogen before RNA isolation.

Cell lines and culture

Human non-TNBC cell lines SKBR-3, T47D and MCF-7 and MCF-10A (normal mammary epithelial cell line) were purchased from ATCC (Manassas, VA, USA). SKBR-3, MCF-7 and T47D cells were grown in DMEM (Gibco, Carlsbad, CA, USA) supplied with 10% fetal bovine serum. MEBM BulletKit (Lonza, Basel, Switzerland) was used for the culture of MCF-10A cells. All the cells were maintained at 37°C in a humidified incubator with 5% CO₂.

qRT-PCR

Total RNA was extracted with TRIzol (Takara, Dalian, China). The isolated RNA was synthesized into cDNA with PrimeScript RT Reagent Kit (Takara), then amplified using TB Green Premix Ex Taq (Takara).

The expression of transcripts was determined by $2^{-\Delta\Delta CT}$. GAPDH was used as internal controls. The primer sequences were listed in **Table S1**.

Western blot assay

Total proteins of BC cells were isolated with RIPA containing PMSF and subjected to SDS-PAGE. The separated proteins were then transferred to the PVDF membranes, which were maintained in blocking buffer and then incubated with anti-USP14 (1:1000, Proteintech, Wuhan, China) or anti- β -actin (1:1000, Proteintech) at 4 °C overnight. After that, the bands were incubated with HRP-conjugated secondary antibodies (1:2000, Proteintech) followed by ECL detection.

RNA in situ hybridization (ISH)

ISH was carried out with a double digoxin-labeled probe specific for circAGFG1 (Digoxin-5'-ATTTAATCCTCGCCTGCATGACTGTTGAAA-3'-Digoxin) to evaluate the expression of circAGFG1 on tissue microarrays (Outdo Biotech, Shanghai, China) which contained 346 non-TNBC tissues. Briefly, the tissue microarrays were dewaxed and rehydrated, then digested with proteinase K followed by hybridization with above mentioned probe at 37°C for 18 h. After that, the tissue microarrays were incubated with anti-digoxin-AP (Roche, Basel, Switzerland) at 4°C overnight, followed by staining with NBT/BCIP (Roche). The ISH score of circAGFG1 of each sample was obtained by multiplying the score of the percentage of positive-stained cells (<5% = 0, 5-25% = 1, 26-50% = 2, 51-75% = 3, >76% = 4) and the scores of the intensity of staining (strong = 3, moderate = 2, weak = 1 and negative = 0). The samples were defined as low or high expression groups by the mean of ISH score of circAGFG1.

Plasmids

The construction of circAGFG1 overexpression and knockdown vectors as well as cell transfection were conducted as previously reported [19].

Cell growth and Transwell assays

CCK-8, colony formation, EdU and Transwell assays were executed as previously described [19].

Xenograft Model

MCF-7 cells stably expressing circAGFG1 or sh-circ and their control groups were inoculated into the dorsal flank of female nude mice (n=3). The volume of each tumor was measured every week and determined with the formula, $0.5 \times \text{length} \times \text{width}^2$. After four weeks, the tumor-bearing mice were sacrificed. The tumors were excised and weighed. The survival analyses of tumor-bearing mice were carried out in parallel with the tumor growth study (n=10).

Immunohistochemistry

The paraffin-embedded sections were dewaxed and rehydrated, followed by incubating with antibodies specific for Ki67 (1:100, Proteintech) at 4 °C for 12 h and biotin-labeled secondary antibodies at 37°C for about 0.5 h. Then, the slides were stained with diaminobenzidine and hematoxylin.

Dual-luciferase reporter assay

For luciferase reporter construction, the sequences of circAGFG1 and 3'UTR of USP14 as well as their mutants with no miR-143-5p complementary sites were subcloned into psiCHECK2, respectively. All these plasmids were finally verified by sequencing. The inhibitors and mimics of miR-143-5p were obtained from RiboBio (Guangzhou, China). The relative activities of luciferase were determined by Dual Luciferase Assay Kit (Hanbio, Shanghai, China).

Fluorescent in situ hybridization (FISH)

Cy3-labeled probes targeting circAGFG1 and FITC-labeled probes targeting miR-143-5p were synthesized by Geneseeed (Guangzhou, China). Hybridization was executed with a FISH kit (RiboBio) and the nucleus was then stained with DAPI.

Pull-down assay

Biotin-labeled circAGFG1 probes and control probes were obtained as previously documented [19]. Pull-down assay was performed with a Pull-Down Kit (Thermo Fisher Scientific, Waltham, MA, USA). The retrieved RNA was followed by reverse transcription and qRT-PCR.

RNA immunoprecipitation (RIP)

RIP assay was performed using antibodies specific for AGO2 (CST, Beverly, MA, USA), IgG control (CST) and RIP kit (Geneseeed) under the conditions recommended by the manufacturer and followed by qRT-PCR analyses.

Statistical analyses

Student's t test or one-way ANOVA were applied in the comparison of differences between two or three groups, respectively. The survival curves of non-TNBC patients were created using Kaplan-Meier method and analyzed using log-rank test. Multivariate analysis was applied to identify the risk factor of the overall survival of non-TNBC patients. The diagnostic ability of circAGFG1 was assessed by Receiver operating characteristic (ROC) curve. The relevance between groups were examined by χ^2 test or Pearson correlation. The results were deemed as statistically significant when P value < 0.05. All these analyses were executed with SPSS 21.0 software.

Results

circAGFG1 is elevated in non-TNBC tissues and correlated with poor clinical outcomes

To gain insights into the expression pattern of circAGFG1 in non-TNBC, qRT-PCR analyses were performed in 30 pairs of non-TNBC tissues and adjacent non-cancerous tissues, as well as non-TNBC cell lines (SKBR-3, MCF-7 and T47D) and normal breast epithelial cell line (MCF-10A), indicating that circAGFG1 was substantially elevated in both non-TNBC tissues and cell lines (**Fig. 1a-b**). Further, ROC curve revealed that BC tissues could be sensitively distinguished from non-cancerous tissues by the level of circAGFG1 (**Fig. 1c**). We then determined the expression of circAGFG1 by ISH on TMAs, which contained a cohort of 346 non-TNBC samples (**Fig. 1d**). The relevance between the clinicopathological features of these non-TNBC patients and the expression level of circAGFG1 was listed in **Table S2**. Survival curve generated from Kaplan-Meier method suggested that patients suffering from non-TNBC with higher expression levels of circAGFG1 exhibited a shorter survival time than those with lower circAGFG1 levels (**Fig. 1e**). Similar results were observed in patients in stage I-II or stage III (**Fig. 1f-g**). Moreover, multivariate analysis uncovered that high level of circAGFG1 might be a standalone risk factor for patients with non-TNBC (**Fig. 1h**). These findings demonstrated that circAGFG1 was also increased in non-TNBC tissues and high level of circAGFG1 heralded poor clinical outcomes of non-TNBC patients.

circAGFG1 enhances the malignance of non-TNBC *in vitro* and *in vivo*

To probe whether circAGFG1 could also increase the malignance of non-TNBC cells, we transfected the overexpression and knockdown vectors of circAGFG1 into SKBR-3 and MCF-7 cells, finding that circAGFG1 was successfully overexpressed or silenced in by these vectors, respectively, compared with their empty vectors (**Fig. 2a-b**). The growth curves derived from CCK-8 assays revealed that ectopic expression of circAGFG1 accelerated the proliferation of SKBR-3 and MCF-7 cells, whereas circAGFG1 depletion suppressed cell proliferation (**Fig. 2c**). Consistently, EdU and colony formation assays further verified that the viability of SKBR-3 and MCF-7 cells was enhanced or impaired by overexpressing or silencing of circAGFG1, respectively (**Fig. 2d-e**). Moreover, Transwell assays revealed that ectopic expression of circAGFG1 facilitated the invasion abilities of SKBR-3 and MCF-7 cells, meanwhile, down-regulation of circAGFG1 compromised the invasion capacities of these two cells (**Fig. 2f**). To further study the biological role of circAGFG1 on tumorigenesis of non-TNBC *in vivo*, MCF-7 cells stably overexpressing or silencing circAGFG1 were established and subcutaneously injected to the dorsal flank of randomly grouped nude mice. Four weeks later, it was found that ectopic expression of circAGFG1 significantly promoted the tumor growth *in vivo*, while depletion of circAGFG1 produced the opposite (**Fig. 2g-i**). Additionally, the overall survival time of the tumor-bearing mice was monitored and analyzed by Kaplan-Meier method. The results suggested that enforced expression of circAGFG1 could evidently shorten the survival time of the tumor-bearing mice (**Fig. 2j**). IHC staining assays revealed that the tumors generated from circAGFG1 overexpression group had a higher level of proliferation index Ki67, whereas a lower level of Ki67 protein was observed in circAGFG1 deficiency group (**Fig. 2k**). These data uncovered that circAGFG1 was a critical mediator in tumor progression of non-TNBC.

circAGFG1 sponges miR-143-5p in non-TNBC cells

To study the potential mechanism of the tumor-promoting effect of circAGFG1 in non-TNBC, we analyzed the potential miRNA target of circAGFG1 using TargetScan and miRanda, finding that miR-143-5p could directly interact with circAGFG1 (**Fig. 3a**). To validate this prediction, we implemented FISH assays in MCF-7 cells using Cy3-labeled circAGFG1 probes and FITC-labeled miR-143-5p probes, finding that both circAGFG1 and miR-143-5p were mainly distributed in the cytoplasm of MCF-7 cells (**Fig. 3b**). Dual-luciferase reporter assay was consequently performed with luciferase reporters of circAGFG1 and miR-143-5p mimics or negative controls in MCF-7 cells. Compared with control group, miR-143-5p mimics markedly reduced the luciferase activity of wild-type circAGFG1 reporter, rather than the mutant version (**Fig. 3c-d**). Considering that miRNAs usually decrease the expression of their target genes in a AGO2-dependent way, therefore, we performed RIP assay using antibodies specific for AGO2 and IgG control to enrich circAGFG1 and miR-143-5p. As a result, both miR-143-5p and circAGFG1 were precipitated by the antibody specific for AGO2, implying the combination of miR-143-5p and circAGFG1 in MCF-7 (**Fig. 3e**). Moreover, pull-down assay using biotin-labeled probe targeting circAGFG1 further validated the direct interaction between circAGFG1 and miR-143-5p in MCF-7 cells (**Fig. 3f**). We then detected the impact of circAGFG1 on miR-143-5p expression in SKBR-3 and MCF-7 cells, finding that enforced expression of circAGFG1 substantially decreased miR-143-5p levels while depletion of circAGFG1 elevated the expression of miR-143-5p (**Fig. 3g**). Additionally, we analyzed miR-143-5p levels in BC of TCGA cohort, finding that miR-143-5p was markedly decreased in BC tissues (**Fig. 3h**). Consistently, qRT-PCR analysis further confirmed the expression level of miR-143-5p in 30 cases of non-TNBC tissues and paired non-tumorous tissues (**Fig. 3i**). These findings proved that circAGFG1 could sponge miR-143-5p in non-TNBC.

USP14 is a direct target of miR-143-5p

By using Targetscan, miRDB and miRanda database, we found that USP14 shared the same miRNA response element of miR-143-5p with circAGFG1 (**Fig. 4a**). Previous studies have reported that USP14 was upregulated and played an oncogenic role in many types of cancers [20]. Consistently, USP14 was evidently elevated in BC tissues in comparison with non-tumor tissues according to TCGA database (**Fig. 4b**), which was further confirmed in 30 pairs of non-TNBC tissues and para-cancerous tissues through qRT-PCR (**Fig. 4c**). To verify the bioinformatic prediction, we executed dual-luciferase reporter assay in MCF-7 cells and uncovered that miR-143-5p significantly suppressed the luciferase activity of wild-type USP14 reporters, rather than the mutant types (**Fig. 4d**). Moreover, miR-143-5p could negatively modulate both mRNA and protein levels of USP14 (**Fig. 4e-f**). These data indicated that USP14 was a target of miR-143-5p in non-TNBC cells.

circAGFG1 contributes to the progression of non-TNBC by modulating USP14 expression through miR-143-5p

To understand whether circAGFG1 exerted its pro-tumor effect via miR-143-5p/USP14 axis, we firstly confirmed that circAGFG1 expression was positively associated with the level of USP14 in 20 non-TNBC tissues by Pearson correlation (**Fig. 5a**). In addition, enforced expression of circAGFG1 markedly elevated both USP14 mRNA and protein, whereas silencing circAGFG1 reduced USP14 mRNA and protein in SKBR-

3 or MCF-7 cells (**Fig. 5b-c**). And as anticipated, ectopic expression or silencing of circAGFG1 induced upregulation or downregulation of USP14 was partially rescued by mimics or inhibitors of miR-143-5p, respectively (**Fig. 5b-c**). Nevertheless, CCK-8 and Transwell assays demonstrated that enforced expression or depletion of circAGFG1 produced tumor-promoting or -inhibiting effects were effectively rescued by the mimics or inhibitors of miR-143-5p, respectively (**Fig. 7d-e**). Taken together, the aforementioned findings demonstrated that circAGFG1 served as a sponge of miR-143-5p to modulate the expression of oncogene USP14, leading to the progression of non-TNBC.

Discussion

Recent years, with the rapid development and application of next generation sequencing and bioinformatic techniques for circRNA identification and quantification, plenty of circRNAs were discovered in various human cell lines and tissues. CircRNAs, as a novel type of noncoding RNA, has been characterized with high stability, abundance, evolutionary conservation as well as cell- and tissue-specific expression pattern, indicating that they are important in the pathophysiological and physiological processes, thus attracted much attention of the researchers [21]. It has been documented that circRNAs were dysregulated in many kinds of cancers, including BC [22]. However, only a few circRNAs have been well studied in BC, the biological function and underly mechanisms of the majority of circRNAs are yet to be elucidated.

Previously, we confirmed the pro-tumor effect of circAGFG1 in TNBC [19]. To understand the biological function of circAGFG1 in greater detail in BC, we expanded our work to non-TNBC in the current study. We found that circAGFG1 was also substantially elevated in non-TNBC cell lines and tissues in comparison with normal breast epithelial cell line and adjacent normal tissues. The expression of circAGFG1 was negatively associated with the overall survival of patients suffering from non-TNBC and high level of circAGFG1 might be a standalone risk factor for the patients with non-TNBC. In addition, functional studies demonstrated that enforced expression of circAGFG1 facilitated the malignance of non-TNBC cells *in vitro*, while circAGFG1 deficiency produced the opposite effects. Consequent *in vivo* studies also supported the tumor-promoting effect of circAGFG1 in non-TNBC. Our findings suggested that circAGFG1 played an oncogenic role not only in TNBC but also in non-TNBC, improving the possibility of circAGFG1 as a prognostic marker in BC.

It has been documented that circRNAs exert their biological function primarily through serving as miRNA sponges, scaffolds promoting protein complex assembly, RBP-binding partners, regulators of gene expression and even being translated themselves [9, 23]. However, miRNA sponge is the most widely reported mechanism of circRNA function in various cancers [24]. For an example, circMTO1 was decreased in hepatocellular carcinoma and could bind with miR-9 to attenuate the inhibitory role of miR-9 on the expression of its target p21 [21]. Subsequent studies revealed that circMTO1 could also sponge miR-760, miR-182-5p, miR-630, miR-92, miR-221, miR-3200-5p, miR-19b-3p, miR-17, miR-199a-3p and miR-6893 [25]. Other circRNAs function as competing endogenous RNA (ceRNA) in cancers have also been studied, such as CDR1as binding with miR-671-5p [26], circPRKCI binding with both miR-545 and miR-589

[27], and circHIPK3 interacting with miR-558 [28]. It indicates that one single circRNA is able to sponge multiple miRNAs to modulate the expression of different targets. In our study, according to the bioinformatic predictions, circAGFG1 could not only bind with miR-195-5p but also miR-143-5p. Consequent FISH, pull-down, RIP and dual-luciferase reporter assays strongly validated the binding of circAGFG1 and miR-143-5p in non-TNBC cells. It has been proved that miR-143-5p functioned to suppress tumor progression and was decreased in pancreatic cancer [29], gastric cancer [30], esophageal squamous cell carcinoma [31], lung adenocarcinoma [32] and BC [33]. Consistently, miR-143-5p was substantially decreased in BC of TCGA cohort, which was further confirmed in 30 cases of paired non-TNBC and para-tumorous tissues via qRT-PCR. In addition, circAGFG1 could negatively regulate the expression of miR-143-5p. Our data suggested that circAGFG1 exerted its tumor promoting effect via miR-143-5p/USP14 axis in non-TNBC.

As a miRNA sponge, circRNA binds with and inhibits the activities of miRNAs, leading to the alleviation of the suppressive effect of miRNAs on their target genes. Therefore, we analyzed the targets of miR-143-5p by using miRDB, Targetscan and miRanda database, and found that USP14 harbored two miRNA response elements of miR-143-5p, indicating that USP14 was a probable target gene of miR-143-5p. USP14 belongs to the deubiquitinating enzymes (DUBs) family and was documented to take part in diverse human diseases, especially in cancer [20]. Recent research revealed that USP14 was highly expressed in BC tissues as well as cell lines, and the combination of USP14 inhibition and enzalutamide might be a novel therapeutic strategy for BC [34]. Besides, inhibition of USP14 at both genetic and pharmacological levels significantly suppressed the viability of BC cell through triggering cell cycle arrest and eliciting apoptosis [35]. Moreover, USP14 could interact with and deubiquitinate CCNB1, leading to an increased in malignant phenotypes of BC cells, which provided a clue for the application of anticancer drugs targeting USP14 [36]. These studies suggested that USP14 served as an oncogene in BC and has been a promising target for the treatment of BC. Consistently, USP14 was evidently elevated in BC tissues by analyzing the TCGA database. Similar result was obtained in qRT-PCR analyses of 30 pairs of non-TNBC tissues. Then, dual-luciferase reporter assays provided strong evidence for the direct interaction between USP14 and miR-143-5p in non-TNBC cells. Moreover, miR-143-5p could negatively modulate both mRNA and protein levels of USP14 in non-TNBC cells. Our findings supported that USP14 was indeed a downstream target gene of miR-143-5p in non-TNBC cells. To understand whether USP14 could be regulated by circAGFG1 through miR-143-5p in non-TNBC, we firstly observed a positive correlation between the expression of circAGFG1 and USP14. Furthermore, the increase or decrease of USP14 mRNA and protein induced by ectopic expression or deficiency of circAGFG1 was partially attenuated by the mimics or inhibitors of miR-143-5p, respectively. As expected, the alteration of cell phenotypes produced by circAGFG1 were also reversed by miR-143-5p. These data demonstrated the hypothesis that circAGFG1 modulates the expression of USP14 through sponging miR-143-5p, thus leading to the progression of non-TNBC.

In summary, we demonstrate that circAGFG1 is also substantially elevated in non-TNBC tissues and negatively associated with the overall survival of patients suffering from non-TNBC. Nevertheless, circAGFG1 promotes the malignance of non-TNBC by adsorbing miR-143-5p to modulate the level of

oncogene USP14. Our former work together with the present study increase the feasibility of circAGFG1 to serve as a potential target in the diagnosis and treatment of both TNBC and non-TNBC.

Declarations

Funding

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Competing Interests

The authors declared there is no conflict of interest.

Author Contributions

JXC and RY designed the study. RY conducted all experiments and analyzed the data. HC was responsible for clinical sample collection and animal study. RY constructed the manuscript. All authors read and approved the final manuscript.

Ethics approval

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Chongqing Medical University (protocol code 20190405). The animal study protocol was approved by Chongqing Medical University Animal Care and Use Committee (pro-tocol code 20200315).

Consent to participate

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

Consent to publish

Written informed consent has been obtained from the patients to publish this paper.

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Figures

Figure 1

circAGFG1 is elevated in non-TNBC and associated with unfavorable clinical outcomes of patients with non-TNBC. **a-b** qRT-PCR analyses of circAGFG1 levels in 30 cases of non-TNBC tissues and paired non-tumor tissues as well as indicated cell lines. **c** Diagnostic ability of circAGFG1 in non-TNBC was assessed by ROC curve. **d** Representative images of ISH staining of circAGFG1 on TMAs which containing 346 non-TNBC tissues. **e-g** Kaplan-Meier survival curves of non-TNBC patients. **h** Multivariate analysis of overall survival in non-TNBC patients. Error bars, SD. $**P < 0.01$, $***P < 0.001$.

Figure 2

circAGFG1 enhances the malignance of non-TNBC cells *in vitro* and *in vivo*. **a-b** The effects of circAGFG1 overexpression (**a**) and knockdown (**b**) vectors on circAGFG1 expression were verified by qRT-PCR analyses. **c-e** CCK-8 (**c**), EdU (**d**) and colony formation (**e**) assays were carried out to examine the viability of MCF-7 and SKBR-3 cells. Scale bar, 100 μ m. **f** Transwell assays to determine the invasion abilities of MCF-7 and SKBR-3 cells. Scale bar, 100 μ m. **g** Photographs of xenograft tumors separated from nude

mice (n=3). **h-i** The weight (**h**) and volume (**i**) of tumors were calculated. **j** Survival analyses of the tumor-bearing mice (n=10) using Kaplan-Meier method. **k** IHC staining to examine the expression of Ki67 in xenograft tumors. Scale bar, 100 μ m. Error bars, SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3

circAGFG1 sponges miR-143-5p in non-TNBC. **a** Complementary bases of miR-143-5p and circAGFG1 was analyzed by miRanda and TargetScan. **b** FISH assays to probe the distribution of indicated transcripts in MCF-7 cells. **c** Schematic diagram illustrating the wild-type (WT) as well as the mutant-type

(MT) circAGFG1 reporter plasmids. **d** Dual-luciferase reporter assay using indicated vectors and mimics in MCF-7 cells. **e** RIP assay was conducted with anti-AGO2 or IgG control. **f** Pull-down assay was carried out using biotin-labeled probe targeting circAGFG1, followed by qRT-PCR analysis. **g** qRT-PCR to analyze the expression of miR-143-5p in MCF-7 cells expressing indicated vectors. **h** Expression of miR-143-5p in BC of TCGA cohort. **i** qRT-PCR to examine miR-143-5p levels in 30 cases of non-TNBC tissues and paired non-tumorous tissues. Error bars, SD. ** $P < 0.01$, *** $P < 0.001$.

Figure 4

USP14 is a target of miR-143-5p. **a** Binding sites of miR-143-5p on the 3'UTR of USP14. **b** Expression of USP14 in BC of TCGA cohort. **c** qRT-PCR to evaluate USP14 levels in 30 cases of non-TNBC tissues and

paired non-tumorous tissues. **d** Dual-luciferase reporter assay using indicated reporters and mimics in MCF-7 cells. **e** and **f** The mRNA (**e**) and protein (**f**) levels of USP14 were assessed by qRT-PCR and western blot analyses, respectively, in SKBR-3 and MCF-7 cells after transfection of indicated mimics or inhibitors. Error bars, SD. * $P < 0.05$, ** $P < 0.01$.

Figure 5

circAGFG1 promotes the progression of non-TNBC through circAGFG1/miR-143-5p/USP14 axis. **a** Pearson correlation analysis to evaluate the relevance of circAGFG1 and USP14 in expression in 20 cases of non-TNBC tissues. **b-c** USP14 mRNA (**b**) and protein (**c**) levels in SKBR-3 and MCF-7 cells after co-transfection of corresponding plasmids and mimics or inhibitors. **d-e** CCK-8 (**d**) and Transwell invasion (**e**) assays were conducted to examine the viability and motility of SKBR-3 and MCF-7 cells after co-transfection of corresponding plasmids, and mimics or inhibitors, respectively. Scale bar, 100 μ m. Error bars, SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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