MicroRNA-598 inhibits the growth of triple negative breast cancer cells by targeting JAG1

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Abstract. Triple-negative breast cancer (TNBC) has an aggressive phenotype and a poor outcome. The discovery that dysregulated microRNAs (miRNAs) play an important role in tumor progression has led to the suggestion that miRNAs (miRs) could be a potential target for the treatment of TNBC. In the present study, it was demonstrated that miR-598 expression was significantly decreased in TNBC tissues and was related to the degree of lymph node metastasis of patients with TNBC. Ectopic expression of miR-598 suppressed viability and colony formation, as well as increased the apoptosis of TNBC cells. To further understand the functional mechanism of action underlying miR-598 in TNBC, targets of miR-598 were predicted with the miRDB bioinformatics tool. Jagged 1 (JAG1) was identified as a direct target of miR-598, possessing a binding site for miR-598 in its 3'-untranslated region. Overexpression of miR-598 inhibited the expression of JAG1 in TNBC cells. In addition, JAG1 was highly expressed in TNBC tissues and its expression was negatively correlated with the expression of miR-598. Overexpression of JAG1 significantly attenuated the inhibitory effects of miR-598 on the proliferation and colony formation of TNBC cells. Collectively, these results provided novel insights into the functional mechanism of action for the miR-598/JAG1 pathway in the development of TNBC.

Introduction

Triple negative breast cancer (TNBC) is a heterogeneous subclass of breast cancer, characterized by the lack of expression of epidermal growth factor receptor, estrogen receptor and progesterone receptor (1-3). Compared with other types of breast cancer, patients with TNBC present a higher risk of metastasis and recurrence, as well as a poorer prognosis (3). Due to the absence of a specific biomarker, the current strategies for early-stage or advanced TNBC remain chemotherapy or radiotherapy, and the clinical outcomes for patients with TNBC are still uncertain; however, patients tend to exhibit more aggressive features compared with other forms of breast cancer (4). The genomic and molecular aberrations that contribute to the initiation and progression of TNBC remain largely unknown. Therefore, identifying novel factors and characterizing the related functional mechanism of actions are critical for the diagnosis and treatment of patients with TNBC.

MicroRNAs (miRNAs) are small non-coding, single stand RNA molecules that negatively modulate gene expression at the post-transcriptional level by binding to the 3'-untranslated region (UTR) of target mRNAs (5-7). Previous studies have reported that miRNAs play a pivotal role in cancer prognoses and drug resistance (8-12). Moreover, aberrant expression of miRNA (miR) has been demonstrated in patients with TNBC, which is correlated with the clinical outcomes of the patient (13,14). A recent study revealed that miR-890 was downregulated in TNBC and that it inhibited the proliferation and invasion of TNBC cells (15). Inhibition of miR-214 also significantly attenuates the migration and invasion of TNBC cells (16). Furthermore, previous studies have identified the downregulation and tumor suppressive functions of miR-598 in multiple cancer types, including gastric cancer, non-small cell lung cancer (NSCLC) and colorectal cancer (17-19). Overexpression of miR-598 also suppresses the malignant features of cancer cells (17-19). Therefore, developing miRNA-based therapeutics may improve the treatment of cancer, particularly for patients with TNBC who show early relapse and poor survival. However, the expression and functional mechanism of action of miR-598 in TNBC remains largely unknown.

Jagged 1 (JAG1) is a canonical ligand that functions primarily in the highly conserved Notch signaling pathway (20). Notch signaling plays important roles in the determination of cellular fate and organ development (21). The classic interaction between JAG1 and Notch leads to a cascade of proteolytic

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cleavages that induces the transportation of Notch intracellular domain into the nucleus to activate the transcription of target genes (22-24). Previous studies have shown that frequent upregulation of JAG1 in various types of cancer is associated with a poor survival rate (21,25). However, downregulation of JAG1 inhibits the progression of cancer types, suggesting the clinical significance of JAG1 as a potential target for cancer treatment (25). Therefore, the present study aimed to detect the expression of miR-598 in TNBC and characterize the functional mechanism of miR-598 in the malignancy of TNBC.

Materials and methods

Cell lines. The TNBC cell lines MDA-MB-231, HCC-1937, MDA-MB-468 and BT-549 and normal human breast cell line MCF-10A were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.). Cells were cultured at 37˚C with 5% CO_2.

Tissue samples. A total of 50 patients (age range, 44-70 years) who were diagnosed as TNBC with negative expressions of estrogen receptor, progesterone receptor and human epidermal growth factor receptor were enrolled in this study. TNBC tissues and adjacent healthy tissues were obtained via surgical resection at Shanxi Provincial Cancer Hospital (Taiyuan, Shanxi, China) between November 2012 and September 2014. Patients who were subjected to neoadjuvant chemotherapy or radiotherapy before the surgery, or those without adjacent healthy tissues were excluded in this study. The lymph node metastasis of patients was determined via hematoxylin and eosin staining by three independent pathologists. All tissues were frozen immediately and stored at -80˚C until use. Written informed consents were received from all patients. The study was approved by the Ethics Committee of Shanxi Provincial Cancer Hospital. The relevant clinical characteristics of the patients enrolled in the present study are provided in Table SI.

Cell transfection. miR-598 mimics (5'-UACGUCAUCGUUG UCAUCGUCA-3') and miR-negative control (NC; 5'-GUUC GUACGUACACUGUUCA-3') were purchased from Shanghai GenePharma Co., Ltd. The overexpression plasmid of JAG1 was generated by amplifying the cDNA of JAG1 and inserting it into the pcDNA-Myc vector (Addgene, Inc.). For cell transfection, MDA-MB-231 and BT-549 cells ($1x10^5$ cells/well) were plated into 6-well plates. After culturing overnight, miRNA (50 nM) was transfected using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. After transfection for 48 h, cells were harvested for further analysis.

Cell proliferation assay. The Cell Counting Kit-8 (CCK-8) assay was performed to determine the cell viability according to the manufacturer's protocol (Beyotime, Institute of Biotechnology). TNBC cells transfected with miR-598 mimics or miR-NC were plated into 96-well plates with 2,000 cells per well. Following incubation with 10 μl CCK-8 regent at 37˚C for 4 h at the indicated time points (1, 2, 3, 4 and 5 days), the absorbance of each well at 450 nm was measured using the microplate reader (Roche Diagnostics).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from tissues or cells was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and quantified using the NanoDrop-2000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). Then, 1 μg RNA was reverse transcribed into cDNA using the PrimeScript RT Reagent kit (Takara Bio, Inc.) at 37˚C for 10 min and 85˚C for 10 sec. The expression of miR-598 was determined using qPCR assays with the TaqMan miRNA PCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) on the Option RT-qPCR detection system (ABI 7500; Thermo Fisher Scientific, Inc.). The PCR cycles were set as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec, 60˚C for 1 min and preservation at 4˚C. The expression of miR-598 was calculated using the comparative quantification cycle ($2^{-\Delta\Delta Cq}$) method (26). The expression of GAPDH was used for normalization. The following primer pairs were used for the qPCR: miR-598 forward, 5'- TACGTCA TCGTTGTCATCGTCA-3' and reverse, 5'-GCATAGACCTG AATGGCGGTA-3'; U6 forward, 5'-GCTTCGGCAGCACAT ATACTAAAAT-3' and reverse, 5'-CGCTTCAGAATTT GCGTGTCAT-3'; JAG1 forward, 5'- ATCGTGCTGCCTTTC AGTTT-3' and reverse, 5'-GATCATGCCCGAGTGAGAA-3' and GAPDH forward, 5'-CACCTGCGCTGTGTGGACT-3' and reverse, 5'-GGATGGCTGATGTGTCGGGTGG-3'.

Western blot analysis. The proteins cells were extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology) containing protease inhibitor. The protein concentration was assessed using the bicinchoninic acid Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Equal amounts of protein (20 μg/lane) were separated by 15% SDS-PAGE and transferred onto the PVDF membrane (EMD Millipore). After blocking with 5% non-fat milk for 1 h at room temperature, the membrane was probed with primary antibodies targeting JAG1 (1:1,000; cat. no. ab109536; Abcam) or GAPDH (1:3,000; cat. no. ab8245; Abcam) at 4˚C overnight. The membrane was washed three times with PBS-Tween-20 (0.1%) and then incubated with Goat anti-Mouse IgG (H+L)-horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000; cat. no. 170-6516; Bio-Rad Laboratories, Inc.) or Goat anti-Rabbit IgG (H+L)-HRP-conjugated secondary antibodies (1:5,000; cat. no. 170-6515; Bio-Rad Laboratories, Inc.) at room temperature for 1 h. Following an extensive wash with PBST, the enhanced chemiluminescence western blotting kit (Pierce; Thermo Fisher Scientific, Inc.) was used to visualize the bands. Densitometric analysis was performed using ImageJ software (version 1.8.0; National Institutes of Health). The expression of GAPDH was used as the loading control.

Targets prediction. The potential targets of miR-598 were predicted using the miRDB online database (version 6.0; http://mirdb.org/).

Dual-luciferase reporter assay. The wild-type (WT) or mutant (MT) JAG1 3'-UTR was amplified and cloned into

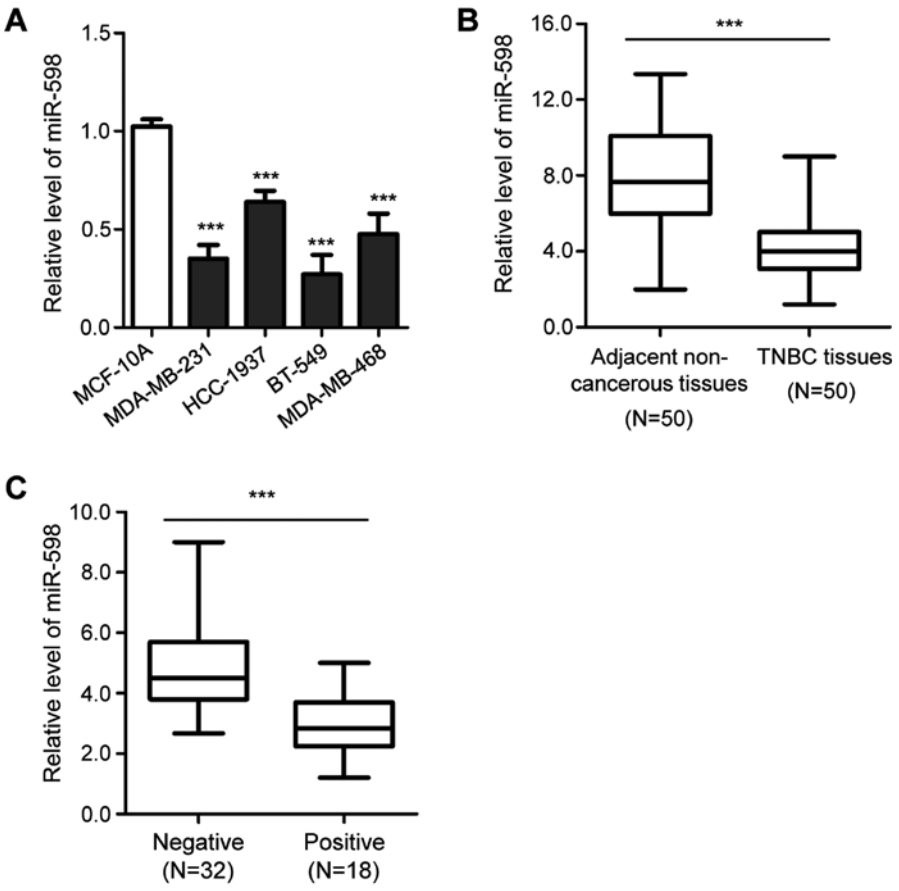

Figure 1. Expression of miR-598 is decreased in TNBC tissues. (A) Expression of miR-598 in TNBC cells was downregulated compared with normal MCF-10A cells. (B) Expression of miR-598 in TNBC tissues and paired adjacent healthy tissues was compared using reverse transcription-quantitative PCR. (C) Expression of miR-598 was lower in patients with TNBC with lymph node metastasis. ***P<0.001 vs. control group. miR, microRNA; TNBC, triple negative breast cancer.

the pMIR-REPORT Luciferase reporter vector (Promega Corporation) to generate the pMIR-JAG1-3'-UTR-WT or pMIR-JAG1-3'-UTR-MT, respectively. Cells ($1x10^4$ cells/well) were plated into the 96-well plate and co-transfected with the luciferase plasmid carrying WT or MT 3'-UTR of JAG1 (100 μg) and miR-598 mimics or miR-NC (50 nM) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After transfection for 48 h, cells were harvested and the luciferase activity was assessed using the Dual-Luciferase Reporter Assay system (Promega Corporation) according to the manufacturer's instructions. The luciferase activity of firefly was also detected for normalization.

Colony formation. TNBC cells transfected with miR-598 mimics or miR-NC were seeded into the 6-well plate with a density of 500 cells per well. Cells were cultured with RPMI-1640 medium containing 10% FBS at 37˚C with 5% CO_2. Following incubation in the CO_2 incubator for 10 days, cells were washed with PBS and fixed with 100% methanol at room temperature for 15 min. The colonies were stained with 0.5% crystal violet (Beyotime Institute of Biotechnology) at room temperature for 10 min and counted manually using a light microscope at x40 magnification.

Statistical analysis. Data are presented as the mean ± SD from three independent experiments. Statistical analysis was performed using SPSS software 13.0 (SPSS, Inc.). Paired and unpaired Student's t-tests was used to analyze the significance between two groups. Difference among multiple groups was determined using one-way ANOVA followed by Tukey's post hoc test. The correlation between the expression levels of miR-598 and JAG1 was assessed using the Pearson's test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-598 expression is downregulated in TNBC tissues and cell lines. To evaluate the potential involvement of miR-598 in TNBC, RT-qPCR analysis was performed to analyze the expression of miR-598 in TNBC cells. Compared with the normal cell line, MCF-10A, the expression of miR-598 was significantly decreased in TNBC cells (Fig. 1A). Moreover, the expression of miR-598 in TNBC tissues was evaluated, and was found to be significantly downregulated in TNBC tissues compared with corresponding healthy adjacent tissues (Fig. 1B). The results also suggested that the expression of miR-598 was significantly lower in patients with lymph node metastasis compared with patients without lymph node metastasis (Fig. 1C). These findings indicated that the downregulation of miR-598 may play an important role in the progression of TNBC.

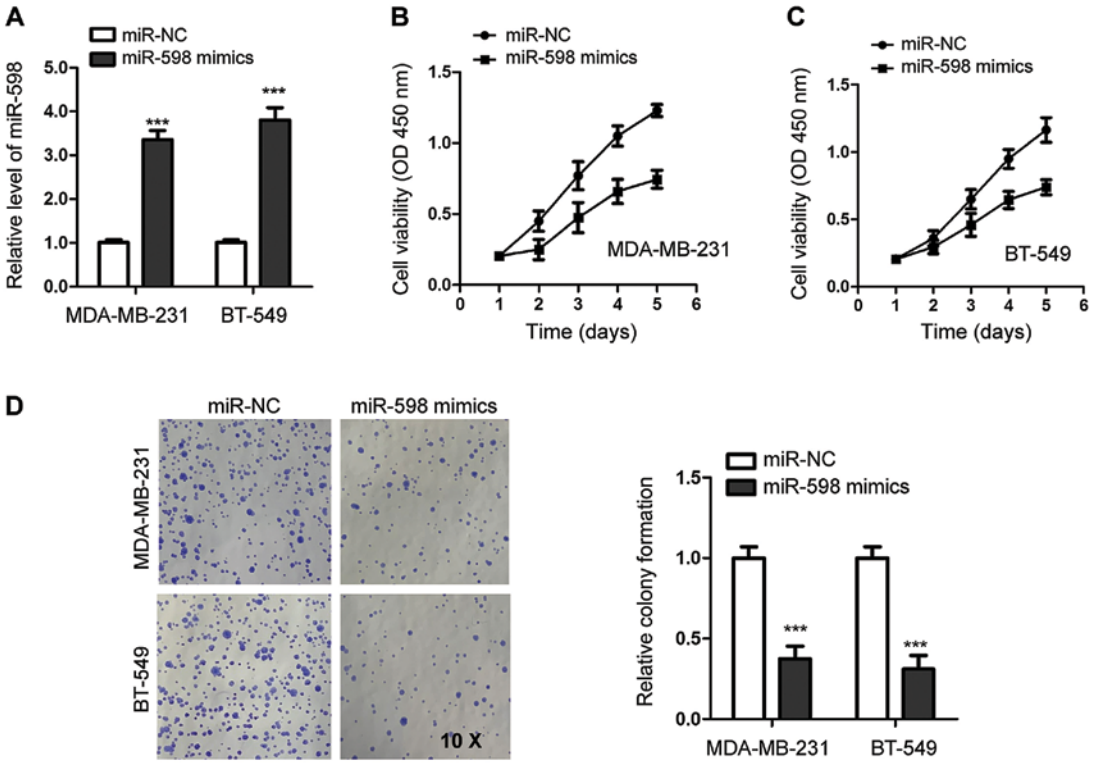

Figure 2. Overexpression of miR-598 inhibits the proliferation of TNBC cells. (A) Transfection of miR-598 mimics increased the expression of miR-598 in MDA-MB-231 and BT-549 cells. Overexpression of miR-598 decreased viability in (B) MDA-MB-231 and (C) BT-549 cells. (D) Transfection of miR-598 significantly inhibited the colony formation abilities of both MDA-MB-231 and BT-549 cells. Magnification, x10. ***P<0.001 vs. control group. miR, microRNA; TNBC, triple negative breast cancer; NC, negative control; OD, optical density.

miR-598 inhibits the viability and colony formation of TNBC cells. To investigate the function of miR-598 in TNBC, MDA-MB-231 and BT-549 cells, which expressed lower levels of miR-598 compared with the other tested TNBC cell lines, were transfected with miR-598 mimics or miR-NC. The overexpression of miR-598 was detected using RT-qPCR (Fig. 2A).

To investigate the effects of miR-598 on the viability of TNBC cells, CCK-8 assays were performed. The results demonstrated that miR-598 overexpression decreased the viability of MDA-MB-231 cells compared with cells expressing miR-NC (Fig. 2B). The inhibitory function of miR-598 overexpression on viability was also observed in BT-549 cells (Fig. 2C).

Colony formation assays were performed to evaluate the influence of miR-598 on the proliferation of TNBC cells. Compared with the control cells, overexpression of miR-598 significantly decreased the colony-formation ability of both MDA-MB-231 and BT-549 cells (Fig. 2D). Thus, these results suggested a tumor suppressive role for miR-598 in TNBC.

miR-598 suppresses cell cycle progression and induces apoptosis of TNBC cells. To further illustrate the function of miR-598 in the progression of TNBC, the cell cycle progression of MDA-MB-231 and BT-549 cells overexpressing miR-598 was detected using fluorescence-activated cell sorting (FACS). The results suggested that overexpression of miR-598 significantly increased the number of cells in the G1 phase compared with cells expressing miR-NC (Fig. 3B), which suggested that G1 cell cycle arrest was induced by miR-598.

In addition, to investigate whether overexpression of miR-598 regulated the apoptosis of TNBC cells, cells transfected with miR-598 mimics or miR-NC were stained with Annexin/FITC and propidium iodide. The FACS analysis indicated that overexpression of miR-598 significantly enhanced the apoptosis of both MDA-MB-231 and BT-549 cells (Fig. 3A). Therefore, the results indicated that miR-598 induced cell cycle arrest and apoptosis of TNBC cells.

JAG1 is a target of miR-598 in TNBC. To understand the molecular mechanism of action underlying the anti-tumor functions of miR-598 in TNBC, the potential targets of miR-598 were predicted using the miRDB website. The prediction analysis identified JAG1 as a possible target of miR-598, as the 3'-UTR of JAG1 contained complementary binding sites for miR-598 (Fig. 4A). To assess this potential association, dual-luciferase reporter assays were performed by transfecting luciferase reporter vectors that harbored WT or MT 3'-UTR of JAG1. The results indicated that overexpression of miR-598 significantly reduced the luciferase activity of cells expressing WT, but not MT 3'-UTR of JAG1 (Fig. 4B), suggesting that there may be specific binding between miR-598 and the 3'-UTR of JAG1.

To further analyze the effect of this interaction, both RT-qPCR and western blotting assays were performed to evaluate the expression of JAG1 after miR-598 overexpression. It was demonstrated that transfection of miR-598 mimics led to a corresponding significant decrease in JAG1 expression in MDA-MB-231 and BT-549 cells (Fig. 4C and D).

To support the negative regulation of JAG1 by miR-598, the expression of JAG1 in TNBC tissues was detected using RT-qPCR. Expression of JAG1 was significantly upregulated in TNBC tissues compared with the healthy tissues (Fig. 4E).

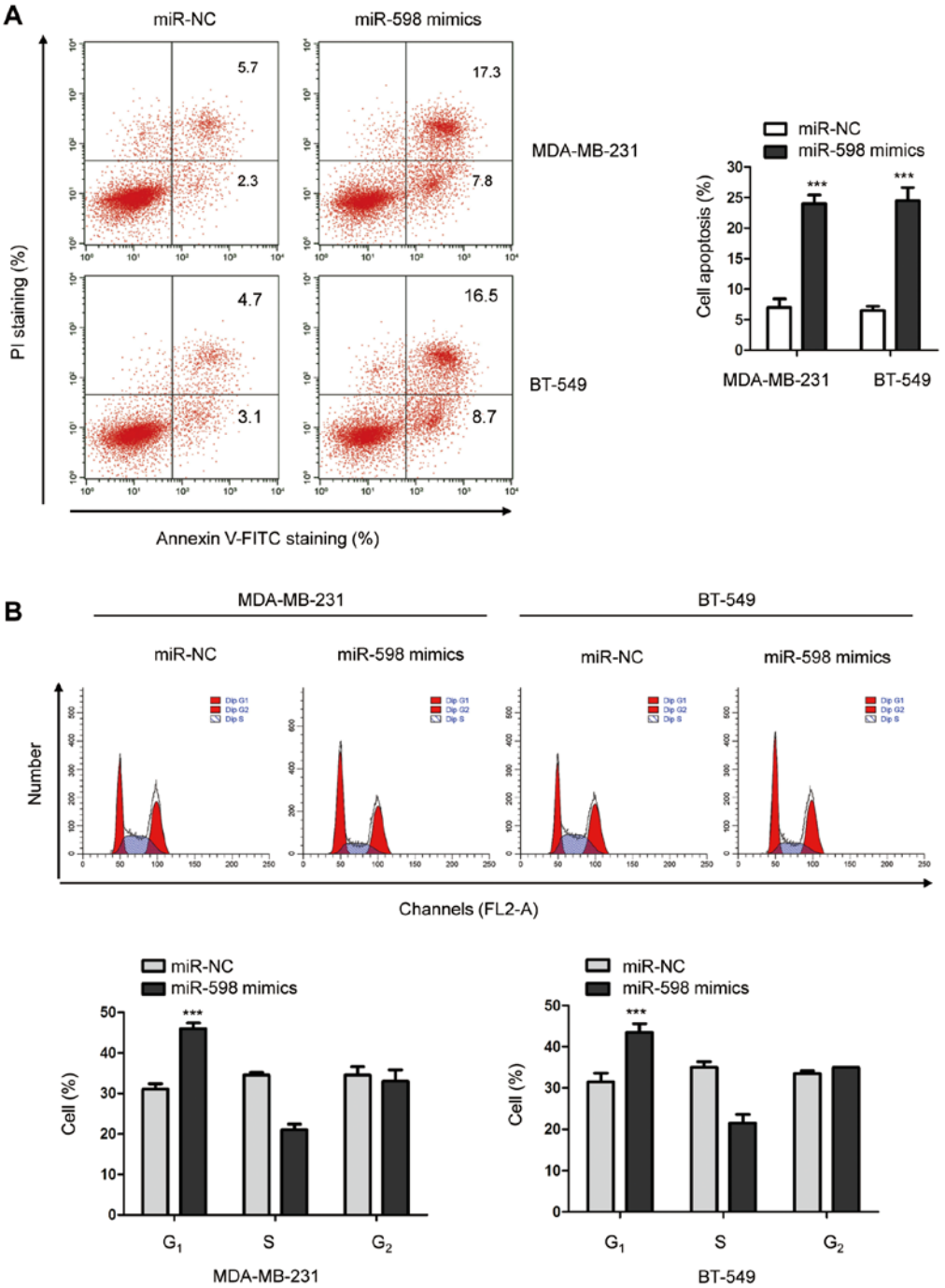

Figure 3. miR-598 overexpression induces apoptosis and cell cycle arrest of TNBC cells. (A) Overexpression of miR-598 increased the apoptotic rate of both MDA-MB-231 and BT-549 cells. (B) Ectopically expressed miR-598 in TNBC cells led to cell cycle arrest in the G1 phase. ***P<0.001 vs. control group. miR, microRNA; TNBC, triple negative breast cancer; NC, negative control; PI, propidium iodide.

Furthermore, the correlation between the expression levels of miR-598 and JAG1 was analyzed using the Pearson's test, which demonstrated that miR-598 expression was moderately inversely correlated to JAG1 expression in TNBC tissues (Fig. 4F). Collectively, these findings indicated that miR-598 targeted JAG1 and inhibited the expression of JAG1 in TNBC cells.

miR-598 suppresses the proliferation of TNBC cells by targeting JAG1. To further investigate whether miR-598 inhibited the viability of TNBC cells by targeting JAG1, JAG1 was overexpressed by transfecting pcDNA-Myc-JAG1 into MDA-MB-231 and BT-549 cells (Fig. 5A). TNBC cells were co-transfected with miR-598 mimics and JAG1, and the CCK-8 assays indicated that the viability of TNBC cells was increased following co-transfection of JAG1 compared with cells transfected with miR-598 alone (Fig. 5B and C). For the colony formation assay, TNBC cells formed fewer colonies when overexpressing miR-598. However, cells co-transfected with miR-598 mimics and pcDNA-JAG1 exhibited more colonies (Fig. 5D). These results suggested that JAG1 plays an important role in miR-598-induced proliferation defects of TNBC cells.

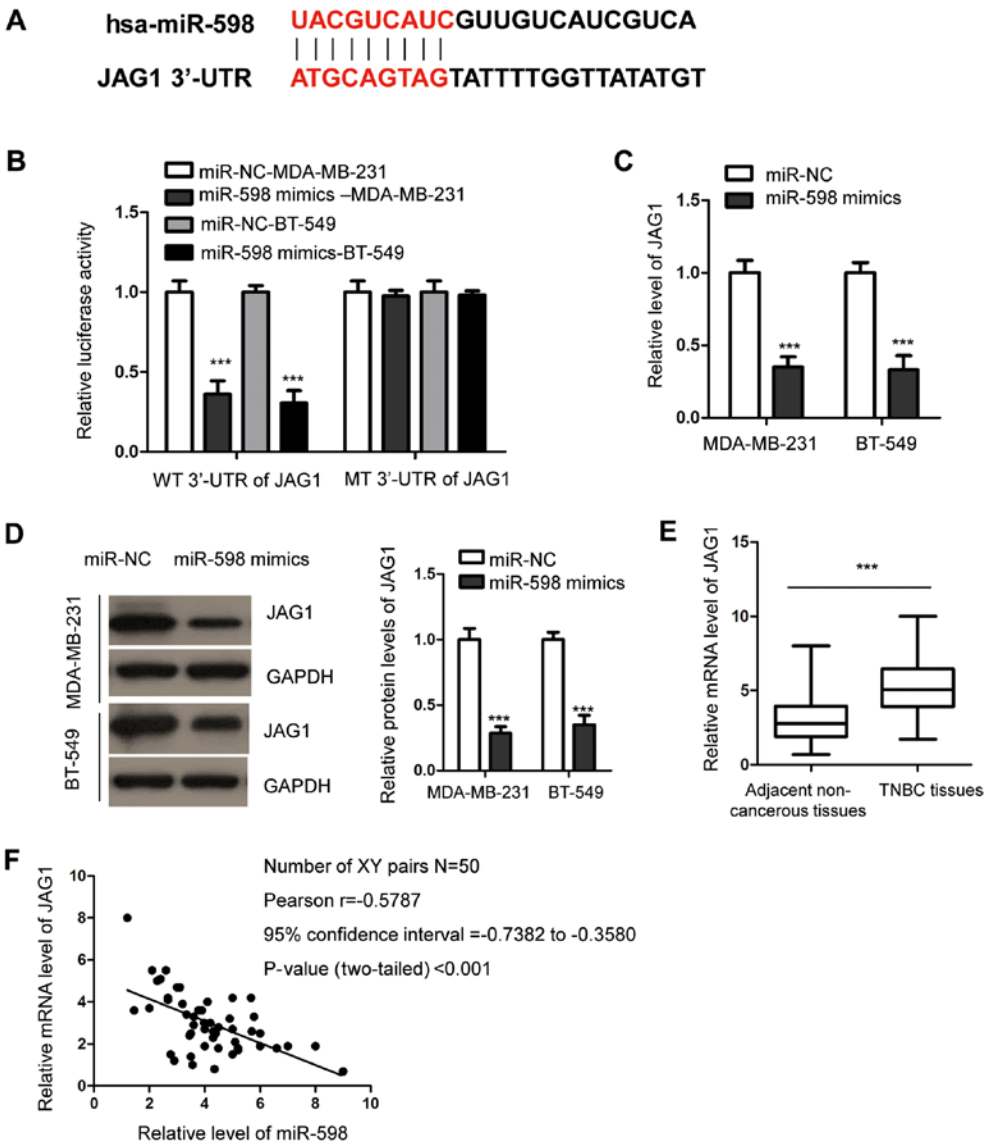

Figure 4. JAG1 is a target of miR-598. (A) Predicted complementary binding site of miR-598 on the 3'-UTR of JAG1. (B) Transfection of miR-598 mimics reduced the luciferase activity in TNBC cells expressing the WT, but not MT, 3'-UTR of JAG1. (C) Overexpression of miR-598 inhibited the mRNA expression of JAG1 in both MDA-MB-231 and BT-549 cells. (D) Transfection of miR-598 decreased the protein expression of JAG1 in TNBC cells (left panel). The densitometry values of the blots were presented in the right panel. Data were obtained from three independent experiments. (E) Expression of JAG1 in paired TNBC tissues and adjacent healthy tissues was detected using reverse transcription-quantitative PCR. (F) Expression of miR-598 was negatively correlated with that of JAG1 in TNBC tissues. ***P<0.001. JAG1, jagged 1; miR, microRNA; MT, mutant; TNBC, triple negative breast cancer; WT, wild-type; UTR, untranslated region; NC, negative control.

Discussion

Due to the lack of precise targets, chemotherapy has remained the main therapeutic strategy for the treatment of TNBC (27-29). However, increased chemoresistance and worse prognoses have been reported in patients with TNBC compared with other subtypes of breast cancer (30,31). Thus, the discovery of novel factors that can be used as potential targets for the diagnosis and treatment of TNBC is critical. Previous studies have reported that frequent aberrant expression of miRNAs is correlated with the initiation and progression of TNBC (32,33). In the present study, miR-598 was downregulated in TNBC tissues and cell lines. Furthermore, highly expressed miR-598 levels significantly inhibited the malignant features of TNBC cells. Thus, these findings provided novel insights into the anti-tumor effects of miR-598 in TNBC.

Abnormal expression of miR-598 is implicated in multiple cancer types and contributes to the malignant phenotypes of cancer cells (17-19). miR-598 inhibits the proliferation and metastasis of ovarian cancer cell (34). In addition, the expression of miR-598 is significantly downregulated in NSCLC, which is negatively correlated with the TNM stage and lymph node metastasis of patients with NSCLC (18). The tumor suppressive role of miR-598 has also been reported in gastric cancer, which may serve as a promising anti-cancer target (17). A recent study also revealed an anti-cancer function of miR-598 in glioblastomas by directly targeting MET transcriptional regulator MACC1 (35). In the present study, miR-598 was decreased in TNBC tissues and cell lines. Furthermore, downregulation of miR-598 was significantly correlated with lymph node metastasis of patients with TNBC, suggesting a potential involvement of miR-598 in the

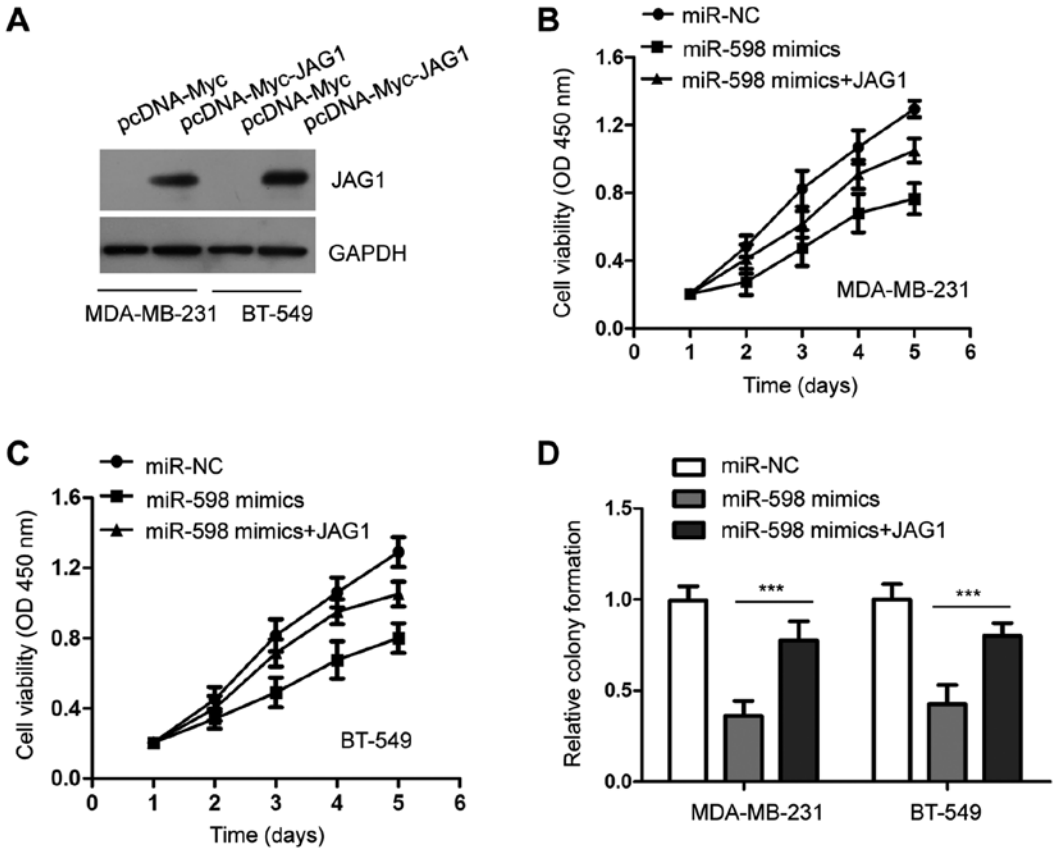

Figure 5. Restoration of JAG1 reverses the suppressed effects of miR-598 on the proliferation of TNBC cells. (A) MDA-MB-231 and BT-549 cells were transfected with Myc-JAG1 and the protein expression of JAG1 was assessed using western blotting. Transfection with JAG1 significantly reversed the impacts of miR-598 on the viability of (B) MDA-MB-231 and (C) BT-549 cells. (D) Overexpression of JAG1 attenuated the suppressed role of miR-598 on the colony formation capabilities of both MDA-MB-231 and BT-549 cells. ***P<0.001. JAG1, jagged 1; miR, microRNA; TNBC, triple negative breast cancer; NC, negative control; OD, optical density.

development of TNBC. However, further research with larger samples size is required to assess the correlation between miR-598 expression and the 5-year overall survival of patients with TNBC to highlight the clinical significance of miR-598. Overexpression of miR-598 suppressed the viability, colony formation and induced apoptosis of TNBC cells. Based on the key roles of metastasis and invasion in the development of TNBC, the effects of miR-598 on the migration of TNBC cells should be further examined in future studies. Consistent with the role of miR-598 in other types of cancer, the present results indicated that miR-598 acted as a tumor suppressor in TNBC and may be a possible target to inhibit the development of TNBC. To support this possibility, the tumor suppressive function of miR-598 should be investigated in in vivo studies with mice models. In addition, the complexity of tumor microenvironment, the side effects of miR-598 introduction, as well as the delivery of miR-598 into the tumor sites require further examination.

JAG1 mediates multiple signaling pathways and is involved in both physiological and pathological conditions (21). As an oncogene, upregulation of JAG1 has been identified in various cancer types and is associated with the malignant progression and a poor prognosis (36). JAG1 has also been reported to be the target of miRNAs in several types of cancer. For example, miR-186 suppresses the proliferation of myelomas by targeting JAG1 (37). A recent study showed that JAG1 was sponged by miR-377-3p and that JAG1 inhibited the proliferation of ovarian cancer cells (38). Additionally, it has been reported that miR-34a attenuated the paclitaxel resistance by directly suppressing JAG1 in prostate cancer (39). In the present study, JAG1 was identified as a downstream target of miR-598 and was inhibited by miR-598. Decreased miR-598 expression was significantly inversely correlated with the expression of JAG1 in TNBC tissues. As a cell surface ligand, JAG1 activates the Notch signaling pathway by interacting with Notch receptors (20). The Notch pathway promotes the metastasis of various types of cancer cells. Moreover, a recent study showed that miR-598 regulated the epithelial-mesenchymal transitions of colorectal cancer cells via directly targeting JAG1 to inactivate the Notch signaling (19). However, further investigation is required to assess the influence of miR-598 on the Notch pathway to explain how decreased JAG1 expression is involved in TNBC. As miRNAs usually have multiple targets, the involvement of other targets along with JAG1 in the progression of TNBC should also be further elucidated.

In conclusion, the present results suggested that miR-598 was downregulated in TNBC tissues and cells. miR-598 exerted its anti-cancer effects on the proliferation of TNBC cells, at least partially, by targeting JAG1. Thus, these findings identified a novel mechanism of action for miR-598 in the malignancy of TNBC, suggesting miR-598 may be a potential target for the treatment of TNBC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GHH, XDB and QH conceived and designed the study, and drafted the manuscript. GHH, XDB and HCJ collected and analyzed the data. All authors have revised and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shanxi Provincial Cancer Hospital. Written informed consents were received from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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