lncRNA NEAT1 regulates gastric carcinoma cell proliferation, invasion and apoptosis via the miR-500a-3p/XBP-1 axis

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Abstract. Gastric cancer is a serious malignant tumor. Despite progression in gastric cancer research in recent years, the specific molecular mechanism underlying the pathogenesis of the disease is not completely understood. Long non-coding RNA (lncRNA) nuclear paraspeckle assembly transcript 1 (NEAT1) affects the proliferation and metastasis of multiple types of tumor cells in colorectal cancer and breast cancer but its specific role in gastric cancer requires further investigation. The aim of the present study was to analyze the role of NEAT1 in gastric cancer. The expression of endoplasmic reticulum stress marker proteins and apoptosis-related proteins in gastric cancer tissue and cell lines was analyzed using western blotting. The targeting relationship of NEAT1 and miR-500a-3p was analyzed using dual-luciferase reporter assay. Cell proliferation was analyzed using CCK8 assay and colony formation assay while cell invasion was detected using Transwell assay. Cell apoptosis was analyzed using TUNEL staining and LC3 expression through immunofluorescent staining (IF). The results showed that lncRNA NEAT1-overexpression gastric cancer cells were established to determine its effects on cell proliferation, invasion, apoptosis, autophagy and endoplasmic reticulum stress. Subsequently, microRNA (miR)-500a was overexpressed in lncRNA NEAT1-overexpression cells. Compared with the vector group, lncRNA NEAT1 overexpression significantly inhibited gastric cancer cell proliferation and invasion, but significantly promoted cell apoptosis. Furthermore, the results indicated that lncRNA NEAT1 targeted and downregulated the expression of miR-500a-3p, and miR-500a-3p targeted X-box binding protein-1 (XBP-1) mRNA. lncRNA NEAT1 overexpression-mediated inhibition of gastric cancer cell proliferation and invasion was significantly reversed by miR-500a-3p overexpression. Furthermore, compared with the vector group, the expression levels of endoplasmic reticulum stress-related proteins (XBP-1S/XBP-1U ratio and 78-kDa glucose-regulated protein) and apoptosis-related proteins (Bax and cleaved-caspase-3) were significantly upregulated by lncRNA NEAT1 overexpression; however, miR-500a-3p overexpression reversed lncRNA NEAT1 overexpression-mediated effects on protein expression. The present study demonstrated that lncRNA NEAT1 inhibited gastric cancer cell proliferation and invasion, and promoted apoptosis by regulating the miR-500a-3p/XBP-1 axis.

Introduction

Gastric cancer is a major global health concern and is one of the major causes of cancer-related mortality (1). Over the past few years, the incidence and mortality of gastric cancer in East Asia have markedly increased (2). Despite advances in diagnostic technology and optimization of surgical treatment plans providing novel treatment strategies for patients with gastric cancer, patient prognosis remains poor (3,4). Therefore, identifying the molecular mechanism underlying gastric cancer is important for the development of novel treatment strategies.

Endoplasmic reticulum stress has been observed during the development of various tumors, such as breast and lung cancer (5-7). In several cell types, endoplasmic reticulum stress can lead to an increase in protein unfolding and cell apoptosis (8). The Cancer Genome Atlas database analysis (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) demonstrated that the expression of the key gene GPR78 for endoplasmic reticulum stress in gastric cancer samples was significantly increased. It has also been reported that endoplasmic reticulum stress could induce the repeated use of abnormally folded proteins in cells, which may promote proliferation and metastasis of

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tumor tissues (9). Therefore, the aforementioned studies indicated that endoplasmic reticulum stress might promote cancer development.

X-box binding protein-1 (XBP-1) serves an important role in the occurrence and development of endoplasmic reticulum stress and the ratio of the two splicing mutants (XBP-1S and XBP-1U) caused by differential mRNA translation in cells often determines the main physiological functions of the cells (10). A previous study revealed that the unfolded protein response, which is induced by endoplasmic reticulum stress, might promote the development of multiple types of cancer, such as neck squamous carcinoma cells and epidermoid carcinoma (11). The active form of XBP-1 (XBP-1S) may promote the development of endoplasmic reticulum stress, thus inducing the unfolded protein response. By contrast, the expression of XBP-1U represses the development of endoplasmic reticulum stress, inhibiting the unfolded protein response (12).

Furthermore, it has been reported that microRNA (miRNA/miR)-500a may promote tumor development and progression by downregulating XBP-1U expression and upregulating XBP-1S expression (13). Further research using StarBase database (http://starbase.sysu.edu.cn/) revealed that lncRNA NEAT1 might target and downregulate the expression of miR-500a-3p (14). However, whether lncRNA NEAT1 alters the expression levels of miR-500a-3p and XBP-1 or influences the development of gastric carcinoma is not completely understood. Therefore, the present study investigated the roles and regulatory mechanism underlying lncRNA NEAT1 in gastric carcinoma cells.

Materials and methods

Cell culture and treatment. Normal epithelial cells of the gastric mucosa (GES1) and gastric carcinoma cell lines (AGS, HST2 and FU97) were obtained from American Type Culture Collection. Cells were cultured in RPMI-1640 (HyClone; Cytiva) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. lncRNA NEAT1 overexpression vector (GV369, $1x10^8$ TU/ml) and its negative control (empty vector), miR-500a-3p mimics (25 μl, AUGCACCUG GGCAAGGAUUCUG) and its control (mimic NC, ACA GCAGUGCCAAUUGGUGGUCUGC) were purchased from Shanghai GeneChem Co., Ltd. Polybrene reagent (Shanghai GeneChem Co., Ltd.) was applied to promote transfection efficacy of lncRNA NEAT1 overexpression vector in AGS cells ($2x10^4$ cells/well). After transfection for 24 h at 37˚C, the cells were used for further experiment. When the confluence of AGS cells was 30%, the transfection of mimic was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h at 37˚C and cells were used for further experiment.

Collection of clinical samples. A total of 30 clinical gastric cancer tissue samples and 30 adjacent healthy tissue samples were obtained from patients from the Zhongda Hospital Southeast University (Nanjing, China) between September 2018 and May 2020. Inclusion criteria were that the gastric cancer had clear pathological diagnosis and no distant metastasis, the patient had not undergone radiation and chemotherapy and no other malignant tumors were found. After gastrectomy, tumor and adjacent healthy tissue (normal gastric mucosal tissue 5 cm from gastric cancer tissue) samples were collected. All experimental procedures were approved by the Ethics Committee of Zhongda Hospital Southeast University (approval number 2018ZDSYLL136291). Written informed consent was obtained from all patients. The clinical characteristics of the patients are summarized in Table I.

Cell Counting Kit-8 (CCK-8) assays. AGS cells ($6x10^3$ cells/well) were seeded into 96-well plates. Following culture for 24 h, 10 μl CCK-8 solution (Dojindo Molecular Technologies, Inc.) was added to each well and incubated for 1.5 h. Absorbance was measured at a wavelength of 450 nm using a spectrophotometer (Thermo Fisher Scientific, Inc.).

Dual-luciferase reporter assay. The AGS cells were seeded into 12-well plates at 60% confluence and transfected within 24 h with luciferase reporter gene plasmids (pGL3-basic, 1 μg) containing the 3'UTR of NEAT1 or XBP1, and miR-500a-3p mimic (20 μM) or miR-NC. Subsequently, 48 h after cell transfection (Shanghai GeneChem Co., Ltd.) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) the luciferase activities were detected according to the instructions of the Dual-Luciferase reporter gene assay kit (Thermo Fisher Scientific, Inc.). *Renilla* luciferase plasmids (0.02 μg) was used as internal reference.

Apoptosis assay. AGS cells ($4x10^5$) were plated into 60-mm culture dishes and their apoptosis levels were evaluated using Annexin V-FITC/PI kit (Thermo Fisher Scientific, Inc.). Cell suspensions were prepared and washed with PBS. Subsequently, cells were incubated with Annexin V (5 μl) at room temperature for 10 min in the dark and then PI (10 μl) in an ice-bath (Beyotime Institute of Biotechnology) in the dark for 30 min. Following washing with PBS, flow cytometry was performed to detect cell apoptosis using CytoFLEX flow cytometry instrument (Beckman Coulter, Inc.). The apoptosis levels were analyzed using FlowJo 10.0 software (FlowJo Software). The upper right quadrant ($FITC^+/PI^+$) were late apoptotic cells and lower right quadrant ($FITC^+/PI^-$) were early apoptotic cells. The apoptosis levels were evaluated through calculating the percentage of late apoptotic cells and late apoptotic cells.

Immunofluorescence. AGS cells were seeded onto a circular glass sheet. Cells were fixed using 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100 and blocked with normal goat serum (Gibco; Thermo Fisher Scientific, Inc.) for 30 min at room temperature. The anti-LC3II/I antibody (cat. no. ab192890; 1:50, Abcam) was used to incubate overnight at 4˚C, followed by an incubation at room temperature for 1 h with a goat secondary antibody to Rabbit IgG (Alexa Fluor 488; ab150081; 1:10,000) to detect LC3II/I protein expression using a confocal microscope (Leica Microsystems GmbH). DAPI was used for labelling nuclear DNA for incubation for 15 min at room temperature. All experiments were performed according to the manufacturer's protocol.

Western blotting. Total protein from cells was extracted using RIPA buffer (Beyotime Institute of Biotechnology).

Table I. Characteristics of patients with gastric cancer.

Characteristic	Cases (n=30)
Age (year)	58.2±6.1
Male, n (%)	17 (56.7)
Smoking, n (%)	10 (33.3)
Drinking, n (%)	13 (43.3)
Family history of cancer (any type), n (%)	12 (40.0)
Hypertension, n (%)	16 (53.3)
Diabetes, n (%)	17 (56.7)

Protein concentrations were determined using the BCA assay (Beyotime Institute of Biotechnology). Subsequently, proteins (50 μg) were uploaded and separated via 10% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred to PVDF membranes (EMD Millipore). Following blocking with 5% skimmed milk powder dissolved into PBST containing 0.05% Tween-20 for 1 h at 4˚C, the membranes were incubated at 4˚C overnight with primary antibodies (all purchased from Abcam) targeted against: 78-kDa glucose-regulated protein (GRP78; 1:100; cat. no. ab21685), XBP-1S (1:1,000; cat. no. ab220783), XBP-1U (1:1,000; cat. no. ab37152), caspase-4 (1:1,000; cat. no. ab238124), C/EBP homologous protein (CHOP; 1:200; cat. no. ab11419), Bcl-2 (1:1,000; cat. no. ab32124), Bax (1:1,000; cat. no. ab32503), cleaved-caspase-3 (1:500; cat. no. ab32042), LC3I/II (1:50; cat. no. ab232940), beclin-1 (1:1,000; cat. no. ab210498), autophagy-related gene 5 (1:1,000; Atg5; cat. no. ab109490) and GAPDH (1:5,000; cat. no. ab8245). Following washing with PBST, the membranes were incubated with secondary antibodies (goat anti-mouse IgG; cat. no. ab216772; Goat anti Rabbit IgG; cat. no. ab216777, 1:10,000; Abcam) for 2 h at 4˚C. Proteins bands were visualized using ECL reagents (Pierce; Thermo Fisher Scientific, Inc.). GAPDH was used as the loading control. The grey value of protein bands was analyzed using ImageJ software 1.46r (National Institutes of Health).

Reverse transcription-quantitative PCR (qPCR). Total RNA from gastric tissue of patients or cells was isolated using TRIzol® reagent (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using the reverse transcription kit (Transcriptor cDNA Synth. Kit 2; cat. no. 4897030001, Roche Diagnostics) for 15 min at 37˚C. Subsequently, qPCR was performed with Applied Biosystems PowerUp SYBR Green using the ABI 7500 system (Thermo Fisher Scientific, Inc.). The following primers were used for qPCR: lncRNA NEAT1 forward, 5'-TTGGGACAGTFFACGTGTGG-3' and reverse, 5'-TCAAGTCCAGCAGAGCA-3'; miR-500a-3p forward, 5'-UAAUCCUUGCUACCUGGGUGAGA-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'; GRP78 forward, 5'-CATCACGCCGTCCTATGTCG-3' and reverse, 5'-CGTCAAAGACCGTGTTCTCG-3'; XBP-1S, forward, 5'-TGTGCTGAGTCCGCAGCAG-3' and reverse, 5'-TGGTTGCTGAAGAG-3'; XBP-1U, forward, 5'-TGGTTGCTGAAGAG-3' and reverse, 5'-GAGATGTTCTGGAGGGGTGACAACTG-3'; caspase-4 forward, 5'-CAAGAGAAGCAACGTATGGCA-3' and reverse, 5'-AGGCAGATGGTCAAACTCTGTA-3'; Bax forward, 5'-CCCGAGAGGTCTTTTTCCGAG-3' and reverse, 5'-CCAGCCCATGATGGTTCTGAT-3'; Bcl-2 forward, 5'-GGTGGGGTCATGTGTGTGG-3' and reverse, 5'-CGGTTCAGGTACTCAGTCATCC-3'; Atg5 forward, 5'-AAAGATGTGCTTCGAGATGTGT-3' and reverse, 5'-CACTTTGTCAGTTACCAACGTCA-3'; GAPDH forward, 5'-TGACGTGCCGCCTGGAGAAC-3' and reverse, 5'-CCGGCATCGAAGGTGGAAGAG-3'; and U6 forward, 5'-TGCTGGGGCTTTCCGGCAGCGC-3' and reverse, 5'-CCCAGTGAGGTCCGGAGGT-3'. The PCR reaction conditions were 95˚C pre-denaturation for 2 min, 95˚C for 15 sec and 60˚C for 30 sec for 40 cycles. miRNA and mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (15) and normalized to the internal reference genes U6 and GAPDH, respectively.

Colony formation assay. AGS cells were seeded ($4x10^2$ cells/well) into 12-well plates. Following culture for 2 weeks, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and stained with crystal violet solution at room temperature for 10 min (Thermo Fisher Scientific, Inc.). The images were captured using an inverted microscope (Olympus Corporation).

Transwell assays. AGS cells ($5x10^5$ cells/ml) were cultured in serum-free medium for 12 h. Matrigel was used to pre-coat upper chamber at 37˚C for 30 min. Subsequently, cells were prepared into single-cell suspension using serum-free medium and plated into the upper chamber of Transwell inserts (Corning, Inc.). Serum-containing medium was added to the lower chamber. Following culture for 24 h, invading cells were fixed with 4% paraformaldehyde at room temperature for 15 min and stained with crystal violet solution at room temperature for 20 min (Thermo Fisher Scientific, Inc.). Invading cells were visualized using an inverted phase contrast microscope (Olympus Corporation).

Statistical analysis. Statistical analyses were performed using GraphPad Prism software (version 6.0; GraphPad Software Inc.). Data are presented as the mean ± SD. All the experiments were repeated three times. Comparisons among multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. Comparisons between two groups were analyzed using a paired t-test. Multiple linear regression and linear regression were performed to assess the association between expression levels of different proteins. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Endoplasmic reticulum stress-related protein expression levels are increased in gastric cancer tissues. Gastric cancer and adjacent healthy tissues were collected for the detection of endoplasmic reticulum stress- and apoptosis-related protein expression levels. A decrease in XBP-1 expression levels may indicate decreased GRP78 expression levels, leading to ER stress and apoptosis, which alters protein folding and can lead to protein accumulation (16,17). The results of the present study demonstrated that GRP78 and Caspase4 expression levels were significantly increased in gastric cancer tissues

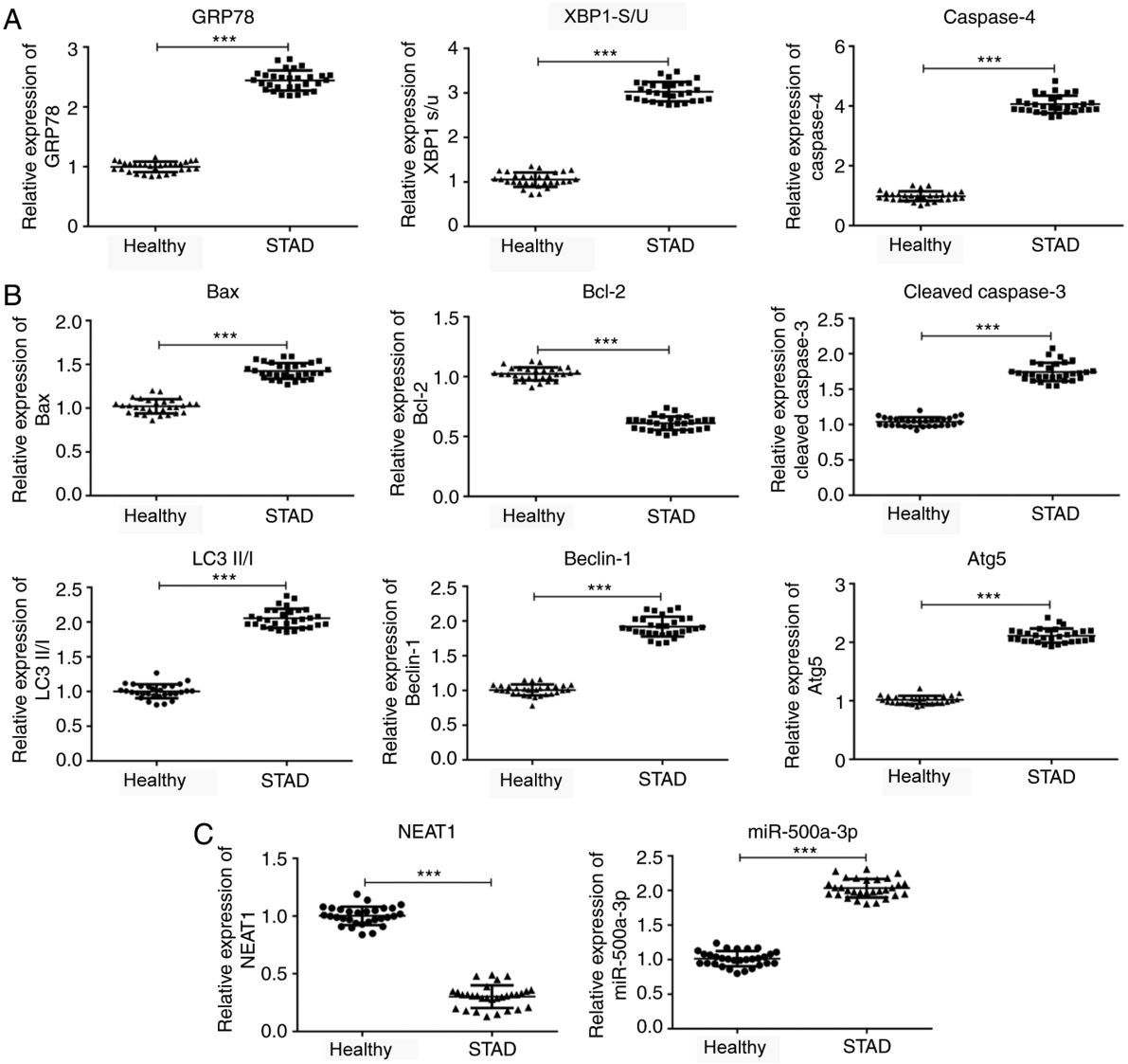

Figure 1. lncRNA NEAT1 expression is upregulated in gastric cancer tissues. Expression levels of (A) endoplasmic reticulum stress-, (B) apoptosis- and autophagy-related proteins in gastric cancer tissues were determined via RT-qPCR and western blotting. (C) lncRNA NEAT1 and miR-500a expression levels in gastric cancer tissues were determined via RT-qPCR. ***P<0.001. lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1; RT-qPCR, reverse transcription-quantitative PCR; miR, microRNA; GRP78, 78-kDa glucose-regulated protein; XBP-1, X-box binding protein-1; Atg5, autophagy-related gene 5; STAD, gastric cancer tissue.

compared with adjacent healthy tissues (Fig. 1A). Similarly, the ratio of XBP-1S/XBP-1U was also significantly increased in gastric cancer tissues compared with adjacent healthy tissues. Bax and cleaved-caspase-3 expression levels were significantly increased, whereas Bcl-2 expression levels were significantly decreased in gastric cancer tissues compared with adjacent healthy tissues (Fig. 1B). Moreover, the expression levels of autophagy-related proteins (LC3II/I, Beclin-1 and Atg5) were significantly increased in gastric cancer tissues compared with adjacent healthy tissues. Furthermore, lncRNA NEAT1 expression levels were significantly downregulated, whereas miR-500a-3p expression levels were significantly upregulated in gastric cancer tissues compared with adjacent healthy tissues (Fig. 1C).

lncRNA NEAT1 expression is negatively associated with the occurrence of endoplasmic reticulum stress, apoptosis and autophagy. Multiple linear regression was used to analyze the association between the expression of lncRNA NEAT1 or miR-500a-3p and the occurrence of endoplasmic reticulum stress, autophagy and apoptosis. The results demonstrated that lncRNA NEAT1 expression was negatively associated with the expression of endoplasmic reticulum stress-related proteins (caspase-4 and GRP78), apoptosis-related proteins (Bax and cleaved-caspase-3) and autophagy-related proteins (LC3II/I, Beclin-1 and Atg5) (Fig. 2A). lncRNA NEAT1 expression was also negatively associated with the XBP-1S/XBP-1U ratio. However, miR-500a-3p expression was positively associated with the expression of endoplasmic reticulum stress-related proteins (caspase-4 and GRP78), apoptosis-related proteins (Bax and cleaved-caspase-3) and autophagy-related proteins (LC3II/I, Beclin-1 and Atg5) (Fig. 2B). Moreover, miR-500a-3p expression was also positively associated with the XBP-1S/XBP-1U ratio.

Endoplasmic reticulum stress is increased in gastric cancer cell lines. Normal gastric mucosal epithelial cells (GES1) and

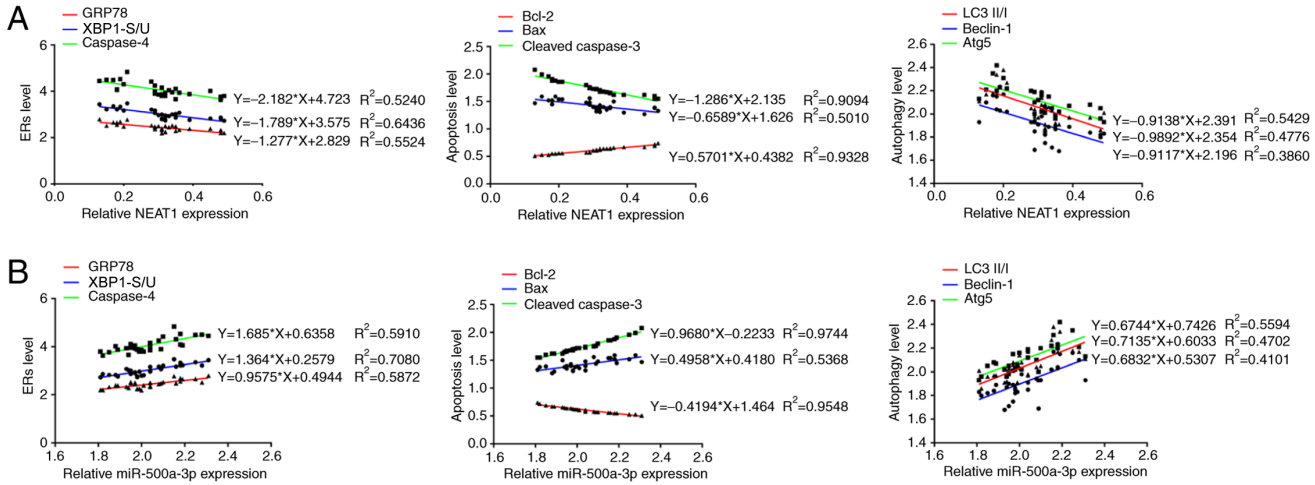

Figure 2. Endoplasmic reticulum stress-related protein expression levels are negatively associated with lncRNA NEAT1 expression in gastric cancer tissues. (A) Linear regression was performed to detect the association between endoplasmic reticulum stress-, autophagy- and apoptosis-related protein expression levels and lncRNA NEAT1 expression levels. (B) Linear regression was performed to detect the association between endoplasmic reticulum stress-, autophagy- and apoptosis-related protein expression levels and miR-500a expression levels. lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1; miR, microRNA; GRP78, 78-kDa glucose-regulated protein; XBP-1, X-box binding protein-1; Atg5, autophagy-related gene 5; ERs, endoplasmic reticulum stress.

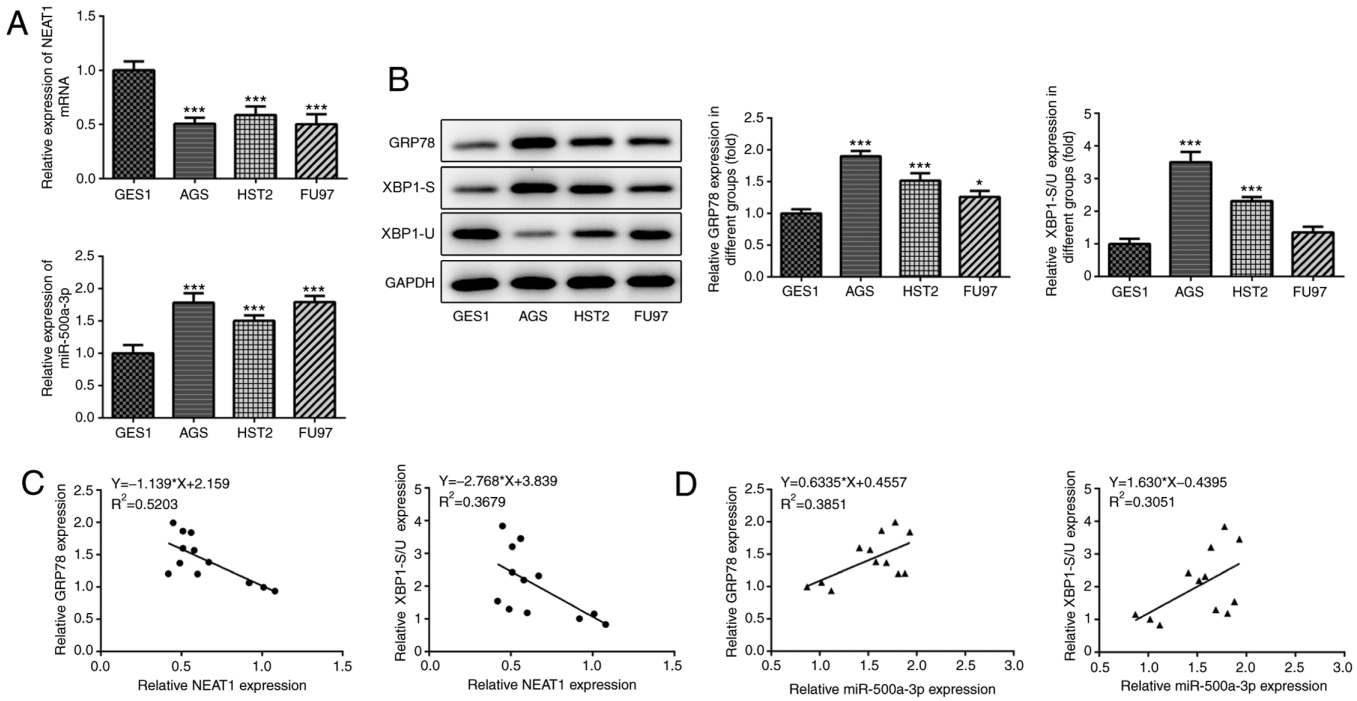

Figure 3. Endoplasmic reticulum stress-related protein expression levels are negatively associated with lncRNA NEAT1 expression in gastric cancer cells. (A) lncRNA NEAT1 and miR-500a expression levels in gastric cancer cells were determined via reverse transcription-quantitative PCR. (B) GRP78, XBP-1S and XBP-1U protein expression levels in gastric cancer cells were determined via western blotting. (C) Linear regression was performed to detect the association between lncRNA NEAT1 expression and the expression levels of endoplasmic reticulum stress-related proteins in gastric cancer cells. (D) Linear regression was performed to detect the association between miR-500a expression and the expression levels of endoplasmic reticulum stress related-proteins in gastric cancer cells. *P<0.05 and ***P<0.001 vs. GES1. lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1; miR, microRNA; GRP78, 78-kDa glucose-regulated protein; XBP-1, X-box binding protein-1.

gastric cancer cells (AGS, HST2 and FU97) were selected for subsequent experiments. Compared with normal gastric mucosal epithelial cells, lncRNA NEAT1 expression levels were significantly downregulated, whereas miR-500a-3p expression levels were significantly upregulated in gastric cancer cells (Fig. 3A). Subsequently, the expression levels of endoplasmic reticulum stress-related proteins were determined via western blotting. The expression levels of GRP78 and XBP-1S were markedly increased, whereas the expression levels of XBP-1U were markedly decreased in gastric cancer cells compared with normal gastric mucosal epithelial cells (Fig. 3B). The results also demonstrated that the XBP-1S/XBP-1U ratio was significantly decreased in gastric cancer cells compared with normal gastric mucosal epithelial

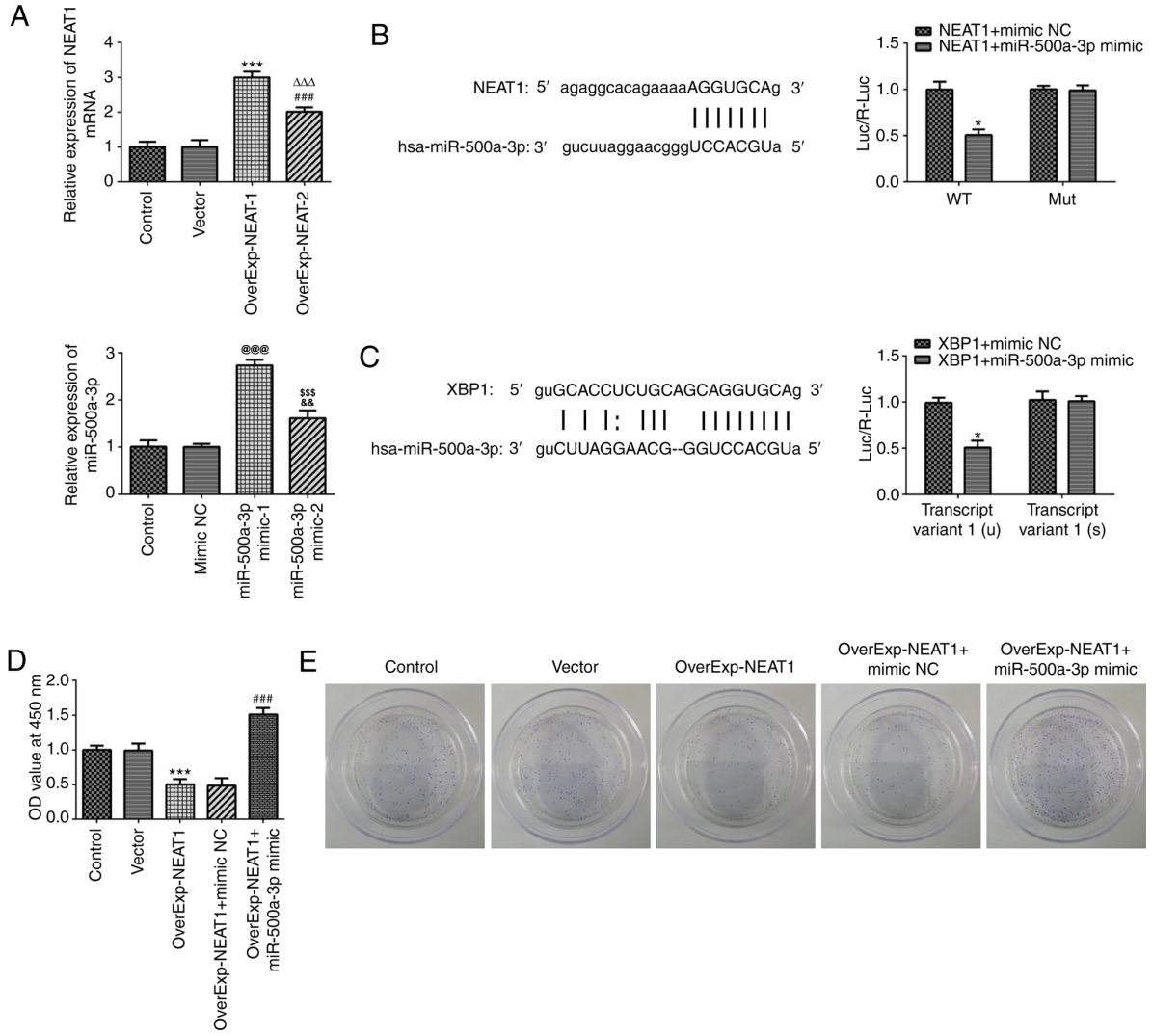

Figure 4. lncRNA NEAT1 targets miR-500a in gastric cancer cells. (A) Transfection efficiency of OverExp-NEAT-1, OverExp-NEAT-2, miR-500a-3p mimic-1 and miR-500a-3p-2 as determined via reverse transcription-quantitative PCR. ***P<0.001 vs. vector; $^{###}$P<0.001 vs. vector; ^{ΔΔΔ}P<0.001 vs. OverExp-NEAT-1. $^{@@@}$P<0.001 vs. mimic NC; $^{&&}$P<0.01 vs. mimic NC; $^{$$$}$P<0.001 vs. miR-500a-3p mimic-1. Luciferase reporter assays were performed to assess the interactions between miR-500a and (B) lncRNA NEAT1, *P<0.05 vs. NEAT1 + mimic NC or XBP1 + mimic NC or (C) XBP-1, *P<0.05 vs. XBP1 + mimic NC. (D) Cell Counting Kit-8 assays were performed to assess the effect of lncRNA NEAT1 overexpression on gastric cancer cell proliferation. ***P<0.001 vs. vector; $^{###}$P<0.001 vs. OverExp-NEAT1 + mimic NC. (E) Colony formation assays were performed to assess the effect of lncRNA NEAT1 overexpression on gastric cancer cell proliferation. lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1; miR, microRNA; OverExp, overexpression; NC, negative control; XBP-1, X-box binding protein-1; OD, optical density; WT, wild-type; Mut, mutant.

cells (Fig. 3B). The present study observed decreased levels of NEAT1 and increased levels of miR-500a-3p, GPR 78 and XBP1 s/u in AGS cells compared with GES1 cells. The increased trend in the expression of GPR78 when compared with GES1 group appeared to be higher in AGS cells compared with other cancer cell lines used in the present study (Fig. 3B). Therefore, AGS cells were used for subsequent experiments. The linear regression analysis results indicated that lncRNA NEAT1 expression was negatively associated with the expression of GRP78 and the XBP-1S/XBP-1U ratio (Fig. 3C), whereas miR-500a-3p expression levels were positively associated with the expression of GRP78 and the XBP-1S/XBP-1U ratio in gastric cancer cells (Fig. 3D).

lncRNA NEAT1 inhibits gastric cancer cell proliferation and invasion, but promotes apoptosis. Subsequently, lncRNA NEAT1- and miR-500a-3p-overexpression gastric cancer cells were established to investigate the effects of lncRNA NEAT1 and miR-500a-3p on gastric cancer (Fig. 4A). Luciferase reporter assays were performed to investigate the association between miR-500a-3p and lncRNA NEAT1 or XBP-1. The results revealed that the fluorescein intensity was significantly decreased in the wild-type lncRNA NEAT1 + miR-500a-3p mimic group compared with the wild-type NEAT1 + mimic NC group (Fig. 4B). In addition, the fluorescein intensity was also significantly decreased in the wild-type XBP-1 + miR-500a-3p mimic group compared with the wild-type XBP-1 + mimic NC group (Fig. 4C). CCK-8 and colony formation assays were performed to detect the alterations in cell proliferation. AGS cell proliferation was significantly inhibited by lncRNA NEAT1 overexpression compared with the vector group (Fig. 4D). However, lncRNA NEAT1 overexpression-mediated effects on cell proliferation were significantly reversed by miR-500a-3p overexpression. Similar

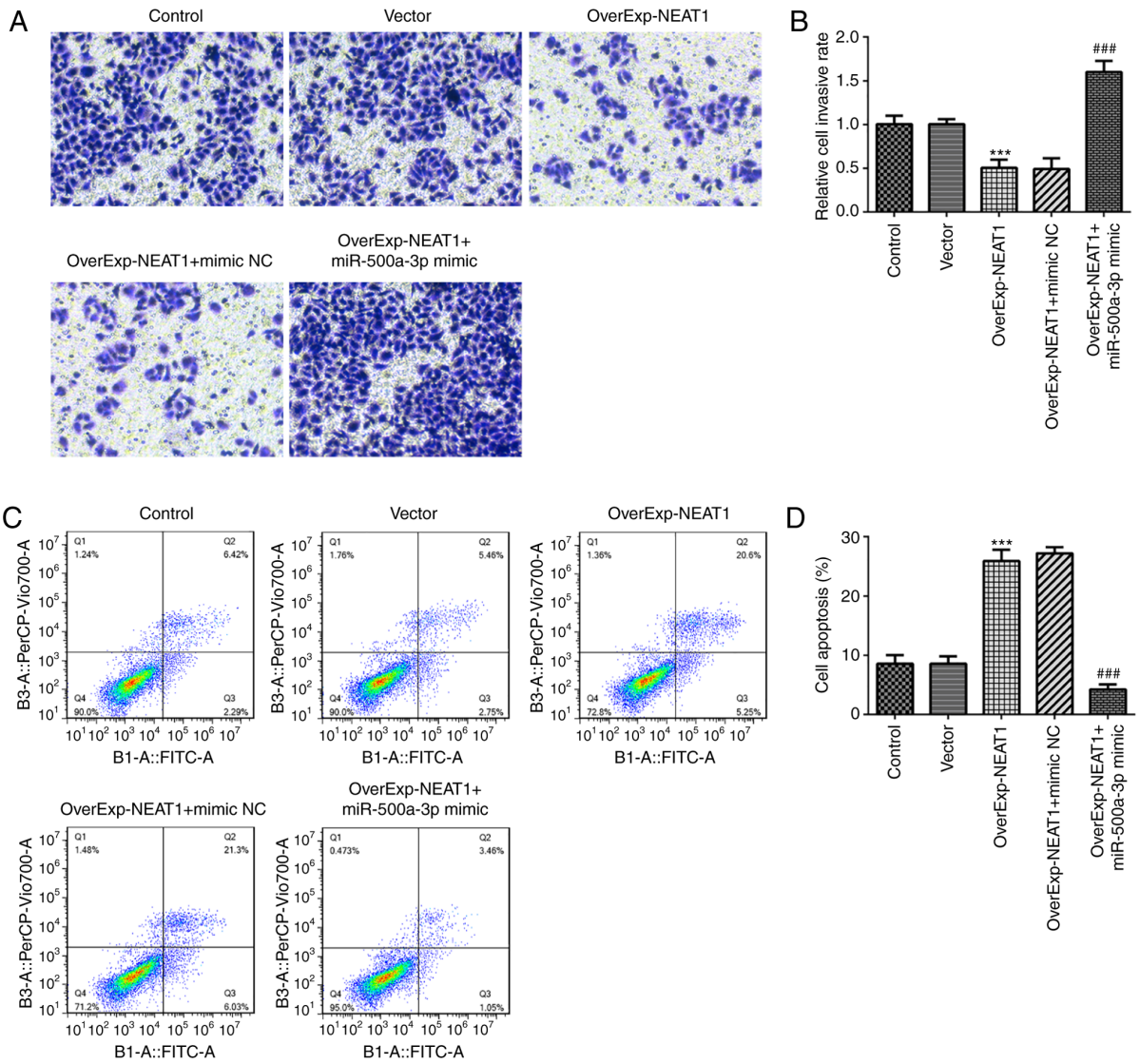

Figure 5. lncRNA NEAT1 inhibits gastric cancer cell invasion, but promotes apoptosis. Effect of lncRNA NEAT1 overexpression on cell invasion was (A) assessed by performing Transwell assays and (B) quantified. Effect of lncRNA NEAT1 overexpression on cell apoptosis was (C) determined via flow cytometry and (D) quantified. ***P<0.001 vs. vector; ###P<0.001 vs. OverExp-NEAT1 + mimic NC. lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1; OverExp, overexpression; miR, microRNA; NC, negative control.

results were obtained from the colony formation assays. Moreover, cell invasion was significantly inhibited by lncRNA NEAT1 overexpression compared with the vector group, which was also significantly reversed by miR-500a-3p overexpression (Fig. 5A and B). In addition, cell apoptosis was significantly increased by lncRNA NEAT1 overexpression compared with the vector group, but miR-500a-3p overexpression significantly reversed lncRNA NEAT1 overexpression-induced cell apoptosis (Fig. 5C and D). Finally, immunofluorescence assays were performed to detect the expression of autophagy-related protein LC3II/I. The results demonstrated that the expression of LC3II/I was markedly downregulated by lncRNA NEAT1 overexpression compared with the vector group, which was obviously reversed by miR-500a-3p overexpression (Fig. 6).

lncRNA NEAT1 overexpression inhibits endoplasmic reticulum stress and apoptosis in gastric cancer cells. The expression levels of endoplasmic reticulum stress-related proteins were detected via western blotting. The results revealed that GRP78 and Caspase4 expression levels were significantly downregulated, whereas CHOP expression levels were significantly upregulated by lncRNA NEAT1 overexpression compared with the vector group (Fig. 7A). In addition, lncRNA NEAT1 overexpression significantly decreased the XBP-1S/XBP-1U ratio compared with the vector group. However, miR-500a-3p overexpression reversed lncRNA NEAT1 overexpression-mediated effects on endoplasmic reticulum stress-related protein expression levels. Furthermore, compared with the vector group, lncRNA NEAT1 overexpression significantly decreased the expression levels of Bcl-2, LC3II/I, Beclin-1 and Atg5, but significantly increased the expression levels of Bax and cleaved-caspase-3. miR-500a-3p overexpression significantly reversed lncRNA NEAT1-overexpression mediated effects on apoptosis- and autophagy-related protein expression levels (Fig. 7B). The present study confirmed increased NEAT1 and decreased miR-500a-3p levels following the induction of NEAT1 overexpression, while their levels could be reversed by miR-500a-3p overexpression (Fig. 7C).

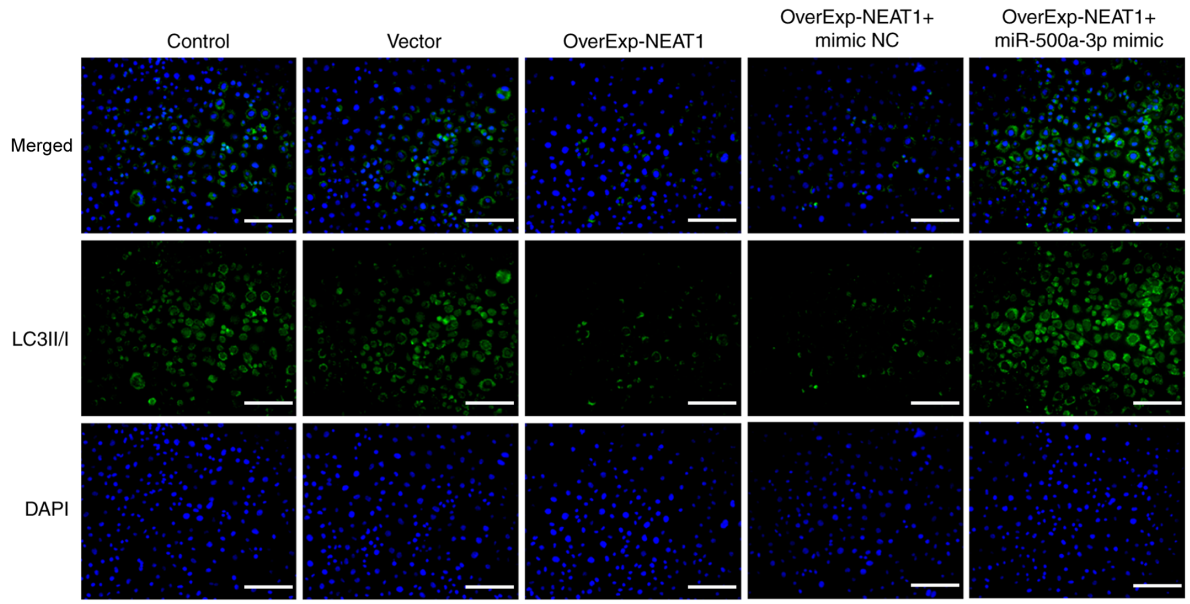

Figure 6. lncRNA NEAT1 suppresses the autophagy process in gastric cancer cells. Immunofluorescence assays was performed to detect the expression of LC3II/I in gastric cancer cells, magnification, x200. lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1; OverExp, overexpression; NC, negative control; miR, microRNA.

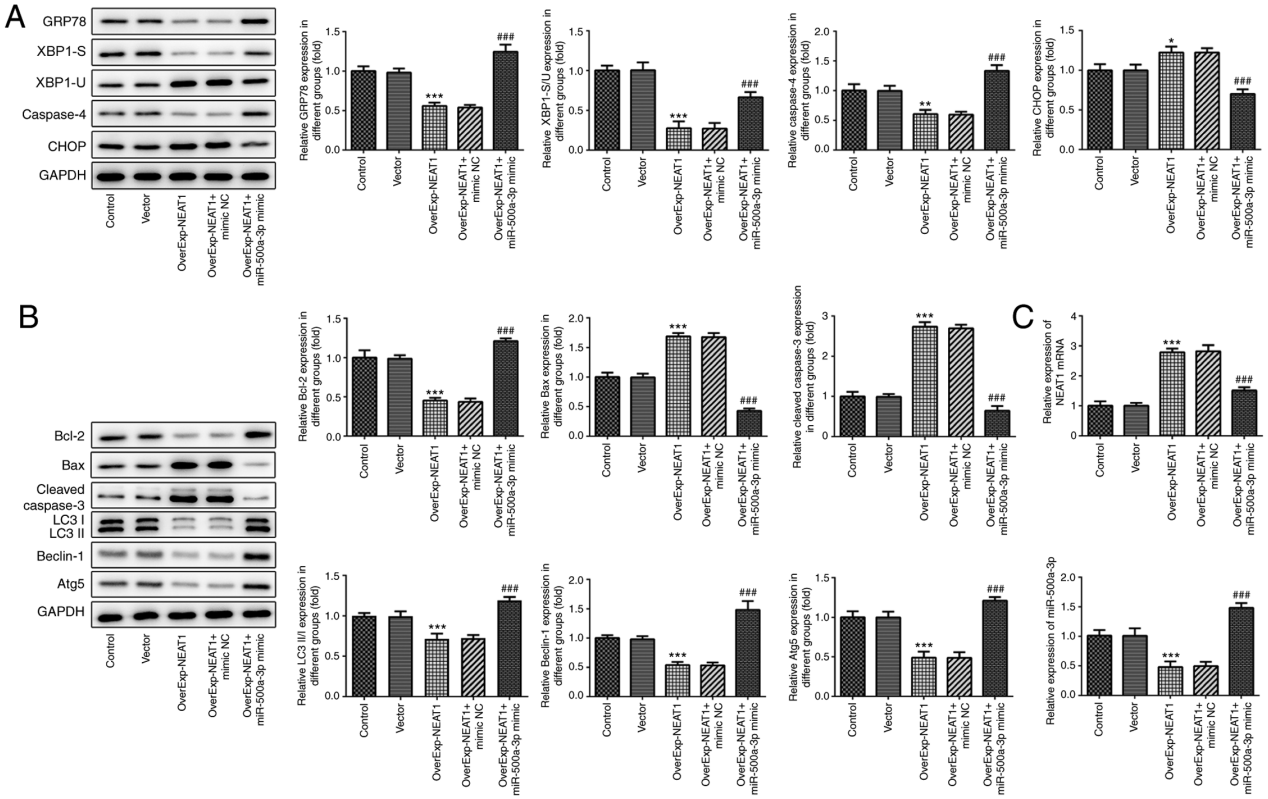

Figure 7. lncRNA NEAT1 downregulates the expression levels of endoplasmic reticulum stress-related proteins. Expression levels of endoplasmic reticulum stress-, (A and B) apoptosis- and autophagy-related proteins were detected via western blotting. (C) lncRNA NEAT1 and miR-500a expression levels were determined via reverse transcription-quantitative PCR. *P<0.05, **P<0.01 and ***P<0.001 vs. vector; $^{\#\#\#}$P<0.001 vs. OverExp-NEAT1 + mimic NC. lncRNA, long non-coding RNA; NEAT1, nuclear paraspeckle assembly transcript 1; OverExp, overexpression; miR, microRNA; NC, negative control; GRP78, 78-kDa glucose-regulated protein; XBP-1, X-box binding protein-1; CHOP, C/EBP homologous protein; Atg5, autophagy-related gene 5.

Discussion

Gastric cancer is the fifth commonest cancer and is the third commonest cause of cancer-related mortality (18). Despite significant progress in the elucidation of the pathogenesis and clinical research of gastric cancer, increasing cases of stomach cancer can be expected in the future due to an aging population (19). Previous studies have demonstrated that the

development and progression of gastric cancer is associated with the expression of several genes, such as miR-17-5p/20a and hosphoinositide 3-kinase/Akt, which affect tumor cell proliferation, migration and invasion (20,21).

In addition, lncRNAs, a class of genes that do not encode proteins, serve a key role in cell metabolism, senescence and apoptosis (22). It has been reported that lncRNA NEAT1 expression might induce colorectal cancer progression by interacting with DEAD-box helicase 5 (23). However, the role of lncRNA NEAT1 in gastric cancer is not completely understood. The present study demonstrated that lncRNA NEAT1 overexpression significantly inhibited gastric cancer cell proliferation and invasion, but significantly promoted cell apoptosis compared with the vector group. Furthermore, lncRNA NEAT1 expression was significantly downregulated in gastric cancer tissues compared with adjacent healthy tissues.

In addition, miR-500a also promotes hepatocarcinoma cell proliferation and invasion (24). miR-500a enhanced hepatocellular carcinoma cell migration and invasion by activating the Wnt/β-catenin signaling pathway (25). In the present study, miR-500a-3p overexpression significantly promoted gastric cancer cell proliferation and invasion, but significantly suppressed apoptosis in lncRNA NEAT1-overexpression gastric cancer cells. In addition, the results indicated that lncRNA NEAT1 targeted and downregulated the expression of miR-500a-3p.

Furthermore, previous studies have suggested that the occurrence of endoplasmic reticulum stress may induce the development of multiple types of cancer, such as carcinomas of the breast, stomach, esophagus and liver (26,27). XBP-1 is a protein that serves a key role during the development of endoplasmic reticulum stress (28). Due to differences in splicing, XBP-1 has two subtypes, XBP-1S and XBP-1U. During the development of multiple tumors, higher expression levels of XBP-1S induce the unfolded protein response and enhance tumor proliferation and metastasis. However, the expression of XBP-1U restricts tumor development by suppressing the unfolded protein response and endoplasmic reticulum stress (29). The present study indicated that miR-500a-3p might affect tumor proliferation and metastasis by regulating the expression of XBP-1. The results demonstrated that miR-500a-3p targeted XBP-1S and its overexpression affected the expression of XBP-1S. Therefore, the results of the present study indicated that miR-500a-3p mediated gastric cancer cell proliferation and invasion affected by NEAT1, which possibly was associated with the inhibition of endoplasmic reticulum stress.

Furthermore, autophagy is a spontaneous auto-degradation process of cells, and the survival of tumor cells often depends on autophagy (30). The present study demonstrated that lncRNA NEAT1 overexpression significantly downregulated autophagy-related protein (LC3II/I, Beclin-1 and Atg5) expression levels compared with the vector group, which was significantly reversed by miR-500a-3p overexpression. The results indicated that lncRNA NEAT1 inhibited the autophagy process to restrict the development of gastric cancer. Cancer cell apoptosis also inhibits cancer development (31). In the present study, compared with the vector group, lncRNA NEAT1 overexpression significantly increased the expression levels of apoptosis-related proteins (Bax and cleaved-caspase-3), which was significantly reversed by miR-500a overexpression. The results indicated that the lncRNA NEAT1 might suppress the development of gastric cancer by promoting gastric cancer cell apoptosis. Previous studies have identified miR-500a-3p as a potential prognostic predictor in certain types of cancer, including hepatocellular carcinoma and glioblastoma, and have reported an association between miR-500a and TNM stage in lung cancer and conjunctival malignant melanoma (32-35). Other studies have reported that XBP-1 is closely associated with clinical outcome (36,37). A key limitation of the present study was that the prognostic impact and cancer staging applications of lncRNA NEAT1, miR-500a-3p and XBP-1 in gastric cancer were not investigated; therefore, further investigation is required. To conclude, the present study demonstrated that the regulatory role of the lncRNA NEAT1/miR-500a-3p axis in endoplasmic reticulum stress, apoptosis and autophagy in gastric cancer cells was potentially mediated by XBP-1. Therefore, the present study identified several potential diagnostic markers for gastric cancer.

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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 or first author on reasonable request.

Authors' contributions

YZ, ZS and HC made substantial contributions to the conception and design of the study, acquired, analyzed and interpreted the data, and drafted and revised the manuscript for important intellectual content. YZ, ZS, YY, SW and HC performed the experiments and interpreted the data. YZ, ZS and HC confirm the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures were approved by the Ethics Committee of Zhongda Hospital Southeast University (approval no. 2018ZDSYLL136291). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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