Original Article
Exosomal long noncoding RNA AGAP2-AS1 regulates trastuzumab resistance via inducing autophagy in breast cancer

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Abstract: Trastuzumab has been widely used for treatment of HER-2-positive breast cancer patients, however, the clinical response has been restricted due to emergence of resistance. Recent studies indicate that long noncoding RNA AGAP2-AS1 (lncRNA AGAP2-AS1) plays an important role in cancer resistance. However, the precise regulatory function and therapeutic potential of AGAP2-AS1 in trastuzumab resistance is still not defined. In this study, we sought to reveal the essential role of AGAP2-AS1 in trastuzumab resistance. Our results suggest that AGAP2-AS1 disseminates trastuzumab resistance via packaging into exosomes. Exosomal AGAP2-AS1 induces trastuzumab resistance via modulating ATG10 expression and autophagy activity. Mechanically, AGAP2-AS1 is associated with ELAVL1 protein, and the AGAP2-AS1-ELAVL1 complex could directly bind to the promoter region of ATG10, inducing H3K27ac and H3K4me3 enrichment, which finally activates ATG10 transcription. AGAP2-AS1-targeting antisense oligonucleotides (ASO) substantially increased trastuzumab-induced cytotoxicity. Clinically, increased expression of serum exosomal AGAP2-AS1 was associated with poor response to trastuzumab treatment. In conclusion, exosomal AGAP2-AS1 increased trastuzumab resistance via promoting ATG10 expression and inducing autophagy. Therefore, AGAP2-AS1 may serve as a predictive biomarker and therapeutic target for HER-2+ breast cancer patients.

Keywords: Breast cancer, trastuzumab resistance, AGAP2-AS1, ATG10, ELAVL1

Introduction
Human epidermal growth factor receptor 2 (HER-2) positive breast cancer (BC) is one of the most common types of BC, and HER-2 is amplified or overexpressed in 15 to 20% of all BC patients [1]. Currently, the use of adjuvant trastuzumab is the standard of care for these patients [2]. However, a very limited part of patients responds well to trastuzumab and over 60% become resistance [3]. Thus, finding novel therapeutic approaches and new targets to promote trastuzumab cytotoxicity are urgently needed.

Long noncoding RNAs (lncRNAs) are now described to have many functions and involved in tumor progression and response to therapy [4]. Exosomes (70-120 nm) are microvesicles that originate from multivesicular bodies (MVBs) and are released into the extracellular milieu upon fusion of MVBs with cytomembrane. Evidence is mounting that numerous lncRNAs contained in exosomes (exosomal lncRNAs) are involved in regulating cancer resistance, such as GAS5, UCA1 and ATB [5-8]. Previously, we demonstrated the essential role of AFAP1-AS1 in trastuzumab resistance. AFAP1-AS1 could interact with AUF1, and then activate ERBB2 translation [9]. RNA-binding proteins (RBPs) are playing important functions in cancers by regulating the messenger RNA (mRNA) stability and splicing [10]. RBPs regulate the step of RNA processing to modulate the progression of BC [11].
For instance, RNA-binding protein CUGBP1 controls the differential INSR splicing in molecular subtypes of BC cells and affects cell aggressiveness [12]. And RNA-binding protein RBM38 increases PTEN expression via enhancing its mRNA stability in BC [13]. Those studies along with ours indicate that IncRNAs are critical regulators for trastuzumab resistance by binding with RBPs.

Autophagy is a well-known cellular process which is accompanied by various pathologic processes and biological reactions, including hypoxia, cancer, nutritional deficiencies and other cellular stresses [14]. Thus, it is easily understood that autophagy plays important roles during drug treatment and resistance. Learning how autophagy participates in trastuzumab resistance could provide new insights into overcoming resistance and developing new therapeutic targets [15]. By modulating key autophagy-related proteins expression, IncRNAs plays essential roles in regulating autophagy [16]. Therefore, finding the regulatory relationships between IncRNAs and autophagy in trastuzumab resistance may provide new thoughts in BC treatment.

In our previous study, we revealed a critical role of AGAP2-AS1 in trastuzumab resistance [17]. Here, we continue to explore the underlying regulatory mechanism of AGAP2-AS1 in BC trastuzumab resistance through binding with RBPs and regulation of autophagy, which may help us obtain new insights into the clinical potential of AGAP2-AS1 in BC therapy.

Materials and methods

Patient samples

Overall, a cohort of serum samples obtained from 90 HER-2 positive (pathological confirmed) patients (female, range of age (median): 38-67 (55)) were included from March 2012 to January 2015, to determine the expression of serum exosomal AGAP2-AS1 for trastuzumab response. Patients include 45 responding (female, range of age (median): 38-65 (54)) and 45 non-responding patients (female, range of age (median): 39-67 (56)) according to iRECIST criteria [18]. Patients who received radiotherapy and chemotherapy before surgical treatment were excluded. Meanwhile, the general clinical information and detailed pathological records were collected. Written-informed consent was obtained from all patients and the study protocol was approved by the Research Scientific Ethics Committee of The First Affiliated Hospital of Zhengzhou University, The First People’s Hospital of Chenzhou City, Tangdu Hospital, The Fourth Military Medical University and Hainan General Hospital.

Cell culture and treatment

The human HER-2 positive BC cell lines SKBR-3 and BT474 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco, CA, USA) supplemented with 10% fetal bovine serum (Australia Origin, Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Solarbio, Beijing, China). The cells were grown in a humidified atmosphere of 5% CO2 and 95% air at 37°C. Trastuzumab (Herceptin) was purchased from Roche (Basel, Switzerland) and used by dissolving in phosphate-buffered saline (PBS). The trastuzumab-resistant cell lines, SKBR-3-TR and BT474-TR, originated from SKBR-3 and BT474 cells, respectively, were established as previously described [19].

RNA extraction and qRT-PCR analysis

Total RNA was isolated from the cultured cell lines or clinical samples by use of Trizol reagent (Invitrogen, Carlsbad, CA), and subjected to reverse transcription as requested (Takara, Shiga, Japan). The RNA concentration and purity were measured by Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA). The expression levels of mRNA and IncRNA were detected by qRT-PCR using the SYBR Premix Ex Taq™ II (Takara, Dalian, China) and standardized using GAPDH as reference gene. All reactions were carried out in triplicate. The relative expression was calculated using the 2^−ΔΔct method. PCR primers were listed in Additional file 1: Table S1.

Plasmid construction and cell transfection

For knockdown of AGAP2-AS1 and ATG10, small interfering RNA (siRNA) was purchased from GenePharma (Shanghai, China). To construct a plasmid overexpressing AGAP2-AS1, the full-length human AGAP2-AS1 sequence was synthesized and subcloned into the pEX-3 vector (GenePharma, Shanghai, China). The pENTER-ATG10 vector used for overexpression of ATG10
mRNA was provided by Vigene BioSciences, followed by transfection into BC cells using Lipofectamine 2000 for 48 h (Invitrogen). For stably overexpressing or silencing AGAP2-AS1, we constructed lentivirus-based vectors from Genechem (Shanghai, China). The sequences of oligonucleotides are presented in Additional file 1: Table S1.

**CCK8 assay**

The cells transfected accordingly were seeded in a 96-well plate, the cells were cultured for 72 h with different dose of trastuzumab. After that, 10 mg/mL of CCK8 reagent (Dojindo, Kumamoto, Japan) was supplemented for cell viability detection according to manufacturer’s protocols. The absorbance at 450 nm was measured with a microplate reader. The dose for the half survival was represented the half-maximal inhibitory concentration (IC50) of trastuzumab in SKBR-3-TR and BT474-TR cells.

**Isolation and identification of exosomes**

Cell supernatant was collected and sequentially centrifuged under 3000 g for 15 min at 4°C after taken out from -80°C and filtered through a 0.45-µm pore polyvinylidene fluoride filter (Millipore, Billerica, Mass). Then, 63 µl ExoQuick™ exosome precipitation solution (SBI, USA) was added into 250 µl serum followed by incubation at 4°C for 50 min. After centrifugation at 1500 g for 30 minutes, exosomes were collected. 50 µl phosphate-buffered saline (PBS) was used to resuspend the exosome pellets obtained from the former step. Exosomes isolated from serum of BC patients were done with similar procedures.

The sample of exosomes was identified through transmission electron microscopy, nanoparticle tracking analysis (NTA, Malvern Panalytical). Exosomes were loaded on a Formvar-carbon-coated electron microscope grid (Polysciences) for 30 min. Then the grid was washed in PBS and fixed in 2% glutaraldehyde (Sigma Aldrich) for 10 min. The grid was subsequently washed in PBS for 5 times and counter-stained with 2% uranyl acetate (Sigma Aldrich) for 1 min. Air-dried grids were viewed with a Hitachi transmission electron microscope.

**RNA sequencing analysis**

Total RNA was extracted from wild type or AGAP2-AS1-knock out SKBR-3 cells using RNeasy mini kit (Qiagen, Germany). The paired-end libraries were constructed by using TruSeq™ RNA Sample Preparation Kit (Illumina, USA) according to the manufacturer’s sample preparation guide. The mRNA expression profiles of the treated cells were determined using the Illumina NovaSeq 6000 (Illumina, USA) following the manufacturer’s instructions. The library construction and sequencing were performed at Shanghai Sinomics Corporation (Shanghai, China). Cuffdiff was used to evaluate differentially expressed genes. The differentially expressed genes were selected using the following filter criteria: \( P \leq 0.05 \) and fold change ≥2.

**Subcutaneous tumorigenicity assay**

4-week-old female BALB/c nude mice were purchased from the Beijing Vital River Laboratory Animal Technology and housed in specific pathogen-free barrier facilities. Fifteen mice were randomly assigned to two exosome-treated groups and one control group (5 mice/group). Mice were subcutaneous injected SKBR-3-TR cells \( (1 \times 10^6 \text{ cells in 0.1 ml physiological saline}) \) in the flank. Each group received PBS, SKBR-3-TR-EXO\_vector, or SKBR-3-TR-EXO\_AGAP2-AS1 treatments every 3 days for 5 weeks. Meanwhile, each group received intra-peritoneal treatment of trastuzumab once every two days for 4 weeks. After 4 weeks post-injection, mice were imaged using a luminescence imaging system. At the end of imaging, all mice were euthanized by carbon dioxide inhalation, followed by cervical dislocation to ensure death. Then, tumors were surgically dissected and weighted. Tumor volume \( (\text{mm}^3) = 0.5 \times \text{width}^2 \times \text{length} \).

To test the therapeutic role of AGAP2-AS1, ASO was used for treatment of xenograft tumors through tail vein injection. Briefly, \( 5 \times 10^5 \text{ SKBR-3-TR cells in 100 µL of sterile PBS} \) were injected directly into the mammary fat pads of mice to establish in situ xenograft. Then, mice were divided into 3 groups \( (n = 5 \text{ in each group}) \) after the xenograft was established. ASO was used at the concentration of 10 nmol, twice a week.

All experimental procedures were conducted in conformity with the ethical standards of national and international guidelines and policies. This study was approved by the Institute Animal Care and Use Committee of The First Affiliated Hospital of Zhengzhou University (Zhengzhou, China).
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**RNA pulldown**

AGAP2-AS1 was transcribed using T7 RNA polymerase in vitro (Ambio Life) followed by the purification with RNeasy Plus Mini Kit (Qiagen) treatment with DNase I (Qiagen). The cell lysates were freshly prepared using Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific, Cat# 20164). Cell protein extracts were collected and mixed with the biotinylated.

RNA probes for AGAP2-AS1 in magnetic beads. Results were analyzed by silver staining and mass spectrometry (ekspertTMnanoLC; AB Sciex TripleTOF 5600-plus; SCIEX). Data were analyzed using Proteinpilot software (https://omic-tools.com/proteinpilot-tool). The retrieved protein ELAVL1 was further validated by standard western blot.

**RNA immunoprecipitation (RIP) and chromatin immunoprecipitation (ChIP)**

Using EZ-Magna RIP RNA Binding Protein Immunoprecipitation Kit, RIP assay was implemented as instructed by supplier (Millipore, Billerica, MA). Cell lysates were reaped for incubation with the ELAVL1 (cat. no. ab200342, Abcam, Cambridge, MA) antibody in magnetic beads, using IgG antibody as negative control. All precipitates were examined by qRT-PCR.

For ChIP assay, the Millipore EZ ChIP Kit (Milliprep) was used according to the manufacture’s guideline. After fixation with 4% formaldehyde, cells were lysed by using RIPA buffer (Beyotime, Shanghai, China). Then, DNA fragments (length of 200-1000 bp) were obtained by treatment with ultrasonic followed by immunoprecipitation using anti-H3K27ac antibody (Abcam, cat. no. ab4729), H3K4me3 antibody (Abcam, cat. no. ab8580) and the negative control IgG antibody (EMD Millipore, cat. no. 12-371) overnight at 4°C. The cross-linked chromatin was then acquired and detected via qRT-PCR after route elution. Experiments were performed in triplicate.

**RNA fluorescent in situ hybridization (RNA-FISH)**

The colocalization of AGAP2-AS1 and ELAVL1 were confirmed by fluorescence staining. Briefly, SKBR-3 cells were seeded on a glass-bottomed confocal plate and cultured overnight. After fixation with 4% PFA and permeabilization with 0.5% Triton, hybridization was carried out overnight with the AGAP2-AS1 probes conjugated with Alexa Fluor 555 (Invitrogen, CA, USA) at 37°C in 2 × SSC, 10% formamide and 10% dextran. Subsequently, Anti-ELAVL1 was incubated in the dark overnight followed by incubation with secondary antibody for 1 h. Finally, the nuclei were stained by DAPI and the images were captured under a Nikon A1Si Laser Scanning Confocal Microscope (Nikon Instruments Inc, Japan).

**Immunohistochemistry (IHC) analysis**

For IHC, the formalin-fixed, paraffin-embedded sections were dewaxed and rehydrated as previously described [17], and then treated with 3% hydrogen peroxide followed by EDTA buffer for antigen retrieval. The sections were then blocked in goat serum for 30 min, incubated with rabbit anti ATG10 antibody (Abcam, cat. no. ab229728) at 4°C overnight and subsequent with horseradish peroxidase-conjugated secondary antibodies for 30 min at room temperature. Finally, the sections were stained with the DAB substrate and hematoxylin. Images were recorded by Nikon Eclipse Ti microscope.

**Western blot analysis**

Protein extraction were performed using RIPA lysis buffer (Pierce, IL, USA) containing protease inhibitor (Roche, CA, USA). Protein extracts were subjected to 10% SDS-polyacrylamide gel electrophoresis followed by electro-transfer to polyvinylidene difluoride membrane. After 1 h of pre-membrane blocking with 5% BSA, the proteins were incubated with respective primary antibodies at 4°C overnight followed by secondary antibodies incubation at room temperature for 1 h. The detection of proteins was carried out using ECL reagent.

**Statistical analysis**

Each experiment contained at least 3 individual bio-replications. Data were all given as the mean ± standard deviation (SD) and processed with Prism Version 5.0 (GraphPad Software, La Jolla, CA). Statistical analysis in form of Student’s t-test or one-way analysis of variance (ANOVA), was considered to be significant when p-values below 0.05.
Results

AGAP2-AS1 was enriched in BC cell-secreted exosomes

Previously, we identified an upregulation of AGAP2-AS1 in trastuzumab-resistant BC cells compared to parental cells [17]. However, whether AGAP2-AS1 can be packaged into extracellular materials, such as exosome, is not well known. By using SKBR-3-TR cells, we extracted exosomes from the cell culture medium and confirmed by transmission electron microscopy (TEM) (Figure 1A) and NanoSight analysis (Figure 1B). The exosomes were further confirmed by western blotting analysis of

![Figure 1. Cellular AGAP2-AS1 was incorporated into exosomes. A. Exosomes was identified under transmission electron microscopy. B. NanoSight analysis showed the size distribution of extracellular vesicles. C. Exosomal marker proteins were identified by western blot. D. qRT-PCR was used to detect the expression of exosomal AGAP2-AS1 in sensitive and resistant breast cancer cells. E. The expression of cellular or exosomal AGAP2-AS1 was determined by qRT-PCR. F, G. Both total and exosomal AGAP2-AS1 were silenced by specific silencing vectors. H, I. Both total and exosomal AGAP2-AS1 were overexpressed by transfection of overexpression vector. *P<0.05, **P<0.01, ***P<0.001.](image-url)
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exosomal markers, TSG101 and HSP70 (Figure 1C). qRT-PCR indicated that the expression of AGAP2-AS1 packaged in exosome was significantly increased in trastuzumab-resistant cells in contrast to the respective parental cells (Figure 1D). Moreover, AGAP2-AS1 was more enriched in exosomes in contrast to intracellular portions, indicating that AGAP2-AS1 may function via incorporating into exosomes (Figure 1E). Importantly, knockdown of cellular AGAP2-AS1 via transfection of AGAP2-AS1 small interfere vectors induced a dramatically decrease of exosomal AGAP2-AS1 expression (Figure 1F and 1G), while overexpression of cellular AGAP2-AS1 showed opposite effects (Figure 1H and 1I), strengthening our conclusion that AGAP2-AS1 was enriched in exacellular exosomes.

Exosomal AGAP2-AS1 was internalized by BC parental cells to induce trastuzumab resistance

To directly probe exosomal AGAP2-AS1 induces trastuzumab resistance in recipient cells, we incubated SKBR-3 and BT474 cells with exosomes derived from SKBR-3-TR cells. As shown in Figure 2A, SKBR-3-TR-secreted exosomes significantly suppressed trastuzumab-induced cytotoxicity. However, treatment with exosomes isolated from SKBR-3-TR cells which are silenced with AGAP2-AS1 lost the ability to induce trastuzumab resistance of parental cells (Figure 2B). On the other hand, incubation SKBR-3 and BT474 cells with AGAP2-AS1-enriched SKBR-3-TR-derived exosomes significantly increased the cellular expression of AGAP2-AS1 of recipient cells while AGAP2-AS1-silenced exosomes showed no influence on AGAP2-AS1 expression level (Figure 2C), suggesting that exosomal AGAP2-AS1 may be internalized to induce trastuzumab resistance.

To provide direct evidence that exosomal AGAP2-AS1 was internalized by BC cells, we labeled extracted exosomes with PKH67 green dye. As expected, a strong green fluorescence signal was observed in recipient cells while no signal was observed in PBS control group (Figure 2D). To eliminate the possibility that endogenous AGAP2-AS1 induced trastuzumab resistance of BC parental cells. We built AGAP2-AS1-knockout SKBR-3 and BT474 cells from using the CRISPR-Cas9 approach with double-strained sgRNAs targeting AGAP2-AS1. A successful deficiency of AGAP2-AS1 was confirmed by our qRT-PCR analysis (Figure 2E). CCK8 assay proved that AGAP2-AS1-overexpressed cell-secreted exosomes promoted trastuzumab resistance of AGAP2-AS1-knockout cells (Figure 2F). These results were consistent with that of AGAP2-AS1-wild type BC cells (Figure 2G). Taken together, we demonstrated that AGAP2-AS1-overexpression cells could spread trastuzumab resistance by transferring exosomal AGAP2-AS1 to sensitive cells.

Exosomal AGAP2-AS1 promotes trastuzumab resistance via inducing autophagy

It is well demonstrated that autophagy may decrease the cytotoxicity of drugs upon cancer cells, inducing resistance [20]. Interestingly, the Gene Ontology (GO) analysis showed that autophagy may be involved in the function of AGAP2-AS1 (Figure 3A). To find whether AGAP2-AS1 regulates trastuzumab resistance via inducing autophagy, we firstly measured the autophagy activity of BC cells. Remarkably, compared to BC parental cells, trastuzumab-resistant cells showed higher activity of autophagy, as evidenced by increased expression of LC3-II, decreased expression of p62 protein and increased formation of LC3 puncta, than the corresponding parental cells, confirming the close association between autophagy and drug resistance in cancer (Figure 3B, 3C). To identify the direct role of autophagy in trastuzumab resistance, we used chloroquine (CQ), a well-known autophagy inhibitor [21]. As expected, CQ treatment inhibited autophagy activity and deteriorated trastuzumab resistance (Figure 3D, 3E).

We then explored whether AGAP2-AS1 influence autophagy of BC cells. Compared to control group, knockdown of AGAP2-AS1 caused decreased LC3-II, elevated p62 expression and reduced number of LC3 puncta in SKBR-3-TR and BT474-TR cells (Figure 3F, 3G). Importantly, an increased autophagy activity was observed in SKBR-3 and BT474 cells when incubated with exosomes derived from SKBR-3-TR cells, however, the pro-autophagic effect was not observed when we incubated with exosomes derived from si-AGAP2-AS1-transfected SKBR-3-TR cells (Figure 3H, 3I). Moreover, treatment with CQ eliminated the elevated autophagic effect, suggesting the high level of autophagy activity were caused by increased autophagic...
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Figure 2. Exosomal AGAP2-AS1 was internalized by BC parental cells. (A, B) Incubation of SKBR-3 and BT474 cells with SKBR-3-TR-secreted exosomes induced suppression of cytotoxicity induced by trastuzumab. However, this effect was not obvious when AGAP2-AS1 was silenced in SKBR-3-TR cells. (C) Incubation SKBR-3 and BT474 cells with AGAP2-AS1-enriched SKBR-3-TR-derived exosomes significantly increased the cellular expression of AGAP2-AS1 of recipient cells while AGAP2-AS1-silenced exosomes showed no influence on AGAP2-AS1 expression level. (D) A strong PKH67 fluorescence signal was observed in recipient cells while no signal was observed in PBS control group. Scale bars: 10 μm. (E) A successful deficiency of AGAP2-AS1 was confirmed by qRT-PCR analysis. (F, G) CCK8 assay proved that AGAP2-AS1-overexpressed cell-secreted exosomes promoted trastuzumab resistance of AGAP2-AS1-knockout cells (F). These results were consistent with that of AGAP2-AS1-wild type BC cells (G). *P<0.05.
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Figure 3. Exosomal AGAP2-AS1 promotes trastuzumab resistance via inducing autophagy. (A) Gene Ontology analysis showed that autophagy may be involved in the function of AGAP2-AS1. (B, C) Trastuzumab-resistant cells showed higher LC3-II, lower p62 protein levels (B) and increased formation of LC3 puncta (C) in contrast to sensitive cells. (D) Western blotting showed that autophagy inhibitor, CQ, significantly inhibited autophagy activity and deteriorated trastuzumab resistance. (E) CQ treatment deteriorated trastuzumab resistance. (F, G) Knockdown of AGAP2-AS1 caused decreased LC3-II, elevated p62 expression and reduced number of LC3 puncta in SKBR-3-TR and BT474-TR cells. (H, I) Incubation with SKBR-3-TR-derived exosomes increased autophagy activity of SKBR-3 and BT474 cells. However, the pro-autophagic effect was not observed when we incubated with exosomes derived from si-AGAP2-AS1-transfected SKBR-3-TR cells. *P<0.05.
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flux (Figure S1). Collectively, we proved that exosomal AGAP2-AS1 transmit trastuzumab resistance via regulating autophagy activity.

Exosomal AGAP2-AS1 upregulates ATG10 expression to induce trastuzumab resistance in vitro

To clarify the functional target of AGAP2-AS1 in autophagy and trastuzumab resistance, we performed RNA sequencing with AGAP2-AS1 knockout SKBR-3 cells and wild-type controlled cells. Preliminarily, 1247 genes were identified with statistical significance (Fold change > 2) in both cell lines (Figure 4A). Given the fact that AGAP2-AS1 may influence trastuzumab resistance via regulating autophagy, we focus on 27 autophagy-related genes, including ATG10 and ATG5 (Figure 4B). By performing qRT-PCR, we identified five genes were significantly altered upon silence of AGAP2-AS1 in trastuzumab resistant cells (Figure 4C). Western blotting showed that the protein levels of two of those genes, ATG10 and ATG5 were silenced by AGAP2-AS1 knockdown (Figure 4D). Moreover, ATG10 was upregulated in trastuzumab-resistant cells compared to parental cells in both transcript and protein levels (Figure 4E), whereas ATG5 was not altered (data not shown). Functionally, knockdown of ATG10 significantly reversed the AGAP2-AS1-induced autophagy activity and trastuzumab resistance (Figure 4F and 4G), while silence of ATG5 showed no effect (data not shown), suggesting that ATG10 may be directly targeted by AGAP2-AS1 and mediates the AGAP2-AS1-induced autophagy and trastuzumab resistance.

Exosomal AGAP2-AS1 promotes trastuzumab resistance via ATG10 in vivo

To further confirm the essential role of exosomal AGAP2-AS1 in autophagy and trastuzumab resistance, we established xenografts in BALB/c nude mice models. Mice were inoculated subcutaneously with SKBR-3 cells and randomly divided into three groups. Mice of each group received PBS, SKBR-3-TR-EXO\textsubscript{Vector} or SKBR-3-TR-EXO\textsubscript{AGAP2-AS1} treatments accordingly, once every 3 days for 5 weeks (Figure 5A). Meanwhile, each group received intraperitoneal treatment of trastuzumab once every two days for 4 weeks (Figure 5B). Mice treated with SKBR-3-TR-EXO\textsubscript{AGAP2-AS1} showed increased growth of xenografts, greater size and weight compared to PBS and SKBR-3-TR-EXO\textsubscript{Vector}-treated mice (Figure 5C-G), suggesting that exosomal AGAP2-AS1 increased trastuzumab resistance of mice. Moreover, IHC analysis showed that SKBR-3-TR-EXO\textsubscript{AGAP2-AS1} treatment increased ATG10 expression compared to PBS and SKBR-3-TR-EXO\textsubscript{Vector} groups (Figure 5H). Consistently, tumors in SKBR-3-TR-EXO\textsubscript{AGAP2-AS1} group showed increased formation of LC3 puncta (Figure 5I). These results suggest that exosomal AGAP2-AS1 induced trastuzumab resistance via promoting ATG10 expression and autophagy in vivo.

AGAP2-AS1 was interacted and stabilized by ELAVL1

To verify the RNA binding protein of AGAP2-AS1, we performed RNA pulldown assay using biotinylated AGAP2-AS1 and sense control. An obvious band of 35-50 kDa was identified with clear significance between AGAP2-AS1 and sense control (Figure 6A). According to mass spectrometry analysis and western blotting after RNA pulldown, we identified it was embryonic lethal abnormal version like RNA binding protein 1 (ELAVL1) (Figure 6B-D). Consistently, RNA-FISH verified that AGAP2-AS1 and ELAVL1 colocalized mostly in the cytoplasm of SKBR-3 cells (Figure 6E). RIP assay further confirmed the direct interaction between AGAP2-AS1 and ELAVL1 (Figure 6F). Furthermore, serial deletion analysis revealed that the 900-1200 nt region of AGAP2-AS1 was essential for binding with ELAVL1 (Figure 6G). Prediction by POSTAR2 (http://lulab.life.tsinghua.edu.cn/postar/index.php) showed a binding motif of ELAVL1 at the 937-957 nt region of AGAP2-AS1 (Figure 6H). According to RNAfold, an online software predicting spatial structure [22], the potential binding area formed stem-loop structure (Figure 6I), which strongly suggests a direct binding.

It is reported that ELAVL1 play a role in stabilizing mRNAs by binding AU-rich elements (AREs) [23]. To verify whether ELAVL1 stabilized AGAP2-AS1, we silenced ELAVL1 expression in BC cells (Figure 6J). Silence of ELAVL1 caused downregulation of AGAP2-AS1 in trastuzumab resistant cells (Figure 6K). Importantly, when we blocked RNA transcription process by using actinomycin D (ActD), the stability of AGAP2-AS1 RNA was dramatically suppressed in
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Figure 4. AGAP2-AS1 regulates trastuzumab resistance via upregulating ATG10 expression. (A, B) Scatter plot and volcano plot showed the differentially expressed genes regulated by AGAP2-AS1, including 27 autophagy-related genes, including ATG10 and ATG5. (C) qRT-PCR verified five genes that were significantly altered upon knockdown of AGAP2-AS1. (D) Western blotting showed that knockdown of AGAP2-AS1 decreased ATG10 and ATG5 expression. (E) ATG10 was upregulated in BC trastuzumab-resistant cells compared to parental cells in both transcript (upper panel) and protein levels (lower panel). (F, G) Knockdown of ATG10 significantly reversed the AGAP2-AS1-induced autophagy activity (F) and trastuzumab resistance (G). *P<0.05, **P<0.01.

Figure 5. Exosomal AGAP2-AS1 promotes trastuzumab resistance via ATG10 in vivo. A. The schematic diagram and protocols to establish mouse xenografts. B. The schematic diagram showed plan of trastuzumab treatment. C. The stripped xenografts from different groups were shown. D, E. Tumor size and tumor weight of stripped xenografts were calculated. F, G. Dual BLI/NIRF imaging were generated and the luciferase intensity were quantified. H. ATG10 expression was detected in established tumors from different groups by using IHC analysis. I. GFP LC3 signaling was evaluated in xenografts to determine autophagy activity. **P<0.01.

ELAVL1-silenced cells compared to that in control cells (Figure 6L). Our results indicate that ELAVL1 directly binds to AGAP2-AS1 and maintain its stability.
AGAP2-AS1-ELAVL1 complex promote H3K4 trimethylation and H3K27 acetylation at the ATG10 promoter

By analyzing the transcriptional modification region at http://genome.ucsc.edu/, we found potential H3K4 trimethylation (H3K4me3) and H3K27 acetylation (H3K27ac) binding areas at ATG10 promoter region (Figure 7A). To probe AGAP2-AS1-ELAVL1 complex increases ATG10 expression by transcription activation, we performed ChIP assay. As expected, both H3K4me3 and H3K27ac enrichment were identified at ATG10 promoter region (Figure 7B). Moreover, ELAVL1 was also enriched at ATG10 promoter region (Figure 7C).
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A

B

C

D

E

F
To further confirm AGAP2-AS-ELAVL1 interacted with ATG10 promoter to induce histone modification, we evaluated the role of AGAP2-AS1 and ELAVL1 in H3K27me3 and H3K27ac enrichment. Figure 7D showed that knockdown of AGAP2-AS1 decreased H3K27me3 and H3K27ac enrichment in SKBR-3-TR and BT474-TR cells. Enrichment of the ATG10 promoter sequences associated with H3K4me3 and H3K27ac were increased in SKBR-3 and BT474 cells incubated with exosomes secreted by AGAP2-AS1-transfected SKBR-3-TR cells (Figure 7E). Interestingly, the effect caused by ectopic expression of AGAP2-AS1 was significantly reversed in cells silenced with ELAVL1, indicating that ELAVL1 is essential for AGAP2-AS1-induced transcription activation (Figure 7F). Although ELAVL1 could participate in the regulation of RNA stability, ActD treatment showed that ELAVL1 had little effect on half-life of ATG10 mRNA (data not shown).

Targeting AGAP2-AS1 with ASO showed potential in reversing trastuzumab resistance

Various in vitro, in vivo and pre-clinical studies proved that antisense oligonucleotide (ASO) may serving as promising small molecular drugs by generating specific vectors against diverse RNAs [24]. To investigate the potential of ASOs targeting AGAP2-AS1 in overcoming trastuzumab resistance, three ASOs against AGAP2-AS1 and one negative control were designed (Figure 8A). AGAP2-AS1 was silenced by ASO-1 and ASO-2 in trastuzumab resistant BC cells (Figure 8B). Cell suppression induced by trastuzumab was promoted by ASO-interfered AGAP2-AS1 (Figure 8C, 8D). ATG10 expression and autophagy activity were also suppressed when AGAP2-AS1 was silenced (Figure 8E). By optimizing the ASOs, we tested their functional efficiency in vivo. Free uptake assay revealed that two ASOs inhibited AGAP2-AS1 expression compared to negative control in SKBR-3-TR cells in a dose-dependent manner (Figure 8F). SKBR-3-TR cells were planted into mammary fat pads. When orthotopic xenograft tumor was established, mice were separated into 3 groups and injected via tail vein with ASO-1, ASO-2 or negative control ASO twice a week for 4 weeks (Figure 8G). Meanwhile, each group received intraperitoneal treatment of trastuzumab. Tumor growth was significantly suppressed in two ASO-treated groups compared to ASO-control group, suggesting that trastuzumab combined with AGAP2-AS1 knockdown via ASO could induce better anti-cancer effects, when compared to trastuzumab treatment only (Figure 8H). ATG10 expression was also suppressed in tissues treated with the two ASOs compared to control group (Figure 8I). Taken together, our results indicate that targeting AGAP2-AS1 with ASO combing drugs might be a promising therapeutic approach to overcome trastuzumab resistance.

Circulating exosomal AGAP2-AS1 predicts response to trastuzumab treatment of BC patients

To determine the predictive value of exosomal AGAP2-AS1 in trastuzumab response of BC patients, we analyzed its expression in 90 serum samples from patients receiving trastuzumab treatment (45 responding and 45 non-responding patients). As shown in Figure 9A, exosomal AGAP2-AS1 is upregulated in non-responding patients compared to responding patients. Moreover, receiver operator characteristic (ROC) curve showed a relatively high predictive value of exosomal AGAP2-AS1 in discriminating responding and non-responding patients, with an area under the curve (AUC) at 0.784, and diagnostic sensitivity and specificity at 77.8% and 73.3%, respectively (Figure 9B). By stratifying patients into a high or low exosomal AGAP2-AS1 expression group with a stratification criteria (4.06) obtained from the ROC curve, we found that the percent of patients showing response to trastuzumab therapy was much lower in high AGAP2-AS1-expressed group than that from low AGAP2-AS1-expressed group (Figure 9C), further strengthening the predictive value of exosomal
AGAP2-AS1 in discriminating responding and non-responding patients.

Discussion

Despite current chemotherapeutic regimens that evidently improved the survival of metastatic tumors, nearly all HER-2+ BC patients finally develop into resistance [25, 26]. Lnc-RNAs are dysregulated in various cancers, and modulates drug resistance by interacting with RNA and proteins [27]. Until now, the precise regulatory mechanisms by which lncRNAs regulate trastuzumab resistance in BC is still not clearly defined. In this study, we investigated the pivotal role and post-transcriptional regulation of lncRNA AGAP2-AS1 in trastuzumab resistance. We revealed that AGAP2-AS1 promotes trastuzumab resistance via packaging into exosomes. In addition, AGAP2-AS1 directly interacted with ELAVL1 which in turn stabilizes AGAP2-AS1; the AGAP2-AS1-ELAVL1 complex could directly bind to promoter of ATG10, inducing an enhanced transcriptional activity of ATG10 through increasing H3K4me3 and H3K27ac enrichment, and finally lead to the late trastuzumab resistance in BC.
activation autophagy and trastuzumab resistance (Figure 9D).

During recent years, more useful therapeutic methods were developed for the treatment of HER-2+ breast cancer [28], however, trastuzumab resistance still occurs and dramatically reduced the clinical practicality and response, which has become one of the most urgent challenges for promoting breast cancer outcome. Furthermore, lacking of effective prognostic biomarkers that could be used for predicting therapeutic response to trastuzumab also greatly hindered the improvement of HER-2 positive patients [29]. In addition to the molecular alterations discussed above, there is increased expression of a number of RTKs-IGF-1Rβ, c-Met, and EGFR-that are implicated in trastuzumab resistance [30]. Currently, very limited biomarkers are useful in guiding therapeutic decisions among HER-2+ patients except for HER-2 itself [31]. Hence, more and more studies needs to be devote to finding promising therapeutic targets and prognostic biomarkers [32, 33].

In recent years, the functions of lncRNAs have drawn more and more attention. A great number of studies have indicated that lncRNAs play important roles in all steps of carcinogenesis and tumor progression, and lncRNAs could be used as new prognostic markers [34, 35]. More recently, emerging evidence has indicated that exosomes regulate malignancy by transferring multiple classes of cargo molecules to recipient cells to mediate intercellular communication, including lncRNAs. Exosomal lncRNAs participate in tumor formation, proliferation, metastasis and chemotherapy resistance by regulating oncogenes or tumor suppressor genes [9, 36, 37]. However, communication in BC cells with different drug response via exosomes is obscured. In this study, we proved that AGAP2-AS1 lncRNA could be incorporated into exosomes and further internalized by recipient cells to transmit resistant property.
LncRNA AGAP2-AS1 and trastuzumab resistance

Autophagy can be a double-edged sword for resistant tumors: it participates in the development of drug resistance and protects cancer cells from chemotherapeutics but can also kill cancer cells in which apoptosis pathways are inactive. Therefore, research on the regulation of autophagy to combat resistance is expanding and is becoming increasingly important [38]. By screening potential targeted mRNAs, we identified ATG10 as one potentially regulated by AGAP2-AS1 and may play pivotal roles in trastuzumab resistance via regulating autophagy. Further experimental data proved that ATG10 expression is positively regulated by AGAP2-AS1 and essential for AGAP2-AS1-induced autophagy and trastuzumab resistance. ATG10 is an autophagic E2-like enzyme that interacts with ATG7 to recruit ATG12 and modulates the conversion of LC3-I to LC3-II [39]. Thus, ATG10 plays a critical role in autophagosome formation. Emerging evidence has emphasized that ATG10 displayed higher expression level in tumors of malignancies such as CRC [40] and lung cancer [41]. In addition, increased expression of ATG10 is positively linked with lympho-vascular invasion and predicts decreased overall survival times [42].

Another finding is the identification of the RBP, ELAVL1 (also known as Hu Antigen R, HuR), which binds to AGAP2-AS1 and regulates stability. ELAVL1 is a member of the RBP ELAV (embryonic lethal abnormal version) family, well studied for its role in RNA splicing and mRNA post-transcriptional regulation [43]. Various studies reported the critical role of ELAVL1 in cancer initiation, progression and resistance, via stabilizing targeted mRNAs. For example, Luo et al. revealed that ZEB1-AS1 IncRNA interacts with ELAVL1, thereby maintaining stability of ZEB1 mRNA in breast cancer [44]. Another study by Chen et al. found that IncRNA HOXB-AS1 promotes cell growth in multiple myeloma via FUT4 mRNA stability by ELAVL1 [45]. Our results suggest that there exists a direct interaction between AGAP2-AS1 and ELAVL1 protein, and the binding of AGAP2-AS1 by ELAVL1 increased the stability of AGAP2-AS1. In addition, AGAP2-AS1-ELAVL1 complex further binds to the promoter of ATG10, altering histone modifications and activating transcription. Moreover, the AGAP2-AS1-induced formation of H3K27ac and H3K4me3 was abolished by knockdown of ELAVL1, suggesting that ELAVL1 is essential for this epigenetic regulation. Reci-
LncRNA AGAP2-AS1 and trastuzumab resistance

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References


LncRNA AGAP2-AS1 and trastuzumab resistance


[44] Luo N, Zhang K, Li X and Hu Y. ZEB1 induced-upregulation of long noncoding RNA ZEB1-AS1 facilitates the progression of triple negative breast cancer by binding with ELAVL1 to maintain the stability of ZEB1 mRNA. J Cell Biochem 2020; 121: 4176-4187.

### Table S1. Information of the qPCR primer, probe and silencing RNA sequences

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<tr>
<th>qPCR primer name</th>
<th>Sequence (5’-3’)</th>
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<td>AGAP2-AS1 (Reverse)</td>
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![Figure S1. Autophagic activities were detected via western blotting in BC cells treated with CQ.](image-url)