

Exosomal Long Noncoding Rna AGAP2-AS1 Regulates Trastuzumab Resistance via Inducing Autophagy in Breast Cancer

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Abstract

Background: 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.

Methods: Trastuzumab resistant cells were established. RNA sequencing and qRT-PCR were performed to identify the target gene of AGAP2-AS1. Mass spectrometry, RNA pulldown and RNA immunoprecipitation assays were performed to verify the direct interactions among AGAP2-AS1 and other associated targets, such as embryonic lethal abnormal version like RNA binding protein 1 (ELAVL1) and autophagy related 10 (ATG10). In vitro and in vivo experimental assays were done to clarify the functional role of exosomal AGAP2-AS1 in trastuzumab resistance.

Results: AGAP2-AS1 promotes and 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. 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 antisenseoligonucleotides (ASO) substantially increased trastuzumab-induced cytotoxicity. Clinically, increased expression of serum exosomal AGAP2-AS1 was associate with poor response to trastuzumab treatment.

Conclusion: ExosomalAGAP2-AS1 increased trastuzumab resistance via promoting ATG10 expression and inducing autophagy. Therefore, AGAP2-AS1 may serve as predictive biomarker and therapeutic target for HER-2+ breast cancer patients.

Background

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]. Evidence is mounting that numerous lncRNAs are involved in regulating trastuzumab 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 lncRNAs 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, lncRNAs plays essential roles in regulating autophagy [16]. Therefore, finding the regulatory relationships between lncRNAs 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 patients were included from March 2012 to January 2015, to determine the expression of serum exosomal AGAP2-AS1 for trastuzumab response. Patients include 45 responding and 45 non-responding patients according to iRECIST criteria [18]. 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 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 Trizolreagent (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 lncRNA were detected by the PrimeScriptTMRT reagent kit (Takara, Dalian, China). qRT-PCR was carried out using the SYBR Premix Ex TaqTM (Takara, Dalian, China) and standardized using GAPDH as reference gene. All reactions were carried out in triplicate. The relative expression was calculated using the $2^{-\Delta\Delta ct}$ 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 ExoQuickTM 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. Exosome 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 reviewed 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 (1×10^6 cells in 0.1ml physiological saline) in the flank. Each group received PBS, SKBR-3-TR-EXO_{Vector}, or SKBR-3-TR-EXO_{AGAP2-AS1} treatment every 3 days for 5 weeks. Meanwhile, each group received intraperitoneal 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 (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, 5×10^5 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 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).

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 (ekspert™ nanoLC; AB Sciex TripleTOF 5600-plus; SCIEX). Data were analyzed using

Proteinpilot software (<https://omictools.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 (Millipore) was used according to the manufacturer'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 1h. 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 1h 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 \pm 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) (Fig. 1A) and NanoSight analysis (Fig. 1B). The exosomes were further confirmed by western blotting analysis of exosomal markers, TSG101 and HSP70 (Fig. 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 (Fig. 1D). Moreover, AGAP2-AS1 was more enriched in exosomes in contrast to intracellular portions, indicating that AGAP2-AS1 may function via incorporating into exosomes (Fig. 1E). Importantly, knockdown of cellular AGAP2-AS1 via transfection of AGAP2-AS1 small interfere vectors induced a dramatically decrease of exosomal AGAP2-AS1 expression (Fig. 1F and 1G), while overexpression of cellular AGAP2-AS1 showed opposite effects (Fig. 1H and 1I), strengthening our conclusion that AGAP2-AS1 was enriched in extracellular 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 Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 2E). CCK8 assay proved that AGAP2-AS1-overexpressed cell-secreted exosomes promoted trastuzumab resistance of AGAP2-AS1-knockout cells (Fig. 2F). These results were consistent with that of AGAP2-AS1-wild type BC cells (Fig. 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 (Fig. 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 (Fig. 3B-C). 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 (Fig. 3D-E).

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 (Fig. 3F-G). 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 (Fig. 3H-I). 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 (Fig. 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 (Fig. 4B). By performing qRT-PCR, we identified five genes were significantly altered upon silence of AGAP2-AS1 in trastuzumab resistant cells (Fig. 4C). Western blotting showed that the protein levels of two of those genes, ATG10 and ATG5 were silenced by AGAP2-AS1 knockdown (Fig. 4D). Moreover, ATG10 was upregulated in trastuzumab-resistant cells compared to parental cells in both transcript and protein levels (Fig. 4E), whereas ATG5

was not altered (data not shown). Functionally, knockdown of ATG10 significantly reversed the AGAP2-AS1-induced autophagy activity and trastuzumab resistance (Fig. 4F and G), 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_{Vector}, or SKBR-3-TR-EXO_{AGAP2-AS1} treatments accordingly, once every 3 days for 5 weeks (Fig. 5A). Meanwhile, each group received intraperitoneal treatment of trastuzumab once every two days for 4 weeks (Fig. 5B). Mice treated with SKBR-3-TR-EXO_{AGAP2-AS1} showed increased growth of xenografts, greater size and weight compared to PBS and SKBR-3-TR-EXO_{Vector}-treated mice (Fig. 5C-G), suggesting that exosomal AGAP2-AS1 increased trastuzumab resistance of mice. Moreover, IHC analysis showed that SKBR-3-TR-EXO_{AGAP2-AS1} treatment increased ATG10 expression compared to PBS and SKBR-3-TR-EXO_{Vector} groups (Fig. 5H). Consistently, tumors in SKBR-3-TR-EXO_{AGAP2-AS1} group showed increased formation of LC3 puncta (Fig. 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 (Fig. 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) (Fig. 6B-D). Consistently, RNA-FISH verified that AGAP2-AS1 and ELAVL1 colocalized mostly in the cytoplasm of SKBR-3 cells (Fig. 6E). RIP assay further confirmed the direct interaction between AGAP2-AS1 and ELAVL1 (Fig. 6F). Furthermore, serial deletion analysis revealed that the 900-1200 nt region of AGAP2-AS1 was essential for binding with ELAVL1 (Fig. 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 (Fig. 6H). According to *RNAfold*, an online software predicting spatial structure [22], the potential binding area formed stem-loop structure and showed MFE (Fig. 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 (Fig. 6J). Silence of ELAVL1 caused downregulation of AGAP2-AS1 in trastuzumab resistant cells (Fig. 6K). Importantly, when we blocked RNA transcription process by using actinomycin D (ActD), the stability of AGAP2-AS1 RNA was dramatically suppressed in ELAVL1-silenced cells compared to that in control cells (Fig. 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 theATG10 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 (Fig. 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 (Fig. 7B). Moreover, ELAVL1 was also enriched at ATG10 promoter region (Fig. 7C).

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. Fig. 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 (Fig. 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 (Fig. 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 unreversing 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 (Fig. 8A). AGAP2-AS1 was silenced by ASO-1 and ASO-2 in trastuzumab resistant BC cells (Fig. 8B). Cell suppression induced by trastuzumab was promoted by ASO-interfered AGAP2-AS1 (Fig. 8C-D). ATG10 expression and autophagy activity were also suppressed when AGAP2-AS1 was silenced (Fig. 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 adose-dependent manner (Fig. 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 (Fig. 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 (Fig. 8H). ATG10 expression was also suppressed in tissues treated with the two ASOs compared to control group (Fig. 8I). Taken together, our results indicate that targeting AGAP2-AS1 with ASOcombing 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 Fig. 9A, exosomal AGAP2-AS1 is upregulated in nonresponding 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 (Fig. 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 (Fig. 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]. LncRNAs 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 activation autophagy and trastuzumab resistance (Fig. 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-1R β , 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 need to be devoted 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.

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 lncRNA interacts with ELAVL1, thereby maintaining stability of ZEB1 mRNA in breast cancer [44]. Another study by Chen et al. found that lncRNA 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. Reciprocally, overexpression of ELAVL1 only partly reversed the decrease of H3K4me3 and H3K27ac enrichment in AGAP2-AS1 silencing cells, indicating that the enhanced transcriptional activity at ATG10 promoter is dependent on the histone modification induced by AGAP2-AS1-ELAVL1 complex.

We need to point out two limitations of our study. First, we explored the experimental role of ASO targeting AGAP2-AS1 in reversing trastuzumab resistance, however, this may need further validation in clinical trials. Second, we preliminarily confirmed the function of AGAP2-AS1/ELAVL1/ATG10 in

autophagy and trastuzumab resistance, however the precise regulatory model, and the ongoing interactions with other cellular factors in tumor microenvironment during this process need more meaningful studies.

Conclusions

In summary, we demonstrated the crucial role of AGAP2-AS1 in trastuzumab resistance of BC by epigenetically increasing ATG10 expression and thereby promoting autophagy. Our discovery not only help us get a better understanding the regulatory potential of AGAP2-AS1 in drug resistance of HER-2+ BC, but also useful for finding promising drug targets and developing novel therapeutic strategies to overcome resistance.

List Of Abbreviations

ASO: Antisense Oligonucleotides;

ATG10: Autophagy related 10;

ATCC: American Tissue Culture Collection;

ANOVA : Student's t-test or one-way analysis of variance;

BC: Breast cancer;

ChIP: Chromatin immunoprecipitation;

DAPI: 4',6-diamidino-2-phenylindole;

DMEM: Dulbecco's modified Eagle medium;

ELAVL1: Embryonic Lethal Abnormal Version Like RNA binding protein 1;

FBS: Fetal bovine serum;

FISH: Fluorescence *in situ* hybridization;

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase;

HER-2: Human epidermal growth factor receptor 2;

IHC: Immunohistochemistry;

LncRNAs: Long noncoding RNAs;

mRNA: messenger RNA;

MFE: minimum free energy;

NC: Negative control;

OS: Overall survival;

PCR: Polymerase chain reaction;

PVDF: Polyvinylidene fluoride;

PFS: Progressive-free survival;

PBS: phosphate-buffered saline;

RIP: RNA immunoprecipitation;

RBPs: RNA-binding proteins;

siRNA: small interfering RNA;

SD: Standard deviation;

TEM: Transmission electron microscopy.

Declarations

Ethics approval and consent to participate

This study was approved by Research Scientific Ethics Committee of The First Affiliated Hospital of Zhengzhou University, The First People's Hospital of Chenzhou City and Hainan General Hospital. All participants signed informed consent prior to using the tissues and serum samples for scientific research.

Consent for publication

Not applicable.

Availability of data and material

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Huaying Dong, Mingli Han, Hongbo Qu, and Xueke Qian mainly did the experiment and wrote the paper. Hui Cao, Xiangke Li, Dongwei Dou, Xue Yang, Yunqing Yang, Na Han, Mulin Ye, Jing Han, Fan Zhang, Wei Wang helped do the cell experiment. Yichao Ding and Mingwei Xie did the statistical work.

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Figures

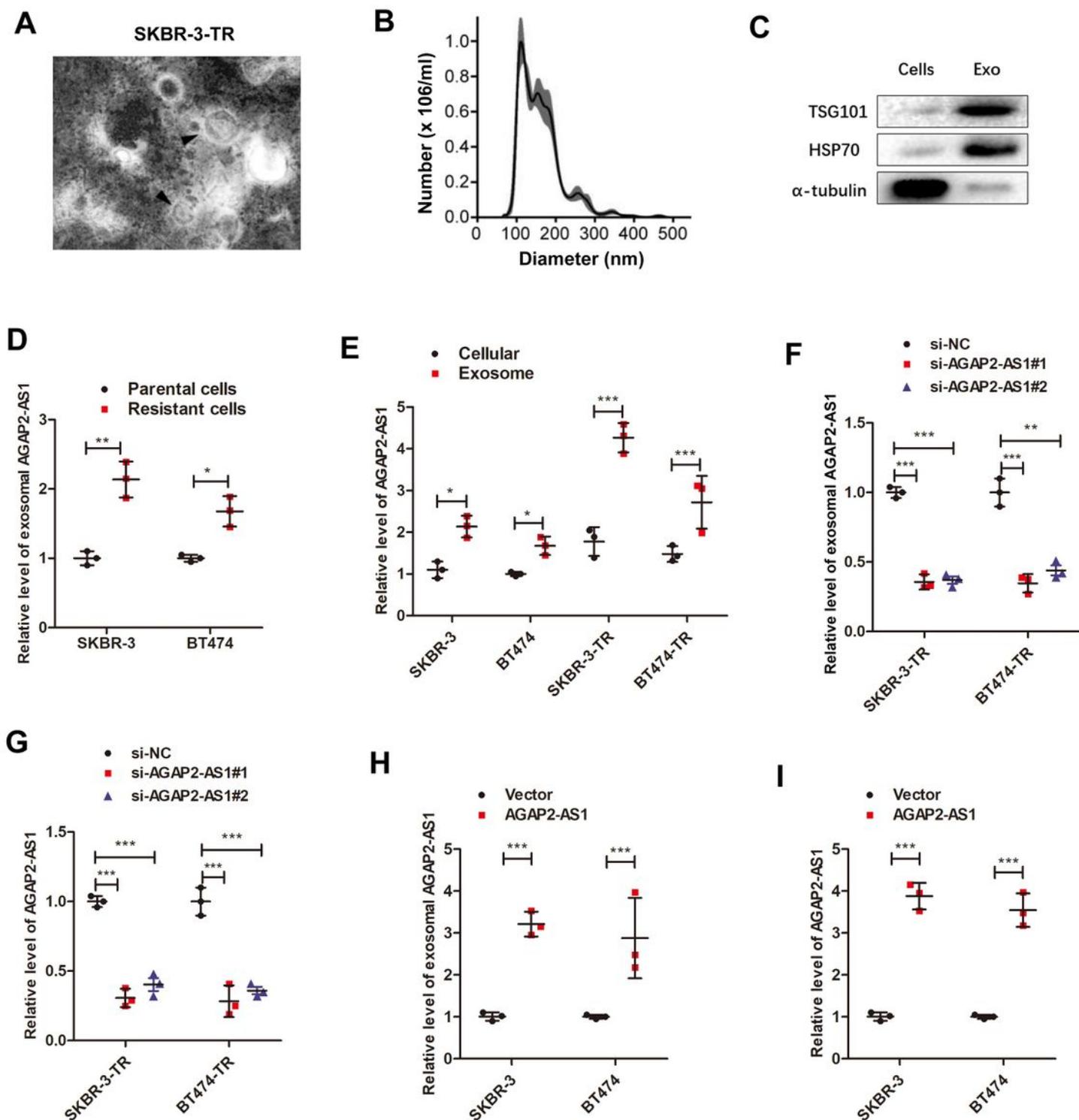


Figure 1

Extracellular AGAP2-AS1 was incorporated into exosomes. (A) Exosomes were 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. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

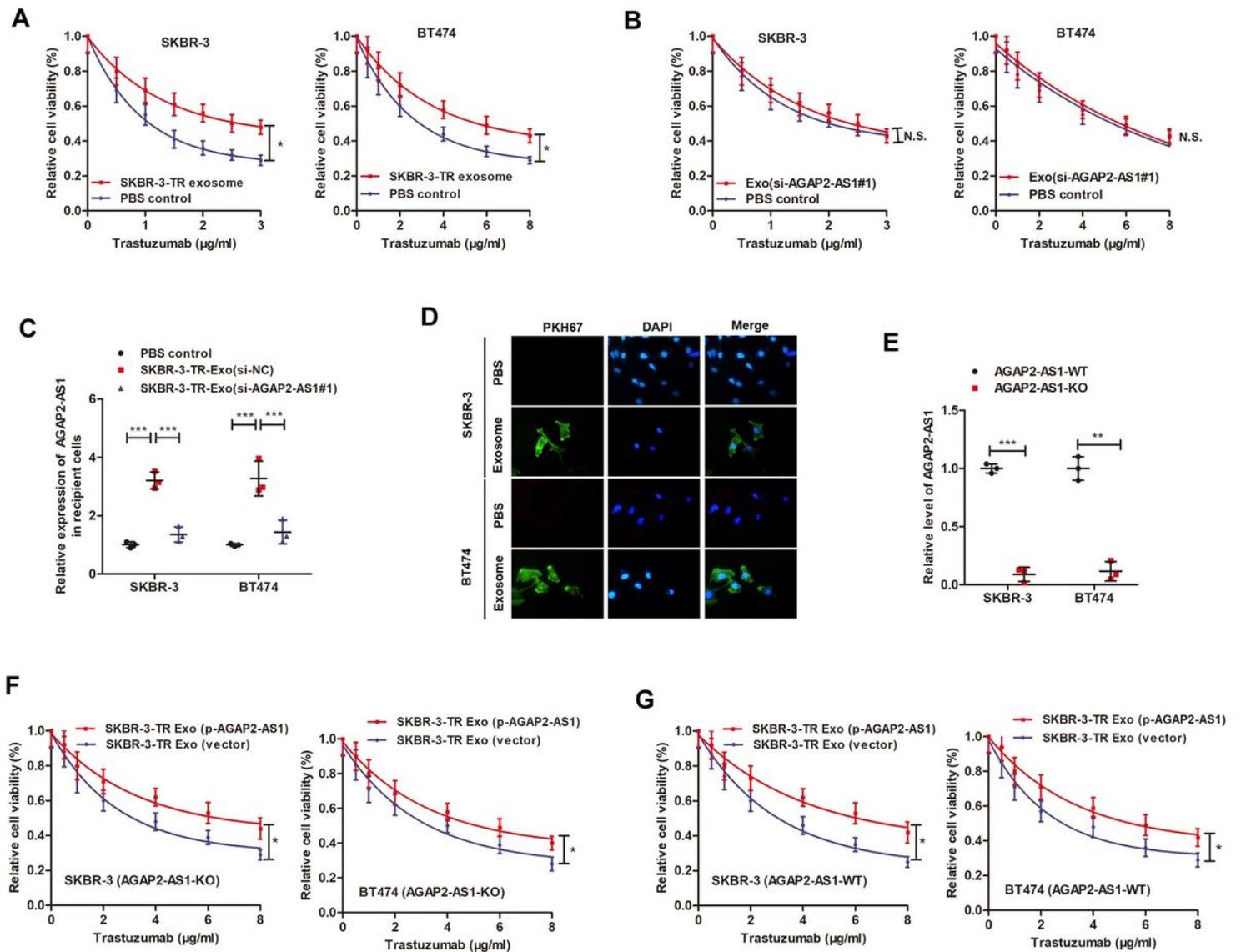


Figure 2

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. (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). * $P < 0.05$

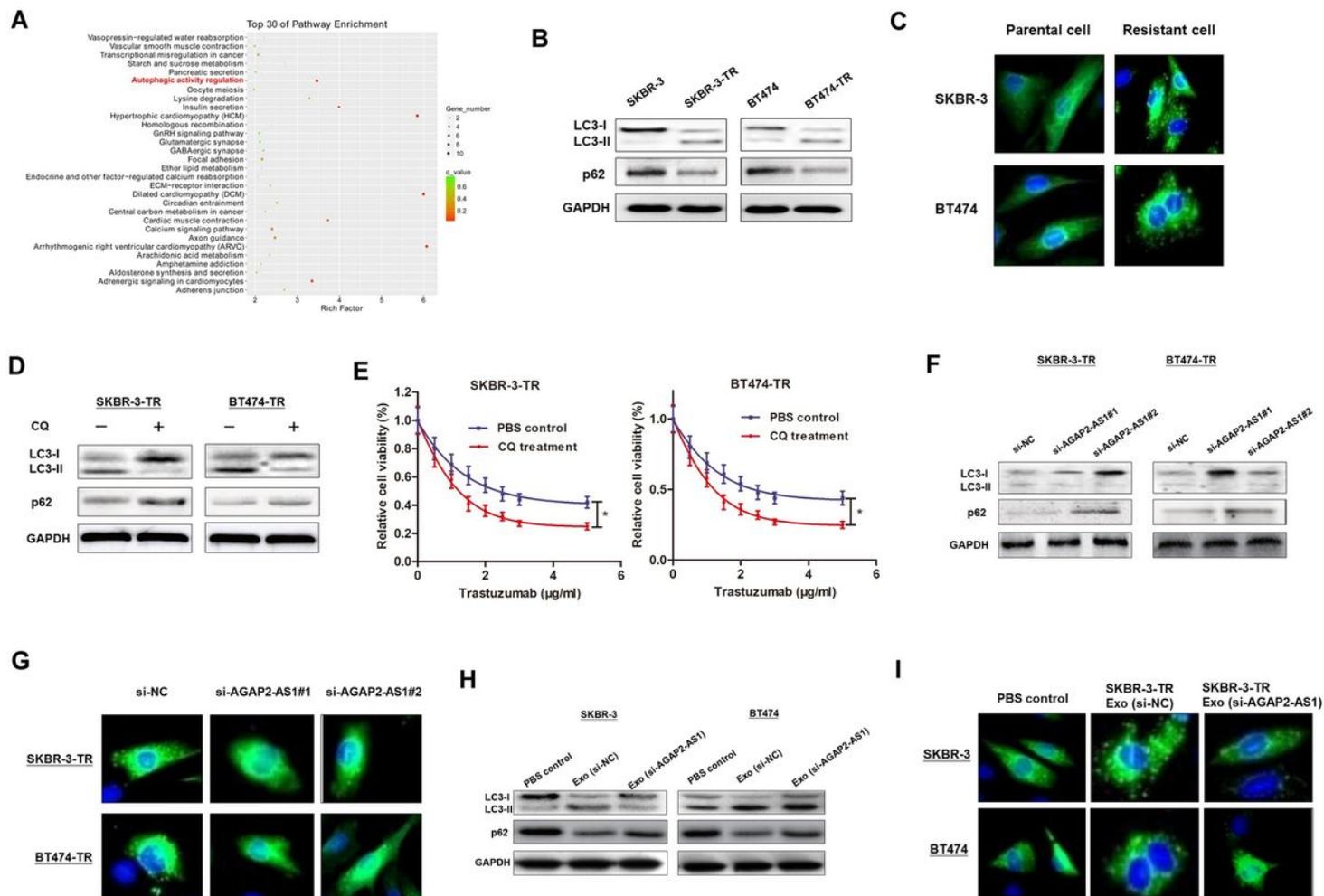


Figure 3

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. * $P < 0.05$

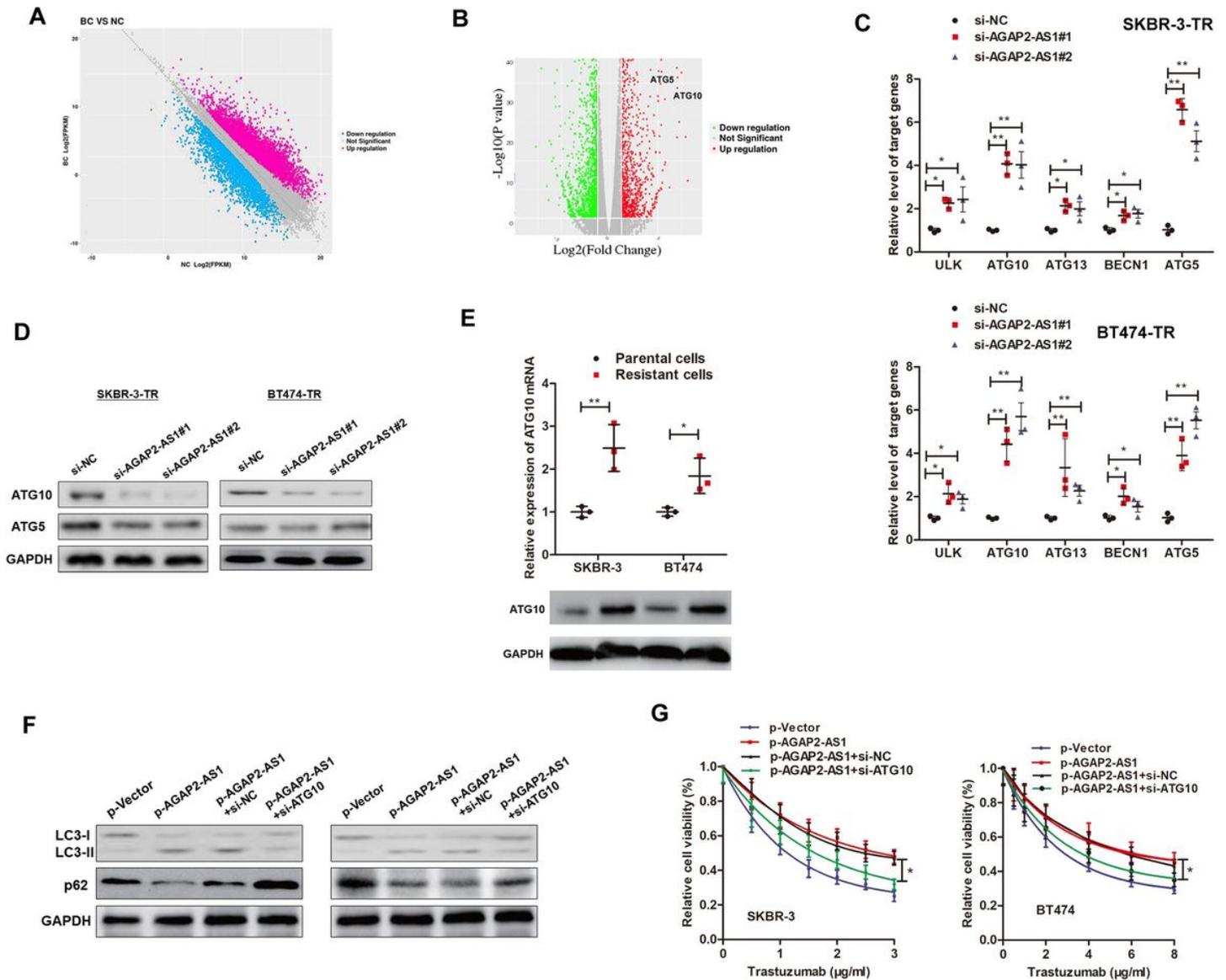


Figure 4

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, such as 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 trastuzumab-resistant cells compared to parental cells in both transcript and protein levels. (F-G) Knockdown of ATG10 significantly reversed the AGAP2-AS1-induced autophagy activity and trastuzumab resistance. * $P < 0.05$, ** $P < 0.01$

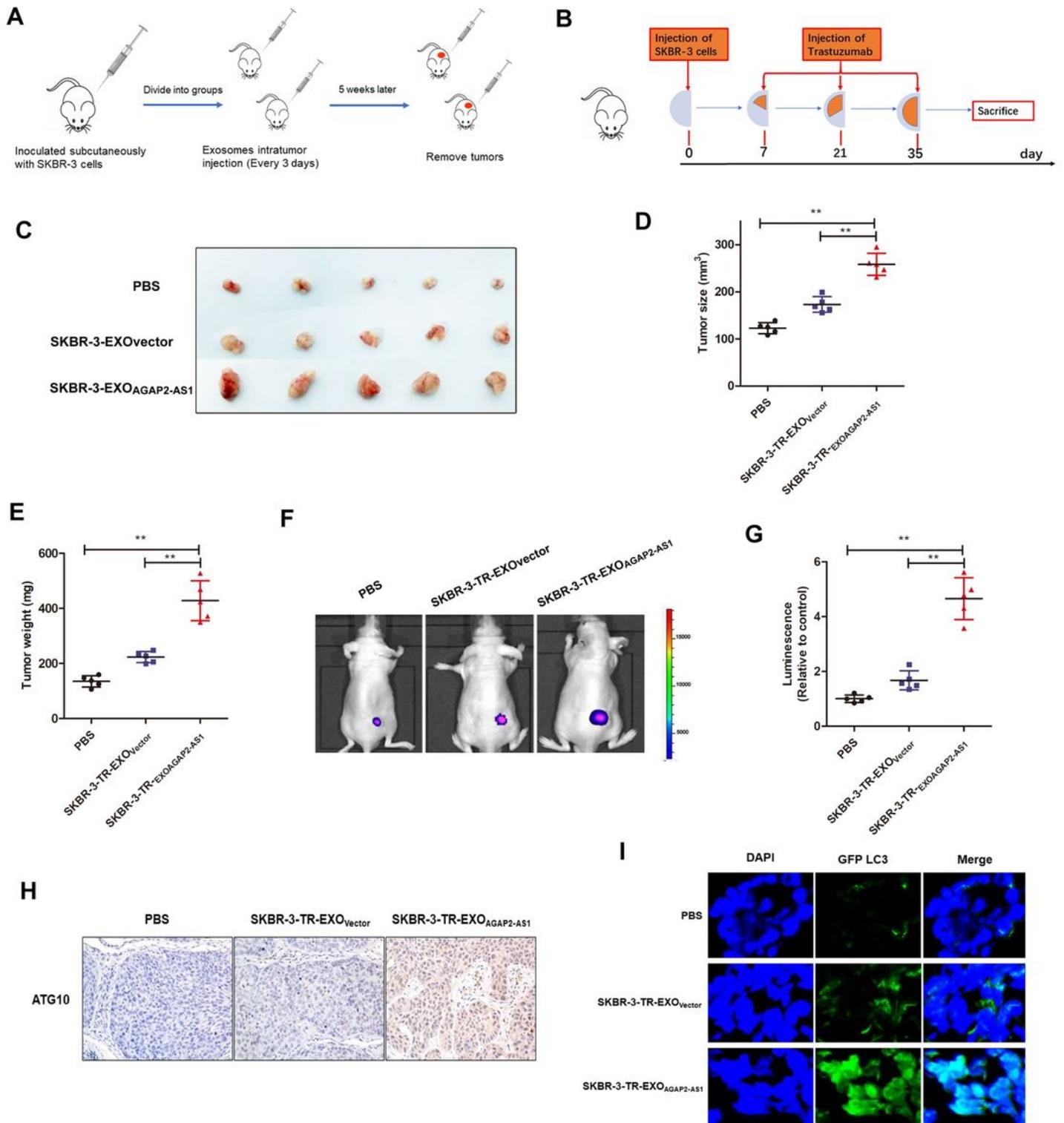


Figure 5

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. ** $P < 0.01$

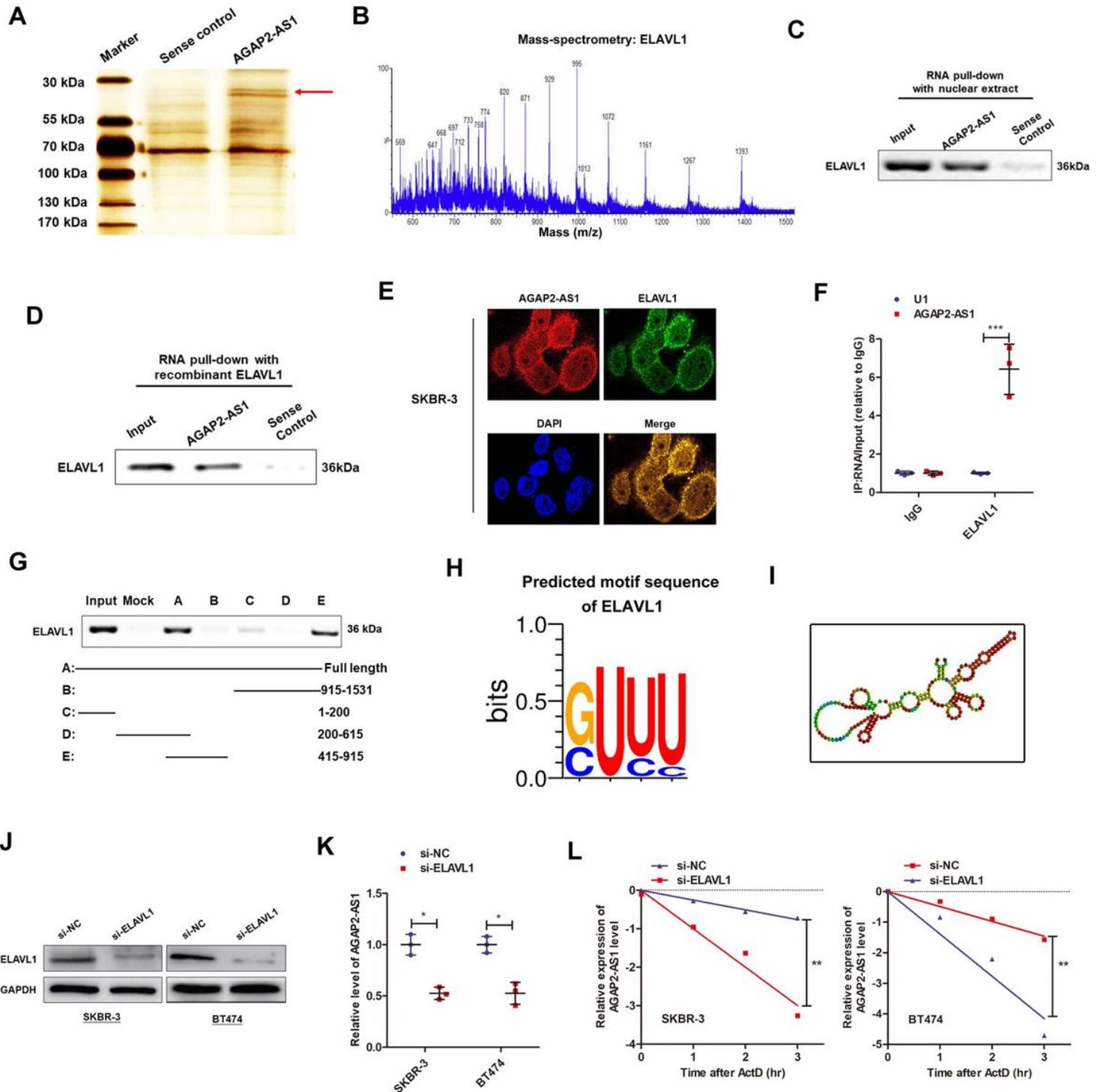


Figure 6

AGAP2-AS1 was interacted and stabilized by ELAVL1 (A) Silver-stained RNA pull-down assay using biotinylated AGAP2-AS1 and sense control were performed, and a specific band was identified. (B) Mass spectrometry analysis of proteins from RNA pull-down, and the ELAVL1 atlas was shown. (C-D) Western blotting confirmed the interacted protein was ELAVL1. (E) RNA-FISH with specific probe verified

that AGAP2-AS1 and ELAVL1 colocalized mostly in the cytoplasm of SKBR-3 cells. (F) RIP assay with ELAVL1 protein further confirmed the direct interaction between AGAP2-AS1 and ELAVL1. (G) Serial deletion analysis revealed that the 900-1200 nt region of AGAP2-AS1 was essential for binding with ELAVL1. (H) Predicted motif sequence of ELAVL1 was shown according to POSTAR2 (<http://lulab.life.tsinghua.edu.cn/postar/index.php>). (I) The 937-957 nt region of AGAP2-AS1 formed a stem-loop structure as predicted by RNAfold (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi?>). (J) ELAVL1 was silenced after transfection with specific silencing vectors. (K) qPCR showed that knockdown of ELAVL1 decreased AGAP2-AS1 expression in SKBR-3 and BT474 cells. (L) The half-life of AGAP2-AS1 RNA was dramatically decreased in ELAVL1-silenced cells compared to that in control cells. * $P < 0.05$, *** $P < 0.001$

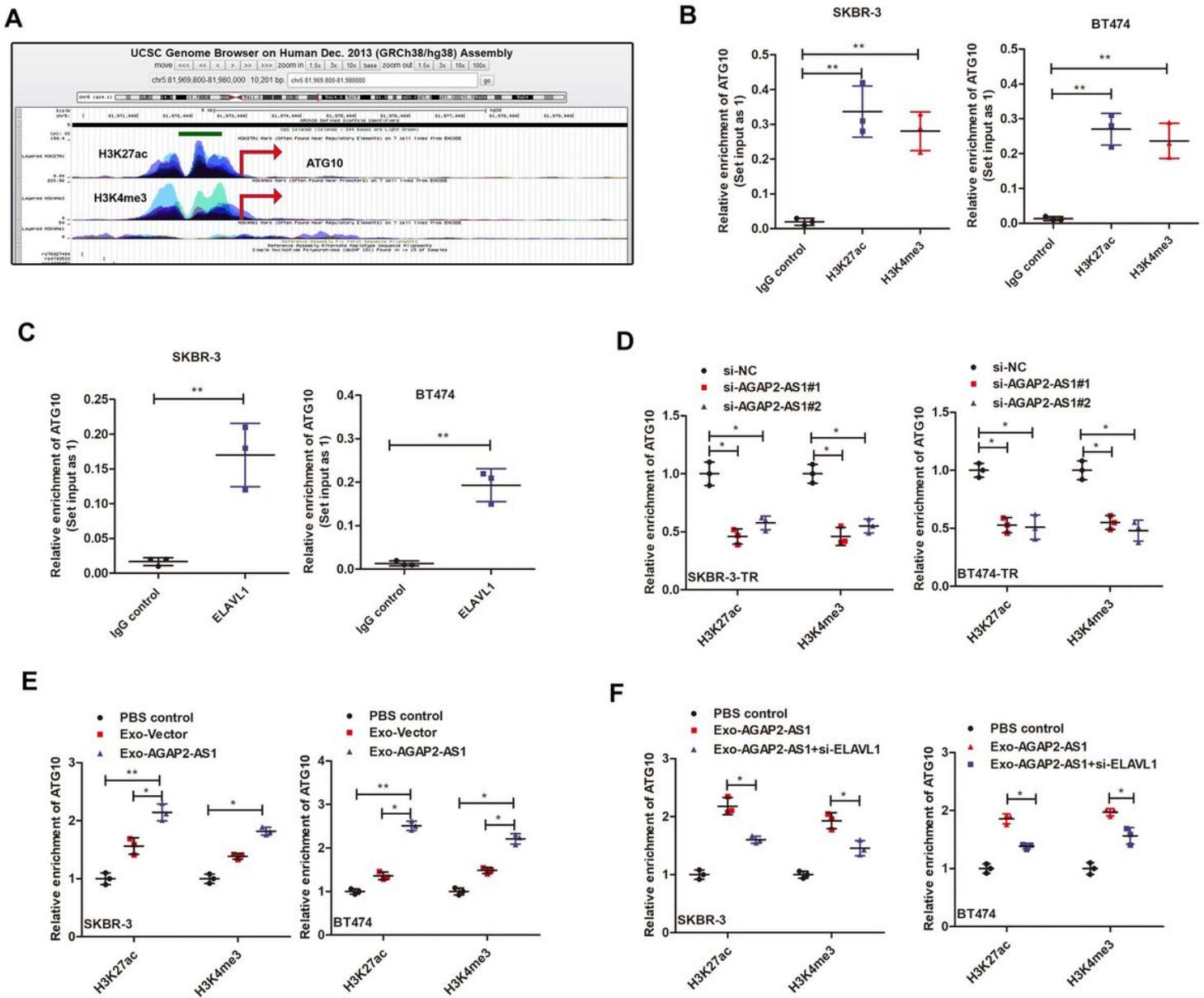


Figure 7

AGAP2-AS1-ELAVL1 complex promote H3K4 trimethylation and H3K27 acetylation at ATG10 promoter region (A) Predictive H3K27ac and H3K4me3 binding sites were shown according to UCSC online website. (B) ChIP-qPCR showed a direct enrichment of H3K27ac and H3K4me3 at the promoter region of ATG10. (C) ChIP-qPCR verified that ELAVL1 was enriched at ATG10 promoter region. (D) knockdown of AGAP2-AS1 decreased H3K27me3 and H3K27ac enrichment in SKBR-3-TR and BT474-TR cells. (E) Enrichment of H3K4me3 and H3K27ac at the ATG10 promoter were increased upon treatment with exosomes secreted by AGAP2-AS1-transfected SKBR-3-TR cells. (F) Silencing ELAVL1 reversed AGAP2-AS1-induced enrichment of H3K27me3 and H3K27ac. * $P < 0.05$, ** $P < 0.01$

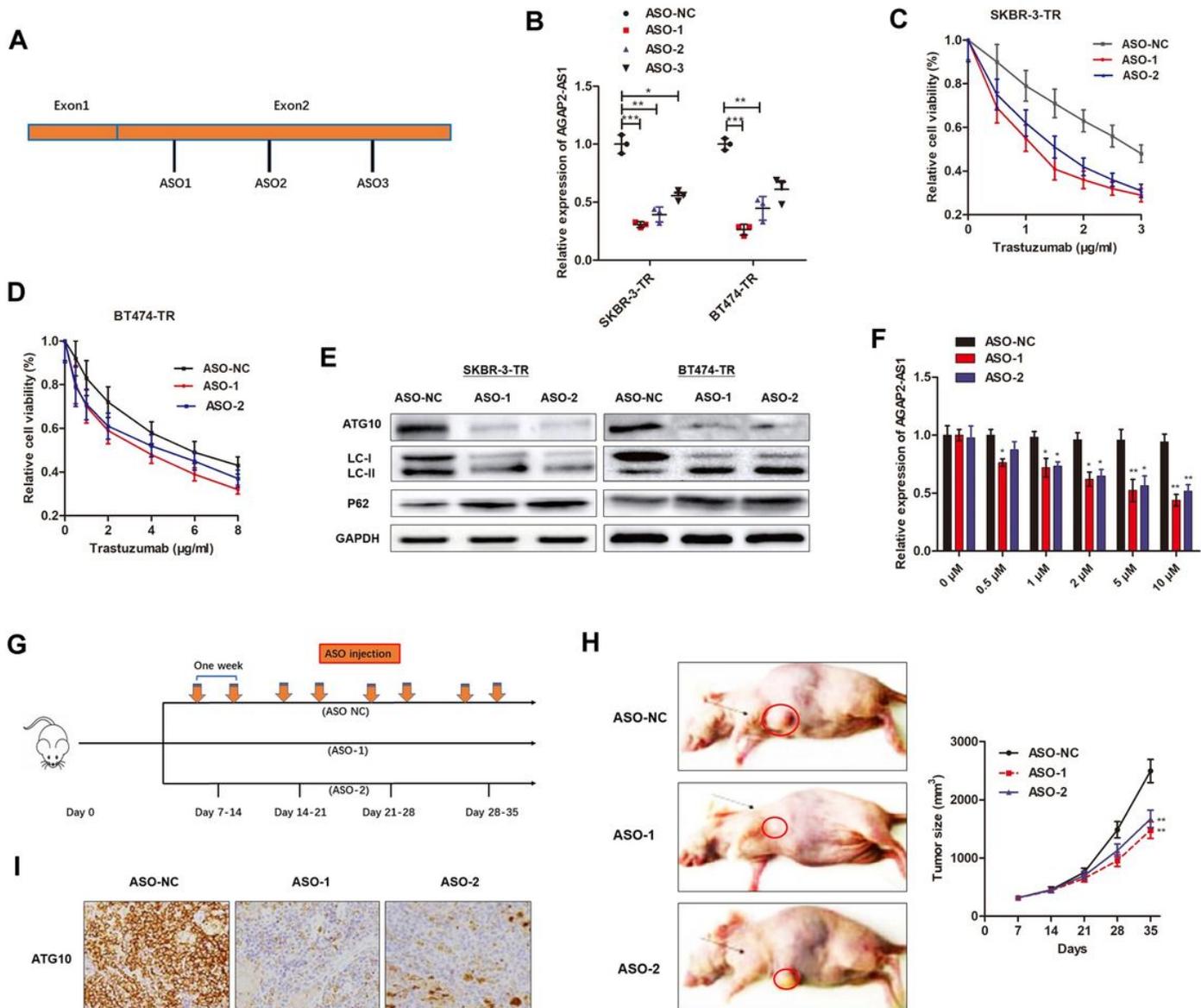


Figure 8

Potential therapeutic role of AGAP2-AS1 in BC. (A) Schematic view of ASO targets on AGAP2-AS1 gene. (B) Relative expression of AGAP2-AS1 48 h after ASO transfection were determined by qRT-PCR. (C-D) ASO treatment increases trastuzumab-induced cell cytotoxicity in SKBR-3-TR and BT474-TR cells. (E)

Western blotting showed the suppressive effect of ASOs in autophagy activity. (F) qRT-PCR showed AGAP2-AS1 expression after ASO delivery without transfect reagents. ASO were added into SKBR-3-TR cells at the concentration as indicated. After 48 h, RNA was extracted, and RT-qPCR was performed. (G) Schematic view of ASO treatment xenograft model. (H) Tumor growth was significantly suppressed in two ASO-treated groups compared to ASO-control group. (I) IHC analysis showed that ATG10 expression was suppressed in tissues treated with the two ASOs compared to control group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

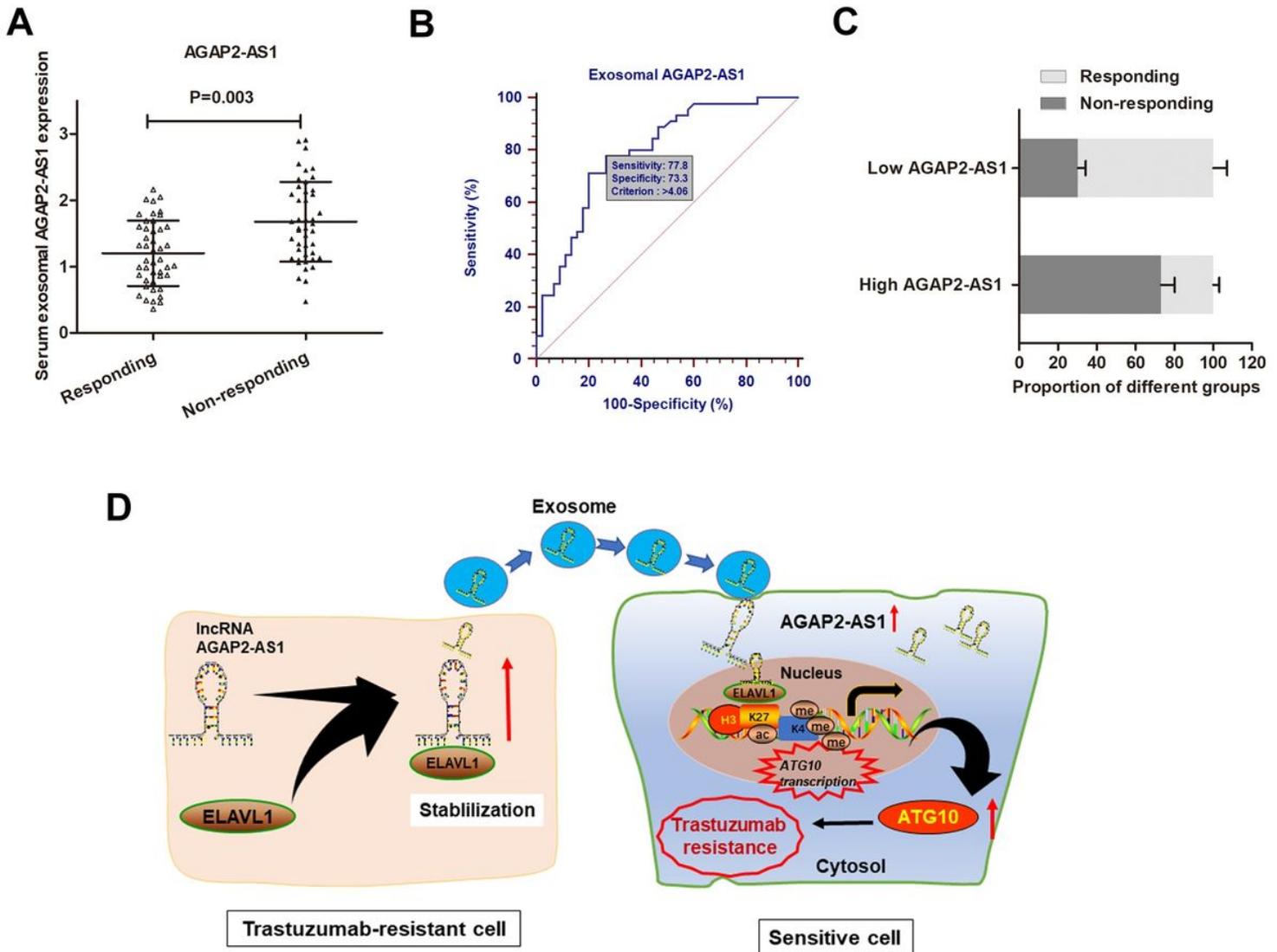


Figure 9

The clinical predictive role of circulating exosomal AGAP2-AS1 (A) Serum exosomal AGAP2-AS1 expression was detected in responding and non-responding patients. (B) ROC curve was established to evaluate the predictive role of AGAP2-AS1 in discriminating responding and non-responding patients. (C) The proportion of high or low-expressing AGAP2-AS1 patients in responding or non-responding groups were shown. (D) Schematic diagram of ELAVL1-AGAP2-AS1-ATG10 axis regulating trastuzumab resistance in BC via exosome.

Supplementary Files

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