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Increased Expression of Exosomal AGAP2-AS1 (AGAP2 Antisense RNA 1) In Breast Cancer Cells Inhibits Trastuzumab-Induced Cell Cytotoxicity

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Statistical Analysis C
Data Interpretation D
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Background: Trastuzumab therapy is important for patients with HER2-positive breast cancer, but more and more patients have experienced trastuzumab resistance during recent years. Accumulating evidence from recent studies showed that long non-coding RNAs (lncRNAs) play essential roles in chemoresistance of various cancer types, but the precise role of lncRNAs in trastuzumab resistance is unclear. In the present study, we aimed to identify the biofunction of lncRNA APAP2-AS1 in tranastuzumab resistance and to reveal the underlying regulatory mechanism.

Material/Methods: By culturing HER2-positive SKBR-3 and BT474 cells with transtuzumab-containing medium, we built trastuzumab-resistant cells. Quantitative real-time PCR was used to test the expression of AGAP2-AS1 in the built trastuzumab-resistant cells. Cell viability assay and TUNEL assay were used to test the cell viability and apoptosis in each group. Exosomes were purified from cells cultured in exosomes-depleted FBS and identified by transmission electron microscopy.

Results: qRT-PCR assay suggested that AGAP2-AS1 was upregulated in the built trastuzumab-resistant cells when compared with parental sensitive cells. Cell viability assay showed that silencing of AGAP2-AS1 enhanced the cytotoxicity induced by trastuzumab treatment. Mechanistically, we revealed that AGAP2-AS1 was secreted outside cells by incorporation into exosomes in an hnRNPA2B1-dependent manner. More importantly, co-culture AGAP2-AS1-containing exosomes with sensitive cells reduced the trastuzumab-induced cell death, and silencing of AGAP2-AS1 from exosomes reversed this effect. In summary, AGAP2-AS1 promotes trastuzumab resistance of breast cancer cells through packaging into exosomes.

Conclusions: Knockdown of AGAP2-AS1 may be helpful for improving the clinical outcome for HER2+ breast cancer patients and could serve as a therapeutic target.

MeSH Keywords: Breast Neoplasms • Exosomes • RNA, Long Noncoding

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Background

Breast cancer is one of the most common cancers and causes high rates of cancer-related mortality among women worldwide [1]. Moreover, about 15% of breast cancer cases are HER2-positive (HER-2+) type. During recent years, substantial advances have been made in the treatment of HER-2+ breast cancer [2,3]. The use of trastuzumab dramatically improved the clinical prognosis of HER2-positive breast cancer patients. However, 27–42% of patients treated with adjuvant trastuzumab finally became resistant and experienced progression [4,5]. The underlying mechanism by which trastuzumab resistance occurs is still unclear. Thus, there is an urgent need to find new molecular markers and reveal the functional mechanism.

During recent years, whole-genome transcriptomic analysis revealed many dynamically expressed lncRNAs, many of which are involved in a variety of biological activities [6]. So far, few lncRNAs have been functionally characterized, but several examples are shown to play a critical role in physiological and pathological processes of cancer by sponging microRNAs [7] or chromatin modification [8,9] at multiple levels. AGAP2-AS1, which is transcribed from a gene located at 12q14.1 and is 1567 nt in length, has been found to be overexpressed in human non-small cell lung cancer (NSCLC) and gastric cancer. A previous study reported that increased AGAP2-AS1 repressed LATS2 and KLF2 expression through interacting with EZH2 and LSD1 in NSCLC [10]. Another study demonstrated that AGAP2-AS1 is upregulated in gastric cancer and promotes cell proliferation and invasion by suppressing P21 and E-cadherin [11]. However, whether AGAP2-AS1 participates in trastuzumab resistance, and if so, the underlying regulatory mechanism, is still unclear.

Exosomes are small extracellular vesicles with a significant role in most processes associated with cancer, and cancer exosomes carry malignant information in the form of proteins, lipids, and nucleic acids that can reprogram recipient cells [12]. Exosomes provide a relatively stable environment for the therapeutic agent of choice, have the potential to be modified to improve cell-specific homing, and have the ability to incorporate into recipient cells to transfer useful information from host cells [13]. However, the roles of lncRNAs contained in exosomes in cancer treatment are largely unknown.

In the present study, we hypothesized that lncRNA AGAP2-AS1 may be secreted by packaging into exosomes, and exosome AGAP2-AS1 may exert a functional role in trastuzumab resistance. To verify this hypothesis, we established 2 trastuzumab-resistant cell lines and confirmed the existence of AGAP2-AS1 in exosomes. By performing a series of functional assays, we explored the functional role of exosome-delivered lncRNA AGAP2-AS1 in trastuzumab resistance of breast cancer cells.

Material and Methods

Cell culture

The human breast cancer cell lines (SKBR-3 and BT474) and 1 normal epithelial cell line (MCF-10A) were purchased from the American Type Culture Collection (ATCC) and were cultured in RPMI 1640 medium (BioWhittaker, Lonza, USA) supplemented with 10% FBS (fetal bovine serum; Shanghai ExCell Biology, Shanghai, China) and 1% penicillin/streptomycin. To remove exosomes, media was centrifuged at 104 492×g overnight at 4°C, followed by filtering through a 0.2-μm filter. Depleted media were stored at 4°C for no longer than 4 weeks. Cells were grown in a humidified atmosphere of 5% CO_2 at 37°C. The cell lines used in this study were not contaminated by mycoplasma. The trastuzumab-resistant cells were constructed by culturing breast cancer cells with 5 mg/mL trastuzumab (Roche, Shanghai, China) for 6 months, as previously reported [14].

Exosomes isolation, labeling and RNA extraction

Exosomes were purified from cells cultured in exosomes-depleted FBS. The purified exosomes were washed with PBS, followed by ultracentrifugation and a further filtration of the supernatant with 0.25-μm filters [15]. A Zetasizer device (Malvern Panalytical, Ltd., UK) was used to determine the size of the exosomes. PKH26 dye was used to label the exosomes. Exosomes used for RNA extraction were treated with Proteinase K and RNase prior to lysis of exosomes, followed by using commercial miRNeasy Serum/Plasma kit (Qiagen, Waltham, MA), according to the instructions provided by the manufacturer.

Transmission electron microscopy (TEM)

Samples containing exosomes were fixed and exposed to a formvar-carbon-coated 300-mesh grids (Electron Microscopy Sciences, Hatfield, USA) for 10 min. Exosomes were then stained with 1.75% uranyl acetate. Finally, samples were dried and imaged with a transmission electron microscope (Hitachi H7500 TEM, Tokyo, Japan). Micrographs were used to identify and quantify exosomes.

Quantitative real-time PCR (qRT-PCR)

For assaying lncRNA expression levels, qRT-PCR was performed using SYBR Premix Ex Taq™ kit (Takara) and a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The values of $2^{-\Delta\Delta CT}$ relative to one of the samples was calculated to analyze relative expression levels of the genes. GAPDH were used to normalize the relative expression levels of circRNAs and miRNAs, respectively. The relative expression level of different genes was calculated using the ΔΔCT method [16]. The primer sequences were: AGAP2-AS1 (Forward) 5'-TACCTTGACCTTGCTGCTCTC-3',

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(Reverse) 5'-TGTCCCTTAATGACCCCATCC-3'; GAPDH (Forward) 5'-GCACCGTCAAGGCTGAGAAC-3', (Reverse) 5'-ATGGTGGTGAAGACGCCAGT-3'.

Cell transfection

The silencing RNA against AGAP2-AS1 (si-AGAP2-AS1) and hnRNPA2B1 (si-hnRNPA2B1) were purchased from RiboBio (Guangzhou, China). Negative control siRNA was purchased from Invitrogen (CAT#12935-110, Shanghai, China). Green fluorescence protein (GFP) was used to show transfection efficiency. Cell lines were transfected using Lipofectamine 2000 (Life Technologies, USA) at the concentration of 100 nM, according to the manufacturer's instructions. The sequences of small interfering RNAs were: si-AGAP2-AS1#1 5'-CCACTCCACCTCAAACTCTTACCTT-3'; si-AGAP2-AS1#2 5'-GGGTCATTAAGGGACAGAGTTCAAG-3'; si-AGAP2-AS1#3 5'-CAGGTGGACTCACAATTCCAAATAT-3'; si-hnRNPA2B1 5'-GCGGAAUUAAAGAAGAUACTT-3'.

TUNEL assay

Trastuzumab-induced apoptosis and DNA fragmentation was performed using TUNEL assay. After transfection or trastuzumab treatment, cells were fixed in 4% (w/v) paraformaldehyde at 4°C for 15 min. TUNEL staining was examined using the TUNEL kit (Roche, Shanghai, China), and the nuclei were stained with DAPI for 10 min. TUNEL-positive cells were counted with a fluorescence microscope (Olympus, Japan).

RNA immunoprecipitation (RIP)

RIP assay was performed by using the Magna RIP™ Kit (Millipore, Billerica, MA). Briefly, breast cancer cells were lysed with complete RNA lysis buffer followed by incubation with immunoprecipitation buffer containing human anti-hnRNPA2B1 antibody (Abcam, 1: 50, ab31645) or negative control mouse IgG (Millipore).

Cell viability assay

The proliferation of transfected cells after trastuzumab treatment was detected using a Cell Counting Kit-8 (Dojindo, Japan). Approximately 2×10^3 cells were seeded in 96-well plates in triplicate. CCK-8 solution (10 ul) was added into each well and incubated at 37°C for 2.5 h. The OD value at 450 nm was measured using a SpectraMax M5 microplate reader (MD, USA).

Western blot and antibodies

The total proteins were prepared, and their concentrations were detected using a Total Protein Extraction Kit (Solarbio, Beijing, China). Cell lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare, USA). Then, 5% non-fat milk in TBST buffer was used for the blockage of membranes containing protein. The primary antibodies used in this study were: anti-HSP70 (1: 1000, Abcam, ab79852), anti-hnRNPA2B1 (1: 1000, Abcam, ab31645), and anti-CD9 (1: 1000, Abcam, ab92726). HRP-conjugated secondary goat anti-mouse (1: 5000, Proteintech, Rosemont, IL, SA00001-1) or goat anti-rabbit (1: 5000, Proteintech, SA00001-2) antibodies were incubated for 2 h at room temperature. The relative grey values of immunoreactive bands were calculated based on GAPDH.

Statistical analysis

All data were analyzed using GraphPad Prism 6.0 (La Jolla, USA) unless otherwise stated. All data are expressed as means ± standard error of the mean (SEM). The parametric paired *t* test was performed to assess differences between 2 groups. One-way analysis of variance was performed to evaluate difference among multiple groups. $P<0.05$ was set as the level of significance.

Results

AGAP2-AS1 expression is increased in trastuzumab-resistant cells

By culturing SKBR-3 and BT474 cells with trastuzumab-contained medium, we generated 2 sub-lines, SKBR-3R and BT474R, which showed resistance to trastuzumab treatment. Compared to parental cells, the built chemo-resistant cells exhibited specifc morphologic changes, including decrease of cell polarity and interaction, and increased pseudopodia presentation (Figure 1A). In addition, we found that the built trastuzumab-resistant cells showed significantly higher viability compared to the respective parental cells that received trastuzumab ($P<0.01$, Figure 1B). More importantly, when cells were treated with trastuzumab at gradient concentrations, the median inhibition concentration (IC50) of trastuzumab was much higher for SKBR-3R cells (0.93 mg/mL) when compared to SKBR-3 cells (0.30 mg/mL). BT474R cells also showed much higher resistance to trastuzumab than did BT474 cells (0.88/0.31, Figure 1C). qRT-PCR showed that AGAP2-AS1 was significantly upregulated in breast cancer cells when compared with MCF-10A cells (Figure 1D). Interestingly, a dramatically increased expression of AGAP2-AS1 was identified in SKBR-3R and BT474R cells when compared to SKBR-3 and BT474 cells, respectively (Figure 1E).

Knockdown of AGAP2-AS1 resensitized trastuzumab resistance in breast cancer cells

To investigate the functional role of AGAP2-AS1 in trastuzumab resistance, we silenced AGAP2-AS1 by generating 3 small interfering RNAs against AGAP2-AS1. As shown in Figure 2A,

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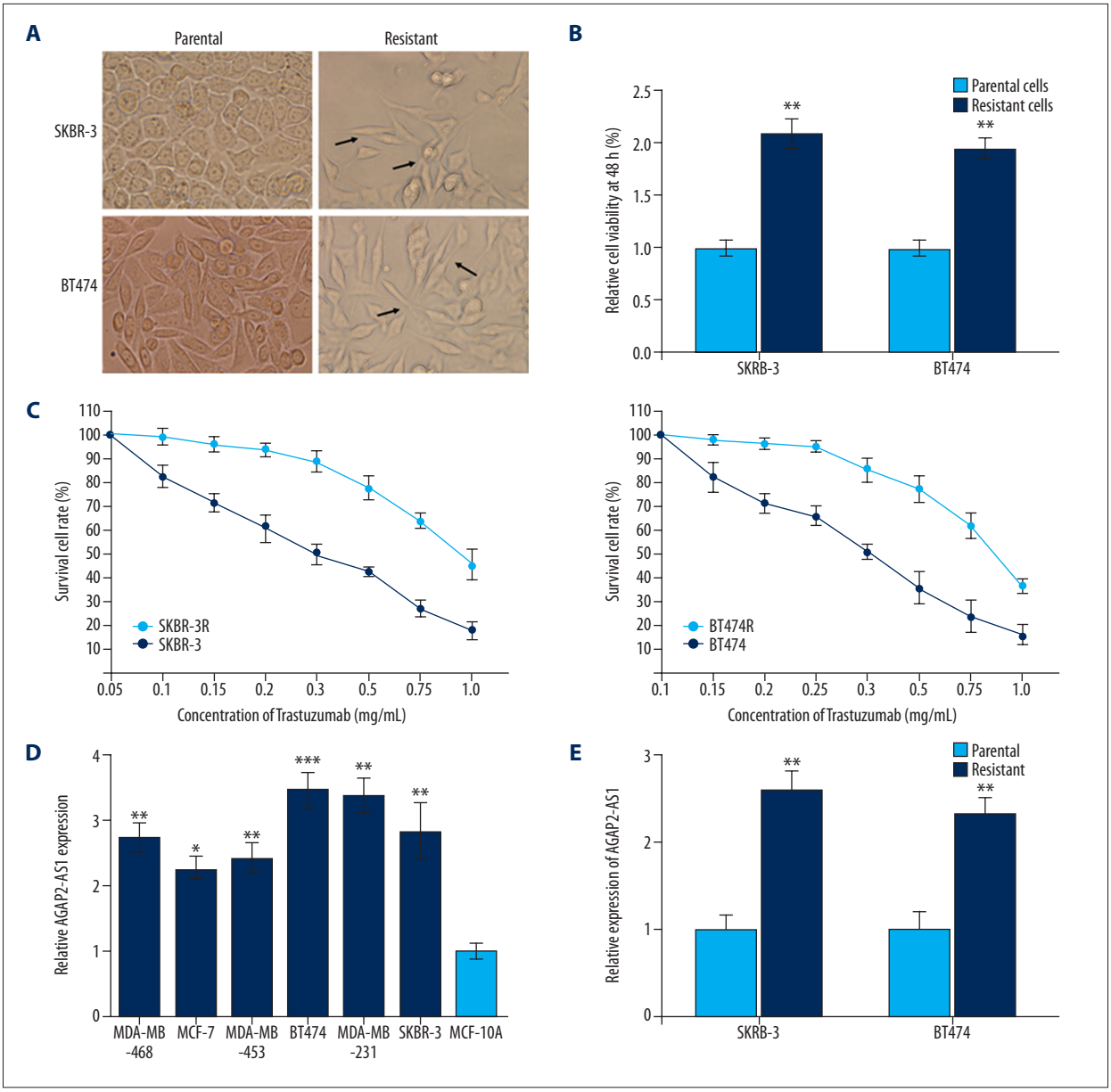

Figure 1. Trastuzumab resistance induces high expression of AGAP2-AS1 in breast cancer. (**A**) The established trastuzumab-resistant cell lines showed specific morphologic changes, including decreased cell polarity and cell interaction, and increased pseudopodia formation (as indicated by arrows). (**B**) The cell viability was measured by using CCK8 (cells treated with trastuzumab for 48 h, ** P<0.01). (**C**) The cell survival rate was determined by CCK8 assay in cells cultured with trastuzumab at different concentrations. (**D**) The expression levels of AGAP2-AS1 in indicated cell lines were measured with qRT-PCR assay, P<0.05, ** P<0.01 and *** P<0.001. (**E**) qRT-PCR determination of AGAP2-AS1 expression in trastuzumab-resistant cells and parental cells, ** P<0.01 compared to parental cells.

si-AGAP2-AS1#2 showed the best silencing efficiency and was used for the subsequent loss-of-function assays. The transfection efficiency was validated by the GFP label (Figure 2B). Cell viability assay indicated that knockdown of AGAP2-AS1 enhanced the cell death induced by trastuzumab treatment (Figure 2C). In addition, flow cytometry experiments clearly revealed that silencing of AGAP2-AS1 promoted trastuzumab-induced cell apoptosis when compared with control cells (Figure 2D). Knockdown of AGAP2-AS1 increased the trastuzumab-induced DNA fragmentation of SKBR-3R and BT474R cells (Figure 2E).

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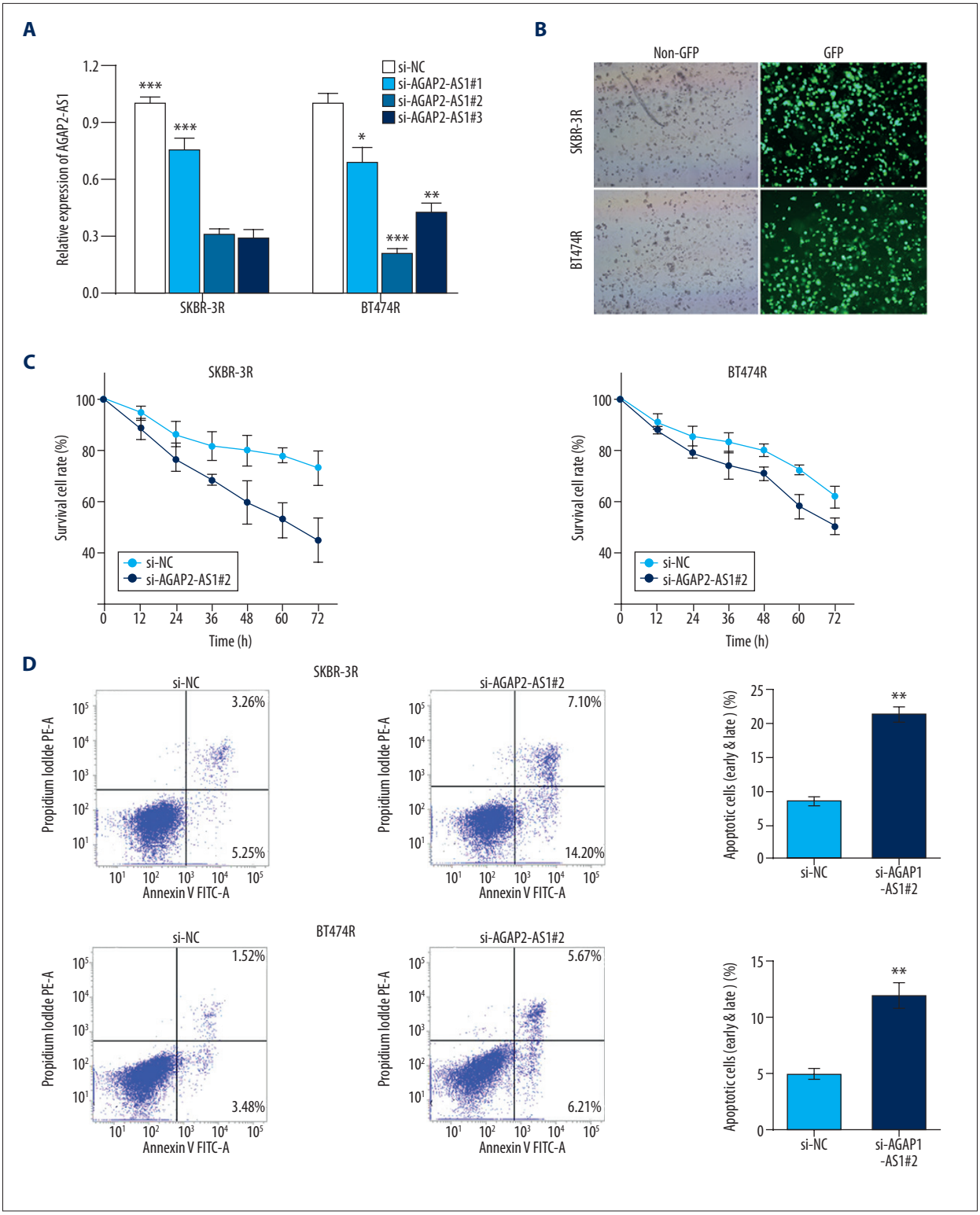

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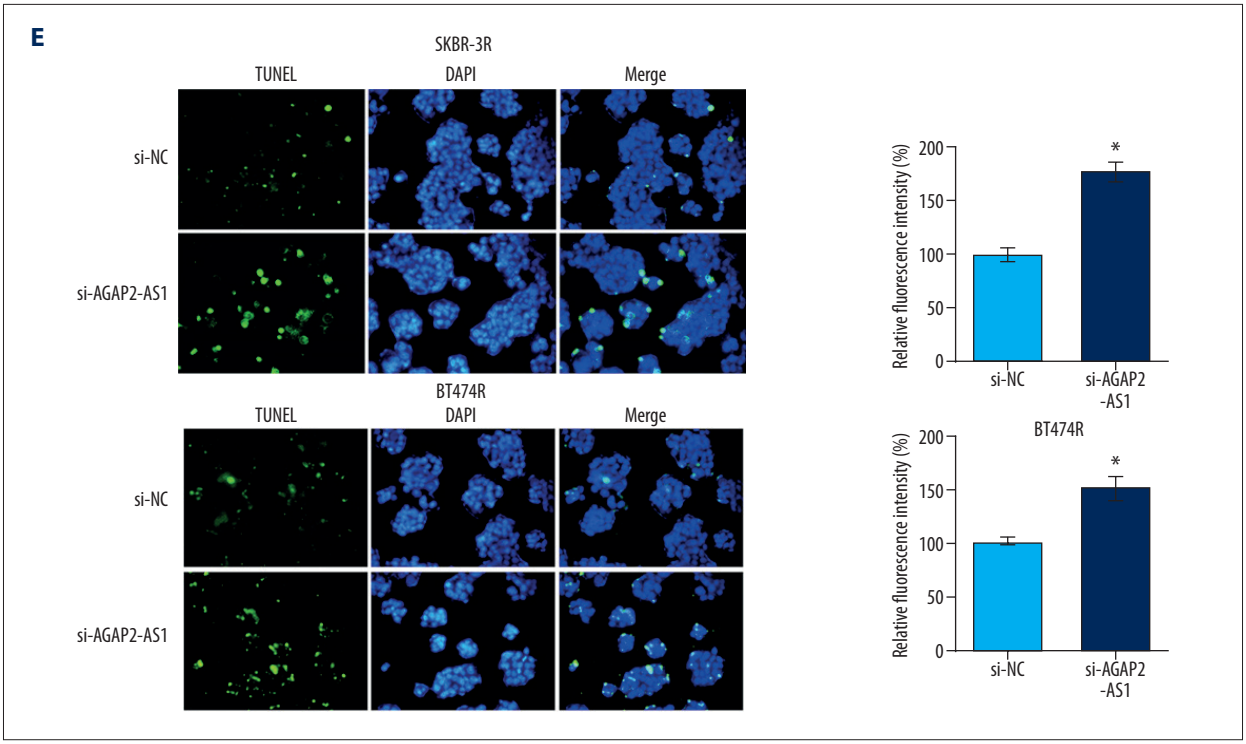

Figure 2. AGAP2-AS1 promoted trastuzumab resistance of breast cancer cells. (**A**) Three siRNAs against AGAP2-AS1 were generated and transfected accordingly, * P<0.05; ** P<0.01; *** P<0.001. (**B**) The transfection efficiency was shown by labeling cells with GFP. (**C**) Cell viability was evaluated by performing CCK8 assay in cells silenced with AGAP2-AS1 or not. (**D, E**) Cell apoptosis induced by trastuzumab was determined by using flow cytometry (**D**) and TUNEL assay (**E**), * P<0.05, ** P<0.01 compared to si-NC.

AGAP2-AS1 is excreted by incorporating into exosomes

We predicted the sub-cellular location of AGAP2-AS1 with lncLocator online software (*http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/*). Figure 3A shows that AGAP2-AS1 was mainly distributed in exosome. As RNA contained in exosome is resistant to RNase treatment, we treated the culture medium with RNase. Figure 3B shows that RNase treatment had no effect on AGAP2-AS1 expression, but a significantly downregulated AGAP2-AS1 was verified when treated with RNase and Triton ×100 simultaneously, indicating that AGAP2-AS1 may be secreted through incorporating into exosomes. Furthermore, TEM scanning validated the existence of exosomes in cell culture medium (Figure 3C). In addition, 78% of the exosomes were 40–150 nm (median value: 58.33 nm) (Figure 3D). The expression of HSP70 and CD9 proteins, which are well-known exosome markers [17,18], are enriched in culture medium, as evidenced by the Western blot experiment (Figure 3E). To directly validate that AGAP2-AS1 is detectable in exosomes, we isolated exosomes, followed by qRT-PCR assay. We found that AGAP2-AS1 is detectable in exosomes, and is upregulated in culture medium from SKBR-3R and BT474R compared to culture medium from parental cells (Figure 3F).

hnRNPA2B1 mediates the incorporation of AGAP2-AS1 into exosomes

Previous studies have demonstrated that RNA-binding hnRNPA2B1 can mediate the packaging of RNAs into exosomes [19]. To clarify whether hnRNPA2B1 is essential for AGAP2-AS1 loading into exosomes, we performed RIP assay with antibody against hnRNPA2B1. As shown in Figure 4A, AGAP2-AS1 was pulled down by hnRNPA2B1 antibody in both SKBR-3R and BT474R cells (Figure 4A). By overexpressing or silencing hnRNPA2B1 (Figure 4B), we revealed that exosomal AGAP2-AS1 expression was upregulated by hnRNPA2B1 overexpression and suppressed by hnRNPA2B1 knockdown in SKBR-3R cells (Figure 4C), which proves the critical role of hnRNPA2B1.

Exosome-mediated transfer of AGAP2-AS1 disseminates trastuzumab resistance

To prove that AGAP2-AS1 promotes trastuzumab resistance through packaging into exosomes, we first examined whether the recipient cells could take up AGAP2-AS1-containing exosomes. By labeling with PKH26 dye and incubating exosomes with trastuzumab-sensitive parental cells, we detected a strong PKH26 red signal (Figure 4D). Subsequently, we tested

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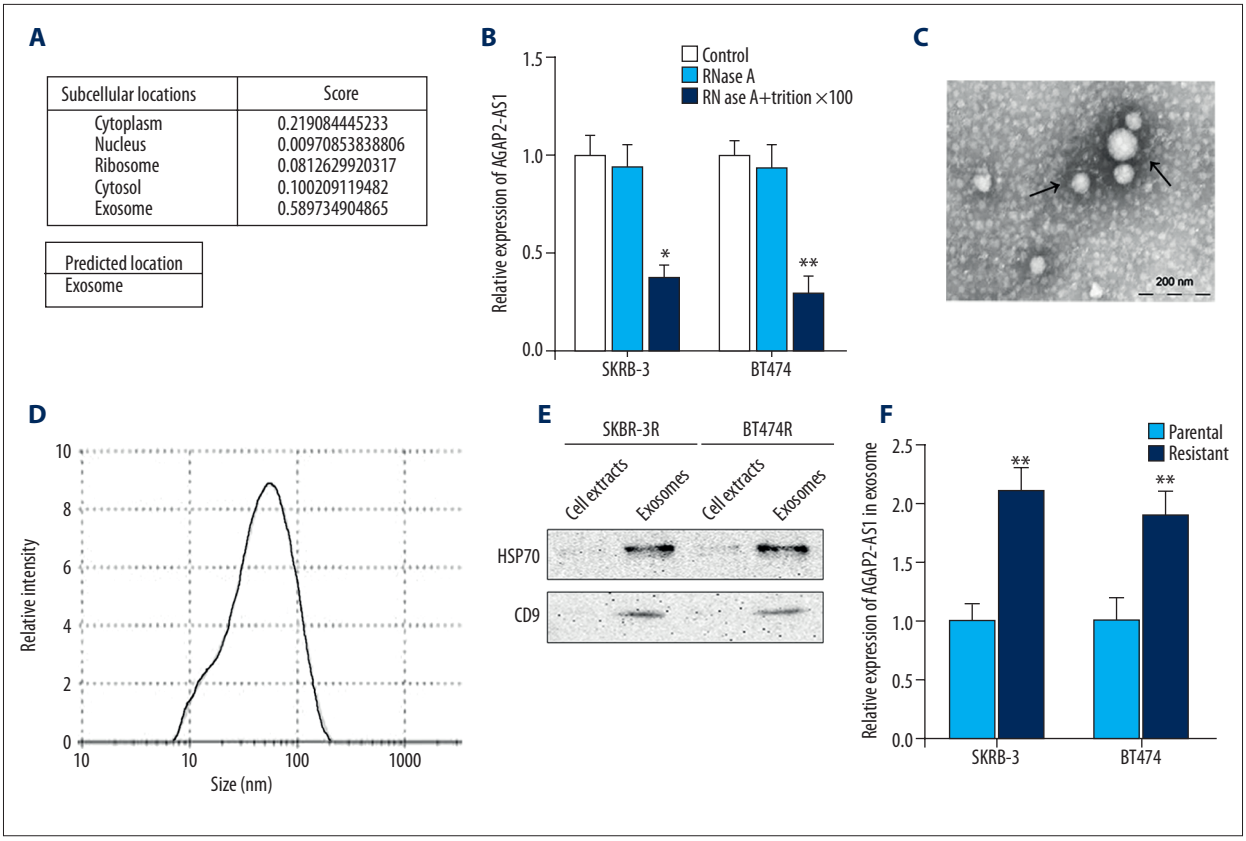

Figure 3. AGAP2-AS1 is excreted through incorporating into exosomes. (**A**) LncLocator (*http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/*) online software was used to predict the relative scoring of AGAP2-AS1 in different subcellular locations. (**B**) lncRNA AGAP2-AS1 expression was measured with qRT-PCR, * P<0.05; ** P<0.01 compared to control. (**C**) The exosomes released from breast cancer cells were imaged and presented by TEM scanning. (**D**) A Zetasizer device was used to determine the size distribution of purified exosomes. (**E**) Western blot analysis was performed to verify the existence of HSP70 and CD9 protein. (**F**) The expression of AGAP2-AS1 contained in exosomes was measured by qRT-PCR, ** P<0.01.

whether the AGAP2-AS1-containing exosomes fused with recipient cells, as previously reported [20,21]. We measured the relative expression of intracellular AGAP2-AS1 of recipient cells via qRT-PCR, and identified upregulated AGAP2-AS1 expression compared to control cells (Figure 4E), which is consistent with our hypothesis. More importantly, the recipient cells treated with AGAP2-AS1-containing exosomes showed increased trastuzumab resistance. However, this effect was reversed by silencing of AGAP2-AS1 (Figure 4F). Overall, we proved that lncRNA AGAP2-AS1 promotes the trastuzumab resistance of breast cancer cells through incorporating into exosomes.

Discussion

Numerous studies have been performed to better understand the biological characteristics and functional roles of lncRNAs in cancer chemoresistance; however, their specific roles are still largely unknown, such as trastuzumab resistance in breast cancer [22]. Thus, better understanding the mechanism of trastuzumab resistance in breast cancer is essential to optimizing current therapeutic strategies and further improve clinical outcomes. In the present study, we demonstrated the function and mechanism of lncRNA AGAP2-AS1 in trastuzumab resistance. We built 2 trastuzumab-resistant breast cancer cell models, on which basis we discovered that AGAP2-AS1 was mainly located in exosomes under extracellular conditions. More importantly, we showed that high-AGAP2-AS1-expressing exosomes isolated from trastuzumab-resistant cells could promote trastuzumab resistance of sensitive cells. Taken together, our findings show that AGAP2-AS1 can disseminate trastuzumab resistance in breast cancer cells through packaging into exosomes.

The HER2 receptor is transcribed from the HER2 gene and is expressed on the surface of epithelial cells to play key roles in breast cancer progression. An elevated number of HER2 receptors is correlated with a worse survival in cancers, including breast cancer [23]. The advent of ant-HER2 therapy has dramatically reduced disease progression and prolonged the survival

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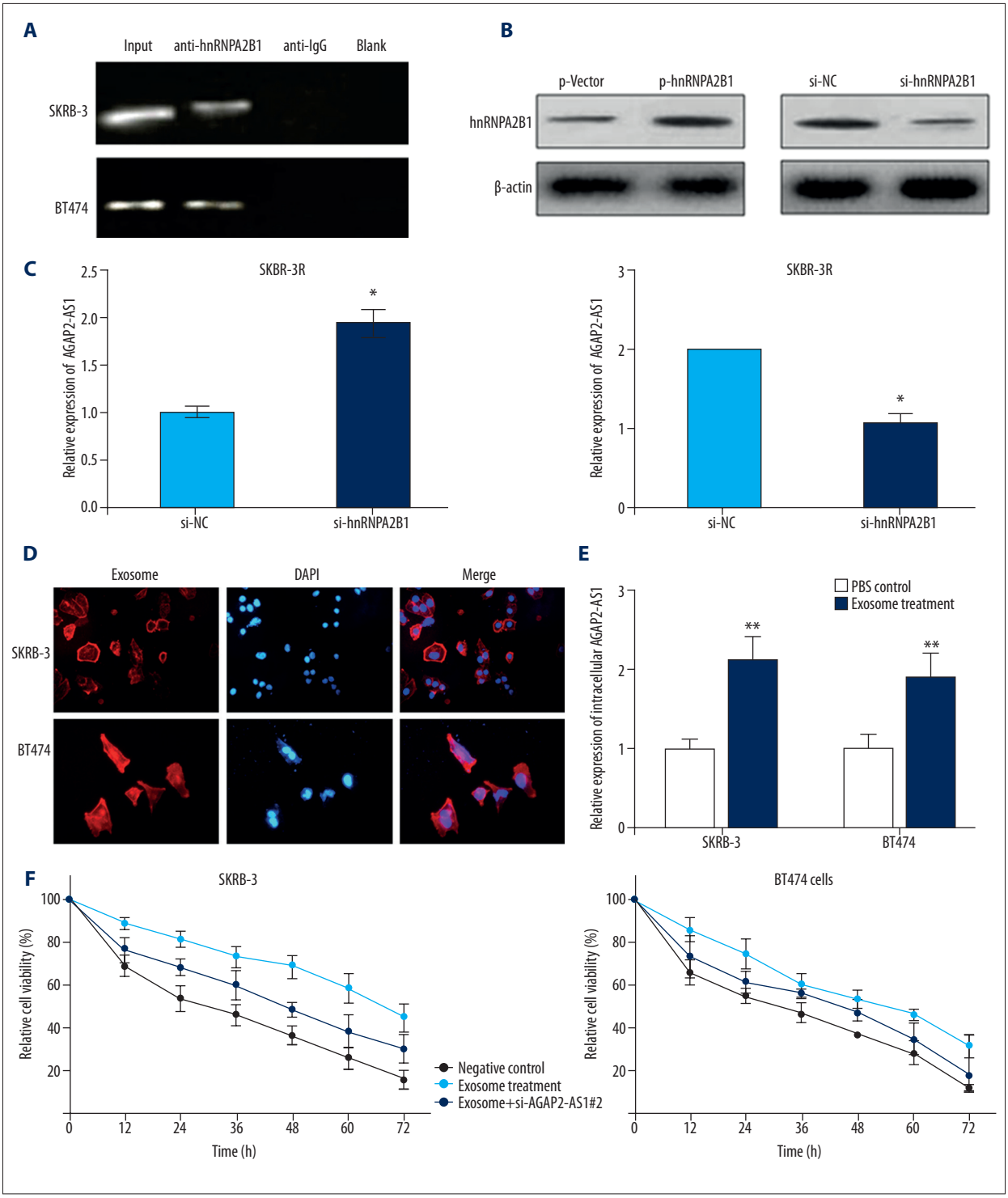

Figure 4. hnRNPA2B1 mediates the incorporation of AGAP2-AS1 into exosomes, which disseminates trastuzumab resistance. (**A**) The enrichment of AGAP2-AS1 by hnRNPA2B1 was measured by performing RNA immunoprecipitation (RIP). (**B**) Specific vectors were used to dysregulate the expression of hnRNPA2B1 in breast cancer cells. (**C**) qRT-PCR was used to detect the influence of hnRNPA2B1 on expression of exosomal AGAP2-AS1, * P<0.05 compared to control vector. (**D**) The existence of exosomes in recipient cells was tracked by labeling with PKH26 dye. (**E**) qRT-PCR was performed to determine the expression level of intracellular AGAP2-AS1 in cells co-cultured with exosome or not, ** P<0.01. (**F**) Cell viability of cells in indicated groups was detected by performing CCK8 assay.

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time of HER2+ cancer patients [24]. Slamon et al. reported that HER2 overexpression is a useful biomarker for early diagnosis and treatment prognosis [25]. However, due to differences in environmental factors, the outcome and therapeutic efficiency of anti-HER2 targeted therapy vary, and some resistance develops, which has become a major clinical problem. Trastuzumab, a first-line treatment of advanced HER2+ breast cancer patients, has paved the way for the era of targeted therapy; however, the median duration of response is modest due to various mechanisms of resistance during the course of treatment [26]. The precise mechanisms underlying trastuzumab resistance are not fully understood.

The trastuzumab treatment-induced resistance may be due to the dysregulation of lncRNAs, and lncRNA expression may be critical for the treatment of HER2+ breast cancer [27]. lncRNA AGAP2-AS1 was previously reported to be an oncogene in gastric cancer and lung cancer [10,11,28], but its expression and function in breast cancer have received little research attention. Our data suggest that AGAP2-AS1 is dysregulated in trastuzumab-resistant cells, and plays key roles in trastuzumab resistance through packaging into exosomes. Extracellular vesicles, such as exosomes, have recently emerged as critical cell-to-cell mediators for transferring important oncogenic or anti-oncogenic information in cancer. Studies have proved that bioactive proteins or RNAs contained in exosomes could stay stable and effectively exert multiple biofunctions [29]. Moreover, the identification of exosomes as information carriers has led us to find its critical roles in the field of drug delivery, as it may be possible to harness these vesicles for therapeutic delivery of miRNA, siRNA, mRNA, lncRNA, peptides, and synthetic drugs [30]. Among the functional links between exosomes and molecular genes, a focus on lncRNA within exosomes may uncover new regulatory mechanisms [31]. As an initial step in this endeavor, our study proved that extracellular AGAP2-AS1 is contained in exosomes, by systematically using 2-steps approaches.

Exosome-mediated interactions between different cancer cells generate signals for cancer progression, therapy resistance, and inflammatory responses [32]. After validating the direct interaction between AGAP2-AS1 and exosomes, we proved that the transport of exosomes can disseminate signals from trastuzumab-resistant cells to recipient-sensitive cells. Importantly, knockdown of AGAP2-AS1 abrogated this effect, which suggests that high AGAP2-AS1 levels within exosomes play a role in trastuzumab resistance. The secretion of exosomal RNAs was reported to be highly selective among different cell types [33], but the mechanisms controlling specific RNA sorting into extracellular exosomes are still poorly understood. Human hnRNPA2B1 is an RNA-binding protein that plays important roles in many biological processes, including maturation, transport, and gene regulation lncRNAs, and controls movement of miRNAs into exosomes by forming RNA-RBP complexes [34]. In this study, we found that hnRNPA2B1 is essential for the process of loading AGAP2-AS1 into exosomes. However, the precise regulatory mechanism of hnRNPA2B1 needs further investigation.

Conclusions

In summary, our data revealed that enhanced expression of lncRNA AGAP2-AS1 promotes trastuzumab resistance of breast cancer cells by incorporating into exosomes in an hnRNPA2B1-dependent manner. This study of the functional role of lncRNAs contained in exosomes will lead to discovery of new therapeutic strategies for cancer patients.

Ethics approval and consent to participate

The study protocol was approved by the Research Ethics Committee of Taizhou Hospital of Zhejiang Province.

Conflicts of interest

None.

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