Exosomes synthesizing HER2 miRNA and engineered to adhere to HER2 on surface tumor cells exhibit enhanced anti-tumor activity

Lei Wang  
Guangzhou Medical University

Xusha Zhou  
Guangzhou Medical University

Weixuan Zou  
Guangzhou Medical University

Yinglin Wu  
Guangzhou Medical University

Jing Zhao  
Shenzhen International Institute for Biomedical Research

Xiaoqing Chen (✉ chennancy@siitm.org.cn)  
Shenzhen International Institute for Biomedical Research  https://orcid.org/0000-0001-5225-4676

Grace Guoying Zhou  
Guangzhou Medical University

Research

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Abstract

Introduction: Exosomes are small vesicles derived from cellular membranes with a diameter of 50–150 nm. Exosomes are considered to be ideal drug delivery systems with a wide range of application in various diseases including cancer. However, nonspecific delivery of therapeutic agents by exosomes in vivo remains a challenging. Human epidermal growth factor receptor 2 (HER2) is an epidermal growth factor receptor tyrosine kinase. Overexpression of HER2 is usually associated with cancer survival and progression in various cancers. In this study, we aimed to develop the novel exosomes with dual HER2-targeting ability as nanoparticle delivery vehicle to enhance anti-tumor efficacy in vivo.

Results Here we report the construction of two kinds of exosomes carrying designed miRNA to block the synthesis of HER2 and as a consequence to kill the tumor cells. The 293-miR-HER2 exosomes package and deliver designed miRNA to cells to block HER2 synthesis. These exosomes kill cancer cells dependent on HER2 for survival but have no effect on cells lacking of HER2 or which were engineered to express HER2 but do not depend on it for survival. The 293-miR-XS-HER2 exosomes carry one more peptide, which enables the exosome to adhere HER2 on the surface of the cancer cells. In consequence these exosomes preferentially enter and kill cells exhibiting HER2 on their surface. The 293-miR-XS-HER2 exosomes are significantly more effective in shrinking the size of HER2-positive tumors implanted in mice than the 293-miR-HER2 exosomes.

Conclusion Collectively, as novel anti-tumor drug delivery vehicles, the HER2 dual-targeting exosomes has increased target-specific delivery efficiency, which can be further utilized to develop new nanoparticle targeted therapy.

Introduction

In this communication we report the construction of exosomes designed to preferentially enter and ultimately kill cancer cells dependent on HER2 for their survival. Relevant to this report are the following:

HER2 (human epidermal growth factor receptor 2) is a member of the human epidermal growth factor receptor family [1–3]. The protein is expressed at high levels on the surface of human breast cancer cells. Its role in the oncogenesis of these cells is supported by numerous observations [4–6]. Thus treatment of HER2 positive cells with antibody was found to block G1-S progression of the cell cycle, decreases levels of cyclin-dependent kinase 2 (CDK2), cyclin E, and CDK6 proteins and reduces cyclin E-CDK2-associated kinase activity [7–10]. Administration of anti-HER2 antibody is a standard of treatment of human HER2 positive breast cancer patients [11–13].

Exosomes are small extracellular vesicles averaging 100–150 nm in diameter. They serve as a means of intercellular communication. Typically they consist of structural proteins as well as selected proteins, miRNAs, mRNAs, and long noncoding RNAs [14–17]. The RNAs exhibit a short nucleotide sequence recognized by the proteins that transport the RNAs to the cytoplasm and package them into exosomes [18–20]. Thus in an earlier report this laboratory designed an miRNA targeting a major herpes simplex
virus regulatory protein. As predicted by the nucleotide packaging signal the miRNAs were packed in exosomes and on exposure to infected cells significantly reduced virus yields [21].

This report consists of three parts. First, we report on the construction of a miRNA targeting the synthesis of HER2 both in cells constitutively expressing HER2 and in cells transfected with a plasmid encoding HER2. Second, we report that the miRNA targeting the synthesis of HER2 reduced the viability of HER2 positive cancer cells both in cell culture and in implanted tumors. Lastly, we enhanced the anti-tumor activity of the exosomes by binding to the exosome surface a ligand with affinity for the HER2 on the surface of tumor cells. The peptide linked exosomes binds to the cell surface and preferentially enter HER2 positive cells.

Results

Design and construction of exosomes carrying miRNAs capable of suppressing the synthesis of HER2.

The objective of the first series of experiments was to design miRNAs targeting HER2. To this end we have constructed 7 miRNAs from which to select a miRNA that was the most effective in blocking the synthesis of HER2 in a HER2 positive cancer cell line and in a cell line transfected with a plasmid encoding HER2. The miRNAs were cloned in a miRNA expression vector named pcDNA6.2-GW/EmGFP-miR-neg downstream of an open reading frame encoding EGFP as described in Materials and Methods.

In the initial screening, SK-OV-3, a HER2-high expressing cell line and HEp-2, a HER2 negative cell line transfected with a plasmid encoding HER2 were transfected with the plasmids encoding the miRNAs. Of the 7 miRNAs tested the miRNA No.1 and No.4 were most effective in suppressing the accumulation of HER2. On the basis of these results, miRNAs No.1 and No.4 were selected for further studies and designated as miR-HER2-1 and miR-HER2-4 respectively.

Next, we modified the two miRNAs by the addition of sequences embodying exosome-packaging-associated motifs (EXO-motifs). The efficacy of suppressing HER2 by miR-HER2-E1 and miR-HER2-E4 was then retested in SK-OV-3 cells and in HEp-2 cells expressing HER2. On the basis of the results shown in Fig. 1a and b, we selected miR-HER2-E1 for further studies. The sequences of miR-HER2-1, miR-HER2-4, miR-HER2-E1 and miR-HER2-E4 are listed in Materials and Methods.

Production and characterization of exosomes containing miR-HER2-E1. In all experiments described in this report we used exosomes produced in HEK-293 cells. The properties of these exosomes were investigated as follows. Briefly:

HEK-293 cells were remain untreated (293) or transfected with 10 µg of non-target (NT) or miR-HER2-E1 plasmids. After 52 h the extracellular medium was collected and the exosomes were purified as described in Material and Methods. The purified exosomes were tested for size distribution and for the presence of proteins associated with exosomes. Typically, exosomes contain Alix, CD9, Annexin V, Flotillin-1, and TSG101.
To test for protein content, purified exosomes were lysed by RIPA lysis buffer. Next, 15 µg of solubilized exosome protein subjected to electrophoresis in denaturing gels, and reacted with antibodies to above proteins (Fig. 2a). As expected the exosome proteins were present in purified exosomes derived from HEK-293, NT or miR-HER2-E1 transfected cells.

Exosomes purified from HEK-293 cells transfected with miR-HER2-E1 were analyzed by Izon's qNano technology as described in Materials and Methods. The results (Fig. 2b) show that the size distributions of exosomes generated by the cell lines transfected with NT or miR-HER2-E1 were nearly identical.

Figure 2c shows the expression of mature miR-HER2-E1 by qPCR analysis. As expected the exosomes were purified from parental HEK-293 cells and the NT-transfected cells did not contain detectable amounts of miR-HER2-E1.

**Exosome-delivered miR-HER2-E1 downregulated the accumulation of HER2 and reduced the viability of cells expressing high levels of HER2.** In this series of experiments, we examined whether miR-HER2-E1 produced in HEK-293 cells and delivered via exosomes effectively blocked the accumulation of HER2. We report 2 series of experiments.

In the first replicate cultures each containing $2.5 \times 10^5$ SK-OV-3 cells were exposed for 72 h to (i) 0.1 µg, 5 µg, or 20 µg of purified exosomes carrying miR-HER2-E1, or (ii) 20 µg of purified exosomes produced in NT transfected HEK-293 cells, or (iii) remain untreated (Con). The HER2 cell lysates were subjected to electrophoresis in denaturing gels and reacted with antibodies to HER2. Figure 3a shows that the accumulation of HER2 decreased in SK-OV-3 exposed to exosomes containing miR-HER2-E1 in a dose dependent manner.

The second series of experiments was designed to determine whether the miR-HER2-E1 expressing exosomes were also able to suppress the accumulation of exogenous HER2. HEp-2 cells were transfected with a plasmid encoding HER2 for 36 h, then exposed for 36 h to 20 µg of purified exosomes produced in miR-HER2-E1 or NT transfected HEK-293 cells. Figure 3b shows that the expression of exogenous HER2 decreased in cells exposed to exosomes containing miR-HER2-E1.

**HER2 positive cancer cells respond to treatment with anti-HER2 antibody suggesting that HER2 is an essential cell surface protein.** To test the hypothesis that the exosome-delivered miR-HER2-E1 kill HER2 dependent cells by blocking the replenishment of the protein in HER2-positive SK-OV-3, HCT116 cancer cells and HER2-negative MDA-MB-231, HEp-2 cells were each seeded in 96-well plates and exposed to 1 µg of exosomes purified from HEK-293 cells transfected with miR-HER2-E1. After 72 h of incubation, the relative cell viability was determined by the CCK8 assay. The obtained values were normalized with respect to the values of mock-treated control group as described in Materials and Methods. The results (Fig. 4) show the following:

1. SK-OV-3 cells exposed to exosome carrying miR-HER2-E1 exhibited 60% cell viability as compared to the viability of SK-OV-3 cells treated with NT-packaged exosomes;
2. HCT116 cells exposed to exosomes carrying HER2-E1 exhibited 40% cell viability as compared to 90% cell viability of HCT116 cells treated with NT-packaged exosomes;

3. HEp-2 and MDA-MB-231, the two HER2-negative cells exhibited 90–100% cell viability on exposure to exosomes carrying miR-HER2-E1.

These results suggest the following: (i) miR-HER2-E1 kills HER2 dependent cells by blocking the replenishment of the protein. (ii) miR-HER2-E1 had no effect on the viability of cells that were not dependent on HER2 for viability.

**Anti-tumor efficacy of miR-HER2-E1 delivered by administration of exosomes** in vivo. In this series of experiments Balb/c derived nude mice were injected subcutaneously into flanks with $5 \times 10^6$ SK-OV-3 cells (HER2 positive), HCT116 (HER2 positive) or MDA-MB-231 (HER2 negative) cells. Once the tumors averaged 90 mm$^3$ in size they were injected intratumorally with 50 µl containing 10 µg of exosomes purified from miR-HER2-E1, or NT transfected HEK-293 cell 6 times in total on days 1, 4, 7, 10, 13, 16 respectively. The size of tumors was measured on days 1, 4, 7, 10, 13, 16, 19 and 22. The results (Fig. 5a, b and c) show that the exosomes carrying miR-HER2-E1 caused a reduction in the volume of SK-OV-3 tumors (Fig. 5a) or in HCT116 tumors (Fig. 5b), but not in tumors induced by MDA-MB-231 tumor cells (Fig. 5c). These results are consistent with the results obtained in cell culture studies.

**Enhancement of anti-tumor activity by targeting exosome to HER2 positive cells.** The experiments described earlier in the text showed that exosomes carrying miR-HER2-E1 entered into both HER2 positive and HER2 negative cells ultimately killed only HER2 positive cells. The objectives of the studies described below were to enhance the entry of the exosomes into HER2 positive cells. To achieve this objective, we constructed two cell lines designed to produce novel exosomes. The 293-miR-XS HER2 exosome carried on their surface a peptide which enabled exosome to adhere to the HER2 on the surface of HER2 positive cells. The 293-miR-XS exosome lacked the HER2 adhesion peptide. Both exosomes packed miR-HER2-E1. Details of the construction and properties of the two cell lines are described in *Materials and Methods*.

Two series of control experiments are relevant here. First, verified the presence of miR-HER2-E1 in exosomes produced in both cell lines. As illustrated in Fig. 6a, the exosomes produced by both cell lines carried miR-HER2-E1 as determined by analyses of cell pellets and purified exosomes.

Next, the exosomes produced by the two cell lines were purified and tested by ELISA to verify that they differed in their ability to adhere to HER2 protein. In brief, exosomes purified from 293-miR-XS and 293-miR-XS-HER2 stable cell lines were coated onto the 96-well ELISA plates. After removal of the coating solution and blocking nonspecific binding by bovine serum albumin (BSA), the coated exosomes were incubated with HER2 protein, and then exposed to HRP-conjugated rabbit anti-HER2 antibody. The plates were then rinsed again, followed by exposure to tetramethyl benzidine substrate (TMB) for color development. The reaction was stopped by the addition of a stop solution. Plates were read on a BioTek microplate reader at a wave length of 450 nm. As shown in Fig. 6b, purified exosomes produced by 293-miR-XS-HER2 preferentially bound HER2 protein.
Anti-tumor efficacy of exosomes carrying miR-HER2-E1 and adhering to HER2. In this series of experiments nude mice derived from Balb/c were injected subcutaneously into flanks with $5 \times 10^6$ SK-OV-3 cells. Tumors averaging $80 \text{ mm}^3$ were injected intravenously on days 1, 4, 7, 10, 13, 16, 19 and 22 with 3 µg/animal (Fig. 7a) or 30 µg/animal (Fig. 7b) of exosomes purified from 293-miR-XS-HER2, 293-miR-XS or parental HEK-293 cells. The sizes of tumors were measured on days 1, 4, 7, 10, 13, 16, 19, 22, 25 and 28.

The results of these experiments are shown in Fig. 7a and b. The results show that exosomes purified from 293-miR-XS-HER2 cells were significantly more effective in reducing the growth of HER2-positive tumors as compared to exosomes purified from HEK-293 or 293-miR-XS cells. The results also show that the shrinkage of tumors injected with 3 µg or 30 µg of 293-miR-XS-HER2 exosomes/animal was virtually identical suggesting that the 3 µg dose was close to or exceeded the amounts required to kill the susceptible cells.

Discussion

The studies presented in this report are the culminations of two discoveries made years ago. The first was the discovery that cells synthesize small RNAs appropriately designated as micro or miRNAs and whose function is to bind to and terminate the translation of specific mRNAs. Control of specific functions by miRNAs is widespread as illustrated by the observation that viruses also encode miRNAs. Thus HSV encodes a diversity of miRNAs including miRNAs made late in infection and which regulate virus yields [22, 23].

The second major discovery centered on communication between cells. It was known for many years that neurons communicate via small vesicles [24, 25]. In more recent times numerous studies have shown that cells communicate by way of small extracellular vesicles or exosomes carrying mRNAs, miRNAs, long on coding RNAs and proteins [14–17]. These vesicles are excreted into extracellular medium by donor cells and taken up by recipient cells. The developments that led to the present studies are twofold. Foremost the design and production of miRNAs directed to specific mRNAs became commonplace. Second and equally important, numerous studies have shown that the packaging of RNAs unto exosome is not random but based on short nucleotide sequences embedded into the RNAs [18–20]. In consequence current technology enables the selective packaging of miRNAs designed to target a specific mRNA. For example, this laboratory has shown that a miRNA designed to target the mRNA encoding a major HSV regulatory protein could be efficiently packaged into exosomes and on delivery to infected cells significantly impacted on viral replication [21].

This report significantly extends the earlier studies. The target in this study was the synthesis of HER2, a protein that defines the oncogenicity of a type of human breast cancer. In cancer cells HER2 is located on the cell surface. Its role is implicit from the observation that anti HER2 antibodies selectively kill HER2 positive cancer cells [26]. Our studies showed that a miRNA designed to target HER2 mRNA and delivered to HER2 positive cells by exosomes blocked the replenishment of HER2 and ultimately killed the cells.
The data generated in this study also showed that the miRNA had no effect on cells that did not express HER2 or that were induce to express HER2 by transfection of HER2 mRNA.

This study went one step further. Tumors are not homogeneous and are likely to consist of both HER2 positive cancer cells as well as HER2 negative cells. While our studies indicate that exosomes carrying miRNA against HER2 have no effect on HER2 negative cells it was nevertheless desirable to increase the uptake of exosomes carrying the HER2 miRNAs by HER2-positive tumor cells. To this end we modified the exosomes to exhibit on their surface a peptide that bound the exosomes to the HER2 on the surface of cancer cells. In consequence we significantly enhanced the uptake of exosomes carrying the miRNAs directed against HER2 by HER2 positive cells.

**Conclusion**

Taken together, our results indicate that as proof of concept, dual targeting exosomes 293-miR-XS-HER2 have been generated and have an increased anti-tumor activity in vivo by systemic delivery. HER2 dual targeting exosomes 293-miR-XS-HER2 was achieved in two steps. Firstly, a HER2 binding peptide is displayed on its membrane, which enable specific HER2 positive cancer entry. In the second step, designed miRNA specific targeting HER2 is released to downregulate HER2 protein expression. These data clearly indicate that as novel anti-tumor drug delivery vehicles, the HER2 dual-targeting exosomes has increased target-specific delivery efficiency, which can be further utilized to develop new nanoparticle targeted therapy.

**Materials And Methods**

**Purchased cell lines.**

HEp-2 cell line (Human laryngeal carcinoma cell) was purchased from ATCC (American Type Culture Collection). HEK-293 cell line (Human embryonic kidney 293 cells) and SK-OV-3 cell line (Human ovarian epithelial cancer cells) were purchased from the Cell Bank of the representative culture preservation committee of the Chinese Academy of Sciences (Shanghai, China). HCT116 (Human colorectal carcinoma cells) and MDA-MB-231 (Human breast cancer cell) cells were kindly provided by Professor Jun Du (Sun Yat-sen University, Guangzhou, China). HEp-2 cells were cultured in DMEM (high glucose) supplemented with 5% (vol/vol) fetal bovine serum (FBS). HEK-293 and MDA-MB-231 cells were maintained in DMEM (high glucose), containing 10% (vol/vol) FBS. SK-OV-3 cells were cultured in McCoy’s 5A Media supplemented with 10% (vol/vol) FBS. All culture media contained 100 U/ml of penicillin, and 100 µg/ml of streptomycin. The cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C.

**Generation of Stable Cell Lines.**
The stable cell line 293-miR-HER2 expressing miRNA targeting HER2 was generated by transfection of the miR-HER2-E1 plasmid into HEK-293 cells. Forty-eight hours after transfection, the cells were selected by the addition of blasticidin (Solarbio Life Sciences) to a final concentration of 6 μg/ml. The cell colony with green fluorescent protein (GFP) was selected and cultured in complete medium with 6 μg/ml of blasticidin. The cell line has been monitored for the expression of GFP and miR-HER2-E1.

To generate cell line which produce exosomes adhering to the surface of HER2 positive cells, the 293-miR-HER2 cells were either infected with lentivector XSTP724PA-1 (XStamp HER2 ligand exosome HER2 receptor targeting lentivector) or infected with control lentivector XSTP710PA-1 according to manufacturer's instructions of XStamp Technology (SBI: XSTP724PA-1/XSTP710PA-1). The two cell lines were re-named as 293-miR-XS-HER2 and 293-miR-XS (control) respectively. The lentivector XSTP724PA-1 contains two copies of the HER2 ligand fused to the 5’N-terminal signal sequence leader and also fused in-frame to the 3’C-terminal C1C2 XStamp domain that directs the entire fusion protein to be displayed on the surface of secreted exosomes [27, 28]. The exosome purified from 293-miR-XS-HER2 was confirmed to its binding ability with HER2 protein by ELISA analysis.

**Antibodies.** The anti-HER2 (Cat No. #2165S) antibody was purchased from Cell Signaling Technology. The anti-GAPDH, anti-Alix, anti-CD9, anti-Annexin V, anti-Flotillin-1, and anti-TSG101 have been described elsewhere [29].

**Plasmid Construction.** The miRNA sequences targeted HER2 gene were designed using Life Technologies’ BLOCK-iT™ RNAi Designer and synthesized by Ige Biotechnology (Guangzhou, China). The synthesized miRNA fragments were digested with BamHI and XhoI restriction enzymes and cloned into the corresponding sites of pcDNA6.2-GW/EmGFP-miR-neg control plasmid (Invitrogen). The sequences of miRNAs were as follows:

- miR-HER2-1: 5’-AACTCAAGCAGGAAGGTGTTTTGGCCACTGACACCTCCCTCTGCTTGAGTT-3’
- miR-HER2-4: 5’-TGTGAGAGCCAGCTGGTTGTGTTTTGGCCACTGACTGACCTCCCTCCATGGCTCTCACA-3’
- miR-HER2-E1: 5’-AACTCAAGCAGGAAGGTGTTTTGGCCACTGACTGACCTCCCTCTGCTTGAGTT-3’
- miR-HER2-E4: 5’-TGTGAGAGCCAGCTGGTTGTGTTTTGGCCACTGACTGACCTCCCTCCATGGCTCTCACA-3’

The underlined sequences identify the mature miRNA sequence.
miR-HER2-E1 and miR-HER2-E4 are modified versions of miR-HER2-1 and miR-HER2-4 respectively in that they contain the exosome-packaging-motifs (EXO-Motifs).

The HER2 expressing plasmid contained HER2 gene 3′-UTR sequence was generated as follow: The Histagged HER2 coding was obtained by PCR using primers:
Forward: 5′-CCCAAGCTTATGGAGCTGGCGGCCTTGTG
Reverse: 5′-ATAAGAATGCGGCCGCTTATCAGTGATGGTGATGGTGATGCACTGGCACGTCCAGACCCAG

The PCR fragment was then subcloned into pcDNA3.1(+) HindIII/NotI sites to generate pHER2-His. The 3′-UTR sequence was synthesized by Ige Biotechnology (Guangzhou, China), and then digested with NotI and XbaI restriction enzymes and cloned into corresponding sites of pHER2-His.

Exosomes Isolation and Quantification. HEK-293 cells seeded in T150 flask with a density of 1 × 10^7 cells were mock transfected or transfected with 10 μg of non-target (NT) or miR-HER2-E1 plasmid. After 4 h incubation, the cells were extensively rinsed with phosphate buffered saline (PBS) and incubated with serum-free medium for an additional 48 h. For the stable cell lines, the cells seeded in T150 flask for 24 h were extensively rinsed with PBS and then incubated in serum-free medium for another 48 h. Cell-free extracellular medium containing exosomes was harvested by centrifugation at 300 × g for 10 min to remove the cells. The supernatant medium was then centrifuged at 10,000 × g for 30 min to remove dead cells and cell debris. Finally, the clear supernatant was centrifuged for 70 min at 100,000 × g to pellet the exosomes. All the centrifugation steps were carried out at 4°C. For immunoblotting, the pelleted exosomes were resuspended in RIPA buffer. For treatment of cells or mice, the pelleted exosomes were resuspended in PBS. The exosomes were quantified by a BCA assay using the Enhanced BCA Protein Assay Kit (Beyotime) according to manufacturer’s instructions.

Exosomes Size Analysis. Exosome size distribution analyses was done using the qNano system (Izon, Christchurch, New Zealand). Izon's qNano technology (www.izon.com) was employed to detect extracellular vesicles passing through a nanopore by way of a single-molecule electrophoresis. In procedure yielded accurate particle-by-particle enumeration of exosomes ranging from 75 to 300 nm in size. Specifically purified exosomes were diluted 1:10 in PBS containing 0.05% Tween-20, vigorously shaken, and measured by using an NP200 (A53942) nanopore aperture according to the manufacturer’s instructions. The results were analyzed using Izon Control Suite software v3.3 (Izon Science).

Quantitative RT-PCR for miRNA. Total RNAs from cells and suspended exosomes were isolated using TRIzol reagent (Thermo Fisher Scientific) and TRIzol LS reagent (Thermo Fisher Scientific) according to
the manufacturer’s instructions, respectively. The miRNAs tested were reverse-transcribed from 50 ng total RNA in duplicate by specific stem-loop primers as described in the TaqMan miRNA reverse transcription kit (Applied Biosystems, Inc.). The expression of miRNA was determined by real-time PCR using TaqMan Universal Master Mix II kit purchased from Applied Biosystems, Inc. miRNA copy number was normalized by comparison with cellular 18s rRNA. The primers of miR-HER2-E1 were designed according to Chen et al [30], and synthesized by Ige Biotechnology. The sequences are as follows:

miR-HER2-E1 stem loop primer:

5'-GTCGGTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTCCTCCT-3';

Forward primer: 5'- AACCAAGCAGGAAGGAGG -3';

Reverse primer: 5'- GTGCAGGGTCCGAGGT -3';

Probe: 5'-(6-FAM) TCGCACTGGATACG (MGB)-3'.

**Immunoblot Assays.** Cell pellets or purified exosomes were harvested and lysed with a RIPA lysis buffer (Beyotime) supplemented with 1 mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Beyotime) and phosphatase inhibitor (Beyotime). Cell lysates and exosomes were heat denatured, and separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The proteins were identified by incubation with appropriate primary antibody, followed by horseradish peroxidase-conjugated secondary antibody (Pierce). The Blots were exposed to the ECL reagent (Pierce) and images were captured using a ChemiDoc Touch Imaging System (Bio-Rad) and analyzed with Image Lab software. The densities of corresponding bands were quantified using Image J software.

**Cell Viability Assay.** SK-OV-3, HCT116, HEp-2 and MDA-MB-231 cells were seeded into 96-well plates at a density of $1 \times 10^4$ per well one day before exposure to exosomes. The cells were exposed to exosomes for 72 h, then CCK8 (Beyotime) assays were performed according to manufacturer’s protocol to quantify cell viability.

**Animal models.** Balb/c nude mice 6-7 weeks of age, were purchased from Vital River Laboratory Animal Technologies Co. Ltd (Beijing, China). The nude mice were injected subcutaneously into flank with $5 \times 10^6$ cells of SK-OV-3, HCT116 or MDA-MB-231 cells. Mice bearing tumors reaching volumes described in individual experiments were randomized and injected intratumorally (i.t.) or intravenously (i.v.) with purified exosomes. Tumor size was measured every three days after injection. The results are displayed as mean tumor volume (mm$^3$) ± standard deviation of 6 mice.
ELISA assays. Exosomes purified from 293-miR-XS or 293-miR-XS-HER2 stable cell lines were coated in amounts of 1 μg/well in triplicate onto the 96-well ELISA plates (Corning). After removal of the coating solution, nonspecific binding sites were blocked by incubation with 2% bovine serum albumin (BSA) at 37°C for 1 h. The plates were then rinsed, and exposed to HER2 protein (Sino Biological, China) for 2 h. The plates were rinsed and then exposed to HRP-conjugated rabbit anti-HER2 antibody (Cat No. 1004-R205, Sino Biological) for an additional 1 h. Plates were then rinsed again, followed by exposure to tetramethyl benzidine substrate (TMB) for color development. The reaction was stopped by addition of stop solution. Plates were read on a BioTek microplate reader at a wavelength of 450 nm.

**Declarations**

**Authors’ contributions**

L.W., J.Z., X.C., and G.Z. designed research; L.W., X.Z., W.Z., Y.W. performed research; L.W., X.Z., X.C. and G.Z. analyzed data and wrote the paper.

**Author details**

1School of Basic Medical Sciences, Guangzhou Medical University, Guangzhou, Guangdong 511436 China

2Shenzhen International Institute for Biomedical Research, 1301 Guanguang Rd. 3F Building 1-B, Silver Star Hi-tech Park, Longhua District, Shenzhen, Guangdong 518116, China

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**Availability of data and materials**

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

**Statement of ethics approval**

Ethics approval for the study was obtained from the Guangzhou Medical University Health Research Ethics Board and all animal experiments were performed in full compliance with the National Institute of
Health Guide for the Care and Use of Laboratory Animals.

**Competing interests**

The authors declare that they have no competing interests.

**References**

1. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science. 1989;244:707–12.

2. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol. 2001;2:127–37.

3. Wang J, Xu B. Targeted therapeutic options and future perspectives for HER2-positive breast cancer. Signal Transduct Target Ther. 2019;4:34.

4. Gutierrez C, Schiff R. HER2: biology, detection, and clinical implications. Arch Pathol Lab Med. 2011;135:55–62.

5. Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. Nat Rev Mol Cell Biol. 2006;7:505–16.

6. Moasser MM. The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis. Oncogene. 2007;26:6469–87.

7. Le XF, McWatters A, Wiener J, Wu JY, Mills GB, Bast RC Jr. Anti-HER2 antibody and heregulin suppress growth of HER2-overexpressing human breast cancer cells through different mechanisms. Clin Cancer Res. 2000;6:260–70.

8. Mittendorf EA, Liu Y, Tucker SL, McKenzie T, Qiao N, Akli S, et al. A novel interaction between HER2/neu and cyclin E in breast cancer. Oncogene. 2010;29:3896–907.

9. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med. 2005;353:1659–72.

10. Zhang K, Hong R, Kaping L, Xu F, Xia W, Qin G, et al. CDK4/6 inhibitor palbociclib enhances the effect of pyrotinib in HER2-positive breast cancer. Cancer Lett. 2019;447:130–40.

11. Larionov AA. Current Therapies for Human Epidermal Growth Factor Receptor 2-Positive Metastatic Breast Cancer Patients. Front Oncol. 2018;8:89.

12. Marty M, Cognetti F, Maraninchi D, Snyder R, Mauriac L, Tubiana-Hulin M, et al. Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. J Clin Oncol. 2005;23:4265–74.

13. Waks AG, Winer EP. Breast Cancer Treatment: A Review. Jama. 2019;321:288–300.
14. Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. Nat Rev Immunol. 2002;2:569–79.

15. Kowal J, Tkach M, Thery C. Biogenesis and secretion of exosomes. Curr Opin Cell Biol. 2014;29:116–25.

16. Zhang J, Li S, Li L, Li M, Guo C, Yao J, et al. Exosome and exosomal microRNA: trafficking, sorting, and function. Genomics Proteomics Bioinformatics. 2015;13:17–24.

17. Mittelbrunn M, Gutierrez-Vazquez C, Villarroya-Beltri C, Gonzalez S, Sanchez-Cabo F, Gonzalez MA, et al. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. Nat Commun. 2011;2:282.

18. Villarroya-Beltri C, Gutierrez-Vazquez C, Sanchez-Cabo F, Perez-Hernandez D, Vazquez J, Martin-Cofreces N, et al. Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. Nat Commun. 2013;4:2980.

19. Statello L, Maugeri M, Garre E, Nawaz M, Wahlgren J, Papadimitriou A, et al. Identification of RNA-binding proteins in exosomes capable of interacting with different types of RNA: RBP-facilitated transport of RNAs into exosomes. PLoS One. 2018;13:e0195969.

20. Squadrito ML, Baer C, Burdet F, Madema C, Gilfillan GD, Lyle R, et al. Endogenous RNAs modulate microRNA sorting to exosomes and transfer to acceptor cells. Cell Rep. 2014;8:1432–46.

21. Wang L, Chen X, Zhou X, Roizman B, Zhou GG. miRNAs Targeting ICP4 and Delivered to Susceptible Cells in Exosomes Block HSV-1 Replication in a Dose-Dependent Manner. Mol Ther. 2018;26:1032–9.

22. Han Z, Liu X, Chen X, Zhou X, Du T, Roizman B, et al. miR-H28 and miR-H29 expressed late in productive infection are exported and restrict HSV-1 replication and spread in recipient cells. Proc Natl Acad Sci U S A. 2016;113:E894–901.

23. Huang R, Wu J, Zhou X, Jiang H, Guoying Zhou G, Roizman B. Herpes Simplex Virus 1 MicroRNA miR-H28 Exported to Uninfected Cells in Exosomes Restricts Cell-to-Cell Virus Spread by Inducing Gamma Interferon mRNA. J Virol. 2019;93:01005–19.

24. Del Castillo J, Katz B. Quantal components of the end-plate potential. J Physiol. 1954;124:560–73.

25. Miller TM, Heuser JE. Endocytosis of synaptic vesicle membrane at the frog neuromuscular junction. J Cell Biol. 1984;98:685–98.

26. Baselga J, Albanell J. Mechanism of action of anti-HER2 monoclonal antibodies. Ann Oncol. 2001;12(Suppl 1):35–41.

27. Delcayre A, Estelles A, Sperinde J, Roulon T, Paz P, Aguilar B, et al. Exosome Display technology: applications to the development of new diagnostics and therapeutics. Blood Cells Mol Dis. 2005;35:158–68.

28. Hartman ZC, Wei J, Glass OK, Guo H, Lei G, Yang XY, et al. Increasing vaccine potency through exosome antigen targeting. Vaccine. 2011;29:9361–7.

29. Zhou X, Wang L, Zou W, Chen X, Roizman B, Zhou GG. hnRNPA2B1 associated with recruitment of RNA into exosomes plays a key role in HSV-1 release from infected cells. JVI. 2020;00367 – 20.
Figures

Figure 1

Down-regulation of HER2 by miR-HER2-E1 and miR-HER2-E4 in SK-OV-3 (a) and HEp-2 cells (b). SK-OV-3 cells grown in 12-well plate were transfected with 0.5 μg plasmids expressing miR-HER2-E1, miR-HER2-E4 or non-target miRNA (NT). HEp-2 cells in 12-well plate were co-transfected with 0.5 μg plasmids expressing miR-HER2-E1, miR-HER2-E4 or non-target miRNA (NT) and 0.2 μg plasmid encoding a His-tagged HER2. The cells were harvested at 72 h after transfection. HER2 protein yields were measured as described in Materials and Methods. GAPDH served as a loading control.
Characterization of exosomes containing miR-HER2-E1. a. Characterization of purified exosomes with respect to the presence of exosome-associated proteins. T150 flasks containing $1 \times 10^7$ HEK-293 cells were mock transfected or transfected with $10 \mu$g of non-target (NT) or miR-HER2-E1 plasmids. After 4 h of incubation, the cells were extensively rinsed with PBS and incubated with serum-free medium for another 48 h. Exosomes were then purified as described in Material and Methods. Purified exosomes and cell pellets were lysed with RIPA lysis buffer, subjected to electrophoresis in denaturing gels and reacted with antibodies against exosomes marker proteins Alix, CD9, Annexin V, Flotillin-1 or TSG101, respectively. b. Particle size distribution and number of isolated exosomes extracted from miR-HER2-E1 and non-targeting (NT) plasmid transfected HEK-293 cells. T150 flasks containing $1 \times 10^7$ HEK-293 cells were treated as described in panel A. Exosomes were purified as described in Materials and Methods. Exosome size distribution and number of isolated exosomes were determined by Izon's qNano technology. c. Quantification of miR-HER2-E1 from purified exosomes. Exosomes were purified from HEK-293 cell supernatant as described in panel A. The amounts expression of miR-HER2-E1 in exosomes was quantified and normalized with respect to 18s rRNA.
Figure 3

Inhibition of HER2 protein accumulation by exosome-delivered miR-HER2-E1. a. Accumulation of HER2 protein in SK-OV-3 cells exposed to exosomes containing miR-HER2-E1. Replicate cultures containing 2.5 × 105 SK-OV-3 cells were remain untreated (Con) or incubated with indicated amounts of exosomes purified from HEK-293 cells transfected with miR-HER2-E1 or non-target (NT) miRNAs. After 72 h the cells were harvested and the cells lysates were electrophoretically separated in an 8% denaturing gel, and reacted with indicated antibodies. The band densities were normalized with respect to GAPDH. b. Accumulation of HER2 protein in HEp-2 cells exposed to exosomes containing miR-HER2-E1. Cultures containing 2.5 × 105 HEp-2 cells were transfected with HER2 expression plasmid for 36 h, then either left untreated (Con) or incubated with 20 µg of purified exosomes produced in HEK-293 cells transfected with plasmids encoding miR-HER2-E1 or non-target (NT) miRNAs. After 36 h of incubation, the cells were
harvested and the cells lysates were electrophoretically separated in an 8% denaturing gel, and reacted with indicated antibodies. The band densities were normalized with respect to GAPDH.

Figure 4

miR-HER2-E1 delivered to HER2-positive cancer cells by exosomes reduces their viability. HER2-positive (+) SK-OV-3 and HCT116 cancer cells or HER2-negative (-) HEp-2 and MDA-MB-231 cells were seeded on 96-well plates with a density of 1 × 10⁴ per well. The cells in triplicate were mock treated (Mock) or incubated with 1 µg of purified exosomes produced in HEK-293 cells transfected with plasmids encoding miR-HER2-E1 or non-target (NT) miRNA. After 72 h of incubation, the relative cell viability was determined by CCK8 assay. The results normalized to the value of mock-treated control group. **p = 0.006 in SK-OV-3 group, **p = 0.004 in HCT116 group. N.S. = no significant difference.
Figure 5

Anti-tumor efficacy of exosome-delivered miR-HER2-E1 in vivo. Nude mice derived from Balb/c mice in groups of 6 mice were injected subcutaneously into flanks with $5 \times 10^6$ SK-OV-3 (a), HCT116 (b) or MDA-MB-231 (c) cells. Tumors averaging 90 mm$^3$ were injected intratumorally every three days, 6 times in total with 10 μg of purified exosomes per injection. Tumor size was measured every three days. Results are shown as mean tumor volume (mm$^3$) ± standard deviation. ***p = 0.0007, *p = 0.045, N.S. = no significant difference.
Figure 6

Derivation of stable cell lines expressing both a ligand to HER2 on tumor cell surface and a miRNA targeting HER2. a. Accumulation of miR-HER2-E1 in stable cell lines. The miR-HER2-E1 isolated from 293-miR-XS and 293-miR-XS-HER2 cell pellets and exosomes were quantified and normalized with respect to 18s rRNA. b. The relative affinity of 293-miR-XS and 293-miR-XS-HER2 exosomes for binding HER2 protein. One microgram purified exosomes from 293-miR-XS or 293-miR-XS-HER2 stable cell lines were coated onto the ELISA plates in triplicate, and then incubated with 1.5 ng HER2 protein. HRP-conjugated rabbit anti-HER2 antibody was used as a detection antibody. The absorbance readings (OD450 nm) are shown on the Y axis.
Figure 7

Anti-tumor efficacy of exosomes adhering to HER2 and expressing miR-HER2-E1. Nude mice implanted with SK-OV-3 tumors averaging 80 mm3 in volume were injected intravenously (i.v.) with exosomes purified from parental HEK-293 cell line (293) or miR-HER2-E1 expression stable cell line (293-miR-XS) or concurrent expression of HER2 protein ligand and miR-HER2-E1 stable cell line (293-miR-XS-HER2). Exosomes were injected at 3 μg /animal (a) or 30 μg /animal (b) every three days for a total of 8 injections. Tumor size was measured every three days. The results are shown as mean tumor volume (mm3) ± standard deviation of 6 mice. ***P = 0.0009 and *P = 0.032 compared with mice treated with exosomes purified from 293-miR-XS group.