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Original Articles

Survivin-targeting miR-542-3p overcomes HER3 signaling-induced chemoresistance and enhances the antitumor activity of paclitaxel against HER2-overexpressing breast cancer

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ABSTRACT

Elevated expression of HER3, which interacts with HER2 in breast cancer cells, confers chemoresistance via phosphoinositide 3-kinase (PI-3K)/Akt-dependent upregulation of Survivin. However, the underlying mechanism is not clear. Ectopic expression or specific knockdown of HER3 in HER2-overexpressing breast cancer cells did not alter *Survivin* mRNA levels and Survivin protein stability, supporting the notion that HER3 signaling may regulate specific miRNAs that target *Survivin* to alter its protein translation. Here we showed that overexpression and specific knockdown of HER3 reduced and enhanced expression of two *Survivin*-targeting miRNAs, miR-203 and miR-542-3p, in breast cancer cells, respectively. While the specific inhibitor of either miR-203 or miR-542-3p attenuated an anti-HER3 antibody-induced downregulation of Survivin, inhibition of miR-542-3p exhibited a better efficacy than miR-203 inhibition did. Consistently, miR-542-3p mimic was much more effective than miR-203 mimic not only in inhibition of Survivin, but also in enhancement of paclitaxel-induced apoptosis in HER2-overexpressing breast cancer cells. Moreover, the combination of miR-542-3p mimic and paclitaxel, as compared with either agent alone, significantly inhibited *in vivo* tumor growth of HER2-overexpressing breast cancer cells. Collectively, our data indicated that the HER3/PI-3K/Akt signaling upregulates Survivin via suppression of miR-203 and miR-542-3p. Because miR-542-3p has three binding sites on the 3′-UTR of *Survivin* mRNA, its mimic was able to effectively downregulate Survivin *in vitro* and *in vivo*. Thus, miR-542-3p-replacement therapy is an excellent approach to overcome HER3-mediated paclitaxel resistance and significantly enhances the antitumor activity of paclitaxel against HER2-overexpressing breast cancer.

1. Introduction

The HER3 receptor is a unique member of the erbB family of receptor tyrosine kinases (RTKs), which are often aberrantly activated in a wide variety of human cancers [1,2]. Unlike other family members, HER3 has no or little intrinsic kinase activity [3,4]. It frequently co-expresses and interacts with other RTKs in cancer cells to activate oncogenic signaling, such as the PI-3K/Akt pathway, MEK/MAPK pathway, Src kinase, etc. [3,5,6]. Although recent studies have identified oncogenic *erbB3* gene mutations in colon and gastric cancers [7], overexpression of HER3 receptor is still the major mechanism for its enhanced signaling, which is associated with poor clinical outcomes in patients with solid tumors [8]. HER3 signaling has been shown to play a pivotal role in the development of *erbB2*-altered (gene amplification and/or overexpression) breast cancer [9,10], castration-resistant prostate

Abbreviations: RTKs, Receptor tyrosine kinases; PI-3K, Phosphoinositide 3-kinase; CHX, Cycloheximide; ELISA, Enzyme-linked immunosorbent assay; IHC, Immunohistochemistry; i.p., Intraperitoneal.

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cancer (CRPC) [11], and ovarian cancer [12,13]. Mechanistic studies have indicated that one of the major functions of HER3 signaling is to cause cancer treatment failure [14,15]. We have shown that elevated expression of HER3 renders HER2-overexpressing breast cancer cells resistant to tamoxifen [16], trastuzumab (Herceptin), lapatinib [17,18], and the chemotherapeutic drug paclitaxel [19]. Thus, effective inhibition of HER3 is thought to be required to overcome drug resistance and to effectively treat HER2-overexpressing breast cancer. In studying the molecular basis of HER3-induced paclitaxel resistance, we found that overexpression of HER3 led to a PI-3K/Akt-dependent upregulation of Survivin [19]. Specific knockdown of Survivin significantly enhanced paclitaxel-induced apoptosis in the otherwise resistant, HER3-expressing breast cancer cells [19], suggesting that Survivin is a valuable target for chemosensitization to abrogate HER3-mediated paclitaxel resistance. Nonetheless, the precise mechanism by which HER3 signaling upregulates Survivin in HER2-overexpressing breast cancer cells remains unclear.

Survivin is a dual-function protein acting as a critical inhibitor of apoptosis (IAP) and a key regulator of cell cycle progression [20]. It is produced in embryonic tissues and undetectable in most adult tissues. Overexpression of Survivin is observed in almost all human malignancies and increased Survivin correlates with poor clinic outcomes, tumor recurrence, and drug resistance in cancer patients [21,22]. Because of its selective expression in tumor tissues, Survivin has been recognized as an attractive therapeutic target [20,23]. Several approaches, including use of transcriptional inhibitor YM155, antisense oligonucleotide, gene therapy, and immunotherapy, have been designed to target Survivin [21,22,24,25]. YM155, a leading inhibitor, has become the first choice in Survivin-related studies and is being used in clinical trials of cancer treatment (https://clinicaltrials.gov/ct2/results?term=YM155&Search=Search). However, concerns have been raised regarding the mechanism of action and specificity of YM155 [26]. A recent study revealed that YM155 failed to improve response rates to paclitaxel and carboplatin in patients with advanced non-small cell lung cancer (NSCLC) [27]. This failure is probably due to the lack of specificity of YM155 and its insufficient inhibition of Survivin in patients [24]. It is believed that novel strategy/agents that can effectively downregulate Survivin *in vivo* are required to increase chemotherapeutic efficacy, thereby reducing the risk of relapse and improving the survival of cancer patients.

In the current study, we focused on elucidating the molecular basis of HER3 signaling-induced upregulation of Survivin in HER2-overexpressing breast cancer cells. Using both an *in vitro* cell culture system and an *in vivo* tumor xenograft model, we also investigated whether the newly identified mechanism-based strategy, miRNA-replacement therapy, can effectively inhibit Survivin, thereby overcoming HER3-mediated paclitaxel resistance and significantly enhancing the antitumor activity of paclitaxel against HER2-overexpressing breast cancer.

2. Materials and methods

2.1. Reagents and antibodies

The miRIDIAN has-miR-203 and has-miR-542-3p specific inhibitors, mimics, and their negative controls were purchased from Thermo Scientific Dharmacon (Lafayette, CO). In vivo-jetPEI® *in vivo* DNA and siRNA delivery reagent was purchased from Polyplus-transfection® SA (New York, NY). The Akt inhibitor VIII was purchased from EMD Chemicals, Inc. (Gibbstown, NJ). Paclitaxel (Ben Venue Labs, Inc., Bedford, OH) was obtained from University of Colorado Hospital pharmacy. The fully human anti-HER3 antibody MM-121 was kindly provided by Merrimack Pharmaceuticals Inc. (Cambridge, MA).

The primary antibodies used for western blot analyses were obtained as follows: Survivin (6E4) (Abcam, Cambridge, MA); Mcl-1 (Santa Cruz Biotechnology, Inc., Dallas, TX); Bcl-xl, caspase-8 (1C12), caspase-3 (8G10), and PARP (Cell Signaling Technology, Inc., Beverly, MA); β-actin (AC-75, Sigma-Aldrich, St. Louis, MO). All other reagents were purchased from Sigma-Aldrich unless otherwise specified.

2.2. Cells and cell culture

Human breast cancer cell lines SKBR3, BT474, MDA-MB-453, and HCC1954 were obtained from the American Type Culture Collection (Manassas, VA). The trastuzumab-resistant subline BT474-HR20 was described previously [17]. The identity of all cell lines was confirmed with DNA profiling by the University of Colorado Cancer Center's DNA Sequencing Core facility. Cell lines were free of mycoplasma contamination, as determined by the MycoAlert™ Mycoplasma Detection Kit (Lonza Group Ltd., Basel, Switzerland) once every three months. All cell lines were maintained in DMEM/F-12 (1:1) medium containing 10% FBS, cultured in a 37 °C humidified atmosphere containing 95% air and 5% CO_2, and split twice a week.

2.3. Transfection of cells with miRNA mimic or inhibitor

Cell transfection with miRNA mimic, inhibitor, or controls was carried out using HiPerFect Transfection Reagent (QIAGEN Inc., Valencia, CA) as described previously [28].

2.4. Quantification of apoptosis

An apoptotic enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics Corp., Indianapolis, IN) was used to quantitate cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) as previously reported [17,19,28].

2.5. Western blot analysis

Protein expression was determined by western blot assays as described previously [18,19,28]. Equal amounts of total cell lysates were boiled in Laemmli SDS sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA), and probed with the primary antibodies described in the figure legends.

2.6. Reverse transcription (RT)-PCR and quantitative real-time (qRT)-PCR

Total RNA was extracted using a modified chloroform/phenol procedure (TRIZOL®, Invitrogen, Carlsbad, CA). First-strand cDNA was generated using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Human *Survivin* mRNA expression was examined by conventional RT-PCR as we described previously [28–30]. To quantify the human *Survivin* and *erbB3* mRNA levels, qRT-PCR was performed using the Absolute* Blue qPCR Master Mixes (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's protocol. The expression of *β-actin* was used as an internal control for both conventional RT-PCR and qRT-PCR. All qRT-PCR reactions were carried out on a 7500 Fast Real-Time PCR system (Applied Biosystems). Sequences of the specific primers were reported previously [28,31].