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Preliminary validation of ERBB2 expression regulated by miR-548d-3p and miR-559

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ABSTRACT

ERBB2 overexpression occurs in numerous types of primary human tumors and alterations in microRNA (miRNA) expression have been associated with tumor suppression or tumorigenesis in human cancer, nevertheless, little is known about natural miRNAs acting on ERBB2. In this study, bioinformatical analysis of the 3′-UTRs of ERBB2 revealed the target elements for miR-548d-3p and miR-559. Moreover, a predicted miRNA/mRNA interaction experimental validation showed that both miR-548d-3p and miR-559 can interact specifically with the 3′-UTR of the ERBB2 mRNA. And miR-548d-3p plus miR-559 transfection showed a cooperative regulation of translationally repressing ERBB2 mRNA rather than by either miR-548d-3p or miR-559 alone. These results not only support the idea that different miRNAs can simultaneously and cooperatively repress a given target mRNA but also preliminarily validate the role of miR-548d-3p and miR-559 in regulating the ERBB2 expression. These data provide molecular basis for the application of miRNAs in ERBB2-targeted therapy.

ERBB2 (V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, alias HER-2/NEU), is a protooncogene that encodes a transmembrane glycoprotein with extensive homology to the human epidermal growth factor receptor known as the ERBB2 protein. It is well known that ERBB2 protein expression is regulated by both transcriptional and post-transcriptional mechanisms, and is involved in the development of numerous types of human cancer such as ovarian cancer, breast cancer, renal and prostate cancers. The abnormal (high) expression of ERBB2 protein correlates with more aggressive clinicopathologic features, drug resistance or sensitivity to specific chemotherapy and specific hormonal therapy regimens in breast cancer [1].

MicroRNAs (miRNAs) are a class of noncoding RNA species of ~22 nt that induce post-transcriptional gene silencing through base pairing with their target mRNAs, that is, in the predominant mechanism, an mRNA sequence complementary to the 'seed sequence' (positions 2–7 of the mature miRNA), within the 3′-UTR of the target mRNAs [2,3], which leads to mRNA cleavage, translational inhibition or cleavage-independent mRNA degradation [4]. Conservation of individual miRNAs across different species suggests their functional importance. Functional studies indicate that miRNAs participate in cell fate determination, pattern formation during embryonic development, and in the control of cell proliferation, cell differentiation, and cell death [5]. Studies have shown that miRNAs are aberrantly expressed or mutated in cancer, suggesting their role as a novel class of oncogenes or tumor suppressor genes. The finding that miRNAs have a role in human cancer is further supported by the fact that more than 50% of miRNA genes are located at chromosomal regions, such as fragile sites, and regions of deletion or amplification that are genetically altered in human cancer [6]. Given the involvement of miRNAs in cancer development, the manipulation of cellular miRNA levels has emerged as a potential therapeutic strategy. Efforts to predictably alter intracellular transcript profiles by increasing specific miRNA levels either through transfection or viral delivery methods have demonstrated the potential of this strategy to modulate cellular physiology [7,8]. Conversely, attempts to reduce miRNA levels using biologically stable antisense moieties such as 2′-O-methyl oligonucleotides (2′-O-Me) or "antagomirs" have also proven capable of altering intracellular transcript profiles [9].

In terms of the miRNAs and ERBB2 status, study was undertaken to examine whether the miRNAs expression profile varied

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according to ERBB2+/ERBB2−, the results were compared between ERBB2+ and ERBB2− of lobular and ductal carcinomas, and did not reveal differentially expressed miRNAs [10]. A more recent study reported the miRNAs expression profiling in 193 primary breast cancer tissues and did not find that specific miRNAs were strongly associated with tumor stage, vascular invasion, and ERBB2 status [11]. Since little is known about natural miRNAs acting on ERBB2, some researchers designed dsRNAs targeting ERBB2 [12,13], and others designed miRNAs and corresponding dsRNA targeting the 3′-UTR of ERBB2 [14]. In this study, we utilized bioinformatic comparison of the 3′-UTR of ERBB2 which revealed the target element of both miR-548d-3p and miR-559, and thus the functional importance of a predicted miRNA/mRNA interaction should be validated to quickly eliminate any interaction sites that are not functional. The pMIR-REPORT™ miRNA Expression Reporter Vector System was used. The rationale for using this assay is that the binding of a given miRNA to its specific mRNA target site will repress reporter protein production and thereby reducing activity/expression that can be measured and compared to a control [15]. The aim of this study was to identify and preliminarily validate putative miRNA/mRNA targets of ERBB2 gene, then provide molecular basis for the application of miRNAs in ERBB2-targeted therapy.

Materials and methods

Bioinformatic prediction of miRNA target sites of ERBB2 gene and ΔG analysis (miRNAs accessibility). Two bioinformatic algorithms—were utilized to predict miRNAs target sites of ERBB2 gene: miRanda (http://microrna.sanger.ac.uk) and TargetScan (http://www.targetscan.org) [16]. It is important to reiterate that multiple algorithms be utilized since these programs often predict distinct miRNAs binding sites and provide us more and better choices. Therefore, in our research, we would only pursue miR-548d-3p and miR-559 as potential regulators of ERBB2 gene expression. Data demonstrated that a common feature of most validated targets is that miRNAs preferentially target 3′-UTR sites that do not have complex secondary structures and are located in accessible regions of the RNA based on favorable thermodynamics. Since RNA accessibility may be a critical feature of miRNAs target recognition, the free energy (ΔG) of the 70 nucleotides flanking the 5′ and 3′ sides of miR-548d-3p or miR-559 binding sites were determined using mFold as described by Zhao et al. [17,18].

Cell culture. Hela cells which are ERBB2-negative human cervical carcinoma [19] were purchased from Shanghai Cell Bank, Chinese Academy of Sciences, and routinely grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco), 100 units/ml penicillin, and 100 μg/mL streptomycin. All cells were maintained in a humidified atmosphere of 5% CO2 at 37 °C.

Recombinant constructs. A 618-bp fragment encompassing the entire ERBB2 3′-UTR. An 611-bp fragment with a deletion of miR-548d-3p 7-bp seed binding site (GTTTTTA) or miR-559 7-bp seed binding site (TTACTTT) from the entire ERBB2 3′-UTR was artificially synthesized by Invitrogen Corporation Shanghai Representative Office, with overhanging Hind III restriction sites to both 5′ and 3′ end. The artificially synthesized genes were subcloned into the pMD18-T Vector following the manufacturer's protocol, (TAKARA) respectively. Plasmid DNA was subsequently isolated from recombinant colonies and sequenced to ensure authenticity. The artificially synthesized inserts were removed from the pMD18-T plasmid by Hind III digestion. The fragments were gelpurified, filled in, and sticky end ligated into a filled-in Hind III site which is located at the downstream of the firefly luciferase (f-luc) reporter gene (pMIR-REPORT™, Ambion). The authenticity and orientation of the inserts relative to the luciferase gene were confirmed by sequencing. The resulting plasmids were designated as pMIR/618/5′ → 3′, pMIR/618/3′ → 5′, pMIR/618/5′ → 3′/ΔmiR-548d-3p and pMIR/618/5′ → 3′/ΔmiR-559, respectively.

Transfection. The following partially double-stranded RNAs that mimic endogenous precursor miRNAs, hsa-miR-548d-3p, hsa-miR-559 and miRNAs Negative Control #1 were obtained from Ambion. Transfection of Hela cells with miRNAs was optimized utilizing SiPORT NeoFX (Ambion). HeLa cells were transfected with the luciferase reporter constructs described above (200 ng), pMIR-REPORT β-gal Control Plasmid (200 ng) and the appropriate miRNAs precursor or the same volume of Nuclease-free Water using siPORT NeoFX Transfection Agent (Ambion). After 48 h, cells were lysed with Reporter Lysis Buffer (Beyotime Institute of Biotechnology) and f-luc and β-gal activities were determined using the Luciferase Rreporter Gene Assay Kit (Beyotime Institute of Biotechnology) and β-gal Assay Kit (Beyotime Institute of Biotechnology). The relative reporter activity was obtained by normalizing to the β-gal activity.

Statistical analysis. All data were expressed as means ± S.E., multiple comparisons were made, and statistical significance was determined using one-way analysis of variance. All statistical analyses were performed using the software package SPSS 13.0, and statistical significance was defined as $p < 0.05$ or $p < 0.01$.

Results

Bioinformatic data suggest that miR-548d-3p and miR-559 may play a biologically relevant role in regulating the expression of ERBB2

On-line search demonstrated that more than 50 putative miRNAs target sites were harbored in the 3′-UTR of the ERBB2 mRNA depending upon the species analyzed. For this study, we have focused on two miRNAs (i.e. miR-548d-3p, and miR-559) that may target the ERBB2 3′-UTR since these two miRNAs target sites of ERBB2 gene are both unique and overlapping, though they have different conservation (Fig. 1).

When the ΔG was calculated using nucleotide sequence surrounding the two predicted ERBB2 miRNAs binding sites, both miR-548d-3p and miR-559 binding sites had a lower ΔG than randomly expected ($\Delta G = -14.13$ kcal/mol) [20] (Table 1), suggesting that these sites may be accessible to miRNAs. Taken together, the bioinformatic data suggested that miR-548d-3p and miR-559 may play a biologically relevant role in regulating the expression of ERBB2 and should be further pursued.

miR-548d-3p, miR-559 can interfere with recombinant luciferase mRNA translation via direct interaction with the ERBB2 3′-UTR

To experimentally validate the computational data, the ERBB2 3′-UTR (i.e. 618 bp) was subcloned downstream of the f-luc open reading frame (Fig. 2). This reporter construct (pMIR/618/5′ → 3′) was cotransfected in the Hela cell line with pMIR-REPORT β-gal Control Plasmid (to normalize for transfection differences) and the miRNA precursor—Negative Control #1 (miR-Control) as a baseline for evaluating the effect of the experimental miRNA precursor on the target gene expression or miR-548d-3p, miR-559 or miR-548d-3p plus miR-559 precursor RNA oligonucleotides. Identical control experiments were also performed utilizing a reporter construct (pMIR/618/3′ → 5′) in which the ERBB2 3′-UTR was cloned in the opposite orientation and luciferase activities were determined. Interestingly, the relative luciferase activity was markedly diminished in cells cotransfected with the pMIR/618/5′ → 3′ construct and miR-548d-3p, miR-559 or miR-548d-3p plus miR-559, especially miR-548d-3p plus miR-559 (50 nM final concentration, Fig. 3A).

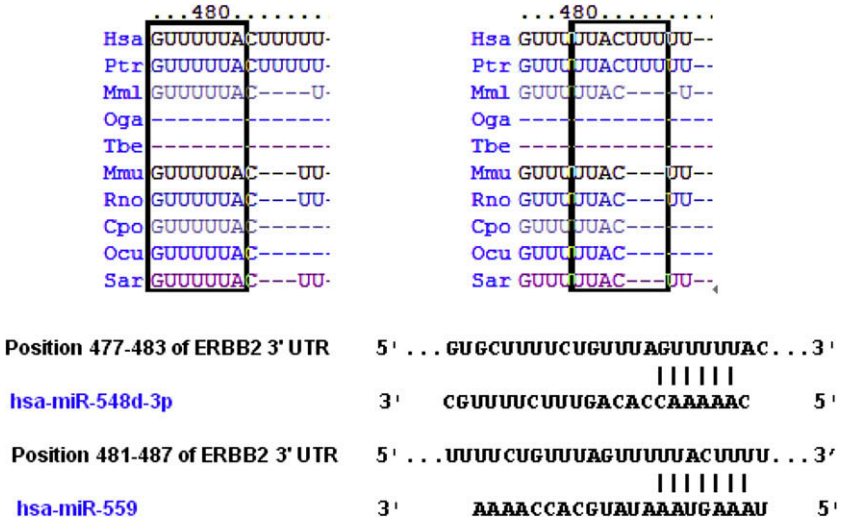

Fig. 1. Two putative miRNAs binding sites located the ERBB2 3′-UTR. One complementarity between miR-548d-3p and the putative ERBB2 3′-UTR site targeted (477–483 bp downstream from the ERBB2 stop codon). Also shown are the conserved bases of the putative miR-548d-3p target sequence present in the ERBB2 3′-UTR. The other complementarity between miR-559 and the putative ERBB2 3′-UTR site targeted (481–487 bp downstream). Also shown are the conserved bases of the putative miR-559 target sequence present in the ERBB2 3′-UTR. The targeted sites of miR-548d-3p and miR-559 are overlapping.

Table 1
Predicted ΔG (−kcal/mol) of the 70 nucleotides flanking the 5′ and 3′ regions of the potential miRNA target sites.

Predicted human ERBB2 3′-UTR miRNA binding sites	5′ 70 bp (ΔG)	3′ 70 bp (ΔG)
miR-548d-3p	−12.70	−13.10
miR-559	−9.97	−13.10

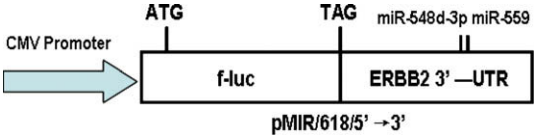

Fig. 2. Schematic representation of the f-luc reporter constructs utilized. The pMIR/618 plasmids contain the full-length 3′-UTR of the ERBB2 in either the 5′ → 3′ or 3′ → 5′orientation with respect to the f-luc open reading frame and were designated pMIR/618/5′ → 3′and pMIR/618/3′ → 5′, respectively. The firefly luciferase start and stop codons are also shown.

To test the potency of the miRNAs, pMIR/618/5′ → 3′ was cotransfected into Hela cells with increasing concentrations of each specific RNA oligonucleotide. Dose response experiments demonstrated that relative luciferase activity was significantly decreased with the increase of concentrations of these RNA (Fig. 3B). In contrast, increasing concentrations of miR-Control (Fig. 3B) had no effect on luciferase activity.

To demonstrate that miR-548d-3p or miR-559 interacts with a specific target sequence localized in the ERBB2 3′-UTR, an additional reporter recombinant construct was generated in which the 7 bp "seed" sequence (i.e. GTTTTTA or TTACTTT), which is complementary to the 5′ end of miR-548d-3p or miR-559 (Fig. 1), was deleted. The resulting construct, pMIR/618/5′ → 3′/ΔmiR-548d-3p or pMIR/618/5′ → 3′/ΔmiR-559, was cotransfected into Hela cells as described above and luciferase activity was measured. Importantly, miR-548d-3p or miR-559 could no longer decrease luciferase activity of the new reporter construct, even increasing concentrations of each specific RNA oligonucleotide (Fig. 3C). Taken together, these results indicate that two potential miR targets (miR-548d-3p, miR-559) identified in the ERBB2 3′-UTR by bioinformatic analyses can interfere with recombinant luciferase mRNA translation via direct interaction with the ERBB2 3′-UTR, miR-548d-3p plus miR-559 can enhance the silencing effect compared with only one of them alone.

Discussion

The ERBB2 oncogene encodes a 185 kDa transmembrane receptor tyrosine kinase and plays an important role in oncogenesis [21]. ERBB2 overexpression occurs in numerous types of primary human tumors, including 25–30% of breast and ovarian carcinomas, and has been associated with aggressive tumor growth and an adverse prognosis [22,23]. These observations implicate ERBB2 overexpression as a factor in the pathogenesis of some human cancers and have led to the development of new therapies that specifically target cancer cells expressing high levels of ERBB2 [24].

Targeted therapies in cancer treatment, represent a new generation of drugs that interfere with specific molecular targets (typically proteins). Examples of approved targeted agents include agents directed against the human epidermal growth factor receptor 2 (HER2, i.e., ERBB2) such as trastuzumab and lapatinib and the anti-VEGF bevacizumab [25], which is, without any doubt, an effective anti-cancer treatment for these patients, where the benefit clearly exceeds the risk in the majority. Furthermore, these targeted agents are not 100% efficient and both de novo and acquired resistance can occur. Strategies to overcome this problem by developing more potent agents and/or synergetic combinations will be necessary in future research [26]. Targeted cancer therapies require development of approaches to inhibiting expression of tumor protein. In recent years, a class of genes that encode tiny RNAs called miRNAs have been found to be altered in human cancer, which are derived from endogenous gene and regulate the expression of endogenous mRNA, not by cleaving but by repressing their translation at a step after translating initiation, accordingly interfere with protein synthesis and constitute a mechanism of protection against tumorigenesis, malignant transformation and tumor progression or vice versa [14]. In this study, two specific miRNAs (i.e. miR-548d-3p, miR-559) were chosen and utilized to investigate whether they could mediate the translational repression of a luciferase/ERBB2 3′-UTR reporter construct. Interestingly, both miR-548d-3p and miR-559 were validated to interact specifically

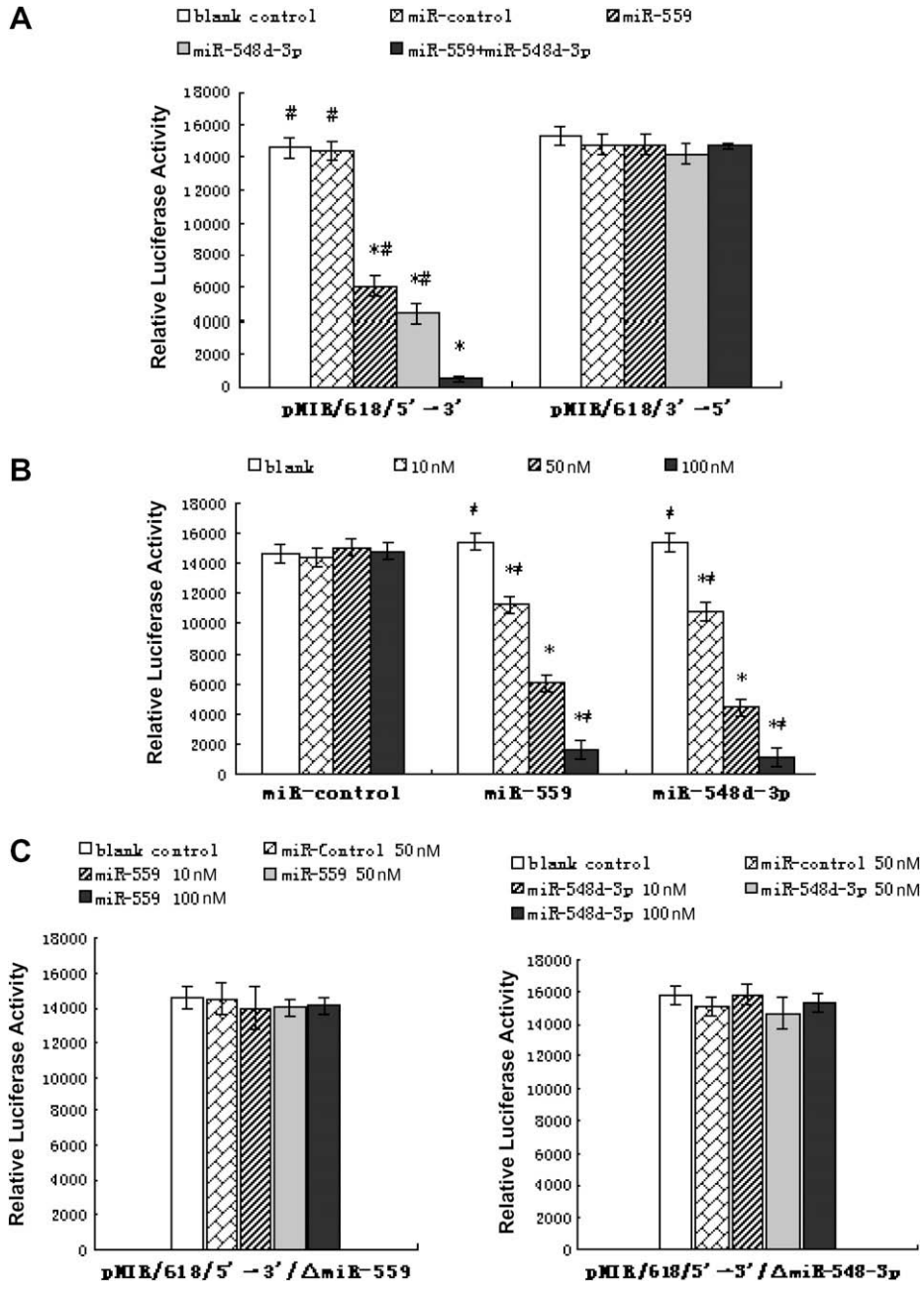

Fig. 3. miR-548d-3p or miR-559 can inhibit reporter activity. (A) Hela cells were cotransfected with pMIR/618/5′ → 3′or pMIR/618/3′ → 5′, β-gal Control Plasmid, and a 50 nM concentration of a given RNA oligonucleotide as shown. Forty-eight hours after transfection, luciferase activities were measured. f-luc activity was normalized to β-gal activity named relative luciferase activity. The mean activities ±S.E. from five independent experiments are shown ($^{*}p < 0.01$ vs. blank transfected; $^{\#}p < 0.05$ vs. miR-559 + miR-548d-3p). (B) Hela cells were cotransfected with pMIR/618/5′ → 3′, β-gal Control Plasmid, and miR-Control, miR-559 or miR-548d-3p, respectively, at the concentrations indicated, and luciferase activities were calculated as described above. The mean activities ±S.E. from four independent transfection experiments are shown ($^{*}p < 0.05$ vs. blank-control at each concentration shown; $^{\#}p < 0.05$ vs. 50 nM of miR-559 or miR-548d-3p). (C) Hela cells were cotransfected with pMIR/618/5′ → 3′/ΔmiR-548d-3p or pMIR/618/5′ → 3′/ΔmiR-559, β-gal Control Plasmid, and a given RNA oligonucleotide, at the concentrations indicated. Forty-eight hours after transfection luciferase activities were calculated as described above. The mean activities ±S.E. from five independent transfection experiments are shown.

with the 3′-UTR of the ERBB2 mRNA, and the potency were increasing when the concentrations of each specific RNA oligonucleotide increased. Although delivery of a single miRNA can clearly produce a phenotypic disturbance, it has been suggested that miRNAs regulation of a given transcript is most efficient when multiple miRNAs work in concert [27,28]. In our study, miR-548d-3p plus miR-559 transfection showed an obviously cooperative regulation of translationally repressing ERBB2 mRNA rather than by either miR-548d-3p or miR-559 alone. These results not only support the idea that different miRNAs can simultaneously and cooperatively repress a given target mRNA but also preliminarily validate the role of miR-548d-3p and miR-559 in regulating the ERBB2 expression. These data provide molecular basis for the application of miRNAs in ERBB2-targeted therapy.

Although we preliminary validated that ERBB2 expression regulated by miR-548d-3p and miR-559, there are a lot of work need to do to achieve the authenticity of a functional miR-548d-3p/ERBB2 mRNA or miR-559/ERBB2 mRNA target pairs. Next, we plan to prove whether miR-548d-3p or miR-559 have an effect on ERBB2 protein expression or not and if their-mediated regulation

of ERBB2 gene expression should equate to altered biological function [15].

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