RESEARCH ARTICLE

Ginsenoside Rh2 differentially Mediates microRNA Expression to Prevent Chemoresistance of Breast Cancer

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Abstract

Chemoresistance is the most common cause of chemotherapy failure during breast cancer (BCA) treatment. It is generally known that the mechanisms of chemoresistance in tumors involve multiple genes and multiple signaling pathways,; if appropriate drugs are used to regulate the mechanisms at the gene level, it should be possible to effectively reverse chemoresistance in BCA cells. It has been confirmed that chemoresistance in BCA cells could be reversed by ginsenoside Rh2 (G-Rh2). Preliminary studies of our group identified some drug-resistance specific miRNA. Accordingly, we proposed that G-Rh2 could mediate drug-resistance specific miRNA and corresponding target genes through the gene regulatory network; this could cut off the drug-resistance process in tumors and enhance treatment effects. G-Rh2 and breast cancer cells were used in our study. Through pharmaceutical interventions, we could explore how G-Rh2 could inhibit chemotherapy resistance in BCA, and analyze its impact on related miRNA and target genes. Finally, we will reveal the anti-resistance molecular mechanisms of G-Rh2 from a different angle in miRNA-mediated chemoresistance signals among cells.

Keywords: Breast cancer - chemoresistance - G-Rh2 - miRNA - cell

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Introduction

Breast cancer is one of the most common cancer in women, drug-resistance and toxicity are the leading causes that limit the success of aggressive breast cancer therapy (Deng et al., 2013; Liu et al., 2013; Gong et al., 2014; Wang et al., 2014). Ginsenoside Rh2 has been used for more than 2, 000 years in oriental countries. It is the major pharmacological active component of ginseng that includes a similar basic structure, which consists of a gonane steroid nucleus that holds 17 carbon atoms settled in a four-trans-ring (Zhang et al., 2012) (Figure 1). As a potent novel class phytoestrogen that exhibits a multi-target, multisystem and a significant low toxicity, Studies shows that G-Rh2 treatment can effect human breast cancer cells and G-Rh2 might be an effective drug-resistance reversing agent in breast cancer chemotherapy, However, the underline mechanism has not been fully elucidated (Choi et al., 2011; Zhang et al., 2012).

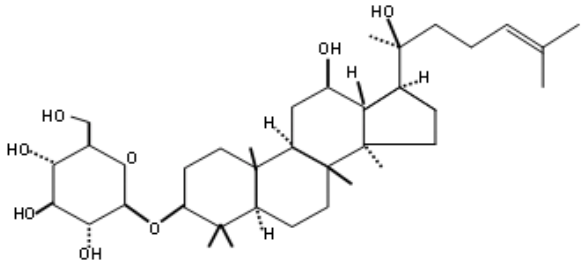

Figure 1. The Structure of G-Rh2

MicroRNAs (miRNAs) are involved in the regulation of various biological processes: tumorigenesis, development, cellular migration, apoptosis, signal transduction and carcinogenesis etc. Recently, increasing evidence revealed that miRNAs act an important role in anticancer drug resistance and miRNAs expression profiling can be related with the development of anticancer drug-resistance, such as miR-181a, miR-34a, miR-222, etc. There are several mechanisms have been shown to be targeted by miRNAs in drug-resistant breast cancer such as DNA repair (Chen et al., 2012; Ratert et al., 2013; Chan et al., 2013; Kutanzi et al., 2011).

Some studies showed G-Rh2 mediates changes in the miRNA expression profile of human lung cancer A549 cells. It also inhibits glioma cell proliferation by targeting miRNA-128 (Wu et al., 2011; Jiao et al., 2013). Adriacin (Adr) and Docetaxel (Doc) are two of the most common chemotherapeutic drugs in BCA. There has been no report to demonstrate the mechanisms of G-Rh2 to reverse drug-resistance in BCA from miRNA levels. Our team conducted the profiling of miRNAs expression

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in human BCA Adr-resistant MCF-7 cells (MCF-7/Adr) and Doc-resistant MCF-7 cells (MCF-7/Doc), we are the first to report the findings and testify that 5 miRNA is related to drug-resistance (An et al., 2013; Zhong et al., 2013). We propose a logical hypothesis: G-Rh2 could mediate the drug-resistance specific miRNA, which could then regulate corresponding target genes through the gene regulatory network -- cutting-off the process and development of drug-resistant tumor cells and improving the treatment effect.

Materials and Methods

Cell culture

Human breast cancer cell line MCF-7 was purchased from ATCC (Rockville, MD). The resistant sublines, selected at 100nm Docetaxel (MCF-7/Doc) or at 500nm Adriamycin (MCF-7/Adr), were successfully established from human breast cancer parental cell line MCF-7 by exposing MCF-7 to gradually increasing concentrations of Doc or Adr in vitro in our laboratory. All cell lines were cultured in DMEM high glucose (HyClone), supplemented with 10% fetal bovine serum (Gibco) in a humidified atmosphere containing 5% CO_2 at 37°C.

Cell proliferation assay

Cells were seeded into 96-well plates (6×10^3 cells/well), treated with different concentrations of G-Rh2 and incubated for 24 hours. Then 20μl of MTT solution (5mg/ml) was added to each well and the cells were maintained in a humidified atmosphere for 3-4 hours at 37°C. The MTT-containing medium was removed and 150lL of DMSO (AMRESCO, America) was added to each well; each experiment was performed in quAdruplicate. The absorbance was measured at 570 nm using CliniBio128 (ASYS-Hitech, Austria)

Real-time quantitative PCR

Total RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA); afterwards a reverse transcription was done using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA); mature miRNA was spotted using TaqMan® MicroRNA Assay (Applied Biosystems, Foster City, CA); all procedures were done according to manufacturer's instructions. Relative expression levers were calculated using the ΔΔCt method, normalized with endogenous control and was presented along with negative control. Clustal X software was used to analyze measured miRNA sequences; the sequences were similarity not high in a reverse transcription system. All reverse transcriptions and PCR assays were presented in triplicate.

MiRNA microarray

Total RNA including miRNAs was extracted using MirVana miRNA Isolation Kit (Ambion, AM1560). The concentration and quality of the RNA were measured by the UV absorbance at 260 and 280 nm (260/280 nm) on Nanodrop 2000 spectrophotometry (Thermo Scientific) and by formaldehyde denaturing gel electrophoresis. The RNA was labeled using the FlashTag RNA Labeling Kit (Genishere), according to Affymetrix manufacturer's recommendations. Hybridization and washing were performed using the Affymetrix Fluidics Station 450 and Hybridization Oven 640 under standard conditions. Image processing was conducted using the Affymetrix GeneArray 3000 scanner. The Affymetrix GeneChip miRNA 2.0 Array contains 15, 644 probe sets including 1105 human mature miRNAs. The raw data was treated using miRNA QC tool software (Affymetrix). The data output was received in Excel spreadsheets containing the normalized micro-RNA expression profiles. Differentially expressed miRNAs were filtered to exclude those changes less than 2.0-fold compared with MCF-7/S.

Establish stable expression of green fluorescent protein in breast cancer cell lines.

In order to facilitate observation, a recombinant lentiviral vector stable expression of green fluorescent protein was used in breast cancer cell lines. MCF-7/Doc or Adr and MCF-7 cells in logarithmic growth phase were seeded in 24-well plates (3×10^4 cells/well) after digestion until cell fusion becomes 50% to 60%; which was carried out in accordance with reagent instruction lentivirus infections. After 72 hours, the collected fluorescence was stronger in each well, which resurfaced after digestion was covered with 50% to 60%; added 2μg/ml puromycin to screen. After one week, 1μg/ml puromycin was added to maintain the pressure; three generations were continued to be cultured to observe the expression of the green fluorescence. Then, the green fluorescence MCF-7 cells, MCF-7/Adr cells and MCF-7/Doc cells were inoculated for 24 hours in equal amounts with G-Rh2 intervention; then afterwards intervened with Doc and Adr for 24 hours.

Western blot

Total protein was extracted and lysed in the RIPA buffer (Beyotime, Jiangsu, China). Equal amounts of proteins were separated by 10% SDS-PAGE and transferred to the polyvinylidene difluoride membranes (Sigma, Germany). After blocking with 5% skim milk, the membranes were incubated with primary antibodies against human Bax (1:500, Santa Cruz, CA) overnight at 4°C; after washing with TBS, the horseradish peroxidase-conjugated secondary antibody (Kangwei Ltd., Beijing, China) was further incubated; the protein band was visualized by Chemiluminescence with pierce ECL kits (Millipore, Billerica, MA). β-actin (1:4000, Bioworld, MN) was used as an internal load to normalize the expression patterns of each sample. Three separate experiments were performed to show the protein expression.

Statistical analysis

All experiments were performed in triplicate and a representative data was shown from three separate experiments. A statistical analysis was performed using a t-test or One-way ANOVA and Spearman rank test with a SPSS 16.0 statistic. All experiments were performed in triplicate;a level of $p<0.05$ was considered statistically significant.

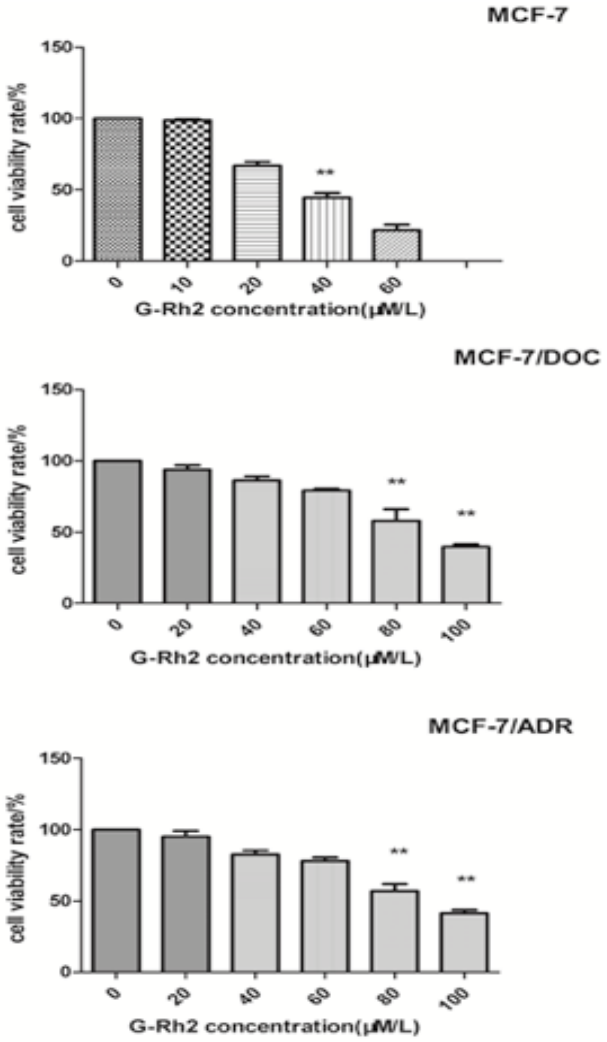

Figure 2. G-Rh2 Treatment Restrains the Viability of Human Breast Cancer Cells by MTT-Cytotoxic. MCF-7 **A)**, MCF-7/Doc **B)** and MCF-7/Adr **C)** cell lines were treated with DMSO (control) or different concentrations of G-Rh2 (10, 20, 40, 60, 80 and 100 μM/l) for 24 hours. All data corresponds to the mean ± SD of three independent experiments. Significantly different compared from with control by one-way ANOVA, $^{**}p < 0.05$, 40 μM/l *vs* 0-20 μM/l, 80 μM/l *vs* 0-60 μM/l, 100 μM/L *vs* 0-80 μM/l

Results

G-Rh2 treatment decreases the viability of breast cancer cells

We utilized different ginsenoside Rh2 concentrations to determine its antiproliferative effects. As shown in Figure 2 ABC, the viability of MCF-7, MCF-7/Doc and MCF-7/Adr cells gradually decreased with the increase of G-Rh2 concentration in a dose-dependent manner. MCF-7 cells with G-Rh2 (40 μM/l) treatment; MCF-7/Adr cells with G-Rh2 (80 μM/l) treatment and MCF-7/Doc cells with G-Rh2 (80 μM/l) treatment for 24 hours compared to MCF-7, MCF-7/Adr and MCF-7/Doc dramatically decreased cell viability with statistical significance; In particular, MCF-7/Doc and MCF-7/Adr cells exhibited a relatively high sensitivity to G-Rh2; indicating that G-Rh2 has a strong anti-proliferative activity in MCF-7 cell lines.

As shown in Figure 3, The three cells with G-Rh2 (80μM/l) treatment for 0, 12, 24, 48 hours dramatically

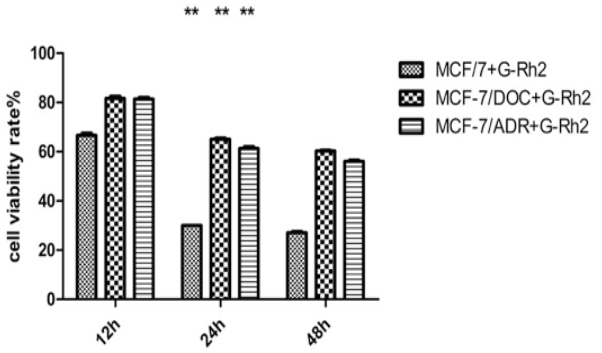

Figure 3. Effect of G-Rh2 Treatment on Viability of MCF-7, MCF-7/Doc and MCF-7/Adr Cell Lines. Each group were treated with G-Rh2 (80μM/l) during in the corresponding period (0h, 12h, 24h, 48h). All data corresponds to the mean±SD of the three independent experiments. Significantly different compared from with control by one-way ANOVA, $^{**}p < 0.01$, MCF-7/Doc *vs* MCF-7/Doc +G-Rh2, MCF-7/Adr vs MCF-7/Adr +G-Rh2

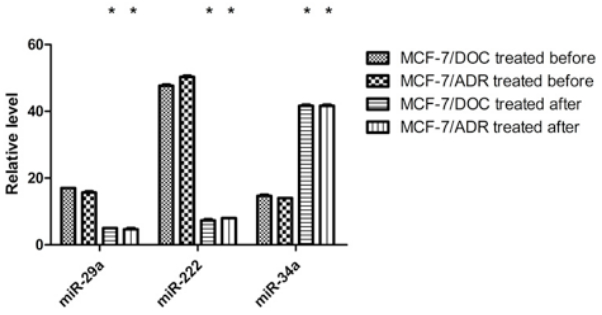

Figure 4. The miR-29a, miR-222 and miR-34a Levels were Significantly Lower After G-Rh2Intervention (80μM/l) for 24h. $^{**}p<0.01$, MCF-7/Doc *vs* MCF-7/Doc +Rh2, MCF-7/Adr vs MCF-7/Adr + G-Rh2

decreased cell viability, In a word, it shows G-Rh2 treatment decreases the viability of breast cancer cells in a dose and time-dependent manner.

G-Rh2 mediating drug-specific miRNA

In previous study, we testified that 5 drug-resistance specific miRNA is related to drug-resistance in MCF-7/Doc and MCF-7/Adr cells (miR-34a↓, miR-130a↑, miR-29a↑, miR-222↑and miR-452↑) (11, 12), Compared to the two cells without intervention, three miRNAs (miR-222, miR-34a and miR-29a) have significant reversal on resistance to change among the five drug-specific miRNAs after G-Rh2 intervention. (Figure 4)

G-Rh2 can reverse drug resistance

MCF-7, MCF-7/Doc, MCF-7/Adr, MCF-7/Doc or MCF-7/Adr with G-Rh2 intervention divided into two groups: the Doc or Adr treated and untreated group, by Doc and Adr, remnants of green fluorescent cells had significant differences between MCF-7/Doc, MCF-7/Adr and MCF-7 cells; proving the existence of MCF-7/Doc and MCF-7/Adr drug resistance. However, the residual green fluorescent cells of the MCF-7/Doc and MCF-7/Adr cells with G-Rh2 intervention coped with the drug was significantly reduced more than the two cells without intervention; suggesting that drug resistance can be reversed by G-Rh2 (Figure 5AB).

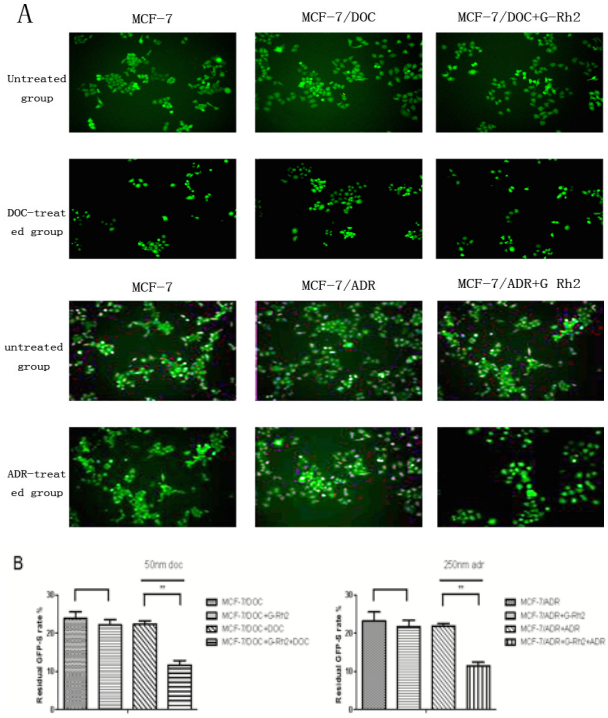

Figure 5. The Green Fluorescent Cells Expression in MCF-7, MCF-7/Doc, MCF-7/Adr cells and MCF-7/Doc and MCF-7/Adr Cells with G-Rh2 Intervention (80μM/l, 24h). A). The residual GFP-S rate was determined after cell mixture was treated with 50nm Doc or 250nm Adr for 24h. B)***p*<0.01, MCF-7/Doc or MCF-7/Adr vs MCF-7/Doc +G-Rh2 or MCF-7/Adr+G-Rh2

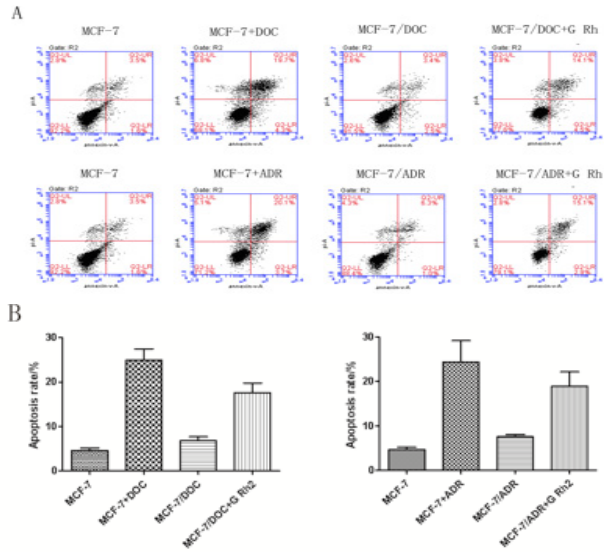

Figure 6. G-Rh2 Induced Apoptotic Cells in MCF-7 Cells, MCF-7/Doc Cells, MCF-7/ADR Cells, G-Rh2 Induced Apoptosis in MCF-7/Doc Cells was Compared with the Negative Control. Similarly, G-Rh2 induced apoptosis in MCF-7/ADR cells was compared with the negative control (A). As shown in (B), the apoptotic cells rate was significant differences. The experiment was performed three times, each in triplicate. Each point represented the means±SD ***p*<0.01, MCF-7/Doc or MCF-7/Adr vs MCF-7/Doc +G-Rh2 or MCF-7/Adr+G-Rh2

G-Rh2 induces apoptotic cell

As shown in Figure 6AB, by flow cytometry analysis, G-Rh2 (80μM/l) induced a significantly increased

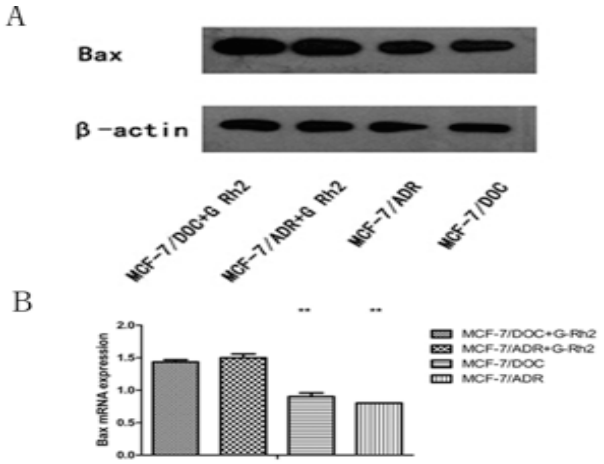

Figure 7. G-Rh2 Treatment Alters Levels of Bax Proteins (A) and mRNA (B) Levels in Breast Cancer Cells. MCF-7/Doc and MCF-7/ADR cells treated with DMSO (control) or 80μM/l G-Rh2 for 24 hours, β-actin was used as a loading control in western blot. A representative pattern is shown from two independent experiments, and the results were consistent

apoptosis rate in MCF-7/Doc cells and MCF-7/Adr cells, compared with negative controls. The apoptosis rate in MCF-7/Doc cells: control, 4.63%±0.32% *vs*. MCF-7/Doc cells, 6.83%±0.50% ($p<0.05$) *vs*. MCF-7+Doc, 24.53%±1.35% ($p<0.05$) *vs*. MCF-7/Doc +G-Rh2, 18.97%±1.20% ($p<0.05$), and mean±SD. Similarly, in Figure 7D, the apoptosis rate in MCF-7/Adr and MCF-7/Doc cells: control, 4.63%±0.32% *vs*. MCF-7/Adr cells, 7.53%±0.28% ($p<0.05$) *vs*. MCF-7+Adr, 24.40%±2.73% ($p<0.05$) *vs*. MCF-7/Adr +G-Rh2, 18.93%±1.85% ($p<0.05$), mean±SD.

G-Rh2 treatment alters the expression of Bax protein in breast cancer cells

As we all know, the gene Bax expression has a role in inducing apoptosis and anti-chemoresistance, study shows some miRNAs related to Bax in it, such as miR-34a, miR-511, miR-365, miR-214, etc. we utilized western blot to detect Bax expresses. As shown in Figure 7AB, the results revealed the Bax expression in MCF-7/Doc and MCF-7/Adr cells significantly decreased, compared with MCF-7 cells; however, the Bax expression in MCF-7/Doc and MCF-7/Adr cells with G-Rh2 intervention significantly increased, compared with the two cells with intervention.

Discussion

One important factor which limits the application of advanced breast cancer treatment is acquired drug-resistance. G-Rh2 has showed a great diversity of biological activities, including immunomodulatory effects, antioxidative, anti-inflammatory, anti-tumor and vasorelaxing activities; In this study, we proved G-Rh2 could mediate the MDR specific miRNA, then regulate the corresponding target genes -- reversing the drug-resistant of BCA cells.

We researched the miRNA expression profiles and explored miRNAs that could be associated to drug resistance. There are several mechanisms have recently

been shown to be targeted by miRNAs in drug-resistant breast cancer, including: decreased intracellular drug concentrations; mediated by drug transporters and metabolic enzymes; impaired cellular responses that affect cell cycle arrest, apoptosis; DNA repair and alterations in the availability of drug targets.

To further verify whether the differential miRNAs expression has a major influence in preventing the process of acquiring drug resistance with G-Rh2 intervention, We tested the five drug-resistance specific miRNAs from both MCF-7/Adr and MCF-7/Doc cells. The results showed that only miR-222, miR-34a and miR-29a changed the characteristics of drug resistant breast cancer cells to Doc and ADR, out of the five selected miRNAs. We therefore conclude that G-Rh2 could mediate the miRNA expression to reduce the drug resistance of breast cancer.

The residual green fluorescent cells of the G-Rh2-mediated cells groups was lesser than another groups; suggesting that anticancer effect and reverse the drug resistance of G-Rh2 has a synergistic effect.

MDR is often related with lowerexpression of Bax, leading to chemotherapeutic failure. Study shows miR-34a regulatory ensures Bax-dependent apoptosis of non-small cell lung carcinoma cells. In order to further explore a possible role of Bax in the effect of G-Rh2 on reversing drug resistance, we assessed Bax expression in beast cancer cells. Our results indicate that G-Rh2 effectively activated drug-resistant BCA cells to Doc and Adr through upregulation of Bax.

In summary, G-Rh2 causes a strong anti-chemoresistance effect in BCA cells, therefore, miRNA plays a major influence in the G-Rh2-mediated effect, particularly, the three specified drug-resistant miRNAs. Moreover, this study provides a novel insight of the molecular mechanisms of G-Rh2 -- reversing drug-resistance by using the latest technology to detect miRNA communication mechanisms. However, this study still has some shortcomings, if animal models are embraced into our study -- through pharmaceutical interventions on the above subjects -- we could further explore how Chinese medicine can inverse and inhibit chemotherapy resistance in BCA from a clinical perspective; which could make our conclusions more persuasive.

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