Ginsenoside Rh2 mediates changes in the microRNA expression profile of human non-small cell lung cancer A549 cells

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Abstract. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer insensitive to chemotherapy. Efforts are, therefore, directed toward understanding the molecular mechanisms of chemotherapy insensitivity and the development of new anticancer drugs. Ginsenoside Rh2, one of the components in ginseng saponin, has been shown to have anti-proliferative effect on human NSCLC cells and is being studied as a therapeutic drug for NSCLC. microRNAs (miRNAs) are small, non-coding RNA molecules that play a key role in cancer progression and prevention. However, the miRNA portrait of ginsenoside Rh2-treated NSCLC cells has not yet been studied. In this study, we identified a unique set of changes in the miRNA expression profile in response to Rh2 treatment in the human NSCLC cell line A549. Using miRNA microarray analysis, we identified 44 and 24 miRNAs displaying changes in expression greater than 2-fold in Rh2-treated A549 cells. In addition, using an miRNA target prediction program, we discovered that these miRNAs are predicted to have several target genes related to angiogenesis, apoptosis, chromatic modification, cell proliferation and differentiation. Thus, these results may assist in the better understanding of the anticancer mechanism of Rh2 in NSCLC.

Introduction

Although several subtypes of lung cancer have been reported to date, there are mainly two types, small-cell and non-small cell lung cancer (NSCLC) (1). NSCLC is the most common type of lung cancer, accounting for approximately 80% of all lung cancer cases, and is highly associated with cigarette smoking. The incidence rate for this type of cancer is increasing dramatically (1). Efforts to cure NSCLC through the development of specific anticancer drugs have failed since NSCLC cells demonstrate relatively high insensitivity to these drugs as an early or a late response (2). Parallel to the drug development, there have been attempts to investigate the molecular portraits of existing anticancer drugs to understand the various drug-related mechanisms at the cellular and molecular levels. This information is being effectively used to develop novel anticancer drugs, early detection markers and diagnostic factors for the successful management of NSCLC (3). However, previous studies have focused mainly on the effectiveness of drugs in NSCLC and not on the underlying molecular mechanisms of the drug action. In order to overcome the issue of drug insensitivity in NSCLC, it is important that the drug-related molecular mechanisms be studied.

microRNAs (miRNAs) are small, non-coding RNA molecules, which are known as important regulators of almost all cellular signaling pathways including normal cell development as well as disease development (4). miRNAs may directly bind to the 3' untranslated regions of their target genes, thereby inhibiting protein synthesis by inhibiting translation (4). Recently, miRNAs have been reported to determine malignancy in lung cancer; low levels of miR-200c in NSCLC cells have been associated with aggressive, invasive growth and metastasis (5). Moreover, miR-21 is overexpressed in NSCLC tissues compared to adjacent non-tumor tissues and it represses the tumor suppressor PTEN and stimulates growth and invasion of NSCLC cells (6,7). Furthermore, miR-126, which is downregulated in NSCLCs, is involved in regulating the response of NSCLC cells to cancer chemotherapy (8). miR-126 may strongly enhance the drug sensitivity of the cells to anticancer agents, including adriamycin and vincristine, through the negative regulation of its target mRNA, which is vascular endothelial growth factor A (VEGF-A) (8). These reports suggest that miRNAs directly regulate not only NSCLC growth, but also anticancer drug sensitivity.

Ginsenoside Rh2 is one of the bioactive components extracted from ginseng, which is a traditional herbal medicine originally used in Asia (9). Several health benefits of Rh2 have been reported due to its anti-inflammatory, anti-

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osteoclastogenic, anti-hyperglycemic and anticancer effects (10-14). The anticancer effect of Rh2 has been particularly observed in NSCLCs. Rh2 is able to block cell proliferation, cause G1 phase arrest, enhance the activity of capase-3 and induce apoptosis in NSCLC A549 cells (15,16). Additionally, combined treatment with Rh2 and betulinic acid synergistically induces apoptosis in A549 cells (17). Notably, the tumor-inhibiting effects of Rh2 have also been induced by hypersensitizing multidrug-resistant cancer cells (18). Rh2 also promotes the reversal of the resistance of NSCLC A549/DDP cells to cisplatin through the mitochondrial apoptotic pathway (19). These studies indicate that Rh2 exerts its anti-cancer effects through the induction of an apoptotic pathway; however, miRNA-based molecular profiling of Rh2-treated NSCLC cells has not yet been conducted to study this process.

By using miRNA expression profiling, to the best of our knowledge, we report the first study demonstrating that the anticancer effect of Rh2 on NSCLC cells is mediated through changes in miRNA expression. We further report that the differentially expressed miRNAs are predicted to have several target genes with anticancer properties.

Materials and methods

Cell cultures. The NSCLC cell lines A549, H1299, Lu-99, EBC-1 and H460 were purchased from ATCC (Manassas, VA, USA) and cultured in RPMI-1640 (Gibco-BRL; Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and penicillin/streptomycin at 37°C in a humidified chamber containing 5% CO_2. For 96-well plate-based experiments, $2x10^3$ cells were seeded into each well and for 60-mm plate-based experiments, $7x10^5$ cells were seeded into each dish.

Ginsenoside Rh2 treatment and cell viability analysis. Ginsenoside Rh2 (analytical grade with 97% purity) was purchased from Sigma-Aldrich, dissolved in ethanol at a concentration of 10 and 40 mg/ml and stored at -20°C until use. Cell viability was determined using the water-soluble tetrazolium salt-1 (WST-1) assay (EZ-Cytox cell viability assay kit; Itsbio, Seoul, Korea) according to the manufacturer's protocol. In brief, cells were incubated in 96-well plates and treated with ethanol or various concentrations of Rh2 for 24 h. After incubation, the kit solution was added, and the cells were again incubated at 37°C for 2 h. Cell viability was measured using an iMark microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm (620-nm reference filter).

miRNA microarray analysis. NSCLC cells treated with or without Rh2 were collected, and then total RNA was purified using the TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. The concentration and quality of each RNA sample were measured using MaestroNano®, a micro-volume spectrophotometer (Maestrogen, Las Vegas, NV, USA). The integrity of each RNA sample was verified using an Agilent 2100 Bioanalyzer® (Agilent Technologies, Santa Clara, CA, USA).

A total of 100 qualified RNA samples were dephosphorylated and labeled with Cyanine 3-pCp using T4 RNA ligase by incubating at 16°C for 2 h. After the labeling reaction, the
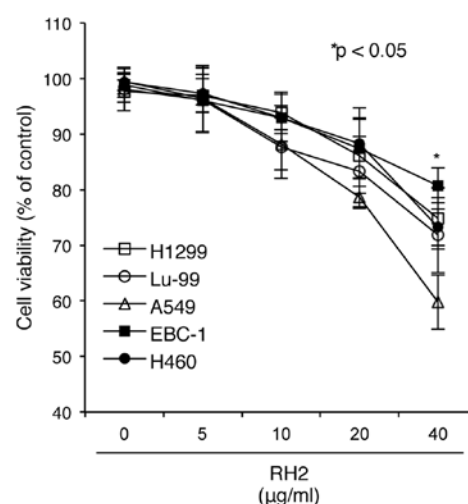

Figure 1. Ginsenoside Rh2 has an anti-proliferative effect on NSCLC cell lines. H1299, Lu-99, H460, EBC-1 and A549 cells ($2x10^3$) were treated with 5, 10, 20 and 40 µg/ml of Rh2 for 24 h. Cell viabilities of the cell lines were detected using the water-soluble tetrazolium salt-1 (WST-1) assay. All experiments were performed in triplicate (n=3). Data are represented as the means ± standard deviation. The Student's t-test was performed to determine statistical significance (*p<0.05).

samples were completely dried using a vacuum concentrator at 55°C for 4 h. The dried samples were treated with GE blocking agent. The SurePrint G3 Human v16 miRNA 8x60K array that contains probes for 1,205 human and 144 human viral miRNAs was used for miRNA profiling. The blocked samples were hybridized to the probes on the microarray at 55°C with a constant rotation at 20 rpm in the Agilent microarray hybridization chamber for 20 h. The microarray slide was washed and scanned using the Agilent scanner to obtain the microarray image. The numerical data for the miRNA profiles were extracted from the image using the Feature Extraction program. These data were analyzed with the aid of the GeneSpring GX software version 7.3 (all were from Agilent Technologies).

The raw data were filtered using FLAG and t-tests. Differentially expressed miRNAs were determined using the fluorescence ratio between the control and Rh2-treated samples, and miRNAs displaying an increase or decrease >2-fold were selected for analysis.

Bioinformatic analysis of miRNAs. Changes in miRNA expression of 2-fold and more between the control and Rh2-treated groups were selected and their putative cellular target genes were predicted using MicroCosm Target version 5 (www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/). Using the gene ontology (GO) analysis tool AmiGO (amigo.geneontology.org/cgi-bin/amigo/browse.cgi), the target genes were categorized into the following three groups: apoptosis, cell proliferation and angiogenesis.

Statistical analysis. Statistical analysis was performed using the χ^2 test or the Fisher's exact test and Spearman's rank correlation coefficient analysis. $p<0.05$ was considered to indicate a statistically significant difference. All values were expressed as the means ± standard deviation.
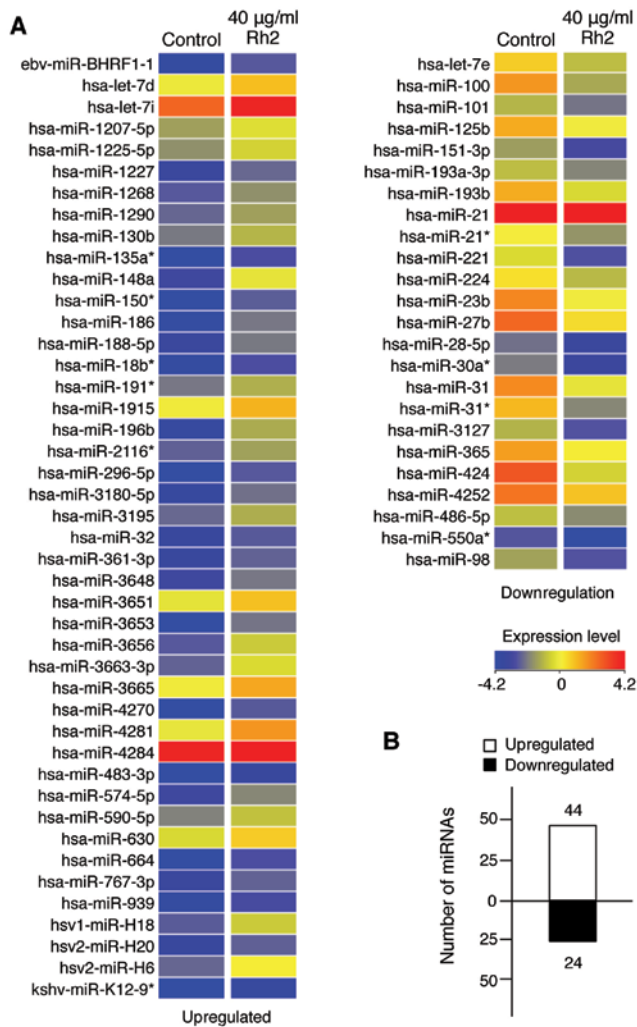
Figure 2. Ginsenoside Rh2 promotes changes in the miRNA expression profiles of NSCLC A549 cells. (A) The Rh2-treated and untreated (control) A549 cells were collected, and total cellular RNA was purified. The RNA samples were re-labeled at the 3'-end with pCp-Cy3. The labeled RNA samples were subjected to miRNA microarray analysis. miRNAs showing >2-fold changes in their expression levels compared to the control were selected. The types and the expression levels of miRNAs that were upregulated (left panel) and downregulated (right panel) following Rh2 treatment in A549 cells are represented by a heat map image. (B) The graph represents the number of upregulated and downregulated miRNAs in Rh2-treated A549 cells. *, Passenger strand from the miRNA duplex.

Table I. Expression levels of miRNAs exhibiting >2-fold expression changes in response to ginsenoside Rh2 in A549 cells[a].

miRNA name	FC	miRNA name	FC
Upregulated			
ebv-miR-BHRF1-1	2.63	miR-32	2.16
let-7d	2.21	miR-361-3p	2.29
let-7i	2.99	miR-3648	2.14
miR-1207-5p	2.40	miR-3651	2.38
miR-1225-5p	2.41	miR-3653	6.04
miR-1227	2.29	miR-3656	4.24
miR-1268	2.03	miR-3663-3p	4.82
miR-1290	2.05	miR-3665	2.79
miR-130b	2.19	miR-4270	4.41
miR-135a	4.88	miR-4281	3.97
miR-148a	9.59	miR-4284	2.19
miR-150*	9.29	miR-483-3p	2.20
miR-186	4.02	miR-574-5p	2.66
miR-188-5p	2.79	miR-590-5p	2.36
miR-18b	2.20	miR-630	2.51
miR-191*	2.13	miR-664	3.75
miR-1915	2.34	miR-767-3p	2.11
miR-196b	7.33	miR-939	2.06
miR-2116*	2.30	hsv1-miR-H18	4.27
miR-296-5p	3.54	hsv1-miR-H20	2.21
miR-3180-5p	2.95	hsv1-miR-H6	7.40
miR-3195	2.49	hsv1-miR-K12-9*	2.31
Downregulated			
let-7e	3.60	miR-27b	4.54
miR-100	9.25	miR-28-5p	2.33
miR-101	2.35	miR-30a	2.61
miR-125b	2.64	miR-31	4.76
miR-151-3p	3.18	miR-31*	9.58
miR-193a-3p	2.11	miR-3127	3.46
miR-193b	3.68	miR-365	2.72
miR-21	4.16	miR-424	12.44
miR-21*	3.88	miR-4252	2.73
miR-221	6.11	miR-486-5p	2.01
miR-224	2.96	miR-550a*	3.02
miR-23b	4.19	miR-98	2.78

[a]The list was selected to demonstrate miRNAs representing >2-fold expression change after flag sorting. FC, fold-change. *, Passenger strand from the miRNA duplex.

Results and Discussion

Since the anti-proliferation properties of ginsenoside Rh2 have been previously investigated only in A549 cells (15,16,19), in the present study, we examined whether the anticancer effect of Rh2 could be demonstrated with other NSCLC cell lines. Therefore, NSCLC cell lines, Lu-99, H1299, H460, EBC-1 and A549, were exposed to various concentrations of Rh2 for 24 h, following which the proliferation of the cells was measured. The WST-1 assay revealed that the Rh2-mediated decrease in cell viability was induced in a dose-dependent manner in these NSCLC cell lines (Fig. 1). In particular, A549 cells exhibited a relatively high sensitivity to Rh2. Treatment with 40 μg/ml of Rh2 decreased the A549 cell viability up to 59.7% compared to the control, indicating that ginsenoside Rh2 exerted a strong anti-proliferative activity in NSCLC cells.

Previous studies on the molecular pathways related to Rh2-mediated anticancer effect have been limited to the activation of caspase-3 and -8 and the upregulation of pRb2 and TRAIL-RI death receptor (16,17). In order to identify additional molecular pathways participating in the anticancer effect

Table II. Predicted targets of miRNAs exhibiting an upregulation in response to Rh2 in A549 cells.

| miRNA name | Function of target genes | | |
	Angiogenesis	Apoptosis	Cell proliferation
has-let-7d	FASLG	CDKN1A, FAS, TP53, CASP3, IRS2, TNFSF9, NME6, TAF9B, FASLG, MAP3K1, TGFBR1	CDKN1A, FAS, TP53, CASP3, IRS2, TNFSF9, IL13, NAP1L1, EIF2S2, OSMR, FASLG, TGFBR1
hsa-miR-32	TWIST1, GATA6	TWIST1, SGK3, BCL11B, RAD21, MAP2K4, ACTC1, TRAF3, ADRB1, APPL1, USP28, GATA6, PAX3	TWIST1, SGK3, BCL11B, EVI5, CDKN1C, NKX2-3, BOX4, CDC27, FBXW7, APPL1, USP28, TSC1, GATA6, PAX3
hsa-miR-148a	TEK, ROCK1, DDAH1, KLF4, BAI3	TEK, ROCK1, MITF, ROBO1, TGFA, WNT1, KLF4, BCL2L11, ROBO2, PTEN, ERBB3, SOS2, IGF1	TEK, MITF, ROBO1, TGFA, WNT1, NRAS, KLF4, PTEN, ERBB3, IGF1
hsa-miR-186	VEGFA, CXCL13	VEGFA, CXCL13, XIAP, PRDX5, SORT1, TERF1, IGF1R, TGFBR2, PDCD4	VEGFA, XIAP, PID1, PDGFC, HOOK3, IGF1R, TGFBR2, JAG1
hsa-miR-296-5p	CXCL10	MEF2D	CXCL10
hsa-miR-130b	BTG1	BTG1, IRF1, PPARG, SPHK2, FOSL1, RUNX3, RNF41, TRIM2, SLTM, PHF17, TP53INP1	BTG1, IRF1, PPARG, SPHK2, FOSL1, RUNX3, HOXA3, WNT2B
hsa-miR-196b	HOXA5, TSPAN12	HOXA5, CDKN12, BIRC6, ABL1, RASSF5	HOXA5, CDKN1B, BIRC6, ING5
hsa-miR-483-3p	HIPK2	HIPK2, SATB1, BAG1	HIPK2, SATB1
hsa-miR-767-3p	NPR1	AGT, BLOC1S2, PDCD6IP, CSNK2A1, TRAF7	NRP1, AGT, BLOC1S2, PDPN, EVI1, UBR5, TIMP2, CNOT8, CDC25C
hsa-miR-361-3p	-	PIM2, RAD9A, USP47, MED1, OGT, MBD4	PIM2, RARA, MED1
hsa-miR-1227	-	ECE1, RYBP, TNFAIP8	-
hsa-miR-1207-5p	PRKCA, GPR124	PRKCA, EGLN2, MKL1, TNS4, CBL, FGFR1	PRKCA, ACVRL1, FGFR1
hsa-miR-1290	GTF2I	BECN1	BECN1, CHUK, CREBBP, MLL2
hsa-miR-664	FOXO4	CUL3, CYCS, BMX, TAF9	FOXO4, CUL3, SMAD4, IRF2

of Rh2, we performed miRNA expression profiling analysis with the aim to detect specific regulators of the Rh2-mediated anticancer properties in NSCLCs. On the basis of a recent report that Rh2 inhibits glioma cell proliferation by targeting miR-128, it is considered a novel mechanism for the anticancer effect of Rh2 (13). In the present study, we discovered that the expression of specific miRNAs in Rh2-treated A549 cells was significantly altered when compared to that in the untreated A549 (control) cells (Fig. 2A). The color bar in Fig. 2 represents altered fluorescence intensity corresponding to miRNAs that were either upregulated (red color) or downregulated (blue color) following Rh2 treatment. A total of 44 miRNAs were upregulated and 24 miRNAs were downregulated in our experiment (Fig. 2B). The fold changes in miRNA expression are shown in Table I. In particular, the expression level of miR-148a was significantly upregulated by 9.59-fold, whereas the

level of miR-424 was significantly downregulated by 12.4-fold in Rh2-treated NSCLC A549 cells.

Noteworthy, although there are no reports regarding the role of miR-148a in NSCLC, miR-148a may prove a novel Rh2 target in NSCLC. miR-148a was originally shown to be downregulated in human breast cancer; however, it is associated with improved response to chemotherapy in esophageal cancer cell lines, attenuates paclitaxel-resistance of hormone-refractory, drug-resistant prostate cancer PC3 cells, suppresses gastric cancer cell invasion and metastasis, and promotes apoptosis in colorectal cancer cells (20-24). Therefore, future studies should be directed towards elucidating the relationship between Rh2-mediated anticancer properties and miR-148a upregulation in NSCLC.

Since the cellular functions of miRNAs are directly mediated by controlling their target gene expression (25), we

Table III. Predicted targets of miRNAs exhibiting a downregulation in response to Rh2 in A549 cells.

miRNA name	Function of target genes		
	Angiogenesis	Apoptosis	Cell proliferation
has-let-7e	FASLG, THBS1, PLXND1	FASLG, MAPK3K1, THBS1, CDKN1A, IGF1R, NGF, TP53, CASP3, IRS2, TGFBR1, FAS	FASLG, THBS1, CDKN1A, IGF1R, FAS, NGF, TP53, CASP3, IRS2, IL13, NRAS, MLL2, CD86, TGFBP1
hsa-miR-21	KRIT1, RHOB, FASLG, FGF1	KRIT1, RHOB, IL2A, PDCD4, MSH2, FASLG, MAP3K1	KRIT1, IL12A, JAG1, TGFBI, DDX11, FASLG, NFIB
hsa-miR-31	-	KIF1B, RASA1, MAP3K1, CD28	IL34, NUMB, CREG1, CD28
hsa-miR-101	PTGS2	PTGS2, JAK2, MITF, DDIT4, USP47, RAC1, ROBO2, TGFBR1, TIAM2, SGK1	PTGS2, JAK2, MITF, TGFBR1, RAP1B, SGK1
hsa-miR-221	HIPK1, ANGPTL4	HIPK1, ANGPTL4, CDKN1B, BCL2L11, AKAP13, SOCS3, ERBB4	HIPK1, CDKN1B, KIT, CCDC88A, MBD2, ERBB4, ARNT
hsa-miR-23b	FGF2, TNFAIP3	FGF2, PRDX3, IL6R, IL12B, FAS, PPARGC1A, MAPK3K9, NKX3-2, MAP3K1, PAK6, PDPK1, CASP7, HSP90B1, PROK2, ERBB4	FGF2, PRDX3, IL6R, IL12B, CNN2, ADRA2A, MAP7, IGSF8, PROK2, ELF5, TGFBR3, DDX11, ERBB4
hsa-miR-27b	SFRP1, ADORA2B, GATA2, RUNX1, FGF1, PLXND1	SFRP1, XIAP, PPARG, BMI1, CHEK2, CREB1, MAGI3, BCL3, MAPK2K4, BAK1, CD28	SFRP1, ADORA2B, GATA2, XIAP, RUNX1, PPARG, RXRA, BMI1, INSR, TSC1, VEGFC, RNF139, FGF1, MET, BAK1, LIFR, E2F7
hsa-miR-125b	COL4A3, AGGF1, WAR5, TNFAIP3	COL4A3, MAP3K10, MAP3K11, CASP2, TIAF1, TP53INP1, TNFAIP3	AGGF3, WARS, MAP3K11, FBXW4, TNFSF4, BAP1, TNFAIP3
hsa-miR-193a-3p	-	ERBB4, MCL1, TNFRSF21, SIAH1	ERBB4, ARNT, ING5, LAMC1, SKAP2, CCND1
hsa-miR-365	ADM	E2F2, TIAM2, SGK1, BCL11B	ADM, PAX6, HDAC4, NFIB, SGK1, BCL11B
hsa-miR-151-3p	HIF1A	HIF1A	HIF1A, DDX11
hsa-miR-424	NF1, VEGFA	NF1, VEGFA, PDCD6IP, IKBKB, YAP1, SIAH1, RAF1, NOTCH2, BDNF, BFAR, WNK3, CD28, SGK1	NF1, VEGFA, YAP1, NOTCH2, RAF1, BDNF, IRS2, WNT5, MAP2K1, EVI5, PPAP2A, CDC27, PRDM4, CD28, CCND1, SGK1
hsa-miR-486-5p	-	FOXO1, PIM1, PTEN, BCL11B	FOXO1, PIM1, PTEN, SMAD2, BCL11B

further analyzed the putative target genes of the miRNAs and the functional relationship between the gene and anticancer properties using bioinformatic tools. First, the miRbase target database tool, MicroCosm, revealed that 827 genes were potentially targeted by Rh2-specific miRNAs. Moreover, since Rh2 promotes the death of cancer cells through its anti-proliferative, anti-angiogenic and apoptotic activities, we selected genes having functions related to apoptosis, cell proliferation and angiogenesis (26) by using the GO analysis tool, AmiGO. Consistent with the previous finding, the GO analysis results showed that several target genes of the Rh2-responsive miRNAs were functionally involved in anticancer pathways. These sets of genes are listed in Tables II and III.

In summary, the present study demonstrates that a subset of human miRNAs reveals significant changes in expression in response to ginsenoside Rh2 in the NSCLC A549 cell line. Given the strong anticancer effects of Rh2 on NSCLCs, it is likely that miRNAs play an important role in the Rh2-mediated anticancer effect. Future bioinformatic studies will highlight the role of miRNAs in Rh2-mediated functions. In the light of the fact that the mechanism of the anticancer effect of Rh2 on NSCLC cells is largely unknown, this study provides novel insight into a possible molecular mechanism through which Rh2 exerts its anticancer effect on NSCLC cells.

Acknowledgements

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