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Original Paper

Expression Profiles of microRNAs in Drug-Resistant Non-Small Cell Lung Cancer Cell Lines Using microRNA Sequencing

Shousen Hu[a] Yongliang Yuan[b,c] Zhizhen Song[b,c] Dan Yan[b,c] Xiangzhen Kong[b,c]

[a]Department of Otolaryngology Head and Neck Surgery, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, [b]Department of Pharmacy, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, [c]Henan Key Laboratory of Precision Clinical Pharmacy, Zhengzhou University, Zhengzhou, China

Key Words
miRNAs • Drug resistance • Non-small cell lung cancer • hsa-mir-561 • hsa-mir-1293

Abstract
Background/Aims: Drug resistance remains a main obstacle to the treatment of non- small cell lung cancer (NSCLC). The aim of this study was to identify the expression profiles of microRNAs (miRNAs) in drug-resistant NSCLC cell lines. ***Methods:*** The expression profiles of miRNAs in drug-resistant NSCLC cell lines were examined using miRNA sequencing, and the common dysregulated miRNAs in these cell lines were identified and analyzed by bioinformatics methods. ***Results:*** A total of 29 upregulated miRNAs and 36 downregulated miRNAs were found in the drug-resistant NSCLC cell lines, of which 26 upregulated and 36 downregulated miRNAs were found to be involved in the Ras signaling pathway. The expression levels, survival analysis, and receiver operating characteristic curve of the dysregulated miRNAs based on The Cancer Genome Atlas database for lung adenocarcinoma showed that hsa-mir-192, hsa-mir-1293, hsa-mir-194, hsa-mir-561, hsa-mir-205, hsa-mir-30a, and hsa-mir-30c were related to lung cancer, whereas only hsa-mir-1293 and hsa-mir-561 were not involved in drug resistance. ***Conclusion:*** The results of this study may provide novel biomarkers for drug resistance in NSCLC and potential therapies for overcoming drug resistance, and may also reveal the potential mechanisms underlying drug resistance in this disease.

Xiangzhen Kong, Ph.D

Department of Pharmacy, the First Affiliated Hospital of Zhengzhou University, No.1 Jianshe road, Zhengzhou, Henan (China)
Tel. +86-371-6629-5652, E-Mail kongxiangzhen118@126.com

Introduction

Non-small cell lung cancer (NSCLC) is the predominant subgroup of lung cancer, which is the most common malignancy worldwide and is characterized by high recurrence, metastasis, and mortality rates [1]. Chemotherapy and targeted therapies are two major treatments for this disease, but long-term survival of NSCLC patients is only 20–35% [2]. The major reason for treatment failure in lung cancer is that the vast majority of patients gradually acquire resistance to chemotherapy or targeted drugs, even after initially responding well [3]. There are many mechanisms of acquired resistance, but efficient means for overcoming drug resistance are still lacking. Therefore, it is necessary to identify novel methods to reverse drug resistance.

MicroRNAs (miRNAs), a class of non-coding RNAs with approximately 18–25 nucleotides, are involved in various aspects of cellular functions such as cell proliferation, differentiation, migration, and apoptosis [4-6]. Increasing evidence has shown that miRNAs play a vital role in drug resistance [7, 8], which leads to treatment failure in cancer. For example, miR-497 sensitizes osteosarcoma cells to cisplatin (DDP), in part via the PI3K/Akt pathway [9]. MiR-17 and miR-92 families play a role in DDP resistance and can be considered novel biomarkers for platinum-based chemotherapy in NSCLC [10]. Although miRNAs have diagnostic, prognostic, and therapeutic implications for drug-resistant cancer patients [11, 12], it is necessary to further study their roles in drug resistance.

In this study, we developed two drug-resistant NSCLC cell lines (A549/DDP and HCC827/gefitinib resistant [GR]) by dose gradient treatments of the A549 cell line with DDP and the HCC827 cell line with gefitinib, respectively. Then, we used miRNA sequencing (miRNA-Seq) to detect the miRNA expression profiles in A549, A549/DDP, HCC827, and HCC827/GR cells. Finally, we characterized the dysregulated miRNAs by bioinformatics methods and identified potential novel drug targets for drug-resistant NSCLC patients. The results of this study may provide novel biomarkers and potential therapies for drug resistance in NSCLC.

Materials and Methods

Cell culture

The DDP-resistant NSCLC cell line A549/DDP and the gefitinib-resistant cell line HCC827/GR were established by dose gradient treatment for almost 6 months. The drug-resistant and parental cell lines were cultured in RPMI-1640 (Hyclone, Marlborough, MA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 100 units/mL penicillin, and 100 mg/mL streptomycin. The drug concentrations used for maintaining the drug resistance of A549/DDP and HCC827/GR were 8.3 μM and 2.5 μM, respectively. The drugs were withdrawn 1 week before the experiment.

Assessment of chemotherapy sensitivity

To calculate the half maximal inhibitory concentration (IC_{50}) of the parental and drug-resistant cells, MTT assays were used to evaluate the inhibitory effects on cell proliferation. To this end, A549, A549/DDP, HCC827, and HCC827/GR cells were plated in 96-well plates and then treated with concentration gradients of DDP or gefitinib for 72 h. Then, 10 μL of 5 mg/mL MTT reagent was added to 100 μL cultured cells and withdrawn after 4 h of incubation. Finally, 150 μL dimethyl sulfoxide was added to the cells, and the absorbance was detected at a wavelength of 570 nm using a microplate spectrophotometer.

RNA isolation and purification

Total RNA was isolated from the cell culture using TRIzol reagent (Invitrogen, Carlsbad, CA) and chloroform according to the manufacturer's protocol. Total RNA concentration and purity were measured with the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA integrity was measured using the 2100 Bioanalyzer (Agilent, Santa Clara, CA) and agarose electrophoresis. All sequenced samples were chosen from high-quality RNA samples with an optical density (OD) of 260/280 ≥ 1.8, OD 260/230 ≥ 1.5, RNA integrity number ≥ 8, and ribosomal RNA (rRNA) 28S/18S ≥ 0.7.

Library construction and miRNA-Seq

Approximately 1 μg total RNA was used to prepare a small RNA library according to the protocol of TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, CA). Then, single-end sequencing (36 base pairs [bp] or 50 bp) was performed on the Illumina HiSeq 2500 System (LC Sciences, Houston, TX) according to the vendor's recommended protocol.

miRNA-Seq data analysis

The raw reads were first subjected to an in-house program, ACGT101-miR (LC Sciences) to remove adapter dimers, junk, low complexity, common RNA families (rRNA, transfer RNA, small nuclear RNA, small nucleolar RNA), and repeats. Subsequently, unique sequences of 18–26 nt in length were mapped to specific species precursors in miRBase 21.0 by conducting a BLAST search to identify known miRNAs and novel 3p- and 5p-derived miRNAs. Length variation at both the 3′ and 5′ ends and one mismatch inside the sequence were allowed in the alignment. The remaining sequences were mapped to other selected species precursors (with the exclusion of specific species) in miRBase 21.0 by a BLAST search, and the mapped pre-miRNAs were further BLASTed against specific species genomes to determine their genomic locations. The above two were defined as known miRNAs. The unmapped sequences were BLASTed against the specific genomes, and the hairpin RNA structures containing sequences were predicted from the flanking 80 nt sequences using RNAfold software (http://rna.tbi.univie.ac. at/cgi-bin/RNAfold.cgi).

Real-time quantitative PCR

Total RNA (1 μg) from A549 and A549/DDP cells was extracted with TRIzol reagent (Invitrogen) and synthesized to the cDNA of miRNA using miRNA First Strand cDNA Synthesis with tailing reaction (Shenggong, Shanghai, China), according to the manufacturer's instructions. Real-time quantitative PCR (qPCR) reactions were performed using SYBR Green Kits (Roche, Mannheim, Germany) on the Applied Biosystems QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). The relative miRNA expression level was calculated by the $2^{-\Delta\Delta CT}$ method, and U6 was used as the internal control. The real-time quantitative PCR primers used were hsa-mir-1293 forward: 5′-TGGGTGGTCTGGAGATTTGTG-3′, hsa-mir-561-5p forward: 5′-CGCGATCAAGGATCTTAAACTTTGCC-3′, hsa-mir-194-5p forward: 5′-CTGTAACAGCAACTCCATGTGGA-3′, hsa-mir-205-5p forward: 5′-TCCTTCATTCCACCGGAGTCT-3′, hsa-mir-30a-5p forward: 5′-CGTGTAAACATCCTCGACTGGAAG-3′, hsa-miR-30c-2-3p forward: 5′-CCTGGGAGAAGGCTGTTTACTCT-3′.

Gene ontology enrichment and Kyoto Encyclopedia of Genes and Genomes pathway analysis

To explore the functional roles of the dysregulated miRNAs, the DAVID bioinformatics resource (v6.8; https://david.ncifcrf.gov/) was used for gene ontology (GO) analysis, and the KOBAS web server (v3.0; http://kobas.cbi.pku.edu.cn/) was employed for Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis with $P < 0.05$.

The Cancer Genome Atlas data analysis

The Cancer Genome Atlas (TGCA) data of patients with lung adenocarcinoma were downloaded from the TGCA website (https://cancergenome.nih.gov/), which is a public funded database that aims to catalogue major cancer-causing genomic alterations to create a comprehensive list of cancer genomic profiles. The expression levels of representative miRNAs between normal and tumor tissues were calculated using the R package and were delineated by box-plot analysis.

Statistical analysis

SPSS software (v13.0; SPSS Inc., Chicago, IL) was employed for statistical analysis. The results are expressed as the mean ± standard deviation (SD). The Student's *t*-test was used to estimate significant differences between groups. $P < 0.05$ was considered statistically significant.

Results

Verification of DDP/gefitinib-resistant cell lines

After 72 h of drug exposure, the IC_{50} values for A549, A549/DDP, HCC827, and HCC827/GR were 2.48, 25.03, 5.72, and 36.48 μM, respectively (Fig. 1A, B), showing a 10.09-fold increase for A549/DDP and a 6.38-fold increase for HCC827/GR. These results verified that the A549/DDP cell line was resistant to DDP and the HCC827/GR cell line was resistant to gefitinib.

Identification of miRNA candidates in A549, A549/DDP, HCC827, and HCC827/GR cell lines by miRNA-Seq

miRNA-Seq was conducted in A549, A549/DDP, HCC827, and HCC827/GR cell lines. A Venn diagram was employed to show the numbers of expressed miRNAs in the cells (Fig. 2A), and heat map analysis was used to visually depict the miRNA expression levels (Fig. 2B).

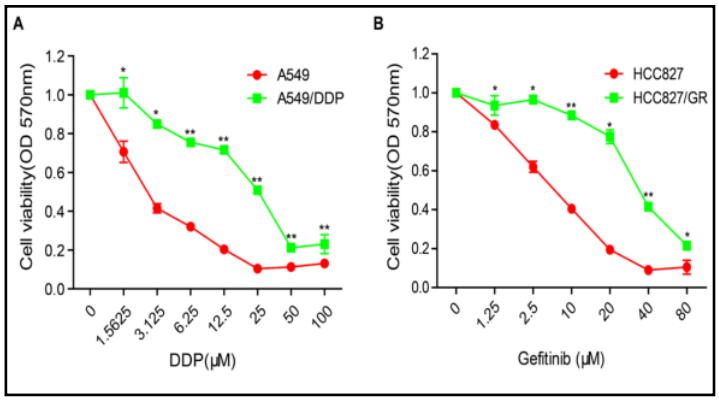

Fig. 1. Verification of the DDP/gefitinib-resistant cell lines. MTT assays of A549, A549/DDP, HCC827, and HCC827/GR cells incubated with concentration gradients of DDP or gefitinib for 72 h. Results are presented as the mean ± SD (n = 3). *P<0.05 and **P<0.01.

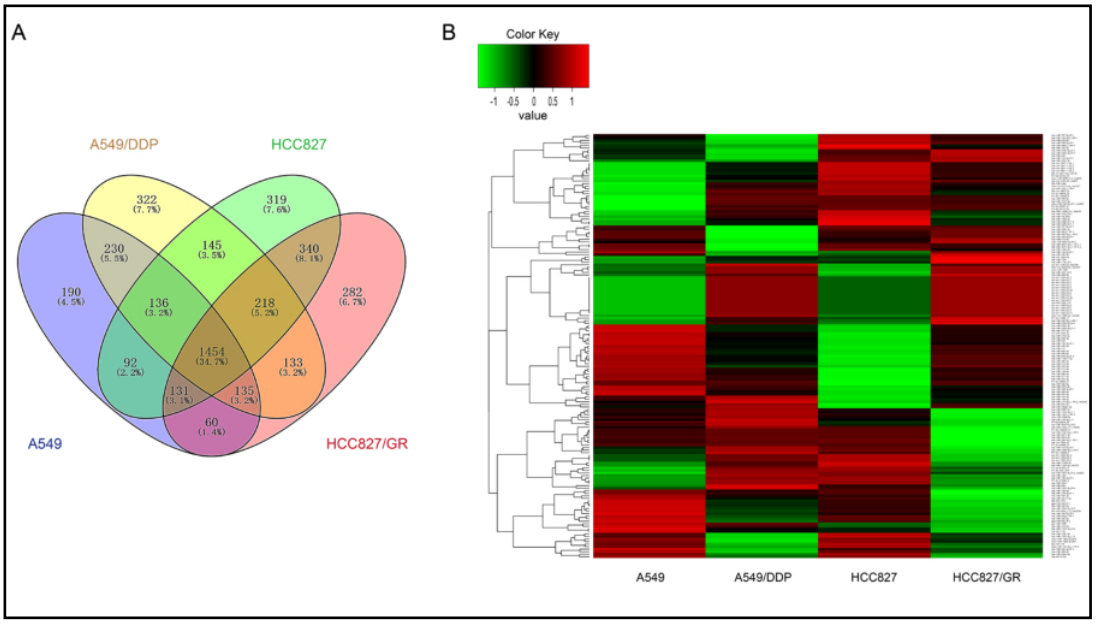

Fig. 2. Differentially expressed miRNAs in A549, A549/DDP, HCC827, and HCC827/GR cells. (A) Venn diagram showing the numbers of expressed miRNAs in A549, A549/DDP, HCC827, and HCC827/GR cells. (B) Heat map displaying the differential expression and hierarchical clustering of miRNAs in A549, A549/DDP, HCC827, and HCC827/GR cells.

Identification and characterization of the same trends in miRNA changes in A549 and A549/DDP, HCC827, and HCC827/GR

To identify differentially expressed miRNAs in the A549, A549/DDP, HCC827, and HCC827/GR cell lines, we defined different miRNAs between A549 and A549/DDP as Group 1, and HCC827 and HCC827/GR as Group 2. Then, we chose the same miRNAs between Group 1 and Group 2 with the criteria of fold change ≥ 2 and $P < 0.05$. Venn analysis showed that there were 29 upregulated miRNAs and 36 downregulated miRNAs (Fig. 3A, B). Heat map analysis visually displayed the dysregulated miRNAs expression levels (Fig. 3C, D).

Validation of miRNA expression by real-time quantitative PCR

To validate miRNA-Seq data, three upregulated and three downregulated miRNAs were detected by real-time quantitative PCR, and the results are shown in Fig. 4. The expression profiles of these miRNAs were in line with the miRNA-Seq data, indicating that data obtained by miRNA-Seq are highly reliable.

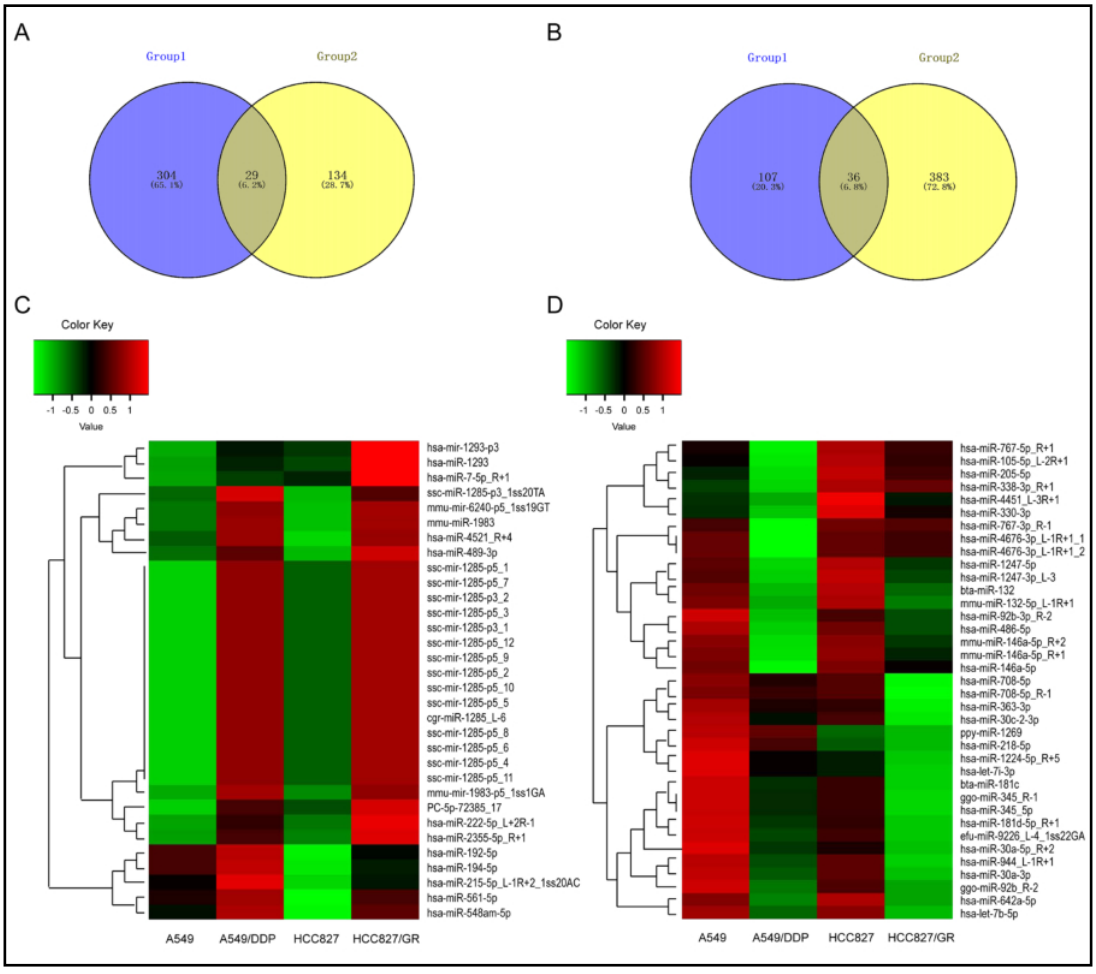

Fig. 3. Expression of the same change trends of miRNAs in A549, A549/DDP, HCC827, and HCC827/GR cells. Venn diagram showing the numbers of upregulated miRNAs (A) and downregulated miRNAs (B) between Groups 1 and 2 with the criteria of fold change ≥ 2 and P<0.05. Group 1 represents the different miRNAs between A549 and A549/DDP. Group 2 represents the different miRNAs between HCC827 and HCC827/GR. Heat map displaying the expression and hierarchical clustering of the upregulated miRNAs (C) and the downregulated miRNAs (D).

GO enrichment and KEGG pathway analysis of the dysregulated miRNAs

Recent studies have shown that miRNAs may regulate their parental genes to participate in biological processes [13, 14]. To determine the potential functions of the dysregulated miRNAs, we employed GO and KEGG pathway analysis. The results of GO analysis of the upregulated and downregulated miRNAs and their identified target genes are shown in Fig. 5. GO analysis indicated that numerous target genes were involved in the regulation of transcription and protein binding. KEGG analysis revealed that 12 pathways, such as autophagy, vascular endothelial growth factor signaling, and Ras signaling, may be involved in both the upregulation and downregulation of miRNAs (Fig. 6).

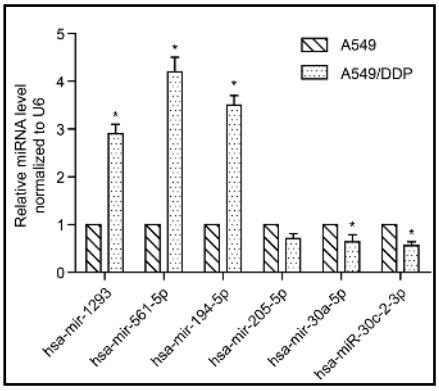

Fig. 4. Validation of miRNA expression by qPCR. The results are expressed as the mean ± SD deviation. The Student's t-test was used to estimate significant differences among groups. P<0.05 was considered statistically significant.

Prediction and annotation of miRNA-mRNA co-expression networks involved in Ras signaling pathway

Among the predicted KEGG pathways, Ras signaling pathway is reportedly involved in drug resistance [15]. We predicted the miRNAs involved in the Ras signaling pathway, including 26 upregulated and 36 downregulated miRNAs. Combined with the data of lung adenocarcinoma from the TCGA database, seven candidate miRNAs, including the upregulated miRNAs (hsa-mir-192, hsa-mir-1293, hsa-mir-194, and hsa-mir-561) and downregulated miRNAs (hsa-mir-205, hsa-mir-30a, and hsa-mir-30c), were taken as representative miRNAs. miRNA-mRNA co-expression networks were constructed using Cytoscape to visualize the regulatory network (Fig. 7).

Expression levels, survival analysis, and receiver operating characteristic curve analysis of representative miRNAs in lung adenocarcinoma based on TCGA database

To assess the expression levels, survival analysis, and receiver operating characteristic (ROC) curve of the representative miRNAs in lung cancer, data from the TCGA database for lung adenocarcinoma were used. As shown in Fig. 8, hsa-mir-192, hsa-mir-1293, hsa-mir-194, hsa-mir-561, and hsa-mir-205 were upregulated and hsa-mir-30a and hsa-mir-30c were significantly downregulated in lung cancer based on the TCGA database. Survival analysis of those representative miRNAs in lung adenocarcinoma showed that there was a positive correlation between the expression of hsa-mir-192, hsa-mir-1293, hsa-mir-194, hsa-mir-561, and hsa-mir-205 and patient survival, and a negative correlation between the expression of hsa-mir-30a and hsa-mir-30c and patient survival (Fig. 9). ROC curve analysis was conducted to assess the discriminatory ability of those representative miRNAs. The area under the curve of those seven representative miRNAs was more than 0.6 (Fig. 10).

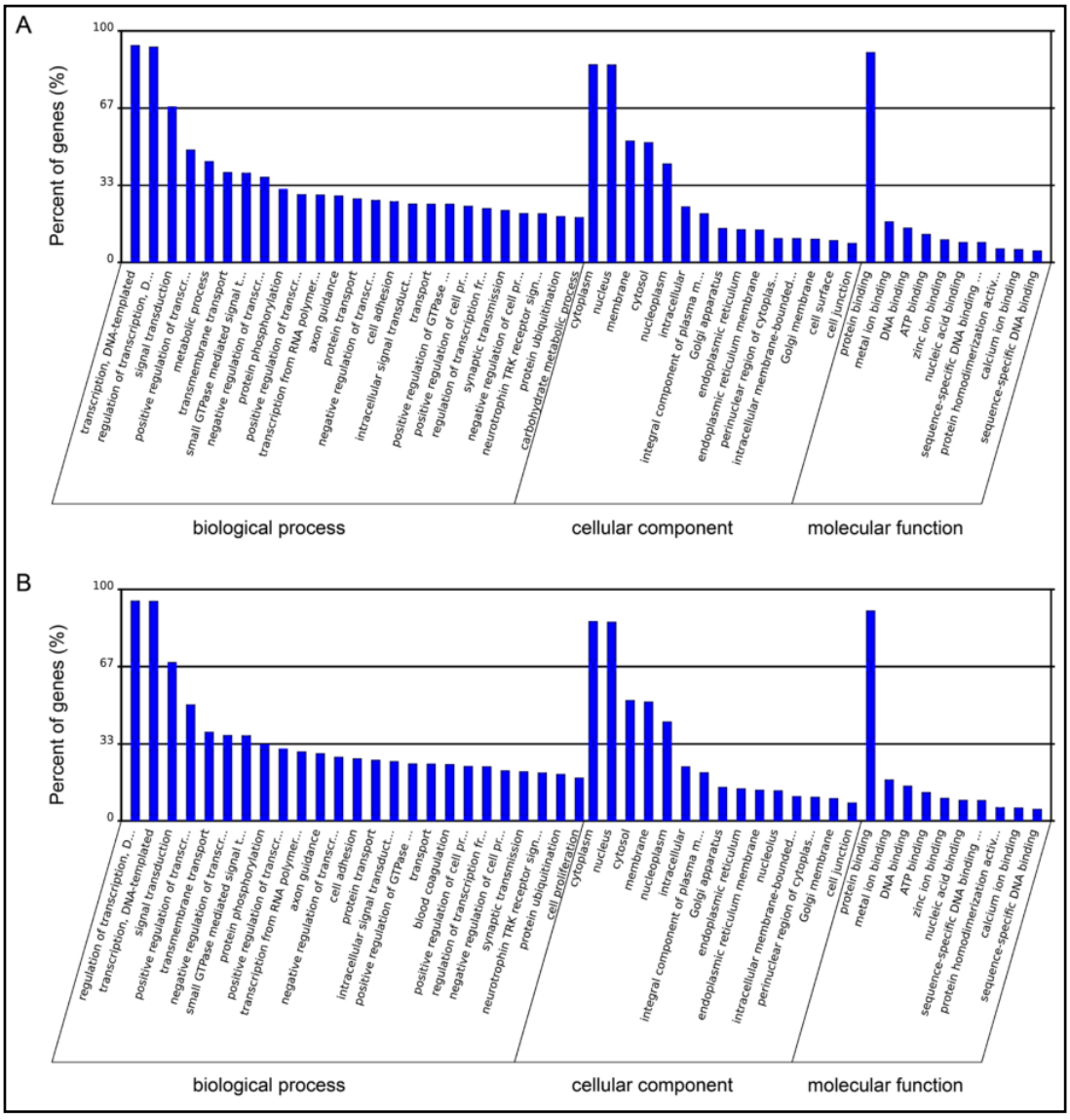

Fig. 5. GO enrichment analysis of the dysregulated miRNAs. GO enrichment analysis of the target genes corresponding to the upregulated miRNAs (A) and the downregulated miRNAs (B).

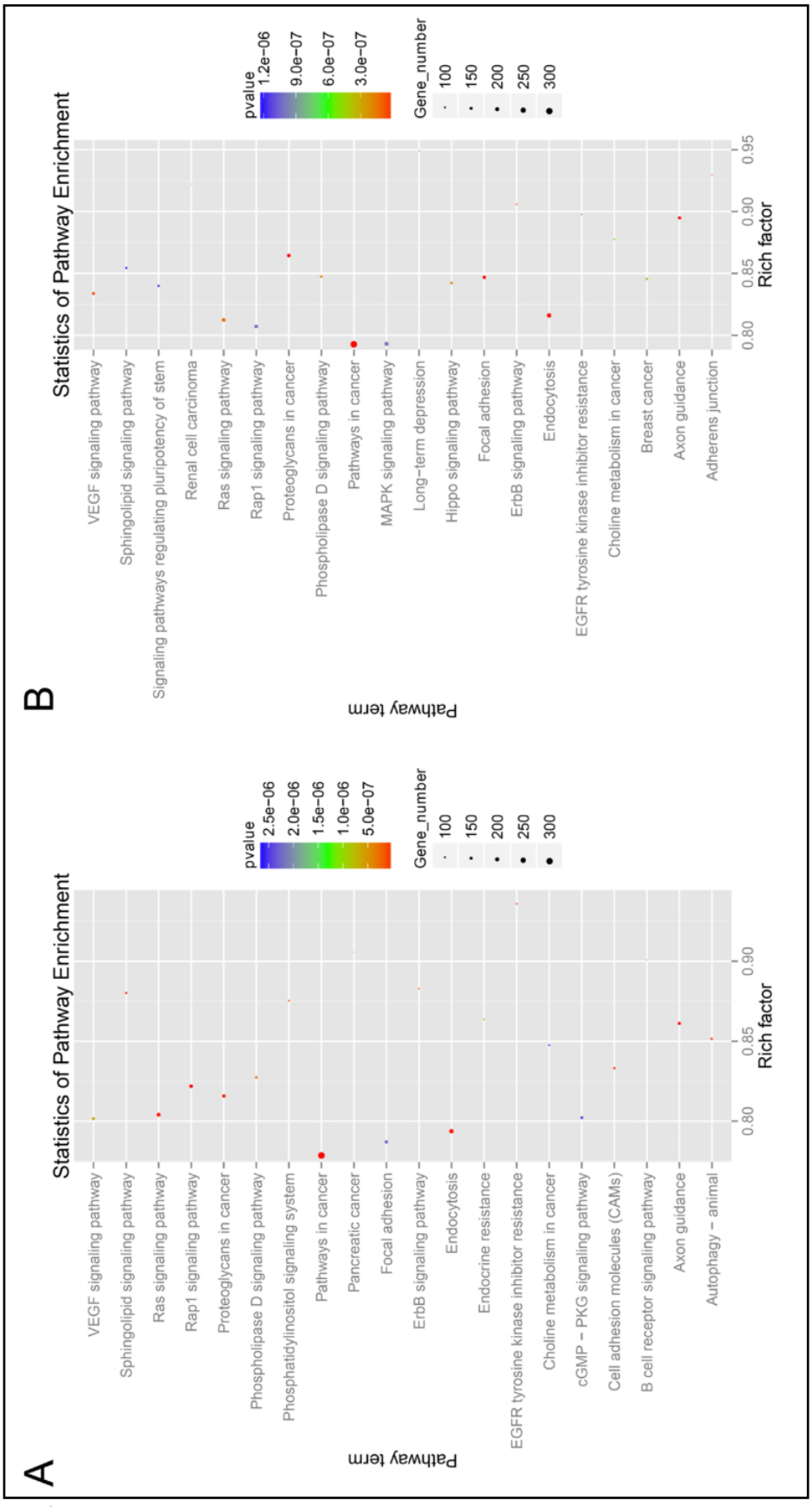

Fig. 6. KEGG pathway analysis of the dysregulated miRNAs. KEGG pathway analysis of the target genes corresponding to the upregulated miRNAs (A) and the downregulated miRNAs (B).

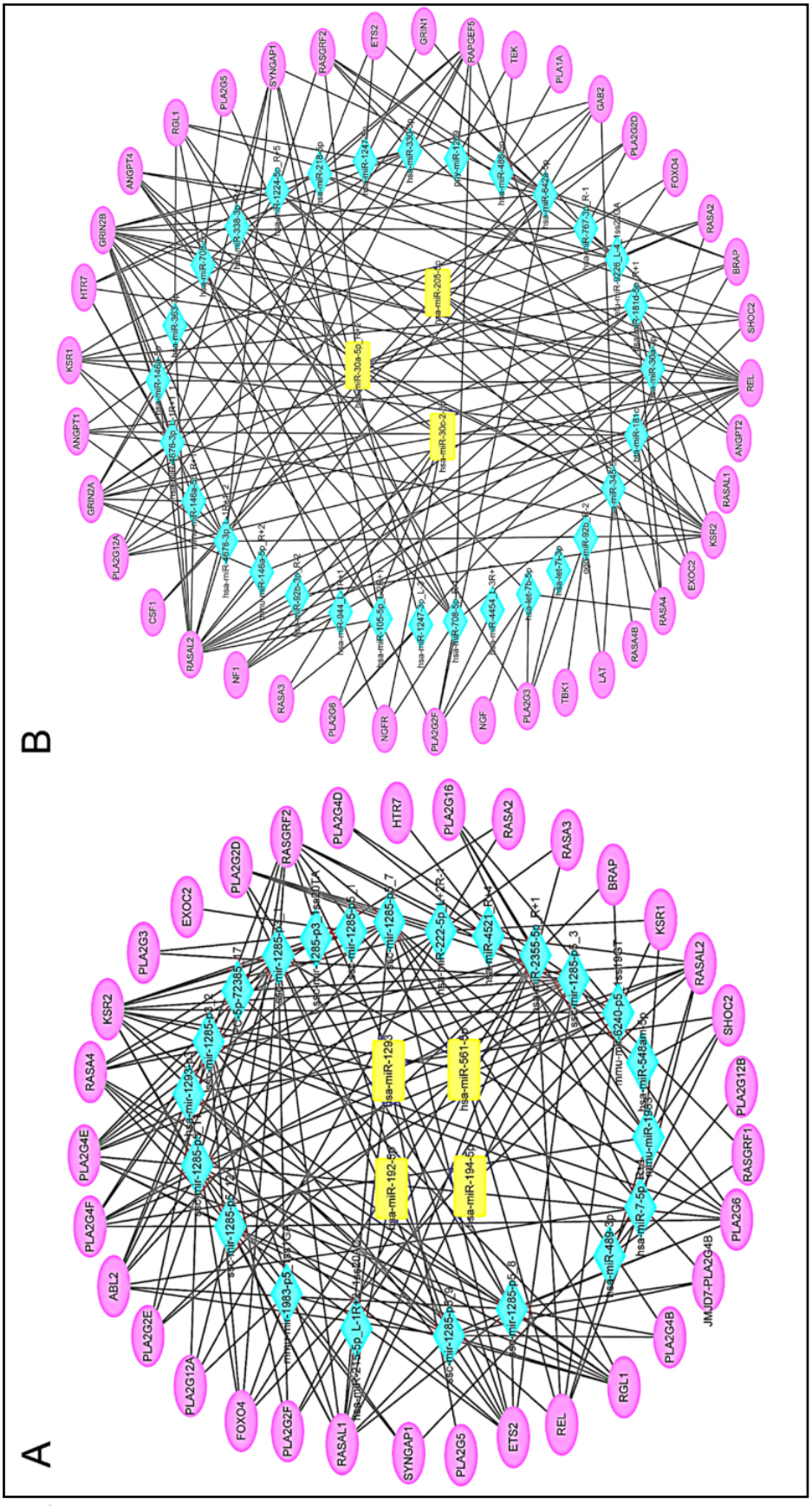

Fig. 7. miRNA-mRNA co-expression network analysis involved in the Ras signaling pathway. The miRNA-mRNA co-expression network analysis of the upregulated miRNAs (A) and downregulated miRNAs (B) was constructed by Cytoscape. Each yellow square represents representative miRNAs, each blue rhombus represents other miRNAs involved in the Ras signaling pathway, and each purple ellipse represents mRNA.

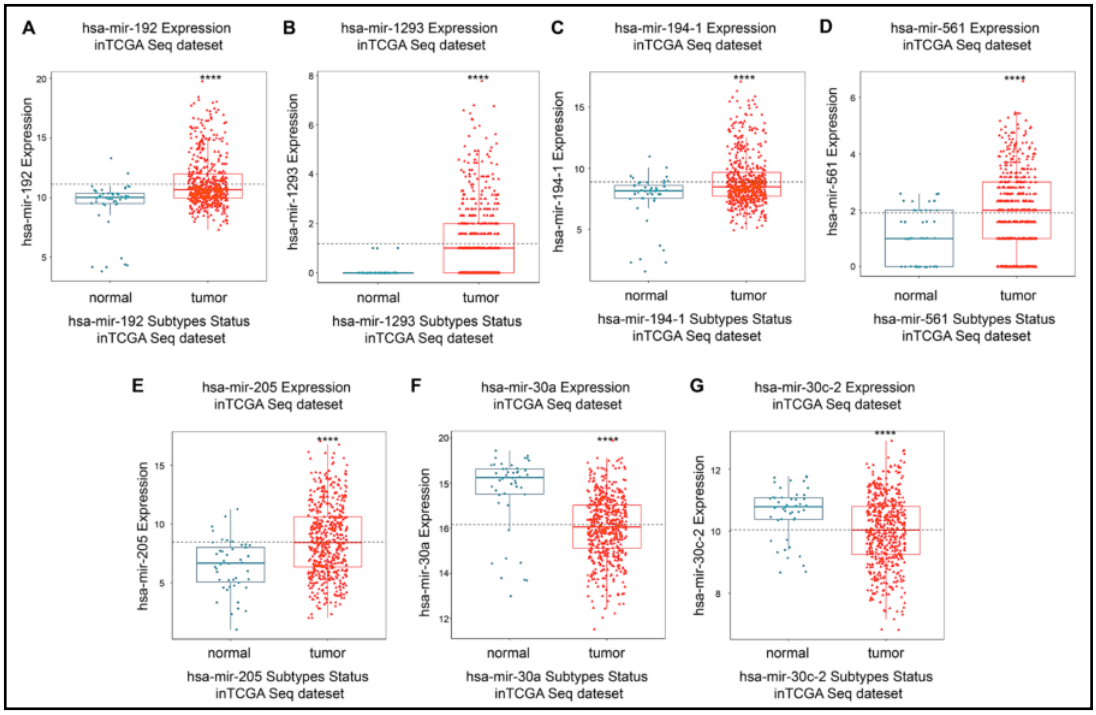

Fig. 8. Expression levels of representative miRNAs in lung adenocarcinoma based on the TCGA database. (A) hsa-mir-192; (B) hsa-mir-1293; (C) hsa-mir-194; (D) hsa-mir-561; (E) hsa-mir-205; (F) hsa-mir-30a; (G) hsa-mir-30c. The normal group and tumor group indicated adjacent non-tumor tissues and lung adenocarcinoma tissues, respectively. ***P<0.001, ****P<0.0001.

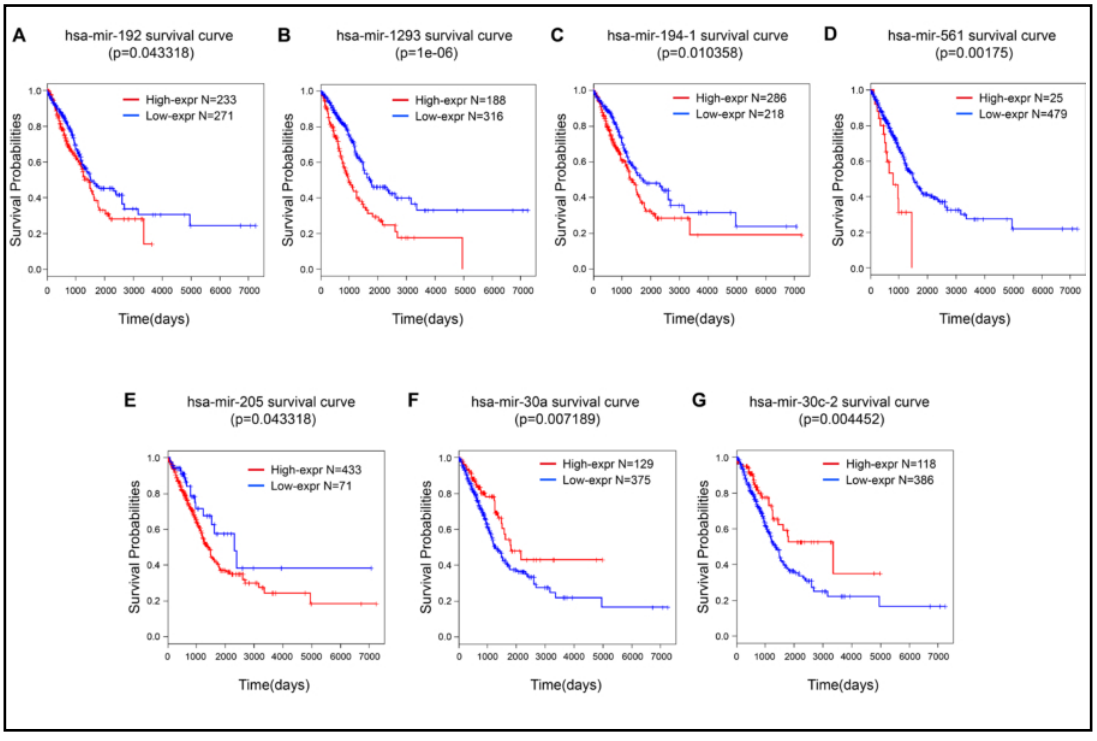

Fig. 9. Survival analysis of representative miRNAs in lung adenocarcinoma based on the TCGA database. (A) hsa-mir-192; (B) hsa-mir-1293; (C) hsa-mir-194; (D) hsa-mir-561; (E) hsa-mir-205; (F) hsa-mir-30a; (G) hsa-mir-30c. Statistics were calculated by the log-rank test.

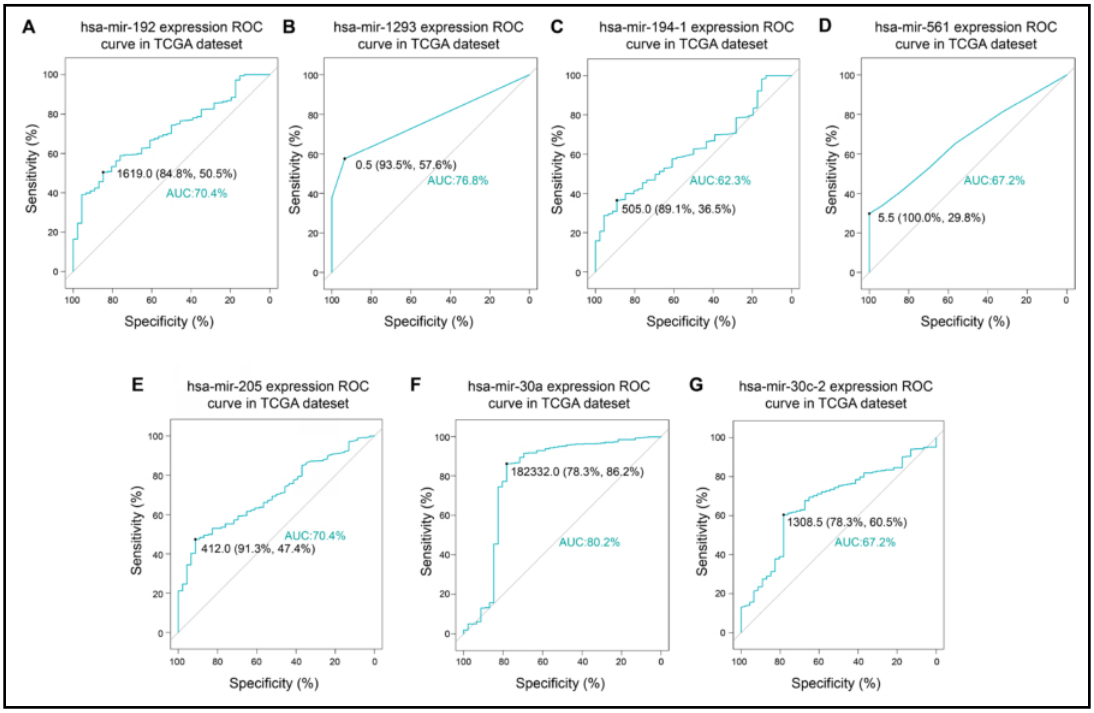

Fig. 10. ROC analysis of representative miRNAs in lung adenocarcinoma based on TCGA database. (A) hsa-mir-192; (B) hsa-mir-1293; (C) hsa-mir-194; (D) hsa-mir-561; (E) hsa-mir-205; (F) hsa-mir-30a; (G) hsa-mir-30c.

Discussion

Although chemotherapy and targeted therapy are the main methods of treating lung cancer, acquired resistance is one of the main reasons for treatment failure. The A549/DDP cell line is resistant to the chemotherapy drug DDP, and the HCC827/GR cell line is resistant to the targeted drug gefitinib. In this study, we detected the miRNA profiles in A549, A549/DDP, HCC827, and HCC827/GR cell lines by miRNA-Seq to evaluate the mechanisms underlying acquired resistance in lung cancer. The same change in trends among the dysregulated miRNAs involved in DDP or gefitinib resistance was identified. KEGG analysis revealed that both upregulated and downregulated miRNAs might be involved in the Ras signaling pathway, which plays an important role in drug resistance [15, 16]. The expression levels, survival analysis, and ROC curve analysis of those dysregulated miRNAs in lung cancer based on data from the TCGA database showed that hsa-mir-192, hsa-mir-1293, hsa-mir-194, hsa-mir-561, hsa-mir-205, hsa-mir-30a, and hsa-mir-30c were related to lung cancer and might be effective biomarkers.

Recent reports have suggested that most of the identified miRNAs in our study have important effects on drug resistance in many different cancers. For example, miR-192 is a potential miRNA involved in chemoresistance to DDP in ovarian cancer cells, to DDP and 5-fluorouracil in esophageal cancer cells, and to docetaxel in lung cancer [17-19]. miR-194 is dysregulated in doxorubicin, carboplatin, and mitomycin-resistant hepatocellular carcinoma cells and docetaxel-resistant lung cancer [19, 20]. Downregulation of miR-205 confers glioma cells with DDP resistance and pancreatic cancer cells with gemcitabine resistance [21, 22]. miR-30a influences chemoresistance in melanoma cells by targeting insulin-like growth factor 1 [23], regulates etoposide/DDP resistance in small cell lung cancer by targeting Beclin-1 [24], sensitizes NSCLC cells to paclitaxel through B-cell lymphoma 2 inhibition [25], and enables acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors to be overcome in NSCLC cells by reducing phosphoinositide-3-kinase regulatory

submit 2 [26]. miR-30a decreases multidrug resistance in human gastric cancer cells by modulating cell autophagy [27]. miR-30a-5p and miR-30c-5p are markedly downregulated in DDP-resistant ovarian cancer cells by overexpressing DNA methyltransferase 1 [28]. However, there have been no reports indicating that hsa-mir-1293 and hsa-mir-561 are related to drug-resistant cancer, suggesting that they may be potential targets for improving anti-cancer treatment.

This study had some limitations. First, the results of cell line experiments are not always relevant in clinical settings, due to the heterogeneity of tumor tissues from patients. Hence, the miRNAs identified in our study may not be consistent with those found in a clinical setting; thus, it is necessary to confirm the results using human tumor tissues. However, most of the miRNAs identified in our study have been reported to be related to drug resistance in other cancers, suggesting that the data from this study may be reliable. Second, only one drug-resistant cell line was used for different NSCLC cell lines, so future studies should be conducted in other (sensitive and resistant) NSCLC cell lines. In addition, validation and functional analyses of potential miRNAs in drug resistance were not conducted in this study, and should be conducted in the future.

The primary goal of this study was to identify potential miRNAs or common biomarkers involved in both chemotherapy resistance and targeted therapy resistance, to provide novel ideas or fields for altering drug resistance. Although this goal was achieved, additional studies are needed to define the roles and mechanisms of the identified miRNAs.

Conclusion

This study evaluated the miRNA expression profile in both chemotherapy-resistant and targeted-therapy resistant NSCLC cell lines, although there are some limitations to the applicability of the results due to the *in vitro* nature of the experiments. However, the results do lay the foundation for future *in vivo* studies, and provide strong support for the possibility that miRNAs may be novel biomarkers of drug resistance in NSCLC, and may even be potential targets for overcoming drug resistance in this disease.

Acknowledgements

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Disclosure Statement

The authors declare that no conflicts of interest exist.

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