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Article

Analysis of Polyphyllin II alters microRNA expression profile in lung cancer A549 cells

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Abstract: Non-small cell lung cancer (NSCLC) is a malignant tumor that threatens the whole world, previous studies found that Polyphyllin II (PPII) can suppress NSCLC cells growth, exhibits significant antitumor activity. MicroRNAs (miRNAs) can regulate the expression of other genes and play a crucial role in the prevention and development of cancer. However, the miRNA portrait of ginsenoside PPII-treated NSCLC A549 cells has not yet been studied. In this work, miRNA-seq analysis was used to determine the changes in miRNA expression profile of NSCLC A549 cells PPII-treaded. In addition, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed on differentially expressed miRNA target genes. Then, predicted the target genes of miRNA and performed functional enrichment analysis on them. We identified 58 up-regulated and 24 down-regulated miRNAs displaying changes their expression in PPII treated A549 cells were greater than 2-fold. In addition, the expression levels of miRNAs were validated by real-time PCR. Therefore, this work has a certain promoting effect on the study of the anti-cancer mechanism of PPII in lung cells.

Keywords: polyphyllin II; miRNA; lung cancer; A549 cells

1. Introduction

According to the 2022 National Cancer Report, there were 9.96 million deaths from cancer, and lung cancer resulted in 1.8 million deaths, ranking first among cancer deaths worldwide [1]. There are mainly two types of cancer: small-cell and non-small cell lung cancer (NSCLC) [2], with NSCLC accounting for approximately 80% [3]. In recent years, although lung cancer patients can be treated with a series of standard treatments, the 5-year survival rate for advanced cancer cases is only about 20% [4]. Therefore, actively seeking effective treatment methods and drugs is crucial for reducing the mortality rate of lung cancer.

Previous studies have shown that many Chinese herbal medicines and their active ingredients are effective in inhibiting cancer and play an important role in the occurrence development of cancers [5,6]. The Chinese herb Paridis Rhizoma, commonly known as Chonglou, is included in the 2020 edition of the Chinese Pharmacopoeia as the dried rhizome of Paridis Rhizoma or Paridis Rhizoma [7]. Chonglou is a commonly used drug in traditional Chinese medicine anticancer formulas, modern pharmacology has confirmed that it has antitumor [8], hemostatic, analgesic, antibacterial, and anti-inflammatory properties, as well as cardiovascular protective effects [9], In recent years studies have found that the saponins in Paris

polyphyllin have a certain inhibitory effect on cancer such as liver cancer, gastric cancer, rectal cancer, breast cancer and other cancer cells [10,11]. Luo et al. [12] found that Polyphyllin I induces an increase in ROS levels and down-regulates the AKT/mTOR signaling pathway to inhibit SW480 cell proliferation. PPII is a steroidal saponin extracted from Chonglou, which exhibited different anti-tumor mechanisms and had a strong synergistic anti-tumor activity against lung cancer [13]. The anti-cancer molecular mechanisms of PPII have also been studied, the researches mainly focus on some signaling pathway, such as PI3K/AKT/mTOR [14], STAT3 and AKT/ NF-κB signaling [15]. However, as far as current research is concerned, the anticancer mechanism of PPII has not been fully elucidated.

MicroRNAs (miRNAs) are small and 21–23 nucleotides long noncoding RNA molecules that participates in the occurrence, development, and metastasis of tumors [16]. They are considered important regulatory factors for almost all cellular signaling pathways, miRNAs can directly bind to the 3' untranslated regions of target genes and regulated the expression of target genes and proteins at the post transcriptional level. Recently, Xu et al. [17] found that Ligustrazine inhibits growth, migration and invasion of medulloblastoma Daoy cells by up-regulating the expression of miRNA-211. Jin et al. [18] proposed that curcumin inhibits the proliferation of human NSCLC cells by the up-regulating the expression of miRNA-192-5p. Xu et al. [19] found that Sinomenine can inhibit the proliferation, migration, invasion, and promote apoptosis of prostate cancer cells by regulating miRNA-23a. Liang et al. [20] found that neferine inhibits the proliferation, migration and invasion of U251 glioma cells by down-regulation miR-10b and deactivating the PTEN/PI3K/AKT and p38MAPK signal pathways. Yuan et al. [21] suggested that Polyphyllin I inhibits IL-1β—induced apoptosis of intervertebral disc nucleus pulposus cells through the miR-503-5p/Bcl-2 axis. In summary, miRNA is closely related to the anti-tumor effects of active ingredients in traditional Chinese medicine. However, so for, there is currently no literature focusing on the changes in miRNA expression profiles in PPII-treated NSCLC cells, and the regulatory relationships between target genes and varied miRNA in PPII-treated NSCLC are still unclear.

This study was designed to differentially expressed miRNAs in PPII-treated lung cancer cells. Firstly, we conducted a miRNA microarray experiment with analysis of PPII-treated NSCLC A549 cells. Secondly, in order to investigate the potential related functions of miRNAs in lung cancer cells, we predicted the target genes of miRNAs and conducted functional enrichment analysis on the target genes. In addition, the expression levels of some miRNAs were verified through real-time PCR. Our data indicates that several miRNAs exhibit different expression changes, suggesting that miRNAs are involved in the cellular responses to PPII. These results will contribute to the study of the expression profile of miRNA in lung cancer cells treated with PPII, and help to understand how miRNA regulates the inhibitory effect of PPII on NSCLC A549 cells. May be to provided a new approach and suggestion for the treatment of NSCLC cancer.

2. Materials and methods

2.1. Reagents and chemicals

PPII was purchased from the Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China), the A549 cell line purchased from the Shanghai Cell Bank, Chinese Science Academy (Shanghai, China), and Fetal bovine serum (FBS) was purchased from CellMax (Beijing, China), the dimethyl sulfoxide (DMSO) and RPMI-1640 were purchased from GIBCO (Grand Island, USA). The 0.25% trypsin ethylene diamine tetra acetic acid and Cell Counting Kit-8 (CCK-8) were purchased from (Sigma-Alrich, USA).

2.2. A549 cell culture

The cells cultured in RPMI-1640 medium containing 10% FBS at 37 °C with 5% CO_2. For the 96 well plate experiment, 5×10^3 cells were seeded into each well and 5 parallel control wells were set up. Before the experiment, PPII was prepared as a 10 mM stock solution.

2.3. Polyphyllin II treatment and cell viability analysis

Human A549 cells were cultured in RPMI-1640 medium containing 1% penicillin/streptomycin and 10% FBS at 37 °C in a humidified chamber containing 5% CO_2. When the cell growth density was 80%–90%, they were sub cultured at 1:3. A549 cells in logarithmic growth stage were digested, centrifuged and inoculated into 96-well culture plate. After cultured in the incubator for 24 h, add various concentrations of PPII (2, 4, 6, 8 μmol) at various time points (24 h, 48 h and 72 h), add 0.1% DMSO to the control group culture medium, and 5 multiple wells were set in each group. Add 10 per hole μL CCK-8 solution, continue to culture in the cell incubator for 2 h, shake and mix evenly, and measured the OD value of each well at a wavelength of 450 nm using a microplate reader to calculate cell survival rate.

2.4. RNA preparation and quality assessment

The total cellular RNA was extracted using the QIAGEN miRNeasy Mini Kit (Cat No./ID: 217004), and the RNA integrity was assessed by an Agilent 4200 TapeStation, three biological replicates were prepared for each cell sample, and 1% agarose gel was used to detect RNA degradation and contamination, only samples of high-quality RNA (RNA Integrity Number ≥ 7.0, 28S/18S rRNA ≥ 1.3) contamination were used in the subsequent construction of RNA-seq libraries.

2.5. High-throughput small RNA library construction

This paper uses the database building method of QIAseq, The QIAseq miRNA library kit is a new generation sequencing library kit that can accurately quantify miRNA through second-generation sequencing (NGS). The kit does not require gel screening and can maximize the detection of miRNA in samples. The pre-adenylated adapter 3’ and 5’ Ligations were sequentially added to miRNA by ligase, the reverse transcription (RT) primer contains unique Molecular Index (UMI) tag sequence, the RT primer binds to a region of the 3’ Ligation to promote the conversion of miRNA into cDNA (each miRNA molecule is assigned to a UMI). Then the library was amplified with universal forward primers and reverse primers, the products were purified by magnetic beads and used for quality control analysis and computer

sequencing.

2.6. Bioinformatic analysis of miRNAs

The software bowtie (version 1.2.2) was used to compare the reads of miRBase, piRNAbank and Rfam databases for microRNA quantification, after comparison and quantification, the UMI counts of miRNA corresponding to each sample are obtained. Then, the CPM (counts per million) value is used to standardize the quantitative UMI counts, and the CPM value is log 2 processed. Calculate the expression difference of the obtained expression UMI counts matrix according to the grouping information. The changes in miRNA expression between control group and the PPII treatment group were 2-fold or even more, the differential miRNAs were analyzed and the differential gene volcano map was made. The CPM values corresponding to different miRNAs were selected for cluster Heatmap analysis. Using miRBase database to predict and annotate the target genes of different miRNAs. Finally, the different miRNA target genes were analyzed by GO and KEGG pathway enrichment analysis.

2.7. Real-time quantitative PCR detection of miRNA

The expression levels of miRNAs were amplified using SYBR green technique. extracted total RNA from cells using Trizol Reagent (Invitrogen, USA), the U6 snRNA was used as endogenous control. The primers of the selected miRNAs were synthesized by Tiangen Biotechnology Co., Ltd., Incubated 20 μL PCR reactions in a 96 well plate at 95 °C for 5 minutes, followed by 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute. All the samples were repeated three times for each miRNA. Based on the threshold cycle (CT) was defined as the fractional cycle number the, relative expression levels of the assay genes were calculated using the $2^{-\triangle\triangle Ct}$ method, and indicate the relative expression levels of genes detected in the experimental group compared to the control group.

2.8. Statistical analysis

The statistical difference of data between control and PPII-treated groups was analyzed by Student's *t* test. The differential changes with a $p < 0.05$ and a fold change (FC) $\geq \pm 1.5$ was considered statistically significant. The data were expressed as means ± standard deviation. All statistical analyses were performed using GraphPad Prism v 7.00 (GraphPad Software, Inc.) and SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. cell viability analysis

Previous studies have found that PPII inhibits liver cancer cell and human bladder cancer cells proliferation, migration and invasion [22,23]. To evaluate PPII-induced changes in miRNA expression in human A549 cells, in this work, we investigated whether the anticancer effect of PPII could be demonstrated with A549 cells, were exposed to various concentrations of PPII (0, 2, 4, 6, 8 μmol) for 24

hours, 48 h and 72 h, respectively. Then, the anti-proliferation effect of PPII on A549 cells was determined using the CCK-8 method. As shown in **Figure 1A**, the cell proliferation rate of A549 cells was decreased with increasing in PPII treatment time and concentration. The results showed that PPII significantly reduced the proliferation of A549 cells in a time-dependent and dose-dependent manner. Whereas the inverted microscopy indicated the morphological changes of A549 cells were observed after 48h treatment. As shown in **Figure 1B**, after PPII treatment, the Morphogenesis of A549 cells changed, the A549 cells became elongated. And as the concentration of PPII increasing, the changes in cell morphology become more widespread.

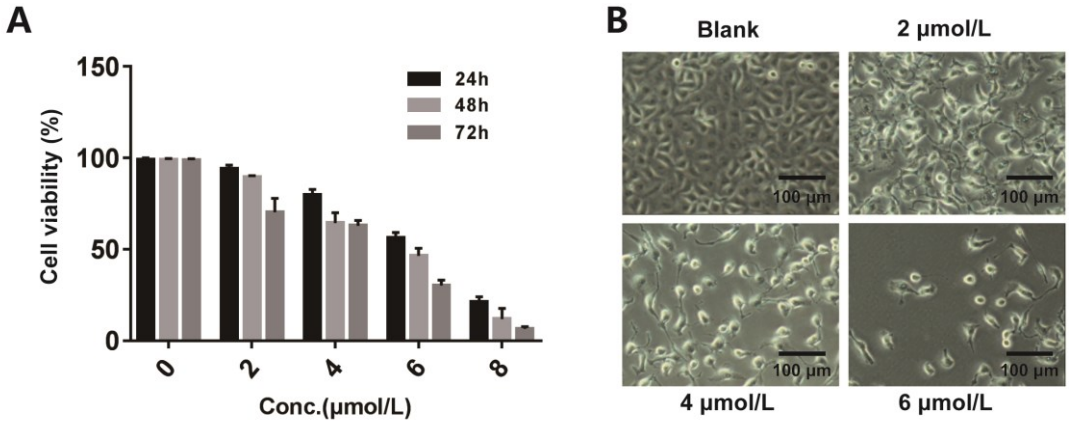

Figure 1. Effects of PPII on cell proliferation of A549 cells. **(A)** Changes in proliferation levels of A549 cells treated with PPII at different time points and concentrations; **(B)** after treated A549 cells with different concentrations of PPII for 48 hours, the changes in cell morphology were observed using an inverted microscope.

3.2. miRNA expression profiling

In the present study, we used to microarray platform to evaluate the change of miRNAs. Results showed that the expression profiles of 82 miRNAs changed significantly (> 2-fold), with 58 up-regulated miRNAs and 24 down-regulated miRNAs detected in the A549 cells, as compared with the parent cells (**Table 1**). The miR-12131 exhibited maximum up-regulation (fold change 6.82), and the miR-4454 exhibited maximum down-regulation (fold change 3.47) in the PPII-treated compared to the control group. Based on the negative binomial distribution, the expression levels of known miRNAs are shown by volcano plots respectively (**Figure 2**), the results indicated that there are more up-regulated miRNAs than down-regulated miRNAs.

Table 1. The expression levels of miRNAs in A549 cells that exhibit > 2-fold expression changes in response to PPII.

miRNA name	FC	miRNA name	FC
Up-regulated			
hsa-miR-12131	6.82	hsa-miR-4790-5p	2.25
hsa-miR-5681a	4.24	hsa-miR-1237-5p	2.24
hsa-miR-6822-3p	3.58	hsa-miR-4533	2.24
hsa-miR-4449	3.08	hsa-miR-6079	2.21

Table 1. (*Continued*).

miRNA name	FC	miRNA name	FC
hsa-miR-1251-5p	3.06	hsa-miR-6726-3p	2.20
hsa-miR-543	2.94	hsa-miR-4720-3p	2.19
hsa-miR-4652-3p	2.89	hsa-miR-4740-3p	2.19
hsa-miR-3621	2.84	hsa-miR-4670-5p	2.19
hsa-miR-6878-5p	2.84	hsa-miR-6883-5p	2.19
hsa-miR-1180-5p	2.81	hsa-miR-525-5p	2.18
hsa-miR-651-3p	2.78	hsa-miR-4724-3p	2.18
hsa-miR-4430	2.76	hsa-miR-4305	2.18
hsa-miR-4427	2.72	hsa-miR-4759	2.18
hsa-miR-518e-3p	2.69	hsa-miR-214-3p	2.17
hsa-miR-4438	2.57	hsa-miR-2861	2.16
hsa-miR-4291	2.51	hsa-miR-873-5p	2.16
hsa-miR-562	2.51	hsa-miR-134-3p	2.15
hsa-miR-943	2.50	hsa-miR-874-5p	2.15
hsa-miR-3074-3p	2.47	hsa-miR-190b-3p	2.13
hsa-miR-3620-3p	2.44	hsa-miR-4636	2.12
hsa-miR-3117-3p	2.44	hsa-miR-4654	2.08
hsa-miR-6735-5p	2.43	hsa-miR-758-5p	2.08
hsa-miR-4498	2.40	hsa-miR-4770	2.08
hsa-miR-1184	2.38	hsa-miR-5194	2.07
hsa-miR-4308	2.37	hsa-miR-4446-5p	2.05
hsa-miR-655-3p	2.36	hsa-miR-4761-3p	2.02
hsa-miR-5683	2.30	hsa-miR-4717-5p	2.01
hsa-miR-4735-5p	2.29	hsa-miR-4797-3p	2.01
Down-regulated			
hsa-miR-4454	−3.47	hsa-miR-216b-5p	−2.21
hsa-miR-520g-3p	−3.46	hsa-miR-4649-3p	−2.21
hsa-miR-1271-3p	−3.24	hsa-miR-6736-5p	−2.19
hsa-miR-7975	−2.98	hsa-miR-6837-5p	−2.19
hsa-miR-4701-5p	−2.69	hsa-miR-33b-3p	−2.18
hsa-miR-4257	−2.51	hsa-miR-6741-3p	−2.11
hsa-miR-4510	−2.42	hsa-miR-1233-5p	−2.08
hsa-miR-670-5p	−2.38	hsa-miR-6085	−2.08
hsa-miR-4793-3p	−2.30	hsa-miR-371a-5p	−2.05
hsa-miR-206	−2.29	hsa-miR-4269	−2.05
hsa-miR-6895-5p	−2.27	hsa-miR-4757-5p	−2.05
hsa-miR-1915-5p	−2.22	hsa-miR-1224-3p	−2.01

Notes: FC: fold-change, Fold change = resistance/parent.

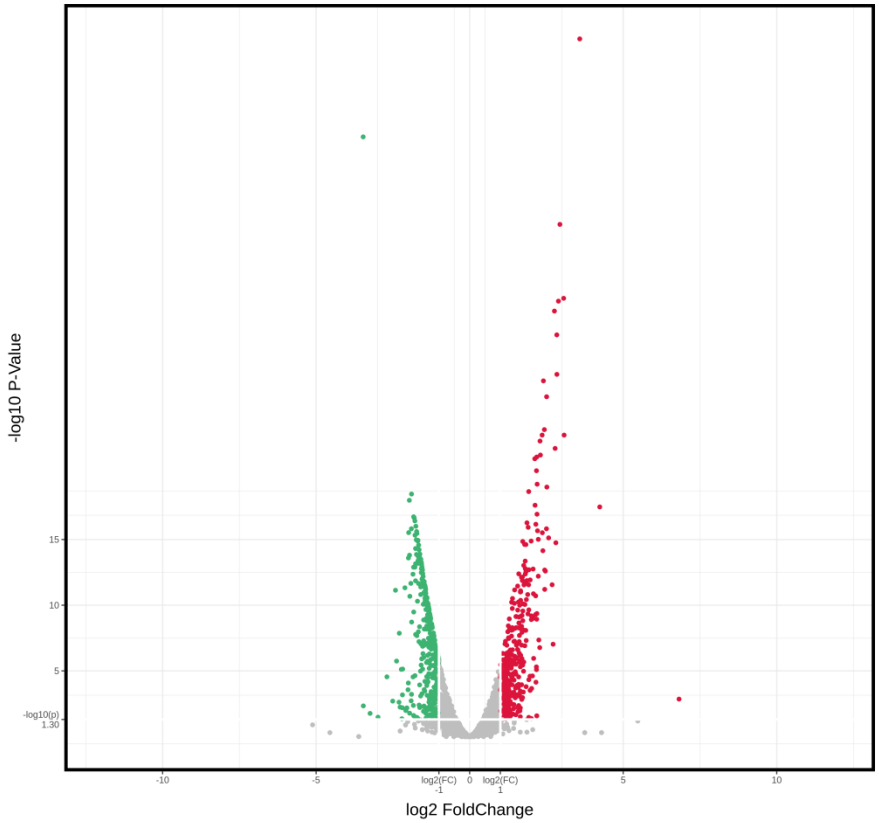

Figure 2. Volcano plots of detected miRNAs.

Notes: The red represents up-regulated miRNAs, and green represents down-regulated miRNAs. while the control sample represents untreated A549 cells, and the polyphllin samples represent A549 cells treated with PPII for 48 h.

3.3. miRNA target gene prediction

Research has proved that miRNA exert their biological functions by participating in the regulation of its downstream gene translation process [24]. In this study, the miRTarBase database is used to predict and annotate the target genes of dysregulated miRNAs. For each miRNA, approximately 20–800 potential target genes were predicted, because there are many target genes, all data are not completely displayed in the paper. Finally, we selected 14 miRNA target genes are listed in **Tables 2** and **3**. We found that more of the predicted targets had functions related to apoptosis and cell growth, in addition, the data showed that some genes were predicted as targets of multiple miRNAs, this is consistent with the literature report [25], a single messenger RNA can recognize multiple messenger RNA targets, while messenger RNA targets can be recognized by multiple miRNAs.

Table 2. Predicted target genes for miRNAs exhibiting up-regulation of PPII in A549 cells.

miRNA name	Target.Gene
hsa-miR-4449	GOLGA8J, F8A3, CARD10, STXBP2, GK5, ZNF487, F8A2, PTGES2, GOLGA8IP, SLC29A2, FAM212B, C6orf141, AKT1S1, VPS37B, TOR2A, BSG, NF2, ZFHX3
hsa-miR-4652-3p	GLIPR1L2
hsa-miR-3621	APOBEC3C, LTBP4, KCNK3, SGMS1, SUGT1, MAPK8IP2, ZNF770, TFCP2L1, PXN, ATG9A, CENPO, CAMK2N2, NRARP, GGCX, KCNN1, ADAMTS17, MAF, TMEM109, SEPT4, PTMA, INSIG1, MFRP, FBXW8, TMEM109, ABL1

Table 2. (*Continued*).

miRNA name	Target.Gene
hsa-miR-518e-3p	PGM2L1, TYRO3, NCKAP5, VSNL1, CSNK2A1, DMGDH, ZNF275, CAPS2, ZNF275, MINOS1, C16orf58
hsa-miR-4790-5p	TRIM6, CCR6, TKFC, ATXN7L1, RNF149, SGO1, SOX4, ZYG11B, SWSAP1, FHDC1, MTO1, ERBB2, CDKN2B, TSC22D2, ZBTB7A, POLE
hsa-miR-6726-3p	ZNF878, PLCG2, KLF13, LPP, BARX2, PRLR, KIAA1143, UHMK1, PAN2, DDX19B, METTL7A, DNAJB13, TAF1D, ESR2, FAM83D
hsa-miR-4670-5p	CXorf21, PDPK1, TFRC, SCUBE3, KRR1, PDPK1, ELK4, GREM2, GRIN2D
hsa-miR-4724-3p	SERINC3, EIF4G3, BPTF, TM9SF4, PURA, ZNF107, RUFY2, KCNN3, PURA, YES1, MYLIP, DCAF8, ERI1, PALM2-AKAP2, ANTXR2, MAPK6, KIF5C, SRSF10, HPS4, ZNF208, MYLIP, DUSP8, DPYSL2, FBXO47, ZNF117, COA4, ACER3, CD46, STAM, AKAP2, TWF1, NARS, TBX4, RXRA, ZNF589, RPS6KA3, MIA3, ZNF562, SRSF2, XPOT, RBM15B, ADD1, ARHGAP9, SERINC3, CCDC121
hsa-miR-4759	SNRPB, ATP9A, DR1, MTRNR2L1, SPOP, DR1, ZNF449, XRCC2, TMEM30B, C2orf69, RAB5B, MTRNR2L11, AZI2, HMGN1, PSD3, CREM, TCF7L2, C9orf78, ZNF460, VSIG2, ZNF514, SYNGR1, TRIM71, CSNK1G2, ABCD2, ZNF740, SNRPB, NUCKS1, SIGLEC9, UBE2G1, RPL22, PPP2R3A, KCNK1, AMFR
hsa-miR-4636	PET117, MTMR3, ACACA, YIPF4, MIB1, LUZP1, CD209, GIPC1, PATZ1, NACC2
hsa-miR-6080	POP7, SMC1A, IFT140, ATPAF1, BLVRA, MSANTD4, HIPK1, KPNA4, AGAP1, TMEM198, ZNF107, NDUFA12, USP36, ADAM22, TULP4, IFNB1, ACVR1, RNF157, TROVE2, KLHL28, OIP5, NDUFA12, PDE4D, SRSF2, ZNF620, USP13, ABCG8, SYNM, TRIM37

Table 3. Predicted target genes for miRNAs exhibiting down-regulation of PPII in A549 cells.

miRNA name	Target.Gene
hsa-miR-7975	C8orf33, SELENON, CASZ1, RPS21, FBXL20, B3GNT7, TYRP1, FRK, CYLD, KANSL1, RAD51, ZFP14, KBTBD8, TNFRSF13C, SFT2D3, ZNF34, SMC1A, HOXD12, GALNT10, MIA3, SCN2B, AMPD3, FAM109A, OSTF1, ABCG8, DIO2, LAX1, TRIM35, LILRB2, DR1, TOX4, FSTL1, FREM1, RNF157, LRAT, GULP1, ACVR1, MYOZ3, NFATC3, SDHD
hsa-miR-33b-3p	BARHL2, GALNT2, MYO3A, TMEM169, MKI67, FAM102B, GIGYF1, RORA, WAC, CDKN1B, TWIST1, SALL4, SMC1A, FLT1, SPN, EVX2, ESCO2, LMBRD2, AFF1, SYNM, DHODH, CHML, APLN, YTHDC1, F2RL2, STX16, THY1, ZNF75A, APLP2, ZNF726, ADD1, SLC12A7, PCF11, UEVLD, HSPA12B, NSA2, TLK1, PRKAR1A, ADAM22, ZC3H12B, SAR1B, COX15, PIGO, PIWIL2, OIP5, OMA1, GLIS2, PIWIL2, AGPAT4, POU2F1, F2R, CLOCK, ABCA1
hsa-miR-6085	BANP, SEMA3F, MYBL2, SEC22C, PLAGL2, GIGYF1, TMEM184B, PTDSS2, RTBDN, STAMBP, VHLL, FSCN1, ALDOC, PHB2, ANKRD13B, TOB2, ANKRD42, MKNK2, KCTD15, AP1B1, MCM7, ZC3H7B, TMEM91, SOX4, SLC5A6, SPRY4, MEF2D, SYNGR2, SLC7A5, HBP1, MAPK1, CCDC121, LIPG, PTPA, RNF40, NCEH1, STIP1, IDS, UBQLN4, KANK4, ANKRD52, TMEM127, KMT2B, FSTL3, APOBEC3C, MLLT11, KCNH2, SLC25A22, SF1, BARHL1, TRAF7, INTS3, METTL6, OR2C3, RPS6KA4, GJB1, ASB16, HSD3B7, POLR3G, MTRF1L, DLG5, PRRC2B, FAM129B, KMT2A, GRID1, NECTIN1

3.4. The GO enrichment analysis of miRNA target genes

The GO is the international standard classification system for gene function. GO is divided into three parts: molecular function (MF), biological process (BP) and cellular component (CC). For GO plotting, we used the GO seq R software package and REVIGO analysis. Through GO enrichment analysis, important functions that cause trait changes can be identified, and corresponding genes for this function can be found. The above shows a group of different GO enrichment analysis. The GO analysis results of miRNA target genes are indicated in **Figure 3**. The figure shows that the significantly enriched GO terms were mainly distributed in the BP and CC categories. The target genes rich in the BP categories mainly involve the following aspects: cadherin binding and cell adhesion molecule binding. In CCs, the main differences involve the following aspects: focal adhesion, cell-substrate junction, cell-substrate adherens junction and nuclear chromatin. In MFs, the main difference

involved the following terms: ribonucleoprotein complex biogenesis, proteasomal protein catabolic process and histone modification.

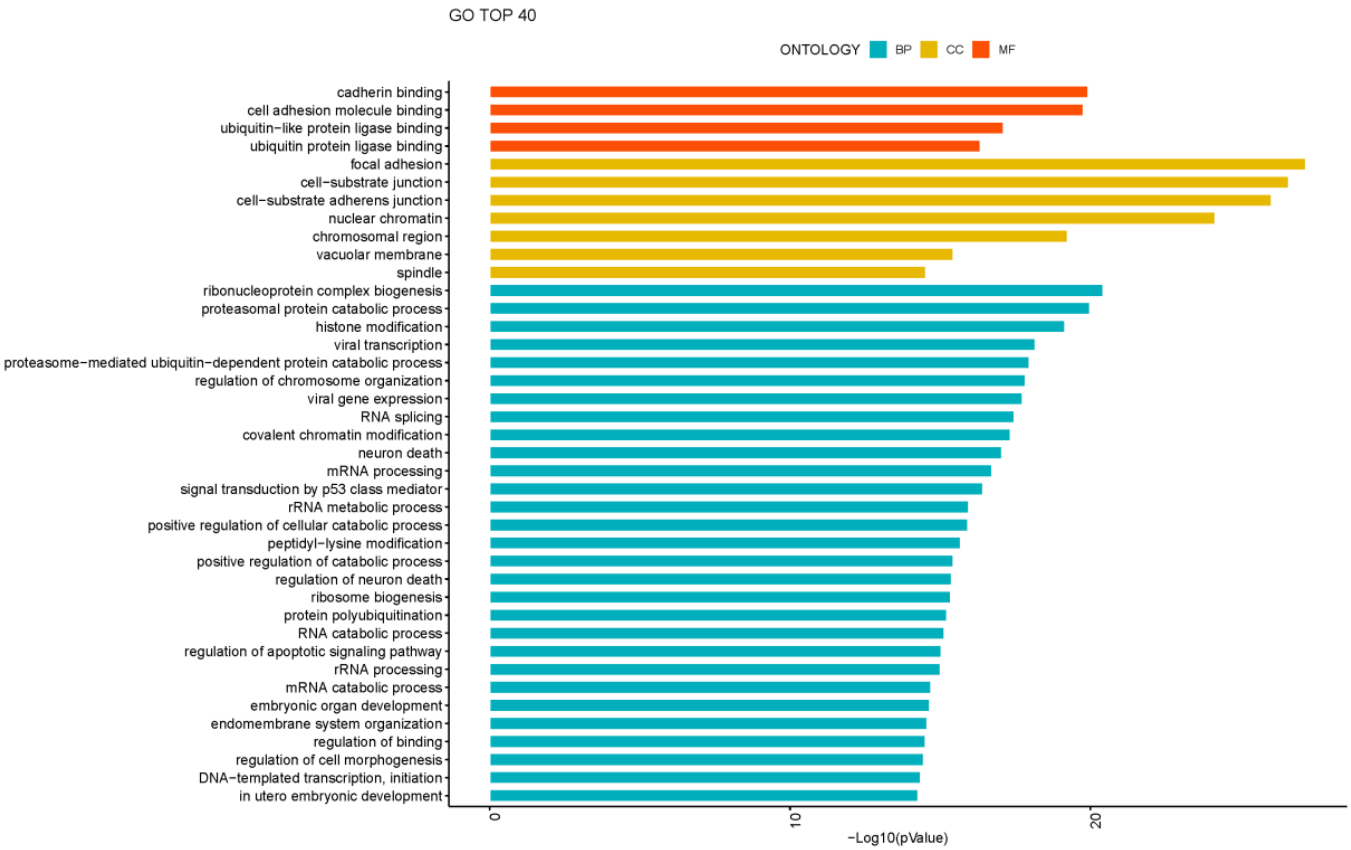

Figure 3. GO enrichment analysis of the miRNAs target genes.

3.5. The KEGG pathway enrichment analysis of miRNA target genes

The KEGG is a bioinformatics resource for understanding biological functions from a genomic perspective. For KEGG plotting, we used the KOBAS software analysis. In this work, we selected KEGG pathway enrichment analysis to clarify the biological pathway of A549 cells inhibition involved in the treatment of PPII. As shown in **Figure 4**, the top 40 pathways with statistical significance. **Figure 4** shows that some of these enrichment pathways were previously found to be involved in invasion, proliferation, tumor growth and metastasis of various cancer cells. For example, its most important enrichment pathways in this study are the Hippo and MAPK signaling pathways, the Hippo signaling pathway has been found to play a critical role in regulating tissue growth, maintaining tissue homeostasis and regeneration and repair. Hippo signal pathway disorder can lead to many diseases including cancer [26]. The numerous studies have shown that the activation of the MAPK signaling pathway involved multiple crucial cellular functions, including proliferation, differentiation, survival, and death [27,28].

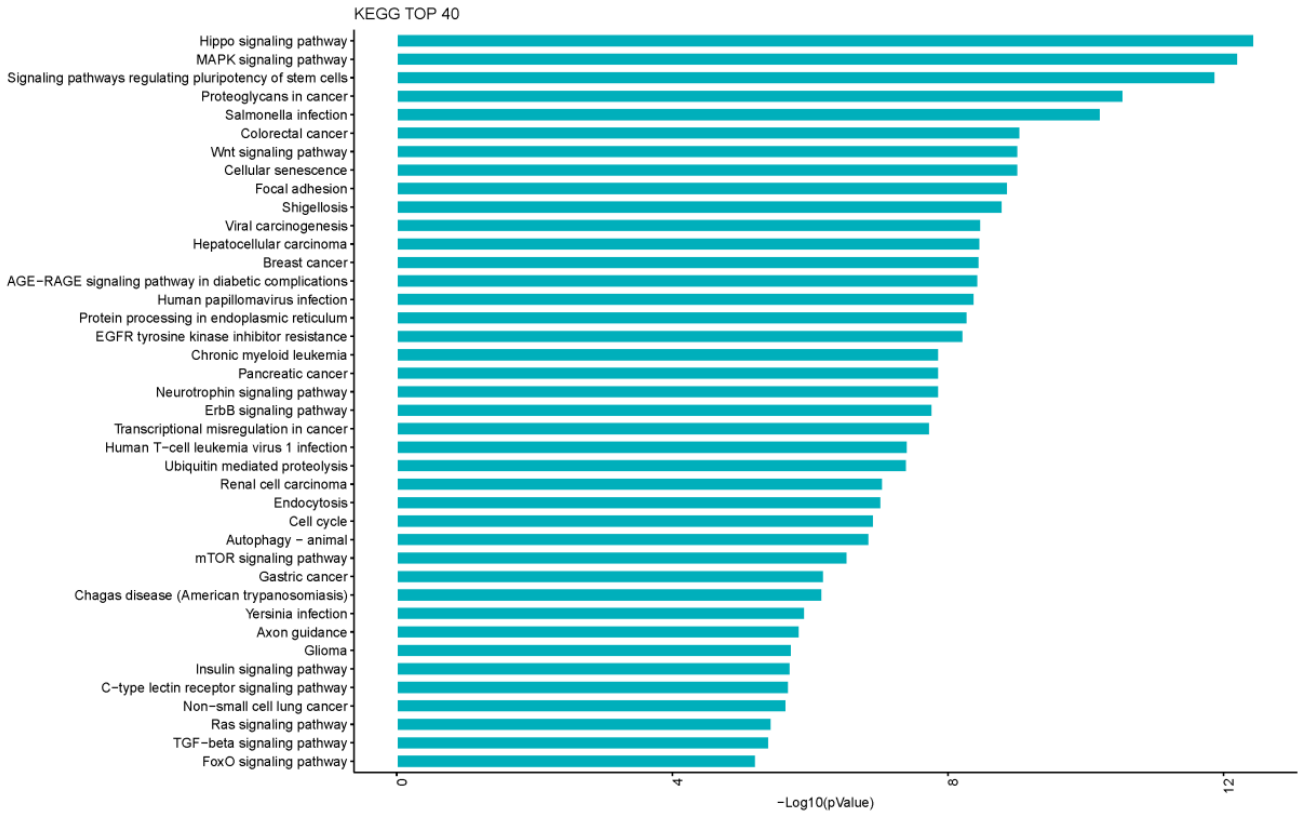

Figure 4. KEGG enrichment analysis of the miRNAs target genes.

3.6. The qRT-PCR validation of important miRNAs

To validate the results of the microarray, we further chose the 8 most differentially expressed miRNAs (including 4 up-regulated miRNAs miR-12131, miR-5681a, miR-6822-3p and miR-4449, 4 down-regulated miRNAs miR-4454, miR-520g-3p, miR-1271-3p and miR-7975) in the A549 cells for qRT-PCR. Primer sequences are shown in **Table 4**. The qRT-PCR results showed that the differential expression trend of 8 miRNAs was consistent with the microarray results and reached statistical significance ($P < 0.05$) (**Figure 5A,B**).

Table 4. Primers used for RT-qPCR.

miRNA	Forward primer (5'-3')
MiR-12131	UUUGGAGAGGUGUACUCCCA
MiR-5681a	AGAAAGGGUGGCAAUACCUCUU
MiR-6822-3p	AGGCUCUAACUGGCUUUCCCUGCA
MiR-4449	CGUCCCGGGGCUGCGCGAGGCA
MiR-4454	GGAUCCGAGUCACGGCACCA
MiR-520g-3p	ACAAAGUGCUUCCCUUUAGAGUAU
MiR-1271-3p	AGUGCCUGCUAUGUGCCAGGCA
MiR-7975	AUCCUAGUCACGGCACCA
U6	CTCGCTTCGGCAGCCACA

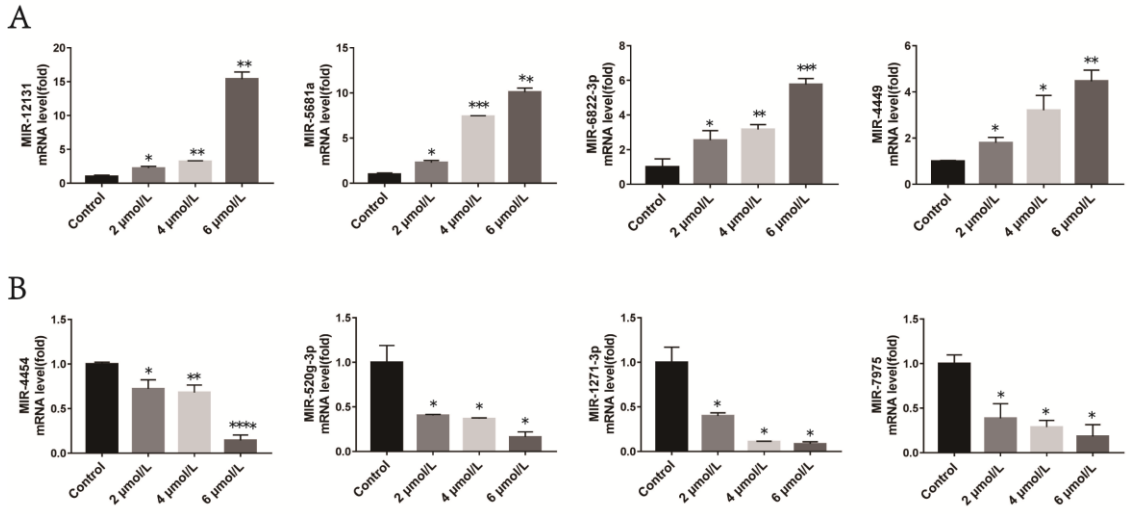

Figure 5. Quantification and confirmation of miRNA expression levels by qRT-PCR. **(A)** the 4 up-regulated miRNAs miR-12131, -5681a, -6822-3p and miR-4449 expression levels were confirmed by qRT-PCR; **(B)** the 4 down-regulated miRNAs miR-4454, -520g-3p, -1271-3p and miR-7975 expression levels were confirmed by qRT-PCR.

The average of three repeated experiments; bar chart, standard error (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and **** $P < 0.0001$ vs. control).

4. Discussion

PPII has significant pro-apoptosis and anti-proliferation effects of NSCLC cells [22]. In this study, we first validated through CCK-8 experiments that PPII can inhibit the proliferation of lung cancer A549 cells. Compared with the control group, treatment with 6 μmol PPII for 24 hours resulted in a 53.8% decreased in A549 cell viability. Previous research on the anticancer effect mediated to PPII-mediated have been limited to some signaling pathways. In recent years, studies have shown that miRNAs play a crucial role in regulating biological functions and gene expression, predicting miRNA tumor associations can help understand the pathogenesis of tumors. Therefore, in order to further investigate other molecular pathways involved in the anticancer effect of PPII, we used miRNA-seq technology to investigated the mechanism of anticancer effects at the transcriptional level.

Among the most recent developments in this field, Polyphyllin I inhibits apoptosis of intervertebral disc nucleus pulposus cells through miR-503-5p/Bcl-2 [21]. In this work, we found that 58 miRNAs were up-regulated, whereas 24 miRNAs were down-regulated with PPII treatment. Specifically, the expression level of miR-12131 was importantly up-regulated by 6.82-fold, while the level of miR-4454 was importantly down-regulated by 3.47-fold in PPII-treated NSCLC A549 cells. We further chose the 8 most differentially expressed miRNAs (including miRNAs miR-12131, miR-5681a, miR-6822-3p, miR-4449, miR-4454, miR-520g-3p, miR-1271-3p and miR-7975) in the A549 cells for qRT-PCR. The differential expression levels of these miRNAs suggested that a role for these miRNAs in the anti-tumor process of drugs.

Existing literature reports indicate that miR-4449 regulates the expression of pro-inflammatory cytokines, ROS levels, and cell apoptosis [29]. Zhao et al. [30] found that M2 macrophage-derived lncRNA NORAD in EVs promotes NSCLC progression through the miR-520g-3p/SMIM22/GALE axis. Maurel et al. [31]

propose that GPC3 over expression and its associated oncogenic effects are related to the down-regulation of miR-1271 in liver cancer. Park et al. [32] suggested that expression of miR-4454 was up-regulated in non muscle invasive bladder cancer (NMIBC) cells, further research found that the up-regulated miR-4454 reduced the expression of tumor suppressor genes DNAJB4 and SASH1, thereby promoting the progress of NMIBC. Moreover, literature research has found that miR-4454 is expressed in different tumors and acts as a tumor suppressor, including cervical cancer, colorectal cancer, lung adenocarcinoma and ovarian cancer [33–36]. The above prompts suggested that PPI regulation of miRNA up-regulation or down-regulation may be closely related to the regulation of inflammatory cytokines, down-regulation of tumor suppressor genes, and inhibition of cell differentiation. In summary, PPII may improve the progression of NSCLC A549 cells by up-regulating or down-regulating miRNA, but its potential role and mechanism still need further in-depth research.

Current research indicates that miRNA can regulate gene expression by binding to target gene mRNA inhibiting protein translation or inducing mRNA degradation [37]. In this study, bioinformatics tools were used to further predict the target genes of miRNA. Firstly, the miRbase target database tool Microcosm was used to predict each miRNA, approximately 800 potential target genes were predicted. In this study, we selected 14 miRNA target genes are listed in **Tables 2** and **3**. The analysis results show that more predictive targets have functions related to cell apoptosis and growth. Next, the GO enrichment and KEGG pathway were conducted. Analysis shows that many signaling pathways are involved in invasion, proliferation, tumor growth and metastasis of various cancer cells. The analysis results identified several pathways related to the miRNA identified in this study. Therefore, future research on the anticancer mechanism of PPII may focus on the cross pathways of miRNAs and its target genes in cancer.

In summary, our series of studies have shown that miRNAs exhibit significant expression changes in response to PPII in the NSCLC cells. PPII has a strong anticancer effect on NSCLC, and miRNA profiling analysis suggests that miRNA may play a role in the PPII mediated anticancer effect. It is important to note that although there is no evidence in this article that potential miRNA targets are directly regulated by the miRNAs identified in this work. We have emphasized the possibility of miRNA mediated PPII regulation in lung cancer cells. Therefore, these research results provided a new research directions and ideas for the possible molecular mechanisms through which PPII exerts anticancer effects on NSCLC cells. In the future, in-depth research is needed on specific miRNAs to determine the exact anticancer mechanism of PPII.

5. Conclusions

To sum up, this study proved for the first time that PPII alters miRNA in cancer A549 cells, we found that the expression of miRNA has produced significant changes. Meanwhile, we further predestined the target genes of the differentially expressed miRNAs, as well as GO and KEGG analysis. It is important to note that, this article only proposes that miRNA plays an important anti-cancer role in PPII

mediated NSCLC. The specific regulatory mechanism of miRNA is unknown in this study, but this study provides new ideas for the study of the anti-cancer mechanism of PPII on NSCLC.

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Data availability: All data used for the results of this study can be obtained from the corresponding author.

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