MiR-17-5p/RRM2 Regulated Gemcitabine Resistance in Lung Cancer A549 Cells

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Research Article

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Abstract

Background

To investigate the roles of miR-17-5p/RRM2 in A549/G+.

Methods

Cell survival was analyzed by CCK8 kit and clone formation experiment; The mRNA expression level was detected by qRT-PCR, and the protein level was compared by Western blotting; And flow cytometry for cell cycle detection; The target gene of miR-17-5p was verified by double luciferase activity experiment.

Results

CCK8 assay displayed that miR-17-5p's overexpression can let A549/G + cells turn into sensitive ones; in turn, in the case of inhibition of expression, miR-17-5p can make A549/G- cells turn into resistance ones. And similar results were obtained in cell clone formation experiment. Cell cycle analysis showed that miR-17-5p's overexpression increased the number of G_1 phase cells and reduced the number of S phase cells in A549/G + cells; Conversely, lowing expression level of miR-17-5p yielded the opposite results in A549/G- cells. Western blot results showed that when miR-17-5p was expressed highly, the expression levels of cell cycle related proteins, CCNE1, CCNA2 and P21 decreased in A549/G + cells; Conversely, inhibition of miR-17-5p expression yielded the opposite results too in A549/G- cells. Western blot analysis of signal pathway proteins and the results showed that PTEN and PI3K expressed higher, but p-PTEN expressed lower in A549/G + cells. After miR-17-5p overexpressed in A549/G + cells, the level of p-PTEN increased, and the level of p-AKT decreased; when miR-17-5p expressed low in A549/G-cells, the expression of p-PTEN and p-AKT were opposite. The dual-luciferase experiment demonstrated that RRM2 was the target gene of miR-17-5p. The restorative experiments of RRM2 verified it.

Conclusion

This article suggested that the miR-17- 5p/RRM2 axis could adjust gemcitabine-resistance in A549 cells and might associate with p-PTEN/PI3K/AKT signal pathway.

Background

In recent fifty years, lung cancer has been becoming the highest incidence rate and fatality rate in China and even in the global. Lachgar A et al.[1]studies showed that most patients with lung cancer had no clinical symptoms in the early stage, while they were already in the middle-to-advanced stage (III or IV) of lung cancer, missing the best surgical treatment time. Therefore, conventional radiotherapy and chemotherapy are still the available treatment methods for this part of lung cancer patients [2]. However,

drug-resistance is an important obstacle restricting the curative effect. Unfortunately, the mechanism of drug-resistance in lung cancer chemotherapy is complicated and still unclear totally. The clinical treatment effect is extremely poor, and the five-year survival rate is only about 15%[3].

At present, the most important and commonly used clinical method for the treatment of NSCLC is chemotherapy[4].Since the 1990s, third-generation chemotherapeutics have appeared on the market, such as gemcitabine (Gem), vinorelbine (NVB), paclitaxel and docetaxel (TXT), etc. It has gradually become the first-line drug for the clinical treatment of NSCLC.

Gemcitabine is a cytosine nucleoside derivative and is a cell cycle specific anti-metabolite. It can inhibit the ribonucleotide reductase, thereby inhibiting DNA synthesis and preventing cells from entering S phase from G_1 phase, and finally are deactivated by CDA (cytidine deaminase, CDA)[5]

We have successfully induced a gemcitabine resistant A549 lung cancer cell line; The differential expression of miRNAs was analyzed in A549/G + and A549/G- cell line. Our results hinted that miR-17-5p expressed significantly different between A549/G + cells and A549/G- cells[6]. MiR-17-5p is one of the miR-17-92 gene family. This family members often expressed highly in multifarious hematological and solid tumors[7, 8].

Studies have shown that miR-17-5p is an oncogene in most tumors, and its expression is up-regulated, which can boost cell proliferation and inhibit apoptosis; but a small number of studies on breast cancer, cervical cancer, prostate cancer and other tumors showed miR-17-5p is a cancer suppressor gene [9]. Meng and other studies found that miR-17-5P may promote lung cancer cells A549, SPCA-1 and GLC-82 to proliferate, migrate and invade by up-regulating CCND1, Vimentin, and N-CA[10].This indicates that miR-17-5p is likely to play an major role in the resistance mechanism of lung cancer.

Therefore, on the basis of the above experiments, this article focuses on the role of miR-17-5p and its target gene in the resistance of lung cancer cell A549 to gemcitabine. The present article aims to clarify the resistant mechanism of A549 cells to gemcitabine and provide more experimental basis for the study of tumor resistance mechanisms.

Methods

Culture of cells

A549 (Human lung adenocarcinoma cell) was bought from Institute of oncology, Peking Union Medical College (Beijing, China). Our team established A549/G + cell lines by exposing A549 to low-dose gemcitabine mixed medium. All cells were cultured in DMEM with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin and maintained at 37˚C in 5% CO_2.

Mimics, siRNA and inhibitor

The miR-17-5p mimics, inhibitor and their control substance were synthesized from RIBOBIO (Guangzhou, China). Their sequence is: miR-17-5p mimics, 5'-UUCCCUUUGUCAUCCUAUGCCU-3', and miR-17-5p inhibitor, 5'-UGGCUUUGGUUGUCUUUGGGUU-3'. P21 siRNA and RRM2 siRNA were also synthesized from RIBOBIO (Guangzhou, China). The most effective siRNA sequence is: P21 siRNA, 5'-GAATGAGAGGTTCCTAAGA – 3' and RRM2 siRNA 5'-GCCTCACATTTTCTAATGA-3'. The cells were transfected with 50 nmol/L miR-17-5p inhibitor and mimics, 50 nmol/L P21 siRNA or RRM2 siRNA. The control reagent is Lipofectamine® 3000 (Life Technologies, Carlsbad, CA, USA). The transfection protocol accords to the operation's instructions.

miRNA profiling and real time quantitative polymerase chain reaction (qPCR)

A549/G + cells were transfected with miR-17-5p mimics, RRM2 siRNA to establish the NC group, siNC group, miR-17-5p mimics group and siRRM2 group. And A549/G- cells were transfected with miR-17- 5p inhibitor, set up in-NC group and inhibitor group. All the following experimental groups follow this grouping pattern.

Culture A549/G- and A549/G + cells in the logarithmic growth phase, and after transfect mimics, inhibitors and siRNAs for 24 hours, total RNA was extracted, reverse transcription (RT) and qPCR reaction carried out successively. The RT and qPCR was used the PIKOREAL 96 Real-Time PCR System (ThermoFisherScientific, USA). U6 and GAPDH were used as endogenous reference for data standardization. The relative expression level of gene was calculated with $2^{-\Delta\Delta CT}$ method. The sequences of primers were seen in Table 1.

Table 1
The sequences of primers of genes

Gene's Name	Primer	Sequence
U6	forward primer	5'-agagaagattagcatggcccctg-3'
	reverse primer	5'-agtgcagggtccgaggtatt-3'
	RT primer	5'-cgtatccagtgcagggtccgaggtattcgcactggatacgacaaaagc-3'
miR-17-5p	forward primer	5'-gcgcaaagtgcttacagtgc-3'
	reverse primer	5'-agtgcagggtccgaggtatt-3'
	RT primer	5'-gtcgtatccagtgcagggtccgaggtattcgcactggatacgac-3'
GAPDH	forward primer	5'-caggaggcattgctgatgat-3'
	reverse primer	5'-gaaggctggggctcattt-3'
RRM2	forward primer	5'-agtggaaggcattttcttttcc-3'
	reverse primer	5'-gcaaaatcacagtgtaaaccct-3'

CCK-8 assay

CCK-8 (Dojindo, Japan) was used to determine cell viability and calculated the IC_{50}. The lysed cells were incubated in 96-well plates at 3×10^3 cells per well. After 24 hours of culture in complete medium, the cells were cultured in medium with different doses of gemcitabine for 72 hours. Then, the medium with drug was discarded, and add 100 μL 10% CCK-8 medium to each well. Absorbance was obtained at 450 nm using microplate reader (Bio Tek Gene5, US). Each drug dose was repeated in three wells and at least three times.

Clonogenic survival assay

The cells were well-distributed inoculated in a six well plate with 1×10^3 cells per well. Different doses of gemcitabine were added to each well. After incubated the cells for 72 hours. The cells were then cultured in complete medium until clones were visible to the naked eye. After formaldehyde fixation, the cell clones were stained with 0.1% crystal violet for half hour.

Cell cycle analysis

The cells were well-distributed inoculated in a six well plate with 2×10^5 cells per well. After 24 hours of transfection, the cells were obtained, and fixed with 500 μL 70% ethanol at 4°C for 12 hours. The next day the cells were washed with PBS. Add 500 μL PI/RNase A (Jiangsu KeyGEN BioTECH, China) staining working solution prepared in advance to each sample, and keep it away from light for 40min at 25°C. The cell samples were inhaled and analyzed by flow cytometry.

Western blotting

Every groups cells were well-distributed inoculated in a six well plate with 2×10^5 cells per well, transfected in groups for 48 hours. The cells were lysed in RIPA buffer (Beyotime Biotechnology, Beijing, China). Separation of all protein samples was by SDS-PAGE and the protein samples on the gel were transferred to a poly vinylidene fluoride membrane. The specific antibodies used include: Rabbit mAbP21 antibody (CST, NO.2947S, USA), Mouse mAb CyclinA2 antibody (CST, NO. 4656T), Mouse mAb CyclinE1 antibody (CST, NO. 4129T), Rabbit mAb PTEN antibody (CST, NO. 9188S), Rabbit mAb P-PTEN antibody (CST, NO. 9554S), Rabbit mAb Phospho-Akt (Ser473) antibody (CST, NO. 9271S), Mouse mAb PI3K antibody (CST, NO. 13666S), Rabbit mAb RRM2 antibody (Abcam, NO.EPR11820) All specific antibodies were used after 1:1000 dilution, and the anti-specific antibody (Beyotime Biotechnology, Beijing, China) was also used after 1:1000 dilution. The anti-specific antibodies bound to PVDF membrane were detected by ECL detection system. And then used Image J software to perform protein band grayscale analysis.

Plasmids

The 3'-UTR of human RRM2 gene cDNA contains a hypothetical binding site of miR-17-5p. The sequence was synthesized chemically from Sangon Biotech (Shanghai, China). For synthetic wild-type (RRM2 WT) gene fragments, we also designed three different corresponding mutants (RRM2 non-seed, RRM2 MUT2, RRM2 MUT4) linked to the downstream of luciferase gene in pmirglo vector (Promega, Madison, WI, USA), and the original pmirGLO as negative controls.

The gene fragment prepared by SangonBiotech and the plasmid pmirGLO purchased by us were subjected to double digestion, 37°C, 60 min, and then digest the product Agarose gel electrophoresis and gel recovery. According to the DNA ligase system, the DNA insert fragment and the plasmid vector fragment were ligated at a molar ratio of 1:20 at 25°C for half hour. Then these recombinants are transformed into host cells, the required clones are selected, the clones are amplified and the recombinants is extracted, and stored at -20°C.

Dual luciferase activity assay.

The mature sequence of miR-17-5p is: 5' CAAAGUGCUUACAGUGCAGGUAG 3'. Among them, the 5 'end of miRNA has miRNA seed sequence, including 2–8 nucleotide sequences. Based on the seed sequence and predicted binding sites, we designed RRM2 WT, RRM2 MUT non-seed, RRM2 MUT2 and RRM2 MUT4 gene fragments as shown in Table 2, synthesized by Shanghai Sangon Biotech. We use the dual luciferase plasmid pmirGLO (Fig. 1A) to express both firefly luciferase and Renilla luciferase in A549/G + cells, using Renilla luciferase as an internal reference and luciferin as the substrate. For dual luciferase activity assay, RRM2 WT, RRM2 non seed, RRM2 MUT2, RRM2 MUT4 and pmirGLO were co-transfected with miR-17-5p inhibitor or mimics. After 48 hours, lyse these cells and place the lysate in a 96-well plate. Then according to the operation introduction (Dual luciferase® Reporter analysis system, Life, USA) measures report activity. And calculate the activity ratio of luciferase of Firefly/Renilla.

Table 2 The sequence of RRM2 gene and RRM2 mutation gene

Gene Name	Sequence
RRM2 WT	gaagtgttacgagctccaccatgaattgtccgtaatgttcattaacagcatctttaaaactgtgtagctacctcacaaccgtgt aagctgggttttatattacatggcatttcctgttttgtaacactttgcagttctttcttatggtattttcccgactttaatgtttacttaa atataaacctggcactttacaaacaaataaacattgtttgtactcatagtagagccaggagcggtggctcatgcctgtaatc ccagcactttggtaggccgaggcgggtggatcactgcaggtatgtgctaccacacctggctaatttttgtatttttagtagag atggagtttca cctgcaggtcaggctgg
RRM2 MUT non-seed	gaagtgttacgagctccaccatgaattgtccgtaatgttcattaacagcatctttaaaactgtgtagctacctcacaaccgtgt aagctgggttttatattacatggcatttcctgttttgtaatctggggcagttctttcttatggtattttcccgactttaatgtttactta aatataaacctgatctgggccaaacaaataaacattgtttgtactcatagtagagccaggagcggtggctcatgcctgtaat cccaatctgggggtaggccgaggcgggtggatcactgcaggtatgtgctaccacacctggctaatttttgtatttttagtag agatggagtttcacctgcaggtcaggctgg
RRM2 MUT2	gaagtgttacgagctccaccatgaattgtccgtaatgttcattaacagcatctttaaaactgtgtagctacctcacaaccgtgt aagctgggttttatattacatggcagggttgaggggagcctctgggacagttctttcttatggtattttcccgactttaatgttta cttaaatataaacctgatctgggccaaacaaataaacattgtttgtactcatagtagagccaggagcggtggctccgattga gccgtttcatctgggagtaggccgaggcgggtggatcactgcaggtatgtgctaccacacctggctaatttttgtatttttag tagagatggagtttcacctgcaggtcaggctgg
RRM2 MUT4	gaagtgttacgagctccaccatgaattgtccggccgaggtcggcctcatcgtgggaaaactgtgtagctacctcacaacc gtgtaagctgggttttatattacatggcagggttgaggggagcctctgggacagttctttcttatggtattttcccgactttaat gtttacttaaatataaacctgatctgggccaaacaaataaacattgtttgtactcatagtagagccaggagcggtggctccg attgagccgtttcatctgggagtaggccgaggcgggtggatcactgcaggtatgtgctaccctcttgaatgccgggggag cgggggagtagagatggagtttcacctgcaggtcaggctgg

Data statistical test

Data statistical test was carried out using SPSS 11.0 and GraphPad Prim 7.0. The mean of the two samples was analyzed by t-test and expressed as *P* Value. The test level is 0.05.

Results

MiR-17-5p's overexpression can increase the sensitivity of A549/G + cells to gemcitabine

The qRT-PCR results proved that miR-17-5p expressed higher in A549/G- cells than in A549/G + cells ($P < 0.01$, Fig. 1B), which is consistent with our previous microarray analysis results[6].

On the premise of successful transfection, in A549/G + and A549/G- cells, overexpression and inhibition of miR-17-5p were carried out for CCK8 test. The inhibitory rates of gemcitabine at each concentration on the experimental group and the control group were as follows (Fig. 1C and 1D). The results indicated that the IC_{50} of the mimics experimental group is lower than that of the NC control group (115 μmol/L *vs.* 475 μmol/L, $P < 0.001$); the IC_{50} of the inhibitor experimental group is higher than that of the NC control group

(281 μmol/L *vs.* 9 μmol/L, $P < 0.05$). Similarly, the clonogenic survival assay also proved that under different gemcitabine doses, miR-17-5p's overexpression can reduce the number of clone formation in A549/G + cells (Fig. 1E); miR-17-5p's low-expression can increase the number clones in A549/G- cells (Fig. 1F). The above results explained that miR-17-5p mimics can reduce the resistance of drug-resistant cell lines to gemcitabine and the inhibitor can increase the resistance of sensitive cell lines to gemcitabine.

MiR-17-5p mimics reduce drug-resistance of A549/G + cells to gemcitabine, which is related to cell cycle regulation

Cell cycle was analyzed by flow cytometry. The number of cells of the G_1 phase increased and the number of cells in the S phase decreased (Fig. 2A) compared with the NC control group in the mimics experimental group. In the same way, the number of cells in the G_1 phase decreased and the number of cells in the S phase increased in the miR-17-5p inhibitor experimental group (Fig. 2B) compared with the NC control group. The above results stated clearly that miR-17-5p mimics or inhibitor can prevent or promote the cells from G_1 to S phase.

In order to confirm the results of flow cytometry, the protein expression level related to cell cycle was used to analyze by Western blotting

Western blotting showed that the protein expression level of CCNE1, CCNA2 and P21 were reduced in the miR-17-5p mimics experimental group compared with the NC control group (Fig. 2C). Similarly, the protein expressions level of CCNE1, CCNA2 and P21 increased after transfection with miR-17-5p inhibitor (Fig. 2D). The above results showed that miR-17-5p is closely related to the resistance of A549 cells to gemcitabine, and it is likely to proceed by regulating the cell cycle.

RRM2 is verified one of miR-17-5p's target genes in A549/G + cells

According to the prediction of miR-17-5p target genes in the three databases of miRTarBase, miRDB, Target Scan and references, RRM2 was proposed as one of the miR-17-5p's target genes. It was verified by double luciferase activity assay.

The results of dual-luciferase experiment are showed that only the miR-17-5p mimics and RRM2 WT group have the lowest dual-luciferase ratio ($P < 0.01$, Fig. 3A). The ratios of the other groups are not statistically significant. The results indicated that RRM2 is one of miR-17-5p's target genes.

At meantime, the mRNA expression level of RRM2 was detected by qPCR. The results displayed that the expression level of RRM2 was reduced or increased in the miR-17-5p mimics group or inhibitor group compared with the respective control groups ($P < 0.01$, $P < 0.001$, Fig. 3B and 3C). The above results showed that miR-17-5p negatively regulates the RRM2 gene at the mRNA level.

Interference with RRM2 gene expression can increase the sensitivity of A549/G + cell to gemcitabine

The restorative assay was used to verify further that RRM2 is one of miR-17-5p's target gene. CCK8 experiments hinted that at the same concentration, the cell survival rate was lower in the siRRM2 experimental group than in of siNC group ($P < 0.05$, Fig. 3D). IC_{50} of siRRM2 experimental group was lower than one of siNC group (772 μmol/L *vs*. 4362 μmol/L, $P < 0.001$). And the cell clone formation experiment was found that the number of cell clonal plaques formed was significantly more in siNC group than in the siRRM2 experimental group (Fig. 3E).

All this suggested that interference with RRM2 gene expression can reduce the tolerance of drug-resistant A549/G + cells to gemcitabine, and reduce the clone formation ability of A549/G + cells, and weaken proliferation ability of A549/G + cells, which accords with the previous results of miR-17-5p's overexpression.

Interfering with RRM2 gene expression may affect the cell cycle

After transfected RRM2 siRNA, observed the changes of cell cycle in A549/G + cells. These results displayed that number of cells in the G_1 phase of the cell cycle increased in experimental group compared with control group; while number of cells in the S phase decreased (Fig. 4A). This indicated that inhibition of RRM2 expression can block cells from G_1 to S phase.

Western blot experiments demonstrated that the expression level of RRM2 in the miR-17-5p mimics or inhibitor experimental group was significantly reduced ($P < 0.001$, Fig. 4B) or increased ($P < 0.001$, Fig. 4C) compared with control group. The above results suggested that miR-17-5p negatively regulates the target gene RRM2 at the protein level.

Then cell cycle protein expression was observed whether is affected when RRM2 is interfered. The results showed that siRRM2 could reduce the RRM2 protein level significantly, and the expression levels of P21, CCNE1 and CCNA2 protein were all reduced ($P < 0.01$, Fig. 4D). These results accord with the results of overexpression of miR-17-5p. This indicated that RRM2, as a miR-17-5p's target gene, takes part in the drug resistance process in A549/G + cells. And P21, CCNE1 and CCNA2 are most likely to be RRM2 downstream genes.

PI3K/AKT pathway activation caused by P-PTEN reduction leads to drug resistance in A549 cells

Western blotting was used to measure protein expression level in A549/G + cells and A549/G- cells. These results displayed that the PTEN and PI3K protein expression level in the A549/G + group were higher and the P-PTEN protein expression level was lower compared with the A549/G- group ($P < 0.05$, Fig. 5A). This

demonstrated that the drug-resistance of A549/G + cells is not related to the total of PTEN protein, but is related to the decrease of activated PTEN protein—p-PTEN.

The protein expressions of related proteins PTEN, P-PTEN, PI3K and P-AKT (Ser) (AKT protein phosphorylated at serine site) were measured. And the results showed that protein expression level of p-PTEN increased or reduced significantly in the miR-17-5p mimics or inhibitor experimental group, while p-AKT (Ser) decreased ($P < 0.01$, Fig. 5B) or significantly increased ($P < 0.01$, Fig. 5C). However, there had no change in the protein expression levels of PI3K and PTEN (Fig. 5C).

Similarly, to observe whether the change of RRM2 expression affects the expression of these proteins. The results displayed that the protein expression of p-PTEN was increased ($P < 0.001$, Fig. 5D) in the siRRM2 experimental group, while the expression level of PI3K, PTEN and p-AKT (Ser) proteins was unchanged (Fig. 5D). Combined with the results before, it further showed that the drug-resistance of A549/G + cells is associated with p-PTEN, and it can be speculated that the expression of p-PTEN protein may increase when RRM2 expression of is inhibited, thereby reducing the resistance of A549 cells to gemcitabine, increasing sensitivity of A549 cells to one. The above results indicated that there may be PI3K/AKT resistance pathway activated by decrease of p-PTEN in A549/G + cells, and this pathway is regulated by miR-17-5p and its target gene RRM2.

Discussion

This paper verifies that the miR-17-5p's expression level in A549/G + was lower than that in A549/G-. We first verified that RRM2 was one of miR-17-5p's target genes in A549/G + cells with double luciferase gene report experiment, and then verified this conclusion by qRT-PCR.

CCK-8 assay and clone formation survival experiment showed that overexpression of miR-17-5p or low-expression of RRM2 in A549/G + cells, the cell survival rate decreased, IC_{50} decreased, clone formation ability decreased, and the cells became sensitive to gemcitabine. On the contrary, low-expression of miR-17-5p or overexpression of RRM2 in A549/G- cells, the survival rate of cells increased, IC_{50} increased, clonogenic ability increased, and the cells became drug-resistant. This suggests that the tolerance of A549 to gemcitabine is related to miR-17-5p/RRM2 pathway.

Coincidentally, Duxbury et al. [11] studies suggest that over expression of RRM2 in pancreatic cancer cells can cause resistance to gemcitabine chemotherapy. When siRNA inhibited RRM2 expression, it could enhance the cytotoxicity induced by gemcitabine and improve the sensitivity of cells to gemcitabine; It coincides with our results. Similarly, Mah et al. [12] used tissue microarray analysis to conclude that RRM2 is a very useful disease-specific predictor, which can predict the survival outcome of different subtypes of non-small cell lung cancer. High levels of RRM2 expression were associated with poor survival outcomes. This is the same as the result of "RRM2 is highly expressed and cells become drug resistant".

Similar studies as Grolmusz et al [13] found gemcitabine can strongly inhibit the proliferation of ACC and increase their apoptosis. However, after a period of treatment, RRM2 was up-regulated, resulting in cell resistance to gemcitabine, which was consistent with the clinical therapeutic effect of ACC. This also shows that the high expression of RRM2 can make cells develop drug resistance. In addition, Jin et al [14] found that expression level of RRM2 was much higher in tumor tissues of patients with lung adenocarcinoma than that in normal tissues; The high expression of RRM2 can boost A549 cells proliferate. On the contrary, inhibiting the expression of RRM2 can also inhibit A549 cells proliferate. This is consistent with our results.

The results of flow cytometry showed that overexpression of miR-17-5p or low-expression of RRM2 in drug-resistant cells A549/G+, the number of G_1 phase cells rose and the number of S phase cells declined. Instead, when interfering with the expression of miR-17-5p or overexpressing RRM2 in sensitive cells A549/G-, the number of G_1 phase cells dropped and the number of S phase cells went up. Based on the previous results, under the same miR-17-5p (or RRM2) expression conditions, when the number of G_1 phase increases, the cells become sensitive; When the number of G_1 phase decreased, the corresponding cells became resistant. This suggests that the tolerance of A549 to gemcitabine is related to the cell cycle.

Then, we selected the G_1/S related proteins CCNE1, CCNA2 F and P21 for Western blotting detection. These results presented that expression levels of CCNE1, CCNA2 and P21 down-regulated at overexpression of miR-17-5p or low-expression of RRM2 in drug-resistant A549/G+. On the contrary, the expression levels of CCNE1, CCNA2 and P21 up-regulated at low-expression of miR-17-5p or overexpression of RRM2 in sensitive cells A549/G-. This indicates that the tolerance of A549 to gemcitabine is related to cell cycle related proteins CCNE1, CCNA2 and P21.

CCNE1 appears in the late stage of G_1 phase, and its expression can be increased by cytokines and certain tumors. CCNE1 can promote the G_1/S phase transition, shorten the G_1 phase, and reduce the cell's requirement for growth factors [15, 16]. CyclinE-CDK2 complex is a key kinase for cells to enter S phase from G_1 phase [17–19]. It can phosphorylate its substrate proteins, such as retinoblastoma protein (Rb), Rb family members P107, CDC6, etc. CCNA2 is mainly expressed in S phase and mediates initiation of the S phase [20], which is a key cell cycle regulator and essential for DNA synthesis and G_1/S transition [21]. Therefore, when miR-17-5p is overexpressed (or low-expression of RRM2), when the expression level of CCNE1 decreases, it is difficult for cells to complete G_1/S transformation, resulting in cell arrest in G_1 phase, cell proliferation is blocked, and cells become sensitive to gemcitabine; However, when the expression level of miR-17-5p (or RRM2) is opposite, the cells become resistant to gemcitabine.

Then we detected the expression level of PI3K/AKT signaling pathway protein. Firstly, Western blotting suggested that PTEN and PI3K expressed higher level in A549/G + cells than did in A549/G- cells, but the level of p-PTEN (phosphorylated PTEN) was lower. This shows that the activated PTEN (p-PTEN) is at a low level in A549/G + cells and has a weak inhibitory effect on AKT. AKT is activated and can activate

downstream signal molecules and play its physiological function, including cell growth, survival and proliferation, etc. All these help tumor cells survive better under drug pressure and show drug resistance [22, 23]. Secondly, PI3K level and PTEN level did not change, but p-PTEN level increased and p-AKT (phosphorylated AKT) level decreased at overexpression of miR-17-5p in A549/G + cells. When miR-17-5p was inhibited in A549/G- cells, the levels of PI3K and PTEN remained unchanged; The level of p-PTEN decreased and the level of p-AKT increased. This suggested that the miR-17-5p's expression level was consistent with that of p-PTEN, but opposite to that of p-AKT. However, it had no change the expression level of PI3K and PTEN. That is to say that miR-17-5p could positively regulate p-PTEN, while p-PTEN negatively regulates p-AKT. After overexpression of miR-17-5p in A549/G + cells, the level of p-PTEN increased and inhibited the level of p-AKT, thus inhibiting the downstream signal pathway function of AKT, inhibiting the drug resistance of cells, and finally the cells became sensitive. Otherwise, knocking down the expression of miR-17-5p in A549/G- cells, the level of p-PTEN also decreases, and the effect on p-AKT is weakened. p-AKT can start the function of downstream signal pathway and promote the resistance of cells to drugs [23, 24]. When we use RRM2 instead of miR-17-5p for restorative experiment, we can reproduce the previous experimental results. This indicates that miR-17-5p/RRM2 is involved in the regulation of PI3K/AKT signaling pathway and regulates the cell response to drugs by regulating PI3K/AKT pathway. Such similar results were also reported in the study of Shah et al[25]. Their experiments have shown that high expression of AKT can induce breast cancer cells to resist tamoxifen. It is also indicated that knockdown of RRM2 expression in AKT induced tamoxifen resistant breast cancer cells can inhibit the proliferation of these breast cancer cells under tamoxifen, that is, the reduction of RRM2 expression can reverse AKT induced tamoxifen resistance. This indicates that AKT induced breast cancer cell tamoxifen is closely related to RRM2. RRM2 and PI3K/AKT pathway may play an important role not only in drug resistance of lung cancer, but also in drug resistance of other tumors.

Conclusions

In brief, this article found that RRM2 is one target gene of miR-17-5p in A549 cells. It was demonstrated that miR-17-5/RRM2 changed cell cycle and cell tolerance to gemcitabine by affecting the expression level of CCNE1, CCNA2 and P21. This change is related to the change of p-PTEN expression level through miR-17-5/RRM2, which affects PI3K/AKT signal pathway and finally affects various effects downstream of AKT. We can change the drug resistance of A549 cells to gemcitabine by changing expression level of miR-17-5p/RRM2, which provides an experimental basis and points out the research direction for overcoming the drug resistance of lung cancer. However, the relationship between miR-17-5/RRM2 axis and PI3K/AKT signaling pathway in affecting cell cycle remains to be further studied; In addition, the role of P21 and the relationship between cell resistance and DNA damage and repair will be described in next research paper.

Abbreviations

A549/G+

gemcitabine-resistance lung cancer cell line A549
A549/G-
gemcitabine-sensitive lung cancer cell line A549
RRM2
ribonucleotide reductase catalytic subunit M2
NSCLC
non-small cell lung cancer
Gem
gemcitabine
NVB
vinorelbine
TXT
paclitaxel and docetaxel
CDA
cytidine deaminase
qPCR
quantitative polymerase chain reaction
RT
reverse transcription
CCK-8
Cell Counting Kit-8
RIPA
radio-immunoprecipitation assay
SDS-PAGE
sodium dodecyl sulfate polyacrylamide gel electrophoresis
UTR
untranslated region
CCNE1
human Cyclin E1
CCNA2
human Cyclin A2
(ACC
adrenocortical cancer.

Declarations

Acknowledgments

Not applicable.

Author’s contribution

Conception and design: Ai-Ling Liang and Yong-Jun Liu; Collection and assembly of data: Xuan Ma, Tian Fu, Zhi-Yin Ke, Shen-Lin Du, Xue-Chun Wang, Ning Zhou; Data analysis and interpretation: Xuan Ma, Tian Fu, Ai-Ling Liang and Yong-Jun Liu; Manuscript writing: Xuan Ma, Tian Fu; All authors read and approved the final manuscript.

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Availability of data and materials

The dataset generated and analyzed used in the current study is available from the corresponding author on reasonable request

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors have no conflicts of interest to declare.

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Figures

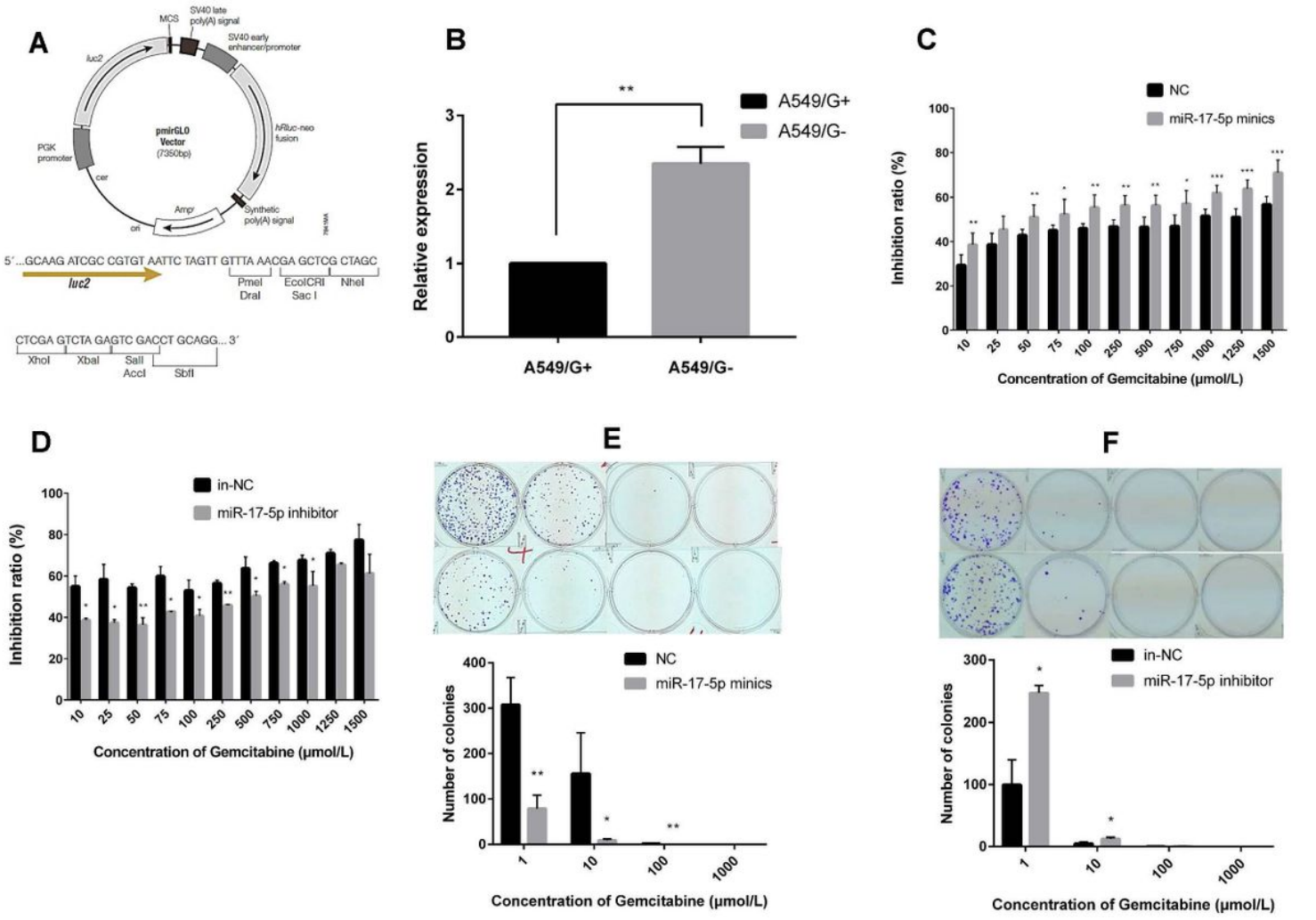

Figure 1

pmirGLO vector structure map and the expression of miR-17-5p affects the effects of gemcitabine on the growth and proliferation of A549/G+ and A549/G- cells

A. pmirGLO Vector structure map; B. The expression of miR-17-5p in A549/G- and A549/G+ cells; C. The inhibition rate of gemcitabine at each concentration after transfection miR-17-5p mimics; D. The inhibition rate of each concentration of gemcitabine after transfection miR-17-5p inhibitor; E. The clone formation changes after transfection miR-17-5p mimics; F. The clone formation changes after transfection miR-17-5p inhibitor.

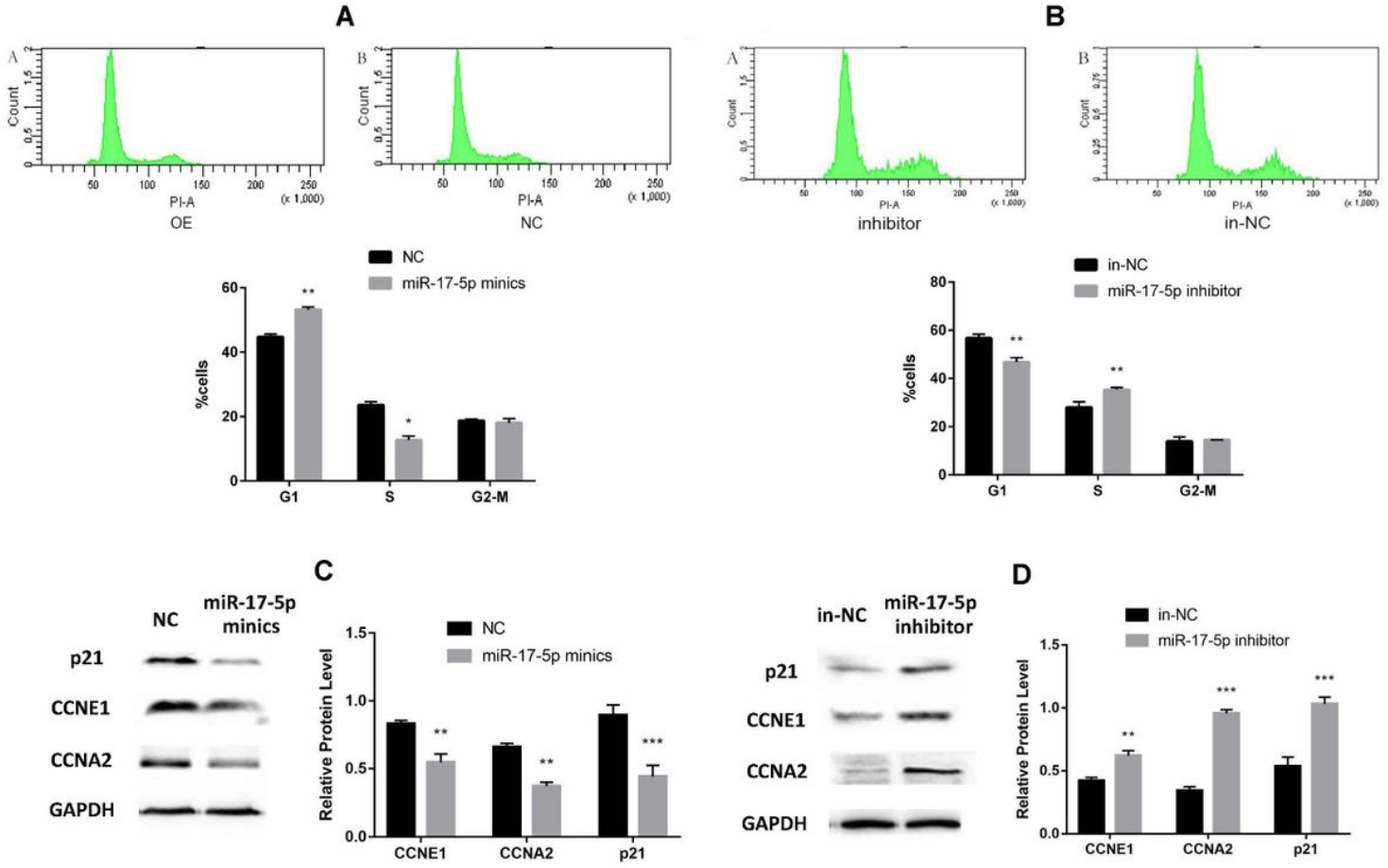

Figure 2

The results of cell cycle and its relative proteins expression

A. The cell cycle changes after transfection miR-17-5p mimics; B. The cell cycle changes after transfection miR-17-5p inhibitor; C. The protein changes after transfection miR-17-5p mimics; D. The protein changes after transfection miR-17-5p inhibitor.

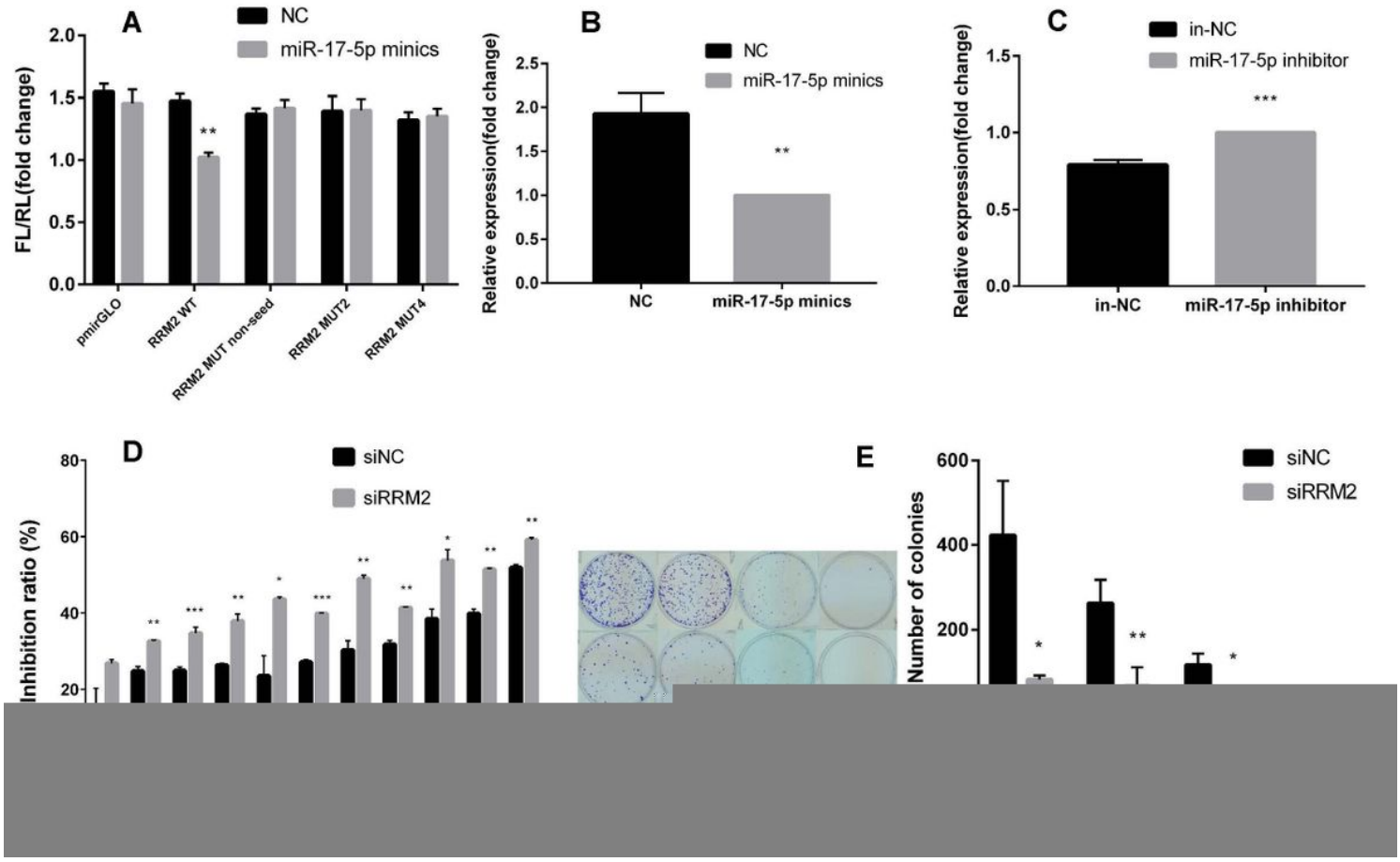

Figure 3

The results of dual luciferase reporter gene experiment, the expression of RRM2 after transfection of miR-17-5p mimics and inhibitor and growth & clone formation of treated by siRRM2.

A. The results of dual luciferase reporter gene experiment; B. The expression changes of RRM2 after transfection of miR-17-5p mimics; C. The expression changes of RRM2 after miR-17-5p inhibitor transfection; D. The inhibition rate of gemcitabine at each concentration on the experimental group (siRRM2) and the control group (siNC); E. The changes in clone formation after transfection siRRM2.

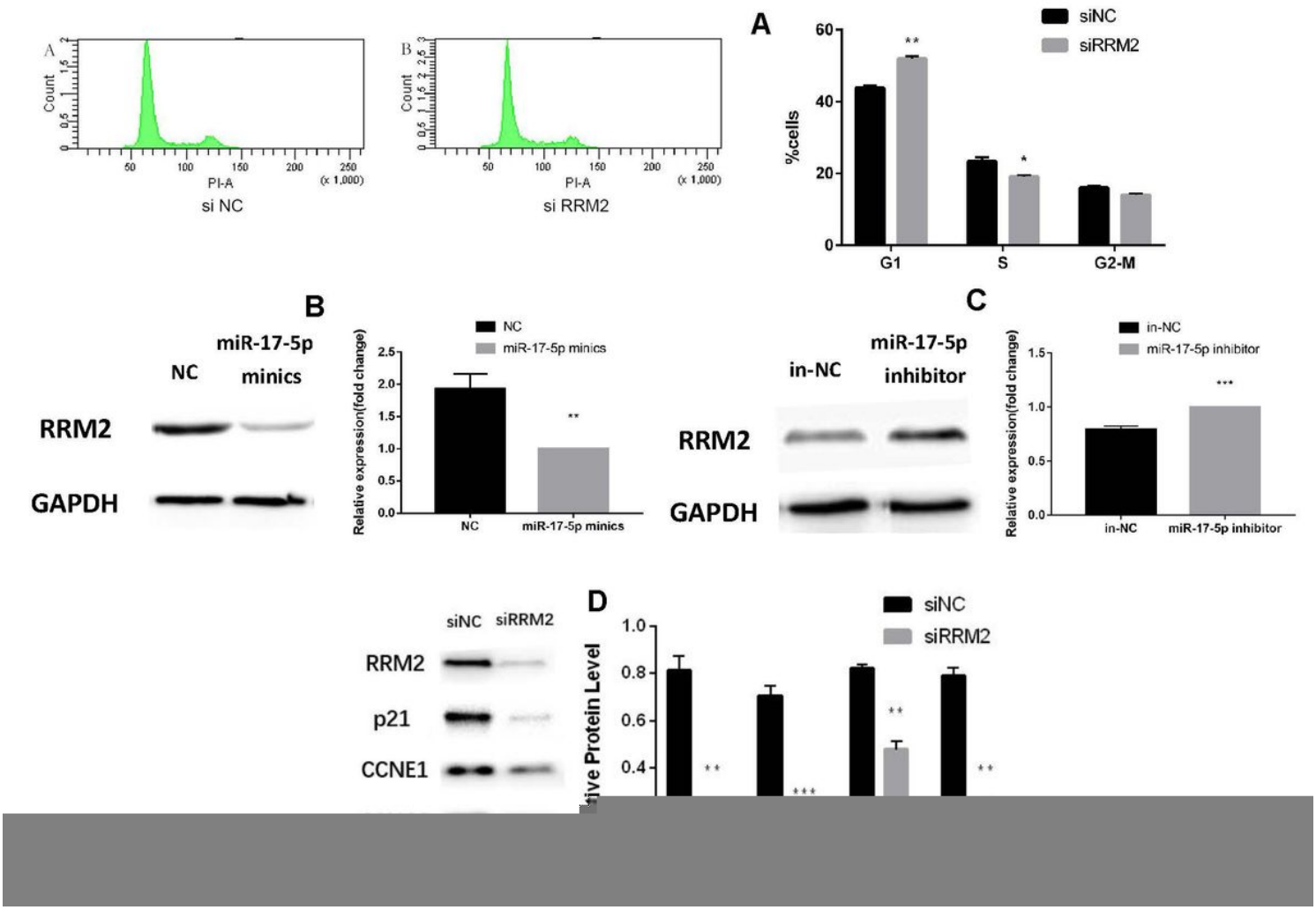

Figure 4

The cell cycle changes, the protein changes of RRM2 and cell cycle relative protein

A. The cell cycle changes treated by siRRM2; B. The changes of RRM2 protein expression treated by miR-17-5p mimics; C. The changes of RRM2 protein expression treated by miR-17-5p inhibitor; D. The protein expression changes of RRM2, P21, CCNE1 and CCNA2 treated by siRRM2.

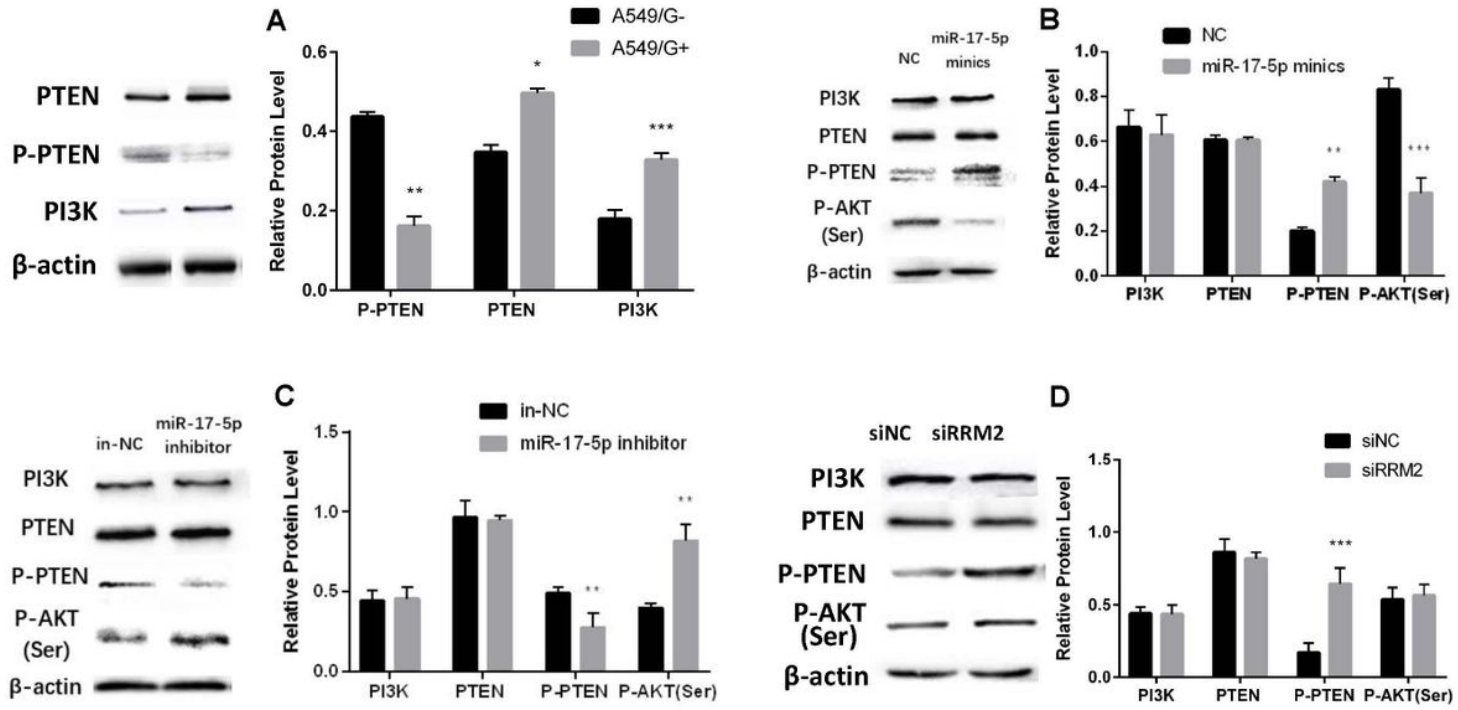

Figure 5

The protein expression difference PTEN, p-PTEN and PI3K under different conditions

A. The protein expression difference PTEN, p-PTEN and PI3K in A549/G- group and A549/G+ group; B. The changes of protein expression PI3K, PTEN, p-PTEN and p-AKT (Ser) after transfection miR-17-5p mimics; C. The changes of protein expression PI3K, PTEN, p-PTEN and p-AKT (Ser) after transfection miR-17-5p inhibitor; D. The changes of protein expression PI3K, PTEN, p-PTEN and p-AKT (Ser) after transfection siRRM2.