

## **Upregulation of miR-202-3p reduces Icotinib resistance in lung carcinoma A549/Ico cells.**

**Hong Yang, Cui-Ying Zhang\***

Department of Oncology, Inner Mongolia People's Hospital, Hohhot, PR China

### **Abstract**

**Icotinib exerts a good anti-tumor efficacy on non-small cell lung cancer (NSCLC), while Icotinib resistance has become an important limiting factor in the clinical application. Studies have certified that a great quantity of microRNAs are involved in the development of cancer. Here we found that miR-202-3p was observably down-regulated in A549/Ico cells, which is a kind of Icotinibresistant lung cancer cells. Transfection of A549/Ico cells with miR-202-3p reversed the Icotinib resistance and increased cell apoptosis. Subsequently, we examined the expression of ADP-Ribosylation Factor-like 5A (ARL5A), the functional target of miR-202-3p, as well as the multi-drug resistant (MDR) related genes glutathione s transferase  $\pi$  (GST- $\pi$ ), Multi Drug Resistance 1 (MDR1), multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP), which were down-regulated following the upregulation of miR-202-3p in A549/Ico cells. Our study demonstrated that miR-202-3p transfection reduced Icotinib resistance in human lung cancer A549/Ico cells by modulation of its downstream target ARL5A and MDR-related genes expression. These findings suggest that miR-202-3p may function as a novel therapeutic candidate in patients with MDR lung cancer.**

**Keywords:** Icotinib resistance, miR-202-3p, Lung carcinoma, A549/Ico cells.

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### **Introduction**

MicroRNAs (miRNAs) are a class of endogenous RNAs which contain 18–25 nucleotides in noncoding single-strand [1]. The number of verified miRNAs has grown rapidly since their discovery in 1993 [2]. Over the past few years, studies have shown that miRNA regulate up to one third of human genes at the posttranscriptional level [3]. As miRNAs involve in biological development, as well as cell proliferation, differentiation and apoptosis, dysregulation of miRNAs plays a crucial role in the pathologic processes of tumorigenesis [4,5]. Although miRNAs perform vital functions in regulation of developmental gene expression, their role in tumor progression remains poorly understood. MiR-202-3p is located within a chromosomal fragile site in 10q26 [6]. Related reports have clarified that miR-202-3p is downregulated in colorectal cancer [7], breast cancer [8] and cervical squamous cell carcinoma [9], it also can inhibit cell proliferation in neuroblastomas [10]. Furthermore, overexpression of miR-202-3p can suppress cell proliferation and induce cell apoptosis in cancer cells.

Nowadays, NSCLC has been one of the leading causes of cancer-related mortality in the world, accounting for about 80% of all lung cancer cases [11]. Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) have proven to be an indispensable method in the treatment of advanced NSCLC [12], due to its exact curative effect, mild adverse reactions and oral administration convenient [13]. Icotinib is a

kind of EGFR-TKIs, developing entirely by Chinese scientists independently, exerts an excellent anti-tumor efficacy on NSCLC [14]. However, although the treatment of Icotinib can prolong the life of patients with NSCLC, the majority of patients develop an acquired resistance to Icotinib 8–10 months following the initiation of treatment [15].

Usually, tumor cells undergo self-adaptation through contacting with chemotherapeutic drugs in MDR [16]. MDR-related protein and lung resistance-related protein can reduce drug absorption while increase drug efflux, along with the enhancement of cellular detoxification capability. ARL5A is a Protein Coding gene which belongs to the ARF family of GTP-binding proteins, and studies have explored that MiR-202-3p can inhibit cell proliferation by directly targeting ARL5A in colorectal cancer [2]. GST- $\pi$  is an important cell detoxifying enzyme [17], MDR1, MRP1 and BCRP are important drug resistance genes, upregulation of these genes is a signal for the emergence of drug resistance [18].

In the present work, we aimed to explore the effect of miR-202-3p expression on Icotinib resistance in human lung cancer A549/Ico cells. We confirmed that the expression of miR-202-3p was downregulated in A549/Ico cells, and transfection of miR-202-3p reversed Icotinib resistance and increased cell apoptosis relatively. Furthermore, upregulation of miR-202-3p decreased ARL5A and MDR gene expression

in A549/Ico cells, indicating that miR-202-3p might play an important role in inhibiting Icotinib resistance.

## Materials and Methods

### Cell culture

Human A549 cells (originated from lung adenocarcinoma) were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 100 µg/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (FBS; Invitrogen) at 37°C in an atmosphere containing 5% CO<sub>2</sub>. Icotinib was purchased from Zhejiang Beida Pharmaceutical Co., Ltd. (Beijing, China). The Icotinib-resistant A549 cell line (A549/Ico) was established by exposure to increasing concentrations (0.5, 1, 5, 10, and 20 µM) of Icotinib. A549 cells were allowed to grow for 2 weeks at each concentration. The Icotinib-resistant cells were maintained in the medium containing 10 µM of Icotinib.

### MTT assay

Cells were seeded in 96-well plates at a density of  $5 \times 10^3$  cells per well and incubated for 48 h. The cell culture fluid was added with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution (Sigma-Aldrich) at a final concentration of 0.5 mg/mL and incubated at 37°C for additional 4 h. Dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to each well to dissolve the formazan product. After incubating for 10 minutes at 37°C, absorbance was measured at a wavelength of 570 nm. The half-maximal inhibitory concentration (IC<sub>50</sub>) was estimated by plotting the concentration-cell viability curve.

### Cell transfection

A549/Ico cells were seeded in 6-well plates at a density of  $5 \times 10^5$  cells per well the day before transfection. Cells were transfected with 1 µg of the corresponding plasmid DNA, using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. After transfection for 48 h, cells were harvested for gene expression analysis or exposure to Icotinib for further detection.

### Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted in each cell group according to kit instructions based on Trizol method. RNA was formed into cDNA *via* TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA), and cDNA was amplified as a template in each group through ABI 7900 TaqMan Sequence Detection System (Applied Biosystems). The sequences of the PCR primers used were as follows: MiR-202-3p, TTCCCATGCCCTATA; ARL5A forward, 5'-CGCTGCCCCGAGAATGGGAA-3' and reverse, 5'-TTGGCCACCAATATCCCACAT-3'; GST-π forward, 5'-GGACCTCCGCTGCAAATAC-3' and reverse, 5'-

GAAGGTCTTGCCTCCCTGG -3'; MDR1 forward, 5'-AAAAAGATCAACTCGTACCCTC-3' and reverse, 5'-GCACAAAATACACCAACAA-3'; MRP1, 5'-ACTTCCACATCTGCTTCGTCAGTG-3' and reverse, 5'-ATTCAGCCACAGGAGGTAGAGAGC-3'; BCRP forward, 5'-ATCAGCTGGTTATCACTGTGAGGCC-3' and reverse, 5'-AGTGGCTTATCCTGCTTGGAAAGGC-3'; GAPDH forward, 5'-CGACCACTTTGTCAAGCTCA-3' and reverse, 5'-AGGGGTCTACATGGCAACTG-3'. Amplification conditions were as follows: 30 sec at 95°C, 60 sec at 65°C and 60 sec at 72°C for 40 cycles, the extension was 5min at 72°C. The relative mRNA expression was determined according to the 2<sup>-ΔΔC<sub>t</sub></sup> method after normalization against GAPDH.

### Apoptosis detection by flow cytometry

Following cell transfection, after treatment of 3 µM of Icotinib for 24 h, cells were harvested and stained with AnnexinV/PI double staining kit (BD biosciences, USA) according to the manufacturer's protocol. Apoptotic cells were assessed by flow cytometry on a FACScan (Beckman Instruments, Fullerton, CA, USA).

### Western blot analysis

Total proteins were extracted via RIPA lysis buffer (Thermo Scientific, Rockford, IL, USA) containing Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA). Then, quantitation of protein was determined by BCA protein quantification kit. Protein samples (20 µg per lane) were subjected to electrophoresis. After applying 10% SDS-PAGE electrophoresis to separate total proteins, the proteins were transferred onto a PVDF membrane. Then, 5% non-fat milk in 1 × TBST (10 mM Tris-HCl, 150 mM NaCl, pH 8.0, and 0.1% Tween 20) was used to block out for 30 min at room temperature, and incubated in primary antibody overnight at 4°C. The primary antibodies included: Bcl-2 (1:2000, EPITMICS, USA), cleaved Caspase-3 (1:1000, BD Biosciences, USA), GAPDH (1:20000, Abcam, UK), ARL5A (1:500, Abgent, USA), GST-π (1:1000, Sigma-Aldrich, USA), MDR1 (1:1000, Abcam, UK), MRP-1 (1:1000, BD Biosciences, USA), BCRP (1:5000, BXP-53 Enzo LifeSciences, Farmingdale, NY). Next, the PVDF membrane was washed 10 min with TBST solution and repeated three times. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. The membrane was washed 10 min with TBST solution and repeated three times. Membranes were developed using the ECL chemiluminescent kit (Amersham Biosciences, Piscataway, NJ, USA).

### Statistical analysis

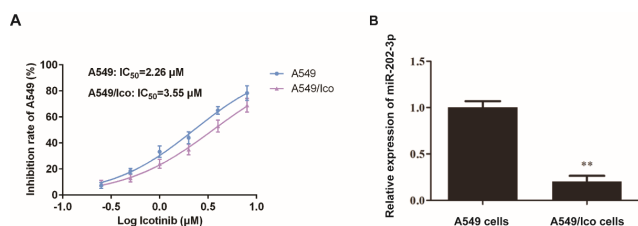
Data were presented as means ± SEM. All statistical calculations were carried out using SPSS 11.7 software (SPSS, Chicago, IL, USA). Continuous variables were compared using the Student's t test for normally distributed variables. Statistical differences among multiple groups were analyzed by

one-way analysis of variance. A P-value of <0.05 was considered statistically significant.

**Results**

**MiR-202-3p is down-regulated in A549/Ico Icotinibresistant lung cancer cells**

We established the Icotinibresistant A549/Ico cells in the previous stage, to test whether the establishment was successful, the Icotinib resistance of A549 and A549/Ico cells was detected by MTT assay after Icotinib treatment for 24 h. As the Figure 1A shown, the half maximal inhibitory concentration (IC<sub>50</sub>) of the A549 cells was 2.26 μM and the IC<sub>50</sub> of the A549/Ico cells was 3.55 μM, with resistant fold change 1.57, which indicated that the A549/Ico cells was available for further research. The mRNA expression of miR-202-3p was detected in A549 and A549/Ico cells respectively. The results revealed that the mRNA level of miR-202-3p was significantly reduced in A549/Ico cells as compared with A549 cells (P<0.05; Figure 1B). This reminded us that miR-202-3p might have a potential connection with Icotinib resistance.

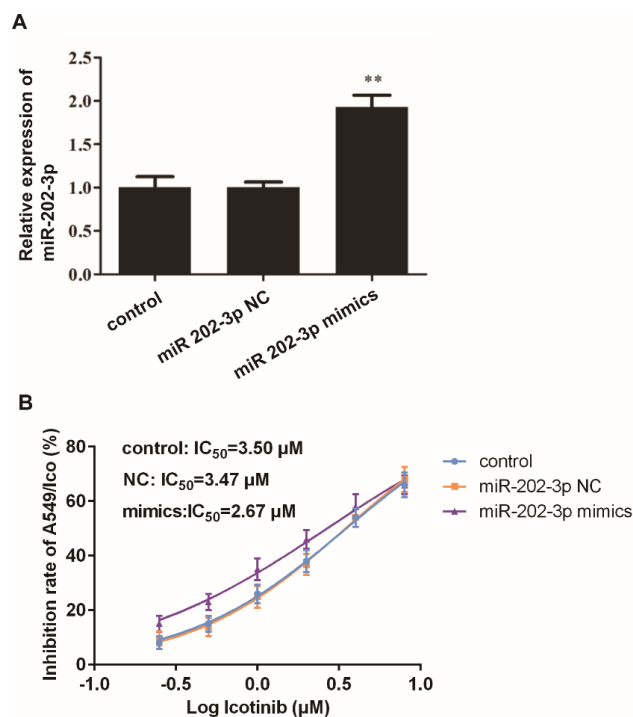


**Figure 1.** MiR-202-3p is down-regulated in A549/Ico Icotinibresistant lung cancer cells. (A) The MTT assay was used to evaluate Icotinib resistance in the A549 and A549/Ico cells. (B) The mRNA level of miR-202-3p were analyzed by RT-PCR analysis in A549 and A549/Ico cells. The data are presented as the means ± SEM. \*P<0.05, \*\*P<0.01, as compared with the A549 cells group (n=4).

**Icotinib resistance is reversed following the transfection of miR-202-3p**

According to the above results, we speculated that miR-202-3p was involved in the regulation of Icotinib resistance. To verify the conjecture, we transfected A549/Ico cells with miR-202-3p mimics or negative control, and cells were analyzed for the expression of putative miR-202-3p through RT-PCR after 48 h. The results demonstrated that the quantity of miR-202-3p was significantly increased in the miR-202-3p-mimics A549/Ico cells in comparison with the normal A549/Ico cells (P<0.01; Figure 2A), while it showed no difference between the miR-202-3p-NC A549/Ico cells and normal A549/Ico cells. Subsequently, we examined Icotinib resistance in A549/Ico cells following miR-202-3p transfection. As shown in Figure 2B, subsequent to transfection of miR-202-3p, the Icotinib resistance of A549/Ico cells was significantly decreased after 24 h Icotinib treatment, as detected by the MTT assay. The IC<sub>50</sub> of the miR-202-3p-mimics A549/Ico cells was 2.67 μM and the IC<sub>50</sub> of the control A549/Ico cells was 3.50 μM, with

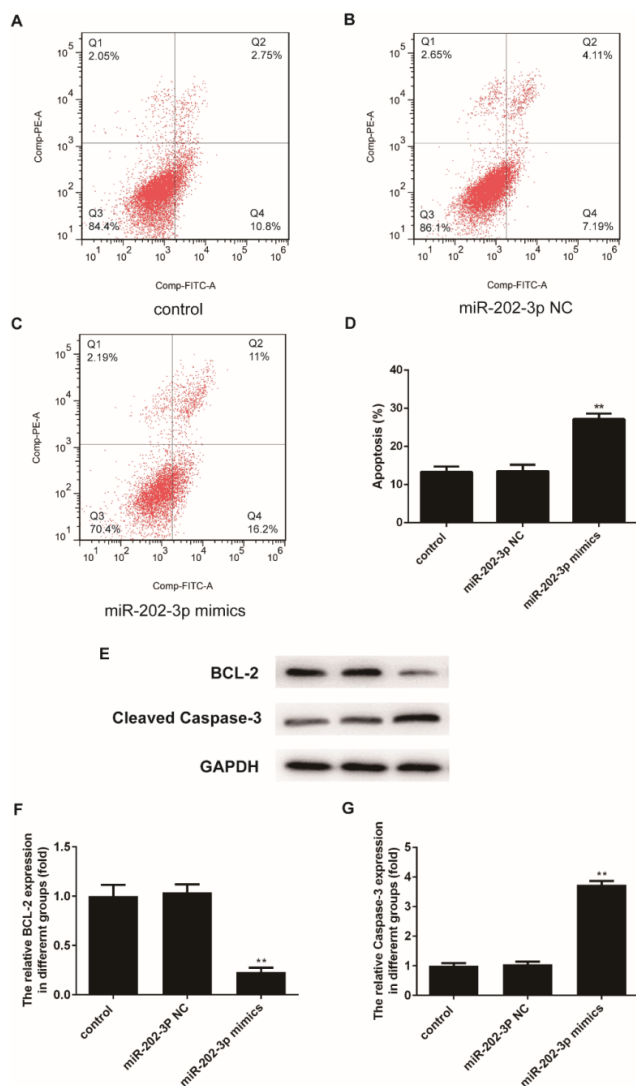
reversal fold change 0.76. While the IC<sub>50</sub> of the miR-202-3p-NC A549/Ico cells was as high as the control A549/Ico cells. These results suggested that miR-202-3p was important in the formation of Icotinib resistance in A549/Ico cells.



**Figure 2.** Icotinib resistance is reversed following the transfection of miR-202-3p. (A) The mRNA level of miR-202-3p was up-regulated in miR-202-3p-mimics A549/Ico cells compared to the A549/Ico cells. The mRNA levels of miR-202-3p was analyzed by RT-PCR analysis. (B) IC<sub>50</sub> of the control A549/Ico cells, the miR-202-3p-NC A549/Ico cells and the miR-202-3p-mimics A549/Ico cells was analyzed by MTT assay. The data are presented as the means ± SEM. \*P<0.05, \*\*P<0.01, as compared with the control group (n=4). Control refers to the A549/Ico cells; miR-202-3p NC refers to the miR-202-3p negative control A549/Ico cells, miR-202-3p mimics refers to the miR-202-3p mimics A549/Ico cells.

**MiR-202-3p overexpression in A549/Ico cells increases cell apoptosis**

Studies have indicated that Icotinib can significantly inhibit the proliferation and increase the apoptosis rate of tumor cells, so that exerts a tumor suppressive effect. Therefore, we examined the impact of miR-202-3p on Icotinib-mediated apoptosis. As the miR-202-3p overexpressed, the apoptosis rate in A549/Ico cells was significantly increased (P<0.05; Figures 3A-3D), meanwhile we detected the protein expression of the apoptosis related protein BCL-2 and cleaved caspase-3, the results showed that the expression of BCL-2 (P<0.05; Figures 3E and 3F) was significantly reduced, and the cleaved caspase-3 (P<0.05; Figures 3E and 3G) expression was significantly improved in the miR-202-3p mimics group, as compared with the control group. This suggested that miR-202-3p mitigated Icotinib resistance by increasing apoptosis.

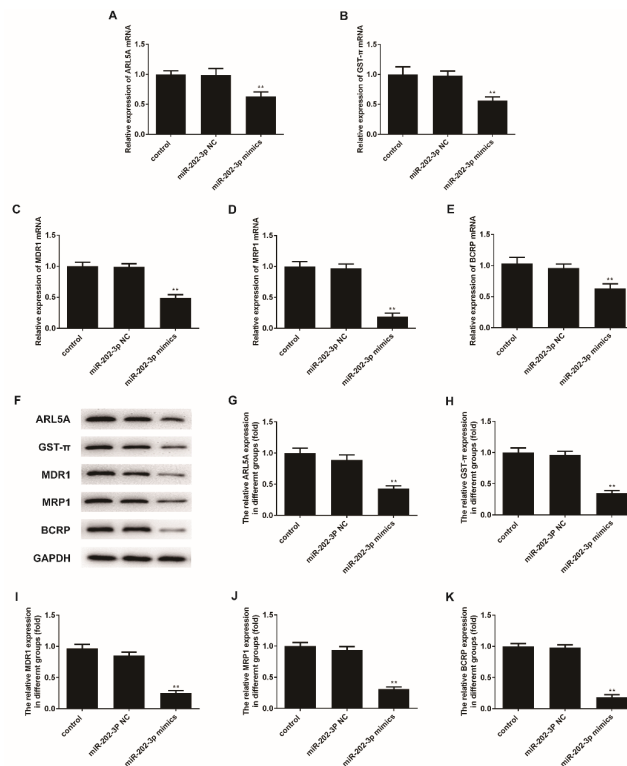


**Figure 3.** MiR-202-3p overexpression in A549/Ico cells increases cell apoptosis. (A, B, C, D) Flow cytometric results showed increased apoptosis in the miR-202-3p mimics group as compared with the control group. (E) The protein of BCL-2 and cleaved caspase-3 was detected by Western blot analysis, with the quantitative analysis of BCL-2 (F) and cleaved caspase-3 (G). The data are presented as the means ± SEM. \*P<0.05, \*\*p<0.01, as compared with the control group (n=4).

**MiR-202-3p overexpression decreases ARL5A and MDR gene expression in A549/Ico cells**

ARL5A is a direct target of miR-202-3p involved in cell proliferation, GST-π, MDR1, MRP1 and BCRP are important cancer associated drug resistance genes, increased expression of these genes is a common cause of drug resistance. In this research, we detected the mRNA and protein expression of ARL5A, GST-π, MDR1, MRP1 and BCRP through RT-PCR analysis and Western blot analysis, respectively. As the Fig.4A shown, the mRNA expression of ARL5A was significantly reduced following the overexpression of miR-202-3p in A549/Ico cells, and the mRNA level of GST-π, MDR1, MRP1 and BCRP showed a similar trend. Likewise, the protein

expression of the above genes detecting via Western blot analysis was consistent with mRNA expression. All the results indicated that overexpression of miR-202-3p in A549/Ico cells inhibited ARL5A and drug resistance genes expression, which contributed to the reverse effect of miR-202-3p on Icotinib resistance (Figure 4).



**Figure 4.** MiR-202-3p overexpression decreases ARL5A and drug resistance genes expression in A549/Ico cells. RT-PCR analysis showed reduced ARL5A (A), GST-π (B), MDR1 (C), MRP1 (D) and BCRP (E) mRNA expression levels in the miR-202-3p mimics group. (F) The protein expression was detected by Western blot. Quantitative analysis revealed reduced ARL5A (G), GST-π (H), MDR1 (I), MRP1 (J) and BCRP (K) protein expression in the miR-202-3p mimics group. The data are presented as the means ± SEM. \*P<0.05, \*\*P<0.01, as compared with the control group (n=4).

**Discussion**

It has been reported that EGFR-TKIs can suppress tumor growth by binding to the intracellular domain of EGFR, thus inhibiting the phosphorylation of the tyrosine kinase and blocking downstream signal transduction pathways [19]. Icotinib, as a kind of EGFR-TKIs, has become an important therapeutic drug for NSCLC [20]. However, it is likely for patients to develop an acquired resistance to Icotinib. How to overcome the Icotinib resistance is a vital problem to the treatment of NSCLC. MiR-202-3p has been observed to be disordered in breast cancer, follicular lymphoma, colorectal cancer, and leukemia. Moreover, miR-202-3p is frequently decreased in human gastric cancer [6]. Although existing researches on miR-202-3p are limited, the above reports still give us some suggestion that miR-202-3p may play an indispensable role in the cancer development. At present,

whether miR-202-3p is involved in drug resistance still remains unclear.

In this study, we found that miR-202-3p expression in A549/Ico cells was dramatically lower than that in A549 cells, which indicated that miR-202-3p was associated with Icotinib resistance. Given miR-202-3p was down-regulated in A549/Ico cells, we conjectured that overexpression of miR-202-3p in A549/Ico cells might reverse Icotinib resistance. In the following study, we discovered that transfection of miR-202-3p decreased IC<sub>50</sub> in A549/Ico cells, which meant a higher sensitivity of A549/Ico cells to Icotinib. Meanwhile, overexpression of miR-202-3p in A549/Ico cells increased cell apoptosis, the expression of BCL-2 was reduced, while caspase-3 expression was increased. These results demonstrated that the decreased miR-202-3p expression in A549/Ico cells should be a cause of Icotinib resistance rather than a concomitant phenomenon. Icotinib resistance may be involved in various mechanisms, such as the activation of detoxifying systems, a reduction in intracellular drug concentration and the inhibition of apoptosis. In this study, we detected some drug resistance related genes.

ARL5A is a member of the ARF family of GTP-binding proteins [21]. ARFs are vital in eukaryotic vesicular trafficking pathways and have a crucial part in the activation of phospholipase D. It is reported that miR-202-3p can inhibit cell proliferation by directly targeting ARL5A [2]. Analogously, in our study, ARL5A was detected to be reduced as a result of miR-202-3p upregulation, suggesting that ARL5A might be a target of miR-202-3p mediated Icotinib resistance, so that suppressed cell viability. GST- $\pi$  is a kind of isoenzymes, highly expressed in most tumor cells [22]. GST- $\pi$  catalyzes the complex formation of chemotherapeutic drugs with GSH, which can be excreted by multidrug resistance associated proteins, thus producing tumor resistance [23]. Our results showed that miR-202-3p also decreased the expression of GST- $\pi$ , it meant that miR-202-3p might reverse drug resistance through inhibiting cell detoxification. MDR1, MRP1 and BCRP are three ABC transporters, belonging to the family of multidrug resistance proteins, play a crucial role in the oncology drug resistance. In this study, overexpression of miR-202-3p in A549/Ico cells was discovered to decrease the expression of the above genes, indicating that miR-202-3p reversed the resistance of Icotinib in A549/Ico cells involving in the mechanism of inhibiting the drug efflux from cells.

In conclusion, our study revealed that miR-202-3p played a crucial part in lung cancer Icotinib resistance. In the Icotinib-resistant tumor cells (A549/Ico cells), the expression of miR-202-3p was significantly reduced. Transfection of miR-202-3p was found to raise sensitivity of A549/Ico cells to Icotinib and increase Icotinib-induced cell apoptosis. The reverse effect of miR-202-3p on Icotinib resistance might involve in the following mechanisms: reducing the expression of ARL5A to inhibit cell proliferation, reducing the expression of GST- $\pi$  to inhibit cell detoxification as well as reducing the expression of MDR1, MRP1 and BCRP to inhibit drug efflux from the cells. All the results indicated that miR-202-3p might

act as an important role in reversing Icotinib resistance in A549/Ico cells, and we tentatively explored its mechanisms. While the Icotinib resistant cancer cells used in our study is limited to A549/Ico, whether the findings applying to other lung cancer cells still remains unknown. Furthermore, concerning to the mechanisms of miR-202-3p on reversed drug resistance, we carried out a shallow exploration, the subsequent downstream functions are worth of further research.

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**\*Correspondence to**

Cuiying Zhang

Department of Oncology

Inner Mongolia People's Hospital

PR China