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Original Article

MicroRNA-375 confers cisplatin resistance by regulating KRAS expression in non-small cell lung cancer cell

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Abstract: Cisplatin is a classic chemotherapeutic drug that has been widely used for non small cell lung cancer (NSCLC) treatment, but NSCLC cells are often resistant to primary or acquired cisplatin therapy. Several studies have shown that miR-375 is significantly elevated in tumor and suggests that this miRNA might play a role in this resistance. In this study, we investigated this possibility and the possible mechanism underlying this role. We showed that forced expression of miR-375 significantly inhibited apoptosis, enhanced cell proliferation and increased the resistance of tumor cells to cisplatin in NSCLC H1975 cells. Furthermore, knock-down of miR-375 reversed these effects on H1975 cells and increased the sensitivity of H1975 to cisplatin chemotherapy. Finally, we showed that miR-375 targeted the KRAS gene, and directly regulated the expression of KRAS mRNA. These results demonstrate that miR-375 may play an important role in the cisplatin resistance of NSCLC.

Keywords: miR-375, NSCLC, cisplatin, KRAS

Introduction

Non small cell lung cancer (NSCLC) is one of the most common types of lung cancers. Despite advances in surgery, chemotherapy and molecular targeted therapy, the mortality rate has only decreased slightly over the past years [1]. Among classic chemotherapeutic drugs, cisplatin is one of the widely used chemotherapies for the adjuvant and metastatic therapy of NSCLC. However, primary or acquired resistance to platinum-based chemotherapy is a major clinical obstacle to the successful treatment of NSCLC [2]. Tremendous efforts have been taken to understand molecular and cellular mechanisms for the action of cisplatin over the past decades, but it remains unclear how these NSCLC cells become resistant to cisplatin. Thus, a better understanding of molecular events underlying this drug resistance is important and necessary for achieving effective therapy against this type of cancer.

MicroRNAs (miRNA) are a class of small RNA molecules that regulate the translation and degradation of mRNAs. MiRNAs bind to complementary sequences in the 3'-untranslated regions (UTRs) of their target mRNAs to promote mRNA degradation or translational repression [3]. MiRNAs function in negative gene regulation. These molecules silence gene expression by interfering with mRNA stability or protein translation. MiRNAs participate in numerous biological processes, such as proliferation, apoptosis, differentiation and invasion [4]. During tumourigenesis, miRNAs act as an oncogene or a tumor suppressor gene and contribute to cancer initiation and progression by regulating target genes. MiR-375 is up-regulated in most of human malignancies and involved in each stage of cancer development and progression, including transformation, neoplasia, invasion, metastasis and drug resistance [5]. Moreover, miR-375 over-expression is associated with poor prognosis of cisplatin chemotherapy, including lymph node and liver metastasis [6]. In addition, miR-375 modulates radiosensitivity of HR-HPV-positive cervical cancer cells by targeting UBE3A through the p53 pathway [7]. Therefore, those studies provide part of molecular mechanisms for how miR-375 contributes to drug resistance.

The relationship between miR-375 dys-regulation and human cancer resistance has attracted increasing attention. However, the association of miR-375 with the sensitivity of NSCLC cells to cisplatin has yet to be explored. The present study aims to provide insights into the association of miR-375 expression with cisplatin resistance in NSCLC. As a result, inhibition of this miR-375 expression led to hypersensitivity of NSCLC cell H1975 to cisplatin, whereas further over-expression of miR-375 promoted cell proliferation. Also, we found that KRAS gene is a direct target of miR-375. These results suggest that miR-375 might be one of the key cellular components important for the development of NSCLC cisplatin resistance. Our study also implies that targeting miR-375 may overcome the resistance of NSCLC to cisplatin and thus serve as a potential target for the development of cancer therapy specifically against cisplatin resistant cancer cells.

Materials and methods

Cell culture and infection

Non-small cell lung cancer cell line H1975 was cultured in RPMI1640 medium with 10% fetal bovine serum (FBS). Pre-miR-375 and miR-375-antisense oligonucleotide (ASO) lentiviral vector were synthesized by Nanjing genscript co., LTD, the nonsense sequence lentiviral vector (NC) as a negative control, lentiviral vector-U6 snRNA as an endogenous control. H1975 cells were transfected with each group. We used puromycin to screen stable cell lines.

TaqMan quantitative real-time PCR analysis of miR-375 expression

miRNA was isolated from the cell lines with miRNA isolation Kit (Omega), reverse transcribed using TaqMan® miRNA reverse transcription kit, and subjected to real-time PCR using TaqMan® miRNA Assay kit [8]. Real-time PCR was performed using Step One Plus Real-Time PCR System (Applied Biosystems, USA) by standardized protocol. To normalize the expression levels of miR-375, U6 was used as a reference. The relative amount of miR-375 to internal control U6 was calculated by using $2^{-\Delta\Delta CT}$.

Cell proliferation assay and cell apoptosis assay

To determine the biological effect of miR-375 on cell proliferation, cell counting CCK-8 kit was used following manufacturer's protocol [9]. 1×10^4 cells per well were plated into 96 well plates and each day harvested a 96 well plates, a total of observation for 7 days. Then, 10 μl of CCK-8 solution was added to each well, and cells were incubated for 2 h at 37°C. Absorbance at 450 nm was read on a microplate reader. All experiments were performed for 3 times. Apoptosis was detected using a DNA fragmentation ELISA kit (Roche, Indianapolis, IN, USA).

Cisplatin sensitivity assays

Each group of cells was harvested by trypsinization. To adjust the cell suspension to 1×10^4 cells/ml, 100 μl per well were plated into 96 well plates in quadruplicate. The total of cisplatin concentration were set up to 7 different groups, each group was 0, 5, 10, 15, 20 25, and 30 (μM). Then, 10 μl of CCK-8 solution was added to each well, and cells were incubated for 2 h at 37°C. Absorbance at 450 nm was read on a microplate reader.

TaqMan quantitative real-time PCR for mRNA expression

Total RNA from cells was isolated using mRNA isolation Kit (Omega) according to the instructions supplied by the manufacturer. Reverse transcription (invitrogen) was performed with (2 μg) of total RNA. Real-time quantitative RT-PCR was performed using TaqMan human miRNA assay kit. The relative amount of KRAS to internal control (β-actin) mRNA was calculated by using $2^{-\Delta\Delta CT}$. Each experiment was performed in triplicate.

Western blotting

Each group of cells was seeded into 6-well plates and the cells were allowed to grow until 100% confluency, then lysed in lysis-buffer on ice. Proteins were separated by 12% SDS-PAGE and blotted to nitrocellulose membranes. Membranes were blocked with 10% non-fat milk powder at room temperature for 2 h and incubated overnight with primary antibodies: Cleaved-PARP, PARP, KRAS and β-actin (Cell signaling, USA). After three 5 min washes in TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 4 h at room temperature, then washed again in TBST and the membranes were developed with an ECL plus western blotting detection system.

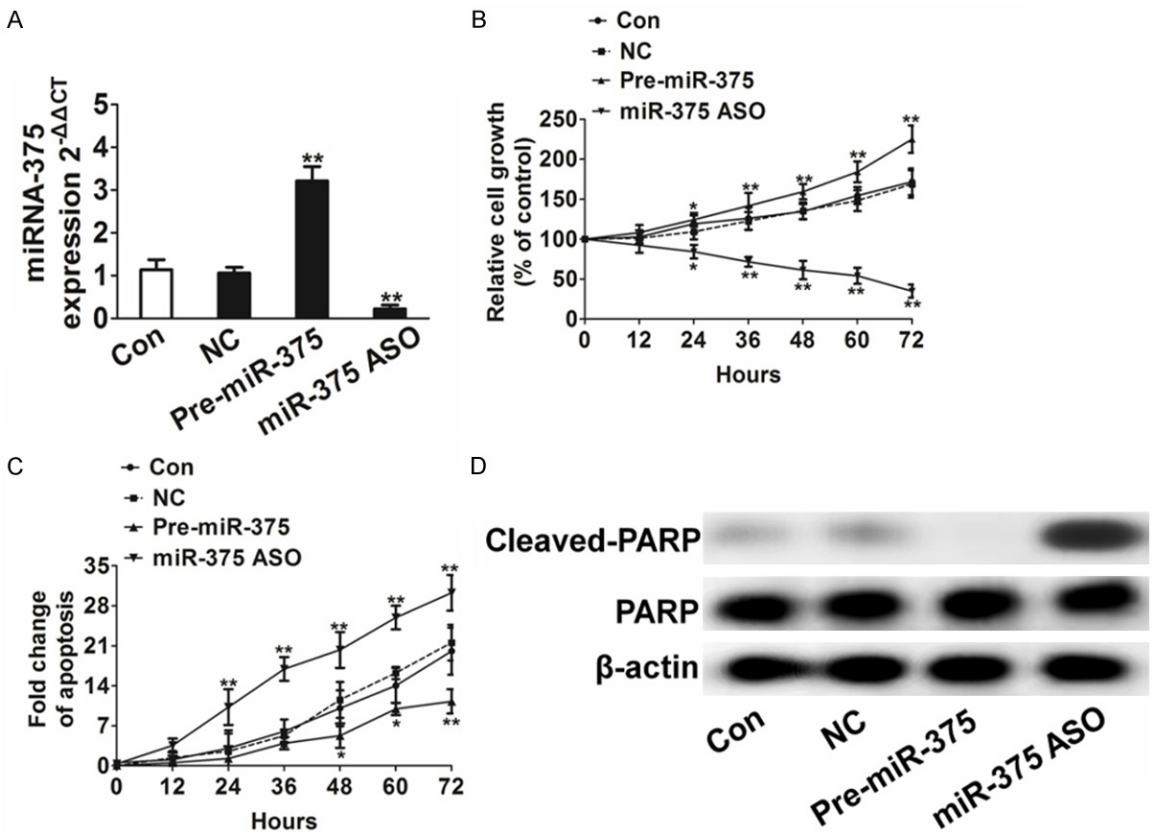

Figure 1. miR-375 increases cell proliferation and anti-apoptotic in H1975 cancer cells. A. Analysis of TaqMan qRT-PCR showed that a decrease or increase after H1975 cells infected with miR-375 ASO and pre-miR-375 lentiviral vector, the nonsense sequence lentiviral vector (NC) as negative control. All data are shown as mean ± SD, $^{**}P < 0.01$ compared to control cells. B. miR-375 increased H1975 cells proliferation. Cells were infected with miR-375 ASO and pre-miR-375 lentiviral vector, and were plated into 96 well plates and harvested at different times. All data were shown as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control cells. C. Impact of miR-375 on cell apoptosis. Apoptosis was analyzed by cell flow cytometry. The cells undergoing apoptosis were Annexin-V-PE-positive and 7-AAD-negative. All data are shown as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control cells. D. Western blot analysis showed that cleaved PARP was higher in miR-375 ASO transfection cells than in control cells and pre-miR-375 lentiviral vector transfection H1975 cells.

Luciferase reporter assay

The 3'-UTR of KRAS containing miR-375 binding site was amplified using the primers: (Forward) 5'-CCGCTCGAGATCCCAGTAATGGAATGAAG-3'; (Reverse): 5'-ATAAGAATGCGGCCGCCATCACTTATTATTGCCTATGT-3'. As a negative control, the mutated binding site of the 3'-UTR sequence (using the reverse complement of the binding site) was amplified using the primers: (Forward) 5'-CCGCTCGAGTGAAGGTAATATTGTGAAGCTATTGTC-3'; (Reverse): 5'-ATAAGAATGCGGCCGCCA TCACTTATTATTGCCTATGT-3'. Products were reclaimed from agarose gel electrophoresis, and cloned into the luciferase reporter PsiCHECK vector (Promega). All constructs were verified by sequencing. Each group cell was seeded in 5 × 10^5 cells per 6-well plates and transfected plasmid when cells reached 70% confluence, luciferase reporters using lipofectamine™ 2000 (Invitrogen) following the instructions. Cells were harvested 48 h after transfection, being analyzed for luciferase activity using the Dual-Luciferase Reporter Assay system (Promega).

Animal experiment

Experiments involving animals were performed according to the Guide for the Care and Use of Laboratory Animals and the institutional ethical guidelines of Huaihe Hospital of Henan University for animal experiments. Scramble-transfected and miR-375-overexpressing H1975 cells (6 × 10^6 cells) were inoculated s.c. into the dorsal flanks of BALB/c nude mice

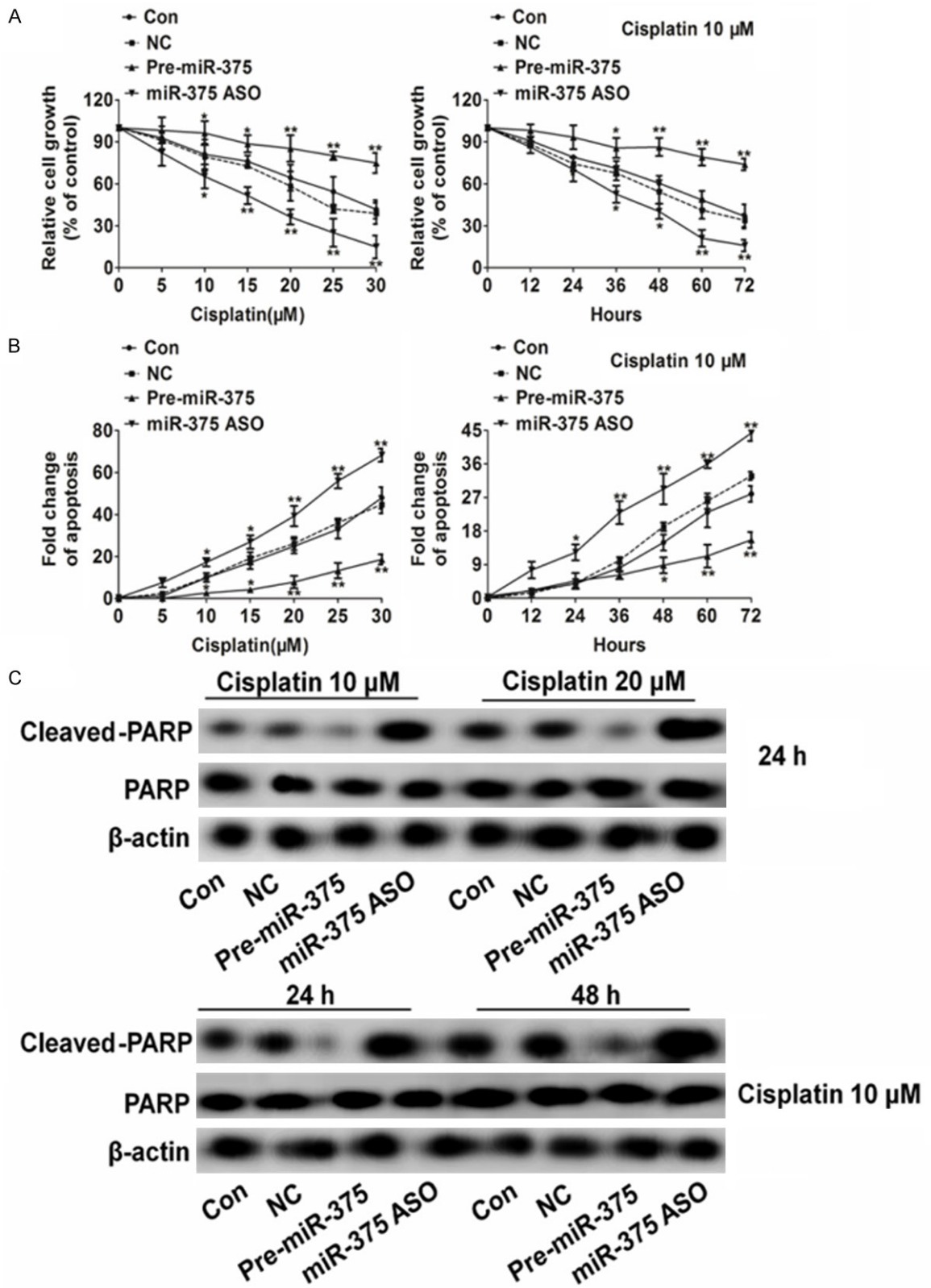

Figure 2. Effect of miR-375 on the sensitivity of H1975 cells to cisplatin. A. Effect of miR-375 on the sensitivity of H1975 cells to cisplatin chemotherapy. The total cisplatin concentration was set up to 7 different groups, each group was 0, 5, 10, 15, 20, 25 and 30 μM. In another experiment, the growth index was assessed after 0, 12, 24, 36, 48 and 72 h in the presence of 10 μM cisplatin. Data were collected from three independent experiments

and were average ± SD. values. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control cells. B. miR-375 down-expression promoted cell apoptosis in H1975 cells after treatment with cisplatin. Data were collected from three independent experiments and were average ± SD. values. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control cells. C. Western blot analysis showed that cleaved PARP was higher in miR-375 ASO lentiviral vector transfection cells than in NC transfection H1975 cells.

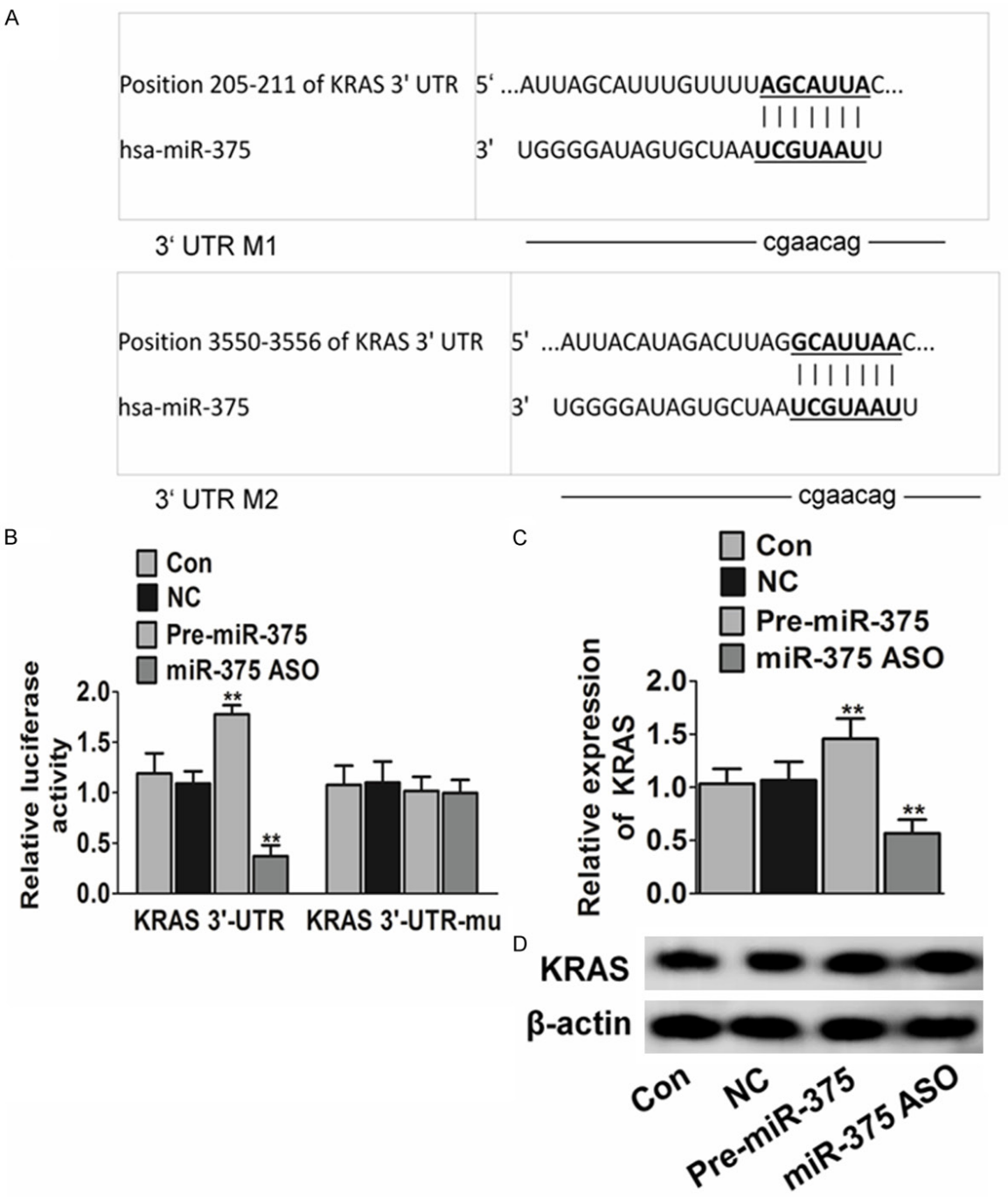

Figure 3. miR-375 regulates the expression of mRNA level of KRAS. A. Sequence of the miR-375 binding sites within the human KRAS 3'-UTR and a schematic of the reporter construct showing the KRAS 3'-UTR sequence and the mutated KRAS 3'-UTR sequence. B. Luciferase activity of the KRAS reporter in the presence of pre-miR-375 lentiviral vector or miR-375-ASO ($^{**}P < 0.01$). Data were collected from three independent experiments and were average ± SD. values. $^{**}P < 0.01$, compared to control cells. C. Analysis of expression levels of endogenous KRAS mRNA in

H1975 cancer cells infected with miR-375 ASO, pre-miR-375 lentiviral vector and the nonsense sequence lentiviral vector (NC) by using qRT-PCR. Data were collected from three independent experiments and were average ± SD. values. $^{**}P < 0.01$ compared to control cells. D. Analysis of expression levels of endogenous KRAS protein in H1975 cells infected with miR-375 ASO, pre-miR-375 lentiviral vector and negative control (NC) by western blot.

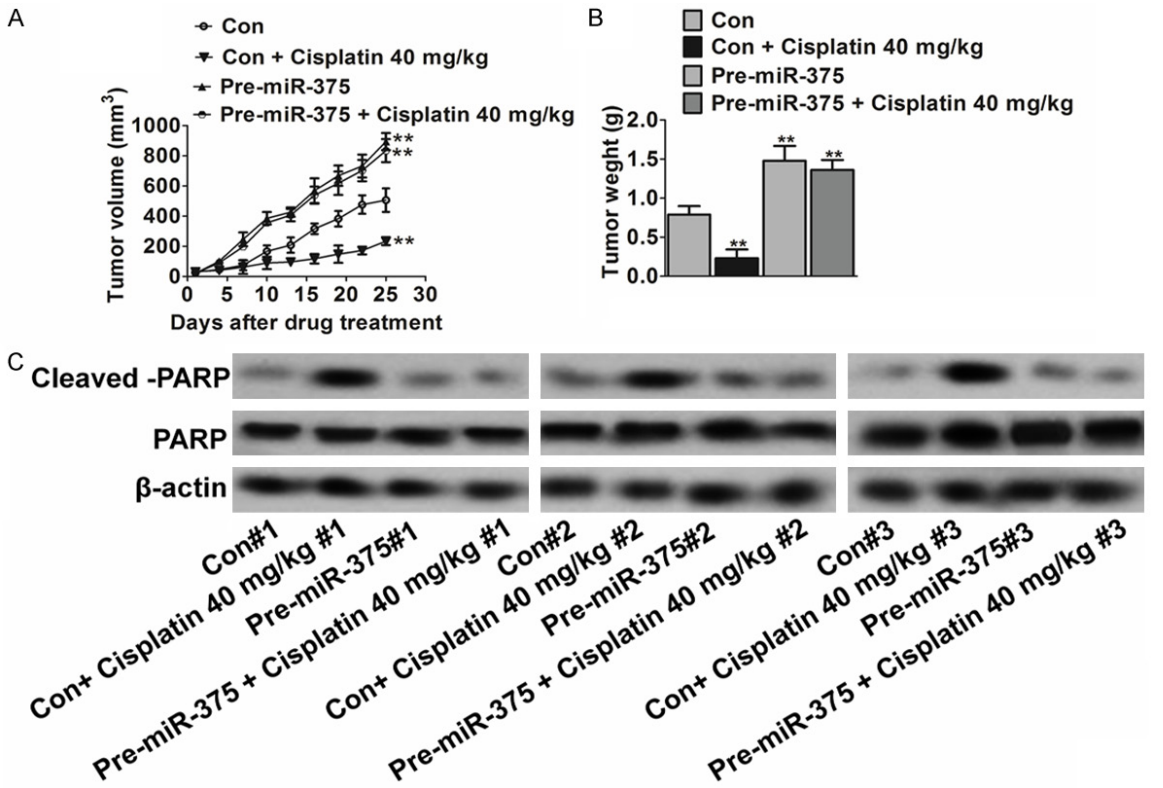

Figure 4. miR-375 over-expression antagonizes tumor growth inhibition induced by cisplatin. MiR-357 decreased the effectiveness of cisplatin in the inhibition of tumor growth in vivo. Xenograft tumor growth curves (A) and tumor weights (B). (C) Western blot analysis showed that cleaved PARP was higher in control tumor treated with cisplatin than in pre-miR-375 tumor and pre-miR-375 treated with cisplatin.

(female, Nu/Nu, six week old). The tumor volume was measured for 25 days. All mice were killed, s.c. tumors were resected, and weights were recorded.

Statistical analysis

Statistical analysis was performed using SPSS 18.0. Data's were presented as the mean ± standard deviation. Statistical analyses were done by analysis of variance (ANOVA) or Student's test. *P* value < 0.05 was considered statistically significant.

Results

miR-375 increases cell proliferation

In order to investigate the function of miR-375 in NSCLC cells, we tested miR-375 expression in H1975 cell line using TaqMan real-time PCR after infection 48 h. The result showed that a decrease or increase after infected with miR-375 antisense oligonucleotide (ASO) and pre-miR-375 lentiviral vector (**Figure 1A**). We observed a significantly increased in proliferation after infection of pre-miR-375 lentiviral vector, In contrast, miR-375 ASO lentiviral vector significantly decreased H1975 cell proliferation (**Figure 1B**). These results indicate that H1975 cell proliferation ability can be significantly enhanced by the increase of miR-375 expression.

We further investigated the effect of miR-375 on cell apoptosis. The results showed that increase of miR-375 expression could decrease apoptosis dramatically. In contrast, knockdown of miR-375 could increase the apoptosis of

H1975 cells (**Figure 1C**). Meanwhile, the expression of cleavage PARP was significantly up-expression in miR-375 knockdown H1975 cells, while slightly down-expression of cleavage PARP in miR-375-overexpression H1975 cells as shown in western blot analysis (**Figure 1D**). These results suggest that miR-375 could function as a strong anti-apoptotic factor in human H1975 cancer cells.

Effect of miR-375 on the sensitivity of H1975 cells to cisplatin chemotherapy

Next, we investigated whether miR-375 could affect the sensitivity of H1975 cells to cisplatin chemotherapy. Compared with the control group (H1975 cells), up-regulation of miR-375 reduced H1975 cells sensitivity to cisplatin, while down-regulation of miR-375 significantly increase killing ability of cisplatin by dose-dependent and time-dependent (**Figure 2A**). This data indicates that miR-375 is one key player in cisplatin resistance of NSCLC cancer cell H1975. We also investigated the effect of miR-375 on the apoptosis of H1975 cells by DNA fragmentation assay and PARP cleavage assay. Similarly, pre-miR-375 lentiviral vector transfection inhibited the apoptosis in H1975 cells which induced by cisplatin (**Figure 2B**). Meanwhile, in the presence of cisplatin, the expression of cleavage PARP was significantly slight in H1975 cells transfected with pre-miR-375 lentiviral vector when compared to nonsense sequence lentiviral vector transfection (**Figure 2C**). These results indicate that miR-375 can protect H1975 cancer cells survival by anti-apoptosis.

KRAS 3'-UTR is a target for miR-375

We then searched for the potential target of miR-375 by TargetScan, PicTar and miRanda-mirSVR [10]. These programs predicted KRAS as one of the prime targets of miR-375. To test whether or not KRAS is a target of miR-375, the plasmid pMIR-reportor containing the wild-type 3'-UTR region of KRAS downstream of the luciferase coding region (**Figure 3A**, KRAS_WT) was constructed. HEK-293T cells were co-transfected with reporter plasmid (KRAS_WT) and scramble. We performed a luciferase reporter assay and observed a significant decrease or increase in luciferase activity in the presence of pre-miR-375 or miR-ASO [11]. In addition, to validate whether KRAS is a direct target of miR-375, we mutated the miR-375 binding site that was located in the 3'-UTR of KRAS, and this mutant reporter was no longer responsive to this miRNA (**Figure 3B**). These results indicate that KRAS is a direct target of miR-375.

At last, we determined the effect of miR-375 on the expression of KRAS in H1975 cells. The results showed that pre-miR-21 increases the expression of KRAS mRNA. Moreover, miR-375-ASO decreased the expression of KRAS mRNA. Those results suggest that miR-375 presents a positive regulator of KRAS (**Figure 3C**). It may be partly involved in regulation of the expression of these proteins at transcriptional levels. Furthermore, we found that miR-375 had no significant effect on the expression of KRAS (**Figure 3D**). To our knowledge, it is the first time to verify that miR-21 can indirectly target KRAS.

miR-375 confers H1975 cells cisplatin resistance in vivo

To determine whether miR-375 conferred resistance to cisplatin-induced apoptosis in vitro translates to drug resistance in vivo, we used a NSCLC xenograft model for this study. Briefly, H1975 cells expressing the control vector or miR-375 were subcutaneously inoculated into athymic nude mice. Mice were randomly divided into four groups 7 days after innoculation. One group was treated with cisplatin (40 mg/kg per day) administered by i.p. injection and the other with the vehicle. Tumor growth and therapeutic response were monitored during the course of cisplatin treatment.

Xenograft tumor growth curves showed that miR-375 conferred the tumors growth advantage as compared with vector control (**Figure 4A**). More importantly, miR-375 induced a marked difference in the response of tumors to cisplatin treatment. Tumors with control cells (designated as con) were very sensitive to cisplatin treatment and failed to grow during drug treatment, whereas tumors with miR-375-expressing cells (designated as pre-miR-375) were relatively resistant to cisplatin treatment and continued to grow. In addition, compared with control tumors, miR-375 tumors showed considerably smaller reduction of tumor weight (**Figure 4B**) after cisplatin treatment. These results indicated that cisplatin treatment was much less potent in the inhibition of tumor

growth of miR-375-expressing cells than that of control cells. To determine whether miR-375-mediated resistance to apoptosis in vitro was associated with decreased cisplatin effect in vivo, western blot assays were performed to examine the cleaved-PARP in tumors. The results showed that pre-miR-375 tumors have slightly cleaved -PARP than control tumors in the absence of cisplatin (**Figure 4C**). In addition, cisplatin treatment did not increase cleaved -PARP in miR-375 tumors (**Figure 4C**), indicating that miR-375 tumors were more resistant to cisplatin-mediated cell apoptosis than control tumors. Taken together, these in vitro and in vivo results reveal a critical role of miR-375 in drug resistance of NSCLC.

Discussion

The dismal outcome of non-small cell lung cancer cell (NSCLC) patients highlights the need for novel prognostic biomarkers. MiRNAs control the expression levels of particular genes. Therefore, dys-regulation of miRNAs, which occurs in certain diseases, such as cancers, may be attributed to aberrant gene expression [12]. The involvement of microRNAs in cancer and their potential as biomarkers of diagnosis and prognosis are becoming increasingly appreciated. Many studies have reported significant associations between miRNA expression profiles and important clinical features of tumors, as well as patient survival rates. MiR-375 has been identified as oncogenes or tumor suppressor genes which has the potential to the development and growth of many human malignancies, including lung cancer, hepatocellular cancer, glioma and colorectal cancer [13]. A number of studies also indicate that miR-375 plays a very important role in tumor cell proliferation, apoptosis and invasion. However, the limited information concerning the role of miR-375 in NSCLC is available [14]. In consistence with these studies, here we showed that miR-375 can promote cell proliferation and inhibit apoptosis in NSCLC line H1975 cells.

Cisplatin resistances have been the bottleneck in improving the efficacy of chemotherapy. MiR-375 has been considered to function as an oncogenic molecule and may play a role in this drug resistance. Several reports suggest that miR-375 is one of the key miRNAs which plays a broad role in sensitivity to chemotherapeutic agents [15]. A study showed that epigenetic silencing of miR-375 induces trastuzumab resistance in HER2-positive breast cancer by targeting IGF1R [16]. Recently, one report showed that miR-375 is up-regulated in acquired paclitaxel resistance in cervical cancer and might be a therapeutic target in paclitaxel-resistant cervical cancer [17]. Therefore, those studies provide part of molecular mechanisms for how miR-375 contributes to drug resistance. Our discovery of miR-375 as a mediator of drug response in NSCLC provides a potential therapeutic target. Inhibition of miR-375 expression could increase NSCLC cancer chemosensitivity.

The KRAS gene is necessary for the maintenance of tumor progression. Mismatch binding initiates subsequent events, including cleavage and excision of the error-containing strand followed by new synthesis and ligation [18]. KRAS gene mutation increased the risk of a wide variety of cancers, especially in colon cancer. MiR-375 appeared to directly target the 3'-UTR of KRAS mRNA, and correlated to cisplatin drug resistance in NSCLC. We further validated this in H1975 cancer cells. We found that up-regulation of miR-375 could markedly increase the expression of KRAS. Hence, we propose that miR-375 may increase cisplatin resistance by regulating the expression of KRAS. These results verify that miR-375 could regulate KRAS expression, leading to the increase of cisplatin resistance. Our study provides a new piece of evidence supporting the involvement of KRAS in miR-375-caused cisplatin resistance.

In conclusion, we have shown that miR-375 plays a significant role in proliferation, apoptosis, invasion and the response to cisplatin and radiotherapy in H1975 cancer cells. Moreover, this role might be attributed to miR-375 targeted KRAS and influencing cisplatin chemotherapy sensitivity. Our finding suggests miR-375 can be a potentially useful marker for prediction of the clinical response to cisplatin therapy, and miR-375 may be a potential target for NSCLC therapy.

Disclosure of conflict of interest

None.

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