Overexpression of miR-203 increases the sensitivity of NSCLC A549/H460 cell lines to cisplatin by targeting Dickkopf-1

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Abstract. The number of new lung cancer cases diagnosed yearly is high, and the mortality rate has not substantially declined. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, and adenocarcinoma accounts for the largest proportion of NSCLC. Currently, platinum-based combined chemotherapy, particularly cisplatin (DDP) is still the main form of treatment for advanced NSCLC. However, cisplatin resistance often occurs in patients who receive chemotherapy. Previous studies offer various explanations for how miRNAs affect cisplatin resistance, but the underlying mechanism remains largely unknown. The present study was designed to focus on miR-203 and Dickkopf-1 (DKK1), investigating the potential mechanisms involved in cisplatin resistance in tissues of lung adenocarcinoma and A549/H460 cell lines. In DDP-sensitive NSCLC samples, miR-203 was expressed at a higher level when compared with this level in DDP-insensitive samples, while DKK1 mRNA was expressed at a relatively low level as indicated by qRT-PCR. Dual luciferase reporter assay revealed that DKK1 is a target gene of miR-203 in A549 and H460 cells. Upregulation of miR-203 reduced the IC50 value of cisplatin in the A549 and H460 cells by inhibiting cell growth and promoting cell apoptosis. Similar effects of tumor inhibition and cisplatin sensitization were verified in vivo. Further research showed that both overexpression of miR-203 and knockdown of DKK1 increased the sensitization to DDP with a lower IC50 value. Upon DKK1 knockdown, overexpression of miR-203 had no added effects on the sensitivity of the cells. In addition, miR-203 was unable to sensitize cells with DKK1 that lacked the 3’ untranslated region (3’UTR). We conclude that miR-203 targets the 3’UTR of DKK1, and increases cisplatin sensitivity in A549/H460 cell lines.

Introduction

According to estimations published by the American Cancer Society in 2016, lung and bronchial cancer are the leading causes of death in both male (27%) and female (26%) cancer patients. Based on an estimated number of new cases (224,390), lung cancer is second only to breast cancer (1). Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, and adenocarcinoma makes up the largest proportion of NSCLC (2). The median age of diagnosis of lung and bronchial cancer is 70, and the 5-year relative survival is as low as 17% (3). Unfortunately, with its high incidence and mortality, the early diagnosis of lung cancer is difficult, and most cases are diagnosed at the advanced stages, at which point tumor resection is no longer an effective option (4). Even though the clinical application of molecular-targeted treatment has improved the situation of advanced lung adenocarcinoma to an extent, combination chemotherapy that contains cisplatin (DDP) and another chemotherapeutic agent is still the primary treatment (5). However, the response rate of cisplatin-based chemotherapy is not favorable, with an average overall survival period of less than one year and a high percentage of intrinsic and acquired drug resistance (6,7). There is an urgent need for researchers to determine the mechanisms underlying DDP resistance and develop the required measures to improve this condition.

miRNAs are non-coding small RNAs 19-25 nucleotides in length that can regulate the translation or degradation of target mRNAs in the human body (8). In cancer, miRNAs play an important role in cell proliferation, invasion and apoptosis and may influence DDP resistance (9-11). Various surveys suggest that miR-203 exhibits abnormal expression in tumor tissues and takes part in the regulation of proliferation, invasion, metastasis and prognosis of tumor cells (12-18). miR-203 has shown potential in the modulation of DDP sensitivity of human cancer cells (9,19-21).

DKK1 is a secreted protein and inhibitor of canonical Wnt/β-catenin signaling (22). It plays an important role in the...
mechanism underlying DDP resistance (23). In many types of cancer, DKK1 plays a role in the promotion of cell proliferation and poor prognosis and affects DDP sensitivity (24-27), but there are also various studies indicating the different functions of DKK1 (28,29). Since both miR-203 and DKK1 may modulate DDP sensitivity, the aim of the present study was to verify the expression levels of miR-203 and DKK1 in lung cancer tissues, lung cancer cell lines, and xenograft tumors in nude mice and investigate the effects of miR-203 on cell proliferation, apoptosis and DDP sensitivity.

Materials and methods

Ethics statement. The present study was approved by the Medical Research Ethics Committee of The First Affiliated Hospital of Zhengzhou University for use of patient tissues as well as for the use of animals in research. All patients who participated in the present study provided written informed consent.

Human tissue samples. In the present study, 30 tissue samples from patients with advanced lung adenocarcinoma were obtained from CT-guided percutaneous puncture or bronchoscopic biopsy. Tissue samples were preserved in liquid nitrogen for the next isolation of RNA or protein. All patients accepted a chemotherapy regimen of pemetrexed combined with DDP (both from Qilu Pharmaceutical Co., Ltd., Jinan, China). The gender, age, level of differentiation, tumor-node-metastasis (TNM) stage and the reaction to two cycles of chemotherapy were recorded and evaluated according to WHO standards.

RNA extraction and qRT-PCR. TRIzol (Invitrogen, Carlsbad, CA, USA) was used to isolate the total RNA of tissues and cells. Quantitative real-time PCR (qRT-PCR) was used to assess the relative expression level of miR-203 and DKK1 mRNA with the 7500 Fast PCR instrument (Applied Biosystems) and were calculated by evaluating the threshold cycle number (Ct).

Western blotting. The protein to be detected was obtained from lysed cells through the action of RIPA lysis buffer (Solarbio, Beijing, China). A BCA kit (Solarbio) was used to detect the protein concentration. SDS-PAGE gel (10%) was made for electrophoreses after protein specimens were added, and then the gel was transferred to a polyvinylidene fluoride (PVDF) membrane (Invitrogen) with a sandwich structure. Skimmed milk powder was used as a blocking agent to block the nonspecific binding site on the PVDF membrane for 2 h at room temperature. Subsequently, anti-human DKK1 rabbit polyclonal antibody at a 1:1,000 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was incubated overnight at 4°C. Then, the PVDF membrane was washed 4 times with phosphate-buffered saline with 0.1% Tween-20 (PBST) (10 min/time). Then, goat anti-rabbit secondary antibody at a dilution of 1:10,000 (Santa Cruz Biotechnology, Inc.) was incubated for 2 h at room temperature. After antibody incubation and washing, ECL substrate was used to detect immunoreactive protein by a chemical luminescence imaging system (FluorChen E; ProteinSimple, San Jose, CA, USA). Quantified data were analyzed using IPP image analysis software. β-actin (Beijing Dingguo Changsheng Biotechnology Co., Ltd.) was used as an endogenous protein.

Cell culture. The human lung adenocarcinoma cell lines A549 and H460, and the NHBE cell line were purchased from the Cell Bank of Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences. All the cells were cultured in an incubator (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at constant temperature of 37°C in 5% CO2 in a water-saturated atmosphere. The cell culture medium was Dulbecco’s modified Eagle’s medium (DMEM) high glucose medium with 10% fetal bovine serum (FBS) (both from Gibco Life Technologies, Carlsbad, CA, USA), and double antibody concentration of penicillin (100 U/ml) and streptomycin (100 µg/ml).

Dual luciferase reporter assay. Dual luciferase reporter gene assay was used to verify whether miR-203 targets DKK1. First, point mutations at the seed region of the DKK1 3’ untranslated region (3’UTR) were established. These had complementary base pairing between miR-203 and the DKK1 mRNA 3’UTR sequence. Then, overlap PCR was used to collect mutant copies of the DKK1 3’UTR (Fig. 2A). Wild-type DKK1 3’UTR was amplified from human genomic DNA. Secondly, recombinant reporter gene vectors pmirGLO-wt-3’UTR DKK1 and pmirGLO-mut-3’UTR DKK1 were constructed (the combined vectors were purchased from Shanghai Biological Technology Co., Ltd.), and then co-transfected with scramble or miR-203 mimics (Shanghai GenePharma Co., Ltd.) and then co-transfected with scramble or miR-203 mimics (Shanghai GenePharma Co., Ltd., Shanghai, China) into A549 and H460 cells, respectively, using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's recommendations. The confluence of the cells was 70-80%. The luciferase activity of the cells was determined using a luciferase assay kit (Promega, Madison, WI, USA) 24 h after transfection.

Lentivirus transfection. Pre-miR-203 and negative control lentivirus vectors were constructed and packaged by Shanghai GenePharma Co., Ltd. and transfected into A549 and H460 cells into 6-well plates, respectively. After transfection, the cells demonstrated puromycin resistance, and different
Concentrations of puromycin (Sigma-Aldrich, St. Louis, MO, USA) were used to select transfected and stably expressed cells 48 h after transfection. We finally used puromycin at the concentration of 2 µg/ml for 14 days to select the stably transfected cells. qRT-PCR was used to verify the expression of miR-203 in the three groups (miR-203, NC and blank).

CCK-8 assay. A Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) assay was performed to examine cell growth after transfection in the three groups in accordance with the manufacturer's recommendations. After 24 h of treatment with different concentrations of DDP ranging from 4 to 20 µM, absorbance of the cells was measured using a fluorescence microplate reader, representing the proliferative activity of the cells. The inhibition rate was calculated using the following formula: Inhibition rate = (control group - experimental group)/control group x 100%. IC50 is the half inhibitory rate, and stands for the concentration of DDP when the inhibition rate is 50%.

Cell apoptosis assays. The apoptosis of A549 and H460 cells was measured by flow cytometry (BD Biosciences, San Diego, CA, USA) using an Annexin V-FITC/propidium iodide (PI) staining assay. Each group of cells (miR-203, NC and blank) was divided into two subgroups, one treated with 4 µM DDP and the other not treated with DDP. Apoptotic cells were calculated by gating PI- and Annexin V-positive cells using flow cytometry with fluorescence-activated cell-sorting (FACS). The activity of caspase-3/-7 in each subgroup was determined using a caspase-3/-7 kit (Promega).

Animal experiment. Mice were bred in a temperature-controlled sterile environment with a 12-h light and dark cycle. Twenty BALB/c nude mice (female, 4-6 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Center (Beijing, China). Two groups of cells (miR-203, NC and blank) were subcutaneously injected under the left forelegs of the mice which were randomly distributed (n=10/group). In each group, half of the mice randomly received DDP (2 mg/kg in 0.2 ml of normal saline for 5 days) by intraperitoneal injection one week after implantation, and the others received 0.2 ml of normal saline alone. Thus, 4 groups were generated and the growth conditions of the xenografts were measured and recorded using an in vivo small animal imaging instrument every week for a total of 4 weeks, represented by the luciferase signals. Then, the mice were sacrificed by cervical dislocation. Each tumor was extracted and weighed.

siRNA transfection. siRNA-DKK1 was purchased from Shanghai GenePharma Co., Ltd. DKK1 lacking the 3'UTR (pcDNA3.1-DKK1 vector) was constructed by Shanghai Biological Technology Co., Ltd. siRNA-DKK1, miR-203 mimics, scramble and pcDNA3.1-DKK1 were transfected into A549/H460 cells alone or the combination using Lipofectamine™ 2000 in accordance with the manufacturer's recommendations. The samples were divided into corresponding groups and CCK-8 assays (IC50) were performed to assess the effects of miR-203 and DKK1 on DDP sensitivity in the A549/H460 cell lines.

Statistical analysis. Data were analyzed using SPSS 21 software. Data showing a normal distribution are expressed as mean ± standard deviation (SD). One-way ANOVA was used for comparison across three or more groups; a two-tailed unpaired Student's t-test was used to compare measurement data from pairs of independent samples. Pearson correlation analysis was used to evaluate the correlation of two groups. P<0.05 was considered to indicate a statistically significant result.

Results

Expression of DKK1 mRNA and miR-203 in cisplatin-sensitive and -insensitive lung adenocarcinoma tissues. qRT-PCR was used to examine the expression levels of DKK1 mRNA and miR-203 in lung cancer tissues from 30 patients with advanced-stage lung adenocarcinoma; the clinical characteristics of the patients are shown in Table I. The results showed that in DDP-sensitive NSCLC samples, miR-203 was expressed at a higher level when compared with the level noted in the DDP-insensitive samples (P<0.05; Fig. 1B), while DKK1 mRNA was expressed at a lower level in the DDP-sensitive NSCLC samples (P<0.05; Fig. 1A). In addition, statistical analysis revealed that expression of DKK1 mRNA was negatively correlated with miR-203 (P<0.05; Fig. 1C; R²=0.532). The different levels of expression of miR-203 and DKK1 in the DDP-sensitive and -insensitive samples suggest that miR-203 is associated with the mechanism of cisplatin resistance.

Confirmation of DKK1 as a target gene of miR-203 in A549 and H460 cells. In the present study, DKK1 was predicted to
be a target gene of miR-203 by searching target gene prediction databases (microRNA.org, miRDB and TargetScan). A dual luciferase reporter gene assay was used to determine whether miR-203 targets DKK1. Western blot analysis showed that there was lower DKK1 expression in the miR-203 group than that in the blank and scramble groups of the A549 and H460 cells (P<0.05; Fig. 2B), which suggests that upregulation of miR-203 may lower the expression level of DKK1 protein. Under a dual reporter gene assay, the relative luciferase activity showed a nearly 1-fold decrease in wild-type DKK1 with upregulated miR-203. However, the relative luciferase activity showed no significant difference in the group with mutant DKK1 with upregulated miR-203 (P>0.05; Fig. 2C). The results were observed both in the A549 and H460 cells, which indicate that miR-203 may downregulate the expression level of DKK1 by binding to the 3'UTR of DKK1 mRNA.

**Upregulation of miR-203 increases cisplatin sensitivity of A549 and H460 cells.** qRT-PCR was used to examine the relative expression of miR-203 in the different groups after A549 and H460 cells were transfected with the lentiviral vectors. Cells in the group transfected with miR-203 revealed a ~7- to 8-fold higher expression of miR-203 when compared with the level in the blank and NC groups (Fig. 3A and B). Following treatment with different concentrations of DDP, ranging from 4 to 20 µM, the cells with miR-203 overexpression demonstrated a significant inhibition of cell growth not observed in the blank and NC groups (P<0.05). Inhibition of cell growth became more extensive upon increases in the concentration of DDP (Fig. 3C and D). Furthermore, the IC_{50} value for the miR-203 group was significantly lower than that in the other two groups (P<0.05; Fig. 3E and F). IC_{50} reflects the sensitivity of DDP in lung cancer cells. These results indicate that upregulation of miR-203 increased the sensitivity of lung adenocarcinoma A549 and H460 cells to DDP.

**Upregulation of miR-203 increases the apoptosis of A549 and H460 cells.** Cell apoptosis was detected using flow cytometry, and caspase-3/-7 activity was measured using a caspase-3/-7 kit. The results indicated that overexpression of miR-203 significantly increased cell apoptosis of the A549 and H460 cells with and without DDP treatment (P<0.05; Fig. 4A and B). Similar results were observed when the activity of caspase-3/-7 was examined (P<0.05; Fig. 4C and D). The increase in caspase-3/-7 activity was more significant following treatment with DDP. Upregulation of miR-203 was found to increase the
Figure 3. Upregulation of miR-203 increases the cisplatin sensitivity (DDP) of A549 and H460 cells. (A and B) The expression level of miR-203 was 7- to 8-fold higher in the A549 and H460 cells after miR-203 lentivirus vector transfection. (C and D) Following treatment with different concentrations of DDP ranging from 4 to 20 µM, the cells with miR-203 overexpression demonstrated significant inhibition of cell growth compared with the blank and NC groups. (E and F) Cells with miR-203 overexpression had a significantly lower IC₅₀ than the other two groups.

Figure 4. Upregulation of miR-203 was found to increase apoptosis of the A549 and H460 cells. (A and B) Cell apoptosis was detected using flow cytometry. Overexpression of miR-203 was found to significantly increase cell apoptosis in the A549 and H460 cell lines with and without cisplatin (DDP) treatment. (C and D) Upregulation of miR-203 increased the activity of caspase-3/-7 in the A549 and H460 cells.
activity of caspase-3/-7 and promote apoptosis in the A549 and H460 cells.

**Upregulation of miR-203 inhibits tumor growth of the nude mouse tumor xenografts.** A nude mouse tumor xenograft model was established to study the effects of miR-203 on tumor growth. The luciferase signal detected by in vivo small animal imaging was in the present study considered representative of tumor growth. The tumor growth curve (Fig. 5A) clearly showed that overexpression of miR-203 inhibited the tumor growth, and this function was also observed under the treatment with DDP. Over time, the tumor inhibition effect of miR-203 and miR-203 combined with DDP was more significant. Moreover, tumors with upregulated miR-203 were more sensitive to DDP and showed a more pronounced decline in tumor weight (P<0.05; Fig. 5B). In this way, upregulation of miR-203 inhibits tumor growth and increases the sensitivity to DDP of the nude mouse tumor xenograft.

**Upregulation of miR-203 sensitizes A549 and H460 cells to DDP through DKK1.** A great deal is known concerning the effects of miR-203 on the inhibition of cell growth and DDP sensitization. More experiments were designed to determine how miR-203 exerts its function. Both overexpression of miR-203 and knockdown of DKK1 resulted in the sensitization to DDP with a lower IC_{50}. In this way, upregulation of miR-203 inhibits tumor growth and increases the sensitivity to DDP of the nude mouse tumor xenograft.
overexpression of miR-203 had no added effects on the sensitivity of the cells (P<0.05, Fig. 6A and C). Overexpression of miR-203 increased the sensitivity of the A549 and H460 cells to DDP compared to the control group, while DKK1 lacking 3'UTR decreased the sensitivity (P<0.05). In addition, miR-203 was not found to sensitize cells to DKK1 lacking the 3'UTR (P>0.05; Fig. 6B and D). In summary, miR-203 was found to exert the function of sensitization to DDP exclusively by targeting the 3'UTR of DKK1.

Discussion

In the present study, miR-203 and DKK1 were differentially expressed in DDP-sensitive and -insensitive lung adenocarcinoma tissues, and a negative correlation was observed between them, suggesting that miR-203 and DKK1 may predict DDP sensitivity. Cell and animal experiments were designed to further investigate the effects of miR-203 and DKK1 on cell proliferation, apoptosis and xenograft growth and the relationship between miR-203 and DKK1. Overexpression of miR-203 was found to inhibit the growth of A549/H460 cells, and also to increase the activity of caspase-3/-7 and promote cell apoptosis, which were considered to contribute to sensitize the response of lung cancer cells to DDP. Dual luciferase reporter assay verified DKK1 as a target gene of miR-203, which was also identified by restore assay. Restore assay showed that miR-203 was not able to sensitize cells with DKK1 lacking the 3'UTR. miR-203 could bind to the 3'UTR of DKK1 and then regulate the characteristics of lung cancer cells.

The present study indicates that DKK1 may participate in the mechanism underlying cisplatin resistance, which is consistent with the findings of previous studies (23-27). Since platinum-based chemotherapy is still the main strategy for treating advanced lung cancer, research into drug resistance is ongoing. However, the mechanism underlying the action of DDP is quite complex and may involve many factors and signaling pathways. More and more evidence has shown that abnormal expression of miRNAs, such as miR-184 (30), miR-1244 (31), miR-196a (32) and miR-141 (33), may be related to DDP expression of miRNAs, such as miR-184 (30), miR-1244 (31), miR-203 increased the cisplatin sensitivity of A549/H460 cell lines by targeting the 3'UTR of DKK1. Whereas miRNAs have complicated interactions with various mRNA targets, and the mechanisms underlying the regulation of cisplatin resistance are intricate and complex, further fundamental research is needed to be conducted before clinical applications become practical.

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References


