Abstract. The present study aimed to investigate the association between microRNA-152 and cisplatin resistance in non-small cell lung cancer. A549 and cisplatin-resistant A549 cells (A549/cis) were maintained in vitro. Reverse transcription-quantitative PCR (RT-qPCR) was performed to analyze differences in microRNA-152 levels between A549 and A549/cis cells, and changes in Bcl-2 and NF-κB expression levels were analyzed via RT-qPCR and western blot analyses. MicroRNA-152 was overexpressed in A549/cis cells via transfection of a microRNA-152 mimic. Upon treating transfected or untransfected A549/cis cells with 2 µg/ml cisplatin for 24 h, a Cell Counting Kit-8 assay, morphological analysis and flow cytometry analysis were performed to evaluate the effect of microRNA-152 on the inhibition of cell proliferation and induction of apoptosis. Furthermore, changes in Bcl-2 and NF-κB expression levels in microRNA-152-overexpressing A549/cis cells were also analyzed. MicroRNA-152 was significantly downregulated and Bcl-2 and NF-κB were significantly upregulated in A549/cis cells (P<0.05). MicroRNA-152 upregulation enhanced the inhibitory effect of cisplatin on A549/cis cells. These results suggest that microRNA-152 downregulates Bcl-2 and NF-κB. MicroRNA-152 downregulation may induce cisplatin resistance in non-small cell lung cancer cells, whereas microRNA-152 upregulation may improve cisplatin sensitivity among A549/cis cells via downregulation of Bcl-2 and NF-κB.

Introduction

In the majority of patients with non-small cell lung cancer (NSCLC), stage progression occurs before the cancer is diagnosed, thereby delaying the patient receiving radical surgery (1). In such cases, non-invasive treatment methods are required (2). Epidermal growth factor receptor (EGFR) mutations in patients with positive NSCLC are the preferred therapeutic targets (3); however, in those not harboring the EGFR mutation, NSCLC treatment methods are limited; thus, chemotherapy is preferred in these patients (4,5). Platinum-based chemotherapy is extensively used, and the efficacy of cisplatin therapy has been demonstrated (6). However, resistance to platinum-based drugs in patients with NSCLC cannot be disregarded; thus, it is important to understand the molecular mechanism underlying platinum-based pharmacotherapeutic resistance among patients with NSCLC (7).

MicroRNAs are small non-coding RNAs that regulate various cellular processes via binding to the 3'-untranslated region (3'-UTR) of target mRNAs, thus degrading mRNAs or inhibiting their translation (8). The role of post-transcriptional regulation by microRNAs has attracted increasing attention. Numerous studies have reported that microRNA expression is associated with tumorigenesis and progression, as well as chemotherapeutic resistance (9,10), and that microRNA-152, specifically, is downregulated in patients with NSCLC (11,12). However, to the best of our knowledge, few studies have investigated the association between microRNA-152 expression and chemotherapeutic resistance to platinum-based drugs in NSCLC. Therefore, the present study aimed to investigate the changes in microRNA-152 expression in cisplatin-resistant A549 cells and its effects on cisplatin sensitivity.

Materials and methods

Cells and cell culture. The A549 cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences. The cisplatin-resistant A549 (A549/cis) cell line was obtained from the Cell Culture Center. Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). All cells were maintained in a humidified incubator at 37°C and 5% CO₂.

Transient transfection. Once the A549/cis cells reached 80% confluence, they were transfected with microRNA-152 mimics or unrelated mimics (negative control), using Lipofectamine®
GAPDH was considered an endogenous control. ECL substrates (Pierce; Thermo Fisher Scientific, Inc.) were used: Forward, 5′-UCAGUGCAUGACAGA ACUUGG-3′; reverse, 5′-AGUUCUGUCAUGCAGU AUU-3′. The working concentration of microRNA-152 mimics was 50 nM, the concentration of unrelated microRNA-152 mimics was the same as that of microRNA-152 mimics. To avoid the influence of cis to A549/cis cells, cells (1x10^5 cells/ml) were cultured in a drug-free medium for at least 2 weeks, and then transfected. Subsequent experiments were conducted 48 h later.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA of A549 cells and A549/cis cells was isolated using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. MicroRNA was isolated using the mirVana miRNA Isolation kit (Ambion; Thermo Fisher Scientific, Inc.). MicroRNA-152 expression was detected using TaqMan MicroRNA Assay primers (Applied Biosystems; Thermo Fisher Scientific, Inc.; http://www.targetscan.org/). The thermocycling conditions are as follows: Initial activation of Taq polymerase at 95°C for 10 min, 40 cycles of PCR amplification at 95°C for 15 sec, 55°C for annealing/extension, and 4°C for cooling. The primers were: Forward, 5′-UCA GUG CAU GAC AGA-3′; reverse, 5′-AGUUGAUCACAGUCAGA AUG-3′. The working concentration of microRNA-152 mimics was 50 nM, the concentration of unrelated microRNA-152 mimics was the same as that of microRNA-152 mimics. To avoid the influence of cis to A549/cis cells, cells (1x10^5 cells/ml) were cultured in a drug-free medium for at least 2 weeks, and then transfected. Subsequent experiments were conducted 48 h later.

Flow cytometry. Apoptosis was assessed via flow cytometry. Briefly, six groups as aforementioned, cells were cultured in 24-well plates at a density of 1x10^5 cells/well, and then trypsinized, harvested, washed and stained with Annexin V-fluorescein isothiocyanate and propidium iodide (PI) for 15 min at 4°C using the apoptosis detection kit (BD Biosciences). The proportion of cells at each stage of the cell cycle was analyzed in each cell group by Cell Quest software version 5.1 (Becton, Dickinson and Company). After 24 h of treatment, 500 µl of PI was added in each group for 15 min at room temperature to stain the nuclei, and cell cycle analysis was performed using a FACstar Plus cytometer (Becton, Dickinson and Company).

Cell proliferation assay. A549/cis cells were divided into 6 groups: Cells without cisplatin treatment and transfection (untreated group); cells without cisplatin treatment transfected with unrelated microRNA mimics (miR control group); cells without cisplatin treatment transfected with microRNA mimics (miR mimics group); untransfected cells with cisplatin treatment (cis group); cells with cisplatin treatment transfected with unrelated microRNA mimics (cis+miR control group); cells with cisplatin treatment transfected with microRNA mimics (cis+miR mimics group). Cells were treated with 10 µl CCK-8 reagent (Abmole Bioscience, Inc.) 24 h after transfection and incubated at 37°C for 1.5 h. The absorbance was measured at 450 nM, using a microtiter plate reader. Meanwhile, cell viability was measured. Cell viability (%) was calculated as follows: (OD sample-OD blank)/(OD control- OD blank) x100. The half maximal inhibitory concentration (IC50) was also calculated. Morphological changes and apoptosis in cells were assessed using a terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay. Briefly, the cells were seeded at a concentration of 1x10^5 cells/ml in 24-well plates and incubated for 24 h. Cells were then washed with ice-cold PBS three times for 5 min and fixed with 4% paraformaldehyde at room temperature for 1 h, followed with acetic acid/ethanol (1:3) for post-fixation -20°C for 5 min. The TUNEL assay was performed using the In Situ Cell Death Detection kit (Roche Applied Science), according to the manufacturer's protocol. Cell nuclei were stained with DAPI (Beyotime Institute of Biotechnology) at 25°C for 10 min. Glycerinum (Beyotime Institute of Biotechnology) was used to mount the slides. TUNEL-positive nuclei were defined as those with dark green fluorescent staining and were identified via fluorescence microscopy. To quantify TUNEL-positive cells, the number of green fluorescence-positive cells was counted in 4-6 random fields at x200 magnification. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole at 25°C for 10 min (Beyotime Institute of Biotechnology). The experiments were repeated 3 times.

Western blot analysis. Mouse anti Bcl-2 (cat. no., B9804; dilution, 1:1,000), NF-κB (cat. no., N8523; dilution, 1:1,000), GAPDH (cat. no., G9295; dilution, 1:3,500) were purchased from Sigma-Aldrich (Merck KGaA), the secondary antibody Goat Anti-Mouse IgG (H+L) (cat. no., SA00001-1; dilution, 1:8,000) was purchased from ProteinTech Group, Inc. Cultured A549 cells, A549/cis cells, A549/cis cells transfected with microRNA-152 mimics, and A549/cis cells transfected with unrelated microRNA-152 mimics were lysed in RIPA buffer with 1% phenylmethylsulfonyl fluoride; the latter two were cultured for 48 h after transfection. Total protein was determined using BCA method. Extracted proteins were loaded 50 µg and separated via SDS-PAGE and electro-transferred onto a PVDF membrane. The blocking reagent was 5% skim milk, then the PVDF membrane was incubated overnight at 4°C in freshly prepared TBST. The blots were probed with primary antibodies at 4°C overnight and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Signals were visualized using ECL substrates (Pierce; Thermo Fisher Scientific, Inc.). GAPDH was considered an endogenous control.

Student's t-test was used for comparison between two groups. One-way analysis of variance was used for comparisons between multiple groups, followed by the Dunnett's method as a post hoc test, using SPSS software (version 21.0; IBM Corp.) P<0.05 was considered to indicate a statistically significant result.
Results

Expression of microRNA-152, Bcl-2, and NF-κB in A549/cis cells. After 48 h of incubation with cisplatin, the IC$_{50}$ of A549 cells and A549/cis cells was 3.128±0.12 µg/ml and 14.107±0.35 µg/ml, respectively, which was significantly different (P<0.05). The resistance index was approximately 4.51 (Fig. 1A). MicroRNA-152 was significantly downregulated (P<0.05) in A549/cis cells compared with that in A549 cells (Fig. 1B). RT-qPCR and western blotting revealed that Bcl-2 and NF-κB were significantly upregulated in A549/cis cells compared with that in A549 cells (all P<0.05; Fig. 1C-F). Further analysis revealed that these improvements were 1.53±0.21-fold (Bcl-2) (Fig. 1C) and 1.37±0.13-fold (NF-κB) (Fig. 1D).

MicroRNA-152 increases cisplatin sensitivity in A549/cis cells. In order to verify the transfection efficiency, unrelated microRNA-152 mimics (negative control) and microRNA-152 mimics were transfected into the A549/cis cells. Cells transfected with the microRNA-152 mimics exhibited significantly increased levels of microRNA-152 expression compared with untreated cells and cells transfected with the miR control (P<0.05; Fig. 2A). In order to further determine the role of microRNA-152 in chemotherapeutic resistance in NSCLC, A549/cis cells were transfected with microRNA-152, and proliferation was assessed using a CCK-8 assay in the present study. Cell inhibition rates of miR control, miR mimics, cis, cis+miR control and cis+miR mimics groups were 3.8±1.3, 4.4±0.9, 4.8±1.1, 14.5±2.2, 13.2±1.9 and 22.3±2.1%, respectively (Fig. 2B). The inhibition rate of the cis+miR mimic group was significantly greater than that of cis and cis+miR control groups (both P<0.05; Fig. 4).

MicroRNA-152 downregulates Bcl-2 and NF-κB in A549/cis cells. In order to determine the effect of microRNA-152 on Bcl-2 and NF-κB expression in A549/cis cells, microRNA-152 mimics were transfected into A549/cis cells. Consequently, Bcl-2 and NF-κB were significantly downregulated, as demonstrated by the results of the RT-qPCR and western blot analyses (all P<0.05; Fig. 5).

Discussion

Systemic chemotherapy helps to comprehensively treat lung cancer; however, chemotherapeutic resistance among tumors started to form granules, and diffuse fluorescence was observed in the nucleus and cytoplasm of cells, leading to the formation of apoptotic bodies (Fig. 2C).
often results in chemotherapeutic failure, and is the primary reason underlying tumor recurrence and metastasis. Cisplatin is a first-line chemotherapeutic drug for NSCLC. However, cisplatin resistance is one of the primary obstacles to its curative effect (14-16); thus, it is important to overcome issues including chemotherapeutic and intervention resistance in tumor cells.

The molecular mechanism underlying cellular cisplatin resistance is complex, being primarily involved in drug transport, drug detoxification, apoptosis and a number of other aspects, with the following common effects: First, molecular pumps on the cell membrane are aberrantly expressed and cause the efflux of the majority of drugs, thereby decreasing the intracellular drug concentration. Resistance can be induced by overexpression of P-gp on the cell membrane, for example, encoded by multi-drug resistance (MDR1), a member of the multidrug resistance gene MDR family, an energy-dependent molecular pump that actively transports cisplatin or other chemotherapeutic drugs outside the cell, thus decreasing the intracellular drug concentration or its redistribution in tumor cells (17). Secondly, the effect of drug detoxification in cells is enhanced; accordingly, drug metabolism and excretion are increased, potentially accompanied by a decrease in drug toxicity, e.g., drug resistance caused by aberrant expression of glutathione S-transferase PI family members, which can increase drug polarity, eliminate drug metabolites, prevent DNA binding of drugs in tumor cells, and catalyze the interaction of glutathione and electrophiles, thereby eliminating reactive oxygen species-mediated injury resulting from chemotherapeutic drugs, accompanied by prevention of lipid oxidation on the cell membrane (18). Thirdly, abnormalities in the DNA repair capacity, e.g., mutation in topoisomerase TOPOII, can result in cisplatin resistance (19). Finally, aberrant activity of apoptosis regulators may cause resistance (20). Cisplatin may enhance apoptosis via certain apoptotic regulators, thereby potentially suppressing the efficacy of cisplatin treatment upon aberrant expression (21).

Recently, numerous studies on different types of tumor have focused on the association between microRNA and tumorigenesis, tumor progression and chemotherapeutic resistance (22-24). MicroRNAs are a class of small non-coding RNAs of ~21-25 nucleotides in length, which suppress target mRNAs at the post-transcriptional level, thereby regulating cell proliferation and apoptosis (25-27).
Accumulating evidence has indicated a close association between microRNA and tumors, which may affect tumor progression, differentiation, metastasis and chemotherapeutic resistance (28). Furthermore, chemotherapeutic resistance is a bottleneck issue warranting urgent resolution in clinical oncology, and potential microRNA targets to reverse chemotherapeutic resistance need to be identified. Upregulation of microRNA-200c reportedly increased the chemosensitivity of breast cancer cells to epirubicin (29), and overexpression of microRNA-1915 reversed multidrug resistance in colon cancer cells (30). By contrast, downregulation of microRNA-93 enhanced apoptosis in cisplatin-resistant ovarian cancer cells (31), and inhibition of microRNA-328 also induced cellular apoptosis and decreased cell proliferation in A549/cis cells treated with cisplatin (32). There have been a number of studies that focus microRNA-152 and cancer in recent years (33,34), which have demonstrated that microRNA-148a and microRNA-152 decrease tamoxifen resistance in estrogen receptor-positive breast cancer via downregulating activated leukocyte cell adhesion molecule. MicroRNA-152 has been demonstrated to function as a tumor suppressor in cervical cancer (35).

In the present study, microRNA-152 was downregulated in cisplatin-resistant A549/cis cells compared with in non-resistant A549 cells; hence, it may be concluded that microRNA-152 downregulation suppressed apoptosis in tumor cells, which is not conducive to the treatment of the tumor. In addition, these data are concurrent with previous reports, for instance, microRNA-152 reportedly inhibited the growth and invasiveness of NSCLC, while microRNA-152 downregulation increased tumor cell proliferation (12).

Thus, the present study preliminarily reports an association between cisplatin resistance and microRNA expression in NSCLC. Although numerous studies have reported that
microRNA-152 is downregulated in tumors and serves as a tumor suppressor (36,37), the association between microRNA-152 and chemotherapeutic resistance has been unclear. In the present study, microRNA-152 levels were lower in A549 cells than in A549/cis cells; thus, A549/cis cells exhibited adequate cisplatin resistance. However, following transfection of microRNA-152 mimics into A549/cis cells, the susceptibility of these cells to cisplatin improved. Therefore, it can be speculated that upregulation of intracellular microRNA-152 resulted from transfection of microRNA-152 mimics into A549/cis cells, which triggered corresponding signaling pathways, thereby enhancing cisplatin resistance in cells. This was further confirmed via TUNEL staining. Following transfection of mimics and cisplatin treatment, dense granular fluorescence was observed in the nucleus or cytoplasm of A549/cis cells. Furthermore, Annexin V-FITC/PI double staining flow cytometric analysis was performed in order to determine the apoptotic rate of cultured cells in vitro. Flow cytometric analysis revealed that the apoptotic rate of group cis+mimics significantly increased, suggesting that transfection of microRNAs into A549/cis cells increased their sensitivity to cisplatin.

However, it is currently unclear which signaling pathway is involved in the underlying molecular mechanisms. Hence, the present study analyzed the levels of Bcl-2 and NF-κB in different cells. Bcl-2 and NF-κB are well-known anti-apoptotic proteins. Numerous studies have reported that Bcl-2 and NF-κB are upregulated in various different types of tumor, both exerting significant anti-apoptotic effects in the proliferation of malignant tumor cells and are closely associated with tumor cell invasiveness and metastasis, including NSCLC, gastric cancer, ovarian cancer, and colon cancer (38,39). Hall et al (40) reported that Bcl-2 serves as a potential chemotherapeutic target in tumors of the reproductive system, with Bcl-2 upregulation suggesting a poor prognosis. Sun et al (41) reported that NF-κB was upregulated in lung adenocarcinoma A549 cells, and it suppressed the chemotherapeutic sensitivity of A549 cells. In the present study, microRNA-152 upregulation suppressed Bcl-2 and NF-κB and weakened their anti-apoptotic effects, thereby providing a potential explanation behind the improvement in the chemotherapeutic sensitivity of A549 cells to cisplatin.

Figure 5. MicroRNA-152 downregulated Bcl-2 and NF-κB in A549/cis cells. (A) A549/cis cells were transfected with microRNA-152 mimics, and Bcl-2 mRNA levels were detected via RT-qPCR analysis. (B) A549/cis cells were transfected with microRNA-152 mimics, and NF-κB mRNA levels were determined via RT-qPCR analysis. (C) Western blotting. Changes in Bcl-2 and NF-κB protein expression levels in A549/cis cells induced by microRNA-152 mimics. A549/cis cells, cisplatin-resistant A549 cells; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control.
However, further studies are required in order to determine whether Bcl-2 and NF-κB are direct targets of microRNA-152 or are simply components of the microRNA-152 regulatory machinery.

To further understand apoptosis in A549/cis cells transfected with microRNA-152, PI staining was performed in order to analyze the effect of microRNA-152 on the cell cycle distribution. Consequently, cells were arrested in the G0/G1 phase of the cell cycle. However, whether Bcl-2 and NF-κB are direct target genes of microRNA-152 or downstream of microRNA-152 regulatory pathways needs to be further confirmed.

The present study revealed that microRNA-152 is downregulated in A549/cis cells, with concomitant upregulation of Bcl-2 and NF-κB. Overexpression of microRNA-152 strengthened the inhibitory effects on cell proliferation and increased the apoptotic rate, accompanied by significant downregulation of Bcl-2 and NF-κB in A549/cis cells. The present results indicate that microRNA-152 upregulation may decrease cisplatin resistance in NSCLC, its effects potentially mediated via regulation of Bcl-2 and NF-κB signal transduction.

Acknowledgements

The authors would like to thank Dr Bing Bai (Department of Endocrine, The Fifth Affiliated Hospital of Zhengzhou University) and Dr Youcai Tang (Laboratory of Rehabilitation Medicine, The Fifth Affiliated Hospital of Zhengzhou University) for coordinating the project and technical assistance throughout the course of this work.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

WZ and HL conceived and designed the study. WZ, SY and DG acquired the data. WZ, JC and SM analyzed the data. WZ, WL, SY, DG, JC, SM, YX and ML contributed to the interpretation of the data. WZ and HL wrote and revised the paper. YX and ML provided administrative, technical, or material support. WZ and HL supervised the study.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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