miR-133a enhances the sensitivity of Hep-2 cells and vincristine-resistant Hep-2v cells to cisplatin by downregulating ATP7B expression

XURUI WANG1,2, WEI ZHU1, XIAODONG ZHAO2 and PING WANG1

1Department of Otolaryngology-Head and Neck Surgery, The First Hospital of Jilin University, Changchun, Jilin 130021; 2Department of Otorhinolaryngology, Jilin Province Cancer Hospital, Changchun, Jilin 130012, P.R. China

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Abstract. The expression levels of the copper transporter P-type adenosine triphosphatase (ATP7B) are known correlate with tumor cell sensitivity to cisplatin. However, the mechanisms underlying cisplatin resistance remained poorly understood. Therefore, in the present study, we treated Hep-2 cells and in-house-developed vincristine-resistant Hep-2v cells with 50, 100, or 200 µM cisplatin and assessed cell viability after 24 or 48 h. Hep-2v cells were shown to be resistant to 50-200 µM cisplatin. Furthermore, using immunofluorescence staining and western blot analysis, we noted that ATP7B, but not copper-transporting ATPase 1 (ATP7A), expression was significantly increased in Hep-2v cells, and this increase was maintained at a higher level compared with Hep-2 cells. As ATP7B is a target of microRNA 133a (miR-133a), the ability of miR-133a to influence cisplatin sensitivity in Hep-2v cells was then assessed by CCK-8 assay. We noted that miR-133a expression was lower in both Hep-2 and Hep-2v cells compared with epithelial NP69 cells. Following treatment with 50 µM cisplatin, in Hep-2v cells expressing exogenous miR-133a we noted reduced ATP7B expression, and these cells had a significantly lower survival rate compared with the control. The present study demonstrates that miR-133a enhances the sensitivity of multidrug-resistant Hep-2v cells to cisplatin by downregulating ATP7B expression.

Introduction

Multidrug resistance is a major cause of cancer chemotherapy failure, and reversing this resistance is an emerging area of interest in cancer treatment. MicroRNAs (miRNAs or miRs) have the potential to act as mediators of multidrug resistance reversal. miRNAs are a class of small non-coding RNAs of about 19-22 nucleotides that play an important role in cell occurrence, development, differentiation, proliferation, aging and apoptosis (1). miRNAs are evolutionarily conserved and are located in the introns or exons of protein-coding genes, or in intergenic regions. Mature miRNAs regulate gene expression by inhibiting protein translation and promoting mRNA degradation.

Differential expression of miRNAs has been described in multiple cancer cell types, and miRNAs commonly function as either tumor suppressors or oncogenes (2,3). The expression of miR-133a is low in the majority of types of tumor cells, and particularly low in squamous cell carcinoma of the maxillary sinus, renal cell carcinoma, and rhabdomyosarcoma (4-6). High miR-133a expression induces the death of multiple cancer cells, including squamous cell carcinomas of the lungs (7), tongue (8) and esophagus (9), as well as prostate carcinoma (10) and bladder carcinoma (11). In squamous cell carcinoma of the head and neck, increased miR-133a expression has been shown to exert anticancer effects through downregulation of the scaffolding protein caveolin-1 (12).

The expression of copper transporting P-type adenosine triphosphatase (ATP7B) has previously been implicated in cisplatin resistance (13). ATP7B transports copper to the Golgi complex to promote the synthesis of copper-containing enzymes, or it mediates the direct elimination of copper from cells. Furukawa et al and others (14,15) have noted that the presence of ATP7B in the Golgi complex is associated with cisplatin resistance and that copper transport is implicated in multidrug resistance. Others have reported that ATP7B-positive esophageal and oral carcinoma patients had poorer outcomes and a noticeably lower long-term survival rate following chemotherapy with cisplatin-containing drugs when compared with patients with ATP7B-negative lesions (16). Nakayama et al (17) reported that ATP7B-transfected KB/WD cells exhibited reduced accumulation of cisplatin compared with KB/CV control cells. Additionally, a cisplatin-resistant oral squamous cell carcinoma cell line established by cultivation of cells in gradually increasing concentrations of cisplatin demonstrated high levels of ATP7B and copper-transporting ATPase 1 (ATP7A) expression (18,19).
In the present study, we report on ATP7B expression in the cell line Hep-2 and the vincristine-resistant cell line Hep-2v. We investigated the influence of exogenous miR-133a on ATP7B expression in both cell lines, and correlated ATP7B expression with sensitivity to cisplatin chemotherapy in Hep-2 cells.

Materials and methods

Cell lines and reagents. The Hep-2 and NP69 cell lines were purchased from Guangzhou Jinibio Biotechnology Co., Ltd. (Guangdong, China). The vincristine-resistant 1 Hep-2v cells were developed in our laboratory (Institute of Otolaryngology-Head and Neck Surgery, Changchun, China). RPMI-1640, fetal calf serum, Alexa 555-labeled goat anti-rabbit IgG (A-21428), and TRIzol reagent were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Antibodies and reagents for western blot were obtained from Signalway Antibody LLC (College Park, MD, USA). The miRcute miRNA cDNA first-strand synthesis kit and miRcute miRNA isolation kits were purchased from Signalway Antibody LLC (College Park, MD, USA). The SYBR-Green I PCR kit was supplied by Roche Biochemicals (Indianapolis, IN, USA).

Cell culture. Hep-2 cells were recovered from frozen storage and sub-cultured in RPMI-1640 medium supplemented with 10% fetal calf serum at 37°C, in an atmosphere with 5% CO2, and 95% relative humidity. Culture medium was refreshed daily. When cells reached 90% confluence, they were detached with 0.25% trypsin and seeded into a new culture flask at a ratio of 1:3. The vincristine-resistant cell line Hep-2v was produced by incubation with gradually increasing concentrations of vincristine (Sigma, St. Louis, MO, USA) for 8 months, with cells developing sensitivity to cisplatin chemotherapy in Hep-2 cells.

Plasmids and transfection. pEZX-miR-133 and pEZX-control lentiviral vectors were from Guangzhou FulenGen Co., Ltd. (Guangdong, China). The sequence of mature miR-133a-1 was identified using the query hsa-miR-133a in GenBank (http://www.ncbi.nlm.nih.gov). The gene ID is MMAT0000427 and the sequences is: 5'-UUUGGUCUCUUCAACACCAGCUG-3'. Precursor miRNA expression constructs were prepared in a feline immunodeficiency virus-based lentiviral plasmid vector system (Guangzhou FulenGen Co., Ltd.). H1 RNA polymerase III promoter-driven pre-miRNA has a stem loop of 5'-ACAUGCUUGCUAGCUAGGUAAUUGAACC AAUUCGCCUCUUCAUGUUGGUCUUCCUACAC GACGCGUAGCUAGGAUGA-3' which is processed into mature miR-133a-1 by the RNAi enzyme system (Guangzhou FulenGen Co., Ltd.). The vector co-expresses reporter gene eGFP under the control of cytomegalovirus (CMV) promoter (Guangzhou FulenGen Co., Ltd.).

Hep-2v cells were detached using 0.25% trypsin and collected by centrifugation at 800 x g for 5 min at room temperature. Sediments were re-suspended in RPMI-1640 culture medium and then seeded onto a 24-well plate (2x10⁴ cells/well). Upon reaching 70-80% confluence, cells were cultured with antibiotic-free serum-containing RPMI-1640 medium overnight. For transfection, 3 µg pEZX-miR-133a plasmid was diluted in 50 µl serum-free culture medium (both self-prepared with FCS and RPMI-1640), centrifuged on a short spin, and slightly mixed. Subsequently, 3 µl GenEscort™ transfection reagent (Wisenge Biotechnology Co., Nanjing, China) was diluted in 50 µl serum-free culture medium and then lightly shaken. Transfection reagent-containing- and pEZX-miR-133a plasmid-containing solutions were then combined. The resulting mixture was lightly shaken and then incubated at room temperature for 15 min. Cells seeded onto 24-well plates were cultured with 100 µl of the resulting mixture at 37°C. Culture medium was refreshed with serum-containing complete culture medium after 4 h, and cells were further cultured for 48-72 h.

Immunofluorescence staining. After removal of the culture medium, glass coverslips with cultured cells were washed with 0.1 M phosphate-buffered saline (PBS), treated with 4% paraformaldehyde at room temperature for 30 min, washed three times with PBS for 10 min each wash, treated with 0.1% Triton X-100 for 10 min, and washed a further three times with PBS. After the addition of 5% goat serum, cells were incubated at room temperature for 1 h, and then incubated with primary antibody (ATP7A or ATP7B) at 4°C overnight. Cells were then washed three times with PBS, incubated with Alexa-555 goat anti-rabbit IgG secondary antibody (1:200 dilution) at room temperature for 1 h in the dark, and washed three times with PBS for 10 min each wash. Hoechst 33342 was used for staining. Cells were mounted in glycerol and observed by laser confocal microscopy (FluoView FV1000; Olympus, Tokyo, Japan) to determine protein expression.

CCK-8 assay. Hep-2 and Hep-2v cells were seeded onto a 96-well plate at 1x10⁴ cells/well. Cells were then treated with indicated concentrations of cisplatin for indicated time periods followed by addition of 100 µl culture medium and 10 µl/well CCK-8 solution. Cells were incubated at 37°C for 1-4 h, and optical density (OD) at 450 nm was determined. Cell viability was calculated as OD value in the treated group/OD value in the control group x 100. The control group was Hep-2v or Hep-2 cells which were not treated with cisplatin.

Reverse transcription-quantitative PCR (RT-qPCR). miRNA was isolated from Hep-2, Hep-2v, and NP69 cells using the miRcute miRNA isolation kit from Signalway Antibody LLC (College Park, MD, USA). Briefly, 5 µl total RNA, 2 µl 10X poly(A) polymerase buffer, 4 µl 5X rATP solution, 8.6 µl RNase-free ddH₂O, and 0.4 µl E. coli poly(A) polymerase were combined in an RNAse-free reaction tube, which was pre-cooled on ice. Next, 2 µl poly(A) reaction solution was used for first-strand cDNA synthesis in a 20-µl reaction containing 2 µl 10X RT primer, 2 µl 10X RT buffer, 1 µl ultrapure dNTP mixture (2.5 mM each), 1 µl RNasin (40 U/ml), 0.5 µl Quant KTaSe, and 11.5 µl RNase-free ddH₂O. After thorough mixing, the mixture was incubated at 37°C for 60 min. qPCR was performed using the miRcute miRNA fluorescent quantitative detection kit (Signalway Antibody LLC, Co., Ltd.) with an upstream primer for hsa-miR-133a, 5'-TTGGTCCCCCTCACCAG-3', and the generic down-
stream primer provided in the kit. A 20-µl reaction containing 10 µl 2X miRcute miRNA Premix, 0.4 µl hsa-miR-133a upstream primer (10 µmol/l), 0.4 µl downstream primer, 2 µl cDNA, and 7.2 µl RNase-free ddH₂O was established. qPCR involved denaturation at 94˚C for 2 min and 40 cycles of 94˚C for 30 sec and 60˚C for 45 sec. The fluorescence value for each cycle was recorded during extension at 75˚C. Relative changes in miRNA levels were calculated using 5S RNA [Tiangen Biotech (Beijing) Co., Ltd.] as an internal control.

RT-qPCR-based analysis of mRNA expression. Cell culture medium was removed, cells were washed twice with PBS and total RNA was extracted using TRIzol. RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA synthesis was then performed using a cDNA first-strand synthesis kit [Tiangen Biotech (Beijing) Co., Ltd.] in 20-µl reactions, each with 2 µl 5X avian myeloblastosis virus (AMV) buffer, 2 µl dNTP mixture, 1 µl AMV, 1 µl Oligo dT, 1 µl RNase Inhibitor, and 13 µl total RNA (2 µg). Reverse transcription was performed by incubation at 42˚C for 1 h, followed by 75˚C for 10 min to inactivate AMV. PCR primers for ATP7B were designed based on the GenBank sequence (http://www.ncbi.nlm.nih.gov) using Primer 3.0 software: 5' -GGTGTTTCTCTCCGTGTTGTGGT-3' and 5'-GGCTGCACAGGAAAGAGTCTCCGTGTTGGT-3'. qPCR was performed using FastStart Essential DNA Green Master (Roche). Each reaction contained 10 µl Master SYBR-Green I mix, 5 µl primer mixture (1.25 nmol/l), 2 µl cDNA, and 3 µl H₂O. qPCR involved denaturation at 95˚C for 10 min and 40 cycles of 94˚C for 1 min, 60˚C for 50 sec, and 72˚C for 50 sec. The fluorescence value for each cycle was recorded during extension at 75˚C. Values for ATP7B were normalized against β-actin. Relative values for mRNA levels were calculated using the ΔΔCt method: 2−ΔΔCt, where ΔCt = Ct(target) - Ct(β-actin) and Δ(ΔCt) = ΔCt(treated) - ΔCt (untreated).

Western blot analysis. Cells were collected and lysed in buffer containing 50 mM HEPES, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, 1 mM PMSF, 1 mM DTT and supplemented with protease inhibitor cocktail). Following centrifugation (10,000 x g for 10 min at 4˚C), the supernatant was discarded and protein concentration was determined using BCA protein assay. Protein samples (4:1 in SDS loading buffer) were denatured at 95˚C for 5 min, subjected to SDS-PAGE, electrically transferred onto PVDF membranes using a semi-dry transfer system, and blocked in 5% dry milk for 1 h. Protein samples were incubated with anti-ATP7B primary antibody (dilution 1:200) at 4˚C overnight and then with HRP-labeled goat anti-rabbit IgG secondary antibody (sc-2004; dilution 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed three times with TBST for 15 min after each incubation. Blots were developed using ECL reagent (Thermo Fisher Scientific) and images obtained using a Dolphin-Chemi Mini image system (Wealtcorp., Sparks, NV, USA). Gray-scale values for each band were analyzed, and relative protein levels were calculated using β-actin as an internal control.

Statistical analysis. All data were collected from at least three independent experiments. SPSS 17.0 software was used for statistical analysis. One-way ANOVA was used for analysis of cell viability and mRNA or protein expression, and Tukey's test was used for multiple pairwise comparisons. A p-value <0.05 was considered to indicate a statistically significant difference.

Results

Cisplatin resistance in Hep-2v cells. A CCK-8 assay was performed to investigate the effects of cisplatin on Hep-2 and Hep-2v cell proliferation (Fig. 1). Following treatment with 50 µM cisplatin for 24 h, the survival rate of Hep-2 cells was 61.7%, while that of Hep-2v had not changed. The difference between the two cell lines was statistically significant (p<0.01). Following treatment with 100 µM cisplatin for 24 h, the survival rate of Hep-2 cells was closer to that of those treated with 50 µM cisplatin, while the survival rate of Hep-2v cells was 80%. However, this difference was also statistically significantly (p<0.05). Treatment with 200 µM cisplatin for 24 h resulted in survival rates of 51.2% and 72% for Hep-2 and Hep-2v cells, respectively. Similar results were obtained when 48-h cisplatin treatment was undertaken (Fig. 1B). These findings suggest that vincristine-treated Hep-2 cells exhibit cisplatin resistance, particularly at 50 µM.
ATP7A and ATP7B expression in Hep-2v cells. We subsequently assessed the expression of cisplatin resistance-related molecules ATP7A and ATP7B by immunofluorescence staining to determine the molecular mechanism underlying cisplatin resistance of Hep-2v cells. Both Hep-2 and Hep-2v cells expressed ATP7A and ATP7B protein. Quantitative fluorescence analysis showed that ATP7B protein expression in Hep-2v cells was significantly higher than that in the Hep-2 cells; however, there was no significant difference in ATP7A expression (Fig. 2). Furthermore, treatment with 50 µM cisplatin increased levels of ATP7B protein expression in both Hep-2 and Hep-2v cells (Fig. 3). In Hep-2v cells, ATP7B protein levels reached a peak at 12 h and began to decrease at 24 h following treatment. Hep-2 cells expressed significantly higher levels of ATP7B than Hep-2 cells at all time points observed (Fig. 3). RT-qPCR revealed that 50 µM cisplatin increased ATP7B gene expression in both Hep-2 and Hep-2v cells. ATP7B expression in Hep-2 cells began to increase at 3 h, peaked at 6 h, and began to decrease at 12 h after cisplatin treatment. Moreover, ATP7B expression in Hep-2v cells followed a similar trend but remained at peak level until 24 h after cisplatin treatment (Fig. 4A). Western blot analysis revealed that ATP7B protein expression in both Hep-2 and Hep-2v cells was significantly greater than that in control cells at 12 h after cisplatin treatment (P<0.05; Fig. 4B and C).

ATP7B expression in miR-133a-overexpressing Hep-2v cells. We subsequently used RT-qPCR, western blot analysis and immunofluorescence staining to assess ATP7B expression in Hep-2v cells overexpressing miR-133a. Hep-2v cells were transfected with either pEZX-miR-133 or pEZX-control plasmid vectors. Transfected cells exhibit reporter gene GFP expression, while ATP7B expression is indicated by red fluorescence following immunostaining (Fig. 5A). ATP7B staining in pEZX-miR-133a-transfected cells was significantly weaker than that in pEZX-control transfected cells (Fig. 5B).
Western blot analysis and RT-qPCR results showed that exogenous expression of miR-133a reduced ATP7B mRNA and protein expression in Hep-2v cells (Fig. 5C-E).

miR-133a overexpression increases the sensitivity of drug-resistant Hep-2v cells to cisplatin. Hep-2v cells were transfected with a recombinant lentivirus vector expressing miR-133a and then treated with 50, 100 or 200 µM cisplatin 48 h later. After a further 24-48 h, cell viability was evaluated by CCK-8 assay. Compared with control cells, Hep-2v cells expressing exogenous miR-133a had significantly significantly reduced survival following treatment with 50 or 100 µM cisplatin (Fig. 6). These results suggest that upregulation of miR-133a enhances the cytotoxic effects of cisplatin on Hep-2v cells, particularly at 50-100 µM concentrations.

Discussion

We previously created a vincristine-resistant cell line and found that these cells were resistant to cisplatin. Therefore, Hep-2v cells are multidrug-resistant.

The dynamic balance of heavy metals in living organisms is maintained by a series of proteins: e.g., in vivo copper metabolism involves the membrane protein copper transporter receptor 1, which selectively imports copper into cells. Following importation, at least three further proteins, homolog of Ace1 activator (HAA1), ATOX1, copper chaperone for superoxide dismutase (CCS), are involved in transporting copper to the Golgi complex and mitochondria (21). HAA1 transports copper to ATP7B through a unique copper-binding site (22). ATP7B transports copper and other heavy metal...
molecules such as cisplatin outside cells, and ATP7B has been implicated in cellular resistance to stibium and arsenite (23, 24). Schilsky et al. (25) found that chromium resistance in hepatoblastoma cell lines involves ATP7B upregulation. Cisplatin functions by inhibiting DNA synthesis through a platinum-DNA reaction (26), and intracellular accumulation of platinum-based compounds is closely related to the degree of cell sensitivity to these drugs.

In the present study, we demonstrated that ATP7B expression was low in Hep-2 cells, and increased in vincristine-resistant Hep-2v cells. Following cisplatin treatment, ATP7B expression in these two cell lines was increased. While ATP7B expression in Hep-2 cells began to decrease at 12 h, expression in Hep-2v cells remained higher. This suggests that the resistance of Hep-2v cells to cisplatin is mediated by persistent high expression of ATP7B. Cisplatin-resistant cells exhibited reduced intracellular accumulation of cisplatin and increased extracellular outflow of cisplatin, and cisplatin resistance is associated with increased ATP7B expression in ovarian carcinoma cell lines (27, 28).

miR-133a functions as a tumor inhibitor and is expressed at low levels in a variety of tumor tissues; exogenous miR-133a induces tumor cell apoptosis and inhibits tumor cell metastasis (29); however, whether miR-133a is involved in chemotherapeutic drug resistance remains unknown. Yuan et al. (30) and others (31) have found that decreased miR-133a expression in adriamycin-resistant MCF-7 cells led to an increase in target gene UCP-2 expression. Previously (32), we compared differential expression levels of miRNAs in laryngeal carcinoma and normal tissues and found that miR-133 expression was low in laryngeal carcinoma tissues.

Investigations utilizing miRanda/miRBase and miRNA.org identified ATP7B as a target of miR-133a. Therefore, we questioned whether the high ATP7B expression in Hep-2v cells was influenced by miR-133a. In the present study, we noted that miR-133a expression in Hep-2 and Hep-2v cells was significantly lower than that in NP69 cells, but there was no marked difference between expression in Hep-2 and Hep-2v cells (Fig. 7). This suggests that high ATP7B expression in Hep-2v cells involves factors additional to miR-133a downregulation.

We transfected a recombinant lentiviral vector expressing miR-133a into Hep-2v cells and detected intracellular ATP7B expression using RT-qPCR and immunofluorescence staining. ATP7B expression was significantly lower in the miR-133a-transfected group compared with the control. These findings indicate that exogenous miR-133a transfection negatively regulates ATP7B expression.

Hep-2v cells expressing exogenous miR-133a exhibited low ATP7B expression, and cell viability was significantly decreased after cisplatin treatment. This provides further evidence that ATP7B promotes multidrug resistance in Hep-2v cells. However, there was no significant change in the viability of Hep-2 cells overexpressing miR-133a. We suggest that this was due to the increase in target gene expression, as it is transient in non-drug-resistant Hep-2 cells, and ATP7B expression rapidly returns to a low level. Therefore, decreased ATP7B expression after miR-133a transfection was not obvious (data not shown).

Hep-2v cells persistently express high levels of ATP7B, and cisplatin treatment stimulates increased ATP7B expression of ATP7B in these cells. ATP7B contributes to the removal of intracellular cisplatin to the extracellular space, thereby promoting cell survival. However, ATP7B expression was significantly decreased following exogenous expression of miR-133a. Reduced levels of ATP7B likely impaired the transportation of cisplatin to the extracellular space, thereby increasing the sensitivity of Hep-2v cells to cisplatin. These
findings suggest that ATP7B is a useful target in cisplatin-resistant tumors.

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References