SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 41: 1131-1139, 2019

miR-138-5p modulates the expression of excision repair cross-complementing proteins ERCC1 and ERCC4, and regulates the sensitivity of gastric cancer cells to cisplatin

JIE NING[1], YANG JIAO[1], XIAOQUE XIE[2], XUEFEI DENG[3], YIYIN ZHANG[1], YUEXIN YANG[2], CHENCHEN ZHAO[1], HUA WANG[1] and KANGSHENG GU[1]

Departments of [1]Oncology and [2]Radiation Oncology, The First Affiliated Hospital of Anhui Medical University; [3]School of Basic Medical Sciences, Anhui Medical University, Hefei, Anhui 230032, P.R. China

Received January 29, 2018; Accepted September 27, 2018

DOI: 10.3892/or.2018.6907

Abstract. The microRNA (miR)-138-5p affects the chemotherapeutic sensitivity of several human cancer types. In the present study, the expression and regulatory mechanisms of miR-138-5p were investigated in the gastric cancer cell line SGC7901 and its cisplatin-resistant derivative SGC7901/DDP. Gene microarray and reverse transcription-quantitative polymerase chain reaction analyses revealed that miR-138-5p was expressed at significantly lower levels in SGC7901/DDP compared with SGC7901 cells. Using computational predictive algorithms, two proteins involved in the nuclear excision repair pathway were identified, excision repair cross-complementing (ERCC)1 and ERCC4, as putative miR-138-5p target genes. Western blot analysis confirmed that ERCC1 and ERCC4 expression levels were inversely proportional to miR-138-5p levels in SGC7901 and SGC7901/DDP cells. Furthermore, ERCC1 and ERCC4 were upregulated in SGC7901 cells expressing miR-138-5p-targeting short hairpin RNA and, conversely, downregulated in SGC7901/DDP cells overexpressing miR-138-5p, confirming that this miRNA regulates ERCC protein levels. Notably, miR-138-5p silencing enhanced the cisplatin resistance of SGC7901 cells, while miR-138-5p overexpression partially reversed the cisplatin resistance of SGC7901/DDP cells. Taken together, these data suggest that miR-138-5p regulates the sensitivity of gastric cancer cells to cisplatin, possibly by modulating expression of the DNA repair proteins ERCC1 and ERCC4.

Introduction

Gastric cancer is one of the most common and lethal types of malignancy worldwide, ranking fourth in morbidity and third in mortality (1). China has the highest incidence of gastric cancer (2). Although there has been progress regarding the diagnosis and treatment of gastric cancer over the past few decades, the prognosis is generally poor, partially due to the highly invasive and metastatic nature of this tumor. Treatment of advanced gastric cancer primarily relies on palliative chemotherapy, with platinum drugs remaining the cornerstone of treatment. However, the majority of antineoplastic protocols that include platinum-based drugs have a <50% efficacy rate, and the development of primary and secondary resistance restrict the applications of these drugs (3). Therefore, the identification of novel biomarkers able to predict the efficacy of platinum-based therapy and guide clinical treatment is required.

Excision repair cross-complementing (ERCC) proteins are key components of the nuclear excision repair (NER) signaling pathway, which is a primary cause of cisplatin [also known as diamminedichloroplatinum (II), DDP] resistance. Previous studies have confirmed that increased expression of ERCC1 is associated with cisplatin resistance in SGC7901/DDP cells (4). ERCC1 forms a heterodimer with ERCC4 to excise the 5' end of damaged DNA as part of the NER signaling pathway. ERCC4- and ERCC1-deficient cells have been reported to be 40 times more sensitive to cisplatin compared with the parental cells (5), supporting the role of these enzymes in the NER signaling pathway and cisplatin resistance. Increasing evidence suggests that microRNAs (miRNAs/miRs) influence the efficacy of chemotherapeutic drugs by regulating gene expression (6). miR-138 is abnormally expressed in various tumors, and has been associated with drug resistance in lung cancer (7-9), cervical cancer (10) and osteosarcoma (11) cell lines. miR-138 promotes chemotherapeutic sensitivity by targeted regulation of DNA repair, induction of apoptosis and inhibition of the epithelial-mesenchymal transition (EMT) (8-12), Nevertheless, little is known regarding the association between miR-138-5p expression and chemotherapeutic sensitivity of gastric cancer. In the present study, the expression of miR-138-5p was examined in a gastric cancer cell line SGC7901 and its cisplatin-resistant derivative SGC7901/DDP. The expression of two putative miR-138-5p target genes and the effects of miR-138-5p modulation on cisplatin sensitivity in the cell lines were also determined.

Correspondence to: Professor Kangsheng Gu, Department of Oncology, The First Affiliated Hospital of Anhui Medical University, 218 Jixi Road, Hefei, Anhui 230032, P.R. China
E-mail: guksahmu@163.com

Key words: gastric cancer, microRNA-138-5p, cisplatin resistance

Materials and methods

Cell culture. The human gastric adenocarcinoma cell lines SGC7901/DDP and SGC7901 were obtained from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). The cisplatin-resistant SGC7901/DDP cell line was developed from the parental SGC7901 line by exposure to increasing concentrations of cisplatin over ~12 months. The cells acquired resistance at 1 μg/ml cisplatin. We have also previously confirmed that SGC7901/DDP cells are highly resistant to cisplatin using cell viability assays (13). All cells were maintained in RPMI-1640 containing 10% fetal calf serum, 100 U/ml penicillin, and 100 U/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified atmosphere with 5% CO_2 at 37˚C. Cells were cultured in the absence (SGC7901) or presence (SGC7901/DDP) of 800 ng/ml cisplatin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

RNA isolation. Total RNA was extracted from the gastric cancer cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. RNA concentrations were measured using a NanoDrop-2000 spectrophotometer with the absorbance set at 260 nm. Aliquots of total RNA were used for the microarray and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses.

Lentiviral infection. miR-138-5p-depleted or -overexpressing cell lines were constructed using lentiviral-mediated short hairpin (sh)RNA targeting or overexpression of miR-138-5p. Vectors encoding miR-138-5p (pLV-miR-138-5p) or a negative control sequence (pLV-miR-138-5p-NC) and the corresponding viruses ($1x10^8$ plaque-forming units) were all purchased from GeneCopoeia, Inc. (Rockville, MD, USA). Lentiviral infection was performed according to the manufacturer's protocol. Briefly, SGC7901/DDP cells were cultured overnight in 12-well plates at a density of $1x10^5$ cells/well, and then infected with the appropriate lentivirus at a multiplicity of infection of 10 plaque-forming units/cell. A total of 72 h after the transduction, SGC7901/DDP cells were transferred to RMPI-1640 containing 2 μg/ml puromycin and cultured for 3 days. Cells that survived were selected and used for subsequent experiments.

A similar approach was used to knock down miR-138-5p in SGC7901 cells, except that the cells were infected with lentiviral vectors encoding a control sequence or short hairpin (sh)RNA specific for miR-138-5p and then cultured for 5 days in RPMI-1640 containing 150 μg/ml hygromycin. Cells that survived were selected and used for subsequent experiments.

The lentiviral vectors co-expressed green fluorescent protein (GFP) for overexpression or red fluorescent protein (RFP) for silencing to allow verification of transduction efficacy by fluorescence microscopy.

RT-qPCR analysis. RT-qPCR was performed on an Mx3000P qPCR system with a SYBR Green single-step kit RT-qPCR kit (Biomics Biopharma, Nantong, China) according to the manufacturer's protocol. Aliquots of cDNA were amplified under the following conditions: 42˚C for 30 min followed by 40 cycles of 20 sec at 95˚C, 30 sec at 60˚C and 30 sec at 72˚C; and an extension step for 2 min at 72˚C to obtain the fluorescent signals. All miRNA levels were normalized to U6 expression. Primers used in this study were: miR-138-5p forward, 5'-AGCTGGTGTTGTGAATCAGGCCG-3' and reverse, 5'-TGGTGTCGTGGAGTCG-3'; U6 forward, 5'-CTC GCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCA CGAATTTGCGT-3'. The relative differences were quantified using the $2^{-\Delta\Delta Cq}$ method, where $\Delta\Delta$Cq is calculated as the: Experimental ($Ct_{miR-138-5p}$-Ct_{u6})-control ($Ct_{miR-138-5p}$-Ct_{u6}). The procedure was performed in triplicate (14).

Microarray analysis. Total RNA was extracted from triplicate biological samples of SGC7901/DDP and SGC7901 cells as aforementioned and sent to Beijing CapitalBio Technology Co., Ltd. (Beijing, China) for microarray analysis.

Expression profiling was performed using an Affymetrix miRNA 4.0 Array, which contains 775 mature human miRNA probe sets. The output data, which contained normalized miRNA expression profiles, were analyzed in Excel spreadsheets. The two groups of data were analyzed by t-tests to obtain P-values, and false discovery rate adjustment was performed to generate corrected P-values. The fold change in differentially expressed miRNAs between SGC7901/DDP and SGC7901 cells was calculated, and Cluster 3.0 software (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) was used to display the differential expression patterns. Genes potentially targeted by the differentially expressed miRNAs were identified using the online tools DIANAmT http://diana.imis.athena-innovation.gr/), miRanda (http://www.microrna.org/microrna/home.do), miRDB (http://www.mirdb.org/), miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html), RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), PICTAR4 (http://pictar.mdc-berlin.de), PICTAR5 (http://pictar.mdc-berlin.de), PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), RNA22 (https://cm.jefferson.edu/rna22/) and TargetScan (http://www.targetscan.org/).

Western blotting. Total proteins were extracted from cultured SCG7901 cells and SGC7901/DDP cells with or without lentivirus infection by cell lysis in radioimmunoprecipitation assay buffer containing protease inhibitors (Beyotime Institute of Biotechnology, Shanghai, China). Protein concentrations were quantitatively determined using a BCA Protein Assay kit (Beyotime Institute of Biotechnology). Lysate samples containing 60 μg total protein were electrophoresed on a 10 or 8% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Beyotime Institute of Biotechnology). The PVDF membrane was blocked with 5% non-fat milk for 1.5 h at room temperature and probed overnight at 4˚C with the following primary antibodies: Rabbit anti-ERCC1 (1:200 dilution; cat. no. ab129267); rabbit anti-ERCC4 (XPF) (1:300 dilution; cat. no. ab76948); rabbit anti-β-actin (1:1,000 dilution; cat. no. ab8227); and rabbit anti-GAPDH (1:1,000 dilution; cat. no. ab9485) (all from Abcam, Cambridge, UK). Following washing with Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution; cat. no. 7074S; Shanghai Youningwei Biotechnology Co. Ltd., Shanghai, China) for 4 h at 4˚C. Protein bands were visualized with an ECL Plus chemiluminescent kit (Thermo Scientific Fisher, Inc.). Protein levels were quantified by

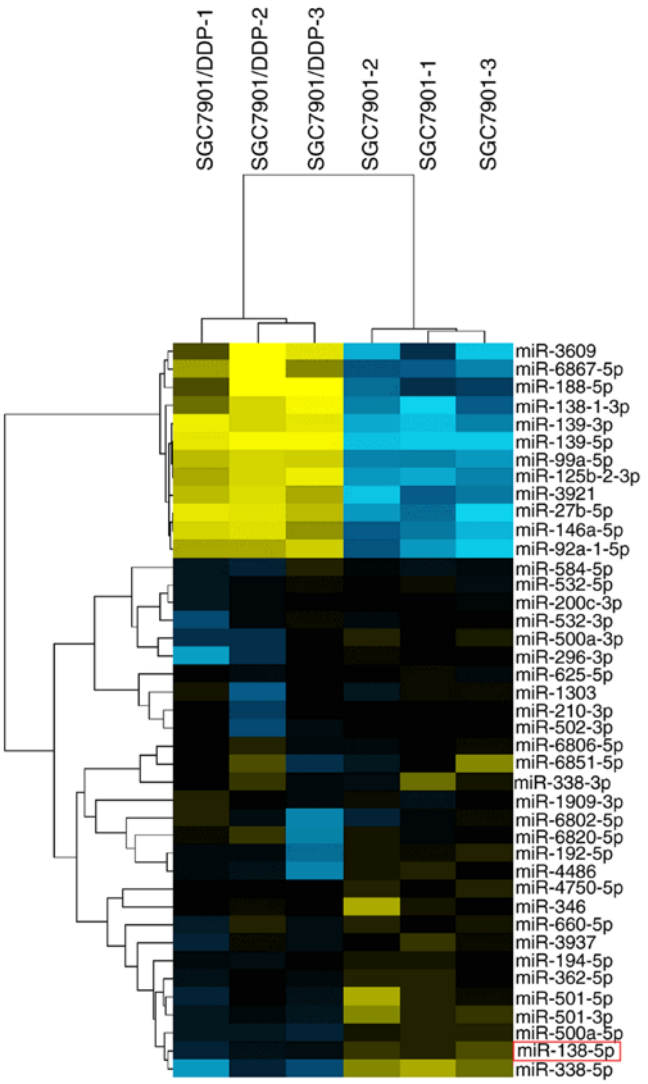

Figure 1. Microarray analysis of differentially expressed miRNAs in SGC7901/DDP and SGC7901 cells. Two-dimensional cluster analysis identified 41 significantly differentially expressed miRNAs (P<0.05, fold change ≥2 or ≤0.5 fold change). Each mRNA is represented by a row in the heat map. Blue and yellow represent miRNAs that are downregulated and upregulated, respectively, in SGC7901/DDP compared with SGC7901 cells. The magnitude of the difference is indicated by the scale bar. miR, microRNA; DDP, cisplatin.

densitometry (Tanon2500 Fully Automatic Digital Gel Image Analysis system; Tanon Science and Technology Co., Ltd., Shanghai, China) and are presented as the fold change in expression after normalization to GAPDH and β-actin.

Cisplatin sensitivity assay. Cisplatin sensitivity was determined using the colorimetric MTT assay. SGC7901/DDP and SGC7901 cells, their miR-138-5p-overexpressing or -silenced counterparts, and control cells were resuspended at $1x10^5$ cells/ml. Aliquots of 100 μl were distributed into 96-well plates, and the volumes were made up to 200 μl/well with RPMI-1640. The cells were incubated for 24 h, and 180 μl of supernatant/well was then removed and replaced with freshly prepared DDP at final concentrations of 40, 20, 2, 0.2 and 0.02 μg/ml for DDP-resistant groups; and at final concentrations of 4, 2, 0.2, 0.02 and 0.002 μg/ml for DDP-sensitive groups. These concentrations were selected because the peak

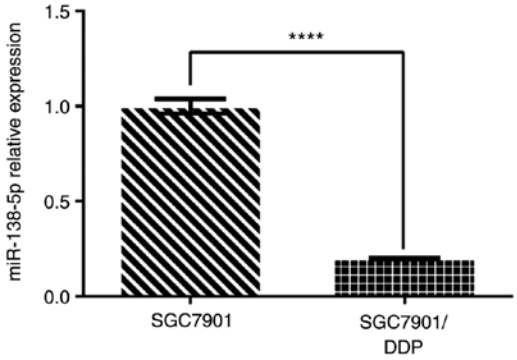

Figure 2. Reverse transcription-quantitative polymerase chain reaction analysis of miR-138-5p in gastric cancer cells. miR-138-5p was significantly downregulated in SGC7901/DDP cells compared with the parental SGC7901 cells. Data are presented as the mean ± standard deviation of experiments performed in triplicate. ****P<0.0001. miR, microRNA; DDP, cisplatin.

concentration of DDP in human plasma is 2.0 μg/ml (15). A total of 48 h later, 180 μl/well of supernatant was removed and replaced with 180 μl fresh medium plus 20 μl of MTT/well (5 mg/ml, Sigma-Aldrich; Merck KgaA). The plate was incubated for 4 h in a humidified atmosphere at 37˚C, the medium was removed, and 150 μl/well DMSO (Sigma-Aldrich; Merck KGaA) was added. The plates were mixed for 10 min at 72˚C to dissolve the formazan crystals and the absorbance at 490 nm was then measured using a spectrophotometer. The 50% inhibitory concentration (IC_{50}) of DDP was calculated based on the relative viability of control wells lacking DDP. The assay was performed in triplicate.

Statistical analysis. Statistical analysis was performed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). All data are presented as the mean ± standard deviation. A two-tailed Student's t-test was used to compare differences between two groups. One-way analysis of variance and the Bonferroni post hoc test was used to compare differences between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-138-5p in SGC7901/DDP and SGC7901 cells. To identify miRNAs potentially involved in cisplatin resistance, miRNA microarray analysis was performed on triplicate samples of SGC7901 and SGC7901/DDP cells. As shown in Fig. 1 and Table I, 41 miRNAs were significantly differentially expressed between SGC7901 and SGC7901/DDP cells, using a threshold of ≥2-fold or ≤0.5-fold change and P<0.05. For further analysis, miR-138-5p was selected, which exhibited the largest difference in expression between the cisplatin-sensitive and -resistant cell lines. This miRNA was of particular interest as it has previously been reported to modulate drug resistance in lung cancer by acting on the NER pathway (9,16). The expression of miR-138-5p was ~3-fold lower in SGC7901/DDP cells compared with SGC7901 cells (P<0.05; Fig. 1).

To confirm these findings, the expression of miR-138-5p was examined by RT-qPCR. The analysis demonstrated that miR-138-5p expression in SGC7901/DDP cells was ~5-fold lower compared with that in SGC7901 cells (P<0.0001; Fig. 2).

Table I. Fold change of differentially expressed miRNAs in SGC7901/DDP and SGC7901 cells.

miRNAs	Fold change	*q*-value (%)
hsa-miR-139-5p	3.9627	0
hsa-miR-139-3p	2.8456	0
hsa-miR-3609	2.6297	1.0853785
hsa-miR-27b-5p	2.6106	0
hsa-miR-138-1-3p	2.3803	1.0853785
hsa-miR-125b-2-3p	2.3502	0
hsa-miR-188-5p	2.2048	3.1418851
hsa-miR-99a-5p	2.0877	0
hsa-miR-6867-5p	2.0662	2.7032068
hsa-miR-3921	2.0584	0
hsa-miR-146a-5p	2.0484	0
hsa-miR-92a-1-5p	2.0356	1.0853785
hsa-miR-584-5p	0.4914	2.7203157
hsa-miR-1909-3p	0.4902	1.4046075
hsa-miR-6806-5p	0.4859	0
hsa-miR-532-5p	0.4509	0
hsa-miR-625-5p	0.4481	0
hsa-miR-6851-5p	0.4475	3.0810745
hsa-miR-200c-3p	0.444	0
hsa-miR-338-3p	0.4315	0
hsa-miR-532-3p	0.4282	0
hsa-miR-6802-5p	0.4274	2.7203157
hsa-miR-4750-5p	0.4255	0
hsa-miR-1303	0.4198	2.7203157
hsa-miR-6820-5p	0.4108	1.5801834
hsa-miR-210-3p	0.3935	0
hsa-miR-346	0.3795	0
hsa-miR-660-5p	0.3795	0
hsa-miR-194-5p	0.3763	0
hsa-miR-502-3p	0.3732	0
hsa-miR-362-5p	0.3497	0
hsa-miR-3937	0.3483	0
hsa-miR-500a-3p	0.3071	0
hsa-miR-296-3p	0.3033	0
hsa-miR-192-5p	0.2989	0
hsa-miR-500a-5p	0.2901	0
hsa-miR-4486	0.2887	0
hsa-miR-501-5p	0.2836	0
hsa-miR-138-5p	0.2686	0
hsa-miR-501-3p	0.2683	0
hsa-miR-338-5p	0.1619	0

q-value (%) is a false discovery rate when the gene is identified as a differential gene, similar to P-value, the difference is more significantly, the *q*-value (%) is more smaller. miR, microRNA.

Thus, the PCR results were consistent with the microarray analysis, confirming that miR-138-5p was downregulated in cisplatin-resistant SGC7901/DDP cells compared with the cisplatin-susceptible parental cells.

Predicted miR-138-5p target genes. To investigate potential target genes of miR-138-5p, the predictive algorithms miRanda, miRWalk and PicTar, which screen for complementary binding sequences between miRNAs and known gene transcripts were used. These analysis identified the excision repair proteins ERCC1 and ERCC4 as possible target genes of miR-138-5p (Fig. 3).

Expression of ERCC1 and ERCC4 in SGC7901/DDP and SGC7901 cells. Western blot analysis was performed to determine the expression levels of ERCC1 and ERCC4 in SGC7901/DDP and SGC7901 cells. As shown in Fig. 4, ERCC1 and ERCC4 proteins were expressed at significantly higher levels in SGC7901/DDP cells compared with that in SGC7901 cells (P<0.01).

Alterations in ERCC1 and ERCC4 expression in miR-138-5p-overexpressing or miR-138-5p-depleted gastric cancer cells Fluorescence microscopy of infected cells. To suppress miR-138-5p expression, SGC7901 cells were infected with lentiviruses encoding RFP and a negative control sequence or an shRNA targeting miR-138-5p, named SGC7901-LV-NC-knockdown (KD) and SGC7901-LV-miR138-5p-KD cells, respectively. Similarly, the effects of miR-138-5p overexpression were investigated by SGC7901/DDP cell infection with lentiviruses encoding GFP and a control sequence or miR-138-5p, named SGC7901/DDP-LV-NC-overexpression (OE) and SGC7901/DDP-LV-miR-138-5p-OE cells, respectively. Fluorescence microscopy of the infected cells revealed successful transduction, as demonstrated by abundant RFP and GFP expression in the corresponding SGC7901 and SGC7901/DDP cells (Fig. 5).

Verification of miR-138-5p expression in transduced gastric cancer cells by RT-qPCR. RT-qPCR was used to verify the miR-138-5p expression in SGC7901 and SGC7901/DDP cells. As shown in Fig. 6A, the levels of miR-138-5p in uninfected SGC7901 cells and control SGC7901-LV-NC-KD cells were similar. However, miR-138-5p was significantly reduced (~5-fold lower) in the SGC7901-LV-miR-138-5p-KD cells compared with both control cell groups (both P<0.0001; Fig. 6A). Similarly, miR-138-5p was significantly upregulated (~4-fold increase) in SGC7901/DDP-LV-miR-138-5p-OE cells compared with either SGC7901/DDP and SGC7901/DDP-LV-NC-OE cells (both P<0.0001; Fig. 6B). Thus, miR-138-5p was successfully knocked down or overexpressed in the transduced gastric cancer cells.

Expression of ERCC1 and ERCC4 following modulation of miR-138-5p expression in gastric cancer cells. As ERCC1 and ERCC4 were identified as potential target genes of miR-138-5p, their expression in SGC7901 or SGC7901/DDP cells was evaluated by western blot analysis. Notably, downregulation of miR-138-5p significantly increased the expression of ERCC1 and ERCC4 compared with uninfected SGC7901 cells or SGC7901-LV-NC-KD cells (P<0.01; Fig. 7).

Conversely, the expression of ERCC1 and ERCC4 was significantly diminished in SGC7901/DDP-LV-miR138-5p-OE cells compared with control SGC7901/DDP and SGC7901/DDP-LV-NC-OE cells (P<0.01; Fig. 8).

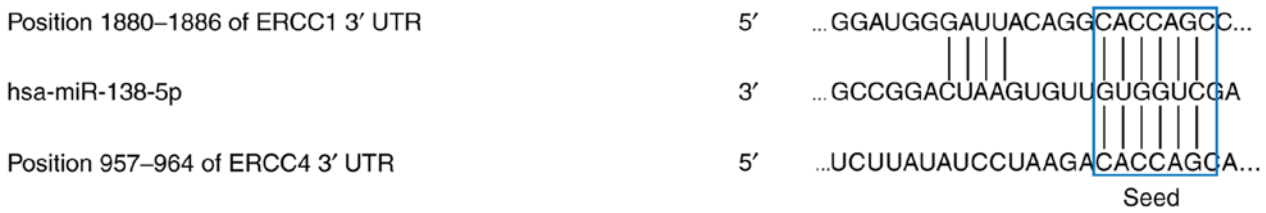

Figure 3. miR-138-5p assumed binding sites in the 3'UTR of ERCC1 and ERCC4 mRNAs. ERCC, excision repair cross-complementing gene; miR, microRNA; UTR, untranslated region.

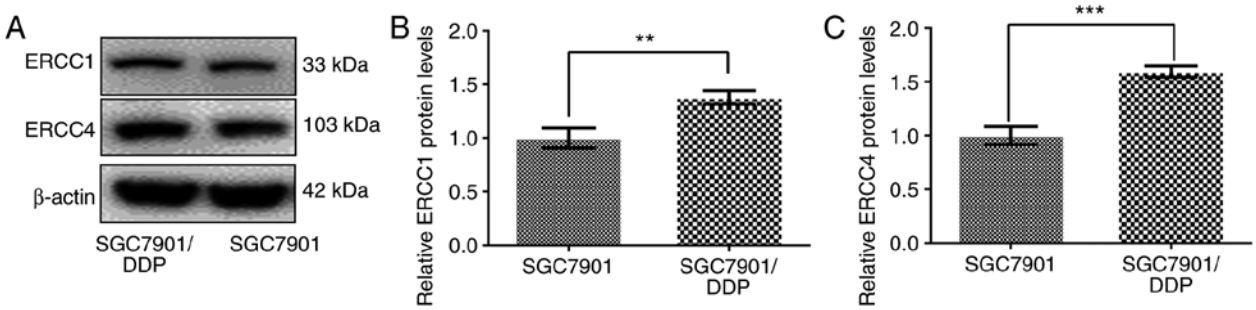

Figure 4. Western blot analysis of ERCC1 and ERCC4 protein levels in SGC7901/DDP and SGC7901 cells. (A) Representative western blot analysis, with β-actin as a loading control. Densitometric quantification of (B) ERCC1 and (C) ERCC4 levels. Data are presented as the mean ± standard deviation of experiments performed in triplicate. **P<0.01 and ***P<0.001. ERCC, excision repair cross-complementing gene; DDP, cisplatin.

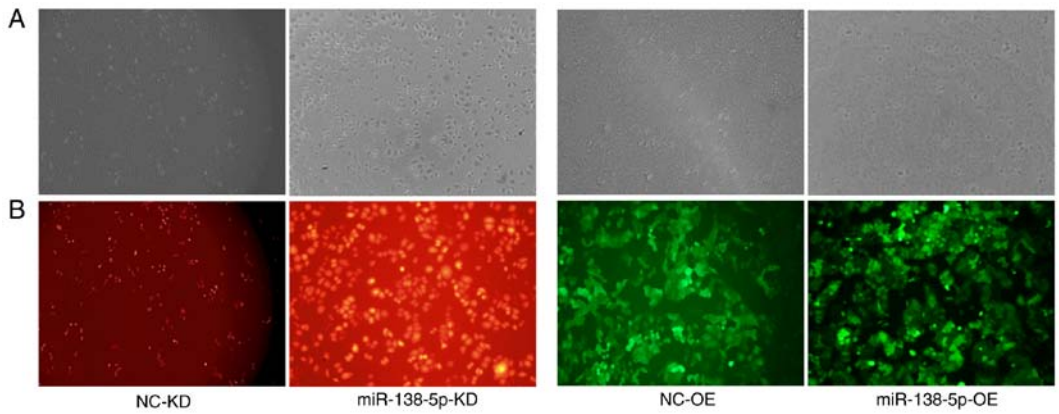

Figure 5. Cell transduction efficiency. (A) Light microscopy and (B) fluorescent microscopy of red fluorescent protein-expressing SGC7901 cells (left panels) and green fluorescent protein-expressing SGC7901/DDP cells (Right panels). Exposure, 600 ms; magnification, x100. OE, overexpression; KD, knockdown; NC, negative control; miR, microRNA; DDP, cisplatin.

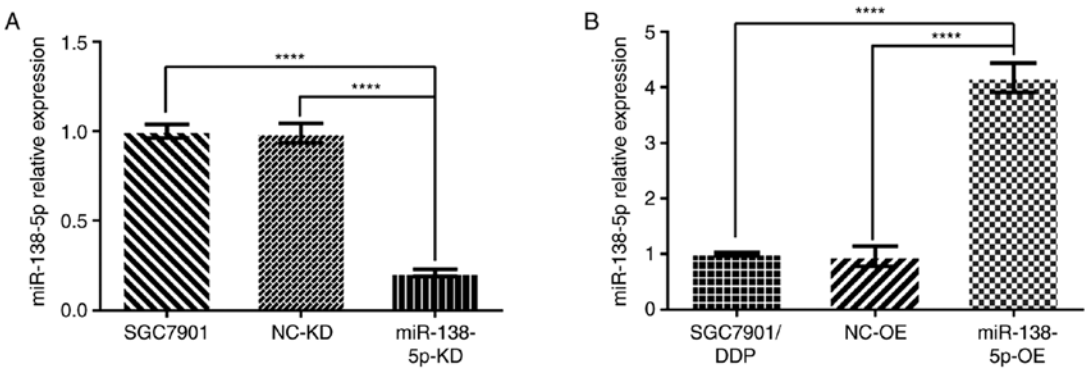

Figure 6. Reverse transcription-quantitative polymerase chain reaction analysis of miR-138-5p expression in lentivirus-infected cells. (A) SGC7901 cells and (B) SGC7901/DDP cells. Data are presented as the mean ± standard deviation of experiments performed in triplicate. ****P<0.0001. OE, overexpression; KD, knockdown; NC, negative control; miR, microRNA; DDP, cisplatin.

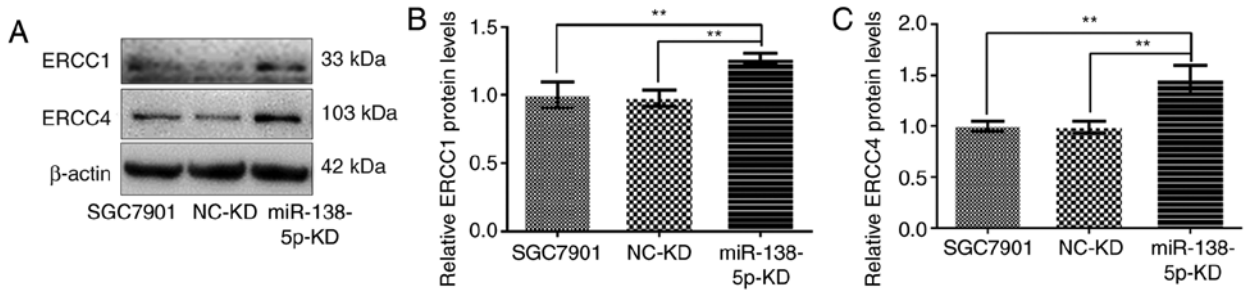

Figure 7. Western blot analysis of ERCC1 and ERCC4 protein levels in SGC7901 cells expressing miR-138-5p-targeting shRNA. (A) Representative western blot, with β-actin as a loading control. Densitometric quantification of (B) ERCC1 and (C) ERCC4 levels in uninfected SGC7901 cells or cells expressing LV-NC-KD or LV-miR-138-5p-KD vectors. Data are presented as the mean ± standard deviation of experiments performed in triplicate. **P<0.01. ERCC, excision repair cross-complementing gene; NC, negative control; miR, microRNA; KD, knockdown.

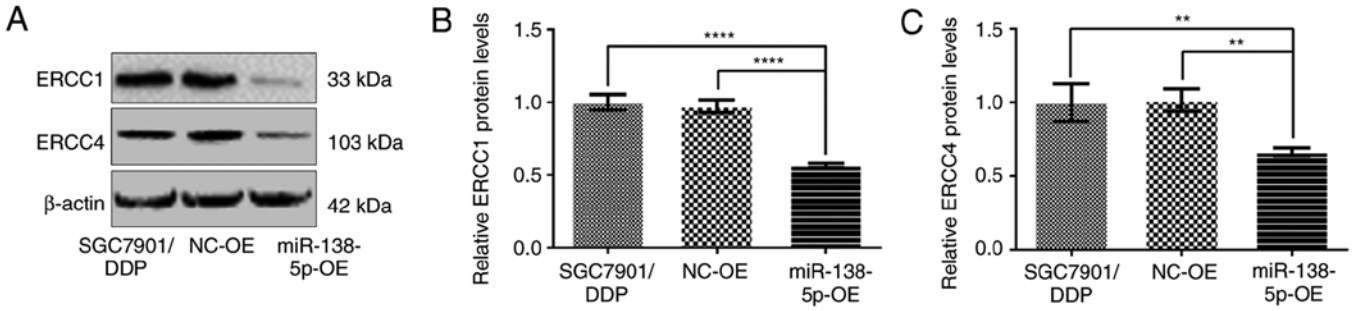

Figure 8. Western blot analysis of ERCC1 and ERCC4 protein levels in SGC7901/DDP cells overexpressing miR-138-5p. (A) Representative western blot, with β-actin as a loading control. Densitometric quantification of (B) ERCC1 or (C) ERCC4 levels in uninfected SGC7901/DDP cells or cells expressing LV-NC-OE or LV-miR-138-5p-OE vectors. Data are presented as the mean ± standard deviation of experiments performed in triplicate. **P<0.01. ****P<0.0001. ERCC, excision repair cross-complementing gene; NC, negative control; miR, microRNA; OE, overexpression.

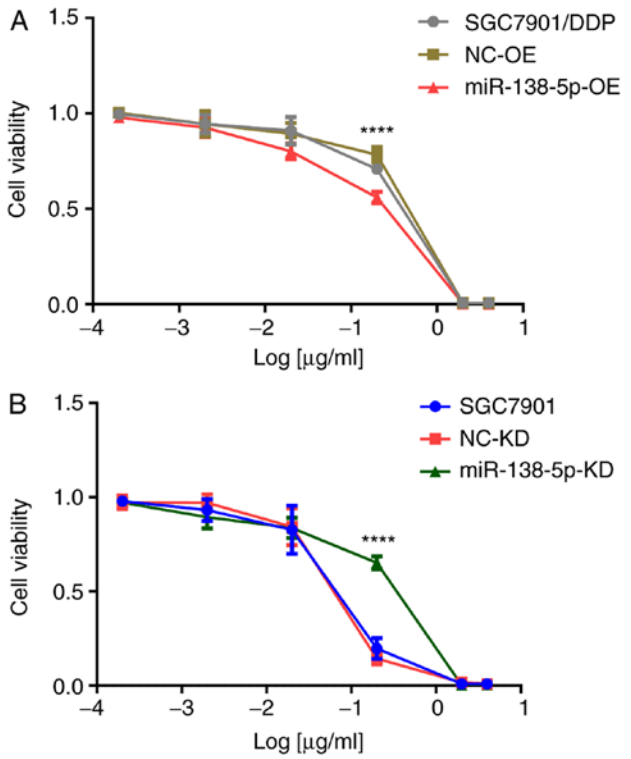

Figure 9. Cell viability of cisplatin-treated gastric cancer cells. (A) SGC7901/DDP cells treated for 48 h with 40, 20, 2, 0.2, and 0.02 μg/ml cisplatin and (B) SGC7901 cells treated for 48 h with 4, 2, 0.2, 0.02 and 0.002 μg/ml cisplatin. Data are presented as the mean ± standard deviation of experiments performed in triplicate. ****P<0.01. NC, negative control; miR, microRNA; KD, knockdown; OE, overexpression; DDP, cisplatin.

Cisplatin sensitivity of gastric cancer cells following modulation of miR-138-5p expression. To evaluate the effects of miR-138-5p downregulation or upregulation on the sensitivity of SGC7901/DDP or SGC7901 cells to cisplatin, cells were treated with varying concentrations (40, 20, 2, 0.2 and 0.02 μg/ml in SGC7901/DDP, SGC7901/DDP-LV-miR138-5p-OE and SGC7901/DDP-LV-NC-OE groups; and with varying concentrations of 4, 2, 0.2, 0.02 and 0.002 μg/ml in SGC7901, SGC7901-LV-NC-KD and SGC7901-LV-miR138-5p-KD groups) of cisplatin for 48 h, and then cell viability was determined using an MTT assay. Drug resistance was evaluated by calculating the IC_{50} of DDP relative to untreated cells. It was demonstrated that miR138-5p-overexpressing SGC7901/DDP cells were significantly more sensitive to cisplatin compared with SGC7901/DDP or SGC7901/DDP-LV-NC-OE cells, as evidenced by the IC50 values of 3.14±0.32, 6.81±0.12, and 6.89±0.14 μg/ml, respectively (P<0.01; Fig. 9A). Conversely, the cisplatin resistance of SGC7901-LV-miR138-5p-KD cells was significantly increased following depletion of miR-138-5p compared with SGC7901 and SGC7901-LV-NC-KD cells (IC50 values 0.39±0.04, 0.20±0.01, and 0.25±0.05 μg/ml, P<0.01; Fig. 9B). Collectively, these data demonstrated that low miR-138-5p levels and high ERCC1 and ERCC4 levels were associated with cisplatin resistance in gastric cancer cells.

Discussion

Cisplatin is a broad-spectrum antineoplastic drug that is widely used in the treatment of cancer, including head and

neck, lung, breast, gastric, ovarian, and testicular cancer; indeed, cisplatin is the cornerstone of numerous combination chemotherapy regimens (17). Primary and secondary resistance to cisplatin restricts its clinical applications. Thus, the identification of novel biomarkers that are able to predict the efficacy of platinum-based therapy is required. The results of the present study demonstrated that miR-138-5p expression modulates the sensitivity of human gastric cancer cells to cisplatin, possibly by suppressing the expression of the NER pathway components ERCC1 and ERCC4. Therefore, the expression level of miR-138-5p and/or the ERCC proteins may be a useful predictor of cisplatin efficacy, thereby guiding the individualized treatment of patients with gastric cancer.

The majority of studies on miR-138-5p performed to date have reported that it is an anti-oncogene (12,18). For instance, Ma *et al* (19) reported that miR-138-5p inhibited the proliferation of gallbladder cancer cells by acting on the anti-apoptotic protein BAG family molecular chaperone regulator 1. Yu *et al* (20) demonstrated that miR-138-5p inhibited the proliferation of pancreatic cancer cells by reducing expression of Forkhead box protein C1. Similarly, Chen *et al* (21) revealed that depletion of miR-138-5p upregulated LIM domain kinase 1 in ovarian cancer, thereby promoting metastasis. However, certain studies have reported conflicting data. For example, in glioma, miR-138-5p is overexpressed compared with normal cells, and promotes tumor occurrence, development, metastasis and infiltration while suppressing apoptosis (22-24). Therefore, miR-138-5p may be a 'dual function' miRNA. A recent report suggested that the expression level of miR-138-5p and its target gene PDK1 are prognostic factors for non-small cell lung cancer (NSCLC) and are associated with tumor-node-metastasis staging and lymph node metastasis (25). Regarding drug resistance, several studies have suggested that miR-138-5p may influence chemotherapy sensitivity via inhibition of DNA repair, induction of apoptosis, and inhibition of EMT. For example, Yang *et al* (16) demonstrated that miR-138 exhibited a direct effect on histone H2AX, thereby inhibiting DNA repair in SCLC cells. Wang *et al* (9) demonstrated that miR-138-5p promoted cisplatin resistance of a NSCLC cell line (A549/DDP) by inhibiting ERCC1 expression and impairing the DNA repair response. In addition, in osteosarcoma, miR-138 increases caspase-3-mediated apoptosis via its target gene histone-lysine N-methyltransferase EZH2, thereby increasing the sensitivity to cisplatin (11). miR-138 has also been reported to enhance the sensitivity of NSCLC cells to adriamycin by targeting E-cadherin, Zinc finger E-box-binding homeobox 2, and vimentin and suppressing EMT (8). Han *et al* (7) revealed that the upregulation of miR-138 and the subsequent effects on G1/S-specific cyclin-D3 enhanced the sensitivity of NSCLC to cisplatin. A similar study have reported that miR-138 enhances the efficacy of 5-fluorouracil and doxorubicin in the cervical cancer cell line HeLa via downregulation of FAK (10). However, the association between miR-138-5p and the resistance of gastric cancer to chemotherapy remains unclear.

Expression of NER pathway constituents is associated with resistance and sensitivity to platinum-based drugs (17,26). ERCC1 is considered to serve a role in platinum-based drug efficacy, since it is predictive of prognosis in lung (27), ovarian (28), cervical (29), bladder (30) and gastric (31) cancer. Indeed, ERCC4- and ERCC1-deficient cells are 40 times more sensitive compared with their parental cells to cisplatin (5). Stevens *et al* (32) demonstrated that the expression of ERCC4 was associated with the sensitivity of ovarian and colon cancer cells to cisplatin. Furthermore, in the hepatic cancer cell line HepG2.2.15, miR-192-mediated regulation of ERCC3 and ERCC4 expression inhibited the NER pathway (33). Finally, depletion of ERCC4 in Chinese hamster mutant cell lines increased cisplatin sensitivity >3-fold compared with depletion of ERCC3, ERCC2, or ERCC5, suggesting an important role for ERCC4 in NER-induced cisplatin resistance (5). However, the involvement of ERCC4 in this process has received little attention to date.

The microarray analysis performed in the current stud identified 41 differentially expressed miRNAs in the two gastric cancer cell lines. miR-138 was chosen for subsequent experiments as it has been previously reported to modulate drug resistance in small cell lung cancer and NSCLC by acting on the NER pathway (9,16), but it has not previously been examined in gastric cancer. The current study demonstrated that miR-138-5p was downregulated in cisplatin-resistant SGC7901/DDP cells, and bioinformatics analysis identified ERCC1 and ERCC4 as possible miR-138-5p target mRNAs. Indeed, the expression of the two proteins was significantly higher in SGC7901/DDP compared with SGC7901 cells. The ERCC proteins were downregulated by miR-138-5p overexpression and, conversely, upregulated by miR-138-5p KD. Furthermore, upregulation of miR-138-5p partially reversed the cisplatin resistance of SGC7901/DDP cells, whereas miR-138-5p downregulation in SGC7901 cells had the opposite effect, rendering the cells more resistant to cisplatin. Collectively, these results suggest that miR-138-5p may regulate DNA damage repair and cisplatin resistance via its effects on ERCC1 and ERCC4. Thus, miR-138-5p may be a novel target for drug-resistant gastric cancer.

There are certain limitations to the present study. First, the study lacks data from animal experiments or patients to validate the *in vitro* findings. Second, the study is based on a single gastric cancer cell line and its platinum-resistant derivative. Third, miR-138-5p was not directly demonstrated to modulate ERCC1/ERCC4 expression; for example, by performing luciferase reporter assays with the wild-type and miR-138-5p-binding mutant forms of the ERCC1 and ERCC4 3'-untranslated regions. Future studies plan to validate these findings by investigating the association between miR-138-5p, ERCC proteins and cisplatin resistance in gastric cancer patients.

In conclusion, microarray and RT-qPCR analysis revealed that miR-138-5p was expressed at significantly lower levels in cisplatin-resistant SGC7901/DDP compared with the parental SGC7901 cells. The expression of ERCC1 and ERCC4 in the cisplatin-resistant cell line was significantly decreased by lentivirus-mediated overexpression of miR-138-5p, suggesting that miR-138-5p may function as a chemosensitizer. In addition, the expression of ERCC1 and ERCC4 in SGC7901 cells was significantly increased by lentivirus-mediated downregulation of miR-138-5p, leading to cisplatin resistance. The expression level of miR-138-5p may be used to predict the chemosensitivity of patients with gastric cancer treated with cisplatin, and may be a potential novel target for reversing drug resistance.

Acknowledgements

The authors would like to thank Ms. Anne M. O'Rourke for editing the English text of a draft of this manuscript.

Funding

The present study was supported by Foreign Science and Technology Cooperation Project of Anhui Province (grant no. 1604b0602027).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HW, KG and XD conceived and designed the study. JN, YJ, XX and YZ performed the experiments. CZ, YY and XX analyzed and interpreted the data. JN and YY wrote the manuscript. JN, YJ and KG reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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.

References

1. Center MM, Jemal A, Lortet-Tieulent J, Ward E, Ferlay J, Brawley O and Bray F: International variation in prostate cancer incidence and mortality rates. Eur Urol 61: 1079-1092, 2012.
2. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J: Cancer statistics in China, 2015. CA Cancer J Clin 66: 115-132, 2016.
3. Schlansky B and Sonnenberg A: Epidemiology of noncardia gastric adenocarcinoma in the United States. Am J Gastroenterol 106: 1978-1985, 2011.
4. Li W, Jie Z, Li Z, Liu Y, Gan Q, Mao Y and Wang X: ERCC1 siRNA ameliorates drug resistance to cisplatin in gastric carcinoma cell lines. Mol Med Rep 9: 2423-2428, 2014.
5. De Silva IU, McHugh PJ, Clingen PH and Hartley JA: Defects in interstrand cross-link uncoupling do not account for the extreme sensitivity of ERCC1 and XPF cells to cisplatin. Nucleic Acids Res 30: 3848-56, 2002.
6. Buhagiar A and Ayers D: Chemoresistance, cancer stem cells, and miRNA influences: The case for neuroblastoma. Anal Cell Pathol 2015: 150634, 2015.
7. Han LP, Fu T, Lin Y, Miao JL and Jiang QF: MicroRNA-138 negatively regulates non-small cell lung cancer cells through the interaction with cyclin D3. Tumour Biol 37: 291-298, 2016.
8. Jin Z, Guan L, Song Y, Xiang GM, Chen SX and Gao B: MicroRNA-138 regulates chemoresistance in human non-small cell lung cancer via epithelial mesenchymal transition. Eur Rev Med Pharmacol 20: 1080-1086, 2016.
9. Wang Q, Zhong M, Liu W, Li J, Huang J and Zheng L: Alterations of microRNAs in cisplatin-resistant human non-small cell lung cancer cells (A549/DDP). Exp Lung Res 37: 427-434, 2011.
10. Golubovskaya VM, Sumbler B, Ho B, Yemma M and Cance WG: miR-138 and miR-135 directly target focal adhesion kinase, inhibit cell invasion, and increase sensitivity to chemotherapy in cancer cells. Anticancer Agents Med Chem 14: 18-28, 2014.
11. Zhu Z, Tang J, Wang J, Duan G, Zhou L and Zhou X: miR-138 acts as a tumor suppressor by targeting EZH2 and enhances cisplatin-induced apoptosis in osteosarcoma Cells. PLoS One 11: e0150026, 2016.
12. Li J, Wang Q, Wen R, Liang J, Zhong X, Yang W, Su D and Tang J: miR-138 inhibits cell proliferation and reverses epithelial-mesenchymal transition in non-small cell lung cancer cells by targeting GIT1 and SEMA4C. J Cell Mol Med 19: 2793-2805, 2015.
13. Xie XQ, Zhao QH, Wang H and Gu KS: Dysregulation of mRNA profile in cisplatin-resistant gastric cancer cell line SGC7901. World J Gastroenterol 23: 1189-1202. 2017.
14. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
15. Sileni VC, Fosser V, Maggian P, Padula E, Beltrame M, Nicolini M and Arslan P: Pharmacokinetics and tumor concentration of intraarterial and intravenous cisplatin in patients with head and neck squamous cancer. Cancer Chemother Pharmacol 30: 221-225, 1992.
16. Yang H, Luo J, Liu Z, Zhou R and Luo H: MicroRNA-138 regulates DNA damage response in small cell lung cancer cells by directly targeting H2AX. Cancer Invest 33: 126-236, 2015.
17. Dasari S and Tchounwou PB: Cisplatin in cancer therapy: Molecular mechanisms of action. Eur J Pharmacol 740: 364-378, 2014.
18. Liu Y, Yang K, Sun X, Fang P, Shi H, Xu J, Xie M and Li M: miR-138 suppresses airway smooth muscle cell proliferation through the PI3K/AKT signaling pathway by targeting PDK1. Exp Lung Res 41: 363-369, 2015.
19. Ma F, Zhang M, Gong W, Weng M and Quan Z: miR-138 suppresses cell proliferation by targeting Bag-1 in gallbladder carcinoma. PLoS One 10: e0126499, 2015.
20. Yu C, Wang M, Li Z, Xiao J, Peng F, Guo X, Deng Y, Jiang J and Sun C: MicroRNA-138-5p regulates pancreatic cancer cell growth through targeting FOXC1. Cell Oncol (Dordr) 38: 173-181, 2015.
21. Chen P, Zeng M, Zhao Y and Fang X: Upregulation of Limk1 caused by microRNA-138 loss aggravates the metastasis of ovarian cancer by activation of Limk1/cofilin signaling. Oncol Rep 32: 2070-2076, 2014.
22. Chan XH, Nama S, Gopal F, Rizk P, Ramasamy S, Sundaram G, Ow GS, Ivshina AV, Tanavde V, Haybaeck J, *et al*: Targeting glioma stem cells by functional inhibition of a prosurvival oncomiR-138 in malignant gliomas. Cell Rep 2: 591-602, 2012.
23. Stojcheva N, Schechtmann G, Sass S, Roth P, Florea AM, Stefanski A, Stühler K, Wolter M, Müller NS, Theis FJ, *et al*: MicroRNA-138 promotes acquired alkylator resistance in glioblastoma by targeting the Bcl-2-interacting mediator BIM. Oncotarget 7: 12937-12950, 2016.
24. Di Pascale F, Nama S, Muhuri M, Quah S, Ismail HM, Chan XHD, Sundaram GM, Ramalingam R, Burke B and Sampath P: C/EBPbeta mediates RNA polymerase III-driven transcription of oncomiR-138 in malignant gliomas. Nucleic Acids Res 46: 336-349, 2018.
25. Han L, Zhang G, Zhang N, Li H, Liu Y, Fu A and Zheng Y: Prognostic potential of microRNA-138 and its target mRNA PDK1 in sera for patients with non-small cell lung cancer. Med Oncol 31: 129, 2014.
26. Amable L: Cisplatin resistance and opportunities for precision medicine. Pharmacol Res 106: 27-36, 2016.
27. Postel-Vinay S and Soria JC: ERCC1 as predictor of platinum benefit in non-small-cell lung cancer. J Clin Oncol 35: 384-386, 2017.
28. Du P, Wang Y, Chen L, Gan Y and Wu Q: High ERCC1 expression is associated with platinum-resistance, but not survival in patients with epithelial ovarian cancer. Oncol Lett 12: 857-862, 2016.
29. Muallem MZ, Marnitz S, Richter R, Kohler C, Sehouli J and Arsenic R: ERCC1 expression as a predictive marker of cervical cancer treated with cisplatin-based chemoradiation. Anticancer Res 34: 401-406, 2014.

30. Li S, Wu J, Chen Y, Tang W, Peng Q, Deng Y, Xie L, Wang J, Huang S, Li R, *et al*: ERCC1 expression levels predict the outcome of platinum-based chemotherapies in advanced bladder cancer: A meta-analysis. Anticancer Drugs 25: 106-114, 2014.
31. Wan J, Chao L, Lee AC and Chen Q: Higher expression of ERCC1 may be associated with resistance to adjuvant platinum-based chemotherapy in gastric cancer. Cancer Invest 35: 85-91, 2017.
32. Stevens EV, Nishizuka S, Antony S, Reimers M, Varma S, Young L, Munson PJ, Weinstein JN, Kohn EC and Pommier Y: Predicting cisplatin and trabectedin drug sensitivity in ovarian and colon cancers. Mol Cancer Ther 7: 10-18, 2008.
33. Xie QH, He XX, Chang Y, Sun SZ, Jiang X, Li PY and Lin JS: miR-192 inhibits nucleotide excision repair by targeting ERCC3 and ERCC4 in HepG2.2.15 cells. Biochem Biophys Res Commun 410: 440-445, 2011.