MicroRNA-218 Increases the Sensitivity of Bladder Cancer to Cisplatin by Targeting Glut1

Peng Li, Xiao Yang, Yidong Cheng, Xiaolei Zhang, Chengdi Yang, Xiaheng Deng, Pengchao Li, Jun Tao, Haiwei Yang, Jifu Wei, Jingyuan Tang, Wenbo Yuan, Xiaoting Xu, Qiang Lu, Min Gu

© 2017 The Author(s). Published by S. Karger AG, Basel

Key Words
MiR-218 • Glut1 • Cisplatin • Chemo-sensitivity • Bladder cancer

Abstract
Background/Aims: MicroRNA-218 (miR-218) is down-regulated in many malignancies that have been implicated in the regulation of diverse processes in cancer cells. However, the involvement of miR-218 in chemo-sensitivity to cisplatin and the precise mechanism of this action remained unknown in bladder cancer. Methods: qRT-PCR was used to detect miR-218 and its target Glut1 expression in bladder cancer cell lines T24 and EJ. CCK-8 method was utilized to measure the cell viability. IC 50 was calculated via a probit regression model. Glut1 was detected by western blotting for analysis of potential mechanism. Luciferase reporter assay was utilized to validate Glut1 as a direct target gene of miR-218. The intracellular level of GSH and ROS were determined using a commercial colorimetric assay kit and 2',7'-dichlorodihydro-fluorescein diacetate, respectively. Results: Over-expression of miR-218 significantly reduced the rate of glucose uptake and total level of GSH and enhanced the chemo-sensitivity of bladder cancer to cisplatin. Mechanistically, Glut1 was found to be a direct and functional target of miR-218. Up-regulation of Glut1 could restore chemo-resistance in T24 and EJ cells. On the contrary, knockdown of Glut1 could generate a similar effect as up-regulating the expression of miR-218. Conclusions: MiR-218 increases the sensitivity of bladder cancer to cisplatin by targeting Glut1. Restoration of miR-218 and repression of glut1 may provide a potential strategy to restore chemo-sensitivity in bladder cancer.
What's more, 10%–20% of all recurring patients are at a high risk of progressing to muscle-invasive bladder cancer (MIBC) [2]. Chemotherapy is one of the most important treatments utilised in bladder cancer to reduce relapse, prevent progress and improve survival. Although cisplatin-containing combination chemotherapy has been the standard of care since the late 1980s [3], the cisplatin/gemcitabine (GC) regimen has a median time-to-progression of only 6 months and has no effect on overall survival after radical cystectomy in high-risk patients [4]. In addition, chemotherapy has failed in a large proportion of patients because of chemotherapy resistance, which leads to the relapse and progression of tumours. Therefore, chemotherapy resistance has become a major obstacle to the success of cancer management.

Cis-diaminedichloroplatinum (II), also known as cisplatin, is a platinum-based drug employed to manage a wide range of malignancies, including bladder cancer [5]. Cisplatin mediates anticancer effects via multiple mechanisms, the most prominent of which is inducing oxidative stress [6]. Nevertheless, many cancers demonstrate chemo-resistance towards cisplatin treatment. Because chemo-resistance, numerous of cisplatin-treated patients must tolerate various side effects without actually benefiting from chemotherapy. Various of mechanisms, such as reduced uptake, increased efflux, increased inactivation of ROS and increased levels of intracellular GSH, amongst others [7] are involved in this phenomenon. Recent studies indicate that microRNAs (miRNAs) may regulate these processes [8].

MiRNAs are endogenous, short non-coding RNAs of approximately 18–25 nt in length. MiRNAs negatively regulate protein-coding gene expression post-transcriptionally by targeting the mRNA 3'-untranslated regions (3'-UTR) [9, 10]. The association between miRNAs and tumour chemo-sensitivity has recently attracted increased attention [8]. Some research has shown that miR-218 acts as a tumour-suppressing miRNA in many cancers, including glioma, cervical cancer and colon cancer [11-13]. Our previous study showed that miR-218 expression was down-regulated in clinical bladder cancer tissues and inhibited the proliferation, migration and invasion of bladder cancer cells [14]. However, the association between miR-218 and the sensitivity of bladder cancer to cisplatin-based treatment remains unrevealed.

Glut1(Glucose transporter isoform 1) is a key rate-limiting enzyme in controlling glycolytic flux in cells [15] and plays a crucial role in tumourigenesis and progression [16]. Glut1 is over-expressed and associated with poor prognosis in varieties of malignancies including bladder cancer [17-19]. Over-expression of Glut1 enhances glycolytic activity, increases cancer cell proliferation and promotes the invasion and metastatic proficiency of cancers [20, 21]. However, the role of Glut1 in the development of cisplatin resistance in bladder cancer remains unknown.

In this study, we investigate the relationship between miR-218 and the sensitivity of bladder cancer cells to cisplatin. Glut1 is also predicted and confirmed as a target gene of miR-218. Our study contributes to the further understanding of the role of miR-218 in bladder cancer and provides a novel option for treatment of bladder cancer.

**Materials and Methods**

**Cell culture**

The human bladder cancer cell lines T24 and EJ were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM medium containing 10% foetal bovine serum (FBS; Gibco, Australia) and 1% penicillin–streptomycin. All cells were cultured in an incubator with humidified 5% CO\(_2\) at 37°C.

**Transfection**

All of the miRNA mimics and siRNA used for transfection were synthesised by GenePharma (Shanghai, China). The Glut1 overexpression and non-sense plasmids were constructed by GeneCopoeia(Shanghai, China). Cells at about 70% confluence were seeded into six-well plates and transfected with 50 nM mature miR-218 mimics, Glut1 over-expression plasmid or Glut1 siRNA and their non-sense controls.
using Lipofectamine 2000 or Lipofectamine 3000 transfection reagent (Invitrogen, USA) according to the manufacturer’s protocol. The cells were harvested 48 h after transfection.

**Plasmid constructs and luciferase reporter assay**

To construct the Glut1 expression plasmids, the wide-type or mutant 3’UTR of Glut1 gene was cloned into the pEZX vector. For the luciferase reporter assay, T24 cells were seeded into 24-well plates and incubated overnight. The T24 cells were then co-transfected with plasmid containing pEZX/Glut1-3’UTR or pEZX/Glut1-3’UTR-mutant and miR-218 mimics or the control using Lipofectamine 2000 (Invitrogen). After 24 h of transfection, the cells were lysed and Firefly and Renilla luciferase activities were measured by a microplate reader using Luc-Pair miR Luciferase Assay (GeneCopoeia, FulenGen, China). Normalised data were obtained by calculating the ratios of Firefly to Renilla luciferase luminescence. All experiments were performed in triplicate.

**IC₅₀ determination**

Twenty-four hours after transfection, cells were digested and seeded into 96-well plates at a density of 5000 cells per well in triplicate and incubated overnight. Then, the cells were treated with a series of dilute concentrations of cisplatin (Sigma). Cell viability was measured after 48 h using the CCK-8 method (CCK8, Dojindo, Japan) according to the manufacturer’s instructions. The probit regression model was utilised to calculate the IC₅₀ as previous described [22]. All experiments were independently performed three times.

**Measurement of intracellular reactive oxygen species**

The intracellular reactive oxygen species (ROS) were measured using the fluorogenic reagent 2’, 7’-dichlorodihydro-fluorescein diacetate (Invitrogen) according to the manufacturer's protocols. DCFH-DA substrate presents no fluorescence by itself and is deacetylated into dichlorofluorescin (DCFH) intracellularly by esterase. DCFH is further oxidised into the fluorescent compound dichlorofluorescein (DCF) by intracellular peroxides. Cells were seeded into 96-well plates at a density of 5000 cells per well in triplicate and incubated overnight. The cells were washed twice with PBS and incubated in 10 μM DCFH-DA at 37°C for 30 min. The fluorescence intensity of the incubation solution was measured at 530 nm using a microplate reader.

**Determination of total cellular glutathione content**

Intracellular levels of reduced glutathione (GSH) were determined using a GSH colorimetric assay kit (BioVision, USA) according to the manufacturer’s instructions. Briefly, 48 h after transfection, cells were harvested, washed twice with PBS, disrupted using ultrasound and centrifuged at 800×g for 10 min at 4°C. Supernatants were isolated and detected for total intracellular GSH by mixing with dithiobisnitrobenzoic acid (DTNB). Absorbance was measured at 405 nm using a microplate reader.

**RNA extraction and qRT-PCR**

Total RNA and miRNA were extracted from transfected cells using Trizol reagent (Invitrogen) and a miRNeasy Mini Kit (Qiagen), respectively. Real-time qRT-PCR was performed to detect the expressions of Glut1 and miR-218 by using SYBR Green PCR Kit (Takara, Japan) according to the manufacturer’s protocol. Briefly, 500 ng total RNA was used for the initial reverse transcription reaction with the gene-specific stem-loop RT primer available in the kit. Here, β-actin and U6 RNAs were used as internal controls for mRNA and miRNA detection, respectively. The qPCR primers were obtained from Sango Biotech (Shanghai, China), and the sequences were as follows: Glut1 (forward: 5’-AAC TCT TCA GCC AGG GTC CAC-3’; reverse: 5’-CAC AGT GAA GAT GAA GAC-3’); β-actin (forward 5’-AGC GAG CAT CCC CCA AAG TT-3’; reverse 5’-GGG CAC GAA GCC TCA TCA TT-3’). Each experiment was replicated three times and data were analysed using the 2⁻ΔΔCT method.

**Protein extraction and Western blot analysis**

The primary antibodies used in this study, including Glut1 and β-actin, were products of Cell Signalling Technology (CST, USA). The transfected cells were lysed and total protein was extracted using RIPA lysis buffer (Beyotime, China). Protein concentration was quantified and a total of 40 μg of proteins was loaded onto 10% SDS-PAGE gels. After electrophoresis, the proteins were transferred to PVDF membranes. The
membranes were blocked for 2 h at room temperature with 5% non-fat milk in TBST and then incubated with primary antibodies at 4°C overnight. The membranes were then washed three times in TBST and incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The blots were developed with ECL solution (Pierce, Rockford, IL, USA) and detected by using a chemiluminescence system (Bio-Rad, USA). Image Lab Software was employed to analyse the intensities of the band signals obtained.

**Glucose uptake assay**

Approximately 24 h before the glucose uptake experiments, cells were plated on six-well plates at a density of $3 \times 10^5$ cells/well, washed twice with phosphate-buffered saline (PBS), and then incubated in 2 ml of Krebs–Ringer–HEPES (KRH) buffer (25 mM Hepes, pH 7.4, 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO$_4$, 1.3 mM CaCl$_2$, 1.3 mM KH$_2$PO$_4$) containing 1 μCi of [3H]-2-deoxyglucose (Perkin Elmer Life Sciences) at 37°C for 20 min to initiate the experiments. The cells were consequently washed twice with ice-cold KRH buffer to halt uptake and lysed in 300 μl of lysis buffer [10 mM Tris–HCl, pH 8.0, 0.2% sodium dodecyl sulphate (SDS)]. Radioactivity was measured by liquid scintillation spectrometry. Disintegrations per minute (DPM) were used to evaluate the intracellular level of [3H]-2-deoxyglucose, and each assay was performed in triplicate.

**Statistical analysis**

All data are presented as mean ± standard deviation. Statistical analysis was performed using SPSS software (version 17.0; IBM Corporation). Student’s $t$ test was utilised to analyse differences between two groups. Statistical significance was considered at 0.05. Graphical presentations were performed using GraphPad Prism 5 software (San Diego, CA). All experiments were repeated more than three times, and each experiment was performed in triplicate.

**Results**

**Over-expression of microRNA-218 restores drug sensitivity**

To investigate the effects of miR-218 on bladder cancer cell lines, we transfected miR-218 mimics or the non-sense control (NC) into T24 and EJ cells. RT-PCR was performed to confirm that miR-218 was significantly increased in the miR-218 mimics group compared with that in the NC group (Fig. 1A). CCK-8 assay showed that compared with NC, over-expression of miR-218 could dramatically inhibit the proliferation of T24 and EJ cells in a series of dilute concentrations of cisplatin (Fig. 1B). These results indicate that increased expression of miR-218 enhanced cisplatin sensitivity in T24 and EJ cells (Fig. 1C).

Altered metabolism (the Warburg effect), which manifests as increased glucose uptake and decreased oxidative phosphorylation, is considered a hallmark of cancer. We determined whether overexpression of miR-218 could affect the metabolism of T24 and EJ cells. Glucose uptake assay suggested that miR-218 over-expression significantly decreased the rate of glucose uptake (Fig. 1D) compared with the control. Reduced glutathione (GSH) is a major cellular metabolite that protects against oxidative and chemical injury and exhibits a variety of other cytoprotective effects. High levels of GSH as well as increased expression of antioxidant enzymes promote cancer cell survival and resistance to anticancer agents [23]. We detected the intracellular level of GSH by utilising a reduced GSH assay and found that over-expressed miR-218 inhibited the generation of intracellular GSH (Fig. 1E). Intracellular ROS levels were also measured and results implied that miR-218 increased the production of ROS (Fig. 1F). All these results imply that miR-218 could promote sensitivity to cisplatin in the T24 and EJ cell lines via various mechanisms.

**MicroRNA-218 directly targets Glut1 3′-UTR and negatively regulates its expression**

Glut1, a key rate-limiting enzyme in the transport of glucose into cancer cells, is over-expressed in many cancers. Our previous study found that Glut1 was dramatically over-expressed in clinical bladder cancer tissue whereas miR-218 was markedly down-regulated [14, 24]. Therefore, we sought to determine whether Glut1 is regulated by miR-218. qRT-PCR and Western blot assay were performed to detect the effect of miR-218 on the expression...
of Glut1, and results demonstrated that miR-218 induced significant decreases in Glut1 compared with the NC group at both the mRNA and protein levels (Fig. 2B, 2D).

Utilising the programs picTar, TargetScan (http://www.targetscan.org) and miRNA (http://www.microrna.org), potential binding sites in the 3'UTR of Glut1 were found (Fig. 2A). To investigate whether the Glut1 3'-UTR is a direct target of miR-218 further, we constructed vectors encoding the full length of the 3'-UTR of Glut1 mRNA to perform a dual luciferase reporter assay (Fig. 2C). Co-transfection experiments implied that miR-218 significantly reduced the luciferase activity of Glut1 containing a wild-type 3'-UTR but not that of Glut1 containing mutant-type 3'-UTR (Fig. 2C). These results confirmed that Glut1 is negatively regulated by miR-218 and is a direct target of miR-218.

**Knockdown of Glut1 increases the sensitivity to cisplatin in T24 and EJ cells**

Our work showed in Fig. 2 confirmed that miR-218 depresses the expression of Glut1 at both the mRNA and protein levels by binding to its 3'UTR. However, the role of Glut1 in the development of cisplatin resistance remains unknown. We performed loss-of-function and gain-of-function experiments to study the Glut1 gene more extensively. T24 and EJ cells
were transfected with small-interfering (si) Glut1 or NC, and the mRNA and protein levels of Glut1 were subsequently detected by qRT-PCR and Western blot. The results showed the depressed expression of Glut1 (Fig. 3A, 3B). Compared with the NC group, cells transfected with si-Glut1 showed significantly inhibited viability and more sensitivity to cisplatin in a series of dilute concentrations (Fig. 3C, 3D). As well, the results of reduced GSH assay suggested that intracellular GSH contents dramatically decreased by knockdown of Glut1 in T24 and EJ cells (Fig. 3E). Blockage of Glut1 also markedly promoted ROS generation (Fig. 3F). These results were consistent in cells over-expressing miR-218 and suggested that miR-218 regulates cisplatin chemo-sensitivity in bladder cancer by targeting Glut1.

Over-expressed Glut1 regains drug-resistance in T24 and EJ cells
To study the function of Glut1 in regulating the chemo-sensitivity of bladder cancer cells to cisplatin, we examined the effect of over-expressed Glut1 in T24 and EJ cells. qRT-PCR and Western blot analysis verified that the expression of Glut1 was significantly enhanced at both the mRNA and protein levels (Fig. 4A, 4B). In addition, up-regulated expression of Glut1 promoted the proliferation of T24 and EJ cells and restored their chemo-resistance to cisplatin (Fig. 4C, 4D). Moreover, increased generation of intracellular GSH and decreased levels of ROS were observed in cells transfected with Glut1 plasmids (Fig. 4E, 4F). All of these results indicated that miR-218 enhances the chemo-sensitivity of bladder cancer to cisplatin by targeting Glut1.
Over-expression of Glut1 rescues chemoresistance in miR-218 transfected EJ and T24 cells

To further confirm that miR-218 regulates cisplatin chemo-sensitivity in bladder cancer by targeting Glut1, we performed a restoration experiment through transfecting Glut1 and miR-218 into bladder cancer cells at the same time. IC50 determination assay showed that upregulation of Glut1 restored the chemo-sensitivity induced by overexpressing of miR-218 in EJ and T24 cells (Fig. 5A). In addition, the decreased generation of intracellular GSH and increased level of ROS due to miR-218 were reversed by transfecting Glut1 into EJ and T24 cells (Fig. 5B, 5C). Moreover, transfection of Glut1 into cells rescued the miR-218-induced downregulation of Glut1 at different concentrations of miR-218 (Fig. 5D). In summary, these results indicated that miR-218 play a crucial role in regulating the sensitivity to cisplatin in bladder cancer cells by directly targeting Glut1.

Discussion

Herein, we demonstrate a novel regulatory signalling mechanism, the miR-218- Glut1 axis, which plays a crucial role in linking miRNAs with cisplatin sensitivity. Our results
indicated that up-regulated miR-218 could increase the cisplatin sensitivity of bladder cancer cells accompanied by reduced glucose uptake, total cellular GSH content and enhanced ROS in T24 and EJ cells. These findings demonstrate that dysregulation of miR-218 could contribute to the decreased sensitivity of bladder cancer cells to cisplatin by glucose metabolism.

Chemotherapy agents, especially cisplatin, are widely used in the management of bladder cancer to reduce relapse, prevent progress and improve survival. However, many tumours have developed chemo-resistance towards cisplatin, a problem that has become a major obstacle in managing bladder cancer. Explorations of the relationship between miRNAs and tumour chemo-sensitivity, in particular, have recently become a research hotspot. Previous studies have confirmed that miR-218 serves as a tumour suppressor in numerous human cancers and over-expressed miR-218 could inhibit the growth and regulate chemo-sensitivity in certain tumours, including gastric cancer and cervical cancer [25, 26]. It is well known that one miRNA may target many genes in different cell systems and miR-218 is no exception. There are several relatively common target genes of miR-218 in cancers such as BMI-1 [14, 27, 28], surviving [29-32] and HMGB1 [26, 33-36]. BMI-1 is a member of the polycomb group (PcG) of genes and is the first identified PcG transcriptional
repressor. Survivin, the smallest member of the inhibitor of apoptosis (IAP) family, serves as an oncogene due to its anti-apoptotic properties. HMGB1 belongs to the high mobility group protein superfamily and plays important roles in the malignant behaviors in cancers. MiR-218 functions as a tumour suppressor by targeting BMI-1, survivin or HMGB1 in different types of cancers. Moreover, miR-218 has been found to regulate chemosensitivity in lung cancer [30], pancreatic cancer [26] and overexpression of miR-218 could induce apoptosis and increase sensitivity to chemotherapeutics in breast cancer by targeting Surviving [32].

In our previous study, we found that miR-218 was significantly down-regulated in bladder cancer tissues and that miR-218 could inhibit bladder cancer cell proliferation, migration and invasion by targeting BMI-1 [14]. In the present study, we investigated the association between miR-218 and the sensitivity of T24 and EJ cells to cisplatin by transfecting miR-218 mimics into T24 and EJ cells.

Our results showed that over-expression of miR-218 could dramatically inhibit the proliferation of T24 and EJ cells and promote their sensitivity to cisplatin. Reduced generation of GSH and increased levels of ROS were also observed in cells transfected with miR-218 mimics. GSH generation depends on the rate of glucose uptake, and Glut1 is a key rate-limiting factor in the transport of glucose into cancer cells, playing important roles in tumourigenesis and tumour progression [16]. Glut1 is over-expressed in a variety of malignancies including bladder cancer, [24, 37-40] and over-expression of Glut1 enhances glycolytic activity, increases cancer cell proliferation and promotes the invasion and metastatic proficiency of cancers [20, 21]. Similarly, our previous study showed that Glut1 was up-regulated in bladder cancer tissues compared with that in adjacent normal tissues [24]. As such, we explored the function of Glut1 and its relationship with the GSH–ROS balance in bladder cancer cells.

A number of studies have recently found that glucose restriction during chemotherapy potentiates sensitivity to chemotherapy agents in breast cancer, melanoma and neuroblastoma in vivo [41]. In addition, research has demonstrated that Glut1 may confer
resistance to chemotherapy agents in a hypoxia-independent manner [42]. Consistent with previous studies, our results showed that over-expression of Glut1 could promote the proliferation of T24 and EJ cells in a series of dilute concentrations of cisplatin and enhance their chemo-resistance to cisplatin. We also observed dramatically increased levels of GSH and reduced ROS in cells over-expressing Glut1. Intracellular GSH is a major endogenous antioxidant that protects cells from oxidative injury. GSH is considered to play a vital role in regulating the chemo-sensitivity of tumour cells [43-48]. Intracellular levels of ROS have also been found to be closely related to the chemo-sensitivity of cancer cells [49, 50]. Our results verify these conclusions to a deeper extent. In contrast, siRNA-induced silence of Glut1 generates opposite effects to those over-expressing Glut1 in T24 and EJ cells. Assays showed more sensitivity to cisplatin, reduced GSH and increased ROS production in T24 and EJ cells transfected with si-Glut1.

Bioinformatics analysis has demonstrated that Glut1 is a potential direct target of miR-218 in bladder cancer. In the present study, dual luciferase reporter assay confirmed that miR-218 directly targets Glut1. qRT-PCR and Western blot experiments further implied that over-expression of miR-218 could prominently down-regulate the expression level of Glut1 in T24 and EJ cells at both the mRNA and protein levels. Moreover, as mentioned above, over-expressed miR-218 shows effects similar to those observed when cells are transfected with si-Glut1. These results indicate that miR-218 could promote sensitivity to cisplatin in T24 and EJ cells targeted by si-Glut1.

Based on our previous study [14, 24] and the present findings, we conclude that restoration of reduced miR-218 levels enhances the sensitivity of bladder cancer cells to cisplatin by directly targeting Glut1. Target miR-218 and Glut1 may provide a potential strategy to restore chemo-sensitivity in bladder cancer.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grants No. 81272832, 81201997 and 81602235), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) and Jiangsu Provincial Special Program of Medical Science (BL2012027). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclosure Statement

Neither author has a competing interest to disclose.

References

Li et al.: Sensitization of Bladder Cancer to Cisplatin by miR-218-Glut1 Pathway

Li et al.: Sensitization of Bladder Cancer to Cisplatin by miR-218-Glut1 Pathway


