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Original Research

miR-214 modulates cisplatin sensitivity of osteosarcoma cells through regulation of anaerobic glycolysis

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Abstract: Osteosarcoma is the most frequent primary bone tumor originating from adolescents and young adults. Despite improvements in the chemo- or radiotherapy of osteosarcoma patients, survival rate has not increased and drug resistance becomes a major factor that limits the effectiveness. Therefore, investigation of new treatment modalities is urgently required to optimize therapeutic options. Our previous study described an oncogenic role of miR-214 through promotion of osteosarcoma cells proliferation. In this study, we report miR-214 contributes to cisplatin resistance in osteosarcoma cells. Overexpression of miR-214 decreased the cisplatin sensitivity. By establishing an osteosarcoma cisplatin resistant cell line, we find miR-214 is significantly upregulated in cisplatin resistant cells. Moreover, we show miR-214 promotes anaerobic glycolysis rates of osteosarcoma cells but suppresses mitochondrial oxidative phosphorylation. Consistently, cisplatin resistant cells exhibit upregulated glycolysis but decreased mitochondrial oxidative phosphorylation, a phenotype called "Warburg effect". Finally, we demonstrate inhibition of glycolysis by either glycolysis inhibitor or miR-214 inhibition significantly re-sensitizes cisplatin resistant osteosarcoma cells. In summary, this study illustrates a miRNA-involved chemosensitivity of osteosarcoma and will contribute to the developments of therapeutic agents for the anti-chemoresistance treatments.

Key words: miR-214; Osteosarcoma cells; Anaerobic glycolysis.

Introduction

Osteosarcoma (OS) is the most frequent primary bone tumor originated from adolescents and young adults (1). The incidence of OS in the general population is three cases per million per year, with peak occurrence in a relative late stage, which need radiotherapy or/and chemotherapy (2). However, osteosarcoma is notorious for the pool response to chemotherapy especially for the young adults and the survival rate has not increased over the last two decades (3). Cisplatin is a well-known chemotherapeutic drug used for treatment of numerous human cancers through crosslink with the purine bases on the DNA to cause DNA damage, and subsequently inducing apoptosis in cancer cells (4). Acquirement of drug resistance is a major factor that limits the effectiveness of cisplatin (5). Currently, the molecular mechanisms of chemoresistance for osteosarcoma treatments have not been fully understood. Thus, improved understanding of the mechanisms underlying osteosarcoma chemoresistance is urgently required to optimize therapeutic options.

MicroRNAs (miRNAs) are a class of small, non-coding, single-stranded RNAs that regulate gene expression through binding to the 3' untranslated region (UTR) of target mRNAs (6, 7). These binding leads to translational repression and/or degradation of their target mRNAs (6, 7). It is well studied that miRNAs regulate a variety of biological processes of cancer, including proliferation, differentiation, migration, metabolism and apoptosis (8). Moreover, specific miRNAs with essential functions were dysregulated in cancers, resulting in aberrant gene expression in tumor initiation, development and metastasis (9). MiR-214 have been implicated in carcinogenesis and tumor progression in OS and other cancers (10, 11, 12). Our previous study reported miR-214 was upregulated in human osteosarcoma tissues and cells (13). We found miR-214 promotes the proliferation and invasion of osteosarcoma cells through direct suppression of LZTS1 (13). In addition, another study described that miR-214 promotes osteosarcoma tumor growth and metastasis by decreasing the expression of PTEN (14), support our previous conclusion that miR-214 acts as an oncogenic miRNA in osteosarcoma. However, the precise role of miR-214 in chemosensitivity of osteosarcoma cells remains unclear at present. In this study, we demonstrate miR-214 promotes the cisplatin resistance in osteosarcoma cells. Through establishing cisplatin resistant cell line from U2OS osteosarcoma cells, the mechanisms for the regulating chemosensitivity will be investigated.

Materials and Methods

Cell culture

Human OS cell lines, Saos-2 and U2OS, were ob-

tained from Cell Bank of Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 100 units/ml of Penicillin-streptomycin (Invitrogen, Carlsbad, CA). Cells were cultured at 37 °C in a humidified incubator containing 5% CO_2.

MiRNA mimics and inhibitor transfection

Cells were seeded into six-well plates and transfected with miR-214 mimics, anti-miR-214 inhibitor or controls using Lipofectamine RNAi MAX (Invitrogen) according to the manufacturer's instructions. Cells were harvested for assay 48 h after transfection.

Generation of cisplatin resistant cell line

The generation of cisplatin resistant U2OS cell line was performed according to the previous report (15). Briefly, U2OS parental cells were treated with gradually increased concentrations of cisplatin from 8-48 μM for the selection of survival cells. After 4 months' treatment, survival cell clones were pooled and characterized as cisplatin resistant cells for the following experiments of this study.

RNA preparation and quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. qRT-PCR for miR-214 detection was performed using 10 ng total RNA and the TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. For glycolytic enzyme mRNAs detection, 1 μg of DNAse-treated RNA was retrotranscribed with the High Capacity cDNA Reverse Transcription Kit, and qRTPCR carried out using gene-specific primers on a 7900HT Fast Real Time PCR System. Quantitative normalization was performed on the expression of RNU6 small nucleolar RNA or GAPDH genes for miR-214 and glycolytic enzyme mRNAs, respectively. Relative expression levels between samples were calculated using the comparative delta CT (threshold cycle number) method ($2^{-\Delta\Delta CT}$). The primers for qPCR are: LDHA: forward: 5'-GGATGAGCTTGCCCTTGTTGA-3'; reverse: 5'-GACCAGCTTGGAGTTCGCAGTTA-3'; PKM2: forward: 5'-GTCTGGAGAAACAGCCAAGG-3'; reverse: 5'-CGGAGTTCCTCGAATAGCTG-3'; HK2: forward: 5'-CAAAGTGACAGTGGGTGTGG-3′; reverse: 5′-GCCAGGTCCTTCACTGTCTC-3′; GAPDH: forward: 5'-AGCTTGTCATCAACGGGAAG-3'; reverse: 5'-TTTGATGTTAGTGGGGTCTCG-3'.

Glucose uptake and lactate production assays

The measurements of glucose uptake and lactate production were performed using the Glucose Uptake Colorimetric Assay Kit (Biovision, # K676 and the Lactate Colorimetric/Fluorometric Assay Kit (Biovision, #K607) according to the manufacturer's instructions. Equal number of cells were analyzed for each test. Experiments were performed at triplicate and data were normalized by protein concentration.

Oxygen consumption

The measurements of oxygen consumption were performed using the Extracellular Oxygen Consumption Assay (Abcam, #ab197243) according to the manufacturer's instructions. Equal number of cells were analyzed for each test. Experiments were performed at triplicate and data were normalized by protein concentration.

Cell viability assays

The viabilities of osteosarcoma cells in response to cisplatin were measured by MTT assay according to previous report (10). Cells were seeded at a density of 2000 cells per well in 96-well plates and cultured for 2 days after transfection. Next, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma, St. Louis, MO) reagent (5 mg/ml) was added, followed by incubation for 4 h. Supernatant fractions were discarded and 150 ll/well of DMSO added to terminate the reaction. Absorbance readings at 490 nm were obtained in triplicate using a spectrophotometric plate reader (Thermo Scientific, Waltham, MA). Results were averaged from three independent experiments.

Western blot analysis

Total proteins were extracted from cells using RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 7.5) plus 1% complete protease inhibitor. Lysates were clarified via centrifugation at 13,000 rpm for 5 min at 4 °C, and the supernatant fractionated using SDS-PAGE and subsequently transferred onto nitrocellulose membrane, followed by blocking with 5% non-fat milk buffer for 1 hour. Proteins were incubated with anti-HK2, anti-LDHA, anti-PKM2 (Cell Signaling #8337) and anti-GAPDH antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for overnight. After washing with TBST buffer, membranes were incubated with secondary antibody for 1 hour at room temperature. Membranes were exposed to LAS 3000 (Fuji Photo Film Co. Ltd., Tokyo, Japan).

Statistical analysis

All data are expressed as means ± standard deviation from three independent experiments. Statistical analyses were performed using SPSS16.0 software (SPSS, Chicago, IL). The differences between groups were analyzed using Student's t-test. *P* values less than 0.05 were considered statistically significant.

Results

Overexpression of miR-214 renders osteosarcoma cells resistant to cisplatin

Our previous study demonstrated an oncogenic role of miR-214 in osteosarcoma (13), suggesting inhibition of miR-214 might be an effective therapeutic approach for the treatments of osteosarcoma patients. To further investigate the function of miR-214 in chemosensitivity of osteosarcoma, we tested the cytotoxicity of osteosarcoma cells Saos-2 and U2OS in response to *in vitro* cisplatin treatments with or without overexpression of miR-214. As an initial experiment, the miR-214 expression levels with or without miR-214 mimic transfection were determined (Fig. 1A). As we expected, Saos-2 and U2OS cells with overexpression of miR-214 showed si-

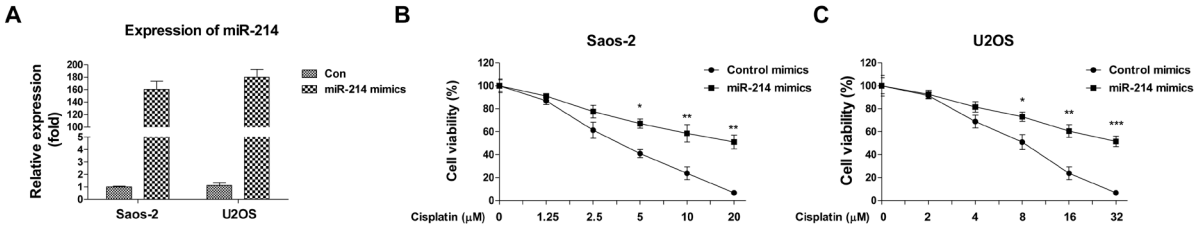

Figure 1. Influence of miR-214 on cell viabilities in response to cisplatin treatments. (A) Saos-2 and U2OS were transfected with control mimics or miR-214 mimics for 48 hours. The expressions of miR-214 were detected by qRT-PCR. (B) Saos-2 cells or (C) U2OS cells were transfected with control mimics or miR-214 mimics for 48 hours, followed by cisplatin treatments at the indicated concentrations for 72 hours. Cell viabilities were measured by MTT assay. Independent experiments were performed in triplicate. *, p <0.05; **, p <0.01; **, p <0.001.

gnificantly resistant to cisplatin treatments (Fig. 1B-1C). Saos-2 and U2OS cells with control miRNAs transfection were sensitive to cisplatin with an IC50 around 3.63 μM and 8.52 μM, respectively. Overexpression of miR-214 renders Saos-2 and U2OS cells resistant to cisplatin with an elevated IC50 around 22 μM and 34 μM (Fig. 1B-1C). Taken together, these results show an oncogenic role of miR-214, overexpression of which decreases the sensitivity of osteosarcoma cells to cisplatin.

MiR-214 is upregulated in cisplatin resistant cells

In order to verify the roles of miR-214, we established a cisplatin resistant cell lines originating from U2OS parental cells. As shown in figure 2A, CDDP resistant cells exhibited an increased IC50 (45.6 μM) compared with parental cells. Moreover, CDDP resistant cells showed less cytotoxicity under the cisplatin treatment at 15 μM (Fig. 2B). Results in figure 2C demonstrated miR-214 was significantly upregulated in U2OS cisplatin resistant cells compared with their parental cells, supporting the above results that miR-214 contributes to the cisplatin resistance.

MiR-214 drives a metabolic switch of osteosarcoma cells

We next investigated the mechanisms for the miR-214 modulated cisplatin resistance. Since cancer cells exhibit a metabolic reprogramming which have profound effects on tumorigenesis, we subsequently assessed the metabolism of osteosarcoma cells. Saos-2 and U2OS cells were transfected with control miR, miR-214 mimics or anti-miR-214, the glucose metabolism and mitochondrial respiration capacity were measured. As we expected, onco-miR-214 promoted glucose uptake and lactate production of SaoS-2 and U2OS cells (Fig. 3A, 3B). To assess the molecular mechanisms of the miR-214 mediated glucose metabolism, we tested the expressions of enzymes in the glucose metabolism pathway. The mRNA levels of Hexokinase 2 (HK2), PKM2 and LDHA were significantly upregulated in miR-214 overexpression osteosarcoma cells (Fig. 3C). In the contrast, the oxygen consumption was decreased in miR-214 overexpression cells (Fig. 3D). Taken together, the above results suggest an oncogenic role of miR-214 in promoting a metabolic switch from oxidative phosphorylation to anaerobic glycolysis.

Glucose metabolism is elevated in cisplatin resistant cells

To determine whether the miR-214 mediated anaerobic glycolysis is associated with cisplatin sensitivity of osteosarcoma cells, we assessed the glucose metabolic rate in cisplatin resistant and sensitive cells. As

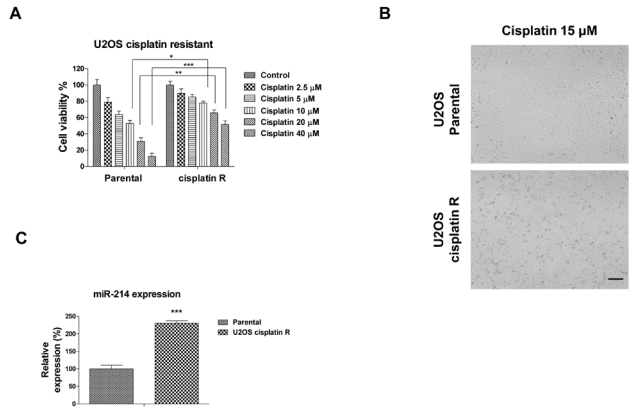

Figure 2. MiR-214 is upregulated in U2OS cisplatin resistant cells. (A) U2OS parental or cisplatin resistant cells were treated with 0, 2.5, 5, 10, 20, or 40 μM cisplatin for 72 hours, followed by the measurements of cell viabilities by MTT assay. (B) U2OS parental or cisplatin resistant cells were treated with 15 μM cisplatin for 72 hours, cell viability and morphological changes were monitored under microscopy. (C) The expressions of miR-214 were measured by qRT-PCR in U2OS parental or cisplatin resistant cells. Independent experiments were performed in triplicate. *, p <0.05; **, p <0.01; **, p <0.001.

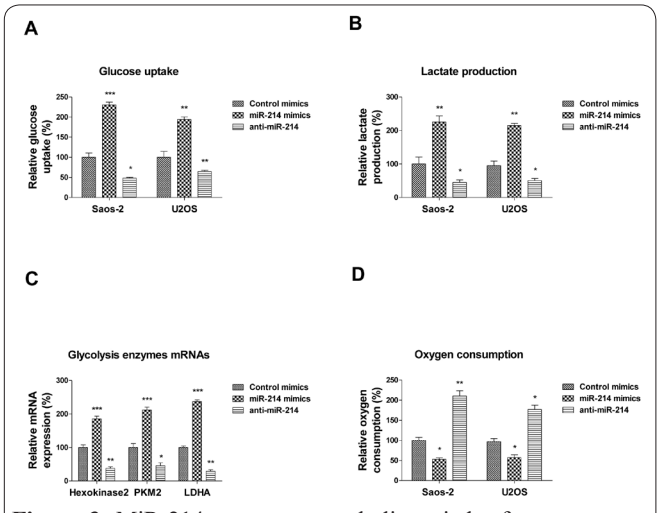

Figure 3. MiR-214 promotes metabolic switch of osteosarcoma cells. Saos-2 and U2OS cells were transfected by control, miR-214 mimics or miR-214 inhibitors for 48 hours. (A) The glucose uptake, (B) lactate production, (C) mRNAs of glycolysis enzymes and (D) oxygen consumption rate were compared. Independent experiments were performed in triplicate. *, p <0.05; **, p <0.01; **, p <0.001.

we expected, the glucose uptake and lactate production were significantly increased in cisplatin resistant U2OS cells compared with its parental cells (Fig. 4A, 4B). Moreover, the protein and mRNA expression of HK2, PKM2 and LDHA were upregulated in cisplatin resistant U2OS cells (Fig. 4C-4D). The oxygen consumption, was decreased in U2OS cisplatin resistant cells (Fig. 4E). These results indicated a tight association between miR-214, glycolysis and cisplatin resistance.

Inhibition of glycolysis by glycolysis inhibitor or miR-214 inhibitor sensitizes cisplatin resistant osteosarcoma cells to cisplatin

The above results revealed the miR-214 mediated glucose metabolism contributes to cisplatin resistance. To evaluate whether inhibition of miR-214 could sensitize cisplatin resistant osteosarcoma through suppression of glycolysis, we tested the cytotoxicity of cisplatin with combination of glycolysis inhibitor, 2-deoxyglucose (2-DG), which is a clinically relevant inhibitor of glucose metabolism (16). Treatments with cisplatin and 2-DG induced a significant growth delay of U2OS cells over the 72-h exposure period compared with control treatment, 2-DG or cisplatin alone (Fig. 5A). Moreover, we tested the effects of inhibiting miR-214 expression on the cell viabilities of cisplatin resistant U2OS cells. Transfection of miR-214 inhibitor significantly suppressed the miR-214 expression in cisplatin resistant cells, compared with control inhibitor (Fig. 5B). As we expected, cisplatin resistant U2OS cells with inhibition of miR-214 displayed a significant sensitization to cisplatin (Fig. 5C). Consistently, we observed the glucose metabolic enzymes were suppressed by miR-214 inhibition in cisplatin resistant U2OS cells (Fig. 5D). Taken together, these data demonstrated targeting miR-214 might be an effective approach for the development of therapeutic agents against chemoresistance.

Discussion

Our previous studies have shown that miR-214 functions as an oncomiR in human osteosarcoma with the

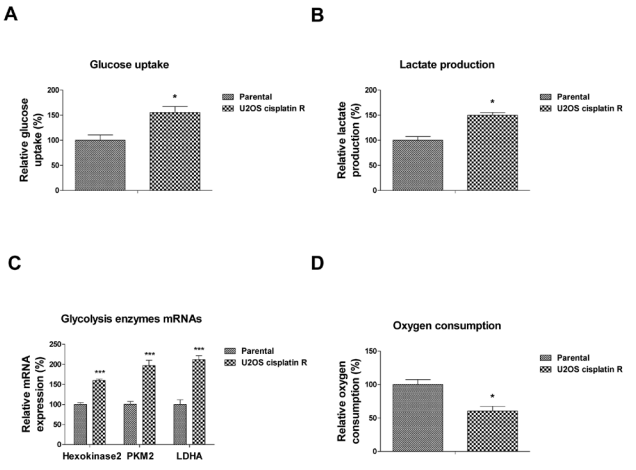

Figure 4. Cisplatin resistant osteosarcoma cells show increased glycolysis and decreased oxygen consumption rate. (A) The glucose uptake, (B) lactate production, (C) mRNAs of glycolysis enzymes and (D) oxygen consumption rate were compared between U2OS parental and cisplatin resistant cells. Independent experiments were performed in triplicate. *, p <0.05; **, p <0.001.

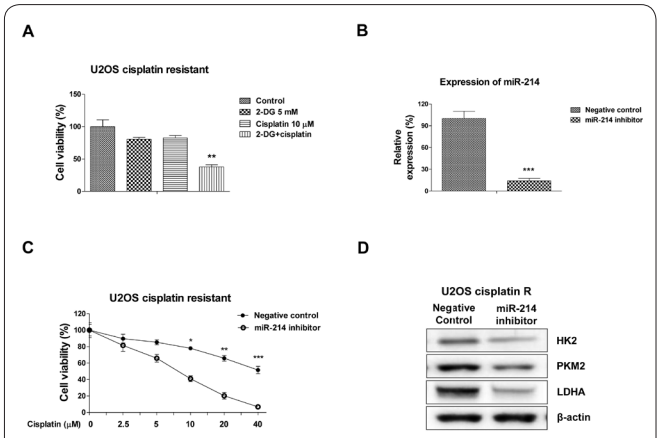

Figure 5. Inhibition the miR-214-mediated glycolysis re-sensitizes cisplatin resistant cells. (A) U2OS cisplatin resistant cells were treated with control, 2-DG alone, cisplatin alone or with the combination of 2-DG and cisplatin for 72 hours. Cell viabilities were measured by MTT assay. (B) U2OS cisplatin resistant cells were transfected with control or miR-214 inhibitor for 48 hours, the expression of miR-214 was detected by q-RT-PCR. (C) U2OS cisplatin resistant cells were transfected with control or miR-214 inhibitor for 48 hours, cells were treated with cisplatin at the indicated concentrations, cell viabilities were measured by MTT assay. (E) The expressions of HK2, PKM2 and LDHA were detected by Western blot. β-actin was a loading control. Independent experiments were performed in triplicate. *, p <0.05; **, p <0.01; **, p <0.001.

observation that miR-214 was dramatically upregulated in osteosarcoma tissues, suggesting its involvement in carcinogenesis (13). The role of miR-214 in chemosensitivity of osteosarcoma is still unclear. This study reports miR-214 promotes cisplatin resistance of osteosarcoma cells, suggesting miR-214 might be a therapeutic target against chemoresistance of osteosarcoma. Consistently, another study has reported the similar roles of miR-214 in cisplatin resistance in human ovarian cancer (17). However, in different tissues, miR-214 acts as reverse roles. It has been reported miR-214 enhances cisplatin-induced cytotoxicity via down-regulation of Bcl2l2 in cervical cancer cells (18). Moreover, miR-214-3p could enhances sensitivity to cisplatin in esophageal squamous cancer cells by different mechanisms (19). The putative explanations of these discrepancy may due to the diversity of direct targets of miR-214. Recently, the pro-survival molecule, Bcl2l2 (18), the inhibitor of apoptosis molecule, Survivin (19) and the oncogene, c-Myc (20) have been reported as direct targets of miR-214, suggesting the miR-214-mediated cisplatin sensitization was through inhibiting survival or oncogenic pathway. In the contrary, tumor suppressive genes such as PTEN (14, 17), LZTS1 (13), p53 (21) and LHX6 (22) have been reported as direct targets of miR-214, suggesting miR-214 contributes to the cisplatin resistance through upregulation of oncogenic pathway. Another explanation of these discrepancy may due to the tissue specific. It has been reported miR-214 promoted the cisplatin-induced cytotoxicity of multiple cancers such as esophageal squamous cancer (19) and cervical cancer (18). On the other way, miR-214 increased the cisplatin resistance of ovarian cancer (17), revealing a tissue specific role of miR-214 in cisplatin sensitivity of multiple cancers.

Cancer cells exhibit important alterations in their

metabolism, providing a survival advantages in unfavorable microenvironments (23). A well-studied metabolic adaptation of tumor cells is a shift to aerobic glycolysis from oxidative phosphorylation (OXPHOS), irrespective of oxygen availability (23). This phenomenon referred to as the "Warburg effect" (24). Moreover, this metabolic switch has been demonstrated to associate with other hallmarks of cancer, such as promotion of apoptosis resistance, the generation of biosynthetic precursors for proliferation and increased chemo- or radio-therapy resistance (25). Currently, the molecular mechanisms for this metabolic switch and how it contributes to behaviors of cancer cells remain elusive. Our study reveals miR-214 promotes cisplatin resistance of osteosarcoma cells. In addition, for the first time, we provided evidence that miR-214 drives a metabolic switch of osteosarcoma cells from mitochondrial oxidative phosphorylation to anaerobic glycolysis, consistent with its oncogenic roles as our early discovery. From comparison of parental osteosarcoma cells and cisplatin resistant cells, we showed cisplatin resistant osteosarcoma cells displayed increased glycolysis and decreased mitochondrial oxidative phosphorylation rate. Importantly, targeting abnormally upregulated glycolysis by glycolysis inhibitor or inhibiting miR-214 re-sensitized cisplatin resistant cells. The detailed mechanisms of how miR-214 regulates glycolysis and oxidative phosphorylation in osteosarcoma are under investigation by us. In our ongoing project, the potential targets of miR-214 involving in the glucose metabolic pathway will be identified. Moreover, how the dysregulated glucose metabolism could contribute to chemoresistance of osteosarcoma will be explored. In general, this study suggests inhibition of miR-214 might contribute to the development of anti-chemoresistant drugs against osteosarcoma.

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