Pathology - Research and Practice 216 (2020) 153-158

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: http://ees.elsevier.com

miR-192 enhances sensitivity of methotrexate drug to MG-63 osteosarcoma cancer cells

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ARTICLE INFO

Keywords
Apoptosis
Chemo-resistance
Methotrexate
miR-192
Osteosarcoma

ABSTRACT

Chemo-resistance remains a considerable obstacle encountered in osteosarcoma (OS) therapy. Evidence has implied that a reduction in the expression of microRNAs (miRs/miRNAs) leads to exacerbated chemo-resistance. Hence, to better understand the role of miR-192 in the pathogenesis of OS during methotrexate (MTX) treatment, we restore miR-192 in the MG-63 cells and investigate the mechanisms, which are associated with MTX-resistance in OS. Exogenetic overexpression of miR-192 was established by transfecting miR-192 mimics into MG-63 cells using Lipofectamine. Trypan blue dye exclusion test was performed to evaluate the proliferation of the MG-63 cells. Chemo-resistance to MTX was determined using the MTT method after 48 h. ELISA cell death assay was performed to evaluate the apoptosis rate. The quantitative RT-PCR (RT-qPCR) was applied to determine the mRNA expression levels before and after the transfection. Our results illustrated that miR-192 is down-regulated in OS tumor cells. Transfection of miR-192 noticeably alleviated the mRNA expression levels of MMP9, c-Myc, K-Ras, CXCR-4, and ADAMTS compared with the control groups (*P-values* < 0.05). MTX Combination treatment with miR-192 noticeably elevated the cytotoxic effect of MTX and alleviated its IC_{50} ($P < 0.05$). Moreover, miR-192 significantly increased the apoptotic effect of MTX. These results implied that miR-192 enhances the sensitivity of MG-63 cells to MTX. Collectively, our results elucidated that miR-192 contributes to chemo-sensitizing MG-63 cells to MTX, and could be considered as a promising agent to overcome MTX-resistance in OS.

1. Introduction

Osteosarcoma (OS) is considered as a highly progressive type of primary malignant bone cancer worldwide, which is distinguished by high prevalence in the metaphysis, such as the distal femur and proximal tibia and occurring mainly in males [1,2]. It typically ranks the 8th top tumor among teenagers, young adults, and adolescents and accounts for 60 % of all malignant childhood bone tumors [3]. The current standard treatment for OS patients includes combining surgery with an adjuvant and neoadjuvant chemotherapy regimen, leading to a 5-year overall survival rate leveled off approximately 60–70 %, during a few decades [4]. Antifolate- and fluoropyrimidine-based chemotherapies such as tomudex (TDX) and methotrexate (MTX) are widely employed to decrease the relapse rates and improve the survival of several malignancies including OS; however, it has reached a plateau [5]. It means that the treatment of OS is often failing due to chemo-resistance growth, and overcoming resistance to chemotherapy is a method to improve the survival rate of OS patients [6]. Therefore, it requires new therapeutic approaches and identifying molecular mechanisms underlying or combination therapy, which enhanced the sensitivity of chemotherapeutic agents to OS.

Identification of microRNAs (miRs/miRNAs) fundamental role in the pathogenesis of diseases and their permanent presence in biological fluids has led to comprehensive studies on the potential role of miRNAs as involved biomarkers in the induction of chemo-resistance/-sensitivity [7,8]. miRNAs belong to the large family of small non-coding

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https://doi.org/10.1016/j.prp.2020.153176
Received 12 April 2020; Received in revised form 6 August 2020; Accepted 9 August 2020
Available online xxx
0344-0338/

RNAs (about 21–23 nt), which are mainly bound imperfectly to the 3′UTR (3′ untranslated region) of target mRNAs, modulating mRNA stability and protein expression at the post-transcriptional levels [9,10]. Abnormal expression of miRNAs in cancers is considered as a biomarker for the diagnosis, prognosis, and therapeutic paradigms. Evidence implies that miRNAs are closely correlated with the progression of sensitivity to chemotherapy or chemo-resistance in tumor cells [11–13]. For instance, miR-497 sensitizes OS cells to cisplatin [14]. Unlike miR-24, which leads to enhancement of MTX resistance [15] or miR-133b, which induces chemo-resistance of OS cells to cisplatin [7].

Previous studies have indicated that miR-192 is significantly down-regulated in malignant cancers, including OS, while the up-regulation of miR-192 could inhibit tumorigenesis of OS [16]. Nevertheless, the potential mechanisms of miR-192 in regulating the development and promoting sensitivity of MTX in OS remains unclear yet.

In the present investigation, we set out to restore miR-192 in OS tumor cells and determine the underlying molecular mechanisms, which are responsible for enhanced sensitivity to MTX in the MG-63 cell line. For this purpose, cell transfection of miR-192 was established, then the effects of miR-192 on proliferation, apoptosis, and specific gene expressions associated with tumor progressives such as K-Ras, c-Myc and MMP9, ADAMTS, and CXCR-4 were investigated compared with control cells. Since established overexpression of these genes, progresses the invasive capability and chemo-resistance in the majority of cancers, hence, it may justify the role of miR-192 in sensitizes of MG-63 cells to MTX. In effect, this study aimed to exhibit the miR-192 as a novel therapeutic target in MTX-resistance in OS patients.

2. Materials and methods

2.1. Cell culture

MG-63 cell line was procured from the American Type Culture Collection (ATCC, USA). Briefly, MG-63 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) basic medium supplemented with 10 % heat-inactivated Fetal Bovine Serum (FBS), with 20 mM HEPES, 1% streptomycin/penicillin G (1% Pen/Strep), and incubated at 37 ˚C with carbon dioxide (CO_2) 5%. The number of viable cells was defined by performing the trypan blue dye exclusion test. RT-qPCR was used to determine miR-192 expression.

2.2. miRNA transfection

MG-63 cells (5×10^5 cells/2 ml/well) were seeded into six-well plates until 40–50 % confluence was reached. Control miR (5′-UUUGUACUACACAAAAGUACUG-3′) and miR-192 mimic (5′-CUGACCUAUGAAUUGACAGC-3′) were designed and purchased from Gene Create Biological Engineering (Wuhan, China), then both were used for the transfection of the cells, which was achieved by using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA), at a final concentration of 50 nM, based on the manufacturer's protocol. In brief, miR-192 mimic or the control miR was mixed with 100 µl of Lipofectamine in 12 ml of culture media and divided per well in six-well microplates. Twenty-four hours after transfection, the media was replaced with fresh medium, and the cells were incubated for another 24 h.

2.3. MTT assay

Cytotoxicity effect of MTX in MG-63 cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT; Sigma Aldrich, USA) assay. Briefly, after washing with PBS three times, cells were detached with 0.25 % trypsin and centrifuged at 1500 rpm for 7 min. Cells were seeded in 96-well microplates at the density of 1.5×10^4 and exposed to diverse concentrations (0.01, 0.05, 0.1, 0.5, and 1 µM) of MTX for 48 h. Subsequently, 20 µl (5 mg/mL) of MTT was added to each well and after additional 4 h of incubation 200 µl/well dimethyl sulfoxide (DMSO) was added to dissolve formazan crystals, following by persistent shaking for 20 min. The absorbance was measured at 570 nm using a microplate reader (Bio-Rad, California). Also, the coefficient of drug interaction (CDI) was applied to analyze the effects of drug combinations.

$$CDI = \frac{\text{survival\% (Durg A + Durag B)}}{\text{survival\% (Durg A)} \times \text{survival\% (Durg B)}}$$

2.4. Apoptosis by cell death detection

MG-63 cells were plated at a density of 1.5×10^5 cells/well in twelve-well microplates and then exposed to MTX (0.1 µM) and miR-192, alone or in combination. Twenty-four hours after incubation, cells were harvested, and apoptosis was detected by ELISA cell death kit assay following the manufacturer's directions (Roche Diagnostics, Tokyo, Japan). This assay determines the amount of mono- and oligonucleosomes released into the cytosol during apoptosis. Briefly, the cells were digested by enzyme and centrifuged at 200 × g for 7 min. Upon the addition of 20 µl of the cell supernatant and 80 µl of a mixture of anti-DNA-peroxidase and anti-histone-biotin to each well, HRP streptavidin-coated plate was incubated for 2 h. After rinsing with incubation solution, 100 µl of ABTS [2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] was transferred per well to form a colored product. Ultimately, the reaction was stopped, and absorbance at 405 nm was determined immediately with an ELISA microplate auto-reader (Bio-Rad).

2.5. Quantitative RT-PCR

To determine mRNA expression levels, total RNA was obtained from cells using a TRIzol® reagent, following the supplier's directions (Invitrogen). The RNA concentration was determined by NanoDrop (Thermo Scientific, USA). RNA was treated with DNAse and was reverse-transcribed with a RT reagent Kit (QIAGEN) for complementary DNA (cDNA) synthesis and genomic DNA removal. Then, cDNA was quantified on an iCycler iQ multicolor real-time PCR detection system (Bio-Rad, USA) using SYBR Green PCR Master Mix (ABI, USA). The PCR amplification condition was set as 10 min of initial denaturation "hot start" at 95 ˚C for 10 min, 40 cycles at 94 ˚C for 45 s, 60 ˚C for 15 s and 72 ˚C for 1 min, finally subjecting at 72 ˚C for 10 min. U6 small nuclear RNA (U6 snRNA) and GAPDH were used as internal references. The relative expression levels of genes were measured based on the comparative C_t method ($2^{-\Delta\Delta C_T}$). All experiments were repeated in quadruplicate. The primers were used are included in Table 1.

2.6. Statistical analysis

All experimental data were processed and analyzed using GraphPad Prism v7 software (La Jolla, USA). Student's *t*-test and analysis of vari-

Table 1
Quantitative RT-PCR primers sequences.

Gene	Sense	Anti-sense
miR-192	GGACTTTCTTCATTCACACCG	GACCACTGAGGTTAGAGCCA
CXCR-4	TCTTCCTGCCCACCATCTACTC	TGCAGCCTGTACTTGTCCGTC
K-Ras	GTAGTTGGAGCTGGTGGC	TTTCACACAGCCAGGAG
c-Myc	AGGCTCTCCTTGCAGCTGCT	AAGTTCTCCTCCTCGTCGCA
MMP9	TCACAAATCCTCCCCAAGTGG	GAATGCGCCCTAAATCACTGA
ADAMTS	CCTCAGCCCTTCTGAAAACTTTGCC	GGCACCAAGTCCTCCACACCC
U6 SnRNA	TCCGATCGTGAAGCGTTC	GTGCAGGGTCCGAGGT
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

ance (ANOVA) were applied to ascertain the significance of differences between groups. The results are expressed as the means ± SD (standard deviation), and *P-values* < 0.05 was considered significant between control and blank control.

3. Results

3.1. miR-192 promoted the cytotoxic effect of MTX in MG-63 cells

Combination therapy consisting of MTX and miR-192 was employed to investigate whether overexpression of miR-192 could increase the sensitivity of the MG-63 cells to MTX. MTT assay results revealed that mono treatment with MTX, induced cell toxicity in a dose-dependent manner. For determination of MTX IC_{50} value, 5 different concentrations of MTX ranging from 1×10^{-2} to 1 μM (0.01, 0.05, 0.1, 0.5, and 1 μM) were tested which MTX IC_{50} was 0.12 μM, after 48 h. As shown in Fig. 1, after 48 h of incubation, miR-192 plus MTX significantly diminished the cell survival rate, relative to the MTX alone or control miR plus MTX group ($P < 0.05$). Meanwhile, transfection with control miR plus MTX had an insignificant effect on the sensitivity of the tumor cells compared to the MTX treated cells ($P > 0.05$; Fig. 1).

3.2. Overexpression of miR-192 enhanced the MTX-induced apoptosis in MG-63 cells

To confirm that whether the sensitizing effect of the miR-192 was related to increasing the extent of apoptosis, the effects of miR-192, MTX, and their combination on apoptosis were assessed by an ELISA-based cell death kit assay. As shown in Fig. 2, 48 h after the transfection of miR-192 alone, apoptosis enhanced by 3.49 fold, whereas MTX treatment alone caused 16.21 fold to enhance in apoptosis ($P < 0.05$, relative to the blank control). Surprisingly, combination therapy further enhanced apoptosis to 24.07 fold after 48 h ($P < 0.05$, relative to the blank control). Also, the combination treatment further enhanced apoptosis to 6.89 and 1.49 fold after 48 h ($P < 0.05$, compared with miR-192 and MTX alone treatment, respectively). However, insignificant alterations in the extent of apoptosis were identified for control miR or control miR plus MTX groups relative to the blank control or MTX alone, respectively ($P > 0.05$). Therefore, these results demonstrate that the sensitization effect of miR-192 is partially attributed to the induction of apoptosis.

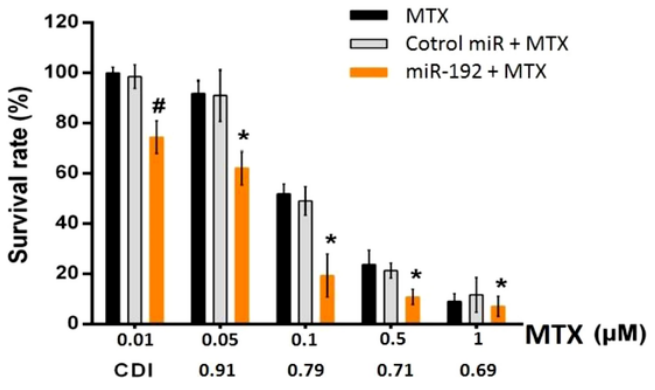

Fig. 1. miR-192 enhanced the MTX-sensitivity of the MG-63 Cells. The cells were treated with MTX, control miR + MTX, and miR-192 + MTX for 48 h and then the cytotoxicities were determined using MTT. The synergistic effect of miR-192 in MTX therapy was established by effect analysis and coefficient of drug interaction (CDI) values. The data are represented as mean ± SD (n = 3); $^{*}P < 0.05$ versus MTX or control miR plus MTX; $^{\#}P < 0.05$ versus blank control.

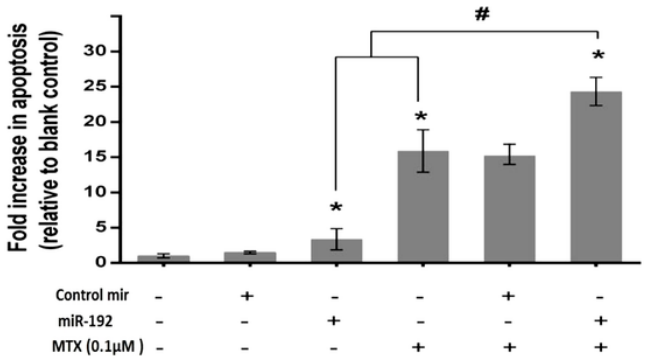

Fig. 2. The impact of miR-192 overexpression on MTX-induced apoptosis. The cells were subjected to MTX (0.1 μM) and mir-192, alone or in combination. After 48 h of transfection, apoptosis was evaluated using ELISA cell death kit assay. The data are represented as mean ± SD (n = 3); $^{*}P < 0.05$ relative blank control or control miR; $^{\#}P < 0.05$ versus miR-192 or MTX.

3.3. Overexpression of miR-192 alleviated gene expression levels of MMP9, c-Myc, K-Ras, CXCR4, and ADAMTS in MG-63 cells

Previous studies have confirmed that miR-192 was remarkably down-regulated in human OS tumor cells. RT-qPCR was performed to determine relative expression levels of MMP9, c-Myc, K-Ras, CXCR-4, and ADAMTS in the MG-63 cell line. As shown in Fig. 3, the miR-192 mimic is significantly higher in MG-63 transfected cells versus the control groups ($P < 0.01$), which revealed that miR-192 was down-regulated in OS tumor cells. These mRNAs expression levels are markedly decreased in transfected cells compared with the control groups ($P < 0.05$), which implied that these genes play critical roles in the progression of OS tumor cells. Indeed, overexpression of miR-192 through the downregulation of these genes may result in an obstacle to the progression of OS.

4. Discussion

Although miR-192 has been reported to have various effects in majority types of cancers [16–18], our results of the present study imply that miR-192 impedes MTX-resistance of MG-63 cells in vitro, which is suggesting that miR-192 promotes chemo-sensitivity in OS. Our investigation is the first evidence showing that miR-192 affects MTX-resistance of OS cells. However, these data provide minimal evidence owing to high variability in expression levels of miRNA and race variation. Therefore, more studies are indispensable to confirm our findings regarding the present research.

Nowadays, with more than 8 million cancer-related mortality per year, notable breakthroughs in cancer treatment are required [19]. Although chemotherapy is a fundamental approach to treat OS, acquired resistance is one of the principal reasons for chemotherapy failure [14]. Thus, identification of prognostic agents that can stratify patients based on clinical and biological markers may help to select satisfactory treatment approaches. Directly or indirectly, miRNAs have been implicated in almost every aspect of cellular functions and biological processes; hence, these potential agents could be employed to overcome chemo-resistance during chemotherapy [20]. However, miRNAs conduct conflicting roles during chemotherapies either as a chemo-sensitization/-resistance, depending on their targets or function mechanisms [21].

Targeting miRNAs to reverse drug resistance may be a hopeful therapeutic approach against OS [22]. miR-192 is deregulated in many kinds of sarcomas, which is associated with chemo-resistance in human cancers [18]. Regarding previous studies, miR-192 is down-regulated in OS, which is a potential agent associated with chemo-resistance to cisplatin in gastric [17] and ovarian cancer cells [23], doxorubicin

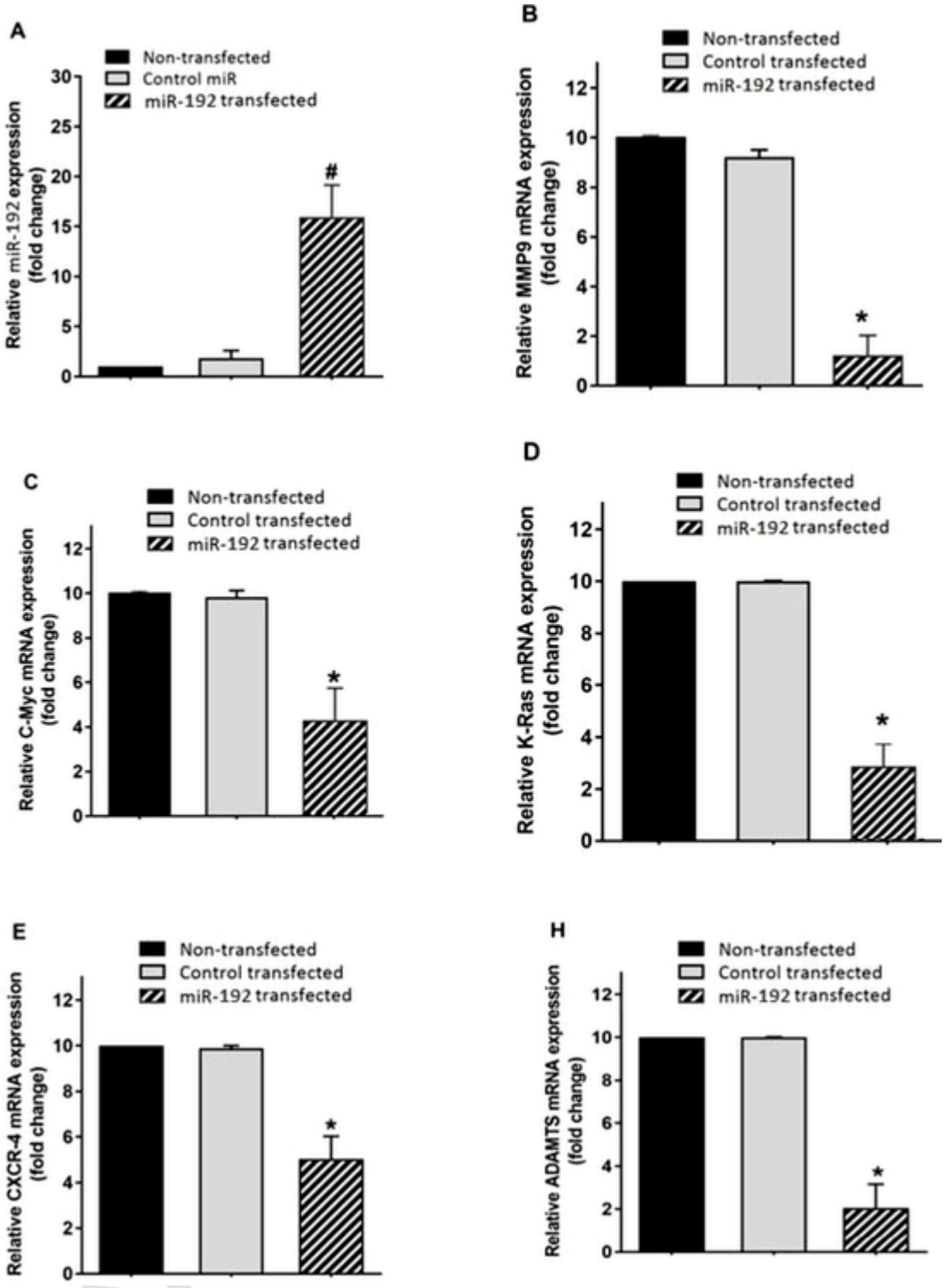

Fig. 3. After 48 h of transfection the expression levels of miR-192 (A), MMP9 (B), c-Myc (C), K-Ras (D), CXCR-4 (E) and ADAMTS (H) in MG-63 cells. The data are stated as mean ± SD (n = 4), $^{\#}P < 0.01$ versus control group, $^{*}P < 0.05$ versus control.

in breast cancer cells [18], cisplatin and 5-fluorouracil in esophageal cancer cells [24], and docetaxel in lung cancer [25]. In this regard, Zhang et al. revealed that miR-192-5p overexpression enhanced sensitivity to DOX, increased apoptosis, elevated JNK activation, and proapoptotic caspase 9 in MCF-7/ADR breast cancer cells [18]. Our data has revealed that miR-192 is significantly down-regulated in MG-63 cells, which is consistent with the earlier studies.

Overexpression of miR-192 enhances the MTX sensitivity of MG-63 cells and a significant decline in IC_{50} and viability of these cells. Indeed, we sought to clarify whether the combination of miR-192 with MTX enhanced the apoptosis in MG-63 cells compared with control cells, in which we received desirable responses from this combination treatment. These results imply that miR-192 plays a crucial role in

the elevated MTX-induced apoptosis in MG-63 cells and might have a potential prognostic role in OS.

Also, to better clarify the role of miR-192 in the sensitivity enhancement to MTX in MG-63 cells, the mRNA expression levels of MMP9, c-Myc, K-Ras, CXCR-4, and ADAMTS were evaluated before and after miR-192 transfection by RT-qPCR. miRNAs can fine-tune angiogenic replies and also modulate the sensitivity of chemotherapeutic drugs [26].

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes (zinc-dependent endopeptidases) with the capability to degrade extracellular matrix (ECM) components, which play pivotal roles in carcinogenesis, proliferation, differentiation, angiogenesis, migration, invasion, immune surveillance, chemo-resistant, and metastasis. Among them, MMP-9 (gelatinase B) has been identified as a potential marker of chemo-resistance, and miRNAs that target this gene might affect the chemo-resistance [27]. In this regard, Lou and colleagues demonstrated that the up-regulation of miR-192 represses the expression levels of MMP-16, MMP-2, and MMP-9 in asthma [28]. Besides, it has been well documented that MMP-9 could be a potential biomarker for OS [29]. We observed that the MMP-9 gene expression was suppressed after the combination treatment with MTX and miR-192 in MG-63 cells.

Furthermore, Zhang et al. demonstrated that miR-192-5p sensitizes breast cancer cells to DOX by targeting peptidylprolyl isomerase A (PPIA) [18]. Also, Xie and colleagues revealed that miR-192-5p partially reversed gastric cancer cells to cisplatin resistance by targeting ERCC3 and ERCC4, which participate in the nucleotide excision repair (NER) pathway, suggesting that miR-192-5p may be a potential biomarker and therapeutic target for these resistance cells [17]. Up-regulation of ADAMTS (a disintegrin-like and metalloproteinase domain with thrombospondin type 1 motifs), CXCR4 (CXC-Chemokine Receptor-4), K-Ras (Kirsten rat sarcoma viral oncogene homolog), and c-Myc induces tumor invasion and metastasis in the majority of cancers including OS [30–33]. Some of the miRNAs such as miR-224 is also involved in chemoresistance to 5-FU in CRC where it's knock-down phenocopied KRAS mutation by enhancing KRAS activity with ERK and AKT phosphorylation increasing 5-FU reverse chemoresistance. Also, K-RAS can induce the expression of c-Myc through its effector signaling pathways. Hence, it is valuable to identify the miRNAs target genes to better understanding the functional roles of them in cancers. Overall, our findings have revealed that miR-192 could down-regulate these gene expressions, which are involved in epithelial-mesenchymal transition (EMT) in cancer cells by sensitization to MTX in MG-63 cells.

5. Conclusion

Herein, we unveiled a strong relationship between miR-192 and enhanced cell sensitivity to MTX, which is frequently used in clinical practice. In effect, there is a negative correlation between the miR-192 level and chemo-resistance in OS tumor cells. Our findings show that the down-regulation of miR-192 is significantly associated with MTX-resistance in MG-63 cells. After the combination treatment of miR-192 with MTX, the mRNA expression levels of MMP9, c-Myc, K-Ras, CXCR4, ADAMTS, survival, and apoptosis analysis showed that miR-192 could considerably elevate the sensitivity of MG-63 cells to MTX. Taken together, our results suggest that miR-192 contributes to chemo-sensitizing MG-63 cells to MTX, and could be considered as a predictive biomarker in diagnosis for OS responsiveness to the chemotherapy.

Consent for publication

All authors have read the manuscript and approved the final version.

Ethical approval

Approved by Tabriz University of Medical Sciences (ethical code: IR.TBZMED.REC.1396.1203)

Funding statement

This study was supported by the Molecular Reseach Center, Tabriz University of Medical Sciences (Grant no: 38845).

CRediT authorship contribution statement

Mohammadreza Bazavar: Conceptualization, Methodology, Software. **Jafar Fazli:** Conceptualization, Methodology, Software. **Amir Valizadeh:** Data curation, Writing - original draft. **Binfang Ma:** Data curation, Writing - original draft. **Erfan Mohammadi:** Visualization, Investigation. **Zatollah Asemi:** Visualization, Investigation. **Forough Alemi:** Writing - review & editing. **Masoomeh Maleki:** Writing - review & editing.

CRediT authorship contribution statement

Mohammadreza Bazavar: Investigation, , . **Jafar Fazli:** Investigation, , . **Amir Valizadeh:** Data curation, Writing - original draft. **Binfang Ma:** Data curation, Writing - original draft. **Erfan Mohammadi:** Visualization, Investigation. **Zatollah Asemi:** Methodology, . **Forough Alemi:** Writing - review & editing. **Masoomeh Maleki:** Writing - review & editingShilong Xing and Bahman Yousefi: Data analysis and critical revisions..

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

The Authors would like to thanks Clinical Research Development Unit, Shohada Hospital, Tabriz University of Medical Sciences for kind supports.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2020.153176.

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