



Research Paper

MiR-193a regulates chemoresistance of human osteosarcoma cells via repression of IRS2

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ABSTRACT

Chemoresistance prevents curative potential of chemotherapy in most cases. MicroRNAs (miRNAs) are key players in regulating chemoresistance in osteosarcoma, which is the most common primary bone cancer. Bisulfite sequencing and quantitative real time PCR analyses showed that miR-193a expression is downregulated by DNA hypermethylation at its promoter region in a chemoresistant cell line, SJSA-1, compared to a chemosensitive cell line G-292. Introduction of a miR-193a mimic in SJSA-1 cells or an antagomir into G-292 cells confirmed the role of miR-193a in osteosarcoma chemoresistance. Bioinformatics together with biochemical assays showed that insulin receptor substrate 2 (IRS2) is a target of miR-193a. Our data concludes that miR-193a plays a role in the osteosarcoma chemoresistance and thus might serve as a useful biomarker for osteosarcoma prognosis.

1. Introduction

MiRNAs are a class of small, non-coding regulatory RNA molecules that participate in a wide range of biological processes. Their dysregulation has been associated with the development of diseases and cancers [1,2]. The abnormal expression of miRNAs contributes to the tumor biogenesis [3], including drug resistance [4], which remains a major obstacle to effective treatment in patients [5]. As a hallmark of cancer, chemoresistance varies drastically among cancer patients of different cancer lesions and even different regions of the same lesions within a single patient [6]. Despite of intensive efforts, our knowledge on the chemoresistance of cancers remains limited [7,8]. To date, much efforts have been exerted in analyzing the role of miRNAs in the initiation and maintenance of chemoresistance in a variety of cancers [9]. The notable examples of miRNAs that contribute to the bladder cancer chemoresistance are miR-30d, miR-181, miR-199a-5p [10] and miR-193a-3p [5,11].

As one of the well-studied miRNAs, miR-193a-3p was reported to be

dysregulated in several types of cancers, such as nonsmall lung cancer (NSCLC) [12], prostate cancer [13], breast cancer [14], head and neck squamous cell carcinomas [15] and colorectal cancer [16]. Notably, miR-193a-3p was also found to be involved in the multidrug resistance of bladder cancer [17]. In addition, DNA methylation is the best-characterized epigenetic mechanism, underlying the faithful transmission of the gene transcription memory through cell division [18]. The hypermethylated state of the promoter and enhancer regions tightly correlates with the transcriptionally silenced state of miRNAs. For example, DNA methylation was implicated in regulating the miR-193a-3p-mediated tumor suppression in oral carcinogenesis [19]. MiR-193a-3p functions as a methylation-silenced tumor suppressor via the repression of the c-kit gene in acute myeloid leukemia [20]. Our recent report also suggests that the hypermethylated miR-193a-3p and miR-193a-5p suppress the metastasis of human osteosarcoma cells by down-regulating Rab27B (a member of RAS oncogene family) and SRR (serine racemase), respectively [21].

Osteosarcoma (OS) is the most common primary bone malignancy

Abbreviations: miR, microRNA; OS, osteosarcoma; BSP, Bisulfite Sequencing PCR; UTR, untranslated region; WT, wild-type vector; Mut, mutation-type vector; Dox, doxorubicin; Etop, etoposide; CDDP, cisplatin; Carb, carboplatin; MTX, methotrexate; 3PM, miR-193a-3p-mimic; 3PA, miR-193a-3p-antagomir; Ago, miR-193a-3p's agomir; Anta, miR-193a-3p's antagomir; IRS2, Insulin Receptor Substrate 2

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in children and young adults [22,23], and the mechanism of OS chemoresistance remains largely unknown. In the present study, we found that miR-193a-3p was epigenetically repressed by promoter hypermethylation in chemoresistant cell line SJS-1 but not in the sensitive OS cell line G-292. In addition, we performed an RNA-seq-based omic analysis for the differentially expressed genes in G-292 versus SJS-1 OS cell lines and showed that miR-193a-3p suppresses the OS chemoresistance via its repression of the IRS2 gene (insulin receptor substrate 2), a newly identified direct target of miR-193a-3p. The IRS2 gene encodes a cytoplasmic signaling molecule that acts as a molecular adaptor to mediate the effects of insulin, insulin-like growth factor 1, and other cytokines. These results suggest a role of methylation-repressed state of miR-193a-3p in OS and provide a pharmacologic rationale for up-regulation of miR-193a-3p expression as a prospective adjunctive therapy for OS.

2. Materials and methods

2.1. Cell lines and cell cultures

Human osteosarcoma cells SJS-1 (ATCC No. CRL-2098) [24] and G-292 (ATCC No. CRL-1423) [25] were purchased from ATCC. Both cell lines were maintained in DMEM (Life Technology) supplemented with 10% fetal bovine serum (Invitrogen) and 1% glutamine at 37 °C in 5% CO₂.

2.2. Methylation analysis

Genomic DNA was extracted from cells using a standard phenol/chloroform purification method, qualified via agarose gel electrophoresis, and treated with an ammonium bisulfate-based bisulfite conversion reagent [26,27]. For bisulfite-sequencing analysis, the 5' upstream flanking sequence from the pre-miR-193a was amplified by PCR using the bisulfite-treated DNA as a template. The PCR fragments from the converted DNA were sequenced and analyzed. Raw sequence data files were processed, and the area ratio (%) of C to C + T of the primary CpG dinucleotide was calculated as the percentage of methylation and was then plotted [5].

2.3. RNA analysis

Total RNA was isolated from the cells at the logarithmic phase using TRIzol (Tiagen Biotech). For mRNA analysis, cDNA primed by oligo-dT was made with a prime Script RT reagent kit (Tiagen Biotech), and the mRNA level of the IRS2 gene was quantified using duplex qRT-PCR analysis in the FTC-3000P PCR instrument (Funglyn Biotech), with the Taqman probe with a different fluorescence label for β-actin (provided by Shing Gene, Shanghai, China) used for a reference. Using the 2^{-ΔΔCt} method, normalization to the β-actin level was performed before the relative levels of the target genes were compared. The sequences of the primers and probes used for the qRT-PCR analysis are:

hIRS2 F: 5'-CATTGACTTCTGTCCCACCAC-3'
 hIRS2 R: 5'-TGAAACAGTGCTGAGCGTCTTC-3'
 hIRS2 probe: 5'-CY5- CTGTCTGGCTTATCACCAGGATGTCAC-3'
 hACTB F: 5'-GCCCATCTACGAGGGGTATG-3'
 hACTB R: 5'-GAGGTAGTCAGTCAGGTCCCG-3'
 hACTB probe: 5'-HEX-CCCCATGCCATCCTGCGTC-3'

2.4. The reagents for the transient transfection assays

The riboFECT CP transfection kit, mimics, antagomiR, siRNA, and negative control (NC) RNA were supplied by ShangHai GenePharma, China. Transfection of these ribonucleic acids and reporter plasmids was performed according to the manufacturer's instruction.

Chemically modified mimic oligonucleotides (agomiRs) were

synthesized to regulate miR-193a-3p expression in vivo. The 3' ends of the oligonucleotides were conjugated to cholesterol, and all nucleotides were 2'-OMe modified. The agomiR oligonucleotides were deprotected, desalted and purified by high-performance liquid chromatography.

The siRNA sequences used for IRS2 interference in this study were as follows:

si-IRS2-1:
 GUACAUCAACAUCGACUUU
 5'GUACAUCAACAUCGACUUU dTdT 3'
 3'dTdT CAUGUAGUUGUAGCUGAAA 5'
 si-IRS2-2:
 CCUCAACAACAACAACAAC
 5'CCUCAACAACAACAACAAC dTdT 3'
 3'dTdT GGAGUUGUUGUUGUUGUUG 5'
 si-IRS2-3:
 CUGCCUCGCGGAUGAAUA
 5'CUGCCUCGCGGAUGAAUA dTdT 3'
 3'dTdT GACGGAGCGACCUACUUUAU 5'

2.5. Chemoresistance profiling (IC50 measurements)

The chemotherapeutic drugs of clinical grade [5,28,29] (NCI Dictionary of Cancer Terms, <http://www.cancer.gov/dictionary>) were as follows: Dox, doxorubicin (Haizheng, Zhejiang, China); Etop, etoposide (Hengrui, Jiangsu, China); MTX, methotrexate (Lingnan, Guangdong, China); CDDP, cisplatin (Haosen, Jiangsu, China) and Carb, carboplatin (Qilu, Shandong, China).

Cells in the logarithmic growth phase were seeded in triplicate in 96-well plates at the density of 1.0 × 10⁴/well and treated with drugs at 2-fold IC₅₀ concentrations for 72 h. Cell survival was then measured using the Cell Counting Kit-8 (CCK-8) (Bimake) according to the manufacturer's instructions. The optical density was determined with a microplate reader (TECAN) at a wavelength of 450 nm.

2.6. Western blot analysis

Total protein was extracted from cells using cell lysates in 1 × SDS loading buffer (60 mM Tris-HCl, pH 6.8; 2% SDS; 20% glycerol; 0.25% bromophenol blue; and 1.25% β-mercaptoethanol). Protein expression was analyzed by western blot using anti-IRS2 antibody (BioSS, China). The anti-GAPDH antibody (San Ying Biotechnology, China) was used to normalize the amounts of analyzed samples. Protein bands were analyzed using the ImageJ software.

2.7. Luciferase reporter assay

A portion of the IRS2 3'-UTR containing the target sequence or the mutant target sequence for miR-193a-3p was cloned at the 3' end of the luciferase-coding sequence of pGL3 (Invitrogen) to construct pGL3-luc-IRS2 WT and pGL3-luc-IRS2 Mut, respectively. The constructs were confirmed by DNA sequencing. Cells were seeded in 96-well plates at approximately 1 × 10⁴ cells per well and transfected with a mixture of 50 ng of pGL3-luc IRS2 WT or Mut, 5 ng of Renilla luciferase vector plus 5 pmol of mimic or NC nucleotides with the riboFECT CP transfection kit according to the manufacturer's instructions. Both the firefly and Renilla luciferase activities were measured 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega) and a Promega GloMax 20/20 luminometer. The relative firefly luciferase activities of the UTR construct and pathway reporter constructs were analyzed as previously reported [5].

2.8. Apoptosis analysis

Cells were harvested and rinsed twice with PBS. Then, 5 μl of FITC-labeled enhanced annexin V and 5 μl (20 μg/ml) of propidium iodide

were added to a 100 μ l cell suspension. After incubation in the dark for 15 min at room temperature, the samples were diluted with 100 μ l PBS. Flow cytometry was performed on a FACSCalibur instrument. The results were analyzed according to the manufacturer's instructions. The experiments were performed independently three times, and the representative results are shown.

2.9. In vivo studies

Four-week-old BALB/C nude mice were purchased from Silaikai Experimental Animal China. All animal experiments were carried out in strict adherence with the regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China. All procedures involving animals and their care in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Science and Technology of China. The results were analyzed as previously reported.

3. Results

3.1. MiR-193a-3p is hypermethylated and down-regulated in the chemoresistant OS cell line SJSA-1

By sequencing study in two human OS cell lines (GEO accession number: GSE89930) [30], drugsensitive G-292 cells and drugresistant SJSA-1 cells, we found that miR-193a-3p was at the top of the list of differentially expressed miRNAs. Quantitative real-time polymerase chain reaction (qRT-PCR) analyses verified that the expression level of

miR-193a-3p was lower in SJSA-1 cells compared with that of G-292 cells (1.00: 12.67 for the sequencing and 1.00:38.55 for the qRT-PCR (Fig. 1(A) and (B)). Moreover, the transfection of miR-193a-3p mimic into SJSA-1 cells increased its expression approximately 62-folds, whereas the transfection of miR-193a-3p antagomiR into G-292 cells significantly decreased its level to 7% (Fig. 1(C) and (D)). To further investigate the underlying mechanism of miR-193a-3p down-regulation in SJSA-1 cells, the methylation status of miR-193a-3p promoter regions in the six OS cells was assessed by Bisulfite Sequencing PCR (BSP) assay. 16 CpG sites among the total 34 CpG sites were found to be methylated at varying ratios (Fig. 2(A)). The average methylation ratios of the miR-193a-3p gene in SJSA-1, MNNG/HOS and Saos-LM7 cells were significantly higher than that in G-292, 143B and MG63 cells (84.34:90.78:89.64:3.31:7.49:9.32, respectively) (Fig. 2(B) and (C)). The methylation level was negatively correlated with the expression level of miR-193a-3p in SJSA-1 and G-292 cells.

3.2. The IRS2 gene is a direct target of miR-193a-3p in OS cells

The miR-193a-3p level was significantly higher in G-292 cells than in SJSA-1 cells. We then predicted the target genes of miR-193a-3p using the following website: microRNA.org (<http://www.microRNA.org/microRNA/getMirnaForm.do>). We subsequently compared the expression patterns of predicted mRNAs between G-292 and SJSA-1 cells by the RNA-seq-based sequencing analysis. Dozens of genes were found to be differentially expressed in the two cell lines. Among them, the expression of IRS2 gene was negatively correlated with the expression of miR-193a-3p. Consequently, the IRS2 level was higher in SJSA-1 cells

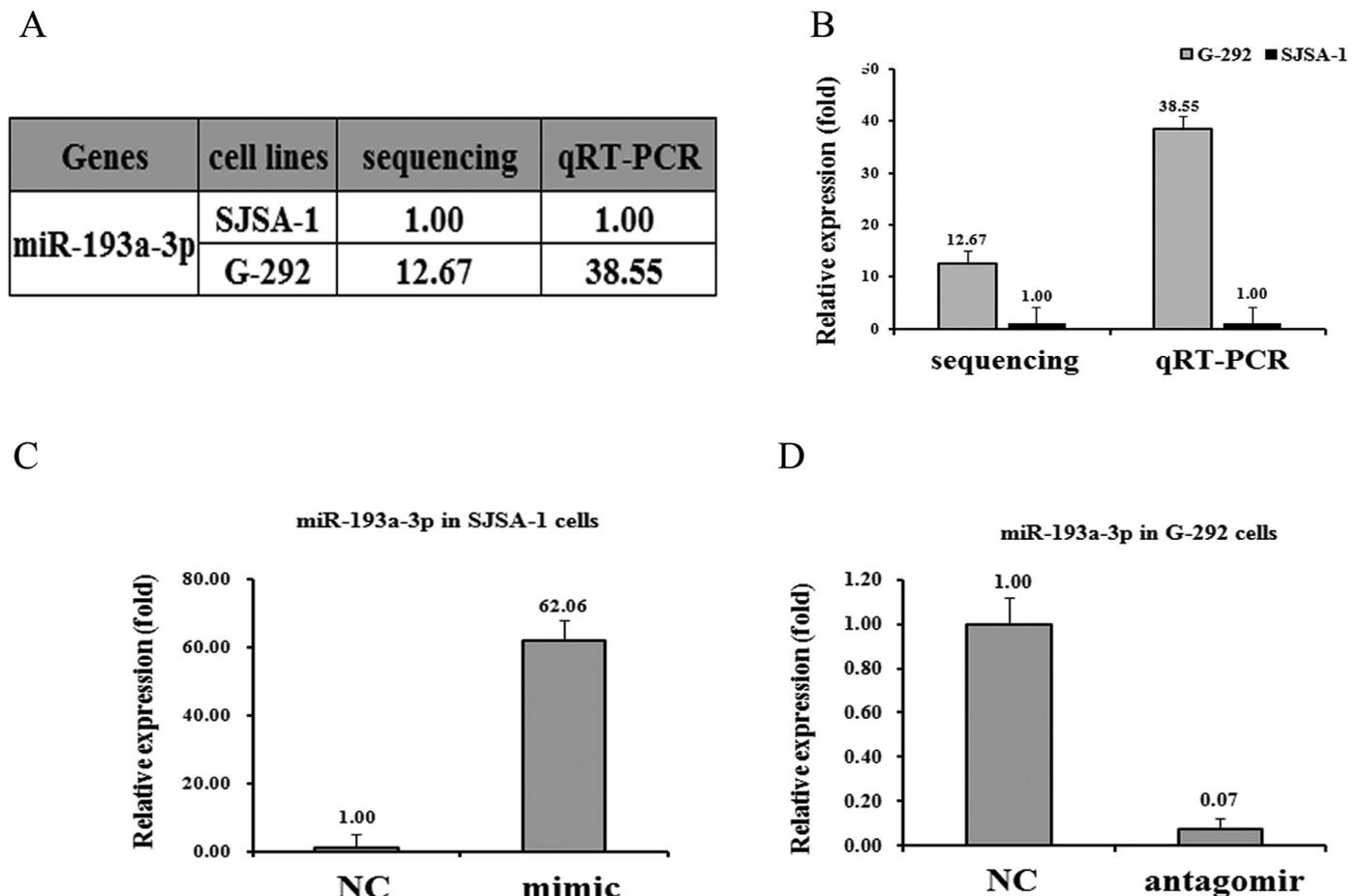


Fig. 1. Expression patterns of miR-193a-3p in SJSA-1 and G-292 cells. miR-193a-3p expression levels in SJSA-1 cells compared with G-292 cells were analyzed by sequencing and qRT-PCR in panels (A) and (B). Expression levels of miR-193a-3p in miR-193a-3p mimic-transfected SJSA-1 cells and miR-193a-3p antagomiR-transfected G-292 cells compared with the negative control (NC) as determined by qRT-PCR (C and D).

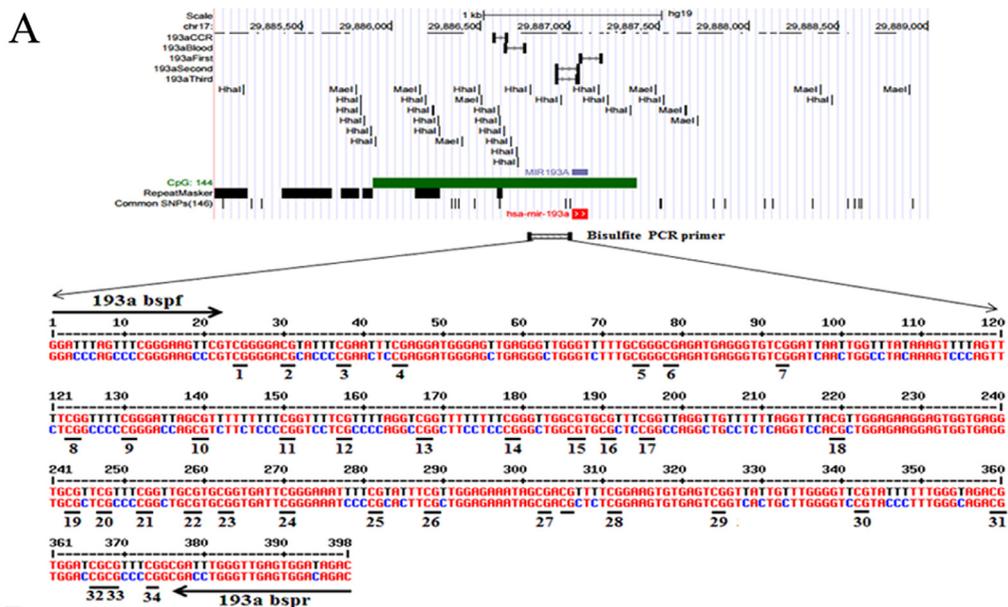


Fig. 2. Differential methylation of the miR-193a gene in six osteosarcoma cells. (A) Design of PCR primers for bisulfate sequencing using the Primer program in the predicted miR-193a CpG islands. Alignment of the sequences from the original genomic DNA (lower row) and bisulfite-treated genomic DNA (upper row); the methylated CpG sites are labeled with numbers. The percentages of the CpG methylation determined with 193a bspf primer are summarized in the table (B) and in the plot (C).

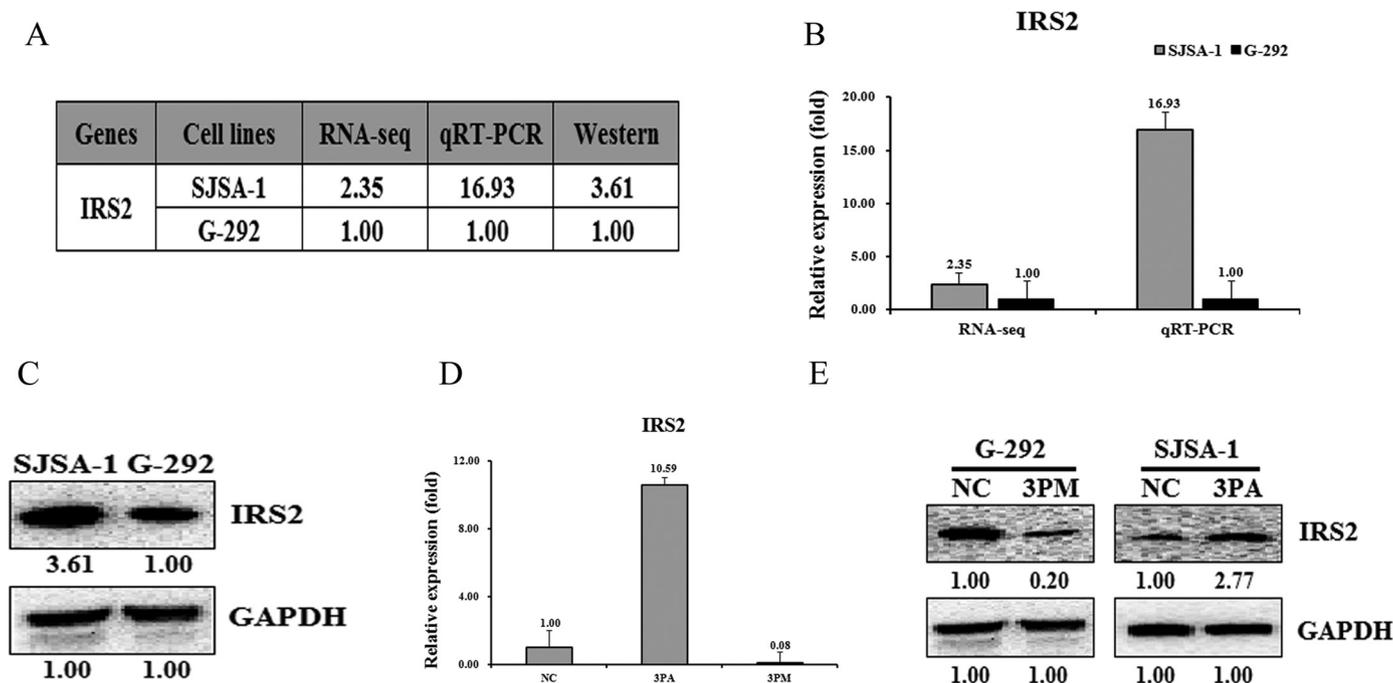


Fig. 3. IRS2 is a potential target gene of miR-193a-3p in OS cells. The higher expression level of IRS2 in SJSA-1 cells than in G-292 cells was determined by miR-seq and qRT-PCR analyses as summarized in the table (A). qRT-PCR and western blot analyses are shown in panels (B) and (C), respectively. Expression levels of IRS2 mRNA and protein in miR-193a-3p mimic (3PM)-transfected SJSA-1 cells and miR-193a-3p antagomir (3PA)-transfected G-292 cells compared with the negative control (NC) as determined by qRT-PCR and western blot analyses (D and E).

than in G-292 cells at both the mRNA (RNA-seq based sequencing: 2.35:1.00, and qRT-PCR analysis: 16.93:1.00) and the protein levels (western blot: 3.61:1.00) (Fig. 3(A), B and 3–(C)).

To check whether IRS2 is one of the authentic targets of miR-193a-3p, we determined the IRS2 levels in the miR-193a-3p mimic-transfected SJSA-1 and the antagomiR-transfected G-292 cells. Following the changes of the miR-193a-3p level, a miR-193a-3p mimic transfection of SJSA-1 cells reduced the IRS2 mRNA level to 8% and the protein level to 20% (Fig. 3(D) and (E)), compared to those in the NC transfection. As expected, miR-193a-3p antagomiR transfection increased the IRS2 mRNA level of 10.59-folds and the protein level of 2.77-folds in G-292 cells compared to NC transfection (Fig. 3(D) and (E)).

To further confirm that IRS2 is a direct target of miR-193a-3p, we cloned the wild-type or the mutant IRS2 gene downstream of the Renilla luciferase gene of pGL3-control vector (Promega) to create pGL3-IRS2 UTR WT or pGL3-IRS2 UTR Mut (Fig. 4(A)). Then the pGL3-IRS2 UTR WT, pGL3-IRS2 UTR Mut constructs or pGL3 enhancer control were transfected into G-292 and SJSA-1 cells. The pGL3-IRS2-UTR WT gave rise to the relative luciferase activities of 0.87 and 0.64 in SJSA-1 and G-292 cells, respectively (Fig. 4(B)). The transfection of miR-193a-3p mimic into SJSA-1 cells significantly reduced the luciferase activity of pGL3-IRS2-UTR WT construct, whereas the control cells showed almost a similar activity upon the transfection of miR-193a-3p mimic (Fig. 4(C)). By contrast, the transfection of the pGL3-IRS2-UTR Mut showed no effect on the luciferase activity. Meanwhile, the transfection of miR-193a-3p antagomiR into G-292 cells raised the luciferase activity of pGL3-IRS2-UTR WT construct. By contrast, a comparable luciferase activity was detected for the pGL3-IRS2-UTR Mut with the transfection of miR-193a-3p antagomiR into G-292 cells, suggesting that miR-193a-3p indeed targets the 3'-UTR region of IRS2 (Fig. 4(D)). Taken together, IRS2 is a direct target of miR-193a-3p and may mediate the miR-193a-3p suppressor effect on the OS drug resistance.

3.3. IRS2 expression negatively correlates with the miR-193a-3p suppressor effect on OS drug resistance

To explore the roles of miR-193a-3p and IRS2 in the OS drug resistance, we first transfected miR-193a-3p mimic into SJSA-1 cells or miR-193a-3p antagomiR into G-292 cells and compared the rates of cell death triggered by drugs at IC₅₀ doses. Transfection of miR-193a-3p mimic into SJSA-1 decreased the cell survival rate to different extents, whereas transfection of miR-193a-3p antagomiR into G-292 increased the cell survival rate after treatment with the following five drugs: Dox, Etop, MTX, CDPP and Carb. These findings suggest that miR-193a-3p suppresses the OS drug resistance (Fig. 5(A)). Then, we transfected si-IRS2 into SJSA-1 cells and tested the level of IRS2. The transfection of si-IRS2 indeed decreased the level of IRS2 at both mRNA (0.63:1) and protein levels (0.42:1), compared to the control cells (Fig. 5(B) and (C)). Then, we compared the rates of cell death triggered by drugs at IC₅₀ doses in the si-IRS2 transfected SJSA-1 cells. The transfection of si-IRS2 into SJSA-1 cells somewhat decreased the chemoresistance against all five drugs, except MTX (Fig. 5(D)). In line with its positive effect on drug resistance, an siRNA-mediated IRS2 repression raised the fraction of apoptotic cells from 3.05% to 6.59%, indicating a decreased cell survival rate upon addition of si-IRS2 into SJSA-1 cells. A similar effect was also found in SJSA-1 cells transfected with the miR-193a-3p mimic, with the fraction of apoptotic cells increasing from 3.92% to 6.12% (Figs. 4(E) and 5(F)). Taken together, the IRS2 gene does contribute a great deal to the suppression effect of miR-193a-3p on the OS drug resistance.

3.4. MiR-193a-3p suppresses both growth and Dox drug resistance of G-292- and SJSA-1-derived tumor xenografts in nude mice

Recently, miR-34a-5p was shown to suppress Dox chemoresistance of OS in tumor xenografts in nude mice via repression of its target gene CD117 [21]. We assumed that miR-193a-3p may perform a function similar to miR-34a-5p. In the present study, we semi-quantified via immunohistological analysis the levels of IRS2 protein in the same set

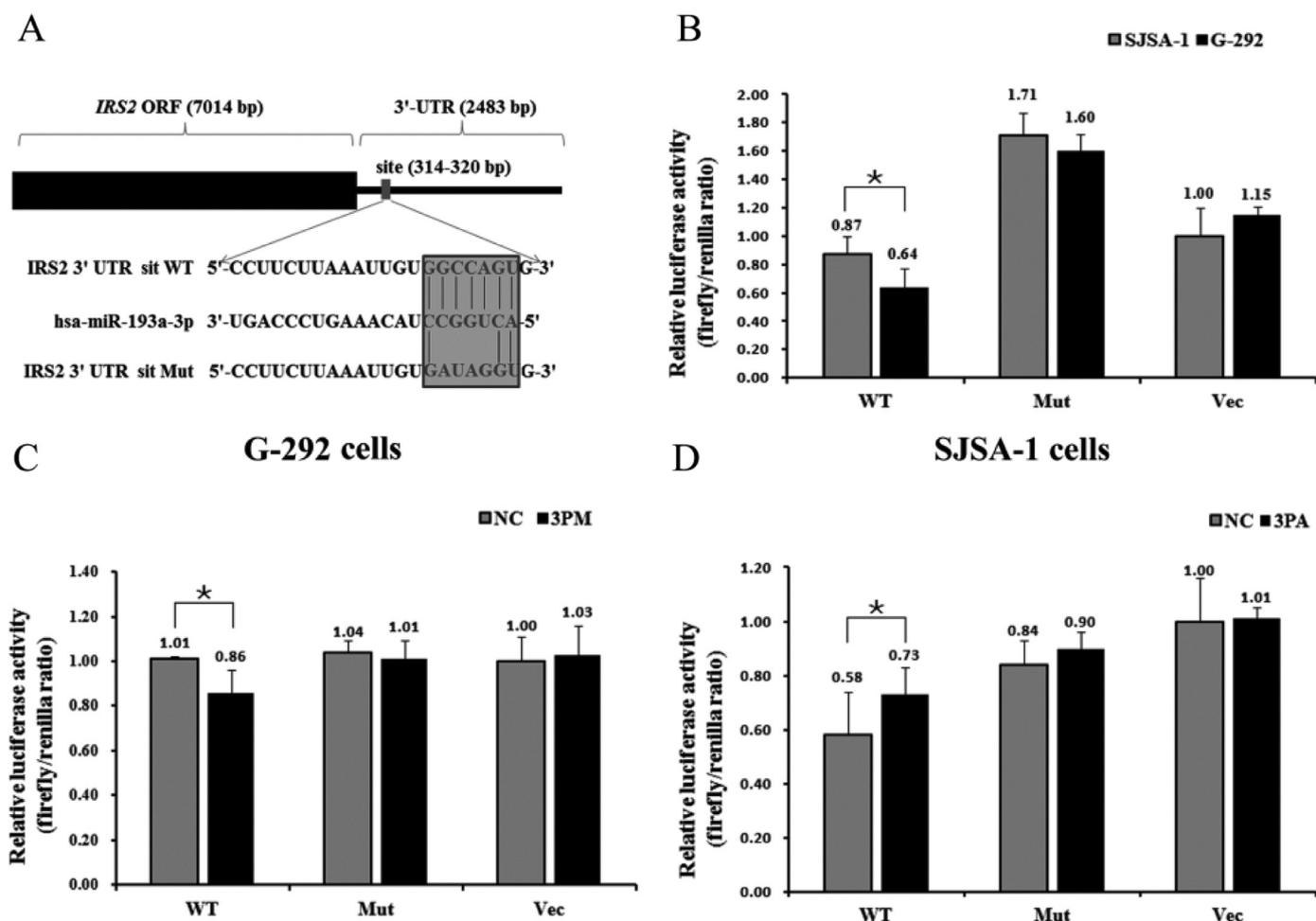


Fig. 4. IRS2 is a direct target of miR-193a-3p in OS cells. (A) Scheme of the luciferase reporter plasmid and the sequences of the WT and Mut 3'-UTR region of the IRS2 gene targeted by miR-193a-3p. The relative luciferase activity (fold-change) of the reporter with WT or Mut IRS2-UTR or with no UTR (Vec) was determined in the miR-193a-3p mimic (in SJSA-1)-, or antagomir (in G292)- or mock-transfected cells (B, C and D). *Renilla* luciferase activity of a cotransfected control plasmid was used as a transfection efficiency control. Representative results from three independent experiments are shown. Error bars represent the s.e.m. * $p < 0.05$ by Student's *t*-test.

of the tumor tissues in mice that were subjected to an injection of Dox or PBS. The intratumoral injection of drugs into miR-193a-3p agomiR SJSA-1 xenograft decreased IRS2 expression. By contrast, in miR-193a-3p antagomiR G-292 xenografts, an increased IRS2 expression was observed in Dox- or PBS-treated mice (Fig. 6). The results further confirmed that miR-193a-3p has a profound negative effect on both the growth and chemoresistance of OS cell-derived tumor xenografts in nude mice.

4. Discussion

Aberrant miR-193a expression has been reported in many types of cancers, including colorectal cancer [16], non-small cell lung cancer (NSCLC) [12], epithelial ovarian cancer cells [31], myeloid leukemia [20] and Wilms' tumor blastema [32]. Our previous works have shown that the miR-193a-3p and miR-193a-5p play important roles in osteosarcoma metastasis through down-regulation of the Rab27B and SRR genes [21]. In addition, previous studies have shown that miR-193a-3p is involved in cancer drug resistance by repressing different genes [11,17]. Such as miR-193a-3p can promote multichemoresistance in both hepatocellular carcinoma and bladder cancer via repressing the expression of its three downstream targets [5,33]. In this study we revealed that miR-193a suppresses chemoresistance by targeting IRS2. Hypermethylation of miR-193a is a cause of chemoresistance in osteosarcoma.

A microRNA executes its biological function via repression of multiple genes at levels of stability and translation mRNA. miR-193a-3p has been reported to target different genes in various types of cancers [5,34]. In the present study, we found that miR-193a-3p is down-regulated in highly chemoresistant SJSA-1 cells. Down-regulation of miR-193a-3p correlates with the hypermethylated state of the promoter and enhancer regions in SJSA-1 cells (Fig. 2). Consistent with previous studies, DNA methylation is the best-characterized epigenetic mechanism and is regarded as a promising molecular indicator for the existence and/or prognostic state of cancer [35,36]. We further investigated the target gene expression in chemoresistant SJSA-1 cells compared to the chemosensitive G-292 cells. As expected, the IRS2 mRNA and protein expression was up-regulated in SJSA-1 cells compared with G-292 cells (Fig. 3(A), B and 3(C)). Moreover, up-regulation of IRS2 correlates with the down-regulation of miR-193a-3p. Hence, miR-193a-3p negatively regulates OS chemoresistance by targeting IRS2. In addition, both the roles and the functional mechanisms of the IRS2 gene in the context of OS drug resistance were systematically addressed in both cultured cells and tumor xenografts in nude mice.

5. Conclusion

Taken together, in line with the findings of the previous studies, here we showed that the expression of IRS2 was associated with the multidrug resistance of OS cell lines. However, the fine mechanism for

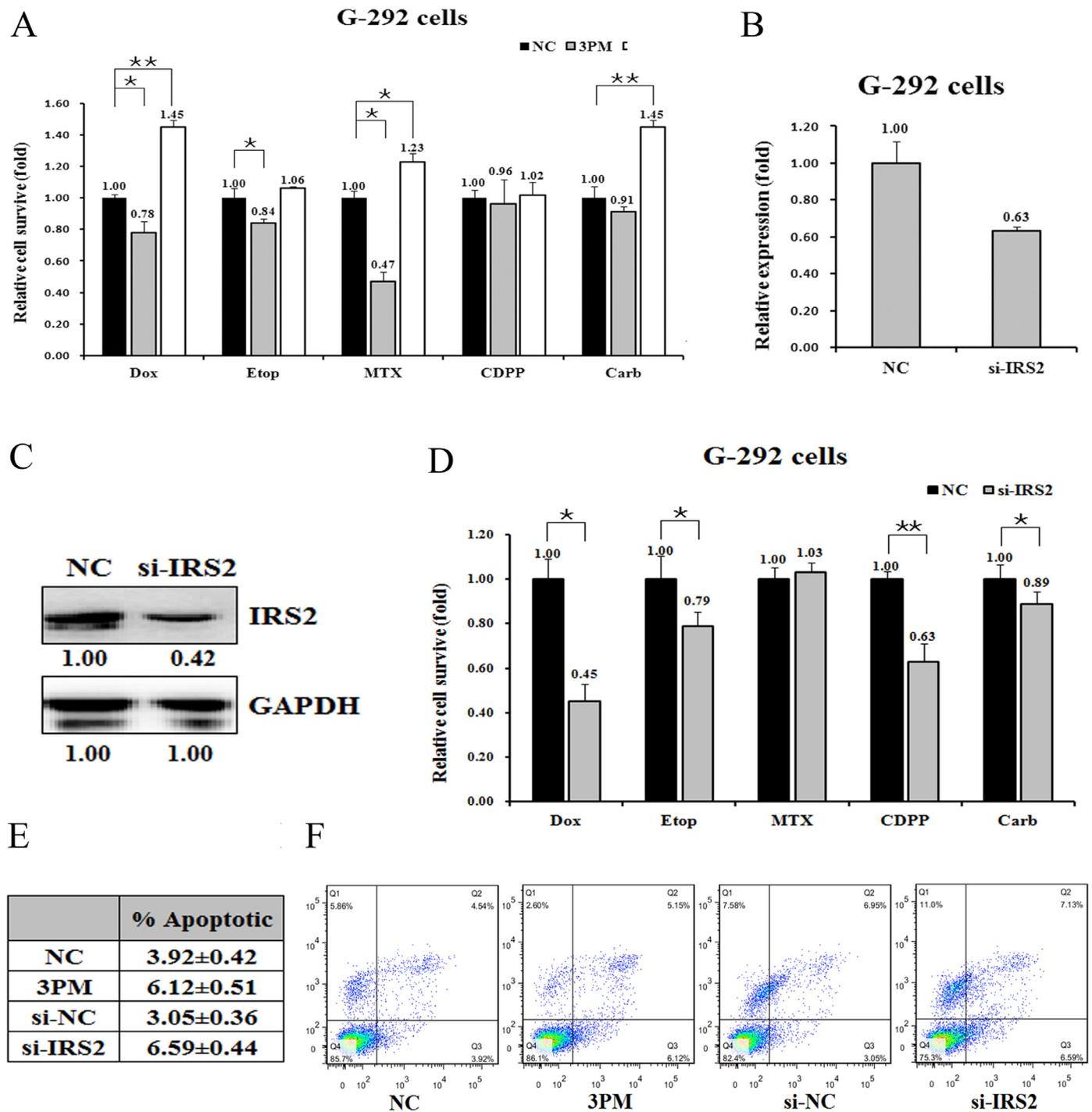


Fig. 5. The effects of a forced reversal of the miR-193a-3p or IRS2 levels on the chemoresistance of G-292 and SJS-A-1 cells. The IC₅₀ doses of drugs induced cell death of SJS-A-1 and G-292 cells transfected with the miR-193a-3p mimic (3PM), antagomir (3PA) or the corresponding negative control (NC) assayed 72 h posttreatment (A). The levels of IRS2 mRNA in the siRNA-transfected SJS-A-1 cells versus the si-NC transfected cells (B). The IRS2 protein level (western blot analysis) in the siRNA-transfected versus the NC-transfected SJS-A-1 cells (C). The relative cell survival of the SJS-A-1 cells transfected with siRNA compared to NC-transfected SJS-A-1 cells 72 h after treatment with drugs at IC₅₀ doses. (D). Effects of forced alteration in both miR-193a-3p and IRS2 levels on apoptosis in SJS-A-1 cells as determined by FACS analysis (E and F). (NC was normalized, *p-value < 0.05; **p-value < 0.01).

the IRS2-mediated OS drug-resistance remains to be clarified.

Ethics approval and consent to participate

Animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal research was approved by the biomedical ethics committee of Anhui Medical University, when we applying for the

National Natural Science Foundation of China (81372868 granted to SBC) in 2013. The animal study proposal was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Science and Technology of China. All of the mouse experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China.

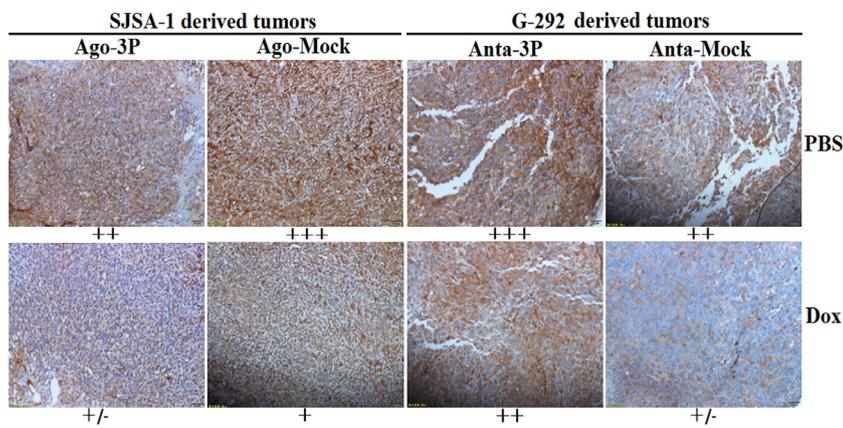


Fig. 6. The IRS2 levels in the miR-193a-3p agomiR-injected SJSA-1 and the miR-193a-3p antagomiR-injected G-292 tumor xenografts versus the NC-injected tumor xenografts determined by immunohistochemical staining. The SJSA-1 and G-292 tumor tissues from each group were fixed on one slide and immunostained with each indicated antibody. The levels of IRS2 protein in each group were determined by immunostaining and are summarized in the table (Magnification: 200×).

Intraperitoneal treatment	SJSA-1 tumors		G-292 tumors	
	Intratumor treatment	anti-IRS2	Intratumor treatment	anti-IRS2
PBS	Ago-Mock	+++	Anta-Mock	++
	Ago-3P	++	Anta-3P	+++
Dox	Ago-Mock	+	Anta-Mock	+/-
	Ago-3P	+/-	Anta-3P	++

Consent to publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed in this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conception and design: YGP and HYW.

Acquisition of data (provided animals, provided facilities, etc.): FFZ, HYW and YGP.

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): SBC and FFZ.

Writing, review, and/or revision of the manuscript: YGP and HYW.

All authors read and approved the final manuscript.

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Not applicable.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jbo.2019.100241.

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