Metformin sensitises osteosarcoma to chemotherapy *via* the IGF-1R/miR-610/FEN1 pathway

Suwei Dong,[1,2*] Yanbin Xiao,[2*] Ziqiang Zhu,[1*] Xiang Ma,[2] Zhuohui Peng,[2] Jianping Kang,[2] Jianqiang Wang,[1] Yunqing Wang,[1] Zhen Li[3]

[1]Department of Orthopaedics, The Second Affiliated Hospital of Xuzhou Medical University, Xuzhou, Jiangsu
[2]Department of Orthopaedics, The Third Affiliated Hospital of Kunming Medical University, Kunming, Yunnan
[3]Department of Medical Oncology, The Second Affiliated Hospital of Xuzhou Medical University, Xuzhou, Jiangsu, China

**These authors contributed equally*

ABSTRACT

Metformin can enhance cancer cell chemosensitivity to anticancer drugs. IGF-1R is involved in cancer chemoresistance. The current study aimed to elucidate the role of metformin in osteosarcoma (OS) cell chemosensitivity modulation and identify its underlying mechanism in IGF-1R/miR-610/FEN1 signalling. IGF-1R, miR-610, and FEN1 were aberrantly expressed in OS and participated in apoptosis modulation; this effect was abated by metformin treatment. Luciferase reporter assays confirmed that FEN1 is a direct target of miR-610. Moreover, metformin treatment decreased IGF-1R and FEN1 but elevated miR-610 expression. Metformin sensitised OS cells to cytotoxic agents, while FEN1 overexpression partly compromised metformin's sensitising effects. Furthermore, metformin was observed to enhance adriamycin's effects in a murine xenograft model. Metformin enhanced OS cell sensitivity to cytotoxic agents *via* the IGF-1R/miR-610/FEN1 signalling axis, highlighting its potential as an adjuvant during chemotherapy.

Key words: osteosarcoma; metformin; IGF-1R; FEN1; chemosensitivity.

Correspondence: Zhen Li, Department of Medical Oncology, The Second Affiliated Hospital of Xuzhou Medical University, Xuzhou, Jiangsu, China. E-mail: lizhenres@163.com

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Conflict of interest: the authors declare that they have no competing interests.

Ethical approval: the Ethics Committee of the Third Affiliated Hospital of Kunming Medical University and the Kunming Medical University Animal Care and Use Committee approved the study (Protocol no. YTH2019-026). Furthermore, per the Declaration of Helsinki, all patients provided written informed consent and authorized biological specimen use.

Availability of data and materials: we declare that the materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes without breaching participant confidentiality.

Introduction

Besides leukaemia and lymphoma, osteosarcoma (OS) is the most frequent primary malignant bone tumour in children and the most common primary malignancy in adolescents.[1] Currently, limb salvage and neoadjuvant chemotherapy are still considered the first-line treatment combination for OS patients; even though many efforts have been made to improve the treatment outcome, the mortality rate remains high.[2] High-dose anti-cancer drug regimens involving adriamycin (ADM), cisplatin (DDP), and methotrexate (MTX) are most commonly used during OS treatment.[3] However, in addition to their association with systemic toxicity and the risk of secondary cancers,[4-6] constitutive or acquired resistance can compromise the effects of many chemotherapeutics.[7] Therefore, preclinical studies are needed to uncover the underlying biological pathways implicated in chemosensitivity.[8-10]

Metformin is the most widely used drug for type II diabetes treatment. It is effective in reducing insulin resistance and decreasing blood glucose[11], and it has recently emerged as a potential agent for tumour prevention and treatment.[12-14] Various mechanisms underly metformin's anti-tumour effect, such as gluconeogenesis and oxidative phosphorylation inhibition, effects on cell growth, mobility, apoptosis, stemness, and autophagy have also been reported.[12,15,16] Intriguingly, several studies have shown that metformin can enhance cytotoxic effects when combined with various cytostatic drugs.[17,18] However, the causal mechanisms for such cooperative effects are still unclear.

Insulin-like growth factor 1 receptor (IGF-1R) is a transmembrane tyrosine kinase receptor in the insulin receptor family that can regulate miRNA expression,[19] and its expression is upregulated in OS. IGF-1R upregulation is associated with tumour stage and metastasis.[20] What's more, IGF-1R blockade increases chemo-sensitivity in multidrug-resistant OS cell lines.[21,22] A recent study demonstrated that metformin could overcome primary resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) with EGFR mutation via targeting the IGF-1R signalling pathway.[23]

Our previous study revealed that flap endonuclease 1 (FEN1) modulates OS chemosensitivity;[24] it is still unknown whether IGF-1R modulates OS cell chemo-sensitivity *via* FEN1. It is well known that miRNAs are responsible for OS chemosensitivity modulation and considered targets for enhancing chemosensitivity,[25] and we demonstrated that miR193b increases the chemosensitivity of OS cells by targeting FEN1.[24] It is possible for mRNA to be targeted by multiple miRNAs. Additionally, miR610 is considered a tumour suppressor in OS.[26] Thus, we hypothesised that IGF-1R promoted FEN1 expression by downregulating miR610. This study aimed to elucidate the potential role of metformin in the IGF1R-miR610-FEN1 signalling pathway, OS cell chemosensitivity modulation and its underlying mechanisms.

Materials and Methods

Ethics statement

This study was approved by The Third Affiliated Hospital of Kunming Medical University Institutional Ethics Committee and conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from each patient for the use of their tissues. Animal experiments were performed following a strict protocol following the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Efforts were made to minimize the suffering of the animals included in the study. The Kunming Medical University Animal Care and Use Committee approved the animal experiment protocols involving mice (Protocol no. YTH2019-026).

Bioinformatics analysis

GeneMANIA (http://genemania.org/) was applied to predict the proteins coexpressed with IGF-1R, and GEPIA (http://gepia.cancer-pku.cn/index.html) was utilised to analyse the correlation between IGF-1R and FEN1 expression in sarcoma tissues. Subsequently, miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/index.php) and TargetScan (http://www.targetscan.org/) were used to assess miRNA binding within FEN1. The dbDEMC 2.0 database (https://www.picb.ac.cn/dbDEMC/index.html) was also used to analyse miR-610 expression in tumour tissues (sarcoma, a subtype of OS).

Tissue samples

Paired tumours and adjacent normal formalin-fixed paraffin-embedded (FFPE) tissue samples were collected from 66 OS patients between 2015 and 2019. A miRNeasy FFPE Kit (Qiagen, Hilden, Germany) was used to extract RNAs from paraffin-embedded samples. Patients with recurrent OS and those undergoing preoperative radiation, chemotherapy or biotherapy were excluded from this study to avoid treatment-related biases when assessing tumour markers.

Cell culture, transfection, antibodies, and reagents

American Type Culture Collection (ATCC, Manassas, VA, USA) provided the human MG-63, U2OS and 143B OS cell lines. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Carlsbad, CA, USA) and maintained at 37°C in a humidified incubator with 5% CO_2. The miR-610 mimic, miR-610 inhibitor, and negative control were designed and synthesised by Guangzhou RiboBio Co., Ltd (Science City, Guangzhou, China), along with the small interfering RNA (siRNA) targeting FEN1 and IGF-1R (si-FEN1, si-IGF1R) and negative control (NC) siRNA. All plasmid constructs were verified by sequencing. Lipofectamine 2000 (Invitrogen, USA) was used for each miRNA or siRNA transfection according to the manufacturer's protocol. Cells were harvested for subsequent experiments 48 h after transfection or anti-cancer drug treatment [metformin 10 mM, ADM (0.7095 μM for MG-63, 0.9787 μM for U2OS), DDP (0.9731 μM for MG-63, 1.018 μM for U2OS), MTX (35.68 μM for MG-63, 33.54 μM for U2OS)]. Metformin, ADM, DDP and MTX were purchased from Sigma-Aldrich (St. Louis, MO, USA). The drugs were prepared immediately before use. The FEN1, IGF-1R, cleaved caspase-3, and GAPDH antibodies were obtained from Abcam (Cambridge, MA, USA).

Luciferase reporter assay

FEN1 3′-untranslated region (3′-UTR) containing wild type (WT) or mutated miR-610 binding site was cloned into the pGL3-basic luciferase reporter vector (Promega, USA), which were named as FEN1 WT and FEN1 MUT, respectively. Briefly, 1×10^5 OS cells/well were seeded into a 24-well plate and co-transfected with FEN1 WT or FEN1 MUT and the miR-610 mimic or NC miRNA by using Lipofectamine 2000 (Invitrogen, USA). Then firefly luciferase activity was measured using a dual-luciferase reporter assay system (Promega, Madison, WI, USA) and normalised to the corresponding *Renilla* luciferase activity.

RT-qPCR

Total RNA was extracted using TRIzol Reagent (Invitrogen)

and purified with an RNeasy Maxi kit (Qiagen) and reverse transcribed using a miScript II RT Kit (Qiagen). The target RNAs were quantified with a Roche LightCycler 480 Real-Time PCR system (Roche Diagnostics, Switzerland), and the relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method;[27] mRNA and miRNA expression was normalised to GAPDH and U6, respectively. The primers used for the qRT-PCR analysis are presented in Table 1.

Western blotting

Proteins were extracted with RIPA lysis buffer (89900, Pierce Biotechnology, Waltham, MA, USA) according to the manufacturer's protocol and quantified using a BCA protein assay reagent kit (23227, Thermo Fisher Scientific). The samples (50 µg protein lysate per well) were electrophoresed in SDS-PAGE slab gels and transferred onto polyvinylidene fluoride (PVDF) membranes by electroblotting. The membranes were pre-treated with 5% non-fat milk in the tris buffered saline (TBS-T) for 2 h and then incubated overnight with primary antibodies at 4°C. The following primary antibodies were used: anti-FEN1, ab17994; anti-IGF-1R, ab263903; anti-cleaved caspase-3, ab49822; all primary antibodies were obtained from Abcam. The membranes were subsequently incubated with horseradish peroxidase (HRP)-labelled secondary antibody (1:10,000, #7076, Cell Signaling Technology, Danvers, MA, USA) for 1 hour. GAPDH was used as an internal loading control (1:1000, ab181602; Abcam). All assays were repeated three times.

Apoptosis analysis

Apoptosis was analysed using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit according to the manufacturer's protocol (BD Biosciences, San José, CA, USA). Cells were seeded into 24-well plates (1×10^5 cells/well), cultured for 24 h, harvested using trypsin, centrifuged at 2500 rpm for 5 min, and washed twice with cold PBS before staining with 1% FITC-labelled annexin V and propidium iodide. After incubation, apoptosis was evaluated using an Aria flow cytometer (BD Biosciences - Immunocytometry Systems) and analysed by Cell Quest software (Becton Dickinson Ltd., Franklin Lakes, NJ, USA). All experiments were performed independently three times.

MTS assay

To determine metformin's cytotoxicity and the combined effect of metformin (10 mM) and chemotherapeutic agents, cells were seeded at a density of 5×10^3 per well in 96-well culture plates in 150 µL of medium with serial doses for 48 h (0, 0.037, 0.111, 0.333, 1, 3, 9, 27, 81, 243 µM) of ADM, DDP or MTX. Each group was assigned six parallel wells that were used as a negative control (without cells). In addition, 30 µL MTS substrate was added to each well and incubated for 2 h in the dark. The absorbance was measured at 490 nm using a plate reader (BMG Labtech, Ortenberg, Germany). The concentrations required to inhibit cell growth by 50% (IC_{50}) were calculated using the Bliss method.[28] These experiments were performed three times independently.

Immunohistochemical analysis

Immunohistochemical (IHC) analysis was performed to determine FEN1 and IGF-1R levels and their distribution patterns. First, 4 µm paraffin-embedded tissue sections were mounted on positively-charged glass slides and baked at 60°C for 2 h. After deparaffinisation in xylene, sections were heated for 10 min in citrate buffer (pH 6.0) for antigen retrieval using a microwave oven, and endogenous peroxidase activity was quenched by incubation in 0.3% H_2O_2. Next, rabbit polyclonal antibodies (FEN1, ab17994; IGF-1R ab263903, Abcam) were diluted 1:250 in phosphate-buffered saline (PBS) to detect FEN1 or IGF-1R proteins. After two washes in PBS, the slides were incubated with ABC (Vector Laboratories, Burlingame, CA, USA), washed, exposed to 3-3'-diaminobenzidine (DAB; Dako Corporation, Carpinteria, CA, USA), and counterstained with haematoxylin. Human lung squamous carcinoma tissue was used as a positive control, while negative controls were obtained by replacing the primary antibody with non-immunized serum. The tissue was considered FEN1 and IGF-1R positive if the staining was detectable in more than 10% of the tumour cells. The slides were independently evaluated to determine the protein levels by three different observers, and the slides with inconsistent scoring were re-evaluated to reach a consensus.

Mice xenograft models

Four to five-week-old athymic female BALB/c nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were randomly divided into four groups (n=5 per group); 5×10^7 143B cells suspended in PBS were injected into the right flank of the nude mice. The xenograft tumour length (a) and width (b) were measured every other day with a Vernier calliper. Tumour volumes (cm^3) were calculated using the following formula: volume = $ab^2/2$. When the tumour volumes reached 0.1 cm^3, the mice were injected intraperitoneally with ADM (5 mg/kg, twice a week), metformin (250 mg/kg, once per day), or ADM (5 mg/kg, twice a week) plus metformin (250 mg/kg, once per day) as stated in previous studies.[29,30] Saline was selected as the drug vehicle. The mice were sacrificed 21 days after inoculation, and tumours were excised and weighed.

Statistical analysis

FEN1 and IGF-1R immunocytochemical labelling were correlated with other clinical pathology parameters using the χ^2 test. Unless indicated otherwise, the quantitative data are expressed as means ± SD relative to the value of the controlled variable. A p-value was considered significant if less than 0.05. All statistical analyses were performed using the SPSS for Windows version 18.0 (SPSS Inc., Chicago, IL, USA).

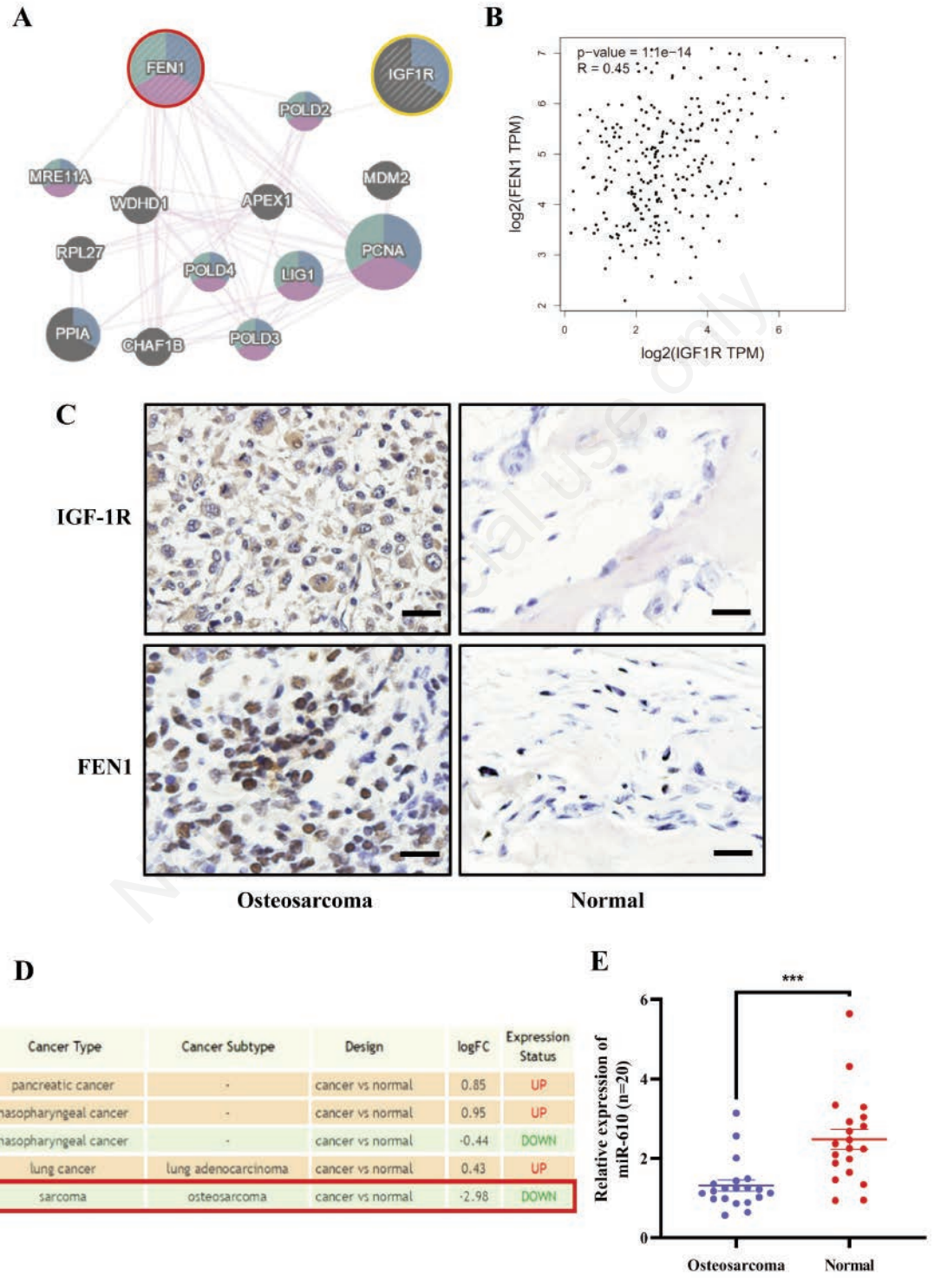
Table 1. RT-qPCR primer sequences.

Target	Primer sequence (5 -3)	
IGF-1R	Forward:	5'-TCATGCCTTGGTCTCCTTGT-3'
	Reverse:	5'-TGCTTTGATGGTCAGGTTGC-3'
FEN1	Forward:	5'-GTGAAGGCTGGCAAAGTCTA-3'
	Reverse:	5'-GCAGTCAGGTGTCGCATTA-3'
GAPDH	Forward:	5'-TCAAGAAGGTGGTGAAGCAGG-3'
	Reverse:	5'-TCAAAGGTGGAGGAGTGGGT-3'
miR-610	Forward:	5'-TGCGCTGAGCTAAATGTGTCC-3'
	Reverse:	5'-CAGTGCGTGTCGTGGAGT-3'
U6	Forward:	5'-GCTTCGGCAGCACATATACTAAAAT-3'
	Reverse:	5'-CGCTTCACGAATTTGCGTGTCAT-3'

Results

IGF-1R/miR-610/FEN1 expression in OS tissues

GeneMANIA was used to predict IGF-1R co-expression protein networks, which indicated an indirect interaction between flap structure-specific endonuclease 1 (FEN1) and IGF-1R (Figure 1A). FEN1 participates in numerous DNA processing pathways,[31] a recent study uncovered that FEN1 inhibition could sensitise cancer cells to drugs.[32] GEPIA demonstrated that IGF-1R expression was



Cancer Type	Cancer Subtype	Design	logFC	Expression Status
pancreatic cancer	-	cancer vs normal	0.85	UP
nasopharyngeal cancer	-	cancer vs normal	0.95	UP
nasopharyngeal cancer	-	cancer vs normal	-0.44	DOWN
lung cancer	lung adenocarcinoma	cancer vs normal	0.43	UP
sarcoma	osteosarcoma	cancer vs normal	-2.98	DOWN

Figure 1. IGF-1R, miR-610 and FEN1 expression patterns in OS tissues. A,B) The correlation between IGF-1R and FEN1. C) Immunohistochemical staining for IGF-1R and FEN1 in OS and paired adjacent normal samples; scale bar: 20 μm. D,E) RT-qPCR detection of miR-610 expression in OS tissues. *** p<0.001 *vs* control.

positively correlated with FEN1 (p<0.001, *r*=0.45, Figure 1B). To validate this analysis, IGF-1R and FEN1 levels were detected in tissues from 66 OS patients with paired adjacent normal tissue. Positive staining IGF-1R staining was confined mainly to the membrane and cytoplasm in OS tissues compared to the negatively stained normal tissue, and FEN1 was expressed primarily on the cellular nucleus (Figure 1C). Moreover, IGF-1R and FEN1 were positively correlated in these samples (p=0.009, *r*=0.319; Tables 2 and 3). To explore the mechanisms underlying this IGF-1R and FEN1 interaction, the miRTarBase and TargetScan databases were used to predict that miR-610 may bind to FEN1 3'-untranslated regions (3'-UTR). The dbDEMC 2.0 database also showed that miR-610 expression decreased in sarcoma and OS subtypes. Twenty OS and paired normal tissues were assessed, which confirmed that miR-610 expression was lower in OS tissues than in normal tissues (Figure 1D).

OS cell apoptosis is modulated by the IGF-1R/miR-610/FEN1 pathway

To further explore the effect of IGF-1R on OS, MG-63 and U2OS cells were transfected with IGF-1R si-RNA, which led to increased miR-610 and decreased FEN1 expression (Figure 2 A-D). A flow cytometry analysis on these cells demonstrated that IGF-1R silencing could induce OS cell apoptosis (Figure 2E). RT-PCR was utilised to assess miR-610 expression in the OS cell lines following transfection with a miR-610 mimic or inhibitor (Figure 3A). Western blotting and real-time PCR demonstrated that FEN1 expression was increased in the OS cells following miR-610 inhibition. In contrast, OS cells treated with the miR-610 mimic exhibited an opposite trend (Figure 3 B,C). FEN1 WT and MUT 3'-UTR sequences were structured based on potential binding sites. Luciferase activity greatly decreased due to miR-610 overexpression when FEN1 WT was co-transfected into 293T cells, whereas co-transfection with MUTt-FEN1-3'UTR did not alter luciferase activity. This data suggested that FEN1 was a direct target of miR-610 in OS cells (Figure 3D). When OS cells were transfected with FEN1 si-RNA (Figure 4A), flow cytometry demonstrated that FEN1 knockdown induced OS cell apoptosis and elevated cleaved caspase-3 levels (Figure 4 B,C). These results indicated that IGF-1/miR-610/FEN1 pathway inhibition could induce OS cell apoptosis.

Metformin regulates IGF-1R/miR-610/FEN1 signalling and chemosensitivity in OS cells

Metformin treatment decreased IGF-1R and FEN1 expression and increased miR-610 expression (Figure 5 A,B). Moreover, when the OS cell lines were treated with metformin, their apoptosis rates were elevated (p<0.05 for both MG-63 and U2OS (Figure 5C). Metformin (10 mM) treatment alone significantly induced MG-63 apoptosis. Furthermore, metformin significantly enhanced ADM-induced apoptosis when combined with ADM (1 μM) (Supplementary Figure 1). Plasmids expressing FEN1 were constructed to explore metformin's anti-cancer activities. Transient transfection increased FEN1 expression in OS cells (Figure 6A), and MG-63 and U2OS sensitivity to ADM, DDP and MTX were evaluated in combination with metformin. The anti-cancer drugs decreased OS cell viability, which was more apparent following co-treatment with metformin. Interestingly, FEN1 overexpression partly inhibited metformin's sensitising effect (Figure 6 B,C; Table 4). This data suggested that the IGF-1R/miR-610/FEN1 signal pathway was involved in metformin-induced chemosensitivity.

Table 2. IGF-1R and FEN1 in paired human OS and adjacent normal specimens.

	Expression	Osteosarcoma	Normal	p
IGF-1R	Positive	46	5	0.000
	Negative	20	61	
FEN1	Positive	50	12	0.000
	Negative	16	54	

Table 3. IGF-1R and FEN1 correlation in human OS specimens.

	IGF-1R			p
	Positive	Negative		
FEN1				
Positive	39	11		p=0.009
Negative	7	9	r=0.319	

Table 4. IC_{50} values for MG-63 and U2OS cells.

	MG-63 IC_{50} (μM)	p	U2OS IC_{50} (μM)	p
ADM	0.707±0.018	0.025	1.01±0.113	0.031
ADM+Met	0.551±0.03		0.596±0.039	
DDP	0.989±0.02	0.017	1.014±0.029	0.005
DDP+Met	0.896±0.005		0.9±0.039	
MTX	35.277±0.971	0.000	32.79±0.691	0.001
MTX+Met	12.3±0.403		10.297±0.499	

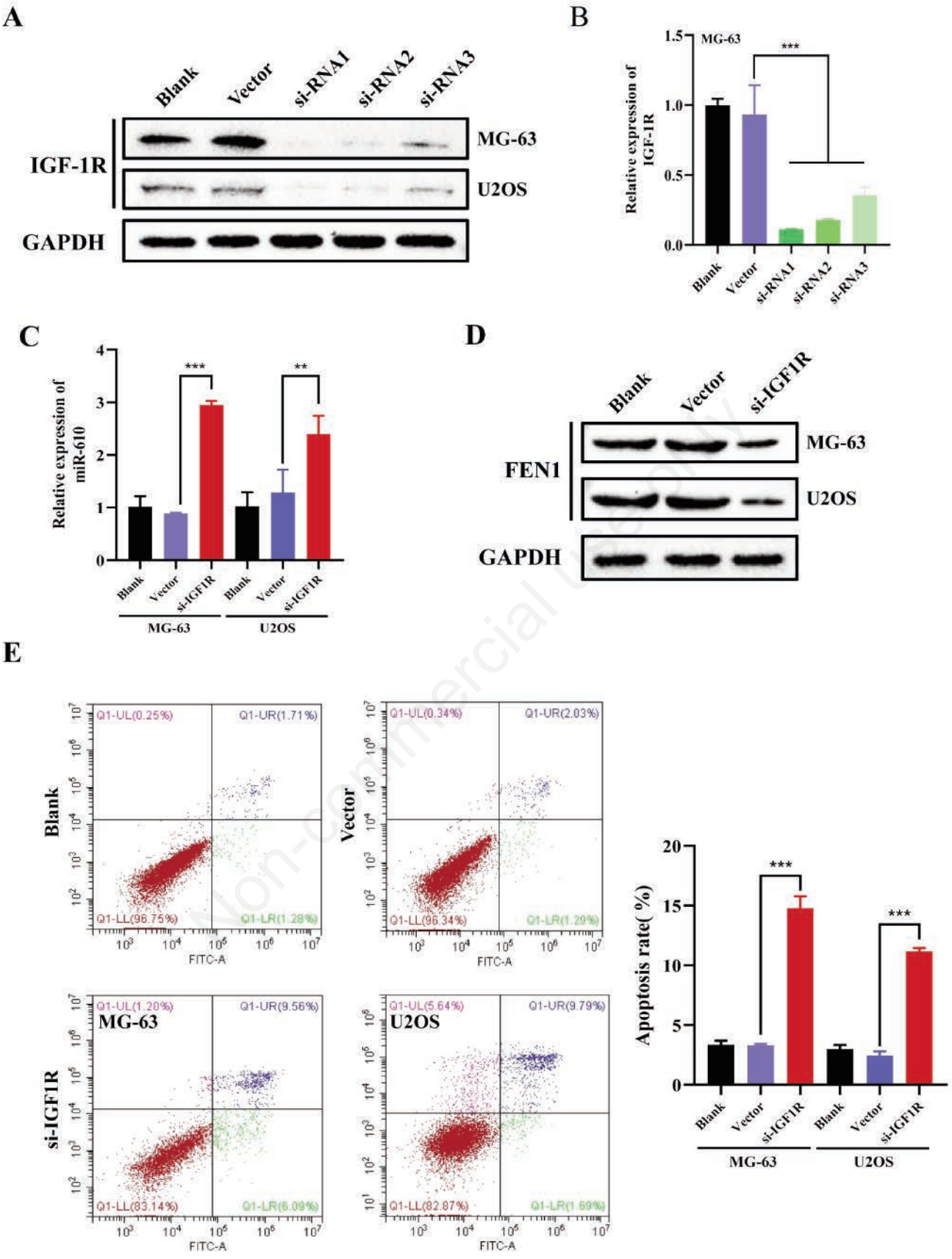


Figure 2. **IGF-1R modulation of miR-610/FEN1 expression and apoptosis in OS cells. A-D) FEN1 and miR-610 expression following IGF-1R expression alteration. E) OS cell apoptosis was determined by flow cytometry after treatment with IGF-1R si-RNA. **p<0.01, ***p<0.001 *vs* control.**

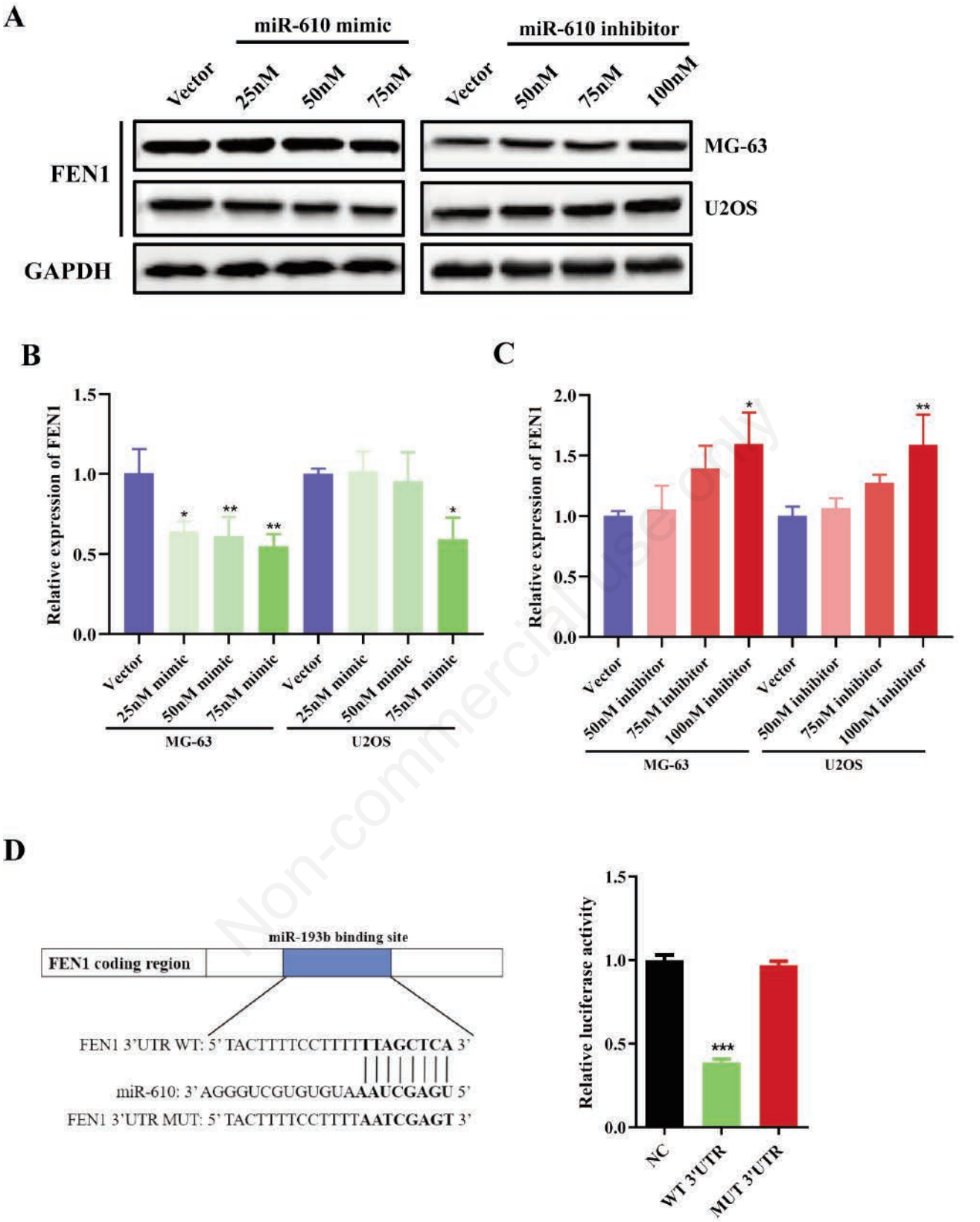


Figure 3. FEN1 is a direct miR-610 target. A-C) FEN1 expression after miR-610 modulation, FEN1 protein levels and mRNA expression in OS cells transfected with either a miR-610 mimic or inhibitor. D) miR-610 binding site in the FEN1 3'-UTR and matched mutated sequence; a luciferase reporter assay in OS cells was conducted to verify the miR-610 and FEN1 binding site interaction. *p<0.05, **p<0.01, ***p<0.001 *vs* control.

Metformin elevates OS chemo-sensitivity *in vivo*

A subcutaneous *in vivo* model was employed to detect the effect of metformin on OS (143B cells) tumorigenicity in female athymic nude mice. The mice were injected intraperitoneally with ADM, metformin or ADM combined with metformin for 21 days once the tumour volume reached 0.1 cm^3. The metformin combination therapy markedly reduced the tumour growth rate ($p<0.01$) and weight ($p<0.05$) compared with chemotherapy alone (Figure 7 A,B). In addition, the metformin treatment group had significantly increased miR-610 and decreased IGF-1R and FEN1 expression (Figure 7C). This data demonstrates that metformin contributes significantly to OS cell chemo-sensitivity *in vivo*.

Discussion

Despite the success of chemotherapy for OS, most patients with OS experience recurrence and have a poor prognosis.[33] In our previous study, FEN1 was shown to be a key regulator of OS cell chemosensitivity,[24] and we demonstrated herein that the IGF-1R/miR-610/FEN1 pathway is involved in OS cell apoptosis modulation. Moreover, metformin has been shown to induce OS cell cycle arrest, apoptosis and autophagy.[34,35] Metformin can also increase EGFR tyrosine kinase inhibitors sensitivity by targeting IGF-1R.[23] Therefore, we hypothesised that metformin could affect OS cell chemosensitivity *via* the IGF-1R signalling pathway.

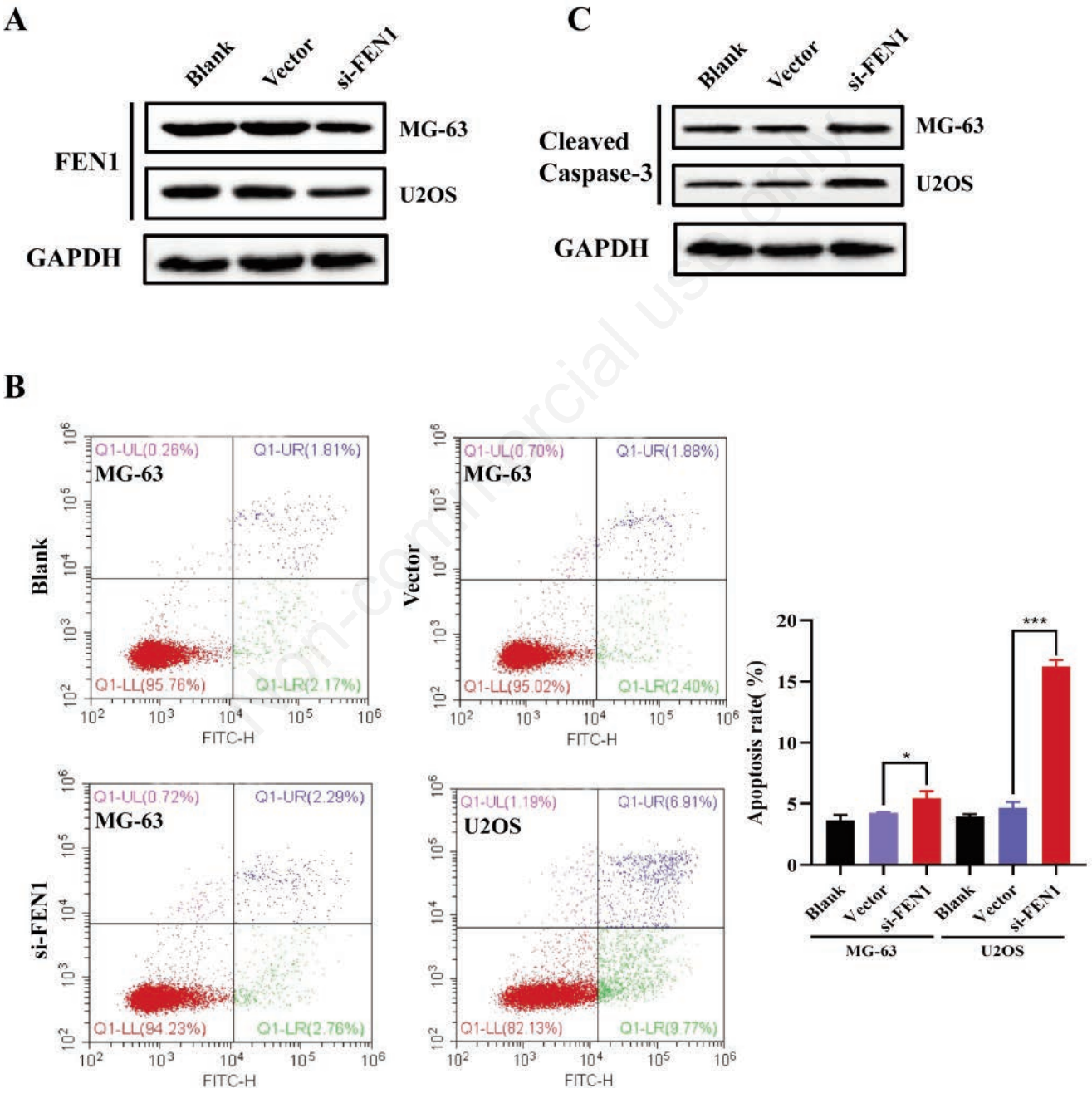


Figure 4. FEN1 modulation of OSC cell apoptosis. A,B) OS cell apoptosis was determined using flow cytometry after FEN1 knockdown. C) Cleaved-caspase3 expression following FEN1 knockdown. *p<0.05, ***p 0.001 *vs* control.

Metformin decreased IGF-1R and FEN1 expression and increased OS cell chemosensitivity *in vitro* and *in vivo*. Recent study demonstrated that metformin could impact head and neck squamous cell carcinoma cell sensitization by modulates a number of genes including FEN1.[36] Thus, FEN1 might be a critical target for metformin to regulate cancer cell chemosensitivity. Although it's uncovered that IGF-1R was targeted by various miRNAs, little is known about miRNAs regulated by IGF-1R. In the present study, we show that IGF-1R impact the expression of miR-610, but the underlying mechanism remains unknown.

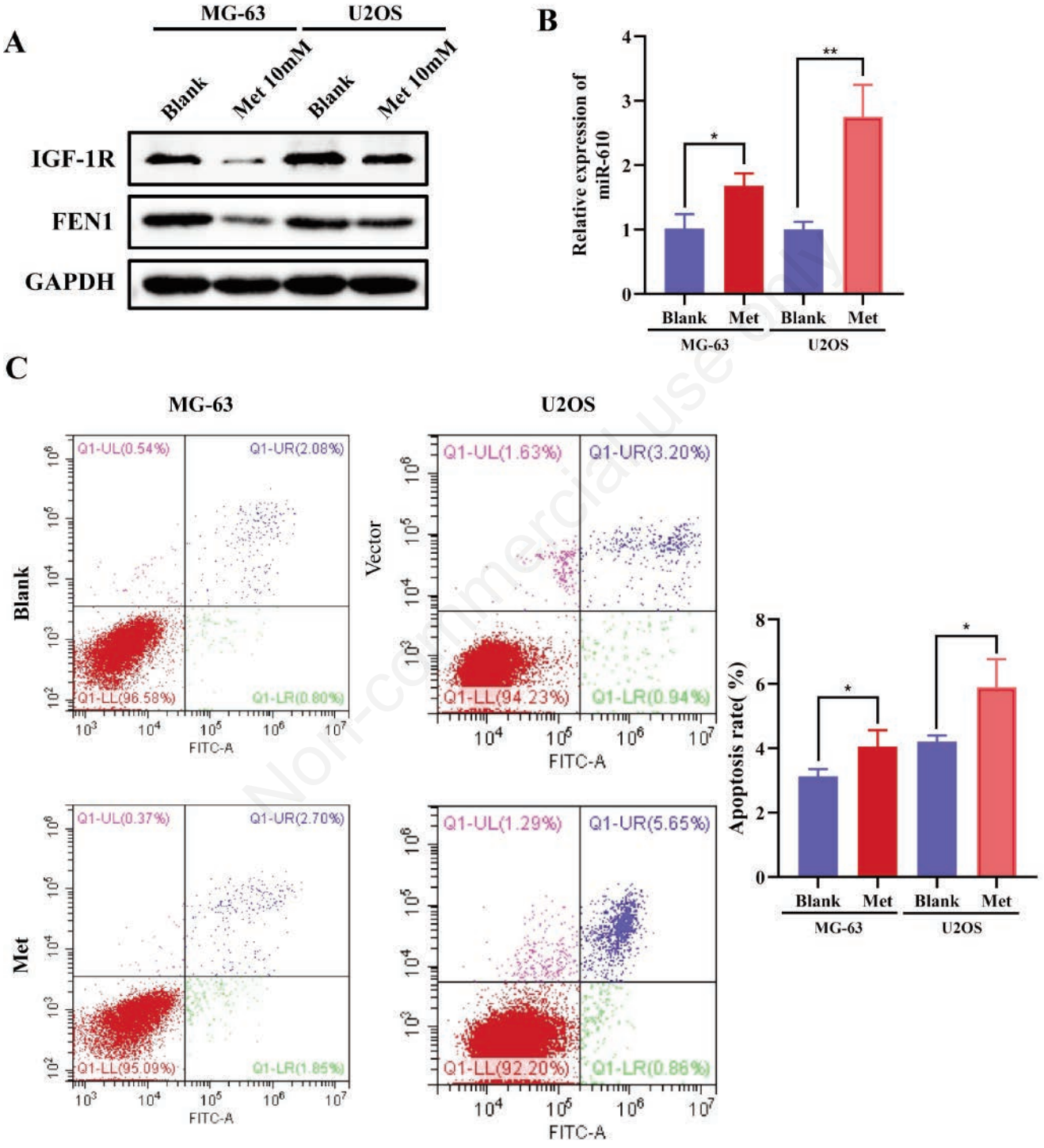


Figure 5. Effect of metformin on IGF-1R/miR-610/FEN1 axis expression and OS cell apoptosis. A,B) IGF-1R, miR-610 and FEN1 expression after metformin treatment. C) OS cell viability was determined by flow cytometry after metformin treatment. *p<0.05, **p<0.01 *vs* control.

Cytotoxic chemotherapy causes severe DNA damage directly by induction of DNA breaks or indirectly due to the formation of reactive oxygen species (ROS) and ultimately induces cell death.[37,38] In response to DNA damage, the cells recruit DNA repair factors to upregulate mutagenic repair pathways to maintain survival.[39] Therefore, decreased double-strand break repair proteins expression can hypersensitise cells to DNA damage agents.[40] Metformin can reduce the expression level of DNA repair proteins.[41] Although FEN1 participates in Top2 mediated DNA repair following agent-induced single-strand breaks (Top2-SSB complex) to maintain DNA stability,[42] FEN1 disruption leads to DNA double-strand break accumulation.[43] Therefore, metformin may sensitise OS cells to chemotherapy agents by disrupting DNA damage repair processes.

Consequently, our study demonstrates that metformin enhances chemotherapy agent-induced apoptosis by regulating the IGF-1R/miR-610/FEN1 signalling pathway in OS. Furthermore, combining metformin with other anti-cancer compounds enhanced the anti-cancer effects, thus providing a potential therapeutic strategy for OS patients.

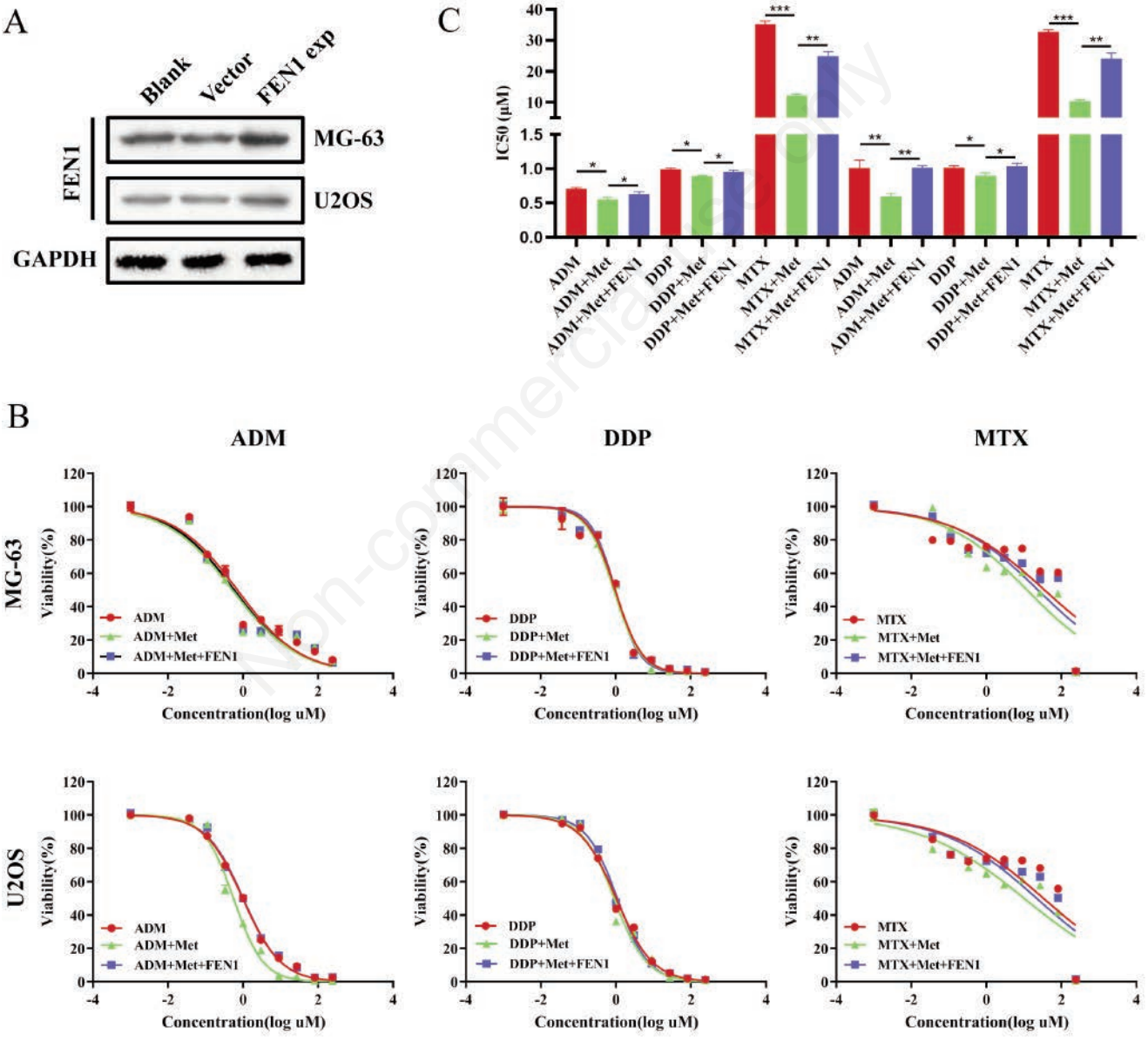


Figure 6. The role of FEN1 in metformin-induced chemosensitivity modulation in OS cells. A) ADM-induced MG-63 cell apoptosis was enhanced by metformin. B,C) Metformin promoted cytotoxic agent-associated induced cell death in OS cells, which was inhibited partly by FEN1 overexpression. *p<0.05, **p<0.01, ***p<0.001 *vs* control.

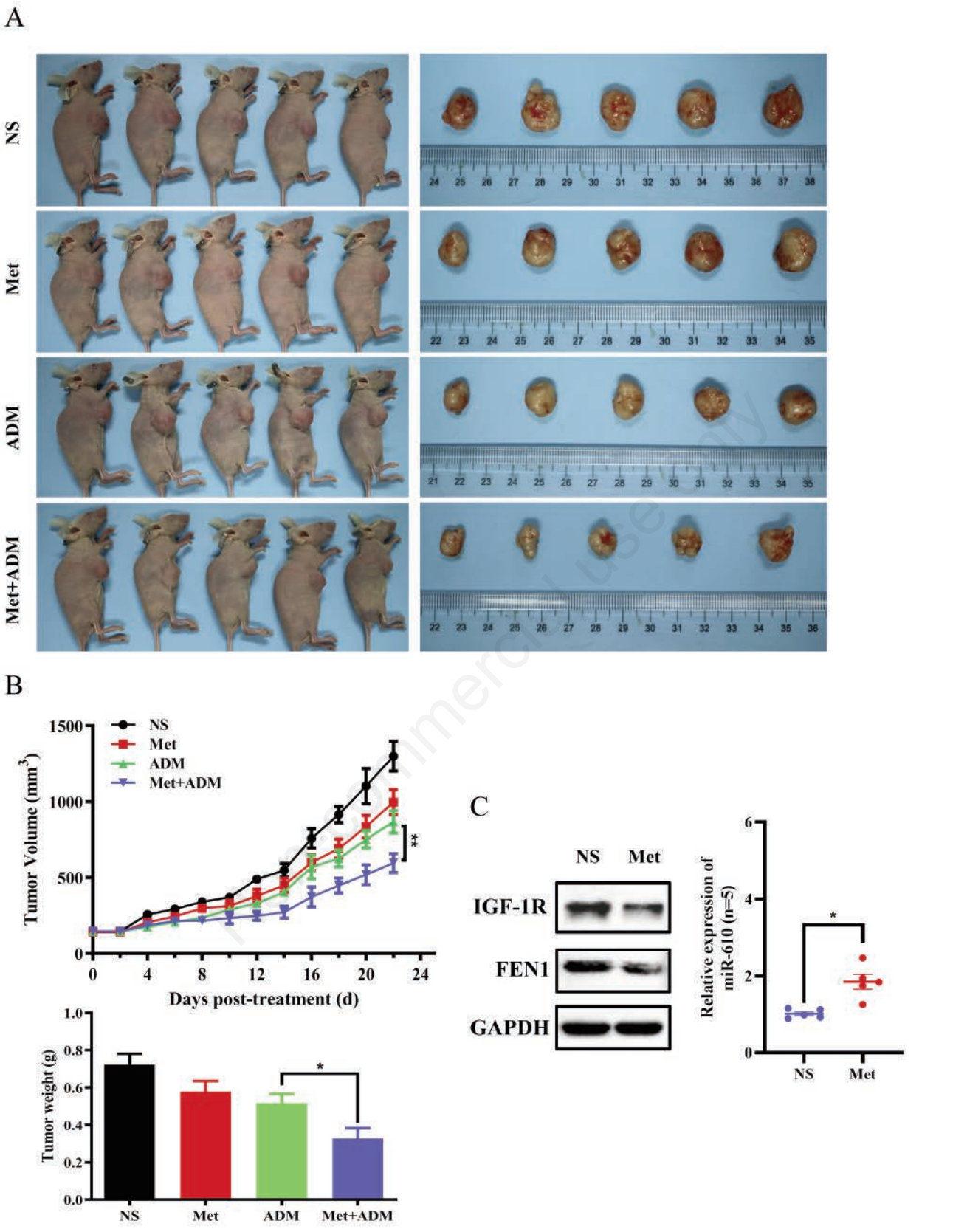


Figure 7. Metformin modulation of OS cell chemosensitivity in a nude mouse model. A) Representative mouse and tumour pictures for each group 21 days after inoculation. B) Tumour volumes and weights in four groups over time. C) *In vivo* IGF-1R, FEN1 and miR-610 expression after metformin treatment. *p<0.05, **p<0.01 *vs* control.

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Online supplementary material:
Figure 1. Flow cytometry assessment of metformin and ADM treatment on MG-63 cell apoptosis; ***p<0.001 *vs* controls.

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