Metformin enhances radiosensitivity in hepatocellular carcinoma by inhibition of specificity protein 1 and epithelial-to-mesenchymal transition

ABSTRACT

Objective: Radiotherapy becomes more and more important in hepatocellular carcinoma (HCC) due to the development of technology, especially in unresectable cases. Metformin has a synergistic benefit with radiotherapy in some cancers, but remains unclear in HCC. This study aims to investigate the effect of metformin on radiosensitivity of HCC cells and the roles of specificity protein 1 (Sp1) as a target of metformin.

Methods: The SMMC-7721 cell line was exposed to various doses of γ-ray irradiation (0, 2, 4, 6, and 8 Gy) and with or without different concentrations of metformin (0, 1, 5, 10, and 20 mM) to measure the radiosensitivity using MTT assay. Flow cytometry was used to determine cell cycle by propidium iodide (PI) staining and apoptosis by Hoechst 33342/PI staining and Annexin V-FITC/PI staining. Real-time polymerase chain reaction and Western blotting were performed to analyze the Sp1 mRNA and protein expressions of Sp1 and epithelial-to-mesenchymal transition (EMT) marker E-cadherin and Vimentin. The invasion capability was measured by the Boyden chamber assay.

Results: In SMMC-7721 cells exposed to irradiation, metformin reduced proliferation and survival cells at various concentrations (0, 1, 5, 10, and 20 mM) and induced cell cycle arrest, apoptosis, and inhibited invasion. In SMMC-7721 cells with irradiation, the mRNA and protein expressions of Sp1 were significantly decreased by metformin as well as a selective Sp1 inhibitor. Metformin attenuated transforming growth factor-β1 induced decrease of E-cadherin and increase of Vimentin proteins.

Conclusion: Metformin demonstrated enhanced radiosensitivity and inhibition of EMT in HCC cells. Sp1 might be a target of metformin in radiosensitization.

KEY WORDS: Epithelial-to-mesenchymal transition, hepatocellular carcinoma, metformin, radiosensitivity, specificity protein 1

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the common cancers and the second cause of cancer-related deaths.[1] Currently, surgery is the standard treatment for HCC, but the prognosis of HCC patients is unsatisfactory, with a low 5-year survival rate.[2] Despite the significant improvements in therapeutic techniques, complete surgical resection is suitable for fewer than 20% of patients with HCC. Furthermore, for advanced HCC patients, little clinical benefits could be achieved by conventional cytotoxic chemotherapy.[3] Among other treatment modalities, radiotherapy can produce sustained local control, especially for unresectable HCC.[4] Therefore, more radiosensitizing agents are needed to improve the clinical radiotherapeutic efficacy of HCC.[5]

Metformin (1,1-dimethylbiguanide hydrochloride), a commonly used drug for the treatment of type 2 diabetes, has been found to demonstrate association with a decreased incidence and mortality in several cancers.[4] Recent evidence has suggested that metformin has antineoplastic effects, especially providing a synergistic benefit with radiotherapy.[7] Metformin shows wide radiosensitizing effects on various cancers, including nasopharyngeal carcinoma, esophageal squamous cell carcinoma, and colorectal cancer.[8-10] The effect of metformin...
Specificity protein 1 (Sp1) is a transcription factor that belongs to Sp/Kruppel-like factor family and high Sp1 expression in tumors shows negative prognosis in glioma, gastric cancer, and lung cancer.[14,15] Furthermore, Sp1-mediated transcriptional activation could confer radiosensitivity for cancer, and inhibition of Sp1 could induce radiosensitization.[14,15] This indicates that Sp1 protein might be a molecule involving radioresistance. However, currently, the role of Sp1 in radioresistance and radiosensitizing effects of HCC is still unclear.

Given the wide radiosensitizing effect of metformin in cancers, in this study, we investigated the effects of metformin on cell viability, cell cycle and apoptosis of HCC cells, and the mRNA and protein expressions of Sp1 by metformin. We also investigate the role of Sp1 and epithelial-to-mesenchymal transition (EMT) in radioresistance of HCC and radiosensitizing effect of metformin. Our study will provide evidence for Sp1 as a target of radiosensitizing effects of metformin.

METHODS

Chemicals
Metformin, mithramycin A, and recombinant human transforming growth factor β1 (TGF-β1) were purchased from Sigma-Aldrich Chemical Corp (St. Louis, MO, USA). High glucose DMEM and fetal bovine serum (FBS) were purchased from Invitrogen-GIBCO (Carlsbad, CA, USA). Trizol® reagent and Superscript III enzyme were purchased from Life Technologies (Carlsbad, CA, USA). Antibodies were purchased as follows: mouse monoclonal antibodies against human Sp1 and β-actin were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and mouse monoclonal antibodies against human E-cadherin and Vimentin were purchased from Cell Signaling Technology (Cell Signaling Technology, Danvers, MA, USA).

Cell culture
The HCC cell line SMMC-7721 was obtained from American Type Culture Collection (Manassas, VA, Virginia, USA) and cultured in high glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin in a 5% CO₂ humid atmosphere at 37°C.

Irradiation
Cells were plated in culture dishes and incubated at 37°C under a humidified 5% CO₂ atmosphere. Cells at 70%–80% confluence were irradiated with a cobalt-60 γ-ray source (Nuclear Power Institute of China) at various doses (0, 2, 4, 6, and 8 Gy). The source–skin–distance technique was 80 cm, and the depth was set at 0.8 cm to the bottom of the culture dishes. After irradiation, cells were treated with metformin for further experiments.

Cell proliferation assay
Cell viability was measured by MTT assay. SMMC-7721 cells were cultured in a 96-well plate (1 × 10⁴ cells/well). After overnight incubation, cells were performed irradiation and (or) cultured with fresh DMEM medium containing various concentrations of metformin (0, 1, 5, 10, and 20 mmol/L) for 24 h. The cells were incubated with MTT solution (5 mg/mL) at 37°C for 4 h and underwent centrifugation to remove the supernatant. Then, the formazan pellet was dissolved with 100 µL DMSO to measure and the absorbance at 570 nm wavelength by a plate reader (Rico RK201, Shenzhen Rico Technology Co., Ltd, Shenzhen, Guangdong, China). The surviving percentage for each group was normalized to those of the control group.

Cell cycle analysis
The cells were seeded in 6-well plates at a density of 3 × 10⁶ cells/well and were incubated with serum-free medium to induce cell quiescence. Then, the cells received irradiation (4 Gy) followed by incubation with metformin (10 mmol/L) for 24 h. The cells were fixed with cold 70% ethanol and stored overnight at −20°C. After centrifugation, the cells were incubated with 100 µg/mL RNase A (Clontech Laboratories, Inc., Mountain View, CA) at 37°C for 30 min, followed by incubation with propidium iodide (PI, 50 µg/mL) for 5 min. Cell cycle was analyzed on a flow cytometry (FACScan, Becton Dickinson, San Francisco, CA, USA).

Apoptosis detection by Hoechst-propidium iodide staining
SMMC-7721 cells were seeded in 6-well plates at a density of 3 × 10⁶ cells/well, followed by treatment with γ-ray irradiation (4 Gy) and (or) metformin (10 mM) for 24 h. After washing twice with PBS (pH 7.4), the cells were stained with Hoechst 33342 (1 µg/mL) and PI (5 µg/mL) for 20–30 min at 4°C in the dark. The morphological changes of cells during apoptosis were observed under a fluorescence microscope.

Apoptosis detection by Annexin V-propidium iodide staining
SMMC-7721 cells were harvested by trypsinization and underwent centrifugation at 1000 g for 5 min. After washing twice with cold PBS, the cells were incubated with 500 µL 1 × binding buffer containing 5 µL Annexin V and 5 µL PI (KeyGEN Annexin V-PE Apoptosis Detection Kit) in the dark for 15 min. Finally, apoptosis was analyzed by flow cytometer (FACScan, Becton Dickinson, San Francisco, CA, USA).

Invasion assay
The Transwell invasion assay was performed to measure in vitro invasion capability of SMMC-7721 cells. About 5 × 10⁴ cells were suspended in 200 µL medium with 1% FBS, and then were plated in the upper chamber of transwell insert, which were coated with 50 µL of 2.0 mg/mL Matrigel. There were 600 µL medium with 10% FBS in the lower chamber, which was used to attract cell migration. After 24 h incubation, cells on the upper surface of the filter were removed. All migrated cells that were on the lower part of
filter were stained with crystal violet and were counted under a light microscope.

**Quantitative reverse transcription-polymerase chain reaction**

SMMC-7721 cells were treated with irradiation (4 Gy), followed by incubation with metformin (0, 1, 5, 10, and 20 mM) or mithramycin A (100 nM) for 24 h. Total RNA was extracted using Triozol® reagent to synthesize complementary DNA by Superscript III enzyme. The Sp1 mRNA level was determined using SYBR Green reagent (TaKaRa, Japan) Real-time PCR System (Applied Biosystems, USA). The polymerase chain reaction (PCR) condition was set as follows: initial denaturation at 95°C for 5 min, followed by 40 amplification cycles of denaturation at 95°C for 5 s and annealing at 60°C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. Sp1 primer: forward, 5'‑AGT TCC AGA CCG TTG ATG GG‑3'; reverse, 5'‑GTT TGC ACC TGG TAT GAT CTT C‑3'. GAPDH primer: forward, 5'‑GGA GTC CAC TGG CGT CTT G‑3'; reverse, 5'‑GCT GAT GAT CTT GAG GCT GTT G‑3'. The 2⁻∆∆Ct method was used to calculate Sp1 mRNA. Relative Sp1 mRNA was normalized to GAPDH.

**Western blotting**

Cells were harvested and lysed with RIPA buffer containing 20% (V/V) cocktail protease inhibitors (Sigma-Aldrich). Proteins (50 µg) were separated by 12% SDS-PAGE gels and electrophoretically transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 3% BSA at 4°C and incubated with antibodies against human Sp1, E-cadherin, or Vimentin (all 1:1000 dilutions), followed by incubation with the horseradish peroxidase-conjugated rabbit antimouse secondary antibodies (IgG) (1:1000 dilutions) at room temperature for 1 h. The bands density in membranes was visualized using chemiluminescent detection system (ECL, Amersham Life Science, Buckinghamshire, England) and was detected by Bio-Rad ChemiDoc XRS (Bio-Rad, USA).

**Statistical analysis**

Data were expressed as the mean ± standard deviation and analyzed by SPSS version 19.0 statistical software (SPSS Inc., Chicago, IL, USA). A two-tailed unpaired Student’s t-test or one-way analysis of variance was performed to compare the differences. *P < 0.05* was considered statistically significant differences.

**RESULTS**

**Metformin sensitized hepatocellular carcinoma cells to radiation**

To evaluate the effects on radiosensitivity of HCC cells by metformin, an MTT assay was performed on SMMC-7721 cells treated with irradiation (0, 2, 4, 6, and 8 Gy) alone or irradiation with metformin (0, 1, 5, 10, and 20 mM) for 24 h. Irradiation decreased survival of SMMC-7721 cells in a dose-dependent manner. Further metformin treatment significantly reduced cell viability following various doses of irradiation (*P < 0.05*) [Figure 1a-d]. Metformin of 10 and 20 mM demonstrated significantly enhanced radiosensitivity on SMMC-7721 cells following all doses of irradiation. This indicates that metformin is an effective radiosensitizer for HCC cells.

**Metformin induced cell cycle arrest and apoptosis and inhibited invasion of hepatocellular carcinoma cells**

To investigate the cytological mechanisms of metformin underlying enhanced radiosensitivity, we measured cell cycle distribution, apoptosis, and tumor invasion of SMMC-7721 by metformin. The inhibition rate on SMMC-7721 cells was about 30% by γ-ray irradiation at 4 Gy dose, so we chose 4 Gy irradiation dose for further experiments. Furthermore, when SMMC-7721 cells were underwent irradiation at 4 Gy, 10 mM metformin treatment reduced cell survival by 30% within 24 h incubation, so we used 10 mM metformin for further experiments. SMMC-7721 cells received irradiation (4 Gy) or irradiation plus metformin (10 mM) for 24 h, and cells treated with equal solution volumes of DEMO served as control. Cell cycle analysis was performed by staining with PI in flow cytometry and was presented as the percentages of SMMC-7721 cells in G0/G1, S, or G2/M phases of total cell population. In SMMC-7721 cells with irradiation, metformin significantly increased the percentage of cells in the G0/G1 phase after 24 h treatment (*P < 0.05*) [Figure 2a]. The percentage of S phase showed slight decreased and G2/M phase remained unchanged after metformin treatment.

To explore whether reduced cell survival by metformin is caused by cell death, we further measured cell apoptosis

![Figure 1: Metformin induces increased radiosensitivity in hepatocellular carcinoma cells. SMMC-7721 cells were plated immediately after γ-ray irradiation (0, 2, 4, 6, and 8 Gy) with metformin of 1 mM (a), 5 mM (b), 10 Mn (c), and 20 Mn (d) for 24 h. The surviving fraction was determined by MTT assay and was normalized to control SMMC-7721 cells which were treated with neither irradiation nor metformin. Each experiment was performed in triplicate and data are shown as mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001 versus irradiation alone group. IR=Irradiation, Met=Metformin](image-url)
Metformin decreased specificity protein 1 to enhance radiosensitivity of hepatocellular carcinoma

To investigate the detailed molecular mechanism underlying enhanced radiosensitivity regulated by metformin on HCC cells, we performed quantitative reverse transcription PCR and Western blotting to determine the expressions of Sp1 mRNA and protein. SMMC-7721 cells received irradiation and then incubated with different concentrations of metformin (0, 1, 5, 10, and 20 nM) or with mithramycin A (100 nM) for 24 h. Results showed that metformin decreased Sp1 mRNA and protein levels in a concentration-dependent manner (P < 0.05) [Figure 3a and b]. The mRNA and protein expressions of Sp1 were also inhibited by mithramycin A, a Sp1 specific inhibitor. However, the same concentration mithramycin A (100 nM) reduced Sp1 mRNA expression by about 15%, but achieved about 66% reduction in Sp1 protein expression.

Specificity protein 1 is involved in enhanced radiosensitivity of hepatocellular carcinoma cells through inhibiting epithelial-to-mesenchymal transition

To investigate whether EMT mediates metformin-induced enhanced radiosensitivity of HCC cells, we applied TGF-β1 to induce radioresistance model. SMMC-7721 cells were treated with DEMO (control group), TGF-β1 (1 ng/mL) (TGF-β1 group), irradiation (4 Gy) (IR group), or irradiation + TGF-β1 (IR + TGF-β1 group). Annexin V/PI staining and Boyden chamber assay were used to determine the impact of metformin on SMMC-7721 cells apoptosis and invasion, respectively. Irradiation significantly increased apoptotic rate compared with control cells; however, this effect could be markedly attenuated by coinoculation with TGF-β1 (P < 0.05) [Figure 4a]. TGF-β1 treatment alone showed no significant change in apoptotic rate of SMMC-7721 cells. Irradiation or TGF-β1 treatment alone significantly increased invasion of SMMC-7721 cells compared with control cells (P < 0.05). Furthermore, in SMMC-7721 cells with irradiation, TGF-β1 significantly increased invasion of SMMC-7721 cells (P < 0.05) [Figure 4b]. This result showed that in SMMC-7721 cells with irradiation, TGF-β1 is an effective

To explore the detailed cellular mechanism regulated by metformin-induced radiosensitization of HCC cells, we aimed to measure the tumor invasion capability by Transwell assay. The invasive SMMC-7721 cells were significantly increased by irradiation compared with control cells (P < 0.05). In irradiation-treated SMMC-7721 cells, metformin significantly decreased cell invasion (P < 0.05) [Figure 2e and f]. Metformin showed no significant change in invasion of SMMC-7721 cells without irradiation. This result showed that irradiation itself can induce invasion of HCC cells, and metformin might increase radiosensitivity through inhibiting irradiation-induced tumor invasion.

Figure 2: Metformin induces cell cycle arrest and apoptosis and inhibits tumor invasion of hepatocellular carcinoma cells. (a) Cell cycle analysis was performed by staining with propidium iodide. (b) SMMC-7721 cells showed typical characteristics of apoptosis. (c) Cell apoptosis was performed by staining with Annexin V and propidium iodide. (d) Metformin induces cell apoptosis in SMMC-7721 cells with irradiation. (e) The invasive cells are stained with crystal violet. (f) Metformin inhibits tumor invasion of SMMC-7721 cells with irradiation. *P < 0.05 versus control group, **P < 0.05 versus irradiation alone group by Hoechst 33342/PI staining to visualize morphological changes of apoptotic cells and by Annexin V/PI staining to determine apoptotic rate. SMMC-7721 cells were treated with γ-ray irradiation (4 Gy) and/or metformin (10 mM) for 24 h. Hoechst 33342/PI staining showed typical characteristics of apoptosis, as demonstrated by strong fluorescent signals (bright blue color) of nuclei. The normal survival cells appeared weakly stained. SMMC-7721 cells with irradiation or metformin alone showed slight more apoptotic cells, and irradiation plus metformin group showed markedly stronger fluorescent signals and reduced cell density due to apoptosis [Figure 2b]. Annexin V/PI staining further confirmed that in SMMC-7721 cells with irradiation, metformin increased apoptotic rate significantly (P < 0.05) [Figure 2c and d]. Irradiation or metformin alone also significantly increased cell apoptotic rate compared with control cell.

To investigate whether Sp1 expression in radiosensitivity of SMMC-7721 cells by metformin, we analyze the radiosensitivity of SMMC-7721 cells by mithramycin A. SMMC-7721 cells were treated with irradiation (0, 2, 4, 6, and 8 Gy) alone or irradiation with treatment of mithramycin A (100 nM). Mithramycin A significantly reduced the cell survival of SMMC-7721 cells after irradiation (P < 0.05) [Figure 3c]. This result showed that Sp1 might confer radioresistance of HCC cells and decreased Sp1 expression might involve the radiosensitivity by metformin.
radioresistance inducer as evidenced by reduced apoptosis and increased invasion. Moreover, irradiation itself can promote SMMC-7721 cell invasion, and this indicates that irradiation may activate some signal pathways which are involved in radioresistance.

We further explored the roles of Sp1 in metformin-induced radiosensitivity. SMMC-7721 cells were treated with DEMO (control group) or irradiation (4 Gy), and the latter then were treated with metformin (10 mM), TGF-β1 (1 ng/mL), TGF-β1 + metformin, and TGF-β1 + mithramycin A (100 nM) for 24 h. Western blot was performed to determine the protein expressions of Sp1 and two EMT marker proteins, E-cadherin and Vimentin. Cells treated with IR or TGF-β1 showed spindled morphological appearance, a characteristic of EMT [Figure 4c]. However, metformin or mithramycin A decreased morphological appearance of EMT induced by TGF-β1. Compared with control group, irradiation alone increased the expressions of Sp1 and promoted EMT process, as evidenced by increased mesenchymal phenotype marker Vimentin and decreased epithelial phenotype marker E-cadherin \( (P < 0.05) \), and these changes in three proteins were all reversed by metformin and enhanced by TGF-β1 [Figure 4d-f]. Furthermore, in TGF-β1-induced radioresistant SMMC-7721 cells, metformin markedly inhibited Sp1 protein and EMT process. Selective inhibition of Sp1 by mithramycin A can enhance radiosensitivity and reverse TGF-β1-induced radioresistance. Therefore, Sp1 might participate in the enhanced radiosensitivity by metformin and might be a signal protein required for TGF-β1-induced EMT and radioresistance of HCC cells.

DISCUSSION

In this study, we have shown that metformin increased radiosensitivity of SMMC-7721 cells, as evidenced by reduced proliferation and survival cells at various concentrations. Metformin also induced cell cycle arrest in G0/G1 phase, promoted apoptosis, and inhibited invasion in HepG2 cells with irradiation. The radiosensitizing effect of metformin was associated with the decreased Sp1 mRNA and protein expressions. Furthermore, in TGF-β1-induced radioresistant SMMC-7721 cells, metformin markedly inhibited Sp1 protein and EMT process. Selective inhibition of Sp1 by mithramycin A can enhance radiosensitivity and reverse TGF-β1-induced radioresistance. Therefore, Sp1 might participate in the enhanced radiosensitivity by metformin and might be a signal protein required for TGF-β1-induced EMT and radioresistance of HCC cells.

Radiotherapy is an important treatment for advanced-stage or unresectable HCC patients. To improve therapeutic efficacy,
combined treatment of radiotherapy plus other treatments should be applied. Metformin is a radiosensitizer in various types of cancers. In this study, we found that metformin enhanced radiosensitivity of HCC cells, which might be associated with reduced proliferation, cell cycle arrest, and apoptosis. Our results are in consistent with other reports showing radiosensitizing effects of metformin on HCC. In these studies, metformin enhanced radiosensitivity through ATP deprivation, inhibition of DNA repair induced by radiation, and suppressing phospho-mTOR and phospho-Akt expressions. Exposure to γ-ray radiation can induce a series of cellular changes, including chromosome aberration, mutations, and cellular damage induced by imbalanced total amount of energy, thereby promoting apoptosis. This indicates that metformin and radiation share common pathways in cell survival and therefore underlie the enhanced apoptosis by metformin.

In this study, we first found that metformin can downregulate Sp1 expression in HCC cells with radiation, which is involved in radiosensitizing effects of metformin. Sp1 is a transcription factor and can regulate the promoter activity of multiple genes involved in oncogenesis. Sp1 is highly expressed in HCC tissues and is associated with poor prognosis. Metformin downregulates Sp1 in various cells types, including breast cancer, pancreatic cancer, and HCC. Our study first demonstrates that metformin downregulates Sp1 expression in radiosensitivity of HCC. In fact, Sp1 protein is involved in radioresistance of various types of cancers. Sp1 overexpression in nasopharyngeal cancer tissue is correlated with lower radiotherapy response. Radiosensitization can be induced by downregulating Sp1 in several cancers, including non-small cell lung carcinoma treated by terameprocol and nasopharyngeal carcinoma treated by MiR-24 or berberine. This indicates that decreased Sp1 protein might also mediate radiosensitization in HCC cells. In fact, Sp1 DNA binding was increased within 30 min after ionizing radiation in radioresistant human malignant melanoma (U1-Mel) cells and indicates that Sp1 might regulate the promoter activity of genes involved in radiosensitivity under radiation condition. Furthermore, Sp1 enhances repair of DNA double-strand break by a nontranscriptional mechanism, thereby promoting survival of cancer cells with radiation. In U251 glioma cells with cobalt-60 γ-ray radiation, SP1 gene expression levels were upregulated.
increased in SMMC-7721 cells after radiation. This indicates that radiation not only initiates apoptosis of tumor cells, but also induces survival signal pathways. Sp1 may be one mediate of these pathways and can provide radioresistance by promoting repair of DNA and transcription of survival genes. Furthermore, suppression of these compensative pathways is a potential strategy for reversing radioresistance and enhancing radiosensitization.

Our study found that decreased Sp1 protein by metformin treatment markedly inhibited EMT process in HCC cells with radiation and TGF-β1-induced radioresistant cells. EMT is a cellular process characterized by loss of cell–cell adhesion and enhanced cell motility and is involved in radioresistance. Our study showed that radiation could enhance EMT process, as proved by decreased E-cadherin and increased Vimentin expressions in SMMC-7721 cells after radiation. This indicates that EMT can be induced by radiation itself to confer radioresistance in HCC cells. Therefore, EMT is a potential target for reversing radioresistance. Metformin can suppress EMT in various types of cancer, and reduced EMT is essential for enhanced radiosensitivity by metformin. Furthermore, our study showed that Sp1 is an upstream regulator of EMT after metformin treatment, as proved by inhibition of EMT in SMMC-7721 cells after radiation by mithramycin A, a selective inhibition of Sp1. The relationship between Sp1 and EMT is complex. One report showed that Sp1 protein is essential to maintain an epithelial state and inhibit EMT process. However, other studies showed that several microRNAs could suppress EMT process through Sp1 silencing in colorectal cancer, gastric cancer, and acute kidney injury. Sp1 can also activate mesenchymal gene and enhance EMT in cooperation with ZEB2. The controversial effect of Sp1 on EMT may be dependent on cell condition that direct Sp1 activates transcription of EMT promotive genes or EMT inhibitory genes. In fact, Sp1 mainly acts as an enhancer of EMT. Thus, Sp1 might be a potential therapeutic target for radioresistance by metformin and other molecules.

**CONCLUSION**

Metformin is a radiosensitizing agent in HCC, with the underlying mechanism of decreased Sp1 protein expression and suppressed EMT process. Our study first reports the downregulation of Sp1 by metformin and confirms Sp1 as an important enhancer of radioresistance in HCC. Our study also provides new evidence that Sp1 lies upstream of EMT in radioresistance. Further study is needed to explore the genes regulated by Sp1 and their roles in EMT and radioresistance of HCC.

**Financial support and sponsorship**
Nil.

**Conflicts of interest**
There are no conflicts of interest.

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