

## Original Article

# Metformin can enhance the radiosensitivity of cholangiocarcinoma through AMPK-FOXO3a axis

Yan Yang<sup>1</sup>, Wei Pan<sup>1</sup>, Sheng Zhang<sup>1</sup>, Yuandong Cao<sup>1</sup>, Hongyan Cheng<sup>2</sup>, Jiayan Chen<sup>1</sup>, Xinchun Sun<sup>1</sup>

<sup>1</sup>Department of Radiation Oncology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China;

<sup>2</sup>Department of Synthetic Internal Medicine, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China

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**Abstract:** This study aims to evaluate the potential of metformin in enhancing the radiosensitivity of cholangiocarcinoma. In this study, we used the methods of proliferation, clonogenic, transfection, western blotting, and cell apoptosis assays to test the human cholangiocarcinoma cells. The Orthotopic tumors in SCID mice were treated with ionizing radiation (IR) and metformin. Our study showed that metformin (5 mM-50 mM) inhibited the proliferation, induced apoptosis and radio-sensitized cholangiocarcinoma cells. Metformin can phosphorylate and activate AMP activated kinase (AMPK), therefore activated the Forkhead Box O3a (FOXO3a) and down-regulated the Forkhead Box M1 (FOXO1) at last. However, the FOXO1 expression was up-regulated when we knocked down the FOXO3a. Metformin or radiotherapy inhibited orthotopic tumor growth and the combined treatment enhanced this effect further than each treatment alone. Metformin can induce the apoptosis of cholangiocarcinoma cells, inhibit tumor growth and sensitise them to IR. Metformin mediated its radiosensitivity enhancement action through the AMPK-FOXO3a-FOXO1 pathway. Our results suggest that metformin can be a useful radiosensitizer in cholangiocarcinoma.

**Keywords:** Metformin, cholangiocarcinoma, ionizing radiation, FOXO3a, FOXO1, radiosensitivity

## Introduction

Cholangiocarcinoma (CC) is a kind of cancer which developing from the intra- or extrahepatic bile ducts. During the past several decades, more deaths were caused by the cholangiocarcinoma worldwide [1-4], which accounted for about 3% of all gastrointestinal cancers [5]. These tumors can arise from anywhere of the biliary tree and develop from the most proximal peripheral intrahepatic ducts to the distal intra-duodenal bile duct. Considering the tumor location, cholangiocarcinoma contains mainly two kinds of bile duct cancers (intrahepatic, and distal extrahepatic), and the perihilar CC is also a subset of extrahepatic CC [6].

Although surgery is still the main treatment for cholangiocarcinoma and sometimes it may be the only choice for potential cure, radiotherapy is becoming more and more important for the non-surgical patients with unresectable or recurrent disease. Among all the factors that

can influence the effect of radiotherapy, the radiosensitivity of tumor cells is becoming a rather important reason. While the chemoradiation therapy has been proved efficient to achieve relatively high local control and to prolong the patients' living time, the serious side effects of chemotherapy were the problems that can never be neglected [7].

In the study [8] of Sanli et al, they showed that ionizing radiation can activate the energy sensor AMP activated kinase (AMPK) pathway, which is a key kinase that mediates a metabolic cell cycle checkpoint. AMPK is a downstream effect of liver kinase B1 (LKB1), a tumor suppressor mutated in Peutz-Jeghers syndrome. It is a heterotrimeric enzyme composed by three subunits:  $\alpha$ -,  $\beta$ - and  $\gamma$ -subunits, which has the ability to sense low energy levels through AMP binding on the  $\gamma$ -subunit and is regulated by the phosphorylation of the  $\alpha$ -subunit Thr172. A large amount of studies have showed that the activation of AMPK is able to inhibit cancer

proliferation in various human cancers [9-11]. Forkhead Box M1 (FOXM1) is a member of the Forkhead Box transcription factors which is essential for the proliferation and apoptosis of cells in the development and function of many organs [12-14]. Some researchers also illuminated that aberrant up-regulation of FOXM1 is associated with the progression of some human cancers [15, 16].

Metformin [1-(diaminomethylidene)-3,3-dimethylguanidine] is derived from the French lilac (*Galega officinalis*), a plant known for several centuries to reduce the symptoms of diabetes mellitus. It is the most widely used oral blood glucose-lowering drug and has been recommended as first line therapy for newly diagnosed type2 diabetes patients [17]. Metformin has been used in the treatment of type2 diabetes for more than 50 years. Metformin has the major clinical advantage of not inducing hypoglycemia and curing hyperglycemia with obvious cardiovascular safety. Recently, lots of studies reported that metformin has the effect of decreasing risk and improving prognosis of different kinds of cancers [18-24].

This study was dedicated to: (1) examine whether metformin can activate the radiosensitivity of cholangiocarcinoma from both in vivo and in vitro experiments and (2) investigate the potential molecular mechanism of the radiosensitivity enhancement of metformin.

## Materials and methods

### *Antibodies and chemicals*

All antibodies were purchased from Cell Signaling Technology (Danver, MA, USA). Metformin HCL was obtained from Sigma-Aldrich. Canada (Oakville, ON, Canada).

### *Cell culture and treatment*

Human cholangiocarcinoma QBC-939 and HCCC-9810 cells were provided by the Liver Surgery Department of the First Affiliated Hospital of Nanjing Medical University and cultured in RPMI 1640 (Sigma Chemical Co., St. Louis, MO, USA) with 10% heat-inactivated fetal bovine serum (Bio Whittaker, Walkersville, MD, USA). After overnight incubations, cells were treated with indicated doses of MET for 24 h before the indicated doses of radiotherapy.

### *Proliferation assay*

Cells were seeded ( $5 \times 10^3$  cells per well) in 96-well plates in triplicate, then adhered overnight and subjected to treatments accordingly. After treatment, cells were washed by the phosphate-buffered saline (PBS). Cell viability was measured by using CCK8 cell proliferation and cytotoxicity assay kit (Beyotime) at 24, and 48 h later. The absorbance was measured at the wavelength of 450 nm.

### *Clonogenic assays*

Cells (500-1000) were seeded into 6-well plates in triplicate for overnight culture and treated with the indicated doses of MET before IR (0, 2 or 8 Gy with 6 MV X-rays). After 10 days culture in 5% CO<sub>2</sub> incubator at 37°C, cells fixed by the 4% PFA and stained with methylene blue were counted. Data were fitted to the linear quadratic model using Graphpad Prism-5 software (GraphPad Prism Software Inc., La Jolla, CA, USA).

### *Flow cytometric analysis of apoptosis*

Apoptosis was quantified with an Annexin V-FITC apoptosis detection kit (Beyotime Biotechnology) as described by the manufacturer's instructions. After exposure to drugs for 48 h, the cells were collected and washed with PBS, then gently resuspended in Annexin V binding buffer and incubated with Annexin V-FITC/PI. Flow cytometry was performed using Cellquest software (BD Biosciences, CA, USA). All experiments were repeated for three times.

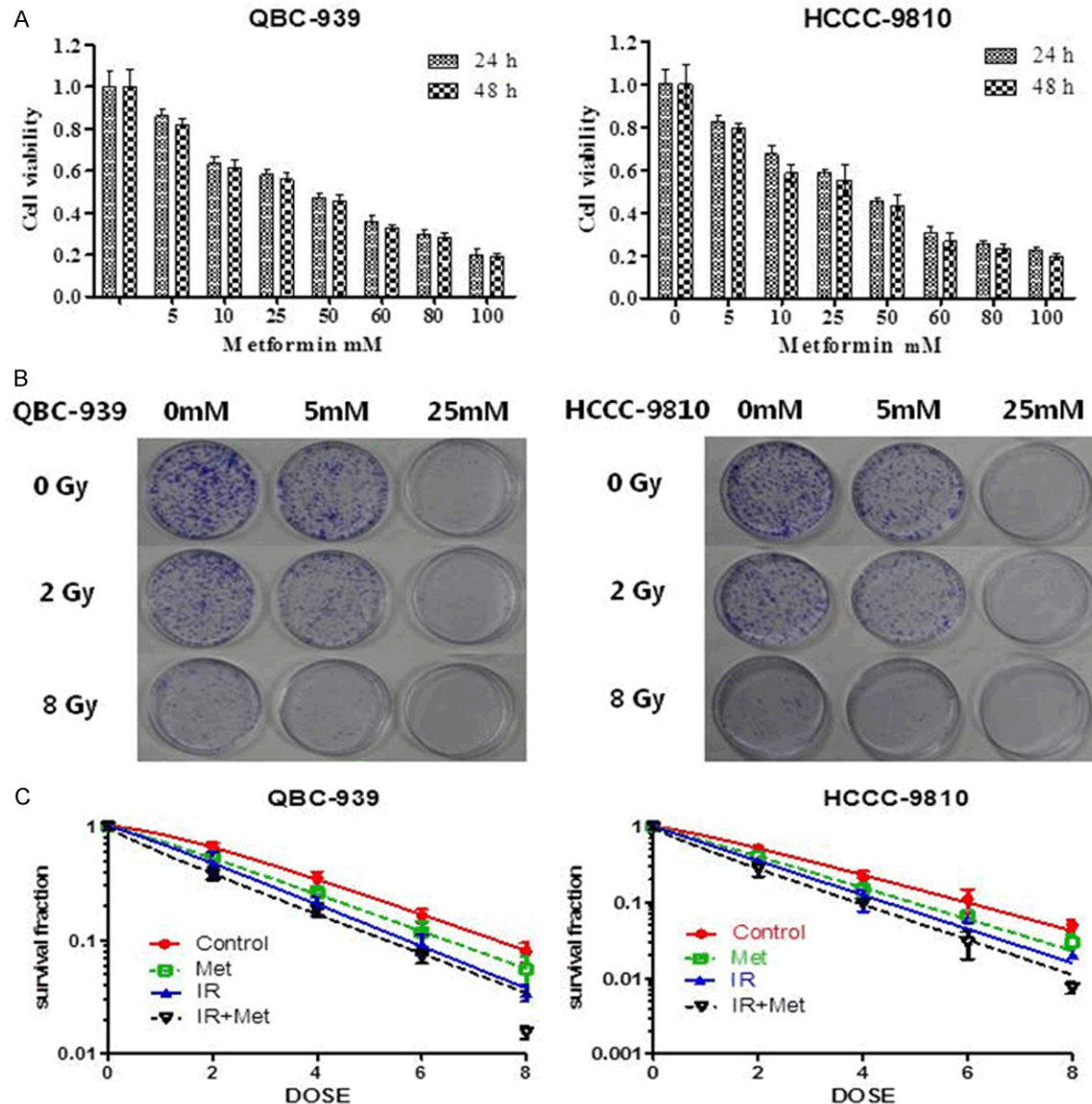
### *Plasmids and cell transfection*

In order to knockdown human FOXO3a, the TriFECTa RNAi Kit was used (purchased from Integrated DNA Technologies, Inc., Iowa, USA). Cell transfection was carried out through using the LipofectAMINETM2000 (Invitrogen) according to the manufacturer's instructions. The transfection effects were tested by Western blotting [15, 16].

### *Western blotting*

Cultured cells were lysed in RIPA lysis buffer (Beyotime Biotechnology) and kept on ice for 20 min. Amount of protein in the lysates was quantified by BCA kit (Beyotime). Equal amounts

## Metformin enhances radiosensitivity of cholangiocarcinoma



**Figure 1.** Metformin sensitizes cholangiocarcinoma (CC) to ionizing radiation (IR). A. Metformin inhibits the proliferation of the CC cells with a time- and dose-dependence. B and C. Clonogenic assay shows that metformin can enhance the radiosensitivity of CC cells.

of proteins were separated by SDS-PAGE and transferred to PVDF membranes, which were probed with the antibodies of AMPK, p-AMPK, FOXO3a, FOXM1 and survivin. The signals were visualized and quantified using the Image J software (National Institute of Health (NIH), Bethesda, MD, USA).

### Animal model

Male SCID mice (CB-17/lcr-scidJc1, CLEA Japan Inc, Tokyo, Japan) at 6-8 weeks of age were used in this study and maintained in the

Laboratory for Animal Experiments. The protocols for all animal experiments were approved by the Ethics Committee of Nanjing Medical University.

### Cell lines

Two types of human cholangiocarcinoma cell lines were used in this study [QBC-939 and HCCC-9810]. The cell lines were cultured in RPMI 1640 (Sigma Chemical Co., St. Louis, MO, USA) with 10% heat-inactivated fetal bovine serum (Bio Whittaker, Walkersville, MD, USA)

**Table 1.** The radiosensitivity of metformin in QBC-939 cells

QBC-939	D0	Dq	SF2 %	SER D0
Control	2.83	0.78	0.81	
Met	2.17	0.12	0.84	
IR	1.90	-0.03	0.84	
Met+IR	1.50	-0.40	0.89	1.27

**Table 2.** The radiosensitivity of metformin in HCCC-9810 cells

HCCC-9810	D0	Dq	SF2	SER D0
Control	3.77	1.92	0.78	
Met	2.97	0.49	0.87	
IR	2.60	0.38	0.85	
Met+IR	2.09	-0.47	0.93	1.25

and were maintained at 37°C in a humidified incubator with 5% CO<sub>2</sub> in air.

#### Orthotopic implantation

QBC-939 and HCCC-9810 cells are directly implanted in SCID mice, respectively. The suspensions containing QBC-939 cells and Matrigel are injected into the bile duct bifurcation region through the interstitial space between the hilar bile duct and portal vein. The HCCC-9810 cells will be injected into the intrahepatic bile duct in the similar way the QBC-939 cells did. We suspend  $1 \times 10^7$  cells in 150  $\mu$ l of serum-free medium. To increase the viscosity of the injected cell suspension, Matrigel was mixed in the cell suspension at a 1:1 (vol/vol) ratio. 0.5-ml syringes with 28.5-gauge needles are used to inject the suspension. We limit the injected suspension volume within 30  $\mu$ l, in which way it can efficiently avoid the leakage of tumor cells from the injection site. The injection performance should be conducted carefully to minimize the damage of the surrounded bile duct tissue. We use the gelfoam to cover the liver surface at the injury site for at least 5 minutes to prevent bleeding and leaking [25].

#### Animal treatment

Animals were divided into four groups after three weeks when the tumor was beginning to arise along the bile duct tree. Drug treatment was delivered via injecting 250 mg metformin body weight or placebo in the same volume per day till euthanasia. Radiotherapy were delivered with a conformal technique (after dosimet-

ric calculations and simulation) using clinical linear accelerators. Orthotopic implantation tumors were subjected to 0 or 8 Gy ionizing radiation while animals were under gaseous anaesthesia.

#### Statistical analysis

The Student 2-tailed t test was used for statistical analysis of data between treatment groups for all experiments. Analysis was performed with SPSS software purchased by SPSS Inc. (Chicago, IL, USA), presently owned by IBM (Armonk, NY, USA). Statistical significance was determined as  $P$ -value<0.05.

## Results

### *Metformin inhibits proliferation and enhances the radiosensitivity of the cholangiocarcinoma cells*

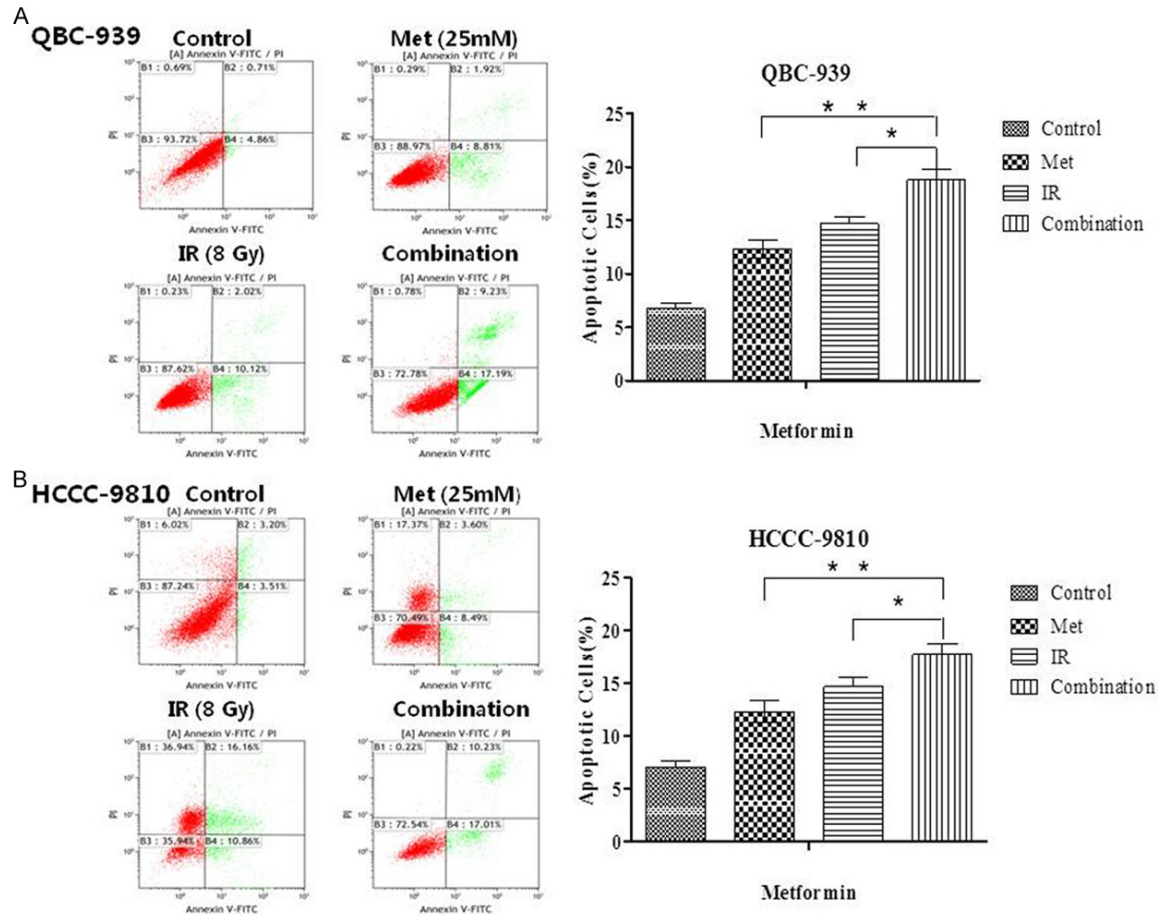
Metformin treatment inhibited the growth of Cholangiocarcinoma (CC) cells with a time and dose dependence (**Figure 1A**). The dose of IC<sub>50</sub> for QBC-939 and HCCC-9810 cells were 52.5 mM and 49.8 mM at 24 h, respectively. And were 48.2 mM and 46.5 mM for 48 h, respectively. According to this, we treated CC cells with low concentration of metformin (5 mM and 25 mM) to investigate the effects of metformin on radiosensitivity of CC cells. The clone formation unit of the two CC cells reduced with the upregulation of the dose of either metformin or irradiation (**Figure 1B**). The results of radiosensitivity of metformin to the cells were illuminated by the clone formation curve (**Figure 1C**).

Then we calculate the SF data which were fitted into the single hit multi target model formula:  $SF = 1 - (1 - e^{-D/D_0})^n$ . The SF2 was 0.84, 0.84 and 0.89 for the three experimental groups of QBC-939 cells, respectively. For HCCC-9810 groups, the data were 0.87, 0.85 and 0.93, respectively. The sensitization enhancement ratios were 1.27 and 1.25, which indicated that treating CC cells with metformin can achieve an obvious radiosensitization effect (**Tables 1 and 2**).

### *Metformin induces the apoptosis of cells and it can enhance this effect when combined with ionizing radiation*

We conducted the flow cytometry assay to evaluate whether metformin has the ability to induce the apoptosis of CC cells. The early





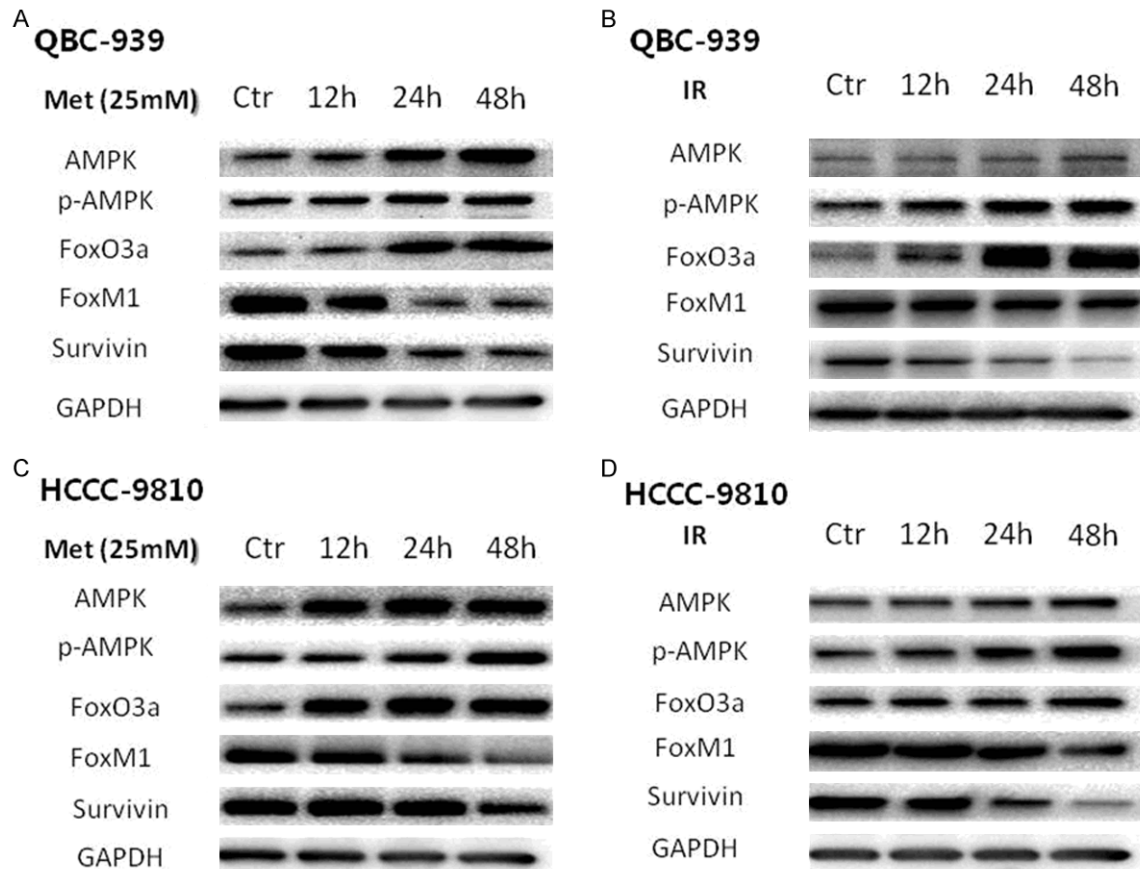
**Figure 2.** Flow cytometric analysis shows that metformin can induce the apoptosis of CC cells. And it can enhance this effect when combined with ionizing radiation (IR). Statistically significant difference was found between treatment groups (\* $P < 0.05$  and \*\* $P < 0.001$ ).

apoptosis rate of three treatment groups were 8.81%, 10.12% and 17.19% for QBC-939 cell lines, respectively (**Figure 2A**). And the data for the HCCC-9810 cell lines were 8.49%, 10.86% and 17.01%, respectively (**Figure 2B**). The results showed that the apoptosis rate of the CC cells were upregulated in the combination treatment groups when compared with the treatment alone groups with a statistical significance. All the data showed that the metformin not only induce the apoptosis rate of the CC cells but also enhance this effect of ionizing radiation.

*The radiosensitivity of metformin to the cholangiocarcinoma cells is correlated with the activation of AMPK-FOXO3a axis*

Ionizing radiation stimulated AMPK phosphorylation and activity. According to the former studies [8, 15, 26], we also found that both metformin and ionizing radiation can induce

the upregulation of AMPK (**Figure 3A**). This study also showed an up-regulation of FOXO3a and a down-regulation of FOXM1 in the three treatment groups. The apoptosis correlation protein-survivin was also down-regulated. Mingo Ming Ho Yung et al [15] had proved that the effect of AMPK-FOXO3a axis in the cervical cancer cells. And we got the similar consequence in CC cells (**Figure 3A-D**). We also found that metformin can enhance the up-regulation of FOXO3a and down-regulation of FOXM1 in CC cells which were treated with ionizing radiation (IR). This effect will get enhanced when we use higher dose of either metformin or IR (**Figure 4A** and **4C**). Thus we use the FOXO3a siRNA to knock down the FOXO3a expression to explore the definite effect of the AMPK-FOXO3a axis. The results showed that the expression of AMPK and p-AMPK was similar as before. However, the FOXO3a was obviously down-regulated. Both FOXM1 and survivin were all up-regulated.



**Figure 3.** A-D. Western blotting assay shows that both metformin and ionizing radiation (IR) can induce the phosphorylation of AMPK of CC cells with a time-dependence. And we found that they can also induce the up-regulation of FOXO3a and the down-regulation of FOXM1.

Then we used the flow cytometry experiment to testify the apoptosis rate of the groups in which all of them had been treated with the siRNA of FOXO3a. The early apoptosis rate were 1.69%, 5.74%, 7.65% and 11.31% of the four groups for QBC-939 cell lines, respectively (**Figure 5A**). And 1.67%, 5.40%, 7.38% and 12.39% for HCCC-9810 cell lines, respectively (**Figure 5B**). The early apoptosis rate for the combination group was higher than the other two experimental groups with a statistical significance for both of the two cell lines. And all of the groups came with an obvious lower early apoptosis rate with a statistical significance when compared with the groups not treated with FOXO3a siRNA.

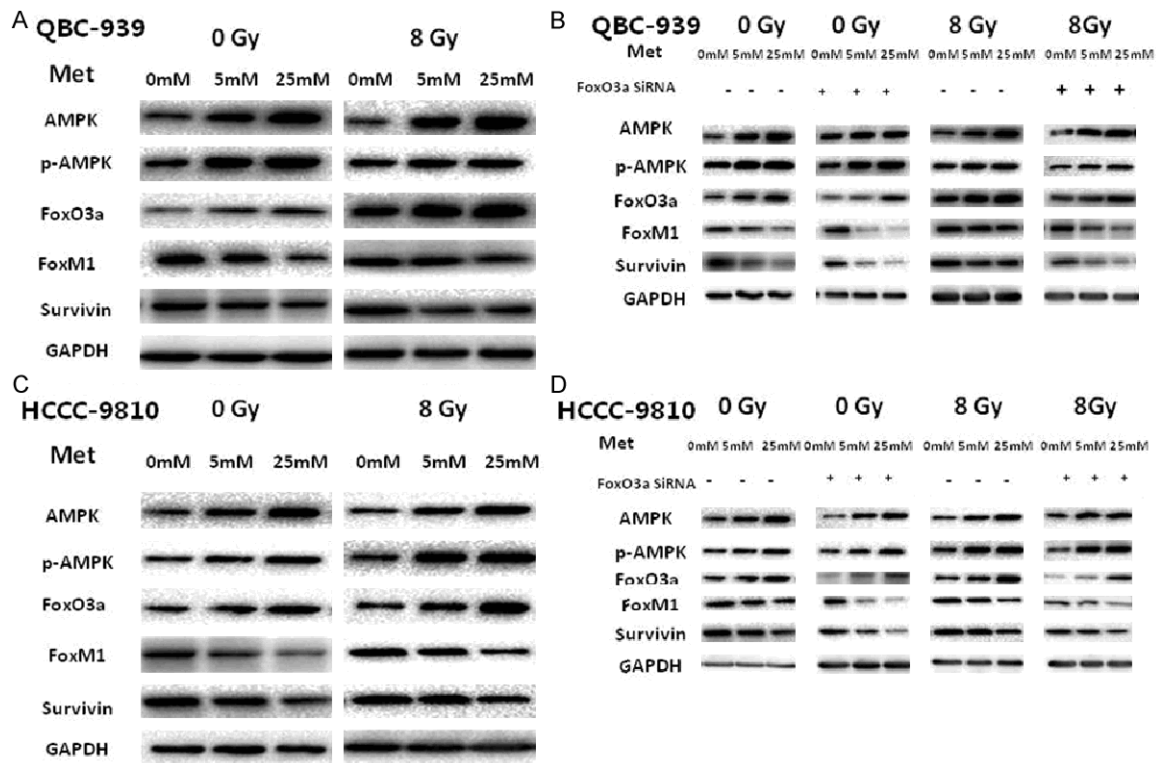
#### *Metformin promotes radiation sensitivity in SCID mice*

The SCID mice were divided into four groups when tumor reached the mean volume of

35 mm<sup>3</sup>: untreated (control), metformin alone, IR alone and combined treatment (MET+IR). To determine the potential radiosensitization effect of metformin on CC in vivo, QBC-939 and HCCC-9810 tumor-bearing mice were treated with a single fraction of 8 Gy irradiation. Mice received intraperitoneal injection of metformin (250 mg/kg) at the same day as the drug alone group. Either irradiation or combination group was obviously effective in delaying tumor growth (**Figure 6C**).

#### **Discussion**

The anti-tumor potential of MET in humans attracted the scientists' attention during the past several years. Large amounts of researches demonstrated that the metformin did truly have the antiproliferative effects for different cancer cells, although at millimole dose concentration of meformin [27, 28]. Considering its anti-tumor effect, similarly, the radiosensitiza-



**Figure 4.** A and C. Western blotting assay shows that metformin can enhance the up-regulation of FOXO3a and down-regulation of FOXM1 in CC cells which were treated with ionizing radiation (IR). And this effect will be enhanced with the up-regulation of the dose of either metformin or IR. B and D. The expression of FOXM1 will be up-regulated when the cells were treated with FOXO3a siRNA.

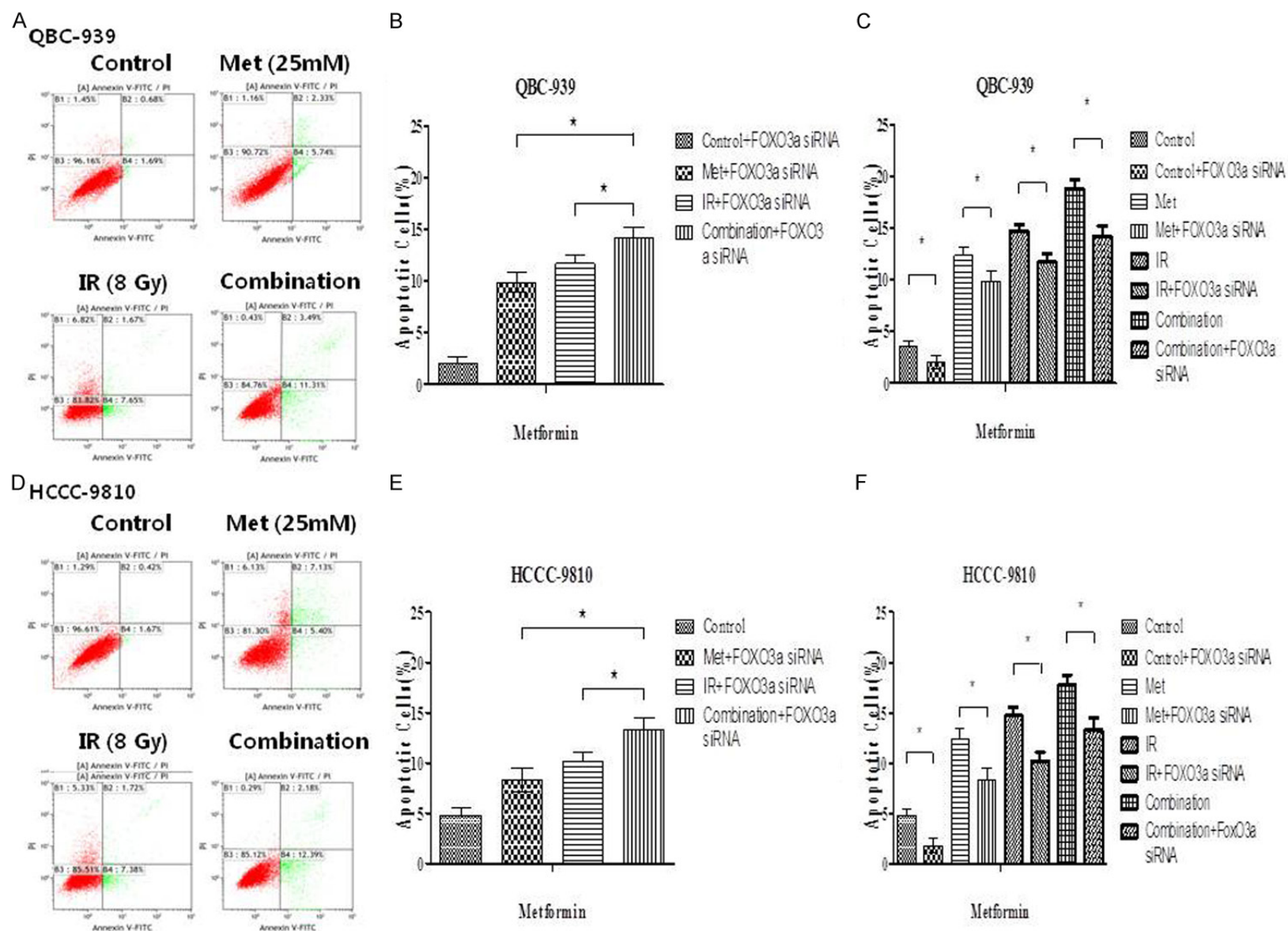
tion effect of metformin also aroused lots of interests worldwide. Song et al [29] found the radiosensitisation of breast cancer and sarcoma cells induced by metformin at millimole dose. Compared to this, Skinner et al and Y Storozhuk [30, 31] found that metformin could decrease the surviving fraction of head-neck and non-small cell lung cancer cells at micromole dose concentration when combined with ionizing radiation. All these results laid the basis of our study, in which we showed obvious antiproliferative effects of 5-50 mM MET in cholangiocarcinoma models.

Metformin was originally developed for the treatment of hyperglycemia and type2 diabetes. This drug can suppress the hepatic glucose production and increases insulin sensitivity, reduces lipolysis in adipocytes and glucose absorption from intestine [32]. According to the report of Giovannucci E et al [33] that metformin treating diabetic cancer patients had substantially (40%) reduced cancer burden compared with other treatments, from which we get a suggestion that the metformin may have positive effects to the cancers cells.

FOX proteins orchestrate the spatio-temporal regulation of a large number of gene transcriptional activities which are necessary to the tissue homeostasis and development. The deregulation of the FOX transcription factors can lead to cancers as FOX proteins are responsible for a wide range of cell-biological activities such as cell proliferation, differentiation, angiogenesis, apoptosis, et al.

In this study, we demonstrated that metformin can induce the cellular apoptosis and enhance sensitivity to radiation in the CC cell lines. And the mechanism of its radiosensitized effect may correlate with the activation of AMPK-FOXO3a-FOXM1 axis. In addition, the decrease of tumor volumes in the orthotopic transplantation models also indicated that the metformin can enhance the radiosensitivity.

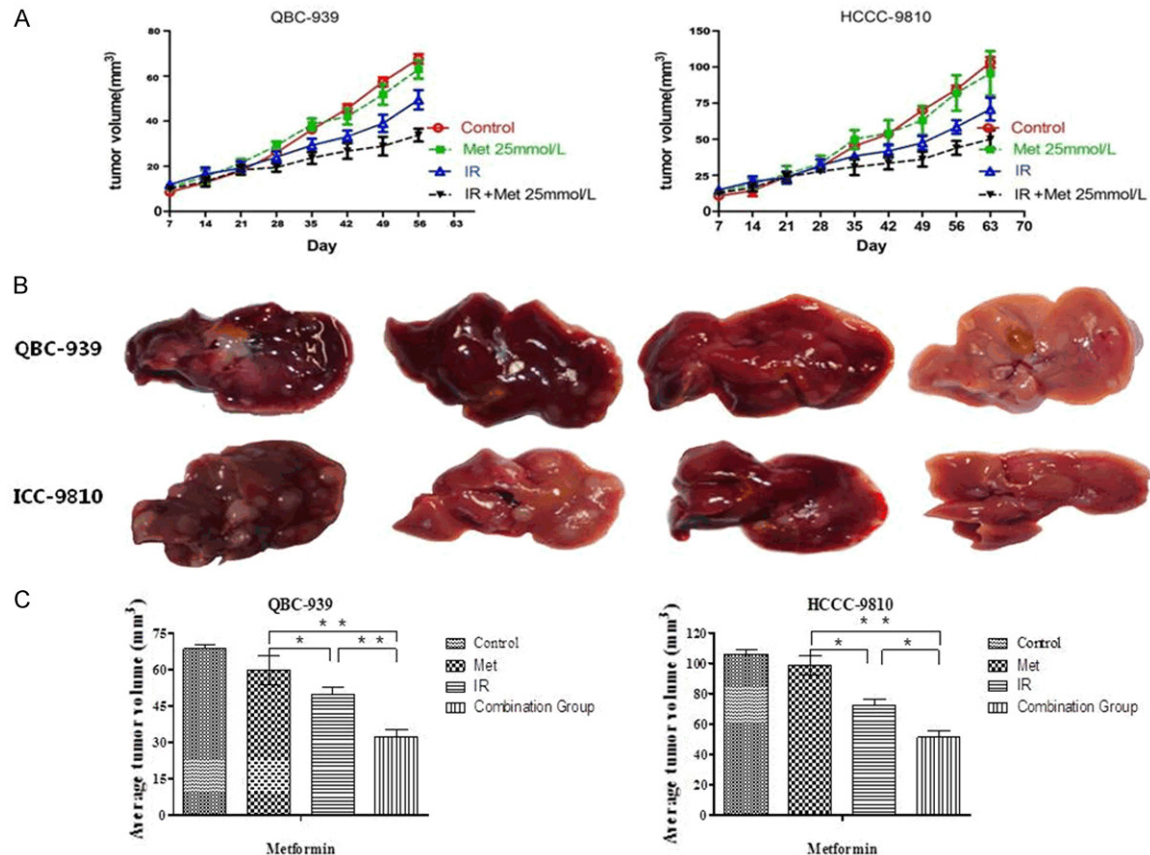
In this study, the apoptosis induced by the metformin in the CC cells had been examined. Previous studies showed that the antineoplastic activity of metformin depends mainly on the AMPK, though other mechanisms have also been illuminated. Similarly, according to some



**Figure 5.** Flow cytometry assay shows that the apoptosis rate will be down-regulated in the four groups which were treated with FOXO3a siRNA when compared with the former groups with no FOXO3a siRNA treatment. However, the apoptosis rate of the combination group is still higher than the other two experimental groups with statistical significance. Statistically significant difference was found between treatment groups (\*P<0.05).



## Metformin enhances radiosensitivity of cholangiocarcinoma



**Figure 6.** A. Effects on orthotopic tumor growth. All the SCID tumor-burdened mice were grafted into the histogram with QBC-939 or HCCC-9810 cells, respectively. The mice were grouped into four: control, MET, IR and combination when the mean tumor volume reached 35 mm<sup>3</sup> which was nearly in the fourth weeks after the tumor cell injection. The tumor volume was measured every 7 days for 8 and 9 weeks, respectively. B and C. Show that the average tumor volume data for each treatment group are shown in mean  $\pm$  SEM. Final tumor volumes were: QBC-939: control: 68.5 $\pm$ 5.1 mm<sup>3</sup>, MET: 59.53 $\pm$ 6.7 mm<sup>3</sup>, IR: 48.90 $\pm$ 3.5 mm<sup>3</sup> and combination: 31.9 $\pm$ 5.2 mm<sup>3</sup> and HCCC-9810: control: 105.39 $\pm$ 4.7 mm<sup>3</sup>, MET: 97.02 $\pm$ 6.13 mm<sup>3</sup>, IR: 61.35 $\pm$ 4.5 mm<sup>3</sup> and combination: 51.25 $\pm$ 4.5 mm<sup>3</sup>. Statistically significant difference was found between treatment groups (\*P<0.05 and \*\*P<0.001).

reports [8, 31], ionizing radiation can induce the activation of AMPK through the same way. In the study of Mingo Ming Ho Yung et al [15], they demonstrated that activated AMPK could reduce FOXM1 expression by blocking the AKT/FOXO3a signaling pathway and thus inhibit the cell growth of cervical cancer.

The activated AMPK had an obvious relationship with the upregulation of FOXO3a, which can induce the downregulation of FOXM1 and survivin. And the inhibition of the FOXM1 protein comes with the increasing of the apoptosis rate which is consistent with the results of other researchers.

The forkhead box class O (FOXO) subfamily of transcription factors (FOXO1, FOXO3a, FOXO4

et al) act downstream of the phosphoinositol-3-kinase (PI3K)-AKT oncogenic signaling cascade, and are responsible for a multitude of cellular functions, including cell apoptosis, proliferation, invasion, migration, and resistance to oxidative stress and DNA damage [34-37]. It is reported that crosstalk of a lot of deregulated signaling cascades ends in the inactivation of the tumor suppressive function of FOXO3a, which shows the antitumor effect of the FOXO3a [38-41]. However, another forkhead subfamily member, forkhead box protein M1 (FOXM1), behaves like a classic oncogene. Like the FOXOs, FOXM1 also has a close relationship with the cellular apoptosis, angiogenesis, cell cycle progression, cell proliferation, tissue repair and homeostasis which has been mani-

tested by many studies [42]. And the overexpression of FOXM1 has also been found in many different types of cancers [43]. More and more evidences from the cancer gene expression studies shed light on that FOXM1 is commonly up-regulated in solid tumors [43-45]. According to the reports, it is easily to find that the expression levels of FOXM1 is proportional to the stages of human cancers. More interesting is that FOXO3a and FOXM1 compete binding to the similar DNA sequences and share a number of downstream target genes [46]. Thus the effect of the FOXM1 can be antagonized by the FOXO3a, including the genes activated by the FOXM1 [45].

The anti-tumor effect of Metformin has been proved in several different kinds of cancers. And the inhibition of FOXM1 through the up-regulation of FOXO3a in the cervical cancer has also been reported recently. Therefore, we investigated the prospect of metformin as a potent FOXM1 inhibitor for the CC, and found that FOXM1 expression in CC cells was suppressed by metformin. These data suggested that metformin could sensitize CC cells to radiotherapy through the AMPK-FOXO3a-FOXM1 axis.

In conclusion, our study indicated that metformin enhances radiosensitivity of CC from both in vitro and in vivo studies. And the mechanism of its radiosensitivity enhancement is correlated with the downregulation of FOXM1 expression through the activation of AMPK-FOXO3a-FOXM1 axis. Metformin appears to be a potential radiotherapy sensitization agent due to its significant antitumor effect.

## Conclusion

Consequently, this study demonstrates that metformin can inhibit tumor growth and enhance the pro-apoptotic effects of radiotherapy. Metformin mediates the activation of tumor suppressor and inhibition of survival pathways of the cells. The radiosensitization effect is the result of convergence effect of ionizing radiation and metformin through the AMPK-FOXO3a-FOXM1 signal pathway. Our work provides a basis to the investigation of metformin in combination with radiotherapy in the patients of cholangiocarcinoma.

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## Disclosure of conflict of interest

None.

**Address correspondence to:** Jiayan Chen and Xinchun Sun, Department of Radiation Oncology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China. E-mail: chenjiayan2008@126.com (JYC); sunxinchun2012@163.com (XCS)

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