

Original Article

MicroRNA-1284 enhances radio-sensitivity in hepatocellular carcinoma cells by regulating SP1

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Abstract: Background: Hepatocellular carcinoma (HCC) is the most common cancer worldwide, with high morbidity and mortality. This study was aimed to explore the role of microRNA-1284 (miR-1284) in radio-sensitivity of HCC cells. Methods: HCC cell lines HepG2 and SMMC-7721 were used in this study and their correspondingly radio-resistant cells were established. The mRNA level expression of miR-1284 in these four cells was monitored by quantitative PCR (qPCR). MiR-1284 mimic or control was transfected into cells, and transfected cells viability and radio-sensitivity were measured by Cell Counting Kit-8 (CCK-8) and clonogenic survival assay. Besides, the expression of Sp1 Transcription Factor (SP1) in miR-1284 overexpressed and suppressed cells were determined by qPCR and Western blot. Further, miR-1284 inhibitor and/or small interfering RNA (siRNA) against SP1 were transfected into cells, and transfected cells viability and radio-sensitivity were measured again. Results: Down-regulation of miR-1284 was found in radio-resistant cells ($P < 0.001$). Overexpression of miR-1284 significantly inhibited cell viability ($P < 0.05$ or $P < 0.01$) and enhanced radio-sensitivity ($P < 0.05$ or $P < 0.01$). SP1 was negatively regulated by miR-1284 ($P < 0.01$ or $P < 0.001$), and knock-down of SP1 significantly abolished the regulatory effects of miR-1284 suppression on cell viability ($P < 0.05$ or $P < 0.01$) and radio-sensitivity ($P < 0.05$ or $P < 0.01$). Conclusion: Overexpression of miR-1284 enhanced the radio-sensitivity of HCC *in vitro*. Of note, SP1 might be involved in the regulatory effects of miR-1284 on HCC cells radio-sensitivity.

Keywords: MicroRNA-1284, hepatocellular carcinoma, SP1, radio-sensitivity

Introduction

Hepatocellular carcinoma (HCC) is the most frequent liver cancer, which is the fourth most common cause of cancer death worldwide [1]. The leading cause of HCC is viral infection with either Hepatitis C virus (HCV) or Hepatitis B virus (HBV) [2]. In addition, alcohol, tobacco, obesity and diabetes are identified as risk factors which are impact on the prognosis of patients with HCC [3, 4]. For HCC treatment, liver transplantation is a curative option in select patients, but it is limited by availability of donor organs and long waiting times [5, 6]. In addition, recent studies have indicated that radiotherapy can play a meaningful role in the management of HCC [7]. Nevertheless, radio-resistance remains one of the serious obstacles to successful treatment, and the overall five-year survival rate remains grim [8]. Therefore, a better understanding of the toler-

ance of the liver to radiation can contribute to the popularity of radiotherapy for treating HCC in clinical practice [9].

MicroRNAs (miRNAs) are a large family of short non-coding RNAs consisting of 18-24 nucleotides, which regulate gene expression at the post-transcriptional level in a sequence-specific manner [10, 11]. MiRNAs are involved in numerous cellular processes, including development, differentiation, proliferation and apoptosis [12]. Besides, several miRNAs are reportedly involved in the tumorigenesis and development of HCC, such as miR-449a, miR-599 and miR-101 [13-15]. MiR-1284 is one of the family members of miRNAs. Recent studies have demonstrated it served as a diagnosis biomarker and auxiliary inhibitor in some cancers. Patnaik *et al.* have proved that the expression of miR-1284 is much lower in lung adenocarcinoma than in their controls [16]. In addition, the

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expression of miR-1284 was down-regulated in gastric cancer tissues with lymph node metastases than in primary gastric cancer tissues [17]. Further, Cao *et al.*, found that miR-1284 overexpression sensitized gastric cancer cells to chemotherapy *in vivo* [18]. However, none literature has reported the role of miR-1284 in HCC.

Thus, this study was aimed to examine the role of miR-1284 in the radio-resistant of HCC. Two kinds of human HCC cell lines, HepG2 and SMMC-7721, were used in this study, and their correspondingly radio-resistant cells were established by multiple fractional irradiations. The expression of miR-1284 in HCC cells and their radio-resistant cells were monitored. MiR-1284 mimic was transfected into HCC cells and the effects of miR-1284 overexpression on cell viability and radio-sensitivity were measured. In addition, we found that Sp1 Transcription Factor (SP1) was negatively regulated by miR-1284. The effects of knock-down of SP1 and miR-1284 suppression on cell viability and radio-sensitivity were detected again, aiming to uncover the underlying mechanism of miR-1284 in HCC.

Materials and methods

Cell culture

Human HCC cell lines HepG2 and SMMC-7721 were obtained from American Type Culture Collection (ATCC; Manassas, USA) and Cell Bank of the Chinese Academy of Sciences (Shanghai, China) respectively. The correspondingly radio-resistant cells were established from HepG2 and SMMC-7721 by multiple fractional irradiations as previously described [19]. All cells were cultured in RPMI-1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) [20]. Cells were incubated in a humidified 5% CO₂ incubator at 37°C.

Cell transfection

HepG2 and SMMC-7721 cells were planted on 60-mm dishes and cultured 24 h. MiR-1284 mimic, inhibitor or control, and/or small interfering RNA (siRNA) against SP1 (GenePharma, Shanghai, China) were transfected into cells. All transfections were performed by using Lipof-

ectamine 2000 (Invitrogen, USA), according to the manufacturer's protocol [21]. After 48 h of transfection, cells were collected for the forthcoming analyses.

Cell viability assay

Cell viability was determined by using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto Prefecture, Kyushu, Japan) according to the manufacturer's protocol. In brief, transfected cells were seeded into 96-well plates at a density of 5×10^3 cells/well. After cells were cultured for 1-5 days, 10 µL of CCK-8 reaction solution was added into each well and incubated for 2 h at 37°C. Subsequently, optical density at 570 nm was measured by a microplate reader (Bio Rad Laboratories, Hercules, CA, USA) [22].

Clonogenic survival assay

Transfected cells were planted into 6-well plates at a density of 1×10^5 cells/well and followed by exposure to a range of radiation doses (0, 1, 2, 3, 4, 5, or 6 Gy), by using 6 MV X-rays generated by linear accelerators (Varian 2300 EX; Varian, Palo Alto, CA) at a dose rate of 3 Gy/min [23]. After 10-16 days of incubation at 37°C, cells were fixed in 100% methanol and stained by 1% crystal violet (Sigma-Aldrich, USA). Microscopic inspection (Olympus IX71; Olympus, Tokyo, Japan) was used for the calculation of surviving fraction and colonies containing more than 50 normal-looking cells were defined as survivors [23].

RNA extraction and real-time quantitative PCR (qPCR)

Total RNA in transfected cells were extracted by Trizol reagent-phenol chloroform (Invitrogen Life Technologies, Carlsbad, CA, USA). Transcriptor First Strand cDNA Synthesis Kit (Roche, USA) was used for reverse transcription, according to the manufacturer's protocol. For real-time qPCR, 20 ng cDNA and SYBR Premix Ex Taq (Takara, Tokyo, Japan) were used and each real-time PCR was carried out on the ABI PRISM 7500 Real-time PCR System (Applied Biosystems, Foster City, CA) [24]. The expression of SP1 was normalized to GAPDH expression in each sample and fold changes were calculated using the classic $2^{-\Delta\Delta Ct}$ method. Primers used in this study were designed and synthesized by GenePharma (Shanghai, China).

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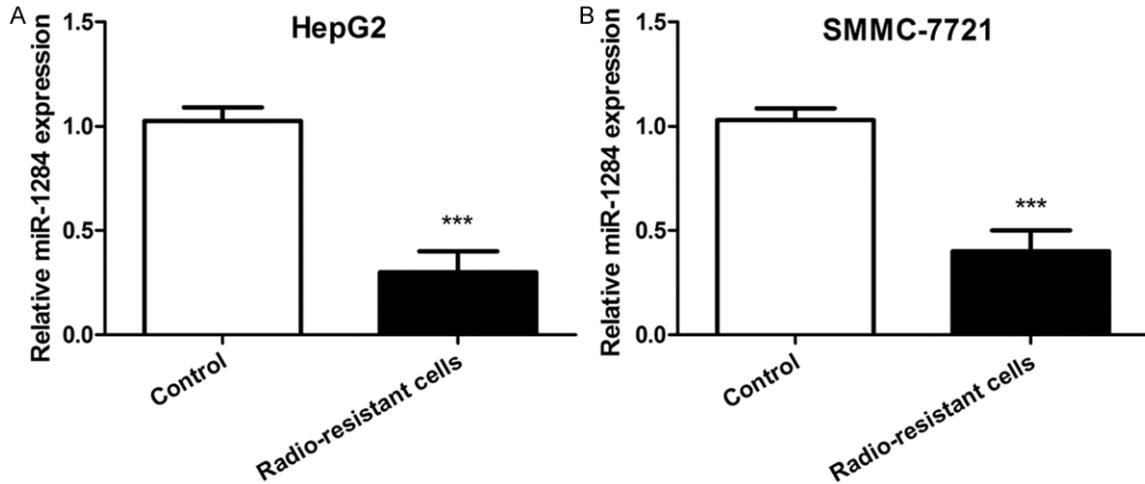


Figure 1. The expression of miR-1284 was reduced in radio-resistant HCC cells. A and B: The mRNA level expression of miR-1284 in two kinds of HCC cells (HepG2 and SMMC-7721) and their correspondingly radio-resistant cells were monitored by qPCR. ***, $P < 0.001$ compared with control group.

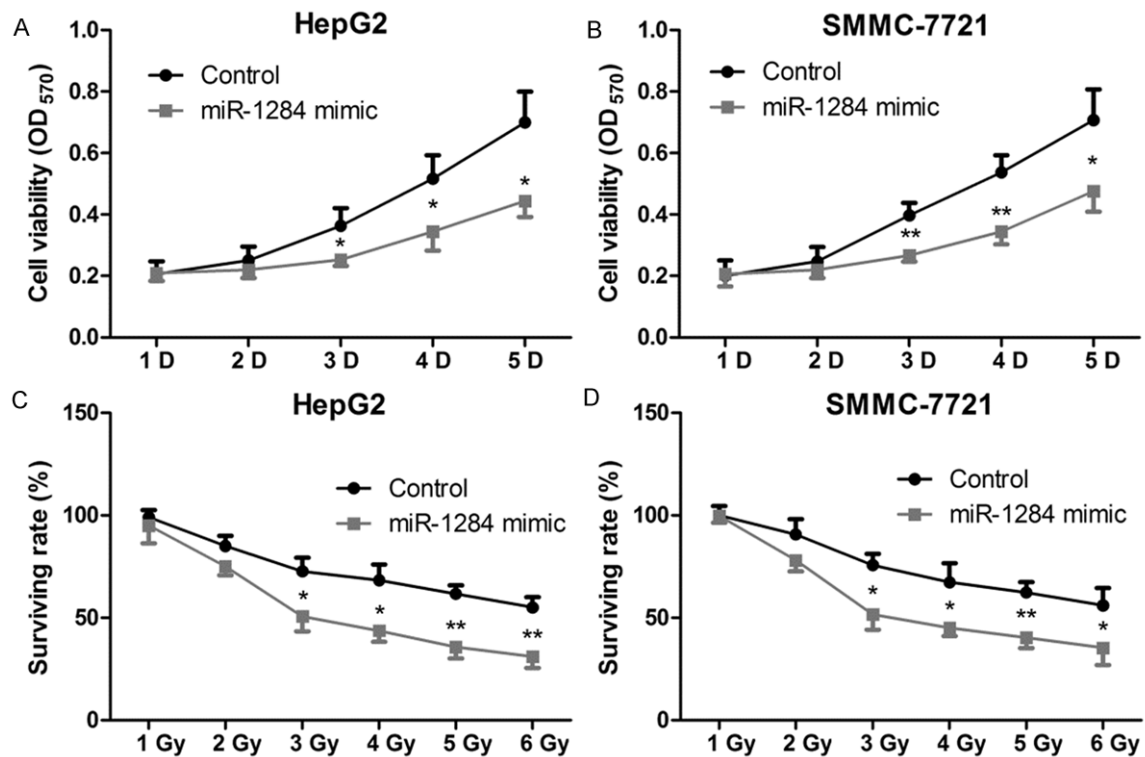


Figure 2. Overexpression of miR-1284 sensitized HCC cells to ionizing radiation. HepG2 and SMMC-7721 cells were transfected with miR-1284 mimic or control respectively. A and B: Transfected cells viability was measured by using CCK-8. C and D: Transfected cells were exposed to a range of radiation doses, and clonogenic surviving rate was calculated. *, $P < 0.05$ compared with control group; **, $P < 0.01$ compared with control group.

Western blot

Transfected cells were collected and lysed in the lysis buffer (Beyotime, Shanghai, China).

Protein concentration of each sample was quantified by BCA Protein Assay Kit (Tiangen, Beijing, China). Equal amount of proteins were loaded on sodium dodecyl sulfate-polyacryl-

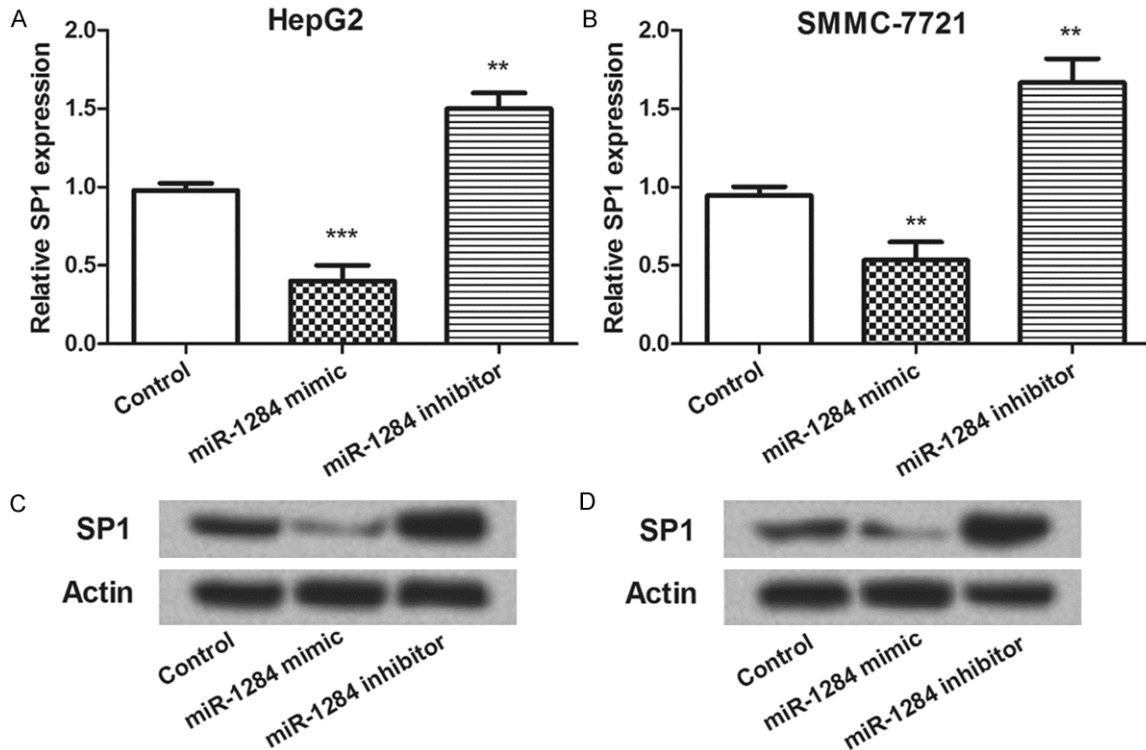


Figure 3. SP1 was negatively regulated by miR-1284. HepG2 and SMMC-7721 cells were transfected with miR-1284 mimic, inhibitor or control respectively. Subsequently, the mRNA and protein level expressions of SP1 in transfected cells were detected by (A and B) qPCR and (C and D) Western blot analysis respectively. **, $P < 0.01$ compared with control group; ***, $P < 0.001$ compared with control group.

amide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, USA) [25]. Membranes were blocked with 5% nonfat dry milk and then probed overnight with primary antibodies: SP1 (sc-14027; Santa Cruz Biotechnology, Santa Cruz, CA) or Actin (sc-7210; Santa Cruz Biotechnology) at 4°C. Afterward, the membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibody (sc-516-087; Santa Cruz Biotechnology) for 1 h at room temperature. The blots were developed by Thermo Scientific Super Signal West Pico Chemiluminescent Substrate (Rockford, USA).

Statistical analysis

Data were expressed as mean \pm standard deviations (SD) from at least three independent analyses. GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA) was used for the data statistical analysis, and data were compared using the Student *t* tests. A level of $P < 0.05$ was considered as statistical significance.

Results

Expression of miR-1284 was reduced in radio-resistant HCC cells

In order to probe the effects of miR-1284 on the radio-sensitivity of HCC cells, the expression of miR-1284 in HepG2 and SMMC-7721 cells and their correspondingly radio-resistant cells, were determined by qPCR. As results showed in **Figure 1A** and **1B**, the mRNA level expression of miR-1284 was significantly down-regulated in radio-resistant cells than in their control groups ($P < 0.001$). These results indicated that miR-1284 might be involved in the radio-resistance of HCC cells.

Overexpression of miR-1284 sensitized HCC cells to ionizing radiation

To explore the detailed impacts of miR-1284 on the radio-sensitivity of HCC cells, HepG2 and SMMC-7721 cells were transfected with miR-1284 mimic or control, and then the transfected cells viability was measured by using CCK-8.

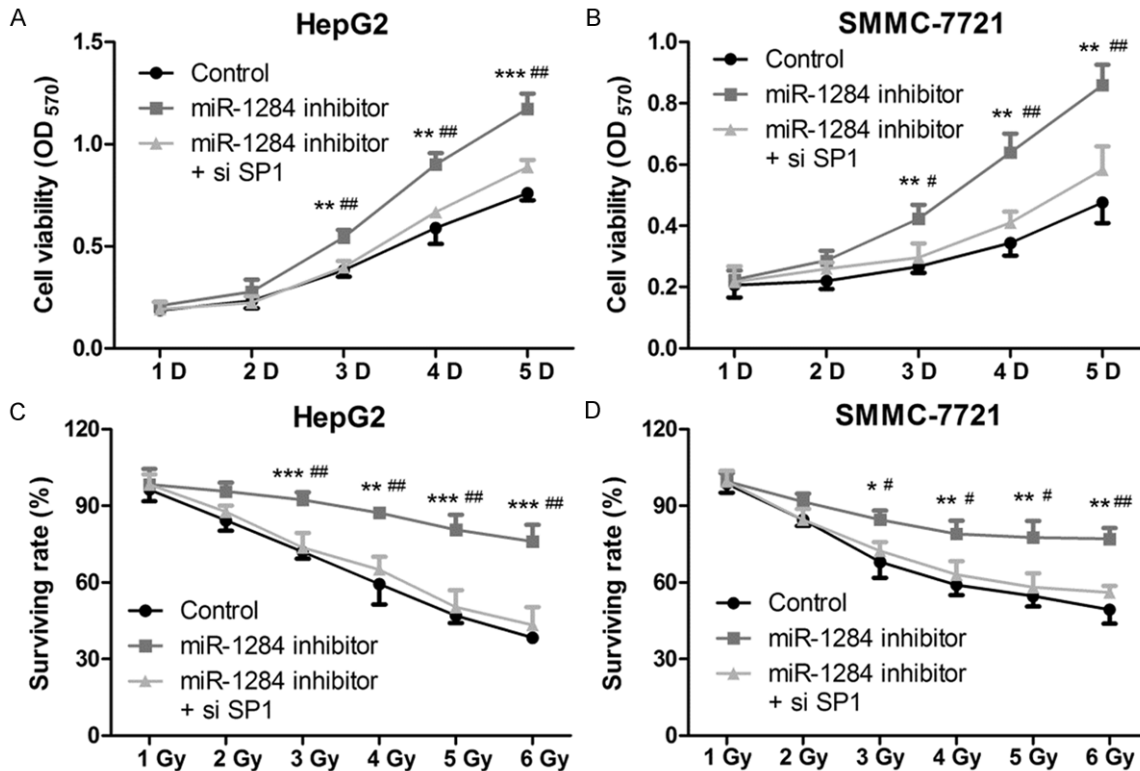


Figure 4. Overexpression of miR-1284 sensitized HCC cells to ionizing radiation via regulating SP1. HepG2 and SMMC-7721 cells were transfected with miR-1284 inhibitor and/or siRNA against SP1. A and B: Transfected cells viability were measured by using CCK-8. C and D: Transfected cells were exposed to various doses of irradiation, and then clonogenic survival was determined. *, $P < 0.05$ compared with control group; **, $P < 0.01$ compared with control group; ***, $P < 0.001$ compared with control group; #, $P < 0.05$ compared with miR-1284 inhibitor + si SP1 group; ##, $P < 0.01$ compared with miR-1284 inhibitor + si SP1 group.

As results showed in **Figure 2A** and **2B**, after the transfected cells were cultured for 3-5 days, miR-1284 overexpression significantly suppressed cell viability of the both two kinds of HCC cells ($P < 0.05$ or $P < 0.01$). Furthermore, the transfected cells were exposed to a range of radiation doses, and we found that (**Figure 2C** and **2D**), the surviving rate was significantly decreased by miR-1284 overexpression at the radiation dose of 3-6 Gy ($P < 0.05$ or $P < 0.01$). According to these findings, we inferred that miR-1284 might be a pivotal regulator in the radio-sensitivity of HCC cells.

SP1 was negatively regulated by miR-1284

To explore the underlying molecular mechanisms of miR-1284 in HCC cells, cells were transfected with miR-1284 mimic, inhibitor or control, and then the mRNA and protein level expressions of SP1 in transfected cells were detected by qPCR and Western blot analyses,

respectively. As results showed in **Figure 3A-D**, both the mRNA and protein level expressions of SP1 were significantly down-regulated in miR-1284 overexpressed cells ($P < 0.01$ or $P < 0.001$), while were significantly up-regulated in miR-1284 suppressed cells ($P < 0.01$). Thus, we deduced that SP1 might be a potential downstream gene of miR-1284 and the expression of SP1 was negatively regulated by miR-1284.

Overexpression of miR-1284 sensitized HCC cells to ionizing radiation via regulating SP1

To explore whether SP1 was involved in the impact of miR-1284 on the radio-sensitivity of HCC cells, cells were transfected with miR-1284 inhibitor and/or siRNA against SP1 and then cell viability were measured again. Results in **Figure 4A** and **4B** displayed that miR-1284 suppression significantly increased cell viability ($P < 0.01$ or $P < 0.001$). However, knock-down

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of SP1 significantly recovered the increased cell viability ($P < 0.05$ or $P < 0.001$). Besides, transfected cells were exposed to radiation and the surviving rate were calculated. Results in **Figure 4C** and **4D** showed that, miR-1284 suppression significantly increased surviving rate ($P < 0.05$, $P < 0.01$ or $P < 0.001$), while knock-down of SP1 significantly recovered the increased surviving rate ($P < 0.05$ or $P < 0.01$). Taken together, miR-1284 sensitized HCC cells to ionizing radiation might be via regulating SP1.

Discussion

HCC is the most common cancer worldwide, with high morbidity and mortality [1]. One of the main problems of treatment failure in HCC is the resistant of tumor cells to irradiation [23]. In the present study, we found that the miR-1284 expression was reduced in the radio-resistant HCC cells, implying that miR-1284 might be involved in the radio-resistance of HCC. Further investigations demonstrated that overexpression of miR-1284 remarkably decreased HCC cells viability and sensitized HCC cells to ionizing radiation. Moreover, SP1 was negatively regulated by miR-1284, and knock-down of SP1 significantly recovered the effects of miR-1284 suppression on HCC cells viability and radiosensitivity.

Currently, an increasing number of literatures have reported miRNAs are pivotal regulators in the therapeutic efficacy of cancer radiotherapy. For instance, Khan *et al.* have demonstrated that high miR-21 base plasma expression was associated poor clinical outcome in patients with locally advanced pancreatic cancer, who were treated with chemo-radiotherapy [26]. Shiiba *et al.* found that miR-125b overexpression enhanced radio-sensitivity of oral squamous cell carcinoma cells [27]. However, few studies have focused on radio-resistance of miRNAs in HCC. Lin *et al.* have revealed that loss of miR-149 expression was involved in the pathogenesis of HCC and overexpression of miR-149 was effective in sensitizing radiotherapy [28]. Jin *et al.* demonstrated that miR-26b enhanced the radio-sensitivity of HCC cells by targeting EPH Receptor A2 (EphA2) protein [29]. In the present study, down-regulation of miR-1284 was found in radio-resistant HCC cells. Further, our study provided the first evidence that miR-1284 could enhance the radio-sensitivity of HCC cells *in vitro*.

SP1 is an 875-amino-acid, 100 to 110 kDa nuclear transcription factor which plays an important regulatory role in multiple cellular processes, such as apoptosis, fibrosis and inflammation [30, 31]. In terms of HCC, SP1 has been reported to be abnormally expressed and activated in HCC tissues, and high expression of SP1 is associated with poor prognosis [32]. In addition, down-regulation of SP1 mediated the inhibitory function of miR-324-5p on HCC migration and invasion [33]. In the current study, we found that the expression of SP1 was negatively regulated by miR-1284. Besides, functional analyses have indicated that knock-down of SP1 notably abolished the regulatory effects of miR-1284 suppression on HCC cells radio-sensitivity. These data implying that miR-1284 enhanced the radio-sensitivity of HCC cells might be via regulating SP1. Similarly, Kang *et al.* have reported that miR-24 suppressed cell proliferation and enhanced radio-sensitivity of nasopharyngeal carcinoma cells by directly targeting SP1 [23].

In conclusion, this study revealed that overexpression of miR-1284 enhanced the radio-sensitivity of HCC *in vitro*. Of note, SP1 was negatively regulated by miR-1284, and SP1 was involved in the regulatory effects of miR-1284 on HCC cells radio-sensitivity. These findings suggested that miR-1284 might be a potential target of radiotherapy, and provided a better understanding of the mechanisms of radio-sensitivity in human HCC. However, further studies are urgently needed to elucidate the deep mechanisms of which miR-1284 enhances HCC sensitivity to radiotherapy.

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Disclosure of conflict of interest

None.

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