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m6A methylation pattern of SLC7A11 mediates the effects of cinobufagin on hepatocellular carcinoma cell proliferation and radioresistance

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► ***Original article***

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ABSTRACT

Background: To determine whether and how cinobufagin regulates hepatocellular carcinoma (HCC) cell proliferation and radioresistance. ***Materials and Methods:*** Radiosensitive (HepG2-NC) and radioresistant (HepG2-SR) HCC cells were treated with cinobufagin, X-ray ionizing radiation (IR) or a combination of cinobufagin and IR at different doses. Cell counting was performed using the Cell Counting Kit-8 assay. Key ferroptosis marker levels were determined using the indicated methods. RNA immunoprecipitation using an anti-m6A antibody followed by quantitative polymerase chain reaction was performed to determine the m6A levels in SLC7A11 mRNA. ***Results:*** Cinobufagin inhibited the proliferation of HepG2-NC and HepG2-SR cells. Exposure to X-rays decreased the HepG2-NC cell count in a time- and dose-dependent manner, but did not affect HepG2-SR cells. A low dose of cinobufagin did not change the cell count without IR exposure, but re-sensitized HepG2-SR cells to IR. The combination of low-dose cinobufagin and IR increased ferroptosis and decreased SLC7A11 expression levels. Mechanistically, the combination of a low dose of cinobufagin and IR decreased m6A levels in the 3' UTR of SLC7A11 in a METTL3-dependent manner. ***Conclusion:*** A low dose of cinobufagin exerted synergistic effects with IR and re-sensitized radioresistant HCC cells to IR via a METTL3/m6A-dependent pathway.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide [1]. Poor response to X-ray ionizing radiation (IR) due to IR resistance remains a clinical challenge in the treatment of HCC [2]. Although research on checkpoint inhibitors as potential radiosensitizers in hepatocellular carcinoma has made significant progress in recent years, the clinical approaches to address radiation resistance remain limited due to the unclear molecular mechanisms underlying radioresistance [3].

The importance of traditional Chinese medicine (TCM) for tumors treatment has been increasingly recognized [4]. Huachansu is a kind of anti-tumor TCM, mainly composed of dried toad skin extract [5]. Cinobufagin is the major bioactive component of huachansu [6]. Cinobufagin has been reported to decrease oncogene expression levels, block the cell cycle, trigger apoptosis, decrease angiogenesis, and enhance the immune response. These are the main mechanisms through which cinobufagin inhibits HCC growth [6].

IR resistance arises from intricate molecular mechanisms that encompass DNA repair, antioxidant defense, cell cycle regulation, and genetic adaptations to counteract radiation-induced damage and maintain cellular integrity [7]. A series of specific expression genes for IR resistance are also targets of cinobufagin, indicating that cinobufagin may regulate tumor IR resistance [8]. However, the relevant molecular mechanisms still need further clarification.

Cell death, specifically, cell suicide, is a fundamental process in the development and prevention of cancer [9]. Apoptosis is the spontaneous programmed cell death that is an important mechanism in tumor elimination [10]. In recent years, new types of cell death, especially ferroptosis, have been widely studied [11-14]. Ferroptosis has been reported to mediate IR function against tumor [14]. So far, it is not clear how cinobufagin regulates the ferroptosis process.

SLC7A11 is an inhibitory factor of ferroptosis, and its expression can reflect the level of ferroptosis (negative correlation) [14]. As we know, the first step in gene expression is mRNA transcription, which is regulated both transcriptionally and post-transcriptionally. As a novel post-transcriptional regulation, N6-methyladenosine (m^6A)

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epitranscriptional modification has recently attracted substantial attention [15]. This study firstly confirmed that cinobufagin could regulate SLC7A11 levels through m^{6}A dependent manner. The novel finding of this study is too explored whether m^{6}A patterns of SLC7A11 mediate the regulatory effects of cinobufagin on HCC cell proliferation.

MATERIALS AND METHODS

Cells and reagents

HepG2-NC (wide type HepG2) is the widely used HCC cell line. HepG2-NC (radiosensitive) or HepG2-SR (radioresistant) cells were gifted by Dr. Niu Y, and a detailed description of these cells has been previously reported [16]. Cinobufagin was purchased from Sigma-Aldrich, USA (cat no. SML3135). Lipid Peroxidation (MDA) Assay Kit and Iron Assay kit (ab83366) were purchased from Abcam, UK. SLC7A11 antibody was purchased from Cell Signal, USA; BCA protein quantitative kit and GAPDH antibody were purchased from Shanghai Biyuntian Company, China. Fer-1 (A13247) was purchased from Adooq Bioscience, USA.

IR exposure and transfection of cells

Cells were exposed to IR in X-RAD 320 cabinet irradiator with indicated radiation energy. Transfections were performed using Lipofectamine® 2000 (Thermo Fisher Scientific; USA). The pmir-GlO luciferase reporters containing wild-type (WT) and mutant (Mut) (GGAC to GGCC) SLC7A11 3′UTR was synthesized by Fitgene (Guangzhou, China).

Real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA was reverse-transcribed using the Eppendorf RealPLEX4 System under the following conditions: 65°C for 5 min, 37°C for 15 min, and 98°C for 5 min. The quantitative PCR (qPCR) parameters were 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60 °C for 5 s, and 72°C for 30 s.

Cell counting detection using CCK-8 reagent

HepG2-NC or HepG2-SR cells were digested using trypsin. Cells were inoculated in a 96-well plate, and then different treatments were performed based on their grouping. The 96-well plate culture medium was discarded after 48 h in the incubator, and CCK-8 reagent were added. Cell counting was determined according to the instruction.

Western blot detection

Cells were lysed, and SDS-PAGE loading buffer was then added to the lysate in the proportion of 1:2, incubated at 100°C in a metal bath for 10 min, and then SDS-PAGE electrophoresis was carried out. After that, trarsmembrane was performed using 250 mA constant current with rotation for 90 min. After sealing with a TBST solution containing 5% milk for 2 h, primary antibodies and second antibodies were used to incubate the membrane in certain order. Thereafter, the membrane was developed by the chemiluminescence method using a gel imager.

MeRIP-qPCR assay

The m^{6}A immunoprecipitation (MeRIP) process was carried out following a reference [17]. To summarize, fragmented RNA was subjected to immunoprecipitation using an anti-m^{6}A antibody linked to Dynabeads from Invitrogen (USA). This was done in an immunoprecipitation buffer at a temperature of 4°C for a duration of 2 hours. The m^{6}A -containing mRNAs were then eluted twice with a solution containing 6.7 mM N6-methyladenosine 5'-monophosphate sodium salt from Sigma-Aldrich, USA, for 1 hour at 4°C. Subsequently, they were precipitated using 5 mg of glycogen from Life Technologies (AM9510, USA) along with one-tenth volumes of 3 M sodium acetate from Sigma-Aldrich, USA, in 2.5 volumes of 100% ethanol at -80°C overnight. The level of m^{6}A enrichment was assessed through qPCR analysis.

Statistical analysis

Statistical comparisons between the two groups were performed using a two-tailed Student's t-test. For comparisons among more than two groups, a one -way ANOVA test was utilized. The statistical analysis was conducted using SPSS software (Armonk, NY, USA). P-values below 0.05 were regarded as statistically significant.

RESULTS

Cinobufagin inhibits HCC cell proliferation regardless of the IR resistance status

HepG2-NC and HepG2-SR were exposed to X-rays for 3 h and cultured for another 12, 24, and 48 h. Exposure to X-rays decreased the HepG2-NC cell count in a time- and dose-dependent manner (figure 1A), but did not affect HepG2-SR cells (figure 1B).

Subsequently, we exposed HepG2-NC and HepG2-SR cells to different doses of cinobufagin for 12, 24, and 48 h and found that cinobufagin decreased the cell counts in a time- and dose-dependent manner (figure 1C-D). These results indicated that cinobufagin inhibited HCC cell proliferation, regardless of the cells' IR resistance status.

Cinobufagin re-sensitizes radioresistant HCC cells to IR

We next exposed HepG2-NC and HepG2-SR cells to different doses of X-rays for 3 h and cultured the cells with or without a low dose of cinobufagin (50 ng/L) for another 48 h. First, treatment with 50 ng/L cinobufagin did not change the cell count without IR exposure. Second, in HepG2-NC cells, the cell counts

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were significantly lower in the cinobufagin (50 ng/L) + IR (8 Gy) treatment group than that in the single IR (8 Gy) treatment group, indicating that cinobufagin exerted a synergistic effect with IR in HepG2-NC cells (figure 2A). Third, in HepG2-SR cells, the dose-dependent inhibition of IR was restored by treatment with cinobufagin (50 ng/L), indicating that cinobufagin re-sensitized radioresistant HCC cells to IR (figure 2B).

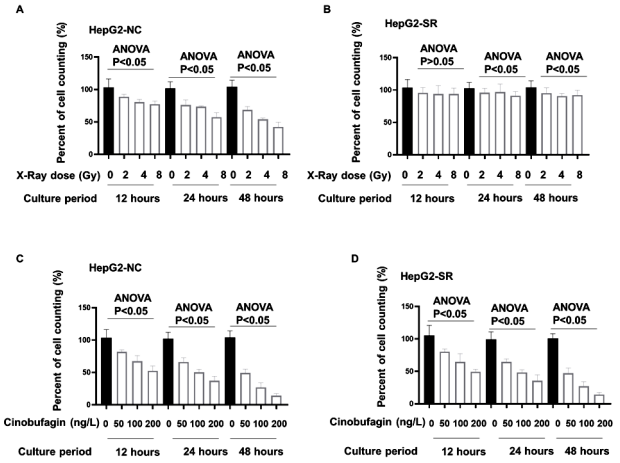

Figure 1. In vitro anti-tumor effect of IR and cinobufagin against HepG2-NC and HepG2-SR cells. **(A-B)** We exposed HepG2-NC and HepG2-SR cells to different doses of X-rays for 3 h and cultured the cells for another 12, 24, and 48 h. Percent of cell counting (%) were determined in **(A)** HepG2-NC and **(B)** HepG2-SR cells. **(C-D)** We exposed HepG2-NC and HepG2-SR cells to different doses of cinobufagin for 12, 24, and 48 h. Percent of cell counting (%) were determined in **(C)** HepG2-NC and **(D)** HepG2-SR cells.

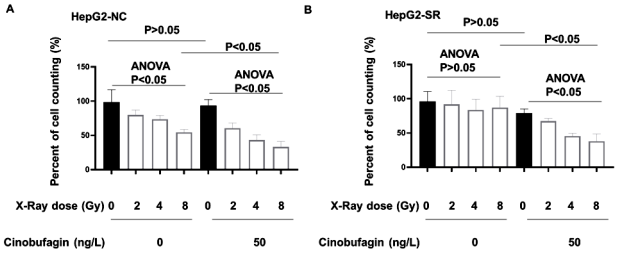

Figure 2. Low dose of cinobufagin and IR had a synergistic anti-tumor effect against HepG2-NC and HepG2-SR cells. **(A-B)** We exposed HepG2-NC and HepG2-SR cells to different doses of X-rays for 3 h and cultured the cells with or without a low dose of cinobufagin (50 ng/L) for another 48 h. Percent of cell counting (%) were determined in **(A)** HepG2-NC and **(B)** HepG2-SR cells.

The combination of cinobufagin and IR triggers ferroptosis in radioresistant HCC cells

Next, we assessed the ferroptosis levels in HepG2-SR cells after specific treatments. IR treatment alone had little effect on iron (figure 3A) or malondialdehyde levels (figure 3B), which are important ferroptosis markers [14]. At a low dose of cinobufagin (50 ng/L), IR treatment increased the levels of these two ferroptosis markers in a dose-dependent manner. Similarly, IR treatment alone had little effect on cell necrosis, whereas treatment with a low dose of cinobufagin (50 ng/L) increased necrosis in a dose-dependent manner (figure 3C). We next exposed HepG2-SR cells to different doses of X-rays for 3 h and cultured them with or without a low dose of cinobufagin (50 ng/L), together with or without fer-1 (a ferroptosis inhibitor), for another 48 h. In the presence of fer-1 treatment, a low dose of cinobufagin (50 ng/L) was no longer able to resensitize HepG2-SR cells to IR (figure 3D). These results demonstrated that the combination of a low dose of cinobufagin and IR triggered ferroptosis in radioresistant HCC cells, and therefore resensitized HepG2-SR cells to IR.

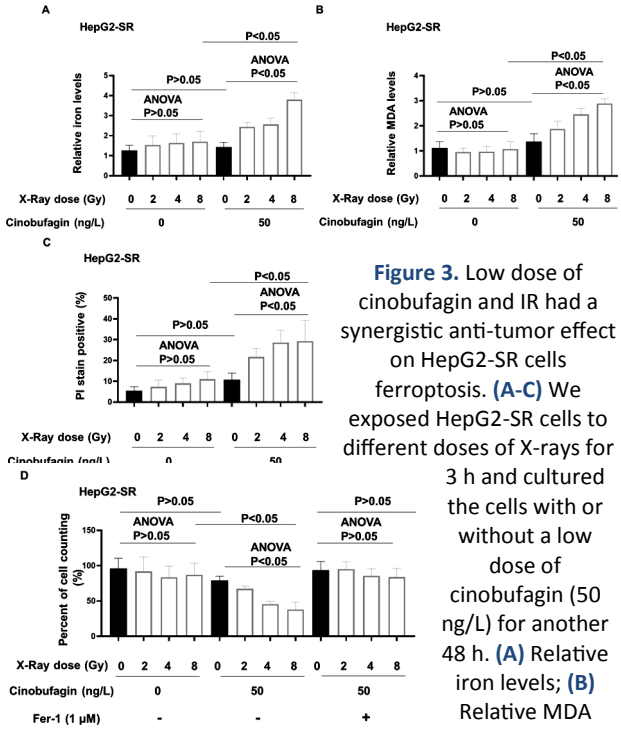

Figure 3. Low dose of cinobufagin and IR had a synergistic anti-tumor effect on HepG2-SR cells ferroptosis. **(A-C)** We exposed HepG2-SR cells to different doses of X-rays for 3 h and cultured the cells with or without a low dose of cinobufagin (50 ng/L) for another 48 h. **(A)** Relative iron levels; **(B)** Relative MDA levels; and **(C)** Percent of PI stain positive cells were determined. **(D)** We exposed HepG2-SR cells to different doses of X-ray for 3 h and cultured the cells with or without a low dose of cinobufagin (50 ng/L), together with or without fer-1 (ferroptosis inhibitor) for another 48 h. Percent of cell counting (%) were determined.

SLC7A11 mediates ferroptosis signaling induced by cinobufagin and IR

SLC7A11 is an important ferroptosis inhibitor [14]. Previous studies have reported that IR inhibits SLC7A11 expression, thereby promoting ferroptosis. Here, we found that IR treatment alone had little effect on SLC7All protein or mRNA levels in HepG2-SR cells (Figure 4A-B). However, in the presence of a low dose of cinobufagin (50 ng/L), IR treatment decreased SLC7All protein and mRNA levels in a dose-dependent manner (figure 4A-B). Next, we exposed HepG2-SR cells to different doses of X-rays for 3 h and cultured the cells with or without a low dose of cinobufagin (50 ng/L), together with or without transfection of a plasmid overexpressing SLC7All for another 48 h. We found that, with SLC7All

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overexpression, a low dose of cinobufagin (50 ng/L) lost the ability to resensitize HepG2-SR cells to IR (figure 4C). These results demonstrated that a decrease in SLC7All levels plays a key role in mediating the effect of the combination of low doses of cinobufagin and IR.

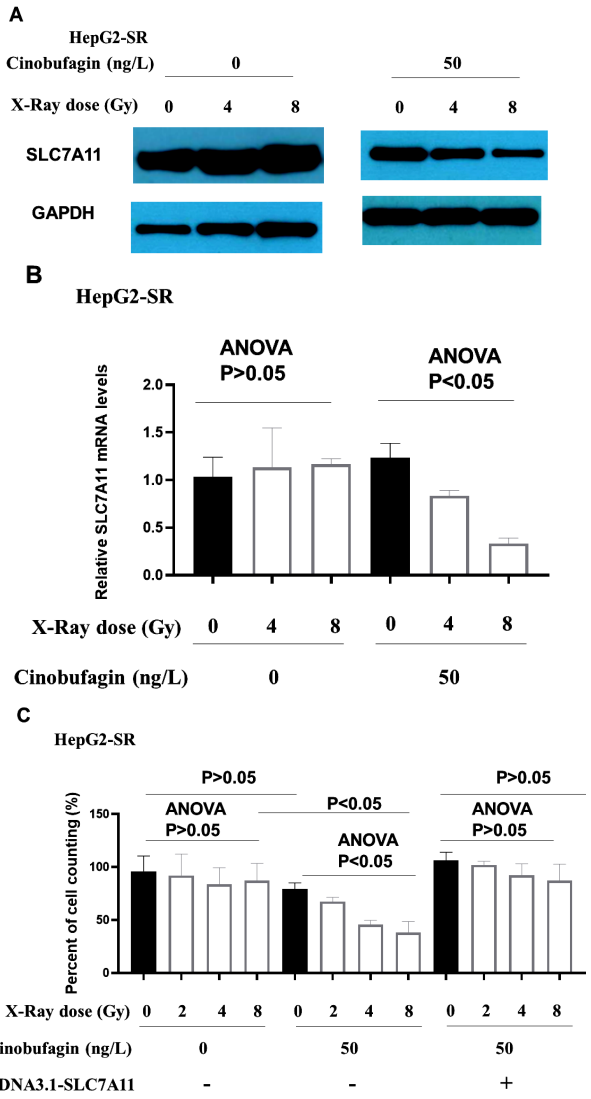

Figure 4. SLC7A11 mediates the ferroptosis signaling regulation induced by low dose of cinobufagin and IR in HepG2-SR cells. **(A-B)** We exposed HepG2-SR cells to different doses of X-rays for 3 h and cultured the cells with or without a low dose of cinobufagin (50 ng/L) for another 48 h. **(A)** the SLC7A11 protein levels were determined; **(B)** the SLC7A11 mRNA levels were determined. **(C)** We transfected the HepG2-SR cells with pcDNA3.1-SLC7A11 plasmid or with pcDNA3.1-NC plasmid for 24 hours, and then exposed the cells to different doses of X-ray for 3 h and cultured the cells with or without a low dose of cinobufagin (50 ng/L) for another 48 h. Percent of cell counting (%) were determined.

The combination of cinobufagin and IR reduces SLC7All levels through the METTL3/m⁶A Pathway

Next, we found that IR treatment alone had little effect on the m6A levels of SLC7All transcripts, and the m⁶A levels of SLC7All transcripts were down-regulated by IR in a dose-dependent manner when a low dose of cinobufagin (50 ng/L) was added (figure 5A, the IgG antibody group was set as the control). METTL3/m⁶A mediates SLC7All expression [18]. When we transiently transfected cells with the METTL3 overexpression plasmid, we found that the m⁶A levels of SLC7All transcripts were not downregulated by the combination of a low dose of cinobufagin and IR, indicating that METTL3 mediates the regulation of m⁶A patterns by low doses of cinobufagin and IR in radioresistant HCC cells (figure 5B; the IgG antibody group was set as a control).

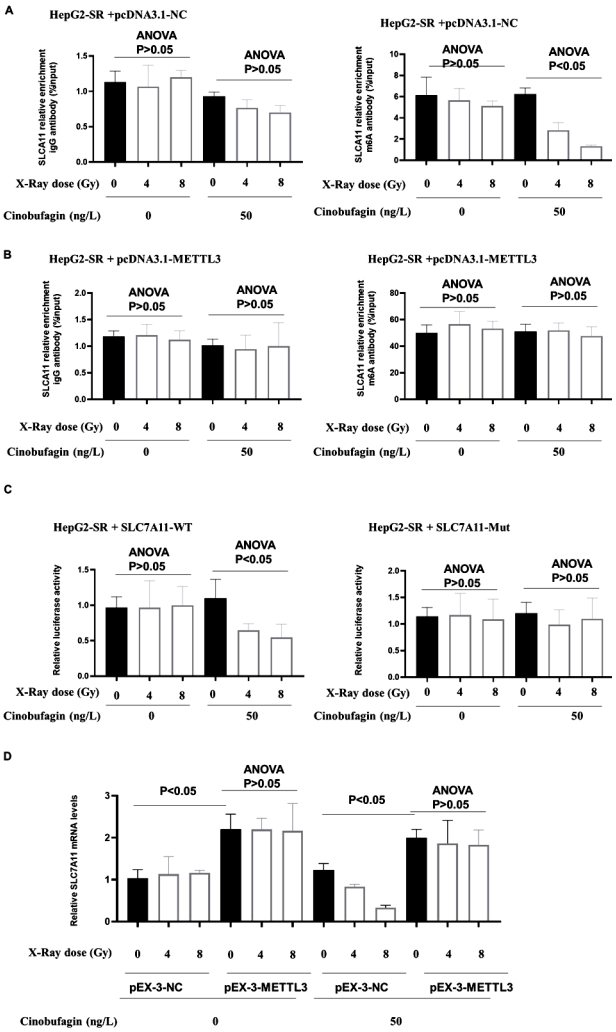

Figure 5. Low dose of cinobufagin and IR modifies SLC7A11 m6A methylation. **(A-B)** We transfected the HepG2-SR cells with **(A)** pcDNA3.1-NC plasmid or with **(B)** pcDNA3.1-SLC7A11 plasmid for 24 hours, and then exposed the cells to different doses of X-rays for 3 h and cultured the cells with or without a low dose of cinobufagin (50 ng/L) for another 48 h. The cells were harvested and the m6A levels of SLC7A11 were determined by meRIP-qPCR (right). Normal igG antibody group was set as controls (left). **(C)** We transfected the HepG2-SR cells with the pmir-GlO luciferase reporters containing WT or Mut SLC7A11 mRNA 3'UTR (named SLC7A11-WT or SLC7A11-Mut, respectively) for 24 hours, and then exposed the cells to different doses of X-rays for 3 h and cultured the cells with or without a low dose of cinobufagin (50 ng/L) for another 48 h. The cells were harvested and the luciferase activities were determined. **(D)** We transfected the HepG2-SR cells with pEX-3-METTL3 plasmid or with pEX-3-NC plasmid for 24 hours, and then exposed the cells to different doses of X-ray for 3 h and cultured the cells with or without a low dose of cinobufagin (50 ng/L) for another 48 h. The SLC7A11 mRNA levels were determined.

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Next, WT and Mut SLC7A11 3′UTRs were cloned downstream of the firefly-luciferase-encoding region in the pmir-GLO vector, according to a previous report [19]. Firefly luciferase reporter assays demonstrated that IR treatment alone had little effect on luciferase activities of either WT SLC7A11 3′UTR or Mut SLC7A11 3′UTR in HepG2-SR cells, whereas treatment with a low dose of cinobufagin and IR reduced the luciferase activity of WT SLC7A11 3′UTR, but not Mut SLC7A11 3′UTR, in HepG2-SR cells, but this reduction was reversed when the METTL3-overexpression plasmid was introduced (figure 5C). These results indicate that a low dose of cinobufagin and IR function on the putative m^{6}A site.

The METTL3/m^{6}A axis plays a key role in stabilizing SLC7A11 mRNA expression.

METTL3 overexpression significantly upregulated SLC7A11 mRNA expression. Furthermore, METTL3 overexpression reversed the effect of the combination of low-dose cinobufagin and IR on SLC7A11 levels. Taken together, our results indicated that the regulation of SLC7A11 by low doses of cinobufagin and IR was mediated by the METTL3/m^{6}A axis.

DISCUSSION

Cinobufagin exhibited potential anticancer in research studies. In the study by Bai *et al.* [20], it was shown that cinobufagin exerted a suppressive effect on colorectal cancer growth through the inhibition of the STAT3 pathway. And, Kim *et al.* [21] discovered that cinobufagin could also suppress the growth of melanoma cells by inhibiting LEF1. Feng *et al.* proved that cinobufagin had strong inhibitory activity against HCC [22]. Our study is in accordance with these previous reports [20-22]. Our findings, combined with previous reports, indicate that cinobufagin exerted strong antitumor effects on different cancer cells through multiple signal pathways.

Cinobufagin enhances cancer treatment when combined with other therapies, showing promising synergistic effects in preclinical studies. More importantly, cinobufagin can re-sensitize some drug-resistant tumors. Ma *et al.* found that cinobufagin could reduce doxorubicin resistance in osteosarcoma [23]; Yuan *et al.* revealed that cinobufagin had the reversal effect on multidrug resistance in colon cancer [24]. Here, we found that cinobufagin and IR had synergistic effects against HCC cells, and cinobufagin could re-sensitize HepG2-SR cells to IR, which further increased the cognition of the function of cinobufagin.

Reduced ferroptosis causes radioresistance [25]. Lei *et al.* conducted a study and provided evidence that treatment with IR (ionizing radiation) stimulates ferroptosis in cancer cells by upregulating the expression of ACSL4. Additionally, they observed that IR also induces the expression of ferroptosis blockers, such as GPX4 and SLC7A11, implying that the effect of IR on ferroptosis is multifaceted [26]. As shown previously, increased expression of SLC7A11 in cancer cells leads to resistance to ferroptosis, which in turn affects cancer growth, invasion, and metastasis, ultimately contributing to an unfavorable prognosis [27]. Our results indicated that the combination of cinobufagin and IR inhibited SLC7A11 expression, thereby eliminating the negative effects of IR on ferroptosis and enhancing ferroptosis, which might be an important mechanism by which cinobufagin re-sensitized IR resistant HCC cells.

SLC7A11 expression can be regulated through multiple mechanisms both at the transcriptional level and post-transcriptionally [28]. Here, we found that the combination of cinobufagin and IR decreased SLC7A11 expression levels, which was mediated by dynamic m^{6}A modification of SLC7A11 transcripts. Several studies have explored the relationship between SLC7A11 levels and m6A methylation patterns. Liu *et al.* conducted a study that revealed the involvement of the METTL3/IGF2BP1/m6A in promoting the stability of SLC7A11 mRNA, leading to an upregulation of its expression [19]. Xu *et al.* demonstrated that METTL3 recruits YTHDF1 to regulate the stability and translation of SLC7A11 [18]. These findings indicated that METTL3 plays a key role in SLC7A11 m^{6}A regulation. When we overexpressed METTL3, SLC7All expression was enhanced and was not affected by cinobufagin or IR, indicating that the synergistic effect of cinobufagin and IR on SLC7A11 was mediated via an METTL3-dependent pathway.

CONCLUSION

Overall, our data provide evidence for re-sensitizing effect of cinobufagin in radioresistant HCC cells. These results implicate cinobufagin as an effective supplement to the current treatment of radioresistant HCC. With low toxicity and no known side effects, cinobufagin may be considered a critical candidate for inclusion in HCC treatment regimens.

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***Conflict of interest*:** The authors declare no conflict of interest.

***Ethics approval and consent to participate*:** The ethics committee of Linyi People's Hospital confirms that this study does not need to be ethically approved because no animal or human samples were included.

All authors declare that they have consented for publication.

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***Authors' contributions*:** All authors contributed equally to the design of the study, data collection and

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analysis, and the writing of the manuscript. All authors read and approved the final manuscript.

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