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Original Research Article

Excessive miR-30a-5p increases the radiosensitivity of hepatoma cells by inhibiting GRP78

Rongyao Xiao[1], Fangzhi Zhou[2], Shuang Gui[3]*

[1]Department of Surgical Oncology, Wenzhou Hospital of Integrated Traditional Chinese and Western Medicine, Wenzhou, Zhejiang Province 325000, [2]Department of Surgery, Wenzhou Hospital of Integrated Traditional Chinese Medicine, Wenzhou, Zhejiang Province 325000, [3]Department of Radiology, Chongqing Qianjiang Central Hospital, Chongqing 409000, China

For correspondence: **Email: guishuang666@163.com; **Tel:** +86-02379225106*

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Abstract

Purpose: *To determine the effect of miR-30a-5p on hepatoma cell radiosensitivity and elucidate the underlying mechanism.*

Methods: *Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to measure miR-30a-5p expression in HepG2 and THLE-3 cells. After 4-Gy X-ray irradiation or miR-30a-5p mimic transfection, the miR-30a-5p level in HepG2 cells was determined using qRT-PCR. Luciferase reporter assay was used to confirm the correlation between miR-30a-5p and glucose-regulated protein 78 (GRP78) levels, while the effects of miR-30a-5p on the viability of HepG2 cells were determined using clone formation and 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assays. Apoptotic cells were evaluated by flow cytometry whereas the protein levels of GRP78, B-cell lymphoma-2 (Bcl-2), BCL2-Associated X Protein (Bax), and cleaved-caspase-9 were quantified by immunoblotting.*

Results: *MicroRNA-30a-5p expression was decreased in HepG2 cells but reduced after 4-Gy x-ray treatment, while miR-30a-5p mimic transfection upregulated miR-30a-5p expression ($p < 0.05$). Cell viability was inhibited after x-ray irradiation or miR-30a-5p mimic transfection and further inhibited by irradiation + miR-30a-5p ($p < 0.05$). Irradiation or miR-30a-5p transfection triggered cell apoptosis; however, irradiation + miR-30a-5p induced more apoptosis, upregulated Bax and cleaved-caspase-9 expression, and reduced Bcl-2 expression ($p < 0.05$). MicroRNA-30a-5p also suppressed GRP78 expression.*

Conclusion: *MicroRNA-30a-5p may enhance HCC x-ray radiosensitivity by inhibiting GRP78., and may be useful in developing treatment strategies for HCC patients*

Keywords: *Liver cancer, MicroRNA-30a-5p, Glucose-regulated protein 78, Radiosensitivity*

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common liver cancer and fourth most common cause of cancer-related death worldwide. Ultrasonic monitoring every six months, with or without alpha fetoprotein, is associated with improved early detection and overall survival rates [1]. Additionally, HCC is relatively resistant to chemotherapy, and surgery remains the only

realistic therapeutic option for patients with HCC [2]. However, because of the limitations of detection, HCC is difficult to detect early, leading to fewer than 40% of patients being eligible for surgery [2]. Therefore, an effective detection strategy with higher sensitivity is necessary for early diagnosis, dynamic monitoring, and drug screening for HCC.

MicroRNAs (miRNAs), a class of short RNA molecules, can modulate posttranscriptional gene expression by modulating mRNA degradation or mRNA translation [3]. Compelling evidence has confirmed that miRNAs are essential in radiation therapy, and miR-21 may be a new promising target in cancer radiation therapy [4]. Additionally, miR-302 replacement therapy in breast cancer promotes breast cancer cell sensitivity to ionizing radiation [5]. Furthermore, miRNAs are potential factors that mediate radiosensitivity in HCC. Although miR-30a-5p represses HCC cell growth [6], its role in radiosensitivity of hepatoma cells remains elusive. Therefore, this study investigated the effect of miR-30a-5p on the sensitivity of HCC cells to interventional radiation therapy to develop a novel target for improving the radiotherapy of HCC. Glucose-regulated protein 78 (GRP78) is a key regulator in the tumor microenvironment, and it is associated with cancer cell growth, apoptosis inhibition, immune escape, metastasis, and angiogenesis. Additionally, anti-GRP78 autoantibodies have been used as serological markers for HCC diagnosis [7], and GRP78 is considered as a novel contributor to the acquisition of resistance to sorafenib in HCC [8]. Unfortunately, the relationship between miR-30a-5p and GRP78 remains unclear. Thus, we investigated the effects of miR-30a-5p on the radiosensitivity of HCC and the underlying mechanism.

EXPERIMENTAL

Cell culture

Human THLE-3 cells (Y-00598) were purchased from FuHeng Biology Co., Ltd (Shanghai, China), and HepG2 cells (cl-0103) were obtained from Procell Life Science & Technology Co., Ltd (Wuhan, China). The cells were kept in RPMI1640 medium (pm150110; Procell Life Science & Technology) containing 10 % fetal bovine serum (FBS; 164220; Procell Life Science & Technology) at 37 °C in 5 % CO_2.

Cell transfection and irradiation treatment

HepG2 cells (2 × 10^5 cells/well) were incubated in 6-well plates at 37°C in 5% CO_2 for 24 h. Two micrograms each of miR-30a-5p mimic or miR-NC mimic (Gene Pharma, Shanghai, China) was transfected into HepG2 cells for 2 h using Lipofectamine™ 2000 transfection reagent (11668019; Thermo Fisher Technology, USA). After 48 h of transfection, the cells were harvested.

Next, untransfected HepG2 cells and miR-30a-5p-transfected cells were vertically irradiated using 6-MV X-rays (4 Gy) at 25 °C. Cells in the control group, 4-Gy X-ray group, miR-NC mimic group, and 4-Gy X-ray+miR-30a-5p mimic group were incubated at 37 °C in 5 % CO_2 for 24 h.

Real-time fluorescent quantitative PCR

TRIzol® (Invitrogen, USA) was used for total miRNA extraction from THLE and HepG2 cells, and the miRNA was elongated using E. coli poly A polymerase. After polyadenylation, the miRNA was reverse transcribed into cDNA using an miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) (MR101-01; Vazyme Biotech, Nanjing, China), following the manufacturer's recommendations. Next, HiScript II Q Select RT SuperMix for qPCR (R232-01) and AceQ Universal SYBR qPCR Master Mix (Q511-02) were used for PCR using the following parameters: 95 °C for 60 s, with 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 sec. U6 was the internal reference for miR-30a-5p, and glyceraldehyde-3-phosphate dehydrogenase served as a control for GRP78. The relative levels of GRP78 and miR-30a-5p were quantified using the $2^{-\Delta\Delta CT}$ method [9]. The specific primer sequences are shown in Table 1.

Clone formation assay

HepG2 cells (5 × 10^3 cells/well) were seeded in 6-well plates and incubated at 37°C overnight.

Table 1: Primer sequences used for RT-qPCR

Gene	Sequences (5´-3´)	
	Forward	Reverse
miR-30a-5p	CGCGATGTGTAAACATCCTCGAC	ATCCAGTGCAGGGTCCGAGG
U6	CTCGCTTCGGCAGCACA	AACGCTTCA CGAATTTGCGT
GRP78	CGTCCTATGTCGCCTTCACT	AATGTCTTTGTTTGCCCACC
GAPDH	CATGTTCGTCATGGGTGTGAA	GGCATGGACTGTGGTCATGAG

After 4-Gy X-ray irradiation, the cells were cultured in an incubator at 37°C for 9 days. Subsequently, the cells were fixed with 1 mL of methanol for 10 min, and then the fixative was discarded. Next, 1 mL of Giemsa staining solution (g1015; Solarbio Technology, Beijing, China) was added to cells for 20 min. The colony formation rate = (number of clones/number of cells inoculated) × 100%.

Determination cell viability

After transfection and 4-Gy X-ray irradiation, HepG2 cells at 1 × 10^5 cells/mL were incubated for 24, 48, and 72 h at 37°C. Next, 20 µL of MTT (ab211091; Abcam, USA) was added, the cells were cultured for 4 h at 37°C, and then 100 µL of DMSO was added. The absorbance was measured at 570 nm.

Dual luciferase reporter assays

TargetScan (Release 7.1) and miRDB were used to obtain the binding sites of miR-30a-5p and the GRP78 3'-UTR. HepG2 cells at of 5 × 10^3 cells/well were seeded in 6-well plates overnight. The PcDNA3.1 empty vector (100 ng) or PcDNA3.1-GRP78-3'-UTR (100 ng) (wt/mut) (Invitrogen, USA) was transfected into HepG2 cells by Lipofectamine 2000 according to the manufacturer's instructions. Luciferase activity was evaluated using a dual luciferase reporter assay kit (ab228530; Abcam) according to the manufacturer's instructions after 24 h of transfection. Renilla served as an internal normalization control.

Annexin V / PI assay

Apoptotic cells were measured using the Annexin V/PI kit (ac12l033; life-ilab Biotechnology, Shanghai, China) according to the manufacturer's instructions. HepG2 cells at 1 × 10^5 cells/well were placed in 6-well plates and then were stained with Annexin V and PI for 15 min in the dark. Apoptotic cells were assessed by FACS Caliber flow cytometry (BD Bioscience).

Western blot assay

Total protein was isolated using RIPA lysis buffer (GenStar BioSolutions, Beijing, China). Next, 30 µg of protein was separated by 10% SDS-PAGE and transferred to PVDF membranes, followed by blocking with 5% skimmed milk for 2 h. The membranes were then probed with rabbit antibodies against GRP78 (1:1000; ab21685), Bax (1:1000; ab32503), Bcl-2 (1:1000; ab32124), cleaved-caspase-9 (1:1000; ab2324), and GAPDH (1:2500; ab9485) at 4°C overnight. Horseradish peroxidase-labeled Goat antirabbit IgG (1:3000; a0208; Beyotime Biotechnology, Shanghai, China) was incubated with the membranes at 25°C for 1 h. Enhanced chemiluminescence reagent (Millipore) was used to analyze the bands.

Statistics

Statistical analysis was performed using Graphpad Prism 8 software (La Jolla, CA, USA). The data of each group were presented as means ± standard deviation. Differences between groups were compared using t test, and one-way analysis of variance was employed to compare among multiple groups. $P < 0.05$ was considered statistically significant.

RESULTS

X-ray irradiation induces decreased expression of miR-30a-5p in HepG2 cells

MicroRNA-30a-5p expression was significantly decreased in HepG2 cells compared with that in THLE-3 cells ($p < 0.05$), and X-ray irradiation induced a significant reduction in miR-30a-5p expression compared with that in HepG2 cells ($p < 0.01$) (Figure 1 A). MicroRNA-30a-5p mimic transfection led to upregulated miR-30a-5p expression compared with the control or NC mimic transfection (Figure 1 B; $p < 0.001$).

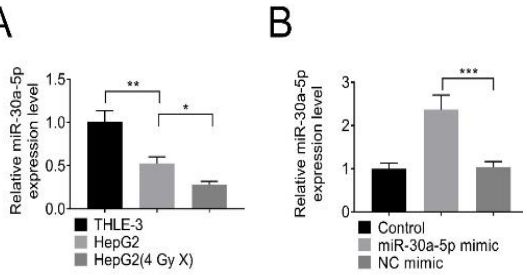

Figure 1: X-ray irradiation induces the downregulation of miR-30a-5p in HepG2 cells. (A) Relative expression of miR-30a-5p in THLE-3 cells, HepG2 cells and X-ray-treated HepG2 cells, as measured using RT-PCR. (B) The relative expression of miR-30a-5p was determined in miR-30a-5p mimic-transfected HepG2 cells. $^{*}P < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$

MicroRNA-30a increases the radiosensitivity of HepG2 cells

Compared with the control or NC mimic transfection, X-ray irradiation or miR-30a-5p transfection inhibited colony formation ($p < 0.01$). X-ray irradiation + miR-30a-5p mimic transfection decreased the colony formation rate ($p < 0.05$) (Figure 2 A and B). The MTT assay revealed that

cell viability was markedly repressed after X-ray irradiation or miR-30a-5p transfection compared with that in the control or NC mimic group ($p < 0.01$ or $p < 0.05$). Additionally, X-ray irradiation + miR-30a-5p mimic transfection significantly inhibited cell viability compared with X-ray irradiation or miR-30a-5p mimic transfection alone ($p < 0.05$ or $p < 0.001$) (Figure 2 C).

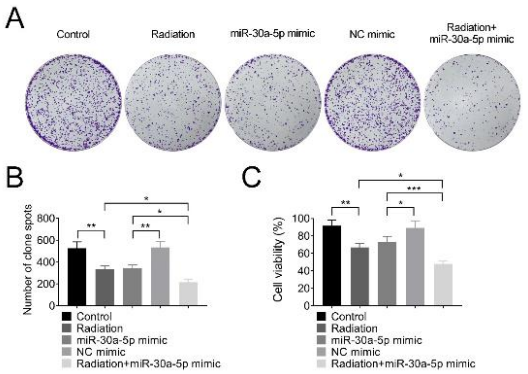

Figure 2: miR-30a enhances the radiosensitivity of HepG2 cells. (A-B) The colony formation assay was performed after X-ray irradiation or miR-30a-5p mimic transfection, and the colony formation rate was calculated. (C) Cell viability as measured by MTT assay after x-ray irradiation or miR-30a-5p mimic transfection. $^{*}P < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

MicroRNA-30a promotes x-ray-induced apoptosis of HepG2 cells

X-ray irradiation or miR-30a-5p transfection significantly promoted apoptosis compared with the control or NC mimic transfection ($p < 0.001$). X-ray irradiation + miR-30a-5p mimic further enhanced apoptosis compared with X-ray irradiation or miR-30a-5p mimic transfection alone ($p < 0.05$, Figure 3 A and B). Additionally, the levels of Bax and cleaved-caspase-9 were significantly upregulated after X-ray irradiation or miR-30a-5p mimic transfection alone ($p < 0.01$), while the Bcl-2 level was downregulated ($p < 0.05$). X-ray irradiation + miR-30a-5p mimic further promoted Bax ($p < 0.01$ or $p < 0.001$) and cleaved-caspase-9 ($p < 0.05$ or $p < 0.001$) expression, but X-ray irradiation + miR-30a-5p mimic transfection further decreased Bcl-2 expression compared with X-ray irradiation or miR-30a-5p mimic transfection alone ($p < 0.01$ or $p < 0.001$) (Figure 3 C).

MicroRNA-30a-5p targets GRP78

The predicted binding sequences between miR-30a-5p and GRP78 3'-URT were ACAAAUG and UGUUUAC (Figure 4 **A**). The relative luciferase activity of GRP78 3'-UTR in the transfected miR-30a-5p group was significantly reduced ($p < 0.001$), while that of GRP78 3'-UTR mut was not significantly changed (Figure 4 B), suggesting that miR-30a-5p targeted the GRP78 3'-UTR region and then inhibited GRP78 expression. Immunoblotting confirmed that miR-30a-5p downregulated GRP78 expression ($p < 0.05$) (Figure 4 C).

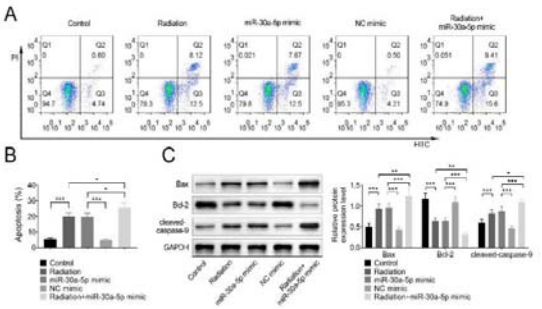

Figure 3: miR-30a promotes X-ray-induced apoptosis of HepG2 cells. (A-B) Cell apoptosis was measured by flow cytometry, and apoptosis was quantified. (C) The protein levels of Bax, Bcl-2, and cleaved-caspase-9 were determined using immunoblotting. $^{*}P < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$

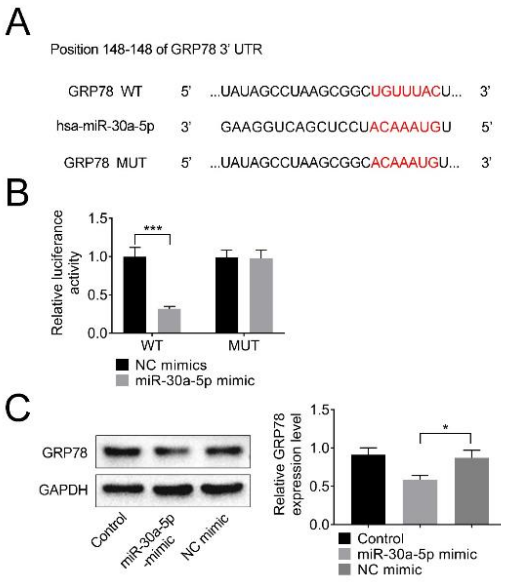

Figure 4: miR-30a-5p targets GRP78. (A) The predicted binding sequences between miR-30a-5p and the GRP78 3'-UTR, as obtained from TargetScan and miRDB. (B) Luciferase activity as assessed using dual luciferase reporter assays. (C) Protein level of GRP78 as determined using immunoblotting. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

DISCUSSION

HCC is a common malignancy, and radiotherapy is an important strategy to treat advanced HCC [10]. The enhancement of radiosensitivity and

improvement of radiation efficacy have been research hotspots recently. It is essential to explore the mechanism of radiosensitivity in HCC cells and identify key genes. In the present study, 4-Gy X-ray irradiation or miR-30a-5p transfection was performed in HepG2 cells, followed by cell viability and apoptosis assays. Furthermore, luciferase reporter assays were performed.

MiR-30a-5p is downregulated in various malignancies, is markedly reduced in HCC tissues compared with that in non-cancerous liver tissues, and inhibits liver cancer cell proliferation [11]. The present study demonstrated that miR-30a-5p in HepG2 cells is decreased compared with that in human normal liver cells, indicating that miR-30a-5p is essential in the occurrence and development of HCC.

The abnormal expression of miRNAs is not only implicated in the growth and apoptosis of tumor cells but also has a close relationship with the radiosensitivity of cancers [12]. Liu and colleagues demonstrated that miR-1271-5p reduced cell proliferation but increased radiosensitivity in SMMC-7721 and HuH-7 cells, effects that were reversed by cyclin-dependent kinase 1 [13]. Another study revealed that miR-30a-3p inhibited esophageal carcinoma (EC) cell metastasis and invasion and promoted the radiosensitivity of EC cells [14]. In addition, a previous study showed that hypoxia led to decreased levels of miR-30a and miR-205 in prostate cancer. Furthermore, miR-30a and miR-205 enhanced the sensitivity of prostate cancer cells to irradiation by regulating TP53INP1 [15]. In the current study, 4-Gy X-ray irradiation significantly repressed miR-30a-5p expression in HepG2 cells. X-ray irradiation or miR-30a-5p significantly suppressed cell viability and colony formation, which were further decreased by X-ray irradiation + miR-30a-5p. In addition, X-ray irradiation + miR-30a-5p transfection induced apoptosis of HepG2 cells compared with X-ray irradiation or miR-30a-5p transfection. Thus, miR-30a-5p inhibits cell viability of X-ray-treated hepatoma cells, enhances apoptosis, and increases radiosensitivity.

Expression of GRP78 was positively correlated with cancer progression, tumor size, and poor prognosis. Recently, Chen found that forkhead box P-2 (FOXP2) promoted tumor proliferation and metastasis by modulating GRP78 in triple-negative breast cancer [16]. Furthermore, miR-495-3p exerted inhibitory effects on multidrug resistance by regulating autophagy by suppressing the GRP78/mTOR pathway in gastric cancer [17]. Another study showed that miR-495 increased the sensitivity of nasopharyngeal carcinoma cells during radiotherapy by modulating GRP78 [18]. The present study suggested that GRP78 expression was increased in HepG2 cells. Furthermore, miR-30a-5p targeted the GRP78 3'-UTR region and inhibited GRP78. Additionally, transfection with miR-30a-5p decreased GRP78 expression, suggesting that miR-30a-5p repressed GRP78 and enhanced the radiosensitivity of X-ray-treated HCC cells.

CONCLUSION

MiR-30a-5p enhances HCC radiosensitivity to x-ray by inhibiting GRP78 expression. This finding suggests a new approach to improve radiotherapy in patients with HCC.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Rongyao Xiao designed the study and supervised the data collection. Fangzhi Zhou analyzed and interpreted the data. Shuang Gui prepared the manuscript for publication and reviewed the draft of the manuscript. All the authors have read and approved the manuscript.

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