Original Research Article

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

Rongyao Xiao1, Fangzhi Zhou2, Shuang Gui3*
1Department of Surgical Oncology, Wenzhou Hospital of Integrated Traditional Chinese and Western Medicine, Wenzhou, Zhejiang Province 325000, 2Department of Surgery, Wenzhou Hospital of Integrated Traditional Chinese Medicine, Wenzhou, Zhejiang Province 325000, 3Department of Radiology, Chongqing Qianjiang Central Hospital, Chongqing 409000, China
*For correspondence: Email: guishuang666@163.com; Tel: +86-02379225106

Sent for review: 4 November 2021
Revised accepted: 11 February 2022

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)-dimethylthiazol(-z-y1)-3,5-di-phenyltetrazoliumromide (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

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% CO2.

Cell transfection and irradiation treatment

HepG2 cells (2 x 10^5 cells/well) were incubated in 6-well plates at 37°C in 5% CO2 for 24 h. Two micromolars 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% CO2 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 a 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^-ΔΔCT method [9]. The specific primer sequences are shown in Table 1.

Clone formation assay

HepG2 cells (5 x 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     | Forward         | Sequences (5’-3’)            | Reverse         |
|----------|-----------------|-------------------------------|-----------------|
| miR-30a-5p| CGCGATGTGTAAACATCCTCGAC | ATCCAGTGAGGGTCCGAGG |                 |
| U6       | CTCGCTTCGGACGACCA | AAGCGTCTCAAGATTTGCCGT |                 |
| GRP78    | CGTCCTATGTCGCCTTCACT | AATGTCTTTGTTTGCCCACC |                 |
| GAPDH    | CATGTTTCGTCATGGGTGAA | GGCATGAGCACTGCTGTAAGAG |                 |
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 3ʹ-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-GRP78-3ʹ-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 (ac121033; 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](image-url)

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 increased 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′-UTR were ACAAAUG and UGUUUUAC (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).

**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.

**Open Access**

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0) and the Budapest Open Access Initiative (http://www.budapestopenaccessinitiative.org/read), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

**REFERENCES**

1. Singal AG, Lampertico P, Nahon P. Epidemiology and surveillance for hepatocellular carcinoma: New trends. J Hepatol 2020; 72(2): 250-261.
2. Li J, Han X, Yu X, Xu Z, Yang G, Liu B, Xu P. Clinical applications of liquid biopsy as prognostic and predictive biomarkers in hepatocellular carcinoma: circulating
tumor cells and circulating tumor DNA. J Exp Clin Canc Res 2018; 37(1): 1-13.

3. Han Q, Xu Z, Zhang X, Yang K, Sun Z, Sun W, Liu W. MiR-486 protects against acute myocardial infarction via regulation of PTEN. Trop J Pharm Res 2021; 20(9).

4. Liu J, Zhu H, Yang X, Ge Y, Zhang C, Qin Q, Lu J, Zhan L, Cheng H, Sun X. MicroRNA-21 is a novel promising target in cancer radiation therapy. Tumor Biol 2014; 35(5): 3975-3979.

5. Liang Z, Ahn J, Guo D, Votaw JR, Shim H. MicroRNA-302 replacement therapy sensitizes breast cancer cells to ionizing radiation. Pharm Res 2013; 30(4): 1008-1016.

6. He R, Yang L, Lin X, Chen X, Lin X, Wei F, Liang X, Luo Y, Wu Y, Gan T. MiR-30a-5p suppresses cell growth and enhances apoptosis of hepatocellular carcinoma cells via targeting AEG-1. Int J Clin Exp Patho 2015; 8(12): 15632.

7. Shao Q, Ren P, Li Y, Peng B, Dai L, Lei N, Yao W, Zhao G, Li L, Zhang J. Autoantibodies against glucose-regulated protein 78 as serological diagnostic biomarkers in hepatocellular carcinoma. Int J Oncol 2012; 41(3): 1061-1067.

8. Chiou J-F, Tai C-J, Huang M-T, Wei P-L, Wang Y-H, An J, Wu C-H, Liu T-Z, Chang Y-J. Glucose-regulated protein 78 is a novel contributor to acquisition of resistance to sorafenib in hepatocellular carcinoma. Ann Surg Oncol 2010; 17(2): 603-612.

9. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 2001; 25(4): 402-408.

10. Lee M, Noh S, Yoon K, Lee S-W, Yoon SM, Jung J, Jeong C, Kwak J. Feasibility Study of Polymer Gel Dosimetry Using a 3D Printed Phantom for Liver Cancer Radiotherapy. J Korean Phys Soc 2020; 76(6): 453-457.

11. Li W-f, Dai H, Ou Q, Zuo G-q, Liu C-a. Overexpression of microRNA-30a-5p inhibits liver cancer cell proliferation and induces apoptosis by targeting MTDH/PTEN/AKT pathway. Tumor Biol 2016; 37(5): 5885-5895.

12. Li K, Zhu X, Li L, Ning R, Liang Z, Zeng F, Su F, Huang S, Yang X, Qu S. Identification of non-invasive biomarkers for predicting the radiosensitivity of nasopharyngeal carcinoma from serum microRNAs. Sci Rep 2020; 10(1): 1-9.

13. Liu H-M, Tan H-Y, Lin Y, Xu B-N, Zhao W-H, Xie Y-A. MicroRNA-1271-5p inhibits cell proliferation and enhances radiosensitivity by targeting CDK1 in hepatocellular carcinoma. J Biochem 2020; 167(5): 513-524.

14. Fan Y, Bian X, Qian P, Wen J, Yan P, Luo Y, Wu J, Zhang Q. miRNA-30a-3p inhibits metastasis and enhances radiosensitivity in esophageal carcinoma by targeting insulin-like growth factor 1 receptor. Mol Med Rep 2019; 20(1): 81-94.

15. Xu C, Yang M, Fan J, Wang W. MiR-30a and miR-205 are downregulated in hypoxia and modulate radiosensitivity of prostate cancer cells by inhibiting autophagy via TP53INP1. Eur Rev Med Pharmacol Sci 2016; 20(8): 1501-1508.

16. Wu J, Liu P, Tang H, Shuang Z, Qiu Q, Zhang L, Song C, Liu L, Xie X, Xiao X. FOXP2 promotes tumor proliferation and metastasis by targeting GRP78 in Triple-negative breast Cancer. Curr Cancer Drug Tar 2018; 18(4): 382-389.

17. Chen S, Wu J, Jiao K, Wu Q, Ma J, Chen D, Kang J, Zhao G, Shi Y, Fan D. MicroRNA-495-3p inhibits multidrug resistance by modulating autophagy through GRP78/mTOR axis in gastric cancer. Cell Death Dis 2018; 9(11): 1-12.

18. Feng X, Lv W, Wang S, He Q. miR-495 enhances the efficacy of radiotherapy by targeting GRP78 to regulate EMT in nasopharyngeal carcinoma cells. Oncol Rep 2018; 40(3): 1223-1232.