World Journal of *Gastroenterology*

World J Gastroenterol 2017 April 7; 23(13): 2269-2452





Published by Baishideng Publishing Group Inc

United Sector of World Journal of Gastroenterology

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| NAME OF JOUKNAL World Journal of Gastroenterology ISSN ISSN 2019-2840 (online) LAUNCH DATE October 1, 1995 FREQUENCY Weekly EDITORS-IN-CHIEF Damian Garcia-Olmo, MD, PhD, Doctor, Professor, Surgeon, Department of Surgery, Universidad Autonoma de Madrid; Department of General Surgery, Fundacion Jimenez Diaz University Hospital, Madrid 28040, Spain Stephen C Strom, PhD, Professor, Department of | CA 90822, United States EDITORIAL BOARD MEMBERS All editorial board members resources online at http:// www.wignet.com/1007-9327/editorialboard.htm EDITORIAL OFFICE Jin-Lei Wang, Director Yuan Qi, Vice Director Ze-Mao Gong, Vice Director World Journal of Gastroenterology Baishidenal Publishing Group Inc 8226 Regency Drive, Pleasanton, CA 94588, USA Telephone: +1-925-2238243 E-mail: editorialoffice@wignet.com Help Desk: http://www.f6publishing.com/helpdesk http://www.ignet.com PUBLISHER | http://www.wignet.com PUBLICATION DATE April 7, 2017 COPYRIGHT © 2017 Baishideng Publishing Group Inc. Articles pub- lished by this Open-Access journal are distributed under the terms of the Creative Commons Attribution Non- commercial License, which permits use, distribution, and reproduction in any medium, provided the original work is properly cited, the use is non commercial and is otherwise in compliance with the license. SPECIAL STATEMENT All articles published in journals owned by the Baishideng Publishing Group (BPG) represent the views, and opin- ions of their authors, and not the views, opinions or policies of the BPG, except where otherwise explicitly indicated. |
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DOI: 10.3748/wjg.v23.i13.2308

Basic Study

World J Gastroenterol 2017 April 7; 23(13): 2308-2317

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

CMA down-regulates p53 expression through degradation of HMGB1 protein to inhibit irradiation-triggered apoptosis in hepatocellular carcinoma

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Author contributions: Wu JH participated in literature search, study design, data collection, data analysis, data interpretation, and wrote the manuscript; Guo JP, Shi J and Wang H carried out the data collection and analysis, and provided the critical revision; Li LL, Guo B, Liu DX and Cao Q conceived of the study, and participated in its design and coordination; Yuan ZY participated in study design and provided the critical revision.

Supported by Natural Science Foundation of Hebei Province, China, No. H2016209007.

Institutional review board statement: The study was reviewed and approved by the North China University of Science and Technology Affiliated Hospital Institutional Review Board.

Institutional animal care and use committee statement: The study was approved by the Institutional Animal Care and Use Committee of North China University of Science and Technology Affiliated Hospital. All operations were performed according to international guidelines concerning the care and treatment of experimental animals.

Conflict-of-interest statement: All authors in this paper declared that there is no conflicts of interest to this work.

Data sharing statement: No additional unpublished data are available.

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Received: January 9, 2017 Peer-review started: January 10, 2017 First decision: February 23, 2017 Revised: February 27, 2017 Accepted: March 6, 2017 Article in press: March 6, 2017 Published online: April 7, 2017

Abstract

AIM

To investigate the mechanism of chaperone-mediated autophagy (CMA)-induced resistance to irradiationtriggered apoptosis through regulation of the p53 protein in hepatocellular carcinoma (HCC).

METHODS

Firstly, we detected expression of lysosome-associated



membrane protein 2a (Lamp-2a), which is the key protein of CMA, by western blot in HepG2 and SMMC7721 cells after irradiation. We further used shRNA Lamp-2a HCC cells to verify the radioresistance induced by CMA. Next, we detected the HMGB1 and p53 expression after irradiation by western blot, and we further used RNA interference and ethyl pyruvate (EP), as a HMGB1 inhibitor, to observe changes of p53 expression. Finally, an immunoprecipitation assay was conducted to explore the interaction between Lamp-2a and HMGB1, and the data were analyzed.

RESULTS

We found the expression of Lamp-2a was increased on irradiation while apoptosis decreased in HepG2 and SMMC7721 cells. The apoptosis was increased markedly in the shRNA Lamp-2a HepG2 and SMMC7721 cells as detected by western blot and colony formation assay. Next, we found p53 expression was gradually reduced on irradiation but obviously increased in shRNA Lamp-2a cells. Furthermore, p53 increased the cell apoptosis on irradiation in Hep3B (p53-/-) cells. Finally, p53 levels were regulated by HMGB1 as measured through RNA interference and the EP treatment. HMGB1 was able to combine with Lamp-2a as seen by immunoprecipitation assay and was degraded *via* the CMA pathway. The decreased HMGB1 inhibited p53 expression induced by irradiation and further reduced the apoptosis in HCC cells.

CONCLUSION

CMA pathway activation appears to down-regulate the susceptibility of HCC to irradiation by degrading HMGB1 with further impact on p53 expression. These findings have clinical relevance for radiotherapy of HCC.

Key words: Hepatocellular carcinoma; Radioresistance; Chaperone-mediated autophagy; HMGB1; p53

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Core tip: The activation of chaperone-mediated autophagy plays an important role in reducing hepatocellular carcinoma (HCC) cell apoptosis in response to irradiation through degraded HMGB1 protein which reduces p53 expression. The discovery of this mechanism will be beneficial for inhibiting radioresistance of HCC and has a promising value in clinical treatment strategy.

Wu JH, Guo JP, Shi J, Wang H, Li LL, Guo B, Liu DX, Cao Q, Yuan ZY. CMA down-regulates p53 expression through degradation of HMGB1 protein to inhibit irradiation-triggered apoptosis in hepatocellular carcinoma. *World J Gastroenterol* 2017; 23(13): 2308-2317 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i13/2308.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i13.2308

INTRODUCTION

Hepatocellular carcinoma (HCC) is a rapidly progressive fatal malignancy, with an increasing incidence of HCC-related morbidity demonstrated at present^[1]. Radiotherapy is regarded as a therapeutic modality and has achieved efficacious tumor control as well as the lengthening of patients' lives in advanced disease^[2,3]. However, HCC has commonly been regarded as a radioresistant tumor for a long time^[4], because varieties of cellular processes are activated in radiotherapy involving essential proteins which make a difference in the curative effects^[5]. Recent research has found that autophagy is not only fundamental for the cellular response to stress^[6], but also strongly links with cancer resistance in therapy^[7]. Chaperone-mediated autophagy (CMA) is a selective form of autophagy and mainly recognizes and degrades KFERQ sequence motifs^[8]. There is a growing interest to investigate CMA function as well as the related mechanism in tumor therapy. It has been shown that CMA activation significantly reduced therapeutic efficacy in tumor therapy^[9]. Nevertheless, the related specific mechanism remains to be elucidated.

p53 is functional when cell damage such as irradiation occurs in the DNA resulting in the inducing of growth arrest in G1 or G2 phases of the cell cycle or leading to cell apoptosis, thus protecting cells from uncontrolled proliferation and inhibiting tumor development^[10,11]. Generally, the loss of p53 function is responsible for increased tumor resistance. It has been reported that CMA can regulate p53 protein expression, but not degrade p53 protein, to influence the Bcl-2 and Bax levels in cytoplasm^[12]. Therefore, there must be another molecule to regulate the p53 expression.

High-mobility group box 1 (HMGB1), a chromatin associated nuclear protein and extracellular damage associated molecular pattern molecule (DAMP), is a protein that has complicated functions in cancer. Reports have shown that HMGB1 expresses highly in varieties of cancers including HCC^[13], lung^[14], gastric^[15,16] etc., which means HMGB1 is closely related with tumor development, infiltration and metastases. Besides, HMGB1 can bind many proteins involved in autophagy including Beclin1, Atg5^[17,18]. Some studies showed that p53 and HMGB1 could form a complex that regulates the cytoplasmic localization of the binding protein and impacts on cell autophagy as well as apoptosis^[19]. This means that HMGB1 could impact the p53 protein expression and then regulate the anti-apoptotic function of p53. Hence, this paper will focus on CMA-induced radioresistance as well as the connection among CMA activation, p53 and HMGB1 protein expression, and ascertain the mechanism of CMA-induced radioresistance in HCC cells through degrading HMGB1 and further regulating p53 expression.

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MATERIALS AND METHODS

Reagents and antibodies

Antibodies targeted to Bcl-2, Caspase 3(cleaved), HMGB1, p53 (rabbit), p21, and Tubulin were obtained from Cell Signaling Technology (United States). Anti-p53 Ab (mouse) was obtained from Santa Cruz Biotechnology, Inc. (United States). Antibodies targeted to Lamp-2a were obtained from Abcam (United States). Ethyl pyruvate (EP) (CAS: 617-35-6) was obtained from SIGMA (United States). Protein A/G sepharose beads were obtained from SIGMA (United States). Lamp2a shRNA (sh-Lamp2a)-expressing lentivirus (target sequence 5 -GCAGTGCAGATGACGACAA-3) and a nonsilencing sequence-expressing lentivirus (sh-NC) (5-TTCTCCGAACGTGTCACGTTTC-3) were supplied commercially by GenePharma Co. Ltd. (Suzhou, China).

Cell lines and culture conditions

The human SMMC7721 (wt p53), HepG2 (wt p53), Hep3B (p53-/-) cell lines were cultured at 37 $^{\circ}$ C in a humidified atmosphere containing 5% CO₂ in the following media: high-glucose DMEM (Gibco) supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin and 100 mg/mL streptomycin.

Irradiation

Cells were cultured as described above and irradiated with a 6-MV X-ray linear accelerator (BJ6B/400 AFC system; Beijing, China) at a dose rate of 360 cGy/min; the total dosage was 0-10 Gy. The control cells were cultured with medium alone. At 48 h after the irradiation, the cells were used for further experiments.

Plate clone formation assay

About 2000 cells were planted to each well of a 6-well culture plate, and each group contained three wells. After incubation at 37 $^{\circ}$ C for 14 d the cells were washed twice with PBS and stained with Crystal Violet Staining Solution. The number of colonies containing 50 cells was counted under a microscope.

shRNA knockdown of Lamp-2 $\!\alpha$ and generation of cell lines

Short hairpin RNA (shRNA) expressing stable Lamp-2 α transformants were generated by infecting the cells with lentiviral expressing specific shRNA (sh-Lamp2aexpressing lentivirus or sh-NC-expressing lentivirus). Western blots were performed to determine the knockdown efficiency.

RNA interference

Cells were cultured and transfected with purified, annealed and desalted double-stranded siRNA (40 μ g/2 × 10⁶ cells) using the Amaxa nucleofection system (kit V, program G-16). siRNA targeted against /HMGB1. (HMGB1 sense strand siRNA: UGUUAC AGAGCGGAGAGAGUU, HMGB1 antisense strand

siRNA: CUCUCUCCGCUCUGUAACAUU. Control sense strand siRNA: GAUGAUCUAAUGGC, Control antisense strand siRNA: GUCUCACUCGCU CUCUAUACU).

Western blotting

Cells were collected and lysed in whole-cell lysate (containing PMSF and a phosphatase inhibitor). Equal amounts of cell lysate were separated by SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes. Proteins were detected using an ECL system (Pierce, Thermo, United States). Each experiment was repeated three times, and similar results were obtained.

Immunoprecipitation

The cell lysate (500 μ g total protein) was combined with 30 μ L of protein A/G sepharose beads and 2 μ g of primary antibody followed by continuous rotation at 4 °C overnight. Beads were washed four times with immunoprecipitation (IP) buffer for 5 min at 2500 rpm and at 4 °C. Next, 1 × SDS buffer was added to the samples, which were then treated at 100 °C for 10 min. Immunoprecipitated samples were analyzed by Western blotting using anti-Lamp-2a, anti-HMGB1.

Statistical analysis

Statistical analysis was performed with SPSS 17.0 software. Graphs were analyzed using Image Lab system. The data are expressed as mean \pm SD of the values from three independent determinations and statistical analysis was conducted using either the Student's *t*-test or one-way analysis of variance in comparison with corresponding controls. Probability values less than 0.05 were considered statistically significant.

RESULTS

CMA conferred resistance to irradiation in HCC cells

One of the major therapeutic mechanisms of irradiation is to induce target cell apoptosis^[20]. In this study, the apoptosis of HepG2 and SMMC7721 after irradiation increased in a dose-dependent manner (Figure 1A). The apoptosis also took on a dynamical change: it increased at 6-12 h, and decreased at 24-48 h (Figure 1B). In consideration of the declining apoptosis induced by radiotherapy, we thought there must exist some factors to reduce the irradiationinduced apoptosis. Studies showing that CMA pathway activation plays a role in regulating cancer cell proliferation or apoptosis gave rise to our attention^[21]. To determine whether CMA pathway activation impacts on the irradiation-induced cancer cell apoptosis, we firstly detected the activation of the CMA pathway. Lamp-2a, the key protein in the CMA pathway^[22], was gradually increased on irradiation and peaked at 48 h (Figure 1B). Contrary to activation of the CMA pathway, the apoptosis levels decreased at 24-48 h. To





Figure 1 Irradiation-induced chaperone-mediated autophagy pathway activation regulated the apoptosis in hepatocellular carcinoma cells. A: HepG2 and SMMC7721 cells were irradiated at different doses and the ability of proliferation was detected by clone formation assay; B: HepG2 and SMMC7721 cells were irradiated at doses of 6 Gy; the levels of Lamp-2a, Caspase 3 (cleaved) and Bcl-2 were determined by western blot at different post-irradiation times; C: sh-Lamp-2a HepG2 and sh-Lamp-2a SMMC7721 cells were irradiated at doses of 6 Gy; the levels of Lamp-2a, Caspase 3 (cleaved) and Bcl-2 were detected by clone formation assay; B: HepG2 and SMMC7721 cells were irradiated at doses of 6 Gy; the levels of Lamp-2a, Caspase 3 (cleaved) and Bcl-2 were detected at 48 h by western blot; D: The ability of propagation in sh-Lamp-2a HepG2 and sh-Lamp-2a SMMC7721 cells was detected by clone formation assay at 48 h after 6 Gy irradiation. ^aP < 0.05, vs control groups or sh-NC groups. Each experiment was repeated three times and similar results were obtained.

confirm whether CMA activation has functions in downregulating irradiation-induced apoptosis, we used sh-Lamp-2a cells to investigate the effects of CMA induced by irradiation in HCC cells. The results showed that the apoptosis increased obviously in sh-Lamp-2a cells (Figure 1C), and the clone formation assay results provided more evidence (Figure 1D). Taken together, from these results, we believe that the activated CMA pathway plays an important role in down-regulating the apoptosis in HCC cells on irradiation.

CMA induced radioresistance through impacting on p53 protein expression in HCC cells

It is well known that p53, an important tumor suppressor, is able to impact on cell apoptosis through a variety of pathways. To find out the role of p53 in HCC cell irradiation, we firstly detected the p53 expression in irradiated HepG2 and SMMC7721 cells. The results showed p53 gradually increased in 6-12 h, and began to decrease in 24-48 h on irradiation (Figure 2A). Meanwhile, HepG2 and SMMC7721 cell apoptosis greatly reduced at 24-48 h after radiotherapy (Figure 1B). From the similar tendency between down-regulated apoptosis and decreased p53 expression on irradiation, we wondered whether the reduced p53 expression induced the down-regulated apoptosis on irradiation. In order to confirm this hypothesis, we detected the apoptosis and growth of HepG2, Hep3B (p53-/-) cells on irradiation. The results demonstrated that the susceptibility to irradiation of Hep3B (p53-/-) was lower than HepG2 (Figure 2B and C). Therefore, we confirmed p53 played key roles in radioresistance. As shown in Figure 1B and Figure 2A, we found the level of p53 protein was just the opposite of the increased CMA pathway activation. This result made us speculate whether there were somehow links between p53 reduction and CMA pathway activation. To confirm whether the reduced levels of p53 had some links with the CMA pathway activation, we carried out the following experiments. We constructed the sh-Lamp-2a HepG2 and sh-Lamp-2a SMMC7721 cells and treated them with irradiation. We found expressions of the p53 and its downstream effector protein p21 were both higher than those in wild type cells (Figure 2D). These results revealed that p53 expression was regulated by the CMA pathway.

p53 expression was regulated by HMGB1 in HCC cells on irradiation

Previous studies showed that p53 was not degraded by the CMA pathway but rapidly degraded by the activity of the p53-targeting ubiquitin ligase MDM^[23]. Nevertheless, we had confirmed that p53 expression was regulated by the CMA pathway activation from our results. So we thought there must be another protein which connected p53 reduction and CMA pathway activation on irradiation. It was reported that p53 could interact with HMGB1 in cells to regulate cell autophagy and apoptosis^[24]. That led us to suspect that HMGB1 may be the key molecule to regulate the p53 reduction on irradiation. So we firstly detected HMGB1 expression in HepG2 and SMMC7721 cells treated with irradiation at 6 Gy. The results showed that HMGB1 increased at 6-12 h and decreased at 24-48 h on irradiation (Figure 3A). To further investigate whether HMGB1 plays a role in regulating apoptosis of HCC cells on irradiation, we performed the experiments described below. Firstly, we used EP, the specific inhibitor of HMGB1, to suppress HMGB1

expression and detected the apoptosis at 48 h after irradiation at 6 Gy. The result revealed that cell apoptosis significantly declined (Figure 3B); meanwhile the colony-forming efficiency was markedly increased (Figure 3C). Next, we further tested and verified the role of HMGB1 by RNA interference. In si-HMGB1 cells, cell colony-forming efficiency increased in contrast to that seen in HCC cells or si-NC cells (Figure 3D). The above data affirmed that HMGB1 was able to regulate HCC apoptosis on radiotherapy. When EP and siRNA of HMGB1 were applied again to detect the change in p53 expression, we found that the expression of p53 and p21 had declined distinctly (Figure 3E and F). Previous studies had shown that knock-out p53 could reduce the apoptosis on irradiation, so HMGB1 could influence HCC cell apoptosis through regulation of p53 expression on radiotherapy.

HMGB1 degraded by the CMA pathway mediated the reduced apoptosis in HCC cells

The above experiments confirmed CMA pathway activation could reduce p53 expression. What's more, p53 was also regulated by HMGB1 on irradiation. So we speculated as to whether HMGB1 was degraded by CMA pathway activation and then reduced the p53 expression. To confirm our hypothesis, we first tested the HMGB1 levels in sh-Lamp-2a HepG2 and sh-Lamp-2a SMMC7721 cells. As Figure 4A shows, the HMGB1 increased obviously in sh-Lamp-2a cells on irradiation, which indicated CMA pathway activation has a function in regulating the HMGB1 expression. Through immunoprecipitation analysis, we found that HMGB1 could interact with Lamp-2a and formed a complex in HCC cells (Figure 4B). The HMGB1 combined with Lamp-2a increased gradually from 12 to 48 h on irradiation. Meanwhile, the HMGB1 in cytoplasm was decreased (Figure 4C). This meant that HMGB1 degraded by CMA resulted in low cytoplasm HMGB1 levels. Further, we continued to detect HMGB1 combined with Lamp-2a in the presence of ethyl pyruvate (EP, which was applied as an inhibitor of HMGB1) by immunoprecipitation assay. As results show in Figure 4D, the HMGB1 reduced markedly in the presence of EP; meanwhile HMGB1 combined with Lamp-2a also decreased. This provides just one more testament to the HMGB1 degradation through CMA pathway activation.

DISCUSSION

HCC is a common malignant tumor and radioresistance is a critical issue in clinical treatment^[25]. In radiotherapy, induction of apoptosis is the major therapeutic mechanism. Once the apoptosis induced by irradiation declines, the tumor appears to have comparatively low sensitivity to irradiation. Recently, CMA has been gaining more and more attention with regard to tumor progression and therapy because of its function in



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Figure 2 p53 was regulated through chaperone-mediated autophagy pathway activation in hepatocellular carcinoma cells on irradiation. A: HepG2 and SMMC7721 cells were irradiated with doses of 6 Gy. At different post-irradiation times, the levels of p53 were determined by western blot; B: HepG2 and Hep3B (p53-/-) cells were irradiated at different doses and the ability of proliferation was detected by clone formation assay; C: HepG2 and Hep3B (p53-/-) cells were irradiated at different doses and the ability of proliferation was detected by clone formation assay; C: HepG2 and Hep3B (p53-/-) cells were irradiated at doses of 6 Gy; the levels of Caspase 3 (cleaved) and Bcl-2 were detected at 48 h by western blot; D: sh-Lamp-2a HepG2 and sh-Lamp-2a SMMC7721 cells were irradiated at doses of 6 Gy; the levels of p53 and p21 were determined after 48 h. ^aP < 0.05 vs control group or sh-NC group; ^cP < 0.05 vs HepG2 groups. Each experiment was repeated three times and similar results were obtained.

regulation of apoptosis. In our study, we found the CMA pathway was activated gradually in radiotherapy of HCC cells, especially in the late stage of irradiation. Inhibition of CMA pathway activation could sensitize tumor cells to undergo apoptosis, implying CMA could protect hepatocarcinoma cells from radioactive harm, and induce cell production of radioresistance. Besides the CMA pathway, there are varieties of cellular processes that determine the success of treatment, including p53 protein^[26]. p53 is a transcription factor associated with more than 50% of human tumors and a key molecule involved in response to cell apoptosis

in radiotherapy^[27-29]. In our study, apoptosis was much lower on irradiation in Hep3B cells (p53-/-) than in HepG2 cells. This made us convinced that on irradiation, p53 plays an important role in apoptosis regulation. Since p53 protein and CMA pathway activation had important functions in HCC cells irradiation, we thought there maybe existed an interaction between CMA pathway activation and p53 protein expression. In sh-Lamp-2a HepG2 and SMMC7721 cells, p53 protein and the downstream p21 protein both increased sharply. From this, we confirmed CMA pathway activation did regulate p53 expression on irradiation. However, Wu JH et al. CMA induced irradiation resistance



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Figure 3 p53 expression was regulated by HMGB1 in hepatocellular carcinoma cells on irradiation. A: HepG2 and SMMC7721 cells were irradiated with doses of 6 Gy. At different post-irradiation times, the levels of HMGB1 were determined by western blot; B: In the presence of EP (10 μ g/mL). HMGB1, Caspase3 (cleaved) were detected by western blot in representative cells irradiated at 6 Gy after 48 h; C: HepG2 and SMMC7721 were exposed to EP(10 μ g/mL) and the ability of proliferation was detected by clone formation assay; D: The ability of proliferation in HepG2 and SMMC7721 cells affected by HMGB1 siRNA was detected by clone formation assay after 6 Gy irradiation; E: In the presence of EP (10 μ g/mL), the levels of p53 and p21 were detected in HepG2 and SMMC7721 cells irradiated at 6 Gy after 48 h; F: After si-HMGB1 HepG2 and si-SMMC7721 cells were irradiated at doses of 6 Gy, the levels of p53 and p21 were determined after 48 h. ^a*P* < 0.05, *vs* control group or si-NC group; ^c*P* < 0.05, *vs* control group or control + EP group; ^e*P* < 0.05, *vs* IR group. Each experiment was repeated three times and similar results were obtained.



Figure 4 HMGB1 was degraded through chaperone-mediated autophagy pathway in hepatocellular carcinoma cells. sh-Lamp-2a HepG2 and sh-Lamp-2a SMMC7721 cells were irradiated at doses of 6 Gy, (A) the levels of HMGB1 were determined after 48 h by western blot and (B) the interaction of Lamp-2a with HMGB1 was detected by immunoprecipitation (IP); C: Lamp-2a and HMGB1 were detected at different post-irradiation times by IP; D: In the presence of EP (10 μ g/mL), the levels of HMGB1 were detected after 48 h by IP. Each experiment was repeated three times and similar results were obtained.

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Vakifahmetoglu-Norberg^[12] mentioned that p53 was not directly degraded through the CMA pathway; so there must be another way to regulate the p53 expression.

It is well known that many proteins in cells can interact with p53 protein and further influence cell progression and apoptosis. The interactions are important for its activity and function. Studies have shown that p53 interacts with HMGB1 proteins in cells and further regulates the cell proliferation and migration^[30-32]. In our study, we found that HMGB1 was significantly expressed in irradiated HCC cells but markedly reduced in the late stage on irradiation. At the same time, the HCC cell apoptosis also reduced in line with the HMGB1 reduction. When HepG2 and SMMC7721 had HMGB1 expression blocked with EP or RNA interference, p53 and its downstream molecule p21 were obviously decreased. Combined with previous p53 regulation of apoptosis results, we confirmed that in the late stage of irradiation, HMGB1 plays a vital role in inducing apoptosis through regulation of p53 expression. Although some studies have revealed that the CMA pathway could impact the HMGB1 expression, the mechanism has been unclear. In our study, we confirmed that HMGB1 could interact with Lamp- 2α and form a complex through immunoprecipitation assay. This means that HMGB1 induced by irradiation maybe contains KFERQ-like sequence motifs which might permit Hsc70 to recognize and in turn promote the degradation of HMGB1 in a lysosome-dependent manner.

To summarize the above, CMA pathway activation could down-regulate HCC cell susceptibility to apoptosis on irradiation through degradation of HMGB1 protein which reduced p53 expression. These findings suggest that CMA plays a critical role in the irradiation treatment of HCC and that it has a promising value in clinical treatment strategy.

COMMENTS

Background

The activation of chaperone-mediated autophagy (CMA) plays an important role in reducing hepatocellular carcinoma (HCC) cell susceptibility to apoptosis during irradiation. In this paper, we mainly explore the possible mechanism of radioresistance of HCC induced by the CMA pathway and the findings will be beneficial to clinical treatment strategy.

Research frontiers

HCC is a common malignant tumor and radioresistance is critical in clinical treatment. Recent research has found that autophagy is not only fundamental for the cellular response to stress, but also strongly links with cancer resistance in therapy. There is a growing interest to investigate CMA function as well as the related mechanism in tumor therapy.

Innovations and breakthroughs

This paper mainly focuses on CMA-induced radioresistance as well as the connection among CMA, p53 and HMGB1 proteins, and ascertains the mechanism of CMA-induced radioresistance in HCC cells through degradation of HMGB1 and further regulating p53 expression.

Applications

This study revealed that CMA plays a critical role in the reduction of apoptosis of HCC cells in irradiation treatment. The discovery of this mechanism will be beneficial to inhibiting radioresistance of HCC and has a promising value in clinical treatment strategy.

Peer-review

It is well-known that p53 is one of the important molecules for anticancer mechanism. The authors showed the CMA activation was associated with reduced p53 and apoptosis. They showed that CMA (not MDM) could decrease p53 protein through the reduction of HMGB1. This paper is generally well-written.

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P- Reviewer: Enomoto H S- Editor: Ma YJ L- Editor: Logan S E- Editor: Zhang FF







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