miR-199b-3p Promotes Radiation-induced Oral Mucositis via DDIT4-mediated mTOR Signal Pathway

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Abstract

Background: Radiation-induced oral mucositis (RIOM) is an adverse reaction in patients of head and neck cancer after radiotherapy. However, the key regulatory factors in the pathogenesis of RIOM remain largely unclear. In this article, we discover a novel role of DNA damage-inducible transcript 4 (DDIT4) in regulating RIOM pathogenesis.

Methods: We established RIOM in vitro and in vivo models to mimic the biological processes of RIOM. The level of DDIT4 in RIOM was analyzed by real-time PCR and Western blot. Through the bioinformatics analysis and luciferase assay, the relationship between miR-199b-3p and DDIT4 was performed. The level of mTOR signaling were explored by Western blot. Besides, Clone Formation and EDU assay were performed to investigate the effects of miR-199b-3p/DDIT4 on cell proliferation. H&E and immunohistochemistry experiments examined the effects of miR-199b-3p/DDIT4 on RIOM in vivo.

Results: We found that the level of DDIT4 was significantly reduced during the RIOM formation, and up-regulated of DDIT4 suppressed the progression of RIOM in vitro and in vivo. Besides, we found DDIT4 was a direct target of miR-199b-3p. Ectopic expression of miR-199b-3p repressed the level of DDIT4 and activated mTOR signal conduction to promote RIOM progress, whereas the silencing of miR-199b-3p promoted the DDIT4-mediated RIOM regulation both in vitro and in vivo.

Conclusion: Collectively, our studies not only identified the novel functional role of DDIT4 in modulating pathogenic processes of RIOM but also provided new directions and ideas for the future treatment of radiotherapy oral mucositis.

1. Introduction

Radiation-induced oral mucositis (RIOM) is the main treatment for nasopharyngeal carcinoma. Nevertheless, radiotherapy has a direct damage on oral mucosa[1, 2]. Excessive inflammation and epithelial ablation are the main features of oral mucositis. Severe oral mucositis may require feeding tubes to control severe pain or stopping radiotherapy prematurely[3]. The occurrence of RIOM is related to the effects of radiation, oxidative stress, transcription factors, pro-inflammatory cytokines and pathogenic microorganisms. The pathological process can be divided into five phases: initiation, up-regulation, amplification, ulceration and healing[4]. The key regulatory factors in the pathogenesis of RIOM remain largely unknown. Therefore, we need to comprehend the pathogenesis and risk actors in order to provide basis for prevention and control of RIOM.

Ionizing irradiation leads to fatal damage in cellular DNA, principally DNA double strand breakage and the production of reactive oxygen species[5]. In RIOM, the activation of NF- κ b and production of inflammatory factors cause downstream signaling pathways to be amplified, which ultimately lead to tissue damage and ulcer formation[6]. DNA Damage Inducible Transcript 4 (DDIT4) is a highly conserved stress-response protein, which is also regarded as REDD1 or RTP801[7]. In previous research, DDIT4 can protect BMSC away from miR-22-mediated cell damage induced by ionizing radiation[8]. DDIT4 suppress

osteoblast cells away from premature senescence induced by gamma radiation[9]. DDIT4 plays a protective role against radiotherapy in glioblastoma. In addition, DDIT4 is a suppressor of mammalian target of rapamycin (mTOR), which could regulate cell growth in response to environmental inputs[10]. The mechanism targets of mTOR are dual specific protein kinases with phosphorylated serine/threonine and tyrosine residues[11]. Nonetheless, the function of DDIT4 in RIOM is still unclear.

In this article, we investigated the function of DDIT4 in regulating the pathogenesis of RIOM. We discovered that DDIT4 was decreased during RIOM in the mouse model and the normal NHKs after irradiation. We also discovered that silencing of DDIT4 could activate mTOR signaling and promote the progress of RIOM in vivo and vitro. We further demonstrated that DDIT4 was a direct target of miR-199b-3p. Ectopic expression of miR-199b-3p repressed DDIT4 and activated mTOR signaling to promote the formation of RIOM. Meanwhile, up-regulated of DDIT4 or down-regulation of miR-199b-3p enhanced RIOM cell model proliferation. In conclusion, our studies provide new directions and ideas for the research of RIOM.

2. Materials And Methods

2.1 Animal experiment

All animal experimental procedures were approved by the Animal Trials of the Affiliated Hospital of ZunYi Medical University. We anesthetized C57/BL6 mice (18-20g; 6-8 weeks) and exposed them to irradiation on the head and neck at a dose of 10 Gy for 3 times. After 7 days of irradiation, the mice were euthanized by exsanguination prior after intraperitoneal anesthesia. The tongue tissues were immediately cut out and fixed in 4% formaldehyde solution, embedded in paraffin and sectioned[12].

The miR-199b-3p inhibitors, DDIT4 OE and DDIT4 KD (Ribobio, Guangzhou, China) were employed to assess the impacts of miR-199b-3p or DDIT4 on the model of RIOM. In brief, miR-199b-3p inhibitors (5'-TAACCAATGTGCAGACTACTGT-3') (10 nmol) in 50 μ L PBS were administered into RIOM mouse caudal veins once for 3 days to conduce miR-21 knockdown. In order to achieve up-regulated or down-regulated of DDIT4 in RIOM mice, Adeno-associated viral vectors (DDIT4 OE) or lentivirus-loaded short hairpin RNA (DDIT4 KD) were injected into RIOM mice by caudal vein. The sequence of the DDIT4 OE was consistent with the coding sequence of mouse DDIT4. The sequence of the DDIT4 KD was designed and synthesized by Ribobio (China).

2.2 Hematoxylin-Eosin (H&E) Staining

Tongue tissues were fixed in 4% paraformaldehyde for 24 h. Then, the samples were embedded in paraffin, and sectioned. The slices were immersed in 0.5% hematoxylin for 5 minutes and eosin solution for 3 minutes. Images of stained sections were observed under a light microscope (Nikon, Japan).

2.3 Immunohistochemistry (IHC) assay

Tongue tissues were fixed with 4% paraformaldehyde, sliced after paraffin embedding. The sections were incubated with antibody CD45 (Abcam, ab281586) or PCNA (Abcam, ab92552) at 4°C overnight treatment. The sections were washed with PBS and then incubated with the secondary antibody of goat anti-rabbit HRP conjugate (CST) at room temperature for 1 h, after which haematoxylin was used for the counterstain. Images of each group were viewed under a light microscope (Nikon, Japan).

2.4 Cell Culture and transfection

Human Oral keratinocytes cell line HOK was purchased from QiYi biotechnology, Shanghai. HOK was cultured in DMEM (Gibco, USA) contained 10% FBS (Gibco, USA) at 37°C in a humidified incubator under 5% CO₂. si-DDIT4 (5'-ACCUUUAU ACUCCAAUUCUUUUU-3'), si-NC (5'-CACUGAUUUUCAAUUGGUGCUAUU-3'), miR-199b-3p mimic (5'-TGTCATCAGACGTGTAACCAAT-3'), miR-199b-3p inhibitor (5'-TAACCAATGTGCAGACTACTGT-3') and NC sequence of miR-199b-3p (5'-ACAGUAGUCUGCACAUUGGUUA-3') were supplied by GenePharma (Shanghai, China). The pcDNA3.1 vector was used to construct DDIT4 over expression plasmid (DDIT4 OE). HOK cells were transfected with si-DDIT4 or its negative control. (When cells confluence upon to 70%, the transfection was carried out with Lipofectamine 2000 (Invitrogen, USA) or RNAiMax transfection (Invitrogen, USA). NHKs were exposed to 10 doses of gamma rays three times (10 Gy*3).

2.5 Clone Formation Assay

NHKs Cells were seeded into 6-well plates with 500 cells/well and incubated with 37°C, 5% CO₂ for 14 days. Subsequently, cells were fixed with 10% formaldehyde, treated with 0.1% crystal violet. At last, the number of colonies were taken by a light microscope (Olympus, Japan).

2.6 EdU assay

Cellular proliferation rate was measured by EdU assay. NHKs cells (5×10^4 /well) were cultured in 24-well plates and transfected for 48 h. Then NHKs cells were fixed with 4% paraformaldehyde, Triton X-100 was used to permeabilize the nuclear membrane, and NHKs cells were blocked with goat serum for 1 h. Further, NHKs cells were stained according to the manufacturer's suggestions.

2.7 Immunofluorescence analyses

The NHKs cells were incubated on the coverslip for 24 hours. Washing by PBS for 3 times, the NHKs cells were fixed in 4% paraformaldehyde for 15 minutes, and then fixed in 0.2% TritonX-100 for 10 minutes at 20°C. After blocking with 3% BSA for 30 minutes, incubated the DDIT4 antibody at -4°C overnight. The Alexa Fluor 488 conjugated second antibody was incubated at 20°C for 1 h. Afterwards, NHKs Cells were stained by rhodamine phalloidin for 25 mins. The nuclei of NHKs were stained with SlowFade® Gold Antifade Mountant (Thermo Fisher, S36942) for 30 mins. Finally, the samples added Anti-fade solution to prevent quenching. Imaging was performed by confocal microscopes (Leica TCS SP8).

2.8 RNA extraction and qRT-PCR

Total RNA was extracted and reverse-transcribed to cDNA by Trizol reagent and PrimeScript™ RT kit (Takara, Japan). The cDNA reactions were amplified using SYBR Premix Ex TaqII (Takara, USA) by fluorescent quantitative PCR 7500 (ABI, USA). All target gene transcripts were normalized to U6 or β -actin using the $2^{-\Delta\Delta CT}$ method. The sequence was shown in **Supplementary table 1**.

2.9 Western blotting analysis

Tissues and cells were fully lysed with 500 μ L protein lysate of RIPA:PMSF=9:1 (Beyotime, China) and placed on ice for 15 minutes. The protein concentration of the samples was determined using the BCA kit (Beyotime, China). Then, 20 μ g protein samples were added to loading buffer at 95°C for 5 minutes. Protein samples were placed in SDS-PAGE gel and shifted into PVDF membranes. After blocking in 5% skim milk for 2 h, membranes incubated with the following primary antibodies at 4°C overnight, DDIT4 (ab106356, Abcam, 1:1000), TSC1/2 (ab32554, Abcam, 1:1000), p-mTOR (ab109268, Abcam, 1:1000), mTOR (ab32028, Abcam, 1:1000), p-P70S6(sc-8416, Abcam, 1:1000), P70S6(sc-8418, Abcam, 1:1000), P-4EBP1 (ab278686, Abcam, 1:1000), 4EBP1 (ab32024, Abcam, 1:1000) and GAPDH (ab9485, Abcam, 1:1000). After incubation with secondary antibodies for 1 h, protein signal was detected by BeyoECL Moon (Beyotime, China) and quantified using ImageJ software.

2.10 miRNA prediction and dual-luciferase reporter assay

The candidate miRNA of DDIT4 was predicted with miRDB, miRwalk and TargetScan, and mmu-miR-199b-3p was chosen as a target miRNA. The wild type and mutant DDIT4 3'-UTR dual-luciferase reporter vectors were constructed. 80 ng luciferase reporter vectors and miR-199b-3p mimic or inhibitor using the lipofectamine 2000 (Invitrogen, CA, USA) were transfected into NHKs. Luciferase activity was measured after 24 h.

2.11 Data analysis

All Experimental results were repeated three times and the data were presented mean $\pm$ standard deviation (SD) and analyzed by GraphPad Prism 8. The student's t-test or one-way ANOVA with Turkey's test was used to measure statistical significance of differences between two groups or multiple groups, respectively. A P value < 0.05 was considered statistically significant.

3. Results

3.1 Effects of DDIT4 overexpression on RIOM

In order to investigate the effect of DDIT4 in RIOM, western blot analysis was performed to assess DDIT4 expression profile in the tongue tissue of RIOM mouse. The results showed that DDIT4 level in RIOM mouse tongue tissues was significantly decreased compared with non-irradiated (Figure.1a).

Subsequently, H&E experiments were performed and showed that there was no change of the tongue tissues in the DDIT4 overexpressed mice under non-irradiation treatment. However, the ulcer areas were

significantly reduced and the stratified squamous keratinized epithelium complete depletion in the group of DDIT4 overexpressed mice under 10 Gy*3 (Figure.1b-c). CD45 is a cell surface tyrosine phosphatase which could regulate T cell and B cell activation and maturation to facilitate radiation damage repair. [13]. Proliferating cell nuclear antigen (PCNA) is a key factor that initiates recombination-associated DNA synthesis after irradiated injury[14]. Immunohistochemical experiments indicated that up-regulated DDIT4 in RIOM in vivo could decrease infiltration of inflammatory cells at the sub-epithelial connective tissue and promote cell proliferation (Figure.1d).

3.2 DDIT4 modulates proliferation in NHKs exposed to irradiation

To confirm whether DDIT4 was also down-regulated by irradiation in vitro, NHKs were exposed to 10 Gy irradiation three times. qRT-PCR analyzed the mRNA level of DDIT4 was no difference at irradiation. Western blot analyzed the protein level of DDIT4 was down-regulation. In addition, immunofluorescence staining showed that the fluorescence intensity of DDIT4 in 10 Gy*3 group was significantly weaker than Non-irradiation (Figure.2a). NHKs were transduced with lentivirus expressing DDIT4, the result of EDU experiment showed that up-regulated of DDIT4 increased the survival fraction of NHKs subjected to irradiation (Figure.2b). Colony-forming assay showed that up-regulated of DDIT4 suppressed the sensitivity of NHKs when exposed to irradiation (Figure.2c).

3.2 DDIT4 suppressed the formation of RIOM by regulating the mTOR pathway

DDIT4 was reported to promote cell survival under radiation via suppressing mTOR pathway, therefore we inferred the involvement of DDIT4 mediated mTOR pathway during the formation of RIOM[11, 15]. DDIT4 as an inhibitor of mTOR can be activated the tuberous sclerosis 1/2 (TSC1/2) complex to interfere glioblastoma cell death induction by radiotherapy. Western blot detected the expression of TSC1/2, mTOR and mTOR downstream protein, including P-mTOR, mTOR, P-P70S6K, P70S6K, P-4EBP1 and 4EBP1 in the model of RIOM[16]. The results showed that TSC1/2 was down-regulated, mTOR and its downstream protein were up-regulated in the model of RIOM (Figure.3a). However, up-regulated of DDIT4 in RIOM mouse model promoted TSC1/2 and suppressed mTOR and its downstream pathway proteins (Figure.3b). Up-regulated of DDIT4 or treated with mTOR inhibitor in RIOM cell model, and discovered that DDIT4 promoted TSC1/2 and suppressed mTOR and downstream pathway proteins, consistent the results with in vivo experiment (Figure.3c).

3.3 DDIT4 is a direct target of miR-199b-3p in RIOM

The mRNA level of DDIT4 had no change significantly under 10 Gy irradiation and combined with the changes of DDIT4 protein in Fig. 2a, we speculated that there was a post-transcriptional regulation mechanism to regulate the expression of DDIT4 in RIOM. It was reported that miRNA could regulate post-transcriptionally by mRNA cleavage or translational repression. We performed three online databases (miRDB, Targetscan, miRWalk) to predict miRNAs, and a total of 15 intersecting miRNAs were found

(Figure.4a). qRT-PCR analysis indicated that the expression of miR-199b significantly increased in RIOM in vivo and vitro (Figure.4b). In addition, the predicted miR-199b-3p binding sites in the 3'UTR of DDIT4 were shown in Figure.4c. Moreover, luciferase reporter was performed to confirm the interaction between DDIT4 and miR-199b-3p. The results indicated that miR-199b-3p mimic suppressed the luciferase activity of wild-type DDIT4, and miR-199b-3p inhibitor promoted the luciferase activity of wild-type DDIT4. Meanwhile, miR-199b-3p mimic and inhibitor had no effects on the mutant group (Figure.4d). Western blot and qRT-PCR further showed that miR-199b-3p negatively regulated the expression of DDIT4 (Figure.4e). These data indicated that DDIT4 was a target of miR-199b-3p.

3.4 MiR-199a accelerated RIOM through targeting DDIT4 in vitro

To evaluate the therapeutic potential of miR-199b-3p in vitro, NHKs were divided into twelve groups: Non-treat group, NC mimic group, miR-199b-3p mimic group, miR-199b-3p mimic+DDIT4 OE group, miR-199b-3p inhibitor group, miR-199b-3p inhibitor+DDIT4 KD group, Non-treat+10 Gy*3 group, NC mimic+10 Gy*3 group, miR-199b-3p mimic+10 Gy*3 group, miR-199b-3p mimic+DDIT4 OE+10 Gy*3 group, miR-199b-3p inhibitor+10 Gy*3 group, miR-199b-3p inhibitor+DDIT4 KD+10 Gy*3 group. The results of EDU assay and clone formation assay showed that up-regulation of miR-199b-3p could suppress proliferation of RIOM cell model and clarified up-regulation of DDIT4 could restore the proliferation inhibition of miR-199b-3p mimic on RIOM cell model. As for Non-irradiated NHKS cells, the level of miR-199b-3p or DDIT4 was no associate with the cells proliferation(Figure.5a-b). Western blot detected TSC1/2, mTOR and its downstream protein, the results discovered that up-regulation of miR-199b-3p in RIOM could suppress TSC1/2 and activate mTOR and its downstream. Up-regulated of DDIT4 could activate TSC1/2 and inhibit the activation of mTOR and its downstream protein by miR-199b-3p mimic(Figure.5c). These results indicated that miR-199b-3p targets DDIT4 and regulates DDIT4 at post-transcriptional level in RIOM.

3.5 MiR-199a accelerated RIOM through targeting DDIT4 in vivo

To evaluate the therapeutic potential of miR-199b-3p in vivo, mice were divided into eight groups: Non-treat group, NC inhibitor group, miR-199b-3p inhibitor group, miR-199b-3p inhibitor +DDIT4 KD group, Non-treat+10 Gy*3 group, NC inhibitor group+10 Gy*3 group, miR-199b-3p inhibitor group+10 Gy*3, miR-199b-3p inhibitor +DDIT4 KD+10 Gy*3 group. As similar to the results of the in vitro study, H&E indicated that down-regulation of miR-199b-3p with no change in Non-irradiated group. However, down-regulation of miR-199b-3p caused the inflammatory cells and ulcer areas were significantly reduced, the stratified squamous keratinized epithelium complete depletion in the group of 10 Gy*3. In addition, down-regulation of DDIT4 could restore the RIOM inhibition of miR-199b-3p inhibitor on RIOM mouse models (Figure.6a-b). CD45 and PCNA were detected by immunohistochemical assay and found that down-regulation of miR-199b-3p in RIOM in vivo could decrease infiltration of inflammatory cells at the sub-epithelial connective tissue and promote cell proliferation and up-regulation of DDIT4 could decrease the

function of down-regulated miR-199b-3p in RIOM in vivo. Western blot analyzed TSC1/2, mTOR and its downstream protein and discovered that down-regulation of miR-199b-3p in RIOM in vivo could accelerate TSC1/2 and suppress mTOR and its downstream protein. Down-regulation of DDIT4 could inhibit the suppression of mTOR and its downstream protein by miR-199b-3p inhibitor (Figure.6d). These results suggested that miR-199b-3p regulated RIOM through DDIT4/TSC1/2/mTOR pathway, and then participated in regulating the development of RIOM in vivo.

4. Discussion

Radiation-induced oral mucositis (RIOM) is a common toxicity in patients receiving radiotherapy for head and neck cancer[17]. The clinical manifestations in the acute stage range from mild erythema to deep mucosal ulceration[18]. RIOM not only significantly affects the oral function of patients, but also affects the short-term and long-term quality of life. In severe cases, it can reduce the tumor control rate and thus impact the survival of patients[1, 19]. The key regulatory factors in the pathogenesis of RIOM remain largely unknown. Therefore, preventing or reducing radiation injury of the oral cavity is very necessary. Previous studies have shown that the level of DDIT4 was related to the sensitivity of human cancer cells to chemotherapy and radiotherapy[9, 10, 20, 21]. In addition, DDIT4 could protect BMSC from damage induced by ionizing radiation[8]. We discovered direct evidence that DDIT4 serves as a key regulator of the pathogenesis of RIOM for the first time.

Our data revealed that DDIT4 was significantly decreased in RIOM mouse and cells model. Furthermore, the level of DDIT4 related to the formation of RIOM. Functionally, up-regulated of DDIT4 accelerated cell proliferation of RIOM in vitro. DDIT4 was a negative regulator of mTOR signal pathway in cellular response to stress. It has been confirmed that irradiation generates excessive amounts of reactive oxygen species (ROS) resulting in oxidative stress which is the reason for the formation of RIOM[22]. Oxidative stress-related critical protein kinase mTOR which could regulate cell growth, cell death, cancer and metabolism[23–25]. Previous study has shown that inhibition of mTOR can protect normal tissues via suppression of radiation-induced senescence[26]. DDIT4 as an inhibitor of mTOR which can be inactivated by the tuberous sclerosis 1/2 (TSC1/2) complex to interfere glioblastoma cell death induction by radiotherapy[10]. Ribosomal protein S6 kinases (P70S6K) and eukaryotic initiation factor 4E (eIF4E)-binding protein (4EBP1) are the characteristic downstream effectors of mTOR. P70S6K and 4EBP1 play particularly important roles in the mTOR signaling pathway growth acceleration function[27]. In this work, we speculated that DDIT4 inhibited the formation of RIOM by activating TSC1/2 to inhibit mTOR and downstream proteins (P70S6K). Western blot detected that the down-regulated of TSC1/2, up-regulated of mTOR and its downstream protein in RIOM mouse and cell model. However we transfected DDIT4 OE could up-regulated TSC1/2, down-regulated mTOR and its downstream proteins at the model of RIOM in vitro and in vivo. The results confirmed our conjecture that DDIT4 suppresses the formation of RIOM by activating TSC1/2 to inhibit the mTOR signaling pathway related to oxidative stress.

MicroRNAs (miRNAs) are small non-coding RNA molecules, which only contain 18-25 nucleotides in length[28–30]. MiRNAs participate in many physiological and pathological processes through completely

or incompletely complementary between seed region and 3'-UTR of target genes[31]. For instance, down-regulated of miR-200c reduced radiation-induced ROS generation and DNA damage in the initiation stage of RIOM[32, 33]. In this article, we detected that the mRNA level of DDIT4 without significantly changes in RIOM. However, the results of western blot indicated the protein level of DDIT4 was downregulated. Therefore, we speculated that the post-transcriptional regulation of DDIT4 was regulated by miRNA. Therefore, we used TargetScan, miRDB, miRwalk software to predict, and found 15 common miRNAs which could combine with DDIT4-3'UTR. qRT-PCR analysis indicated only miR-199b-3p was up-regulated in RIOM. Bioinformatics and luciferase reporter assays suggested that miR-199b-3p specifically bound to DDIT4. miR-199b-3p is a member of the miR-199 family, which could inhibit head and neck squamous carcinoma cell migration and invasion[34]. In addition, miR-199b-3p can suppress the apoptosis of cerebral microvascular endothelial cells in ischemic stroke[34]. In this research, we found that miR-199b-3p negatively regulates the expression of DDIT4 protein. Soon afterwards, we further explored the role of miR-199b-3p in RIOM. We proved that miR-199b-3p promoted the formation of RIOM by suppressing DDIT4 and activating mTOR pathway.

5. Conclusion

In summary, our data provided the evidence that DDIT4 modulates the pathogenesis of RIOM. We identified the miR-199b-3p promotes RIOM through DDIT4/TSC1/2/mTOR pathway. This may provide a new perspective for the treatment and prognosis of RIOM.

Declarations

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Conflicts of interest

The authors declared that there are no conflicts of interest.

Availability of data and material

The data used to support the findings of this study are available from the corresponding author upon request.

Code availability

Not applicable.

Authors' contributions

Jiantang Yang: Conceptualization, Methodology, Writing-Original draft preparation, Software; **Lili Fu:** Visualization, Investigation; **Yi Zeng:** Data curation, Writing- Reviewing and Editing; **Chen Yuan:** Supervision, Software, Validation; All of them read and approved the final manuscript.

Ethics approval

All animal experimental procedures were approved by the Animal Trials of the Affiliated Hospital of ZunYi Medical University.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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References

1. O.M. Maria, N. Eliopoulos, T. Muanza, Radiation-Induced Oral Mucositis, *Front Oncol* 7 (2017) 89.
2. S. Mallick, R. Benson, G.K. Rath, Radiation induced oral mucositis: a review of current literature on prevention and management, *Eur Arch Otorhinolaryngol* 273(9) (2016) 2285-93.
3. C.K. Lambertz, J. Gruell, V. Robenstein, V. Mueller-Funaiole, K. Cummings, V. Knapp, NO STOPS: Reducing treatment breaks during chemoradiation for head and neck cancer, *Clin J Oncol Nurs* 14(5) (2010) 585-93.
4. S.T. Sonis, Pathobiology of mucositis, *Semin Oncol Nurs* 20(1) (2004) 11-5.
5. N. Fischer, E.J. Seo, T. Efferth, Prevention from radiation damage by natural products, *Phytomedicine* 47 (2018) 192-200.
6. Y. Hou, H. Liang, E. Rao, W. Zheng, X. Huang, L. Deng, Y. Zhang, X. Yu, M. Xu, H. Mauceri, A. Arina, R.R. Weichselbaum, Y.X. Fu, Non-canonical NF-kappaB Antagonizes STING Sensor-Mediated DNA Sensing in Radiotherapy, *Immunity* 49(3) (2018) 490-503 e4.
7. J. Su, M. Wang, Y. Yan, S. Ju, J. Chen, X. Wu, Increased REDD1 facilitates neuronal damage after subarachnoid hemorrhage, *Neurochem Int* 128 (2019) 14-20.
8. Z. Liu, T. Li, F. Zhu, S. Deng, X. Li, Y. He, Regulatory roles of miR-22/Redd1-mediated mitochondrial ROS and cellular autophagy in ionizing radiation-induced BMSC injury, *Cell Death Dis* 10(3) (2019) 227.

9. X.H. Li, C.T. Ha, D. Fu, M. Xiao, REDD1 protects osteoblast cells from gamma radiation-induced premature senescence, *PLoS One* 7(5) (2012) e36604.
10. M. Foltyn, A.L. Luger, N.I. Lorenz, B. Sauer, M. Mittelbronn, P.N. Harter, J.P. Steinbach, M.W. Ronellenfitsch, The physiological mTOR complex 1 inhibitor DDIT4 mediates therapy resistance in glioblastoma, *Br J Cancer* 120(5) (2019) 481-487.
11. Y. Yin, H. Hua, M. Li, S. Liu, Q. Kong, T. Shao, J. Wang, Y. Luo, Q. Wang, T. Luo, Y. Jiang, mTORC2 promotes type I insulin-like growth factor receptor and insulin receptor activation through the tyrosine kinase activity of mTOR, *Cell Res* 26(1) (2016) 46-65.
12. L. Wang, Y. Chen, Y. Yan, X. Guo, Y. Fang, Y. Su, L. Wang, J.L. Pathak, L. Ge, miR-146a Overexpression in Oral Squamous Cell Carcinoma Potentiates Cancer Cell Migration and Invasion Possibly via Targeting HTT, *Front Oncol* 10 (2020) 585976.
13. S.H. Frost, B.W. Miller, T.A. Back, E.B. Santos, D.K. Hamlin, S.E. Knoblaugh, S.L. Frayo, A.L. Kenoyer, R. Storb, O.W. Press, D.S. Wilbur, J.M. Pagel, B.M. Sandmaier, alpha-Imaging Confirmed Efficient Targeting of CD45-Positive Cells After 211At-Radioimmunotherapy for Hematopoietic Cell Transplantation, *J Nucl Med* 56(11) (2015) 1766-73.
14. E. Bartova, J. Suchankova, S. Legartova, B. Malyskova, M. Hornacek, M. Skalnikova, M. Masata, I. Raska, S. Kozubek, PCNA is recruited to irradiated chromatin in late S-phase and is most pronounced in G2 phase of the cell cycle, *Protoplasma* 254(5) (2017) 2035-2043.
15. J.A. Pinto, C. Rolfo, L.E. Raez, A. Prado, J.M. Araujo, L. Bravo, W. Fajardo, Z.D. Morante, A. Aguilar, S.P. Neciosup, L.A. Mas, D. Bretel, J.M. Balko, H.L. Gomez, In silico evaluation of DNA Damage Inducible Transcript 4 gene (DDIT4) as prognostic biomarker in several malignancies, *Sci Rep* 7(1) (2017) 1526.
16. E. Lopez-Rivera, P. Jayaraman, F. Parikh, M.A. Davies, S. Ekmekcioglu, S. Izadmehr, D.R. Milton, J.E. Chipuk, E.A. Grimm, Y. Estrada, J. Aguirre-Ghiso, A.G. Sikora, Inducible nitric oxide synthase drives mTOR pathway activation and proliferation of human melanoma by reversible nitrosylation of TSC2, *Cancer Res* 74(4) (2014) 1067-78.
17. Z. Zheng, X. Zhao, Q. Zhao, Y. Zhang, S. Liu, Z. Liu, L. Meng, Y. Xin, X. Jiang, The Effects of Early Nutritional Intervention on Oral Mucositis and Nutritional Status of Patients With Head and Neck Cancer Treated With Radiotherapy, *Front Oncol* 10 (2020) 595632.
18. O.M. Maria, A. Syme, N. Eliopoulos, T. Muanza, Single-Dose Radiation-Induced Oral Mucositis Mouse Model, *Front Oncol* 6 (2016) 154.
19. A. Trotti, L.A. Bellm, J.B. Epstein, D. Frame, H.J. Fuchs, C.K. Gwede, E. Komaroff, L. Nalysnyk, M.D. Zilberberg, Mucositis incidence, severity and associated outcomes in patients with head and neck cancer receiving radiotherapy with or without chemotherapy: a systematic literature review, *Radiother Oncol* 66(3) (2003) 253-62.
20. Y. Li, L. Tao, Z. Zuo, Y. Zhou, X. Qian, Y. Lin, H. Jie, C. Liu, Z. Li, H. Zhang, H. Zhang, X. Cen, S. Yang, Y. Zhao, ZY0511, a novel, potent and selective LSD1 inhibitor, exhibits anticancer activity against solid tumors via the DDIT4/mTOR pathway, *Cancer Lett* 454 (2019) 179-190.

21. H.O. Jin, S.E. Hong, J.Y. Kim, M.R. Kim, Y.H. Chang, Y.J. Hong, J.K. Lee, I.C. Park, Induction of HSP27 and HSP70 by constitutive overexpression of Redd1 confers resistance of lung cancer cells to ionizing radiation, *Oncol Rep* 41(5) (2019) 3119-3126.
22. T. Kuilman, C. Michaloglou, W.J. Mooi, D.S. Peeper, The essence of senescence, *Genes Dev* 24(22) (2010) 2463-79.
23. H. Hua, Q. Kong, H. Zhang, J. Wang, T. Luo, Y. Jiang, Targeting mTOR for cancer therapy, *J Hematol Oncol* 12(1) (2019) 71.
24. D. Mossmann, S. Park, M.N. Hall, mTOR signalling and cellular metabolism are mutual determinants in cancer, *Nat Rev Cancer* 18(12) (2018) 744-757.
25. T. Suto, T. Karonitsch, The immunobiology of mTOR in autoimmunity, *J Autoimmun* 110 (2020) 102373.
26. B. Farhood, M. Ashrafizadeh, E. Khodamoradi, M. Hoseini-Ghahfarokhi, S. Afrashi, A.E. Musa, M. Najafi, Targeting of cellular redox metabolism for mitigation of radiation injury, *Life Sci* 250 (2020) 117570.
27. J. Chen, C.F. Hu, J.H. Hou, Q. Shao, L.X. Yan, X.F. Zhu, Y.X. Zeng, J.Y. Shao, Epstein-Barr virus encoded latent membrane protein 1 regulates mTOR signaling pathway genes which predict poor prognosis of nasopharyngeal carcinoma, *J Transl Med* 8 (2010) 30.
28. M. Diaz-Martinez, L. Benito-Jardon, L. Alonso, L. Koetz-Ploch, E. Hernando, J. Teixido, miR-204-5p and miR-211-5p Contribute to BRAF Inhibitor Resistance in Melanoma, *Cancer Res* 78(4) (2018) 1017-1030.
29. M.Q. Cao, A.B. You, X.D. Zhu, W. Zhang, Y.Y. Zhang, S.Z. Zhang, K.W. Zhang, H. Cai, W.K. Shi, X.L. Li, K.S. Li, D.M. Gao, D.N. Ma, B.G. Ye, C.H. Wang, C.D. Qin, H.C. Sun, T. Zhang, Z.Y. Tang, miR-182-5p promotes hepatocellular carcinoma progression by repressing FOXO3a, *J Hematol Oncol* 11(1) (2018) 12.
30. M. Correia de Sousa, M. Gjorgjieva, D. Dolicka, C. Sobolewski, M. Foti, Deciphering miRNAs' Action through miRNA Editing, *Int J Mol Sci* 20(24) (2019).
31. L.L. Sun, J. Wang, Z.J. Zhao, N. Liu, A.L. Wang, H.Y. Ren, F. Yang, K.X. Diao, W.N. Fu, E.H. Wan, X.Y. Mi, Suppressive role of miR-502-5p in breast cancer via downregulation of TRAF2, *Oncol Rep* 31(5) (2014) 2085-92.
32. J. Tao, M. Fan, D. Zhou, Y. Hong, J. Zhang, H. Liu, S. Sharma, G. Wang, Q. Dong, miR-200c Modulates the Pathogenesis of Radiation-Induced Oral Mucositis, *Oxid Med Cell Longev* 2019 (2019) 2352079.
33. M. Pu, J. Chen, Z. Tao, L. Miao, X. Qi, Y. Wang, J. Ren, Regulatory network of miRNA on its target: coordination between transcriptional and post-transcriptional regulation of gene expression, *Cell Mol Life Sci* 76(3) (2019) 441-451.
34. Y.X. Yong, H. Yang, J. Lian, X.W. Xu, K. Han, M.Y. Hu, H.C. Wang, L.M. Zhou, Up-regulated microRNA-199b-3p represses the apoptosis of cerebral microvascular endothelial cells in ischemic stroke through down-regulation of MAPK/ERK/EGR1 axis, *Cell Cycle* 18(16) (2019) 1868-1881.

Figures

Figure 1

DDIT4 is down-regulated in vitro model of RIOM and inhibited the formation of RIOM

(A) The protein level of DDIT4 in the Non-irradiated and 10Gy*3 group. (B) HE staining analysis of the influence of up-regulated DDIT4 on the tongue tissues of Non-irradiated and 10Gy*3 group. (C) The effect

of up-regulated DDIT4 on the area of tongue ulcer in the Non-irradiation and 10Gy*3 groups. (D) IHC staining of CD45 or PCNA of tongue tissues from up-regulated DDIT4 at Non-irradiated or 10Gy*3 group. *** $P \leq 0.001$ vs. Non-irradiated group.

Figure 2

DDIT4 modulates proliferation in NHKs exposed to irradiation

(A) qRT-PCR, western blot and immunofluorescence analyzed the expression of DDIT4 in NHKs exposed to irradiation. (B-C) EdU method and colony formation assays evaluated the effect of DDIT4 on the proliferation of NHKs , *** $P \leq 0.001$ vs. Non-irradiated group.

Figure 3

DDIT4 suppressed the formation of RIOM by regulating the mTOR pathway

(A) The expression levels of mTOR and its downstream proteins in RIOM mouse model were assessed by western blot assay, $***P < 0.001$ vs. Non-irradiated group. (B) The levels of mTOR and its downstream proteins in RIOM mouse model with up-regulated DDIT4 were assessed by western blot assay, $**P < 0.01$, $***P < 0.001$ vs. vector group. (C) The levels of mTOR and its downstream proteins in RIOM cell model with up-regulated DDIT4 or down-regulated mTOR pathway were assessed by western blot assay. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control group.

Figure 4

DDIT4 is a direct target of miR-199b-3p in RIOM

(A) Three online databases Targetscan, miRDB, and miRWalk were performed to predict intersecting miRNAs. (B) qRT-PCR assay was used to detect intersecting miRNAs expression. (C) The potential binding sites of miR-199b-3p with 3'UTR of DDIT4. (D) Luciferase reporter assay was carried out to prove the relationship between miR-199b-3p and DDIT4. (E) Western blot analysis the expression of DDIT4, *** $P < 0.0001$ vs. miR-199b-3p mimic, ### $P < 0.0001$ vs. miR-199b-3p inhibitor.

Figure 5

MiR-199a accelerated RIOM through targeting DDIT4 in vitro

(A-B) EdU method and colony formation assays evaluated the effect of miR-199b-3p on the proliferation of NHKs. (C) The expression levels of mTOR and its downstream proteins detected by western blot assay in RIOM cell model, *** $P < 0.001$ vs. miR-199b-3p mimic, ### $P < 0.0001$ vs. miR-199b-3p inhibitor.

Figure 6

MiR-199a accelerated RIOM through targeting DDIT4 in vivo

(A) HE staining analysis of the effects of miR-199b-3p on the tongue tissues of non-irradiated and 10Gy*3 group. (B-C) The effect of miR-199b-3p on the area of tongue ulcer in the non-irradiation and 10Gy*3 groups. (D) The expression levels of mTOR and its downstream proteins in RIOM mouse model by western blot assay. *** $P \leq 0.001$ vs. miR-199b-3p inhibitor.

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