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Original Article

TP53 induced glycolysis and apoptosis regulator knockdown radiosensitizes glioma cells through oxidative stress-induced excessive autophagy

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Abstract: *TP53* induced glycolysis and apoptosis regulator (TIGAR) knockdown has been suggested to be a feasible solution to radiosensitize glioma cells. The mechanisms inside it were not fully understood. In this study, human malignant glioma cells, A172 and T98G, were treated with TIGAR siRNA and the relationship between TIGAR interfering-induced radiosensitization and autophagy promotion in irradiated glioma cells was investigated. In order to explore whether TIGAR knockdown-promoted autophagy was protective or not, glioma cells were treated with 3-methyladenine. It was indicated that inhibition of autophagy diminished the radiosensitizing effect of TIGAR knockdown. NADPH content was determined to demonstrate the mechanism of TIGAR interfering-induced autophagy in irradiated glioma cells. It was revealed that N-Acetylcysteine treatment abrogated TIGAR knockdown-induced NADPH depletion and inhibited the autophagy activity, while buthionine sulfoximine further increased the autophagy flux in cells suffered by TIGAR interfering and irradiation. In conclusion, our data revealed an excessive autophagy was induced by TIGAR knockdown in irradiated glioma cells, which was dependent on NADPH depletion.

Keywords: TIGAR, NADPH, autophagy, glioma, radiosensitization

Introduction

Gliomas represent 80% of primary malignant brain tumors, and are mainly categorized into four grades: grade I, grade II, grade III and grade IV (glioblastoma) [1]. Glioblastoma is the most devastating malignant form of primary brain tumors and is extremely difficult to surgically eradicate. Although radiation therapy has benefited a lot of glioma patients, most glioblastomas are highly radioresistant and the efficacies are still far from satisfactory [2]. As a result, it is critical to develop novel therapeutic strategies for this disease.

Autophagy or "self-eating" digests proteins and functionless cell organelles to reuse [3]. One of the major functions of autophagy is maintaining cellular homeostasis [4]. That means damaged or dispensable proteins and organelles are removed to make cell survival. On the other side, if autophagy is over-activated, it may cause much more cell apoptosis or cell death [5]. Autophagy has recently emerged as an essential mechanism in irradiated human glioma cells to orchestrate cellular response to ionizing radiation (IR)-induced DNA damage and oxidative stress [6].

TP53-induced glycolysis and apoptosis regulator (TIGAR) is a p53-target gene structured and functioning as fructose-2, 6-bisphosphatase (FBPase-2), which inhibits glycolysis and activates the pentose phosphate pathway (PPP) [7, 8]. In vitro analysis showed that TIGAR could be upregulated under oxidative stress induced by chemo-oxidants [9], nutrient starvation or metabolic stress [10], and radiotherapy [11, 12].

TIGAR knockdown was proved to radiosensitize both A172 (p53 wild-type) and T98G (p53 mutant) glioma cells via inhibition of thioredoxin-1 (TRX1) nuclear translocation and abrogation of DNA damage repair process [12]. The central mechanism of TIGAR knockdown-induced radiosensitization of glioma cells might be NADPH depletion-induced ROS accumulation.

TIGAR interfering also might up-regulate the autophagy activity in cells with nutrient starvation [10]. It was reported that either nutrient starvation or metabolic stress could result in a strong activation of autophagy, which was significantly increased by TIGAR interfering and reduced in TIGAR over-expressed cells.

Since TIGAR knockdown could significantly activate autophagy in cells suffered by oxidative stress, we hypothesized that TIGAR interfering-induced reduction of radioresistance in glioma cells may be correlated with autophagy promotion. In this study, we demonstrated the relationship between the radiosensitization and promotion of autophagy activity in glioma cells suffered by TIGAR interfering. Moreover, suppression of TIGAR knockdown-promoted autophagy by pharmacological approach could diminish the radiosensitivity of glioma cells, which indicated that the autophagy induced by both TIGAR knockdown and IR treatment was traumatic.

Materials and methods

Cell culture and radiation conditions

Human glioma cell line A172 and T98G were cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS). Cells were irradiated with an accelerator for X-ray therapy at a dose rate of 1.2 Gray (Gy)/min.

TIGAR knockdown

Small-interfering RNA (siRNA) sequence (5'-GCAGCAGCTGCTGGTATAT-3') was synthesized as an antisense, and a scramble sequence (5'-TTACCGAGACCGTACGTAT-3') was synthesized as a negative control. siRNAs were transfected using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA).

Vector construction, transfection and TIGAR overexpression

TIGAR was cloned using the following primers: 5'-GCAGGTACCATGGCTCGCTTCGCTCTG-3' (sense) and 5'-GCACTCGAGTTAGCGAGTTTCAGTCAGTCC-3' (antisense), then subcloned into pcDNA3.1 vector (Invitrogen) to create pcDNA3.1-*TIGAR*. The recombination plasmid was transfected using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). And the transfection efficiency was determined by Western blot assay 48 h post-transfection.

Clonogenic survival assay

Twenty-four hours after transfection, cells were plated in triplicate into six-well plates. Plates were irradiated 24 hours after plating. Fourteen days after IR, cells were fixed and stained with Giemsa. Colonies consisting of more than 50 cells were counted as a single colony.

Western blot analysis

The cells were harvested and lysed on ice. Then, the cell lysates were centrifuged at 12,000 rpm for 15 min. Identical amounts of protein (60 μg) from each sample were loaded and run on 12% SDS-PAGE gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA) by Semi-Dry Electrophoretic Transfer (Bio-Rad, US). After membrane blocking with 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) at room temperature for 1 h, the membranes were incubated with specific primary antibodies at 4°C overnight.

Fluorescent LC3 plasmid transfection

A172 glioma cells were transfected with a pEX-GFP-LC3 expressing plasmid (GenePharma, Shanghai, China) by Lipofectamine 2000 Reagent. The formation of green fluorescence protein (GFP)-LC3 punctate structures was examined using a Leica DM IL LED microscope (Leica Microsystems, Wetzlar, Germany).

NADPH analysis

NADPH content was detected by NADP/NADPH quantitation kit (Biovision, Milpitas, CA) according to the manufacture's introduction.

Statistical analysis

Results are expressed as means ± standard error in independent experiments. Differences among samples were analyzed with the one-way ANOVA. A p value less than 0.05 were considered statistical significant.

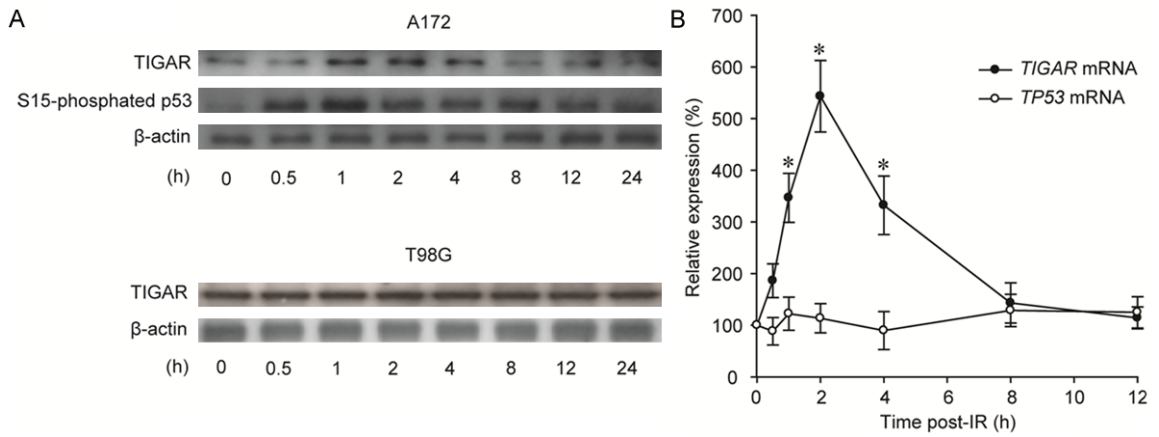

Figure 1. Ionizing radiation results in TIGAR activity in glioma cells. A: A172 and T98G cells were irradiated by 8 Gy IR and IR-induced TIGAR and (Ser-15) phosphorylated p53 expression was examined by Western blot. B: A172 glioma cells were treated with 8 Gy IR, and the mRNA levels of *TIGAR* and *p53* were determined by real-time PCR.

Results

TIGAR expression is up-regulated by ionizing radiation

As shown in **Figure 1A**, both TIGAR and (Ser-15) phosphorylated p53 were increased in irradiated A172 cells. TIGAR expression was enhanced in 1-4 h post-IR while phosphorylated p53 was up-regulated earlier than TIGAR activation in 0.5 h post-IR. However, in mutant-p53 (M237I) expressing T98G glioma cells, there was no IR-induced TIGAR expression in 0.5-48 h post-IR. Real-time PCR analysis showed that, in A172 cells, TIGAR mRNA level went up in 1-4 h post-IR, which indicated an increased transcriptional activity of TIGAR (**Figure 1B**). While no up-regulation of p53 mRNA was observed in irradiated A172 glioma cells.

TIGAR interfering diminishes the radioresistence of glioma cells and promotes IR-induced autophagy

In order to determine whether the up-regulation of TIGAR transcription was benefit for irradiated glioma cells or not, A172 glioma cells were suffered by TIGAR siRNA 48 h before IR. Western blot assay revealed a significant inhibition of TIGAR expression in TIGAR siRNA transfected glioma cells (**Figure 2A**). Clonogenic assay illustrated that the survival fraction of A172 cells was notably decreased by TIGAR abrogation at 2-8 Gy X-ray irradiation (**Figure 2A**). The TIGAR-abrogation effect on the radiosensitivity of T98G cells showed the same pattern as that of A172 cells.

Autophagy is considered to play an important role in the response of radiation therapy. To examine whether autophagy was induced by TIGAR inhibition and/or IR treatment in A172 glioma cells, western blot analysis was performed to show the conversion of LC3-I (18 kDa) to LC3-II (16 kDa). As shown in **Figure 2B**, LC3-II expression in A172 cells was up-regulated by IR exposure alone. Meanwhile, p62 expression was decreased in 12-24 h post 8-Gy irradiation and went up to the basal level in 48 h post-IR, which also revealed an IR-induced autophagy in A172 glioma cells. TIGAR knockdown further enhanced the ratio of LC3-II to LC3-I in 12- 24 h post-IR (**Figure 2C**). Contrarily, TIGAR over-expression diminished the LC3-II level in A172 cells being irradiated (**Figure 2C**), and the expression of p62 was significantly up-regulated by TIGAR transfection. Fluorescent assay using LC3 plasmid transfection illustrated that, the percentage of GFP-LC3-positive cells displaying GFP puncta which indicated the autophagy activity was increased by IR treatment. TIGAR interfering further increased the GFP puncta while TIGAR over-expression decreased it (**Figure 2D**).

TIGAR abrogation-induced radiosensitization of glioma cells is related with the traumatic autophagy

In order to examine the relationship between TIGAR interfering-promoted radiosensitization

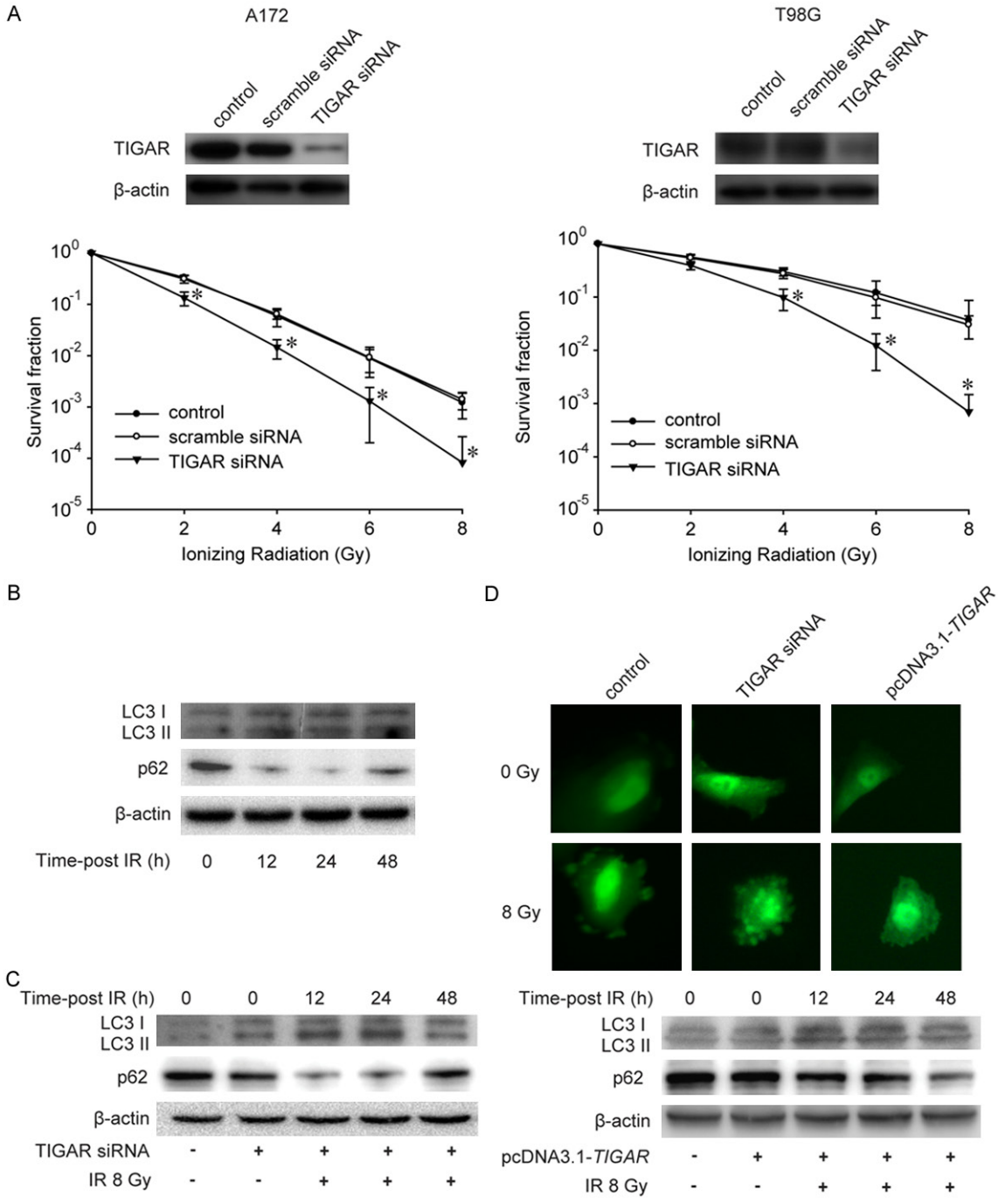

Figure 2. TIGAR interfering radiosensitizes glioma cells and promotes IR-induced cell autophagy. A: Cells were transfected with TIGAR siRNA for 48 h and TIGAR expression was examined by Western blot. Clonogenic survival of cells transfected with scramble siRNA and TIGAR siRNAs 48 h before IR were investigated after a range of radiation doses. *$P < 0.05$, scramble siRNA vs TIGAR siRNA. B: A172 cells were exposed to 8 Gy IR, and then expressions of p62 and LC3 at indicated time post IR were determined by Western blot. C: Cells were transfected with TIGAR siRNA (left panel) or pcDNA3.1-*TIGAR* (right panel) 48 h before IR. Western blot was performed to indicate p62 and LC3 protein levels in response to 8 Gy IR. D: A172 cells are transfected with GFP-LC3 plasmid, and then treated with 8 Gy of X-ray and/or TIGAR siRNA or pcDNA3.1-*TIGAR*. Representative photos show the formation of punctate LC3.

and autophagy in irradiated glioma cells, A172 or T98G cells were co-treated with IR and 3-methyladenine (3-MA) which could block autophagosome formation via the inhibition of

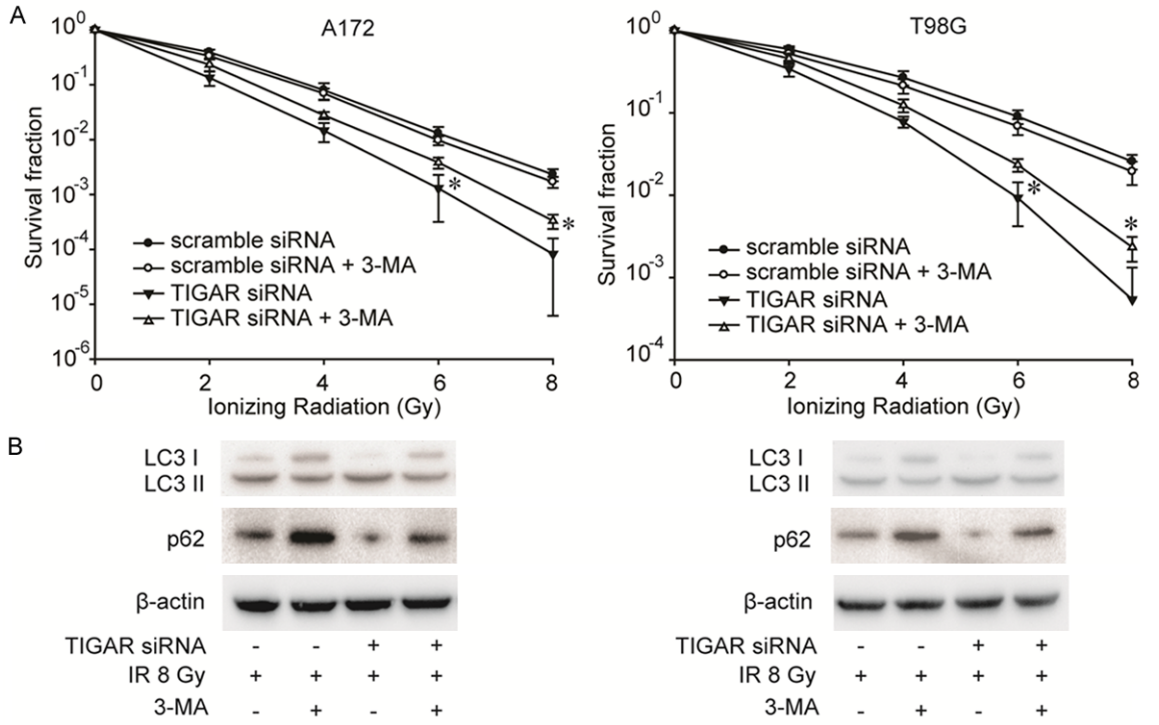

Figure 3. TIGAR abrogation induces excessive autophagy in irradiated glioma cells. A: A172 and T98G glioma cells were transfected with scramble or TIGAR siRNA 48 h before IR. Then cells were exposed to a range of radiation doses as indicated, with or without 3-MA (10 mM) co-treatment for 24 h, and clonogenic assay was performed. *P < 0.05. B: A172 (left panel) and T98G (right panel) glioma cells treated with TIGAR siRNA or 3-MA (10 mM) were irradiated by 8-Gy X-ray. Expressions of p62 and LC3 were detected by Western blot 24 h post-IR.

phosphatidylinositol 3-kinases (PI3K) [13]. Clonogenic assay revealed that 3-MA could significantly diminish the radiosensitization effect of TIGAR knockdown in both A172 and T98G glioma cells (**Figure 3A**). Western blot assay showed that both IR-induced autophagy and TIGAR-promoted autophagy in irradiated glioma cells were notably blocked by 3-MA treatment (**Figure 3B**). These results above indicated that when glioma cells were suffered from TIGAR abrogation, IR-induced autophagy seemed to be unfavorable for cloning forming efficiency, which indicated an excessive and traumatic autophagy induced by TIGAR interfering in irradiated glioma cells.

TIGAR knockdown-induced excessive autophagy was dependent on the disruption of the pro-oxidant-antioxidant balance

TIGAR functions to inhibit glycolysis and activates the pentose phosphate pathway (PPP) which increases NADPH generation under oxidative stress. And TIGAR knockdown was proved to aggravate IR-induced oxidative stress in glioma cells [12]. In this study, the NADPH content in A172 glioma cells treated with IR and/or TIGAR abrogation was determined. As shown in **Figure 4A**, radiation treatment could decrease the NADPH content in A172 glioma cells to about 70% of the original level. TIGAR knockdown further aggravated the oxidative stress of the irradiated A172 cells. While the NADPH content was further abrogated by BSO (right panel) and rescued by NAC (left panel) co-incubation. Western blot revealed that the autophagy activity was negatively correlated with the NADPH level in A172 glioma cells being irradiated. NAC treatment could inhibit the autophagy activity of glioma cells suffered by TIGAR interfering and IR (**Figure 4B**), while BSO further promoted autophagy in A172 cells.

Discussion

Our previous study has demonstrated the main mechanisms of TIGAR abrogation-induced radiosensitivity of glioma cells. TIGAR silencing disturbed the pro-oxidant-antioxidant balance and depleted the NADPH content in irradiated

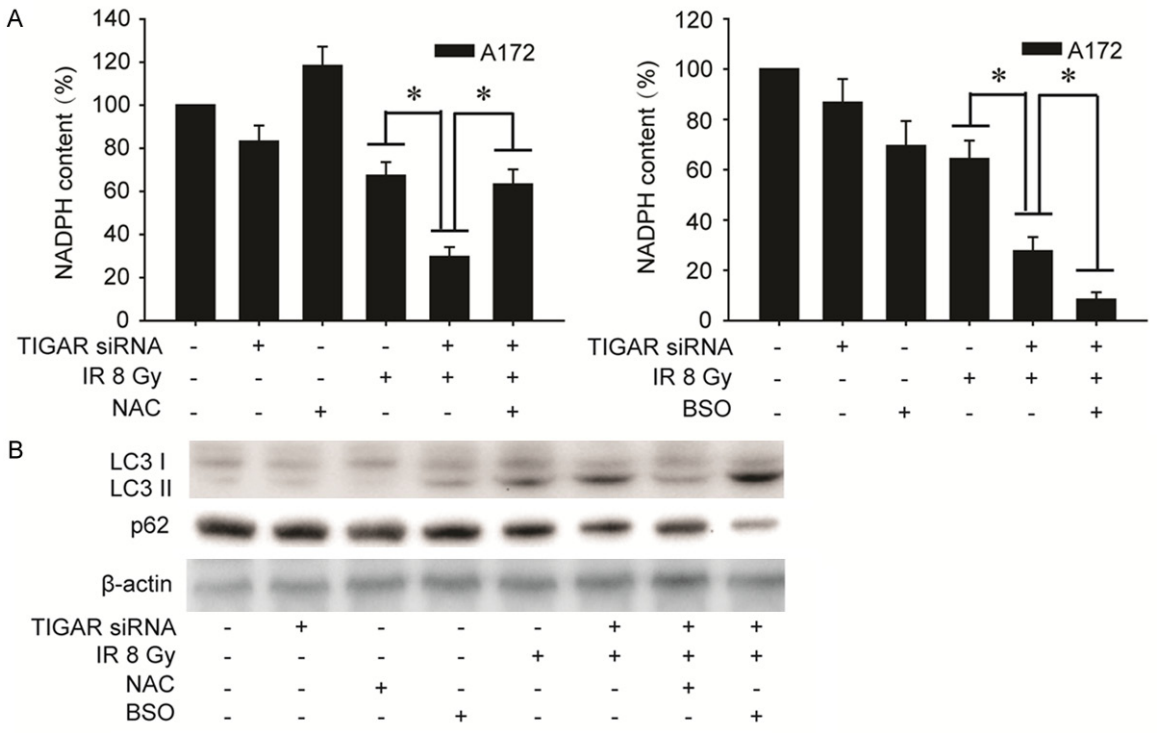

Figure 4. TIGAR knockdown-induced excessive autophagy in irradiated glioma cells is dependent on NADPH depletion. Glioma cells were transfected with TIGAR siRNA 48 h before IR. Then cells were exposed to 8 Gy irradiation and NAC (left) or BSO (right) treatment for 1 h. A: Cellular NADPH production was measured 1 h post-IR. *$P < 0.05$. B: The expressions of LC3 and p62 protein were determined by Western blot 24 h post IR.

glioma cells, which made thioredoxin-1 (TRX1) over-oxidized and IR-induced TRX1 nuclear translocation inhibited. Since reduced TRX1 played an important role in nuclear signal transduction for DNA damage repair, the inhibition of TRX1 nuclear translocation significantly postponed the DNA repair process and radiosensitized glioma cells [12].

In this study, IR-induced TIGAR over-expression was determined by Western blot in A172 glioma cells. Ser-15 phosphorylated p53 expression was also observed, which indicated an inhibition of p53 degradation and activation of p53 by ATM/ATR/DNA-PK mediated DNA damage signal transduction [14, 15]. Real-time PCR analysis revealed that TIGAR mRNA was up-regulated in 1-4 h post-IR, indicating an increase of transcriptional activity of TIGAR. The results above demonstrated that, the transcriptional factor p53 was activated by ionizing radiation, which increased the transcriptional activity of *TIGAR* thus enhanced TIGAR expression in A172 glioma cells. However, in p53-mutant (M237I) T98G glioma cells, there was no increase in TIGAR expression post-IR. Clonogenic assay revealed that, TIGAR interfering could diminish the radioresistence of both A172 and T98G cells, indicating even the basal level of TIGAR was essential for cell survival in glioma cells being irradiated.

The p62 protein recognizes cellular waste and functionless cell organelles, which is then scavenged by a sequestration process known as self-eating or autophagy [16]. During the autophagy process, LC3 system is important for transport and maturation of the autophagosome [17]. Once an autophagosome has matured, it fuses its external membrane with lysosomes to degrade the waste inside it [18]. In this study, both p62 and the ratio of LC3 II/LC3 I were determined by Western blot. Ionizing radiation enhanced the LC3 II/LC3 I ration while decreased p62 expression in 12-24 h post-IR, indicating an increase of autophagy in A172 glioma cells. TIGAR knockdown further promoted the autophagy activity of irradiated A172 cells, while TIGAR over-expression significantly inhibited IR-induced autophagy.

In order to discover whether TIGAR knockdown and IR induced autophagy in glioma cells was traumatic or not, a common specific inhibitor of autophagic/lysosomal degradation called 3-methyladenine (3-MA) was used to block autophagy. Expression of both LC3 and p62 revealed an inhibition of autophagy in glioma cells treated with 3-MA. Since 3-MA could only diminish the radiosensitivity of glioma cells treatment with TIGAR abrogation, it was concluded that TIGAR knockdown induced excessive autophagy which made more cell death.

Finally, to demonstrate the mechanism of TIGAR interfering-induced excessive autophagy in irradiated glioma cells, N-Acetyl Cysteine (NAC) was used to abrogate TIGAR knockdown-induced NADPH depletion in irradiated glioma cells. Buthionine sulfoximine (BSO) which reduced levels of glutathione was used to further disturb the pro-oxidant-antioxidant balance of irradiated glioma cells. Surprisingly, the autophagy level indicated by the expression of p62 and LC3 was negatively correlated with the NADPH level whenever TIGAR was abrogated or not.

As a result, it could be concluded that TIGAR interfering-induced excessive autophagy in irradiated glioma cells might be dependent on the NADPH depletion and disturbance of the pro-oxidant-antioxidant balance.

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Disclosure of conflict of interest

None.

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References

[1] Thomas AA, Brennan CW, DeAngelis LM, Omuro AM. Emerging therapies for glioblastoma. JAMA Neurol 2014; 71: 1437-1444.

[2] Van Meir EG, Hadjipanayis CG, Norden AD, Shu HK, Wen PY, Olson JJ. Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma. CA Cancer J Clin 2010; 60: 166-193.

[3] Kuo SY, Castoreno AB, Aldrich LN, Lassen KG, Goel G, Dančík V, Kuballa P, Latorre I, Conway KL, Sarkar S, Maetzel D, Jaenisch R, Clemons PA, Schreiber SL, Shamji AF, Xavier RJ. Small-molecule enhancers of autophagy modulate cellular disease phenotypes suggested by human genetics. Proc Natl Acad Sci U S A 2015; 112: E4281-7.

[4] Perera RM, Stoykova S, Nicolay BN, Ross KN, Fitamant J, Boukhali M, Lengrand J, Deshpande V, Selig MK, Ferrone CR, Settleman J, Stephanopoulos G, Dyson NJ, Zoncu R, Ramaswamy S, Haas W, Bardeesy N. Transcriptional control of autophagy-lysosome function drives pancreatic cancer metabolism. Nature 2015; 524: 361-5.

[5] Kimura T, Takabatake Y, Takahashi A, Isaka Y. Chloroquine in cancer therapy: a double-edged sword of autophagy. Cancer Res 2013; 73: 3-7.

[6] Palumbo S, Comincini S. Autophagy and ionizing radiation in tumors: the "survive or not survive" dilemma. J Cell Physiol 2013; 228: 1-8.

[7] Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, Gottlieb E, Vousden KH. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell 2006; 126: 107-120.

[8] Li H, Jogl G. Structural and biochemical studies of TIGAR (TP53-induced glycolysis and apoptosis regulator). J Biol Chem 2009; 284: 1748-1754.

[9] Lui VW, Wong EY, Ho K, Ng PK, Lau CP, Tsui SK, Tsang CM, Tsao SW, Cheng SH, Ng MH, Ng YK, Lam EK, Hong B, Lo KW, Mok TS, Chan AT, Mills GB. Inhibition of c-Met downregulates TIGAR expression and reduces NADPH production leading to cell death. Oncogene 2011; 30: 1127-1134.

[10] Bensaad K, Cheung EC, Vousden KH. Modulation of intracellular ROS levels by TIGAR controls autophagy. EMBO J 2009; 28: 3015-3026.

[11] Pena-Rico MA, Calvo-Vidal MN, Villalonga-Planells R, Martinez-Soler F, Gimenez-Bonafe P, Navarro-Sabaté À, Tortosa A, Bartrons R, Manzano A. TP53 induced glycolysis and apoptosis regulator (TIGAR) knockdown results in radiosensitization of glioma cells. Radiother Oncol 2011; 101: 132-139.

[12] Zhang H, Gu C, Yu J, Wang Z, Yuan X, Yang L, Wang J, Jia Y, Liu J, Liu F. Radiosensitization of glioma cells by TP53-induced glycolysis and apoptosis regulator knockdown is dependent on thioredoxin-1 nuclear translocation. Free Radic Biol Med 2014; 69: 239-248.

[13] Artero-Castro A, Perez-Alea M, Feliciano A, Leal JA, Genestar M, Castellvi J, Peg V, Ramón Y Cajal S, Lleonart ME. Disruption of the ribosomal P complex leads to stress-induced autophagy. Autophagy 2015; 11: 1499-519.

[14] Tibbetts RS, Brumbaugh KM, Williams JM, Sarkaria JN, Cliby WA, Shieh SY, Taya Y, Prives C, Abraham RT. A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev 1999; 13: 152-157.

[15] Ohtsuka T, Jensen MR, Kim HG, Kim KT, Lee SW. The negative role of cyclin G in ATM-dependent p53 activation. Oncogene 2004; 23: 5405-5408.

[16] Komatsu M, Waguri S, Koike M, Sou YS, Ueno T, Hara T, Mizushima N, Iwata J, Ezaki J, Murata S, Hamazaki J, Nishito Y, Iemura S, Natsume T, Yanagawa T, Uwayama J, Warabi E, Yoshida H, Ishii T, Kobayashi A, Yamamoto M, Yue Z, Uchiyama Y, Kominami E, Tanaka K. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell 2007; 131: 1149-1163.

[17] Tanida I, Ueno T, Kominami E. LC3 conjugation system in mammalian autophagy. Int J Biochem Cell Biol 2004; 36: 2503-2518.

[18] Pattingre S, Espert L, Biard-Piechaczyk M, Codogno P. Regulation of macroautophagy by mTOR and Beclin 1 complexes. Biochimie 2008; 90: 313-323.