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ORIGINAL ARTICLE

TRIAP1 knockdown sensitizes non-small cell lung cancer to ionizing radiation by disrupting redox homeostasis

Chun-cheng Hao[1], Jia-ning Luo[1], Cui-yang Xu[1], Xin-yu Zhao[1], Zhen-bin Zhong[1], Xiao-nan Hu[1], Xiao-ming Jin[2] & Xiaofeng Ge[1]

1 Department of Radiotherapy, Harbin Medical University Cancer Hospital, Harbin City, China
2 Department of Pathology, Harbin Medical University, Harbin City, China

Keywords
Non-small cell lung cancer; radioresistance; redox homeostasis; TRIAP1.

Correspondence
Xiao-ming Jin, Department of Pathology, Harbin Medical University, No. 157 Health Road, Nangang District, Harbin City, Heilongjiang 150086, China.
Tel: +86-13199461038
Email: jinxm623@163.com

Xiao-feng Ge, Department of Radiotherapy, Harbin Medical University Cancer Hospital, No. 150 Haping Road, Nangang District, Harbin City, Heilongjiang 150040, China.
Tel: +86-13199461038
Email: gexiaofeng121@163.com

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Abstract

Background: Radioresistance of some non-small cell lung cancer (NSCLC) types increases the risk of recurrence or metastasis in afflicted patients, following radiotherapy. As such, further improvements to NSCLC radiotherapy are needed. The expression of oncogene TP53-regulated inhibitor of apoptosis 1 (TRIAP1) in NSCLC is increased following irradiation. Furthermore, gene set enrichment analysis (GSEA) has suggested that TRIAP1 might be involved in maintaining redox homeostasis. This in turn might enhance cell radioresistance.

Methods: In this study we irradiated human NSCLC cell lines (A549 and H460), while knocking down TRIAP1, to determine whether a disrupted redox homeostasis could attenuate radioresistance.

Results: Irradiation notably increased both mRNA and protein levels of TRIAP1. In addition, TRIAP1 knockdown decreased the expression of several antioxidant proteins, including thioredoxin-related transmembrane protein (TMX) 1, TMX2, thioredoxin (TXN), glutaredoxin (GLRX) 2, GLRX3, peroxiredoxin (PRDX) 3, PRDX4, and PRDX6 in A549 and H460 cells. In addition, silencing TRIAP1 impaired the radiation-induced increase of the aforementioned proteins. Continuing along this line, we observed a radiation-induced reduction of cell viability and invasion, as well as increased apoptosis and intracellular reactive oxygen species following TRIAP1 knockdown.

Conclusions: In summary, we identified TRIAP1 as a key contributor to the radioresistance of NSCLC by maintaining redox homeostasis.

Introduction

Lung cancer remains the leading cause of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC) is the most common type of this malignancy, accounting for approximately 85% of all lung cancer cases.[1] NSCLC has a very poor prognosis, with five-year survival rates below 20%. Radiotherapy is one of the major therapeutic strategies to counter early stage and locally advanced NSCLC.[2] Radiation exposure generates reactive oxygen species (ROS) via the radiolysis of water, which can in turn cause damage to biological macromolecules such as DNA. Such genetic damage has been associated with the activation of cell cycle checkpoints, which may in turn delay cellular growth and could potentially activate apoptotic pathways, thus leading to cell death. However, the response of NSCLC to radiation treatment is variable. Some NSCLC cells show intrinsic radioresistance, which can lead to recurrence or metastasis in lung cancer patients, following radiotherapy.[3] This phenomenon is a challenge for the further improvement of NSCLC radiotherapy.

TP53-regulated inhibitor of apoptosis 1 (TRIAP1) is an evolutionarily conserved protein, mainly regulated by TP53. Whereas TP53 is a well-known tumor suppressor, TRIAP1 functions as an oncogene because it mechanistically inhibits the interaction of cytochrome c with the apoptotic protease activating factor 1, thus preventing cancer cell apoptosis.[4,5] A previous transcriptomics study found that radiation significantly increased TRIAP1 expression in

A549 cells.[6] Furthermore, gene set enrichment analysis (GSEA) in this study highlighted an implication of TRIAP1 in the regulation of redox homeostasis, which implies that TRIAP1 might be linked to the radioresistance of NSCLC cells by maintaining redox homeostasis. Our study explored the role of TRIAP1 in the radiosensitivity of NSCLC cells and the underlying mechanisms.

Methods

Cell culture

We used human NSCLC cell lines A549 and H460, derived respectively from lung carcinoma and large-cell lung cancer (American Type Culture Collection, Rockville, Maryland, US). We cultured the cells in RPMI 1640 medium (Invitrogen, Carlsbad, California, USA), supplemented with 10% fetal bovine serum (Gibco, CA, USA), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco) in a 5% CO_2 humidified incubator at 37°C.

Ionizing radiation (IR) exposure

We X-irradiated A549 and H460 cells using a linear accelerator (Primus K, Siemens, Munich, Bayern, Germany) with 6 MV photons/100 cm focus-surface distance (2.0 or 4.0 Gy/minute). Cell viability, apoptosis, and other assays were performed 48 hours after irradiation.

Short hairpin RNA transfection

TRIAP1-targeting short hairpin RNA (shRNA) and negative control shRNA were purchased from GenePharma (Shanghai, China). We cultured A549 and H460 cells in 12-well plates until confluence was approximately 80%. Next, we performed cell transfection with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after transfection, we evaluated the expression of TRIAP1 in A549 and H460 cells using quantitative real-time polymerase chain reaction (PCR) and western blot. The cells with the TRIAP1 knockdown were in turn irradiated or kept as control.

PCR analysis

We extracted RNA from cells using TRIzol (Invitrogen), followed by reverse transcription using the SuperScript First-Strand Synthesis System (Invitrogen). PCR conditions were as follows: 95°C for five minutes, followed by 38 cycles of 95°C for 15 seconds and 60°C for 40 seconds. The primer sequences that were used in this assay are listed in Table 1. We determined the relative mRNA levels using the $2^{-\Delta\Delta Ct}$ method with normalization against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, internal control).

Western blot assay

Cell lysates were prepared using ice-cold radioimmunoprecipitation assay buffer (Invitrogen) and we subsequently measured the protein concentrations using a

Table 1 The primer sequences for PCR

Gene name	Primer orientation	Sequences	Temperature	Amplification size
TRIAP1	Forward	5′-AGGATTTCGCAAGTCCAGAA-3′	60	132
	Reverse	5′-GCTGATTCCACCCAAGTAT-3′		
TMX1	Forward	5′-TTGCGAAAGTAGATGTCACAGAG-3′	60	107
	Reverse	5′-CTGATAGCGCCTAAATTCACCAT-3′		
TMX2	Forward	5′-GTCTTGGCACCTCTAATTGCT-3′	60	79
	Reverse	5′-ACAGAAGGTAGTAAGGTTGGGC-3′		
TXN	Forward	5′-GTGAAGCAGATCGAGAGCAAG-3′	60	87
	Reverse	5′-CGTGGCTGAGAAGTCAACTACTA-3′		
GLRX2	Forward	5′-TCTTTGGAGAATTTAGCGACGG-3′	60	180
	Reverse	5′-CTGGTTTCCATATTCAAGCAGGT-3′		
GLRX3	Forward	5′-GTCCCTCCTTGTGGTCCATTT-3′	60	75
	Reverse	5′-AACTCTGCCATAACTTCGTTCAT-3′		
PRDX3	Forward	5′-ACAGCCGTTGTCAATGGAGAG-3′	60	152
	Reverse	5′-ACGTCGTGAAATTCGTTAGCTT-3′		
PRDX4	Forward	5′-AGAGGAGTGCCACTTCTACG-3′	60	103
	Reverse	5′-GGAAATCTTCGCTTTGCTTAGGT-3′		
PRDX6	Forward	5′-GACTCATGGGGCATTCTCTTC-3′	60	241
	Reverse	5′-CAAGCTCCCGATTCCTATCATC-3′		
GAPDH	Forward	5′-GGAGCGAGATCCCTCCAAAAT-3′	60	197
	Reverse	5′-GGCTGTTGTCATACTTCTCATGG-3′		

protein assay kit (Beyotime biotechnology company, Nanjing, China). Next, we separated the protein samples by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by a transfer onto nitrocellulose membranes. To detect our proteins of interest we incubated the membranes (4°C, overnight) with the following primary antibodies: anti-TRIAP1 antibody (1:500, Abcam, Cambridge, Massachusetts, UK), anti-thioredoxin-related transmembrane protein 1 (TMX1) antibody (1:500, Abcam), anti-TMX2 antibody (1:500, Abcam), anti-thioredoxin (TXN) antibody (1:500, Abcam), anti-glutaredoxin (GLRX) 2 antibody (1:500, Abcam), anti-GLRX3 antibody (1:500, Abcam), anti-peroxiredoxin (PRDX) 3 antibody (1:500, Abcam), anti-PRDX4 antibody (1:500, Abcam), anti-PRDX6 antibody (1:500, Abcam) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:2000, Abcam). This was followed by an incubation (one hour, room temperature) with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). We visualized the immune complexes using an enhanced chemiluminescence kit (Abcam) and quantified the protein bands by densitometry, using the Quantity One software (Bio-Rad Laboratories).

Cell viability measurement

We measured cell viability with a cell counting kit-8 (CCK8). Following an incubation (one hour, 37°C) of cells with CCK8 solution (Boster, Wuhan, China), we measured absorbance at 450 nm using a multimode plate reader (EnSpire, PerkinElmer, USA).

Apoptosis assay

We harvested A549 and H460 cells using trypsin and suspended the cells in binding buffer. Fluorescein isothiocyanate-conjugated annexin V and propidium iodide (Beyotime biotechnology company) were added to the cell suspension and incubated (15 minutes, room temperature) in a dark chamber. In turn, we quantified the stained cells using a flow cytometer (FACSCalibur; Becton Dickinson Biosciences, San Jose, California).

Invasion assay

We performed the invasion assays in transwell plates containing polycarbonate filters (8.0 μm pore size). First, we coated the transwell inserts with 50 uL of 1 mg/mL Matrigel matrix (Becton Dickinson, Franklin Lakes, NJ, USA) and stored the inserts at 37°C (four hours) to elicit gelling, according to the manufacturer's recommendations. We seeded A549 and H460 cells at a density of 1.5×10^5 cells in 200 uL of medium (without fetal bovine serum) in the upper chamber. Next, we removed the inserts and washed with phosphate-buffered saline (PBS). The nonmigrating cells in the upper chamber were removed using a cotton swab. The inserts were then fixed in cold methanol (10 minutes, room temperature) and stained with hematoxylin. Cells that invaded the lower surface were counted in 10 fields at 200× magnification.

Endogenous ROS measurement

We incubated (30 minutes, 37°C) A549 and H460 cells with 7′-dichlorodihydrofluorescein diacetate (c-H2DCFDA, 10 μM, Invitrogen), a ROS-detecting molecular probe. When ROS is present, oxidation of c-H2DCFDA takes place and forms a fluorescent compound. This fluorescence was measured at an excitation wavelength of 480 nM and an emission wavelength of 525 nM, using a FAC Scan flow cytometer (Becton Dickinson, San Jose, CA).

Statistical analysis

Data are expressed as mean ± standard deviation. All statistical differences were analyzed using one-way analysis of variance, followed by a Tukey post-hoc test. A *P*-value of <0.05 was considered statistically significant.

Results

TRIAP1 expression is negatively correlated with overall survival of lung adenocarcinoma patients

To the best of our knowledge, the expression and role of TRIAP1 in NSCLC has not been systemically investigated until now. However, the TCGA database has already collected a vast amount of information regarding gene expression and its correlation with patient survival for a variety of tumors. By data mining the TCGA database (http://ualcan.path.uab.edu/analysis.html), we discovered that TRIAP1 expression is upregulated in lung adenocarcinoma, lung squamous cell carcinoma, and various other cancer types (Fig 1a,b). Moreover, Asian lung adenocarcinoma patients showed a relatively higher TRIAP1 expression than Caucasian and African-American patients (Fig 1c). TRIAP1 expression appeared more prominent as clinical stages of lung adenocarcinoma became more advanced (Fig 1d). We used the Kaplan-Meier plotter (http://gepia.cancer-pku.cn/) to investigate the correlation between individual TRIAP1 mRNA levels and overall survival (OS) of lung adenocarcinoma patients. Here, we observed that TRIAP1 expression is negatively correlated with the OS of lung adenocarcinoma patients (Fig 1e).

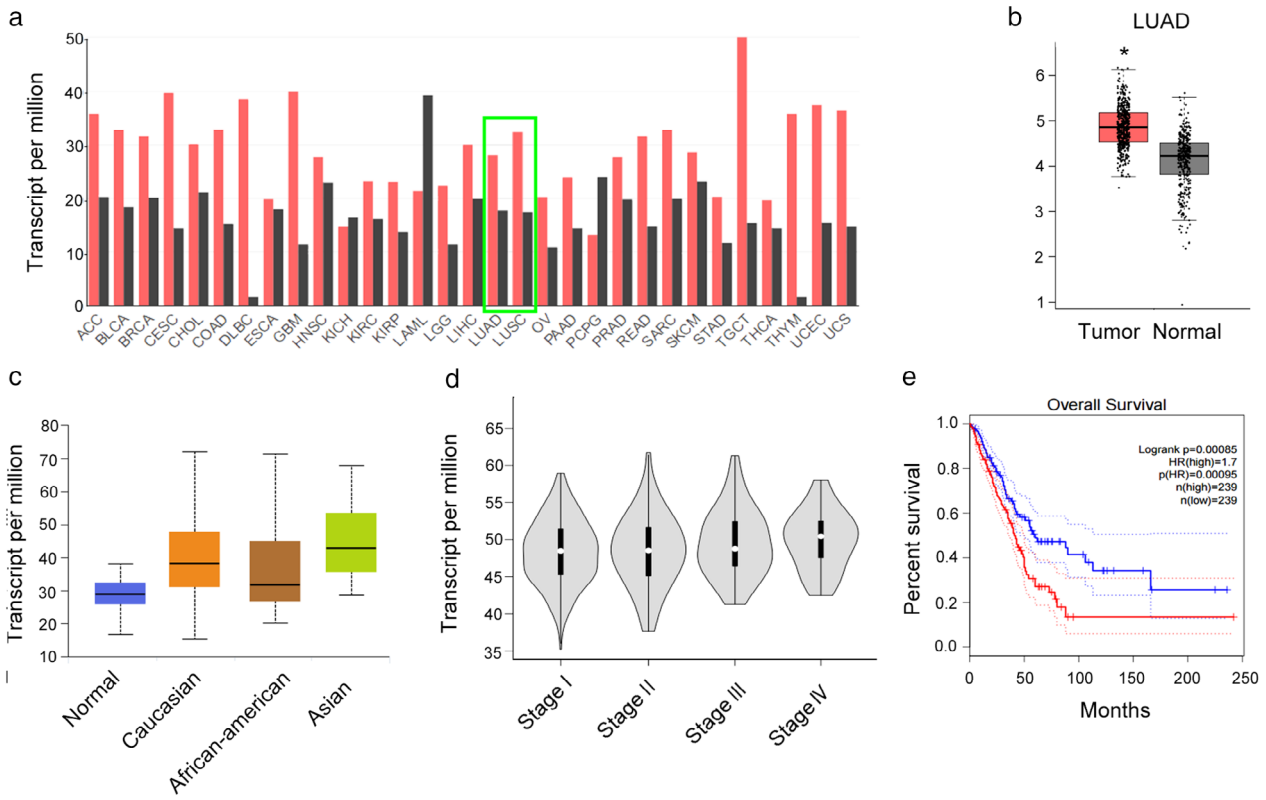


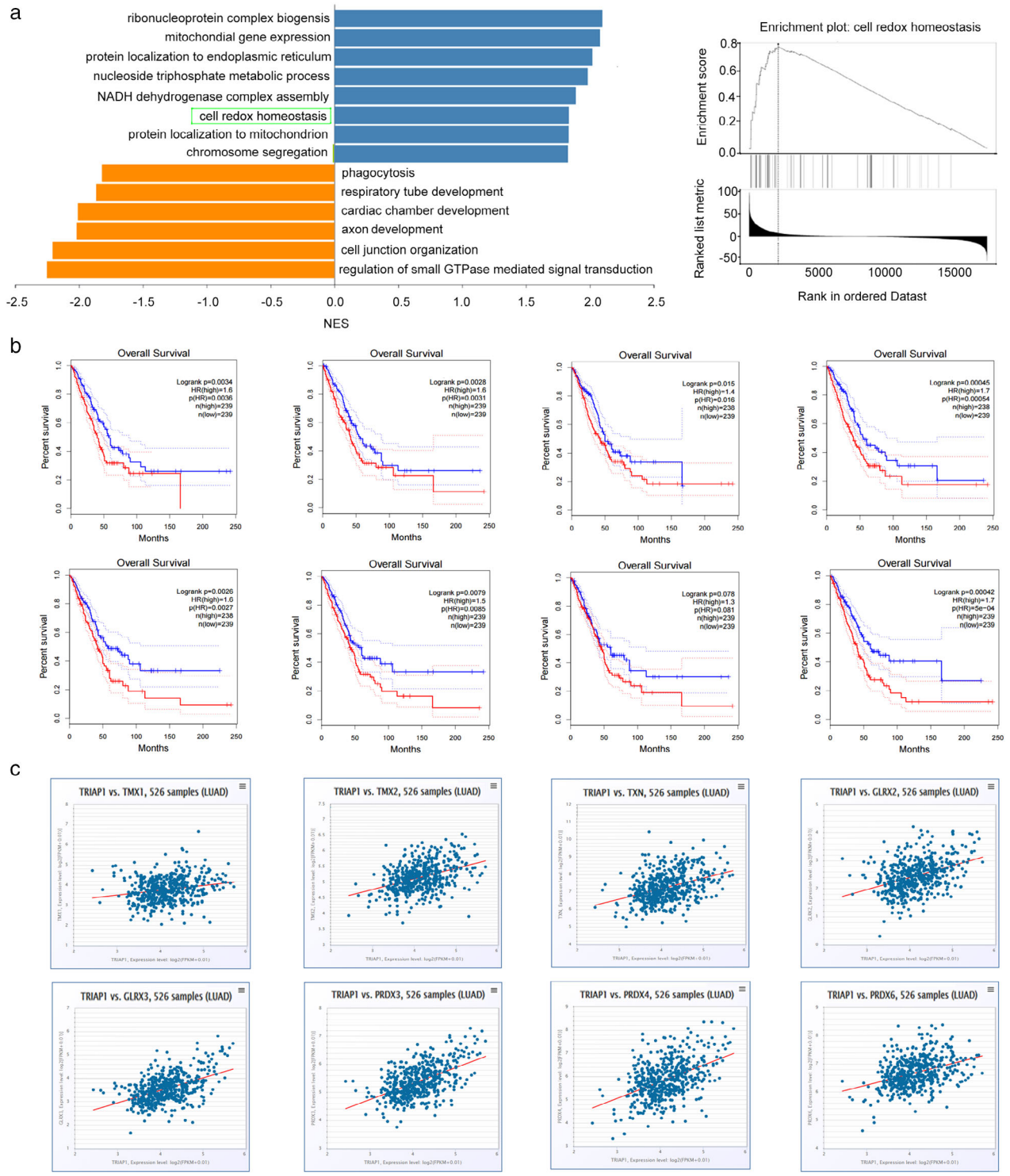
Figure 1 Bioinformatics analysis of the role of TRIAP1in the pathogenesis of lung adenocarcinoma. (**a**) The TCGA database highlighted the expression of TRIAP1 in various cancer types and corresponding healthy tissues (**a**) (■) Tumour, and (■) Normal. (**b**) Among the investigated tissues, lung adenocarcinoma showed higher TRIAP1 expression as compared to healthy tissues. (**c**) Moreover, Asian patients with lung adenocarcinoma demonstrated higher TRIAP1 expression than the Caucasian and African-American patients. (**d**) TRIAP1 expression showed an increasing trend as the clinical stages of lung adenocarcinoma progressed. (**e**) The Kaplan-Meier curves illustrated that TRIAP1 expression was inversely correlated with the OS of lung adenocarcinoma patients (——) Low TRIAP1 TPM, and (——) High TRIAP1 TPM. *$P < 0.05$ vs. normal tissues.

TRIAP1 is a regulator of cell redox homeostasis according to GSEA

High throughput sequencing data (http://www.linkedomics.org/admin.php) highlighted various genes that are correlation with TRIAP1 expression in lung adenocarcinoma. GSEA of these genes might predict the biological processes influenced by TRIAP1. It is likely that TRIAP1 affects various biological processes, such as maintenance of redox homeostasis, which might be crucial for cancer cell radioresistance (Fig 2a). Twenty five genes that were correlated with TRIAP1 expression are involved in the maintenance of redox homeostasis. Of these, there are eight genes with an inverse correlation between expression and OS in lung adenocarcinoma patients, as is indicated in the Kaplan-Meier plot (http://gepia.cancer-pku.cn/) (Fig 2b). Data that we extracted from the TCGA database (http://starbase.sysu.edu.cn/index.php) further confirmed a positive correlation between TRIAP1 and these eight genes regarding their expression, as was demonstrated via Pearson correlation analysis (Fig 2c). As a result, we decided to further study these eight genes (TMX1, TMX2, TXN, GLRX2, GLRX3, PRDX3, PRDX4, and PRDX6).

Irradiation promoted TRIAP1 expression in NSCLC cells

As was indicated by PCR, the mRNA levels of TRIAP1 were increased in A549 and H460 cells following exposure to 2 Gy ($P < 0.01$) and 4 Gy ($P < 0.01$ or $P < 0.05$). Of note, the increase was most prominent with the 2 Gy dose (Fig 3a). Transfection with shRNA-TRIAP1 decreased TRIAP1 mRNA expression in both A549 and H460 cells ($P < 0.01$, Fig 3b) and prevented the previously described radiation-induced (2 Gy) increase of TRIAP1 mRNA. Western blot results highlighted a very significant increase in TRIAP1 protein expression (in both A549 and H460 cells) after irradiation with 2 Gy ($P < 0.001$, Fig 3c). Conversely, TRIAP1 protein levels in both A549 and H460 cells were decreased after transfection with shRNA-TRIAP1 ($P < 0.01$). The treatment of shRNA-TRIAP1 in combination with IR did not



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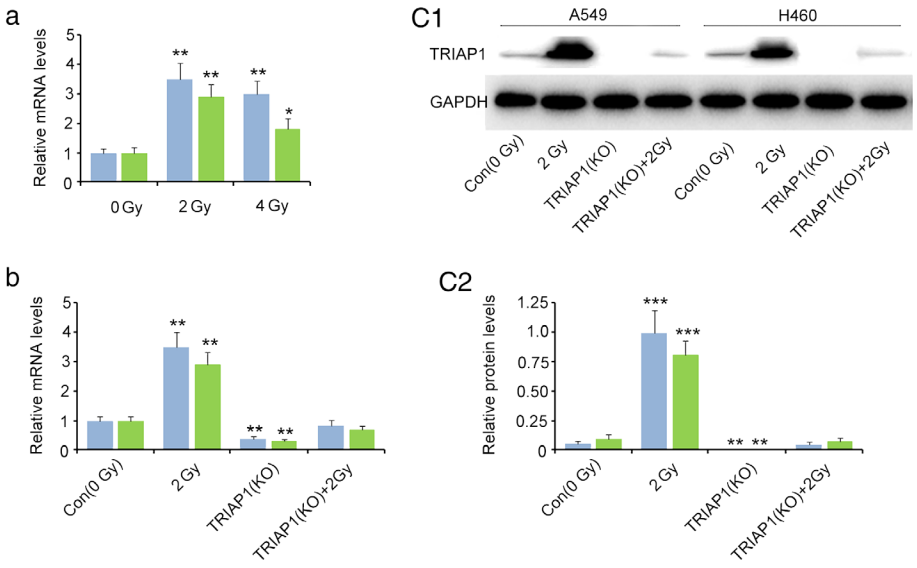

Figure 3 Irradiation promoted TRIAP1 expression in NSCLC cells. (**a**) A549 and H460 cells were exposed to X-rays a dose rate of 2.0 or 4.0 Gy/min. TRIAP1 mRNA levels in A549 and H460 cells were assessed by PCR. (■) A549, and (■) H460. (**b**) TRIAP1 was knocked down by transfecting (before irradiation) a shRNA, which targeted TRIAP1. TRIAP1 mRNA and protein levels in A549 and H460 cells were assessed respectively by PCR (■) A549, and (■) H460 and (**c**) estern blot.(■) A549, and (■) H460. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control group.

significantly decrease TRIAP1 protein expression compared to control.

TRIAP1 mediated upregulation of various antioxidative proteins in NSCLC cells following irradiation

PCR analysis demonstrated that mRNA levels of TMX1, TMX2, TXN, GLRX2, GLRX3, PRDX3, PRDX4, and PRDX6 in A549 and H460 cells were significantly increased after irradiation ($P < 0.05$, $P < 0.01$, or $P < 0.001$, Fig 4a). Next, we observed that TRIAP1 knockdown was associated with decreased TMX1, TMX2, TXN, GLRX2, GLRX3, PRDX4, and PRDX6 mRNA levels in A549 cells. In addition, TMX1, GLRX2, GLRX3, PRDX3, PRDX4, and PRDX6 mRNA was reduced in H460 cells ($P < 0.05$ or $P < 0.01$). Furthermore, TRIAP1 knockdown prevented the radiation-induced increase of TMX1, TMX2, TXN, GLRX2, PRDX3, PRDX4, and PRDX6 in A549 cells. Similarly, the radiation-induced expression of TMX1, TMX2, TXN, GLRX2, GLRX3, PRDX3, and PRDX6 was prevented in H460 cells ($P < 0.05$ or $P < 0.01$ vs. irradiation group). Building upon these findings, we explored expression at the protein level as well, using western blot. Both A549 and H460 cells showed radiation-induced increases in TMX1, TMX2, TXN, GLRX2, GLRX3, PRDX3, PRDX4, and PRDX6 protein levels ($P < 0.05$, $P < 0.01$, or $P < 0.001$, Fig 4b). Silencing TRIAP1 in A549 cells reduced TMX1, TMX2, GLRX2, PRDX4, and PRDX6 protein levels ($P < 0.05$), while knocking down TRIAP1 in H460 cells reduced the amount of TMX1, GLRX2, GLRX3, PRDX3, PRDX4, and PRDX6 ($P < 0.05$ or $P < 0.01$). Radiation-induced increases of TMX1, TMX2, TXN, GLRX2, PRDX3, PRDX4, and PRDX6 proteins in A549 cells were inhibited by TRIAP1 knockdown ($P < 0.05$ or $P < 0.01$ vs. irradiation group). Furthermore, in H460 cells, it was apparent that TRIAP1 knockdown prevented the radiation-induced increases of TMX1, TMX2, TXN, GLRX2, GLRX3, PRDX3, and PRDX6 proteins ($P < 0.05$ or $P < 0.01$ vs. irradiation group).

Figure 2 GSEA demonstrated the involvement of TRIAP1 in the regulation of cell redox homeostasis. (**a**) High throughput sequencing data (http://www.linkedomics.org/admin.php) highlighted various genes of which the expression is correlated with TRIAP1 expression in lung adenocarcinoma. GSEA of these genes suggested that various biological processes might be influenced by TRIAP1, such as the maintenance of redox homeostasis. This process might be an important factor in cancer radioresistance. (**b**) A total of 25 genes that were correlated with TRIAP1 expression are involved in the maintenance of redox homeostasis. From all these, it is the expression of eight specific genes that is inversely correlated with the OS of lung adenocarcinoma patients, as indicated by the Kaplan-Meier curve (http://gepia.cancer-pku.cn/). (——) Low TMX1 TPM, (——) High TMX1 TPM; (——) Low TMX2 TPM, (——) High TMX2 TPM; (——) Low TMN TPM, (——) High TMN TPM; (——) Low GLRX2 TPM, (——) High GLRX2 TPM; (——) Low GLRX3 TPM, (——) High GLRX3 TPM; (——) Low PRDX3 TPM, (——) High PRDX3 TPM; (——) Low PRDX4 TPM, (——) High PRDX4 TPM; (——) Low PRDX6 TPM, (——) High PRDX6 TPM. (**c**) Data from the TCGA database (http://starbase.sysu.edu.cn/index.php) further confirmed the positive correlation between TRIAP1 and these eight genes, regarding their expression, via Pearson correlation analysis. (——) Regression (y = 0.2399x + 2.7822) and (●) $r = 0.207$, *P*-value = 1.72e–06; (——) Regression (y = 0.3382x + 3.7549) and (●) $r = 0.401$, *P*-value = 9.33e–22; (——) Regression (y = 0.6028x + 4.7771) and (●) $r = 0.375$, *P*-value = 4.72e–19; (——) Regression (y = 0.4282x + 0.6806) and (●) $r = 0.397$, *P*-value = 2.86e–21; (——) Regression (y = 0.5347x + 1.3424) and (●) $r = 0.485$, *P*-value = 1.99e–32; (——) Regression (y = 0.5690x + 3.0335) and (●) $r = 0.531$, *P*-value = 1.23e–39; (——) Regression (y = 0.7181x + 2.9036) and (●) $r = 0.464$, *P*-value = 2.17e–29; (——) Regression (y = 0.3831x + 5.0952) and (●) $r = 0.373$, *P*-value = 7.50e–19.

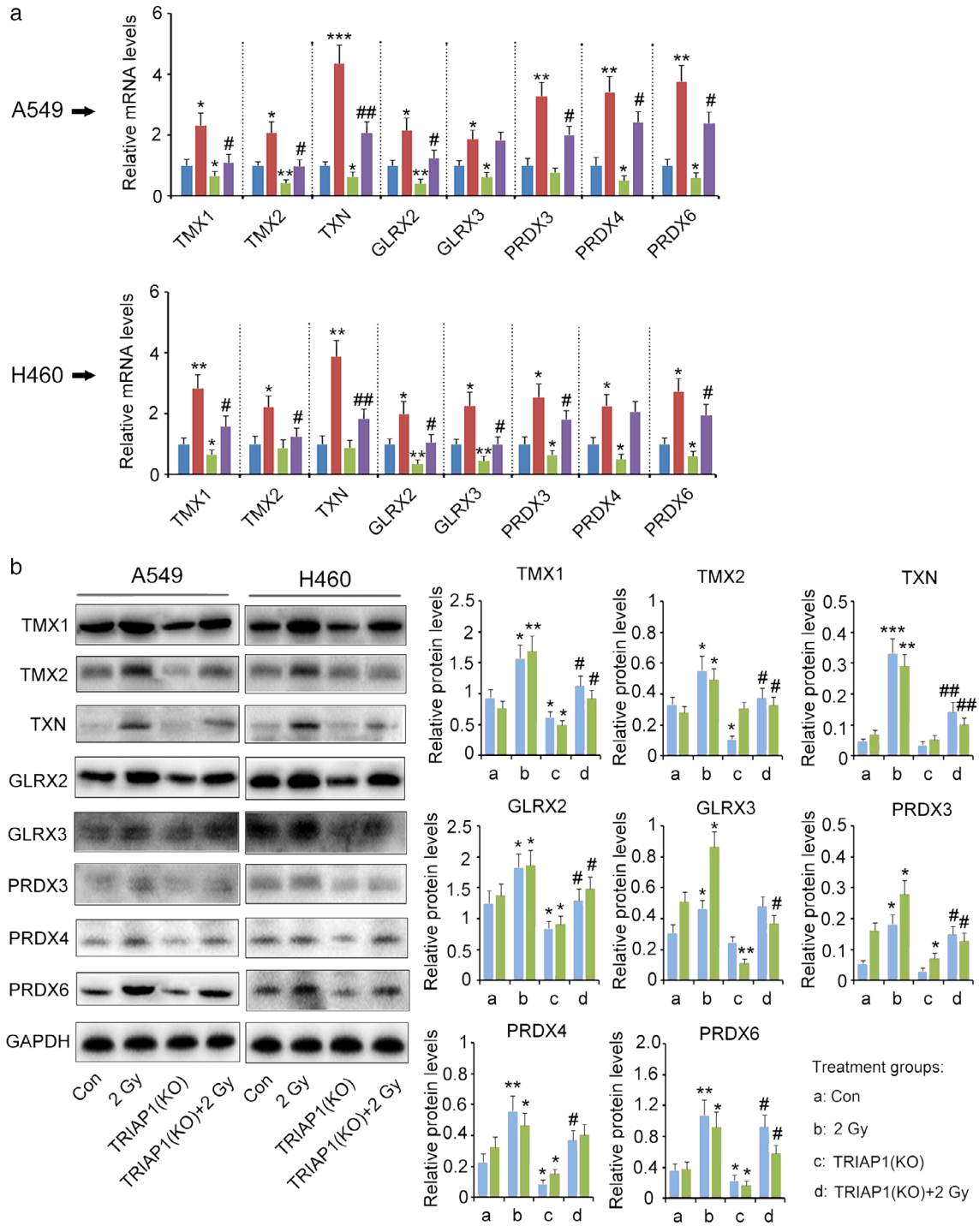

Figure 4 TRIAP1 mediated the upregulation of various antioxidative proteins in NSCLC cells following irradiation. Bioinformatics analysis showed that antioxidative proteins (TMX1, TMX2, TXN, GLRX2, GLRX3, PRDX3, PRDX4, and PRDX6) were positively regulated by TRIAP1. To validate this prediction, TRIAP1 was knocked down by transfecting shRNA which targets TRIAP1, before irradiation. The mRNA and protein levels of these antioxidative proteins were assessed respectively by by (**a**) PCR (■) Con, (■) 2 Gy, (■) TRIAP1 (KO), and (■) TRIAP1 (KO)+2 Gy; and (■) Con, (■) 2 Gy, (■) TRIAP1 (KO), and (■) TRIAP1 (KO)+2 Gy and (**b**) estern blot. (■) A549, and (■) H460. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 vs. control groups; $^{\#}P$ < 0.05 and $^{\#\#}P$ < 0.01 vs. radiation group.

TRIAP1 knockdown increased the toxicity of ionizing radiation to NSCLC cells

We found that 2 Gy exposure decreased cellular (A549 and H460) viability ($P < 0.05$, Fig 5a). Comparably, silencing TRIAP1 also reduced A549 and H460 cell viability ($P < 0.05$). Combining TRIAP1 knockdown and 2 Gy irradiation resulted in an even more pronounced inhibition of cell viability, as compared to both treatments separately ($P < 0.01$). In addition, 2 Gy irradiation increased the rate of apoptosis in both A549 and H460 cells ($P < 0.05$, Fig 5b). Here, knocking down TRIAP1 also led to an increased rate of apoptosis in both A549 and H460 cells ($P < 0.01$).

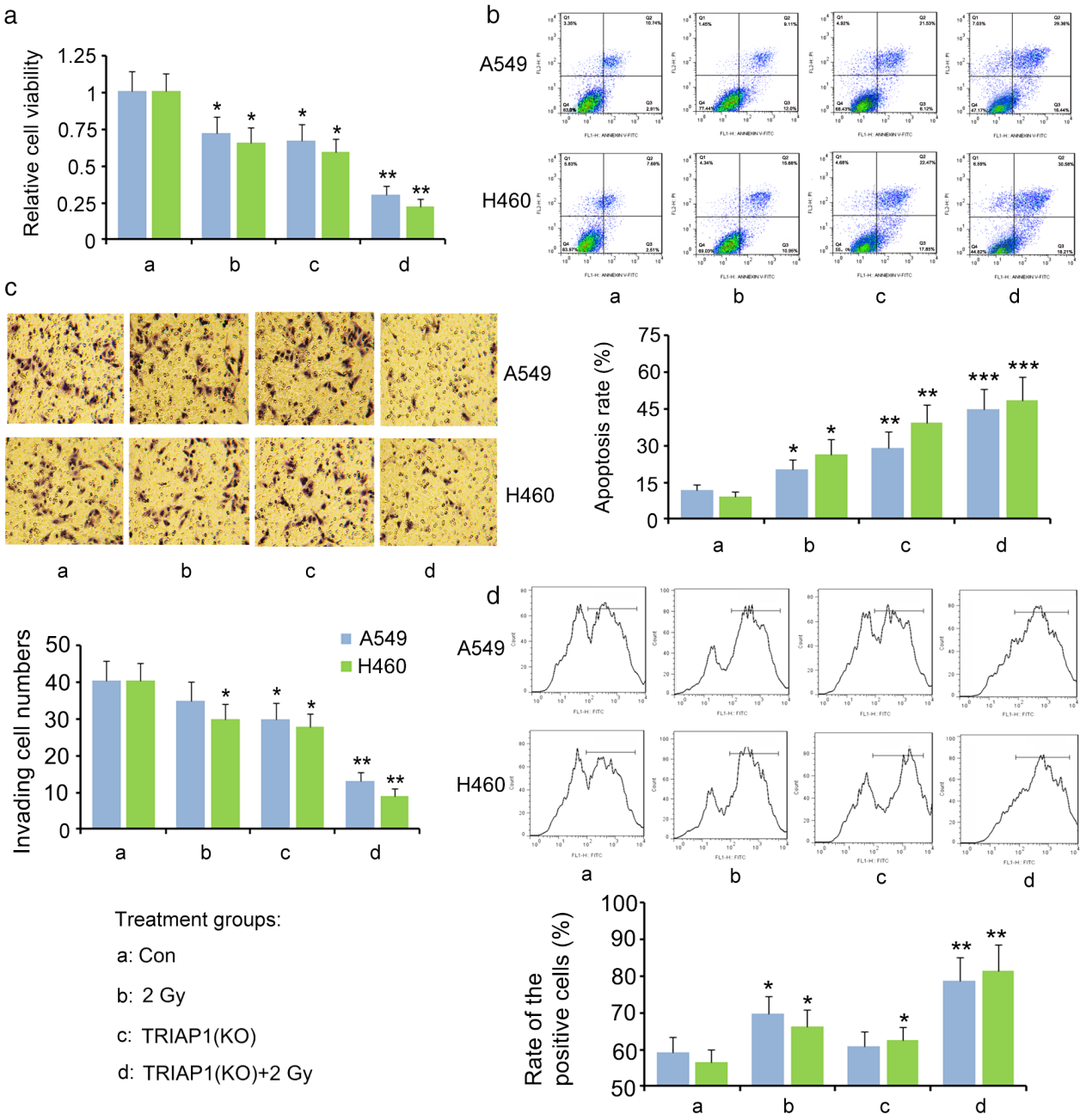

Figure 5 TRIAP1 knockdown increased the toxicity of radiation exposure to NSCLC cells. TRIAP1 was knocked down by transfecting shRNA, which targeted TRIAP1, before irradiation. Next, (**a**) cell viability (■) A549, and (■) H460, (**b**) apoptosis rate (■) A549, and (■) H460, (**c**) cell invasion (■) A549, and (■) H460, and (**d**) intracellular reactive oxygen species levels were assessed (■) A549, and (■) H460. $^{*}P < 0.05$ and $^{**}P < 0.01$ $^{***}P < 0.001$ vs. control groups; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. radiation group.

TRIAP1 knockdown in combination with 2 Gy irradiation further aggravated the rate of apoptosis in both A549 and H460 cells ($P < 0.001$). Furthermore, 2 Gy irradiation decreased the invasion of H460 cells ($P < 0.05$, Fig 5c), while no such effect was observed in A549 cells. TRIAP1 knockdown reduced the number of invading A549 and H460 cells ($P < 0.05$). Silencing TRIAP1 prior to 2 Gy irradiation caused a marked inhibition of invading A549 and H460 cells ($P < 0.01$).

In this study, we also evaluated the impact of TRIAP1 knockdown and 2 Gy irradiation (potentially both combined) on cellular ROS levels. We determined that 2 Gy irradiation increased the ROS levels in A549 and H460 cells ($P < 0.05$, Fig 5d), and TRIAP1 knockdown increased ROS levels in H460 cells, while ROS levels in A549 cells remained unaltered. Silencing TRIAP1 prior to 2 Gy irradiation notably increased ROS levels in A549 and H460 cells ($P < 0.01$).

Discussion

We performed a bioinformatics analysis on data collected from the TCGA database, which demonstrated that TRIAP1 is upregulated in lung adenocarcinoma and that its expression level is inversely correlated with overall patient survival. This suggests that TRIAP1 might play a cancer-promoting role in lung adenocarcinoma. Previous studies demonstrated that both microRNA-18a and microRNA-320b target TRIAP1. Of note, abnormal downregulation of these microRNAs results in an elevated TRIAP1 expression, thus promoting the progression of ovarian cancer and nasopharyngeal carcinoma.[4,5] The scientific consensus states that the cancer-promoting role of TRIAP1 is probably associated with its inhibitory effect on apoptosis. Fook-Alves *et al.* discovered that permanent silencing of TRIAP1 promoted late apoptosis in multiple myeloma cells, by increasing caspase 9 and APAF1 expression.[7] In addition, stable transfection of TRIAP1 expressing plasmids in breast cancer cells increased the number of doxorubicin-resistant clones, while shRNA targeting TRIAP1 had an adverse effect.[8]

In our study, we demonstrated that TRIAP1 expression in NSCLC was increased following irradiation. The increase of TRIAP1 expression is probably a response of cells to radiation. Radiation has been reported to change cell signal, epigenetic modification, expression of transcription factors and miRNAs, which are probably implicated in the change of TRIAP1 expression in response to radiation.[9–12] The solid mechanism is needed to be identified in further studies. A GSEA study showed that TRIAP1 expression is correlated with several proteins that play key roles in maintaining the redox homeostasis. As such, TRIAP1 might also be implicated in the modulation of cellular redox equilibrium. Because this function of TRIAP1 has not previously been reported, we decided to further investigate which of these proteins are regulated by TRIAP1 and whether the regulatory effect on redox equilibrium is involved in the radioresistance of NSCLC. Knocking down TRIAP1 in NSCLC cells decreased the expression of TMX1, TMX2, TXN, GLRX2, GLRX3, PRDX3, PRDX4, and PRDX6 at both the mRNA and protein level, suggesting that these proteins are indeed regulated by TRIAP1.

TMX1, TMX2, and TXN are important members of the thioredoxin antioxidant system. TMX1 and TMX2 belong to the TMX subfamily and are localized to the membrane of the endoplasmic reticulum (ER). These proteins participate in various redox reactions, via the reversible oxidation of its active center (dithiol) to a disulfide.[13,14] This will in turn catalyze dithiol-disulfide exchange reactions. TMXs have antiapoptotic properties in response to ER stress.[13,14] Radiation-induced ROS leads to the accumulation of misfolded or abnormal proteins in the ER, which causes ER stress. Because ER stress is a critical inducer of apoptosis, previous studies focused on the link between ER stress and the enhanced radiosensitivity of cancer cells.[15–18] Of note, Matsuo *et al.* found that overexpression of TMXs in 293 cells significantly attenuated ER stress and delayed the onset of ER stress-induced cell death.[13] These findings suggest that TMX1 and TMX2 might protect NSCLC against radiation-induced apoptosis. TXN1, also known as Trx1, is a small redox with a highly conserved redox-active motif (-Cys-Gly-Pro-Cys-). TXN1 overexpression in NSCLC is indicative of a more aggressive tumor phenotype, which in turn is associated with bad prognostic features and a poorer outcome.[19] Inhibiting the expression and function of TXN1 has been shown to increase the radiosensitivity of various cancer types.[20–22]

GLRX2 and GLRX3 are thiol-disulfide oxidoreductases, which consist of an N-terminal Trx homology region followed by two tandem repeats of glutaredoxin domains. They play a pivotal role in protecting the cell from oxidative stress-induced apoptosis. For example, GLRX2 overexpression can prevent H_2O_2-induced apoptosis by protecting the mitochondrial activity of complex I.[23] Furthermore, cancer cells with GLRX2 overexpression are more resistant to the doxorubicin and phenylarsine oxide, which induce oxidative stress.[24,25] More importantly, GLRX2 can maintain an antioxidative and antiapoptotic state, even when other antioxidant enzymes such as superoxide dismutase 1 and thioredoxin reductase might be dysfunctional.[26,27] Decreased levels of GLRX3 render cancer cells susceptible to death following oxidative stress.[28] Furthermore, GLRX3 expression has also been associated with the migration, metastasis, and progression of oral squamous cell carcinoma and nasopharyngeal carcinoma.[29,30]

The aforementioned studies suggest that the upregulation of GLRX2 and GLRX3 by TRIAP1 confers resistance of NSCLC to ROS-induced death following radiation.

The PRDX family of antioxidant enzymes comprises six identified mammalian isoforms (PRDX1-6). These proteins primarily protect cells from oxidative damage via the oxidation of a cysteine residue, which then forms sulfenic acid. This is subsequently reduced by thioredoxin. In addition to its antioxidant role, overexpression of PRDXs in cancer tissue appears strongly correlated with more aggressive cancer behavior, tumor metastasis and tumor-node-metastasis (TNM), indicating a possible role in the progression of tumorigenesis.[31–34] The cancer-promoting effects of the PRDXs are probably associated with the regulation of several reductant-oxidant-sensitive cellular processes, such as cell proliferation, apoptosis, and cell signaling.[31–34] In our study, TRIAP1 mediated the radiation-induced upregulation of PRDX3, PRDX4, and PRDX6 in NSCLC. They might enhance the cellular antioxidant capacity and promote the survival of NSCLC following irradiation.

TRIAP1 knockdown is associated with a more profound inhibition of radiation-induced cell viability, reduced cell invasion and an increased apoptosis rate of NSCLC cells. This suggests that the toxic effects of radiation are enhanced. Ketteler *et al.*[35] unveiled that TRIAP1 (secreted by fibroblasts) was taken up by adjacent prostate cancer cells, PC3 and LNCaP, resulting in a pronounced cancer radioresistance.[36] Studies have illustrated that TRIAP1 exerts antiapoptotic effects via its interaction with HSP70, which inhibits cytochrome c to activate the apoptotic protease activating factor 1 and in turn the downstream caspase-9.[4,5] Moreover, we discovered that TRIAP1 is involved in the upregulation of several antioxidant proteins, which can help remove radiation-induced ROS. Because excessive ROS production is the leading cause for radiation-induced cell death, an increased antioxidative capacity might be responsible for TRIAP1-mediated radioresistance in NSCLC cells. However, it remains unclear how TRIAP1 induces the upregulation of these antioxidant proteins in NSCLC cells. Previous studies reported that TRIAP1 also regulates various signaling pathways and biological processes.[36,37] Our in-depth analysis will investigate the mechanism of regulation of antioxidant proteins by TRIAP1 in near future.

In summary, this study is the first to demonstrate that TRIAP1 contributes to the scavenging of radiation-induced ROS by upregulating several antioxidant proteins. Because TRIAP1 knockdown enhanced the radiosensitivity of NSCLC cells, we conclude that TRIAP1 is a key contributor to the radioresistance of NSCLC by maintaining redox homeostasis.

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Disclosure

The authors declare there are no potential conflicts of interest.

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