

Down-Regulation of PER2 Increases Apoptosis of Gliomas after X-Ray Irradiation

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Abstract

Period2 (*PER2*), a core circadian gene, not only modulates circadian rhythm but also may play an important role in other biological processes including pathways involved in the proliferation and apoptosis of tumor cells. In this study, we investigated the mechanism by which downregulated expression of *PER2* promotes apoptosis of wild-type *TP53* human glioma U343 cells exposed to X-rays. U343 cells were irradiated with 6mV 10Gy X-ray irradiation after infection with shRNA lentivirus to reduce expression of *PER2*, and then analyzed by several methods such as SCGE analysis, flow cytometry, RT-PCR, and western blotting. Compared with controls, U343 cells expressing low levels of *PER2* showed serious DNA damage when exposed to X-ray irradiation in SCGE analysis ($P < 0.05$), and higher death rates in flow cytometry assay ($P < 0.05$). RT-PCR and western blot analysis both revealed decreased expression of *ATM* and *TP53*, which regulate DNA damage and repair via the ATM-TP53 pathway, and an increased expression of *C-MYC*, which is related to cell apoptosis. Thus, our research suggests that *PER2* may play an important role in tumor radiotherapy, which is attributable to enhanced ATM-TP53 signaling and pro-apoptotic processes. These findings provide a new target for the clinical treatment of glioma, and a reliable basis for post-radiation therapy and gene therapy for glioma and other cancers.

Keywords: Glioma; *PER2*; shRNA; TP53; U343 cells

Introduction

The clinical management of malignant glioma is the most difficult faced by the practicing neurologist. Patients have a very poor prognosis, largely because of the paucity of druggable targets amenable to therapeutic intervention. However, recent progress at the molecular level has identified candidate targets, many of which have potential to become future treatment options.

The Period2 (*PER2*) gene is an indispensable component of the mammalian circadian clock [1]. In 2002, the murine *PER2* gene was reported to play an important role in tumorigenesis. A higher incidence of tumor development was observed in *PER2*-deficient mice than in wild-type mice [2]. In addition, clear differences in *PER2* expression are evident in tumor tissue and non-involved peripheral tissues [3-7]. Elsewhere, overexpression and/or mutations in the *PER2* gene have been reported to correlate with enhanced tumor growth in breast cancer, colon cancer and lymphoma, corresponding with altered expression of *TP53* and the oncogenes *BCLx1*, *BCL-2*, cyclinB1, cyclin D, cyclin E and *C-MYC* [8-15]. *PER2* has also been linked with DNA damage response pathways [16]. X-ray-induced DNA damage in tumor cell lines with mutated *PER2* exhibited increased sensitivity to damage affecting cell proliferation, apoptosis, and the TP53 pathway [17-19].

While it is evident that *PER2* is critical to cell proliferation, apoptosis and the *TP53* pathway in neoplasia, the mechanism remains poorly understood. Based upon previous studies, we have examined the role of *PER2* in the cellular response to X-ray-induced DNA

damage in the human glioma U343 cell model. RNA interference technology was used to downregulate the expression of *PER2*. We observed that U343 cells with low-level expression of *PER2* exposed to low dose X-ray irradiation exhibited serious DNA damage and an enhanced death rate. Our data indicate that *PER2* interacts with the TP53 pathway and contributes to increased *TP53* activity, rescuing DNA-damaged cells from TP53-mediated apoptosis.

Material and Methods

Cell lines and reagents

The U343 glioma cell line was purchased from Ji Ni Biotechnology Co. Ltd. (Guangzhou, China). Cells were maintained according to the supplier's directions and guidelines, and were cultivated at 37°C in 100% Rh 5% CO₂/95% air atmosphere. ShRNA-*PER2* lentivirus was obtained from the Genechem Chemical Technology Co. Ltd. (Shanghai, China). Oligonucleotide primer and probe sequences were designed and provided by Sangon Biotech Co. Ltd. (Shanghai, China). The Annexin V-EGFP Apoptosis Detection kit (KeyGEN Biotechnology, Njing, China), Comet Assay for DNA Damage Detection Kit (KeyGEN Biotechnology, China), puromycin (Solarbio Technology Co. Ltd., China), and Maxima SYBR Green Qpcr Master Mix (Fermentas, USA) were used according to the manufacturers' instructions.

Transfection with shRNA lentivirus

The expression of *PER2* was regulated using lentiviral transfection of shRNA. U343 glioma cells were stably transformed with shRNA-

PER2 and shRNA-control constructs according to the manufacturers' protocol. U343 cells were plated in 6-well plates at a density of 2×10^4 cells/well in RPMI-1640 medium (Gibco, USA) and allowed to attach for 18 h prior to transfection with shRNA lentivirus vectors. Cells were permitted to integrate for 24 h after infection. Puromycin (3 μ g/ml) was added to select cells with integrated retrovirus and stable cell lines were established after one week. Interference sequence of shRNA-PER2 are showed in Table 1.

Sense strand	5'CCGGGCCAGAGTCCAGATACCTTTACTCGAGTAAAGG TATCTGGACTCTGGCTTTTTG 3'
Antisense strand	5'AATTCAAAAAGCCAGAGTCCAGATACCTTTACTCGAG TAAAGGTATCTGGACTCTGGC 3'

Table 1: Interference sequence of shRNA-PER2.

X-ray irradiation

Stably-transfected glioma U343 cells were exposed to 124 cGy/min, an exposure field of 25×25 cm² and spacing of 80 cm, and an accumulated dose of 10 Gy X-Rays (Varian Medical Systems Inc., California, USA).

Comet-FISH assay

The alkaline comet assay was performed following the standard protocol of McKelvey-Martin et al. [20-22]. U343 glioma cells were harvested and washed twice with phosphate buffered saline (PBS) and mixed with 0.7% low-melting point agarose. An agarose gel sandwich was prepared comprising of a base layer 0.5% of standard agarose and a second layer of 0.7% low-melting point agarose with cells, and overlaid with 0.7% low-melting point agarose to a depth of 0.5 mm. The assay was incubated at 4°C in a high humidity environment for 30 min. Gels were immersed in alkaline lysis solution (90% lysis buffer pH10 and 10% DMSO) and maintained in the dark for 2 h at 4°C. Gels were briefly rinsed with PBS, equilibrated in 300 mM NaOH and 1 mM EDTA (pH13) for 40 minutes, and then subjected to 100 mA at 25 mV for 30 min. Gels were stained with propidium iodide, and DNA comet tails visualized with a fluorescent microscope at an excitation wavelength of 530 nm and emission wavelength of 620 nm.

Apoptosis assay

U343 human glioma cells were harvested using trypsin-EDTA, resuspended to a concentration of 1×10^6 cells/ mL and stained with 5 μ g Annexin-V-EGFP (for apoptosis) and 5 μ g propidium iodide (for ds-DNA) for 5 min at 4°C. Cells were analyzed using a flow cytometer (BD Bioscience, USA) (excitation 488 nm; emission 525 nm (GFP) and 620 nm (PI)).

Real-time PCR

Gene expression was quantified using real-time PCR analysis and SYBR Green (Fermentas, USA). Total RNA was extracted from U343 cells with Trizol and reverse-transcribed into cDNA using RevertAid First Strand cDNA Synthesis (Fermentas, USA). Samples were analyzed in triplicate using a CFX96 Real-time PCR System (Bio-Rad, Hercules, Ca, USA). A total of 500 ng of sample cDNA was subjected to the following: 2 min at 50°C (annealing), 10 sec at 95°C (melting), followed by 40 cycles of 15 sec at 95°C and 30 sec at 56°C and 30 sec

extension at 72°C. Primers and probes were designed to hybridize with *ATM*, *TP53*, *C-MYC* and *PER2*, as shown in Table 2.

Per 2	forward	5' CCCTGGTGTCTGGGAAGAT 3'
	reverse	5' GGAGGTGAAACTGTGGAACA 3'
ATM	forward	5' TGTGACTTTTCAGGGGATTG 3'
	reverse	5' ATAGGAATCAGGGCTTTTGGA 3'
P53	forward	5' CTCCTCAGCATCTTATCCGAGT 3'
	reverse	5' GCTGTTCCGTCCCAGTAGATTA 3'
c-myc	forward	5' CCTCCACTCGGAAGGACTATC 3'
	reverse	5' TGTTGCGCTGACATTCTC 3'
β -actin	forward	5' TGACGTGGACATCCGCAAAG 3'
	reverse	5' CTGGAAGGTGGACAGCGAGG 3'

Table 2: Primers and probes of ATM, TP53, C-MYC and PER2.

Western blotting

U343 glioma cells were harvested and total protein was extracted at 24 h after X-ray irradiation. Isolated proteins (40-60 mg) were separated using SDS-PAGE, and transferred to nitrocellulose membranes (Millipore, USA). Non-specific binding sites were blocked with albumin and membranes probed with antibodies specific for *ATM*, *PER2*, and *C-MYC* (Abcam, USA), *TP53* (Proteintech, USA), or β -actin (TransGen Biotechnology, China). Each assay was repeated at least three times. Images and relative expression levels of target genes were captured and quantified using Gel-Pro analyzer (Media Cybernetics, USA).

Statistical Analysis

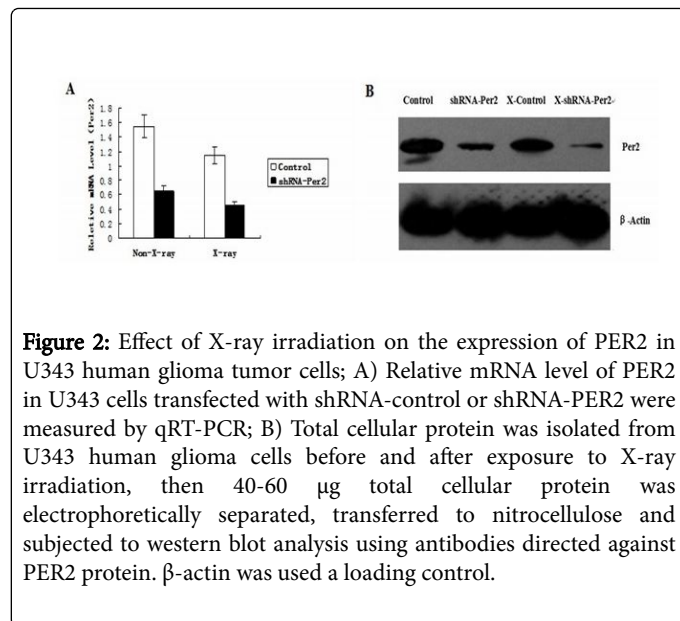
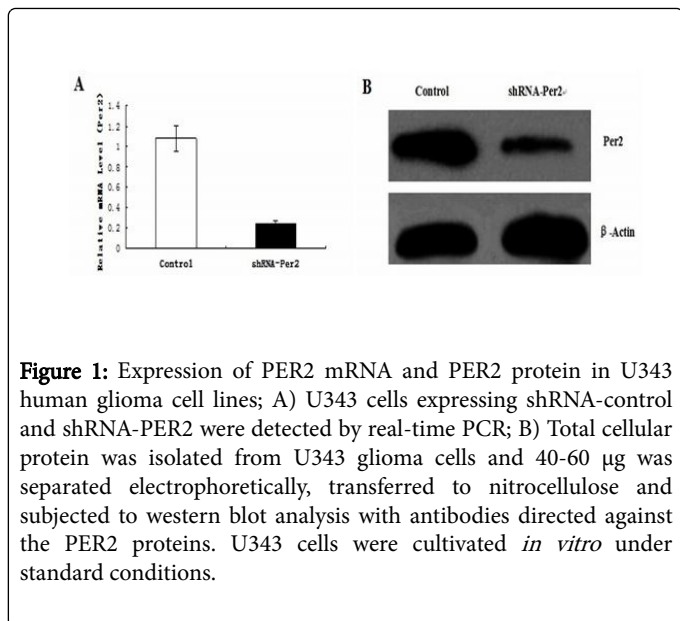
Data were analyzed where relevant using univariate analysis of variance using SPSS version 11.0 (IBM Corporation, USA). Statistical significance was accepted when $P < 0.05$.

Results

Expression of PER2 in U343 glioma cells

Quantitative real-time PCR (qRT-PCR) and western blotting were applied to assay *PER2* expression at the RNA and protein levels, respectively. Both RNA and immunoreactive protein were notably reduced in U343 cells transfected with shRNA-PER2 relative to shRNA-transfected control U343 cells ($P < 0.05$; $n = 3$) (Figure 1). The expression level of *PER2* mRNA was reproducibly reduced by more than 70% in shRNA-PER2 cells (Figure 1A). Cells transfected with shRNA-PER2 also expressed reduced *PER2* protein levels compared with control cells (Figure 1B).

Having demonstrated that lentiviral mediated RNA interference technology was an effective inhibitor of *PER2* expression, subsequent experiments utilized this technology to examine the role of *PER2* in glioma cells subjected to low dose X-ray irradiation.

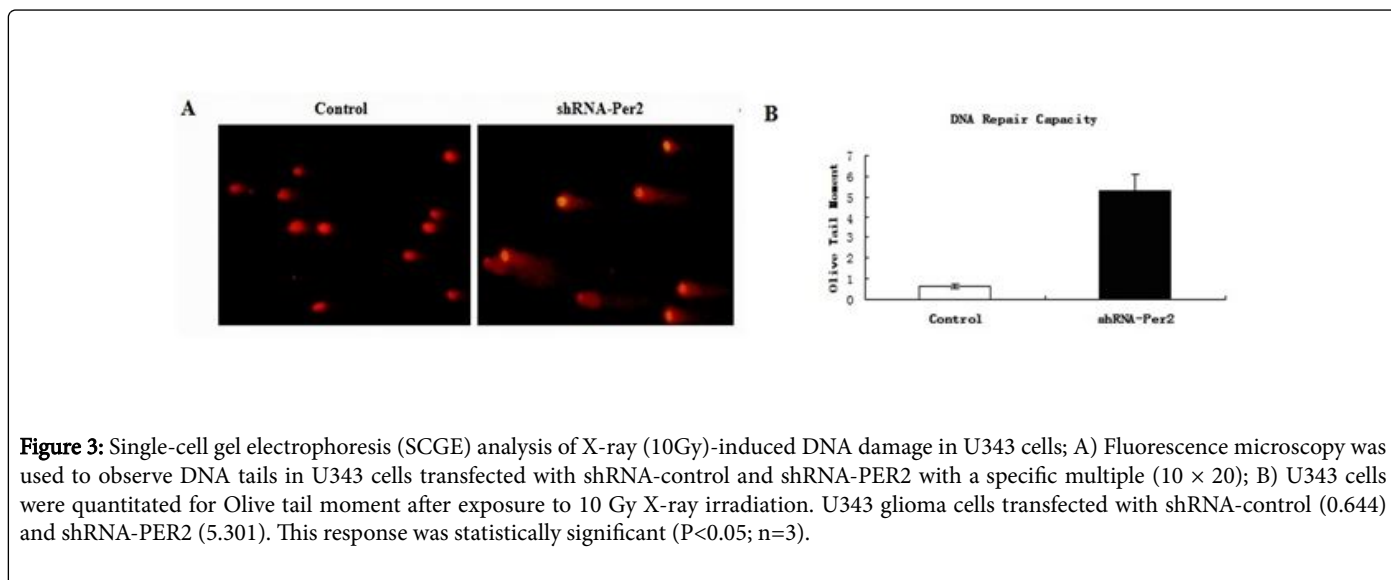


X-ray irradiation attenuates PER2 expression in U343 glioma cells

This experiment is intended to study the role of *PER2* in the apoptosis mechanism which is induced by X-ray irradiation. Prior to this, we should to verify whether there was a relationship between *PER2* and X-ray radiation. We measured the expression of *PER2* before and after irradiation. *PER2* expression was observed to decrease, both at the mRNA level (qRT-PCR) and at the protein level (western blot) (Figures 2A and 2B).

Downregulation of PER2 enhances radiosensitivity and DNA damage in U343 glioma cells

After exposure to 10 Gy of X-rays, U343 cells were selected with puromycin for 7 days prior to analysis for DNA damage. Single cell gel electrophoresis (SCGE) revealed the presence of differential DNA damage. We noted DNA tailing was more prominent in cells transfected with shRNA-PER2: the tail was eight times longer than the group transfected with shRNA-control, suggesting decreased DNA repair capacity in the shRNA-PER2 group ($P < 0.05$; $n = 3$) (Figure 3A and 3B). These data indicated that the downregulation of *PER2* increased glioma cell sensitivity to ionizing radiation, resulting in increased DNA damage. The observed increase in DNA was significant ($P < 0.05$).



Downregulation of PER2 increases apoptosis in X-ray-irradiated U343 glioma cells

To determine the possible effects of irradiation on the apoptosis of glioma cells, U343 cells were double-stained with Annexin V-EGFP and propidium iodide and analyzed using flow cytometry. In shRNA-control-transfected U343 cells, 1.81% of the population was double-positive for EGFP and PI, indicative of apoptosis. In contrast, U343 glioma cells expressing *PER2* (transfected with shRNA-PER2) were found to be 9.98% double-positive i.e. apoptotic (Figures 4A and 4B). A similar situation was also observed in the double-staining cells by epifluorescent microscopy, which confirmed double-staining cells were apoptotic. (Figure 4C). These data suggest that downregulation of *PER2* is associated with a significant increase in apoptosis of the U343 cell population after exposure to X-ray ionizing radiation.

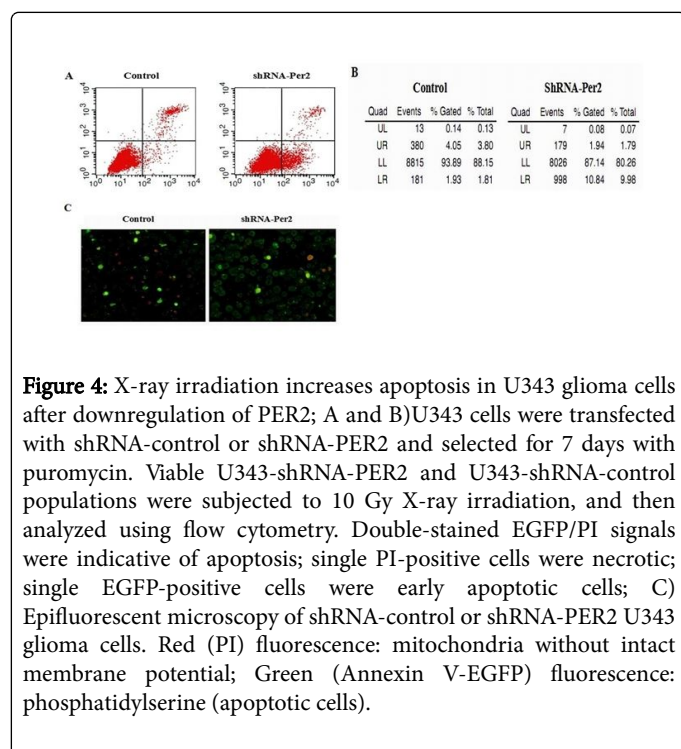


Figure 4: X-ray irradiation increases apoptosis in U343 glioma cells after downregulation of PER2; A and B) U343 cells were transfected with shRNA-control or shRNA-PER2 and selected for 7 days with puromycin. Viable U343-shRNA-PER2 and U343-shRNA-control populations were subjected to 10 Gy X-ray irradiation, and then analyzed using flow cytometry. Double-stained EGFP/PI signals were indicative of apoptosis; single PI-positive cells were necrotic; single EGFP-positive cells were early apoptotic cells; C) Epifluorescent microscopy of shRNA-control or shRNA-PER2 U343 glioma cells. Red (PI) fluorescence: mitochondria without intact membrane potential; Green (Annexin V-EGFP) fluorescence: phosphatidylserine (apoptotic cells).

Downregulation of PER2 reduces the expression of ATM and TP53 but increases the expression of C-MYC in X-ray-irradiated U343 glioma cells

Because our data suggested that *PER2* protected U343 glioma cells from programmed cell death, we subsequently examined the expression of genes known to be important in DNA damage repair, programmed cell death and proliferation, *ATM*, *TP53*, and *C-MYC*. The expression of *ATM* and *TP53* were found to be significantly downregulated in U343 glioma cells after exposure to 10 Gy X-irradiation. In contrast, the expression of the oncogene *C-MYC* was increased in irradiated shRNA-PER2 U343 cells. Changes in mRNA expression were found to correlate with similar changes in immunoreactive proteins detected by western analysis (Figure 5A and 5B).

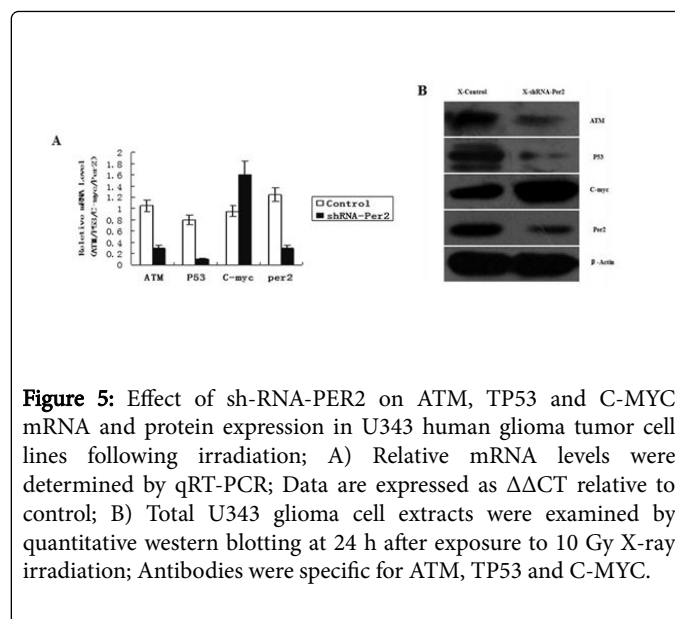


Figure 5: Effect of sh-RNA-PER2 on ATM, TP53 and C-MYC mRNA and protein expression in U343 human glioma tumor cell lines following irradiation; A) Relative mRNA levels were determined by qRT-PCR; Data are expressed as $\Delta\Delta CT$ relative to control; B) Total U343 glioma cell extracts were examined by quantitative western blotting at 24 h after exposure to 10 Gy X-ray irradiation; Antibodies were specific for ATM, TP53 and C-MYC.

Discussion

In recent years, research of the core clock circadian gene *PER2* has revealed its contribution to the pathology of diseases such as liver cancer, breast cancer, colon cancer, lymphoma, and glioma. We have investigated the role of *PER2* in glioma. Evidence from initial research implicated *PER2* as a regulator of the circadian clock [14,23-25]; more recently evidence has expanded on the function of *PER2* function to include the promotion of apoptosis in cancer [9,11]. We hypothesized that *PER2* is also a tumor suppressor gene.

Experimental evidence from our previous studies indicated that the expression of *PER1* and *PER2* closely correlates with the incidence and development of glioma. Moreover, biological rhythm has also become an important prognostic indicator of survival of glioma patients [6]. However, our data from C6 glioma cells studied *in vitro* also indicated its involvement. Following administration of radiation at different time points, rhythm points expressing high levels of *PER2* showed a death rate significantly higher than other points. C6 cells transfected with plasmid pcDNA3.1 (+) *PER2* were mostly arrested at G2/M phase, which downregulated proliferation and increased apoptosis of glioma cells. In addition, we detected upregulation of PCNA expression following downregulation of *PER2*, which further verified that *PER2* and PCNA are both closely related to the degree of differentiation of glioma. All of the above data suggested that *PER2* plays an important role in promoting apoptosis of glioma cancer cells [20]. These findings are consistent with our data in human glioma cancer specimens [6].

As for the specific mechanisms, apoptosis was induced by X-ray after DNA damage. When DNA damage occurs, it continues to activate several checkpoints such as *ATM/ATR*, *CHK1/2*, and so on [21-22]. Then, *TP53*, an important protein in the regulation of DNA damage and repair and the cell cycle also is activated [26-28], which results in the arrest of the cell cycle at G1/S phase, S or G2/M phase so as to provide sufficient time for repair of DNA damage [21,22]. If DNA damage is not properly repaired, chromosomal deletions, duplications or translocations can occur, thus *TP53* protein will instantly activate *P21 (CDKN1A)*, *BAX* and other related genes to prevent gene mutation and carcinogenesis [21,22,26-28]. In addition, *TP53* is also

connected to promoter binding of the oncogene *C-MYC* and inhibits its transcription through promoting deacetylase [29]. Conversely, *TP53* in the cytoplasm can directly act on pro-apoptotic *BCL* family members to enhance transduction of apoptotic signals [10].

In this study, we focused on how *PER2* is involved in DNA damage, apoptosis and radiosensitivity of glioma cells induced by X-ray irradiation. After comprehensively surveying the links between *PER2* and *TP53* in previous reports [2,4,9-15], we selected wild-type *TP53* human U343 glioma cells, then RNA interference technology and shRNA-lentivirus were used to downregulate *PER2*. However, we found that following treatment with 6 mV, 100cGy of X-ray irradiation, DNA damage was observed in both two groups after 24 h, and shRNA-*PER2*-transfected cells showed more serious DNA tailing phenomena and significantly increased death rate compared with control cells. Following exposure to X-ray irradiation, U343 cells with low *PER2* expression levels had serious DNA damage and a higher death rate. We mainly focused on the mechanism by which *PER2* regulates the apoptosis of U343 cells through the ATM-TP53 pathway to initiate DNA damage repair. Unexpectedly, the important checkpoint, *ATM*, in DNA damage should be activated a large number when exposure to X-ray irradiation, then start the DNA repair system mediated by TP53, to ensure maximum repair damaged DNA. However, in our research, the expression of *ATM* was significantly decreased in U343 cell with low *PER2* expression levels when exposure to X-ray irradiation, then causing a corresponding declined expression of *TP53*. It shows that in addition to the effect of X-ray irradiation, *ATM* also can be affected by other factors, such as *PER2*, in our experiments, which play the most direct impact factor. We found that ensuring other disturbing factors consistently, downregulation of the expression of *PER2* can decrease expression of activated *ATM* and *TP53*. So we deem it not only involved in the regulation of DNA damage, but regulated cell apoptosis by ATM-TP53 pathway as the upstream gene of *ATM* and *TP53*.

These results indicated the important effect of *PER2* in apoptosis of U343 cells caused by irradiation: On the one hand, downregulation of *PER2* can enhance the radiosensitivity of U343 glioma cells, while expression of important checkpoint in DNA damage, such as *ATM*, was decreased by regulation of *PER2*, which reduced the effect of the ATM-TP53 pathway activated by DNA damage corresponding. Moreover, *C-MYC* is also controlled by the circadian clock [30]; together with the two proteins described above, it can induce the apoptosis of U343 cells.

Conclusions

In summary, it can be considered from our present study that downregulation of *PER2* can increase the sensitivity of U343 glioma cells exposed to X-ray irradiation and promote apoptosis. The mechanism by which it does this can be summarized as changes expression of *PER2*, which can activate the ATM-TP53 pathway and other genes associated with cell cycle arrest and apoptosis caused by X-ray such as *ATM*, *TP53* and *C-MYC*, thus further inducing apoptosis. Therefore, we hypothesize that *PER2*, the core circadian gene, is not only a tumor suppressor gene but can also be regarded as an upstream regulatory gene of *TP53*, which plays an important role in inhibiting tumor growth and promoting apoptosis of cancer cells by regulating *TP53* expression, DNA damage repair and apoptosis. These findings provide a new target for the clinical treatment of glioma, and a reliable basis for post-radiation therapy and gene therapy for glioma and other cancers.

Conflict of Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled "Down-regulation of PER2 increases apoptosis of U343 glioma cells after X-ray irradiation by ATM-TP53 pathway".

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