Tropical Journal of Pharmaceutical Research June 2023; 22 (6): 1205-1210
**ISSN:** 1596-5996 (print); 1596-9827 (electronic)


Available online at http://www.tjpr.org
**http://dx.doi.org/10.4314/tjpr.v22i6.9**

**Original Research Article**

# Period circadian regulator 3 (PER3) enhances sensitivity to radiotherapy in gastric cancer via Wnt/β-catenin pathway

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*Sent for review: 27 February 2023* *Revised accepted: 28 May 2023*

## Abstract

***Purpose:*** *To investigate a novel radiotherapeutic biomarker for gastric cancer (GC) treatment.*
***Methods:*** *Radioresistant gastric cancer cells (NCI-N87-RR and MKN45-RR) were established using Gy. Protein levels were determined using western blots. Clone formation was evaluated and cell viability assessed by cell counting kit-8 (CCK-8). Apoptosis was determined by flow cytometry.*
***Results:*** *Period circadian regulator 3 (PER3) was down-regulated in both cell lines (NCI-N87-RR and MKN45-RR). Moreover, PER3 over-expression enhanced sensitivity to radiation in radioresistant gastric cancer cells. Cell proliferation was inhibited, while PER3 promoted cell apoptosis. Furthermore, PER3 over-expression suppressed β-catenin and cellular myelocytomatosis oncogene (c-myc) and enhanced axin protein level induced by Gy, regulating transduction of Wnt/β-catenin signaling. In addition, PER3 contributed to the sensitivity of drug-resistant GC cells to cisplatin.*
***Conclusion:*** *Period circadian regulator 3 enhances the sensitivity of GC to radiation through Wnt/β-catenin signaling pathway, providing a potential therapeutic strategy for the management of GC.*

***Keywords:*** *Gastric cancer, Radiation sensitivity, Radiotherapy, PER3, Wnt, β-catenin*



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## INTRODUCTION

Gastric cancer (GC) is known as the one of the most common malignant tumors of the digestive system all over the world [1,2]. The incidence of GC is at 42 % in East Asian countries [3]. Approximately 720,000 people die every year of GC [4]. To date, tumor diagnosis, surgical techniques, and novel molecular drugs targeting GC have significantly improved. However, the total five-year survival rate of GC patients is poor [5]. Chemo-radiotherapy is an effective method for GC treatment and has been found to improve survival rate and local effect [6]. However, a serious concern is the occurrence of radio-resistance, and therefore the application of radiotherapy is clinically limited [7]. Thus, it is necessary to identify a novel radiotherapeutic biomarker for enhancing the efficacy of radiotherapy for GC treatment.

The period genes (PER1, PER2, and PER3) regulate circadian rhythm. Circadian rhythm contributes to the physiological and pathological processes of tumor development [8]. Accumulating evidence has shown that circadian

clock genes (PER1 and PER2) promote sensitivity of human tumor cells to apoptosis via DNA damage [9]. Moreover, period circadian regulator 3 (PER3) has a role in carcinogenesis-related cellular processes [10]. A decrease in PER3 expression has also been observed in tumors in colon cancer [10]. Period circadian regulator 3 polymorphisms also play an essential role in various cancers. A previous study revealed that PER3 could inhibit the sternness of prostate cancer stem cells in the tumor microenvironment [11]. However, the relationship between PER3 and GC sensitivity to radiation is unclear.

The effects of PER3 on the sensitivity of gastric cancer cell lines to radiation was investigated, thus providing a new target for GC treatment.

## EXPERIMENTAL

### Cell culture and treatment

Gastric cancer cells (NCI-N87 and MKN45) were purchased from Peking Union Medical College PMUC (Beijing, China) and grown in RPMI-1640 containing 10% fetal bovine serum (FBS). Cisplatin (CDDP) was harvested from Sigma (St. Louis, MO, USA). Cells were exposed to CDDP at concentrations of 0.1, 1, 5, 10, 20, 40, 80, and 160 μM prior to use.

### Establishment of radio-resistant GC cells

Radio-resistant GC cells (NCI-N87-RR as well as MKN45-RR) were subjected to 2-Gy X-rays at 300 Gy/min (at an irradiation range of 20 × 20 cm). The total exposure dose and time were 60 Gy and 6 months, respectively. After stable passage, a colony-formation assay was used to analyze the radio-resistance of the RR cells. The cells were thereafter seeded in six-well plates, and then exposed to various radiation doses (0, 2, 4, and 6 Gy). Fixing was done using 4 % paraformaldehyde after irradiation for ten days and stained using 0.5 % crystal violet. Colonies were photographed and counted. Plating efficiency (PE) and survival fraction (SF) was calculated using Eqs 1 and 2.

$$PE = (Cn/N)100 \quad \ldots\ldots\ldots\ldots (1)$$

$$SF = (Cc/Cs)(PE/100) \quad \ldots\ldots\ldots\ldots (2)$$

where Cn is colony number, N is the number of inoculated cells, Cc is colonies counted and Cs is the number of cells seeded.

Colonies and SF of the PER3 protein overexpressed in radio-resistant GC cells by exposure to 4 Gy-radiation dose was also determined.

### Western blot analysis

Total protein was isolated by radioimmunoprecipitation assay (RIPA) lysis buffer. The samples (30 - 50 μg) were loaded into sodium doceyl-sulfate polyacrylamide gels. After blocking with 5 % skim milk at 37 °C for 1 h, membranes were covered with primary antibodies, including PER3 (1:1000), β-catenin (1:1000), Axin (1:1000), c-myc (1:1000), and GAPDH (1:5000) (all from Abcam, Cambridge, MA, USA) at 4 °C overnight. Subsequently, secondary antibody was used to incubate the membranes. Signals were determined with enhanced chemiluminescence substrate (Takara, Dalian, China). The bands were visualized via Image J software.

### Immunofluorescence

The cells were fixed with 4 % paraformaldehyde, treated and blocked with Triton X-100 and goat serum respectively. Cells were then covered with primary antibody against γ-H2AX or RAD51 (1:200, all from CS) for 2 h, and incubated with the secondary antibody along with 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining. Finally, cells were visualized via a confocal laser scanning microscope.

### Cell counting kit-8 (CCK-8)

A CCK-8 kit (Beyotime, Shanghai, China) was used to analyze cell viability. Cells were seeded into 96-well plates, and 10 μL of CCK-8 solution was added into each well. After a 3h incubation period, the optical density value was examined using an MRX II microplate reader at 450 nm. The 50 % inhibiting concentration ($IC_{50}$) value was calculated.

### Cell apoptosis assay

The cells were harvested and re-suspended using 100 μL of a binding buffer. To stain the cells, 5 μL of both Annexin V-FITC and propidium iodide (PI) were added to the tube for 15 min. Cell apoptosis was then determined with a flow cytometer.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0. Data are presented as mean ± standard deviation (SD). Unpaired *t*-test, one-way ANOVA and Tukey's test were used to

evaluate statistically significant differences. $P < 0.05$ was considered statistically significant.

## RESULTS

### Radio-resistant gastric cancer cells down-regulates PER3

There was a dose-dependent decrease in clone formation in GC cells and radio-resistant GC cells exposed to Gy. Additionally, the SF in GC cells was lower compared to radio-resistant GC cells (Figure 1 A). Western blot verified that the expression of PER3 was decreased in both NCI-N87-RR as well as MKN45-RR cells (Figure 1 B). These results indicate that PER3 is down-regulated in radio-resistant GC cells.

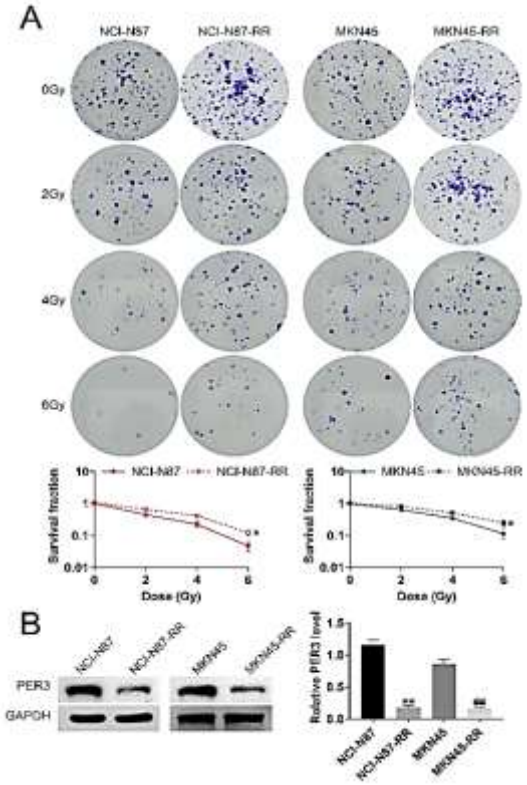


**Figure 1:** PER3 is downregulated in radio-resistant gastric cancer cells. (A) Clone formation assay used to determine clone formation and SF determined. (B) the expression of PER3 and GAPDH protein analyzed using western blot. Number of replicates = 3. *$P < 0.05$, **$p < 0.01$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, *vs NCI-N87, $^{\#}$vs MKN45

### PER3 contributes to sensitivity of radio-resistant GC cells to radiation

The PER3 over-expression vector was transfected into radio-resistant GC cells (NCI-N87-RR and MKN45-RR). There was a dose-dependent down-regulation in clone formation in radio-resistant GC cells treated with Gy. The SF in GC cells transfected with PER3 was high (Figure 2 A). Immuno-fluorescence analysis of the radio-resistant GC cells showed that PER3 over-expression or Gy treatment increased γ-H2AX and decreased RAD51 expression. Additionally, PER3 over-expression promoted Gy-induced γ-H2AX and inhibited Gy-caused RAD51 expression (Figure 2 B). These data suggest that PER3 enhances the sensitivity of radio-resistant GC cells to radiation.

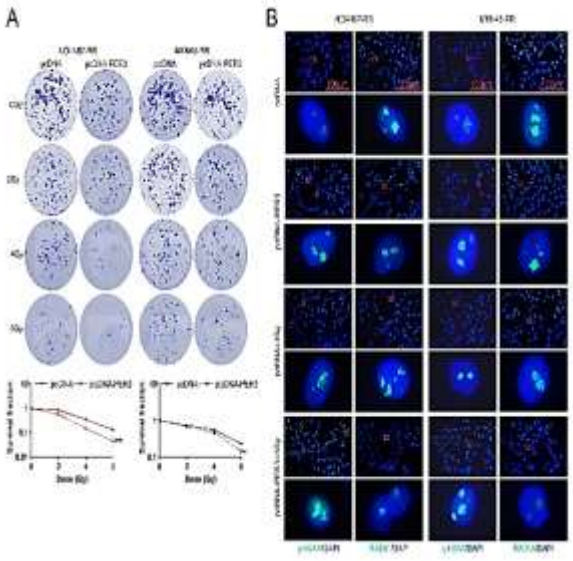


**Figure 2:** PER3 promotes the sensitivity of radioresistant GC cells to radiation. (A) Clone formation measurement and SF analysis were conducted using clone formation assay. (B) Immunofluorescence was used to determine γ-H2AX and RAD51 expression. Number of replicates = 3. *$P < 0.05$ **$p < 0.01$

### PER3 facilitates apoptosis of radio-resistant gastric cancer cells

Over-expression of PER3 or Gy treatment suppressed cell viability in radio-resistant GC cells (NCI-N87-RR and MKN45-RR). In contrast, PER3 over-expression further inhibited the Gy-induced decrease in cell viability (Figure 3 A). Flow cytometry verified that PER3 over-expression or Gy treatment promoted cell apoptosis in radio-resistant GC cells. Gy-induced cell apoptosis was further enhanced by PER3 over-expression (Figure 3 B). These results imply that PER3 mediates cell apoptosis in radio-resistant GC cells.

### PER3 regulates Wnt/β-catenin signaling pathway

Over-expression of PER3 or Gy treatment in NCI-N87-RR inhibited β-catenin and c-myc

protein expressions and promoted axin protein expression. Additionally, PER3 over-expression repressed Gy-induced down-regulation of β-catenin and c-myc and enhanced Gy-induced axin protein levels. The results were also replicated in MKN45-RR. The results indicate that PER3 regulates transduction of Wnt/β-catenin signaling (Figure 4).

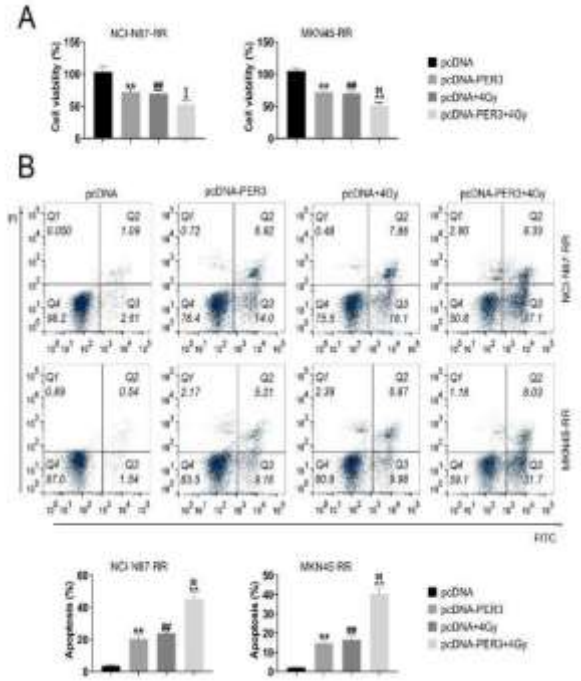


**Figure 3:** PER3 facilitates cell apoptosis in radioresistant gastric cancer cells. (A) Cell viability was evaluated using CCK-8 assay. (B) Cell apoptosis was analyzed with flow cytometry. Number of replicates = 3. ***P* < 0.01, $^{\#\#}p$ < 0.01, ^^*p* < 0.01, $^{\$\$}p$ < 0.01, *vs pcDNA, $^{\#}$vs pcDNA, ^vs pcDNA, $^{\$}$vs pcDNA + 4Gy

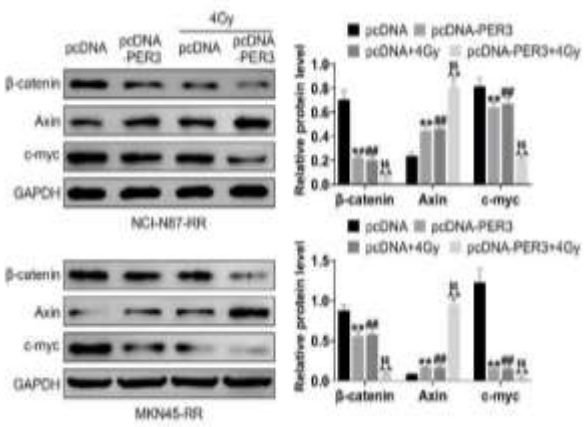


**Figure 4:** PER3 regulates transduction of Wnt/β-catenin signaling β-catenin, Axin, c-myc, as well as GAPDH protein levels were analyzed using western blot. Number of replicates = 3. ***P* < 0.01 $^{\#\#}p$ < 0.01, ^^*p* < 0.01, *vs pcDNA, $^{\#}$vs pcDNA, ^vs pcDNA, $^{\$}$vs pcDNA + 4Gy

### PER3 facilitates the sensitivity of drug-resistant GC cells to CDDP

The CCK-8 assay showed an increase in cell viability and $IC_{50}$ upon CDDP treatment (Figure 5A). Furthermore, PER3 over-expression facilitated $IC_{50}$ value in NCI-N87 as well as MKN45 cells exposed to CDDP (Figure 5 B). These findings imply that PER3 stimulates the sensitivity of drug-resistant GC cells to CDDP.

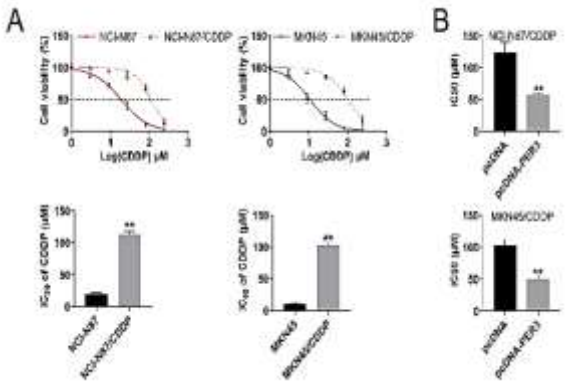


**Figure 5:** PER3 facilitates the sensitivity of drug-resistant GC cells to CDDP. (A) CCK-8 assay was applied for cell viability and $IC_{50}$ value was measured. (B) $IC_{50}$ was determined and analyzed using the CCK-8 assay; n = 3. **P* < 0.05, ***p* < 0.01

## DISCUSSION

This study investigated the role of PER3 in the sensitivity of GC to radiation in radio-resistant GC cell lines (NCI-N87-RR, and MKN45-RR). The study revealed that PER3 is down-regulated in radio-resistant GC cells, and enhances the sensitivity of the cells to radiation. Cell apoptosis was also increased in radio-resistant GC cells upon PER3 over-expression. These results showed that PER3 functions in the transduction of Wnt/β-catenin signaling.

Previous study reported that PER3 reverses paclitaxel resistance in paclitaxel-resistant prostate cancer cells via down-regulating the notch pathway, decreased proliferation, and aided apoptosis [12]. Additionally, PER3 over-expression has been shown to reduce chemo-resistance of colorectal cancer stem-like cells by suppressing notch and β-catenin signaling [13]. In this study, PER3 expression was repressed in radio-resistant GC cells, indicating that PER3 regulated the chemo-resistance of radio-resistant GC cells. Additionally, PER3 promoted the sensitivity of cells to radiation and facilitated cell

apoptosis in radio-resistant GC cells. These results indicate that PER3 may exert critical roles in oncogenesis and radio-resistance.

Evidence has shown that Wnt signaling regulates biological processes, including proliferation, migration, differentiation, as well as stem cell self-renewal [14]. The Wnt signaling pathway contains the canonical and non-canonical pathways. It has been reported that the canonical Wnt signaling pathway is referred to as the Wnt/β-catenin signaling pathway [15]. Wnts suppressed degradation of β-catenin, stabilized β-catenin, as well as contribute to β-catenin accumulation, regulating downstream target genes [16].

In the Wnt/β-catenin pathway, abnormal Wnt/β-catenin signaling is closely associated with the oncogenesis of several cancers. For example, Chen *et al* [17] found that lncRNA NEAT1 promote glioblastoma progression by regulating Wnt/β-catenin signaling. Moreover, Wnt/β-catenin signaling has been found to influence cancer radio-resistance by mediating DNA damage repair [18]. Thus, the Wnt/β-catenin signaling may play a vital role in the oncogenesis and radio-resistance of cancers. Additionally, via Wnt/β-catenin signaling, PER3 was found to negatively regulate the sternness of prostate cancer stem cells [11].

The over-expression of PER3 further inhibited Gy-induced β-catenin and c-myc protein expression and promoted Gy-induced axin protein expression, indicating that PER3 participated in regulating the transduction of Wnt/β-catenin signaling. Ciplastin (CDDP) treatment contributed to cell viability and $IC_{50}$, and PER3 over-expression was found to enhance $IC_{50}$ in GC cells exposed to CDDP. These data imply that PER3 controls the sensitivity of GC to radiation via regulation of Wnt/β-catenin pathway, thereby facilitating the sensitivity of drug-resistant GC cells to CDDP.

## CONCLUSION

The findings of this study reveal the effects of PER3 on the sensitivity of GC to radiation, apoptosis, and proliferation in radio-resistant gastric cancer cells. Period circadian regulator 3 (PER3) promotes the sensitivity of GC to radiation by regulating Wnt/β-catenin pathway. It further contributes to the sensitivity of drug-resistant GC cells to CDDP, providing a new therapeutic strategy for treating patients with GC. The role of PER3 radioresistant GC cell lines (NCI-N87-RR, and MKN45-RR) was done *in vitro* and not clinically. Since other signaling pathways may also be affected by PER3 in GC, it is essential to investigate the mechanism underlying PER3 in regulating the sensitivity of GC to radiation in further experiments.

## DECLARATIONS

***Acknowledgements***

None provided.

***Funding***

None provided.

***Ethical approval***

None provided.

***Availability of data and materials***

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

***Conflict of Interest***

No conflict of interest associated with this work.

***Contribution of Authors***

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors contributed to conception and design. Material preparation and experiments were performed by Zhiyi Shangguan. Qian Zhang and Tian Luan performed data collection and analysis. The first draft of the manuscript was written by Hongtao Hu, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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