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Functional Interplay Between TDR1 and the XRCC5-Mediated DNA Repair Pathway in Tardigrades under Ionizing Radiation Exposure

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Abstract. Tardigrades exhibit exceptionally prominent tolerance to ionizing radiation, while the functional correlation between the specific protein TDR1 and XRCC5, a core factor in their DNA repair pathway, remains unclear. In this study, Hypsibius exemplaris was selected as the model organism. Optimal experimental conditions were screened through radiation gradient experiments, and combined with CRISPR/Cas9 gene knockout technology, the mRNA expression of TDR1 and XRCC5, their protein levels, and the number of γ-H2AX foci (a DNA damage marker) were detected to investigate the synergistic mechanism of the two in radiation resistance. The results showed that 2000 Gy γ-rays and 4 h post-irradiation were the optimal conditions, under which both TDR1 and XRCC5 were significantly upregulated. Knockout experiments demonstrated that XRCC5 plays an essential role in the repair pathway, while TDR1 acts as its auxiliary factor; the deficiency of either protein induces compensatory upregulation of the other at both transcriptional and translational levels, confirming a bidirectional synergistic regulatory mechanism. This study breaks through the previous research limitation of focusing on the single function of stress-resistant proteins in tardigrades, clarifies the synergistic mechanism between TDR1 and XRCC5, and lays a foundation for the research on the extreme environmental adaptation mechanism of tardigrades.

Keywords: Tardigrade (Hypsibius exemplaris), Radiation resistance, TDR1, XRCC5, CRISPR/Cas9

1. Introduction

1.1. DNA damage induced by ionizing radiation

Ionizing radiation (IR) damages cellular DNA through direct and indirect effects, posing a significant threat to the health and life of organisms. This threat exists not only in extreme environmental fields such as space exploration but also in daily life-related scenarios including clinical radiotherapy and the nuclear industry. A deep understanding of the mechanisms of radiation damage is fundamental to addressing these challenges.

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The direct effect involves high-energy particles acting on DNA, causing single-strand breaks (SSBs) and double-strand breaks (DSBs)—the latter leads to chromosomal aberrations, genomic instability, or cell death, and is the most lethal type of damage due to its difficulty in repair [1]. The indirect effect is mediated by reactive oxygen species (ROS) produced from water radiolysis; ROS diffuse and attack DNA, causing base oxidation and exacerbating strand breaks, significantly reducing repair efficiency [2].

Overall, among these types of damage, the repair efficiency of DSBs directly determines the upper limit of an organism's radiation resistance.

1.2. Radiation resistance of tardigrades

1.2.1. The extraordinary tolerance of tardigrades

Among known multicellular organisms, tardigrades exhibit particularly remarkable radiation resistance. Previous studies have confirmed that typical tardigrade species, such as Hypsibius exemplaris and Ramazzottius varieornatus, can withstand γ-ray irradiation doses of 3000–5000 Gy [3], which is approximately 1000 times the lethal dose for humans. Even more astonishingly, they maintain genomic stability and reproductive capacity after exposure to such high radiation doses [4]. These observations raise a scientific question: what mechanisms enable tardigrades to achieve such robust DNA damage repair?

1.2.2. Classical repair pathways

Eukaryotes primarily rely on two DNA repair pathways to combat radiation: non-homologous end joining (NHEJ) and homologous recombination (HR). The former rapidly ligates double-strand break (DSB) ends through DNA end-binding proteins Ku70/XRCC6 and Ku80/XRCC5, while the latter relies on RAD51 and RPA proteins to mediate strand invasion of homologous templates, thereby achieving accurate DSB repair with sister chromatids as templates [5,6]. However, in mammals, the DNA repair system exhibits a clear dose threshold in response to radiation. Gradient dose studies indicate that when radiation doses reach 10 Gy, some DSBs cannot be effectively repaired, leading to mitotic catastrophe and cell death [7]. Additionally, the transcriptional response in mammals is extremely limited, with post-radiation upregulation typically only reaching 1.5–4 fold, making it insufficient to cope with ultra-high dose damage [8].

This finding stands in stark contrast to the extreme tolerance observed in tardigrades. Notably, tardigrades retain the classic NHEJ core factor XRCC5/Ku80, and its transcript levels are enriched up to 315-fold after radiation exposure. RNAi-mediated knockdown of XRCC5 drastically reduces tardigrade survival, directly demonstrating that high expression of XRCC5 plays a critical role in their radiation resistance [8].

However, this does not fully explain the extreme radiation resistance of tardigrades. Since XRCC5 did not function completely in heterologous expression experiments in E. coli [8], it may suggest that the function of XRCC5 is not carried out independently. Combined with recent studies revealing that the TRID1 protein carried by tardigrades dynamically enhances the function of repair complexes by recruiting repair factors through phase separation [9], the strong upregulation of XRCC5 suggests that tardigrades may have evolved certain cofactors that, by cooperating with DNA repair pathways, achieve efficient repair of ultra-high-dose damage.

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1.2.3. TDR1

Recent studies have reported a tardigrade-specific protein called TDR1. This protein not only shows strong upregulation after irradiation in tardigrades but also binds to DNA in vitro and colocalizes with nuclear DNA in vivo. Moreover, TDR1 orthologs are present in three tardigrade species and are significantly expressed after IR exposure. This evolutionary conservation suggests that TDR1 may play an important functional role in radiation resistance [10].

1.3. Project description

1.3.1. Research question

We aim to investigate: whether TDR1 and XRCC5 are merely in simple co-expression or function synergistically? Whether the loss of TDR1 affects the expression of XRCC5, and whether it impacts the efficiency of DNA repair?

1.3.2. Hypothesis

By utilizing CRISPR/Cas9 knockout technology [11], a tardigrade model with TDR1 and XRCC5 knockouts was established to detect the mRNA levels, protein levels, and γ-H2AX signals of TDR1 and XRCC5, and to investigate the synergistic relationship between TDR1 and the classical repair factor XRCC5.

2. Methods

2.1. Radiation gradient experiment

2.1.1. Tardigrade culture

In this experiment, Hypsibius exemplaris will be adopted as the model organism, with rearing conditions set at 16°C, a light cycle of 14 hours of light and 10 hours of darkness, and microalgae as the food source. For large-scale centralized cultivation, the bottom of untreated Petri dishes can be pre-coated with 0.2% linseed oil emulsion and 4% (+/-)-α-tocopherol, followed by the addition of algal solution and water [10,12].

2.1.2. Tardigrade radiation treatment

Cultured tardigrades will be irradiated with γ-rays using a ^{137}Cs radiation source at a dose rate of 6.04 Gy/min. We will set up two dose groups: 0 Gy, 100 Gy, and 2000 Gy. Samples will be collected at 4 hours and 24 hours post-irradiation for analysis [4].

2.1.3. Gene expression analysis

Total RNA will be extracted from samples and subjected to quality control. Qualified RNA samples will be reverse-transcribed into cDNA. Specific primers will be designed for the target genes XRCC5 and TDR1 [13,10], with actin selected as the reference gene. Amplification will be performed on a real-time quantitative PCR instrument. After the reaction, Ct values of each sample will be exported from the instrument to correct for differences in cDNA input among samples. The

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relative expression abundances of the two target genes will be calculated, followed by statistical analysis and comparison.

2.2. Gene knockout experiment

2.2.1. Construction of gene knockout strains

We will design two gRNA targets for each of the TDR1 and XRCC5 genes, targeting specific exon regions. Cas9 ribonucleoprotein (RNP) complexes will be prepared following the method described by Kumagai et al. [14]. Healthy 7–10-day-old adult female *H. exemplaris* will be selected, and Cas9 RNP complexes will be injected into the ventral body cavity using a microinjection system. The injected individuals will then be transferred to fresh culture medium for recovery, and surviving individuals will be screened 1 hour later [11]. Eggs laid by these individuals will be collected and cultured to generate offspring. Small tissue samples from offspring will be obtained via microdissection, and DNA will be extracted for Sanger sequencing to screen for successfully gene-edited individuals for subsequent experiments.

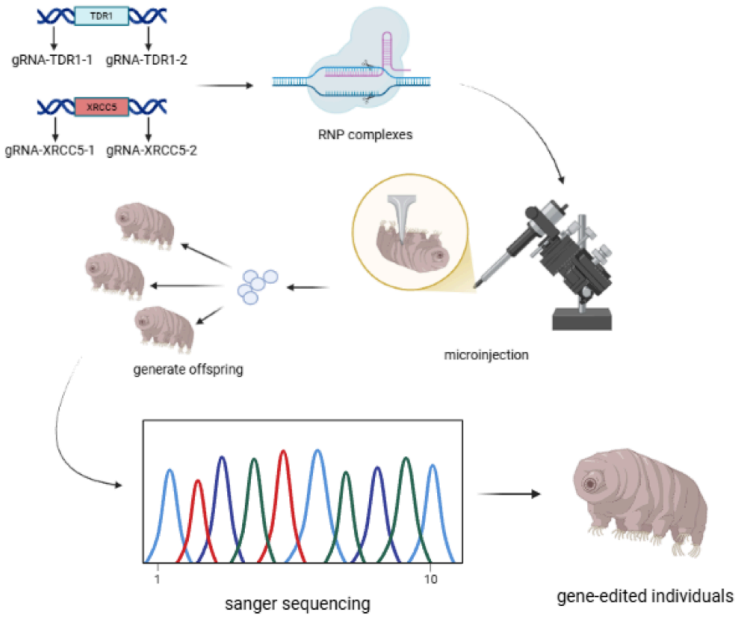

Figure 1. Experimental procedure for TDR1/XRCC5 gene editing in H. exemplaris

2.2.2. TDR1 knockout experiment

An experimental group (TDR1⁻), a wild-type control group, and a negative control group (injected with empty RNP) will be established. The experimental group will be irradiated with 2000 Gy as described above, and samples from the three groups will be collected at 4 hours for analysis.

Some samples will be fixed on slides, incubated with anti-phosphorylated H2AX antibody for 1.5 hours, followed by incubation with fluorescently labeled secondary antibody. After washing, nuclear staining will be performed, and slides will be mounted. Imaging will be conducted using a confocal microscope, and the number of γ-H2AX foci per nucleus will be counted.

mRNA expression levels of XRCC5 in some samples will be detected as described previously. Proteins from remaining samples will be extracted following the protocol by Schokraie et al. (2010)

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for Western blot assay, and fluorescent signals will be acquired using a chemiluminescence imaging system. With actin as the reference, band gray values will be analyzed, and the ratio of XRCC5 to β-actin gray values will be used as the relative expression level for comparison among the three groups [15].

2.2.3. XRCC5 knockout experiment

An experimental group (XRCC5^{-}), a wild-type control group, and a negative control group (injected with empty RNP) will be established. The above procedures will be repeated to count the number of γ-H2AX foci per nucleus and detect the mRNA and protein expression levels of TDR1.

3. Expected results

3.1. Predicted results of the radiation gradient experiment

3.1.1. Effect of radiation dose on the expression of TDR1 and XRCC5

We predict that after 100 Gy radiation treatment, the relative expression abundances of the two genes will show no significant change compared with the control group. We hypothesize that this dose does not reach the DNA damage threshold required to trigger a clear response of TDR1 and XRCC5. In contrast, after 2000 Gy radiation treatment, the relative expression abundances of both TDR1 and XRCC5 will be significantly upregulated, with the upregulation magnitude of XRCC5 possibly higher than that of TDR1. This indicates that 2000 Gy can capture the response signal more accurately and is suitable as the condition for subsequent experiments.

3.1.2. Effect of detection time on the expression of TDR1 and XRCC5

We predict that under the 2000 Gy dose, the relative expression abundances of TDR1 and XRCC5 will be relatively high at 4 hours post-radiation. However, at 24 hours post-radiation, the relative expression abundances of XRCC5 and TDR1 will decrease compared with those at 4 hours. We hypothesize that by 24 hours, the DNA repair process of tardigrades will have been initiated for a period of time, and part of the DNA damage will have been repaired—this leads to a reduced response intensity of repair-related genes, which is not conducive to accurately capturing the response signal. Based on the predicted results of dose and time, we will select 2000 Gy and 4 hours as the conditions for subsequent knockout experiments.

3.2. Predicted results of the gene knockout experiment

3.2.1. Predicted results of the TDR1 knockout experiment

The number of γ-H2AX foci per cell nucleus in the TDR1^{-} experimental group should be higher than that in the wild-type control group and the negative control group. This suggests that after TDR1 gene knockout, the efficiency of DNA double-strand break repair in tardigrades decreases under moderate to high radiation doses, confirming that TDR1 is involved in the radiation-induced DNA damage repair process of tardigrades.

The relative expression level of XRCC5 in the TDR1^{-} experimental group should be slightly higher than that in the wild-type control group and the negative control group. We hypothesize that

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after TDR1 deletion, tardigrades compensate by upregulating the transcriptional level of XRCC5 to partially make up for the deficiency in DNA repair function

The relative expression level of XRCC5 protein in the TDR1⁻ experimental group should also be higher than that in the wild-type control group and the negative control group, which is consistent with the compensatory upregulation trend of XRCC5 mRNA.

3.2.2. Predicted results of the XRCC5 knockout experiment

The number of γ-H2AX foci per cell nucleus in the XRCC5⁻ experimental group should be significantly higher than that in the wild-type control group and the negative control group, with a greater magnitude of upregulation than that in the TDR1⁻ experimental group. This is because XRCC5 is a core gene in the DNA repair pathway, and its deletion will lead to severe impairment of DNA double-strand break repair ability.

The relative expression level of TDR1 in the XRCC5⁻ experimental group should be higher than that in the wild-type control group and the negative control group. We hypothesize that after XRCC5 deletion, TDR1—acting as an auxiliary factor in DNA repair—may participate in damage repair through compensatory upregulation.

The relative expression level of TDR1 protein in the XRCC5⁻ experimental group should be higher than that in the wild-type control group and the negative control group, which is consistent with the expression trend of TDR1 mRNA.

4. Discussion

4.1. Functional division of labor and synergistic mechanism between TDR1 and XRCC5

Regarding the functional division of labor, the magnitude of upregulation of γ-H2AX foci number in the XRCC5⁻ experimental group is significantly higher than that in the TDR1⁻ experimental group, which confirms the core role of XRCC5 in DNA repair. In contrast, after TDR1 knockout, the number of γ-H2AX foci increases but with a relatively low magnitude of increase, accompanied by the compensatory upregulation of XRCC5—this suggests that TDR1 may act as an "auxiliary factor" in the repair pathway.

In terms of the synergistic mechanism, the deletion of TDR1 leads to the upregulation of XRCC5 at both transcriptional and protein levels, while the deletion of XRCC5 results in the compensatory expression of TDR1. This indicates that when the function of a gene in the repair pathway is impaired, other genes compensate for pathway defects by enhancing their expression. This synergistic mechanism may be a crucial adaptive strategy for tardigrades to cope with extreme radiation environments.

4.2. Research limitations

Currently, stress-resistant proteins of tardigrades such as Dsup and CAHS have been identified, and their individual functions are clearly understood [16,17]. Our study aims to break through the limitations of studying individual proteins and instead explain the radiation resistance of tardigrades from the perspective of dynamic synergistic regulation.

However, although the experimental design of this study refers to existing literature and the physiological characteristics of tardigrades, there are still several limitations in this research: the current predicted results are based on the "complete knockout" assumption, but in actual experimental operations, insufficient knockout efficiency or chimeric individuals may occur; our

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study can only confirm the synergistic effect between TDR1 and XRCC5, yet the specific molecular mechanisms remain unclear.

5. Conclusion

This study focuses on the model organism Hypsibius exemplaris and explores the functional association between TDR1 and XRCC5 in the DNA repair pathway under ionizing radiation. The radiation gradient experiment identified 2000 Gy radiation dose and 4 hours post-irradiation as the optimal research conditions, under which both TDR1 and XRCC5 exhibit significant transcription. Gene knockout experiments revealed the functional division of labor—with XRCC5 serving as the repair core and TDR1 as an auxiliary factor—and a bidirectional compensatory repair regulatory mechanism.

This study breaks through the previous limitations of researching the single function of stress-resistant proteins in tardigrades and shifts to the level of pathway synergy. However, there are still limitations: the predicted results are based on ideal assumptions, and the specific molecular mechanisms underlying the synergy remain unclear. Future studies need to be further improved by optimizing experimental design and technical methods.

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