Reducing TR4 gene expression enhances the sensitivity of prostate cancer cell lines to olaparib by inhibiting the ATM/ATR pathway

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Abstract
Prostate cancer (PC) has risen to become the second most common neoplasm in men, trailing only lung cancer [1]. Individuals with advanced prostate cancer, particularly those who are resistant to androgen deprivation therapy (ADT), also known as castration-resistant prostate cancer (CRPC), have very few treatment options and a poor prognosis. Notably, advances in phase III clinical trials have highlighted the pronounced efficacy of poly adenosine diphosphate-ribose (ADP-ribose) polymerase (PARP) inhibitors in CRPC patients harbouring homologous recombination repair (HRR)-related genetic aberrations. However, HRR-related mutations occur in only 20–25% of CRPC patients. It is crucial to reduce resistance to PARP inhibitors and make them available to other CRPC patients. Testicular nuclear receptor 4 (TR4), an important molecular player involved in tumorigenesis, metastasis and chemoresistance, has emerged as a focus of investigation. Our study indicated that in prostate cancer cell lines treated with olaparib treatment, TR4 expression increased as olaparib concentrations increased. Targeting TR4 expression can alter the in vitro sensitivity of prostate cancer cell lines to olaparib. Further investigation of the molecular mechanisms revealed that down-regulating TR4 gene expression can enhance the in vitro sensitivity of prostate cancer cell lines to olaparib, which is mediated by the inhibition of the Ataxia Telangiectasia Mutated (ATM)/Ataxia Telangiectasia and Rad3-related (ATR) pathway. This study provides a novel sensitization therapeutic strategy for CRPC patients who are resistant to PARP inhibitors.

Keywords
ATM/ATR pathway; CRPC; DNA damage response; NR2C2; Olaparib; PARP inhibitor; TR4

1. Introduction

Prostate cancer has risen to become the second most common malignancy in men worldwide, trailing only lung cancer [1]. When individuals are ineligible for surgery or experience post-operative recurrence and choose further active treatment, ADT is the primary treatment modality in addition to conventional radiation therapy, with an initial response rate of approximately 80% for metastatic prostate cancer. In recent years, with the development and clinical application of androgen receptor signalling pathway inhibitors (ARSI) such as enzalutamide and apalutamide, ADT + ARSI has gradually replaced ADT alone as the new standard of care for these patients [2, 3]. Despite its effectiveness in the early years of treatment, the inevitable progression of patients to CRPC poses a formidable clinical challenge. Despite recent advances in anti-androgenic chemotherapy development, therapeutic strategies for CRPC have been limited by low efficacy and poor outcomes, necessitating urgent refinements or novel approaches [4].

DNA damage response (DDR) is a critical mechanism for cellular survival that coordinates the repair of damaged cells. In contrast, disrupting the DDR in tumour cells causes cell death as DNA damage accumulates. DNA damage refers to both single-strand breaks (SSBs) and double-strand breaks (DSBs) [5]. PARP, an essential protein family in the DDR, is primarily involved in SSBs repair. PARP inhibitors reduce PARP enzymes catalysis and trapping at the site of DNA damage, impeding DSB repair [6]. The repair of DNA double-strand breaks is carried out via dual pathways: HRR and nonhomologous end-joining repair (NHEJR). ATM, ATR, Breast cancer 1/2 (BRCA1/2), RAD51 Recombinase (RAD51), RAD52 homolog, DNA repair protein (RAD52), and other proteins play critical roles in the HRR process [7]. The proteins involved in the NHEJR process include Ku70, Ku80, X-Ray Repair Cross-Complementing Protein 4 (XRCC4), DNA-PKcs, DNA ligase IV, Artemis and others [8].

Recent investigations have shown that PARP inhibitors are
effective in patients with breast or ovarian cancer who have inherent mutations in the BRCA1 or BRCA2 genes. Deleterious mutations in these genes significantly impair the HRR process in tumour cells, making them more susceptible to PARP inhibitors [9].

This suppresses both the SSB and DSB pathways in tumour cells, resulting in a typical synthetic lethality effect. A synthetic lethality effect occurs when two nonlethal genes are impaired at the same time, resulting in cell death. The cells can survive as long as at least one of these genes is functional [10]. The recent approval of the PARP inhibitor olaparib (OLA) for prostate cancer patients with HRR mutations marks a significant breakthrough [11]. However, the prevalence of HRR-related gene mutations in CRPC remains around 25%, which limits the efficacy of PARP inhibitors in patients who do not have HRR gene defects [12].

TR4, a member of the nuclear receptor superfamily, has been linked to a variety of physiological processes, including reproduction, cerebellar development, the regulation of glucose and lipid metabolism, responses to oxidative stress, and gene damage repair, as revealed by gene knockout mouse studies [13]. Recent research has highlighted the crucial role of TR4 in prostate cancer metastasis and radiotherapy resistance, and its expression levels are closely linked to prognosis [14, 15]. Aberrant TR4 protein levels may also be closely associated with drug sensitivity [16]. This study investigated how TR4 gene expression correlated with the sensitivity of prostate cancer cell lines to PARP inhibitors. We intended to figure out if this correlation is mediated through TR4’s involvement in DNA damage repair, which affects prostate cancer’s response to PARP inhibitors.

2. Materials and methods

2.1 Cell culture and inhibitors

Human prostate cancer cell lines (DU145 and PC3) were obtained from the Cell Bank of the Chinese Academy of Sciences. PC3 cells were grown in RPMI 1640 medium (Servicebio, Wuhan, China) supplemented with 10% fetal bovine serum (FBS; Dakewe, 6021061, Shenzhen, China), whereas DU145 cells were maintained in Dulbecco’s modified eagle medium (Servicebio, Wuhan, China) supplemented with 10% FBS. All cell lines were kept in a 5% CO2 cell incubator at 37 °C.

Olaparib (MedChemExpress, HY-10162, USA) was sourced from MedChemExpress and was stored in a DMSO solution.

2.2 Antibodies

Proteintech (Wuhan) provided antibodies against ATM (27156-1-AP), NR2C2 (20981-1-AP), γH2AX (29380-1-AP), β-actin (66009-1-Ig), ATR (19787-1-AP) and RAD52 (28045-1-AP).

2.3 Quantitative real-time Polymerase Chain Reaction (PCR)

Total RNA from PC cells was carefully extracted using the Cell Total RNA Extraction Kit (19202ES60, Yeasen, Shanghai, China), and Strand cDNA Synthesis SuperMix obtained from Yeasen was used for reverse transcription into complementary DNA (cDNA) according to the instructions provided. To measure the relative expression levels of TR4 and β-actin mRNA, qRT-PCR was performed using 2 × RealStar Fast SYBR qPCR Mix (A301, GeneStar, Beijing, China) and a Slan-965 real-time PCR system (Hongshi, Shanghai). The qPCR primer sequences for β-actin and TR4 were synthesized by General Biol (AnHui) and are as follows:

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\begin{align*}
TR4: & \quad \text{Forward: 5'}-\text{GGCTCTGAACCTGCTCTG-3'} \\
& \quad \text{Reverse: 5'}-\text{AGGATGAACTGCTTGGG-3'} \\
\beta-\text{Actin:} & \quad \text{Forward: 5'}-\text{CAGCTCTCCAGCTTCC-3'} \\
& \quad \text{Reverse: 5'}-\text{GTACAGTCTTTGCG-3'}
\end{align*}
\]

2.4 Western blot analysis

Radio immunoprecipitation assay (RIPA) lysis buffer-strong (20101ES60, Yeasen, Shanghai, China) was used to lyse the harvested cells and extract their proteins. The lysates were treated with a proteinase inhibitor cocktail (G2006, Servicebio, Wuhan, China). The protein concentrations were determined using a Bicinchoninic acid (BCA) protein concentration detection kit (20201ES76, Yeasen, Shanghai, China). After diluting the samples to the same concentration, a quarter volume of loading buffer (G2075, Servicebio, Wuhan, China) was added. Proteins were separated using 8%, 10% and 12.5% sodium dodecyl sulfate-polyacrylamide quick gel electrophoresis (SDS-PAGE) (20325ES62, Yeasen, Shanghai, China). The proteins were transferred to a Polyvinylidene Fluoride (PVDF) membrane (IPVH00010, Immobilon, Shanghai, China) with a pore size of 0.2 μm. Following skim milk blocking and antibody incubation, the protein membrane was visualized with an enhanced chemiluminescence substrate kit (36222ES60, ECL, Yeasen, Shanghai, China) and then exposed to an exposure machine. The grayscale values of the protein membrane were analysed with ImageJ software.

2.5 Silencing and overexpression of TR4

PC cells (1 × 10⁵ cells/well) were placed in a six-well plate, and LipoFectamine 3000 reagent (Invitrogen, L3000001, USA) was used for transfection with TR4-targeting small interfering RNA (siRNA) sequences (General Biol; siTR4: CCGGA-GAAACCAAGAA) or nontargeting siRNA according to the instructions. P3000 reagent (Invitrogen, L3000001, USA) was used to transfect PC cells with TR4-overexpressing plasmids.

2.6 Cell viability assay

The Cell Counting Kit-8 (CCK-8; Yeasen, 40203ES60, Shanghai, China) was used to evaluate cell viability and growth. PC cells were plated at a density of 3 × 10⁵ cells per well in a 96-well plate. The cells were then treated with various concentrations of drug reagents based on experimental design, and cell samples were collected at different time intervals. After removing the cell culture medium from the 96-well plate, a 10% CCK-8 reagent solution made with phosphate-buffered saline (PBS; Yeasen, 41403ES76, Shanghai, China) was added. The cell plate was then incubated at 37 °C in
a 5% CO₂ cell incubator for 3 hours. The optical density was measured at 450 nm with a microplate reader (Thermo Scientific, 1410101, USA).

2.7 Colony formation assay

In the colony formation assay, cells were evenly distributed in a six-well plate, with approximately 800 cells per well. These cells were then incubated for two weeks in a complete medium supplemented with olaparib, and the medium was replenished every 3 days. To visualize and quantify colony formation, cultured cells were fixed in a 4% paraformaldehyde solution, stained with 20% crystal violet, dried and imaged. The acquired results were quantified using ImageJ software.

2.8 Transwell migration assay

The transwell migration assay used Transwell inserts (Corning, USA) with a cell density of 2 × 10⁴ per well. The upper chamber was filled with 200 µL of 1% FBS medium, while the lower chamber provided with 600 µL 20% FBS medium. After 24 to 48 hours of incubation, the cells were fixed with a 4% paraformaldehyde solution, stained with 20% crystal violet, air-dried and examined under a microscope. ImageJ software was then used to quantify the experimental outcomes.

2.9 Statistical analysis

All the experimental data were statistically analysed using GraphPad Prism 9.5.0 software (GraphPad Software, USA). T-tests and one-way analysis of variance (ANOVA) were used based on the composition of the data groups. A p-value < 0.05 indicates statistical significance. The notations for reporting significance levels are as follows: NS, for no statistical significance; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

3. Results

3.1 Relationship between TR4 expression and PC cell lines migration and proliferative capacity

To test the effect of altered TR4 expression levels on the in vitro behaviour of PC cell lines, we used plasmid transfection methods to introduce TR4-cDNA and TR4-siRNA into DU145 and PC3 cells, resulting in cell lines with TR4 overexpression and TR4 knockdown, respectively. TR4 expression changes were validated at the mRNA level using qRT-PCR (Fig. 1A, p < 0.05), followed by protein confirmation via Western blotting (Fig. 1B, p < 0.05). CCK-8 experiments were also performed to assess the proliferative capacity of PC cell lines with TR4 overexpression or knockdown, which revealed that alterations in TR4 expression levels had minimal impact on their proliferative abilities (Fig. 1C, NS). Similarly, colony formation assays yielded consistent results (Fig. 1D, NS). However, in Transwell migration assays evaluating the migratory capabilities of the aforementioned cell lines, we observed a significant increase in migration ability with TR4 overexpression, while lowering TR4 expression levels resulted in decreased migratory capabilities (Fig. 1E, p < 0.05). As a result, for the rational design of future experiments, we used cell proliferative capacity as an indicator to assess the response of PC cell lines to olaparib.

3.2 Concentration-dependent TR4 overexpression in olaparib-treated PC cell lines

We exposed DU145 and PC3 cells to gradient concentrations of olaparib for 48 hours and measured cell sensitivity to different concentrations using the CCK-8 assay. PC cell lines expressed resistance to olaparib, maintaining proliferative activity even at higher concentrations (80 µM) (Fig. 2A). Furthermore, both mRNA and protein levels of TR4 showed concentration-dependent upregulation in response to olaparib treatment (Fig. 2B,C). As a result, we hypothesized that TR4 expression levels are strongly related to the in vitro sensitivity of PC cell lines to olaparib.

3.3 Targeting TR4 expression alters in vitro sensitivity to olaparib

To investigate the potential mediation of PC cell line sensitivity to olaparib by TR4 expression, we transfected DU145 and PC3 cells with plasmids that silence TR4 expression. TR4-silenced PC cell lines showed altered proliferation in the presence of 40 µM olaparib, as demonstrated by colony formation assays (Fig. 2D, p < 0.001). The CCK-8 assays revealed that olaparib has stronger inhibitory effects on the proliferation of TR4-silenced PC cell lines (Fig. 2E, p < 0.05). Furthermore, rescue experiments revealed reintroducing TR4 expression via TR4-cDNA transfection into TR4-knockdown PC cell lines restored olaparib resistance to untreated levels (Fig. 2F, p < 0.01; Fig. 2G, p < 0.01). These results supported the link between TR4 expression and olaparib sensitivity in vitro in PC cell lines.

3.4 TR4 affects the in vitro sensitivity of olaparib via the ATM/ATR pathway

PARP proteins are essential components of DDR, with the primary function of SSBs. The PARP inhibitor olaparib can block the DDR pathway by reducing catalysis or trapping of PARP enzymes at the site of DNA damage [6].

We examined the levels of the DNA damage marker γH2AX in PC cell lines subjected to TR4 knockdown, olaparib treatment, or a combination of both to see if it enhances the sensitivity of prostate cancer cell lines to PARP inhibition by inhibiting the HRR process in the DSB repair pathway. Western blot analysis showed that the combined treatment significantly increased γH2AX expression in the PC cell lines compared to single-drug or TR4 knockdown alone (Fig. 3A–C, p < 0.01).

Given the importance of the ATM/ATR pathway in signal transduction within the HRR pathway and the key role of RAD52 in HRR, we investigated the protein expression of ATM, ATR and RAD52 in the treated cell lines. In PC cell lines subjected to the combined treatment, the expression of the aforementioned proteins was significantly lower than that of the individual treatments (Fig. 3A–C, p < 0.05).

To increase the reliability of our findings, we performed
FIGURE 1. Modulation of TR4 expression and its impact on prostate cancer cell migration and proliferation. (A,B) Examination of changes in TR4 levels in DU145 and PC3 cells at the mRNA and protein levels. (C) Results of continuous 5-day CCK-8 experiments to assess changes in cell viability after targeting changes in TR4 levels in DU145 and PC3 cells. (D) Results of the colony formation assay after adjusting TR4 levels in PC cell lines and cultivating for two weeks. (E) The effect of TR4 targeting changes on DU145 and PC3 cell migration. Cells were incubated in transwell inserts for 24 hours (DU145) and 48 hours (PC3). siNC/siControl, siRNA-negative control; siTR4, siRNA-TR4; OE-NC, overexpression-negative control; OE-TR4, overexpression-TR4; OLA, olaparib; NS, for no statistical significance; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.
**FIGURE 2.** Correlation of TR4 expression with *in vitro* sensitivity to olaparib in PC cell lines. (A) Viability of DU145 and PC3 cells treated with olaparib (0–100 µM) for 48 hours using CCK-8 reagent. (B,C) TR4 expression was measured at mRNA and protein levels in DU145 and PC3 cells cultured in media containing varying concentrations of olaparib (0–60 µM). (D) Silencing the TR4 gene and treating PC cell lines with 40 µM olaparib resulted in changes in proliferative capacity. The colony formation assays were incubated continuously for two weeks. (E) CCK-8 assay to determine changes in cell sensitivity to olaparib gradient concentrations after silencing the TR4 gene in PC cell lines. (F) After two days of treatment with olaparib at a gradient concentration, sensitivity to olaparib in cell lines that re-overexpressed TR4 following TR4 knockdown returned to normal levels as detected by the CCK-8 assay. (G) After 2 weeks of treatment with 40 µM olaparib, colony formation assay revealed that proliferative capacity returned to normal levels in PC cell lines re-overexpressed TR4 following TR4 knockdown. siNC/siControl, siRNA-negative control; siTR4, siRNA-TR4; OE-NC, overexpression-negative control; OE-TR4, overexpression-TR4; OLA, olaparib; NS, for no statistical significance; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.
rescue experiments in which TR4 was re-expressed in TR4-knockdown PC cell lines and co-administered olaparib. The analysis revealed that the protein expression levels of γH2AX, ATM, ATR and RAD52 were restored (Fig. 3D–F, *p < 0.05). In conclusion, our findings suggest that knocking down TR4 can improve the in vitro responsiveness of PC cell lines to the PARP inhibitor olaparib by down-regulating the ATM/ATR pathway.

4. Discussion

Prostate cancer is a malignancy drove by androgens, which play crucial role in its development and progression. In addition to traditional radiotherapy, targeted therapy for androgens and their receptors is an important treatment modality for CRPC, particularly in patients who are unable to undergo radical prostatectomy or who experience postoperative recurrence. The combined usage of ADT and ARSI has become the crucial treatment for advanced-stage prostate cancer [2, 3]. However, a significant number of patients develop CRPC, making endocrine therapy significantly less effective or even ineffective, posing a significant therapeutic challenge in CRPC [17]. Recent advances in personalized cancer treatment include obtaining patients’ unique genetic information via sequencing and tailoring individualized targeted therapy accordingly [18].

PARP inhibitors, which inhibit PARP function in breast and ovarian cancer patients with HRR gene defects, can cause DNA damage to accumulate in tumour cells, resulting in synthetic fatalities. According to the preliminary data from a recent phase 3 clinical trial (PROpel trial), combining the PARP inhibitor olaparib with abiraterone showed significant efficacy in metastatic CRPC (mCRPC) patients with HRR
gene defects [19]. Meanwhile, in the Phase 3 MAGNITUDE and Phase 3 TALAPRO-2 trials, we discovered that niraparib and talazoparib, both PARP inhibitors, demonstrated similar efficacy when combined with ARSI in mCRPC patients [20, 21]. These trials open up new opportunities for developing treatment strategies for CRPC patients. However, HRR gene defects account for approximately one-quarter of CRPC patients, and olaparib has been approved by the Food and Drug Administration (FDA) for use in them in 2020. Olaparib, a PARP inhibitor, has extremely limited efficacy in patients without HRR gene mutations [12]. Furthermore, in the long run, prostate cancer resistance to PARP inhibitors evolves in cancer cells and clinical trials via a variety of mechanisms. As a result, the urgent task at hand is to sensitize patients to PARP inhibitors and broaden their clinical applications.

Our research focused on TR4, a member of the nuclear hormone receptor family, due to its critical role in a variety of physiological processes. Aberrant expression and activity of TR4 can lead to disruptions and defects in several processes, including cell proliferation, embryonic development, cellular tissue differentiation, and cell cycle progression [22]. Notably, TR4 has been linked to prostate cancer metastasis, with clinical specimens demonstrating elevated TR4 expression in high Gleason score samples [22]. TR4 has been shown to influence prostate cancer migration and invasion at the cellular level by regulating C-C Motif Chemokine Ligand 2 (CCL2) or miR-373-3p expression [14]. Other studies have found that downregulating TR4 gene expression can improve prostate cancer sensitivity to chemotherapy and radiotherapy [15, 16]. Thus, TR4 is an important molecule involved in tumorigenesis, tumour metastasis and tumour resistance.

In our study, we first validated previous research findings that showed that targeting changes in TR4 expression significantly altered the migration ability of PC cell lines while having no apparent effect on their proliferation. Following the treatment of PC cell lines with a gradient of olaparib concentrations, we observed that TR4 expression increased with increasing concentrations of olaparib. As a result, by manipulating TR4 expression levels, we demonstrated a link between TR4 and the in vitro sensitivity of PC cell lines to olaparib. We designed experiments and measured the expression levels of relevant proteins, including ATM, ATR, γH2AX and RAD52, to understand the molecular mechanism underlying the increased sensitivity of PC cell lines to the PARP inhibitor olaparib induced by TR4 inhibition. The results indicated that lowering TR4 levels could enhance the in vitro sensitivity of PC cell lines to the PARP inhibitor olaparib by inhibiting the ATM/ATR pathway and suppressing the HRR process, inducing synthetic lethality in tumour cells.

However, it is essential to recognize the limitations of the study. First of all, this article only investigates and demonstrates the cellular mechanism by which TR4 increases the sensitivity of PC cell lines to PARP inhibitors. These experimental results still need to be validated through animal experiments to increase the reliability of the conclusions. Secondly, we used the androgen receptor negative (AR-negative) prostate cancer cell lines DU145 and PC3. The role of TR4 in the co-inhibition of AR and PARP can continue to be explored using AR-positive cell lines such as LNCap and C4-2.

Our study did not go into great detail about the molecular mechanisms underlying the impact of TR4 expression changes on the ATM/ATR pathway. Recent literature has shed light on how TR4-mediated Quaking (QKI)/circle Zinc Finger E-Box Binding Homeobox 1 (circZEB1)/miR-141-3p/ZEB1 can improve radiation sensitivity in prostate cancer. ZEB1, as mentioned in this context, interacts directly with Ubiquitin Specific Peptidase 7 (USP7), enhancing its stability and Checkpoint Kinase 1 (CHK1) capabilities and thus promoting the HRR process [15]. Furthermore, ZEB1’s mechanism for mediating HRR in colorectal cancer cells involves Nijmegen Breakage Syndrome 1 (NBS1), Ring Finger Protein 8 (RNF8) and RNF168 [23]. The role of ZEB1 as a TR4 downstream gene in the DDR of prostate cancer cells deserves further investigation.

NHEJR, unlike HRR, is an error-prone repair pathway that operates throughout the cell cycle and cannot completely replace for the error-free repair function of HRR [24]. TR4 regulates cell HRR via the ATM/ATR pathway. Given the complexities of DDR, there’s reason to speculate that TR4 may influence NHEJR through this network. Further research is required to elucidate the specific mechanisms.

Regarding the future experimental direction of this study, firstly, PARP inhibitors work by inhibiting PARP-induced Poly (ADP-ribosyl) ation (PARylation) and/or trapping PARP at DNA damage sites [8]. Assessing PARylation levels may be a better way to evaluate the efficacy of PARP inhibitors in cells. Furthermore, in studies related to ovarian and triple-negative breast cancers, Salt Inducible Kinase 2 (SIK2) inhibition improves PARP inhibitor sensitivity by impeding the DSB process, inducing synthetic lethality, and triggering apoptosis [25]. TR4 inhibition combined with olaparib may have final effects such as inducing apoptosis and altering the cell cycle. More extensive research is required for detailed mechanisms.

In terms of clinical application, the effect of Bexarotene as an antagonist of TR4 has been demonstrated in cell experiments in vitro in mCRPC [16]. Therefore, the combination of Bexarotene and olaparib in CRPC patients is probably the research direction of future prostate cancer treatment strategies. Moreover, recent studies have shown that co-inhibition of AR and PARP can cause synthetic lethality, potentially bringing new combination therapy strategies to patients without DDR-associated mutations [26].

5. Conclusions

Knocking down TR4 can improve the in vitro sensitivity of PC cell lines to the PARP inhibitor olaparib by down-regulating the ATM/ATR pathway.

AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on reasonable request from the corresponding author.
AUTHOR CONTRIBUTIONS

GAZ—developed the project; QQX and KL—conducted the experiment and analyze the data; PL—wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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