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ORIGINAL RESEARCH

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Targeting TR4 nuclear receptor-mediated p21 downregulation to increase apoptosis for reverse of the Enzalutamide-resistance in prostate cancer

Xuedong Chen[1], Ken Liu[2], Qiangqiang Xu[2], Peng Li[1], Qingfeng Ji[1], Junjie Ye[1,*]

[1]Urology, Wenzhou Medical University Lishui Hospital (Lishui City People's Hospital), 323000 Lishui, Zhejiang, China
[2]Wenzhou Medical University, 325000 Wenzhou, Zhejiang, China

***Correspondence**
18957093216@163.com
(Junjie Ye)

Abstract

Background: Enzalutamide (enz), also known as MDV3100, is a crucial treatment for advanced Castration-Resistant Prostate Cancer (CRPC). However, many patients develop enz resistance, leading to disease progression and recurrence. Testicular Nuclear Receptor-4 (TR4) has been implicated in regulating prostate cancer initiation, progression, metastasis and radiosensitivity. p21, a suppressor of cell growth, is crucial in caspase-activated apoptosis through both p53-dependent and -independent pathways. This study aimed to explore how TR4 contributes to enz-resistance in CRPC and the involvement of p21. **Methods**: Enz-resistant cell lines were generated by culturing C4-2 cells with increasing enz doses from 10 μM to 80 μM for 100 days. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (MTS) and Immunohistochemistry (IHC)-morphology assay confirmed the establishment of resistant cells. After *TR4* knockdown and overexpression, Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Western Blot (WB) were used to detect TR4 and p21 expression changes and flow cytometry was employed to assess apoptosis. **Results**: C4-2 MDV-R cells were spindle-shaped and had a lower apoptosis rate than parental C4-2 cells under enz treatment. *TR4* expression was elevated in C4-2 MDV-R cells. Overexpressing *TR4* decreased the apoptosis rate of C4-2 cells treated with 25 μM enz, while knocking down *TR4* increased it. Simultaneously, TR4 negatively regulated p21 expression. Knocking down *TR4* in C4-2 MDV-R cells enhanced apoptosis during enz treatment. **Conclusions**: TR4 could be a potential therapy target in combination with enz as the promising treatment for CRPC.

Keywords

TR4; p21; Enzalutamide; CRPC

1. Introduction

In 2023, approximately 290,000 American men were diagnosed with prostate cancer (PCa), accounting for nearly 30% of all newly diagnosed male cancers, with an estimated at 35,000 deaths, representing 11% of cancer deaths occur in men [1]. Because prostate cancer cells require androgens to thrive and persist, Androgen Deprivation Therapy (ADT) is the cornerstone of prostate cancer treatment as prostate cancer cells rely on androgens [2]. However, most patients develop ADT resistance with 2 to 3 years, resulting in CRPC [3]. Actually, researchers have revealed that CRPC continues to rely on androgen signaling [4]. Consequently, numerous novel drugs targeting the androgen receptor (AR) have been created, with enzalutamide (enz) being one of them. Developed by Anstai Pharmaceutical, enz was approved by the Food and Drug Administration (FDA) in 2012 and is considered a promising breakthrough drug. The enz has been a favorable drug for it has better affinity to the AR than bicalutamide and lacks agonist activity [5]. A double-blind phase 3 trial showed it could extend the survival of CRPC patients by 4.8 months [6, 7].

Recently, a study indicated that approximately 20% to 40% of CRPC patients are unresponsive to enz [7, 8]. Patients who exhibit an initially respond after long-term treatment with enz will inevitably develop drug resistance over time [7]. However, the cause of enz resistance in CRPC is still not well understood. Hence the mechanism dissection of enz resistance becomes one of the hottest issues in the battle against prostate cancer. The *TR4* (also known as NR2C2), was initially identified from cDNA libraries of the prostate and testis in humans and rats [9]. TR4 can be detected in various tissues and may play a role in important biological processes such as bone formation, reproductive functions, metabolic processes and programmed cell death [10]. Through direct transcriptional regulation of genes involved in several key signaling pathways. Besides, TR4 has the capability to engage with distinct nuclear

receptors including the androgen receptor, estrogen receptor, vitamin D receptor and Peroxisome Proliferator-Activated Receptor 17 (PPAR17) [9]. In the context of prostate cancer, TR4 has demonstrated its role in controlling the initiation, advancement and spread of the disease [11]. Additionally, it has the ability to influence the radiosensitivity of prostate cancer cells [12]. Previous research has shown that high levels of TR4 expression are associated with a decreased response to docetaxel treatment [13]. Alterations in TR4 may influence the effectiveness of chemotherapy in PCa [14]. Building upon these results, we sought to explore the potential connection between TR4 and enz resistance.

This study indicated enz-resistant C4-2 cells showed elevated TR4 expression relative to enz-sensitive counterparts. The analysis of cell apoptosis demonstrated that an increase in TR4 expression in C4-2 cells led to a decrease in apoptosis, while silencing *TR4* enhanced apoptosis under enz treatment. These results suggest an inverse relationship between TR4 expression level, and the apoptotic rate induced by enz exposure. The analysis of mechanisms indicates that TR4 may influence the sensitivity of the enzyme through the p21 pathway, which is related to the regulation of cell apoptosis. Targeting *TR4* signals with *TR4*-siRNA may be used to increase survival rates for CRPC patients who already acquired enz resistance.

2. Methods

2.1 Cell and drug-resistant cell lines cultures

Provided by the University of Rochester Medical Center, C4-2 human PCa cells were grown in 1640 medium (C11875500BT, Gibco, Shanghai, China) containing 10% Fetal Bovine Serum (FBS). The experiments only utilized cells in the log-phase, while Enzalutamide (S125016, Selleck, Shanghai, China) was kept at −80 °C until needed. The enz-resistant cell lines were cultured with increasing the enz dose from 10 μM to 80 μM for 100 days in C4-2 cells. The results from MTS proliferation assay as well as IHC-morphology assay confirmed the establishment of such enz-resistant C4-2 cells.

2.2 Cell transfection

The siRNA and TR4 gene were inserted into pLentiviral Knockout 1 (pLKO.1) and lentivirus expression vector plasmids (pWPI), respectively. Lentiviruses encoding either control or *TR4*-targeting constructs were prepared by transfecting 293T with Lipofectamine 2000 (the ratio of pKLO.1/pWPI, Packaging Plasmid 2 and Envelope Plasmid was 4:3:2). Following a 48-h incubation period, the Lentiviruses were collected for infection of C4-2 PCa cells. RNA will be extracted 24 to 48 hours, and protein extraction will be carried out between 48 to 72 hours after transfection. Western blot verification identified stable clones, which were labeled as C4-2 Scr, C4-2 si*TR4*, C4-2 Vector, C4-2 TR4.

2.3 RT-PCR

The standard PCR protocols were followed, which encompassed RNA extraction, conversion of RNA into cDNA and the subsequent PCR amplification. TRIzol reagent (Invitrogen) was used to isolate RNA. Transcriptor First Strand cDNA Synthesis Kit (04896866001, Roche, Beijing, China) was applied to reverse transcription. SYBR Green Mix was used for qPCR. Primers were designed by Premier 6.0 (Tongyong, Shanghai, China). The primer sequences: (TR4 forward) 5′-GGCTCTGAACCTGCCTCTG-3′, (TR4 reverse) 5′-AGGATGAACTGCTGTTTGGG-3′, (p21 forward) 5′-TCCACAGCGATATCCAGACA-3′, (p21 reverse) 5′-GGACATCACCAGGATTGGAC-3′, (Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) forward) 5′-GGAGTCAACGGATTTGGT-3′, (GAPDH reverse) 5′-GTGATGGGATTTCCATTGAT-3′. The qPCR setup: Step 1: 95 °C—2 min; Step 2: 95 °C—30 s; 60 °C—30 s; 68 °C—1 min; repeated 40 cycles; Step 3: 72 °C—10 min. Data analysis method: $2^{-\Delta\Delta Ct}$.

2.4 Western blot

Protein concentration was measured with the BCA Kit (KGB5303-100, KeyGEN BioTECH, Nanjing, Jiangsu, China) after cell washed by Phosphate-Buffered Saline (PBS) and digested by Radioimmunoprecipitation Assay Buffer (RIPA). And 30 μg of proteins were taken for subsequent experiments, including separated using 8%–12% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels (P0012A, Beyotime, Shanghai, China), transferred to a Polyvinylidene Fluoride (PVDF) membrane (Millipore, American) and blocked with 5% milk. Primary antibodies (TR4, ab109301, Abcam, Shanghai, China and p21, #2947, CST, Shanghai, China) were applied to incubate the membranes at 4 °C for more than 8 hours. Subsequently, the secondary antibodies (HAF008/HAF007, R&D, Shanghai, China) were applied for the second round of incubation at room temperature for approximately 2 hours, followed by protein visualization using the Enhanced Chemiluminescence (ECL) system.

2.5 MTS assay

A density of 5×10^3 PCa cells was distributed evenly among the wells of the 96-well plates, with each type of cell allocated to 6 same wells. Cells were taken enz according to the arrangement after anchorage. MTS was added to each well after 0, 2, 4 or 6 days of culture, and absorbance at 490 nm was measured to assess cell viability. A dose-response curve was plotted.

2.6 Apoptosis assay

Each type of cell was plated into six-well plates, and 25 μM enz was added into each well after they adhered. Apoptosis rate of each well was evaluated using an Apoptosis Detection Kit (40302ES50, Yeasen, Shanghai, China). Data analysis was done with FCS4 Express platform (BD Biosciences, San Jose, CA, American).

3. Results

3.1 TR4 expressed highly in the enz-resistant C4-2 MDV-R cells than in the normal C4-2 PCa cells

The C4-2 MDV-R cells were isolated from the parental C4-2 PCa cells. This cell line was verified with morphology, apoptosis assay and MTS growth assay. When observed under optical microscopy, the C4-2 MDV-R cells display a spindle-like shape, which is different from the original C4-2 cells (Fig. 1A). Next, it was observed that the C4-2 MDV-R cells exhibited lower levels of cell apoptosis compared to the original C4-2 cells when exposed to enz, as determined via flow cytometry ($p < 0.05$; Fig. 1B). The sensitivity of C4-2 MDV-R to enz was obviously lower compared with the parental C4-2 cell lines (Fig. 1C). Western blot analysis was performed to investigate the levels of TR4 protein and its role in conferring acquired resistance to enz in C4-2 cells. The expression of TR4 was significantly elevated in C4-2 MDV-R cells compared to the levels observed in the non-resistant C4-2 PCa cells (Fig. 1D). Thus, an upregulation of TR4 in C4-2 cells may be attributed to enz treatment.

3.2 TR4 expression modifies the apoptosis of enz-treated PCa cells

To investigate the influence of TR4 on enz sensitivity, we manipulated the expression of TR4 in C4-2 cells by introducing active TR4 or using *TR4*-siRNA to silence its function. Western blotting was used to measure TR4 expression (Fig. 2A). The results of the apoptosis test indicated that the reduction of TR4 notably elevated the apoptosis rate induced by enz in normal C4-2 PCa cells, whereas the overexpression of TR4 decreased the apoptosis rate triggered by enz in normal C4-2 PCa cells (Fig. 2B).

The findings suggest that TR4 strengthens enz resistance, possibly through the suppression of cell apoptosis.

3.3 TR4 regulates p21 negatively in C4-2 cells

In order to investigate the mechanisms of how TR4 inhibits the enz-induced apoptosis in C4-2 cells, we examined the crucial genes that participate in cell apoptosis signaling pathways. Given the prominent role of TR4 as a transcriptional factor [14], our investigation focused on discerning

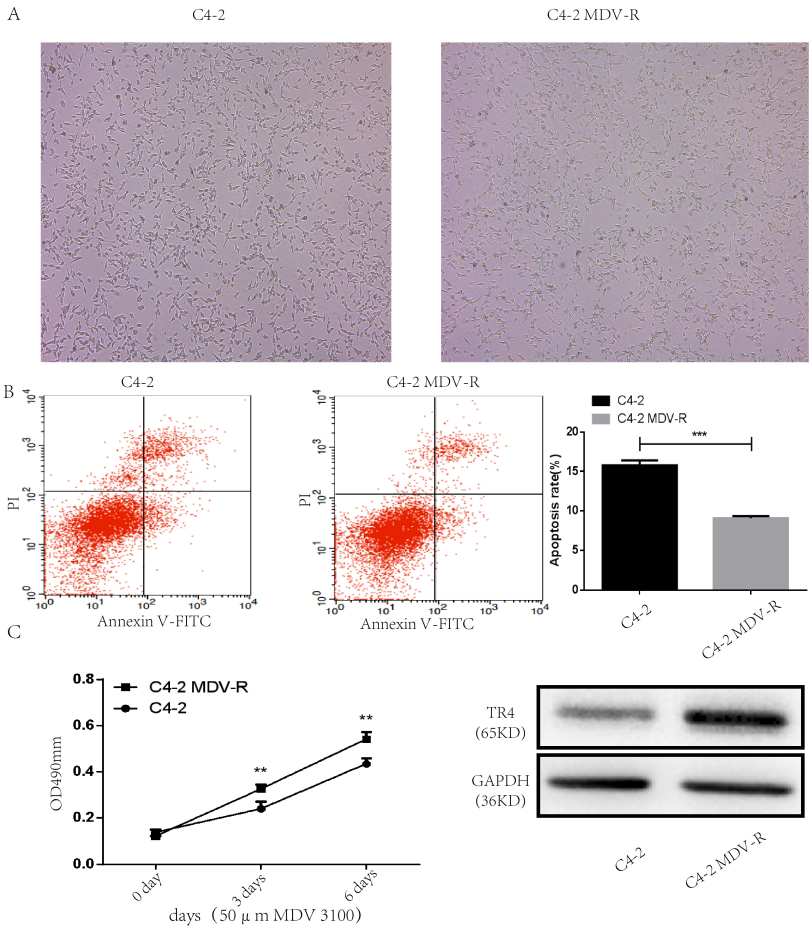

FIGURE 1. TR4 expression is notably higher in enz-resistant C4-2 MDV-R cells than in normal C4-2 PCa cells. (A) Morphological characteristics of normal C4-2 PCa cells and MDV-R cells (70% confluence, 40×). (B) Flow cytometric analysis of cell apoptosis rate in normal C4-2 PCa cells and MDV-R cells after treatment with 25 μM enz. (C) MTS assay was used to assess the sensitivity of MDV-R and normal C4-2 PCa cell lines to enz. (D) Western blot of TR4 expression. $^{**}p < 0.01$, $^{***}p < 0.001$. MDV-R cell: Enzalutamide-resistant cell lines; TR4: Testicular Nuclear Receptor-4; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; OD: Optical Density; PI: Propidium Iodide; Annexin V-FITC: Annexin V-Fluorescein Isothiocyanate.

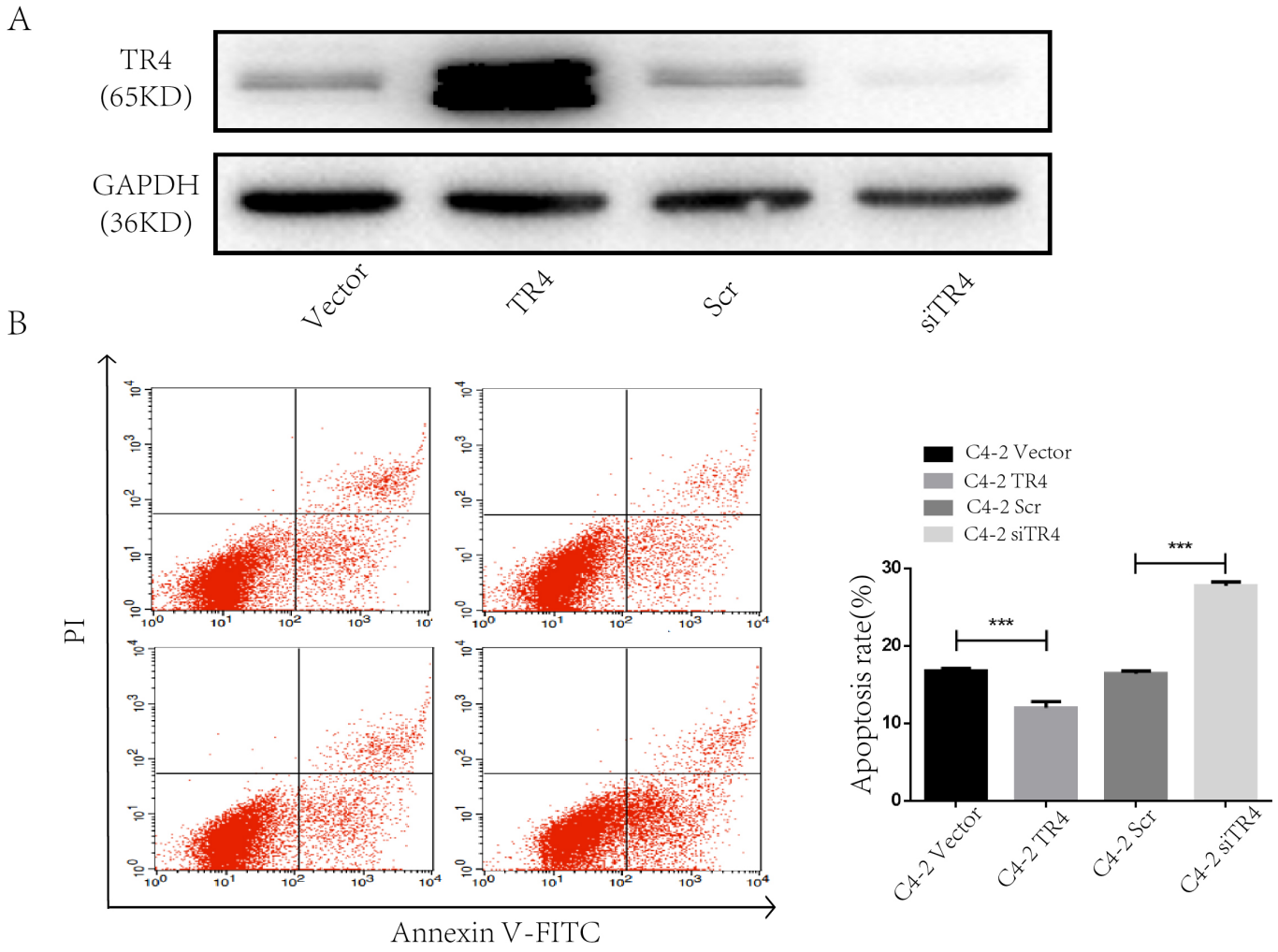

FIGURE 2. TR4 expression modifies apoptosis in enz-treated PCa cells. (A) Alter TR4 expression in C4-2 cells via addition of functional *TR4* or *TR4*-siRNA knockdown (western blot). (B) Flow cytometric evaluation of apoptosis after cells exposed to 25 μM enz. ***: $p < 0.001$. TR4: Testicular Nuclear Receptor-4; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; PI: Propidium Iodide; Annexin V-FITC: Annexin V-Fluorescein Isothiocyanate.

whether TR4 affected the sensitivity of C4-2 cells by suppressing tumor suppressors or apoptosis factors. JASPAR (https://jaspar.elixir.no/) is a commonly utilized open-access database that offers hand-picked, high-quality and non-repetitive DNA-binding profiles for transcription factors across various taxonomic groups [15]. After conducting a search on the JASPAR database, we identified ten possible TR4 response elements (TR4REs) in the −2000 region of the p21 5′ promoter (**Supplementary Table 1**). Thus, TR4 could be concentrated on the p21 promoter. RT-qPCR and Western blotting assays served as initial tools to examine how TR4 expression on the levels of p21 protein. An inverse correlation was noted between TR4 levels and p21 expression in C4-2 cells: Overexpression of TR4 led to a reduction in p21 transcription and protein levels, whereas the suppression of TR4 resulted in elevated p21 protein levels (Fig. 3A,B). The findings indicated that p21 was a target of TR4, potentially crucial for TR4-induced enz resistance in C4-2 cells.

3.4 Targeting TR4 in C4-2 MDV-R cells increase apoptosis induced by enz

On the basis of findings mentioned earlier, our subsequent experiment aimed to investigate the potential enhancement of enz-induced apoptosis and sensitivity to enz by targeting TR4 in C4-2 MDV-R cells. Fig. 4A,B illustrates the reduction of TR4 levels in C4-2 MDV-R cells through *TR4*-siRNA, and the p21 expression was raised as demonstrated by qPCR and Western blot assays. As expected, the downregulation of TR4 resulted in an elevation of enz-induced apoptosis through the upregulation of p21. Thus, downregulation of TR4 may appear to be an effective treatment to reverse enz-resistance in prostate cancer.

4. Discussion

At present, most prostate cancer patients who show an initial positive reaction to ADT are likely to progress to CRPC over

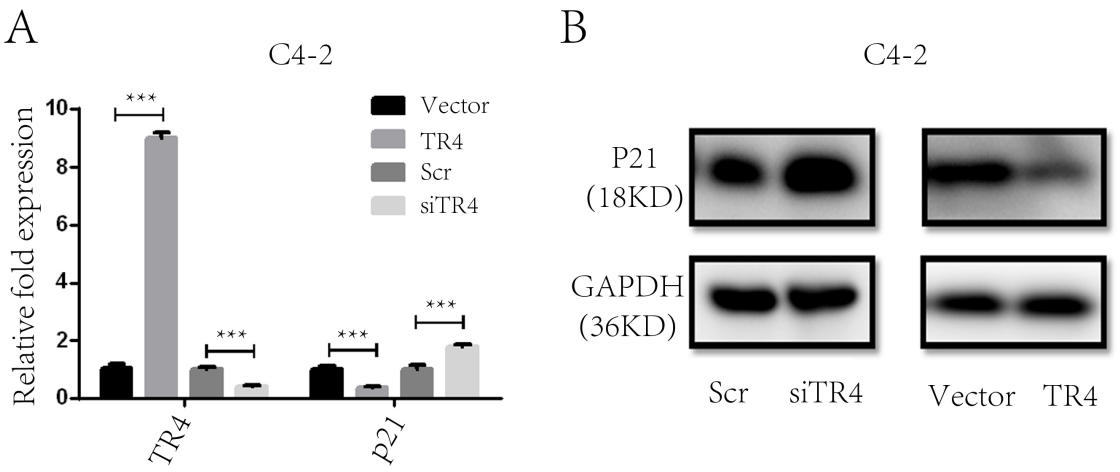

FIGURE 3. p21 mediates TR4-stimulated enz resistance. (A) qPCR assessment of p21 mRNA regulation by TR4. (B) Western blotting to assess TR4's impact on p21 protein expression in C4-2 Scr, si*TR4*, Vector and TR4 cells. ***: $p < 0.001$. TR4: Testicular Nuclear Receptor-4; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase.

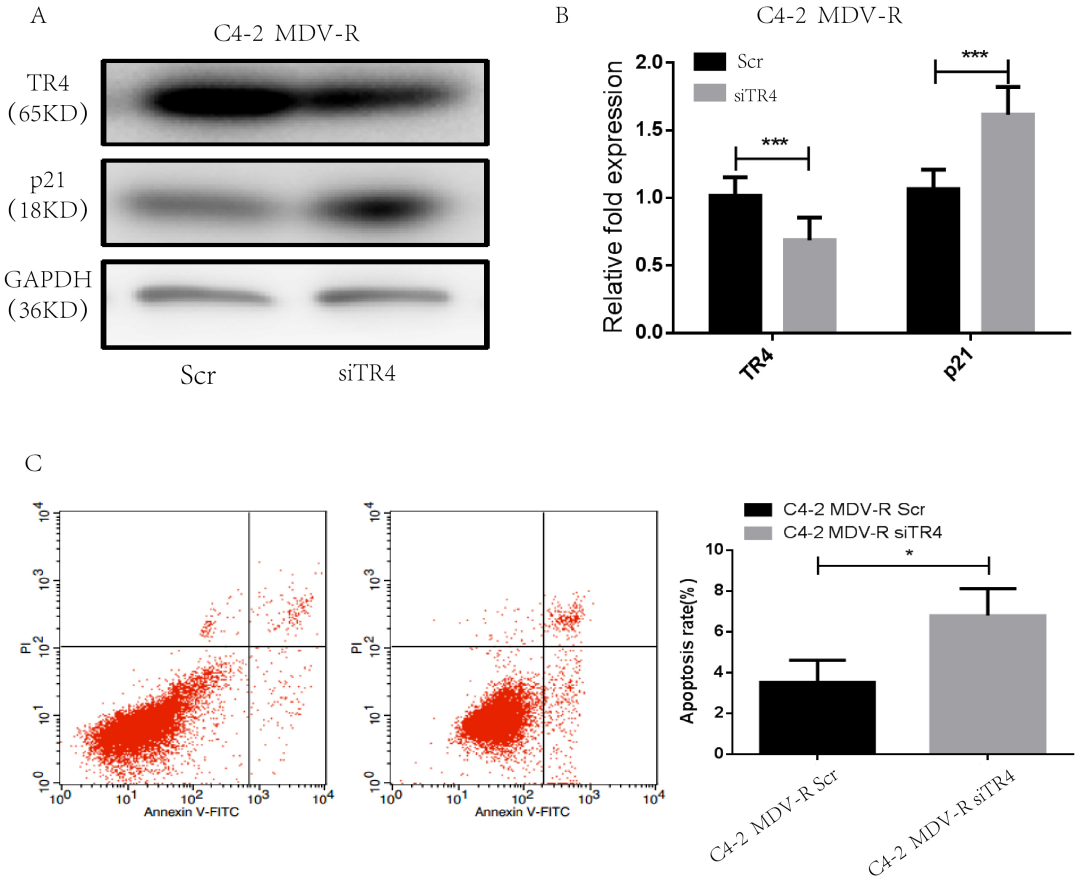

FIGURE 4. Targeting TR4 in C4-2 MDV-R cells increase apoptosis induced by enz. (A,B) TR4 and p21 expression of C4-2 MDV-R cells was measured using qPCR and Western blotting after TR4 knockdown. (C) Flow cytometric assessment of apoptosis in C4-2 MDV-R Scr/C4-2 MDV-R si*TR4* cells. ***: $p < 0.001$. TR4: Testicular Nuclear Receptor-4; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; MDV-R cell: Enzalutamide-resistant cell lines; PI: Propidium Iodide; Annexin V-FITC: Annexin V-Fluorescein Isothiocyanate.

time. Consequently, enz therapy has emerged as the final resort for those with CRPC. Regrettably, as almost all patients develop resistance [16], there is only a marginal increase in overall survival, benefiting around half of the patients for a few additional months. Although common in clinical settings, the mechanisms behind enz resistance remain unclear. Only a few mechanisms have been suggested to explain enz-resistance in CRPC, including constitutive AR signaling activation, AR mutations, bypassing AR signaling, Intratumoral androgen synthesis, microenvironmental effects [5, 17–19]. Lineage plasticity is the capacity of cells to undergo phenotypic conversion, enabling them to shift from one cell lineage to another. In the context of prostate cancer, lineage plasticity allows cancer cells to evade treatment and immune recognition by changing their phenotypes [20]. This plasticity is also a crucial mechanism underlying the development of CRPC [21]. The factors influencing plasticity are multifaceted and varied, encompassing aberrant genes expression (such as Forkhead Box A2, Tumor Protein p53, SRY-Box Transcription Factor 2) [22–24], along with signaling cascades like the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) inflammatory signaling pathway and the Fibroblast Growth Factor Receptor (FGFR) signaling pathway [20]. Nevertheless, the involvement of TR4 in PCa plasticity remains unexplored, setting the course for our forthcoming research endeavors.

p21, also known as, Cyclin-Dependent Kinase (CDK)-interacting protein 1, functions as suppressor of cyclin-dependent kinase activity by inhibiting the formation of all cyclin/CDK complexes [25]. Known for suppressing cell growth, p21 plays a pivotal role in apoptosis triggered by caspase activation, leveraging both p53-dependent and -independent routes [25]. The mechanism by which p21 promotes apoptosis remains uncertain; it could be associated with the augmentation of BCL2-Associated X Protein (BAX), activation of Tumor Necrosis Factor (TNF)-related death receptors, or interference with DNA repair processes. Beyond this, p21 has been shown to suppress tumors in multiple cancers like lung cancer [26], pancreatic cancer [27], breast cancer [28], hepatocellular carcinoma [29] and so on. In this study, we investigated the role of p21 as a key mediator in TR4-induced enz resistance of PCa cell line. This study showed that the suppression of TR4 led to a substantial increase in p21 protein levels in both normal C4-2 and MDV-R cell lines, whereas the upregulation of TR4 notably reduced p21 expression in C4-2 cells. This evidence indicated that TR4 targeted p21 and may impact enz resistance in C4-2 PCa cells by modulating apoptosis via p21 expression. The specific mechanisms of TR4-mediated p21 downregulation remain to be explored.

These data provided in this study emphasize the potential reason for the ineffectiveness of enz therapy following a 4.8-month increase in survival benefit. More importantly, they raise the possibility that TR4 targeting could provide a promising treatment strategy for patients with enz-resistant CRPC. Although the ligands specific to TR4 remain unidentified, previous studies indicate that compounds like metformin, Polyunsaturated Fatty Acids (PUFAs) and thiazolidinediones can modulate TR4 functioning as ligands or activators [30]. More effects are needed to search for additional agents that could suppress TR4 function. We anticipate that the use of a specific and efficient TR4 inhibitor in conjunction with enz will enhance the fight against CRPC in the future. Future considerations may also encompass combination therapies that employ compounds proven to inhibit cell proliferation through autophagy blockade. Furthermore, it remains to investigate whether the effect of TR4 extends to apalutamide and darolutamide, as well as to determine if TR4 plays a role in the adaptive transformation of prostate cancer. Although this study offers a potential avenue for exploration, it is accompanied by several significant challenges. Notably, the instability in the preparation of cellular models may compromise the reliability and reproducibility of subsequent mechanistic investigations. Furthermore, the heterogeneity observed in clinical cases introduces considerable uncertainty into the development and application of TR4-targeted therapeutic strategies.

5. Conclusions

TR4 is upregulated in enzalutamide-resistant C4-2 cells and associated with reduced apoptosis, while its knockdown enhances apoptosis upon treatment. TR4 may regulate enzalutamide sensitivity via p21-dependent apoptotic modulation.

AVAILABILITY OF DATA AND MATERIALS

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

AUTHOR CONTRIBUTIONS

JJY—Research protocol design. KL, QQX and QFJ—experiment. XDC—Manuscript writing. PL—Data analysis. All authors have reviewed the manuscript and consented to its publication.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found, in the online version, at https://oss.jomh.org/

files/article/192827858536095744O/attachment/Supplementary%20material.docx.

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