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TITLE: TRAF4 and Castration-Resistant Prostate Cancer

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The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
It is now well-recognized that AR remains to be a critical player in castration-resistant prostate cancers. It was suggested that the function of AR in CRPC is not to turn on the same transcriptional targeted genes in the absence of androgen but to turn on a distinct set of genes independent of androgen. However, it was not clear what triggers the functional switch of AR. Here we report another pathway to bypass androgen dependency through AR ubiquitination. We found that TRAF4, a RING domain E3 ubiquitin ligase, is overexpressed in CRPCs. Its overexpression promoted androgen-independent cell growth. In this funding period we determined the role of TRAF4 and its regulated AR targeted genes in CRPC development. We further identified TRAF4-mediated AR ubiquitination sites.
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1. Introduction

It is now well recognized that AR remains active in castration-resistant prostate cancers (CRPCs). Post-translational modification, such as phosphorylation, plays a role in ligand-independent activation of AR. Ubiquitination is an important post-translational modification regulating protein degradation, trafficking, activity, and protein-protein interaction. Deregulation of the ubiquitin pathways has been implicated in a number of diseases including cancers. Targeting the ubiquitination system for cancer therapy has gained a broad interest. We recently found that TRAF4, a RING domain E3 ubiquitin ligase is highly expressed in CRPCs. Overexpression of TRAF4 promoted androgen-independent growth of prostate cancer cells and this function requires its E3 ubiquitin ligase activity. We further identified AR as a TRAF4 ubiquitin substrate using mass spectrometry and found that AR was able to regulate a different set of gene transcription when TRAF4 was overexpressed. In this study, we propose to test the hypothesis that TRAF4 mediated AR ubiquitination promotes CRPC development.

2. Keywords: TRAF4, AR, ubiquitination, UBE2C, CRPC

3. Accomplishments:

(1) Major goals of the project:
   a. To understand the function of TRAF4-mediated ubiquitination in castration-resistant prostate cancer cell proliferation
      Expected completion date: 24th month
      Percentage of completion: 50%
   b. To determine the role of TRAF4 in androgen-independent prostate cancer progression
      Expected completion date: 24th month
      Percentage of completion: 5%
   c. Determine the function of TRAF4 overexpression in CRPC development and metastasis using mouse tumor model
      Expected completion date: 36th month
      Percentage of completion: 5%

(2) Accomplishments under these goals:

Major activities: 1) Determining the role of TRAF4 and TRAF4-regulated AR target genes in CRPC cell growth.

2) Identified TRAF4-mediated AR ubiquitination sites
3) Animal protocol approval
4) Generation of TRAF4 overexpression mouse line

Specific objective: To determine whether TRAF4-mediated AR ubiquitination plays a role in androgen-independent growth of prostate cancer cells.
Specific Aim 1: To determine the role of TRAF4-mediated ubiquitination in regulating androgen-independent growth

Subtask 1: To determine the role of TRAF4 downstream targets in androgen-independent growth

(1) TRAF4 plays a role in CRPC cell growth

We previously found that overexpression of TRAF4 promoted androgen-independent growth of LNCaP cells. We also examined the expression levels of TRAF4 in androgen-independent LNCaP derivative, Abl cells, and found that TRAF4 is highly expressed in Abl cells compared to androgen-dependent LNCaP cells (Fig. 1A). To determine whether TRAF4 plays a role in CRPC cell growth, we knocked-down TRAF4 in Abl cells using two different TRAF4-specific siRNA and monitored the cell growth. As shown in Fig. 1B, TRAF4 knockdown significantly reduced Abl cell growth. Similar results were also obtained in another CRPC cell line, LNCaP-C4-2 cells (Fig. 1C). These results suggest that TRAF4 plays a role in CRPC cell growth.

(2) TRAF4-regulated AR target gene plays a role in CRPC cell growth

It was reported previously that AR is able to regulate a distinct set of gene transcription, including UBE2C and CDC20 (mitosis regulating genes), in Abl cells, which are not regulated by AR in LNCaP cells [1]. These genes were believed to contribute CRPC development [1-4]. We found that TRAF4 overexpression in LNCaP cells promoted UBE2C and CDC20 upregulation and this upregulation was abolished
Fig. 2: TRAF4 regulated AR target gene played a role in CRPC growth (A-B) TRAF4 overexpression in LNCaP cell upregulated non-classical AR target genes UBE2C (A) and CDC20 (B). (C) Depletion of UBE2C reduced Abl cell growth. (D) TRAF4 regulated UBE2C and CDC20 gene expression in androgen-independent C4-2 cells.

when AR was knocked-down (Fig. 2A and B). We further found that depletion of UBE2C using specific siRNA reduced Abl cell growth similar to the effect of TRAF4 knockdown (Fig. 2C). To determine whether TRAF4 is able to regulate UBE2C expression in CRPC cells, we knocked-down TRAF4 in C4-2 cells and found that UBE2C and CDC20 expression were reduced (Fig. 2D). These results suggest that TRAF4 can regulate AR targeted CRPC associated gene expression in CRPC cells.

(3) TRAF4 regulated AR recruitment to the UBE2C enhancer

Since AR was found to bind UBE2C enhancer only in androgen-independent cells but not in androgen-dependent cells, we then determined whether TRAF4 plays a role in regulating AR binding to the UBE2C enhancer in CRPC cells. We performed a Chromatin Immunoprecipitation (ChIP) experiment in Abl cells using AR-specific antibody. As shown in Fig. 3A, AR was able to bind the UBE2C enhancer in Abl cells, consistent with the previous report [1]. When TRAF4 was knocked-down, AR recruitment was significantly reduced. In contrast, TRAF4 knockdown did not affect the recruitment of AR to the classical AR target gene, PSA, promoter (Fig. 3B). To determine whether TRAF4 overexpression is sufficient to drive AR binding to the UBE2C enhancer, we carried out the ChIP experiment in LNCaP cells with or without TRAF4 overexpression. As shown in Fig. 3C, we found that TRAF4 overexpression in
LNCaP cells increased AR recruitment to the UBE2C enhancer (Fig. 3C). These results suggest that TRAF4 overexpression promoted the ability of AR to regulate CPRC related genes, leading to a CRPC phenotype.

Fig. 3: TRAF4 regulated AR recruitment to UBE2C enhancer (A) TRAF4 depletion reduced AR recruitment to the UBE2C enhancer in Abl cells (B) TRAF4 depletion did not reduce AR recruitment to the PSA enhancer in Abl cells. (C) Overexpression of TRAF4 in LNCaP cells increased AR recruitment to the UBE2C enhancer. Shown are the ChIP experiment results using specific AR antibody or IgG control.

Subtask 2: Identification of TRAF4 substrate ubiquitination sites

We previously found that deletion of the ligand binding domain (LBD) of AR abolished TRAF4-mediated AR ubiquitination, suggesting that the LBD could be TRAF4-targeted ubiquitination domain. To determine the AR ubiquitination sites we first generated a series of AR LBD deletion mutants and then tested the ability of TRAF4 to ubiquitinate these mutants. As shown in Fig. 4A, wild type TRAF4 but not the RING domain deletion of TRAF4 was able to promote full-length AR ubiquitination, suggesting that the E3-ubquitin ligase activity of TRAF4 was required for this function. However, deletion of the last 53 amino acids of the LBD at the C-terminus of AR abolished TRAF4-mediated ubiquitination. We then generated different AR mutants with lysine residues mutated to arginine residues in this region and then performed an in vivo ubiquitination assay. As shown in Fig. 4B, mutation of K911 and K913 residues completely abolished TRAF4-mediated ubiquitination, suggesting that TRAF4 ubiquitinated AR at the K911 and K913 residues.

Specific Aim 2: To determine the role of TRAF4 in androgen-independent prostate cancer progression

The animal protocol for the proposed research was approved by the USAMRMC Animal Care and Use Review Office (ACURO) on 12/23/2015. The proposed research under this aim will be performed in the following year.

Specific Aim 3: Determine the function of TRAF4 overexpression in CRPC development and metastasis using mouse tumor model
Fig. 4: TRAF4 promoted AR ubiquitination at the C-terminal K911 and K913 residues (A) Deletion of the C-terminal 53 amino acids (1-867aa) of AR abolished TRAF4-mediated ubiquitination. 293T cells were transfected with HA-Ubiquitin, wild type or RING domain deletion of TRAF4, full length or deletion mutant of AR. AR was then immunoprecipitated using specific AR antibody and the ubiquitinated form was detected using HA-specific antibody in the Western blot. (B) Mutation of K911 and K913 residues abolished TRAF4-mediated AR ubiquitination.

Subtask 1: Generation of TRAF4 minigene

We have successfully inserted TRAF4 cDNA into a minigene consisting of a ubiquitous CAGGS (a hybrid chicken β-actin and cytomegalovirus) promoter with a floxed STOP cassette inserted between the promoter and the TRAF4 gene to silence TRAF4 expression.

Subtask 2: Generation of TRAF4 mouse

This minigene was then inserted into the Rosa 26 locus in the mouse embryonic stem cells. After embryo injection, we obtained a chimera with the TRAF4 minigene. We are now examining whether the TRAF4 transgene is able to get germline transmission.

References:


(3) **Opportunities for training and professional development this project provided:** This project provided me the opportunity to attend the Innovative Minds in Prostate Cancer Today Young Investigators Meeting.

(4) **How were the results disseminated to communities of interest?**

Nothing to report

(5) **Plan to do during the next reporting period to accomplish the goals**

a. Since we identified the AR ubiquitination sites, we plan to test whether mutation of TRAF4-targeted AR ubiquitination sites affects AR function and TRAF4-promoted androgen-independent growth.

b. We will further explore the underlying mechanism as to how TRAF4-mediated AR ubiquitination regulates its genomic binding.

c. We will determine the effects of TRAF4 on androgen-independent prostate cancer cell growth in vivo using xenograft mouse model

d. We will generate a TRAF4 overexpression prostate cancer mouse model to examine the role of TRAF4 in CRPC development.

4. **Impact**

   (1) The impact on the development of the principal discipline of the project

   We identified TRAF4 as a novel gene that promotes androgen-independent growth and metastasis of prostate cancer cells through AR ubiquitination. Depletion of TRAF4 reduced CRPC cell growth. Our study reveals a novel pathway regulating AR post-translational modification important for CRPC progression and provides potential therapeutic targets.

   (2) The impact on other discipline

   Nothing to report

   The impact on technology transfer

   Nothing to report

   The impact on society beyond science and technology

   Nothing to report
5. Changes/Problems:

Actual or anticipated problems or delays and actions or plans to resolve them:

We were delayed in the Major Task 3 subtask 2 and 3. The problem was we did not get germline transmission after embryo injection. The embryo injection was performed in the Genetically Engineered Mouse Core at Baylor College of Medicine. Similar problem was also reported with other PIs’ embryo injection performed at the same time. Unstabled embryo stem cells used for injection was believed causing this problem. We reconstructed the TRAF4 minigene and performed another embryo injection using new ES cells.

6. Products:

Publications, conference papers, and presentations:

Poster presentation: Singh, R., Shao, J., Karri, D., Dasgupta, S., O’Malley, B.W. and Yi, P. TRAF4-mediated AR Ubiquitination and Castration-Resistant Prostate Cancer Innovative Minds in Prostate Cancer Today Young Investigators Meeting

7. Participants & other collaborating organizations

(1) Individuals have worked on the project

<table>
<thead>
<tr>
<th>Name</th>
<th>Ping Yi</th>
<th>Ramesh Singh</th>
</tr>
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<tbody>
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<td>Postdoctoral associate</td>
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<tr>
<td>Researcher Identifier</td>
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<td>0000-0001-5052-7925</td>
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<tr>
<td>Nearest person month worked</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Contribution to Project</td>
<td>Dr. Yi designed and supervised the proposed research</td>
<td>Dr. Singh performed the experiment proposed.</td>
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(2) Changes in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period:

Nothing to report

(3) Other organizations involved as partners:

Nothing to report

8. Special Reporting Requirements

9. Appendices