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TITLE: Epigenetic machinery regulates alternative splicing of androgen receptor (AR) gene in castration-resistant prostate cancer

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Androgen receptor pathway inhibition (ARPI) is the primary treatment for metastatic prostate cancer (PCa) since PCa depends on androgen for growth. Although initially responsive, most tumors progress into androgen-independent/castration-resistant PCa (CRPC). No curative therapy is available. One of the ways for PCa cell to evade targeted therapy is the emergence of constitutively active AR variants (AR-Vs) such as AR-V7 that does not depend on androgen. Our research goal is to test the hypothesis that the epigenetic regulator KDM4B, a histone lysine demethylase, promotes AR-V7 via alternative splicing, leading to CPRC. We have made significant progress in the last year on identification of the mechanism by which KDM4B regulates AR-V7 and establishing the efficacy of our newly identified KDM4B inhibitor B3 as a monotherapy or combined with approved anti-androgen agents in AR-V7-expressing CRPC in preclinical mouse models.
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1. INTRODUCTION:
   The subject of our research is to identify the molecular mechanism of the drug resistance in castration-resistant prostate cancer (CRPC). Our preliminary data suggest that one of the mechanisms of the resistance is the emergence of constitutively active androgen-receptor variants such as AR-V7. Our goals are to demonstrate that histone lysine demethylase KDM4B regulates AR-V7 via alternative splicing and to test the efficacy of our newly identified KDM4B inhibitor(s) as a monotherapy or combined with approved anti-androgen agents in AR-V7-expressing CRPC in pre-clinical animal models of CRPC.

2. KEYWORDS:
   Histone lysine demethylase, castration-resistant prostate cancer, alternative splicing, AR-V7, KDM4B, small molecule inhibitors.

3. ACCOMPLISHMENTS:
   What were the major goals of the project?
   List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

   There are two specific aims in this proposal. We have made significant progresses for both aims in the past year. One manuscript for publication is written and is currently under review.

   **Aim 1. To establish that KDM4B promotes AR-V7 expression and identify the regulatory mechanisms.**
   **Major Task 1:** Determine the role of KDM4B in promoting AR-V7 expression in various PCa cell lines, including those resistant to enzalutamide.—completed (6/30/2017).
   **Major Task 2:** Determine how KDM4B binds to the spliceosome associated with pre-mRNA.—completed (6/30/2017).

   **Milestone #1:** Co-author manuscript on KDM4B-RNA interaction.—We have met this milestone. The manuscript was submitted to cancer cell and is currently in 2nd revision (6/30/2018).
   **Major Task 3:** Map RNAPII, H3K9/K36me3 occupancy around AR locus using ChIP-qPCR in several CRPC cells.—in progress, 60% completed.
   **Major Task 4:** To identify potential KDM4B-regulated alternative splice gene(s) using RNA-seq and map KDM4B-RNA interactions with CLIP-seq.—in progress, 60% completed.

   **Milestone #2:** Co-author manuscript on mechanism by which KDm4B regulates AR-V7 at chromatin level---We have met this milestone. The manuscript is under 2nd revision for Cancer Cell (7/30/2018).

   **Aim 2. To evaluate the clinical application of KDM4B inhibitors on CRPC tumors expressing AR-Vs.**
   **Major Task 5:** Identify two lead compounds using CPRC cell lines and optimizing their dosage and schedule in xenograft models.—in progress, 60% completed. Please see the progress report from the partner of the project Dr. JT Hsieh (PC150152P1) for details.

   What was accomplished under these goals?
   For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1) major activities;
   a) We tested the effect of KDM4B knockdown (KD) on 22Rv1 cell growth in vitro.
   b) We tested the effect of KDM4B KD on the sensitivity of 22Rv1 cells to enzalutamide.
c) We tested the effect of KDM4B KD on the tumor growth in vivo. Please see Partnering-PI Dr. JT Hsieh’s progress report.

d) We tested the synergy between B3 and enzalutamide on CRPC cells in vitro. Please see Partnering-PI Dr. JT Hsieh’s progress report.

e) We performed cell fractionation and isolated RNAs from cytosol (cyto), soluble nuclear (snuc), and chromatin fractions (chr). We also estimated the levels of pre-RNA of AR near the CE3 region in VCaP cells transfected with or without KDM4B.

f) We measured the relative occupancy of KDM4B and levels of H3K9me3 on the chromatin around the CE3 locus using chromatin-immunoprecipitation (ChIP)-qPCR assays.

g) We performed chromatin RNA immunoprecipitation (ChRIP) together with ChIP in 22Rv1 cells treated with control vehicle or KDM4B inhibitor B3 to unravel how KDM4B couples spliceosome to the chromatin.

h) We tested the chromatin accessibility around the CE locus using ATAC-qPCR assays.

2) Specific objective;
We made significant progress in specific aim 1, identifying the mechanism by which KDM4B couples spliceosome to the chromatin. In collaboration with partnering PI Dr. Hsieh, we also tested the effect of knocking down KDM4B in 22Rv1 cells on the tumor growth in vivo, and tested the ability of KDM4 inhibitor B3 to sensitize CRPC cell 22Rv1 to enzalutamide inhibition.

3) Significant results or key outcomes;
Significant results.

(1) KDM4B KD inhibited the growth of 22Rv1 cell in vitro under both FBS and CFBS conditions. Under CFBS conditions, the attenuated growth by KDM4B KD can be rescued by re-expression of AR-V7 (Fig. 1A), sensitized 22Rv1 cells to enzalutamide inhibition (Fig. 1B), downregulated genes involved in epithelial-to-mesenchymal transition (Fig. 1C) and cell cycle (Fig. 1D), and the cell membrane staining of E-cadherin (Fig. 1E).

(2) KD of KDM4B in 22Rv1 significantly attenuated the tumor growth in vivo (please see partnering-PI Dr. JT Hsieh’s progress report).

(3) KDM4B overexpression (OE) in LNCaP, VCaP and 22Rv1 cells upregulated preferentially chromatin-associated AR-V7 mRNA and had little effect on chr AR mRNA (Figure 2A and B) whereas KDM4B knockdown (KD) in 22Rv1 cells downregulated chr AR-V7 mRNA (Figure 2C and D). A significant amount of AR-V7 pre-RNA, quantified by qRT-PCR with primers in the intron regions flanking CE3, were found in the chromatin fraction compared to that in the soluble nuclear fraction (Figure 2E), suggesting that the majority of alternative splicing of CE3 occurred at chromatin. KDM4B OE in LNCaP cells resulted in a significantly increased chromatin-bound KDM4B (Figure 2F) and downregulation of H3K9me3 around CE3 (Figure 2G), respectively. Strong KDM4B-ChrIP peaks (Figure 2H, lower panel, 13f and CE3) were observed near the 3΄ss of CE3 but not C4, suggesting that KDM4B preferentially regulates the usage of CE3. A KDM4B-ChIP peak was also observed, which coincides with the KDM4B-ChrIP peak at the I3f position (Figure 2H, upper panel), suggesting a coupling of chromatin DNA and RNA by KDM4B. The KDM4B inhibitor B3 inhibited KDM4B binding to both the chromatin and chromatin-associated RNA (Figure 2H). KDM4B-transfected LNCaP cells showed significantly more open chromatin in the I3f and CE3 regions compared to KDM4Bm or vector-transfected cells (Figure 2I). Together with the results from figure 6, these data suggested the following model (Figure 2J): binding of KDM4B to H3K9me3 slows down AR transcription and opens up the chromatin to expose alternative exons (Figure 2J, I3f). As the transcription continues, KDM4B preferentially binds the pre-mRNA and influences the selection of splicing site together with the spliceosome

Key outcomes. Our studies demonstrate that KDM4B promote progression of CRPC via AR-V7. KDM4B can activate AR-V7 alternative splicing at chromatin level. Targeting KDM4B could inhibit CRPCs that are refractive to current androgen-deprivation therapy.

What opportunities for training and professional development has the project provided?

Nothing to report
How were the results disseminated to communities of interest?
We have submitted a manuscript for publication

What do you plan to do during the next reporting period to accomplish the goals?
We are on target following the plan stated in the SOW. We have worked out the condition for genomic studies including ChIP and CLIP. For the next reporting period, we will continue to map RNAPII, H3K9/K36me3 occupancy around AR locus using ChIP-qPCR in several CRPC cells (major task 3) and to identify potential KDM4B-regulated alternative splice gene(s) using RNA-seq and map KDM4B-RNA interactions with CLIP-seq (major task 4). We will also work with Partnering PI Dr. Hsieh on working out the best therapeutic regimen of delivering B3 alone or in combination with enzalutamide in inhibition of CRPC xenograft.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project? What was the impact on other disciplines?
Alternative splicing is emerging as an important determinant of oncogenesis, response to treatment, and drug resistance, thus representing an important vulnerability with potential to be exploited for therapeutic purposes. Epigenetic changes including histone modifications have long been recognized to play a role in tumor initiation and progression. Here, we identified a novel mechanistic link between alternative splicing and histone lysine demethylase KDM4B. KDM4B is overexpressed in many human cancers including prostate cancer. We show that KDM4B functions as both a chromatin remodeler and a trans-acting splicing factor, brings the splicing machinery to the chromatin. We identified many KDM4B-targeted genes including AR whose alternatively spliced isoforms have been implicated in general tumorigenesis, thus demonstrating the far-reaching significance of this novel mechanism.

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
Nothing to report

5. CHANGES/PROBLEMS: The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:

Changes in approach and reasons for change
Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to report

Changes that had a significant impact on expenditures
Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects
Nothing to report
6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

- Publications, conference papers, and presentations
  Nothing to report
- Books or other non-periodical, one-time publications.
  Nothing to report
- Other publications, conference papers and presentations.
  Nothing to report
- Website(s) or other Internet site(s)
  Nothing to report
- Technologies or techniques
  Nothing to report
- Inventions, patent applications, and/or licenses
  Nothing to report
- Other Products
  Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Name: Zhi-Ping Liu  
Project Role: PI  
Researcher Identifier (e.g. ORCID ID): 0000-0003-1341-3878  
Nearest person month worked: 3  
Contribution to Project: Designed experiments, analyzed data, write progress report, and manuscript.  
Funding Support: Cancer prevention and research institute of Texas (CPRIT), American heart association (AHA), DOD, NIH

Name: LingLing Duan  
Project Role: Research associate  
Researcher Identifier (e.g. ORCID ID): 0000-0001-7291-861X  
Nearest person month worked: 8  
Contribution to Project: designed and performed experiments, analyzed data, write progress report, and manuscript.  
Funding Support: Cancer prevention and research institute of Texas (CPRIT), DOD
Name: Qing-Jun Zhang
Project Role: Research Associate
Researcher Identifier (e.g. ORCID ID): 0000-0002-0749-642X
Nearest person month worked: 4
Contribution to Project: performed experiments and analyzed data
Funding Support: Cancer prevention and research institute of Texas (CPRIT), American heart association (AHA), DOD, NIH

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report

What other organizations were involved as partners?
Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: please see Partnering PI Jer-Tsong Hsieh’s progress report

QUAD CHARTS: N/A
9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Figure 1. KDM4B knockdown inhibits CRPC growth in vitro. 22Rv1 cells were transfected with non-targeting or KDM4B-targeting gRNA. Clonal controls and KDM4B KD (cl4, cl7) cells were selected. (A) Relative growth of ctl and cl4 cells in the presence of vector or AR-V7 expression plasmid. Equal amounts of cells were seeded in medium supplemented with FBS or CFBS (n=5, mean±SEM). *, p<0.05. (B) Relative cell growth of ctl1 or cl4 with or without transfected AR-V7 plasmid in medium containing various concentrations of enzalutamide (n=6, mean±SEM). (C-D) Relative mRNA of genes indicated from ctl, cl4, or cl7 cells in the presence or absence of ectopically expressed AR-V7. mRNAs were normalized against internal GAPDH and expressed relative to control cells transfected with empty vector (n=3, mean±SD). (E) Immunofluorescence micrographs of indicated cells stained with E-cadherin (E-cad, green) and DAPI.
Figure 2. KDM4B regulates AR-V7 alternative splicing at chromatins. (A-D) Relative cytosol, soluble nuclear (snuc), and chromatin-associated (chr) AR-V7 mRNA (A, C) and AR mRNA (B, D) in LNCaP, VCaP, and 22Rv1 cells transfected with control vector or KDM4B (A, B), or 22Rv1 cells transfected with non-targeted or KDM4B-targeted sgRNA (C, D). RNAs were normalized against GAPDH and expressed again cytosol RNA in vector-transfected (A & B) or ctl-transfected (C & D) cells. (E) Relative pre-RNA near CE3 of AR in chromatin and soluble nuclear fractions of VCaP cells transfected with control vector or KDM4B. The PCR primer pairs used are pictured below the graph. (F, G) Relative chromatin-occupancy of KDM4B (F) and H3K9me3 (G) in LNCaP-4B and LNCaP-Bls cells in genomic regions between exon 3 and exon 4 of AR. (H) Relative enrichment of KDM4B-bound chromatin DNA (upper panel) and RNA (lower panel) near the CE3 of AR in 22Rv1 cells treated with veh or B3. Values were expressed as % of input. qPCRs were performed using DNA from ChIP and cDNA from ChRIP cross the whole AR locus. Only representative peaks around CE3 are presented. (I) ATAC-qPCR was used to assess open chromatin near CE3 locus in LNCaP cells transfected with control vector, KDM4B, or KDM4Bm. qPCR signals were expressed relative to vector transfected cells at each locus. (A-I) n=3, mean ± SD, *, p<0.05. (J) Model showing chromatin and its associated RNA near the I3f and CE3 positions.