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14. ABSTRACT

Standard therapy for advanced Prostate Cancer (PCa) consists of anti-androgens, which provide respite from disease progression, but ultimately fail resulting in incurable mCRPC. We recently uncovered the critical TLK1>NEK1>ATR>Chk1 axis in mediating the DDR and cell cycle checkpoint while transiting from Androgen Sensitive to Insensitive growth for LNCaP and TRAMP-C2 cells. However, we did not know the generality of this pathway in PCa progression since there are few cell lines where the transition has been studied. Furthermore, the identification of Nek1, and more importantly the TLK-mediated phosphorylation of T141, has never been studied in PCa biopsies. We reported the first study of a PCa TMA of p-Nek1-T141 and correlation to the Gleason score. In addition, we found that TRAMP mice treated with a novel TLK 1inhbitor we generated (J54), following castration did not recover cancerous growth of their prostates. Moreover, we recapitulated the process of translational increase in TLK1B expression in a naïve PDX model that was established from an AR+ adenocarcinoma. Therefore, we believe that this TLK1-Nek1 mediated DDR axis is likely to be a common adaptive response during the transition of PCa cells toward androgen-insensitive growth, and hence CRPC. progression, which has the potential to be targeted with THD and other TLK or Nek1 inhibitors.

15. SUBJECT TERMS
None listed.

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INTRODUCTION

Prostate Cancer (PCa) is one of the most common urological malignancies in men in the United States. Tousled Like kinases (TLKs) are involved in numerous cellular functions, including the DNA Damage Response (DDR). During the tenure of this grant, through a novel proteomic approach, we have identified that NIMA kinase NEK1 strongly interacts and co-localizes with TLKs and has a role in the DDR, upstream of ATR and Chk1, and regulates the checkpoint in response to oxidative stress or replication arrest. We have then tested Thioridazine (THD), an anti-psychotic drug and specific inhibitor of TLKs in inhibiting the activity of NEK1 in prostate cancer cell lines and LNCaP cell derived xenografts in NOD/SCID mice and the subsequent role of ATR-Chk1 axis following DNA damage. Treatment with THD or J54 (a novel less toxic inhibitor we have generated)suppresses the outgrowth of androgen-independent (AI) colonies of LNCaP cells cultured with bicalutamide.

Administration of THD or Bicalutamide long-term was not very effective in inhibiting tumor growth. In contrast, combination therapy of THD/J54 and Bicalutamide remarkably inhibited the tumor growth in LNCaP xenografts model. Further p-ATR and p-Chk1 axis was inhibited by treatment with THD and in combination with bicalutamide as evident by immunoblotting of residual tumors. In cells, following DNA damage, addition of the TLK inhibitor THD impaired ATR and Chk1 activation, indicating the existence of a TLK1>NEK1>ATR>Chk1 pathway.

We continued with the goals of the SOW, and showed that TRAMP mice treated with THD, following castration did not recover cancer outgrowth of their prostates, had no sign of metastases, and failed to convert to the classic NE phenotype at 28 weeks. In addition, as part of the original proposal, we had suggested that we may generate a pT141-Nek1 specific antiserum(although this was not part of the SOW), which we proposed would become handy in as a biomarker for PCa biopsies. We reported the first study of a PCa TMA of p-Nek1-T141 and correlation to the Gleason score. Finally, we recapitulated the process of translational increase in TLK1B expression in a naïve PDX model that was established from an AR+adenocarcinoma. Hence, in future work (not part of this grant) we can rely on such PDX model that more closely resembles the human disease. More recently, we have found that the TLK1>Nek1 axis is also extremely important for the stabilization and increased expression of YAP, a key mediator of the progression to CRPC. It was reported that ectopic expression of YAP is sufficient to convert LNCaP cells from AS to AI in vitro. It also was recently determined that ERG (and the common TMPRSS2-ERG rearrangement) activates the transcriptional program regulated by YAP, and that prostate-specific activation of either ERG or YAP in mice induces similar transcriptional changes and results in age-related prostate tumors. This work provided direct genetic evidence of a causal role for ERG in prostate cancer and revealed a connection between ERG and the Hippo pathway, but in human PCa it is still unknown what are the key components/regulators of the Hippo pathway. Our preliminary work suggests that the TLKs are novel regulators of this nexus that can be optimally targeted, particularly after we discovered that the expression of TLK1B is specifically increased following Androgen Deprivation Treatment (ADT), and results in Nek1 phosphorylation and YAP stabilization in PCa cell lines and in xenografts.

Keywords

TLK1; Nek1; ATR; Chk1; DNA Damage Response (DDR); Cell Cycle Checkpoint; Androgen (In)Sensitive PCa; Xenograft models

.

Summary of key accomplishments for current year of work

1. **First paper:** Khalil, MD.I., Ghosh, I., Singh, V., Chen, J., Haining, Z., and De Benedetti, A. (2020) NEK1 phosphorylation of YAP promotes its stabilization and transcriptional output. Cancers 12(12)3666 https://doi.org/10.3390/cancers12123666.

   ### ABSTRACT

   Most prostate cancer (PCa) deaths result from progressive failure in standard androgen deprivation therapy (ADT), leading to metastatic castration-resistant PCa (mCRPC); however, the mechanism and key players leading to this are not fully understood. While studying the role of tousled-like kinase 1 (TLK1) and never in mitosis gene A (NIMA)-related kinase 1 (NEK1) in a DNA damage response (DDR)-mediated cell cycle arrest in LNCaP cells treated with bicalutamide, we uncovered that overexpression of wt-NEK1 resulted in a rapid conversion to androgen-independent (AI) growth, analogous to what has been observed when YAP1 is overexpressed. We now report that overexpression of wt-NEK1 results in accumulation of YAP1, suggesting the existence of a TLK1>NEK1>YAP1 axis that leads to adaptation to AI growth. Further, YAP1 is co-immunoprecipitated with NEK1. Importantly, NEK1 was able to phosphorylate YAP1 on six residues in vitro, which we believe are important for stabilization of the protein, possibly by increasing its interaction with transcriptional partners. In fact,

knockout (KO) of NEK1 in NT1 PCa cells resulted in a parallel decrease of YAP1 level and reduced expression of typical YAP-regulated target genes. In terms of cancer potential implications, the expression of NEK1 and YAP1 proteins was found to be increased and correlated in several cancers. These include PCa stages according to Gleason score, head and neck squamous cell carcinoma, and glioblastoma, suggesting that this co-regulation is imparted by increased YAP1 stability when NEK1 is overexpressed or activated by TLK1, and not through transcriptional co-expression. We propose that the TLK1>NEK1>YAP1 axis is a key determinant for cancer progression, particularly during the process of androgen-sensitive to -independent conversion during progression to mCRPC.

2. **Second paper**: Singh, V., Khalil, I., and De Benedetti, A (2020) The TLK1/Nek1 axis contributes to mitochondrial integrity and apoptosis prevention via phosphorylation of VDAC1. Cell Cycle 9: 1-13. DOI: 10.1080/15384101.2019.1711317.

ABSTRACT
The TLK1/Nek1 axis contributes to cell cycle arrest and implementation of the DDR to mediate survival upon DNA damage. However, when the damage is too severe, the cells typically are forced into apoptosis, and the contribution of TLKs in this process has not been investigated. In contrast, it is known that Nek1 may play a role by phosphorylating VDAC1 maintaining proper opening and closure of the channel and thus mitochondrial integrity. We now show that the activating phosphorylation of Nek1-T141 by TLK1 contributes to the phosphorylation and stability of VDAC1 and thereby to mitochondrial permeability and integrity. Treatment of three different cell lines model that overexpress Nek1-T141A mutant with doxorubicin showed exquisite sensitivity to the drug, with implementation of rapid accumulation of cells with subG1 DNA content (apoptotic) and other alterations in the cell cycle. In addition, these cells displayed reduced oxygen consumption under normal conditions and less reliance on mitochondria and more dependence on glycolysis for energy production. Consistent with greater apoptosis, upon treatment with low doses of doxorubicin, cells overexpressing Nek1-T141A displayed leakage of Cyt-C into the cytoplasmic fraction. This suggests that inhibiting the TLK1/Nek1/VDAC1 nexus could sensitize cancer cells to apoptotic killing in combination with an appropriate DNA damaging agent. We in fact have previously reported that Nek1 expression is elevated in advanced Prostate Cancer (PCa) and we now report that VDAC1 expression is elevated and correlated with disease stage, thereby making the TLK1/Nek1/VDAC1 nexus a very attractive target for PCa.

3. **Third paper:** Madere, C., Khalil, MD.I., Ghosh, I., Adam, MR., and De Benedetti, A. (2021) Interaction of TLK1 and AKTIP as a potential regulator of AKT activation in castration-resistant prostate cancer progression *Pathophysiology* 2021, *28*(3), 339-354; **https://doi.org/10.3390/pathophysiology28030023**

ABSTRACT
Prostate cancer (PCa) progression is characterized by the emergence of resistance to androgen deprivation therapy (ADT). AKT/PKB has been directly implicated in PCa progression, often due to the loss of PTEN and activation of PI3K>PDK1>AKT signaling. However, the regulatory network of AKT remains incompletely defined. Here, we describe the functional significance of AKTIP in PCa cell growth. AKTIP, identified in an interactome analysis as a substrate of TLK1B (that itself is elevated following ADT), enhances the association of AKT with PDK1 and its phosphorylation at T308 and S473. The interaction between TLK1 and AKTIP led to AKTIP phosphorylation at T22 and S237. The inactivation of TLK1 led to reduced AKT phosphorylation, which was potentiated with AKTIP knockdown. The TLK1 inhibitor J54 inhibited the growth of the LNCaP cells attributed to reduced AKT activation. However, LNCaP cells that expressed constitutively active, membrane-enriched Myr-AKT (which is expected to be active, even in the absence of AKTIP) were also growth-inhibited with J54. This suggested that other pathways (like TLK1>NEK1>YAP) regulating proliferation are also suppressed and can mediate growth inhibition, despite compensation by Myr-AKT. Nonetheless, further investigation of the potential role of TLK1>AKTIP>AKT in suppressing apoptosis, and conversely its reversal with J54, is warranted.

IMPACT:
Our work, in addition to follow the general guides of the original proposal, has expanded into new areas of research. In addition to having discovered a new role for TLK1 in the anti-apoptotic field, we have made the key discovery that the TLK1>Nek1 axis is also a key regulator of the Hippo pathway, in addition to regulating the DDR>ATR>Chk1 axis.

What were the major goals of the project? We have targeted a specific liability that incurs in Androgen Responsive PCa cells when shifted to ADT, by adding an inhibitor (THD) of the TLK1>Nek1>ATR>Chk1 DDR axis in order to abrogate the checkpoint and promote apoptosis. This axis was initially hypothesized at

the onset of submitting this grant. The new slant in our research is that a bifurcation of this axis is also key to YAP accumulation and hence participant in the conversion to CRPC.

CHANGES/PROBLEMS:

We have not encountered any major problem with the work, except that the breeding of our TRAMP/Nek1-KO mice suffered because of the impact of COVID19. This has resulted in a delay in the collection of prostates. However, we have been able to publish other important work in journals with a strong reputation.

What do you plan to do during the next reporting period to accomplish the goals? The final part of the grant and in line with the SOW was the generation of a Nek1-KO mouse in TRAMP background, so that we can determine genetically that these mice, following castration do not progress to CRPC. This will provide direct genetic evidence for the critical importance of the TLK1>Nek1>DDR axis in progression to CRPC. We are now back on track with the transgenic mice, and have begun analyzing the work, which at this point seems to give really exciting data (to be reported in the final report in 2022).

PRODUCTS: (3 publications – already listed; 5 Abstracts):

Meetings Attended and Papers Presented.

Vibha Singh[1], Praveen Kumar Jaiswal[1], Hari K Koul[1,2], Xiuping Yu[1], Arrigo DeBenedetti[1*]
Over expression of Protein kinase Nek1 and its modulation by Thioridazine in Prostate cancer – AACR 2020

Siddhant Bhoir1,2†, Vibha Singh2†,Rupesh Chikhale3‡, Javeena Hussain4‡, Donard Dwyer5, Richard Bryce3*, Sivapriya Kirubakaran1,4* and Arrigo De Benedetti2*
Targeting Prostate Cancer: The Tousled Way – Gordon Research Conference 2020

Ishita Ghosh and Arrigo DeBenedetti
Role of Tousled like Kinase and LATS1 interaction in DNA damage and repair. –AACR 2020

Imtiaz Khalil and Arrigo De Benedetti
The Implications of TLK1-MK5 Signaling Axis in Prostate Cancer Cell Motility and Invasion – ASBMB 2020

Imtiaz Khalil and Arrigo De Benedetti
TLK1-MK5 signaling axis contributes to prostate cancer cell motility and invasion – AACR 2020

Participants

The following people (all in the Department of Biochemistry and Molecular Biology and the Feist Weiller Cancer Center at LSUHSC) have participated in this research: Vibha Singh, Praveen Kumar Jaiswal, Ishita Ghosh, Imtiaz Khalil, Hari K. Koul, Xiuping Yu, and Arrigo De Benedetti

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

Vibha Singh is a postdoc in my lab, and she had limited training in animal work. During this period she has become proficient in xenografts implants and with surgical castration. She is also now experienced in fluorescent microscopy.
Ishita Ghosh is a graduate student in my lab, and has learned numerous techniques in vitro and with animals.
MD Imtiaz Khalil is a graduate student in my lab, and has learned numerous techniques in vitro and with animals. He is also now experienced in fluorescent microscopy.

Participants & Other Collaborating Organizations

We have engaged in a productive collaboration with scientists in the Department of Molecular and Cellular Biochemistry and Proteomics Core, Center for Structural Biology, University of Kentucky, Lexington, KY 40506, USA. Namely, Drs. Jin Cheng and Haining Zhu.

Appendix (3 papers).