DNA-Dependent Protein Kinase Drives Prostate Cancer Progression through Transcriptional Regulation of the Wnt Signaling Pathway

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Abstract

Purpose: Protein kinases are known to play a prominent role in oncogenic progression across multiple cancer subtypes, yet their role in prostate cancer progression remains under-explored. The purpose of this study was to identify kinases that drive prostate cancer progression.

Experimental Design: To discover kinases that drive prostate cancer progression, we investigated the association between gene expression of all known kinases and long-term clinical outcomes in tumor samples from 545 patients with high-risk disease. We evaluated the impact of genetic and pharmacologic inhibition of the most significant kinase associated with metastatic progression in vitro and in vivo.

Results: DNA-dependent protein kinase (DNAPK) was identified as the most significant kinase associated with metastatic progression in high-risk prostate cancer. Inhibition of DNAPK suppressed the growth of both AR-dependent and AR-independent prostate cancer cells. Gene set enrichment analysis nominated Wnt as the top pathway associated with DNAPK. We found that DNAPK interacts with the Wnt transcription factor LEF1 and is critical for LEF1-mediated transcription.

Conclusions: Our data show that DNAPK drives prostate cancer progression through transcriptional regulation of Wnt signaling and is an attractive therapeutic target in aggressive prostate cancer.

Introduction

Androgen-deprivation therapy is the standard of care for patients with advanced prostate cancer. However, responses are not durable and tumors invariably progress to castration-resistant prostate cancer (CRPC). CRPC has limited treatment options and ultimately undergoes metastatic progression to become a lethal disease. Although the development of next-generation androgen therapy, chemotherapy, and immunotherapy has improved overall survival for metastatic CRPC (mCRPC) patients (1–6), the mortality rates of patients with mCRPC remain high. Thus, there is a clear need to identify novel therapeutic targets to prevent progression to mCRPC and to improve outcomes.


Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-18-2387

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Published OnlineFirst July 2, 2019; DOI: 10.1158/1078-0432.CCR-18-2387

5608 Clin Cancer Res; 25(18) September 15, 2019

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We also show that Wnt3A localized high-risk disease and validated the prognostic value of significantly associated kinase with metastatic progression in prostate cancer. We found that inhibition of DNAPK suppresses growth of both androgen receptor (AR)-positive and AR-negative prostate cancer models. Our results suggest that DNAPK interacts with the Wnt transcription factor LEF1 and promotes disease aggressiveness through Wnt signaling. Together, these findings suggest that DNAPK represents an attractive therapeutic target in prostate cancer, and highlight the potential of DNAPK inhibition as a strategy for treating Wnt-addicted cancers. Our findings have led to the initiation of the first trial of DNAPK inhibition for patients with metastatic prostate cancer.

**Translational Relevance**

Kinases play a vital role in driving oncogenic pathways and have been the target for therapeutic intervention across multiple cancer subtypes (7). However, the role of kinases in aggressive prostate cancer remains poorly understood. Moreover, there are commonly no kinase inhibitors that are known to increase survival of patients with prostate cancer (8). The discrepancy between the utility of kinase inhibitors in multiple cancer subtypes and the lack of effective kinase inhibitors in prostate cancer strongly suggests the need for a more systematic exploration of kinases in prostate cancer.

To identify novel driver kinases involved in prostate cancer progression, we performed an unbiased analysis evaluating the association between expression of all known kinases and clinical outcomes using prostate cancer samples from a large cohort of patients with high-risk disease treated with prostatectomy. We identified DNA-dependent protein kinase (DNAPK) as the most significantly associated kinase with metastatic progression in localized high-risk disease and validated the prognostic value of high levels of DNAPK expression across multiple data sets. DNAPK, a nuclear serine/threonine protein kinase, is well known for its role in DNA repair via the nonhomologous end joining pathway (9). However, numerous studies have demonstrated the importance of DNAPK in a variety of other processes, including the modulation of chromatin structure and transcription through its interaction with a variety of receptors, polymerases, and transcription factors (10–14). We have previously found that DNAPK transcriptionally regulates multiple pathways involved in prostate cancer progression (15); however, the dominant pathways mediated by DNAPK signaling have not been elucidated.

To interrogate the dominant mechanism by which DNAPK drives tumor progression, we utilized Gene Set Enrichment Analysis (GSEA) on gene-expression data from both clinical prostate cancer specimens and prostate cancer cell lines to nominate Wnt signaling as a key DNAPK-regulated pathway. We demonstrate that genetic or pharmacologic inhibition of DNAPK suppresses Wnt3A-induced migration and invasion in prostate cancer cells. We also show that DNAPK interacts with the Wnt transcription factor LEF1 and that inhibition of DNAPK reduces LEF1-mediated transcription. Using multiple DNAPK inhibitors, we demonstrate that inhibition of DNAPK reduces growth of prostate cancer xenografts in vivo as well as proliferation of human prostate cancer explants ex vivo. Together, our data show that DNAPK is a viable prognostic biomarker that drives disease progression through transcriptional regulation of Wnt signaling and support the use of DNAPK inhibitors as a therapeutic strategy in aggressive prostate cancer.

**Materials and Methods**

**Human studies**

Patient demographics are described in Supplementary Table S1. Data collection at each institution (Mayo Clinic and Johns Hopkins Medical Institute) was approved by the corresponding institutional review board (IRB) and patient consent was waived by the IRBs. The studies were conducted in accordance with the U.S. common rule.

**Cell lines**

LNCaP, C4-2B, PC-3, VCaP, and DU-145 cells (all male in gender) were purchased from the ATCC, grown in respective ATCC recommended culture media, and maintained at 37°C in 5% CO2. All cell lines were regularly tested for Mycoplasma contamination and tested negative. For androgen-deprivation studies, LNCaP cells were grown in RPMI media without phenol red supplemented with 5% charcoal-stripped serum (Thermo Fisher Scientific). LNCaP-AR-enza-res cells were generated by treating LNCaP-AR parental cells with increasing doses of enzalutamide (starting from 1 μmol/L to 50 μmol/L) until resistance emerged.

**Clinical cohorts and microarray analysis**

Radical prostatectomy samples were obtained from the Mayo Clinic (MC, N = 545) and Johns Hopkins Medical Institute (JHMI, N = 355). Patient demographics are included in Table S1. MC was a nested case–control study of metastatic progression (16), and JHMI was a cohort study of observation until metastatic progression (17). Stratification of patients into luminal and basal subtypes was determined as previously described (18).

Samples were processed and gene expression was analyzed with the Affymetrix Human Exon 1.0 ST array in a CLIA-certified facility as described previously (16, 17). All human kinases (19) were evaluated for differential expression associated with metastatic progression by Fisher exact test on high and low expression clusters determined by k-medoids clustering (Fig. 1A; refs. 19, 20) using R software. Kaplan–Meier curves for freedom from metastasis (Fig. 1B and C), prostate cancer–specific survival, and overall survival were generated with high expression defined as the 80th percentile in each cohort as described previously (15) and P values were calculated using the log-rank test. The association of DNAPK with metastasis was also examined independently in Oncomine (www.oncogene.org) in prostate cancer versus metastatic samples. One cohort by Chandran and colleagues (21) was excluded because the DNAPK probe information was internally discordant. All statistical analyses were performed in R. A P < 0.05 was considered statistically significant.

**DNAPK knockdown microarray**

RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol and hybridized to Affymetrix Human Gene ST 2.1 microarrays. Expression data were normalized using the robust multiarray average (RMA) (22) and log2
transformed. Expression profiles were analyzed using standard GSEA comparing the controls with the DNAPK knockdown samples (23), the KEGG and Biocarta pathway gene sets, and using the GSEA Preranked algorithm on Pearson correlations between each gene and DNAPK in the clinical samples. The association of DNAPK with metastasis was also examined independently in Oncomine (www.oncomine.org; ref. 24) in prostate cancer versus metastatic samples.

Figure 1.
DNAPK is the most significant kinase associated with metastatic progression in prostate cancer. A, The log odds ratio of having a metastatic event with expression of every kinase is shown. Each kinase is represented by a yellow bar. The enrichment of DNAPK is shown with the red bar. B, Kaplan-Meier curves demonstrate the clinical outcomes (metastasis-free survival, prostate cancer-specific survival, and overall survival) associated with DNAPK expression in discovery (MC cohort, \( N = 545 \)) and validation (JHMI cohort, \( N = 355 \)) cohorts. C, Kaplan-Meier curves demonstrate distant metastasis-free survival associated with DNAPK expression in MC and JHMI cohorts stratified into luminal and basal subtypes. D, Box plots show Oncomine analyses of DNAPK expression in primary (green) vs. metastatic (red) prostate cancer. \( P \) values for Kaplan–Meier survival curves were calculated using the log-rank test.

siRNA treatment and cell proliferation assays
ON-TARGETplus siRNA against DNAPK, LEF1, CTNNB1, and nontargeting control (siNTC) from Dharmacon were used at 100 nmol/L. Cells were transfected in 6-well plates at a density of 50,000 cells per well using Oligofectamine (Invitrogen), according to the manufacturer’s protocol. Transfection was repeated 24 hours later; the cells were grown for an additional 48 hours and replated at a density of 5,000 to 10,000 cells per well.
in 24-well plates. For proliferation assays with NU7441, cells were treated with NU7441 (1 μmol/L) or DMSO for 24 hours before plating. Cells were then harvested and plated at the previously mentioned density in the presence of NU7441 or DMSO. Cells were counted in duplicate at indicated time points using a Beckman Coulter cell counter and growth curves were plotted as the mean ± SD.

Quantitative real-time-PCR assay
RNA was isolated from cell or tissue lysates by the RNeasy Micro Kit (Qiagen), and cDNA was synthesized from 1-μg RNA using SuperScript III (Invitrogen) and Random Primers (Invitrogen), per the manufacturer’s protocol. qRT-PCR was carried out on the StepOne Real-Time PCR system (Applied Biosystems) using gene-specific primers designed with Primer-BLAST (Supplementary Table S4) and synthesized by IDT Technologies. Validation of siRNA-mediated knockdown was carried out using Fast SYBR Green Master Mix (Thermo Fisher Scientific), per the manufacturer’s protocol. The Wnt gene list was adopted from the Wnt classification method and plotted as the mean fold change ± SD compared with the nontargeting siRNA (i.e., relative quantity or RQ) in duplicate.

Immunoblot analysis
Cell lysates were separated on 4% to 12% gradient SDS polyacrylamide gels (Thermo Fisher Scientific) and transferred to polyvinylidene difluoride membranes (GE Healthcare) by wet transfer. Antibodies to phospho-DNAPK (Abcam No. 18192), KU70 (BD Biosciences No. 611892), β-catenin (Cell Signaling Technology No. 5605S), GAPDH (Cell Signaling Technology No. 2118L), total DNAPK (Ab-4, NeoMarkers), active β-catenin (Millipore No. 05-665), and LEF1 (Millipore No. 17-604) were used at 1:1,000 dilutions. Membranes were blocked for 1 hour in 5% BSA or 5% low-fat milk dissolved in 0.05% Tween-20/TBS (TBST) and then incubated with primary antibodies overnight at 4°C. Membranes were washed three times in TBST and incubated with HRP-linked secondary antibodies diluted 1:10,000 in TBST for 1 hour at room temperature. Membranes were then washed three times in TBST and developed using ECL Prime (GE Healthcare) detection reagent according to the manufacturer’s protocol.

Immunoprecipitation studies
For immunoprecipitation studies, nuclear pellets were prepared from cells using NE-PER kit (Thermo Fisher Scientific) according to manufacturer’s protocol. The nuclear pellets were directly lysed in immunoprecipitation buffer (IP buffer) containing 20 mmol/L Tris pH 7.5, 1% Triton X-100, and 300 mmol/L NaCl supplemented with Halt Protease/phosphatase inhibitor cocktail, (Thermo Fisher Scientific). Cell lysates were cleared with MagnaBind Beads (Thermo Fisher Scientific) for 2 hours, and were incubated with antibodies against DNAPK or LEF1 antibodies overnight at 4°C. The following day, MagnaBind Beads were added to the lysates and incubated for 2 hours at 4°C for the bead–antibody cross-linking. Beads were then washed three times with IP buffer and resuspended in 2× loading buffer (Bio-Rad). Immunoblot analysis for detection of DNAPK, KU70, and LEF1 was performed as described previously.

TOPFLASH reporter assay
TOPFLASH TCF/LEF1 reporter was a kind gift from Dr. Chandan Kumar-Sinha (University of Michigan). The reporter construct was cotransfected with Renilla luciferase (1:50 ratio) into PC-3 cells using FuGene HD transfection reagent (Promega). After 24 hours, cells were treated with 1 μmol/L NU7441, or were induced with 200 nmol/L Wnt3A for 3 hours, or were treated with a combination of NU7441 and Wnt3A. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, cat. No. E1910) according to the manufacturer’s protocol. Levels of TOPFLASH firefly luciferase were normalized to Renilla luciferase and graphs were plotted as the mean ± SD in duplicates. Readings were recorded on GloMax 96 microplate luminometer (Promega).

Chromatin immunoprecipitation assay (ChIP)
ChIP assays were performed using the HighCell CHIP kit (Diagenode No. C01010601) following the manufacturer’s protocol. Briefly, PC-3 or C4-2B cells were treated either with DMSO or with 1 μmol/L NU7441 for 24 hours. Cells were trypsinized and washed with PBS and were resuspended in 500-μL PBS. Cells were fixed by adding 13.5 μL of 36.5% formaldehyde for 8 minutes and the fixing was terminated by adding 57-μL 1.25-M glycine. Cells were spun, washed three times with PBS and resuspended in 1 mL of ice-cold buffer L1. After 10 minutes, cells were spun down, and the pellets were resuspended in 1 mL ice-cold buffer L2. Cells were collected by centrifugation after 5 minutes, and pellets were resuspended in 200-μL shearing buffer supplemented with protease inhibitors. Cells were sonicated in BioRuptor (Diagenode) for four runs of 10 cycles (30 seconds on and 30 seconds off). Lysates were resuspended in a total of 1 mL CHIP buffer C1 and were centrifuged. Cleared lysates were collected and 9.5 μL of lystate was used as input while 950-μL lystate was used for ChIP. ChIP was performed using protein G beads coated with 5-μg DNAPK cocktail antibody (Ab-4, NeoMarkers) or IgG (Santa Cruz No. SC-2025) overnight. The next day, beads were collected, washed three times with ChIP buffer C1 and once with wash buffer W1. The input samples and washed beads were incubated at 55°C (15 minutes) then 100°C (15 minutes) in complete DIB buffer, supplemented with 1-μL proteinase K and were incubated. Beads were spun and supernatants containing DNA were collected and performed qRT-PCR. qRT-PCR data were analyzed using the relative quantification method after normalizing with respective input controls and plotted as the mean fold change ± SD compared with the IgG (i.e., RQ). For the ChIP experiments in the backdrops of LEF1 knockdown, cells were treated either with siNTC or with siLEF1 and incubated for 48 hours. ChIP assays were performed as described above.

Boyden chamber–based invasion and migration assays
Boyden Transwell chambers for invasion and migration were purchased from Corning Inc. Cells were transfected with siRNAs as described above or treated with DMSO, 1 μmol/L NU7441 (Selleckchem), 1 μmol/L CC-115 (Celgene), 1 μmol/L IWP-2 (Selleckchem), a combination of IWP-2 and CC-115 (1 μmol/L each), or 10 μmol/L enzalutamide (Selleckchem) for 24 hours. Cells were then trypsinized and resuspended in serum-free media at a density of 30,000 per PC-3 cells and at a density of 200,000 (for invasion, or 100,000 for migration) for LNCaP-AR and C4-2B cells, in the upper chamber. The lower chamber was supplied with media containing 10% FBS, supplemented with DMSO, 1

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Clin Cancer Res; 25(18) September 15, 2019

5611

Published OnlineFirst July 2, 2019; DOI: 10.1158/1078-0432.CCR-18-2387

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Kothari et al.

μmol/L NU17441, 1 μmol/L CC-115, or 200 ng/mL Wnt3A (R&D Systems). For the experiments with IWP-2, lower chambers were supplemented with media containing 10% FBS and IWP-2 and/or CC-115 (1 μmol/L each). Cells were incubated for 48 hours and were then fixed using 4% paraformaldehyde for 30 minutes at room temperature. Wells were washed with PBS, and membranes were stained with 0.25% (wt/vol) crystal violet (Sigma-Aldrich) in 25% (vol/vol) methanol (Fisher Scientific) for 20 minutes and then washed (with PBS). For quantification, crystal violet trapped by the cells was dissolved in 10% acetic acid and absorbance was measured at 560 nm using a Spectramax 384 plus plate reader. Representative images were taken on an Olympus IX17 using DP Controller 3.1.1.267 software. Graphs were plotted as the mean ± SD and P values were calculated by two-way ANOVA using GraphPad Prism software.

In vivo animal studies

Four- to 6-week-old male CB17-SCID mice were provided by our lab’s in-house breeding colony and housed under pathogen-free conditions approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the U.S. Department of Agriculture and Department of Health and Human Services. Animal experiments were approved by the University of Michigan’s University Committee on Use and Care of Animals and carried out in accordance with established guidelines. For xenograft studies involving treatment with NU17441, precastured mice were anesthetized with approximately 2% isoflurane and LNCaP-AR cells suspended in 100 μL of 1:1 mixture of 1× PBS and Matrigel (BD Biosciences) were subcutaneously injected bilaterally into their flanks. When the tumor size reached 24 mm³, mice were randomized into control and treatment groups (n = 6 for the NU17441 arm and n = 5 for the vehicle arm). The DNAPK inhibitor NU17441 (Selleck Chemicals) was administered once daily via IP injection at 25 mg/kg body weight five times per week for 4 weeks. For the CC-115 and enzalutamide xenograft studies, LNCaP-AR cells were injected subcutaneously into the flanks of uncastrated mice as described above. Once tumors reached an average size of 80 mm³, mice were randomized and divided into treatment groups of vehicle control, CC-115 (2 mg/kg), enzalutamide (10 mg/kg), and a combination of CC-115 and enzalutamide. CC-115 and enzalutamide were administered once daily via oral gavage five times per week for 6 weeks. Tumor growth was monitored two to three times per week. After 4 (NU17441) or 6 (CC-115, enzalutamide) weeks of treatment, mice were weighed and euthanized and the tumors harvested. Tumor caliper measurements were converted into tumor volumes using the formula \( V = \frac{1}{2} \text{length} \times \text{width}^2 \text{mm}^3 \) and plotted as mean tumor volume ± SEM using GraphPad Prism software.

IHC

Human prostate ex vivo explant cultures were conducted as previously described (25). Briefly, fresh primary tumor tissue was obtained from a clinical pathologist immediately following radical prostatectomy at Thomas Jefferson University Hospital in accordance with IRB standards and in compliance with federal regulations governing research on deidentified specimens and/or clinical data [45 CFR 46.102(f)]. The specimens were processed under a laminar flow hood using sterile technique and transported in culture media (IMEM, 5% FBS, 0.01 mg/mL insulin (Invitrogen No. 12585-014), 30 μmol/L hydrocortisone (Sigma No. H-0888), and penicillin/streptomycin) on ice. Tissue was subdivided into approximately 1 mm³ pieces and placed in a 24-well plate on presoaked dental sponges (Novaris No. 96002; two to three pieces per sponge) placed into 0.5-ml culture media in the presence or absence of drug (1 μmol/L NU17441). Plates were placed in an incubator at 37°C and 5% CO₂. Media were replaced every 48 hours with appropriate treatment, and explants were harvested on day 6. Tissue was either formalin fixed for IHC analysis or placed in 1-mL RNAlater (Ambion No. AM7020; kept at 4°C for 24 hours, then stored at −80°C until processing) for RNA analysis. RNA extraction was performed using TRIzol (Life Technologies; No. 15956018) per the manufacturer’s instructions. IHC samples were stained using Ki67 antibody (Cell signaling Technologies; No. 9027) and Ki67-positive cells were counted. Graphs were prepared using GraphPad Prism software.

Data availability

Microarray data are available at NCBI GEO accession GSE46691, GSE79956, GSE79957, and GSE116895.

Results

DNAPK is the most significantly associated kinase with metastatic progression in prostate cancer

To identify the kinases that potentially drive progression of aggressive prostate cancer, we first evaluated the association of expression of all known kinases with metastatic progression, using prostate cancer samples from a cohort of patients treated with prostatectomy at the MC. Patient demographics of this cohort are described in Supplementary Table S1. Notable features of this cohort include the long clinical follow-up (median of 13.4 years), large sample size (N = 545), and prevalence of high-risk characteristics as defined by National Comprehensive Cancer Network criteria (www.nccn.org) such as extracapsular extension (50%) and seminal vesicle invasion (32%). Additionally, and consistent with these high-risk features, 39% of the patients in this cohort experienced metastatic progression. In this discovery cohort, we ranked all kinases (39) by the relative enrichment for metastatic progression in cases with high versus low expression of each kinase, with expression cutoffs defined by an unbiased clustering approach as previously described (20). This analysis demonstrated that DNAPK was the most significant kinase that enriched for metastatic progression (OR, 2.19; log₂ OR, 1.314, P < 0.0001; Fig. 1A; Supplementary Table S2). We next used Kaplan–Meier analyses to assess the prognostic value of elevated tumor DNAPK expression in this cohort using the same cutoff as described previously (15), and found that high expression of DNAPK was significantly associated with not only metastatic progression (HR, 2.0; 1.5–2.7, P < 0.0001), but also decreased rates of prostate cancer-specific survival (HR, 2.4; 1.7–3.5, P < 0.0001) and overall survival (HR, 2.0; 1.5–2.6, P < 0.0001; Fig. 1B). We validated these findings in an independent cohort of patients from JHMI (Supplementary Table S1), where high DNAPK expression again was associated with increased metastatic progression (HR = 2.4; 1.7–3.6, P < 0.0001) and decreased prostate cancer–specific survival (HR, 2; 1.1–3.5, P = 0.02), with borderline significance for decreased overall survival (HR, 1.7; 0.97–2.8, P = 0.06; Fig. 1B). Because we have previously found that DNAPK transcriptionally activates the androgen receptor (AR) to potentiate AR function, we next
stratified our patient cohorts into luminal (more AR-driven) and basal (less AR-driven) subtypes (18, 26). As expected, within the luminal subtype of prostate cancer, characterized by elevated AR expression and signaling activity, increased DNAPK expression was associated with poor metastasis-free survival. Unexpectedly, we found that increased DNAPK expression is also a strong prognostic indicator in the basal subtype of prostate cancer, which is thought to be more AR independent (Fig. 1C). After identifying the prognostic value of DNAPK in localized disease and the luminal and basal subtypes of prostate cancer, we examined the expression of DNAPK in metastatic prostate cancer using Oncomine analysis (www.oncomine.org). DNAPK was significantly overexpressed in metastatic versus primary prostate cancer samples in 10 of the 12 cohorts that we examined (Fig. 1D). These results show that DNAPK is strongly prognostic in localized high-risk prostate cancer, the luminal and basal subtypes of prostate cancer, and is associated with development of metastatic disease.

Genetic and pharmacologic inhibition of DNAPK suppresses aggressive cancer phenotypes in vitro

Given the prognostic value of DNAPK expression in localized disease and the luminal and basal subtypes of prostate cancer, we next assessed the functional significance of DNAPK in both AR-dependent and AR-independent prostate cancer cell line models. Genetic (via siRNA-mediated knockdown) or pharmacologic inhibition (via the small-molecule inhibitor NU7441) of DNAPK drastically diminished migration, invasion, and proliferation of both AR-positive cells (LNCaP-AR and C4-2B), as well as AR-negative PC-3 cells (Fig. 2A–D). Efficient knockdown of DNAPK was achieved in these experiments (Fig. 2E). Given our previous findings that DNAPK is a required cofactor for AR (12), our results from the AR-positive LNCaP-AR and C4-2B cells were expected; however, the reduction in aggressive cancer phenotypes in AR-negative PC-3 cells indicate that DNAPK has functions beyond regulating AR activity that may contribute to prostate cancer progression.

The Wnt pathway is associated with DNAPK in prostate cancer samples

Recently, we investigated transcriptional networks that are modulated by DNAPK using cell line models (15) in the context of AR. Guided by our observation that DNAPK is a prognostic indicator in the luminal and basal subtypes of prostate cancer and that DNAPK inhibition significantly reduces aggressive cancer phenotypes in both AR-dependent and AR-independent cells, we extended these studies by investigating DNAPK-associated pathways in clinical samples as well as in preclinical models. We performed guilt-by-association analyses to identify the role of DNAPK in modulating pathways involved in promoting metastasis. We measured changes in gene expression, due to siRNA-mediated DNAPK knockdown in AR-dependent (VCaP and C4-2B) and AR-independent (PC-3 and DU145) cell lines, using microarray analyses and ran standard GSEA analyses on these data. We also correlated each gene with DNAPK expression in clinical samples from the MC cohort and ran GSEA Preranked using the same gene sets. A scatterplot of the GSEA normalized enrichment scores (NES) for each pathway gene set was then generated with in vitro NES values on the y-axis, and in vitro NES scores on the x-axis (Fig. 3A). The Wnt pathway had the highest average NES (knockdown NES = 1.48, MC NES = 2.47, average = 1.97; Fig. 3A; Supplementary Table S3). Furthermore, we found that expression of DNAPK and β-catenin (CTNNB1), a critical Wnt effector, was tightly correlated in clinical samples (Pearson correlation coefficient = 0.7; Supplementary Fig. S1A). These data nominate the Wnt pathway as highly significant pathway associated with DNAPK, supporting a role of DNAPK in mediating the regulation of the Wnt signaling pathway.

siRNA-mediated knockdown of DNAPK suppresses androgen-deprivation therapy (ADT)–induced Wnt signaling

To investigate the relationship between DNAPK and Wnt signaling in prostate cancer, we first evaluated the expression of Wnt pathway genes in prostate cancer across all stages of progression as represented by the following cell lines: (i) LNCaP, which are AR-positive, androgen-sensitive cells that represent hormone-sensitive disease; (ii) LNCaP-AR and C4-2B, which are AR-positive, androgen-insensitive cells that represent CRPC; and (iii) PC-3 cells, which are AR-negative, androgen-insensitive cells that represent androgen-independent disease. We found that increased Wnt pathway gene expression associated with models of more aggressive disease (LNCaP-AR, C4-2B, and PC-3) compared with the hormone-sensitive LNCaP cells (Fig. 3B; Supplementary Fig. S1B), suggesting that progression on ADT could be mediated by Wnt signaling. Consistent with clinical observations that hormone-naïve cells eventually overcome ADT, we found that LNCaP cells continued to grow despite androgen depletion using charcoal-stripped serum, albeit at a much slower rate (Fig. 3C). Interestingly, growth under androgen-depleted conditions triggered the expression of multiple Wnt genes in LNCaP cells (Fig. 3D, gray bars), further supporting a role for Wnt-mediated disease progression in response to ADT.

Given our earlier observation that DNAPK modulates the Wnt pathway, we next asked if DNAPK is involved in ADT-induced Wnt signaling. siRNA-mediated knockdown of DNAPK in LNCaP cells grown under androgen-depleted conditions caused a marked decrease in expression of Wnt genes (Fig. 3D, red bars), suggesting that DNAPK is required for ADT-induced Wnt signaling. Furthermore, DNAPK knockdown (via siRNA) prevented the emergence of ADT-resistant LNCaP cells (Fig. 3E), demonstrating the importance of DNAPK-mediated signaling in hormone resistance. Taken together, these data suggest that DNAPK inhibition is a viable therapeutic strategy to prevent the emergence of ADT resistance in prostate cancer and likely acts through inhibiting Wnt signaling.

DNAPK inhibition reduces Wnt-induced invasion and migration in CRPC cells

Because our results implicate DNAPK-mediated Wnt signaling as a contributor to ADT resistance, we next investigated the relationship between DNAPK and Wnt signaling in cell line models of frank castration-resistance. Genetic or pharmacologic inhibition of DNAPK in CRPC cells (LNCaP-AR and C4-2B) using siRNAs targeting DNAPK or the DNAPK inhibitor NU7441 caused a marked reduction in mRNA levels of Wnt pathway genes (Fig. 4A and B; Supplementary Fig. S2A and S2B), whereas mRNA levels of genes implicated in other pathways remained broadly unchanged (Supplementary Fig. S2C). Genetic and pharmacologic inhibition of DNAPK also reduced steady-state levels of active (dephosphorylated) β-catenin and c-Myc protein (a Wnt target gene; ref. 27) and suppressed Wnt3A-mediated induction of active β-catenin and c-Myc protein levels (Fig. 4C and D). Pharmacologic inhibition using NU7441 or the DNAPK
inhibitor CC-115 (ref. 28; United States Patent No. 8,110,578), which is currently being tested in early clinical trials for solid tumors (NCT01353625, NCT02833883, and NCT02977780), reduced invasion and migration of CRPC cells and suppressed Wnt3A-mediated induction of invasion and migration of CRPC cells (Fig. 4E). Interestingly, a combination of CC-115 and IWP-2 (a laboratory-grade Wnt antagonist) reduces the migration of prostate cancer cells similar to the extent of CC-115
**Figure 3.**

**A,** Scatter plot represents the GSEA NES for all pathways associated with DNAPK in both the in vitro knockdown experiment (using VCaP, C4-2B, PC-3, DU145 cells) as well as in the clinical samples from the MC. The Wnt pathway had the top average NES, with high scores in both the clinical and in vitro knockdown results, and is shown in red. Dots in black denote other pathways. **B,** The bar graph shows the expression of select Wnt pathway genes in LNCaP-AR and C4-2B (CRPC) cells compared with LNCaP (hormone-naïve) cells. GAPDH was used as an internal reference (N = 3 in duplicates). **C,** The growth curves show the effect of androgen depletion on proliferation of LNCaP cells, compared with LNCaP cells grown under normal serum (NS) conditions (N = 3 in duplicates). **D,** Bar graphs represent the expression of Wnt genes in LNCaP cells grown under androgen-depleted (charcoal-stripped serum) conditions, LNCaP cells grown under normal serum conditions, and after DNAPK knockdown in LNCaP cells grown under charcoal-stripped serum conditions, compared with LNCaP cells grown under charcoal-stripped conditions. (N = 3, in duplicates). **E,** Cell growth curves demonstrate the effect of DNAPK knockdown on proliferation of LNCaP cells that developed ADT resistance (from C, N = 2 in duplicates). A subset of these cells was switched to normal serum conditions as a reference. *, P < 0.05; **, P < 0.001. P values were calculated using Student t test (Fig. SB, D) or two-way ANOVA (C and E). All data are represented as mean ± SD RQ, relative quantity.
Figure 4.
DNA PK inhibition reduces Wnt signaling in CRPC cells. A and B, Bar graphs show the expression of Wnt pathway genes after knockdown (via siRNA) or inhibition (via NU7441, 1 μmol/L) of DNA PK in LNCaP-AR cells (N = 2, in duplicates). C and D, Immunoblot analyses show the effect of DNA PK knockdown (via siRNA) or inhibition (via NU7441, 1 μmol/L) on indicated proteins in LNCaP-AR and C4-2B cells. E, Representative images (magnification, 20×) demonstrate the effect of DNA PK inhibition (via NU7441 or the clinical-grade DNA PK inhibitor CC-115, 1 μmol/L) on invasion and migration of LNCaP-AR and C4-2B cells (N = 3 in duplicates). Recombinant human WNT3A (200 ng/mL) was used to stimulate Wnt signaling (C-E). Bar graphs represent the quantification of invaded/migrated cells. F, Images (magnification, 20×) display the effect of DNA PK inhibition (via NU7441 or CC-115) on invasion and migration of enzalutamide-resistant LNCaP-AR (LNCaP-AR-enza-res) cells. Bar graphs show quantification of invaded/migrated cells. All data are represented as mean ± SEM; *, P < 0.05 compared with respective controls and were calculated using two-way ANOVA (A, B, and E) or one-way ANOVA (F). Graphs and statistical analyses were done on GraphPad Prism software. RQ, relative quantity.
DNAPK Regulates Wnt Signaling to Drive Prostate Cancer Progression

DNAPK transcriptionally regulates Wnt signaling through interaction with LEF1 to control metastatic phenotypes in CRPC cells

LEF1 is the primary transcription factor involved in canonical Wnt-mediated signaling (27). To investigate the molecular mechanisms underlying DNAPK-mediated regulation of Wnt signaling, we performed coimmunoprecipitation studies and found that DNAPK interacts with LEF1 in a DNA-independent manner (Fig. 5A). Using a TOPFLASH reporter assay, we found that DNAPK inhibition with NU7441 or CC-115 dramatically reduced invasion and migration of LNCaP-AR-enza-res cells (Fig. 4F). Thus, DNAPK inhibition is a potential therapeutic strategy not only in CRPC but also in enzalutamide-resistant CRPC, for which there are currently limited treatment options.

DNAPK transcriptionally regulates Wnt signaling and promotes metastatic phenotypes in CRPC cells

Inhibition of DNAPK may be a clinically relevant therapeutic target in the current absence of effective and tolerable clinical agents that target β-catenin or Wnt signaling.

Second-generation ADTs, including abiraterone and enzalutamide, provide only modest survival benefits in CRPC, suggesting that DNAPK may be a clinically relevant therapeutic target in the current absence of effective and tolerable clinical agents that target β-catenin or Wnt signaling.

Based on our findings, we hypothesized that Wnt signaling could be an important mechanism of resistance to second-generation ADTs. Interestingly, we found that expression of Wnt pathway genes was broadly increased in enzalutamide-resistant LNCaP-AR cells (LNCaP-AR-enza-res; Supplementary Fig. S4). Similar to our results in CRPC cells presented in Fig. 4E, DNAPK inhibition with NU7441 or CC-115 dramatically reduced invasion and migration of LNCaP-AR-enza-res cells (Fig. 4F). Thus, DNAPK inhibition with NU7441 demonstrated a marked reduction in Wnt signaling in expression of Wnt target genes (Fig. 6F) and a significant decrease in Ki67-positive staining, a marker of proliferative activity (Fig. 6G). Taken together, our results highlight the promise of DNAPK inhibition for the treatment of aggressive prostate cancer.

Discussion

Current clinical prognostic paradigms, using PSA level, Gleason score, tumor stage, and pathologic features provide guidance in the treatment of patients with prostate cancer but are imperfect (29). The genomics era is revolutionizing decision-making in the clinic by providing an unprecedented view into tumor biology and the promise of personalized medicine. Unbiased microarray expression studies have been used to identify numerous prognostic and/or predictive individual-gene biomarkers. More recently, multigene signatures have been developed that have performed strongly in several clinical contexts and are now available commercially, including Decipher and Prolaris (16, 30). Although these tools provide prognostic information, they have no direct association with therapeutic strategies. Currently, androgen-directed therapies remain a standard of care for patients with advanced prostate cancer but are not durable, with inevitable development of resistance to enzalutamide and other second-generation AR-targeting agents (reviewed in ref. 31). Thus, novel therapeutic strategies that target AR as well as other driver pathways are desperately needed. Our findings provide strong preclinical evidence that DNAPK inhibition may be one such effective strategy.

Here, utilizing large discovery and validation cohorts, we identify a highly targetable kinase, DNAPK, as a top prognostic gene for aggressive CRPC. Our preclinical data show that DNAPK inhibition inhibits aggressive cancer phenotypes, including proliferation, migration, and invasion. We further demonstrate that Wnt signaling is activated very early after initiation of ADT and that DNAPK inhibition blocks Wnt signaling to prevent the emergence of ADT resistance. These data provide compelling support for DNAPK inhibition as a promising therapeutic strategy.
Figure 5.
DNAPK interacts with LEF1. A, Immunoblot analyses of DNAPK immunoprecipitates probed with DNAPK, Ku70 and LEF1 antibodies and LEF1 immunoprecipitates probed with DNAPK and LEF1 antibodies in LNCaP-AR cells, in the presence or absence of ethidium bromide is shown. B, Bar graph displays the TOPFLASH reporter activity in PC-3 cells (N = 3). Luminescence is normalized to Renilla luciferase. Data are represented as mean ± SEM. *P < 0.05 compared with DMSO. **P < 0.05 compared with Wnt3A using one-way ANOVA. C, Enrichment of DNAPK on regulatory regions (by chromatin immunoprecipitation assay, using DNAPK antibody) of select Wnt genes is shown after the treatment with DMSO or NU7441. En, enhancer; Prom, promoter; n.s., not significant. Data are represented as mean ± SEM. *P < 0.05 compared with IgG control. **P < 0.05 compared with DMSO treatment. (N = 3 in duplicates). P values were calculated by two-way ANOVA. D, Representative images (magnification, 20×) show the effect of DNAPK, LEF1, or beta-catenin (CTNNB1) knockdown on invasion and migration of LNCaP-AR, C4-2B, and PC-3 cells (N = 2 in duplicates). Bar graphs represent the mean ± SD of migration where siRNA knockdowns are compared with NTC control of respective cell lines. *, P < 0.05 using two-way ANOVA. Graphs and statistical analyses were completed using GraphPad Prism software.
Figure 6.

DNAPK inhibition reduces CRPC xenograft growth in vivo. A, Growth curves demonstrate the growth of LNCaP-AR xenografts, treated daily with vehicle (black, \( n = 5 \)) or 25 mg/kg NU7441 (red, \( n = 6 \)). B, The bar graph shows the fold change in tumor volume at the end of the experiment. C, Kaplan–Meier curves display the tumor doubling time in vehicle or NU7441 (25 mg/kg body weight) treated xenografts. D, Growth curves show the effect of enzalutamide (10 mg/kg, \( n = 16 \)), CC-115 (2 mg/kg, \( n = 14 \)), enzalutamide + CC-115 (\( n = 14 \)) treatment on LNCaP-AR xenografts compared with vehicle (\( n = 14 \)) control. CC115 and enzalutamide were administered once daily via oral gavage five times per week for 6 weeks. The bar graph shows the percent increase in tumor volumes, compared with starting volume, at the endpoint of treatments. E, Kaplan–Meier curves demonstrating the tumor tripling time in vehicle, enzalutamide, CC-115, and enzalutamide + CC-115–treated xenografts. Tumors were measured at indicated time points using caliper measurements. F, The bar graph displays the effect on select Wnt target genes in human explants treated with NU7441 (\( n = 6 \)) or vehicle control (\( n = 6 \)). G, Representative images showing the percent Ki67 nuclei (red arrows) that were scored in the explant tissue after treatment with either vehicle or NU7441 (\( n = 6 \)). All data are represented as mean ± SEM. * \( P < 0.05 \) compared with respective control; ** \( P < 0.01 \) CC-115 compared with vehicle. \( P \) values were calculated using the Student t test for tumor growth studies and log-rank test for Kaplan–Meier survival curves.
during ADT initiation. We also show that DNAPK is a strong prognostic indicator not only for AR-enriched luminal prostate cancer but for the AR-independent basal subtype of prostate cancer as well and show that DNAPK inhibition suppresses aggressive cancer phenotypes in AR-independent cells in vitro and in vivo. Thus, DNAPK inhibition has strong potential for overcoming therapy resistance in AR-independent, advanced prostate cancer. The impact of these findings is amplified by the availability of the clinical-grade DNA PK inhibitor CC-115, which is currently being assessed in patients with metastatic CRPC (clinicaltrials.gov identifier NCT02833883). Moreover, based on our finding that DNA PK inhibition interrupts Wnt signaling at a very distal step, we strongly suspect that DNA PK inhibition will block aberrant Wnt signaling arising from genetic alterations upstream in the pathway, making it effective against a wide variety of driver mutations.

Overcoming therapeutic resistance is dependent on identifying compensatory pathways prostate cancer cells use to evade current therapies. In this regard, our finding that Wnt signaling is activated upon ADT or enzalutamide treatment is consistent with a growing body of literature that Wnt signaling is a mechanism by which prostate cancer cells develop resistance to ADT (32–34). Wnt pathway genes have been shown to be upregulated in patients with locally advanced and metastatic prostate cancer in the months after first receiving ADT (35). Additionally, alterations in the Wnt signaling pathway, including genetic aberrations (36) and elevated expression (37), are virtually absent in treatment-naïve prostate cancer, whereas they are prevalent in advanced disease (35). Furthermore, the Wnt pathway has a known tumor-promoting role in advanced prostate cancer (34, 38) and, interestingly, the androgen receptor itself was reported to be a Wnt target gene (39). The relationship between AR and Wnt signaling remains complex with conflicting interpretations (reviewed in ref. 33); nevertheless, the observations listed above all support or are consistent with the hypothesis that activation of Wnt signaling is a key mechanism of resistance to AR directed therapies. Despite decades of effort, and numerous targets and compounds, pharmacologic inhibitors of the Wnt pathway have not progressed in clinical trials due to toxicity. Although we are actively working to characterize the DNA PK–Wnt axis, we hypothesize, based on our current data, that the inhibition of DNA PK activity interferes with its binding to active transcription sites of Wnt genes and suppresses LFET-mediated Wnt signaling. Thus, the ability of DNA PK inhibition to block Wnt signaling represents a long-awaited opportunity to effectively inhibit the Wnt pathway and potentially AR transcription (12), targeting two dominant pathways in CRPC.

Advanced prostate cancer is further complicated by a growing recognition that ADT might drive transdifferentiation of prostate cancer cells toward a neuroendocrine phenotype (reviewed in ref. 40). This very fast-growing and aggressive stage of disease is androgen/AR-independent and is nearly always lethal due to a lack of effective treatment options. Thus, our observations that DNA PK is strongly prognostic in the basal subtype of prostate cancer and that DNA PK inhibition efficiently reduces the aggressive cancer phenotypes of PC-3 (AR-independent) cells in preclinical studies provide the rationale for DNA PK inhibition as a therapeutic strategy for AR-independent prostate cancer.

In addition to prostate cancer, DNA PK inhibition potentially has significant clinical relevance across several disease sites. In a recent study by the Cancer Genome Atlas Network characterizing the molecular landscape of colon and rectal cancer, aberrations in Wnt signaling pathways were found in approximately 93% of colon and rectal cancer tumors (41). Wnt signaling also appears to be a driver in gastric cancer, one of the leading causes of cancer-related deaths worldwide. Upregulation and genetic or epigenetic alterations of many Wnt genes are known to be associated with poor prognosis and resistance to therapies for this disease (42). Similarly, Wnt signaling is known to be an oncogenic driver in cancers of the lung, breast, liver, and ovary (43).

In summary, by systematically exploring the landscape of kinases in lethal prostate cancer, we uncovered the strong prognostic importance of DNA PK and demonstrated a novel role of DNA PK in regulating Wnt signaling in androgen-resistant and AR-independent models of prostate cancer. Moreover, a clinical-grade DNA PK inhibitor, currently being tested in humans, reduces aggressive cancer phenotypes and resistance. Thus, DNA PK is a unique and exciting “therapeutic targetable prognostic biomarker” in lethal prostate cancer with rapid translational potential.

Disclosure of Potential Conflicts of Interest
S.G. Zhao has ownership interests (including patents) in Celgene and GenomDX Biosciences. L. Chang is an employee of and has ownership interests (including patents) at PFS Genomics. S.A. Tomlins is an employee of and has ownership interests (including patents) at Strata Oncology. B. Sun is a consultant/advisory board member for Janssen, Astellas, Sanofi, Almac Diagnostics and Abbiglie, reports receiving commercial research grants from GenomDX and Astellas/Medivation, and is a coinventor on a patent on ETs gene fusion which has been patented by the University of Michigan and whose diagnostic field of use has been licensed to Hologic/Gen-Probe, Inc., which has sublicensed rights to Roche/Ventana Medical Systems. K. Hege has ownership interests (including patents) in and reports receiving commercial research support from Celgene. E.H. Filvaroff has ownership interests (including patents) in Celgene. J. Barnes has ownership interests (including patents) in GenomDX. D. Rathkopf is a consultant/advisory board member for Janssen, Tecon, and AstraZeneca. D.E. Spratt is a consultant/advisory board member for Janssen and Blue Earth. A. Ashworth has ownership interests in Syncona, Tango, Prolynx, Gladiator and Bluestar, is a consultant/advisory board member for Tango, Pfizer, SPARC, Bluestar, Gladiar, and Genentech, reports receiving commercial research grants from AstraZeneca and SPARC, and is a co-inventor/holds patents on the use of PARP inhibitor held jointly with AstraZeneca from which he has benefited financially and (may do so in the future) through the ICR Rewards to Inventors’ Scheme. G.V. Raj is an employee of and has ownership interests (including patents) at Etezalx, reports receiving speakers bureau honoraria from Astellas, Pfizer and Bayer, and reports receiving commercial research grants from Bayer. K.E. Knudsen is a consultant/advisory board member for and reports receiving commercial research support from Celgene. F.Y. Feng is a co-founder of and has ownership interests (including patents) at PFS Genomics, is a consultant/advisory board member for Bayer, Blue Earth Diagnostics, Celgene, Clovis, Janssen, EMD Serono, Sanofi, Dendreon, Ferrin, Astellas, and Reflexion, and reports receiving commercial research grants from Zenith and Amgen. No potential conflicts of interest were disclosed by the other authors.

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6520 Clin Cancer Res; 25(18) September 15, 2019 Clinical Cancer Research
DNAPK regulates Wnt signaling to drive prostate cancer progression


Acknowledgments

The authors gratefully thank Dr. Rohit Malik (University of Michigan), Ela Davisovic (GenomeDx Biosciences), and members of the Feng and Knudsen laboratories for their input. We thank Steven Kronenberg for help with the figures. This work was supported by a grant to V. Kothari and S.G. Zhao from the PCF, to F.Y. Feng, K.E. Knudsen and S.A. Tomlins from the PCF/Evans Foundation, K.E. Knudsen from PA CRRE and NCI (CA159945, CA167640), F.Y. Feng and V. Kothari from the DOD PCA idea development Award No. W81XWH1810599.

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Received October 30, 2018; revised April 7, 2019; accepted June 20, 2019; published first July 2, 2019.

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