NF-κB gene signature predicts prostate cancer progression

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Abstract

In many prostate cancer (PCa) patients, the cancer will be recurrent and eventually progress to lethal metastatic disease after primary treatment, such as surgery or radiation therapy. Therefore, it would be beneficial to better predict which patients with early-stage PCa would progress or recur after primary definitive treatment. In addition, many studies indicate that activation of NF-κB signaling correlates with PCa progression; however, the precise underlying mechanism is not fully understood. Our studies show that activation of NF-κB signaling via deletion of one allele of its inhibitor, IκBα, did not induce prostatic tumorigenesis in our mouse model. However, activation of NF-κB signaling did increase the rate of tumor progression in the Hi-Myc mouse PCa model when compared to Hi-Myc alone. Using the non-malignant NF-κB activated androgen depleted mouse prostate, a NF-κB Activated Recurrence Predictor 21 (NARP21) gene signature was generated. The NARP21 signature successfully predicted disease-specific survival and distant metastases-free survival in patients with PCa. This transgenic mouse model derived gene signature provides a useful and unique molecular profile for human PCa prognosis, which could be used on a prostatic biopsy to predict indolent versus aggressive behavior of the cancer after surgery.
Introduction

Prostate cancer (PCa) is the most common non-cutaneous malignancy and the second leading cause of cancer death in American men (1). The advent of prostate-specific antigen (PSA) testing has revolutionized early PCa detection. If elevated PSA levels are detected, a needle biopsy of the prostate is recommended to check for histological evidence of PCa. If cancer is detected, the patient can select either active surveillance or one of several definitive treatment options, such as surgery, radiation therapy or brachytherapy. Recent reports, however, have raised concern over the efficacy of PSA screening. The US Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial report found that PSA screening did not reduce the mortality due to PCa (2) and the European Randomized Study of Screening for Prostate Cancer (ERSPC) found that to prevent one death due to PCa, 33 patients would have to be treated (3). These studies suggested that PSA testing cannot distinguish between aggressive versus indolent PCa; therefore, more patients are being treated then necessary. As a result of these and other reports, the US Preventive Services Task Force (USPSTF) made the recommendation to stop routine screening by PSA testing on all men (4). Regardless of individual opinion on the PSA screening controversy, there is general agreement that a definitive test is needed to distinguish patients that have aggressive disease and who should undergo therapy from the patients that have latent or indolent PCa. Therefore, a critical question in the clinical management of PCa is how to separate the patients with indolent PCa from early-stage PCa patients that would benefit from definitive treatment.

As an important class of transcriptional regulators, NF-κB proteins are involved in many events of cancer development and progression. Many studies indicate that activation of NF-κB signaling in PCa cells correlates with PCa progression, including chemoresistance, advanced stage, PSA recurrence and metastatic spread (5-12). Previously, we and other researchers have confirmed that NF-κB signaling
plays a critical role in the progression of PCa to castrate resistant and metastatic cancer (13-17). We have further reported that activation of NF-κB signaling promotes growth of PCa cells in bone (18). However, the detailed mechanism by which NF-κB signaling contributes to PCa development and progression, and whether activation of NF-κB signaling is sufficient to predict poor survival outcome and systemic metastasis in the patients with PCa are not fully understood.

In this study, we investigated the role of NF-κB signaling in PCa initiation and progression using a NF-κB activated PCa mouse model and generated a gene signature from a non-malignant NF-κB activated mouse prostate that distinguishes subsets of human cancer and predicts clinical outcome in PCa. Our study show that NF-κB activation via deletion of one allele of IκBα (inhibitor of NF-κB) does not induce prostatic tumorigenesis, but it does decrease the time required to develop PCa in the ARR2PB-myc-PAI (Hi-Myc) mouse model when compared to Hi-Myc alone. In addition, a gene signature generated from a non-malignant NF-κB activated androgen depleted mouse prostate distinguishes subsets of human cancer and predicts clinical outcome in PCa patients. To our knowledge, this is the first report of a genetically engineered non-cancerous mouse model where the gene expression signature is effective in predicting the clinical outcome in PCa. Most importantly, our ability to identify PCa patients at most risk of disease progression is via a signature that is generated from the increased NF-κB activity and decreased androgen receptor signaling. Generating this signature from the mouse did not require a cancerous phenotype to develop in the prostate. This suggests that primary PCa that shows activation of the NF-κB pathway predisposes these patients to failure of androgen deprivation therapy resulting in metastatic and castrate resistant disease.
Materials and Methods

Cell culture and materials. The human prostate carcinoma cell line LNCaP was obtained from ATCC (Manassas, VA). C4-2B cells were gifts of Dr. Leland Chung (Cedars Sinai Medical Center, Los Angeles, CA)(19). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in the air. Cell lines were routinely cultured in RPMI 1640 (Gibco-BRL) medium containing 5% fetal calf serum (FBS) (Hyclone), 0.1% ITS and 0.1% Glutamine (Gibco-BRL).

Continuously activated NF-κB signaling mouse model. IκBα haploid insufficient mouse (IκBα+/-) (20, 21) was used as the continuously activated NF-κB mouse model. A NF-κB constitutively activated PCA mouse model (Myc/IκBα+/-) was generated by crossing IκBα+/- mouse with the ARR₂PB-myc-PAI (Hi-Myc) PCA mouse line (22). Prostates were harvested at 3 and 6 month of age and fixed in 10% buffered formalin and paraffin-embedded for histological and immunohistochemical analysis. Each experimental group consisted of at least 5 mice.

RNA extraction and microarray analysis. RNAs from prostate tissues (wild-type and IκBα+/- mice; intact and castrated) were used for microarray analysis (prostate tissues from castrated 7 week old IκBα+/- and wild-type mice that were harvested at 2 weeks post-castration; 4 mice for each group). The protocol for mRNA quality control and gene expression analysis was that recommended by Affymetrix (Santa Clara, California, USA). In brief, total RNAs were extracted using Trizol (Gibco-BRL), and residual genomic DNA was removed by DNaseI (Invitrogen) treatment. RNA samples were stored at -80°C. RNA quality was analyzed by the Vanderbilt Microarray Shared Resource (VMSR) using spectrophotometry (NanoDrop Technologies, Wilmington, DE) and bioanalysis (Agilent Technologies,
Santa Clara, CA). RNA samples were submitted to the VMSR for amplification (NuGen Systems, Inc., Traverse City, MI) and labeling, followed by hybridization to Affymetrix GeneChip Expression Arrays.

**Microarray data sources.** The mouse microarray dataset included 4 groups (wild-type and IκBα+/- mice; intact and castrated). Normalized microarray data (Mouse 430 expression arrays) were pre-processed in Vanderbilt Microarray Shared Resource (VMSR). For survival analysis, we downloaded a human PCa dataset from NCBI GEO (GSE10645) (23). The human dataset includes 596 patient samples with three common clinical survival outcomes: no evidence of disease progression (NED), PSA recurrence alone (PSA) and systemic metastasis (SYS) (24).

**Generation of the NF-κB signature.** The microarray data analysis was performed to directly compare prostate tissues from intact/castrated wild-type and IκBα+/- (intact mice group: wild-type versus IκBα+/- mice; castration group: castrated wild-type versus castrated IκBα+/- mice; and wild-type versus castrated wild-type mice). For statistical analysis, we used the Significance Analysis of Microarray (SAM) software package from Stanford University (25), and based on our practical consideration, the SAM false discovery rates (FDR) were adjusted to obtain approximately equal number of significant genes (ca. 500) for various mouse signatures. Unsupervised hierarchical clustering was performed with various extracted signatures using TIGR MeV program (26). In order to define corresponding orthologous human NF-κB signatures for cross-species survival analysis, NCBI Gene and NCBI HomoloGene databases were used to translate mouse array probesets to human homolog gene symbols. Thereby, we generated the human genes NF-κB signatures. Using EXALT validation modular (27) and the human PCa data sets (GSE10645), we performed survival analysis on the human NF-κB signatures.
Cancer-specific survival and metastasis-free survival analysis of human NF-κB signature. A Spearman rank correlation was calculated for the expression data of each patient and each human NF-κB signature gene, and tumor sample profiles were clustered. The group assignments for the patient samples were determined based on the first bifurcation of the clustering dendrograms (28). Disease-specific survival (DSS) and distant metastases-free survival (DMFS) between the two groups (Favorable-prognosis and Poor-prognosis groups) were analyzed and compared by the Kaplan-Meier method (KM) and the Cox proportional hazards model for univariate and multivariate survival analyses. For graphic representation, KM estimated survivor function was plotted for each subgroup. The KM curves helped to assess the relationship between disease-specific survival and survival time. Differences in survival time were tested for statistical significance by the log-rank test and the Cox proportional hazards model. The statistical modules including Spearman rank correlation, log-rank test, and KM plot, and univariate survival analysis were implemented in the iterative EXALT application with the open-source R scripts, version 2.10.1 (www.r-project.org).

Ingenuity Pathway Analysis. Functional annotation networks were generated using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Mountain View, CA) software, which provides a graphical representation of the molecular relationships between genes. The network was generated using the 21 gene set. Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). Direct relationships are indicated by solid lines and indirect through dashed lines. Line beginnings and endings illustrate the direction of the relationship (e.g. arrow head indicates gene A influences gene B).
**Western blot analysis.** NF-κB signaling was activated in PCa cells by infecting with IKK2-EE retroviral vector, in which NF-κB activity was activated with a constitutively active (EE) mutants of IKK2 (29, 30); while NF-κB signaling was inactivated in PCa cells by infecting with IKK2-KD retroviral vector, in which NF-κB activity was inhibited with a kinase dead (KD) IKK2 mutant (29, 30). The cells infected with empty vector were used as controls. A 20μg aliquot of each protein sample from NF-κB signaling activated and inactivated PCa cells was separated on a 4 to 12% Tris-glycine gradient gel (NOVEX™), and then transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked with 5% skim milk in TBS-T (Tris-buffer saline, 1% Tween-20) buffer. The JNK, p38MAPK, p-JNK, p-p38MAPK and E-cadherin antibodies (Santa Cruz) were added at the optimal concentration (1:1000) and the blots were incubated 1 hour at room temperature. After washing three times for 10 minutes in TBS-T, incubation was performed for 1 hour with the secondary horseradish-peroxidase-conjugated antibodies. The signals were detected using the ECL system (Amersham Biosciences).
Results

Activation of NF-κB signaling did not induce prostatic tumorigenesis but it did increase the rate of tumor progression in the Hi-Myc mouse PCa model.

In order to investigate the role of NF-κB signaling in PCa tumorigenesis and progression, we utilized a knockout mouse model of IκBα (20, 21), the major inhibitor of NF-κB function (31). The continuous activation of NF-κB signaling in the prostate of IκBα+/- mice was confirmed by crossing the IκBα+/- mice with NGL, a NF-κB reporter mouse (32, 33) (Supplementary Fig. 1). To investigate the influence of NF-κB signaling activation in prostate development, IκBα+/- and wild-type mice were sacrificed at 3 and 6 months of age and the prostates were harvested. In contrast to wild-type mice, the prostates with activated NF-κB signaling showed multiple layers of epithelial cells within the glandular structures surrounded by extensive fibromuscular stroma (Fig. 1A). However, the prostatic epithelial cells lack nuclear features of dysplasia or malignancy, such as nuclear atypia, enlarged nucleoli, or invasion into the basal and stromal cells layer (Fig. 1A). These results indicate that continuous activation of NF-κB signaling in the mouse is sufficient to cause hyper-proliferation in both the prostate epithelium and stroma, which are histological features characteristic of human benign prostatic hyperplasia. However, activation of NF-κB alone in the prostate does not cause tumorigenesis in this mouse model.

ARR2PB-myc-PAI (Hi-Myc), a transgenic mouse model of PCa, develops invasive adenocarcinoma in the prostate by 6 months of age (22). To investigate if activation of the NF-κB pathway promotes PCa progression, the Hi-Myc mouse was crossed with the IκBα+/- mouse (Myc/IκBα+/- mouse). Activation of NF-κB signaling in the prostate of bigenic mouse (Myc/IκBα+/- mouse) was confirmed by IHC staining of p65-pho antibody (Supplementary Fig. 2). At 3 months of age, as expected, Hi-Myc mice developed prostatic intraepithelial neoplasia (PIN) lesions. However, the Myc/IκBα+/- bigenic mice
developed prostatic adenocarcinoma by 3 months. Histologic analysis showed nuclear atypia, enlarged nucleoli, and frank invasion into the stromal compartment (Fig. 1B). At 6 months of age, Hi-Myc mice developed invasive adenocarcinomas that were mainly limited to the dorsal and lateral prostatic lobes. However, the Myc/IκBα+/- bigenic mice developed a more aggressive cancerous phenotype in the dorsal and lateral lobes as well as in the anterior and ventral prostatic lobes (Fig. 1C). In addition, the prostate from Myc/IκBα+/- bigenic mice showed increased nuclear AR staining and significantly greater numbers of luminal Ki67 positive cells (p=0.042), a proliferation marker, when compared to Hi-Myc prostates (Supplementary Fig. 3A and B). However, significant distant metastasis was not detected in this model by the 6 month of age. These results indicated that continuous activation of NF-κB signaling enhances Myc induced mouse PCa development and progression.

**Use of non-malignant NF-κB activated mouse prostates to identify human orthologs expressed in PCa patients.**

In order to understand how NF-κB signaling contributes to PCa progression, we performed RNA microarray analysis on prostates dissected from intact wild-type and IκBα+/-, as well as androgen depleted (castrated) wild-type and IκBα+/- mice. Changes in gene expression were determined by using the wild-type prostate as the control (wild-type vs. IκBα+/-; castrated wild-type vs. castrated IκBα+/-; and wild-type vs. castrated wild type). Significant differential expression of mouse genes between the wild-type (control) and the experimental groups were identified. Unsupervised hierarchical clustering was performed with the extracted signature. In order to define a corresponding orthologous human NF-κB signature for cross-species survival analysis, NCBI Gene and NCBI HomoloGene databases were used to translate mouse array probesets to human homolog gene symbols. Mouse genes within the NF-κB signature were converted to the species-consistent (orthologous) human NF-κB signature genes. In order to investigate whether the gene expression signatures derived from mouse models can serve as
predictors of progression of human PCa, we identified orthologous members of the human NF-κB signatures within expression data from primary human PCa data sets (PR18846227) published by the Mayo Clinic (24). The Mayo Clinic microarray contains 526 gene targets for RNAs, including genes whose expression is altered in association with PCa progression (24). The comparison of mouse identified/converted human orthologs to the Mayo Clinic data sets generated three lists of common genes as follows: 1) 21 human genes from NF-κB activated (IκBα+/−) androgen depleted (castration) mouse prostate (Table 1); 2) 24 human genes from the NF-κB activated (IκBα+/−) intact (no castration) mouse prostate (Supplementary Table 1); 3) 228 human genes from the androgen depleted (castrated) wild-type mouse prostate (Supplementary Table 2).

**NF-κB gene signature generated from a non-malignant NF-κB activated androgen depleted mouse prostate predicts overall cancer-specific survival of PCa patients**

The human PCa Mayo Clinic microarray data set (PR18846227) consists of 596 tumors from patients that include 200 cases of systemic metastasis, 201 cases of PSA recurrence alone (biochemical recurrence) and 195 cases with no evidence of disease progression (24). Radical Retropubic Prostatectomy (RRP) was performed on all patients. This PCa data set was interrogated by the human NF-κB signature derived from NF-κB activated androgen depleted mouse prostate (21 orthologous genes) (Table 1) as well as the NF-κB activated intact mouse prostate (24 orthologous genes) (Supplementary Table 1). To partition patient samples into two prognostic groups, a Spearman rank correlation was calculated for the expression data of patients and with the 21 and 24 human NF-κB signature genes. The 21 orthologous gene signature was termed NF-κB Activated Recurrence Predictor 21 (NARP21) and the 24 orthologous gene signature was termed NF-κB 24 (NF24).

From this NARP21 gene signature, two group assignments (Favorable-prognosis and Poor-prognosis groups) for the patient samples were determined based on the first bifurcation of the clustering.
dendrograms (27). KM and log-rank analyses demonstrate a significant difference in predicting PCa specific death (DSS) from the human PCa data sets by using the NARP21 gene signature (Fig. 2A). Univariate and multivariate analyses of NARP21 were performed to further evaluate the performance of NARP21 using DSS as clinic endpoint and compared with other known prognostic factors, namely, tumor stage, ploidy, PSA, and Age. The unadjusted (univariate) and adjusted (multivariate) HR of these factors and NARP21 were determined (Table 2).

The prognostic value of NARP21 from unadjusted hazard ratio (univariate HR) (high risk vs. low risk) in PR18846227 was 3.4 (95% CI: 2.2-5.2; p < 0.001) for DSS. Univariate Cox proportional-hazards analysis demonstrated that NARP21 could successfully predict PCa specific death. Multivariate Cox proportional-hazards analysis was used to determine if NARP21 added independent prognostic information to other standard clinicopathological features. In this multivariate Cox proportional-hazards analysis (Table 2), significant associations (p < 0.001) were observed in PR18846227 between NARP21 and disease-free time after adjustment for standard clinical covariates. Thus, NARP21 contributed new and important prognostic information beyond that provided by established clinical predictors.

Although the NF24 was statistically significant in predicting patient outcome (HR: 1.6; 95% Confidence Interval [CI]: 1.1-2.5; p=0.0212) (Supplementary Fig. 4), the NARP21 signature performed the best in predicting PCa patient clinical outcome. Therefore, further analysis of NF24 signature is not presented.

In order to confirm whether the NARP21 signature is a function of loss of androgen signaling or also includes a contribution resulting from the activation of the NF-κB pathway, we analyzed the association between the signature generated from the androgen depleted wild-type mouse prostate (termed AD228) (Supplementary Table 2) and PCa specific survival. The AD228 signature was not associated with PCa
specific death (HR: 1.1; 95% CI: 0.7-1.6; p=0.687) (Fig. 2B). These results indicate that the NARP21 gene signature associated with PCa specific death is not due to the effect of androgen depletion alone.

**NARP21 signature is associated with metastasis-free survival of PCa patients**

In order to determine whether the NARP21 gene signature applied to prostate tissue at the time of radical surgery would predict subsequent development of systemic metastasis in the patients with localized PCa (stage T2 and T3), we analyzed its association with metastasis-free survival. Among 596 cases from the Mayo clinic cohort, 254 and 265 cases were identified as stage T2 and T3, respectively, while 77 cases had lymph node metastasis at the time of RRP. The 77 cases that were lymph node positive at the time of surgery were removed from this analysis and evaluated as a separate cohort (47/77 cases developed systemic metastasis). Among 519 cases of clinically localized PCa (stage T2 and T3; no lymph node metastasis), 153 cases progressed to systemic metastasis disease after prostatectomy (up to 15 years follow up) (24). When we analyzed the NARP21 gene signature of patients with clinically localized PCa at the time of surgery, NARP21 was significantly associated with DMFS (HR: 2.7; 95% CI: 1.9-3.7; p<0.001) (Fig. 3A). Therefore, the NARP21 gene signature identifies changes in gene expression profile in the human primary tumor that have occurred prior to any clinical evidence of metastasis in the patient. Notably, the gene signature AD228 generated from a wild-type androgen depleted mouse prostate was not associated with DMFS in patients with localized PCa at the time of surgery (HR: 1.1; 95% CI: 0.8-1.4; p=0.651) (Fig. 3B).

Univariate and multivariate analyses of NARP21 were also performed to evaluate the association between NARP21 and PCa metastasis (Table 2). The prognostic value of NARP21 from unadjusted hazard ratio (univariate HR) (high risk vs. low risk) in PR18846227 was 2.8 (95% CI: 2.1 - 3.8; p < 0.001) for DMFS. Univariate Cox proportional-hazards analysis demonstrated that NARP21 could successfully predict PCa metastasis. Significant associations (p < 0.001) were also observed in
PR18846227 between NARP21 and patient metastasis-free time after adjustment for standard clinical covariates. Thus, NARP21 contributed new and important predictive information beyond the established clinical predictors.

Next, the NARP21 signature was used to evaluate the PCa patients that had lymph node metastasis at the time of RRP. Among the 77 lymph node positive cases, 47 cases (61%) had systemic metastasis, while 30 cases had no further systemic metastasis at the time of this analysis. By using the NARP21 gene signature to analyze these patients, in the 47 cases which had further systemic metastasis, almost 80% (37/47 cases) would fall in a poor-prognosis group, while only 20% cases (10/47 cases) segregated into the favorable-prognosis group (Supplementary Table 3 and Fig. 3C). Survival analyses showed that the NARP21 gene signature predicts significant differences in the distant metastasis-free survival of the patients that had lymph node metastasis at the time of surgery (HR: 2.1; 95% CI: 1.0-4.3; p=0.0324) (Fig. 3D). This result demonstrates that the association of the NARP21 gene signature with metastatic progression is independent of lymph node status.


In order to further understand the detailed mechanism by which NF-κB signaling contributes to metastatic progression of PCa, we used the 21 genes (Table 1) from the NARP21 gene signature to perform an Ingenuity Pathway Analysis (IPA). IPA showed that this list would re-establish a direct link to the NF-κB pathway (Fig. 4 and Supplementary Fig. 5). In addition, the results showed a highly interconnected network of aberrations along the c-Jun N-terminal kinases (JNK) signaling pathway (Fig. 4 and Supplementary Fig. 5). JNK signaling is one of the important pathways of mitogen-activated protein kinase (MAPK) signaling due to phosphorylation of its activation domain (34). Many published studies have confirmed that activation of the JNK pathway plays a critical role in metastatic progression.
in multiple cancers (35-37). Therefore, these findings strongly indicate that the JNK pathway may be an important downstream target by which NF-κB signaling promotes PCa progression. Our studies further indicate that activation of NF-κB signaling, either by constitutively active (EE) mutants of IKK2 (Fig. 5 and Supplementary Fig. 6) or expression of p65 (Supplementary Fig. 7), increases JNK phosphorylation (but not that of p38MAPK) and decreases E-cadherin expression in PCa cells (Fig. 5). In addition, blocking JNK signaling inhibits NF-κB induced invasive ability efficiently in PCa cells (Supplementary Fig. 8).
Discussion

From the clinical perspective, it is understood that although two patients can be diagnosed with PCa of identical stage and grade, these same two patients can have very different clinical outcomes. One patient may harbor indolent PCa, which will remain non-harmful during his lifetime, while the other patient may harbor a tumor that will progress to lethal metastatic disease (38). The tumors in different patients must be different at the molecular level and the goal of personalized medicine is to generate individual risk profiles from the primary PCa that could identify high risk individuals for aggressive therapeutic treatment and clinical follow up. As well, it is equally important to identify the patients that have indolent PCa in order to save these individuals from undergoing unnecessary treatment. Recently, several groups have investigated molecular and genetic characteristics of PCa in order to develop both prognostic and predictive biomarkers (39-41). However, the use of these models in urologic practice is not standard.

Activation of NF-κB signaling alone by deletion of one allele of IκBα does not induce prostatic tumorigenesis in our mouse model, while, continuous activation of NF-κB signaling enhances earlier development of PCa in the Hi-Myc mouse model of PCa (Fig. 1). In addition to our results, many studies have been reported that NF-κB signaling plays a critical role in the progression to castrate resistant and metastatic prostate cancer (13-17). These results suggest that although development of PCa may require some other driver(s) to induce tumorigenesis, activation of NF-κB signaling is an important factor to contribute to PCa progression. Therefore, our studies were focused on the mechanism by which NF-κB signaling contributes to PCa progression and whether activation of NF-κB signaling is sufficient to predict poor survival outcome and systemic metastasis in the patients with PCa. Using non-tumorigenic prostates from a mouse model with increased NF-κB activity and androgen depletion, we
developed a gene expression signature (NARP21) that discriminated high versus low risk cases of cancer metastasis and death in patient with PCa (Fig. 2 and 3). The data represents a successful, biologically-based translational model demonstrating that cross-species functional genomics approach can yield insights into the molecular mechanisms of human prostate pathogenesis. Most importantly, our ability to identify PCa patients with the most risk of disease progression is achieved via a signature that is generated from a non-cancerous mouse prostate with a single genetic change resulting in elevated levels of NF-κB pathway in an androgen depleted mouse.

Our studies showed that the human NF-κB signature, which derived from NF-κB activated androgen depleted mouse prostate, successfully predicted cancer prognosis (Fig. 2A and 3A). The signatures generated from the NF-κB activated intact mouse prostate (NF24) or the wild-type castrated mouse prostate (AD228) had less or no predictive value. The prostate is an androgen-sensitive organ, and it is well known that androgen activity plays a critical role in PCa development and progression. These results suggest that the contribution of NF-κB signaling in PCa progression may be more significant during treatment of PCa with androgen ablation therapy. This observation is consistent with our previous studies that show the activation of NF-κB signaling contributes to castrate resistant growth (13, 42). In addition, analysis of the NARP21 genes by IPA showed that the 21-genes list reflected a gene network that is linked to the NF-κB pathway. Surprisingly, this NF-κB gene signature does not contain the obvious inflammatory markers associated with the traditional NF-κB pathway. These results suggest that some “non-standard” downstream target genes of NF-κB pathway may play an important role during tumor progression.

IPA studies, using NARP21 gene signature, showed a highly interconnected network of NF-κB and JNK pathways (Fig. 4). It is well known that JNK signaling is an important component of the MAPK pathway and that it plays a critical role in cancer metastasis by affecting cellular migration and invasion.
Recent studies show that the knockout of JNK1 and JNK2 in the phosphatase and tensin homolog (Pten) null mouse model of prostate cancer results in increased tumorigenesis and metastasis (43). This suggests that JNK restrains tumor progression. Our data indicates that NF-κB is upstream of JNK activation (Fig. 5). Further, we see that drug inhibition of JNK increases invasiveness of PCa cells (Supplementary Fig 4). Therefore, inhibition of the NF-κB pathway or altering the JNK pathway alone or in combination may have clinical utility to enhance the treatment of advanced PCa. Clearly, we need to further understand how the NF-κB and JNK pathway affects the development of metastasis and CRPC.

In summary, the NARP21 gene signature generated from a non-malignant NF-κB activated androgen depleted mouse prostate successfully distinguished subsets of human cancer and predicts clinical outcome in PCa patients. This prediction signature can have a significant impact on identifying patients with indolent or aggressive disease.
Acknowledgments

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Authors' Contributions

Conception and design: R. Jin, Y. Yi, R.J. Matusik

Development of methodology: R. Jin, Y. Yi

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Jin, Y Yi, F.E. Yull, T.S. Blackwell

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Jin, Y. Yi, T. Koyama

Writing, review, and/or revision of the manuscript: R. Jin, Y. Yi, F.E. Yull, T.S. Blackwell, P.E. Clark, T. Koyama, J.A. Smith Jr., R.J. Matusik

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Jin, Y. Yi, R.J. Matusik

Study supervision: J.A. Smith Jr., R.J. Matusik

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Notes

R. Jin and Y. Yi contributed equally to this work.
REFERENCES:


TABLES:

Table 1. Matched human homolog genes list from NF-κB activated androgen depleted (castrated) mouse prostate (21 genes).

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<tr>
<th>Gene Symbol</th>
<th>Gene Title</th>
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<tr>
<td>ACP2</td>
<td>acid phosphatase 2, lysosomal</td>
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<td>ACPP</td>
<td>acid phosphatase, prostate</td>
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<tr>
<td>CCNB1</td>
<td>cyclin B1</td>
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<td>DLGAP1</td>
<td>discs, large (Drosophila) homolog-associated protein 1</td>
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<td>EGR3</td>
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<td>ENPP2</td>
<td>Ectonucleotide pyrophosphatase/phosphodiesterase 2</td>
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<tr>
<td>FBXW11</td>
<td>F-box and WD-40 domain protein 11</td>
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<td>GABRG2</td>
<td>gamma-aminobutyric acid (GABA-A) receptor, subunit gamma 2</td>
</tr>
<tr>
<td>GDF15</td>
<td>growth differentiation factor 15</td>
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<td>H1FX</td>
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<td>isovaleryl coenzyme A dehydrogenase</td>
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<td>RAB8A</td>
<td>RAB8A, member RAS oncogene family</td>
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<td>ring finger protein 2</td>
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<tr>
<td>SPINT1</td>
<td>Serine protease inhibitor, Kunitz type 1 // Transcribed locus, moderately similar to XP_217082.2 similar to hypothetical protein FLJ23518 [Rattus norvegicus]</td>
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</table>
**Table 2.** Univariate and multivariate analysis of NARP21 in prostate cancer

<table>
<thead>
<tr>
<th>Endpoints*</th>
<th>Analysis</th>
<th>HR (95% CI)**</th>
<th>HR P***</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSS</td>
<td>Univariate</td>
<td>3.4 (2.2 - 5.2)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Multivariate****</td>
<td>2.7 (1.7 - 4.2)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>DMFS</td>
<td>Univariate</td>
<td>2.8 (2.1 - 3.8)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Multivariate</td>
<td>2.4 (1.8 - 3.2)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

*Endpoints: Clinic endpoints are disease-specific survival (DSS) and distant metastases-free survival (DMFS).

**HR (95% CI): hazard ratio value (95% confidence interval).

***HR P: hazard ratio p-value.

**** Adjusted factors in prostate cancer: tumor stage, ploidy, PSA, and Age.
FIGURES LEGENDS:

Figure 1. Activation of NF-κB signaling did not induce prostatic tumorigenesis but it did increase the rate of tumor progression in the Hi-Myc mouse PCa model. A) Continuous activation of NF-κB signaling induced prostate epithelial and stromal hyper-proliferation. Prostates from IκBα+/− and wild type mice were harvested at 3 and 6 months of age. Histological analysis was performed by H&E staining. B and C) Continuous activation of NF-κB signaling promotes PCa progression in the ARR2PB-myc-PAI transgenic mouse. The prostates from Myc alone (ARR2PB-myc-PAI) and Myc/IκBα bigeneic mice were harvested at 3 (B) and 6 (C) months of age. Histological analysis was performed by H&E staining. (DP: Dorsal Prostate; LP: Lateral Prostate; VP: Ventral Prostate; AP: Anterior Prostate).

Figure 2. The NARP21 gene signature predicts significant difference in the overall cancer-specific survival of PCa patients. (A) KM analyses were used to examine whether there was a significant association between overall cancer-specific survival prediction and the signature generated from NF-κB activated castrated mouse prostate (NARP21) or from the wild type castrated mouse prostate (AD228) (B). Two types of overall cancer-specific survival outcomes were compared in the plot: a poor-prognosis group (black dashed line) and a favorable-prognosis group (red solid line). The disease-specific survival (DSS) time in years is displayed on the X-axis, and the Y-axis shows the probability of overall cancer-specific survival. P value is by log-rank test.

Figure 3. The NARP21 gene signature predicts significant differences in the distant metastasis-free survival of PCa patients. (A and B) KM analyses were used to examine whether there was a significant association between distant metastasis-free survival and the signature generated from NF-κB
activated castrated mouse prostate (NARP21) (A) or from the wild type castrated mouse prostate (AD228) (B). Two types of distant metastasis-free survival outcomes were compared in the plot: a poor-prognosis group (black dashed line) and a favorable-prognosis group (red solid line). The distant metastases-free survival (DMFS) time in years is displayed on the X-axis, and the Y-axis shows the probability of metastasis-free survival. P value is by log-rank test. (C) 47 out of 77 PCa patients who had lymph node metastasis at the time of RRP surgery progressed to systemic metastatic PCa eventually. The time of post-surgery is displayed on the X-axis, and the Y-axis shows the percentage of systemic metastasis from poor-prognosis and favorable-prognosis groups which predicted by the NARP21 gene signature at each time point, respectively. (D) KM plot for the systemic metastasis-free survival of PCa patients who had lymph node metastasis at the time of RRP surgery. Two types of distant metastases-free survival (DMFS) outcomes were compared in the plot: a poor-prognosis group (black dashed line) and a favorable-prognosis group (red solid line) stratified by the NARP21 gene signature gene expression profile. The distant metastases-free survival (DMFS) time in years is displayed on the X-axis, and the Y-axis shows the probability of systemic metastasis-free survival. P value is by log-rank test.

**Figure 4. Molecular network analysis using Ingenuity Pathway Analysis (IPA).** Network analysis showed pathways associated with the NARP21 gene signature genes (21 genes) derived from NF-κB activated androgen depleted mouse prostate. The network was generated using the NARP21 gene set. A simplified diagram from IPA shows the key molecular pathways detected by the NARP21 gene signature genes (red). A full molecular network is presented in Supplementary Fig. 5.

**Figure 5. Activation of NF-κB signaling correlates with increases in JNK phosphorylation (but not in p38MAPK) and decreases E-cadherin expression in PCa cells.** NF-κB signaling was activated in
LNCaP PCa cells by infecting with IKK2-EE retroviral vector, in which NF-κB activity was activated with a constitutively active (EE) mutants of IKK2 (28, 43); while NF-κB signaling was inactivated in C4-2B PCa cells by infecting with IKK2-KD retroviral vector, in which NF-κB activity was inhibited with a kinase dead (KD) IKK2 mutant (28, 43). The cells infected with empty vector were used as controls. Western blotting evaluating total and phosphorylated JNK (A) and p38 (B), and E-cadherin (C) levels in NF-κB activated (LNCaP-EE and C4-2B-EV) or inactivated (LNCaP-EV and C4-2B-KD) PCa cells.
Fig 1.
Fig 2.
Fig 3.
NF-κB gene signature predicts prostate cancer progression

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