NF-κB Gene Signature Predicts Prostate Cancer Progression

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Abstract

In many patients with prostate cancer, 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 prostate cancer would progress or recur after primary definitive treatment. In addition, many studies indicate that activation of NF-κB signaling correlates with prostate cancer 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 prostate cancer model when compared with Hi-Myc alone. Using the nonmalignant 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 prostate cancer. This transgenic mouse model–derived gene signature provides a useful and unique molecular profile for human prostate cancer prognosis, which could be used on a prostatic biopsy to predict indolent versus aggressive behavior of the cancer after surgery. Cancer Res; 74(10); 1–10. ©2014 AACR.

Introduction

Prostate cancer is the most common noncutaneous malignancy and the second leading cause of cancer death in American men (1). The advent of prostate-specific antigen (PSA) testing has revolutionized early prostate cancer detection. If elevated PSA levels are detected, a needle biopsy of the prostate is recommended to check for histologic evidence of prostate cancer. 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 U.S. Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial report found that PSA screening did not reduce the mortality due to prostate cancer (2) and the European Randomized Study of Screening for Prostate Cancer found that to prevent one death due to prostate cancer, 33 patients would have to be treated (3). These studies suggested that PSA testing cannot distinguish between aggressive versus indolent prostate cancer; therefore, more patients are being treated than necessary. As a result of these and other reports, the U.S. Preventive Services Task Force 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 prostate cancer. Therefore, a critical question in the clinical management of prostate cancer is how to separate the patients with indolent prostate cancer from patients with early-stage prostate cancer 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 prostate cancer cells correlates with prostate cancer 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 prostate cancer to castrate-resistant and metastatic cancer (13–17). We have further reported that activation of NF-κB signaling promotes growth of prostate cancer cells in bone (18). However, the detailed mechanism by which NF-κB signaling contributes to prostate cancer development and progression, and whether activation of NF-κB signaling is sufficient to predict poor survival outcome and systemic metastasis in the patients with prostate cancer are not fully understood.
In this study, we investigated the role of NF-κB signaling in prostate cancer initiation and progression using a NF-κB–activated prostate cancer mouse model and generated a gene signature from a nonmalignant NF-κB–activated mouse prostate that distinguishes subsets of human cancer and predicts clinical outcome in prostate cancer. Our study shows that NF-κB activation via deletion of one allele of 1xBα (inhibitor of NF-κB) does not induce prostatic tumorigenesis, but it does decrease the time required to develop prostate cancer in the ARR2PB-myc-PAI (Hi-Myc) mouse model when compared with Hi-Myc alone. In addition, a gene signature generated from a nonmalignant NF-κB–activated androgen-depleted mouse prostate distinguishes subsets of human cancer and predicts clinical outcome in patients with prostate cancer. To our knowledge, this is the first report of a genetically engineered noncancerous mouse model where the gene expression signature is effective in predicting the clinical outcome in prostate cancer. Most importantly, our ability to identify patients with prostate cancer 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 prostate cancer 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 American Type Culture Collection. C4–2B cells were gifts of Dr. Leland Chung (Cedars Sinai Medical Center, Los Angeles, CA; ref. 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 bovine serum (Hyclone), 0.1% insulin, transferrin, and selenium, and 0.1% glutamine (Gibco-BRL).

Continuously activated NF-κB signaling mouse model

15αBα haploid insufficient mouse (15αBα−/−; refs. 20, 21) was used as the continuously activated NF-κB mouse model. A NF-κB constitutively activated prostate cancer mouse model (Myc/15αBα−/−) was generated by crossing 15αBα−/− mouse with the ARR2PB-myc-PAI (Hi-Myc) prostate cancer mouse line (22). Prostates were harvest at 3 and 6 month of age and fixed in 10% buffered formalin and paraffin-embedded for histologic and immunohistochemical analysis. Each experimental group consisted of at least 5 mice.

RNA extraction and microarray analysis

RNAs from prostate tissues (wild-type and 15αBα−/− mice; intact and castrated) were used for microarray analysis (prostate tissues from castrated 7-week-old 15αBα−/− and wild-type mice that were harvested at 2 weeks postcastration; 4 mice for each group). The protocol for mRNA quality control and gene expression analysis was that recommended by Affymetrix. 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) and bioanalysis (Agilent Technologies). RNA samples were submitted to the VMSR for amplification (NuGen Systems, Inc.) and labeling, followed by hybridization to Affymetrix GeneChip Expression Arrays.

Microarray data sources

The mouse microarray dataset included four groups (wild-type and 15αBα−/− mice; intact and castrated). Normalized microarray data (Mouse 430 expression arrays) were preprocessed in VMSR. For survival analysis, we downloaded a human prostate cancer dataset from NCBI Gene Expression Omnibus (GSE10645; ref. 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; ref. 24).

Generation of the NF-κB signature

The microarray data analysis was performed to directly compare prostate tissues from intact/castrated wild-type and 15αBα−/− (intact mice group, wild-type vs. 15αBα−/− mice; castration group, castrated wild-type vs castrated 15αBα−/− mice; and wild-type vs. castrated wild-type mice). For statistical analysis, we used the Significance Analysis of Microarray (SAM) software package from Stanford University (Stanford, CA; ref. 25), and based on our practical consideration, the SAM false discovery rates 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). 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 prostate cancer datasets (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 on the basis of 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 and the Cox proportional hazards model for univariate and multivariate survival analyses. For graphic representation, Kaplan–Meier estimated survivor function was plotted for each subgroup. The Kaplan–Meier
curves helped to assess the relationship between DSS 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 Kaplan–Meier 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) 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 biologic 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 prostate cancer 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), whereas NF-κB signaling was inactivated in prostate cancer 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 20-μg aliquot of each protein sample from NF-κB signaling activated and inactivated prostate cancer cells was separated on a 4% to 12% Tris-glycine gradient gel (NOVEX), and then transferred to nitrocellulose membranes (Schleicher and Schuell). The membranes were blocked with 5% skim milk in TBS-T (1% Tween-20) buffer. The membranes (Schleicher and Schuell) were probed with anti-JNK, p38MAPK, p-JNK, p-p38MAPK, and E-cadherin antibodies (Santa Cruz Biotechnology) were added at the optimal concentration (1:1000), incubation was performed for 1 hour with the second- 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 enhanced chemiluminescence 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 prostate cancer model

To investigate the role of NF-κB signaling in prostate cancer tumorigenesis and progression, we utilized a knock-out 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 (Supplementary Fig. S1; refs. 32, 33). 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 with 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 hyperproliferation in both the prostate epithelium and stroma, which are histologic features characteristic of human benign prostatic hyperplasia. However, activation of NF-κB alone in the prostate does not cause tumorigenesis in this mouse model.

ARR_PBMyc-C6 (Hi-Myc), a transgenic mouse model of prostate cancer, develops invasive adenocarcinoma in the prostate by 6 months of age (22). To investigate whether activation of the NF-κB pathway promotes prostate cancer 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 immunohistochemical staining of p65-pho antibody (Supplementary Fig. S2). At 3 months of age, as expected, Hi-Myc mice developed prostatic intraepithelial neoplasia 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 androgen receptor staining and significantly greater numbers of luminal Ki-67–positive cells (P = 0.042), a proliferation marker, when compared with Hi-Myc prostates (Supplementary Fig. S3A andS3B). However, significant distant metastasis was not detected in this model by 6 months of age. These results indicated that continuous activation of NF-κB signaling enhances Myc-induced mouse prostate cancer development and progression.

Use of nonmalignant NF-κB–activated mouse prostates to identify human orthologs expressed in patients with prostate cancer

To understand how NF-κB signaling contributes to prostate cancer 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. To define a corresponding orthologous human NF-κB signature

www.aacrjournals.org Cancer Res; 74(10) May 15, 2014 OF3

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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 prostate cancer model. A, continuous activation of NF-κB signaling induced prostate epithelial and stromal hyperproliferation. Prostates from IkBa+/− and wild-type mice were harvested at 3 and 6 months of age. Histologic analysis was performed by H&E staining. B and C, continuous activation of NF-κB signaling promotes prostate cancer progression in the ARR2PB-myc-PAI transgenic mouse. The prostates from Myc alone (ARR2PB-myc-PAI) and Myc/IkBa bigeneic mice were harvested at 3 (B) and 6 (C) months of age. Histologic analysis was performed by H&E staining. DP, dorsal prostate; LP, lateral prostate; VP, ventral prostate; AP, anterior prostate.
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. To investigate whether the gene expression signatures derived from mouse models can serve as predictors of progression of human prostate cancer, we identified orthologous members of the human NF-κB signatures within expression data from primary human prostate cancer datasets (PR18846227) published by the Mayo Clinic (Rochester, MN; ref. 24). The Mayo Clinic microarray contains 526 gene targets for RNAs, including genes whose expression is altered in association with prostate cancer progression (24). The comparison of mouse identified/converted human orthologs to the Mayo Clinic datasets generated three lists of common genes as follows: (i) 21 human genes from NF-κB–activated (IkBα:−) androgen-depleted (castration) mouse prostate (Table 1); (ii) 24 human genes from the NF-κB–activated (IkBα:±) intact (no castration) mouse prostate (Supplementary Table S1); (iii) 228 human genes from the androgen-depleted (castrated) wild-type mouse prostate (Supplementary Table S2).

**NF-κB gene signature generated from a nonmalignant NF-κB–activated androgen-depleted mouse prostate predicts overall cancer-specific survival of patients with prostate cancer**

The human prostate cancer Mayo Clinic microarray dataset (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 prostate cancer dataset 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 S1). 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 on the basis of the first bifurcation of the clustering dendrograms (27). Kaplan–Meier and log-rank analyses demonstrate a significant difference in predicting prostate cancer-specific death (DSS) from the human prostate cancer datasets 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) HRs of these factors and NARP21 were determined (Table 2).

The prognostic value of NARP21 from unadjusted HR (univariate HR; high risk vs. low risk) in PR18846227 was 3.4 [95% confidence interval (CI), 2.2–5.2; P < 0.001] for DSS. Univariate Cox proportional hazards analysis demonstrated that NARP21 could successfully predict prostate cancer-specific death. Multivariate Cox proportional hazards analysis used was to determine whether 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 clinic 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% CI, 1.1–2.5; P = 0.0212; Supplementary Fig. S4), the NARP21 signature performed the best in predicting prostate cancer patient clinical outcome. Therefore, further analysis of NF24 signature is not presented.

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

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene title</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACP2</td>
<td>Acid phosphatase 2, lysosomal</td>
</tr>
<tr>
<td>ACPP</td>
<td>Acid phosphatase, prostate</td>
</tr>
<tr>
<td>CCNB1</td>
<td>Cyclin B1</td>
</tr>
<tr>
<td>DLGAP1</td>
<td>Discs, large (Drosophila) homolog-associated protein 1</td>
</tr>
<tr>
<td>EGR3</td>
<td>Early growth response 3</td>
</tr>
<tr>
<td>ENPP2</td>
<td>Ectonucleotide pyrophosphatase/phosphodiesterase 2</td>
</tr>
<tr>
<td>FBXW11</td>
<td>F-box and WD-40 domain protein 11</td>
</tr>
<tr>
<td>GABRG2</td>
<td>γ-aminobutyric acid (GABA-A) receptor, subunit-γ2</td>
</tr>
<tr>
<td>GDF15</td>
<td>Growth differentiation factor 15</td>
</tr>
<tr>
<td>H1FX</td>
<td>H1 histone family, member X</td>
</tr>
<tr>
<td>HMGR</td>
<td>3-hydroxy-3-methylglutaryl-Coenzyme A reductase</td>
</tr>
<tr>
<td>ITPKA</td>
<td>Inositol 1,4,5-trisphosphate 3-kinase A</td>
</tr>
<tr>
<td>IVD</td>
<td>Isovaleryl coenzyme A dehydrogenase</td>
</tr>
<tr>
<td>KIAA0196</td>
<td>RIKEN cDNA E430025E21 gene</td>
</tr>
<tr>
<td>RABBA</td>
<td>RAB8A, member RAS oncogene family</td>
</tr>
<tr>
<td>RNF2</td>
<td>Ring finger protein 2</td>
</tr>
<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>
</tr>
<tr>
<td>TPX2</td>
<td>TPX2, microtubule-associated protein homolog (Xenopus laevis)</td>
</tr>
<tr>
<td>TRPS1</td>
<td>Trichorhinophalangeal syndrome I (human)</td>
</tr>
<tr>
<td>XDH</td>
<td>Xanthine dehydrogenase</td>
</tr>
<tr>
<td>ZNF511</td>
<td>Zinc finger protein 511</td>
</tr>
</tbody>
</table>

**The NF-κB Signature Predicts Prostate Cancer Progression**
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 S2) and prostate cancer-specific survival. The AD228 signature was not associated with prostate cancer-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 prostate cancer-specific death is not due to the effect of androgen depletion alone.

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

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 prostate cancer (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, whereas 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 prostate cancer (stage T2 and T3; no lymph node metastasis), 153 cases progressed to systemic metastasis disease after prostatectomy (up to 15 years follow-up; ref. 24). When we analyzed the NARP21 gene signature of patients with clinically localized prostate cancer 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 before 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 prostate cancer 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 prostate cancer metastasis (Table 2). The prognostic value of NARP21 from unadjusted HR (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 prostate cancer 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.

**Table 2. Univariate and multivariate analysis of NARP21 in prostate cancer**

<table>
<thead>
<tr>
<th>Endpoints(^a)</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(^b)</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>

\(^a\)Endpoints: clinic endpoints are DSS and DMFS.

\(^b\)Adjusted factors in prostate cancer: tumor stage, ploidy, PSA, and age.
Next, the NARP21 signature was used to evaluate the patients with prostate cancer that had lymph node metastasis at the time of RRP. Among the 77 lymph node-positive cases, 47 cases (61%) had systemic metastasis, whereas 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 that had further systemic metastasis, almost 80% (37/47 cases) would fall in a poor-prognosis group, whereas only 20% cases (10/47 cases) segregated into the favorable-prognosis group (Fig. 3C and Supplementary Table S3). 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.

Identification of gene networks and pathways associated with metastatic progression of human prostate cancer

To further understand the detailed mechanism by which NF-κB signaling contributes to metastatic progression of prostate cancer, we used the 21 genes (Table 1) from the NARP21 gene signature to perform an IPA. IPA showed that this list would reestablish a direct link to the NF-κB pathway (Fig. 4 and Supplementary Fig. S5). In addition, the results showed a highly interconnected network of aberrations along the c-Jun NH2-terminal kinases (JNK) signaling pathway (Fig. 4 and Supplementary Fig. S5). 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 prostate cancer progression. Our studies further indicate that activation of NF-κB signaling, either by constitutively active (EE) mutants of IKK2 (Fig. 5 and Supplementary Fig. S6) or expression of p65 (Supplementary Fig. S7), increases JNK phosphorylation (but not that of p38MAPK) and decreases E-cadherin expression in prostate cancer cells (Fig. 5). In addition, blocking JNK signaling inhibits NF-κB-induced invasive ability efficiently in prostate cancer cells (Supplementary Fig. S8).

Discussion

From the clinical perspective, it is understood that although two patients can be diagnosed with prostate cancer of identical stage and grade, these same two patients can have very different clinical outcomes. One patient may harbor indolent prostate cancer, which will remain non-harmful during his lifetime, whereas 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 prostate cancer 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 prostate cancer to save these individuals from undergoing unnecessary treatment. Recently, several groups have investigated molecular and genetic characteristics of prostate cancer 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 IkBα does not induce prostatic tumorigenesis in our mouse model.
model, whereas continuous activation of NF-κB signaling enhances earlier development of prostate cancer in the Hi-Myc mouse model of prostate cancer (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 prostate cancer may require some other driver(s) to induce tumorigenesis, activation of NF-κB signaling is an important factor to contribute to prostate cancer progression. Therefore, our studies were focused on the mechanism by which NF-κB signaling contributes to prostate cancer progression and whether activation of NF-κB signaling is sufficient to predict poor survival outcome and systemic metastasis in the patients with prostate cancer. Using nontumorigenic prostates from a mouse model with increased NF-κB activity and androgen depletion, we developed a gene expression signature (NARP21) that discriminated high- from low-risk cases of cancer metastasis and death in patient with prostate cancer (Figs. 2 and 3). The data represent 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 patients with prostate cancer with the most risk of disease progression is achieved via a signature that is generated from a noncancerous mouse prostate with a single genetic change, resulting in elevated levels of the 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 (Figs. 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 prostate cancer development and progression. These results suggest that the contribution of NF-κB signaling in prostate cancer progression may be more significant during treatment of prostate cancer 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 "nonstandard" downstream target genes of the NF-κB pathway may play an important role during tumor progression. IPA studies, using NARP21 gene signature, showed a highly interconnected network of the 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 (35–37). Recent studies show that the knockout of JNK1 and JNK2 in the Pten null mouse model of prostate cancer results in increased tumorigenesis and metastasis (43). This suggests that JNK restrains tumor progression. Our data indicate that NF-κB is upstream of JNK activation (Fig. 5). Furthermore, we see that drug inhibition of JNK increases invasiveness of prostate cancer cells (Supplementary Fig. S4). 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 prostate cancer. 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 nonmalignant NF-κB–activated androgen-depleted mouse prostate successfully distinguished subsets of human cancer and predicts clinical outcome in patients with prostate cancer. This prediction signature can have a significant impact on identifying patients with indolent or aggressive disease.

Disclosure of Potential Conflicts of Interest
T.S. Blackwell is a consultant/advisory board member of Boehringer Ingelheim. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: R. Jin, Y. Yi, F.E. Yull, P.E. Clark, R.J. Matusik
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Jin, Y. Yi, F.E. Yull, T.S. Blackwell, R.J. Matusik
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Jin, Y. Yi, P.E. Clark, T. Koyama, J.A. Smith, R.J. Matusik
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Jin, R.J. Matusik
Study supervision: R. Jin, J.A. Smith, R.J. Matusik

Acknowledgments
The authors thank Dr. Martin Leverkus (University of Magdeburg, Germany) for providing them with I KK2-EIE and I KK2-KD retroviral vectors, and Tom C. Case and Manik Paul for technical assistance.

Grant Support
This work was supported by the Department of Defense Prostate Cancer Research Program (W81XWH–10–1–0236; R. Jin), the American Cancer Society (IRG–58–009-51, IRG–58–009–53; Y. Yi), the NIH (R01–CA113734; F.E. Yull), and the National Cancer Institute (4R01 CA076142–15; R.J. Matusik).

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Received September 5, 2013; revised January 7, 2014; accepted February 17, 2014; published OnlineFirst March 31, 2014.

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*Cancer Res* Published OnlineFirst March 31, 2014.

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