Molecular Characterization of Neuroendocrine Prostate Cancer and Identification of New Drug Targets

Himisha Beltran1,*, David S. Rickman2,*, Kyung Park2, Sung Suk Chae2, Andrea Sboner2, Theresa Y. MacDonald2, Yuwei Wang8, Karen L. Sheikh2, Stéphane Terr2, Scott T. Tagawa1,3, Rajiv Dhir9, Joel B. Nelson10, Alexandre de la Taille11, Yves Allory11, Mark B. Gerstein6,7, Mark B. Gerstein6,7, Sven Perner13, Kenneth J. Pienta13,14, Arul M. Chinnaiyan14,15, Yuzhuo Wang8, Colin C. Collins8, Martin E. Gleave8, Francesca Demichelis2,4,16, David M. Nanus1,3, and Mark A. Rubin2,3

ABSTRACT
Neuroendocrine prostate cancer (NEPC) is an aggressive subtype of prostate cancer that most commonly evolves from preexisting prostate adenocarcinoma (PCA). Using next-generation RNA sequencing and oligonucleotide arrays, we profiled 7 NEPC, 30 PCA, and 5 benign prostate tissue (BEN) samples and validated findings in tumors from a large cohort of patients (37 with NEPC, 169 with PCA, and 22 with BEN) using immunohistochemistry and FISH. We discovered significant overexpression and gene amplification of AURKA and MYCN in 40% of NEPC and 5% of PCA tumors, respectively, and evidence that they cooperate to induce a neuroendocrine phenotype in prostate cells. There was dramatic and enhanced sensitivity of NEPC (and MYCN overexpressing PCA) to Aurora kinase inhibitor therapy both in vitro and in vivo, with complete suppression of neuroendocrine marker expression following treatment. We propose that alterations in Aurora kinase A and N-myc are involved in the development of NEPC and that future clinical trials will help determine the efficacy of Aurora kinase inhibitor therapy.

SIGNIFICANCE: We report on the largest in-depth molecular analysis of NEPC and provide new insight into molecular events involved in the progression of prostate cancer.

Cancer Discovery; 1(6); 487–95. © 2011 AACR.
expression of neuroendocrine-associated genes (e.g., NSE, SYP) and MYCN, though there were some tumors with mixed molecular features (Fig. 1C).

We evaluated 45 NEPC tumors ranging from pure small cell carcinoma to tumors with mixed features of both PCA and NEPC (Supplementary Fig. S1). The TMPRSS2-ERG gene fusion was detected by FISH break-apart assay in 44% of NEPC tumors. It is important to note that in tumors demonstrating both PCA and NEPC foci, there was perfect concordance with regard to the TMPRSS2-ERG status (Fig. 1A). NEPC foci lacked ERG protein expression by immunohistochemistry (IHC), even in tumors harboring the TMPRSS2-ERG rearrangement, with a sharp margin separating NEPC and PCA components in mixed tumors. This margin also corresponded directly to the presence or absence of AR expression (in PCA and NEPC, respectively), consistent with ERG protein expression being driven by androgen and requiring AR signaling.

Using next-generation RNA sequencing (RNA-Seq) and oligonucleotide arrays, we sequenced 7 NEPC and 30 localized PCA tumors. The clinical characteristics are summarized in Supplementary Tables S1 and S2. There were significant gene expression differences between NEPC and PCA, with 936 of 25,932 evaluated genes showing differential expression after correction for multiple hypothesis testing (Benjamini–Hochberg \( P < 0.001 \)) (Fig. 1B; Supplementary Table S3). There were no global gene expression differences between primary and secondary NEPC. As expected, NEPC showed low expression of known androgen-regulated genes (e.g., KLK3 (encoding PSA), TMPRSS2, NKX3-1) and high expression of neuroendocrine-associated genes (e.g., CGA and SYP), though there were some tumors with mixed molecular features (Fig. 1C). EZH2, a polycomb gene shown to be associated with aggressive behavior in a number of cancer types including prostate (8), was significantly overexpressed in NEPC compared with PCA (\( P = 0.0001 \)). Somatic copy number alteration assessment revealed discrete, statistically significant differences in the number of genomic amplifications and deletions in NEPC compared with PCA (Fig. 1D).

After integration of gene expression and copy number data, we evaluated targetable lesions and discovered significant overexpression and gene amplification of AURKA (Akira kinase A) in NEPC compared with PCA (\( P = 1.46 \times 10^{-2} \)) (Fig. 2A; Supplementary Fig. S2). AURKA mRNA was overexpressed in all 7 cases. AURKA amplification was observed in 4 of 7 NEPC tumors and associated with overexpression (\( P = 0.0006 \)), but AURKA overexpression also occurred without amplification, potentially through other mechanisms. Within NEPC, the level of AURKA overexpression was not differential based on whether AURKA was amplified or not (Supplementary Fig. S3A). AURKA is a serine/threonine kinase involved in mitotic spindle formation, centrosome separation, and the G2-M transition during the cell cycle (9), and it has oncogenic properties (9).

In human neuroblastoma models, AURKA has also been shown to interact with and stabilize the oncogene N-myc (MYCN) (10) independent of its role in mitosis. Because neuroblastoma is another highly aggressive neuroendocrine tumor, we queried the data set for MYCN gene expression and discovered significant overexpression in NEPC compared with PCA (\( P = 0.0005 \)) (Fig. 2B). The Affymetrix 6.0 array does not have adequate coverage of the MYCN locus on 2p24 with the nearest markers 2.5 kb from the 3′ and 5′ end of the gene and, therefore, is suboptimal in evaluating for MYCN copy number gain. N-myc is a transcription factor in the MYC family involved in nervous system development that is not normally expressed in the prostate (11) and has not been previously linked to prostate cancer.

We screened benign prostate tissue and prostate tumors from a larger cohort of patients (22 with benign prostate, 169 with primary PCA, and 37 with NEPC) (Fig. 2C) and found by IHC that Aurora kinase A was overexpressed in none of the benign prostate cases, 12% of PCA cases, and 76% of NEPC cases. AURKB was also overexpressed in NEPC, although to a lesser degree, and AURKC was minimally expressed in both NEPC and PCA (Supplementary Fig. S3B). In NEPC, there was strong cytoplasmic expression of Aurora kinase A in the majority of tumor cells (>50%; Fig. 2D), but in PCA <5% of tumor cells exhibited cytoplasmic Aurora kinase A staining that was generally weaker and speckled. AURKA amplification was detected by FISH in none of the benign prostate cases, 5% of PCA cases, and 40% of NEPC cases (Fig. 2E). Neither AURKB nor AURKC were amplified. MYCN amplification was detected by FISH in none of the benign prostate cases, 4% of PCA cases, and 40% of NEPC cases. In nearly all positive cases (>90%), amplification of AURKA and MYCN was concurrent (Supplementary Fig. S4). Notably, in one patient who progressed from PCA to NEPC after 3 years, there was amplification of AURKA and MYCN in his primary PCA, suggesting that these genomic aberrations can arise early.

To investigate the relationship between AURKA and MYCN in the prostate, we transfected MYCN in benign prostate epithelial cells (RWPE-1) and prostate cancer cells (LNCaP), and found that MYCN induced expression of Aurora kinase A and phosphorylated histone H3 [a downstream marker of Aurora kinase activity (12)] (Fig. 3A, B). Chromatin immunoprecipitation (ChIP) in LNCaP cells stably transfected with MYCN revealed that N-myc does not bind the promoter of AURKA but does bind to E-box binding elements associated with the N-myc-responsive promoter of telomerase reverse transcriptase (hTERT), as previously described (13) (Supplementary Fig. S5). Instead, we found that the N-myc protein physically interacts with Aurora kinase A (as seen by communoprecipitation) and enhances Aurora kinase A protein stability (Fig. 3C).

In order to determine if there is a role of Aurora kinase A or N-myc in neuroendocrine differentiation, we assessed neuroendocrine marker expression after transfection of AURKA and MYCN in RWPE-1. Surprisingly, we found that either AURKA or MYCN induced the expression of the neuroendocrine markers SYP and NSE, which are not normally expressed in benign prostate. Similarly, knockdown of AURKA with siRNA suppressed NSE expression in the NEPC cell line NCI-H660 (Supplementary Fig. S6). Furthermore, LNCaP cells stably transfected with MYCN (LNCaP-n-myc) phenotypically resembled NEPC, with upregulation of the neuroendocrine marker NSE, downregulation of AR and androgen-regulated genes (TMPRSS2,
Molecular Characterization of Neuroendocrine Prostate Cancer

**Figure 1.** Characterization of NEPC. A, tumor with mixed features of NEPC and PCA; hematoxylin and eosin (H&E) staining, immunohistochemical analysis for AR and ERG, and FISH for ERG break-apart (indicating gene fusion). B, gene expression of 936 of 25,932 genes showing differential expression between 7 NEPC cases and 30 PCAs, after Benjamini-Hochberg correction for multiple hypothesis testing of < 0.001 (red = high expression; green = low expression). C, gene expression of select genes comparing NEPC and PCA, including neuroendocrine-associated genes (SYP, CHGB, CHGA), EZH2, MIB1 (Ki67), PSMA, AR, and androgen-regulated genes [NKX3-1, KLK3 (PSA), TMPRSS2]. D, graphic representation of genomic landscape of PCA (in order of increasing Gleason Score) and NEPC, as determined by Affymetrix 6.0 oligonucleotide array (red = copy number gain; blue = copy number loss; white = no change).

*NKX3-1*, and upregulation of EZH2 compared with control LNCaP cells (Fig. 3B). Chromatin immunoprecipitation revealed that N-myc binds the promoters of NSE, SYP, and AR, suggesting direct modulation of the neuroendocrine phenotype by transcription factor binding (Fig. 3D). In MYCN-amplified neuroblastoma cells (IMR-32), N-myc also bound the NSE and SYP promoters, but not the AR promoter, suggesting that N-myc occupancy at the AR promoter may be prostate specific.

Based on these findings, we posited that treatment with an Aurora kinase inhibitor would have a preferential effect on NEPC compared with PCA. To test this hypothesis in vitro, we used two experimental models: LNCaP cells stably transfected with MYCN (which phenotypically resemble NEPC), and the NCI-H660 cell line. NCI-H660 was originally derived at time of autopsy from a patient with small cell carcinoma initially thought to be lung cancer but later classified as prostate cancer (14, 15). RNA sequencing revealed that NCI-H660 has a
molecular signature similar to that of NEPC tumors, including overexpression of AURKA and MYCN. AURKA and MYCN copy number gains (Supplementary Fig. S7A and S7B) were observed in these cells, as well as increased phosphorylated AURKA relative to phosphorylated AURKB and AURKC (Supplementary Fig. S7C), indicative of upregulated AURKA kinase activity.

LNCaP N-myc cells showed enhanced in vitro sensitivity to the Aurora kinase inhibitor PHA-739358 (Nerviano Medical Sciences) compared with control LNCaP cells (LNCaP EV) (Fig. 4A). Similarly, NCI-H660 cells showed enhanced sensitivity to PHA-739358 compared with two PCA cell lines (DU145, VCaP) and noncancerous RWPE-1 cells (Fig. 4B). Knockdown of AURKA in NCI-H660 cells with multiple shR-NAs showed similar results (Supplementary Fig. S8). Given the role of AURKA in the cell cycle, we conducted FACS analysis of cells following PHA-739358 treatment. Polyploidy was induced in all cells (i.e., LNCaP, LNCaP N-myc, NCI-H660), and there was dose-dependent PHA-739358–mediated G2–M arrest in LNCaP and LNCaP N-myc cells. However, there was no significant G2–M arrest in NCI-H660 cells, supporting our hypothesis that AURKA may have alternative mechanisms of action in NEPC (Supplementary Fig. S9).

We then tested PHA-739358 in xenografts using two NEPC models: (i) NCI-H660 xenografts, which are histologically similar to human NEPC, positive for neuroendocrine markers by IHC, and although harboring the TMPRSS2-ERG gene fusion (16) are negative for ERG protein (Fig. 4F; Supplementary Figs. S10 and S11); and (ii) the LTL-352 xenograft, derived from a patient with a history of metastatic PCA that progressed to NEPC after 40 months of androgen deprivation therapy (17). When treated with PHA-739358, there was average tumor shrinkage of 50% to 87% in both NCI-H660 and LTL-352 xenografts ($P < 0.001$) compared with no effect in LNCaP xenografts (Fig. 4C, D) and a cytostatic effect in VCaP xenografts (Supplementary Fig. S12), and was without significant toxicity (Supplementary Fig. S13). Phosphorylated histone H3 expression was significantly inhibited in the treated NCI-H660 xenografts (indicating on-target drug effect) and not in the LNCaP xenografts (Fig. 4E). Notably, SYP expression was also completely suppressed in the treated NCI-H660 xenografts (Fig. 4F), again supporting a role of Aurora kinase A in modulating the neuroendocrine phenotype.

**DISCUSSION**

Although de novo NEPCs are considered uncommon and represent only 0.5% to 2% of all prostate cancers, focal neuroendocrine differentiation is present in 10% to 100% of localized PCAs and increases with disease progression (18, 19). Autopsy studies suggest that a subset of patients with prostate cancer die from pure AR-negative NEPC (20, 21), but this incidence may be underrecognized. Patients are not typically biopsied late in the stages of PCA to evaluate for NEPC progression, but NEPC can be suspected in patients with progressive disease despite normal or modestly elevated PSA and elevated serum markers of neuroendocrine differentiation (i.e., chromogranin A or NSE) (1). Serum markers and the amount of neuroendocrine differentiation in tumors increase with long-term androgen
Molecular Characterization of Neuroendocrine Prostate Cancer

Figure 3. A, immunoblot analysis for protein expression of AURKA, phosphorylated histone H3 (PO4-H3), NSE, and synaptophysin (SYP) after transient transfection of MYCN, AURKA, or empty vector (EV) in RWPE-1 cells. BE(2)N is a neuroblastoma cell line as positive control for NSE. B, stable LNCaP cell line overexpressing N-myc compared with EV. Immunoblot analysis for protein expression of N-myc, AURKA, PO4-H3, NSE, SYP, PSA, AR, and β-actin. Quantitative real-time PCR (qRT-PCR) and microarray (MA) data showing induction of NSE (qRT-PCR) and EZH2 (MA) gene expression and suppression of AR (qRT-PCR) and androgen-regulated genes [NKX3-1, TMPRSS2 (MA)]. C, Left, immunoprecipitation of LNCaP N-myc cell lysates using antibodies directed against N-myc or control IgG antibodies and Western blot using antibodies directed against AURKA (Aurora A) or control IgG antibodies; Right, LNCaP control (LNCaP EV) and LNCaP N-myc cells were treated with cycloheximide for the indicated time (in minutes) and Aurora kinase A or β-tubulin levels were assessed by immunoblotting. The normalized percentage of AURKA is relative to β-tubulin and to time point 0 for LNCaP EV or LNCaP N-myc. D, N-myc directly binds to the SYP, NSE, and AR promoters in LNCaP N-myc cells but not in LNCaP EV cells. Above, a not-to-scale schematic representation of SYP, NSE, and AR promoters with the indicated E-box sites (gray and black circles) is shown. The transcription start site for each gene is indicated with an arrow. Below each schematic, bar graphs represent the amount of enriched DNA (relative to input chromatin preparation) for each E-box site (color-coded) in the indicated cell lines following ChIP using either anti-N-myc (right) or anti-IgG (left) antibodies. IMR-32 is an MYCN-amplified neuroblastoma cell line. In IMR-32 cells, N-myc binds promoters of SYP and NSE, but not AR.
deprivation (18, 22). Depletion of androgen in cell culture also promotes neuroendocrine differentiation of LNCaP adenocarcinoma cells (23). These data suggest that with the recent introduction of new highly potent androgen deprivation therapies into the clinical arena, the incidence of NEPC may escalate.

Although histologically similar, NEPC differs from small cell carcinomas of other primary sites in that there
is evolution from PCA, as suggested by concordance of the TMPRSS2-ERG gene rearrangement and other molecular abnormalities (such as TP53 mutations) in mixed tumors (24). We found dramatic gene expression and copy number differences between PCA and NEPC even though they can coexist within the same tumor foci. It is possible that a few key mutations occur that drive and select for the neuroendocrine phenotype and a number of passenger alterations follow as a result of the phenotype. The molecular events associated with this transformation have not been well defined.

We found AURKA and MYCN to be overexpressed and amplified in NEPC and have provided evidence that they functionally cooperate and can induce a neuroendocrine phenotype in prostate cells. AURKA is best known for its role in mitosis, but its interaction with the oncogene N-myc has been described in neuroblastoma (10) and now NEPC, and it may play a role in other neuroendocrine carcinomas. MYCN is most commonly amplified in neuroblastoma, although it has been reported in other central nervous system tumors as well as up to 40% of small cell lung cancers (25). We have shown for the first time that MYCN is abnormally expressed and amplified in NEPC and in 5% of PCAs and is involved in neuroendocrine differentiation as well as the stabilization of AURKA.

It remains unclear why MYCN and AURKA are coamplified in nearly all PCAs and NEPCs, despite their location on separate chromosomes (2p24 and 20q13, respectively). In neuroblastoma, a positive feedback loop has been described in which AURKA also induces MYCN (10), and perhaps it is cooperation of these two lesions that drives the phenotype. Notably, AURKA and MYCN are also overexpressed and amplified in approximately 5% of individuals with PCA, which may represent a high-risk population that could benefit from early intervention. We did not see any association with standard clinical parameters (Supplementary Fig. S14). Larger studies are needed to determine the prognostic role of AURKA and MYCN amplification in PCA.

Currently, there is no standard treatment for patients with NEPC, accounted for in part by its poor molecular characterization. Based on the biologic role of AURKA in NEPC, its interaction with MYCN, and the dramatic and preferential sensitivity of NEPC preclinical models to AURKA inhibition, we propose that AURKA inhibitors may potentially benefit patients with these molecular alterations. The potential therapeutic role of AURKA inhibitors, either alone or in combination with cytotoxic chemotherapy, should be further explored in this subset of patients with NEPC as well as in patients with other tumors with AURKA and MYCN alterations. Although a recent phase II trial evaluating PHA-739358 for patients with castration-resistant prostate cancer failed to achieve its primary end point of PSA response (26), we propose that it was an unselected population, and that PSA may not be an ideal trial end point because NEPC does not secrete PSA. As new targeted agents are being introduced into the clinical arena, patient selection based on molecular subtyping is essential as we transition into an era of personalized oncology.

METHODS

Clinical Cohort

All tissue samples were collected as part of an Institutional Review Board–approved protocol at Weill Cornell Medical College (WCMC). Deidentified frozen NEPCs were obtained from WCMC (tumor metastases obtained from lung, soft-tissue, and spinal cord metastases), University of Michigan rapid autopsy program (metastases), Henry Mondor Hospital (prostatectomy case), University of Pittsburgh (biobank program), and University of British Columbia (metastatic tumor passed as second-generation xenograft). Additional formalin-fixed paraffin-embedded (FFPE) NEPC tumors for validation studies were obtained from WCMC, University of Tübingen, and University of Pittsburgh. Frozen and FFPE localized PCA and benign prostate tissues were collected at time of radical prostatectomy at WCMC. All cases were reviewed by the study’s pathologist (M.A. Rubin), and high-density tumor foci with < 10% stroma were selected for RNA and DNA extraction. Normal prostate samples were selected from blocks containing no tumor tissue, in order to minimize contamination. Clinical features of the frozen NEPC and PCA tumor cases are summarized in Supplementary Methods.

RNA Sequencing and Copy Number Assessment

The complete transcriptomes of 7 NEPC tumors, 30 prostate adenocarcinomas, 6 normal prostatic epithelial samples, and 6 prostate cell lines were sequenced on an Illumina GA II Sequencer. Paired-end sequencing was done and reads were mapped to human genome (hg18) using ELAND alignment software. Gene expression was quantified using RSEQtools (27). Pflueger and colleagues (28) have provided a full description of the PCA cases. Tumor DNA together with paired blood DNA was extracted from high-density foci, and areas of genomic gain and loss were assessed using the Affymetrix 6.0 SNP array platform. For a complete description of the methodology, see Supplementary Methods.

Immunohistochemistry and Fluorescence In Situ Hybridization

Validation studies were done on 169 primary PCA, 37 NEPC, and 22 benign FFPE prostate tissue samples using IHC for protein expression and FISH for gene amplification or ERG gene rearrangement. For reagents used and detailed methods, see Supplementary Information. IHC was considered positive if > 1% of tumor cells displayed immunoreactivity in cell cytoplasm (Aurora kinase A) or nucleus (Aurora kinase B, ERG). ERG rearrangement was assessed using dual-color break-apart interphase FISH assay as described previously (3, 17). In cases in which FISH did not work, the TMPRSS2-ERG gene fusion was evaluated using reverse transcriptase PCR to screen for fusion transcript expression (as described in ref. 3).

Statistical Analysis

A Wilcoxon test was applied for mRNA differential analysis, followed by Benjamini–Hochberg correction for multiple hypothesis testing. Pearson correlation and Fisher exact test were implemented for gene-gene expression correlation and genomic aberration association analysis, respectively. A Student t test method was used to determine differences in tumor volumes in xenograft studies, with the criterion for significance < 0.05.

Transfection, Quantitative PCR, and Immunoblot Analysis

Functional studies were conducted using the NCI-H660 cell line as a model of NEPC, and VCaP and LNCaP as models of PCA. All cell lines were purchased from ATCC and the 293FT cell line was...
purchased from Invitrogen and maintained according to the manufacturer’s protocols. A complete description of the materials and methods is provided in the Supplementary Methods.

**Drug Treatment**

PHA-739358 was obtained from Nerviano Medical Sciences in powder form (molecular weight = 414.36). Prostate cell lines for this study were obtained from the American Type Culture Collection. RWPE-1 (20 × 10^3 cells/well), NCI-H660 (20 × 10^3 cells/well), DU145 (5 × 10^3 cells/well), VCaP (20 × 10^3 cells/well), and LNCaP (1.5 × 10^3 cells/well) were seeded on 96-well tissue culture plates. At 24 hours, cell lines were treated with vehicle (0.5% dimethyl sulfoxide) or escalating doses of PHA-739358 (5, 50, 100, and 500 nmol/L and 1 and 5 μmol/L). At 48, 72, 96, and 120 hours, viability was assessed by the WST-1 assay (Roche), reading absorbance at 450 nm according to the manufacturer's instructions. Xenografts were prepared by injection of 10^6 NCI-H660 cells, VCaP cells, or LNCaP cells into NU/J mice (Jackson Laboratories). LTL-352 xenografts were established at BC Cancer Center Living Tumor Laboratory as previously described (17).

**Additional Methods**

Detailed methodology is described in the Supplementary Methods.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Naoki Kitabayashi, Juan-Miguel Mosquera, Isabelita Vengo, Derek Oldridge, and Terry Vuong for their technical assistance and Nerviano Medical Sciences for providing PHA-739358.

**Grant Support**

This study was supported by the Ann and William Bresnan Foundation, Prostate Cancer Foundation (PCF), PCF Young Investigator Award (H. Beltran), Department of Defense New Investigator Award (D.S. Rickman), NCI P50 CA69568 (A.M. Chinnaiyan, K.J. Pienta), Department of Defense New Investigator Award (F. Demichelis), NCI R01-CA116337 (F. Demichelis, M.A. Rubin), R01-125612 (F. Demichelis, M.A. Rubin), and NCI EDRN U01 CA111275 (F. Demichelis, A.M. Chinnaiyan, M.A. Rubin).

Received June 7, 2011; revised August 24, 2011; accepted September 13, 2011; published online November 17, 2011.

**REFERENCES**


