**NKX3.1 Haploinsufficiency is Prognostic for Prostate Cancer Relapse Following Surgery or Image-Guided Radiotherapy**

Jennifer A. Locke1,2, Gaetano Zafarana1,2, Adrian S. Ishkanian1,2, Michael Milosevic1,2, John Thoms1,2, Cherry L. Have1, Chad A. Malloff3, Wan L. Lam3, Jeremy A. Squire4, Melania Pintilie1,2, Jenna Sykes2, Varune Rohan Ramnarine1,2, Alice Meng1,2, Omer Ahmed1,2, Igor Jurisica1,2, Theo van der Kwast1,2, Robert G. Bristow1,2,5

1Departments of Radiation Oncology, Medical Biophysics, Laboratory Medicine and Pathology and Dalla Lana School of Public Health, University of Toronto
2Ontario Cancer Institute/Princess Margaret Hospital-University Health Network
3Department of Cancer Genetics and Developmental Biology, British Columbia Cancer Research Centre
4Department of Pathology and Oncology, Queen’s University, Kingston, Ontario

Running Title: **NKX3.1 Is Prognostic For Prostate Cancer Outcome**

Keywords: prostate cancer, NKX3.1, diagnostic biopsies, radiotherapy, aCGH

Correspondence to:
Robert G. Bristow MD, PhD, FRCPC
Radiation Medicine Program
Princess Margaret Hospital
610 University Avenue
Toronto, ON
M5G 2M9
Canada
Phone: 416-946-2936
Fax: 416-946-4586

Email: rob.bristow@rmpuhn.on.ca

Financial Support: This work was supported by the Ontario Institute for Cancer Research through funding provided by the Government of Ontario, the Canada Foundation for Innovation (grant numbers CF1 #12301 and CF1 #203383), the Ontario Research Fund (grant number GL2-01-030), and IBM. Individuals were supported by the Canada Research Chair Program (IJ), the Comprehensive Research Experience for Medical Student program at the University of Toronto (JAL) and a Canadian Cancer Society Research Scientist Career Award (RGB).

DISCLOSURE: The authors do not have any conflicts of interest to disclose.
STATEMENT OF TRANSLATIONAL RELEVANCE

Intermediate-risk prostate cancers show heterogeneity in treatment response. Novel biologic and genetic prognosticators could further sub-categorize patients and help individualize therapy. We determined allelic loss or gain in 44 genes thought to be associated with tumour initiating cell (TIC) status using DNA derived from pre-treatment biopsies. We found significant genetic alterations in $c\text{-}MYC$, PCSA and $NKX3.1$. After adjusting for clinical factors in a multivariate model, $NKX3.1$ haploinsufficiency was significantly associated with bRFR in IGRT patients when tested alone, or when combined with $c\text{-}MYC$ gain. A similar trend for $NKX3.1$ status as a novel prognostic factor was observed in patients following radical prostatectomy. Our results support the use of genomic predictors, such as $NKX3.1$ status in needle biopsies, for personalized approaches to prostate cancer management. Future work should focus on whether $NKX3.1$ haploinsufficiency is a surrogate for differential TIC fraction and validating our finding in prospective prostate cancer treatment cohorts.
ABSTRACT

Background: Despite the use of PSA, Gleason-score, and T-category as prognostic factors, up to 40% of patients with intermediate-risk prostate cancer will fail radical prostatectomy or precision image-guided radiotherapy (IGRT). Additional genetic prognosticators are needed to triage these patients towards intensified combination therapy with novel targeted therapeutics. We tested the role of the NKX3.1 gene as a determinant of treatment outcome given its reported roles in tumor initiating cell (TIC) renewal, the DNA damage response and co-operation with c-MYC during prostate cancer progression.

Methods: Using high-resolution aCGH, we profiled the copy number alterations in TIC genes using tumor DNA from frozen needle biopsies derived from 126 intermediate-risk patients who underwent IGRT. These data were correlated to biochemical relapse-free rate (bRFR) using the Kaplan-Meier method and Cox proportional hazards models.

Results: A screen of the aCGH-IGRT data for TIC genes showed frequent copy number alterations for NKX3.1, PSCA and c-MYC. NKX3.1 haploinsufficiency was associated with increased genomic instability independent of PSA, T-category and Gleason-score. After adjusting for clinical factors in a multivariate model, NKX3.1 haploinsufficiency was associated with bRFR when tested alone (HR=3.05, 95% CI:1.46-6.39, p=0.0030) or when combined with c-MYC gain (HR=3.88, 95% CI:1.78-8.49, p=0.00067). A similar association was observed for patients following radical prostatectomy using a public aCGH database. NKX3.1 status was associated with positive biopsies post-IGRT and increased clonogen radioresistance, in vitro.

Conclusions: Our results support the use of genomic predictors, such as NKX3.1 status, in needle biopsies, for personalized approaches to prostate cancer management.
INTRODUCTION

Men with localized prostate cancer are placed in low, intermediate and high-risk groups that predict for biochemical failure and disease-free survival using clinical prognostic factors. These include pathologic Gleason-score (GS), pre-treatment serum prostate specific antigen (PSA; ng/ml) and TNM staging (1-3). One third of all prostate cancer cases diagnosed present as intermediate-risk disease (i.e. T1-T2; PSA < 20 ng/ml and GS < 8). This category exhibits great variability in outcome as 5-year PSA-based biochemical failure rates range from 9-44% following radical prostatectomy or image-guided radiotherapy (IGRT) (2). Additional biological or genetic sub-classifiers of this group could be used to improve outcome with novel combined modality therapies using molecular-targeted agents (4, 5).

Our group is interested in the role of novel DNA-based genetic prognostic and predictive factors that could predict for local radioresistance or occult systemic metastases (4, 5). We have used high-resolution array comparative genomic hybridization (aCGH) to characterize genetic changes in intermediate-risk patients based on tumor DNA derived from frozen biopsies of men who subsequently underwent IGRT. Using this approach, we have confirmed reports of DNA copy number alterations in prostate cancer for a number of gene loci including amplification of c-MYC and PSCA, TMPRSS2/ERG gene fusions and deletion of PTEN, ATBF1, KLF5, KL6, RB1, NKX3.1, E-cadherin, p16INK4A, p27KIP, and SMAD4 genes (4, 6-8). Amplification of the 8q24.21 locus containing the c-MYC oncogene has been associated with increased relapse following radical prostatectomy (9, 10), and metastatic disease (11). These aggressive clinical phenotypes may be secondary to aberrant c-MYC signalling and its function pertinent to the transcriptional control of genes involved in DNA damage response (DDR) and genetic stability, cell proliferation, apoptosis, cell migration, angiogenesis and metastasis (12, 13).
NKX3.1, a tumor suppressor, is required for prostatic stem cell maintenance (14) and haploinsufficiency of this gene has been associated with prostate cancer progression (15, 16). Recently, NKX3.1 has been implicated in DDR through interactions with topoisomerase I (17) and through facilitating recruitment of phosphorylated-ATM and γH2AX to sites of DNA double strand breaks (DSBs) damage (16). Pre-clinical models have supported a unique cooperation between c-MYC overexpression and NKX3.1 allelic loss in driving prostate cancer progression and aggression in murine prostate tumors (18). Furthermore, amplification of the 8q24.21 locus also encompasses PSCA, which has recently been shown at the mRNA level in preoperative negative prostate biopsies to predict for incidental prostate cancer (19). c-MYC, NKX3.1 and PSCA are all considered to be markers of tumor initiating cells (TICs) or clonogens (14, 20) which in turn can be a determinant of metastasis and therapeutic response due to altered DDR responses (21, 22).

We therefore tested whether allelic gains and losses in TIC-related loci, such as c-MYC, NKX3.1 and PSCA, were prognostic or predictive of outcome after localized treatment following either IGRT (e.g., daily DSBs for 7-8 weeks) and/or radical prostatectomy (e.g., IGRT) (12-14). For this purpose, we analyzed aCGH data from cohorts of patients who underwent IGRT or surgery for copy number changes of c-MYC, NKX3.1 and PSCA and tested whether they were determinants of biochemical failure.
MATERIALS AND METHODS

Patient Cohort

Two hundred and forty-seven men with histologically confirmed adenocarcinoma of the prostate were studied as a prospective clinical study, which was approved by the University Health Network Research Ethics Board and registered (NCT00160979) in accordance with the criteria outlined by the International Committee of Medical Journal Editors. From 1996-2006, pre-treatment biopsies were derived from those patients who had chosen radical radiotherapy for primary treatment (REB#00-0443-C) at the Princess Margaret Hospital (PMH) from the original 247 candidates. Prior to radiotherapy, each patient underwent trans-ultrasound (TRUS)-guided insertion of 3 intra-prostatic gold fiducial markers for radiotherapy planning, as previously described (4). At the same time, they underwent 3 research biopsies (2 for formalin-fixation and 1 flash-frozen in liquid nitrogen (4)). Staging CT and bone scans were not routinely performed for those with low and intermediate-risk disease. Bulk disease, defined by an independent observer as sufficient tumor in biopsies from the measurement sites to permit manual microdissection, was identified in 142 of these patients (tissue was unavailable for remaining patients). Of these 142 patients, 126 patients met intermediate-risk criteria as defined by D’Amico (i.e. T1–T2 disease, a Gleason-score less than 8 and PSA less than 20 ng/ml (3)) and also had information pertaining to long-term biochemical outcome. The final study therefore included 126 patients (see Table I).

Radiotherapy Planning and Delivery

The clinical target volume (CTV) encompassed the prostate gland alone. The planning target volume (PTV) was defined by a 10 mm margin around the CTV except posteriorly where
the margin was 7 mm. All patients were treated with 6-field conformal or intensity modulated radiotherapy. The radiotherapy dose was escalated over the period of accrual in a series of separate phase I/II studies. As summarized in Table I, 33 patients (26%) received a dose of 75.6 Gy in 1.8 Gy daily fractions and 78 (62%) a dose of 78-79.8 Gy in 1.8-2 Gy daily fractions. Fifteen patients (12%) participated in a study of hypofractionated radiotherapy and received 60-66 Gy in 3 Gy daily fractions. Neoadjuvant and concurrent hormonal therapy was used in 33 patients (26%) as bicalutamide 150mg PO OD for 3 months prior to, and 2 month concurrent, with radiotherapy. None of the patients received adjuvant hormonal treatment. Patients were followed at 6 monthly intervals after completing treatment with clinical examination and PSA. Additional tests and the management of patients with recurrent disease were at the discretion of the treating physician. The median follow-up of surviving patients was 6.7 years following the end of treatment.

**aCGH Analysis**

Frozen biopsies were embedded in optimum cutting temperature (OCT) at –80°C and cut into 10-micron sections for manual microdissection and preparation of DNA samples as previously described (4). DNA labeling and hybridization was performed with slight modifications to that described previously (4). In brief, 300 ng of sample and reference DNA were differentially labeled in a random priming reaction with Cyanine 3–dCTP and Cyanine 5–dCTP (Perkin Elmer Life Sciences). The reaction was incubated in the dark at 37°C for 16–18hr. DNA samples were then combined and mixed with 100 µg of human Cot-1 DNA (Invitrogen) followed by removal of unincorporated nucleotides using microcon YM-30 columns (Millipore). The mixture was then applied onto arrays containing 26,819 bacterial artificial chromosome (BAC)-derived amplified fragment pools spotted in duplicate on aldehyde coated glass slides.
Slides were scanned using a dual laser array scanner (Axon) and spot signal intensities determined using the SoftWoRx Tracker Spot Analysis software (Applied Precision). The log2 ratios of the Cyanine 3 to Cyanine 5 intensities for each spot were assessed. Data were filtered based on both standard deviations of replicate spots (data points with greater than 0.075 standard deviation were removed) and signal to noise ratio (data points with a signal to noise ratio less than 3 were removed). Resulting dataset was normalized using a stepwise normalization procedure (23). Areas of aberrant copy number were identified using a robust Hidden Markov Model (24) and classified as either loss, neutral, or gain for all clones processed. Pre/post-processed log2 ratios of intensities for clone regions were visualized using SeeGH software (25, 26). The genomic positions of clones are mapped to the NCBI’s Genome Build 36.1, released in March 2006. Percentage Genome Alteration (PGA) is defined as the cumulative size of the genetic alterations found in each patient DNA sample divided by the total size of the human genome. PGA is a general measure of genetic instability and was utilized in the analyses of this study as a surrogate for DNA ploidy.

**FISH hybridization**

Three color interphase FISH was applied to formalin-fixed paraffin-embedded (FFPE) prostate cancer biopsies to validate the genomic imbalances associated with c-MYC allelic gain and NKX3.1 allelic loss in tumors by aCGH analysis, as previously described by our group (4). Loss of NKX3.1 was defined as one copy of NKX3.1 in the majority of tumor nuclei while gain of c-MYC was defined as greater than two copies of c-MYC in the majority of tumor nuclei (see detailed methods in [Supplemental Table III](#)).
Surviving Fraction determination and Western Blot analysis of various prostate cancer cell lines

Clonogenic assay data on surviving fraction after 3 Gy for LNCaP, PC-3, DU-145 and 22RV1 were previously published from our laboratory and others (27, 28). Data for all the cell lines were available for extraction at 3Gy and used in the analysis. The surviving fraction of the BPH-1 cell line was generated anew using the same methodology (27). For each cell line, protein was quantified (BioRad protein assay) before Western blot analyses for NKX3.1 (Lifespan Biosciences; LS-B3435) and KU-70 (Santa Cruz Biotechnology; SC-5309) and visualized using an Odyssey 3.0 system. All cell lines were obtained from ATCC and authenticated by STR DNA profiling (March 2011).

Statistical methods

The primary outcome was time to biochemical failure as defined by Roach et al. to be a PSA rise of at least 2ng/mL above post-radiation nadir value (29). Differences in PGA between genetically altered groups were compared using the Mann-Whitney-Wilcoxon test. Five-year biochemical relapse-free rates (bRFR) were calculated using the Kaplan-Meier method. The log-rank test was used to evaluate the association of NKX3.1 status (loss or normal), PSCA (gain or normal) and c-MYC status (gain or normal) with bRFR. Multivariate NKX3.1 and c-MYC were also tested adjusting for known clinical factors (T-category (T1 versus T2), pre-treatment PSA (continuous) and Gleason-score (6 versus 7)) using the Cox proportional hazards model. The proportional hazards assumptions of each model were checked by looking at scaled Schoenfeld residuals. The Fisher’s exact test was used to investigate statistical associations between NKX3.1, c-MYC and PSCA. A two-sided p-value of 0.05 was used to assess statistical significance.
RESULTS

(i) *NKX3.1* allelic loss and *c-MYC* allelic gain are common events in intermediate-risk prostate cancer

As *NKX3.1* may be a putative TIC marker in prostate cancer (14), we determined whether *NKX3.1* and other TIC-related genes showed allelic gains or losses in our cohort (see Supplemental Table II). Allelic changes were observed predominantly at loci containing the *NKX3.1*, *c-MYC* and *PSCA* genes. *c-MYC* and *PSCA* are located on chromosome site 8q24 while *NKX3.1* is located on chromosome site 8p21 (Figure 1A). Upon evaluation of 126 biopsies by aCGH, 59/126 or 47% of the intermediate-risk patients displayed an allelic loss in chromosome site 8p21 (*NKX3.1*) while 29/126 or 23% and 23/126 or 18% of the intermediate-risk patients displayed an allelic gain in chromosome site 8q24 corresponding to *c-MYC* and *PSCA*, respectively. A *c-MYC* gain was rarely observed in absence of a *NKX3.1* loss with 27/126 or 21% of the biopsies showing concomitant *c-MYC* gain and *NKX3.1* loss and only 2/126 (2%) of the biopsies showing *c-MYC* gain alone. As *c-MYC* and *NKX3.1* have been shown to cooperate in animal models we audited in secondary FISH analyses three patients exhibiting *NKX3.1* loss and *c-MYC* gain based on aCGH analysis and confirmed associated copy number changes within multiple nuclei (Figure 1A, Supplemental Table III).

(ii) *NKX3.1* allelic loss and *c-MYC* allelic gain are correlated with increased genomic instability

We calculated the relative genetic instability within each tumor DNA specimen using percent genome alteration (PGA). Tumor specimens with an allelic loss of *NKX3.1* were associated with significantly higher PGA (1.2 versus 6.9, p<0.0001) when compared to tumor specimens without
NKX3.1 loss (Figure 1B). Tumor specimens with NKX3.1 altered/c-MYC altered had significantly higher PGA (10.3 for both altered versus 1.1 for both normal, p<0.0001) when compared to specimens without such alterations; tumors with NKX3.1 altered/c-MYC normal also had significantly higher PGA as compared to NKX3.1 normal/c-MYC normal (3.9 versus 1.1, p=0.00066) (Figure 1C). When sub-grouping patients by Gleason-score and NKX3.1/c-MYC status, we observed that specimens with alterations in both NKX3.1 and c-MYC were associated with higher PGA in both GS 6 and GS 7 cancers when compared to those with normal NKX3.1 and c-MYC status (p<0.01) (Figures 1D). Significant relationships between genetic instability in specimens with both allelic changes were independent of pre-treatment PSA (<=10ng/mL and >10 ng/mL) and T-category (T1 and T2) (Supplemental Figure II). Alteration in PSCA was also associated with a significantly higher PGA as compared to normal PSCA (data not shown).

(iii) Patients with NKX3.1 allelic loss are more likely to relapse following image-guided radiotherapy

We next examined the impact of allelic loss of NKX3.1 in our clinical cohort of 126 patients with intermediate-risk prostate cancer on biochemical relapse-free rates (bRFR) following treatment with a DSB-inducing agent: IGRT (mean dose 76.4 Gy). The clinical characteristics of this cohort with 6.7 years median follow-up (range 0.8-10.3) are shown in Table I. Forty-seven patients in this cohort experienced a biochemical relapse; 42 as defined by the Phoenix definition and an additional 5 who were pre-emptively treated with salvage hormones due to a rising PSA by their attending physician. Univariate analysis showed that patients whose tumors displayed NKX3.1 loss had increased failure at 5 years compared to patients with normal NKX3.1 status (bRFR 87% versus 58%, p=0.00015) (Figure 2A). The HR associated with NKX3.1 loss alone
was 3.03 (95% CI: 1.66-5.52). Patients whose tumors displayed a \(c\)-MYC gain also had increased biochemical failure at 5 years compared to patients with normal \(c\)-MYC (bRFR 80% versus 49%, \(p=0.00093\)) (Figure 2B). The HR associated with \(c\)-MYC gain alone was 2.77 (95% CI: 1.48-5.18). Patients with combined allelic changes in \(c\)-MYC and NKX3.1 had a poor prognosis when compared to patients with normal \(c\)-MYC and NKX3.1 status (bRFR 89% versus 51%, \(p=0.00017\)) (Figure 2C). The HR associated with \(c\)-MYC gain and NKX3.1 loss was 3.98 (95% CI: 1.92-8.26) as compared to normal \(c\)-MYC and NKX3.1. Lastly, patients whose tumors displayed a \(PSCA\) gain also had increased biochemical failure at 5 years compared to patients with normal \(PSCA\) (bRFR 79% versus 46%, \(p=0.00058\)) (Supplemental Figure I). The HR associated with \(PSCA\) gain was 3.26 (95% CI: 1.6-6.63).

On a multivariate analysis, NKX3.1 allelic loss alone was a significant independent predictor after correcting for PSA, Gleason-score, T-category, \(c\)-MYC gain and \(PSCA\) gain (HR=3.05, 95% CI: 1.46-6.39, \(p=0.0030\)) (Table II). This effect was also observed when NKX3.1 loss and \(c\)-MYC gain were combined and tested against PSA, Gleason-score and T-category with both normal (HR=3.88, 95% CI: 1.78-8.49, \(p=0.00067\)). The significance for these effects was preserved when factoring in the model pre-hormone treatment, PGA and radiation therapy dose in addition to the pre-treatment PSA, T-category and the Gleason-score (Zafarana et al.; In Press, CANCER, 2011). In contrast, \(c\)-MYC and \(PSCA\) were significant on multivariate analysis when controlling for PSA, Gleason-score and T-category independently; but were no longer significant when NKX3.1 was added to the model (Table IIB).

(iv) NKX3.1 allelic loss and relapse following radical prostatectomy

We next interrogated the publically-available Memorial Sloan Kettering Prostate Cancer aCGH database (http://www.cbioportal.org/cgx/index.do?cancer_type_id=pca) consisting of 131
intermediate-risk patients who underwent radical prostatectomy. The clinical characteristics of this surgical cohort are shown in **Supplemental Table I**. In this cohort 49/131 or 37% of the intermediate-risk patients displayed an allelic loss of chromosome site 8p21 (\(\text{NKX3.1}\)) and 16/131 or 12% displayed an allelic gain of chromosome site 8q24 (\(c\text{-MYC}\)). Univariate analysis showed that patients whose tumors displayed \(\text{NKX3.1}\) loss had a trend towards increased failure at 5 years as compared to patients with normal \(\text{NKX3.1}\) status (bRFR 89% versus 72%, \(p=0.07\)) (**Figure 3A**). The HR associated with \(\text{NKX3.1}\) loss alone was 2.07 (95% CI: 0.93-4.63).

Patients whose tumors displayed \(c\text{-MYC}\) gain did not have a statistically significant difference in failure at 5 years as compared to patients with normal \(c\text{-MYC}\) status (bRFR 83% versus 78%, \(p=0.62\)) (**Figure 3B**). The HR associated with \(c\text{-MYC}\) gain alone was 1.36 (95% CI: 0.4-4.57).

Furthermore, patients whose tumors displayed \(\text{NKX3.1}\) loss and/or \(c\text{-MYC}\) gain had a trend towards increased failure at 5 years as compared to patients with normal loci status (bRFR 90% versus 73%, \(p=0.054\)) (**Figure 3C**). The HR associated with \(\text{NKX3.1}\) loss and \(c\text{-MYC}\) gain was 2.17 (95% CI: 0.97-4.86). On multivariate analysis of the surgical cohort, allelic loss of the \(\text{NKX3.1}\) loci and/or gain of the \(c\text{-MYC}\) loci appeared to be a prognosticator after modeling with Gleason-score, although not statistically significant (HR=2.19, 95% CI: 0.98-4.91, \(p=0.057\)) (**Supplemental Table VB**).

(v) **NKX3.1 status and expression may associate with clinical local control and/or radioresistance *in vitro***

Given our clinical findings, we explored a potential relationship between \(\text{NKX3.1}\) loss and radiotherapy response using a small sub-group of 37 patients that had post-IGRT biopsies at the time of PSA failure. Of the 59 patients with loss of NKX3.1, 14 patients had a post-RT biopsy...
and 9 (64%) of these were associated with local recurrence. This suggests that a component of biochemical failure could be associated with tumor cell radioresistance. We therefore determined whether there was any correlation between endogenous NKX3.1 expression and radioresistance \textit{in vitro} in a panel of five prostate cancer cell lines for which clonogenic radiation survival data were available (Figure 4; see Methods for details). We observed an inverse correlation between the relative protein expression of NKX3.1 and clonogenic survival at 3Gy ($r^2 = 0.9079$, $p=0.0122$). When taken together, our limited clinical biopsy data and radiation clonogenic survival data, suggest that decreased expression of NKX3.1 may associate with the intrinsic radioresistance of prostate cancer cells.
DISCUSSION

Using high-resolution aCGH of tumor DNA obtained from 126 prostate cancer biopsies, we demonstrate for the first time that \textit{NKX3.1} haploinsufficiency in pre-treatment biopsies is an independent prognostic factor in intermediate-risk prostate cancer treated by IGRT. Furthermore, combined \textit{NKX3.1} loss and \textit{c-MYC} gain was prognostic for radiotherapy relapse with a $\sim$4-fold decrease in bRFR. Although not significant, a similar trend was observed in a surgical cohort of 131 intermediate-risk patients obtained through the Memorial Sloan Kettering Prostate Cancer aCGH database. Given the latter observation, it is not clear that NKX3.1 status represents a predictive factor, rather than prognostic factor, for radiotherapy relapse. Indeed, the observation of increased hazard ratios in both treatment groups could be explained by NKX3.1 allelic loss heralding the presence of occult metastases at the time of any local treatment and biochemical failure due to systemic disease. However, our limited data using post-IGRT positive biopsies and pre-clinical data on relative clonogenic survival support the concept that NKX3.1 allelic loss and decreased expression associates with prostate cancer cell radioresistance. Future studies should focus on validating our results and discerning whether NKX3.1 is a prognostic or predictive factor of biochemical failure following modern-era IGRT.

In our prostate cancer biopsy cohort, combined \textit{NKX3.1} loss and \textit{c-MYC} gain was significantly associated with an increased genetic instability as measured by PGA. \textit{c-MYC}’s central roles in prostate cancer initiation and progression are well-established (12, 13). Recently, Iwata \textit{et al.} demonstrated using transgenic models that overexpression of \textit{c-MYC} is crucial for PIN development from luminal epithelial cells and responsible for repressed NKX3.1 expression in these PIN lesions (18). The significant frequency of \textit{NKX3.1} loss and \textit{c-MYC} gain observed in 21\% of biopsies in our study supports the interaction between \textit{c-MYC} and \textit{NKX3.1} for prostate
cancer progression and aggression. Most genomic-based predictors in prostate cancer to date have been based on tissues derived post hoc from radical prostatectomies (5, 30). Our study is clinically significant in that we utilized tumor DNA derived from a single biopsy from the putative index lesion. This is not dissimilar to the material and information acquired through diagnostic biopsies that undergo formalin-fixation with paraffin embedding (FFPE) in many clinics today. Our data suggest that biopsy-derived DNA could be useful in stratifying clinically-heterogeneous intermediate-risk cancers specifically planned for IGRT treatment into sub-categories for improved patient management.

The efficacy of radiotherapy is dependent on the eradication of all clonogenic TICs (21, 22). Wang et al. recently demonstrated using a genetic-lineage approach that NKX3.1 is a marker and regulator of a rare, normal prostate luminal TIC population (termed CARNs; Castration-resistant NKX3.1-expressing cells) that could be important in disease progression in the presence of c-MYC or PTEN alterations (14). At present, the quantification of TICs in prostate cancer biopsies is not possible due to a lack of validated immunohistochemistry TIC markers and the expected rarity of these cells (0.01-10%) within tissue sections (21, 31). It is generally accepted that these rare events would be too few to be detected by many standard techniques such as FISH combined with immunohistochemistry. One potential explanation is that genomic rearrangement events in TICs are clonally expanded during differentiation into transit amplifying cells and is then detectable by aCGH at a heightened frequency in biopsies; however this remains to be proven experimentally. We also focused on NKX3.1 as a key TIC mediator in prostate cancer relapse/progression possibly through altered DNA repair (16, 17). Recently, Bowen et al. utilized a NKX3.1-siRNA approach in LNCaP cells to demonstrate that NKX3.1 protein triggers recruitment of phosphorylated-ATM and γH2AX to sites of DNA
damage in response to radiation (16). We speculate that NKX3.1 expression may regulate TICs through differential DDR and play a role in the response of prostate cancer to radiotherapy (21). Until explicit DDR and stem cell studies with models isogenic for \textit{NKX3.1} are utilized, this hypothesis remains unproven.

There are several caveats to our study. First, the cause of relapse post-radiotherapy (local versus systemic) is not known in our cohort as we did not have access to post-radiotherapy biopsies in all treated patients. Given the fact that failures within this genetic group of patients were early (e.g., starting at 6 months post IGRT); our failure data may, in part, be due to sub-occult metastases at the time of treatment. Second, in the assessment of allelic loss of \textit{NKX3.1} allele, we do not know whether some patients are homozygous null for \textit{NKX3.1} (only a minority of tumor cells with bi-allelic loss of \textit{NKX3.1} were observed using FISH slides in 3 patients (see Figure 1A)). The observed heterogeneity could be due to the inherent limitation of the FISH technique, in that we could only assess part of the nuclear volume present within our 4μ sections. We have addressed this issue by counting at least 100 nuclei in each gland to avoid counting artefacts, but we can not be sure that in some cases, there is loss of the second allele of \textit{NKX3.1} and whether this has further impact on clinical outcome. However, in genetically engineered mice, \textit{NKX3.1} haploinsufficiency is sufficient to drive prostate cancer aggression (32, 33). A comprehensive FISH study on a large series of prostate biopsies needs to confirm relative significance of FISH-based allelic changes compared to aCGH-based allelic changes. Finally, the outcome analysis was based on only one biopsy taken using TRUS-guidance to the index lesion prior to radiotherapy and has not addressed the known intra-prostatic heterogeneity and biology of multifocal disease. We cannot rule out that other important genetic changes are present within
the gland pre- and post-radiotherapy; however clinical data suggests that the dominant lesion is
location for recurrence post-radiotherapy in approximately 80% of local failures (34).

In conclusion, our study using genetic data on frozen biopsies in a modern era cohort is
the first report to show that an allelic loss in a TIC-associated gene is prognostic for relapse
following IGRT in prostate cancer. We speculate that the interrogation of TIC-related gene loci
within biopsies could alter treatment if future pre-clinical and clinical studies support this
endpoint as a surrogate for relative TIC aggression and resistance. NKX3.1 status and other
genetic features may be valuable information in triaging intermediate-risk patients to sub-groups
of patients who may benefit from the use of molecular-targeted agents in combination with IGRT
and surgery.
ACKNOWLEDGEMENTS

We would like to thank C. Sanders and N. Schultze for helpful comments on negotiating the MSKCC cBio portal for the validation of our results with surgical outcome data.
REFERENCES


TABLE

Table I: Clinical characteristics of the intermediate-risk prostate cancer patients n=126 in this study

<table>
<thead>
<tr>
<th></th>
<th>N (%)</th>
<th>How was used in the analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T-category</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>45 (36%)</td>
<td>T1 vs. T2</td>
</tr>
<tr>
<td>T2</td>
<td>81 (64%)</td>
<td></td>
</tr>
<tr>
<td><strong>Gleason-score</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>31 (25%)</td>
<td>6 vs. 7</td>
</tr>
<tr>
<td>7</td>
<td>95 (75%)</td>
<td></td>
</tr>
<tr>
<td><strong>Pretreatment-PSA (ng/ml)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>7.8</td>
<td>Continuous</td>
</tr>
<tr>
<td>Range</td>
<td>0.9 – 19</td>
<td></td>
</tr>
<tr>
<td><strong>Hormone therapy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neo-adjuvant</td>
<td>33 (26%)</td>
<td></td>
</tr>
<tr>
<td><strong>RT dose (Gy/fraction)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60/20</td>
<td>12 (10%)</td>
<td>75.6 vs. 60+66</td>
</tr>
<tr>
<td>66/22</td>
<td>3 (2%)</td>
<td>78+79.8 vs. 60+66</td>
</tr>
<tr>
<td>75.6/42</td>
<td>33 (26%)</td>
<td></td>
</tr>
<tr>
<td>78/39</td>
<td>3 (2%)</td>
<td></td>
</tr>
<tr>
<td>79.8/42</td>
<td>75 (60%)</td>
<td></td>
</tr>
<tr>
<td>Mean = 76.4Gy</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table II: Multivariate results of the association of NKX3.1, c-MYC, PSCA and c-MYC/NKX3.1 alterations on T-category, Gleason-score and Pretreatment PSA with the time to recurrence in the 126 intermediate-risk patients

A)  

<table>
<thead>
<tr>
<th></th>
<th>HR</th>
<th>95% Confidence Interval</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-category: T2 versus T1</td>
<td>0.76</td>
<td>0.41 - 1.41</td>
<td>0.39</td>
</tr>
<tr>
<td>Pretreatment PSA (continuous)</td>
<td>1.11</td>
<td>1.04 - 1.2</td>
<td>0.0036</td>
</tr>
<tr>
<td>Gleason-score: 7 versus 6</td>
<td>1.1</td>
<td>0.54 - 2.22</td>
<td>0.8</td>
</tr>
</tbody>
</table>

B)  

<table>
<thead>
<tr>
<th></th>
<th>HR</th>
<th>95% Confidence Interval</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-category: T2 versus T1</td>
<td>0.76</td>
<td>0.40 - 1.43</td>
<td>0.40</td>
</tr>
<tr>
<td>Pretreatment PSA (continuous)</td>
<td>1.12</td>
<td>1.03 - 1.21</td>
<td>0.0049</td>
</tr>
<tr>
<td>Gleason-score: 7 versus 6</td>
<td>0.82</td>
<td>0.41 - 1.67</td>
<td>0.59</td>
</tr>
<tr>
<td>NKX3.1</td>
<td>3.05</td>
<td>1.46 - 6.39</td>
<td>0.0030</td>
</tr>
<tr>
<td>c-MYC</td>
<td>0.79</td>
<td>0.23 - 2.66</td>
<td>0.70</td>
</tr>
<tr>
<td>PSCA</td>
<td>2.06</td>
<td>0.61 - 6.97</td>
<td>0.25</td>
</tr>
</tbody>
</table>

C)
<table>
<thead>
<tr>
<th>Status</th>
<th>HR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-T-category: T2 versus T1</td>
<td>0.72</td>
<td>0.39</td>
<td>1.36</td>
</tr>
<tr>
<td>Pretreatment PSA (continuous)</td>
<td>1.12</td>
<td>1.03</td>
<td>1.21</td>
</tr>
<tr>
<td>Gleason-score 7 versus 6</td>
<td>0.8</td>
<td>0.39</td>
<td>1.62</td>
</tr>
<tr>
<td>Either c-MYC or NKX3.1 versus both normal</td>
<td>3.46</td>
<td>1.67</td>
<td>7.2</td>
</tr>
<tr>
<td>Both c-MYC and NKX3.1 versus both normal</td>
<td>3.88</td>
<td>1.78</td>
<td>8.49</td>
</tr>
</tbody>
</table>

Results are displayed as HR (hazard ratio), 95% confidence interval and p-value. (A) Clinical model, (B) Effect of NKX3.1 alone, c-MYC alone and PSCA alone when adjusting for the clinical factors and each other, (C) Effect of NKX3.1 and c-MYC when adjusting for the clinical factors.
FIGURE LEGENDS

Figure 1: (A) Regions of DNA copy number gains (red) and losses (green) on chromosome 8 from 126 intermediate-risk prostate cancer samples. Representative FISH images are shown for three prostate cancer biopsies. The panel shows a pseudo-color image with DAPI counterstained nuclei. Original magnification is 63x. Multiple-color FISH shows tumor cells with red signal for NKX3.1 (8p21), orange signal for c-MYC (8q22) and green signal for the control probe (cep8). White arrows depict example tumor nuclei with loss of NKX3.1 signal and gain in c-MYC signals. (B-D) Percentage genome alteration (PGA) levels are displayed for patient biopsies categorized as per their (B) NKX3.1 and (C) c-MYC status; alone or in combination. Also shown in (D) is the effect of the genetic changes within groups of patients with GS 6 or 7. The median PGA for each group is represented by the black line. (GS= Gleason-score; * = significantly different, p<0.05; Mann-Whitney analyses).

Figure 2: Univariate Kaplan-Meier plots of biochemical relapse-free rate of survival versus time to recurrence for patients who underwent IGRT with differential NKX3.1 status (A), c-MYC status (B) or combined NKX3.1/c-MYC status in biopsies are displayed.

Figure 3: Univariate Kaplan-Meier plots of biochemical relapse-free rate of survival versus time to recurrence for the Memorial Sloan Kettering Prostate Cancer cohort of patients who underwent surgery with differential NKX3.1 status (A), c-MYC status (B) or NKX3.1/c-MYC status in biopsies are displayed.

Figure 4: In a panel of prostate cancer cell lines, relative protein expressions of NKX3.1 were inversely correlated to surviving fraction at 3Gy radiation (SF=surviving fraction).
Figure 1

A

NKX3.1

C-MYC

8

Research. on April 14, 2017. © 2011 American Association for Cancer Research.
Figure 2A

NKX3.1 Loss vs. NKX3.1 Normal HR=3.03, 95% CI: 1.66–5.52
Log-rank p-value=0.00015

- NKX3.1 Normal, n=67, 5y RFR=87%
- NKX3.1 Loss, n=59, 5y RFR=58%

RELAPSE-FREE RATE

TIME TO BIOCHEMICAL RELAPSE (MONTHS)
cMYC Gain vs. cMYC Normal HR=2.77, 95% CI:1.48−5.18
Log-rank p-value=0.00093

- cMYC Normal, n=97, 5y RFR=80%
- cMYC Gain, n=29, 5y RFR=49%

TIME TO BIOCHEMICAL RELAPSE (MONTHS)

RELAPSE-FREE RATE
Figure 2C

Log-rank p-value=0.00017

NKX3.1 or cMYC altered vs. NKX3.1 & cMYC normal
HR=2.82, 95% CI:1.42-5.61

NKX3.1 & cMYC altered vs. NKX3.1 & cMYC normal
HR=3.98, 95% CI:1.92-8.26

NKX3.1 & cMYC normal, n=65, 5y RFR=89%
NKX3.1 or cMYC altered, n=34, 5y RFR=61%
NKX3.1 & cMYC altered, n=27, 5y RFR=51%

TIME TO BIOCHEMICAL RELAPSE (MONTHS)

RELAPSE-FREE RATE
Figure 3A

NKX3.1 Loss vs. NKX3.1 Normal, HR=2.07, 95% CI: 0.93-4.63
Log-rank p-value=0.07

- NKX3.1 Normal, n=82, 5y RFR=89%
- NKX3.1 Loss, n=49, 5y RFR=72%

TIME TO BIOCHEMICAL RELAPSE (MONTHS)
Figure 3B

MYC Gain vs. MYC Normal, HR=1.36, 95% CI: 0.4-4.57
Log-rank p-value=0.62

- MYC Normal, n=115, 5y RFR=83%
- MYC Gain, n=16, 5y RFR=78%

TIME TO BIOCHEMICAL RELAPSE (MONTHS)
Figure 3C

MYC Gain and/or NKX3.1 Loss vs. Both Normal, HR=2.17, 95% CI: 0.97–4.86
Log-rank p-value=0.054

- Both Normal, n=78, 5y RFR=90%
- MYC Gain and/or NKX3.1 Loss, n=53, 5y RFR=73%

TIME TO BIOCHEMICAL RELAPSE (MONTHS)

REMISSION-FREE RATE
Figure 4

Surviving Fraction at 3 Gy

\[ r^2 = 0.9079 \]
\[ p = 0.0122 \]
Clinical Cancer Research

NKX3.1 Haploinsufficiency is Prognostic for Prostate Cancer Relapse Following Surgery or Image-Guided Radiotherapy

Jennifer A. Locke, Gaetano Zafarana, Adrian S. Ishkanian, et al.

Clin Cancer Res Published OnlineFirst November 2, 2011.

Updated version
Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-11-2147

Supplementary Material
Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2011/11/02/1078-0432.CCR-11-2147.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.