Clinical Cancer Research

NKX3.1 Haploinsufficiency Is Prognostic for Prostate Cancer Relapse following Surgery or Image-Guided Radiotherapy

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Abstract

Background: Despite the use of prostate specific antigen (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 toward 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 cooperation with *c-MYC* during prostate cancer progression.

Methods: Using high-resolution array comparative genomic hybridization (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) by 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 with 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. *Clin Cancer Res; 18(1); 308–16. ©2011 AACR.*

Introduction

Men with localized prostate cancer are placed in low-, intermediate-, and high-risk groups that predict for biochemical failure and disease-free survival with clinical prognostic factors. These include pathologic Gleason-score (GS), pretreatment serum prostate specific antigen (PSA; ng/mL), and tumor—node—metastasis 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% to 44% following radical prostatectomy or image-guided radiotherapy (IGRT; ref. 2). Additional biological or genetic subclassifiers 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

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Translational Relevance

Intermediate-risk prostate cancers show heterogeneity in treatment response. Novel biologic and genetic prognosticators could further subcategorize patients and help individualize therapy. We determined allelic loss or gain in 44 genes thought to be associated with tumor initiating cell (TIC) status using DNA derived from pretreatment biopsies. We found significant genetic alterations in *c-MYC*, *PCSA*, and *NKX3.1*. After adjusting for clinical factors in a multivariate model, *NKX3.1* haploinsufficiency was significantly associated with bRFR in image-guided radiotherapy patients when tested alone, or when combined with *c-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.

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* signaling 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 (DSB) damage (16). Preclinical 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 (TIC) 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; refs. 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 to 2006, pretreatment biopsies were derived from those patients who had chosen radical radiotherapy for primary treatment (REB#00-0443-C) at the Princess Margaret Hospital from the original 247 candidates. Prior to radiotherapy, each patient underwent transultrasound (TRUS)-guided insertion of 3 intraprostatic 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; ref. 4). Staging CT and bone scans were not routinely carried out 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 GS < 8 and PSA < 20 ng/mL; ref. 3) and also had information pertaining to long-term biochemical outcome. The final study therefore included 126 patients (see Table 1).

Radiotherapy planning and delivery

The clinical target volume (CTV) encompassed the prostate gland alone. The planning target volume was defined by a 10 mm margin around the CTV except posteriorly in which 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 1, 33 patients (26%) received a dose of 75.6 Gy in 1.8 Gy daily fractions and 78 (62%) a dose of 78 to 79.8 Gy in 1.8 to 2 Gy daily fractions. Fifteen patients (12%) participated in a study of hypofractionated radiotherapy and received 60 to 66 Gy in 3 Gy daily fractions. Neoadjuvant and concurrent hormonal therapy was used in 33 patients (26%) as bicalutamide 150 mg po OD for 3 months

Table 1. Clinical characteristics of the intermediate-risk prostate cancer patients, $n = 126$ in this study

	N (%)	How was used in the analysis
T-category		T1 vs. T2
T1	45 (36%)	
T2	81 (64%)	
GS		6 vs. 7
6	31 (25%)	
7	95 (75%)	
Pretreatment-PSA (ng/mL)		Continuous
Median	7.8	
Range	0.9–19	
Hormone therapy		
Neoadjuvant	33 (26%)	
RT dose (Gy/fraction)		75.6 vs. 60 + 66
60/20	12 (10%)	78 + 79.8 vs. 60 + 66
66/22	3 (2%)	
75.6/42	33 (26%)	
78/39	3 (2%)	
79.8/42	75 (60%)	
Mean = 76.4Gy		

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 at −80°C and cut into 10 μ sections for manual microdissection and preparation of DNA samples as previously described (4). DNA labeling and hybridization was carried out 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 to 18 hours. DNA samples were then combined and mixed with 100 μg of Human Cot-1 DNA (Invitrogen) followed by removal of unincorporated nucleotides using micro con YM-30 columns (Millipore). The mixture was then applied onto arrays containing 26,819 bacterial artificial chromosome–derived amplified fragment pools spotted in duplicate on aldehyde coated glass slides (SMRT v.2; BC Cancer Research Centre Array Facility). Slides were scanned with a dual laser array scanner (Axon) and spot signal intensities determined by the SoftWoRx Tracker Spot Analysis software (Applied Precision). The $\log_2$ ratios of the Cyanine 3 to Cyanine 5 intensities for each spot were assessed. Data were filtered based on both SDs of replicate spots (data points > 0.075 SD were removed) and signal to noise ratio (data points with a signal to noise ratio < 3 were removed). Resulting data set 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/postprocessed $\log_2$ ratios of intensities for clone regions were visualized with SeeGH software (25, 26). The genomic positions of clones are mapped to the National Center for Biotechnology Information'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 used 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 whereas gain of *c-MYC* was defined as more than 2 copies of *c-MYC* in the majority of tumor nuclei (see detailed methods in Supplementary Table 3).

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 3 Gy 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 American Type Culture Collection and authenticated by short tandem repeat DNA profiling (March 2011).

Statistical methods

The primary outcome was time to biochemical failure as defined by Roach and colleagues to be a PSA rise of at least 2 ng/mL above postradiation 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), pretreatment PSA (continuous), and GS (6 versus 7)] with the Cox proportional hazards model. The proportional hazards assumptions of each model were checked by looking at scaled Schoenfeld residuals. The Fisher exact test was used to investigate statistical associations between *NKX3.1*, *c-MYC*, and *PSCA*. A 2-sided $P = 0.05$ was used to assess statistical significance.

Results

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 Supplementary Table 2). 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 whereas *NKX3.1* is located on chromosome site 8p21 (Fig. 1A). Upon evaluation of 126 biopsies by aCGH, 59 of 126 or 47% of the intermediate-risk patients displayed an allelic loss in chromosome site 8p21 (*NKX3.1*) whereas 29 of 126 or 23% and 23 of 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 of 126 or 21% of the biopsies showing concomitant *c-MYC* gain and *NKX3.1* loss and only 2 of 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 3 patients exhibiting *NKX3.1* loss and *c-MYC* gain based on aCGH analysis and confirmed associated copy number changes within multiple nuclei (Fig. 1A, Supplementary Table S3).

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 PGA. Tumor specimens with an allelic loss of *NKX3.1* were associated with significantly higher PGA (1.2 vs. 6.9, $P < 0.0001$) than tumor specimens without *NKX3.1* loss (Fig. 1B). Tumor specimens with *NKX3.1* altered/*c-MYC* altered had significantly higher PGA (10.3 for both altered vs. 1.1 for both normal, $P < 0.0001$)

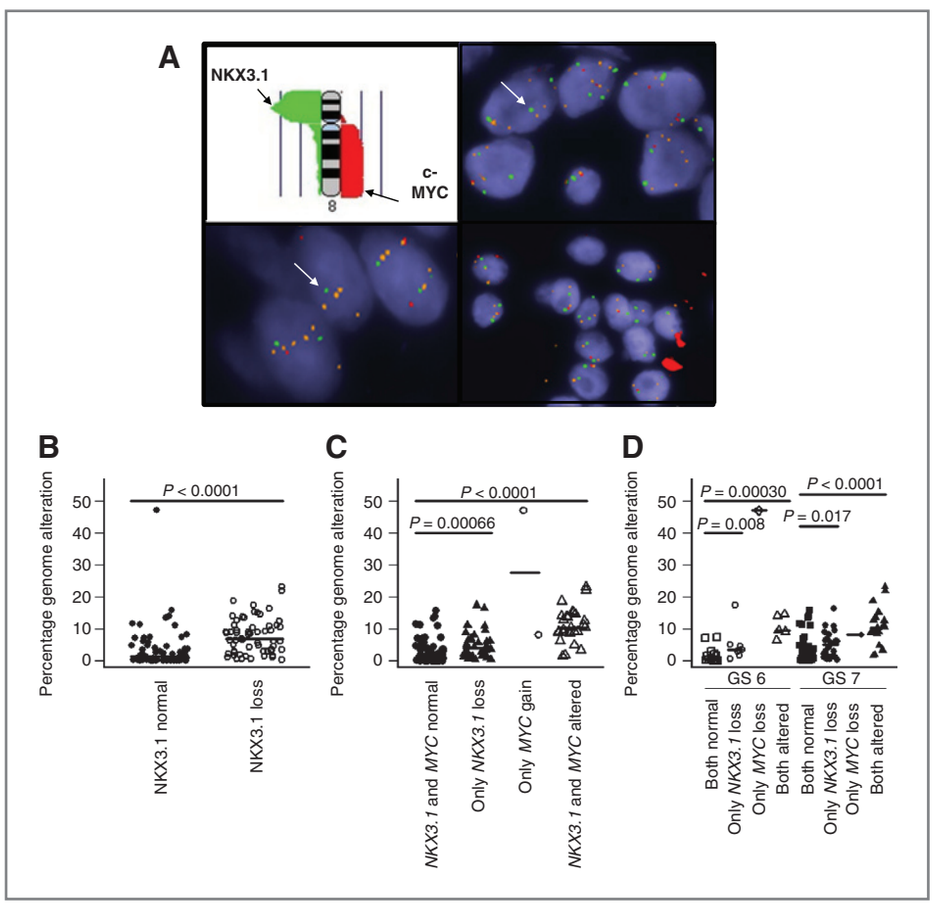

Figure 1. A, regions of DNA copy number gains (red) and losses (green) on chromosome 8 from 126 intermediate-risk prostate cancer samples. Representative FISH images are shown for 3 prostate cancer biopsies. The panel shows a pseudocolor image with 4′, 6—diamidino—2—phenylindole counterstained nuclei. Original magnification is 63×. 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, 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 (*, significantly different, $P < 0.05$; Mann–Whitney analyses).

than specimens without such alterations; tumors with *NKX3.1* altered/*c-MYC* normal also had significantly higher PGA than *NKX3.1* normal/*c-MYC* normal (3.9 versus 1.1, $P = 0.00066$; Fig. 1C). When subgrouping patients by GS 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 than those with normal *NKX3.1* and *c-MYC* status ($P < 0.01$; Fig. 1D). Significant relationships between genetic instability in specimens with both allelic changes were independent of pretreatment PSA ($\leq$10 and >10 ng/mL) and T-category (T1 and T2; Supplementary Fig. S2). Alteration in *PSCA* was also associated with a significantly higher PGA than normal *PSCA* (data not shown).

Patients with *NKX3.1* allelic loss are more likely to relapse following IGRT

We next examined the impact of allelic loss of *NKX3.1* in our clinical cohort of 126 patients with intermediate-risk prostate cancer on 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 1. Forty-seven patients in this cohort experienced a biochemical relapse; 42 as defined by the Phoenix definition and an additional 5 who were preemptively treated with salvage hormones because of 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 with patients with normal *NKX3.1* status (bRFR 87% vs. 58%, $P = 0.00015$; Fig. 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 with patients with normal *c-MYC* (bRFR 80% vs. 49%, $P = 0.00093$; Fig. 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 with patients with normal *c-MYC* and *NKX3.1* status (bRFR 89% vs. 51%, $P = 0.00017$; Fig. 2C). The HR associated with *c-MYC* gain and *NKX3.1* loss was 3.98 (95% CI: 1.92–8.26) as compared with normal *c-MYC* and *NKX3.1*. Last, patients whose tumors displayed a *PSCA* gain also had increased biochemical failure at 5 years compared with patients with normal *PSCA* (bRFR 79% vs. 46%, $P = 0.00058$; Supplementary Fig. S1). 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, GS, T-category, *c-MYC* gain, and *PSCA* gain (HR = 3.05, 95% CI: 1.46–6.39, $P = 0.0030$; Table 2). This effect was also observed when *NKX3.1* loss and *c-MYC* gain were combined and tested against PSA, GS, 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 prehormone treatment, PGA, and radiation therapy dose in addition to the pretreatment PSA, T-category, and the GS (Zafarana and colleagues; In Press, CANCER, 2011). In contrast, *c-MYC* and *PSCA* were significant on multivariate analysis when controlling for PSA, GS, and T-category independently but were no longer significant when *NKX3.1* was added to the model (Table 2B).

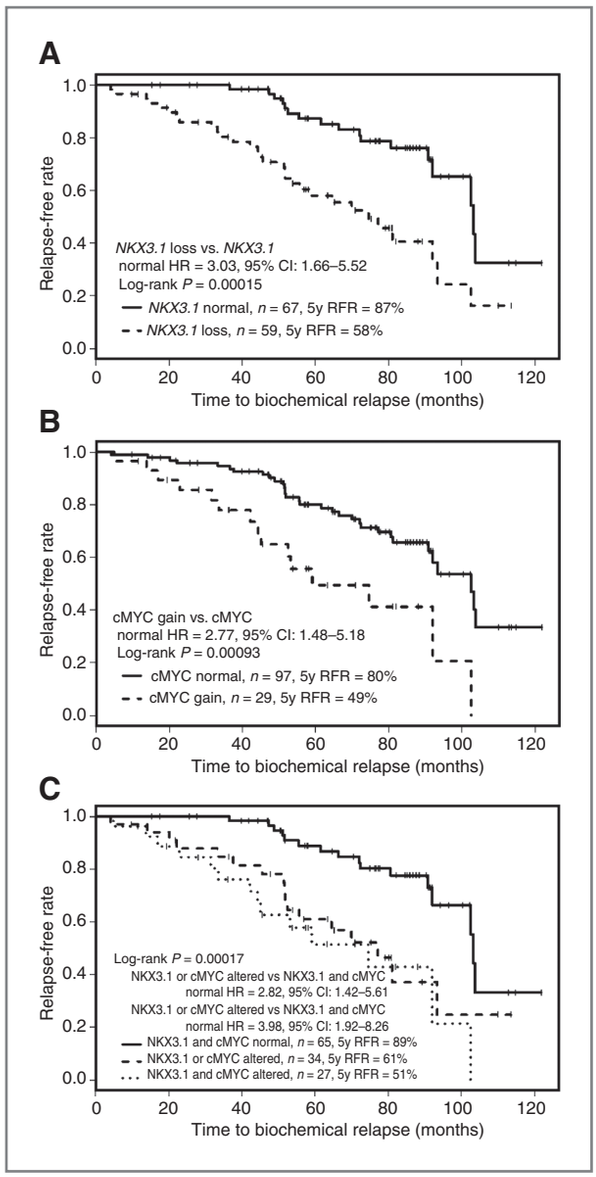

Figure 2. Univariate Kaplan–Meier plots of bRFR of survival versus time to recurrence for patients who underwent IGRT with differential *NKX3.1* status (A), *c-MYC* status (B), or combined *NKX3.1*/*c-MYC* (C) status in biopsies are displayed.

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/public-portal/) consisting of 131 intermediate-risk patients who underwent radical prostatectomy. The clinical characteristics of this surgical cohort

Table 2. Multivariate results of the association of *NKX3.1*, *c-MYC*, *PSCA*, and *c-MYC*/*NKX3.1* alterations on T-category, GS, and pretreatment PSA with the time to recurrence in the 126 intermediate-risk patients

	HR (95% CI)	*P*
(A) Clinical model		
T-category: T2 vs. T1	0.76 (0.41–1.41)	0.39
Pretreatment PSA (continuous)	1.11 (1.04–1.2)	0.0036
GS: 7 vs. 6	1.1 (0.54–2.22)	0.8
(B) Effect of *NKX3.1* alone, *c-MYC* alone, and *PSCA* alone when adjusting for the clinical factors and each other		
T-category: T2 vs. T1	0.76 (0.40–1.43)	0.40
Pretreatment PSA (continuous)	1.12 (1.03–1.21)	0.0049
GS: 7 vs. 6	0.82 (0.41–1.67)	0.59
NKX3.1	3.05 (1.46–6.39)	0.0030
c-MYC	0.79 (0.23–2.66)	0.70
PSCA	2.06 (0.61–6.97)	0.25
(C) Effect of *NKX3.1* and *c-MYC* when adjusting for the clinical factors		
Pre–T-category: T2 vs. T1	0.72 (0.39–1.36)	0.32
Pretreatment PSA (continuous)	1.12 (1.03–1.21)	0.005
GS 7 vs. 6	0.8 (0.39–1.62)	0.54
Either *c-MYC* or *NKX3.1* vs. both normal	3.46 (1.67–7.2)	0.00087
Both *c-MYC* and *NKX3.1* vs. both normal	3.88 (1.78–8.49)	0.00067

NOTE: Results are displayed as HR, 95% CI, and *P* value.

are shown in Supplementary Table 1. In this cohort, 49 of 131 or 37% of the intermediate-risk patients displayed an allelic loss of chromosome site 8p21 (*NKX3.1*) and 16 of 131 or 12% displayed an allelic gain of chromosome site 8q24 (*c-MYC*). Univariate analysis showed that patients whose tumors displayed *NKX3.1* loss had a trend toward increased failure at 5 years as compared with patients with normal *NKX3.1* status (bRFR 89% vs. 72%, $P = 0.07$; Fig. 3A). The HR associated with *NKX3.1* loss alone was 2.07 (95% CI: 0.93–4.63). Patients whose tumors displayed *c-MYC* gain did not have a statistically significant difference in failure at 5 years as compared with patients with normal *c-MYC* status (bRFR 83% vs. 78%, $P = 0.62$; Fig. 3B). The HR associated with *c-MYC* gain alone was 1.36 (95% CI: 0.4–4.57). Furthermore, patients whose tumors displayed *NKX3.1* loss and/or *c-MYC* gain had a trend toward increased failure at 5 years as compared with patients with normal loci status (bRFR 90% vs. 73%, $P = 0.054$; Fig. 3C). The HR associated with *NKX3.1* loss and *c-MYC* gain was 2.17 (95% CI: 0.97–4.86). On multivariate analysis of the surgical cohort, allelic loss of the *NKX3.1* loci and/or gain of the *c-MYC* loci seemed to be a prognosticator after modeling with GS, although not statistically significant (HR = 2.19, 95% CI: 0.98–4.91, $P = 0.057$; Supplementary Table 5B).

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 *NKX3.1* loss and radiotherapy response using a small subgroup 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 *in vitro* in a panel of 5 prostate cancer cell lines for which clonogenic radiation survival data were available (Fig. 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 show for the first time that *NKX3.1* haploinsufficiency in pretreatment biopsies is an independent prognostic factor in intermediate-risk prostate cancer treated by IGRT. Furthermore, combined *NKX3.1* loss and *c-MYC* gain was prognostic for radiotherapy relapse with an approximately 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

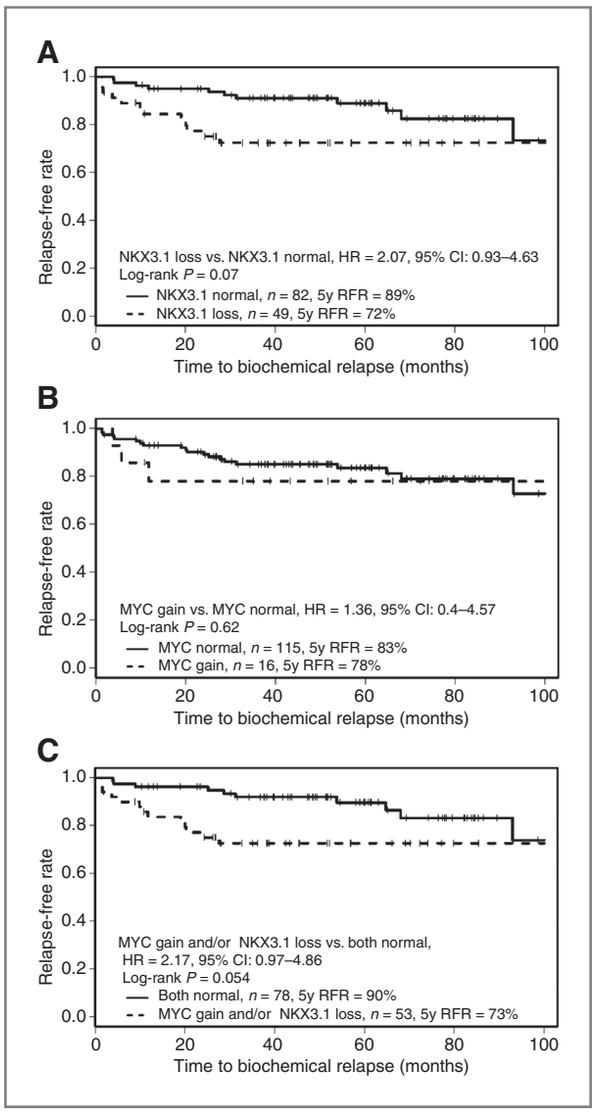

Figure 3. Univariate Kaplan–Meier plots of bRFR of survival versus time to recurrence for the Memorial Sloan Kettering Prostate Cancer cohort of patients who underwent surgery with differential *NKX3.1* status (A), *c-MYC* status (B), or *NKX3.1*/*c-MYC* (C) status in biopsies are displayed.

observation of increased HRs 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 preclinical 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 *NKX3.1* loss and *c-MYC* gain was significantly associated with an increased genetic instability as measured by PGA. c-MYC's

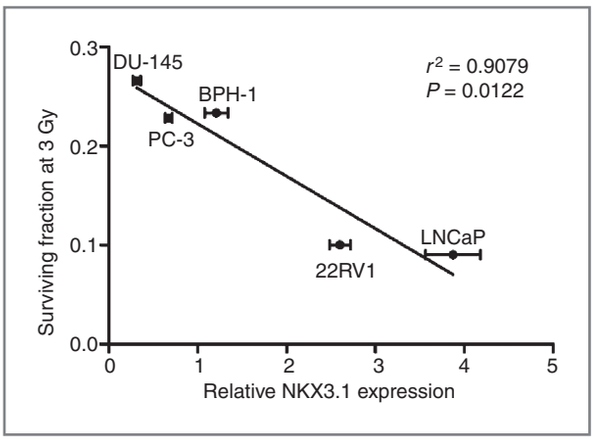

Figure 4. In a panel of prostate cancer cell lines, relative protein expressions of NKX3.1 were inversely correlated to surviving fraction at 3 Gy radiation. SF, surviving fraction.

central roles in prostate cancer initiation and progression are well established (12, 13). Recently, Iwata and colleagues showed using transgenic models that overexpression of c-MYC is crucial for prostatic intraepithelial neoplasia development from luminal epithelial cells and responsible for repressed NKX3.1 expression in these prostatic intraepithelial neoplasia lesions (18). The significant frequency of *NKX3.1* loss and *c-MYC* gain observed in 21% of biopsies in our study supports the interaction between *c-MYC* and *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 used 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 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 subcategories for improved patient management.

The efficacy of radiotherapy is dependent on the eradication of all clonogenic TICs (21, 22). Wang and colleagues recently showed using a genetic-lineage approach that NKX3.1 is a marker and regulator of a rare, normal prostate luminal TIC population (castration-resistant NKX3.1-expressing cells; CARN) 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 and colleagues used a *NKX3.1*–siRNA approach in LNCaP cells to show 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 *NKX3.1* are used, this hypothesis remains unproven.

There are several caveats to our study. First, the cause of relapse postradiotherapy (local versus systemic) is not known in our cohort as we did not have access to postradiotherapy 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 suboccult metastases at the time of treatment. Second, in the assessment of allelic loss of *NKX3.1* allele, we do not know whether some patients are homozygous null for *NKX3.1* [only a minority of tumor cells with biallelic loss of *NKX3.1* were observed using FISH slides in 3 patients (see Fig. 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 4μm sections. We have addressed this issue by counting at least 100 nuclei in each gland to avoid counting artifacts, but we can not be sure that in some cases, there is loss of the second allele of *NKX3.1* and whether this has further impact on clinical outcome. However, in genetically engineered mice, *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 with 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 intraprostatic heterogeneity and biology of multifocal disease. We cannot rule out that other important genetic changes are present within the gland pre- and postradiotherapy; however, clinical data suggests that the dominant lesion is location for recurrence postradiotherapy 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 preclinical 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 subgroups of patients who may benefit from the use of molecular-targeted agents in combination with IGRT and surgery.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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