Nuclear Factor-κB Nuclear Localization Is Predictive of Biochemical Recurrence in Patients with Positive Margin Prostate Cancer

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ABSTRACT

Purpose: **Radical prostatectomy (RP) patients with positive surgical margins are at increased risk for recurrence, emphasizing the need for prognostic markers to stratify probable outcome for optimal patient management decisions. We tested the hypothesis that nuclear localization of nuclear factor (NF)-κB, a transcription factor involved in the regulation of cell growth, angiogenesis, invasion, and apoptosis, is associated with an increased risk of biochemical recurrence after RP.**

Experimental Design: **Analyses addressed data from 42 patients (age range, 52–72 years; mean age, 63.7 years) who exhibited positive surgical margins after RP. Immunohistochemical analysis of NF-κB (p65) was performed on the positive margin tissue. A nuclear staining cutoff of >5% was considered positive. The relation between nuclear NF-κB expression and biochemical recurrence (prostate-specific antigen >0.3 ng/mL and rising) after RP was tested in univariate and multivariate Cox regression models.**

Results: **Biochemical recurrence was recorded in 23 patients (54.8%; median follow-up, 3.2 years). Univariate Cox regression demonstrated a 4.9-fold (95% confidence interval, 1.5–16.7; $P = 0.01$) higher rate of recurrence in men with NF-κB > 5%. In the multivariate model, after controlling for primary ($P = 0.004$) and secondary ($P = 0.7$) Gleason patterns, lymph node ($P = 0.06$) and seminal vesicle invasion ($P = 0.2$), and preoperative prostate-specific antigen ($P = 0.009$), NF-κB > 5% was associated with a 6.2-fold higher risk of biochemical recurrence (95% confidence interval, 1.7–23.5; $P = 0.007$).**

Conclusions: **In univariate and multivariate analysis, NF-κB nuclear expression was strongly predictive of biochemical recurrence in patients with positive surgical margins after RP. We propose that nuclear NF-κB may serve as a useful independent molecular marker for stratifying patients at risk for recurrence.**

INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer, and the second leading cause of cancer-related deaths in North American men. Radical prostatectomy (RP) represents one of two standard treatment options for men with clinically localized prostate cancer. It is still controversial whether immediate adjuvant treatment should be instituted in the presence of pathologically confirmed positive surgical margins after RP. Several candidate molecular markers, including RNASEL, GSTP1, p21, p27, p53, NKX3.1, PTEN, and TERT, have been or are currently being studied in an effort to enhance the accuracy of pathological stage and grade and pretreatment serum prostate-specific antigen (PSA), in predicting the risk of biochemical failure after RP (1–3). To date, none have been adopted into routine clinical practice.

Classical nuclear factor (NF)-κB transcription factor is a heterodimer formed by the p50 and RelA (p65) proteins (4–7). It is expressed in most cells but kept inactive and maintained in the cytoplasm by interaction with IκB inhibitors. Normally, the activation of NF-κB requires signals that lead to the phosphorylation (by the activated IKK complex), ubiquitination, and proteasome-dependent degradation of IκB. NF-κB then translocates to the nucleus and activates the expression of various genes involved in cell growth, differentiation, inflammatory responses, and the regulation of apoptosis. There is growing evidence that NF-κB is implicated in oncogenesis (8, 9). NF-κB is constitutively activated in many lymphoid and nonlymphoid cancers (10, 11). NF-κB can prevent cell death by apoptosis in many cell types (including the human prostate cancer PC3 and DU145 cell lines) after chemotherapy, radiotherapy, or tumor necrosis factor α treatment (12, 13). More recently, constitutive activation of NF-κB has been detected in androgen-independent prostate cancer cell lines as well as in prostate cancer tissues (14–17). In particular, strong nuclear NF-κB staining was observed in prostate cancer lymph node metastases (18). Moreover, NF-κB expression in RP specimen was shown to correlate with progression to bone metastasis (19). NF-κB aberrant acti-

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vation has been shown to promote cell growth, survival, and metastasis in cell lines and xenograft models (20–25).

Whereas the role of NF-κB in prostate carcinogenesis and cancer progression is becoming more apparent, the clinical usefulness of NF-κB as a predictor of disease progression has not yet been fully investigated. We tested the hypothesis that NF-κB could be a prognostic marker for eventual biochemical recurrence in men with positive pathological surgical margins after RP.

PATIENTS AND METHODS

After providing informed consent, 90 consecutive patients with positive margins were initially identified in a patient database, but 48 were excluded for the following reasons: absence of tissue blocks with true positive margins after central pathological review; detectable postoperative PSA; and neoadjuvant hormone therapy or adjuvant therapy before PSA recurrence. Tissue slides of the remaining 42 formalin-fixed paraffin-embedded specimens were immunostained for NF-κB transcription factor by the biotin-streptavidin-peroxidase method using the NF-κB p65 (F-6) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) that recognizes NH_2-terminal sequences of the p65 subunit.

Tissue sections were deparaffinized with toluene, rehydrated through graded EtOH, treated with 3% H_2O_2 to eliminate endogenous peroxidase activity, and incubated for 10 minutes at 95°C in citrate buffer (pH 6.0) for antigen unmasking. Nonspecific antigen was blocked with a protein blocking serum-free reagent (Dako Diagnostics Canada, Inc., Mississauga, Canada) Slides were then incubated for 60 minutes in a humidified chamber with the specific p65 antibody. The optimal concentration (1 μg/mL) was determined by serial dilutions. Twenty-minute incubations with the secondary biotinylated antibody and streptavidin-horseradish peroxidase (Dako Diagnostics Canada Inc.) were performed sequentially. Reaction products were developed using diaminobenzidine containing 0.3% H_2O_2 as a substrate for peroxidase. Hematoxylin counterstaining was done for ease of reading. Substitution of the primary antibody with PBS served as a negative control.

Slides were examined under standard light microscopy, and subcellular staining was identified. NF-κB nuclear localization was evaluated as the overall proportion of tumor cells with positive nuclear staining. An average of 20 fields per tumor (100 cells per field) were analyzed at ×400. Positive nuclear staining

Table 1 Clinical and pathological characteristics of 42 evaluable patients who were treated with RP

	NF-κB > 5%	NF-κB ≤ 5%	*P*
No. of patients	27	15	
Age (y)			0.6
Mean (SE)	64.1 (0.99)	63.1 (1.6)	
PSA (ng/mL)			0.2
Mean (SE)	13.9	10.96 (1.56)	
Gleason sum (%)			
6	4 (14.8)	4 (26.7)	0.1
7	17 (63.0)	10 (66.7)	
8	0	1 (6.7)	
9	6 (22.2)	0	
Seminal vesicle invasion	3	2	0.8
Lymph node metastases	4	0	0.1

NOTE. Data are tabulated according to NF-κB nuclear staining status.

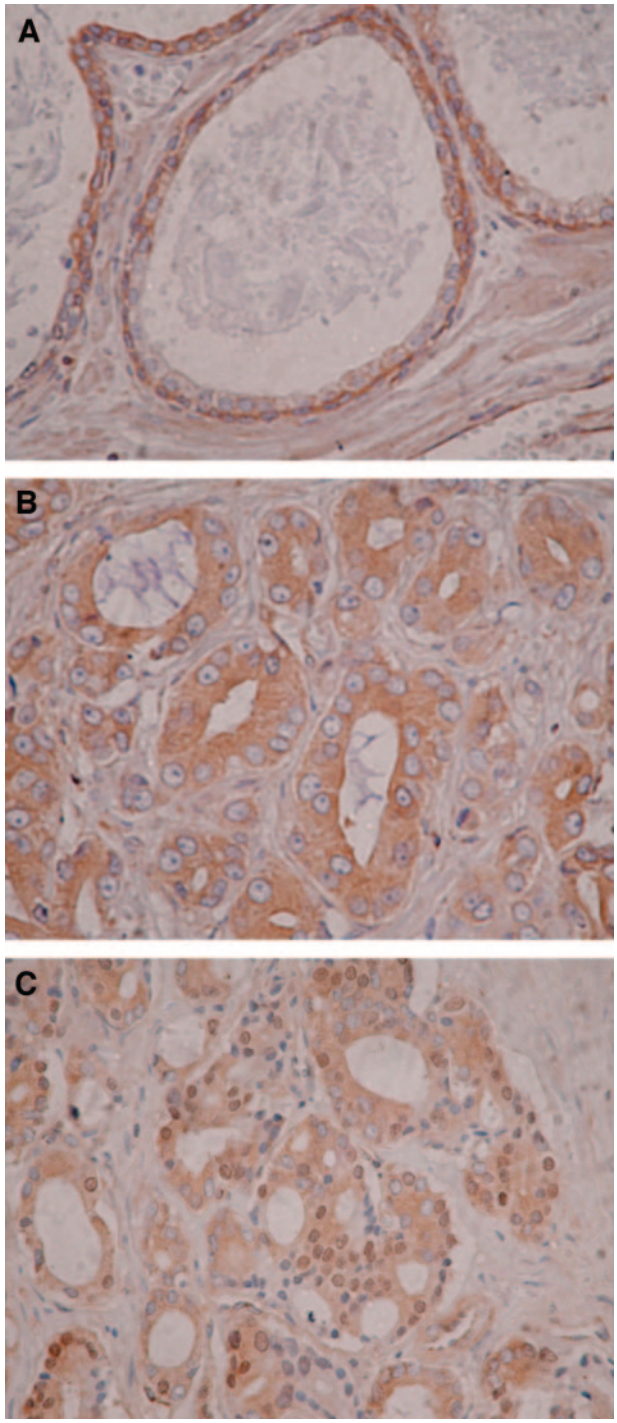

Fig. 1 Immunohistochemical detection of NF-κB (p65 subunit) in prostate tissues. Note the strong cytoplasmic staining in normal prostate basal cells (*A*), whereas tumor tissues show either predominant cytoplasmic staining (*B*) or cytoplasmic and nuclear staining (*C*).

Table 2 Univariate and multivariate Cox survival analyses addressing the association between NF-κB and biochemical prostate cancer recurrence (PSA > 0.3 ng/mL and rising) after RP

Predictors variable	Biochemical recurrence after RP	
	Univariate (rate ratio and 95% CI)	Multivariate (rate ratio and 95% CI)
NF- κB	4.9 (1.47–16.70); $P = 0.01$	6.2 (1.64–23.53); $P = 0.007$
Age (y)	0.97 (0.90–1.05); $P = 0.4$	0.95 (0.88–1.03); $P = 0.2$
pPSA (ng/mL)	0.96 (0.91–1.02); $P = 0.2$	0.91 (0.85–0.98); $P = 0.01$
Gleason sum	$P = 0.009$	$P = 0.03$
Seminal vesicle invasion	2.5 (0.83–7.46); $P = 0.1$	2.2 (0.65–7.40); $P = 0.2$
Lymph node metastases	2.2 (0.64–7.68); $P = 0.2$	4.8 (0.99–23.62); $P = 0.052$

NOTE. Covariates include age, pPSA, Gleason sum, seminal vesicle invasion, and lymph node metastases. Primary and secondary Gleason patterns were used to define the Gleason sum.

was defined as a sharp brown coloration in the nucleus of a cell. All slides were independently analyzed by two blinded observers. Interpretations diverging <10% were independently reanalyzed, resulting in concordance scores. We used >5% positive tumor cell nuclear staining as a cutoff indicating significant NF-κB activation in cancer tissue. Univariate biochemical-free survival analyses revealed that 5% represented the most statistically significant cutoff in this data set.

Pretreatment serum prostate-specific antigen (pPSA), pathological Gleason score, presence of extracapsular extension, presence of seminal vesicle invasion, presence of pelvic lymph node metastasis, and surgical margin status were recorded for each patient. Positive surgical margins were defined by the presence of tumor cells at the inked margin of resection.

Postoperative follow-up consisted of physical examination and serial serum PSA measurements. The Hybritech PSA assay (Hybritech Tandem R, San Diego, CA) was used principally, but not exclusively, for both pretreatment evaluation and disease follow-up. PSA failures were defined as serum levels > 0.3 ng/mL and rising, and failure time was dated back to first PSA > 0.3 ng/mL. PSA assays were generally obtained at 1 month after surgery and then every 3 months for the first year, every 6 months for two subsequent years, and then annually. For statistical purposes, patients who did not relapse were censored at the date of their last undetectable PSA assay.

The probability of remaining free from recurrence [mean with 95% confidence intervals (CIs)] was calculated using the Kaplan-Meier method. Univariate and multivariate Cox proportional hazards regression models were used to test the association of clinical and pathological variables with biochemical failure. All statistical tests were performed using SPSS version 10 (SPSS, Inc., Chicago, IL) and S-PLUS Professional version 1 (MathSoft Inc., Seattle, WA). Two-sided tests with a significance level at 0.05 were used.

RESULTS

Table 1 shows clinical and pathological characteristics of our patient cohort. Age at surgery was 52 to 72 years (mean age, 63.7 years). Preoperative PSA ranged from 3 to 36 ng/mL (mean, 12.9 ng/mL). In all, 64% of patients had a Gleason sum of 7. Follow-up ranged from 0.3 to 12.2 years (mean follow-up, 3.6 years; median follow-up, 3.2 years). Biochemical recurrence occurred in 23 patients (54.8%). RP tissues were evaluated for NF-κB staining and classified as having either >5% or <5% nuclear staining in tumor cells. Normal prostate glands demonstrated cytoplasmic staining mainly in the basal cell layer (Fig. 1*A*). Cancer glands almost always demonstrated cytoplasmic staining of the NF-κB marker, mostly of high intensity (Fig. 1*B*). Nuclear staining was only observed in cancerous glands, although it was quite heterogeneous within the tumor (Fig. 1*C*). We found no correlation between the intensity of the staining and prognosis.

Table 2 shows the univariate and multivariate Cox survival analyses addressing the association between NF-κB and biochemical prostate cancer recurrence (PSA > 0.3 ng/mL and rising) after RP. Covariates include age, pPSA, pathological Gleason score, and seminal vesicle and lymph node metastases. Kaplan-Meier survival curves were constructed to show the biochemical recurrence-free survival difference between patients with positive and negative nuclear NF-KB staining (Fig. 2), and these results indicate a clear difference between the two groups in terms of recurrence.

Univariate Cox regression demonstrated a 4.9-fold (95% CI, 1.5–16.7; $P = 0.01$) higher rate of recurrence in men with >5% NF-κB nuclear staining. In the multivariate model, after controlling for pathological Gleason sum ($P = 0.03$), lymph node metastases ($P = 0.07$), seminal vesicle invasion ($P = 0.6$),

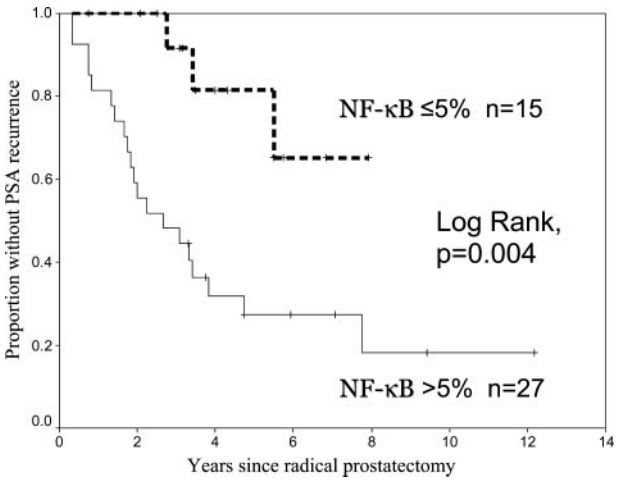

Fig. 2 Kaplan-Meier curve of biochemical recurrence-free survival according to NF-κB status.

and pPSA ($P = 0.01$), NF-κB nuclear staining of >5% was associated with a 6.3-fold higher risk of biochemical recurrence (95% CI, 1.7–23.5; $P = 0.007$; Table 2). These results indicate that NF-κB status appears to represent the most statistically significant predictor of biochemical recurrence in the presence of positive surgical margins after RP.

DISCUSSION

PSA recurrence after RP is a precursor of cancer progression, with a mean time to metastases estimated at 8 years from the time of PSA elevation. Time to biochemical progression, PSA doubling time, and Gleason score have been identified as predictors of progression to metastatic disease. Once metastatic disease has occurred, the median time to death is 5 years (26). Therefore, PSA recurrence represents a valuable surrogate marker of cancer progression.

Patients with positive margins have a 2-fold higher recurrence rate when compared with patients with negative margins. The progression-free probability at 5 years for patients who have negative margins at RP is approximately 80% compared with 42% to 64% for men with positive margins (27, 28). Adjuvant radiation therapy is more effective if administered early before clinical failure. However, radiation may not be required in an appreciable proportion of patients who will not fail, despite having positive surgical margins. The identification of prognostic factors predictive of recurrence may help in developing a risk-adjusted approach to timely adjuvant therapy after RP.

Several pathological features have been shown to predict biochemical recurrence including preoperative PSA level, pathological T stage, Gleason score (biopsy and prostatectomy), margin status, lymph node and seminal vesicle involvement, perineural invasion, and tumor volume (27, 29). Although histologic grading is one of the most important predictors, its usefulness is limited because the majority of cases are moderately differentiated tumors (Gleason score of 6 or 7). The multivariate input of multiple predictors has been used in nomogram format to provide the most accurate and bias-free estimates of recurrence (30, 31). However, restrained input from predictor variables limits the accuracy of these tools. Therefore, there is a clear need for novel molecular markers that can complement standard predictors and increase the accuracy of existing predictive tools.

Several nuclear markers have been evaluated for their prognostic value in prostate cancer. Overexpression of the cell cycle inhibitor protein p21 has been shown as an independent predictor of response to salvage radiotherapy after RP (30). Progression to androgen independence has also been correlated with p21 (31). Expression of p53 and the combined loss of PTEN and p27 have been shown as independent predictors of disease-free survival in multivariate analyses (32, 33). Other promising candidate biomarkers currently under investigation and associated with disease progression include RNASEL, GSTP1, NKX3.1, and TERT (2, 3).

The results of the study we report here demonstrate that positive NF-κB nuclear staining of cancer cells at the margin is associated with a 6-fold increase in the risk of biochemical recurrence in patients with positive RP surgical margins. In a multivariate analysis, we confirmed the value of NF-κB as an independent prognostic variable in this patient population. To date, none of the molecular prognostic markers have been applied to routine clinical practice, although with further confirmatory studies, we believe that the expression nuclear NF-κB, either alone or in combination with other molecular markers, may eventually be useful in improving the value of parameters commonly used to help predict outcome in patients with prostate cancer.

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