

**ORIGINAL ARTICLE**

Determining the prognostic significance of IKK α in prostate cancer

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Abstract

Background: As the survival of castration-resistant prostate cancer (CRPC) remains poor, and the nuclear factor- κ B (NF- κ B) pathways play key roles in prostate cancer (PC) progression, several studies have focused on inhibiting the NF- κ B pathway through generating inhibitory κ B kinase subunit α (IKK α) small molecule inhibitors. However, the identification of prognostic markers able to discriminate which patients could benefit from IKK α inhibitors is urgently required. The present study investigated the prognostic value of IKK α , IKK α phosphorylated at serine 180 (p-IKK α S180) and threonine 23 (p-IKK α T23), and their relationship with the androgen receptor (AR) and Ki67 proliferation index to predict patient outcome.

Methods: A cohort of 115 patients with hormone-naïve PC (HNPC) and CRPC specimens available were used to assess tumor cell expression of proteins within both the cytoplasm and the nucleus by immunohistochemistry. The expression levels were dichotomized (low vs high) to determine the associations between IKK α , AR, Ki67, and patients' survival. In addition, an analysis was performed to assess potential IKK α associations with clinicopathological and inflammatory features, and potential IKK α correlations with other cancer pathways essential for CRPC growth.

Results: High levels of cytoplasmic IKK α were associated with a higher cancer-specific survival in HNPC patients with low AR expression (hazards ratio [HR], 0.33; 95% confidence interval [CI] log-rank, 0.11-0.98; $P = .04$). Furthermore, nuclear IKK α (HR, 2.60; 95% CI, 1.27-5.33; $P = .01$) and cytoplasmic p-IKK α S180 (HR, 2.10; 95% CI, 1.17-3.76; $P = .01$) were associated with a lower time to death from recurrence in patients with CRPC. In addition, high IKK α expression was associated with high levels of T-cells (CD3+ $P = .01$ and CD8+ $P = .03$) in HNPC; however, under castration conditions, high IKK α expression was associated with high levels of CD68+ macrophages ($P = .04$), higher Gleason score ($P = .01$) and more prostate-specific antigen concentration ($P = .03$). Finally, we identified crosstalk between IKK α and

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members of the canonical NF- κ B pathway in the nucleus of HNPC. Otherwise, IKK α phosphorylated by noncanonical NF- κ B and Akt pathways correlated with members of the canonical NF- κ B pathway in CRPC.

Conclusion: The present study reports that patients with CRPC expressing high levels of nuclear IKK α or cytoplasmic p-IKK α S180, which associated with a lower time to death from recurrence, may benefit from IKK α inhibitors.

KEYWORDS

androgen receptor, castration-resistant prostate cancer, noncanonical NF- κ B pathway

1 | INTRODUCTION

Prostate cancer (PC) remains the most commonly diagnosed non-cutaneous malignancy in men and the second most common cause of cancer death worldwide.^{1,2} Active surveillance, radical prostatectomy, brachytherapy, and external beam radiotherapy are currently the most common treatments for localized PC.³ However, androgen deprivation therapy (ADT) and/or chemotherapies are the most indicated remedy to treat advanced or metastatic PC.⁴ Ninety percent of patients have remission of the disease, which always develops into castration-resistant prostate cancer (CRPC) within 2 to 3 years.⁵ Metastatic CRPC associates with poor prognosis and the mean survival time is approximately 18 months.⁶ As androgen receptor (AR) signaling is the main driver of PC cell proliferation and survival, understanding ADT resistance mechanisms and new adjuvant therapies are required to improve patient survival. Some of these mechanisms have been explained during the last few years.⁷ As prostate cells are androgen-dependent, the absence of androgens leads to apoptotic cell death, which promotes inflammation surrounding the tumor, which is related to the constitutive activation of the nuclear factor kappa-light-chain-enhancer of the activated B cell (nuclear factor- κ B [NF- κ B]) pathway.⁸ The NF- κ B family comprises five proteins- RelA/p65, NF κ B1/p50, c-Rel, RelB, and NF- κ B 2/p52. In absence of stimulus, these proteins reside in the cytoplasm forming homo- or heterodimers and typically are bound to an inhibitory protein (inhibitor of κ Bs). Under the stimulus, NF- κ Bs are activated via one of two cascades (canonical and noncanonical). Briefly, canonical NF- κ B signaling is activated by the cytoplasmic inhibitory κ -B kinase (IKK) complex composed of IKK subunits α and β (IKK α and IKK β) and the regulatory subunit NF- κ B essential modulator (NEMO or IKK γ). Upon stimulation, the IKK complex catalyzes the phosphorylation of I κ B α in a manner that is dependent on IKK β . This results in the targeted degradation of I κ B α and the release of the p65-p50 dimer to accumulate in the nucleus. On the other hand, IKK α homodimers and nuclear factor κ B-inducing kinase (NIK) are the main drivers for the activation of the noncanonical NF- κ B pathway. Following their activation, the RelB-p100 heterodimer is processed to RelB-p52.^{9,10} The upregulation of the noncanonical NF- κ B subunit p52 has been described as important in PC. For example, Lessard et al reported RelB-p52 dimers are more expressed in PC cores than the canonical NF- κ B subunits RelA and p50.

In addition, the number of nuclear RelB-positive cores was correlated with higher Gleason scores, suggesting the role of non-canonical NF- κ B subunits in the progression of PC.¹¹ Furthermore, they reported that androgenic stimulation of LNCaP cells (androgen-sensitive cells) with the androgen analog R1881 positively regulates the noncanonical NF- κ B pathway as p52 accumulates both in the nucleus and the cytoplasm.¹² Hence, Nadimity et al demonstrated that the overexpression of the p52 subunit was implicated in castration-resistant growth by inhibiting LNCaP cell cycle arrest and apoptosis in the androgen-deprived condition in vitro and inducing LNCaP cell growth in castrated nude mice in vivo. Furthermore, this was accompanied by continued expression and activation of the AR, providing evidence that p52 may activate AR during CRPC development.¹³ Subsequently, Nadimity et al exhibited that RelB-p52 with AR gene coactivators induce the aberrant activation of AR. In addition, they proved that the knockdown of p52 reduce AR activity in LNCaP cells.¹⁴ Furthermore, they showed that several genes involved in cell growth, proliferation, and movement were potential targets of RelB/p52.¹⁵ Collectively, these findings suggest a role for RelB/p52 in the progression of CRPC. More importantly, the resistance to next-generation anti-androgens (enzalutamide) was associated with AR and AR splice variants activation derived from an increase of RelB/p52 expression.^{16,17} Targeting the noncanonical NF- κ B pathway could, therefore, offer a new treatment paradigm for PC in combination with ADT.

NIK and IKK α are current therapeutic targets under investigation in the noncanonical NF- κ B pathway due to their crucial role in processing p100 to p52.¹⁸ In PC, IKK α -focused studies emphasized its function in controlling invasiveness, metastasis and inflammation, suggesting the therapeutic potential of IKK α inhibition.¹⁹⁻²¹ For example, IKK α inhibition by using synthetic small interference RNAs confirmed its major role in PC invasion and metastasis.¹⁹ In addition, a few studies implicate IKK α in cancer cell proliferation. Karin et al determined epithelial proliferation being regulated by IKK α ,²² although this study was on breast cancer (BCa). In PC, Shukla et al examined the effect of inhibiting IKK α by using apigenin and demonstrated antiproliferative and anti-invasive effects.²⁰

Despite many reports in the literature describing the development of IKK β inhibitors (subsequently abandoned because of reports of target-related toxicity¹⁸), potent and selective IKK α inhibitors with in vivo activity remain unknown. Although we have made some

progress in this respect²³ and a IKK α clinical candidate(s) should emerge from our work, the discovery of prognostic markers able to identify which patients could benefit from this therapy is urgently required. Previously, we have demonstrated that high IKK α expression was associated with reduced time to recurrence (TTR) and cancer specific survival (CSS) in estrogen receptor positive BCa.²⁴ This study aims to assess whether combining IKK α expression and stratification of patients according to their AR status can predict those likely to respond to combination therapy of ADT and an IKK α inhibitor. Furthermore, since as little is known about the involvement of IKK α in PC cell proliferation, and its function has been seen more related to invasive and metastatic capacities,¹⁹ we assessed the association between proliferative index Ki67 and IKK α as well as its prognostic value during the progression to CRPC.

2 | MATERIALS AND METHODS

2.1 | Patient cohort and sample collection

A total of 115 patients were included in this study, diagnosed between 1984 to 2000 at the Edinburgh Western General Hospital, Glasgow Royal Infirmary Hospital and Kilmarnock Crosshouse Hospital. Patients who initially responded to androgen ablation treatment (sub-capsular bilateral orchidectomy or LHRH agonists combined with anti-androgens) and subsequently relapsed (two consecutive rises in prostate-specific antigen [PSA] >10%) were included in the study. All selected patients had both hormone-naïve PC (HNPC) specimens gathered via trans-rectal ultrasound guided biopsies and CRPC specimens gathered via transurethral resection of the prostate to relieve bladder outflow obstruction available. Information relating to clinical diagnosis, treatment and outcome was obtained from the pathology notes including age (median 70 years, interquartile range (IQR), 66-74), PSA at diagnosis (median, 34.5 ng/mL; IQR, 9-126), PSA at recurrence (median 16 ng/mL; IQR, 5-39), Gleason score at diagnosis (median score 7.5; IQR, 6-9), Gleason score at recurrence (median score 9; IQR, 8-9), time to relapse from diagnosis (TTR median 2.6 years; IQR, 1.6-4.3), time to death from relapse (TTDR median 2.2 years. IQR 1.0-3.6) and CSS (median 5.5 years; IQR, 3.4-7.3). The study was approved by the Multicentre Research Ethics Committee from Scotland (MREC/01/0/36) and Local Research and Ethical Committees.

2.2 | Immunohistochemistry

IHC was performed on 4 μ m sections to assess total IKK α , IKK α phosphorylated at serine 180 (p-IKK α S180) and threonine 23 (p-IKK α T23). IHC for IKK β , NEMO, Akt phosphorylated at the serine 473 (p-Akt S473), Ki67 and AR had previously been performed in this cohort. Slides were deparaffinised with xylene and rehydrated through a series of graded alcohols. Heat-induced antigen retrieval was performed using citrate buffer pH 6 (Vector Laboratories, CA) under pressure for 5 minutes. Endogenous peroxidase activity was blocked

using 3% (vol/vol) hydrogen peroxide and nonspecific background staining was blocked using 5% (vol/vol) horse serum in Tris-buffered saline for 20 minutes. Total IKK α (Cat GWB-662250; Genway), p-IKK α S180 (Cat ab138426; Abcam) and p-IKK α T23 (Cat ab38515; Abcam) primary antibodies were used. Slides were then incubated in these primary antibodies overnight at 4°C; with the following antibody concentrations: total IKK α at 1:1000, p-IKK α S180 at 1:200, and p-IKK α T23 at 1:200. Envision (Dako) was added to the sections for 30 minutes at room temperature then slides were visualized using DAB substrate. Harris Haematoxylin counterstaining was performed then slides were dehydrated and mounted using a distrene, plasticizer, xylene.

2.3 | Scoring method

Stained sections were scanned using a Hamamatsu NanoZoomer (Welwyn Garden City, Hertfordshire, UK) at \times 20 magnification, and visualization was carried out using Slidepath Digital Image Hub, version 4.0.1 (Slidepath, Leica Biosystems, Milton Keynes, UK). Cytoplasmic and nuclear protein staining were assessed separately. Slides were scored by two independent observers using the weighted histoscore method, and the interclass correlation coefficient was greater than 0.7 for all antibodies.²⁵ If a difference of more than 50 weighted histoscore units was observed, the core was re-scored by both observers independently, in the majority of the cases this resolved any differences in scores, however, if there still remained a difference, both observers discussed the case and reached a consensus. The weighted histoscore is calculated using the following equation: $0 \times (\% \text{ cells not stained}) + 1 \times (\% \text{ cells weakly stained}) + 2 \times (\% \text{ cells moderately stained}) + 3 \times (\% \text{ cells strongly stained})$. This gives a range of scores from 0 to 300. Ki67 was assessed using a point count and <1% considered high expression.

2.4 | Assessment of the local inflammatory infiltration

The number of T-lymphocytes (CD3+, CD8+, and FOXP3+) and macrophages (CD68+ was determined by IHC for this cohort using the following antibodies: CD3 (Cat RM-9107-S, 1:1000; Thermo Fisher Scientific), CD8 (Cat Clone C8/114B, 1:200; Dako), FOXP3 (Cat 22510, 1:100; Abcam) and CD68 (Cat M0876, 1:200; Dako). Briefly, the density for each immune cell subtype was evaluated and the immune infiltration was graded as absent, weak, moderate or strong using a semi-quantitative method.

2.5 | Statistical analysis

Statistics were performed using the SPSS version 25.0 (IBM, Armonk, NY) and *P* values of less than .05 were considered statistically significant. Cut-off values to dichotomize each protein into low and high expression were determined using receiver operating characteristic

TABLE 1 Overview of patients' characteristics (n = 115)

Clinicopathological parameters	N (%)	Median (IQR)
Age (<70/>70/unknown)	55 (48)/10 (9)/48 (42)/2 (2)	70 (66-74)
Gleason at diagnosis (<7/7/>7/unknown)	28 (24)/24 (21)/52 (45)/11 (10)	7.5 (6-9)
Gleason at recurrence (<7/7/>7/unknown)	5 (4)/12 (10)/90 (78)/8 (7)	9 (8-9)
PSA at diagnosis (≤10 ng/mL/>10 ng/mL/unknown)	29 (25)/69 (60)/17 (15)	34.5 (9-126)
PSA at recurrence (≤10 ng/mL/>10 ng/mL/unknown)	26 (23)/32 (28)/57 (50)	16 (5-39)
Ki67 at diagnosis (≤1% cells/>1% cells/unknown)	14 (12)/47 (41)/54 (47)	3.0 (1.0-6.5)
Ki67 at recurrence (≤1% cells/>1% cells/unknown)	11 (10)/58 (50)/46 (40)	8.0 (3.0-16.0)

Abbreviations: IQR, interquartile range; PSA, prostate-specific antigen.

analysis. The relationship between protein expression and TTR, TTDR, and CSS was determined using Kaplan-Meier survival curves and differences compared using the log-rank test. Hazard ratio (HR) and 95% confidence interval (CI) values were calculated using univariate cox regression survival analysis. The associations between IKKα and clinicopathological characteristics, inflammation features or activated AR signaling was examined using the χ^2 test for linear trend. Correlations between IKKα and PC-deregulated pathways were conducted using the non-parametric Spearman's rank test. Multivariate cox regression survival analysis using a backward conditional elimination model and a statistical significance threshold of P value less than .05 was performed to identify independent prognostic biomarkers.

3 | RESULTS

3.1 | Patient characteristics

We analyzed 115 patients that progressed from HNPC to CRPC to investigate IKKα and AR as biomarkers for combination therapy (Table 1). Forty-eight percent of patients were younger than 70 years of age and 41% patients had a high proliferation rate for HNPC, and this increased to 50% with progression to CRPC. Similarly, for Gleason grade, 45% patients had a high tumor grade for HNPC, and this increased to 78% with progression to CRPC. Conversely, PSA concentration decreased during the transition to CRPC, 60% patients had a high PSA concentration for HNPC, and this decreased to 28% with progression to CRPC. Median follow-up was 4.9 years (IQR, 3.3-7.0) with 68 cancer and 40 non-cancer deaths. All patients presented biochemical relapse (TTR median 2.6 years; IQR, 1.6-4.3).

3.2 | The molecular prognostic profile differed between HNPC and CRPC

We investigated the associations between AR, Ki67, total IKKα, p-IKKα S180, p-IKKα T23 and TTR, TTDR, or CSS during the transition from HNPC to CRPC (Table 2). Ki67 proliferation rate and CSS were associated at the time of diagnosis (HR, 2.5; 95% CI, 1.2-5.0; P = .01);

patients with HNPC presenting high Ki67 expression were associated with significantly shorter CSS. However, following transition to CRPC, high Ki67 expression associated with shorter TTDR (HR, 2.6; 95% CI, 1.3-5.2; P = .01) with patients with a high Ki67 having a reduced 2-year TTDR of 46% compared with 69% for those with low Ki67. In addition, high levels of cytoplasmic p-IKKα S180 (HR, 2.10; 95% CI, 1.17-3.76; P = .01) and nuclear IKKα (HR, 2.60; 95% CI, 1.27-5.33; P = .01) were associated with significantly shorter TTDR.

3.3 | The prognostic effect of cytoplasmic IKKα was associated with low AR expression in HNPC but not CRPC patients

As IKKα has a role in the transition from HNPC to CRPC by enhancing the expression of genes transcribed by AR,^{13,14} we stratified patients based on their AR protein expression. In HNPC, patients with high levels of cytoplasmic IKKα and low AR expression associated with greater CSS (HR, 0.33; 95% CI, 0.11-0.98; log-rank P = .04, Figure 1B). Conversely, cytoplasmic IKKα expression was not associated with survival in the full cohort (HR, 0.72; 95% CI, 0.36-1.43; log-rank P = .34; Figure 1A) or patients with high AR expression (HR, 3.37; 95% CI, 0.95-12.04; log-rank P = .05; Figure 1C). Furthermore, a lower expression of cytoplasmic IKKα was strongly associated with >10 ng/mL PSA in the full cohort (P ≤ .001) as shown in Table 3. In CRPC, no associations were seen in low or high AR expressed patients for cytoplasmic or nuclear IKKα (Table S2).

3.4 | Patient stratification based on their Ki67 proliferation rate enhanced the prognostic effect of IKKα phosphorylation at S180 and T23

As high proliferative index Ki67 is associated with poorer PC survival²⁶ and IKKα suppression possesses an antiproliferative effect,²⁰ we investigated the association of IKKα and patient outcome in patients stratified for low and high Ki67. In HNPC (Table S1), high cytoplasmic p-IKKα S180 associated with better CSS in patients with high Ki67 (HR, 0.8; 95% CI, 0.2-4.1; P = .02). No associations were seen for any

TABLE 2 Associations between androgen receptor, Ki67, total IKK α , p-IKK α S180, p-IKK α T23, and survival during the transition from hormone-naïve to castration-resistant prostate cancer (n = 115)

	Hormone-naïve prostate cancer			Castration-resistant prostate cancer								
	N (%)	2-y CSS (SE)	P	N (%)	2-y TTR (SE)	P	N (%)	2-y CSS (SE)	P	N (%)	2-y TTDR (SE)	P
Androgen receptor			.38			.07			.06			.06
Low	37 (54)	95 (0.04)		36 (54.5)	64 (0.08)		21 (32)	100 (0.00)		19 (30)	71 (0.11)	
High	31 (46)	90 (0.06)		30 (45.5)	60 (0.09)		44 (68)	88 (0.05)		44 (70)	42 (0.08)	
Ki67			.01*			.86			.08			.01*
Low	27 (46)	92 (0.05)		27 (46)	63 (0.09)		24 (36)	91 (0.06)		24 (36)	69 (0.10)	
High	32 (54)	94 (0.04)		32 (54)	59 (0.09)		43 (64)	93 (0.04)		42 (64)	46 (0.08)	
cIKK α			.34			.12			.77			.10
Low	27 (55)	93 (0.05)		25 (52)	68 (0.09)		46 (68)	96 (0.03)		44 (67)	66 (0.07)	
High	22 (45)	86 (0.07)		23 (48)	65 (0.10)		22 (32)	86 (0.07)		22 (33)	48 (0.11)	
nIKK α			.97			.34			.17			.01*
Low	25 (51)	88 (0.07)		23 (48)	65 (0.10)		21 (31)	90 (0.07)		21 (32)	73 (0.10)	
High	24 (49)	92 (0.06)		25 (52)	68 (0.09)		47 (69)	94 (0.04)		45 (68)	54 (0.08)	
c p-IKK α S180			.60			.06			.07			.01*
Low	9 (17)	100 (0.00)		9 (17)	67 (0.16)		39 (59)	97 (0.03)		37 (58)	74 (0.08)	
High	44 (83)	91 (0.04)		43 (83)	70 (0.07)		27 (41)	89 (0.06)		27 (42)	40 (0.10)	
n p-IKK α S180			.86			.83			.56			.51
Low	27 (51)	93 (0.05)		26 (50)	69 (0.09)		33 (50)	94 (0.04)		32 (50)	67 (0.09)	
High	26 (49)	92 (0.05)		26 (50)	69 (0.09)		33 (50)	94 (0.04)		32 (50)	51 (0.09)	
c p-IKK α T23			.99			.67			.31			.85
Low	29 (54)	93 (0.05)		27 (51)	74 (0.08)		38 (54)	95 (0.04)		37 (54)	57 (0.08)	
High	25 (46)	92 (0.05)		26 (49)	73 (0.09)		32 (46)	94 (0.04)		31 (46)	62 (0.09)	
n p-IKK α T23			.59			.77			.63			.75
Low	27 (50)	96 (0.04)		27 (51)	74 (0.08)		35 (50)	97 (0.03)		33 (48.5)	66 (0.09)	
High	27 (50)	89 (0.06)		26 (49)	73 (0.09)		35 (50)	91 (0.05)		35 (51.5)	54 (0.08)	

Abbreviations: c/n, cytoplasmic/nuclear; CSS, cancer specific survival; IKK, inhibitory κ -B kinase; IKK α , IKK subunits α ; TTDR, time to death from relapse; TTR, time to recurrence.

* $P \leq .01$.

other markers in patients with HNPC. In CRPC (Table S2), high expression of nuclear p-IKK α T23 was associated with better CSS (HR, 0.1; 95% CI, 0.01-0.8; $P = .007$; Figure 2B) and greater TTDR (HR, 0.1; 95% CI, 0.02-1.3; $P = .04$; Figure 3B) in patients expressing low Ki67. However, no significant associations were seen for the full cohort (Figures 2A and 3A) or patients with high Ki67 (Figures 2C and 3C). No associations were seen for any other markers for CRPC.

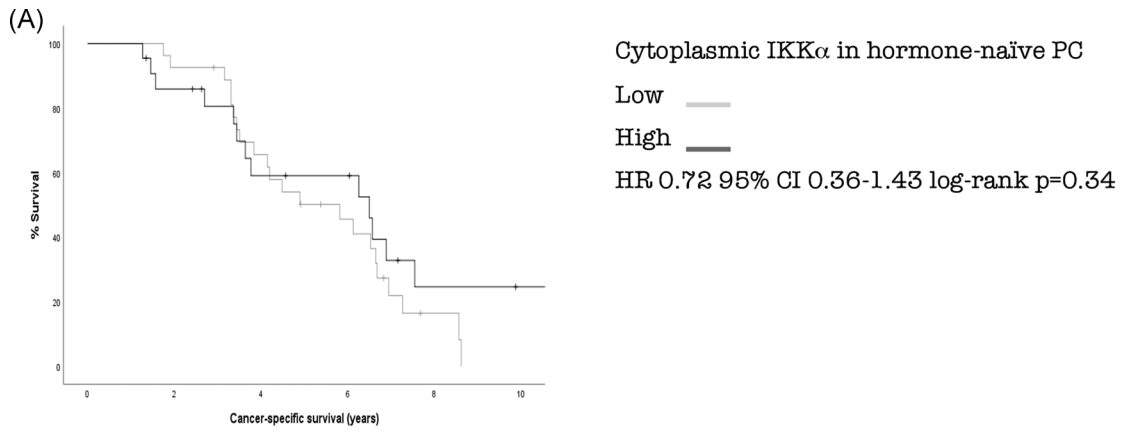
3.5 | IKK α associated with markers of adaptive immunity in HNPC but innate immunity in CRPC

As IKK α is involved in PC progression and CRPC growth due its interactions with inflammatory modulators and AR signaling,^{27,28} we investigated associations between total IKK α , p-IKK α S180 or p-IKK α T23, and clinicopathological parameters and inflammatory regulators (Table 3). For HNPC, low total cytoplasmic IKK α associated with increased PSA levels ($P = .001$), and high cytoplasmic p-IKK α T23 associated with a strong CD3+ ($P = .01$) and CD8+ ($P = .03$) lymphocytic

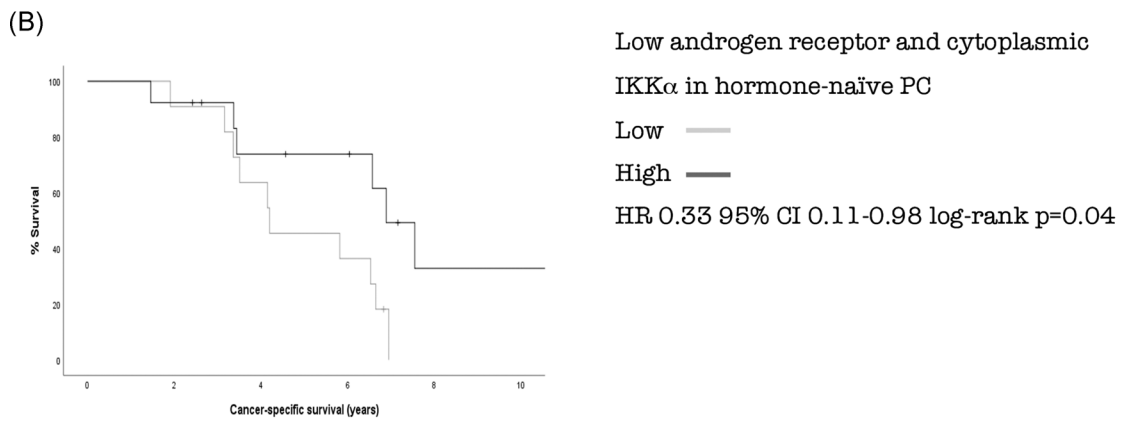
infiltration. No associations were seen for nuclear expression. For CRPC, high total cytoplasmic IKK α was associated with strong CD68+ macrophage infiltration ($P = .04$) whereas high cytoplasmic p-IKK α T23 associated with weak CD3+ lymphocytic infiltration ($P = .04$). Finally, high nuclear p-IKK α T23 associated with increased Gleason score ($P = .01$), and high nuclear p-IKK α S180 associated with increased PSA levels ($P = .03$). No associations were seen for total nuclear IKK α .

3.6 | IKK α was correlated with members of the canonical NF- κ B, PI3/Akt, and AR pathways

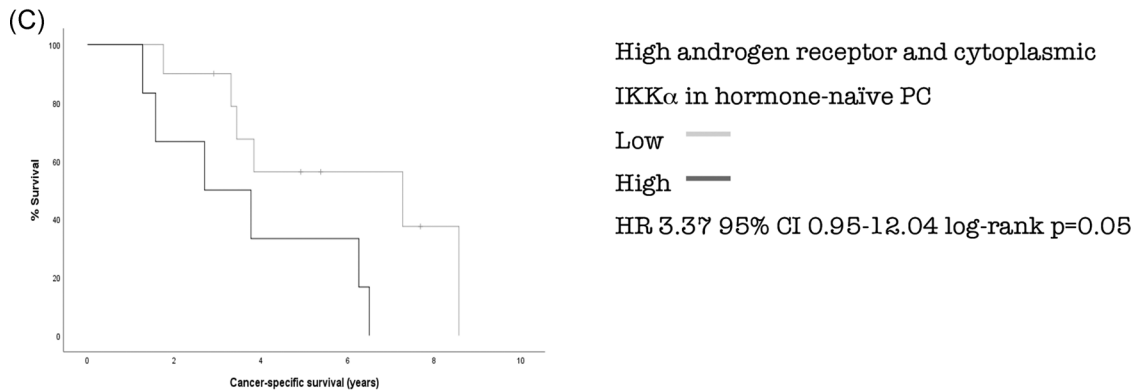
As IKK α has been previously seen to interact with members of other cancer pathways involved in CRPC growth, such as the canonical NF- κ B, PI3K/AKT, and AR pathways,²⁹⁻³² we evaluated potential correlations between total IKK α , p-IKK α S180, and p-IKK α T23, and some of the members of these pathways (Table 4). Total IKK α correlated with NEMO ($P = .04$), and p-IKK α T23 correlated with AR ($P = .02$) in the nucleus of HNPC cells. Otherwise, total IKK α , p-IKK α



Number of patients at risk:
 Low 27(0) 25(0) 17(1) 10(3) 2(5) 0(5)
 High 22(0) 18(1) 11(3) 10(4) 3(6) 2(7)



Number of patients at risk:
 Low 11(0) 10(0) 7(0) 4(0) 0(1) 0(1)
 High 13(0) 12(0) 8(2) 7(3) 2(5) 2(5)



Number of patients at risk:
 Low 10(0) 9(0) 5(1) 3(3) 1(4) 0(4)
 High 6(0) 4(0) 2(0) 2(0) 0(0) 0(0)

FIGURE 1 Cytoplasmic inhibitory κ -B kinase subunits α (IKK α) associated with good prognosis in high AR-expressed patients with hormone-naïve prostate cancer (HNPC). Kaplan-Meier plots showing associations between cancer specific survival (CSS) and (A) cytoplasmic IKK α expression. B, C, Kaplan-Meier curves showing associations between CSS and cytoplasmic IKK α in (B) low and (C) high patients with androgen receptor (AR). CI, confidence interval; HR, hazards ratio

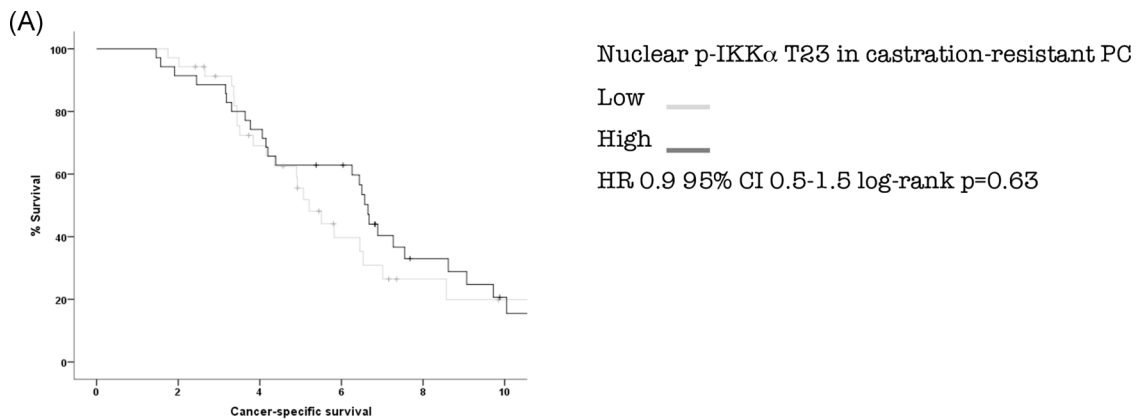
TABLE 3 The relationship between total IKK α , p-IKK α S180, p-IKK α T23, clinicopathological parameters and inflammatory features during the transition from hormone-naïve to castration-resistant prostate cancer (n = 115)

	IKK α				p-IKK α S180				p-IKK α T23							
	Cytoplasmic		Nuclear		Cytoplasmic		Nuclear		Cytoplasmic		Nuclear					
	Low (%)	High (%)	P	Low (%)	High (%)	P	Low (%)	High (%)	Low (%)	High (%)	P	Low (%)	High (%)	P		
Hormone-naïve prostate cancer																
Clinicopathological parameters																
Age, years																
≤70	18 (67)	11 (48)	.18	18 (72)	11 (44)	.05	7 (78)	24 (53)	16 (59)	15 (56)	.78	14 (48)	20 (74)	15 (54)	19 (68)	.27
>70	9 (33)	12 (52)		7 (28)	14 (56)		2 (22)	21 (47)	11 (41)	12 (44)		15 (52)	7 (26)	13 (46)	9 (32)	.95
Gleason score			.93			.05					.26					.99
<7	7 (26)	5 (22)		5 (20)	7 (28)		4 (44)	10 (23)	6 (23)	8 (30)		9 (31)	8 (30)	8 (29)	9 (32)	.80
7	6 (22)	5 (22)		2 (8)	8 (32)		1 (11)	9 (21)	3 (12)	7 (26)		6 (21)	6 (22)	6 (21)	6 (21)	.67
>7	14 (52)	13 (57)		18 (72)	10 (40)	.62	4 (44)	25 (57)	17 (65)	12 (44)	.38	14 (48)	13 (48)	14 (50)	13 (46)	.24
PSA, ng/mL			.001***			.16					.13					.31
≤10	2 (8)	10 (50)		5 (23)	7 (29)		0 (0)	13 (31)	5 (20)	8 (31)		7 (25)	7 (30)	7 (26)	7 (29)	.80
>10	24 (92)	10 (50)		17 (77)	17 (71)		9 (100)	29 (69)	20 (80)	18 (69)		21 (75)	16 (70)	20 (74)	17 (71)	.24
Ki67			.72			.41					.08					.43
≤1% cells	6 (35)	7 (41)		7 (50)	5 (26)		4 (67)	10 (33)	9 (47)	5 (29)		6 (29)	8 (47)	7 (30)	7 (47)	.51
>1% cells	11 (65)	10 (59)		7 (50)	14 (74)		2 (33)	20 (67)	10 (53)	12 (71)		15 (71)	9 (53)	16 (70)	8 (53)	.62
Inflammatory features																
CD68+ macrophages			.31			.41					.41					.51
Low	3 (33)	2 (67)		1 (25)	4 (50)		1 (25)	4 (50)	1 (17)	4 (67)		2 (29)	3 (50)	4 (44)	1 (25)	.43
High	6 (67)	1 (33)		3 (75)	4 (50)		3 (75)	4 (50)	5 (83)	2 (33)		5 (71)	3 (50)	5 (56)	3 (75)	.62
CD8+ T-cells			.62			.74					.74					.003*
Low	4 (50)	2 (67)		3 (60)	3 (50)		3 (75)	3 (43)	3 (60)	3 (50)		6 (75)	0 (0)	4 (50)	2 (67)	.09
High	4 (50)	1 (33)		2 (40)	3 (50)		1 (25)	4 (57)	2 (40)	3 (50)		2 (25)	3 (100)	4 (50)	1 (33)	.01**
CD3+ T-cells			.89			.62					.82					.09
Low	5 (56)	1 (50)		2 (67)	4 (50)		2 (67)	4 (50)	2 (50)	4 (57)		6 (86)	0 (0)	6 (67)	0 (0)	.87
High	4 (44)	1 (50)		1 (33)	4 (50)		1 (33)	4 (50)	2 (50)	3 (43)		1 (14)	4 (100)	3 (33)	2 (100)	.09
FOXP3+ T-cells			.24			.09					.80					.09
Low	4 (40)	3 (75)		1 (20)	6 (67)		2 (50)	5 (56)	3 (50)	4 (57)		3 (38)	5 (83)	5 (56)	3 (60)	.87
High	6 (60)	1 (25)		4 (80)	33 (9)		2 (50)	4 (44)	3 (50)	3 (43)		5 (63)	1 (17)	4 (44)	2 (40)	.09

TABLE 3 (Continued)

	IKK α				p-IKK α S180				p-IKK α T23			
	Cytoplasmic		Nuclear		Cytoplasmic		Nuclear		Cytoplasmic		Nuclear	
	Low (%)	High (%)	P		Low (%)	High (%)	P		Low (%)	High (%)	P	
Castration-resistant prostate cancer												
Clinicopathological parameters												
Age, years												
≤70	24 (52)	15 (65)	.30		11 (52)	28 (58)	.65		26 (67)	13 (46)	.10	
>70	22 (48)	8 (35)	.96		10 (48)	20 (42)	.50		13 (33)	15 (54)	.13	
Gleason score												
<7	2 (5)	1 (5)	.30		0 (0)	3 (7)	.65		2 (5)	0 (0)	.10	
7	5 (11)	2 (9)	.96		2 (10)	5 (11)	.50		6 (16)	1 (4)	.13	
>7	37 (84)	19 (86)	.46		18 (90)	38 (83)	.43		30 (79)	26 (96)	.28	
PSA, ng/mL												
≤10	16 (52)	6 (40)	.67		5 (39)	17 (52)	.15		13 (52)	8 (36)	.74	
>1	15 (48)	9 (60)	.46		8 (62)	16 (49)	.15		12 (48)	14 (64)	.74	
Ki67												
≤1% cells	8 (31)	7 (37)	.67		6 (50)	9 (27)	.15		7 (32)	6 (27)	.74	
>1% cells	18 (69)	12 (63)	.67		6 (50)	24 (73)	.15		15 (68)	16 (73)	.74	
Inflammatory features												
CD68+ macrophages												
Low	5 (56)	0 (0)	.04*		3 (50)	2 (25)	.33		4 (33)	0 (0)	.49	
High	4 (44)	5 (100)	.04*		3 (50)	6 (75)	.33		8 (67)	1 (100)	.49	
CD8+ T-cells												
Low	4 (50)	3 (75)	.41		5 (83)	2 (33)	.08		6 (60)	1 (100)	.43	
High	4 (50)	1 (25)	.41		1 (17)	4 (67)	.08		4 (40)	0 (0)	.43	
CD3+ T-cells												
Low	5 (56)	1 (20)	.20		2 (33)	4 (50)	.53		4 (33)	1 (100)	.19	
High	4 (44)	4 (80)	.20		4 (67)	4 (50)	.53		8 (67)	0 (0)	.19	
FOXP3+ T-cells												
Low	6 (60)	2 (40)	.46		4 (57)	4 (50)	.78		7 (54)	1 (100)	.37	
High	4 (40)	3 (60)	.46		3 (43)	4 (50)	.78		6 (46)	0 (0)	.37	

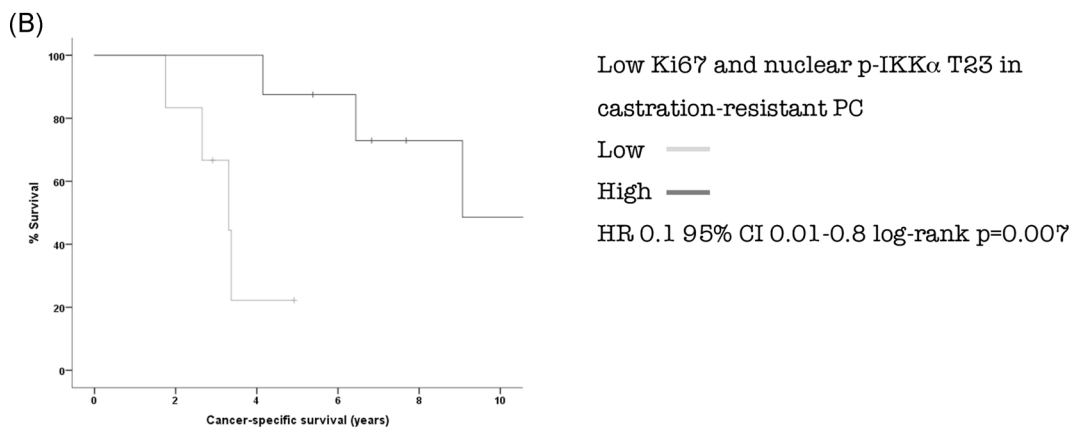
Abbreviations: IKK, inhibitory κ -B kinase; IKK α , IKK subunits α ; PSA, prostate-specific antigen. * $P < .05$. ; ** $P \leq .01$. ; *** $P \leq .001$.



Number of patients at risk:

Low 35(0) 34(0) 21(4) 9(8) 4(10) 2(11)

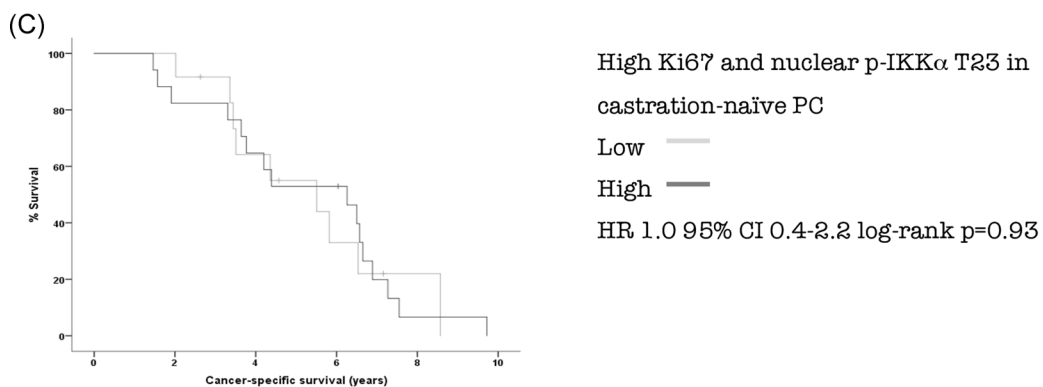
High 35(0) 32(0) 26(0) 21(1) 8(5) 4(6)



Number of patients at risk:

Low 6(0) 5(0) 1(1) 0(2) 0(2) 0(2)

High 8(0) 8(0) 8(0) 6(1) 3(3) 2(3)

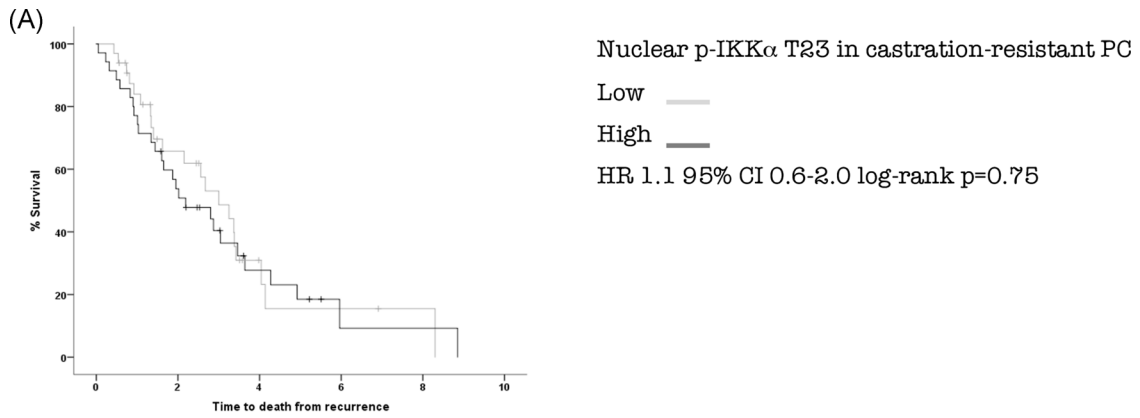


Number of patients at risk:

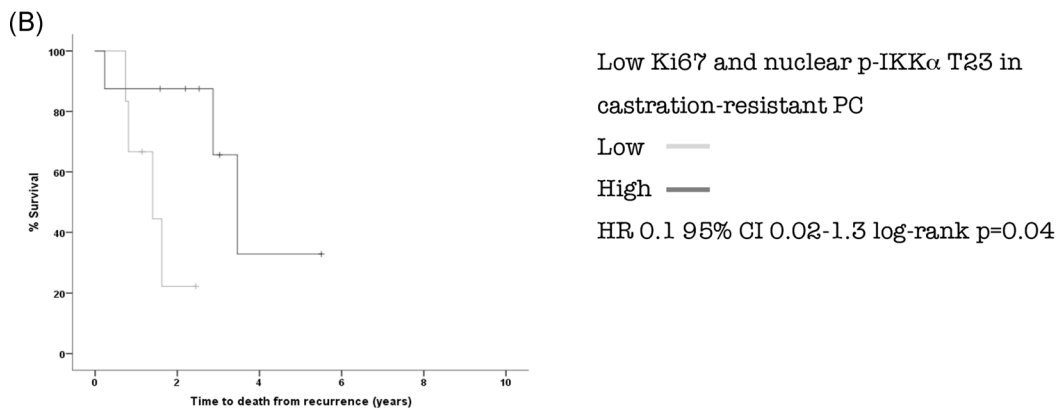
Low 12(0) 12(0) 7(1) 3(2) 1(3) 0(3)

High 17(0) 14(0) 11(0) 9(0) 1(1) 0(1)

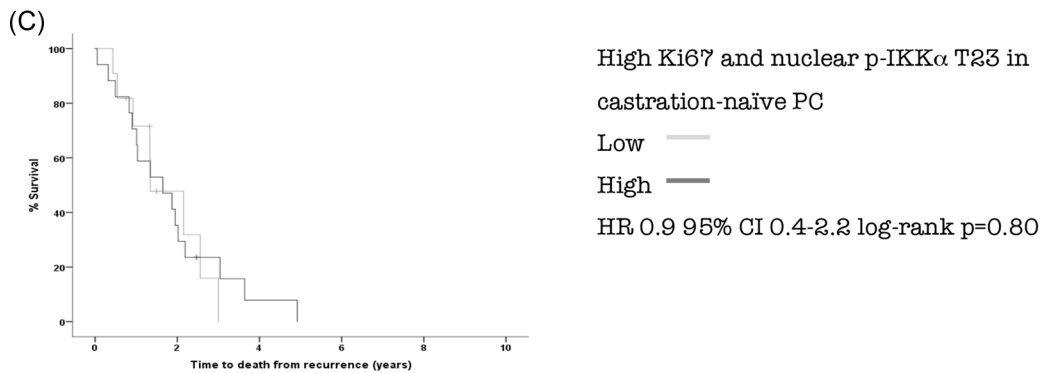
FIGURE 2 Nuclear p-IKK α T23 is associated with good prognosis in castration-resistant prostate cancer (CRPC) patients with low Ki67. A, Kaplan Meier curves showing associations of nuclear p-IKK α T23 and CSS in the full cohort. B, Kaplan Meier curves showing associations of nuclear p-IKK α T23 and CSS in patients with low Ki67. C, Kaplan Meier curves showing associations of nuclear p-IKK α T23 and CSS in patients with high Ki67. CI, confidence interval; CRPC, castration-resistant prostate cancer; CSS, cancer specific survival; HR, hazards ratio; IKK, inhibitory κ -B kinase; IKK α , IKK subunits α



Number of patients at risk:
 Low 33(0) 17(6) 4(11) 2(11) 1(12) 0(12)
 High 35(0) 18(1) 6(6) 1(8) 1(8) 0(8)



Number of patients at risk:
 Low 6(0) 1(1) 0(2) 0(2) 0(2) 0(2)
 High 8(0) 6(1) 1(4) 0(5) 0(5) 0(5)



Number of patients at risk:
 Low 11(0) 3(3) 0(3) 0(3) 0(3) 0(3)
 High 17(0) 6(0) 1(1) 0(1) 0(1) 0(1)

FIGURE 3 Nuclear p-IKK α T23 is associated with good prognosis in CRPC patients with low Ki67. A, Kaplan Meier curves showing associations of nuclear p-IKK α T23 and time to death from relapse (TTDR) in the full cohort. B, Kaplan Meier curves showing associations of nuclear p-IKK α T23 and TTDR in patients with low Ki67. C, Kaplan Meier curves showing associations of nuclear p-IKK α T23 and TTDR in patients with high Ki67. CI, confidence interval; CRPC, castration-resistant prostate cancer; HR, hazards ratio; IKK, inhibitory κ -B kinase; IKK α , IKK subunits α

TABLE 4 Correlations (*P* value) between the expression of total IKK α , p-IKK α S180, p-IKK α T23, NEMO, total IKK β , p-AKT S473 and androgen receptor in the cytoplasm and the nucleus of prostate cancer cells during the transition from hormone-naïve to castration-resistant prostate cancer (*n* = 115)

	Hormone-naïve prostate cancer				Castration-resistant prostate cancer			
	IKK α		p-IKK α S180		p-IKK α T23		p-IKK α S180	
	Cytoplasmic	Nuclear	Cytoplasmic	Nuclear	Cytoplasmic	Nuclear	Cytoplasmic	Nuclear
Canonical NF- κ B pathway								
cNEMO	.22	.48	.20	.64	.06	.57	.25	.68
nNEMO	.33	.04*	.63	.31	.32	.87	.75	.99
cIKK β	.36	.42	.35	.07	.61	.19	<.001***	.83
nIKK β	.60	.96	.20	.23	.27	.29	.39	.50
Akt pathway								
m p-Akt S473	.43	.28	.82	.07	.59	.18	.64	.17
c p-Akt S473	.09	.80	.94	.45	.13	.42	.31	.06
n p-Akt S473	.17	.58	.74	.39	.87	.65	.66	.99
Androgen receptor pathway								
nAR	.16	.88	.09	.76	.30	.02*	.09	.34

Abbreviations: IKK, inhibitory κ -B kinase; IKK β , IKK subunit β ; NEMO, NF- κ B essential modulator; NF- κ B, nuclear factor κ B; m/c/n, membrane/cytoplasmic/nuclear.

P* < .05; *P* \leq .01; ****P* \leq .001.

S180 (noncanonical NF- κ B pathway), and p-IKK α T23 were strongly correlated with IKK β (canonical NF- κ B pathway) (*P* \leq .001 for all markers) in the cytoplasm of CRPC cells. Furthermore, cytoplasmic and nuclear p-IKK α T23 were correlated with membrane and cytoplasmic p-Akt S473, respectively, (*P* = .03 for both markers). Finally, total IKK α and IKK β also correlated in the nucleus of CRPC cells (*P* = .01), and cytoplasmic total IKK α correlated with nuclear NEMO (*P* = .04). Figure 4 summarizes the crosstalk between IKK α and the different cancer pathways.

3.7 | IKK α was not an independent prognostic marker in CRPC

We performed a univariate and multivariate cox regression analysis to determine the effect of clinicopathological parameters, inflammatory features and IKK α expression on patients' survival (Table 5). As IKK α was not associated with prognosis in the total cohort in HNPC, we only conducted the analysis for CRPC patients. Under univariate analysis, PSA (*P* = .02), Ki67 (*P* = .01), total nuclear IKK α (*P* = .01) and cytoplasmic p-IKK α S180 (*P* = .01) were associated with TTDR and taken forward into multivariate analysis. Under multivariate analysis, only PSA (*P* = .05) trended towards significance for TTDR, neither total nuclear IKK α (*P* = .11) nor cytoplasmic p-IKK α S180 (*P* = .26) were independent prognostic factors.

4 | DISCUSSION

Many studies associate IKK α with PC cell proliferation, survival, invasion, androgen-independent growth, and tumor metastasis,^{13,14,19,21,28,33} suggesting IKK α inhibitors as potential therapeutic agents to treat localized and advanced PC.¹⁸ However, differences in IKK α prognostic role between HNPC and CRPC are unclear. For this reason, we should establish prognostic markers able to differentiate which patients will benefit from IKK α inhibition before starting the treatment. This is the first study to investigate this concept by determining the prognostic value of IKK α before and after castration-resistance as well as its relationship with AR expression.

We confirm that total cytoplasmic IKK α is a potential good prognostic marker for HNPC patients with low AR, and it does not associate with members from the canonical NF- κ B pathway, suggesting that it may be working through the noncanonical pathway. On the other hand, IKK α is a poor prognostic maker for all CRPC, with nuclear IKK α and cytoplasmic p-IKK α S180 associating with shorter TTDR. In addition, cytoplasmic p-IKK α S180 correlates with cytoplasmic IKK β to drive bad prognosis, suggesting it may be interacting with the canonical NF- κ B pathway in CRPC. Furthermore, cytoplasmic p-IKK α T23 associates with increased lymphocytic infiltrate in HNPC, whereas total cytoplasmic IKK α associates with increased macrophages in CRPC. These results suggest that the role of IKK α changes during the progression to CRPC from protective to detrimental, potentially due to interactions with inflammatory

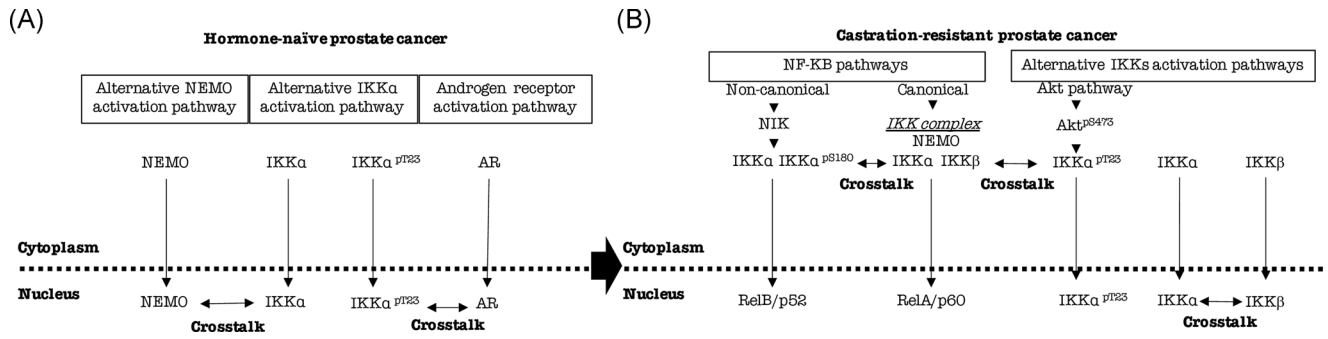


FIGURE 4 IKK α crosstalk with other members of cancer pathways in the cytoplasm and the nucleus of prostate cancer cells during the transition from hormone-naïve to castration-resistant prostate cancer. A, IKK α crosstalk in hormone-naïve prostate cancer. Total nuclear IKK α correlates with nuclear nuclear factor- κ B (NF- κ B) essential modulator (NEMO), and nuclear phosphorylated IKK α at threonine 23 correlates with nuclear androgen receptor. B, Crosstalk in castration-resistant prostate cancer. Phosphorylated IKK α at serine 180 (noncanonical NF- κ B pathway) and phosphorylated IKK α at threonine 23 correlate with IKK β (canonical NF- κ B pathway) in the cytoplasm. In addition, phosphorylated IKK α at threonine 23 correlates with phosphorylated Akt at serine 473 (Akt pathway) in the cytoplasm. Finally, there is a correlation between IKK α and IKK β in the nucleus. IKK, inhibitory κ -B kinase; IKK α , IKK subunits α ; IKK β , IKK subunits β

infiltrate and whether it is acting via the canonical or noncanonical NF- κ B pathway.

Assessing the role of IKK α is complex, given its different biological functions that are dependent on both cellular type and localization. Although the activity of cytoplasmic IKK α is associated with the activation of NF- κ B pathways (canonical and noncanonical), nuclear IKK α has consistently been demonstrated to work

independently in PC.¹⁸ Furthermore, IKK α is a substrate for multiple kinases that phosphorylate it at specific residues. For example, the phosphorylation of IKK α at S180 is known to take place in the cytoplasm by NIK, a prerequisite for activating the noncanonical NF- κ B pathway,³⁴ whereas phosphorylation at T23 is by Akt.³⁵ IKK α can therefore be activated in response to several signaling pathways, which, in turn, interact with each other to further complicate IKK α

TABLE 5 Analysis of the effect of clinicopathological parameters, inflammatory features, and IKK α on the survival of castration-resistant prostate cancer (n = 115)

	Castration-resistant prostate cancer			
	Time to death from recurrence			
	Univariate HR (95% CI)	P	Multivariate HR (95% CI)	P
Clinicopathological parameters				
Age ($\leq 70 / > 70$ y)	1.47 (0.90-2.39)	.12
Gleason score ($< 7 / > 7$)	1.16 (0.69-1.96)	.57
PSA (≤ 10 ng/mL / > 10 ng/mL)	2.22 (1.14-4.33)	.02*	3.00 (1.02-8.81)	.05
Ki67 ($\leq 1\%$ cells / $> 1\%$ cells)	2.60 (1.30-5.20)	.01**	2.40 (0.75-7.66)	.14
Inflammatory features (low/high)				
CD68+ macrophages	0.34 (0.06-1.88)	.22
CD8+ T-cells	1.73 (0.40-7.44)	.46
CD3+ T-cells	3.46 (0.73-16.40)	.12
FOXP3+ T-cells	0.89 (0.24-3.38)	.87
IKKα pathway (low/high)				
c IKK α	1.65 (0.90-3.02)	.11
n IKK α	2.60 (1.27-5.33)	.01**	2.50 (0.81-7.73)	.11
c p-IKK α S180	2.10 (1.17-3.76)	.01**	1.68 (0.69-4.13)	.26
n p-IKK α S180	1.22 (0.67-2.20)	.51
c p-IKK α T23	1.06 (0.59-1.88)	.86
n p-IKK α T23	1.10 (0.62-1.96)	.75

Abbreviations: CI, confidence interval; c/n, cytoplasmic/nuclear; HR, hazards ratio; IKK, inhibitory κ -B kinase; IKK β , IKK subunit β ; NEMO, NF- κ B essential modulator; NF- κ B, nuclear factor κ B; m/c/n, membrane/cytoplasmic/nuclear; PSA, prostate-specific antigen.

*P < .05; **P \leq .01.

functionality and make deconvolution of its roles as instigator and/or responder challenging in an oncogenic setting.³⁶ Several IKK α -related interactions drive PC progression, such as the crosstalk between the canonical and noncanonical NF- κ B pathways, and more importantly, the positive relationship between noncanonical NF- κ B and AR signaling.²⁹ However, how this differs between HNPC and CRPC is not well understood. To help address this question, this study utilized patients able to provide both HNPC and CRPC samples to assess the differences between the two in the same cohort. The results showed that cytoplasmic IKK α was associated with low AR in HNPC, where patients presenting high levels of total cytoplasmic IKK α and low AR protein expression had longer CSS compared with patients expressing low total cytoplasmic IKK α . Furthermore, low total cytoplasmic IKK α -expressing patients with high AR tended to live longer than those with high levels of total cytoplasmic IKK α , indicating that IKK α inhibitors could potentially be of benefit to the latter group. Additionally, we found that the high expression of nuclear IKK α and cytoplasmic p-IKK α S180 was associated with shorter TTDR in patients with CRPC but was not independently prognostic. Furthermore, p-IKK α S180 (noncanonical NF- κ B pathway) was correlated with IKK β (canonical NF- κ B pathway) in the cytoplasm of patient with CRPC samples, suggesting interactions between the canonical and noncanonical pathways. Moreover, cytoplasmic total IKK α correlated with cytoplasmic IKK β , indicating that a proportion of the total IKK α accumulated in the cytoplasm may be involved with the IKK β /NEMO complex, which activates the canonical NF- κ B pathway.³⁷ This is not surprising as IKK α is a critical component of both pathways and suggests that it is acting to drive poor prognosis via the canonical NF- κ B pathway in CRPC, both directly via the IKK complex and indirectly through crosstalk with the noncanonical NF- κ B pathway.

Despite several studies having demonstrated the involvement of nuclear IKK α in PC progression and metastasis, little is known about the mechanistic connection between prostate tumorigenesis and nuclear IKK α activity.^{19,21,22,33} We found nuclear IKK α correlated with nuclear NEMO in HNPC, which supports Margalef's report of a complex comprised of the active isoform of nuclear IKK α (p45-IKK α) and nuclear NEMO to prevent apoptosis and sustain tumor growth, although this study was in colorectal cancer.³⁸ In addition, nuclear IKK α correlated with nuclear IKK β in CRPC to drive bad prognosis, suggesting their crosstalk is through alternative pathways (independent from NF- κ B pathway). Although IKK α can be distributed in the cytoplasm as well as in the nucleus, IKK β is mainly located in the cytoplasm, although it has also been found to have a nuclear function that is related to DNA repair.³⁹

Despite the interaction between nuclear IKK α and AR being already known,⁴⁰ this is the first study demonstrating a positive correlation between the expression of nuclear p-IKK α T23 and AR in HNPC, suggesting crosstalk between these proteins. In addition, IKK α phosphorylation at T23 by Akt has been described as crucial for its translocation into the nucleus³⁵ and the correlation we found between p-IKK α T23 and Akt in CRPC supports this interaction, which is consistent with the study by Luo et al who demonstrated

that nuclear IKK α accumulation correlated with the progression and the clinical grade of PC.²¹ Interestingly, we observed no correlation between p-IKK α T23 and Akt in HNPC, suggesting T23 phosphorylation of IKK α may involve another kinase in this phase of the disease.

Interestingly, these results also suggest an involvement of p-IKK α T23 in the canonical NF- κ B pathway, with cytoplasmic p-IKK α T23 correlating with IKK β in the cytoplasm and the nucleus in patients with CRPC. This is the first study to propose an interaction between p-IKK α T23 and IKK β , potentially via an independent mechanism, but this requires further investigation.

The activity of IKK α is known to be dependent on molecular and cellular changes in the tumor microenvironment, including those promoted by therapeutic interventions. The inflammatory response elicited by androgen deprivation promotes the deregulation of several pathways including NF- κ B and is a major contributor to the emergence of androgen-independent PC.^{27,41,43} Despite most of the studies analyzing macrophages (CD68+) and lymphocytes (CD3+ and CD8+) in PC tissues identifying them being protumorigenic,⁴⁴ their prognostic relevance is unclear in PC. Therefore, we evaluated the association between specific immune cells and IKK α expression during the transition from HNPC to CRPC. The results showed high expression of cytoplasmic p-IKK α T23 associated with high CD3+ and CD8+ tumor-infiltrated lymphocytes (TILs) in HNPC. On the other hand, high cytoplasmic p-IKK α T23 was associated with low CD3+ TILs and high macrophage (CD68+) infiltration in CRPC, suggesting the involvement of macrophages (CD68+) in CRPC progression. Accordingly, macrophage infiltration induced by castration^{45,46} has been related with the acquisition of CRPC.^{47,48}

Despite IKK α being seen as a key mediator of inflammation and metastasis in PC, its relationship with cell proliferation remains unclear. In this study, we showed that high p-IKK α T23 nuclear expression was significantly associated with low Ki67 in CRPC and allied with better prognosis, suggesting that it is not related to proliferation in PC.

5 | CONCLUSION

In conclusion, we have shown that total cytoplasmic IKK α is potentially a marker for good prognosis for HNPC patients with low AR expression, and it does not associate with members from the canonical NF- κ B pathway. Furthermore, IKK α is a marker for poor prognosis for patients with CRPC, with nuclear IKK α and cytoplasmic p-IKK α S180 associating with shorter TTDR. Cytoplasmic p-IKK α S180 also correlate with cytoplasmic IKK β to drive bad prognosis. These results suggest that the noncanonical NF- κ B pathway is dampened by the canonical pathway to promote disease progression. Taken together, these data indicate that patients with CRPC may benefit from treatment with IKK α inhibitors if they were to be developed as therapeutic agents. We note that any expression of IKK α can be used as an independent prognostic marker, and more studies will be necessary to further validate and establish whether combining

IKK α with other markers, such as AR could be used as prognostic biomarkers.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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