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RESEARCH

Classical and Alternative Nuclear Factor-κB Pathways: A Comparison among Normal Prostate, Benign Prostate Hyperplasia and Prostate Cancer

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Abstract Nuclear factor-κB (NF-κB) is controlled by the classical and alternative NF-κB pathways, the role of which in prostate cancer (PCa) is not clearly defined. To provide this missing translational link, we compared the classical and alternative NF-κB pathways in normal prostate, benign prostate hyperplasia (BPH) and PCa. Prostate specimens were divided into three groups: group A, PCa (n=68); group B, BPH (n=60); and group C, normal prostates (n=15). The gene expression levels of NF-κB1 and NF-κB2 were determined by real-time quantitative RT-PCR. Additionally, we analyzed the expression and sub-cellular localization of phosphorylated P50 (p-P50) and phosphorylated P52 (p-P52) proteins by immunohistochemical staining. Furthermore, associations were made between NF-κB pathway proteins and patients' prognosis. Compared with BPH and normal prostate tissues, the expression of NF-κB1 gene was differentially down-regulated by >1.5-fold, whereas NF-κB2 gene was differentially up-regulated by >2-fold in PCa tissues. The proportion of p-P50 positive patients in group A (26.5%) was significantly lower than in group B (88.3%, p=0.005) and C (100%, p=0.002). The proportion of p-P52 positive patients in group A (42.6%) was significantly higher than in group B (11.7%, p=0.009) and C (6.7%, p=0.008). Comparison of the survival curves in group A according to p-P52 expression showed a significant difference between positive and negative patients. The p-P52 positive patients showed worse prognosis (p=0.019). Our findings suggest for the first time that the classical and alternative NF-κB pathways have an important role in PCa. p-P52 might be a predictor of poor prognosis for PCa.

Keywords Immunohistochemistry · Nuclear factor κB · Prognosis · Prostate cancer · Real-time quantitative RT-PCR

Chao Cai, Fu-neng Jiang and Yu-xiang Liang offered equal contributions to this study.

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Introduction

In Western and Asian men, prostate cancer (PCa) is one of the most frequently diagnosed cancers and a leading cause of cancer death. It is well documented that the majority of PCa deaths are due to metastases that are resistant to therapy [1]. The reasons for treatment failure are that some patients dis-sense to androgen withdrawal therapies and someone relapse to androgen deprivation-resistance state at treatment periods. Recent studies also demonstrate that increased androgen expression is both necessary and

sufficient to convert PCa growth from a hormone therapy-sensitive to a resistant state in xenograft models [2,3]. Since androgen mRNA levels are often increased in androgen deprivation-resistance tumors without gene amplification, it is likely mediated by transcription factors and transcription regulating signal transduction pathways that are altered during progression.

Nuclear factor-κB (NF-κB) represents a family of inducible dimeric transcription factors, which in mammals comprises RelA (p65), RelB, Rel (c-Rel), NF-κB1 (p50/p105) and NF-κB2 (p52/p100) [4]. These structurally homologous proteins form various homodimers and heterodimers via their N-terminal Rel homology domains (RHDs). In unstimulated cells, homodimers or heterodimers of NF-κB family members are bound to ankyrinrich regions of inhibitor of NF-κB (IκB) inhibitory proteins [5,6]. This binding serves to retain the dimers in the cytoplasm, which are hence unable to initiate transcription of target genes. The NF-κB1/p105 and NF-κB2/p100 precursor proteins, which encode p50 and p52 in their amino-terminal halves, also behave like IκBs, with ankyrin repeats in their carboxyl-terminal halves being analogous to those of the smaller IκBs. The IκBs and NF-κB2/p100 are important targets of inducible regulatory pathways that mobilize active NF-κB to the nucleus. These pathways are termed the 'classical' pathway and the 'alternative' pathway [7].

The classic NF-KB pathway is thought to be critical for tumorigenesis of various human malignancies, including leukemia, lymphomas, and a number of solid tumors [8]. It is hypothesized to contribute to development and/or progression of malignancy by regulating the expression of genes involved in cell growth and proliferation, anti-apoptosis, angiogenesis, and metastasis. But little is known about the role of the alternative NF-KB pathway in prostate cancer development. Recently, high constitutive nuclear levels of RelB have been observed in human PCa specimens with high Gleason scores [9]. However, whether the two NF-KB pathways contribute to progression of PCa is unknown. To provide this missing translational link, the present study used human PCa specimens to reveal that both NF-KB pathways have an important role in prostate malignant neoplasia.

Materials and Methods

Patients and Tissue Samples

Prostate specimens were obtained from surgeries performed between 2005. Feb. and 2008 Oct at Department of Urology in Guangzhou First Municipal People's Hospital for patients who underwent the operation of transurethral resection prostate and suprapubic radical prostatectomy. None of the patients had received any preoperative therapy. The pathological diagnosis of prostate biopsy was performed preoperatively and confirmed postoperatively. Borderline tumors were excluded from this study. All patients were given a follow-up ranging from 2 to 5 years. For the analysis of survival and follow-up, the date of prostatectomy was used to represent the beginning of the follow-up period. All of the patients whose cause of death was not related with PCa were excluded when we collected investigative cases. The specimens were divided into three groups: group A, PCa (n=68); group B, BPH (n=60); and group C, normal prostates (n=15).

Prior informed consent was obtained from the patients for the collection of specimens in accordance with the guidelines of Guangzhou First Municipal People's Hospital, Guangzhou Medical College, Guangzhou, China, and the study protocols were approved by the Ethics Committee of Guangzhou First Municipal People's Hospital, Guangzhou Medical College, Guangzhou, China. All specimens were handled and made anonymous according to the ethical and legal standards. The clinicopathological characteristics in the study cohort were shown in Table 1.

Quantitative Real-Time PCR

Total RNA of prostate tissues in three groups was prepared, reverse transcribed and quantitative real-time PCR (QRT-PCR) carried out as previously described [11]. The primers 5′- CTG CAG ACA ATT TCC CAC AC -3′ and 5′- GCC AAG TTG AAA AGG TCT CG -3′ were used to amplify 150-bp transcripts of NF-kB1, the primers 5′- GGC TGT ACA AAG AGC AAG GC -3′ and 5′- TGG CTT GGA CAG AGA CCA GG -3′ were used to amplify 181-bp transcripts of NF-kB2 and the primers 5′- GGT GGC TTT TAG GAT GGC AAG -3′ and 5′- ACT GGA ACG GTG AAG GTG ACA G -3′ were used to amplify 161-bp transcripts of β-actin. Product-specific amplification was confirmed by melting curve analysis and agarose gel electrophoresis. Serial dilutions were made using previously generated PCR products, assigned arbitrary values corresponding to the dilutions, and used to construct relative standard curves for NF-kB1 and NF-kB2, respectively. Targets were normalized using β-actin as internal standard.

Immunohistochemistry Analysis

The prostate specimens in three groups were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. The paraffin-embedded tissues were cut at 3 μm and stained following being dried on ProbeOn Plus (Fisher Scientific International, Hampton, NH, USA). Staining was done using avidin- biotin complex with a microprobe

Table 1 p-P50 and p-P52 expression patterns versus clinicopathologic parameters of PCa patients

Characteristics	Cases	p-P50 (n, %)		p	p-P52 (n, %)		p
	(N)	0	1		0	1	
Age (years)							
≤60	11	8(72.7)	3(27.3)		6(54.5)	5(45.5)	
				0.29			0.25
>60	57	42(73.7)	15(26.3)		33(57.9)	24(42.1)	
Serum PSA levels (ng/mL)							
<4	15	10(66.7)	5(33.3)		7(46.7)	8(53.3)	
				0.20			0.15
≥4	53	40(75.5)	13(24.5)		32(60.4)	21(39.6)	
Gleason Score							
≤6	29	16(55.2)	13(44.8)		23(79.3)	6(20.7)	
				0.008			0.008
>6	39	34(87.2)	5(12.8)		16(41.0)	23(59.0)	
TNM Stage							
T1	9	6(66.7)	3(33.3)		7(77.8)	2(22.2)	
T2	19	11(57.9)	8(42.1)		15(78.9)	4(21.1)	
				0.02			0.01
T3	24	19(79.2)	5(20.8)		12(50.0)	12(50.0)	
T4	16	14(87.5)	2(12.5)		5(31.3)	11(68.8)	

manual stainer (Fisher Scientific International). The slide to which a paraffin section was attached went through deparaffinization and hydration, and was then treated with a solution of Peroxidase-blocking reagent (Dako, Glostrup, Denmark) to exhaust endogenous peroxidase activity. It was put in citric acid solution and heated for 10 min in a microwave and then left at room temperature for 20 min to expose antigen hidden inside the tissue due to formalin fixation, and the process was repeated three times. To inhibit non-specific antigen-antibody reactions possible in immunohistochemical staining, reaction was done using a protein blocker (Research Genetics, Huntsville, AL, USA) for 5 min and the slide was washed thoroughly with water. The slides were incubated overnight with the primary antibodies against phosphorylated Ser 907 of P50 (p-P50, 1:150; #sc-101746, 100 μg/ml, Santa Cruz Biotech, Santa Cruz, CA, USA) and phosphorylated Ser 865 of P52 (p-P52, 1:100; #sc-101742, 100 μg/ml, Santa Cruz Biotech, Santa Cruz, CA, USA) at 4°C. Secondary antibodies for the detection of primary antibodies were reacted for 10 min using anti-mouse IgG (Sigma, St. Louis, MO, USA) to which biotin was attached, and then washed with buffer solution and reacted with horseradish peroxidase for 10 min. It was washed thoroughly with buffer solution; chromogen AEC (3-amino-9-ethylcarvazole; Zymed, San Francisco, CA, USA) was then applied and reddish brown response was examined. After hematoxylin contrast staining, the slide was enclosed with Universal Mount (Research Genetics) and examined. In each immunohistochemistry run, the omission of the primary antibody served as negative control.

Following a hematoxylin counterstaining, immunostaining was scored by two independent experienced pathologists, who were blinded to the clinicopathological parameters and clinical outcomes of the patients. The scores of the two pathologists were compared and any discrepant scores were trained through re-examining the stainings by both pathologists to achieve a consensus score. Sections were scored either as positive (1) or negative (0) for unequivocal brown nuclear NF-kB staining in at least 5% of cancer cells [10].

Statistical Analysis

The software of SPSS version 16.0 for Windows (SPSS Inc, IL, USA) and SAS 9.1 (SAS Institute, Cary, NC) was used for statistical analysis. Continuous variables were expressed as $\overline{X} \pm s$. The associations between protein expression and different clinical parameters were evaluated using Fisher's exact test or X^2 test. We used the Kaplan-Meier estimator and univariate Cox regression analysis to assess the marginal effect of each factor. The differences between groups were tested by log-rank analyses. The joint effect of different factors was assessed using multivariate Cox regression. Differences were considered statistically significant when p was less than 0.05.

Results

Differential NF-kB1 and NF-kB2 Expression in PCa, BPH and Normal Prostate Tissues

In the subset of prostate specimens suited for quantitative RT-PCR analyses, we profiled the mRNA expression of NF-kB1 and NF-kB2 in three groups. As shown in Fig. 1, NF-kB1 gene expression in PCa tissues was statistically significantly lower than that in BPH (1.52-fold higher than PCa, p=0.02) and normal prostate (1.96-fold higher than PCa, p=0.01) tissues, whereas NF-kB2 gene expression in PCa tissues was statistically significantly higher than that in BPH (2.28-fold lower than PCa, p=0.01) and normal prostate (3.16-fold lower than PCa, p=0.006) tissues. The difference in the expression levels of NF-kB1 and NF-kB2 between BPH and normal prostate tissues had no statistical significance (p>0.05).

Immunohistochemical Expression and Sub-cellular Localization of P-P50 and P-P52

The expression and sub-cellular localization of p-P50 and p-P52 in the prostate specimens in three groups were examined using immunohistochemical staining. p-P50 and p-P52 were both expressed in prostatic tissues of three groups. Most prostatic tissues in groups B and C were characterized by a strong cytoplasmic and nuclear p-P50 staining of the basal cell constituent and a variable cytoplasmic staining of the secretory (luminal) cells. p-P50 nuclear expression was usually not detected in PCa tissues (Group A). Contrary to p-P50, p-P52 was present in the nuclear of cancer cells in PCa tissues (Group A), whereas only the cytoplasmic expression of p-P52 was detected in prostatic tissues in groups B and C.

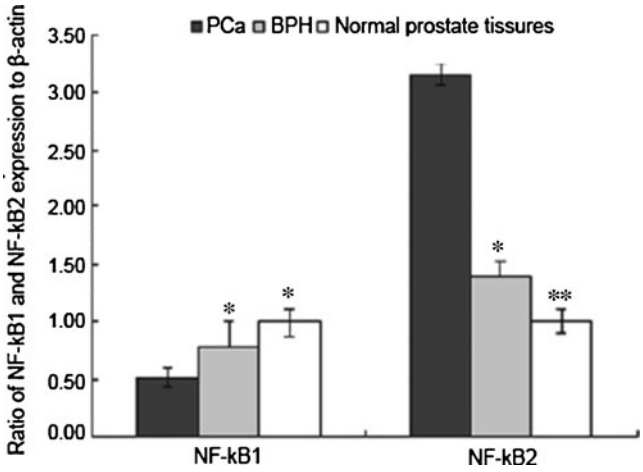

Fig. 1 NF-kB1 and NF-kB2 expression in prostate tissues of three groups. Total RNA was prepared from the prostate tissues, reverse-transcribed and subjected to QRT-PCR using oligonucleotide primers specific for NF-kB1 and NF-kB2. Expression was normalized using β-actin expression as an internal standard. Values shown are $\overline{X} \pm s$. '*' p<0.05, comparison of the expression level of NF-kB1 and NF-kB2 in PCa with that in BPH and normal prostate tissues; '**' p<0.01, comparison of the expression level of NF-kB2 in PCa with that in normal prostate tissues

For the expression of p-P50, there was a significant association among three groups. The proportion of p-P50 positive scored patients in group A (26.5%) was significantly lower than group B (88.3%, p=0.005) and C (100%, p=0.002). Contrary to p-P50, the proportion of p-P52 positive scored patients in group C (6.7%, p=0.009) and group B (11.7%, p=0.008) was significantly lower than group A (42.6%). No significant difference was observed between group B and group C for p-P50 and p-P52 expression, respectively. (Please see details in Fig. 2)

Association of P-P50 and P-P52 Expression with the Clinicopathological Characteristics of PCa

Fisher's exact test or X^2 test (Table 1) showed no significant statistical association of p-P50 and p-P52 immunostaining with age and serum PSA levels of patients (p>0.05), suggesting that these variables might not affect the expression of p-P50 and p-P52. A significant association of p-P50 and p-P52 positive expression rates with Gleason score (p=0.008 and 0.008, respectively) and TNM stage was observed (p=0.02 and 0.01, respectively). When PCa tissues were divided into low- to intermediate grade (Gleason scores≤6) and high-grade (Gleason scores>6) tumors, there was a trend towards decreased p-P50 nuclear staining in high-grade specimens, whereas a trend towards increased p-P52 nuclear staining in high-grade specimens (Table 1). In addition, patients with a higher TNM stage tend to express a low level of p-P50 and a high level of p-P52 (Table 1).

Significant Prognostic Value of P-P50 and P-P52 Expression Patterns for PCa Patients

There was not significant association of the survival curves according to the p-P50 nuclear expression (p=0.262) in group A. Comparison of the survival curves in group A according to p-P52 expression showed a significant difference between p-P52 positive and p-P52 negative patients, whereas the p-P52 positive patients shown worse results (p=0.019). (Please see details in Fig. 3)

Discussion

NF-kB is centrally involved in tumorigenesis and tumor progression in various types of cancer. In our series, we found that the NF-kB subunit NF-kB1 gene expression in

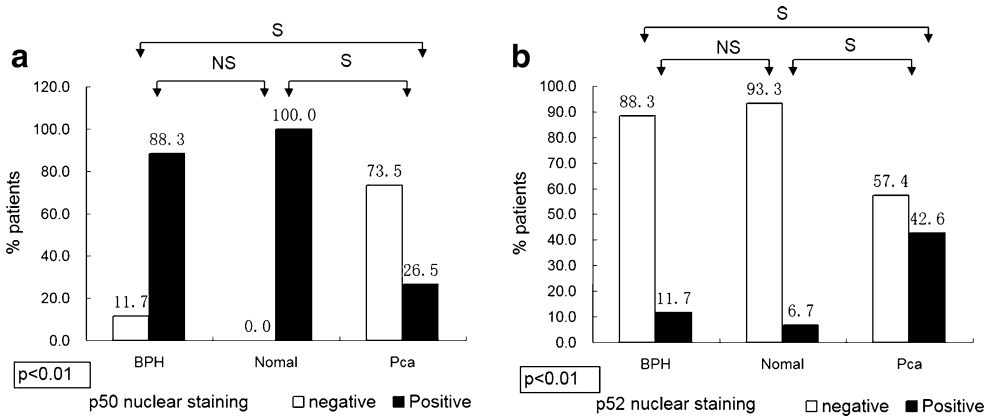

Fig. 2 Expression of p-P50 **a** and p-P52 **b**. A significant proportion of patients from malignant group stained for p-P50 and p-P52

PCa was lower than that in BPH and normal prostate tissues, which was contrary to NF-kB2 gene. These findings were consistent with the immunohistochemical staining patterns of p-P50 and p-P52 in three groups. In addition, there was a trend towards decreased p-P50 nuclear staining in aggressive PCa specimens, whereas a trend towards increased p-P52 nuclear staining in aggressive PCa specimens. Furthermore, we demonstrated a prognostic role for p-P52. Staining for p-P52 in malignant group correlated to worse prognosis. Several aspects following could explain the association of the p-P50 and p-P52 expression patterns with PCa progression.

Recent advances identified several possible mechanisms involved in the progression of hormone refractory PCa cells. PCa cells can survive in low levels of androgens by several mechanisms including androgen receptor (AR) mutation or amplification; or increasing 5a-reductase activity to convert testosterone to dihydrotestosterone [11]. Several different growth factors also stimulate proliferation by ligand-independent mechanisms [12]. In addition, clonal expansion of cells with neuroendocrine differentiation may also be involved in hormone refractory PCa progression [13]. Understanding the interplay between NF-κB and AR signalling is of particular importance considering the role of the two NF-κB pathways in the progression of PCa. Several reports suggest that the classical NF-κB signalling is restrained in androgen-dependent cells but confers a selective survival advantage in androgen-deprived conditions. RelA/p65 which is the primary player in the classical NF-kB pathway has been shown to be constitutively activated in PCa and to have an antagonistic relationship with the AR [14]. However, RelA/p65 has been shown to activate PSA expression and is up-regulated in androgen-independent PCa [15]. With the emergence of new evidence that implicates the alternative NF-κB pathway in the development of lymphoid and solid tumors, and recent results that report the expression and nuclear localization of alternative RelB and p52 subunits in PCa tissues [16]. It also demonstrated that the overexpression of NF-kB2/p52 protected androgen sensitive LNCaP cells from apoptotic cell death and cell cycle arrest induced by androgen-deprivation. NF-kB2/p52 activation induces androgen-independent growth in vitro and in vivo [17].

NF-kB factors are a promising research field for new therapeutic approaches in cancer. It is known that certain drugs act through NF-kB pathways. Recently, in PCa, most studies have been focused on the relationship between NF-κB

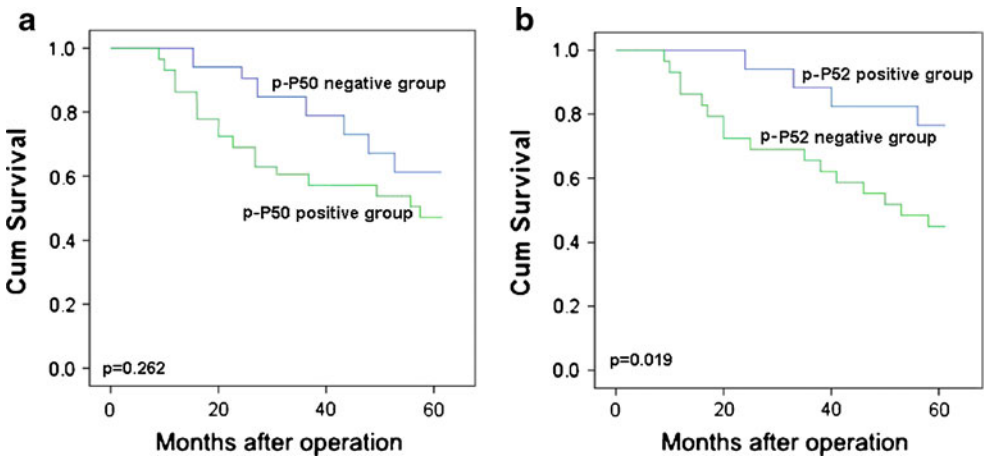

Fig. 3 Kaplan-Meier overall survival curves for p-P50 and p-P52 expression in PCa

factors and AR signalling. In the present study, our findings on the human PCa specimens suggest for the first time that the members of classical (NF-kB1/p50) and alternative (NF-kB2/p52) NF-κB pathways have an important role in the progression of prostate malignant neoplasia. Especially, p-P52 might be a predictor of poor prognosis for PCa.

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