Significance of nuclear factor kappa beta activation on prostate needle biopsy samples in the evaluation of Gleason score 6 prostatic carcinoma indolence

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Abstract

Background. Prostate cancer (PCa) is the most common cancer in men in developed European countries. Majority of men newly diagnosed with PCa are candidates for primary curative therapy, either with radical prostatectomy (RP) or radiation. However, many PCa are low risk, even indolent and these patients are candidates for active surveillance, so the prediction of such cancers is needed to avoid overtreatment. The main goal of our study was to find out whether the immunohistochemical expression of NF-κB p65 in biopsy samples with Gleason score 3+3=6 (GS 6) can be a negative predictive factor for PCa indolence. Methods. Study was based on a retrospective cohort of 178 PCa patients with initial total PSA ≤ 10 ng/ml, number of needle biopsy specimens ≥8, GS 6 on biopsy and T1/T2 estimated clinical stage who underwent laparoscopic radical prostatectomy and whose archived formalin-fixed and paraffin-embedded (FFPE) prostate needle biopsy specimens were used for additional immunohistochemistry staining for detection of NF-κB p65. Both cytoplasmatic and nuclear NF-κB p65 expression in biopsy cores with PCa were correlated with postoperative pathological stage, positive surgical margins, GS and biochemical progression (BP) of disease. The final analysis involved 123 patients regarding the postoperative stage, surgical margins and GS and 118 regarding the BP. Results. Postoperative pathological stage 3 was noticed in 27 (22%) and positive surgical margins were detected in 13 patients (10,6%). After median follow-up of 66 months, BP (PSA ≥ 0,05 ng/ml) occurred in 20 (16,9%) patients, 11 (55%) with GS 6 after RP and 9 (45%) with GS 7. Cytoplasmatic nor nuclear NF-κB p65 expressions were not significantly associated with pathological stage, positive surgical margin and postoperative GS. Patients with positive
cytoplasmic NF-kB reaction had significantly more BP compared to those with negative cytoplasmic NF-kB reaction with PSA 0,2 ng/ml as cutoff point (p=0,015) and a trend towards more BP with PSA ≥ 0,05 ng/ml as cutoff point (p=0,068).

Conclusions. Cytoplasmic expression of NF-κB is associated with more BP and might be an independent prognostic factor for recurrence-free survival (RFS), but further studies including larger patient cohorts are needed to confirm these initial results.

Introduction

Prostate cancer (PCa) is the most common cancer in men in developed European countries, particularly those with a high proportion of elderly population, with incidence rate up to 189 per 100.000 (1). In last two decades the disease has emerged as the most frequent cancer amongst men following rapid increases in the detection of a substantial number of early-stage PCa, particularly due to the increased number of PSA testing. Recognizing that the expected men’s life is obviously increasing, PCa also means increasing financial burden for individual countries (2). Majority of men newly diagnosed with PCa will be candidates for primary curative therapy, either with radical prostatectomy (RP) or radiation, but many PCa, however, are low-grade, even indolent and the number of newly diagnosed PCa far outnumbers the number of lethal cases. Indolent PCa may exist for a long period without causing any symptoms or death, so the prediction of low risk and indolent PCa is needed to avoid overtreatment by unnecessary invasive therapies, and select men for active surveillance (AS) (3–5).

The Nuclear factor-Kappa Beta (NF-κB) family of transcription factors plays a crucial role in inflammation as well as in the development and progression of cancer. Extensive evidence indicates that the NF-κB pathway is implicated in controlling the
expression of genes involved in cell survival, proliferation, angiogenesis, and invasion (6). Many studies indicate that activation of NF-κB signaling in PCa cells correlates with PCa progression, including chemoresistance, advanced stage, biochemical progression (BP), and metastatic spread (7–11). NF-κB is critical for human health, and aberrant NF-κB activation contributes to development of various autoimmune, inflammatory and malignant disorders including rheumatoid arthritis, atherosclerosis, inflammatory bowel diseases, multiple sclerosis and malignant tumors (12). Despite the growing evidence for a role of NF-κB in prostate tumorigenesis and resistance to therapy, the mechanisms underlying the activation of NF-κB in PCa remain only partially understood.

The main goal of our study was to find out whether the immunohistochemical expression of NF-κB p65 in biopsy samples with Gleason score 3 + 3 = 6 (GS 6) is inversely correlated with prostatic carcinoma indolence.

Materials and Methods

Our study was based on a retrospective cohort of 178 PCa patients whose archived formalin-fixed and paraffin-embedded (FFPE) prostate needle biopsy specimens were used for additional immunohistochemistry staining. All consecutive patients underwent the extraperitoneal laparoscopic RP (ELRP) or “nerve-sparing” extraperitoneal laparoscopic RP (N-S ELRP) without lymph node dissection between 2006 and 2012 as a first treatment of PCa. All patients were followed-up for at least five years after surgery at Department of Urology in General Hospital Slovenj Gradec. Hospital patient’s files were used for clinical data.

The inclusion criteria were total PSA ≤ 10 ng/ml, number of biopsy specimen ≥ 8, histopathological result of prostate cancer GS 6 on biopsy and T1/T2 estimated
clinical stage (Table 1). The exclusion criterion was the presence of chronic diseases in which the activation of NF-κB is common. After the screening review of the clinical data and histopathological revision of prostate needle biopsy specimens, done by two unrelated pathologists with extensive experience in PCa, 15 patients were excluded from the study, as they did not meet the criterion of GS. During the additional microtome cutting of archived FFPE prostate needle biopsy specimens for immunohistochemistry due to the lack of the tissue, another 40 patients were excluded. The final analysis in this study involved 123 patients regarding the postoperative stage, surgical margins and GS (Table 2). Based on a PSA levels, two BP were defined, at PSA cutoff point ≥ 0.05 ng/ml and ≥ 0.2 ng/ml, 6 months or more after radical prostatectomy. Recurrence-free survival (RFS) was defined as the period between the surgery and BP (i.e. first increase of PSA above one or both PSA cutoff points). 5 patients were excluded due to the initiation of hormonal treatment immediately after surgery, so regarding the BP 118 patients were analyzed (Table 3).

For control group archived FFPE prostate needle biopsy specimens from 60 patients with PCa GS 7, 30 with 3 + 4 and 30 with 4 + 3, were used.

Study was approved by the Slovene National Medical Ethics Committee.

TISSUE PREPARATION AND IMMUNOHISTOCHEMICAL STAINING

IHC staining for detection of NF-κB p65 was performed on 2–4 μm FFPE tissue sections, dried at 56°C for 2 hours, using fully automated IHC system Ventana Benchmark XT (manufacturer Ventana ROCHE inc.). Epitope was retrieved on board employing heat-mediated epitope retrieval using high pH Cell Conditioning Solution 1 (cat No 950 – 124, manufacturer Ventana ROCHE inc.) for 88 minutes at 100°C. Epitope was detected using commercially available mouse monoclonal antibody NF-
κB p65 (clone F-6; cat No sc-8008; manufacturer Santa Cruz Biotechnology inc.)
directed against amino acids 1-286 of NF-κB p65 of human origin. Primary antibody
was diluted 1:200 using DAKO REAL™ antibody diluent (cat No S2022; manufacturer
DAKO Agilent technologies inc.) and incubated on board for 60 minutes at 37°C.
Primary antibody was visualized using 3-step multimer detection system OptiView
DAB IHC Detection Kit (cat No 760 – 700; manufacturer Ventana ROCHE inc.)
according to manufacturer’s instructions.

IMMUNOHISTOCHEMICAL ANALYSIS

The staining was analysed by pathologist with extensive experience in PCa who was
not familiar with patient’s clinical data. For nuclear staining, positive result was
reported when at least 5% nuclei of cancer cells showed unequivocal brown
coloration (13). To consider reaction as positive, nuclear brown coloration should
exceed the effect of cytoplasmatic overlapping. The intensity of cytoplasmatic
staining was assessed as negative, weak, moderate and strong, and for statistical
analysis grouped as negative (negative, weak) and positive (moderate, strong) (14).

STATISTICAL ANALYSES

Clinical, laboratory and pathological characteristics were summarized using
frequency and percentage for categorical variables, and mean and range for
continuous variables. RFS was calculated from the time of primary tumour excision,
and was censored at the last contact date if there were no events. Survival curves
were calculated by Kaplan-Meier’s method and tested for statistical significance
using log-rang test. Association of NF-kB expression status with pathological
findings was tested using Chi-square test. The differences were considered
statistically significant if the p values were less than 0.05. Software package SPSS
22.0 for Windows was used.

Table 1
Patient characteristics

| Characteristic                        | Value (Range)             |
|--------------------------------------|---------------------------|
| N = 123                              |                           |
| Mean Age, year, (range)              | 63.6 (50–75)              |
| Mean Initial PSA, ng/ml, (range)     | 5.32 (1.32–9.51)          |
| Mean Prostate volume, ml, (range)    | 38.3 (14–97)              |
| Mean Biopsy cores, n, (range)        | 9.6 (8–10)                |
| Total Biopsy cores, n                | 1.180                     |
| Clinical stage                       |                           |
| T1, n, (%)                           | 113 (91.9)                |
| T2, n, (%)                           | 10 (8.1)                  |
| Biopsy GS 3 + 3 = 6, n, (%)          | 87 (70.7)                 |
| Surgery                              |                           |
| ELRP, n, (%)                         | 36 (29.3)                 |
| N-S ELRP, n, (%)                     |                           |

Table 2
Pathological results after radical prostatectomy

| Classification | N of total patients | % of total patients |
|----------------|---------------------|---------------------|
| pT            |                     |                     |
| T2            | 96                  | 78.0                |
| T3            | 27                  | 22.0                |
| Surgical margins |                | 10.6                |
| Positive      | 110                 | 89.4                |
| Negative      | 79                  | 64.2                |
| Gleason score |                     | 30.1                |
| 3 + 3 = 6     | 37                  | 5.7                 |
| 3 + 4 = 7     | 7                   |                     |
| 4 + 3 = 7     |                     |                     |

Table 3
Biochemical progression after radical prostatectomy

| Classification                      | N of total patients | % of total patients | GS 3 + 3 = 6 | GS 3 + 4 = 7 |
|-------------------------------------|---------------------|---------------------|--------------|--------------|
| Biochemical progression             |                      |                     |              |              |
| PSA 0.05–0.19 ng/ml                 | 14                  | 11.8                | 8            | 6            |
| PSA ≥ 0.2 ng/ml                     | 6                   | 5.1                 | 3            | 3            |

Results

Tables 1 and 2 show patients and pathological characteristics and Table 3 shows BP in patients after RP. Postoperative pathological stage 3 was noticed in 27 (22%) and positive surgical margins were detected in 13 patients (10.6%). BP (PSA ≥ 0.05 ng/ml) occurred in 20 (16.9%) patients (11 with GS 6 after RP and 9 with GS 7). Among them, postoperative PSA ≥ 0.2 ng/ml was detected in 6 patients (5.1%), with GS 6 in 3 and GS 7 in 3. Positive cytoplasmic NF-κB staining was detected in 173 (56.9%) and positive nuclear NF-κB staining in 57 (18.7%) of the 304 analyzed biopsy cores with GS 6.
CYTOPLASMATIC NF-κB STAINING

Cytoplasmatic NF-κB p65 expression was not corelated with pathological stage, positive surgical margin and postoperative GS (Table 4). Cytoplasmatic NF-κB p65 expression was significantly more common in BP with PSA cut off point ≥ 0,2 ng/ml (P = 0,015) and there was a trend towards BP with PSA cut off point ≥ 0,05 ng/ml (P = 0.068) (Fig. 1). Cytoplasmatic NF-κB p65 expression was positive in 57/60 control group patients with GS 7 (Table 5).

| Table 4 |
| --- |
| Association of NF-κB p65 expression status in cytoplasm with pathological findings |
| N = 123 | N of total patients | NF-κB p65 expression | P value |
| | | negative | positive |
| pT2 | 96 | 48 | 48 |
| pT3 | 27 | 12 | 15 |
| Negative surgical margins | 110 | 53 | 7 |
| Positive surgical margins | 13 | 7 | 6 |
| GS 3 + 3 = 6 | 79 | 39 | 40 |
| GS 3 + 4 = 7 | 37 | 17 | 20 |
| GS 4 + 3 = 7 | 7 | 5 | 2 |

*pT2 versus pT3, **negative versus positive surgical margin, ***3 + 3 = 6 versus 3 + 4 = 7 versus 4 + 3 = 7

NUCLEAR NF-κB STAINING

Nuclear NF-κB p65 expression was not associated with pathological stage, positive surgical margins and postoperative GS (Table 6), neither with BP (Fig. 2) and did not differ from control group patients with GS 7 (Table 7).
### Table 6

Association of NF-κB p65 expression status in nucleus with pathological findings

|             | N of total patients | NF-κB p65 expression | P value |
|-------------|---------------------|----------------------|---------|
|             | pT2                 | pT3                  |         |
| Negative surgical margins | 96                  | 27                   | 81      | 15 |
| Positive surgical margins | 110                 | 13                   | 92      | 18 |
| GS 3+3 = 6 | 7                   | 6                    | 66      | 2 |
| GS 3+4 = 7 | 7                   | 6                    | 31      | 13 |
| GS 4+3 = 7 | 7                   | 6                    | 6       | 1 |
|             | N=123               |                       |         |

* pT2 versus pT3, **negative versus positive surgical margin, ***3 + 3 = 6 versus 3 + 4 = 7 versus 4 + 3 = 7

### Table 7

Nuclear NF-κB p65 expression status in biopsy group GS 6 and control biopsy group GS 7

|             | N of total patients | NF-κB p65 expression | P value |
|-------------|---------------------|----------------------|---------|
|             | 3+3 = 6             | 3+4 = 7              | 4+3 = 7 |
| negative    | 123                 | 103                  | 17      | 20 |
| positive    | 30                   | 24                   | 13      |
|             | N=103               |                       |         |

* 3+3 = 6 versus 3+4 = 7, **3+3 = 6 versus 4+3 = 7

### Discussion

Increase in incidence of PCa since 1990s mostly starts with PSA testing, either in the form of all types of screening or on the basis of a suspicious digital rectal examination. Nevertheless, the most important part of diagnostic procedure is accurate histopathologic diagnosis, particularly in low-risk PCa where AS could be an option (15). Despite the fact that in our study biopsy samples were evaluated by two experienced uropathologists, we recorded the postoperative upgrade of GS 6 to GS 7 in 44 patients (3+4 in 37 and 4+3 in 7), so biopsy under-grading was present in 35.8%. This is in concordance with literature reports where GS from needle biopsies underestimates the GS of the radical prostatectomy specimen in 28-57% (16). Postoperative pathological stage 3 was noticed in 27 (22%), positive surgical
margins were detected in 13 (10.6%) and clinically significant BP (PSA ≥ 0.2 ng/ml) in 6 (5.1%) patients.

Molecular biomarkers offer the possibility to further stratify patients with similar clinicopathological parameters. Domingo-Domenech and Ross reported that tumors with nuclear NF-κB expression and no additional risk factors (i.e. low GS and low preoperative PSA) had the lowest rate of biochemical recurrence in the nuclear NF-κB positive group (9,10). In addition to nuclear staining we included also the NF-κB cytoplasmic staining and contrary to reported results found that only the cytoplasmic NF-κB variable remained significantly associated (p = 0.015) with worse RFS (PSA ≥ 0.2 ng/ml). The p65 subunit of NF-κB was expressed in the cytoplasm of 173 (56.9%) biopsy cores with GS 6. Only 57 (18.7%) biopsy samples also showed a nuclear staining of NF-κB p65, suggesting a constitutive activation of NF-κB in these tissues. The relationship between NF-κB expression and the risk of BP was assessed using Kaplan-Meier disease outcome analysis. Using a bimodal approach, the presence or absence of NF-κB nuclear staining was not associated with worse RFS. In the present study, NF-κB expression and its subcellular localization were highly variable among different specimens. In another study, nuclear NF-κB was found in 40% of PCa in 40 specimens assayed (8,13). As in the current study, nuclear NF-κB did not significantly correlate with GS. The functional relevance of this immunoreactivity on NF-κB activation is yet unknown. This limitation is based on the fact that p65/NF-κB nuclear translocation is necessary but not sufficient for NF-κB induced transcriptional activity, since both recruitment of NF-κB to target genes and NF-κB-induced transcriptional events after recruitment are needed for this to occur. There should be also noted that the minimum percentage of tumor cells with nuclear p65 staining required to potentially result in detectable NF-κB-induced
transcriptional activity remains uncharacterized. An important limitation of our study is a significant reduction in the size of diagnostic biopsies during microscopic reevaluation and diagnosis of PCa with GS 6 (13,14).

In our group of 20 patients who developed the BP with PSA cutoff point ≥ 0,05 ng/ml cytoplasmic NF-κB staining was detected in 14 (70%) and nuclear NF-κB staining in only 1 (5%), while among those 6 patients who had the BP with PSA cutoff point ≥ 0,2 ng/ml cytoplasmic NF-κB staining was noticed in all 6 (100%) and nuclear NF-κB staining in 1 (16,7%). Among several available selection criteria for AS worldwide, at our institution the EAU AS guidelines were used (17). According to them all patients in our cohort had initial PSA below 10 ng/ml, biopsy GS 6, estimated clinical stage T1c-T2 and 82 of them (66,7%) had ≤ 2 positive cores on biopsy. From clinical point of view the BP of PCa after RP is defined with PSA ≥ 0,2 ng/ml (18). In our group of 6 patients with BP at PSA cutoff point ≥ 0,2 ng/ml, positive cytoplasmic NF-κB staining was present in all 6 and all AS criteria were met in 5 (83,3%) patients. There were no positive nuclear NF-κB staining in any of these 5 patients. As a control group we used 60 patients with biopsy GS 7 and positive cytoplasmic NF-κB staining was present in 57 (95%) of them. Since patients with biopsy GS 7 are not candidates for AS according to EAU AS guidelines there is no need for additional prognostic factor (i.e.positive cytoplasmic NF-κB staining). However, in patients with biopsy GS 6, an additional prognostic factor is needed in order to stratify these patients to a group where only AS is enough. According to our results positive cytoplasmic NF-κB staining could be a negative predictive factor for the GS 6 PCa indolence and these patients are candidates for primary curative therapy.

There are several limitations of our study. Most importantly, all patients underwent surgical treatment, so the significance of NF-kB activation on prostate needle biopsy
samples for disease progression was found only indirectly, based on BP and is most probably underestimated. Another important limitations are small number of patients and single institution results.

Conclusion

Cytoplasmic expression of NF-κB is associated with worse RFS, while it is not significantly associated with standard prognostic factors and as such might be an independent prognostic factor for RFS, but further studies including larger patient cohorts are needed to confirm these initial results.

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17.
Declarations

Ethics approval and consent to participate

Study was approved by National Medical Ethics Committee of Republic Slovenia, Nr. 109/10/14, from 14.9.2014.

Consent for publication

Not applicable.

Availability of data and materials

The datasets analysed during the current study available from the corresponding author on reasonable request.

Competing interests

"The authors declare that they have no competing interests" in this section.

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Authors' contributions

MZ as PhD student suggested designing of the study, collected clinical data, analysed the results and drew conclusions from the study. BP and SC as two unrelated pathologists performed histopathological examinations of prostate tissue specimens. BG and PD performed prostate needle biopsy specimens preparation and immunohistochemical staining. MH advised on the design of the study and supervised the study work as the main mentor. AP performed statistical analysis of
the study.

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Figures

**Figure 1**

RFS in patients with positive and negative NF-κB p65 expression status in cytoplasm (N = 118)

**Figure 2**

RFS in patients with positive and negative NF-κB p65 expression status in nucleus (N = 118)
Figure 3

Immunohistochemistry of NF-κB p65

GS 6: positive cytoplasmatic staining (A), positive nuclear staining (B); GS 7: positive cytoplasmatic staining (C), positive nuclear staining (D)