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ORIGINAL ARTICLE

Primary High-Grade Non-Muscle-Invasive Bladder Cancer: High NFκB Expression in Tumor Specimens Distinguishes Patients Who are at Risk for Disease Progression

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Abstract To investigate the potential prognostic role of NFκB expression in primary high-grade non-muscle-invasive bladder cancer. Patients with primary high-grade non-muscle-invasive bladder cancer who received induction and maintenance BCG therapy were retrospectively included. Recurrence and progression were histologically proven. Intensity and extent of immunochemistry were assessed. The final evaluation of the NFκB staining was done by combining intensity and extent as product and expressing it as low NFκB expression or high NFκB expression . Epidemiological, pathological, clinical parameters and NFκB expression were statistically analyzed for recurrence (REC), progression (PR), recurrence-free survival (RFS) and progression-free survival (PFS). NFκB is significantly associated with disease progression ($p < 0{,}001$ in univariate analysis and $p = 0{,}001$, Odds Ratio = 14,484, 95% Confidence Interval = 3187-65,821 in multivariate analysis), but not with recurrence. The median value of NFκB expression as product is significantly higher for the patients with progression in comparison to patients with recurrence only ($p = 0{,}003$) and patients without recurrence or progression ($p = 0{,}001$). Patients' age is significantly associated ($p = 0{,}001$ in univariate analysis and $p = 0{,}003$, Odds Ratio = 1273, 95% Confidence Interval = 1086–1492 in multivariate analysis) with disease recurrence. High NFκB expression in primary high-grade non-muscle-invasive bladder cancer, treated with postoperative intravesical BCG immunotherapy, could represent an unfavorable prognostic factor.

Keywords NFκB · High-grade · Non-muscle-invasive · Bladder cancer · Progression; BCG

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Introduction

Bladder cancer is the 11th most common cancer and the 14th leading cause of cancer deaths [1]. About 75% of patients are diagnosed with a disease which infiltrates only the mucosa or the submucosa, and not the muscle of the bladder wall. Intravesical instillation of Bacillus Calmette-Guérin (BCG), including induction and maintenance scheme, is the most effective adjuvant treatment for high-grade non-muscle-invasive bladder cancer (HGNMIBC) patients [2]. However, approximately 30% of these tumors recur after the standard induction schedule and 12% (0-35%) become muscle-invasive [3]. Radical cystectomy is an effective treatment for patients in whom BCG therapy has failed. Within this context, efforts have been made to identify the subgroup of patients who will not respond to BCG and who could have a

maximized clinical benefit from the implementation of an early aggressive therapy [4–6].

Several studies examined the potential prognostic role of many factors, including molecular, pathological and immunological ones, for HGNMIBC patients but only few have focused on patients treated with BCG [7]. Some of these factors have been well studied while others have displayed a controversial prognostic role.

A key mediator of the inflammation is the nuclear factor-κB (NFκB). Additionally to inflammation, NFκB contributes to tumorigenesis by inhibiting apoptosis and promoting angiogenesis and metastasis. NFκB is a family of transcription factors, whose members share a Rel homology domain (RHD), subjecting them to a regulation which is focused on a nuclear–cytoplasmic shuttling [8]. The RHD is the dimerization and DNA-binding domain, contains the nuclear localization signal (NLS) and is the binding site of NFκB inhibitors [9]. The NFκB family members are: p50/NFκB1, p52/NF-κB2, c-Rel, RelB and p65/RelA [9]. RelA, RelB and c-Rel are synthesized as mature products and contain transactivation domains. The mature proteins of p50 and p52 are a result of proteolysis and cannot activate transcription on their own. At rest NFκB is localized in cytoplasm and consists of three subunits: p50 and p65 and the inhibiting subunit IκBa, which is bound to p65 [9]. NFκB activation may be caused by several cytokines, mitogens, oxidative stress, cell damage and viral infections [10, 11]. Upon cell activation, the kinase IκB kinase (IKK) phoshorylates IκBa making it a target for degradation via a ubiquitin ligase complex. This permits the liberation of the NLS of NFκB and consequently its translocation in the nucleus [9]. Activated NFκB regulates the transcription of more than 200 genes including anti-apoptotic ones, cell cycle regulators, angiogenic factors and genes which encode adhesion molecules and inflammatory cytokines [9].

The purpose of our study is to define the potential prognostic significance of epidemiological, pathological and clinical parameters as well as of NFκB expression for HGNMIBC patients who were treated postoperatively with intravesical BCG immunotherapy.

Materials and Methods

We retrospectively included patients with transurethrally (TUR) resected, histologically proven, primary, single or multiple, non-muscle-invasive, high-grade, transitional-cell carcinoma of the urinary bladder. The follow-up period started on the day of the first postoperative follow-up cystoscopy and ended on the day of the last follow-up visit or on the day of a patient's death. Exclusion criteria were a present or previous history of upper urinary tract carcinoma and the muscle-invasive disease.

A single immediate postoperative intravesical instillation of chemotherapy (Epirubicin 50 mg) was administered to each patient, if there was no contraindication. Random biopsies of the bladder were performed, if there was a suspicion of Tis (carcinoma in situ) lesion. Whenever biopsy specimen did not contain muscle or tumor resection was incomplete, a TUR was repeated within 4-6 weeks. All patients received an induction course of 6 weekly intravesical BCG instillations and maintenance BCG therapy [12]. The follow-up based on the Guidelines for Non-muscle-invasive bladder cancer by the European Association of Urology/EAU (GEAU) [1].

Any histologically established change from Ta to at least T1 and from T1 to at least T2 stage as well as the presence of Tis, at any point, in histologic reports which were derived from patients with no previous Tis diagnosis was set as progression . The unsuccessful treatment with BCG determined as: BCG failure or BCG-refractory tumor or high-grade recurrence after BCG or BCG intolerance [1] and we made treatment decisions concerning BCG failure and recurrences after BCG according to the GEAU [1].

Patients' Group 1 included consecutive patients with disease recurrence ± progression and Group 2 patients who had neither recurrence nor progression. Group 2 consisted of selected patients with matched baseline characteristics (at diagnosis of bladder cancer and before the beginning of any treatment) with the patients of Group 1.

The pathology staging (TNM classification of malignant tumors UICC, International Union Against Cancer, 7th edition, 2010) and grading (classification of World Health Organization/WHO, 2004) were evaluated by one pathologist specialized on genitourinary tract tumors. Representative samples of good morphology and antigenicity of the primary tumors were obtained for immunohistochemical staining with a mouse monoclonal antibody for NFκB (against the p65 subunit, clone F-6, dilution 1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Staining was performed in the same run, in order to preclude run to run differences in intensity. Staining intensity (SI) of NFκB on a high-power field was classified according to an arbitrary four-tiered scale (negative = 0, mild = 1, moderate = 2, strong = 3) in a manner consistent with previous investigations [13, 14]. The extent of staining (SE), defined as percentage of area which was occupied by positively stained tumor cells, was classified according to an arbitrary two-tiered scale (low positivity = staining area < 70%, high positivity = staining area ≥ 70%). The final evaluation of staining was done by applying the product P of SI and SE as follows: P = (SI) x (SE). The possible values of P are: 0-300. P values <140 and P values ≥ 140 were defined as low NFκB expression (LE) and high NFκB expression (HE) respectively. LE is expected to be associated with a good clinical outcome (neither recurrence nor progression), while HE is expected to be associated with a bad clinical outcome (either recurrence or progression). HE

and LE are shown in Figs. 1 and 2 respectively and they were taken into account for the definitive statistical analysis.

Epidemiological (age, gender, smoking), pathological (stage T, concomitant carcinoma *in situ*/Tis) and clinical parameters (number of tumors, tumor size, patient group) as well as NFκB expression, evaluated as P , were analyzed for disease recurrence (REC), disease progression (PR), recurrence-free survival (RFS) and progression-free survival (PFS).

Univariate and multivariate analyses for REC and PR were based on Chi-Square or Fisher's Exact test and multiple logistic regression respectively. Univariate analysis for RFS and PFS was performed using Log-rank test for categorical variables and Cox regression for scale variables. Multivariate analysis for RFS and PFS was assessed using Cox regression analysis after checking the proportional hazards assumption. We used the independent-samples Kruskal Wallis test to investigate whether the median values of P were significantly different between patients who had recurrence and progression, patients who had only recurrence and patients who had neither recurrence nor progression. The level of statistical significance was set as $p \leq 0.05$. The IBM SPSS Statistics version 21 software was used for the statistical analyses.

The study was approved by the Ethics Committee-Scientific Board of the University Hospital of Larissa and it conforms to the provisions of the Declaration of Helsinki (as revised in Tokyo 2008).

Results

We totally included 80 patients, 40 patients in Group 1 and 40 patients in Group 2. Baseline characteristics for the total number of patients as well as for the patients of Groups 1 and 2 are shown in Table 1.

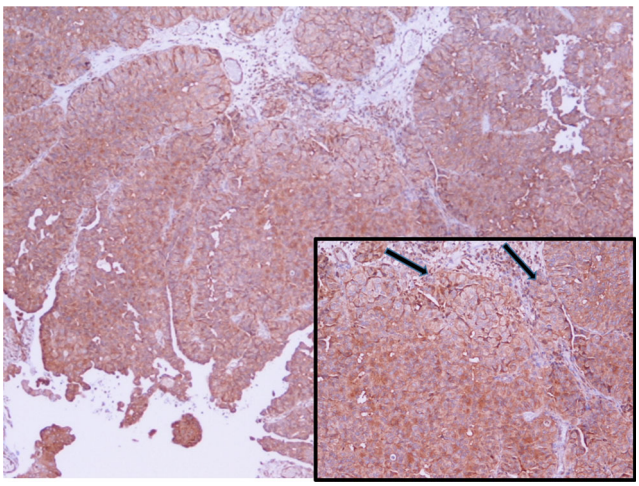

Fig. 1 High-grade papillary urothelial carcinoma, stage pT1 (arrows). High expression HE of NFkB immunostain. NFkB ×100 and NFkB ×400 (inset)

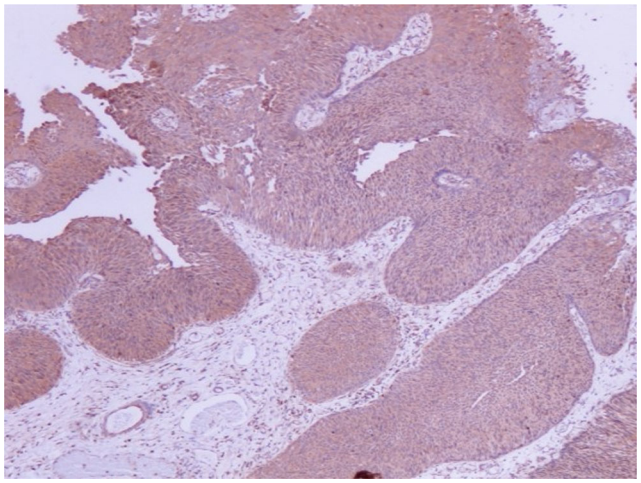

Fig. 2 High-grade papillary urothelial carcinoma, stage pTa. Low expression LE of NFkB immunostain. NFkB ×100

In Group 1, all patients had recurrence and the median RFS was 10.7 months. Twenty-four patients (24/40, 60%) had progression and the median PFS was 39.2 months. Twenty-four patients (24/40, 60%) died. Median follow-up was 62.7 months. In Group 2, no patient had either recurrence or progression by definition. Four patients (4/40, 10%) passed away. Median follow-up, median RFS and median PFS were 87.4 months.

We only observed cytoplasmic staining and the results of the immunostaining for the expression of NFκB as P are presented in Table 2.

In univariate and multivariate analyses, only the ΄΄HE΄΄ was statistically significantly associated (p < 0,001 and p = 0,001 respectively) with disease progression. In particular, patients with HE had a worse clinical outcome, meaning an increased odds ratio of disease progression during the entire observation period (p = 0,001, Odds Ratio/OR = 14,484, 95% Confidence Interval/CI = 3187–65,821), in comparison with patients with LE .

In univariate analysis, only the ΄΄HE΄΄ was statistically significantly associated with PFS (p = 0,001). In particular, patients with HE had a worse clinical outcome, meaning worse progression-free survival, when they were compared with patients with LE during the entire observation period, as it is also shown in Fig. 3 by Kaplan-Meier curves. In multivariate analysis, none of the studied parameters was statistically significantly associated with PFS.

The patients who had recurrence and progression score a median value of 155 (40-270) in P (Fig. 4**)**. *This value is statistically significantly higher (p = 0,003) in comparison to patients who had only recurrence, whose median value is 120* (60-140). *This value is also statistically significantly higher (p = 0,001) in comparison to patients who had neither recurrence nor progression, whose median value is 100* (40-210).

Table 1 Baseline characteristics for patients of Groups 1, 2 as well as for their total number

	TOTAL (n = 80)	GROUP 1 (n = 40)	GROUP 2 (n = 40)	p value
Median age (years)	67.5 ± 8.7 (38-87)	68.5 ± 7.2 (58-87)	64 ± 9.3 (38-77)	0.758 (*)
Gender (♂/♀)	66/14 (82.5%/17.5%)	34/6 (85%/15%)	32/8 (80%/20%)	0.345
Smoker (Yes/No/Ex)	40/13/27 (50%/16.3%/33.7%)	20/5/15 (50%/12.5%/37.5%)	20/8/12 (50%/20%/30%)	0.117
Number of tumors (Single/Multiple)	42/38 (52.5%/47.5%)	20/20 (50%/50%)	22/18 (55%/45%)	0.464
Tumor size (>3 cm/<3 cm)	39/41 (48.8%/51.2%)	21/19 (52.5%/47.5%)	18/22 (45%/55%)	0.361
Concomitant carcinoma *in situ* (Yes/No)	16/64 (20%/80%)	8/32 (20%/80%)	8/32 (20%/80%)	0.637
Tumor stage T (Ta/T1)	15/65 (18.8%/81.2%)	7/33 (17.5%/82.5%)	8/32 (20%/80%)	0.494

p values: Pearson Chi-Square test, (*): T-test

Only the age of patients was statistically significantly associated, in univariate (p = 0,001) and multivariate analyses (p = 0,003, OR = 1273, 95% CI = 1086-1492), with disease recurrence.

In univariate analysis, only age was statistically significantly associated with RFS (p = 0,013). In multivariate analysis none of the studied parameters was statistically significantly associated with RFS.

Discussion

The high-grade, non-muscle-invasive bladder cancer is a high-risk disease [1]. Its natural history is characterized by its tendency to recur and to progress to muscle invasiveness, despite the initial high rate of response to postoperative BCG treatment [15]. A reliable prognosis is difficult to be made, because of the heterogeneity of the tumor. Hence, it is a clinical challenge to identify validly and on time the patients who will not respond to BCG adjuvant intravesical therapy and whose progression and recurrence will occur sooner. Nevertheless, prognosis is important for all patients with high-risk bladder cancer treated with BCG. European Organization for Research and Treatment of Cancer (EORTC) has proposed risk tables for predicting recurrence and progression, after taking into account the six most significant clinical and pathological factors [16]. Although the prognostic value of the EORTC scoring system was confirmed by data from other studies [1], it has not achieved a universal and unanimous acceptance yet. Many molecular markers have been proposed for stratifying patients in terms of clinical course and outcome [9]. Till today no marker has earned a global acceptance and implementation though.

Nakshatri H et al. compared the activity of NFκB in estrogen receptor (+) and estrogen receptor (−) cell lines of human breast cancer and they found that NFκB was constitutively active in the estrogen receptor (−) cell lines [17]. A study with the human colon cancer cell line SW48 and a study with thyroid carcinoma cell lines showed the presence of activated NFκB [18, 19]. Moreover, the activated NFκB was associated with a negative prognostic value in immunohistochemical studies on gastric carcinoma [20, 21]. Another immunohistochemical study on pancreatic adenocarcinoma documented the NFκB activation [22]. All these findings indicate that NFκB seems to be constantly activated and to have an intense potential of transactivation in many types of human malignancy [9]. Furthermore, nuclear translocation was reported to be notably higher in prostatic, gastric and colon adenocarcinoma cells when compared to normal ones [13, 18, 21]. Immunohistochemical studies [13, 23] as well as studies with prostate cancer cell lines [24, 25] report an aberrant activation of NFκB.

Regarding bladder cancer Levidou et al., applying immunohistochemistry in a cohort of 116 bladder cancer patients, found a mainly nuclear p65/RelA immunoexpression in the majority of studied tumors [9]. In another study, based also on

Table 2 The results of the immunostaining for the NFκB expression as product (stain intensity x stain extent)

	Immunostaining score for NFκB as Product = Stain intensity x Stain extent	
	Low NFκB expression (Product < 140%)	High NFκB expression (Product ≥ 140%)
TOTAL GROUP (n = 80)	57 (71.3%)	23 (28.7%)
GROUP 1 (n = 40)	25 (62.5%)	15 (37.5%)
Group 2 (n = 40)	32 (80%)	8 (20%)

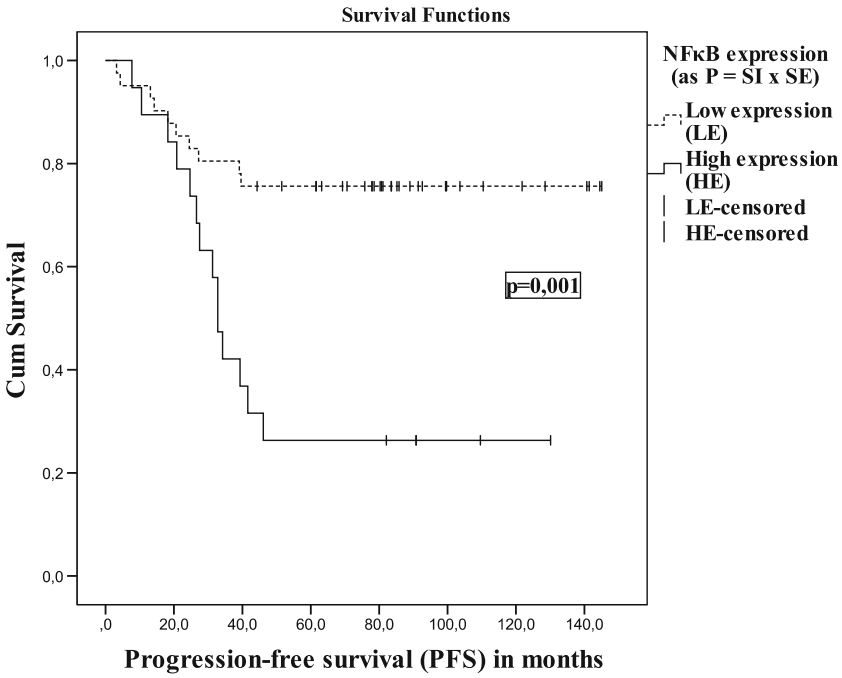

Fig. 3 Kaplan-Meier curves for progression-free survival (PFS). Patients with a high NFκB expression (HE) had a worse clinical outcome (p = 0,001), meaning worse progression-free survival, in comparison with patients with a low NFκB expression (LE) during the entire observation period. (NFκB expression as product P = Staining intensity/SI x Staining extent/SE)

immunohistochemistry, Xie DH et al. reported a higher NFκB expression in bladder urothelial carcinoma cells in comparison to cells of non-neoplastic mucosa [26]. *To our knowledge our study is the first one exploring the potential association of NFκB expression in primary HGNMIBC, treated postoperatively with intravesical BCG, with prognostic parameters*. This entity is considered as a high-risk one for disease recurrence and progression. Consequently, it has a unique clinical interest. *We found that the high expression of NFκB associates significantly with progression but not with recurrence and it has additionally a negative impact on PFS for these specific patients. We consider this finding as important, because it allows us to identify within a group of high-risk patients those who are at greatest risk for bladder cancer progression*. We can postulate that this particular subgroup of patients, meaning patients whose tumors are characterized by the NFκB high expression, could be benefited by implementing an early radical therapy (cystectomy and urinary diversion). Finally, according to our results the patient's age is the most important prognostic factor for recurrence, because it increases the odds

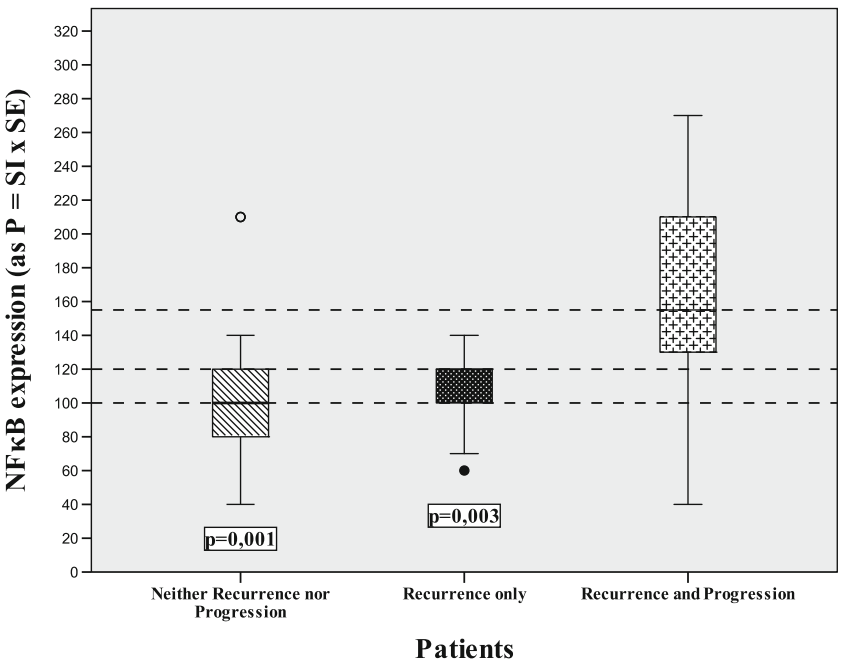

Fig. 4 Boxplot showing the comparison of the median values of NFκB expression as product P (P = SI x SE) between patients who had recurrence and progression, median value equal to 155 (40-270), patients who had only recurrence, median value equal to 120 (60-140), and patients who had neither recurrence nor progression, median value equal to 100 (40-210). Patients who had recurrence and progression have a statistically significantly higher expression of NFκB in comparison to patients who had only recurrence ($p = 0,003$) and in comparison to patients who had neither recurrence nor progression (p = 0,001)

for recurrence and it also has a negative effect on RFS. For this reason, we should keep a strict follow-up program for our elderly HGNMIBC patients.

The potential limitations of our study are its retrospective nature and the small number of enrolled patients. The non-observation of positive nuclear NFκB immunostaining (PNNI) by us creates the impression of a potential antithesis to the abovementioned finding by Levidou et al. [9]. Regarding this point, Levidou et al. comment that bladder tumors of advanced stage T and advanced histological grade demonstrate fiercer PNNI [9]. The fact that we did not enroll patients whose disease combined these two features may explain that we did not detect PNNI. We could hypothesize that the translocation of NFκB in the nucleus, hence the observation of PNNI, dictates an advanced stage (≥pT2) in high-grade tumors. On the other hand, the advantages of our study are the homogeneity of the enrolled patients, the relatively long duration of follow-up period, the use of the product for the assessment of the staining transcends the current heterogeneity in the evaluation of NFκB immunostaining in literature and the immunostaining grading by a pathologist specialized on genitourinary tract malignancies. The current literature on NFκB protein expression in primary HGNMIBC before applying adjuvant BCG treatment is limited and provides no unanimity regarding the way of assessment of the immunohistochemical staining of NFκB. Using the P , we managed to incorporate two parameters (SI, SE) in a new one. Moreover, the evaluation of SI of NFκB was done in a manner consistent with previous investigations [13, 14]. The evaluation of SE was based on the study of Levidou et al. [9], which presented, to our knowledge, the largest cohort of bladder cancer patients in the literature. Levidou et al. used an arbitrary three-tiered scale according to the percentage of neoplastic cells with cytoplasmic staining (absent/low, moderate and extensive). We applied an arbitrary two-tiered scale (low positivity = staining area < 70%, high positivity = staining area ≥ 70%), representing the percentage of area which was occupied by positively stained tumor cells. HE combines a high value of SI (2, 3) with a high positivity of SE, resulting in the cut-off value of 140 . Using this specific cut-off value, we think that the NFκB-expression in our samples cannot be questioned. In addition, we statistically evaluated P as a binary variable, in order to enhance the power of the statistical analysis and to avoid grey zones like moderate . To our point of view, the use of HE and LE provides a more clear answer to the question Is the studied factor expressed in the specimens? . Lastly, SI is a parameter that contains subjectivity, which we believe that we minimized cooperating with a specialized-on-genitourinary-tract-malignancies pathologist.

HGNMIBC is an often disease which can recur or progress, despite the use of postoperative intravesical immunotherapy with BCG. So far, an adequate and safe prognosis, and especially for the patients who are at greater risk, is not achieved. Molecular markers could support the effort in this field. Within this text, our study detects an initial evidence for the usefulness of NFκB expression in HGNMIBC as an alert marker for disease progression. Further studies are indispensable in order to elucidate the potential role of NFκB and confirm or not our promising result.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest regarding the publication of this paper.

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