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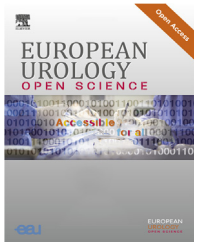

European Association of Urology

Bladder Cancer

Fibroblast Activation Protein-α and the Immune Landscape: Unraveling T1 Non–muscle-invasive Bladder Cancer Progression

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Abstract

Background and objective: The tumor microenvironment (TME) in non–muscle-invasive bladder cancer (NMIBC) plays an important role in the anticancer response. We aimed to identify the prognostic biomarkers in the TME of patients with NMIBC for progression to ≥T2.

Methods: From our institutional database, 40 patients with T1 high-risk NMIBC who progressed were pair matched for Club Urologico Español de Tratamiento Oncologico (CUETO) progression variables with 80 patients who never progressed despite longer follow-up. Progression was defined as ≥T2 or extravesical disease. Patients were treated at least with bacillus Calmette-Guérin (BCG) induction (five or more of six doses). Immunohistochemical (IHC) markers for the TME were used on tissue at first T1 diagnosis: CD8-PanCK, GZMB-CD8-FOXP3, CD163, PD-L1 SP142/SP263, fibroblast activation protein-α (FAP), and CK5-GATA3. Full tissue slides were annotated digitally. Relative marker area (IHC-positive area/total area) or density (IHC-positive cells per area; n/mm^2) was calculated, differentiating between regions of interest (ROIs; T1, Ta, and carcinoma in situ) and between compartments (stromal, epithelial, and combined). Differences in IHC variables were assessed using the *t* test, for continuous variables using analysis of variance and comparisons of more than two groups using Tukey's test. Conditional logistic regression for progression at 5-yr follow-up was performed with clusters based on pair matching.

Key findings and limitations: Only FAP expression (increase per 50%) in T1 (odds ratio [OR]: 1.33; 95% confidence interval [CI]: 1.04–1.70) and all ROIs combined (OR: 1.62; 95% CI: 1.14–2.29) correlated significantly with progression. None of the other clinicopathological/IHC variables correlated with progression.

Conclusions and clinical implications: FAP is a potential prognostic biomarker for progression in high-risk NMIBC. FAP is a marker for cancer-associated fibroblasts and

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is linked to immunosuppression and neoangiogenesis, which makes future investigation clinically relevant.

Patient summary: We found that progression of high-risk non–muscle-invasive bladder cancer to muscle-invasive disease is less in patients with lower fibroblast activation protein-α (FAP) expression, which is a marker for cancer-associated fibroblasts.

1. Introduction

T1 non–muscle-invasive bladder cancer (NMIBC) is a heterogeneous disease with still poorly predictable clinical behavior, due to the lack of reliable biomarkers to aid in patient counseling and treatment decisions [1]. Currently, there are no prognostic biomarkers for progression in clinical use, apart from clinicopathological models, which lack accuracy [2,3].

The tumor microenvironment (TME) plays an important role in the anticancer immune response [4]. This response requires infiltration of immune cells, such as cytotoxic T lymphocytes (CTLs), into the epithelial compartment of the tumor [5]. Furthermore, activation of CTLs seems important, as the sole presence of CTLs in the epithelial compartment did not predict response to immunotherapy, but coexpression of CTLs with granzyme B (GZMB) in the epithelial compartment did [6]. GZMB is a serine protease required for the cytotoxic effect of CTLs as part of the immune response.

In earlier work, we found that the presence of fibroblast activation protein-α (FAP) in T1 disease correlated with progression to ≥T2 bladder cancer (BC) [7]. FAP is a marker for cancer-associated fibroblasts (CAFs) and has been found to be prognostic for worse outcome in ≥T2 BC and other malignancies [8–11]. Basal and luminal molecular subtypes also correlate with clinical outcome in T1 NMIBC [12]. Other components of the TME and markers for immune activation have been identified as prognostic for clinical outcome in BC, such as regulatory T cells, tumor-associated macrophages, and PD-L1 [13].

Based on the unmet need for prognostic biomarkers in T1 NMIBC, the emerging data on the importance of the TME in BC, our earlier work on basal/luminal markers, and our recent data on FAP [7,14], we hypothesized that the TME, characterized by an immunohistochemical (IHC) panel of novel and known markers, might host prognostic biomarkers for progression. To that end, we designed a retrospective nested case-control study with high-grade (HG) T1 NMIBC patients who progressed and who were pair matched with patients who did not progress.

2. Patients and methods

We strictly adhered to the REMARK reporting recommendations for tumor marker prognostic studies (Supplementary material) [15]. Ethical approval was obtained from the Ethics Committee Research UZ/KU Leuven (internal number S59191) in accordance with the Declaration of Helsinki of 1964 and its later amendments, with a waiver of informed consent due to the retrospective nature and secondary use of tissue.

2.1. Study design

We performed a retrospective study of patients diagnosed with T1 HG disease between March 1993 and December 2010. Our primary clinical endpoint was progression, which was defined as ≥T2 disease at transurethral resection of bladder tumor (TURBT) during follow-up or development of extravesical disease on imaging during follow-up. We randomly selected 40 patients who progressed and pair matched them with 80 patients who never progressed despite having longer follow-up, for a case-control ratio of 1:2 [2]. The median follow-up time of nonprogressors was 8.3 yr (interquartile range [IQR]: 4.9, 10.7), and the median follow-up time of progressors was 3.4 yr (IQR: 2.0, 6.5). Matching variables were based on Club Urologico Español de Tratamiento Oncologico (CUETO) progression score variables: age (≤70 vs >70 yr), primary versus recurrent disease, number of tumors ($n \leq 3$ vs $n > 3$), and concomitant carcinoma in situ (CIS; yes vs no). Tumor stage and grade were not included as matching variables, as all patients were initially staged as T1 HG. Recurrence was defined as any stage disease recurrence at TURBT during follow-up. Clinical endpoints were calculated from the first diagnosis of T1 HG at TURBT. Selection of candidate variables for our model, sample size calculation, and assay methods can be found in the Supplementary material.

2.2. Patients

Patients with T1 HG NMIBC were included from our institutional NMIBC database. The inclusion criteria were the following: first diagnosis of T1 HG NMIBC at TURBT. Prior TURBTs and/or intravesical instillations for Ta low-grade or HG disease were allowed. The exclusion criteria were prior ≥T2 disease and presence of extravesical disease. All patients were treated with TURBT and received at least five or more of six doses of an initial bacillus Calmette-Guérin (BCG) induction course.

2.3. Specimen characteristics

Hematoxylin-eosin (H&E) slides and formalin-fixed paraffin-embedded (FFPE) blocks were retrieved from the first diagnosis of T1 HG NMIBC at TURBT. Twelve serial slides were cut from the FFPE block of each patient. A panel

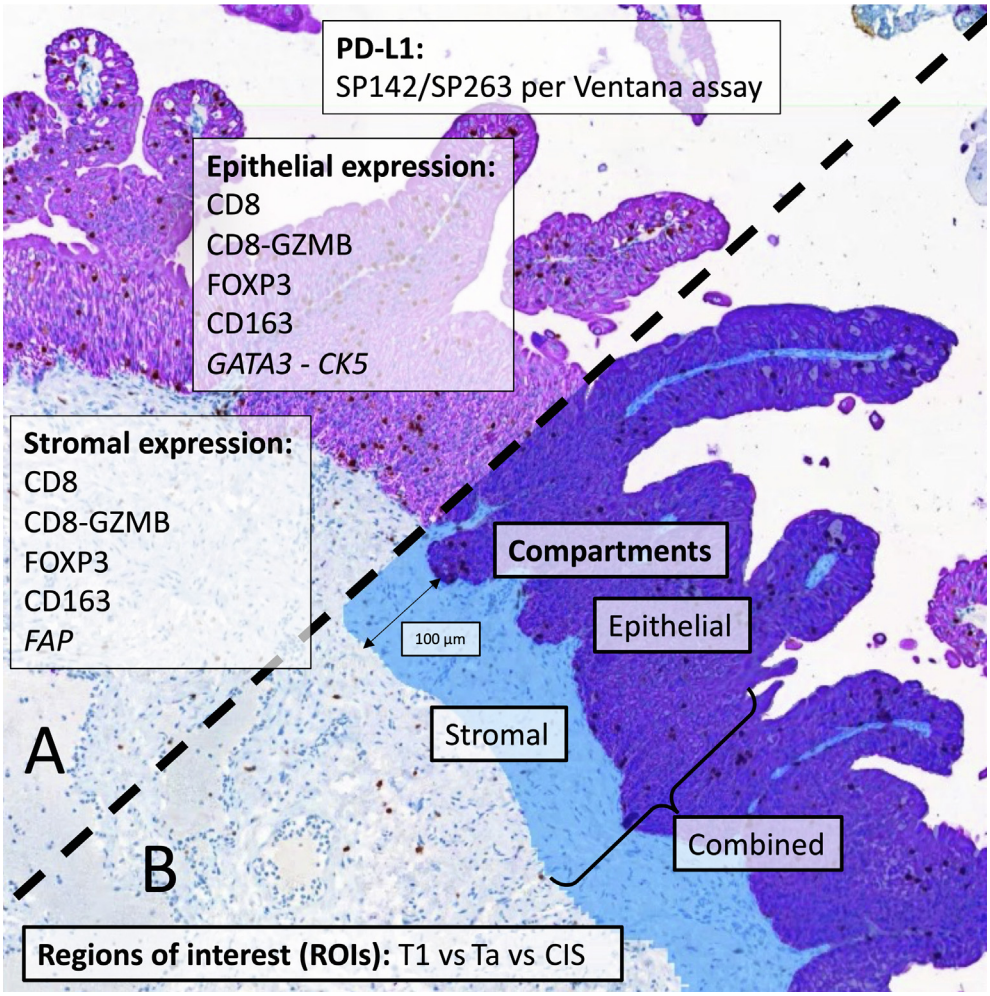

Fig. 1 – A PanCK stain (2.5 × magnification) that illustrates an assessment of immunohistochemical markers. (A) Epithelial and/or stromal expression was assessed depending on each marker. PD-L1 (clones SP142 and SP263) was scored as per Ventana protocol. (B) Using a PanCK stain, we differentiated between compartments: epithelial and/or stromal expression, or a combination of both. In addition, we differentiated expression of markers for regions of interest: T1, Ta, and CIS. A stromal margin of 100 µm was taken as illustrated. CIS = carcinoma in situ; FAP = fibroblast activation protein-α. Manufacturers: 1. PanCK: Ventana, 760-2595, Oro Valley, Arizona, USA; 2. CD8: Abcam, Ab178089, Cambridge, UK.

of well-validated IHC markers for CAFs, immune cells, and tumor cells was used (Fig. 1 and Supplementary Table 1).

2.4. Statistical analysis methods

Differences between progressors and nonprogressors were assessed using the *t* test for continuous variables and the chi-square test for categorical variables. IHC expression was differentiated between the regions of interest (ROIs) using an analysis of variance (T1 vs Ta vs CIS) and post hoc pairwise comparisons using Tukey's test (T1 vs Ta, T1 vs CIS, and Ta vs CIS). Conditional logistic regression was performed at 5-yr follow-up to assess correlations between progression and categorical or continuous variables. Nonprogressors with <5 yr of follow-up were excluded. Clusters for conditional logistic regression were assigned based on pair matching of progressors and nonprogressors (1:2). In case of exclusion of progressors or nonprogressors (due to pT0/pT2 after revision or follow-up <5 yr), nonprogressors were reassigned to progressors if applicable to form new pairs (1:2). In case of missing data in progressors, the matched observation was excluded from the analysis of that specific clinicopathological or IHC variables. In case of missing data in nonprogressors, the matched observation was retained in case of one missing matched variable but excluded in case of two missing matched variables. Analyses were performed in R (v4.0.0) using the "lubridate", "epiDisplay", and "pheatmap" packages.

3. Results

3.1. Characteristics

After single pathologist (T.G.) revision of all slides and tissue quality assessment, we retained a cohort of 111 patients (76 nonprogressors and 35 progressors) for a correlation of progression with all IHC markers (differentiated per compartment and ROI). For an analysis of IHC correlation, the ROI distributions per patient were as follows: T1 83% (n = 92), Ta 86% (n = 96), and CIS 28% (n = 31). The final presence of T1 ROI per patient was not 100% as expected for our cohort, which is due to the limited amount/volume of T1

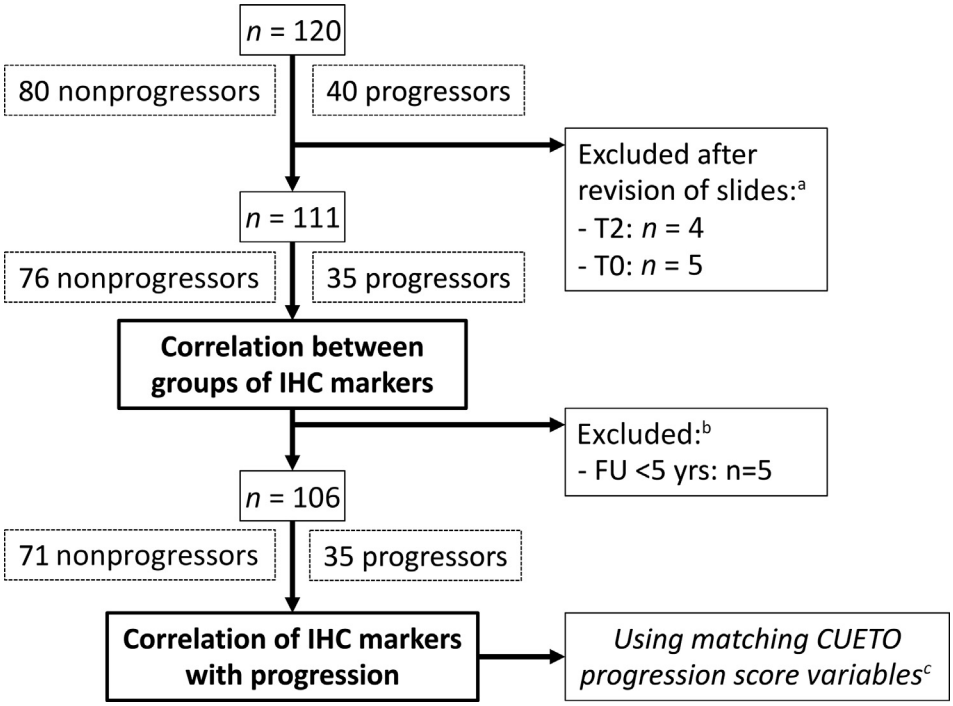

Fig. 2 – Study flowchart of included patients after being diagnosed with high-grade T1 NMIBC (*n* = 120). CUETO = Club Urologico Español de Tratamiento Oncologico; FU = follow-up; IHC = immunohistochemical; NMIBC = non–muscle-invasive bladder cancer. [a]Patients were excluded due to the following reasons: T2: presence of T2 disease at revision; T0: absence of relevant tumor at revision. [b]Patients with a follow-up of <5 yr were excluded from conditional logistic regression. [c]Patients were matched for CUETO progression score variables: age (≤70 vs >70 yr), recurrence (primary vs recurrent), number of tumors (*n* ≤ 3 vs *n* > 3), and presence of carcinoma in situ (CIS; yes vs no).

ROIs in some samples, making these prone to loss of presence of T1 on the last (12th) H&E slide. After excluding patients with <5 yr of follow-up, a final cohort of 106 patients (71 nonprogressors and 35 progressors) was retained to correlate IHC markers with progression on conditional logistic regression (Fig. 2). There were no significant differences in CUETO scores and clinical and pathological variables when breaking down between progressors and nonprogressors, except for the presence of CIS (p = 0.02), lymphovascular invasion (p = 0.03), and HG disease at re-TURBT (p = 0.049; Table 1). T1 substage (extensive vs microinvasion) did not differ significantly when stratified by performance of re-TURBT (Supplementary Table 2). No other clinically meaningful differences, despite being non-significant, were identified.

3.2. Immunohistochemistry

IHC expression of immune cell markers, basal-luminal markers, and the CAF marker FAP is discussed for the following parameters: progression (progressors vs nonprogressors), ROIs (T1 vs Ta vs CIS), and compartment (epithelial vs stromal, and immune cell markers only).

3.3. Immune markers: CD8, CD8-GZMB, FOXP3, CD163, PD-L1 SP142, and PD-L1 SP263

3.3.1. Progression: yes versus no

Stromal (p = 0.02) and combined (stromal and epithelial; p = 0.02) expression of FOXP3 in CIS ROIs was significantly higher in nonprogressors. None of the other IHC markers differed significantly, differentiated per compartment (stromal vs epithelial vs combined) and ROI (T1 vs Ta vs CIS vs ROIs combined).

3.3.2. ROIs: T1 versus Ta versus CIS

The PD-L1 SP142 combined positive score was significantly higher in T1 than in CIS ROIs (p = 0.03). CD163 expression was significantly higher in the epithelial compartment of CIS than in Ta (p = 0.0002), and combined (stromal and epithelial) expression was higher in T1 than in Ta (p < 0.0001) and in CIS than in Ta (p < 0.0001) ROIs. CD8-GZMB expression was significantly higher in the epithelial compartment in CIS than in Ta (p = 0.02) ROIs, and combined (stromal and epithelial) expression was higher in T1 than in Ta (p = 0.04) and in CIS than in Ta (p = 0.01) ROIs. CD8 expression was significantly higher in the epithelial compartment in CIS than in Ta (p = 0.006) ROIs, and combined (stromal and epithelial) expression was higher in T1 than in Ta (p < 0.0001) and in CIS than in Ta (p < 0.0001) ROIs. FOXP3 expression was significantly higher in the epithelial compartment in T1 than in Ta (p = 0.004), and higher in CIS than In Ta (p = 0.004) ROIs, and combined (stromal and epithelial) expression was higher in T1 than in Ta (p < 0.0001) and in CIS than in Ta (p < 0.0001) ROIs.

3.3.3. Compartments

Expression of CD8, CD8-GZMB, FOXP3, and CD163 was significantly higher in the stromal than in the epithelial compartment, which was true for all ROIs (T1 vs Ta vs CIS vs ROIs combined) regardless of progression status (progressor vs nonprogressor vs combined).

Table 1 – Characteristics of included patients for nonprogressors and progressors

Characteristics	Nonprogressors (*N* = 76; 68%)	Progressors (*N* = 35; 32%)	*p* value[a]
Female, *n* (%)	16 (21)	5 (14)	0.4
Age (yr), *n* (%)			
<60	16 (21)	4 (11)	0.2
60–70	19 (25)	14 (40)	
>70	41 (54)	17 (49)	
Smoking history, *n* (%)			
Active	14 (18)	6 (17)	0.3
Never	23 (30)	16 (46)	
Stopped	39 (51)	13 (37)	
Primary T1[b] *n* (%)	63 (83)	28 (80)	0.7
T1 substage[c] (*n* = 92), *n* (%)			
Extensive invasion	35 (58)	18 (56)	0.4
Microinvasion	25 (42)	14 (44)	
Size of lesions: ≥3 cm, *n* (%)	49 (64)	23 (66)	0.9
Number of lesions: >1, *n* (%)	41 (54)	21 (60)	0.6
CIS (prior to revision), *n* (%)	2 (3)	5 (14)	0.02
CIS (after revision), *n* (%)	13 (17)	18 (51)	0.002
EAU risk groups, *n* (%)			
High risk	54 (71)	24 (69)	0.8
Very high risk	22 (29)	11 (31)	
Lymphovascular invasion, *n* (%)	0	2 (6)	0.03
Variant histology, *n* (%)	7 (9)	2 (6)	0.5
Presence of detrusor, *n* (%)	59 (78)	31 (89)	0.09
Prior intravesical therapy, *n* (%)			
BCG	1 (1)	1 (3)	0.5
Chemotherapy	3 (4)	1 (3)	
Re-TURBT, *n* (%)			
Yes, before BCG induction	21 (28)	14 (44)	0.3
Yes, after BCG induction	49 (64)	17 (49)	
No	6 (8)	4 (11)	
High grade at re-TURBT, *n* (% of re-TURBT before BCG)	1 (5)	4 (18)	0.049

BCG = bacillus Calmette-Guérin; CIS = carcinoma in situ; EAU = European Association of Urology; NMIBC = non–muscle-invasive bladder cancer; TURBT = transurethral resection of bladder tumor.
Descriptive statistics are given as median (quartile 1, quartile 3) or frequency (percentage).
[a] The *p* values were calculated using the *t* test for continuous and chi-square test for categorical variables.
[b] Primary T1: no prior NMIBC.
[c] Lamina propria invasion depth cutoff 0.5 mm.

3.4. Basal-luminal differentiation: CK5 and GATA3

3.4.1. Progression: yes versus no

Both CK5 and GATA3 did not differ significantly between progressors and nonprogressors, differentiated per compartment (stromal vs epithelial vs combined) and ROI (T1 vs Ta vs CIS vs ROIs combined).

3.4.2. ROIs: T1 versus Ta versus CIS

Both CK5 and GATA3 did not differ significantly between ROIs (T1 vs Ta vs CIS).

3.5. Cancer-associated fibroblasts

3.5.1. Progression: yes versus no

Stromal expression of FAP in T1 ROIs (p = 0.004), and all ROIs combined (p = 0.002) was significantly higher in progressors than in nonprogressors.

3.5.2. ROIs: T1 versus Ta versus CIS

Stromal FAP expression was significantly higher in T1 than in Ta (p = 0.0002), and higher in T1 than in CIS (p = 0.01) ROIs.

3.6. Conditional logistic regression

We excluded five nonprogressors due to a follow-up of <5 yr for conditional logistic regression (Fig. 2). None of the considered clinicopathological or IHC variables (differentiated per ROI [T1 vs Ta vs CIS vs ROIs combined] and compartment [epithelial vs epithelial vs combined]) could discriminate between progressors and nonprogressors on conditional logistic regression, except for stromal FAP expression (Supplementary Table 3). For every 50% increase in stromal FAP expression in T1 ROIs, the odds of progression at 5-yr follow-up increased by 33% (95% confidence interval [CI]: 1.04, 1.70; p = 0.02), while this was 62% (95% CI: 1.13, 2.29; p = 0.007) for every 50% increase in stromal FAP expression in all ROIs combined.

3.7. Immunohistochemistry signature: heatmap

As FAP expression in T1 ROIs correlated with progression, we created a heatmap to visualize IHC expression in T1 ROIs specifically, in which progressors and nonprogressors are visualized separately (Fig. 3); a heatmap of IHC expression in all ROIs combined can be found in Supplementary Figure 1. Details and comparisons of all IHC markers, per ROI and compartment, can be found in Supplementary Tables 4–6.

4. Discussion

In a context of an unmet need for prognostic biomarkers in high-risk NMIBC, the emerging potential of the TME and our data on the prognostic potential of FAP in NMIBC, we set up

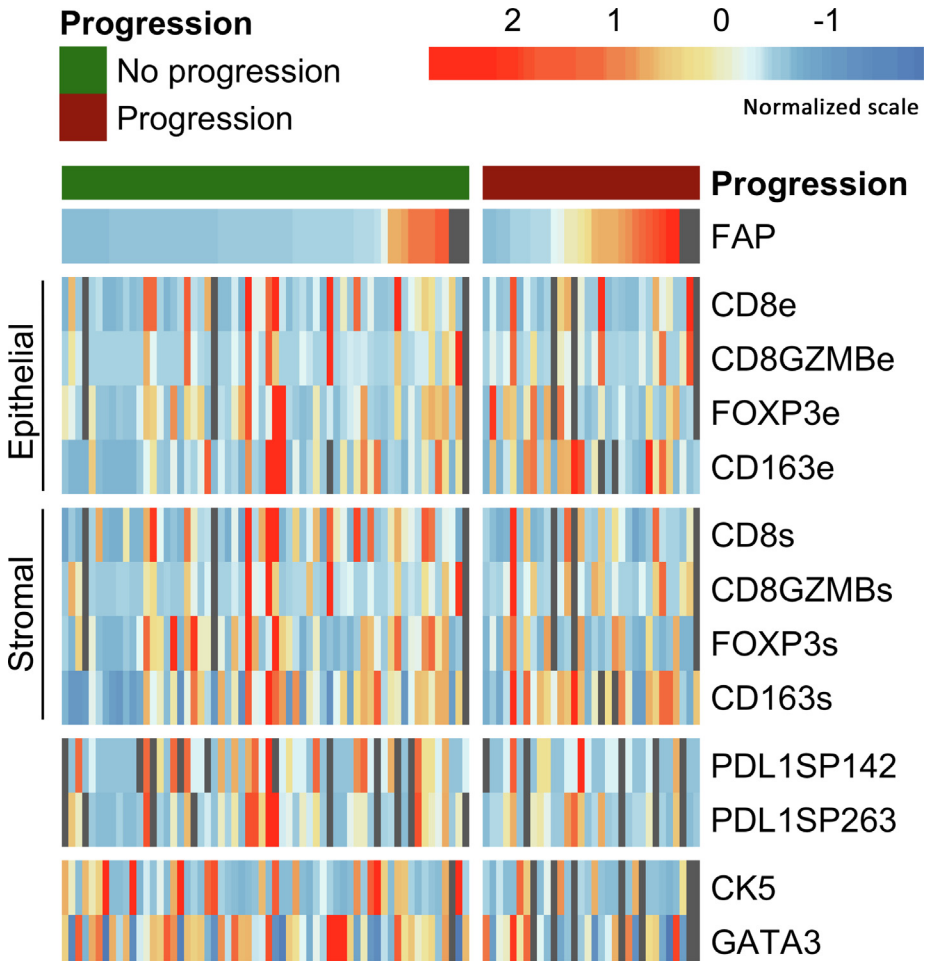

Fig. 3 – Illustrative heatmap of all immunohistochemical (IHC) markers in T1 ROIs per patient (*n* = 92), which were sorted (nonclustered) by progression: yes (green) versus no (red). IHC expression data are visualized as normalized data to illustrate more clearly the relative differences per IHC variable between patients. Gray bars indicate missing IHC expression data. Stromal or epithelial expression of an IHC marker was differentiated using "s" or "e" behind the marker's name (for CD8, CD-8GZMB, FOXP3, and CD163). FAP = fibroblast activation protein-α; ROI = region of interest.

a pair-matched nested case-control study, applied a well-defined panel of markers for CAFs and immune cells, and aimed to identify prognostic markers for progression in patients with high-risk NMIBC [7]. As a result of our matching procedure, none of the clinicopathological variables correlated with progression, which illustrates adequate matching of progressors and nonprogressors. HG disease at re-TURBT differed between progressors and nonprogressors, but did not impact clinical outcome on conditional logistic regression [16]. To our surprise, only FAP expression in T1 ROIs and all ROIs combined (T1, Ta, and CIS) correlated with progression on conditional logistic regression at 5-yr follow-up (Supplementary Table 3). None of the other IHC variables correlated with progression on conditional logistic regression, even when differentiating between ROIs (T1 vs Ta vs CIS vs ROIs combined) and compartments (epithelial vs epithelial vs combined) if applicable. FAP expression was significantly higher in T1 ROIs than in both Ta and CIS ROIs.

In earlier work, we identified FAP as a strong prognostic marker for unfavorable clinical outcome in T1 NMIBC [7]. This finding is confirmed in this study and strengthened, since none of the clinicopathological variables or other IHC markers could discriminate between progressors and nonprogressors on conditional logistic regression. This further stresses both the prognostic value of CAFs and the importance of the stromal tumor compartment [17]. FAP is linked to tumor biology–related processes, such as immunosuppression, immunomodulation, and neoangiogenesis [18]. Moreover, the role of FAP extends to the remodeling of the extracellular matrix and intracellular signaling regulation, which impairs clinical outcome [19]. Several reports show that CAFs can induce immunosuppressive changes in the extracellular matrix of the peritumoral stroma, which prevent infiltration of tumor-infiltrating lymphocytes (TILs) in the tumoral epithelium [20,21]. However, in this study, we also observed high stromal and epithelial CD8+ TILs in several "FAP high" cases. In colon cancer, FAP expression correlates with an increased density of immunosuppressive FOXP3-positive cells and a modified ratio of CD8+ T cells, which indicates an interplay between FAP+ fibroblasts and immune cells [22]. Additionally, FAP influences the immunosuppressive environment through signaling pathways such as FAP-STAT3-CCL2, particularly in desmoplasia-associated cancers [23]. Although speculative, these insights underscore the potential of combining

Table 2 – Clinical outcome of included patients for nonprogressors and progressors

Events at 5-yr follow-up	Nonprogressors (*N* = 76; 68%)	Progressors (*N* = 35; 32%)	*p* value[a]
BCG induction (≥5/6)[b], *n* (%)	76 (100)	35 (100)	1
Adequate BCG as per FDA[c] *n* (%)	34 (45)	4 (11)	<0.001
Follow-up (yr)	8.3 (4.9, 10.7)	3.4 (2.0, 6.5)	<0.001
Recurrence, *n* (%)	28 (37)	35 (100)	
Progression, *n* (%)	0	34 (97)	
Metastatic disease, *n* (%)	0	19 (54)	
Cancer-specific mortality, *n* (%)	0	17 (49)	
All-cause mortality, *n* (%)	15 (20)	23 (66)	

BCG = bacillus Calmette-Guérin; FDA = US Food and Drug Administration.
Descriptive statistics are given as median (quartile 1, quartile 3) or frequency (percentage).
[a] The *p* values were calculated using the *t* test for continuous and chi-square test for categorical variables.
[b] Defined as at least five of six doses of an induction course.
[c] Adequate BCG as defined by the FDA: at least five of six doses of induction plus two of three doses of maintenance, or at least five of six doses of induction plus at least two of six doses of a second induction course [29].

FAP and immune cell interactions as a more accurate prognostic tool in cancer research, since immunosuppression of FAP might only be biologically relevant in the presence of immune cells [24].

We found no prognostic effect for any of the immunological markers analyzed in the present study (CD8, FOXP1, GZMB-CD8, and PD-L1). This is in line with a recent report on CD8+ TILs in NMIBC, although studies report mixed results regarding the prognostic value of immune cells and systemic inflammatory markers in BC [25–28]. Reasons for these mixed results are most likely methodological: differences in sample types (T1 or mixed NMIBC), antibody clones, assessment algorithms, or tumor ROIs included in assessment. We have tried to anticipate these methodological challenges by stringent sample selection, rigorous antibody clone selection and validation, standardized ROI selection, and an automated image analysis.

Our study is not devoid of limitations. First, due to a limited amount of T1 tissue in some samples, we did not retain T1 tissue on all final slides. Therefore, after single pathologist review, only 83% (n = 92) had residual T1 in the last cut H&E slide of the TURBT specimen. Second, all patients received at least five or more of six doses of BCG induction, but only 11% of the progressors and 45% of the nonprogressors (p < 0.001) received adequate maintenance BCG as defined by the US Food and Drug Administration, which could imbalance our cohort (Table 2) [29]. Third, a re-TURBT was not always performed before the start of BCG induction, as advised currently. In a large proportion of patients, re-TURBT was performed after BCG induction, which has been suggested to be oncologically equivalent [30]. The proportion of patients who had re-TURBT did not differ between progressors and nonprogressors, and should therefore not interfere with our analyses (Table 1).

Our study has several strengths. First, we used full tissue slides, an approach that differs from tissue microarrays (TMAs) that are commonly used in this field of research. TMAs have less tissue and less histological variation, and offer no guarantee that T1 disease is still present. Second, we used a segmented histological approach with digital differentiation between tumor stages (ROIs: Ta, T1, and CIS) and differentiation between peritumoral stromal and tumoral epithelial areas, which allowed generation of detailed expression data per ROI and compartment, an approach that differs from the bulk of IHC or molecular studies that do not provide this grade of detail. Finally, we strictly followed the REMARK reporting recommendations, which facilitate consistent, high-quality reporting of tumor marker studies, and aids in the interpretation and application of our results.

5. Conclusions

This study provides additional evidence on the prognostic value of FAP in high-risk NMIBC, with FAP emerging as the sole prognostic marker in our TME panel. In future studies, we will investigate whether FAP represents a stromal epiphenomenon or reflects a real biological processor in relation with tumor and immune cells.

Author contributions: Tim Muilwijk had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Muilwijk, Akand, Kockx, Gevaert, Joniau.
Acquisition of data: Muilwijk, Akand.
Analysis and interpretation of data: Muilwijk, Akand, van Dam.
Drafting of the manuscript: Muilwijk.
Critical revision of the manuscript for important intellectual content: Muilwijk, Akand, Baekelandt, Akand, Van den Broeck, Van Cleynenbreugel, Van der Aa, Gevaert, Joniau.
Statistical analysis: Muilwijk.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.euros.2024.06.011.

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