Original paper

Immunohistochemical differentiation between muscularis mucosae and muscularis propria for improving the staging of bladder cancer in patients undergoing transurethral resection of bladder tumours

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Microscopic differentiation between muscularis mucosae (MM) and muscularis propria (MP) of the bladder in the material obtained during transurethral resection (TUR) remains difficult. The study was aimed at determination of the usefulness of immunohistochemical staining in this context. Forty-seven TUR specimens were stained with 5 mouse anti-human antibodies: anti-desmin, anti-filamin, anti-type IV collagen, anti-smoothelin, and anti-vimentin. Slides were assessed under light microscopy and the intensity of the immune reaction within MM and MP was evaluated on a four-level visual scale as follows: negative (0) and weakly (1), moderately (2), or strongly (3) positive. MM was identified in 27 patients (57.4%). The modal values of reaction intensity in MM and MP was 0 and 2 for desmin ($p > 0.05$), 2 and 2 for filamin ($p = 0.01$), 2 and 2 for type IV collagen ($p > 0.05$), 1 and 2 for smoothelin ($p = 0.03$), and 2 and 0 for vimentin ($p = 0.02$), respectively. Identical intensity within MM and MP was observed in 7.1%, 28.6%, 20%, 30.1%, 5.6%, respectively. Immunohistochemistry can help differentiate between MM and MP in TUR specimens. As of yet, no single marker can reliably differentiate between MM and MP; however, a combination of anti-filamin, anti-smoothelin, and anti-vimentin antibodies may be reasonable for diagnostic purposes.

Key words: bladder cancer, microscopic diagnostics, immunohistochemistry, staging, muscularis mucosae.

Introduction

Microscopic examination of bladder tumours obtained during transurethral resection (TUR) is the most important diagnostic and staging tool, and directly influences therapeutic decisions in patients with bladder cancer [1]. The examination should define the histological origin of the cancer, depth of cancer cell invasion, and grade of the cancer and describe the tumour configuration, provide information on whether a muscle layer is present in the specimen, as well as other information [2, 3]. Despite improvements in surgical technique and histological methods, microscopic examinations of TUR specimens remain difficult. This results from numerous factors of which the most important are the low quantity of

available tissue, especially low quantities of bladder detrusor, tissue artefacts related to the use of diathermy for TUR, reactive and inflammatory phenomena, and, finally, the presence of non-cancerous histological structures hampering the classical texture of the bladder wall. The pathologist must be able to distinguish muscularis propria (MP) from muscularis mucosae (MM), stromal desmoplasia from muscular invasion, and intravesical fat from perivesical adipose tissue. In this context, proper identification of histological structures within the mucosa and MP plays key roles in adequate staging. For differentiation between MM and MP, the International Society of Urologic Pathology Consensus recommends using immunohistochemical staining with anti-smoothelin and anti-vimentin antibodies [4]. However, expert panels highlight important limitations of these recommendations related to insufficient data on this issue. The role of anti-vimentin staining was examined in only a single study, which included only 15 cystectomy specimens [5]. The data on anti-smoothelin staining comes from five studies, including three that investigated only TUR specimens [5, 6, 7, 8, 9]. Moreover, to date many other antigens of diagnostic potential have not been tested. Further investigation in the field of immunohistochemistry in the staging of bladder cancer is therefore needed.

The expression of 12 protein antigens within MM and MP in cystectomy specimens was recently evaluated. Based on this analysis, five antigens that could potentially differentiate these two histological structures were identified: desmin, filamin, type IV collagen, smoothelin, and vimentin [10]. The aim of the present study was to assess the value of immunohistochemical staining of these five selected proteins for differentiating between MM and MP to enable proper staging of bladder cancer in a representative cohort of patients undergoing TUR.

Material and methods

Material

The study was based on 47 TUR specimens obtained from 10 women and 37 men whose mean age was 72.0 years. One specimen with hyperplastic MM that could not be unambiguously distinguished from MP was excluded. In the remaining 46 cases, pathological examination revealed the presence of bladder cancer in 100%, including stage Ta in 17.4%, T1 in 41.3%, and muscle invasive bladder cancer in 41.3%. The institutional review board approved this study (KB252/2010).

Methods

All specimens were fixed in formalin, dehydrated, embedded in paraffin blocks, and serially cut into 3-μm slices with a microtome. Immunohistochemical staining was a manual two-day procedure using mouse anti-human and donkey anti-mouse antibodies as the primary and secondary antibodies, respectively. Thermal incubation in Target Retrieval Solution (Dako, Denmark) was used for antigen retrieval in all cases, while an additional 3-minute enzymatic exposition to proteinase K (Dako, Denmark) was adopted in slides stained with type IV collagen. Endogenous peroxidase was blocked with hydrogen peroxide, while non-specific binding sites were blocked with normal donkey serum. Based on previously published data [10], anti-desmin, anti-filamin, anti-type IV collagen, anti-smoothelin, and anti-vimentin antibodies were selected as primary antibodies in the study. Details regarding these antibodies are presented in Table I. Horseradish peroxidase and diaminobenzidine as the chromogen were applied for the colour reaction. A single pathologist assessed all slides under light microscopy. The intensity of the immunological reaction was evaluated in a four-level subjective visual scale as negative (0) or weakly (1), moderately (2), or strongly (3) positive. From each patient, 10 representative high power field (HPF) images were analysed and the results were averaged. Figure 1 presents examples of microscopic image interpretation. As a negative control, normal mouse immunoglobulins were applied in the same concentration as the primary antibody.

Statistical analysis

Results are presented as modal values, presenting the most commonly noted score of immune reaction

Table I. Details regarding primary antibodies used in the study

Antigen	Manufacturer	Clone	Antigen retrieval method	Concentration	Dilution
Desmin	Dako, Denmark	D33	Thermal	115 μg/ml	1 : 100
Filamin	Leica Biosystems Newcastle Ltd, United Kingdom	PM6/317	Thermal	50 μmol/ml	1 : 100
Collagen IV	Dako, Denmark	CIV22	Thermal and enzymatic	182 μg/ml	1 : 200
Smoothelin	Abcam plc, United Kingdom	R4A	Thermal	1,00 mg/ml	1 : 50
Vimentin	Dako, Denmark	V9	Thermal	50 μg/ml	1 : 100

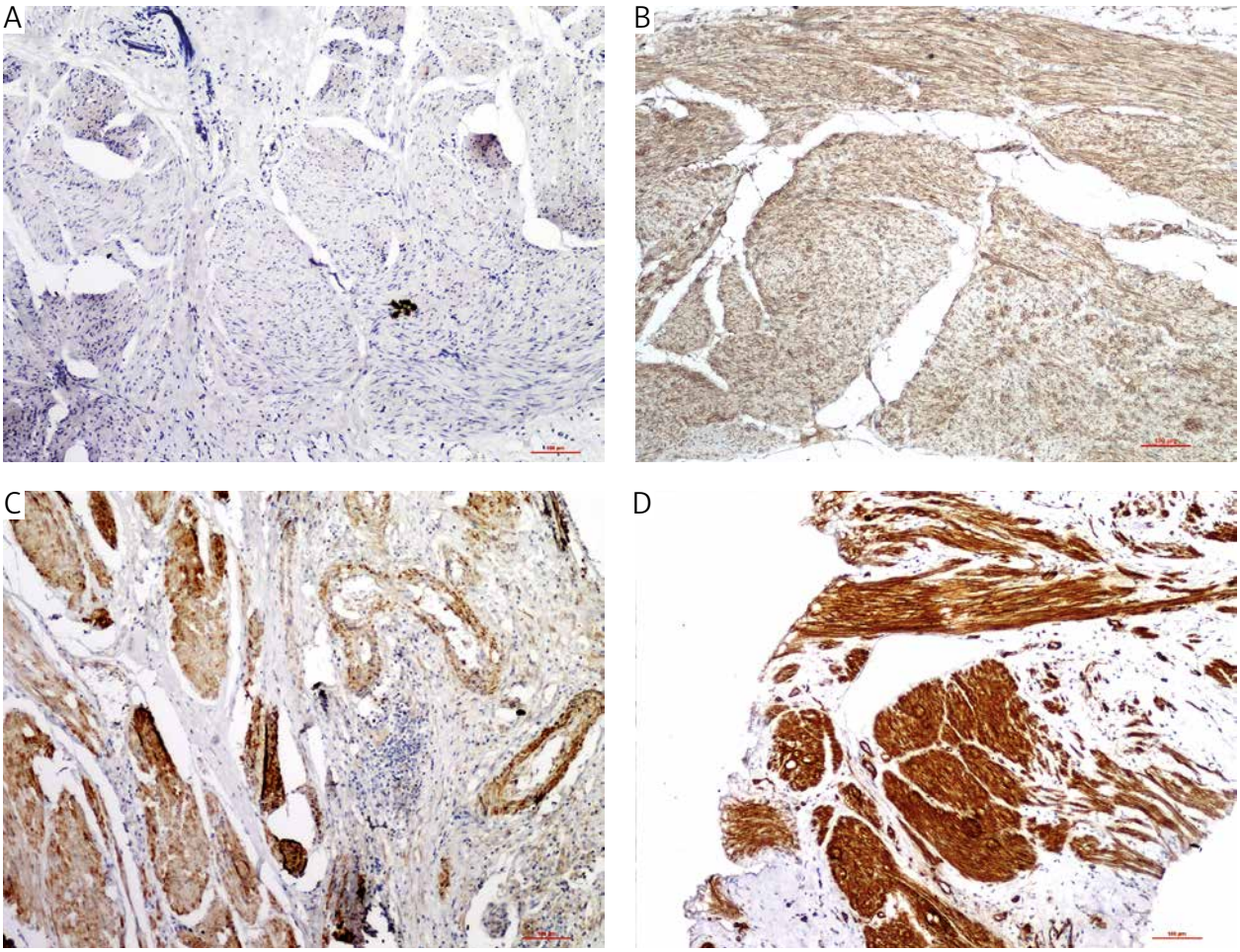

Fig. 1. Examples of reaction intensity interpretation within a muscularis propria. A) Negative reaction (0). B) weakly positive reaction (1). C) Moderately positive reaction (2). D) Strongly positive reaction (3). Light microscope, magnification 100×

intensity, exact percentage of slides with declared reaction intensity, and the percentage of slides with the same or different reaction intensity within MM and MP. For comparison of reaction intensity within MM and MP, the Pearson formula of the χ^2 test was used. Differences were considered statistically significant at $p < 0.05$.

Results

Histological structures identified as MM were observed in 27 patients (57.4%), including 29.8% of slides stained with desmin, 42.6% of slides stained with filamin, 10.6% of slides stained with type IV collagen, 40.4% of slides stained with smoothelin, and 40.4% of slides stained with vimentin. MP was present in 45 patients (95.7%).

Table II presents the detailed results for each antibody. The modal values of reaction intensity in MM and MP were 0 and 2 for desmin ($p > 0.05$), 2 and 2 for filamin ($p = 0.01$), 2 and 2 for type IV collagen ($p > 0.05$), 1 and 2 for smoothelin ($p = 0.03$), and 2 and 0 for vimentin ($p = 0.02$), respectively. Figures 2-5 present microscopic images of MM and

Table II. Detailed results of reaction intensity observed within MM and MP in the study

	Reaction intensity within MM				Reaction intensity within MP				*P* value
	0	1	2	3	0	1	2	3	
Desmin	42.9%	35.7%	21.4%	0%	5.9%	0%	88.2%	5.9%	0.37
Filamin	20%	35%	40%	5%	28.6%	7.1%	50%	14.3%	0.01
Collagen IV	0%	20%	60%	20%	0%	0%	54.5%	45.5%	0.08
Smoothelin	42.1%	57.9%	0%	0%	30.8%	23.1%	42.3%	3.8%	0.03
Vimentin	0%	31.6%	63.2%	5.3%	45.8%	45.8%	8.3%	0%	0.02

MP stained with hematoxylin-eosin, and anti-filamin, anti-smoothelin and anti-vimentin antibodies, respectively.

Reaction intensity within MM and MP differed by at least one point in 92.9%, 71.4%, 80%, 69.9%, and 94.4% of cases, respectively, and was stronger in MM in slides stained for vimentin and MP slides stained for desmin, filamin, type IV collagen, and smoothelin. The datasets analysed during the study are available from the corresponding author on reasonable request.

Discussion

Surgical treatment is the most critical step in the management of patients with bladder cancer [11]. However, microscopic examination of surgical material from TUR remains one of the most difficult in uro-pathology. Orientation in the specimen and proper identification of histological structures is limited by the low volume of the available tissue, cautery-related artefacts, inflammatory responses, and the presence of MM, myofibroblasts, or intravesical adipose tissue. As an indirect result, bladder cancer

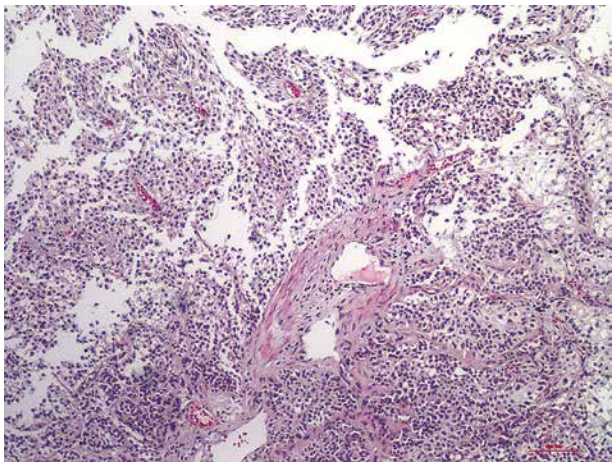

Fig. 2. Muscularis mucosae of the bladder stained with hematoxylin and eosin. Light microscope, magnification 100×

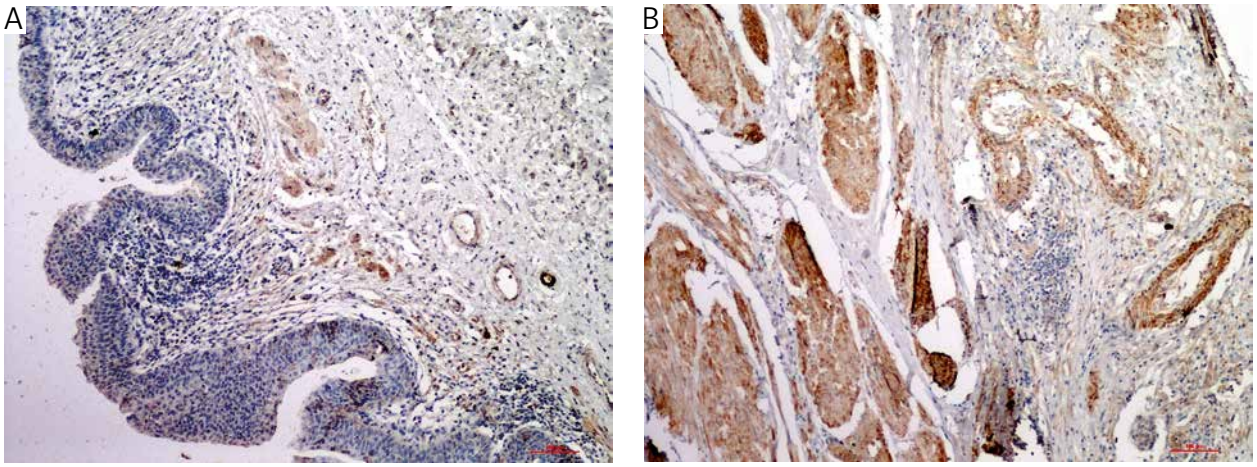

Fig. 3A. Muscularis mucosae of the bladder stained with anti-filamin antibodies. Light microscope, magnification 100×
Fig. 3B. Muscularis propria of the bladder stained with anti-filamin antibodies. Light microscope, magnification 100×

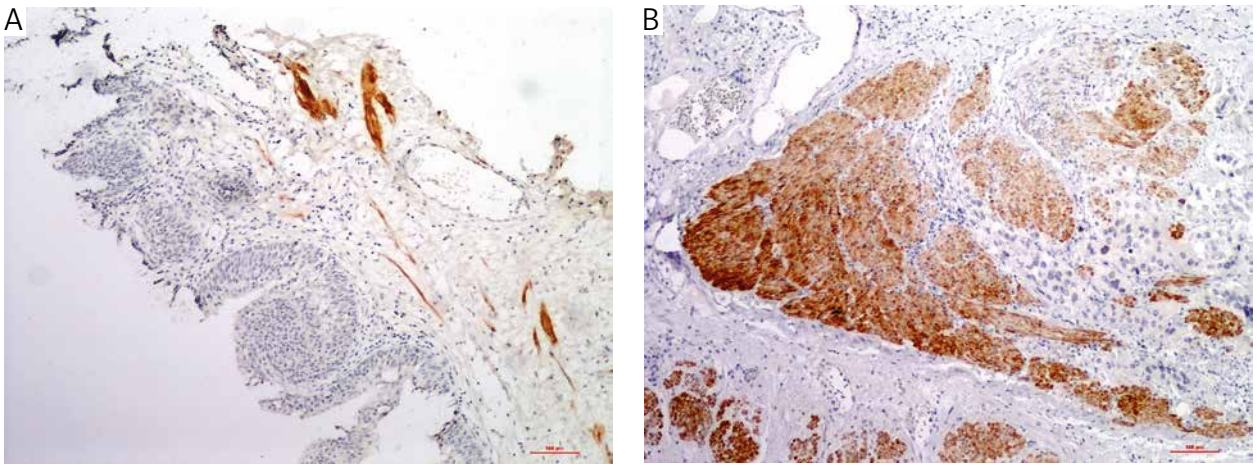

Fig. 4A. Muscularis mucosae of the bladder stained with anti-smoothelin antibodies. Light microscope, magnification 100×
Fig. 4B. Muscularis propria of the bladder stained with anti-smoothelin antibodies. Light microscope, magnification 100×

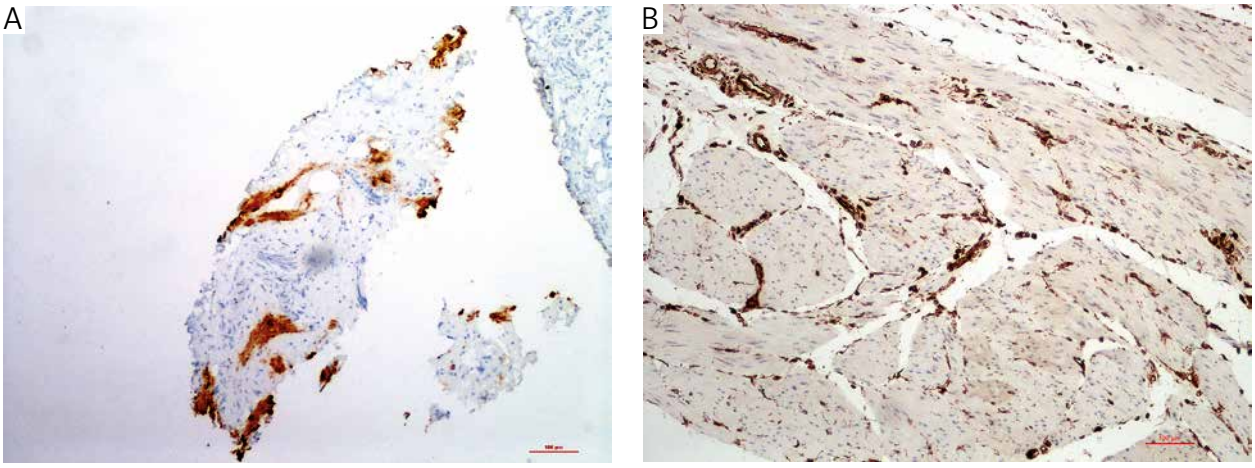

Fig. 5A. Muscularis mucosae of the bladder stained with anti-vimentin antibodies. Light microscope, magnification 100×
Fig. 5B. Muscularis propria of the bladder stained with anti-vimentin antibodies. Light microscope, magnification 100×

diagnosed during microscopic examination of TUR specimens may be up-staged or down-staged after microscopic examination of radical cystectomy specimens in as many as 32–76.2% and 0–46.2% of cases, respectively [12, 13, 14, 15, 16, 17, 18]. Because of this, the opinion of a second pathologist is recommended during the primary diagnosis, especially in questionable cases [1].

Differentiating between non-muscle-invasive and muscle-invasive bladder cancer is of cardinal importance for clinical decision making [1, 19, 20]. Microscopically, this differentiation relies on the exclusion or confirmation of cancer cell invasion into detrusor muscle fibres. The microscopic assessment can be substantially complicated by the presence of MM, which consists of areas of smooth spindle cells located in the central part of subepithelial connective tissue. These structures can mimic MP, especially when they are rich and hyperplastic, and present in limited specimens [21]. Moreover, the presence of MM is not universal. It is observed in 78–100% of patients [21, 22, 23, 24], whereas hyperplastic MM is seen in 17–53% of patients [21, 25]. Inadequate differentiation between MM and MP can contribute to the reasons for high rates of upstaging from T1 to muscle-invasive bladder cancer after restaging TUR [26].

We performed a study aimed at identifying immunohistochemical markers of MM and MP that could be used for pathological staging of bladder cancer. After the selection of five antigens with diagnostic potential in cystectomy specimens [10], we recently examined the value of anti-desmin, anti-filamin, anti-type IV collagen, anti-smoothelin, and anti-vimentin staining in differentiating between MM and MP in 47 TUR specimens. The two main findings of the present study are that no single antigen can be used to reliably distinguish between MM and MP, and that the combination of anti-filamin, anti-smoothelin, and anti-vimentin staining can provide adequate information.

Results obtained for filamin cannot be compared to any data in the literature as none exist. Despite statistically significant differences in the expression of filamin within MM and MP observed in the present study, we emphasize that in one case inversely stronger reaction intensity was observed within MM; therefore, our results must be interpreted with caution. Another limitation of hypothetic staging of bladder cancer using anti-filamin staining is the observation by Malmqvist *et al*., who noticed that desmin expression increases with the development of bladder outlet obstruction [27].

Expression of smoothelin was stronger within MP in 7 of 10 cases in the present study. This antigen is the one most studied in the context of MM and MP differentiation. Paner *et al*. and Council *et al*. initiated the research in 2009. Independently, based on total of 59 cystectomy specimens, they consistently noticed no smoothelin expression within myofibroblasts, no or weak expression within MM, and strong expression within MP [5, 7]. The next year, Paner *et al*. reported moderate, strong, or diffuse expression of smoothelin within MP in 70% of 69 TUR specimens, thereby confirming its potential to indicate this bladder layer [8]. Subsequently, Bovio *et al*. confirmed different expression levels of smoothelin within MP and MM in 26 cystectomy and TUR specimens [6], while Hansel *et al*. confirmed the lack of strong smoothelin expression within MM in bladder diverticula resected from 40 patients [28]. However, the latter research group highlighted a 32% incidence of moderate smoothelin expression in cases of hyperplastic MM, while Miyamoto *et al*. pointed out that in as many as 25% of cases the reaction intensity within MM and MP was similar, concluding that the use of smoothelin immunohistochemistry as a diagnostic tool for MP invasion requires caution [9]. Interestingly, the general reaction intensity for smoothelin within MP was lower in the present study compared to data reported in the liter-

ature. This could be the result of the antigen retrieval method since acid buffer provides more diagnostic microscopic images [29]. The burden of results obtained in the present and/or previous studies on filamin and smoothelin justify the conclusion that a combination of antigens is needed for reliable diagnostics.

In the present study, the most significant results were obtained for vimentin. Its expression was stronger within MM in almost 95% of cases, while the modal value of the reaction intensity within MP was lower by two points. The study by Council *et al*. supports our findings. They also noticed a weaker reaction within MP in 93% of cases in a series of 15 cystectomy specimens [5]. Our study is the first confirming the value of anti-vimentin staining for differentiating between MM and MP in TUR specimens; hence, it justifies International Society of Urologic Pathology Consensus [4]. Apart from staging purposes, vimentin expression in urothelial carcinoma may have a prognostic role for patients [30, 31] as well as a role in a new non-invasive diagnostic urine test for bladder cancer [32, 33].

Statistically insignificant results were obtained for type IV collagen and desmin. At this point, it must be emphasized that the presence of MM was observed only in 10.6% and 29.8% of slides stained with anti-collagen and anti-desmin antibodies, respectively. This could be the reason for the lack of statistical significance since clinically observed differences seem to be important. MM was negative for desmin in over 40% of cases, while MP was moderately or strongly positive in almost 95% of cases. Moreover, reaction intensity within MM and MP differed in 92.9% of cases. However, in the only available study on differentiating MM from MP with desmin, Council et al. observed similarly strong reactions in both structures [5]. For type IV collagen, modal values of the reaction intensity within MM and MP in the present study were identical, while the intensity was stronger by at least one point within MP in 80% of cases. This protein has never before been tested for differentiating between MM and MP. Considering all these data, further studies on desmin and type IV collagen are needed in this setting. Meanwhile, the most useful application of anti-desmin in bladder cancer specimens may be T1 substaging depending on the depth of invasion of the cancer cells into the lamina propria [34]. The most useful application of anti-type IV collagen may be in assessing cancer microinvasion [35, 36].

The limitations of present study include manual staining, subjective assessment of immune reaction intensity and limited number of involved patients. However, a written step-by-step algorithm of the procedure was adopted to minimise the risk of mistakes at the time of preparation. Simultaneously, no alternative method of quantifying reaction intensity is universally accepted, and each of the available systems has important drawbacks. One possible approach that could improve the standardization of results involves the use of an autostainer, with the subsequent morphometric analysis of digital specimen images by a specialized program. Finally, in general the evaluation of immunohistochemical intensity sometimes differs among pathologists, and is affected by the condition of samples, including fixation. This limits practical potential of results presented within the present study.

Differentiation between MM and MP in TUR specimens may be facilitated by immunohistochemistry. Because no single marker is reliable for differentiating between MM and MP, it seems reasonable to use a combination of anti-filamin, anti-smoothelin, and anti-vimentin antibodies for diagnostic purposes. Routine use of immunohistochemistry in cases of newly diagnosed T1 bladder cancer could potentially reduce the number of TURs that are restaged and may improve the survival of patients with muscle-invasive bladder cancer.

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The authors declare no conflict of interest.

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