Diagnostic Utility of Antibody to Smoothelin in the Distinction of Muscularis Propria From Muscularis Mucosae of the Urinary Bladder

A Potential Ancillary Tool in the Pathologic Staging of Invasive Urothelial Carcinoma

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Abstract: Accurate recognition of urinary bladder muscularis propria (MP) invasion by urothelial carcinoma is crucial as it is the critical crossroad between conservative and aggressive management for the patient. It is now widely known that an inconsistent layer of muscularis mucosae (MM) muscle exists in the lamina propria, which can mimic the MP muscle, particularly when hyperplastic, making staging extremely challenging in some limited, unoriented, or highly cauterized specimens. Smoothelin is a novel smooth muscle-specific contractile protein expressed only by fully differentiated smooth muscle cells, and not by proliferative or noncontractile smooth muscle cells and myofibroblasts. We performed immunohistochemical staining in the bladder for smoothelin to: (a) evaluate its expression in MM and MP muscle in cystectomy specimens and by comparing the staining pattern with smooth muscle actin (SMA), (b) study MP variations in the bladder trigone and at the ureteric insertion in the bladder wall, and (c) assess the staining pattern of MM and MP in a representative group of transurethral resection of bladder tumor specimens. In contrast to SMA, which equitably stained both types of muscle fibers, smoothelin displayed striking differential immunoreactivity between MM and MP muscle. With smoothelin, the MM muscle (including hyperplastic forms) typically showed absent (19/42, 45%) or weak and focal (18/42, 43%) staining, whereas the MP muscle typically showed strong and diffuse staining (36/42, 86%). Smoothelin accentuated individual muscle fibers within groups of MP bundles only, a feature which was evident in both MM and MP stained by SMA. When only strong and diffuse immunoreactivity in muscle was set as a threshold for positivity, 100% specificity and positive predictive value of smoothelin for MP (vs. MM) was achieved in our study. Smoothelin staining confirmed the morphologic variations in MP muscle in the bladder wall of the trigone and at the ureteric insertion. In addition to the well-defined muscle layers of MM and MP, SMA staining revealed a continuous band of ill-defined haphazardly oriented compact spindle cells that were immediately subjacent to the urothelium in all cases. These spindle cells blended with the morphologically recognizable thin slender fascicles of the MM muscle. We designate this hitherto uncharacterized thin layer of SMA-positive [muscle-specific actin positive (6/6), Masson trichrome stain predominantly blue (5/6)] and smoothelin-negative cells as suburothelial band of myofibroblasts. In all 10 transurethral resection of bladder tumor sections, smoothelin staining was in agreement with the routine light microscopic presence and absence of MP muscle. In conclusion, the relatively distinct immunohistochemical staining pattern of smoothelin between MP and MM (including its hyperplastic forms) makes it a robust and attractive marker to be incorporated in the contemporary diagnostic armamentarium for the sometimes difficult area of staging bladder urothelial carcinoma.

Key Words: urinary bladder cancer, muscularis mucosae, muscularis propria, smoothelin, immunohistochemistry, invasion, staging, smooth muscle actin, suburothelial cells

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The quintessential role of the surgical pathologist in reporting invasive urothelial carcinoma is to evaluate the level of invasion in the urinary bladder wall, a vital determinant of subsequent therapy and prognosis. In the urinary bladder, invasion of urothelial carcinoma limited to the lamina propria is staged as pT1 and involvement of muscularis propria (MP) is staged as at least pT2.[5] There are 3 potential pitfalls in this assessment. In the lamina

propria, there is an inconsistent layer of muscularis mucosae (MM) muscle which occasionally can be hyperplastic and mimic the MP muscle making staging sometimes extremely difficult in limited or unoriented specimens such as biopsies or transurethral resection of bladder tumor (TURBT) specimens.[1,4,9,11,13] Another compounding factor is the presence of a desmoplastic stromal response in which prominent myofibroblasts may mimic muscle bundles.[6] Further, when urothelial carcinoma infiltrates MP, in addition to tumor surrounding MP, tumor may infiltrate the muscle bundles, fracturing their round contours and splaying the muscle fibers.[3] In these situations, distinction from MM or desmoplastic stromal response may be problematic. Studies to date, predominantly presented in abstract form only, have shown that there are no consistent and reliable immunohistochemical markers to distinguish between the 2 types of muscle in the bladder wall, although desmin, caldesmon, smooth muscle myosin-heavy chain may be used to reliably distinguish muscle bundles from myofibroblasts in a desmoplastic stroma.[2,3]

Smoothelin is a novel smooth muscle-specific marker expressed only in terminally differentiated smooth muscle cells as part of its contractile cytoskeleton.[7,14] Unlike traditional smooth muscle markers [ie, smooth muscle actin (SMA)], smoothelin expression is absent or limited in noncontractile and proliferative smooth muscle cells or cells with smooth musclelike features (ie, myofibroblasts).[7,14] A recent study has demonstrated expression of smoothelin in the MP muscle of normal and overactive bladder.[8] In the current study, we compare the immunohistochemical expression of smoothelin and SMA in the bladder wall to explore their potential use as a discriminatory stain between MM and MP muscle in cystectomy specimens and in a representative group of TURBT specimens. We recently described topographical variations of the bladder MP (ie, trigone and ureteric insertion in bladder wall),[9] and we evaluate smoothelin immunohistochemistry in this muscle to further characterize these MP variations.

MATERIALS AND METHODS

Specimens

Archival formalin-fixed, paraffin-embedded tissue blocks of adult urinary bladder from Loyola University Medical Center, Maywood, IL, and The Methodist Hospital, Houston, TX, were used for the immunohistochemical study. These included nontumoral cystectomy sections of 42 nontrigonal bladder wall (from 34 patients), 5 trigonal bladder wall, 6 bladder wall at the ureteric insertion, and 10 TURBT sections with invasive urothelial carcinoma (from 10 patients). Of the cystectomy sections, 34 of 42 showed at least the focal presence of hyperplastic MM (> 4 muscle fibers thick) distinguishable on hematoxylin and eosin (H&E)-stained slides.[9] The different categories of muscle in the bladder wall were in accordance to our previously described findings.[9]

Immunohistochemistry

Heat-induced epitope retrieval, after deparaffinization and rehydration of tissue sections, was performed in 10 mM citrate buffer (pH 6.0) and heating for 3 times was performed before immunostaining. The following antibodies were used: smoothelin (R4A; 1:150 dilution; Abcam Inc, Cambridge, MA) and SMA (1A4, prediluted, Ventana, Tuczon, AZ). Tissue sections were incubated with primary antibody for 32 minutes at room temperature, washed with phosphate-buffered saline and incubated with a secondary antibody conjugated to horseradish peroxidase (Benchmark IHC/ISH module, Ventana). Hematoxylin was used as a counter stain. The interpretation of immunoreactivity was performed in a semiquantitative manner by analyzing the extent of the staining positivity of the muscle cells. For MP, the inner muscle layer or muscle bundles bordering the lamina propria were selected to evaluate staining of MP bundles of the inner half of the bladder wall because these muscle bundles were most likely to be included in TURBT specimens. Furthermore, we could confidently evaluate these with respect to the overlying urothelium in all available specimens. Staining intensity was evaluated as weak and strong. The staining pattern score was evaluated as follows: 0 or negative $\leq 5\%$; +1 or focal = 5% to 10% positivity; +2 or moderate = 11% to 50% positivity; and +3 or diffuse > 50% muscle cells positivity. During the study evaluation of immunohistochemical stains, a unique SMA-positive layer was identified in the suburothelium in all cases; we additionally performed muscle-specific actin (MSA, HHF-35, prediluted, Ventana) immunohistochemisty, and Masson trichome stain in 6 cases to determine whether this layer is composed of myocytes or myofibroblasts.

RESULTS

Differential Staining of Bladder MM Muscle and MP Muscle

The immunohistochemical staining for smoothelin and SMA in bladder MM with and without hyperplastic MM and MP muscle are summarized in Table 1.

With SMA, strong and diffuse (+3) staining was observed with similar intensity and pattern in MM (42/42, 100%) and MP (42/42, 100%), including hyperplastic MM (34/34, 100%) muscle (Figs. 1A, C, and E). In hyperplastic MM, haphazardly intertwined muscle fibers with irregular contours were highlighted by SMA. In MP, SMA accentuated individual muscle fibers in compact bundles with regular outlines. Interestingly, SMA further highlighted a distinct band of scattered and isolated SMA-positive spindle cells in the suburothelial lamina propria in every section (42/42, 100%), which were most often not readily recognizable except as an ill-defined condensed connective tissue layer on the corresponding H&E slides. On higher power, the cells of this layer were comprised of small, disorganized, and individual spindle cells, which blended below with the thin delicate MM muscle fibers, which were readily recognized by H&E

TABLE 1. Comparative Immunohistochemical Staining of Muscularis Mucosae and Muscularis Propria Muscles of the Urinary Bladder With Smoothelin and SMA

Intensity	Distribution	MM (%)	MM Hyperplastic (%)	MP (Upper MP) (%)
Smoothelin				
Negative	0	19/42 (45)	17/34 (50)	0/42 (0)
Weak	+1	18/42 (43)	12/34 (35)	0/42 (0)
	+2	5/42 (12)	5/34 (15)	6/42 (14)
Strong	+3	0/42 (0)	0/34 (0)	36/42 (86)
SMA				
Negative	0	0/42 (0)	0/34 (0)	0/42 (0)
Weak	+1	0/42 (0)	0/34 (0)	0/42 (0)
	+2	0/42 (0)	0/34 (0)	0/42 (0)
Strong	+3	42/42 (100)	34/34 (100)	42/42 (100)

MM indicates muscularis mucosae; MP, muscularis propria; hyperplastic, >4 muscle fibers thick; SMA, smooth muscle actin; staining: 0, <5% positive; +1, 5% to 15% positive; +2, 16% to 50% positive; +3, >50% positive.

staining. Additional studies performed on 6 cases to characterize this layer supported the myofibroblastic nature of these cells (MSA positive 6/6, Masson trichrome blue staining 5/6, and red staining 1/6). We designate this distinctive layer as suburothelial band of myofibroblasts (SUM) (Figs. 1A, 2A, and 3). The SUM in all sections did not stain with smoothelin (0/42, 0%).

Unlike SMA, smoothelin displayed striking differential immunoreactivity between MM and MP muscles (Figs. 1B, D, and F). The MM muscle predominantly had absent (19/42, 45%) or weak and focal (18/42, 43%) smoothelin staining whereas only few (5/42, 12%) had +2 staining and none (0/42, 100%) had strong or diffuse (+3) staining. The subset of hyperplastic MM represented in the cystectomy specimens had predominant absent (17/34, 50%) and weak and focal (12/34, 35%) smoothelin staining and none showed strong or diffuse (+3) staining (Figs. 2A, B) (0/34, 0%). In contrast to the MM, the MP muscle bundles predominantly (36/42, 86%) showed strong and diffuse (+3) smoothelin staining. There was always a significant and striking disparity in staining intensity between the MM and MP muscle in every section. Smoothelin intensely accentuated individual muscle fibers within groups of MP bundles similar to that observed with SMA staining. Smoothelin occasionally showed +2 staining in MP muscle (6/42, 14%); in all these cases the MM staining was negative to rarely weak and focal (5% to 15%) staining retaining the striking differential staining with MP. Absent or focal staining with smoothelin was not observed in the MP muscle (0/42, 0%). The smooth muscle associated with the lamina propria vascular plexuses occasionally stained weakly with smoothelin.

When all smoothelin positivity regardless of staining intensity and pattern (weak or strong and +1 to +3) was considered, the specificity for MP (vs. MM) was only 50% (Table 2). When weak and focal (+1) smoothelin staining was discounted and only weak and (+2) and strong and diffuse (+3) staining was included, the specificity for MP (vs. MM) improved to 63%. When only strong and diffuse (+3) staining was accounted, the specificity and positive predictive value of smoothelin for MP (vs. MM) was 100%. Complete lack of staining to smoothelin was seen only in MM equivalent to a negative predictive value of 100% to MP for smoothelin. The sensitivity for MP slightly diminished to 86% (from 100%) when only strong and diffuse (+3) smoothelin staining was considered as MP.

Topographical Variations of Bladder MP at the Trigone and Ureteric Insertion

In the trigone, 3 of 5 bladder sections showed irregular small MP bundles with gradual diminution in size as they extended almost to the suburothelial region. This observation was in accordance with our previously published findings of muscle in this region.[9] These superficial extensions of MP in the trigone showed variable moderate (+2, 3/3) immunoreactivity with smoothelin, whereas the subjacent deeper and broader MP bundles stained more diffusely (+3) (Fig. 4A). At insertion of the ureter in the bladder wall, 6 of 6 sections showed the presence of a more superficial (suburothelial) ureteral MP overlying the bladder MP. On cross section, the ureteral MP muscle bundles were smaller in caliber than the bladder MP. All ureter and bladder MP muscle bundles were positive with smoothelin, the ureteral MP muscle varied from moderate (2/6, 33%) to diffuse (4/6, 67%) staining, and the bladder MP consistently showed diffuse staining (Fig. 4B) (6/6, 100%).

Smoothelin in TURBT Specimens

Nine of 10 TURBT sections showed presence of characteristic MP muscle in the H&E stain. Five of 9 were involved and 4 of 9 were free of invasive urothelial carcinoma. In all 9 TURBT sections, smoothelin highlighted MP muscle with strong and diffuse (+3) staining. In 2 of the 9 TURBT sections, in addition to the typical MM and MP muscle there were muscle bundles that were difficult to categorize on H&E as MM or MP. In one case, based on strong and diffuse (+3) smoothelin immunoreactivity with morphologic correlation, the muscle was

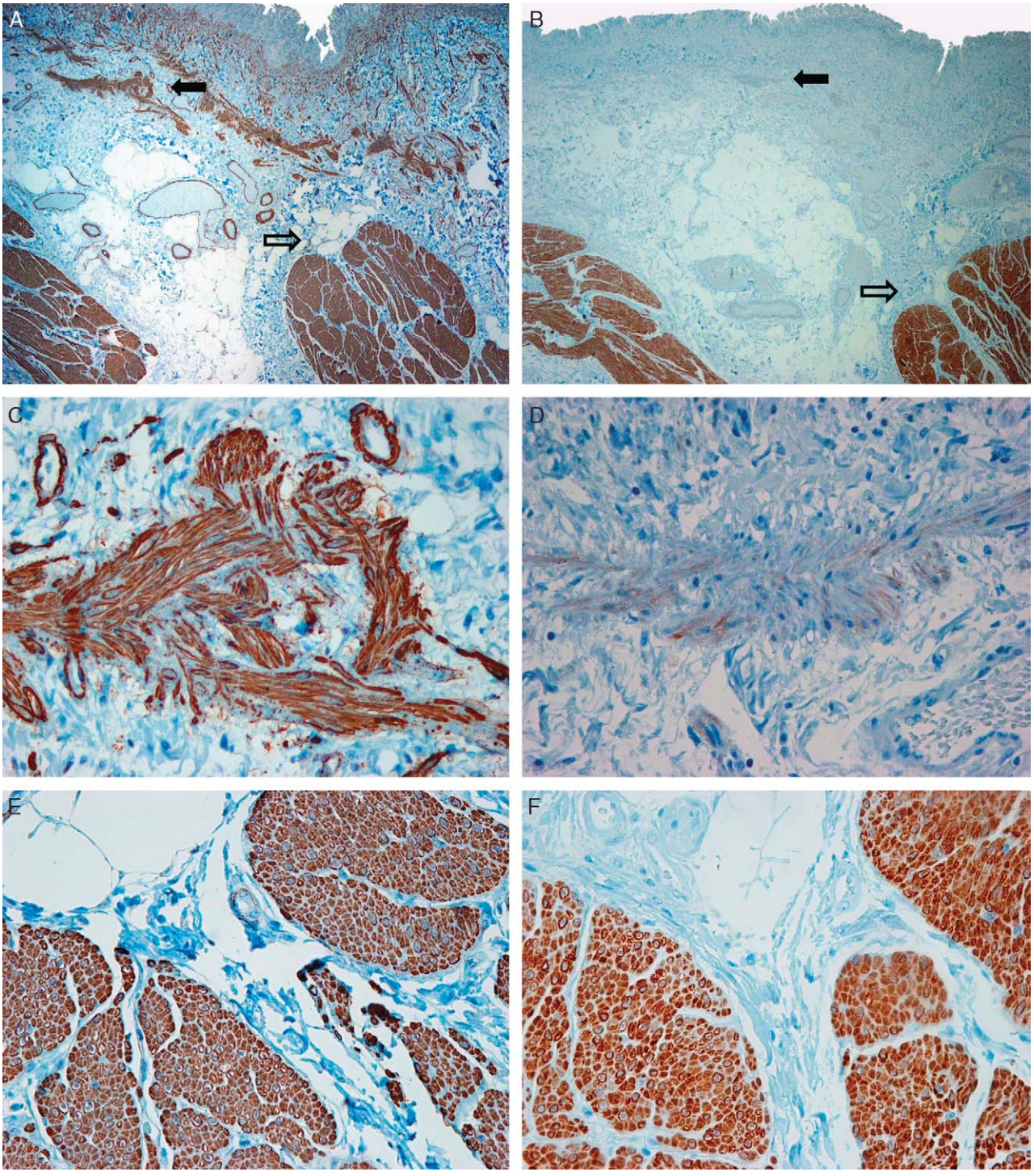

FIGURE 1. Differential Immunohistochemical staining of MM and MP muscle by SMA (A, C, E) and smoothelin (B, D, F). A, SMA highlights both hyperplastic MM (solid arrow) and MP muscle (open arrow) with diffuse strong staining. C, High power region of dark arrow. E, High power region of open arrow. B, In contrast, smoothelin shows only patchy staining in hyperplastic MM (solid arrow) but diffuse strong staining in MP muscle (open arrow). D, High power region of dark arrow. F, High power region of open arrow. MM indicates muscularis mucosae; MP, muscularis propria; SMA, smooth muscle actin.

determined to be MP muscle bundles infiltrated and destroyed partially by invasive urothelial carcinoma (Figs. 5A, B). In the other case, SMA staining outlined the nontypical muscle as MM based on the long slender disposition of the muscle bundles; these were negative for smoothelin (Figs. 5C, D). The single TURBT section

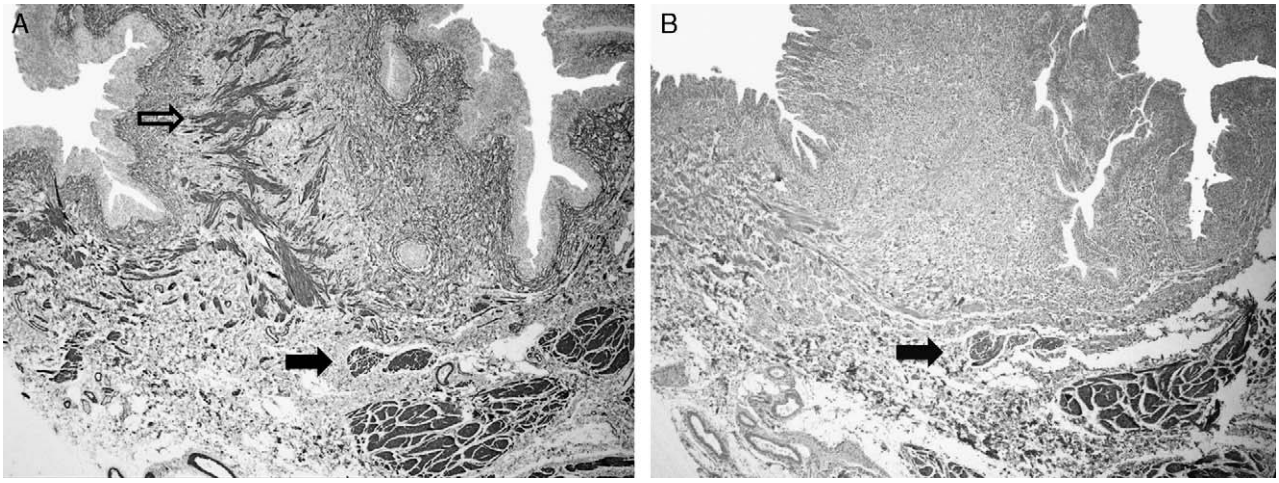

FIGURE 2. Smoothelin versus SMA in markedly hyperplastic MM. A, SMA diffusely stains the hyperplastic MM (open arrow) and MP muscle (solid arrow). B, In contrast, smoothelin shows complete absence of staining in the hyperplastic MM, but maintains diffuse strong staining in MP muscle (arrow). MM indicates muscularis mucosae; MP, muscularis propria; SMA, smooth muscle actin.

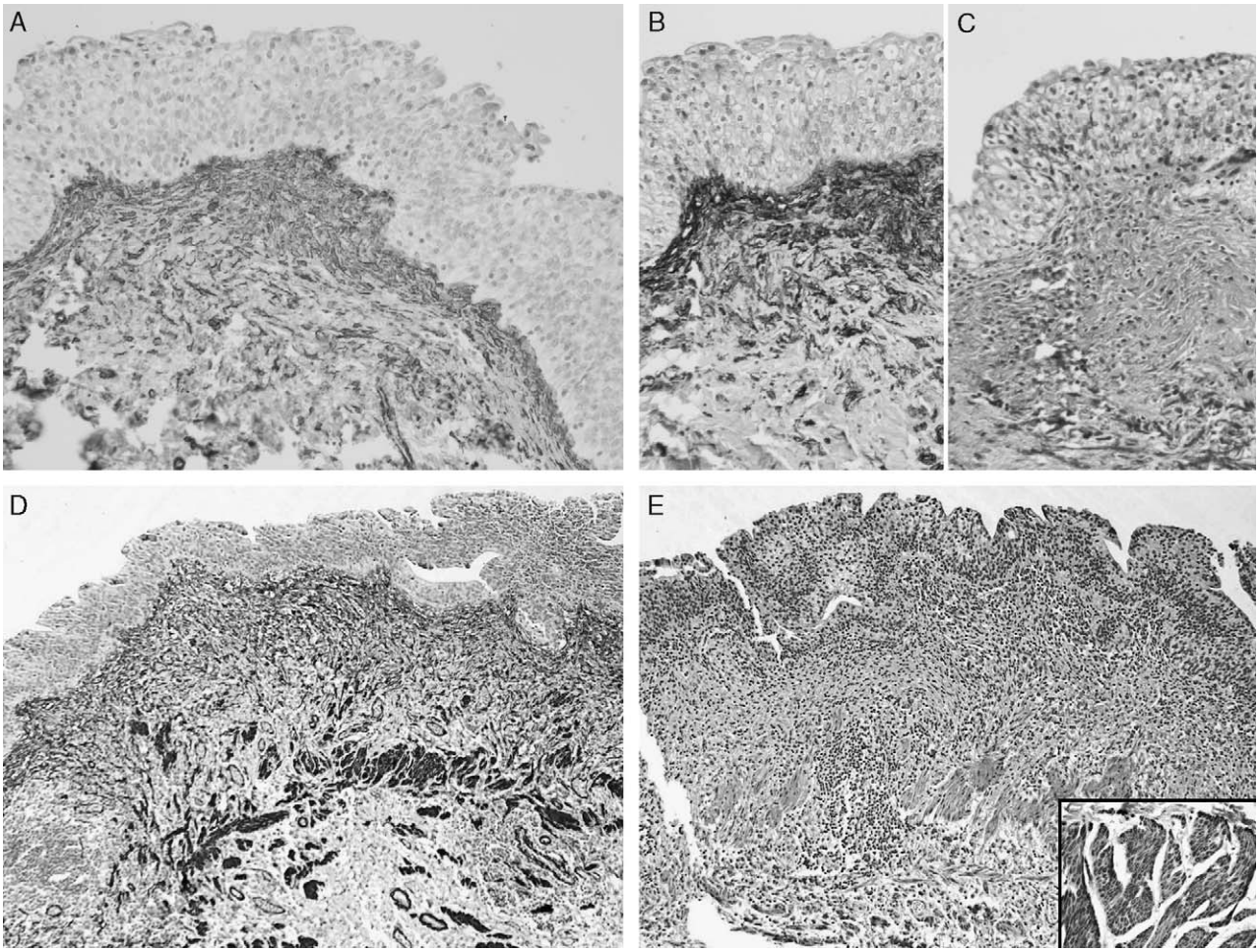

FIGURE 3. SUMs highlighted by (A) SMA and (B) MSA immunohistochemistry and (C) stained blue with Masson trichrome stain. D, The SUM merges with the muscularis mucosae muscle bundles and (E) both are nonreactive with smoothelin [(inset) MP muscle in same section is positive]. MP indicates muscularis propria; MSA, muscle-specific actin; SUM, suburothelial band of myofibroblasts; SMA, smooth muscle actin.

TABLE 2. Smoothelin Staining of Bladder Muscularis Propria

Smoothelin Staining	Specificity for MP (%)	Sensitivity for MP (%)	PPV for MP (%)	NPV for MP (%)
Weak or strong and +1 to +3	50	100	71	100
Weak or strong and +2 to +3	63	100	89	100
Strong and +3	100	86	100	85

MP indicates muscularis propria; PPV, positive predictive value; NPV, negative predictive value; Staining pattern: 0, < 5% positive; +1, 5% to 15% positive; +2, 16% to 50% positive; +3, > 50% positive.

without distinguishable MP on H&E showed absent smoothelin staining.

DISCUSSION

We demonstrate the potential robust utility of smoothelin to discriminate MP muscle from hyperplastic MM muscle in diagnostic surgical pathology. This is a novel finding as no immunohistochemical stain before our study has shown reliable discriminatory power to distinguish between MM and MP muscle. With smoothelin, MM muscle typically shows lack of, or only weak and focal immunoreactivity (88%). In contrast, the MP muscle is consistently strongly and diffusely immunoreactive (86%). This discriminatory capability with smoothelin is not appreciated with the more traditional smooth muscle marker SMA, which shows similar intense and diffuse staining in both MM and MP muscle. When only strong and diffuse (ie, greater than 50% of myocytes) positivity was considered, smoothelin was absolutely specific for distinguishing between MP (positive) and MM (negative). Thus, involvement of urothelial carcinoma by thick muscle bundle, which are of strong and diffusely smoothelin positive in the appropriate architectural context is highly supportive for MP muscle involvement. On the other hand, involvement by urothelial carcinoma by muscle bundles completely lacking smoothelin staining with light microscopic correlation is supportive of hyperplastic MM muscle involvement. Since 12% of MM may show moderate (+2) staining we recommend that attention should be paid to internal positive control (classic MP in slide) or an external positive control which should include a section of the bladder wall with MM and MP.

Recently, we described morphologic variations of bladder MM and MP muscle and the bladder wall[9] beyond the early classic descriptions by Dixon and Gosling,[4] Ro et al,[11] and Philip et al.[10] The MM is typically arranged in individual or small groups of slender and wavy fascicles or wispy fibers.[9] MM occasionally has focal to rarely extensive hyperplastic appearance (53%).[9] Helpful clues to the recognition of hyperplastic MM muscle bundles are that they have haphazard interwining of individual fibers with irregular outlines and typically occur as isolated muscle bundles in the superficial lamina propria, and/or in close association with the lamina propria vascular plexus. In contrast, MP muscle bundles

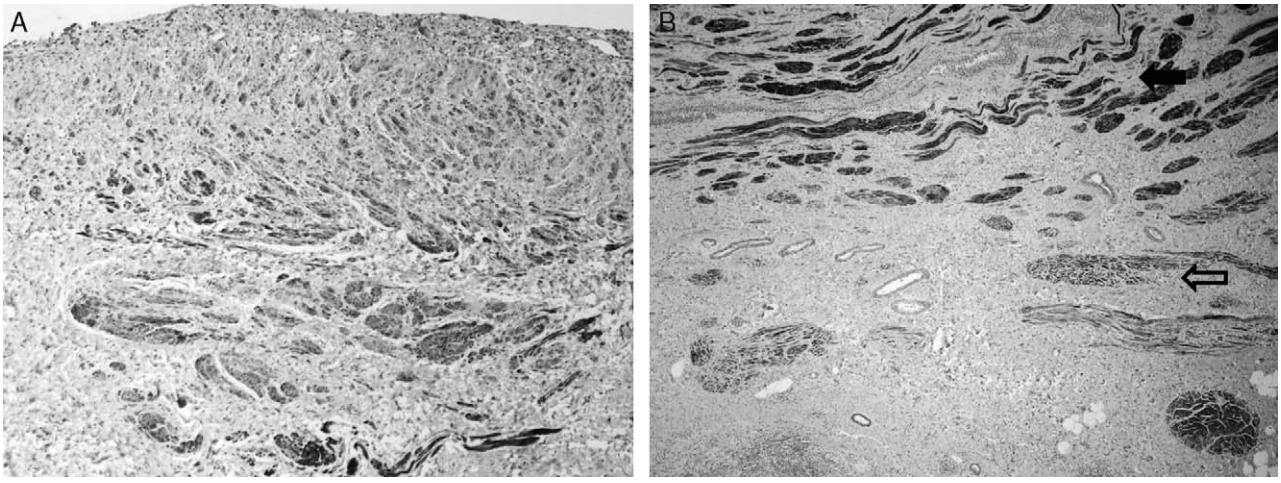

FIGURE 4. Topographic variations of MP muscle at this site. A, Trigonal MP stains with smoothelin showing the occasional variation of MP muscle as there is gradual diminution in muscle bundle caliber that extends almost to the suburothelium. B, At the ureteral insertion into the bladder, smaller caliber or more closely packed ureteral MP muscle (dark arrow) stains intensely with smoothelin similar to the deeper situated larger caliber bladder MP muscle (open arrow). MP indicates muscularis propria.

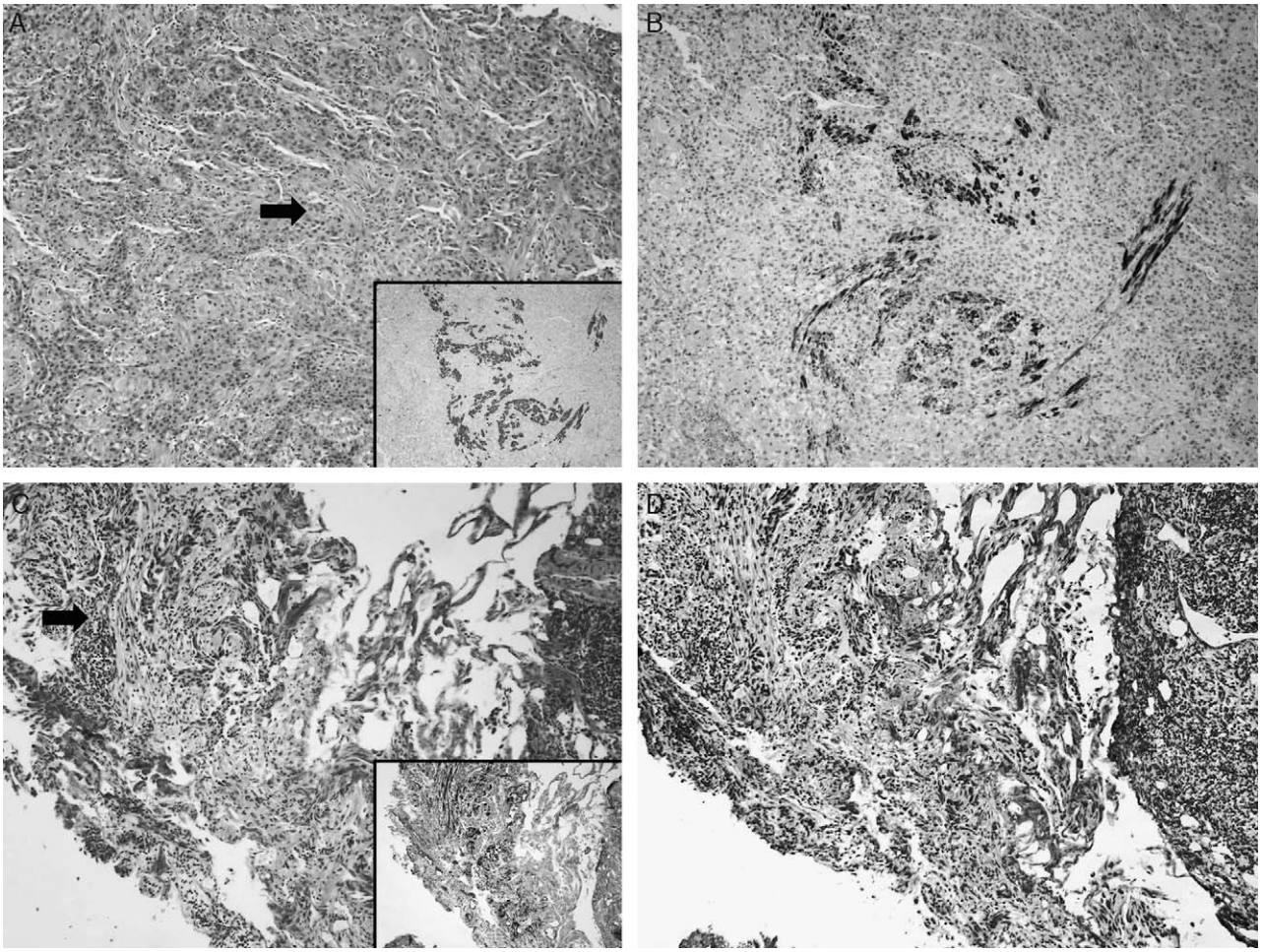

FIGURE 5. Smoothelin staining in TURBT specimens. A, MP muscle fibers (arrow) dispersed by infiltrating urothelial carcinoma, highlighted by SMA (inset) and (B) intense staining with smoothelin. C, Delicate MM muscle fibers (dark arrow) involved by infiltrating urothelial carcinoma in a TURBT specimen with the cautery artifact highlighted by SMA (inset), and (D) lack of staining with smoothelin in MM muscle bundles. MM indicates muscularis mucosae; MP, muscularis propria; SMA, smooth muscle actin; TURBT, transurethral resection of bladder tumor.

are frequently present in groups of compact muscle bundles with a smooth regular outline often arranged in several layers. In the current study, we examined 10 random TURBT sections including 9 with and 1 without morphologically identifiable MP muscle; and in all cases, smoothelin immunohistochemistry findings were in agreement with the typical muscle type by morphologic evaluation. Our pilot investigation in TURBT specimens indicates that smoothelin staining has value in these specimens, which may have crush and/or thermal artifact that may make evaluation of muscle-type difficult. A more detailed study of the utility of smoothelin in TURBT specimens, evaluating the impact of staining in the presence of poor orientation, extensive infiltration, crush, and cautery artifact is underway and will be the subject of a separate report. Overall, knowledge of morphology and variations of MM and MP muscle remains paramount in light microscopic evaluation, which is usually adequate in most cases.[9] Smoothelin immunohistochemistry is a potentially valuable diagnostic tool for pathologic staging in cases where muscle bundle patterns and morphology are not typical.

In this study, we observed scattered, small, disorganized, and individual SMA/MSA-positive cells along the suburothelial lamina propria, which represent scattered myofibroblasts. These cells are not readily recognizable on H&E slides except for a band of condensed connective tissue. On the basis of our study, we designate this layer as SUM. This has been described in more basic science literature[12,15] and the cells in the superficial region have been shown to have a more fibroblastic profile and a myofibroblastic character in the deep aspect based on vimentin and desmin immunoreaction.[15] It has been suggested that these cells may operate as a functional syncytium, integrating signals and responses in the bladder wall.[15] The SUMs in all our cases consistently stained negative by smoothelin.

We have recently elaborated on the topographical variations of the MP muscle bundles in bladder trigone and at the insertion of the ureter.[9] The trigonal MP extends more superficially and often shows gradual diminution of the bundle size as they extend to an almost suburothelial location.[9] In the current study, the MP of the trigone sections with typical morphology stained with smoothelin in all cases. In 3 of 5 cases, there were smaller MP bundles extending to the surface which were also diffusely smoothelin positive confirming our previous morphologic interpretation.[9,11] Our previous study demonstrated that at the ureteral insertion, MP muscle bundles are more superficial and smaller in caliber than the bladder MP.[9] Smoothelin immunohistochemical staining supports that these superficial ureteral muscle bundles are fully differentiated smooth muscle cells similar to those of the bladder MP based on their overall immunopositivity; however, there was greater variability in staining between muscle bundles in ureteral MP verus that of bladder MP.

Several other markers have been considered as being specific for muscle including SMA, metavinculin, calponin, and smooth muscle myosin isoforms, but some of these have been shown to react in other cells such as myofibroblasts, myoepithelial cells, cardiac and striated muscle cells, and most have been found in proliferative stages of smooth muscle cells.[14] A recent immunohistochemical study in abstract form showed similar staining of MM and MP with SMA, MSA, caldesmon, and desmin.[2] Our study demonstrates that smoothelin has discriminatory value as it is exclusively expressed intensely and diffusely only in fully differentiated smooth muscle cells, that is, MP (detrusor muscle).

The primary reason for distinguishing MM for MP muscle bundles is for accurate staging, which has therapeutic and prognostic implications. In most settings this distinction is not problematic; however, there are certain situations where an ancillary marker would be extremely useful. These include (a) a brisk desmoplastic myofibroblastic response which may simulate muscle invasion especially in cauterized specimens; (b) hyperplastic MM muscle bundles especially when invaded by cancer; (c) typical MP muscle bundles which are scant or very superficial; and (d) extensive urothelial carcinoma which infiltrates and disperses the usual round contour of MP muscle bundles where differential diagnosis includes hyperplastic MM and MP muscle. Availability and application of a reliable ancillary marker will be of great help to achieve accurate staging in these difficult scenarios.

In conclusion, the relatively distinct and differential immunohistochemical staining pattern of smoothelin between MP and MM (including its hyperplastic form) makes it an attractive marker to be incorporated in the contemporary diagnostic armamentarium for the sometimes difficult area of staging bladder urothelial carcinoma, which has important prognostic and therapeutic implications. The staining has promise in TURBT specimens based on preliminary analysis. Knowledge of morphology and variations of MM and MP muscle by H&E evaluation remains paramount and must always take precedence when evaluating the depth of urothelial carcinoma invasion. The relative specificity of smoothelin based on preliminary analysis for MP allowed further confirmation of the topographical variations of more superficial MP in the bladder trigone and ureter insertion which may complicate the traditional pT staging evaluation at these sites.

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