**TERT promoter mutation analysis as a surrogate to morphology and immunohistochemistry in problematic spindle cell lesions of the urinary bladder**

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**Aims**: Pseudosarcomatous myofibroblastic proliferations (PSMPs) of the urinary bladder are diagnostically challenging. Diagnostic difficulties are mainly due to frequent cytokeratin expression, variable ALK expression and worrisome morphological features suggestive of malignancy. Conversely, sarcomatoid urothelial carcinoma (UC) may show bland inflammatory myofibroblastic tumour (IMT)-like morphology. TERT promoter mutations are characteristic events in urothelial cancers, but have not been studied in PSMPs.

**Methods and results**: We compared histomorphological and immunohistochemical features and TERT promoter status in 16 PSMPs and 18 sarcomatoid UC. In a subset of PSMPs, RNA sequencing was performed. At least focal IMT-like morphology was seen in nine of 17 sarcomatoid UC. Atypical mitoses, differentiated urothelial component and heterologous elements were the most reliable distinguishing histomorphological features of sarcomatoid UC, if present. A panel of immunohistochemistry (IHC) including ALK (clone D5F3), p53 pattern, p63 and GATA3 reliably distinguished PSMP from sarcomatoid UC. GATA3 (P = 0.001) and p53 patterns (mutant versus wild-type; P < 0.001) were differentially expressed between PSMPs and sarcomatoid UC. Diffuse pancytokeratin staining was significantly associated with PSMPs (10 of 13) compared to four of 14 sarcomatoid UCs (P = 0.012). TERT promoter mutations were found in 17 of 18 sarcomatoid UC versus none of 16 PSMPs (P < 0.001). RNA sequencing revealed ALK genetic rearrangements in one of two ALK-positive and one of 10 ALK-negative PSMPs, which revealed a novel FN1/RET gene fusion.

**Conclusion**: Careful histomorphological analysis and differential IHC reliably distinguish the majority of PSMPs and sarcomatoid UC. In equivocal cases, TERT promoter mutation analysis and/or detection of ALK expression/rearrangements are valuable additional diagnostic adjuncts, strongly supporting sarcomatoid UC and PSMP, respectively.

**Keywords**: ALK gene rearrangement, inflammatory myofibroblastic tumour, pseudosarcomatous myofibroblastic proliferation, sarcomatoid urothelial carcinoma, TERT promoter mutation

**Introduction**

Myofibroblastic proliferations of the urinary bladder encompass several described lesions with overlapping morphology, including inflammatory myofibroblastic
tumour (IMT) and postoperative spindle cell nodule (PSCN). Whether these two lesions represent the same or different entities remains controversial. IMT seems to occur in virtually all locations, in adults most frequently in the lungs, whereas in children, the urinary tract, gastrointestinal tract and soft tissue are more frequently involved. Unlike its soft tissue counterparts, bladder IMT seems to follow a benign clinical course which makes its separation from malignant spindle cell lesions, especially sarcomatoid urothelial carcinomas (UC), most important. Accordingly, organ preservation is the mainstay of surgical treatment of IMT.

The majority of IMTs harbour a translocation of the anaplastic lymphoma kinase (ALK) gene on chromosome 2p23, resulting in constitutive gene activation and nuclear and/or cytoplasmic ALK protein expression in up to 75% of bladder IMTs.1,4,7,9

Introduced by Proppe et al.,10 the term ‘PSCN’ is reserved for myofibroblastic lesions occurring several weeks to months after lower urogenital tract surgery/instrumentation. PSCN closely resembles IMT, including even ALK expression in a subset of cases, but due to its close association with antecedent trauma is considered reactive.1,11

The term ‘pseudosarcomatous myofibroblastic proliferation’ (PSMP) is used differently by different authors, either in a general sense to refer to any myofibroblastic lesion regardless of being neoplastic or reactive in origin or as a term specifically defining only reactive/reparative myofibroblastic lesions of the bladder including PSCN and myofibroblastic proliferations without history of prior surgery or trauma.12

We support the opinion that both neoplastic proliferations with genetic rearrangements (i.e. IMT) and reactive myofibroblastic proliferations without rearrangements (including PSCN and those without prior surgical intervention or trauma) are morphologically similar, with a usually benign clinical course, irrespective of being neoplastic or reactive/reparative in origin. Therefore, we lumped these entities in this study together within the general term PSMP for differential immunohistochemistry (IHC) and TERT promoter mutation analysis to compare them with sarcomatoid UC.

Detrusor muscle involvement in bladder PSMP frequently raises suspicion of invasive cancer. This may be complicated by brisk mitotic activity, foci of ischaemic-type necrosis and pancytokeratin expression. In contrast, focal or occasionally extensive PSMP (IMT)-like morphology in sarcomatoid UC with relatively bland cytology, myxoid stroma and lack of necrosis could give the impression of a low-grade lesion such that distinction from PSMP may be challenging or on occasion even arbitrary. IMT associated with carcinoma and one histologically malignant bladder IMT (inflammatory fibrosarcoma) have been reported.3

Several studies have addressed immunohistochemical features of PSMP, some including the differential diagnosis of sarcomatoid UC. However, previous reports presenting p53 lack information on the p53 phenotype (wild-type versus mutant type) and we did not find any data on GATA binding protein 3 (GATA3). Thus, we chose to study these biomarkers in a panel of previously proposed markers.3,6,14

TERT promoter mutations are frequent in UC, but also occur in several other human malignancies, although exceptionally rare in other genitourinary cancers and most sarcomas.18 TERT promoter mutation analysis serves as an adjunct tool for verification of malignancy in bland-appearing bladder tumours and their separation from benign mimics.19

Until now, there have been no data on TERT promoter mutations in bladder PSMPs. Accordingly, the potential diagnostic value of the detection of these mutations in distinguishing malignant from benign spindle cell urothelial neoplasms of the bladder in equivocal and challenging cases remained unexplored. We performed TERT promoter mutation analysis in PSMPs (including ALK-positive and -negative lesions) and sarcomatoid UC, including nine cases with IMT-like morphology. Additionally, ALK-negative PSMPs were screened for genetic rearrangements by RNA sequencing.

Materials and methods

Thirty-four cases originally diagnosed as IMT (n = 8), PSCN (n = 7), PSMP (n = 1) or sarcomatoid UC (n = 18) according to the histology reports were collected from the records of our department and cooperating institutes. Institutional review board approval (University Hospital Erlangen) was obtained for molecular analysis on archival material. None of the cases was published before. Haematoxylin and eosin (H&E) slides and IHC were critically re-evaluated by two experienced pathologists (A.A., S.B.). Because it is frequently impossible to verify history of antecedent instrumentation in all cases, and as the prior aim of the study was separation of clinically benign from malignant lesions, ALK-positive and -negative PSMPs were lumped together in one PSMP cohort for comparative analysis with sarcomatoid UC in this study. If available, data of fluorescence in-situ hybridisation (FISH) analysis were obtained from previous

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diagnostic reports. Ethical approval for use of formalin-fixed and paraffin-embedded (FFPE) tissue (no. 329_16B) was obtained from the University of Erlangen-Nürnberg.

Histomorphological analysis included documentation of detrusor muscle involvement, IMT-like areas in sarcomatoid UC (defined by myxoid stroma, little to moderate atypia, sometimes low mitotic activity and reduced cellularity associated with background inflammation), differentiated urothelial component (CIS, urothelial dysplasia, papillary carcinoma), heterologous elements, necrosis, mitotic count and atypical mitotic figures.

Supplemental IHC (Table 1) was performed on a BenchMark ULTRA System (Ventana Medical Systems, Inc., Tucson, AZ, USA) if blocks were available. The cytokeratin and smooth muscle actin (SMA) staining patterns were considered ‘diffuse’ if staining was consistently positive in the majority (>80%) of tumour cells or ‘focal’, in the case of heterogeneous, patchy or positive staining restricted to few cells. For Ki67, a proliferation index >15% was considered ‘high’. P53 IHC was interpreted as mutated-type, in tumour cells or ‘focal’, in the case of heterogeneous, patchy or positive staining restricted to few cells. For Ki67, a proliferation index >15% was considered ‘high’. P53 IHC was interpreted as mutated-type, in cases with strong and diffuse nuclear immunoreactivity or loss of expression (null-phenotype)20 and wild-type in cases with patchy–variable and intermediate intensity immunostaining.

All reported FISH results (n = 15 of 16 PSMP; n = 1 of 16 not available) were obtained from analyses previously performed at our institute, according to our laboratory standard protocol with a ZytoLight® SPEC ALK/EML4 TriCheck™ Probe (ZytoVision GmbH, Bremerhaven, Germany) on 2 µm deparaffinised FFPE tissue slides. ALK rearranged cases show one fused (orange/green) and one separate orange and green split-signal in >25 of 50 tumour cells.21 If there was no information available from the diagnostic records we did not perform additional FISH analysis for this study.

For DNA isolation tumour tissue was manually microdissected according to previously marked slides to achieve >80% purity of tumour tissue and in order to separate sarcomatoid and differentiated carcinoma components and processed, as previously described.22

TERT promoter mutation analysis (n = 16 PSMP; n = 18 sarcomatoid UC) addressing hot-spot mutations at positions –146, –124 and –57 base pairs (bp) of the TERT promoter was performed with an ABI Prism 3500 Genetic Analyzer and the SNaPshot Multiplex-Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions. All primers and reaction conditions have been reported.23 As explained above, all cases (n = 16 PSMP; n = 18 sarcomatoid UC) were marked for manual microdissection before DNA isolation in order to achieve >80% purity of tumour tissue. Sarcomatoid and differentiated carcinoma components were separated marked in eight of 18 sarcomatoid UC cases. Separate analysis of both components was performed in three of eight sarcomatoid UC with differentiated component; in five of eight cases only the microdissected sarcomatoid component was analysed, due to very small amounts of the differentiated tumour component.

RNA was isolated from tissue sections and quantified spectrophotometrically. Sequencing analysis (n = 12 of 16 PSMP; four of 16 not available) was performed according to the manufacturer’s protocol on a MiSeq System using the TruSight RNA Fusion panel (Illumina Inc., San Diego, CA, USA) covering 507 fusion-associated genes (Supporting information, Data S1). Detailed information on the analysis has been published previously.24

Statistical analyses were performed with Excel for Windows (Microsoft Excel 2016) and spss for Windows (IBM Statistics, version 24.0) using cross-tabulations (χ² test). For expected values <5, the two-sided Fisher’s exact test was chosen. Results were regarded significant for P-values < 0.05.

Table 1. Antibodies used for immunohistochemistry (IHC)

| Antibody | Company     | Clone          | Dilution |
|----------|-------------|----------------|----------|
| ALK*     | Cell Signaling | D5F3          | 1:100    |
| Pancytokeratin | ZytoMed    | cocktail AE1/AE3 | 1:40    |
| Cytokeratin 18 | Sigma    | CY-90         | 1:500    |
| Cytokeratin 19 | Dako     | RCK108        | 1:300    |
| Ki67     | Dako        | MIB1          | 1:100    |
| p53      | Dako        | DO-7          | 1:50     |
| p63      | DCS         | SFI-6         | 1:100    |
| GATA3    | DCS         | L50-823       | 1:1000   |
| Smooth muscle actin | Dako | 1A4         | 1:400    |

ALK, anaplastic lymphoma kinase; IHC, immunohistochemistry; GATA3, GATA binding protein 3.

*For ALK IHC, on-slide positive controls were used.

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Results

CLINICAL CHARACTERISTICS

Histomorphological and immunohistochemical re-evaluation revealed 16 PSMPs (six ALK-positive; 10
Sixteen of 16 PSMPs showed a wild-type TERT promoter status, whereas 17 of 18 (94%) sarcomatoid UCs were mutated ($P < 0.001$) (Table 5). Mutations (G$\rightarrow$A) were most frequent at position $-124$ (13 of 18) (72%). There was no obvious association between the mutation position and IMT-like morphology in sarcomatoid UC ($P = 0.59$). Separate analysis of the differentiated and the sarcomatoid component was performed in three of eight cases, which revealed corresponding TERT promoter mutations in both components. In five of eight cases analysis of only the sarcomatoid UC component was performed.

**ALK Analysis**

ALK rearrangement was detected by ALK FISH in three of five ALK-positive PSMPs (Figure 3). RNA-based sequencing was performed in two of two ALK-positive PSMPs with negative FISH result and 10 of 10 ALK-negative PSMPs (Table 6). An FN1/ALK fusion was detected in one of the two ALK-positive PSMPs and a novel FN1/RET gene fusion with breakpoints at FN1 chromosome 2:216251411 and RET chromosome 10:43604476 (PSMP 9; Figure 3) in one of 10 ALK-negative PSMP.

**Discussion**

Inflammatory myofibroblastic tumour (IMT) is difficult to distinguish histomorphologically from other reactive PSMPs in the urinary bladder$^{12,25}$ and IMT may also occur after surgical intervention.$^{11}$ Conversely, distinction between PSMP of the urinary bladder and sarcomatoid UC may pose a diagnostic challenge on transurethral resections, especially in ALK-negative lesions with high mitotic count, necrosis$^{6,12}$ and detrusor muscle involvement.$^{3,6}$ Sarcomatoid UC occasionally mimic benign spindle cell lesions, due to areas with bland cytology, compact fascicular growth, low mitotic count, myxoid stroma, background inflammation and lack of consistent cytokeratin expression, giving the impression of non-epithelial origin. In our PSMP cohort, misleading morphological features were present in comparable frequencies, as documented in the literature, e.g. detrusor muscle involvement was present in 50% of PSMPs compared with ~ 60% in previous reports.$^{3,26}$ In contrast, half of our sarcomatoid UC cohort presented with bland appearing IMT-like areas, in some of them to a degree that the malignant character of the lesion was doubtful.

Absence of atypical mitoses in PSMPs, compared to 63% sarcomatoid UCs, was a reliable distinguishing morphological feature, as reported previously.$^{26,27}$ A differentiated urothelial component as a classical argument in favour of sarcomatoid UC was found in 44% of sarcomatoid UC. However, as residual or recurrent high-grade urothelial lesions may be detected in resections for PSMP, and PSMP may clinically mimic recurrent UC, careful interpretation of this finding is mandatory to avoid over interpretation of PSMP associated with CIS or other high-grade urothelial lesions as sarcomatoid component of invasive UC. Another reliable feature was the presence of...
Table 2. Patient characteristics

| Case no. | Sex  | Age at diagnosis | Surgery     | Diagnosis                              | Patient history     |
|---------|------|------------------|-------------|----------------------------------------|---------------------|
| 1       | M    | 81               | TUR-B       | ALK-negative PSMP                      | UC                  |
| 2       | M    | 67               | Cystectomy  | ALK-negative PSMP                      | Urothelial CIS      |
| 3       | F    | 24               | TUR-B       | ALK-positive PSMP                      | NA                  |
| 4       | M    | 74               | TUR-B       | ALK-negative PSMP                      | UC                  |
| 5       | F    | 62               | TUR-B       | ALK-positive PSMP                      | NA                  |
| 6       | M    | 45               | TUR-B       | ALK-positive PSMP                      | NA                  |
| 7       | M    | 52               | TUR-B       | ALK-negative PSMP                      | Urothelial CIS      |
| 8       | M    | 51               | TUR-B       | ALK-positive PSMP                      | NA                  |
| 9       | M    | 79               | TUR-B       | ALK-negative PSMP                      | NA                  |
| 10      | M    | 54               | TUR-B       | ALK-negative PSMP                      | UC                  |
| 11      | F    | 73               | TUR-B       | ALK-positive PSMP                      | NA                  |
| 12      | M    | 57               | TUR-B       | ALK-negative PSMP                      | UC pTaG3, high-grade |
| 13      | M    | 67               | TUR-B       | ALK-negative PSMP                      | UC                  |
| 14      | F    | 52               | Cystectomy  | ALK-negative PSMP                      | UC pT1G3, high-grade |
| 15      | F    | 64               | TUR-B       | ALK-positive PSMP                      | NA                  |
| 16      | M    | 58               | TUR-B       | ALK-negative PSMP                      | BPH                 |

**PSMP**

| Case no. | Sex  | Age at diagnosis | Surgery     | Diagnosis                              | Patient history     |
|---------|------|------------------|-------------|----------------------------------------|---------------------|
| 1       | M    | 81               | TUR-B       | ALK-negative PSMP                      | UC                  |
| 2       | M    | 67               | Cystectomy  | ALK-negative PSMP                      | Urothelial CIS      |
| 3       | F    | 24               | TUR-B       | ALK-positive PSMP                      | NA                  |
| 4       | M    | 74               | TUR-B       | ALK-negative PSMP                      | UC                  |
| 5       | F    | 62               | TUR-B       | ALK-positive PSMP                      | NA                  |
| 6       | M    | 45               | TUR-B       | ALK-positive PSMP                      | NA                  |
| 7       | M    | 52               | TUR-B       | ALK-negative PSMP                      | Urothelial CIS      |
| 8       | M    | 51               | TUR-B       | ALK-positive PSMP                      | NA                  |
| 9       | M    | 79               | TUR-B       | ALK-negative PSMP                      | NA                  |
| 10      | M    | 54               | TUR-B       | ALK-negative PSMP                      | UC                  |
| 11      | F    | 73               | TUR-B       | ALK-positive PSMP                      | NA                  |
| 12      | M    | 57               | TUR-B       | ALK-negative PSMP                      | UC pTaG3, high-grade |
| 13      | M    | 67               | TUR-B       | ALK-negative PSMP                      | UC                  |
| 14      | F    | 52               | Cystectomy  | ALK-negative PSMP                      | UC pT1G3, high-grade |
| 15      | F    | 64               | TUR-B       | ALK-positive PSMP                      | NA                  |
| 16      | M    | 58               | TUR-B       | ALK-negative PSMP                      | BPH                 |

**Sarcomatoid UC**

| Case no. | Sex  | Age at diagnosis | Surgery     | Diagnosis                              | Patient history     |
|---------|------|------------------|-------------|----------------------------------------|---------------------|
| 1       | M    | 88               | TUR-B       | Sarcomatoid UC, IMT-like               |                    |
| 2       | M    | 60               | TUR-B       | Sarcomatoid UC                         |                    |
| 3       | M    | 68               | TUR-B       | sarcomatoid UC                         |                    |
| 4       | M    | 75               | TUR-B       | Sarcomatoid UC, IMT-like areas         |                    |
| 5       | M    | 80               | TUR-B       | Sarcomatoid UC                         |                    |
| 6       | M    | 86               | TUR-B       | Sarcomatoid UC with chondrosarcomatous heterologous component, IMT-like |                    |
| 7       | M    | 83               | TUR-B       | Sarcomatoid UC, IMT-like               |                    |
| 8       | F    | 66               | Biopsy/resection | Sarcomatoid UC, IMT-like               |                    |
| Case no. | Sex | Age at diagnosis | Surgery  | Diagnosis                                      | Patient history                                      |
|---------|-----|------------------|----------|-----------------------------------------------|-----------------------------------------------------|
| 9       | M   | 69               | TUR-B    | Sarcomatoid UC, IMT-like                      |                                                     |
| 10      | M   | 50               | TUR-B    | Sarcomatoid UC                                |                                                     |
| 11      | F   | 57               | TUR-B    | Sarcomatoid UC                                |                                                     |
| 12      | M   | 77               | Cystectomy| Sarcomatoid UC with leiomatous heterologous component |                                                     |
| 13      | M   | 78               | TUR-B    | Sarcomatoid UC                                |                                                     |
| 14      | M   | 57               | TUR-B    | Sarcomatoid UC with myogenic heterologous component (DD epithelioid leiomysarcoma) |                                                     |
| 15      | M   | 65               | TUR-B    | Sarcomatoid UC, IMT-like                      |                                                     |
| 16      | M   | 78               | TUR-B    | Sarcomatoid UC, IMT-like areas                |                                                     |
| 17      | M   | 86               | TUR-B    | Sarcomatoid UC                                |                                                     |
| 18      | M   | 82               | TUR-B    | Sarcomatoid UC                                |                                                     |

TUR-B, transurethral resection of the bladder; PSMP, pseudosarcomatous myofibroblastic proliferation; IMT, inflammatory myofibroblastic tumour; UC, urothelial carcinoma; CIS, carcinoma in situ; ALK, anaplastic lymphoma kinase; BPH, benign prostatic hypertrophy; M, male; F, female; DD, differential diagnosis.

*Rearrangement by RNA-fusion analysis only
†Biopsy of bladder tumour and resection of subcutaneous metastasis.
heterologous elements, which was limited to four of 18 sarcomatoid UC.

Apart from ALK protein expression (discussed below), the most reliable distinguishing immunohistochemical marker is the mutation-type p53 expression pattern, found in 15 of 16 sarcomatoid UC, but not in PSMPs. However, p53 pattern was equivocal in two ALK-positive PSMPs and one sarcomatoid UC. As previously reported, p63 is expressed in a subset of sarcomatoid UC but not in PSMP. The same is true for GATA3, which has not been evaluated in previous studies. Both p63 and GATA3 are of help only if present as negative staining is observed in both entities.

A major problem with most morphological and immunohistochemical features of sarcomatoid UC is that they are not universally found and hence only helpful if present. Moreover, absence of morphological criteria may result from sampling errors or tissue artefacts: common findings, especially in transurethral resection specimens. Similarly, in consultation cases, the choice of representative tumour blocks is crucial.

Cytokeratin expression in PSMPs is a well-known diagnostic pitfall found in up to 80% of cases. As expected, pancytokeratin expression was frequent in both PSMP and sarcomatoid UC (88% each), yet the staining pattern differed significantly ($P = 0.012$), with diffuse and consistent staining observed in PSMP but only focal and patchy staining in the majority of sarcomatoid UCs. However, in the literature and in our study, cases of PSMP with focal cytokeratin staining pattern were observed.

TERT promoter mutations occur in up to 80% of bladder cancers throughout all stages, grades and subtypes. TERT promoter mutations have been recurrently reported in liposarcomas, solitary fibrous tumours and atypical fibroxanthomas, but are exceedingly rare in many other soft tissue cancers.

Figure 1. A,C,E, Morphological spectrum of PSMP and their urothelial mimics. B,D,F, examples for IMT-like morphology in sarcomatoid UC; all haematoxylin and eosin. IMT, inflammatory myofibroblastic tumour; PSMP, pseudosarcomatous myofibroblastic proliferation; UC, urothelial carcinoma.
The diagnostic value of TERT promoter mutations is not only in confirmation of a urothelial origin within genitourinary malignancies, but also in the differentiation of bland-looking UC variants from benign urothelial lesions. To date, there has been only one study on sarcomatoid UC restricted to upper urinary tract tumours, reporting a 35% TERT mutation frequency, yet the frequency in sarcomatoid UC localised in the bladder has not been investigated. The frequency of 94% TERT

| Case no. | ALK (D5F3) | Pancytokeratin | p53 | Ki67 | p63 | GATA3 | SMA |
|----------|-------------|----------------|-----|------|-----|-------|-----|
| PSMP     |             |                |     |      |     |       |     |
| 1        | –           | Focal          | WT  | ≤15  | –   | –     | +   |
| 2        | –           | +              | WT  | ≤15  | –   | –     | +   |
| 3        | +           | +              | WT  | ≤15  | –   | –     | Focal |
| 4        | –           | +              | WT  | >15  | –   | –     | +   |
| 5        | +†          | +              | WT  | NA   | –   | –     | Focal |
| 6        | +†          | +              | WT  | >15  | –   | –     | Focal |
| 7        | –           | +              | WT  | NA   | –   | –     | Focal |
| 8        | +           | –              | WT  | NA   | –   | –     | +   |
| 9        | –†          | –              | Equivocal | >15 | –   | –     | Few cells |
| 10       | –           | Focal          | WT  | ≤15  | –   | –     | +   |
| 11       | +†          | +*             | NA  | NA   | –   | NA    | NA  |
| 12       | –           | Focal          | Equivocal | >15 | –   | –     | +   |
| 13       | –           | +              | WT  | ≤15  | –   | –     | +   |
| 14       | –           | +              | WT  | >15  | –   | –     | +   |
| 15       | +†          | +              | WT  | >15  | –   | –     | Focal |
| 16       | –           | +              | WT  | >15  | –   | –     | +   |
| Sarcomatoid UC |             |                |     |      |     |       |     |
| 1        | –           | –              | MUT | NA†  | Few cells | + | – |
| 2        | NA          | +*             | NA  | >15† | –   | +     | –   |
| 3        | NA          | Focal          | Equivocal | >15† | Focal | Few cells | NA |
| 4        | –           | Focal          | MUT | >15  | –   | –     | Focal |
| 5        | –           | Focal          | MUT | >15  | –   | –     | –   |
| 6        | NA          | Focal          | MUT | >15† | Focal | Focal | NA  |
| 7        | –           | +              | MUT | >15  | –   | Focal | +   |
| 8        | –           | +              | NA  | >15† | –   | +     | –   |
| 9        | –           | Focal          | MUT§ | NA†  | +   | +     | Focal |
| 10       | –           | Focal          | MUT | >15  | +   | –     | –   |
| 11       | NA          | +              | MUT | >15† | +   | –     | NA  |
| 12       | –           | Focal          | MUT | >15  | +   | –     | –   |
| 13       | –           | Focal          | MUT§ | >15† | Focal | – | NA  |
| 14       | –           | +              | MUT | NA   | –   | –     | –   |

The diagnostic value of TERT promoter mutations is not only in confirmation of a urothelial origin within genitourinary malignancies, but also in the differentiation of bland-looking UC variants from benign urothelial lesions. To date, there has been only one study on sarcomatoid UC restricted to upper urinary tract tumours, reporting a 35% TERT mutation frequency, yet the frequency in sarcomatoid UC localised in the bladder has not been investigated. The frequency of 94% TERT

Table 3. Results of immunohistochemistry

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promoter mutations in our sarcomatoid UC cohort is even higher than the frequencies reported in conventional UC.29,30 We did not find TERT promoter mutations in PSMP.

According to our results, the presence of TERT promoter mutation is a highly specific differential marker in favour of sarcomatoid UC; however, the sensitivity needs to be confirmed in larger cohorts.

The frequency of ALK negativity in PSMPs of the bladder (63% in our cohort) is probably influenced by the fraction of included post-surgical (PSCN) subcohort. The high percentage of ALK-negative PSMP may also explain the lack of a statistically significant difference between the PSMP and sarcomatoid UC cohorts regarding ALK expression status. Minimal (<1%) cytoplasmic ALK protein expression was seen in a single sarcomatoid UC with IMT-like morphology. In this case the IMT-like areas were limited, and alternated with unequivocal sarcomatoid UC. A low-grade papillary tumour component was also present. Consequently, we did not perform additional FISH or RNA sequencing in this case, as the ALK pattern was obviously non-specific.

Approximately 25% ALK-positive PSMP cases may be missed if only one method of detection is used.36 Choi et al.11 reported a 91% concordance regarding detection of ALK protein expression with two different antibodies, with a slightly higher sensitivity for the ALK clone D5F3 antibody, which we used in our study. Antibody studies with ALK rearranged lung adenocarcinomas reported a sensitivity of 100% and specificity of 99% and positive and negative predictive values of 96 and 100%, respectively, for this antibody.37 Choi et al. presented three cases positive by ALK IHC but negative by FISH analysis and one case negative by ALK IHC but positive by FISH analysis.11 As the ALK antibody detects overexpression of the ALK protein caused by several alternate mechanisms besides translocation, including activating point mutations, amplification, and post-transcriptional modification, FISH can be negative despite positive IHC,11 which may explain the results in one of our

Table 3. (Continued)

| Case no. | ALK (D5F3) | Pancytokeratin | p53 | Ki67 | p63 | GATA3 | SMA |
|----------|------------|----------------|-----|------|-----|-------|-----|
| 15       | NA         | NA             | MUT | >15 | Focal | Focal | –   |
| 16       | –          | –              | MUT | >15 | +   | Focal | NA  |
| 17       | –          | Focal          | MUT | >15 | –   | –     | NA  |
| 18       | +          | Focal          | MUT | NA  | –   | + (weak) | Focal |

WT, wild-type-pattern; MUT, mutant pattern; NA, not available; FISH, fluorescence in-situ hybridisation; ALK, anaplastic lymphoma kinase; GATA3, GATA binding protein 3; SMA, smooth muscle actin.

*Staining pattern not assessable.
†FISH analysis: confirmed ALK rearrangement.
‡FISH analysis: no ALK rearrangement.
§Null-phenotype.
¶Atypical mitotic figures present.

Table 4. Differential expression of immunohistochemical markers

| ALK (D5F3) | IMT/PSMP | Sarcomatoid UC | P-value
|------------|----------|----------------|---------|
| 6/16 (37.5%) | 1/13 (7.7%) | 0.093 |

P-value < 0.05 significant (bold font indicates statistically significant values).

ALK, anaplastic lymphoma kinase; IMT, inflammatory myofibroblastic tumour; SMA, smooth muscle actin; GATA3, GATA binding protein 3; UC, urothelial carcinoma; DD, differential diagnosis; PSMP, pseudosarcomatous myofibroblastic proliferation.

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cases. We did not find cases with ALK rearrangement and negative IHC. Thus, combined ALK IHC and FISH will not be able to detect all ALK alterations. Moreover, cases with non-ALK gene rearrangements cannot be excluded by these techniques.

Therefore, we tested all PSMPs which stained negative for ALK (D5F3), and ALK-positive cases with a negative FISH result with a commercial RNA-based fusion assay, revealing chromosomal rearrangements in two of 12 cases. Alterations of the ALK gene have been reported in up to 70% of bladder IMTs, mainly rearrangements, but also copy number gains/amplifications.\textsuperscript{3,38} In the bladder, \textit{FN1},\textsuperscript{39,40} \textit{CLTC},\textsuperscript{40} \textit{HNRNPA1},\textsuperscript{41} \textit{TPM4}\textsuperscript{12} and \textit{ATIC}\textsuperscript{43} are documented fusion partners. Besides \textit{FN1}/ALK fusion\textsuperscript{40} in one case, we found a novel \textit{FN1}/RET fusion in one ALK-negative case. This case was consequently reclassified from initially ALK-negative PSMP (prior to RNA sequencing) to PSMP with novel rearrangement in Table 6 (after RNA sequencing). The involvement of \textit{RET} has been reported in two recent cases of a uterine\textsuperscript{44} and a pulmonary IMT\textsuperscript{45} only, the latter with ALK protein expression, despite lack of ALK gene involvement. However, to our knowledge, involvement of \textit{RET}, has not previously been reported in bladder IMT. One case with ALK protein expression was negative for rearrangement by FISH and RNA sequencing, which may be explained by false-positive IHC, gene fusion not covered by the Illumina panel, or genetic alteration other than rearrangement.\textsuperscript{11} Secondary ALK expression has recently been reported in diverse entities carrying non-ALK gene fusions, including angiomatoid fibrous histiocytoma (\textit{EWSR1}-rearranged),\textsuperscript{46} rhabdomyosarcoma (\textit{TFCP2} and other

Figure 2. Differential immunohistochemistry in pseudosarcomatous myofibroblastic proliferations (PSMP) (left column: A,C,E) and sarcomatoid urothelial carcinoma (UC) (right column: B,D,F). First row: anaplastic lymphoma kinase (ALK) expression positive in inflammatory myofibroblastic tumour (IMT), negative in sarcomatoid UC; second row: pancytokeratin (AE1/AE3) expression diffuse in PSMP, focal in sarcomatoid UC; third row: p53 wild-type pattern in PSMP and mutant pattern in sarcomatoid UC.

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### Table 5. Results of TERT promoter mutation analysis (bold font indicates TERT promoter mutations)

| Case no. | TERT-57 | TERT-124 | TERT-146 | Diagnosis                          |
|----------|---------|----------|----------|-----------------------------------|
| PSMP     |         |          |          |                                   |
| 1        | WT      | WT       | WT       | ALK-negative PSMP                 |
| 2        | WT      | WT       | WT       | ALK-negative PSMP                 |
| 3        | WT      | WT       | WT       | ALK-positive PSMP                 |
| 4        | WT      | WT       | WT       | ALK-negative PSMP                 |
| 5        | WT      | WT       | WT       | ALK-positive PSMP                 |
| 6        | WT      | WT       | WT       | ALK-positive PSMP                 |
| 7        | WT      | WT       | WT       | ALK-negative PSMP                 |
| 8        | WT      | WT       | WT       | ALK-positive PSMP                 |
| 9        | WT      | WT       | WT       | ALK-negative PSMP                 |
| 10       | WT      | WT       | WT       | ALK-negative PSMP                 |
| 11       | WT      | WT       | WT       | ALK-positive PSMP                 |
| 12       | WT      | WT       | WT       | ALK-negative PSMP                 |
| 13       | WT      | WT       | WT       | ALK-negative PSMP                 |
| 14       | WT      | WT       | WT       | ALK-negative PSMP                 |
| 15       | WT      | WT       | WT       | ALK-positive PSMP                 |
| 16       | WT      | WT       | WT       | ALK-negative PSMP                 |
| sarcomatoid UC | |          |          |                                   |
| 1        | WT      | WT       | G>A      | Sarcomatoid UC, IMT-like          |
| 2        | WT      | G>A      | WT       | Sarcomatoid UC                    |
| 3        | WT (WT) | G>A (G>A)| WT (WT)  | Sarcomatoid UC (differentiated component) |
| 4        | WT      | G>A      | WT       | Sarcomatoid UC, IMT-like areas    |
| 5        | WT      | G>A      | WT       | Sarcomatoid UC                    |
| 6        | NA      | WT       | G>A      | Sarcomatoid UC with chondrosarcomatous heterologous component, IMT-like |
| 7        | WT (WT) | WT (WT)  | G>A (G>A)| Sarcomatoid UC, IMT-like (differentiated component) |
| 8        | WT      | G>A      | WT       | Sarcomatoid UC, IMT-like*         |
| 9        | WT      | G>A      | WT       | Sarcomatoid UC, IMT-like          |
| 10       | WT      | G>A      | WT       | Sarcomatoid UC                    |
| 11       | WT      | G>A      | WT       | Sarcomatoid UC                    |
| 12       | WT      | G>A      | WT       | Sarcomatoid UC with leiomyomatous heterologous component |
| 13       | A>C     | WT       | WT       | Sarcomatoid UC                    |
| 14       | WT      | WT       | WT       | Sarcomatoid UC with myogenic heterologous component (DD epithelioid leiomyosarcoma) |
| 15       | WT      | G>A      | WT       | Sarcomatoid UC, IMT-like          |
| 16       | WT      | G>A      | WT       | Sarcomatoid UC, IMT-like areas    |

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Table 5. (Continued)

| Case no. | TERT-57 | TERT-124 | TERT-146 | Diagnosis                                      |
|----------|---------|----------|----------|-----------------------------------------------|
| 17       | WT (WT) | G>A      | G>A      | Sarcomatoid UC (differentiated component)     |
| 18       | WT      | G>A      | WT       | Sarcomatoid UC, IMT-like                      |

NA, not available; WT, wild-type; DD, differential diagnosis; UC, urothelial carcinoma; IMT, inflammatory myofibroblastic tumour; PSMP, pseudosarcomatous myofibroblastic proliferation.

* TERT promotor mutation analysis performed on tissue of subcutaneous metastasis.

Figure 3. A. ALK-negative case with FN1/RET fusion (haematoxylin and eosin; B, example for ALK FISH with rearrangement resulting in one split signal (arrows indicate split signals). ALK, anaplastic lymphoma kinase; FISH, fluorescence in-situ hybridisation.

Table 6. Results of ALK analysis (*Case no.* refers to prior tables; bold font indicates cases with rearrangement)

| PSMP with rearrangement and/or ALK protein expression | Diagnosis | ALK (D5F3)* | RNA sequencing | FISH 2p23 break-apart | Case no. |
|-------------------------------------------------------|-----------|-------------|----------------|-----------------------|---------|
| ALK-negative PSMP†                                    | –         | –           | FN1/RET fusion | No rearrangement       | PSMP_9  |
| ALK-positive PSMP                                     | +         |             | FN1/ALK fusion | No rearrangement       | PSMP_15 |
| ALK-positive PSMP                                     | +         | NA          | ALK rearrangement | PSMP_11 |
| ALK-positive PSMP                                     | +         | NA          | ALK rearrangement | PSMP_5  |
| ALK-positive PSMP                                     | +         | NA          | ALK rearrangement | PSMP_6  |
| ALK-positive PSMP                                     | +         | No rearrangement | No rearrangement | PSMP_3  |
| ALK-positive PSMP                                     | +         | NA          | NA              | PSMP_8  |

| PSMP without re-arrangement                          | ALK-negative PSMP | – | No rearrangement | NA | PSMP_1 |
|-------------------------------------------------------|-------------------|---|-----------------|----|------|
| ALK-negative PSMP                                     | –                 | No rearrangement | NA | PSMP_2 |
| ALK-negative PSMP                                     | –                 | No rearrangement | NA | PSMP_4 |
| ALK-negative PSMP                                     | –                 | No rearrangement | NA | PSMP_7 |
| ALK-negative PSMP                                     | –                 | No rearrangement | NA | PSMP_10|
| ALK-negative PSMP                                     | –                 | No rearrangement | NA | PSMP_12|
| ALK-negative PSMP                                     | –                 | No rearrangement | NA | PSMP_13|
| ALK-negative PSMP                                     | –                 | No rearrangement | NA | PSMP_14|
| ALK-negative PSMP                                     | –                 | No rearrangement | NA | PSMP_16|

ALK, anaplastic lymphoma kinase; PSMP, pseudosarcomatous myofibroblastic proliferation; NA, not available; FISH, fluorescence in-situ hybridisation.

* All negative cases with positive on-slide controls
† Final diagnosis after RNA sequencing.
rearrangements)\(^1\) and others. This indicates the need for verification of the exact fusion partners in patients selected for novel therapeutic options targeting ALK.

In conclusion, the combination of morphological features (atypical mitoses, heterologous elements and differentiated urothelial component) with an IHC panel including the p53 phenotype (mutant versus wild-type), GATA3, p63 and ALK (D5F3) should suffice for the differential diagnosis between PSMP and sarcomatoid UC in the majority of cases. ALK (D5F3) expression is usually confirmatory of PSMP in this differential diagnosis. However, ALK (D5F3) negativity does not indicate sarcomatoid UC.

Additionally, our study identifies TERT promoter mutations as a reliable and sensitive molecular surrogate, excluding a diagnosis of PSMP and supporting the diagnosis of sarcomatoid UC and therefore helpful to distinguish sarcomatoid UC from PSMP in extremely difficult cases. This is particularly true in the context of ALK negativity, where diagnostic uncertainty is left after careful assessment of clinical history, histomorphology and IHC. However, TERT analysis alone does not confirm the diagnosis of sarcomatoid UC, as TERT promoter mutations can also be found in other tumours.\(^16\) but combined with IHC in the context of a genitourinary carcinoma makes the diagnosis of UC most likely. In difficult questions regarding organ-preserving surgery, detection of the gene rearrangement using NGS might be mandatory and valuable for reliable biopsy interpretation, especially as ALK IHC and FISH may be negative despite genetic rearrangement.

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Conflicts of interest

The authors declare that they have no conflicts of interest regarding this manuscript.

Author contribution

All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content. S.B. takes responsibility for the integrity of the work as a whole, from inception to published article. Substantial contributions to conception and design: S.B., A.A.; acquisition of data: S.B., A.A., R.S., N.T.G., B.W., A.H.; analysis and interpretation of data: S.B., A.A., R.S.; drafting the article or revising it critically for important intellectual content: S.B., A.A., R.S., N.T.G.; final approval of the version to be published: S.B., A.A., R.S., N.T.G., B.W., A.H.

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Supporting Information
Additional Supporting Information may be found in the online version of this article:
Data S1. Promoter Mutation Analysis.