

# ***TP53* mutational analysis supports monoclonal origin of biphasic sarcomatoid urothelial carcinoma (carcinosarcoma) of the urinary bladder**

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**Sarcomatoid urothelial carcinoma of the urinary bladder is an uncommon neoplasm with biphasic morphology exhibiting both epithelial and sarcomatoid components. Whether this tumor arises from a single cancer stem cell with subsequent differentiation or represents collision of the progeny of two separate cancer stem cells is a matter of controversy. To clarify its clonal origin, we analyzed the *TP53* mutation status of a series of 17 sarcomatoid urothelial carcinomas using single-strand conformation polymorphism, DNA sequencing and p53 immunohistochemistry. Sarcomatoid and epithelial tumor components were separately microdissected using laser capture microdissection. Five out of the 17 sarcomatoid urothelial carcinomas contained *TP53* point mutations in exons 5 and 8. In all five cases, the *TP53* point mutations were identical in both the epithelial and sarcomatoid components. The sarcomatoid and epithelial tumor components in all 17 cases showed concordant p53 expression patterns. Our results suggest that despite their conspicuous divergence at the phenotypic level, the sarcomatoid and carcinomatoid elements of this uncommon tumor share a common clonal origin.**

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Sarcomatoid urothelial carcinoma of the urinary bladder is an uncommon neoplasm with both epithelial and sarcomatoid components. Although some investigators favor the use of the term carcinosarcoma, the current recommendation by the World Health Organization is to use the term sarcomatoid carcinoma for this entity.<sup>1</sup> Patients with this neoplasm are predominantly male. Although most patients present between 60 and 70 years of age, patients as young as 2 years old have been

reported.<sup>2,3</sup> Sarcomatoid carcinoma typically presents at an advanced stage and is associated with a poor prognosis. Etiologic factors are largely unknown, although some believe previous radiotherapy may be a significant contributing factor.<sup>4,5</sup>

The epithelial components most frequently described in sarcomatoid carcinoma include urothelial carcinoma, small-cell carcinoma, squamous carcinoma and adenocarcinoma. Chondrosarcoma, leiomyosarcoma, malignant fibrous histiocytoma, osteosarcoma, rhabdomyosarcoma and undifferentiated sarcoma comprise the most frequently identified mesenchymal components. The origin of this unusual lesion is controversial. Some investigators believe that it represents the collision of two separate malignant tumors occurring independently and synchronously in the same location.<sup>6–11</sup> Others suggest that sarcomatoid carcinoma has a monoclonal origin with subsequent differentiation into its

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carcinomatoid and sarcomatoid elements.<sup>12,13</sup> In this study we analyzed the *TP53* mutation status of a series of 17 sarcomatoid urothelial carcinomas using single-strand conformation polymorphism (SSCP), DNA sequencing and immunohistochemistry to clarify the clonal origin of these different tumor components.

## Materials and methods

### Patients

Seventeen patients (sixteen men and one woman) diagnosed with sarcomatoid carcinoma of the urinary bladder between 1992 and 2006 were included in this study. The age range at tumor resection was 44–83 years, with a mean age of 64 years. Tumors were diagnosed by light microscopy with the criteria established for sarcomatoid carcinoma of the urinary bladder according to the World Health Organization classification system.<sup>1</sup> The tumor, lymph node and metastasis classification system (2002) was used for pathological staging.<sup>14</sup> Among the seventeen cases, seven were diagnosed as stage pT2 and six as pT3. Four patients were managed surgically by transurethral resection, precluding definitive pathologic staging.

### Tumor Samples and Microdissection

Archival paraffin blocks from 17 cases of sarcomatoid carcinoma of the urinary bladder were collected from the participating institutions. Serial 5- $\mu$ m-thick paraffin sections were prepared from each block. Tumor cells from epithelial and sarcomatoid components were separately dissected after routine H&E staining with a PixCell II Laser Capture Microdissection System (Arcturus Engineering, Mountain View, CA, USA) as previously described.<sup>15–17</sup> Approximately 600 cells from each phenotypically different tumor component were microdissected as demonstrated in Figure 1. Adjacent normal urothelium was microdissected for use as a control. The microdissected tissue was incubated in 50  $\mu$ l of digestion buffer containing 10 mM Tris-HCl, 1 mM EDTA, 1% Tween 20 and 5 mg/ml of proteinase K (pH 8.3) at 37°C overnight. The samples were boiled for 10 min to inactivate proteinase K and subjected to a phenol–chloroform extraction (phenol:chloroform:isoamyl alcohol = 25:24:1). The genomic DNA was dissolved in 30  $\mu$ l of ddH<sub>2</sub>O after phenol–chloroform extraction.

### SSCP Analysis

*TP53* mutation screening in exons 5, 7 and 8 was performed using SSCP. *TP53* sequences in exons 5, 7 and 8 were amplified by hot-start PCR using the primers listed in Table 1.<sup>18,19</sup> *TP53* sequences were amplified using 3  $\mu$ l of genomic DNA, 2.3 mM

MgCl<sub>2</sub>, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2  $\mu$ M deoxynucleotide triphosphates, 0.8  $\mu$ Ci  $\alpha$ -<sup>32</sup>PdATP, 0.33  $\mu$ M of each primer and 1 unit of Taq DNA polymerase (Bio-Rad, Hercules, CA, USA) with a final volume of 25  $\mu$ l. Each PCR protocol had an initial denaturing step of 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, which was then followed by a single final extension step at 72°C for 7 min. PCR products were diluted with loading buffer (0.04% blomophenol blue, 0.04% xylene cyanol FF, 5% glycerol) and separated by a non-denaturing 6% polyacrylamide gel at 150 V overnight. After electrophoresis, the gels were fixed in 10% acetate acid containing 20% methanol, dried on blotting paper on a vacuum gel dryer and exposed to X-ray film for 2–18 h. Developed films were evaluated by visual inspection.

### *TP53* Mutation Analysis

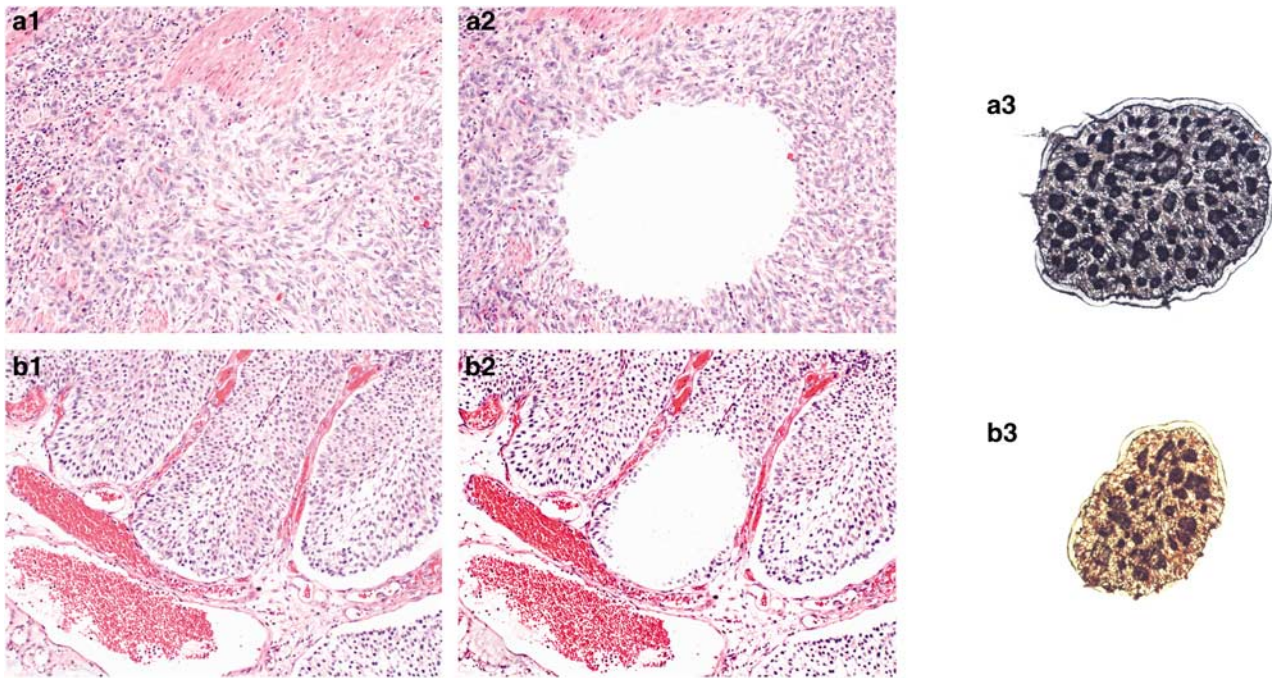
*TP53* sequences in exons 5, 7 and 8 were amplified by PCR using the primers listed in Table 1 according to described protocols. The PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN Sciences, MD, USA) and the DNA concentration adjusted to 20 ng/ $\mu$ l. Purified PCR product was sequenced with the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the designated primer.

### p53 Immunohistochemistry

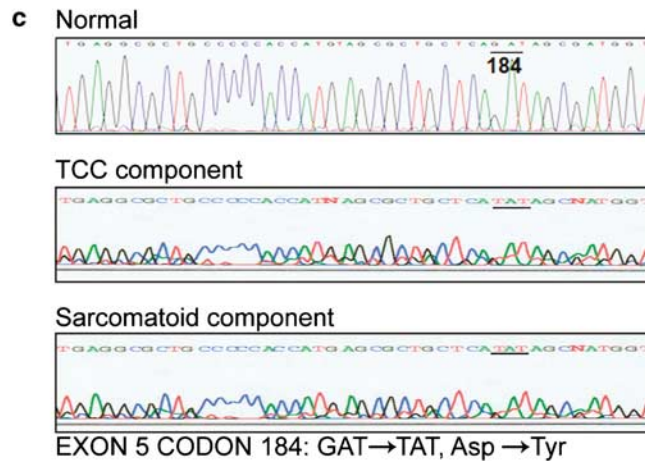
Immunostaining for p53 was performed on paraffin-embedded tissue sections using the peroxidase-labeled streptavidin–biotin method. 5- $\mu$ m-thick tissue sections from each case were deparaffinized in xylene for 5 min and then rehydrated through graded ethanol to distilled water. Antigen retrieval was achieved by heating the slides with DAKO antigen retrieval solution, pH 6.0 (Dako Corporation, Carpinteria, CA, USA) at 95°C for 10 min. Endogenous peroxidase was blocked by incubating the slides in 3% H<sub>2</sub>O<sub>2</sub> for 5 min. Detection of p53 protein was performed using a monoclonal antibody against p53 (Dako Corporation). The primary antibody was incubated for 10 min, followed by biotinylated secondary antibody and peroxidase-labeled streptavidin. 3,3-Diaminobenzidine was used as the chromogen. The staining was evaluated by light microscopy using a three-tiered system (1+, 2+ and 3+), corresponding with the intensity of the nuclear staining. The percentage of tumor cells staining was also recorded.<sup>20,21</sup>

## Results

Point mutations were identified in 5 of 17 cases by SSCP as well as *TP53* gene sequencing of exons 5, 7 and 8. A total of 100% concordance was observed



Case 15



**Figure 1** Laser capture microdissection of sarcomatoid urothelial carcinoma of the urinary bladder (**a1–b3**): hematoxylin and eosin-stained sections showing sarcomatoid (**a1**) and epithelial (transitional cell carcinoma, TCC) (**b1**) components before microdissection; corresponding sarcomatoid component (**a2**) and epithelial component (**b2**) after microdissection; and laser-captured sarcomatoid (**a3**) and epithelial (**b3**) tumor cells. (**c**) Case 15: point mutation at exon 5 codon 184: (GAT→TAT, Asp→Tyr) revealing identical mutations in epithelial components (middle panel) and sarcomatoid components (bottom panel); normal tissue did not harbor the mutation in the corresponding codon (top panel).

between the tumors with mutations detected by SSCP and those with point mutations identified by DNA sequencing. The mutation patterns were identical in the separately microdissected epithelial and sarcomatoid components in all five cases (Table 2). Normal tissue did not show *TP53* mutations in any of the 17 cases studied.

Four tumors exhibited point mutations in exon 8 (codons 261, 268, 272 and 275). Three of these mutations resulted in amino-acid substitutions (codon 268: serine to arginine, codon 272: alanine to proline and codon 275: alanine to valine). The T to C mutation at codon 261 was a silent mutation.

Two tumors had point mutations in exon 5 (both at codon 184) with identical G to T substitutions resulting in an aspartic acid to tyrosine alteration. One of these two tumors had a second mutation in codon 268 in exon 8.

Immunohistochemistry detected moderate to strong p53 expression in eight cases, two of which harbored *TP53* mutations (Table 2; Figure 2). The sarcomatoid and epithelial tumor components showed concordant p53 expression patterns and intensities. The three cases with identified point mutations that did not show increased p53 staining were negative in both the epithelial and sarcomatoid

**Table 1** Primers used for p53 mutation detection

Exon no.	Primer sequence	PCR product (bp)
5	5-IA CCTGACTTTCAACTCTGTCTC	158
	5-IB ACTGCTTGTAGATGGCCA TG	
	5-IIA CAGCTGTGGGTTGATTCCAC	
7	5-IIB CTGGGGACCCTGGGCAAC	176
	7-IA AGGCGCACTGGCCTCATCTT	
	7-IB TCCAGTGTGATGATGGTGAGG	
8	7-IIA CATGTGTAACATTTCCCTGCAT	141
	7-IIB GAGGCAAGCAGAGGCTGG	
	8-IA CCTTACTGCCTCTTGCTTCTC	
8	8-IB CTTGCGGAGATTCTCTTCCTC	130
	8-IIA TTGTGCCTGTCCCTGGGAGAG	
	8-IIB CTCACCGCTTCTTGTCCT	
		127

areas. Of the 12 cases without identified *TP53* mutations, half (six) demonstrated nuclear p53 staining and half had no increased staining. The p53 expression patterns were identical in both the carcinomatoid and sarcomatoid areas.

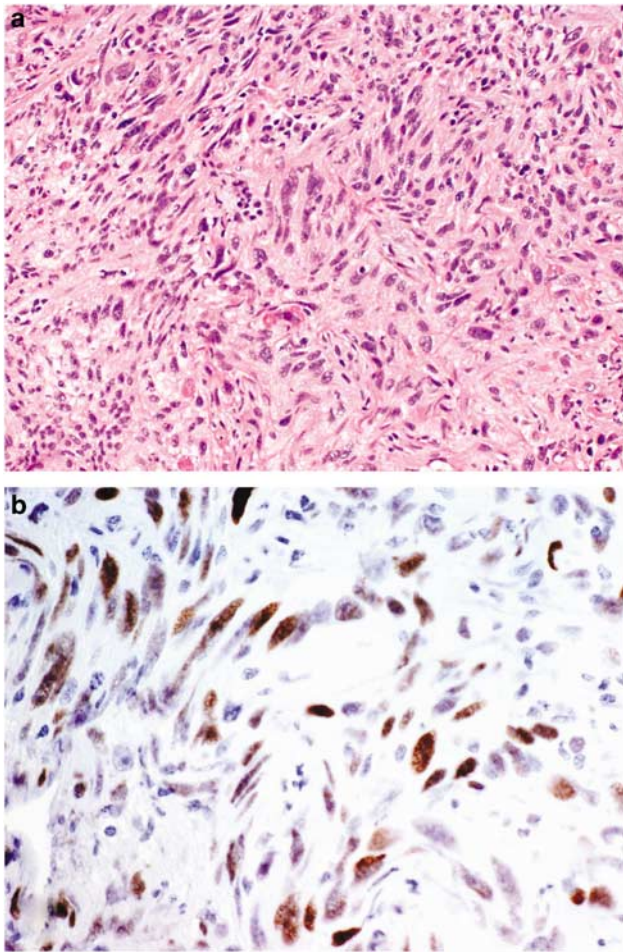
## Discussion

In this study we analyzed *TP53* mutation status and p53 protein expression in both the sarcomatoid and epithelial components of cases of sarcomatoid urothelial carcinoma. Concordant *TP53* mutations and p53 protein expression support the monoclonal origin of both components.

**Table 2** Detection of *TP53* mutations in sarcomatoid urothelial carcinoma

Case no.	Component	SSCP	Exon 5	Exon 7	Exon 8	p53 IHC
1	C	Mutation detected	No mutation	No mutation	Codon 272, GCT → CCT, Ala → Pro	3+, 90%
	S	Mutation detected	No mutation	No mutation	Codon 272, GCT → CCT, Ala → Pro	3+, 90%
2	C	No mutation	No mutation	No mutation	No mutation	3+, 50%
	S	No mutation	No mutation	No mutation	No mutation	3+, 50%
3	C	No mutation	No mutation	No mutation	No mutation	3+, 90%
	S	No mutation	No mutation	No mutation	No mutation	3+, 90%
4	C	No mutation	No mutation	No mutation	No mutation	NEG
	S	No mutation	No mutation	No mutation	No mutation	NEG
5	C	No mutation	No mutation	No mutation	No mutation	NEG
	S	No mutation	No mutation	No mutation	No mutation	NEG
6	C	No mutation	No mutation	No mutation	No mutation	NEG
	S	No mutation	No mutation	No mutation	No mutation	NEG
7	C	No mutation	No mutation	No mutation	No mutation	3+, 90%
	S	No mutation	No mutation	No mutation	No mutation	3+, 90%
8	C	No mutation	No mutation	No mutation	No mutation	3+, 40%
	S	No mutation	No mutation	No mutation	No mutation	3+, 20%
9	C	No mutation	No mutation	No mutation	No mutation	2+, 15%
	S	No mutation	No mutation	No mutation	No mutation	2+, 15%
10	C	No mutation	No mutation	No mutation	No mutation	3+, 90%
	S	No mutation	No mutation	No mutation	No mutation	3+, 90%
11	C	Mutation detected	No mutation	No mutation	Codon 261, GGT → GGC, Gly → Gly	2+, 15%
	S	Mutation detected	No mutation	No mutation	Codon 261, GGT → GGC, Gly → Gly	2+, 15%
12	C	No mutation	No mutation	No mutation	No mutation	NEG
	S	No mutation	No mutation	No mutation	No mutation	NEG
13	C	No mutation	No mutation	No mutation	No mutation	NEG
	S	No mutation	No mutation	No mutation	No mutation	NEG
14	C	Mutation detected	No mutation	No mutation	CODON 275, GCC → GTC, Ala → Val	NEG
	S	Mutation detected	No mutation	No mutation	Codon 275, GCC → GTC, Ala → Val	NEG
15	C	Mutation detected	CODON 184, GAT → TAT, Asp → Tyr	No mutation	No mutation	NEG
	S	Mutation detected	CODON 184, GAT → TAT, Asp → Tyr	No mutation	No mutation	NEG
16	C	No mutation	No mutation	No mutation	No mutation	NEG
	S	No mutation	No mutation	No mutation	No mutation	NEG
17	C	Mutation detected	CODON 184, GAT → TAT, Asp → Tyr	No mutation	Codon 268, AGC → CGC, Ser → Arg	NEG
	S	Mutation detected	CODON 184, GAT → TAT, Asp → Tyr	No mutation	Codon 268, AGC → CGC, Ser → Arg	NEG

C, transitional cell (urothelial) carcinoma component; S, sarcomatoid carcinoma component; IHC, immunohistochemistry; SSCP, single-strand conformation polymorphism; NEG, negative.



**Figure 2** p53 immunohistochemistry in sarcomatoid urothelial carcinoma. Sarcomatoid components showed strong p53 positivity (b); the same components in hematoxylin and eosin-stained sections (a).

The histogenesis of the epithelial and sarcomatoid elements of sarcomatoid carcinoma of the urinary bladder has been a matter of controversy involving two main theories. According to the collision theory, the two components are similar only in their location and synchrony, developing independently from two separate stem cells of epithelial and mesenchymal origin. The divergence hypothesis contends that the lesion develops from a monoclonal origin (a single cancer stem cell) and subsequently diverges into morphologically dissimilar components. Histologic support for the divergence theory focused on identification of a 'transition zone' between the histologically dissimilar areas to indicate divergence from a monoclonal origin.<sup>22</sup> More recently, concordant X-chromosome inactivation and significant overlap in loss of heterozygosity were reported in separately microdissected components of sarcomatoid carcinoma of the urinary bladder in a study by Sung *et al*,<sup>23</sup> supporting a monoclonal origin for both components. Gronau *et al*<sup>24</sup> obtained similar results, finding that the tumor components showed overlapping chromosomal altera-

tions as well as additional nonoverlapping alterations. These secondary mutational aberrations were thought to represent events significant in the divergence occurring after the initial tumorigenesis.<sup>24</sup>

Although *TP53* mutations are common in urothelial carcinomas, particularly in high-grade and high-stage cancers, *TP53* mutation analysis has not previously been utilized to evaluate the molecular characteristics of the morphologically distinct components of sarcomatoid carcinoma of the urinary bladder. *TP53* exons 5–8, corresponding to the DNA-binding domain, are considered to be the region most frequently harboring point mutations in bladder carcinomas.<sup>25–27</sup> Among these exons, 5 and 8 contain the majority of mutations.<sup>28</sup> We focused our screening on exons 5, 7 and 8 allowing us to screen those exons most likely to harbor mutations without significantly decreasing our sensitivity. However, the possibility of additional mutations in less common regions cannot be ruled out.

SSCP and DNA sequencing identified *TP53* mutations in exons 5, 7 or 8 in 5 of the 17 cases we evaluated. The concordance between SSCP and DNA sequencing was 100% in the current investigation. In addition, we found that the mutations identified in the microdissected epithelial and sarcomatoid components were identical in all five cases. The sarcomatoid and epithelial tumor components in all cases showed concordant p53 expression patterns. Our data suggest a common clonal origin of the phenotypically different tumor components. The *TP53* mutations probably occurred early in the common tumorigenesis of these morphologically distinct tumor components. Subsequently, these lesions were likely exposed to additional stimuli giving rise to the biphasic phenotype.

p53 immunohistochemistry revealed the epithelial and sarcomatoid areas of all 17 cases to have similar staining characteristics in both pattern and quantity. This further supports the biological and molecular similarities between these two morphologically distinct components. The fact that only 40% of the cases harboring *TP53* mutations had positive nuclear p53 staining suggests that *TP53* mutations may be only one of the factors resulting in an altered p53 signal system.<sup>25,29,30</sup>

In summary, our discovery of identical *TP53* mutation patterns and nuclear p53 immunohistochemical staining characteristics in both the epithelial and sarcomatoid components suggests a common clonal origin of the phenotypically distinct tumor components of sarcomatoid carcinoma of the urinary bladder.

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