

## Tumorigenesis and Neoplastic Progression

# Molecular Genetic Evidence for a Common Clonal Origin of Urinary Bladder Small Cell Carcinoma and Coexisting Urothelial Carcinoma

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**In most cases, small-cell carcinoma of the urinary bladder is admixed with other histological types of bladder carcinoma. To understand the pathogenetic relationship between the two tumor types, we analyzed histologically distinct tumor cell populations from the same patient for loss of heterozygosity (LOH) and X chromosome inactivation (in female patients). We examined five polymorphic microsatellite markers located on chromosome 3p25-26 (D3S3050), chromosome 9p21 (IFNA and D9S171), chromosome 9q32-33 (D9S177), and chromosome 17p13 (TP53) in 20 patients with small-cell carcinoma of the urinary bladder and concurrent urothelial carcinoma. DNA samples were prepared from formalin-fixed, paraffin-embedded tissue sections using laser-assisted microdissection. A nearly identical pattern of allelic loss was observed in the two tumor types in all cases, with an overall frequency of allelic loss of 90% (18 of 20 cases). Three patients showed different allelic loss patterns in the two tumor types at a single locus; however, the LOH patterns at the remaining loci were identical. Similarly, the same pattern of nonrandom X chromosome inactivation was present in both carcinoma components in the four cases analyzed. Concordant genetic alterations and X chromosome inactivation between small-cell carcinoma and coexisting urothelial carcinoma suggest that both tumor compo-**

**nents originate from the same cells in the urothelium. (Am J Pathol 2005, 166:1533–1539)**

Small-cell carcinoma of the urinary bladder histologically resembles that occurring in the lung and has been reported with an increasing frequency in recent years.<sup>1–10</sup> It has been estimated to represent 0.5% of bladder malignancies and develops more frequently in older men, with hematuria as the most common presenting symptom.<sup>8</sup> Small-cell carcinoma of the urinary bladder behaves aggressively, often with locally advanced or metastatic disease at the time of presentation.<sup>11</sup>

Over the years, three principal theories have been proposed to account for the development of small-cell carcinoma in the urinary bladder. The first theory is that small-cell carcinomas originate from multipotential, undifferentiated cells or stem cells in the urothelium.<sup>5,8,12,13</sup> The frequent association of this tumor with coexisting urothelial carcinoma supports this theory. The second theory is that these tumors arise from neuroendocrine cells within normal or metaplastic urothelium.<sup>14</sup> The third theory is that small-cell carcinomas are derived from an undefined population of submucosal neuroendocrine cells.<sup>1</sup> In this study, we investigated the clonal relationships between small-cell carcinoma and coexisting urothelial carcinoma using loss of heterozygosity (LOH) and X chromosome inactivation analysis.

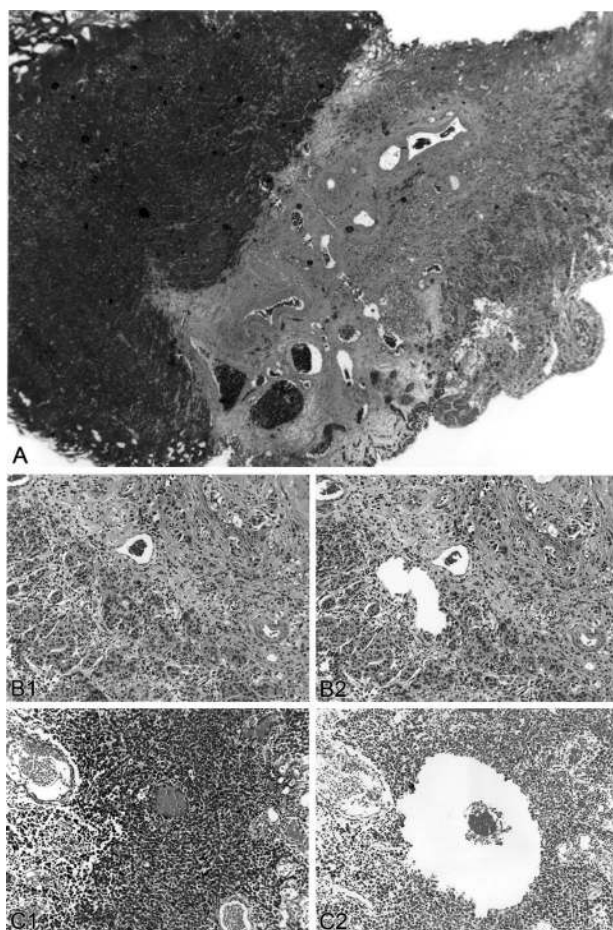
## Materials and Methods

### Patients

Twenty patients with small-cell carcinoma of the urinary bladder and concurrent urothelial carcinoma were included in our study. Archival materials from the 20 cases

Accepted for publication January 13, 2005.

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**Figure 1.** Laser microdissection of concordant small-cell and urothelial carcinoma of the urinary bladder. **A:** Low-power view of a tumor with coexisting small-cell carcinoma and urothelial carcinoma components. **B:** Urothelial carcinoma before microdissection (**B1**) and after microdissection (**B2**). **C:** Urothelial carcinoma before microdissection (**C1**) and after microdissection (**C2**).

were retrieved from the surgical pathology files. Patients ranged in age from 58 to 83 years of age, with a mean age of 69 years. Ten patients were pathological stage pT2; nine were pathological stage pT3; and one was pathological stage pT4. Tumors were diagnosed by light microscopy with each case fulfilling the criteria established for urothelial carcinoma and small-cell carcinoma according to the World Health Organization classification system.<sup>15</sup> The 2002 tumor, lymph node, and metastasis (TNM) classification system was used for pathological staging.<sup>16</sup> This research was approved by the Indiana University Institutional Review Board

### Tissue Samples and Microdissection

Histological sections were prepared from formalin-fixed, paraffin-embedded tissue and were stained with hematoxylin and eosin (H&E) for microscopic evaluation. From these slides, the two different tumors (urothelial carcinoma and small-cell carcinoma) were identified. Laser-assisted microdissection of the two components was performed (Figure 1) on the unstained sections using a PixCell II Laser Capture Microdissection system (Arctu-

rus Engineering, Mountain View, CA), as previously described.<sup>17–19</sup> Approximately 400 to 1000 cells of each component were microdissected from the 5- $\mu$ m histological sections. Normal tissue from each case was microdissected as a control.

### Detection of LOH

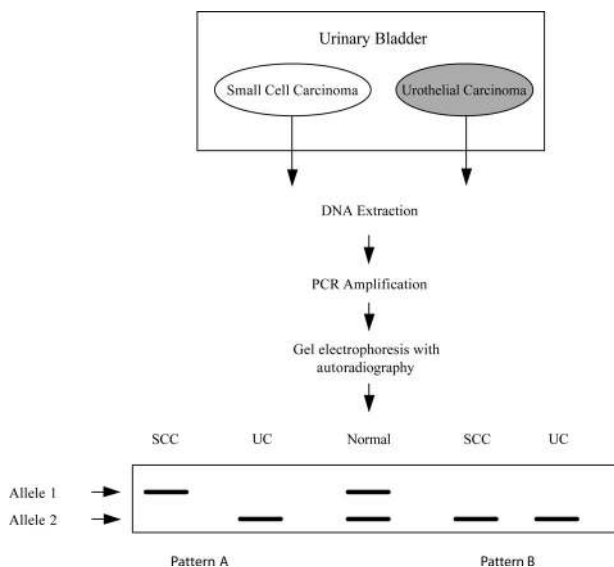
The dissected cells were de-paraffinized with xylene and ethyl alcohol. Polymerase chain reaction (PCR) was used to amplify genomic DNA at various specific loci on chromosome 3p25-26 (D3S3050), chromosome 9p21 (D9S171 and IFNA), chromosome 9q32-33 (D9S177), and chromosome 17p13 (TP53). Previous studies demonstrated frequent allelic instability on these chromosomes in urothelial carcinoma.<sup>20–26</sup> D3S3050 is located in the region near the Von-Hippel Lindau tumor suppressor gene locus. IFNA and D9S171 include regions of the putative tumor suppressor gene p16. D9S177 is located within a putative tumor suppressor gene involved in the carcinogenesis of squamous cell carcinomas and urothelial carcinomas. TP53 contains the tumor suppressor p53 locus. Polymerase chain reaction amplification and gel electrophoresis were performed as previously described.<sup>17,20,27–33</sup> Polymerase chain reactions for each polymorphic microsatellite marker were repeated at least twice from the same DNA preparations, and the same results were obtained.

### Analysis of Allelic Loss Pattern

When the genetic material in a patient was found to be homozygous for the polymorphic markers (ie, showing only one allele in the normal control tissue), the case was considered noninformative. Patients with genetic material that was informative (ie, showing two alleles in the normal control tissue) were divided into two categories.<sup>34</sup> Their DNA may show no allelic deletions in the tumor, retaining two different alleles of similar intensity on autoradiographs, or show absence of one allele. Allelic loss pattern analysis is illustrated in Figure 2. DNA sampled from separate small-cell and urothelial carcinoma cells demonstrating identical allelic loss patterns is compatible with a common clonal origin, whereas different patterns of allelic deletions are compatible with independent clonal origins of these tumors (Figure 2).<sup>17,19,20,25,28,29,32</sup>

### X Chromosome Inactivation Analysis

X chromosome inactivation analysis was performed on the urothelial and small-cell carcinomas from four female patients, as previously described.<sup>25,30–32</sup> DNA samples were prepared from the two tumor types from the same patient. The microdissected tissue was placed in 15  $\mu$ l of buffer (ie, 10 mmol/L Tris, 1 mmol/L ethylenediamine tetraacetic acid, 1% Tween 20, and 0.2 mg/ml proteinase K, pH 8.3) and incubated overnight at 37°C for DNA extraction. Eight-microliter aliquots of the DNA extract were digested overnight with 1 U of *Hha*I restriction endonuclease (New England Biolabs, Inc., Beverly, MA) in



**Figure 2.** Analysis of allelic loss pattern in concordant small-cell and urothelial carcinoma of bladder. Cells from the distinctly separate small-cell and urothelial components were obtained by tissue microdissection, DNA was prepared, and the polymerase chain reaction was used to amplify DNA. DNA sampled from separate small cell and urothelial cells demonstrating identical allelic loss pattern (pattern B) is compatible with a common clonal origin, whereas different patterns of allelic deletions (pattern A) are compatible with independent clonal origins of these two components.

a total volume of 10  $\mu$ l. Control reactions for each sample were incubated in the digestion buffer without *Hha*I endonuclease. Primers used in this reaction were: AR-sense, 5'-TCC AGA ATC TGT TCC AGA GCG TGC-3'; and AR-antisense, 5'-GCT GTG AAG GTT GCT GTT CCT CAT-3'. Three microliters of digested or nondigested DNA was amplified in a 25- $\mu$ l polymerase chain reaction volume containing 0.1  $\mu$ l of  $\alpha$ -<sup>32</sup>P-labeled deoxyadenosine triphosphate (3000 Ci/mmol), 4  $\mu$ mol/L AR-sense primer, 4  $\mu$ mol/L AR-antisense primer, 4% dimethyl sulfoxide, 2.5 mmol/L MgCl<sub>2</sub>, 300  $\mu$ mol/L deoxycytidine triphosphate, 300  $\mu$ mol/L deoxythymidine triphosphate, 300  $\mu$ mol/L deoxyguanosine triphosphate, 300  $\mu$ mol/L deoxyadenosine triphosphate, and 0.13 U of AmpliTaq Gold DNA polymerase (Perkins-Elmer Cetus, Foster City, CA). PCR amplification was performed with an initial denaturation step of 95°C for 8 minutes followed by 32 cycles as follows: 95°C for 40 seconds, 63°C for 40 seconds, 72°C for 60 seconds, and the final extension step at 72°C for 10 minutes. The PCR products were then diluted with 4  $\mu$ l of loading buffer containing 95% deionized formamide, 20 mmol/L ethylenediamine tetraacetic acid, 0.05% bromophenol, and 0.05% xylene cyanole FF (Sigma Chemical Co., St. Louis, MO). The samples were heated to 95°C for 5 minutes and then placed on ice. Three microliters of this mixture was loaded onto 6.5% polyacrylamide denaturing gels without formamide. Electrophoresis was performed at 1600V for 4 to 7 hours, followed by autoradiography with Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY) for 8 to 16 hours.

### Analysis of X Chromosome Inactivation

The cases were considered informative if the control sample displayed two alleles after PCR amplification without *Hha*I digestion. Nonrandom inactivation of the X chromosomes was defined as a complete or nearly complete absence of one or the other allele after *Hha*I digestion, indicating predominance of one androgen receptor allele.<sup>25,30-32</sup>

The clonality of the samples was evaluated on the basis of a polymorphism of the X-linked human androgen receptor gene (HUMARA) locus.<sup>25,30-32</sup> The technique is dependent on digestion of DNA with the methylation-sensitive restriction enzyme *Hha*I, polymerase chain reaction (PCR) amplification of the HUMARA locus, and detection of methylation of this locus. With this method, only the methylated HUMARA allele is selectively amplified by PCR. The random inactive status of X chromosomes is established in all female somatic cells early in embryogenesis.<sup>32</sup> Normal female tissues should be a cellular mosaic, with an equal distribution of cells containing maternal or paternal-derived inactivated X chromosomes. Tumors arising from a single transformed progenitor cell should contain the same inactive X chromosome in each tumor cell. Different patterns of X chromosome inactivation are consistent with independent origin of the cells.

### Immunohistochemistry

Neuroendocrine marker immunostainings were performed on formalin-fixed, paraffin-embedded sections using the avidin-biotin complex technique, as previously described.<sup>35-37</sup> Primary rabbit polyclonal antibodies were used for evaluation of chromogranin A expression (DAKO, Carpinteria, CA; prediluted antibody).

### Results

Eighteen of 20 patients (90%) with small-cell and urothelial carcinoma showed allelic loss in both components (Table 1). All 18 of these cases showed loss of heterozygosity (LOH) in at least 1 locus in each tumor type. The number of specific loci lost ranged from one to five in both tumor types. Figure 3 shows representative LOH results (cases 17 and 19). Two patients did not show allelic loss at any of the five loci examined in either tumor type.

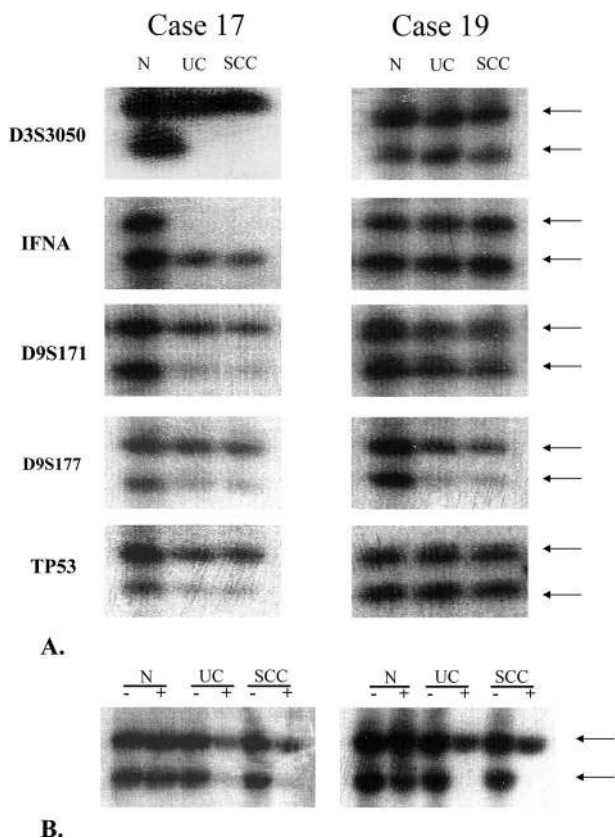
Nearly identical patterns of allelic loss were present in the small-cell and the urothelial carcinoma components in all of the tumors (Table 1). The frequency of allelic loss in the urothelial carcinoma components of informative cases was 47% (9 of 19) with D3S3050, 47% (9 of 19) with IFNA, 30% (6 of 20) with D9S171, 42% (8 of 19) with D9S177, and 41% (7 of 17) with TP53. The frequency of allelic loss in the small-cell carcinoma components of informative cases was 42% (8 of 19) with D3S3050, 47% (9 of 19) with IFNA, 35% (7 of 20) with D9S171, 42% (8 of 19) with D9S177, and 47% (8 of 17) with TP53. Three patients showed different allelic loss patterns in the two



**Table 1.** Comparison of Allelic Loss in Concurrent Urothelial Carcinoma and Small-Cell Carcinoma of the Urinary Bladder

Case no.	Microsatellite markers										X-Chromosome inactivation	
	D3S3050		IFNA		D9S171		D9S177		TP53		HUMARA	
	UC	SCC	UC	SCC	UC	SCC	UC	SCC	UC	SCC	UC	SCC
1	v	v	u	u	v	v	v	v	t	t		
2	v	v	v	v	v	v	v	v	t	t		
3	v	v	u	u	t	t	t	t	t	t		
4	t	t	t	t	v	v	v	v	v	v		
5	t	t	v	v	v	v	v	v	u	u		
6	NI	NI	NI	NI	t	t	v	v	v	v		
7	v	v	u	u	v	v	NI	NI	t	t		
8	t	t	v	v	v	v	v	v	v	v		
9	v	v	u	u	v	t	v	v	NI	NI		
10	v	v	v	v	v	v	u	u	v	v		
11	t	t	v	v	v	v	v	v	NI	NI		
12	v	v	t	t	u	u	t	t	NI	NI		
13	v	v	v	v	t	t	v	v	v	v		
14	t	v	v	v	v	v	t	t	v	v		
15	t	t	t	t	t	t	t	t	t	t		
16	v	v	v	v	v	v	v	v	v	v		
17	t	t	u	u	t	t	t	t	t	t	t	t
18	t	t	t	t	v	v	t	t	v	t	t	t
19	v	v	v	v	v	v	t	t	v	v	t	t
20	v	v	v	v	v	v	v	v	v	v	t	t

U, urothelial carcinoma component; S, small-cell carcinoma component; t, loss of lower allele; u, loss of upper allele; v, both alleles present; NI, noninformative; HUMARA, human androgen receptor gene.



**Figure 3.** Representative results of loss of heterozygosity (LOH) (A) and X chromosome inactivation analysis (B) (cases 17 and 19). DNA was prepared from normal tissue (N), small-cell carcinoma (SCC), and urothelial carcinoma (UC) of the combined tumor; amplified by polymerase chain reaction using polymorphic markers D3S3050, IFNA, D9S171, D9S177, and TP53; and separated by gel electrophoresis. **Arrows:** allelic bands. -: without *HbaI* digestion; +: with *HbaI* digestion.

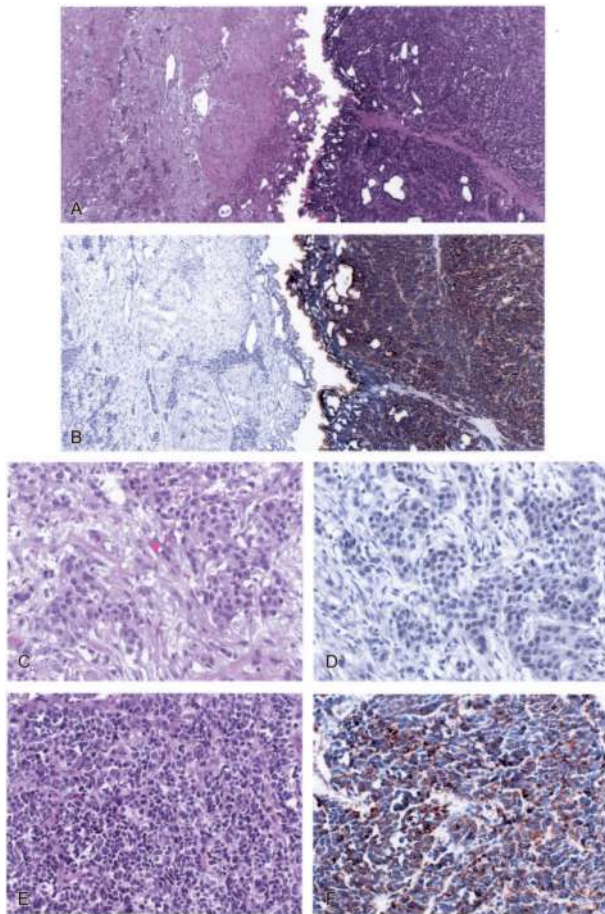
tumor types at a single locus. However, in all three of these patients, the LOH patterns at the other loci were identical. Of these three patients, one showed LOH at the D9S171 locus in the small-cell carcinoma but not in the urothelial carcinoma; one showed allelic loss at the D3S3050 locus in the urothelial carcinoma but not in the small-cell carcinoma; and one showed LOH at the TP53 locus in only the small-cell carcinoma. These were the only discrepancies from an otherwise identical LOH pattern between the tumor types in all of the patients examined. Identical LOH patterns in both tumor types were seen at the IFNA and D9S177 loci.

X chromosome inactivation analysis was performed on the two tumor types in four female patients (Table 1; Figure 3). The same pattern of nonrandom X chromosome inactivation in the urothelial carcinoma and the small-cell carcinoma tumors was seen in the four cases analyzed, providing further evidence of common origin.

Immunostaining for markers of neuroendocrine differentiation was performed in eight cases, and all showed positive staining in the small-cell carcinoma component. The coexisting urothelial carcinoma components were negative (Figure 4).

### Discussion

Small-cell carcinoma of the urinary bladder often coexists with urothelial carcinoma in the same patient.<sup>11</sup> Detailed characterization and comparison of genetic alterations in biologically and morphologically distinct tumor cell populations may provide information about the clonal evolution of concurrent small-cell and urothelial carcinoma of the urinary bladder. Loss of heterozygosity (LOH) has



**Figure 4.** Neuroendocrine marker (chromogranin A) staining in small-cell carcinoma with coexisting urothelial carcinoma. **A:** Left, urothelial carcinoma; right, small-cell carcinoma (H&E staining, original magnification,  $\times 40$ ); **B:** chromogranin A staining (original magnification,  $\times 40$ ); **C:** urothelial carcinoma (H&E staining, original magnification,  $\times 200$ ); **D:** chromogranin A was negative in urothelial carcinoma component (original magnification,  $\times 200$ ); **E:** small-cell carcinoma (H&E staining, original magnification,  $\times 200$ ); **F:** small-cell carcinoma component showed positive cytoplasmic staining for chromogranin A (original magnification,  $\times 200$ ).

been found at various chromosomal loci in many types of human cancers,<sup>38</sup> including urothelial carcinomas.<sup>20–26</sup> The chromosomal regions in which LOH has been detected are thought to contain specific genes that when disrupted lead to either neoplastic transformation or progression. In this study, we analyzed the pattern of allelic loss in coexisting small-cell and urothelial carcinomas in 20 patients using five polymorphic microsatellite markers: DS3050, IFNA, D9S171, D9S177, and TP53. We also assessed the clonality of these tumor types using X chromosome inactivation analysis in four women.

We found a nearly identical pattern of allelic loss in the small-cell carcinoma and the concurrent urothelial carcinoma in all informative cases. In addition, the X chromosome analysis data are consistent with a common origin for these two tumor types. These findings do not support the hypothesis of a nonurothelial endocrine cell as the precursor for small-cell carcinoma of the bladder. Rather, they suggest that the small-cell component and the urothelial component share the same cell of origin, that of

a multipotential, undifferentiated cell or stem cell present in the urothelium. Three patients demonstrated LOH at a single locus in one of the tumor types that was not present in the other coexisting tumor type; however, in each of these three cases, identical allelic loss patterns were present at the other loci, and in one of these cases, X chromosome inactivation analysis was performed and demonstrated an identical pattern of nonrandom X chromosome inactivation in each of the two tumor types. Thus, despite these minor differences in LOH pattern, the data overwhelmingly support a common clonal origin for urothelial and small-cell carcinomas of the urinary bladder. These three differences are most likely attributable to clonal divergence during tumor progression.

The identification of components of different biological aggressiveness within a single neoplasm is a common finding in pathology. These variable components are thought to result from tumor cell dedifferentiation or transformation, with the subsequent evolution of different subpopulations of tumor cells, a concept that is exemplified by the co-existence of small cell and urothelial carcinoma of the bladder. Our study provided evidence of biphenotypic differentiation in the tumorigenesis of small-cell carcinoma of the urinary bladder. We found that neuroendocrine biomarkers were positive in the small-cell carcinoma component but negative in the non-small-cell (urothelial) carcinoma component in the same patient in all cases studied (Figure 4). Thus, the tumor likely undergoes biphenotypic differentiation after carcinogenesis is initiated, a hypothesis that is further supported by our X chromosome inactivation data. The same pattern of X chromosome inactivation (between small-cell carcinoma and coexisting urothelial carcinoma components) was seen in all four women, suggesting a common clonality. The stage of carcinogenesis in which the pathways of differentiation diverge remains to be resolved.

The existence of pure carcinoids of bladder<sup>39</sup> suggests that there are neuroendocrine cells of the bladder that can become neoplastic. Oesterling et al<sup>1</sup> speculated that neuroendocrine stem (Kultschitzky type) cells with neurosecretory granules may exist within the urothelium that could give rise to neuroendocrine tumors, such as small-cell carcinoma. They also suggested that small-cell carcinoma of the bladder may derive from a poorly defined submucosal cell of neural crest origin, the same cells from which pheochromocytomas/paragangliomas<sup>40</sup> and neurofibromas<sup>41</sup> arise in the urinary bladder. Cramer et al<sup>14</sup> suggested that small-cell carcinomas in the bladder arise from a cell that is present in the urothelium as a result of metaplasia. They cited the frequent findings of glandular metaplasia as well as adenocarcinoma and squamous cell carcinoma of the urinary bladder as evidence for their hypothesis. A large fraction of patients with small-cell carcinoma of the urinary bladder have concomitant foci of urothelial carcinoma.<sup>2,6,11</sup> Our data did not support the notion that the small-cell tumor cells develop from neuroendocrine cells within normal or metaplastic urothelium or from an undefined population of submucosal neuroendocrine cells. However, the possibility remains that the small-cell carcinoma is a result of progression of urothelial carcinoma by an unknown

mechanism of carcinogenesis. The current chemotherapeutic management of these tumors is in part predicated on the concept that these tumors should respond as if they are derived from neuroendocrine cells. Our findings raise doubts about this premise. Nonetheless, caution is warranted in interpreting our data. Further delineation of tumorigenesis of these cases would involve not only molecular studies of the malignant neuroendocrine cell population, but also functional studies involving induction of carcinogenesis in non-neoplastic neuroendocrine cells and investigation of the molecular changes that occur in these cells during carcinogenesis.

In summary, concordant genetic alterations between small-cell carcinoma and coexisting urothelial carcinoma as well as X chromosome inactivation analysis data suggest that urothelial and small-cell carcinoma components originate from the same cells in the urothelium.

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