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ORIGINAL ARTICLE

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Histogenesis of nonurothelial carcinomas of the urinary bladder from pre-existent transitional cell carcinomas. A histopathological and immunohistochemical study

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Abstract The histogenesis of nonurothelial carcinomas of the urinary bladder is difficult to understand, since the bladder is normally lined exclusively by transitional cell epithelium. To gain more insights into the pathogenesis of nonurothelial carcinomas, the morphology and immunohistochemistry of transitional cell carcinomas (TCC), mixed transitional cell and nonurothelial carcinomas, and pure nonurothelial carcinomas were comparatively studied. Of papillary and of nonpapillary (solid) TCC (overall incidence 6.8%), 4.8% and 15.4%, respectively, disclosed foci of altered cellular and architectural phenotypes, consisting of squamous epithelium, pseudoglandular formations, and true glands with or without mucus production. The diverse phenotypic variants develop obviously by a metaplastic process as a result of the well-known inherent potential of the urothelium to undergo several pathways of cellular differentiation. There is strong evidence that squamous cell carcinomas arise secondarily from a squamous metaplasia and adenocarcinomas from metaplastic glandular epithelium within pre-existing TCC following complete carcinogenic transformation of the initially bland-looking metaplastic tumor cells. The metaplastic origin of nonurothelial bladder carcinomas is supported by immunohistochemical findings. The high molecular weight cytokeratin 34βE12 identifies tumor cells with squamous characteristics, helping to explain the development of squamous cell carcinomas. Secretion of MUC5AC apomucin is assumed to play a central role in the histogenesis of nonurachal mucus-producing adenocarcinomas, including signet ring cell carcinomas. Metaplastic phenotypic variants of TCC should be recognized as distinct tumor entities with the potential to transform into nonurothelial carcinomas and thus possibly implying a poorer clinical outcome than typical, uniform TCC.

Keywords Urinary bladder · Transitional cell carcinomas · Phenotypic variants · Mixed urothelial and nonurothelial carcinomas · Pure nonurothelial carcinomas · Metaplasia · Histogenesis · Immunohistochemistry · Cytokeratin 34βE12 · MUC5AC apomucin

Introduction

Approximately 7% of carcinomas of the urinary bladder account for nonurothelial carcinomas (squamous cell carcinomas, adenocarcinomas including signet ring cell carcinomas, and undifferentiated carcinomas), the histogenetic development of which seems difficult to understand, since the bladder normally contains neither squamous, columnar, nor glandular epithelium. The most attractive concept to explain the histogenesis of nonurothelial vesical carcinomas presents the capacity of the transitional cell epithelium to undergo several pathways of cellular and architectural differentiation, most probably as a result of the embryologic origin of the urinary bladder from the totipotential tissues of the urogenital sinus and the mesonephric ducts (trigone). Although phenotypic (squamous and glandular) variants of transitional cell carcinomas (TCC) have long been known, little attention has been paid so far to their significance for the development of nonurothelial vesical carcinomas from pre-existing TCC. To elucidate the role of metaplastic processes during bladder carcinogenesis, we studied the morphology of TCC with aberrant phenotypes and by comparison of mixed transitional cell and nonurothelial carcinomas as well as of pure nonurothelial carcinomas. The various tumor types were additionally analyzed by immunohistochemistry in an attempt to identify possible alterations in the expression of gene protein products. The present investigation appears of interest not only from a histogenetic but also

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from a clinical point of view, since urothelial carcinomas with cellular variants may possibly be associated with a poorer prognosis than typical, uniformly differentiated TCC.

Materials and methods

Included in this study were all carcinomas of the urinary bladder retrieved from the files of the Center of Pathology of the University of Göttingen, Germany, between January 1995 and December 1996. The vast majority of the tumors were available from transurethral resection and a few from cystectomy specimens. Sections of 4 μm thickness were prepared from the formalin-fixed, paraffin-embedded samples and routinely stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and alcian blue (AB, pH 2.5); sections of 3 μm thickness were used for immunohistochemical stainings.

Immunhistochemistry for cytokeratin 8 (NCL-CK8 clone C-51, Novocastra, Newcastle upon Tyne, UK), high molecular weight cytokeratin clone 34βE12 (M630, Dako Diagnostik, Hamburg, Germany), MUC1 glycoprotein (NCL-MUC1 clone Ma 695, Novocastra), MUC5AC apomucin (NCL-HGM-45M1, Novocastra), "human secretory component" bound to secretory polymeric immunoglobulin IgA (NCL-SCO5, Novocastra), epithelial sialomucin (clone 140 C1, ICN Biomedicals, Costa Mesa, USA), and p53 tumor suppressor oncoprotein (clone DO-7, Dako Diagnostik) was performed using the alkaline phosphatase-antialkaline phosphatase (APAAP) method. The antisera applied were all monoclonal mouse antihuman. Staining for the cytokeratin 34βE12 and the "human secretory component" followed digestion with trypsin (0.1% in Tris-buffered saline [TBS] with 0.1% $CaCl_2$, adjusted to pH 7.8) for 30 min at 37°C. The sections provided for immunostaining with cytokeratin 8, p53 protein, and MUC1 glycoprotein were pretreated with microwaves (750 W) for 15 min. After thorough washing in TBS (pH 7.4, 0.05 M), the sections were incubated with the primary antibody diluted in TBS (pH 7.4, 0.05 M) for 2 h at room temperature (cytokeratin 8 1:50, cytokeratin 34βE12 1:100, MUC1 glycoprotein 1:50, MUC5AC apomucin 1:50, "human secretory component" 1:20, epithelial sialomucin 1:15, p53 protein 1:20). Following washing in TBS, the secondary antibody (rabbit antimouse immunoglobulins, Dako Diagnostik) diluted 1:50 in TBS containing 10% nonimmune human serum was applied for 30 min. Thereafter, the sections were subjected to the APAAP complex (mouse monoclonal alkaline phosphatase anti-alkaline phosphatase diluted 1:100 in TBS, Dako Diagnostik) for 45 min at room temperature. The color reaction was developed by treating the sections with a freshly prepared solution of hexazotized new fuchsin for 30 min, followed by application of Mayer's hemalaun as counterstain.

The peroxidase-antiperoxidase (PAP) technique was used for immunohistochemical labeling of glutathione-S-transferase Pi (antibody mouse monoclonal antihuman, clone 353–10, Dako Diagnostik). To block endogenous peroxidase activity, the sections were treated with methanol containing 1% hydrogen peroxide for 30 min at room temperature. After thorough washing in TBS (pH 7.4, 0.05 M), the sections were incubated with nonimmune swine serum (Dako Diagnostik) diluted 1:4 in TBS for 10 min. Thereafter, the primary antiserum was applied at a dilution 1:50 in TBS for 2 h at room temperature. The sections were then again rinsed with TBS and incubated with the secondary antibody (swine antirabbit immunoglobulin, Dako Diagnostik) diluted 1:100 in TBS. The PAP complex (mouse monoclonal) was applied at a dilution of 1:100 in TBS. Antibody localization was visualized using 3-amino-9-ethylcarbazole (Sigma Chemical, Munich, Germany) as chromogen in acetate buffer (pH 5.2, 0.1 M) containing 0.1% hydrogen peroxide. The sections were then washed with distilled water and counterstained with Mayer's hemalaun.

Expression of c-myc gene product (anti-c-myc mouse monoclonal antihuman, Ab-1 clone 9E10, Calbiochem, Cambridge, UK) was studied using the standard avidin-biotin technique. After blocking of endogenous peroxidase (see above), the tissues were treated with nonimmune swine serum diluted 1:5 in TBS (pH 7.4, 0.05 M) for 30 min. Subsequently, the sections were incubated with the primary antiserum diluted in TBS (1:20) overnight at 4°C. Thereafter, biotinylated antimouse immunoglobulins (Vector Laboratories, Burlingame, USA) were applied as secondary antibody at a dilution of 1:75 in TBS for 20 min at room temperature. The sections were then treated with the avidin-biotin complex (Vectastain ABC kit, Vector Laboratories) diluted in TBS (1:100) for 20 min. Between each step of the procedure – except between application of the swine serum and the primary antibody – the sections were carefully rinsed with TBS. The color reaction to identify the sites of immunoprecipitation was developed with 3-amino-9-ethylcarbazole as described above.

For immunohistochemical demonstration of c-erbB-2(HER-2/neu) oncoprotein, the tissues were incubated with the primary antibody (mouse monoclonal antihuman, Ab-2 clone 9G6, Calbiochem) diluted in TBS (1:50) for 2 h at room temperature. Thereafter, the sections were covered with biotinylated anti-mouse/antirabbit immunoglobulins (Biogenex, San Ramon, USA) as secondary antibody, followed by treatment with alkaline phosphatase-conjugated streptavidin (Biogenex) for 20 min. The sections were washed with TBS between the various steps and finally stained with hexazotized new fuchsin and Mayer's hemalaun.

A total of 233 carcinomas (67 papillary and 69 nonpapillary TCC with a uniform urothelial differentiation, 48 TCC with focally altered cellular and structural differentiations, 30 pure nonurothelial carcinomas, and 19 mixed urothelial and nonurothelial carcinomas) were studied by immunohistochemistry. Staining was scored as negative, diffusely positive throughout the tumor, or patchy positive in small (microfocal) or large areas. Nuclear reactivity of the p53 protein was stratified semiquantitatively into three categories: less than 10%, between 10% and 50%, and more than 50% of moderately and strongly positive cells.

Results

Histopathological spectrum of carcinomas

Among the 868 reclassified carcinomas of the urinary bladder, there were 641 transitional cell carcinomas (TCC) with a papillary (73.8%) and 143 with a nonpapillary solid (16.5%) pattern of growth showing various grades and stages, 37 (4.3%) pure squamous cell

Table 1. Histological types of mixed transitional cell carcinomas (TCC) and nonurothelial carcinomas ($n = 23$)

Histologic subtype	*N* cases
Solid TCC and squamous cell carcinoma	7
Papillary TCC and squamous cell careinoma	3
Solid TCC and undifferentiated carcinoma	4
Papillary TCC and undifferentiated carcinoma	1
Solid TCC and adenocarcinoma	2
Papillary TCC and adenocarcioma	1
Solid TCC, adenocarcinoma, and undifferentiated carcinoma	2
Squamous cell carcinoma and undifferentiated carcinoma	2
Squamous cell carcinoma and adenocarcinoma	1

carcinomas with or without keratinization, 22 (2.5%) undifferentiated carcinomas (12 of the large, eight of the small cell type, and two of the sarcomatoid type), two (0.2%) pure adenocarcinomas, and 23 (2.7%) mixed transitional cell and nonurothelial carcinomas (Table 1).

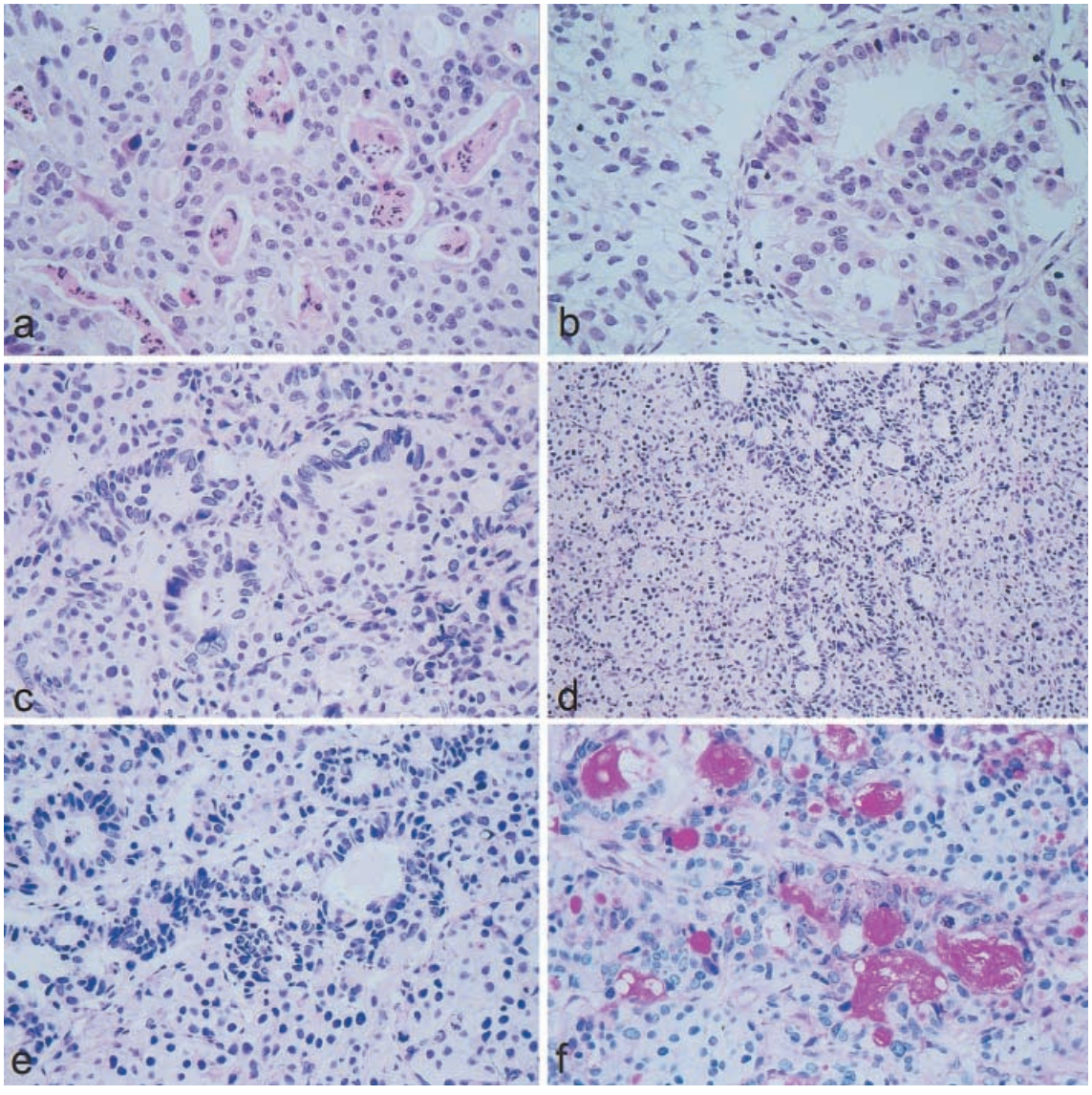
Table 2. Incidence of transitional cell carcinomas (TCC) with foci of altered cellular and architectural phenotypes referred to all papillary ($n = 641$) and solid ($n = 143$) TCC

Phenotypes	*N* cases (%)
Papillary TCC with altered differentiations	31 (4.8)
Squamous	8 (1.2)
Pseudoglandular	11 (1.7)
True glandular	6 (0.9)
Mixed	6 (0.9)
Solid TCC with altered differentiations	22 (15.4)
Squamous	4 (2.8)
Pseudoglandular	8 (5.6)
True glandular	8 (5.6)
Mixed	2 (1.4)

Morphology of transitional cell carcinomas with altered phenotypes

With an overall incidence of 6.8%, 4.8% of the papillary and 15.4% of the solid TCC disclosed foci with diverse types of an altered cellular and architectural differentiation (Table 2). The TCC with a squamous cell differ-

Fig. 1a–f. Solid transitional cell carcinomas (TCC) illustrating pseudoglandular and true glandular structures. **a** Pseudoglandular formations consisting of microcystic spaces containing necrotic cell material (H&E, orig. magnification ×180). **b** Direct conversion of urothelial carcinoma cells into gland-forming, bland-looking columnar cells (H&E, orig. magnification ×180). **c** Group of true glands, one of them arising directly from the urothelial carcinoma cells (H&E, orig. magnification ×180). **d** Larger focus of densely-packed, small-sized true glands (H&E, orig. magnification ×90). **e** Same TCC as demonstrated in Fig. 1**c** and **d** with interspersed true glands lined by columnar cells (H&E, orig. magnification ×180). **f** Beginning formation of true glands with production of extracellular mucus (PAS, orig. magnification ×180)



entiation were characterized by interspersed islands of squamous epithelium without substantial cellular atypias, a finding which helps to distinguish this type of TCC from mixed TCC and squamous cell carcinomas. The areas with squamous cell characteristics were usually well circumscribed, but in some instances the squamous cells merged gradually with the surrounding urothelial carcinoma cells. Pseudoglandular formations consisted of microcystic spaces bordered by flattened or cuboidal urothelial carcinoma cells and commonly filled with necrotic cell material (Fig. 1a). True glands were lined by a single layer of columnar cells (Fig. 1b–e, Fig. 2a, b) with or without production of mucus that stained positively in the PAS (Fig. 1f) but not always in the AB reaction. In rare instances, the glands contained goblet cells with accumulation of abundant mucus positive for both PAS and AB. The glands were arranged in small groups and usually present at several sites of the carcinomas. TCC with interspersed foci of well-formed, bland-looking true glands should be separated from mixed TCC and adenocarcinomas, which show an intimate admixture of urothelial carcinoma cells and glandular structures with unequivocal adenocarcinomatous features. Occasionally, a direct conversion of urothelial carcinoma cells into glandular epithelium could be observed (Fig. 1b, c, Fig. 2c, d). The

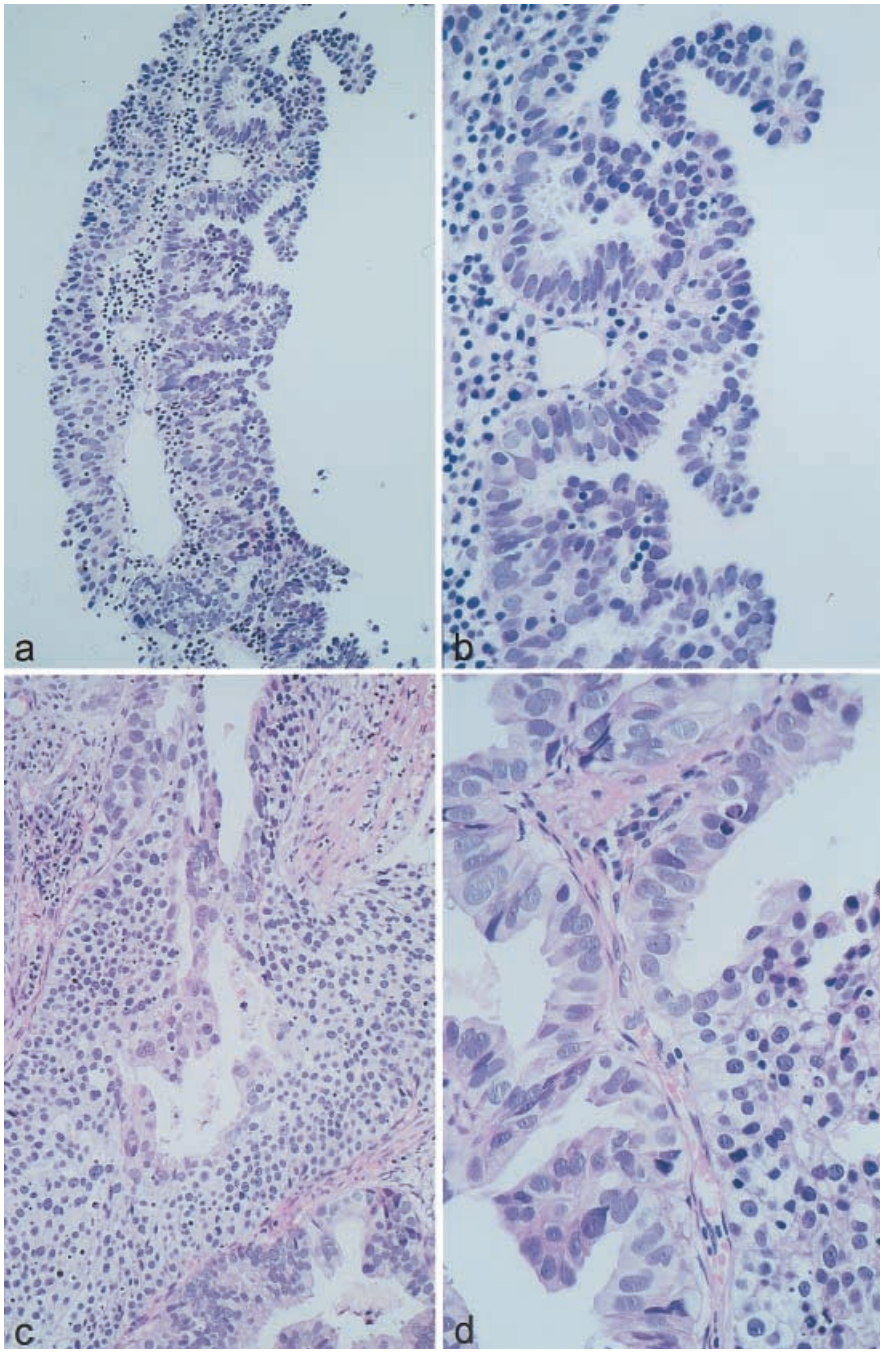


Fig. 2a–d. Transitional cell carcinomas (TCC) demonstrating transition into true glandular formations. **a** Papilla of a papillary TCC covered on the left side by urothelial carcinoma cells and on the right side by gland-forming columnar cells lacking substantial atypias (H&E, orig. magnification ×90). **b** Higher magnification of the TCC shown in Fig. 2a (H&E, orig. magnification ×180). **c** TCC with transition into an adenocarcinoma (H&E, orig. magnification ×180). **d** High-power view of the mixed TCC and adenocarcinoma seen in Fig. 2c documenting a direct conversion of urothelial carcinoma cells into the adenocarcinomatous glands (H&E, orig. magnification ×500)

various phenotypes of cellular and structural differentiation coexisted in some cases.

Immunohistochemistry of transitional cell carcinomas with a uniform urothelial differentiation

Immunohistochemistry was available for 67 papillary TCC (17 grade 1, 38 grade 2, 12 grade 3; 34 pTa, 18 pT1, nine pT2a, and one pT2b, staging not possible in five cases) and for 69 nonpapillary TCC (32 grade 2, 37 grade 3; four pT1, 18 pT2a, 32 pT2b, nine pT3, and three pT4a, staging not possible in three cases) with a uniform urothelial differentiation. In a very few cases, the immunohistochemical reactions did not allow an accurate assessment, resulting in a reduced number of evaluated cases. The findings are summarized in Table 3.

High molecular weight cytokeratin 34βE12 and cytokeratin 8

Positive cytoplasmic staining with the high molecular weight cytokeratin 34βE12 was observed in 17.9% of papillary and in 15.9% of solid TCC (overall incidence 16.9%). The cytokeratin was usually expressed in scattered small areas of tumor cells, most of which displayed beginning squamous features in the H&E stain (Fig. 3a). Only two carcinomas displayed a diffuse immunoreactivity and in two papillary TCC, positivity was restricted to the basally located tumor cells (Fig. 3c). Immunoreactivity was unrelated to tumor grades and stages. The normal-appearing urothelium adjacent to the carcinomas disclosed a positive reaction in 29 of 69 cases (42.0%) available for examination, with positivity confined to the basal cell layer. Cytokeratin 8 was strongly expressed in nearly all papillary and nonpapillary TCC with a predominantly diffuse pattern of reactivity.

C-erbB-2(HER-2/neu) oncoprotein and c-myc oncoprotein

Strong distinctly cell membrane-associated expression of C-erbB-2 oncoprotein was detected in 16.4% of papillary and in 42.6% of solid TCC, with an overall positivity of 29.6% (Fig. 3d). The majority of positive cases

Table 3. Immunohistochemistry of papillary (p) and solid (s) transitional cell carcinomas (TCC) with a uniform urothelial differentiation

Antibody	Total number of cases	*N* positive cases (%)	Pattern of positive immunostaining (*n* cases)		
			Microfocal	Large areas	Diffuse
Cytokeratin 34βEl2					
pTCC	67	12 (17.9)	10	1	1
sTCC	63	10 (15.9)	9	0	1
Cytokeratin 8					
pTCC	67	65 (97.0)	5	9	51
sTCC	68	66 (97.1)	8	6	52
C-erbB-2 oncoprotein					
pTCC	67	11 (16.4)	7	3	1
sTCC	68	29 (42.6)	19	3	7
C-myc oncoprotein					
pTCC	66	19 (28.7)	4	10	5
sTCC	66	43 (65.2)	17	5	21
MUC5AC apomucin					
pTCC	64	9 (14.1)	8	1	0
sTCC	66	5 (7.6)	3	2	0
"Secretory component"					
pTCC	67	38 (56.7)	37	1	0
sTCC	68	36 (52.9)	35	1	0
MUC1 glycoprotein					
pTCC	67	66 (98.5)	11	18	37
sTCC	69	69 (100)	10	10	49
Epithelial sialomucin					
pTCC	67	66 (98.5)	10	18	38
sTCC	69	69 (100)	9	10	50
p53 oncoprotein ($\leq$ 10%)					
pTCC	65	25 (38.5)			
sTCC	65	24 (36.9)			
Glutathione-S-transferase Pi					
pTCC	67	53 (79.1)	19	9	25
sTCC	68	64 (94.1)	23	15	26

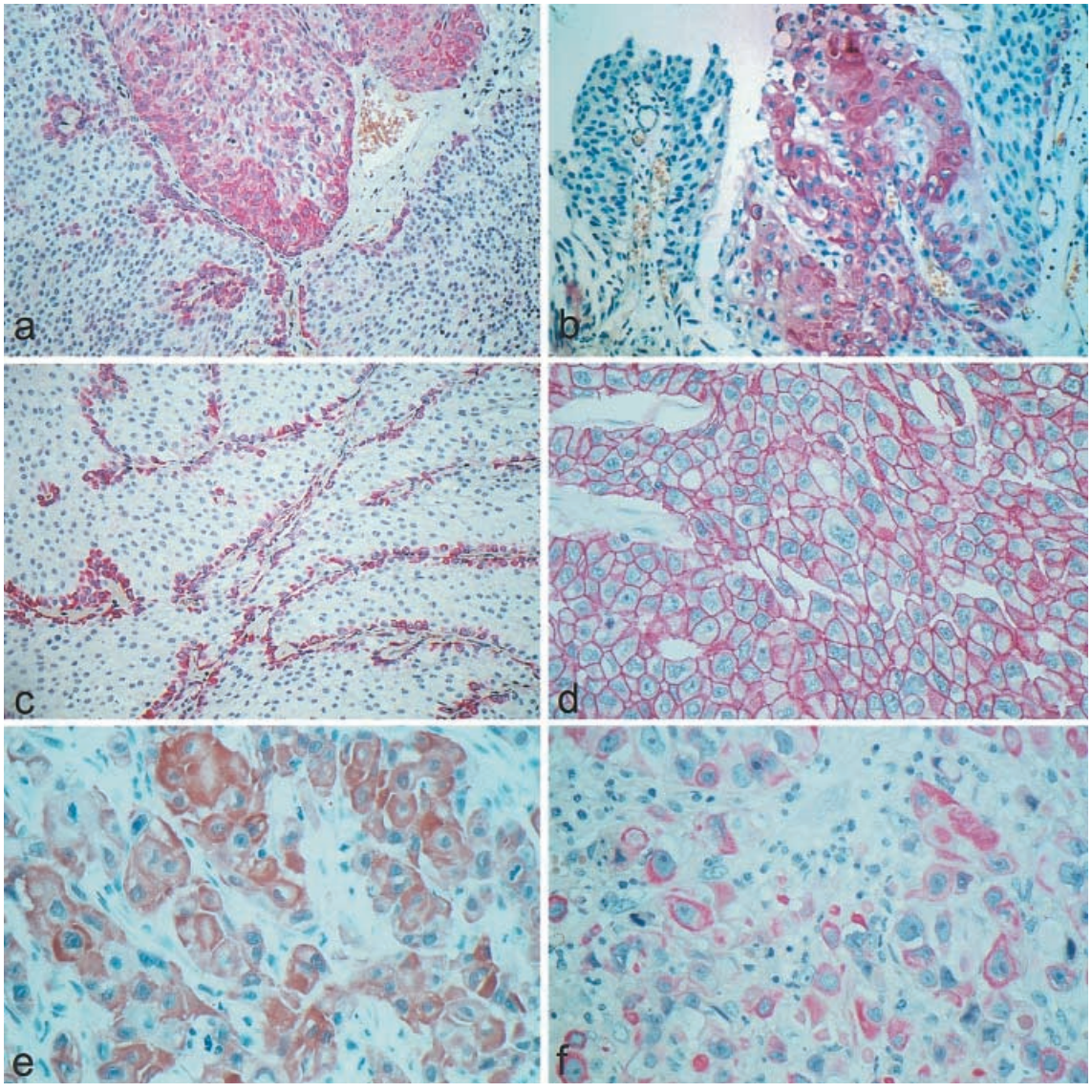


Fig. 3a–f. Immunohistochemistry of transitional cell carcinomas (TCC). **a** Expression of cytokeratin 34βE12 in an area of cells showing a beginning squamous differentiation (orig. magnification ×90). **b** Focus of a fully developed squamous cell metaplasia with strong expression of cytokeratin 34βE12 (orig. magnification ×90). **c** Papillary TCC revealing only basally located tumor cells positive for cytokeratin 34βE12 (orig. magnification ×90). **d** Strong membrane associated immunoreactivity for c-erbB-2 oncoprotein (orig. magnification ×180). **e** TCC with squamous cell features staining positively for glutathione-S-transferase Pi (orig. magnification ×180). **f** Intracytoplasmic positivity for the so-called secretory component (orig. magnification ×500)

demonstrated a heterogeneous patchy staining. One fourth of the solid, but only 1 of 11 papillary TCC showed a diffuse immunoreactivity. Most of the positive papillary (four grade 2, seven grade 3) and nonpapillary (11 grade 2, 18 grade 3) TCC represented grade 3 tumors, and in none of the cases could a grade 1 carcinoma be observed. Only three of the 11 papillary TCC expressing the c-erbB-2 protein were noninvasive, most of the positive solid TCC exhibited muscle-invasive growth (Table 4). The non-neoplastic, normally appearing urothelium adjacent to the tumors stained negatively for c-erbB-2 protein in all 66 cases studied. The c-myc protein was expressed – mainly within the cytoplasm – in 28.7% of the papillary and in 65.2% of the nonpapillary TCC. Half of the solid carcinomas and one fourth of the papillary TCC showed a diffuse immunoreactivity. Nearly all solid TCC but only a few of the papillary carcinomas had invaded the muscle layer (Table 4). Expression was not correlated with the tumor grades. The non-neoplastic transitional cell epithelium stained positively in 25.4% of cases (16 of 63).

MUC5AC apomucin, so-called secretory component, MUC1 glycoprotein, and epithelial sialomucin

Nine of 64 (14.1%) uniformly differentiated papillary and five of 66 (7.6%) solid TCC expressed the MUC5AC apomucin (overall incidence 10.8%) with

Table 4. Correlation between stages of uniformly differentiated transitional cell carcinomas and expression of C-erbB-2, C-myc, MUC5AC apomucin, and glutathione-S-transferase Pi

Pattern of growth	C-erbB-2 ($n=40$)1		C-myc ($n=62$)		MUC5AC ($n=14$)		Glutathione-S-transferase Pi ($n=117$)	
Papillary	3×	pTa	12×	pTa	5×	pTa	29×	pTa
	4×	pT1	4×	pT1	2×	pT1	13×	pT1
	4×	pT2a	3×	pT2a	2×	pT2a	7×	pT2a
							1×	pT2b
							3×	Staging not possible
Solid	2×	pT1	1×	pT1	2×	pT2a	2×	pT1
	7×	pT2a	13×	pT2a	1×	pT2b	18×	pT2a
	14×	pT2b	22×	pT2b	1×	pT3	32×	pT2b
	3×	pT3	4×	pT3	1×	pT4	9×	pT3
	1×	pT4	2×	pT4			2×	pT4
	2×	Staging not possible	I×	Staging not possible			1×	Staging not possible

a heterogeneous pattern of staining (Fig. 4 a–c). Most of the carcinomas showed only small areas of tumor cells and scattered individual cells which contained large amounts of granular deposits of the MUC5AC within their cytoplasm, some of them resembling goblet or signet ring cells (Fig. 4d). The latter always stained strongly positive both for AB and PAS, but not all other cells which had produced the apomucin did so. Most of the positive papillary (two grade 1, five grade 2, and two grade 3) and all solid TCC (two grade 2 and three grade 3) were higher-grade carcinomas. All solid carcinomas showed muscle-invasive growth, while only a few of the papillary TCC did so (Table 4). The normally appearing non-neoplastic transitional cell epithelium adjacent to the tumors revealed a positive patchy reaction, mainly of the superficial cells (Fig. 4f), in nine of 59 cases (15.3%). The "secretory component" was expressed in approximately half of the papillary (56.7%) and nonpapillary (52.9%) TCC and was unrelated to the grades and stages. The pattern of intracytoplasmic immunoreactivity was heterogeneous and confined to small areas (Fig. 3f). The positive carcinoma cells were usually negative in the PAS and AB reaction and not identical with those secreting the MUC5AC apomucin. The non-neoplastic urothelium showed patchy areas of positive cells within the superficial layer in 66.2% of cases. Practically all papillary and solid TCC showed a strong, in most cases diffuse positive intracytoplasmic immunoreactivity for the MUC1 glycoprotein and the epithelial sialomucin. The normally appearing transitional cell epithelium expressed both glycoproteins within all layers of all cases examined.

Tumor suppressor oncoprotein p53

The tumor suppressor oncoprotein p53 was expressed in 38.5% of the papillary TCC (at least 10% of positive cells), the nuclear accumulation increasing with increasing grades and stages, particularly in the category of carcinomas with a positive staining of more than 50% of the tumor cells. Solid TCC expressed the p53 protein in 36.9%, but there was no clear-cut correlation with the grades and stages. The non-neoplastic, normally looking urothelium adjacent to the tumors contained only a very few positive cells in six of 56 cases.

Glutathione-S-transferase Pi

The glutathione-S-transferase Pi was detected focally or diffusely in 79.1% of the papillary and in 94.1% of the solid TCC, independently of their grades (Fig. 3e). The vast majority (96.8%) of the positive solid TCC but only 15.1% of the papillary carcinomas were muscle-invasive (Table 4). The non-neoplastic urothelium surrounding the carcinomas expressed the enzyme in 75.7% of the cases (50 of 66) within all cell layers.

Immunohistochemistry of transitional cell carcinomas with phenotypic variants

The immunohistochemical findings available for 27 papillary and 21 solid TCC with focally altered cellular and architectural differentiations are summarized in Table 5. Foci of cells with a squamous differentiation expressed nearly always the high molecular weight cytokeratin 34βE12 (Fig. 3b). The MUC5AC apomucin was expressed in a considerably higher frequency in TCC with aberrant phenotypic features (overall incidence 43.8%, range between 36.8% and 54.5%) than in uniform TCC (overall incidence 10.8%, Table 3). Intracytoplasmic granular deposits of MUC5AC were observed in urothelial carcinoma cells, in glandular epithelium with production of PAS and AB positive mucus including particularly goblet cells, and occasionally in columnar cells of true glands without mucus secretion as well as in scattered carcinoma cells bordering pseudoglands (Fig. 4e). The so-called secretory component was expressed in 77.1% of TCC with cellular variants (range between 73.7% and 81.8%) but only in 54.1% of uniform papillary and nonpapillary TCC (Table 3). The c-erbB-2 oncoprotein stained

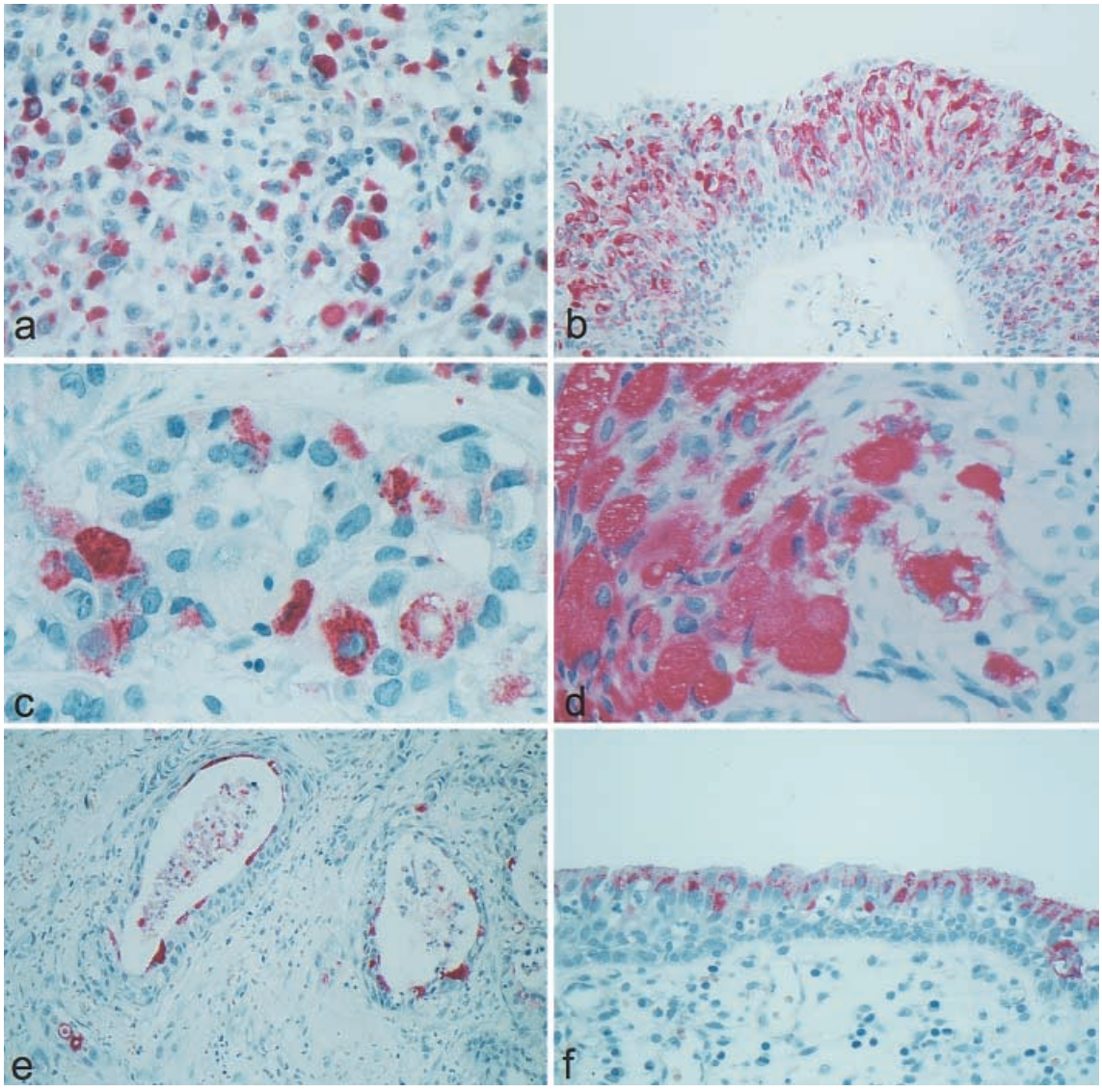


Fig. 4a–f. Transitional cell carcinomas (TCC) with expression of MUC5AC apomucin. **a** Nonpapillary TCC containing numerous tumor cells with abundant intracytoplasmic deposits of MUC5AC (orig. magnification ×180). **b** Part of a papillary TCC showing strong immunoreactivity for MUC5AC (orig. magnification ×90). **c** TCC with some intermingled carcinoma cells containing granular deposits of MUC5AC apomucin within their cytoplasm (orig. magnification ×500). **d** Group of densely packed goblet cells with heavy accumulation of MUC5AC (orig. magnification ×180). **e** Pseudoglands bordered by urothelial carcinoma cells, some of which staining positively for MUC5AC (orig. magnification ×90). **f** Non-neoplastic urothelium adjacent to a carcinoma with superficial cells containing intracytoplasmic granular deposits of MUC5AC (orig. magnification ×180)

positively in a comparatively high percentage of TCC with true glands. Positive immunoreactivity for the c-myc gene product was most commonly observed in TCC with mixed phenotypic cellular characteristics. The p53 oncoprotein stained positively at higher levels in TCC with mixed cellular phenotypes (50%) than in pure TCC (overall positivity 37.7%, Table 3). The glutathione-S-transferase Pi was present in all TCC with focal squamous epithelium and mixed cellular differentiations. Although the immunohistochemical profiles of TCC with focally altered cellular and architectural differentiations differ from those of uniformly differentiated TCC in many aspects, only the expression of cytokeratin 34βE12 and MUC5AC apomucin could be reliably correlated with special cellular phenotypes.

Immunohistochemistry of nonurothelial and mixed urothelial and nonurothelial carcinomas

The immunohistochemical results obtained for pure nonurothelial carcinomas are compiled in Table 6. Nearly all squamous cell carcinomas had expressed the high molecular weight cytokeratin 34βE12 and the glutathione-S-transferase Pi. The percentage of squamous cell carcinomas staining positively for c-erbB-2 oncoprotein (22.2%) and the "secretory component" (21.1%) was substantially lower than that of solid

Table 5. Immunohistochemistry of papillary (27 cases) and nonpapillary (21 cases) transitional cell carcinomas with aberrant phenotypes

Antibody	Type of differentiation			
	Squamous ($n=10$)	Pseudoglandular ($n=19$)	True glandular ($n=11$)	Mixed ($n=8$)
	N cases with positive immunoreactivity (%)			
Cytokeratin 34βE12	9 (90.0)	7 (36.8)	2 (18.2)	1 (12.5)
Cytokeratin 8	8 (80.0)	18 (94.7)	11 (100)	8 (100)
C-erbB-2 oncoprotein	1 (10.0)	4 (21.1)	6 (60.0)	2 (27.1)
C-myc oncoprotein	2 (20.0)	9 (50.0)	3 (30.0)	8 (100)
MUC5AC apomucin	4 (40.0)	7 (36.8)	6 (54.5)	4 (50.0)
"Secretory component"	8 (80.0)	14 (73.7)	9 (81.8)	6 (75.0)
MUCl glycoprotein	10 (100)	18 (100)	11 (100)	8 (100)
Epithelial sialomucin	10 (100)	19 (100)	11 (100)	8 (100)
p53 oncoprotein (≤ 510%)	3 (30.0)	6 (31.6)	3 (27.3)	4 (50.0)
Glutathione-S-transferase Pi	10 (100)	*17* (89.5)	7 (63.6)	8 (100)

Table 6. Immunohistochemistry of pure nonurothelial and mixed urothelial and nonurothelial carcinomas

Antibody	Squamous cell carcinomas ($n=19$)	Undifferentiated carcinomas ($n=10$)	Adenocarcinomas ($n=1$)	Mixed urothelial and nonurothelial carcinomas ($n=19$)
	N cases with positive immunoreactivity (%)			
Cytokeratin 34βE12	18 (94.7)	1 (10)	Negative	11 (57.9)
Cytokeratin 8	12 (63.2)	7 (70)	Focally +	15 (78.9)
C-erbB-2 oncoprotein	4 (22.2)	0	Negative	6 (31.6)
C-myc oncoprotein	15 (83.3)	6 (60)	Negative	11 (57.9)
MUC5AC apomucin	1 (5.3)	0	Focally +	3 (15.8)
"Secretory component"	4 (21.1)	2 (20)	Focally +	8 (42.1)
MUC1 glycoprotein	16 (84.2)	8 (80)	Diffusely +	16 (84.2)
Epithelial sialomucin	18 (94.7)	8 (80)	Diffusely +	16 (84.2)
p53 oncoprotein (≤10%)	9 (47.4)	6 (60)	Positive (20%)	7 (36.8)
Glutathione-S-transferase Pi	18 (94.7)	4 (40)	Negative	16 (84.2)

TCC (42.6% and 52.9%, respectively, Table 3). Undifferentiated carcinomas of all subtypes did not express the c-erbB-2 protein and the MUC5AC apomucin and were positive for cytokeratin 34βE12 in only a single case. Compared with uniformly differentiated TCC, undifferentiated carcinomas showed a significantly lower positivity for the "secretory component" (20%) and the glutathione-S-transferase (40%). The p53 oncoprotein had accumulated at higher levels in squamous cell (47.4%) and undifferentiated carcinomas (60%) than in uniform TCC (37.7%, Table 3). The pure nonurachal adenocarcinoma studied by immunohistochemistry displayed a focal reactivity for MUC5AC and the "secretory component." The only substantial difference in the staining pattern of the various gene protein products between pure TCC and mixed transitional cell and nonurothelial carcinomas consisted in a considerably higher expression of cytokeratin 34βE12 in the mixed carcinomas (Table 3, Table 6).

Discussion

It was the objective of the present study to get more insights in the histogenesis of nonurothelial vesical carcinomas, the occurrence of which appears surprising at first glance, since the urinary bladder is normally lined exclusively by highly specialized urothelial cells. Based upon the well-known inherent potential of the normal transitional cell epithelium to transform into diverse nonurothelial cellular phenotypes such as squamous, columnar, glandular, and so-called nephrogenic epithelium by a metaplastic process (for review of the literature see [35, 36]), it seemed reasonable to assume that the neoplastic urothelium would also be capable of developing metaplastic changes, helping to explain the histogenetic formation of nonurothelial carcinomas from pre-existing TCC.

Histopathological findings

The spectrum of histopathological types of carcinomas observed is similar to that reported by many others. Focally altered cellular and architectural differentiations were shown by 4.8% of the papillary and 15.4% of the nonpapillary (solid) TCC, supporting the concept of an origin of nonurothelial carcinomas secondarily from pre-existent TCC by a metaplastic conversion of urothelial carcinoma cells into other cellular phenotypes. This histogenetic pathway could best be documented for

the development of squamous cell carcinomas and is indicated by the occurrence of islands of cells disclosing squamous characteristics within otherwise typical TCC (squamous variant), as also described by others [3, 45, 64]. The metaplastic conversion of urothelial into squamous carcinoma cells may progress and overgrow with time, leading to mixed transitional and squamous cell carcinomas and ultimately to the formation of pure squamous cell carcinomas. Strong support of this pathogenetic mechanism comes from animal experiments clearly documenting transition of squamous metaplasia within TCC into squamous cell carcinomas [36, 37]. Similarly, nonurachal adenocarcinomas may arise metaplastically from pre-existent TCC, obviously by two pathogenetic mechanisms. The first pathway is initiated by the induction of microfocal necroses of urothelial carcinoma cells – most likely due to apoptosis – resulting in the development of pseudoglands. In a second step, the urothelial epithelium bordering the pseudoglands changes to cuboidal and columnar epithelium with subsequent formation of true glands. In a third step, the glandular epithelium may acquire the ability of producing mucus. This stepwise developmental process is also substantiated by experimental findings obtained in rats [36, 37]. The second possibility consists in a direct conversion of urothelial carcinoma cells into glandular epithelium, as is documented in this study as well. Subsequent complete carcinogenic transformation of the metaplastic glandular epithelium results in the formation of mixed transitional cell carcinomas and adenocarcinomas, which were also observed by other authors [14, 22, 28, 44], and ultimately of pure adenocarcinomas. Although the occurrence of true glandular structures in TCC (glandular variant) is well known, only a very few original studies dealt with this peculiar morphologic phenomenon so far [64]. Histogenetic derivation of nonurachal adenocarcinomas from either an adenocarcinoma in situ [33, 35, 53] or possibly from intestinal (colonic) cystitis glandularis [17, 66] is assumed to play a minor role compared with the metaplastic formation from pre-existing TCC.

Undifferentiated carcinomas of the various subtypes develop from pre-existing TCC most probably by a dedifferentiation of the neoplastic urothelial cells. This histogenetic pathway is clearly suggested by the occurrence of mixed urothelial and undifferentiated carcinomas observed in the present and in some other studies [10, 46, 54], showing gradual transitions between the two tumor cell populations.

Immunohistochemical findings

Previous immunohistochemical studies were mainly concerned with the correlation of oncoprotein expression of TCC with their grades and stages, recurrences, and survival times for identifying prognostic indicators of disease progression, but without regarding peculiar phenotypic variants. The primary intention of the current investigation was to evaluate the significance of possible aberrations of gene protein expression for the histogenesis of nonurothelial bladder cancers.

The cytokeratin clone 34βE12 identifies the high molecular weight cytokeratins 1, 5, 10, and 14 of approximately 66 kD and 57 kD, all of them repeatedly documented to be mainly raised – though not exclusively – against normal stratified squamous epithelium and shown to be strongly positive in squamous cell carcinomas at various sites [26, 27, 48, 50, 62]. Cytokeratin 34βE12 was found to be reliably expressed in areas of a squamous cell metaplasia within otherwise uniformly differentiated TCC as well as in pure squamous cell carcinomas, thus indicating a metaplastic origin of squamous carcinomas from pre-existing TCC. Expression of the cytokeratins 5, 10, and 14 by metaplastic squamous epithelium occurring in TCC has already been reported by others [49, 61]. Routine application of cytokeratin 34βE12 seems useful for detecting TCC with a squamous metaplasia and in distinguishing poorly differentiated squamous carcinomas from undifferentiated carcinomas which were demonstrated to stain negatively, confirming the results of Ordonez and coworkers [54]. Helpap and Köllermann recently reported that all papillary TCC express the cytokeratin 34βE12, low-grade and low-stage carcinomas showing a positivity exclusively of the basally located tumor cells, higher-grade and higher-stage TCC disclosing a diffuse immunoreactivity [30, 31]. We found, by contrast, a considerably lower incidence of positive papillary TCC, and noteworthy were a very few carcinomas revealing diffuse staining patterns or positive reactions of the basal tumor cells only, expression not being linked with the tumor grades and stages. The reasons for this discrepancy are not known, but it is strongly supposed that the used antisera – purchased from two different sources – are not identical in their antigenicity, apart from the fact that Helpap and coworkers applied the antibody in 1:1 dilution as opposed to 1:100 in the current study.

Secretion of MUC5AC apomucin was examined to gain possibly more insights into the histogenesis of mucus-producing adenocarcinomas from pre-existing transitional cell carcinomas. The MUC5AC apomucin belongs to a family of eight different subtypes of mucins which all are produced by the mucus-secreting columnar surface epithelium of the stomach [4, 5, 6, 7, 23]. The antibody 45M1 used recognizes a specific epitope situated on the peptide core of the mucin [6]. We were able to document 10.8% of uniformly differentiated papillary and nonpapillary TCC expressing the MUC5AC apomucin, as recently published in detail [38]. Transitional cell carcinoma with foci of altered cellular phenotypes had produced the apomucin with a comparatively high overall incidence of 43.8%. Since 45M1 antigenicity was reported to be absent in the normal adult urothelium of the urinary bladder but present in the fetal prostatic urethral epithelium and trigonal cells of the bladder [18], resurgence of MUC5AC apomucin in TCC is regarded as a reexpression

of oncofetal antigenicity. We suppose that secretion of MUC5AC apomucin plays an essential role in the formation of mucus-producing nonurachal adenocarcinomas, including signet ring cell carcinomas from pre-existing TCC.

The so-called secretory component – to the best of our knowledge examined in bladder cancer for the first time – has previously been shown to be produced by secretory epithelial cells of various organs, particularly of the small intestine, colon, and breast [8, 12]. It was identified in approximately half of the pure TCC and in a higher incidence of 73.7% to 81.8% in TCC with cellular variants. The "secretory component" is the exodomain of a transmembrane protein receptor on secretory epithelial cells binding polymeric immunoglobulins (pIgA and pIgM) and mediating their transport into exocrine fluids, the external secretions containing the secretory component either attached to the secretory immunoglobulins (sIgA and sIgM) or free [2, 8, 11, 39]. Because metaplastic glandular epithelium did not consistently express the "secretory component," it cannot be regarded as a marker of glandular differentiation in TCC and thus for the histogenetic development of vesical adenocarcinomas, although Brooks and Ernst [12] reported immunoreactive "secretory component" to be present in adenocarcinomas of the colon, breast, and lung.

C-erbB-2 (HER-2/neu) oncoprotein was overexpressed in 29.6% of all uniformly differentiated TCC, nonpapillary TCC staining more commonly positive (42.6%) than papillary TCC (16.4%). Other authors reported an overall positive immunoreactivity ranging between 12% and 47% [19, 24, 32, 40, 43, 51, 58, 68]. Expression was correlated with the grades and stages, high-grade and high-stage more frequently positive than low-grade and low-stage TCC, confirming the results of previous studies [15, 19, 24, 25, 47, 51, 58, 59]. Since positivity lacked to be associated with the divergent cellular phenotypes within TCC as well as in mixed urothelial and nonurothelial carcinomas, the presence or absence of c-erbB-2 does not help to clarify the histogenesis of nonurothelial carcinomas. The c-myc gene product was detected in approximately half of all TCC with a uniform urothelial differentiation, unrelated to the tumor grades, but associated with the pattern of growth, nonpapillary TCC staining positively in two thirds and papillary TCC in less than one third of the cases. The few previous investigations reported an overexpression of between 56% and 75% [34, 42, 60, 63]. Immunoreactivity proved not to be linked with the various types of altered cellular and architectural differentiation in TCC, so that the c-myc oncoprotein cannot serve as a marker of special cellular phenotypes and does not allow any conclusions regarding the pathogenetic development of nonurothelial carcinomas.

Analyzing the status of the p53 tumor suppressor oncoprotein, 36.3% of all TCC with a uniform urothelial differentiation showed a positive nuclear immunoreactivity (at least 10% positive cells), while others determinated p53 overexpression in a wide range of 22% to 78%, most probably reflecting different definitions of positivity [1, 9, 13, 16, 21, 41, 52, 56, 57, 67]. Unlike most previous studies reporting expression of p53 at higher levels in high-grade and high-stage than in low-grade and low-stage carcinomas, we found such a correlation only for TCC with a papillary but not a solid pattern of growth. The increased incidence of p53-positive cases among the TCC with altered phenotypic characteristics (50%) than with pure TCC (36.3%) indicate an increased loss of cell cycle control and is in favor of a progression of carcinomas with cellular variants into mixed transitional cell and nonurothelial carcinomas and finally into pure nonurothelial carcinomas by an increased proliferative activity.

A high percentage of uniformly differentiated TCC expressed the glutathione-S-transferase of the Pi class (placental form), previously demonstrated to be strongly expressed in the normal urothelium [65]. Among the pure nonurothelial carcinomas, squamous cell carcinomas showed the highest and undifferentiated carcinomas the lowest incidence of immunoreactivity. Interestingly, Eimoto and coworkers [20] reported all squamous carcinomas of the lung to stain positively and all undifferentiated small cell carcinomas negatively. The various isoenzymes of the glutathione-S-transferase catalyzes the conjugation of electrophilic compounds and presumably detoxifies chemical carcinogens but evidently does not play a role in the pathogenesis of nonurothelial bladder carcinomas.

Conclusions

The current histopathological and immunohistochemical investigation documented a relatively high incidence of TCC with foci of divergent cellular and architectural phenotypes. They most probably develop by a metaplastic process as a result of the well-known capacity of urothelium to undergo several pathways of cellular differentiation. The morphological findings clearly support the concept of a metaplastic origin of nonurothelial carcinomas of urinary bladder from pre-existent transitional cell carcinoma. The immunohistochemical staining profiles of gene protein products reveal a considerable cellular heterogeneity of TCC, those with altered phenotypic characteristics differing in many aspects from uniform urothelial carcinomas. The high molecular weight cytokeratin 34βE12 identifying tumor cells with a squamous differentiation helps to explain the histogenesis of squamous cell carcinomas. Neoexpression of MUC5AC antigenicity is assumed to play a key role in the formation of mucus-producing adenocarcinomas, including signet ring cell carcinomas. Metaplastic phenotypic variants of TCC should be realized as distinct tumor entities bearing the potential to progress into nonurothelial carcinomas and thus possibly implying a more aggressive behavior and a poorer clinical outcome than typical uniformly differentiated TCC.

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