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Tumor-associated neoexpression of the pS2 peptide and MUC5AC mucin in primary adenocarcinomas and signet ring cell carcinomas of the urinary bladder

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Summary. To gain more detailed insight into the histogenesis of primary nonurachal adenocarcinomas and signet ring cell carcinomas of the urinary bladder, we analyzed by immunohistochemistry the expression of a broad panel of proteins, associated with cell differentiation (pS2 peptide, MUC5AC, MUC6, spasmolytic polypeptide, cyclooxygenases-1 and -2, caveolin-1), and of various novel known or candidate tumor suppressors (14-3-3 sigma, SYK, PTEN, maspin). Included were 12 adenocarcinomas admixed to urothelial carcinomas, 10 pure adenocarcinomas and 5 signet ring cell carcinomas. As the most important finding, the majority of signet ring cell carcinomas and three quarters of the adenocarcinomas (72.7%) expressed the pS2 peptide, and nearly half of the adenocarcinomas (45.5%) as well as most of the signet ring cell carcinomas were observed to secrete the MUC5AC apomucin. Since expression of both proteins was absent in the normal nonneoplastic urothelium, their tumor-associated appearance is regarded as a neoexpression or reexpression, respectively, of normally cryptic antigenic determinants, and is assumed to be involved in the phenotypical formation of vesical adenocarcinomas, including signet ring cell carcinomas. The expression of both pS2 and MUC5AC in variants of urothelial carcinomas with a glandular differentiation and an extracellular mucus production support the concept that adenocarcinomas may histogenetically develop from preexistent TCC. Adenocarcinomas which secrete the pS2 peptide and the MUC5AC glycoprotein are proposed to be subclassified as adenocarcinomas of the intestinal type, as distinguished from those of the common type lacking an expression. The tumor suppressor genes showed a loss of protein expression in adenocarcinomas, ranging from 54.5% (14-3-3 sigma), to 31.8 (PTEN), 27.3% (SYK) and 18.2% (maspin). Similar expression profiles in the coexistent urothelial carcinomas argue against a specific involvement of these genes during the morphogenesis of adenocarcinomas.

Key words: Urinary bladder, Adenocarcinomas, Signet ring cell carcinomas, Immunohistochemistry, pS2 peptide, MUC5AC mucin, Tumor suppressors

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Introduction

Approximately 93% of carcinomas of the urinary bladder represent urothelial carcinomas, the remaining 7% comprise mainly squamous cell carcinomas, undifferentiated small cell carcinomas, adenocarcinomas and rarely signet ring cell carcinomas. The most likely explanation for the occurrence of nonurothelial carcinomas can most likely be explained by the well-known inherent potential of urothelial cells to undergo several pathways of phenotypical differentiation, probably as a result of the embryological derivation of the bladder from the pluripotent cloacal endoderm and the Wolffian ducts. Our previous histomorphological and immunohistochemical studies indicated that adeno-carcinomas may develop either directly from the normal urothelium or, more frequently, secondarily from preexistent urothelial carcinomas by a metaplastic process (Kunze, 1998a,b; Kunze et al., 2001; Kunze and Francksen, 2002). A controversial debate as to whether a cystitis glandularis of the intestinal type, also referred to as intestinal metaplasia, represents a precursor lesion of adenocarcinomas or not is still being conducted (Corica et al., 1997; Sung et al., 2006). The signalling molecular mechanisms underlying the phenotypical transformation of normal and neoplastic urothelial cells into other cell types remain to be elucidated. Nonurothelial vesical cancers merit attention from a clinical point of view because of their high malignant potential and poor outcome.

To gain more insight into the processes resulting in

the formation of adenocarcinomas and signet ring cell carcinomas, we examined by immunohistochemistry a broad spectrum of proteins associated with cell differentiation, possibly involved in phenotypical conversion of urothelial into glandular epithelium. A further question we addressed in the present study was whether the activity of several novel known or candidate tumor suppressors plays a role in modulating the morphogenesis of bladder cancers. Recently, we were able to demonstrate that methylation of normally unmethylated cytosine in CpG dinucleotides at the 5' promoter region of the caveolin-1, 14-3-3 sigma, SYK and CAGE-1 genes differed between the various histopathological carcinoma subtypes, indicating that epigenetic events may be implicated in the formation of nonurothelial carcinomas of the urinary bladder (Kunze et al., 2006a,b; Kunze and Schlott, 2007). This is the first report documenting a tumor-associated de novo expression or reexpression, respectively, of the pS2 peptide and MUC5AC apomucin in adenocarcinomas and signet ring cell carcinomas.

Materials and methods

Specimens

We analyzed archival formalin-fixed and paraffin-embedded samples of primary urinary bladder cancers, consisting of 12 adenocarcinomas coexisting with papillary and nonpapillary (solid) urothelial carcinomas of all grades and stages (mixed urothelial carcinomas and adenocarcinomas), 10 pure adenocarcinomas, 5 primary signet ring cell carcinomas, 6 urothelial carcinomas with a focal glandular differentiation, and 4 urothelial carcinomas with an extracellular production of epithelial mucus. None of the adenocarcinomas originated from urachal remmants and, thus, represented nonurachal adenocarcinomas. The tumor specimens were obtained from patients who had undergone transurethral resection or – rarely – radical cystectomy. Sections of 3 µm thickness were prepared and routinely stained with haematoxylin and eosin (H & E), and Alcian blue. Six samples of normal nonneoplastic bladder mucosa from cystectomy specimens served as controls.

Immunohistochemistry

The antibodies used and the dilutions applied are listed in Table 1. Sections of 2 µm thickness were prepared from the archival formalin-fixed and paraffin-embedded samples, mounted on silane-coated slides, dewaxed in xylene, rehydrated in graded ethanol and washed with distilled water. With the exception of the reactions using the antibodies against the pS2 peptide and the cyclooxygenase-1, the sections were then incubated in citrate buffer (pH 6) and subjected in a chamber over hot water vapor (approximately 90°C) for 45 minutes. Thereafter, the sections were cooled down at room temperature for 20 minutes and rinsed in distilled water for 5 minutes. For examination of the expression of the pS2 protein, the sections were treated with bacterial protease (0.04%; Sigma-Aldrich Chemie, Taufkirchen, Germany) for 10 minutes at 40°C and, thereafter, rinsed in distilled water. For the immunostaining of the cyclooxygenase-1, the sections were neither subjected to hot water vapor nor treated with protease. All specimens were then treated with nonimmune bovine albumin serum (2%) for 10 minutes. The primary antibodies were applied for 30 minutes at room temperature. Following a wash with Tris-buffered saline (TBS; pH 7.4, 0.05 M), the sections were incubated with the biotinylated secondary antibody (ChemMateTM link detection kit alkaline phosphatase, code No. K 5005; DaKoCytomation, Hamburg, Germany) for 20 min. After being washed with TBS, the specimens were treated with streptavidine alkaline phosphatase (ChemMateTM detection kit; code-No. K 5005; DaKoCytomation) for 20 min. The sections were rinsed in TBS, and "Fast Red" was applied as

Table 1. Survey of antibodies used. All antibodies were mouse monoclonal, except against the pS2 peptide.

Antibody	Dilution	Source
pS2	1 : 100	Novocastra[1] Code No. NCL-pS2
MUC5AC	1 : 100	Novocastra Code No. NCL-MUC-5AC Clone CLH2
hSP	1 : 10	Novocastra Code No. NCL-HSP Clone GE 16 C
Sialosyl-Tn	Ready to use	DaKo Cytomation[2] Code No. N 1579 Clone HB-STn1
COX-1	1 : 20	Novocastra Code No. NCL-COX-1 Clone 12 E12
COX-2	1 : 50	Novocastra Code No. NCL-COX-2 Clone 4H12
CAV-1	1 : 250	BD Biosciences[3] Clone 2297
14-3-3 sigma	1 : 50	Abcam[4] Clone 1.N.6
SYK	1 : 100	Biomeda[5] Clone D410.1
PTEN	1 : 200	Novocastra Code No. NCL-PTEN Clone 28H6
Maspin	1 : 25	Novocastra Code No. NCL-Maspin Clone EAW24

[1]: Newcastle upon Tyne, United Kingdom; [2]: Hamburg, Germany; [3]: Heidelberg, Germany; [4]: Cambridge, United Kingdom; [5]: Foster City (CA), USA.

chromogen (ChemMate™ detection kit alkaline phosphatase, code No. K 5005, DaKoCytomation) to visualize the sites of immunoprecipitations. The samples were counterstained with Mayer's haemalaun. All samples were stained simultaneously in the same procedure. Negative control staining was obtained by omission of the primary antibody.

Cytoplasmic immunoreactivity was classified according to the following categories: negative, diffuse (homogeneous) and focal (heterogeneous) positivity. The immunoreactivity of the normal urothelium served as standard for evaluating the intensity of precipitations in the carcinomas. For the assessment of the expression of PTEN, the tumors were grouped into those with a moderate (20-50% positive cells) and a strong (> 50% - 100% positive cells) intranuclear immunostaining. Carcinomas containing less than 20% positive cells were considered to lack gene activity.

Results

Immunostaining of the normal urothelium

All six samples of normal, nonneoplastic bladder mucosa showed strong intracytoplasmic immunoprecipitations of the cyclooxygenases-1 and –2 (COX-1 and COX-2), maspin, 14-3-3 sigma and SYK including all cell layers, and all probes displayed an intranuclear expression of PTEN in 90% to 100% of the cells. Two specimens were focally immunoreactive for the spasmolytic polypeptide (hSP) and the sialosyl-Tn antigen. The pS2 peptide, the MUC5AC apomucin and the caveolin-1 protein were not expressed.

Immunohistochemistry of adenocarcinomas within urothelial carcinomas and of pure adenocarcinomas

Expression of proteins involved in cell differentiation

We analyzed 12 adenocarcinomas coexistent with urothelial carcinomas and 10 pure adenocarcinomas. The immunohistochemical findings are compiled in Tables 2 and 3. Taken together, 16 of the 22 adenocarcinomas (72.7%) revealed an expression of the pS2 peptide with strong intracytoplasmic immunoprecipitations. Pure adenocarcinomas were positive in 90% of the cases with an exclusively homogeneous expression pattern (Fig. 1A), compared to adenocarcinomas admixed to urothelial carcinomas which were immunopositive in

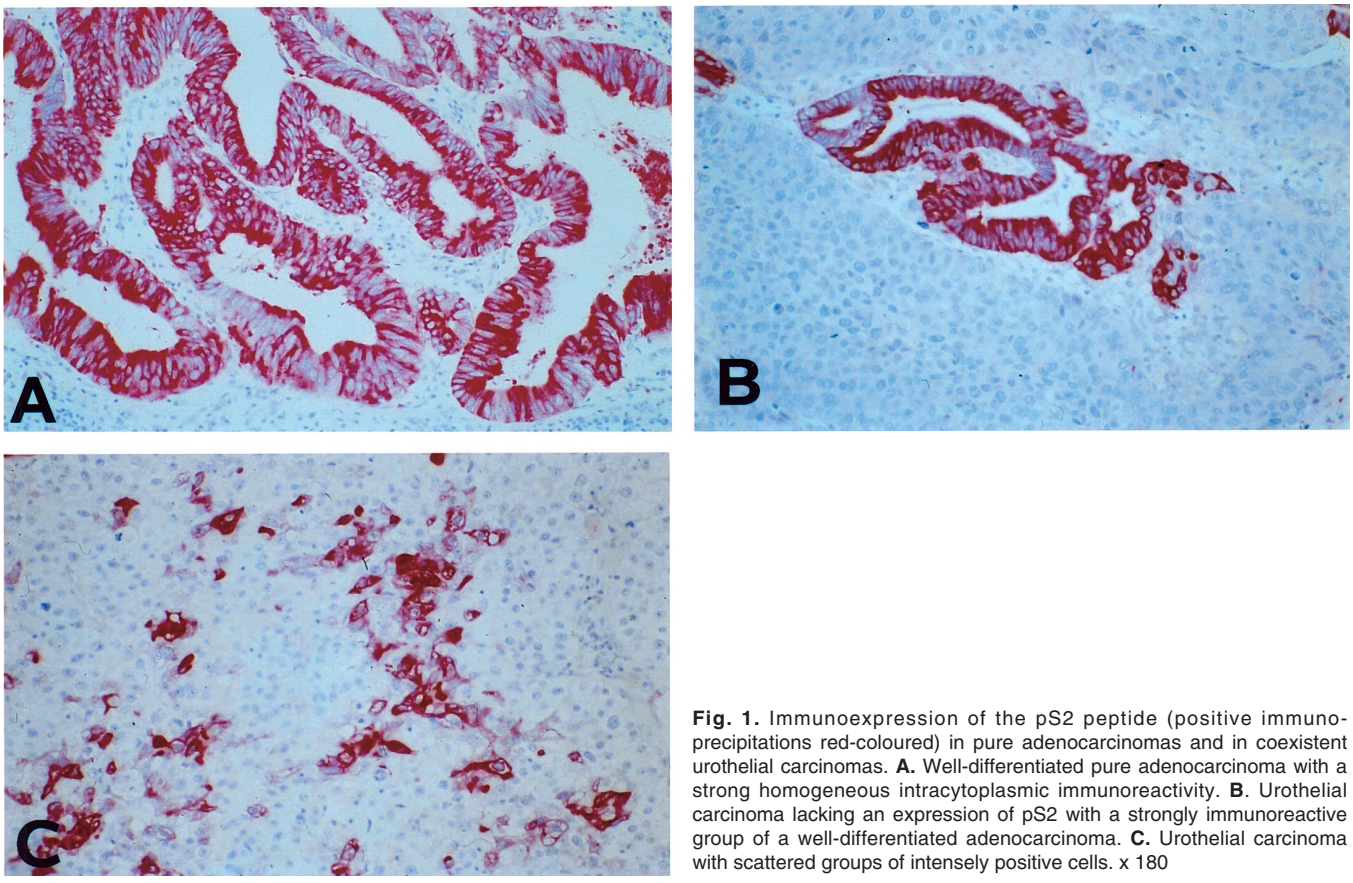

Fig. 1. Immunoexpression of the pS2 peptide (positive immunoprecipitations red-coloured) in pure adenocarcinomas and in coexistent urothelial carcinomas. **A.** Well-differentiated pure adenocarcinoma with a strong homogeneous intracytoplasmic immunoreactivity. **B**. Urothelial carcinoma lacking an expression of pS2 with a strongly immunoreactive group of a well-differentiated adenocarcinoma. **C.** Urothelial carcinoma with scattered groups of intensely positive cells. x 180

only 58.3% and more frequently characterized by a heterogeneous staining (Fig. 1B). The coexistent urothelial carcinomas expressed pS2 rarely (Fig. 1C). The MUC5AC apomucin was secreted predominantly heterogeneously in 45.5% of the 22 adenocarcinomas (Fig. 2A,B). Nine of the 10 MUC5AC positive tumors

Table 2. Immunohistochemical expression patterns of proteins associated with cell differentiation or known/putative tumor suppressor activity in adenocarcinomas coexistent with urothelial carcinomas (UC; 12 cases), pure adenocarcinomas (10 cases) and signet ring cell carcinomas (5 cases).

	Adenocarcinomas within UC			Pure adenocarcinomas			Signet ring cell carcinomas	
Type of protein	No staining	Focal	Homogeneous	No staining	Focal	Homogeneous	No staining	Positive
pS2	5	4	3	1	-	9	1	4
MUC5AC	6	5	1	6	2	2	2	3
hSP	12	-	-	6	4	-	5	-
Sialosyl-TN	2	3	7	4	1	5	1	4
COX-1	-	-	12	1	1	8	4	1
COX-2	4	2	6	4	-	6	4	1
CAV-1	12	-	-	10	-	-	5	-
14-3-3 sigma	6	4	2	6	3	1	5	-
SYK	4	1	7	2	1	7	5	-
PTEN	5	1	6	2	3	5	3	2
Maspin	1	3	8	3	2	5	5	-

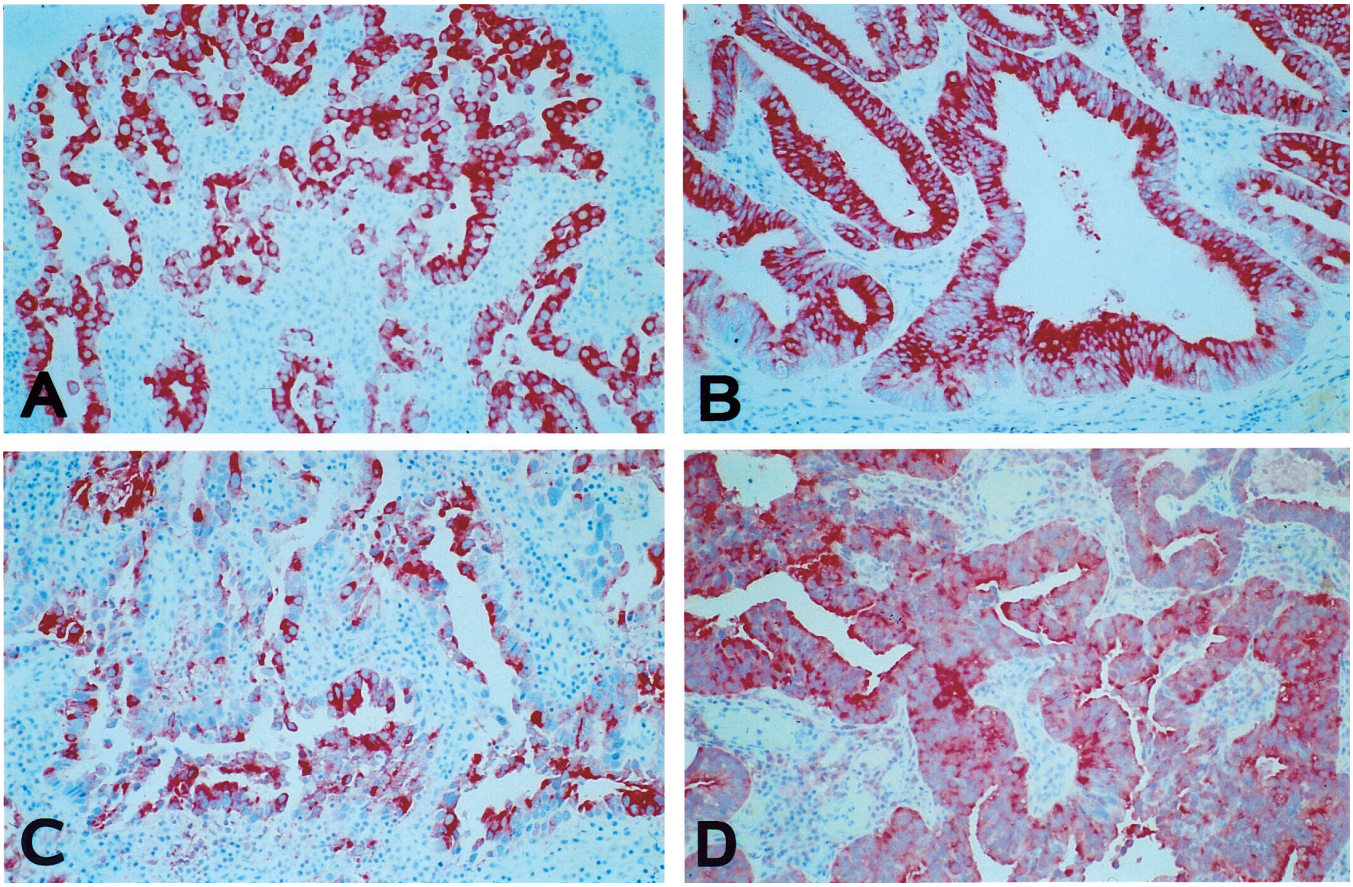

Fig. 2. Expression of the MUC5A glycoprotein, the sialosyl-Tn antigen and the cyclooxygenase-2 in adenocarcinomas. **A.** Moderately differentiated pure adenocarcinoma revealing a strong homogeneous secretion of the MUC5A apomucin. **B.** The same well-differentiated adenocarcinoma as demonstrated in figure 1A with a strong immunoreactivity for MUC5AC. **C.** Adenocarcinoma showing a homogeneous expression of the sialosyl-Tn antigen and **D** of the cyclooxygenase-2. x 180

coexpressed the pS2 protein. Two of the coexisting urothelial carcinomas expressed MUC5AC, coinciding with the production of pS2. The spasmolytic polypeptide was focally present in 18.2% of the adenocarcinomas which were all simultaneously immunoreactive for the pS2 protein and, with one exception, for MUC5AC. Of the 22 adenocarcinomas, 72.7% strongly expressed the sialosyl-Tn antigen, most of them revealing a homogeneous intracytoplasmic immunostaining (Fig. 2C). Twelve of the 16 tumors harbouring the sialosyl-Tn antigen coexpressed the pS2 polypeptide and nine the MUC5AC mucin. Only half of the adjacent urothelial carcinomas were immunopositive for the sialosyl-Tn antigen and these showed, almost exclusively, a patchy pattern of expression, as distinguished from the adenocarcinomas. All adenocarcinomas - except for a single case - and all coexistent urothelial carcinomas showed a homogeneous intracytoplasmic expression of the cyclooxygenase-1, but the intensity of the immunoprecipiations proved to be lower in most tumors compared to the normal urothelium. In constrast, one-third of the adenocarcinomas (36.4%) and 16.7% of the admixed urothelial carcinomas were characterized by a loss of cyclooxygenase-2 activity (Fig. 2D). The caveolin-1 gene product was absent in adenocarcinomas and expressed in only one TCC.

Expression of known or candidate tumor suppressors

The results are summarized in Tables 2 and 3. The 14-3-3 sigma gene protein product was absent in half of the 22 adenocarcinomas (54.5%) and in one-third (33.3%) of the urothelial carcinomas (Fig. 3A). The vast majority of positive carcinomas expressed the protein only focally. The SYK protein was homogeneously expressed in most adenocarcinomas, but absent in one-fourth (27.3%) of the cases. Maspin was not expressed in 18.2% of the adenocarcinomas and 22.7% exhibited only a heterogeneous immunoreactivity (Fig. 3B). A similar maspin expression profile was observed in the coexistent urothelial carcinomas. The PTEN protein was strongly expressed in the normal, nonneoplastic urothelium including all cell layers, while absent or underexpressed (less than 20% positive cells) in one-third (31.8%) of the adenocarcinomas (Fig. 3C) and in 41.7% of the coexisting urothelial carcinomas. Four of the 15 immunoreactive adenocarcinomas (more than 20% positive cells) disclosed in 20% to 50%, the others in >50% to 90% tumor cells with a positive immunostaining.

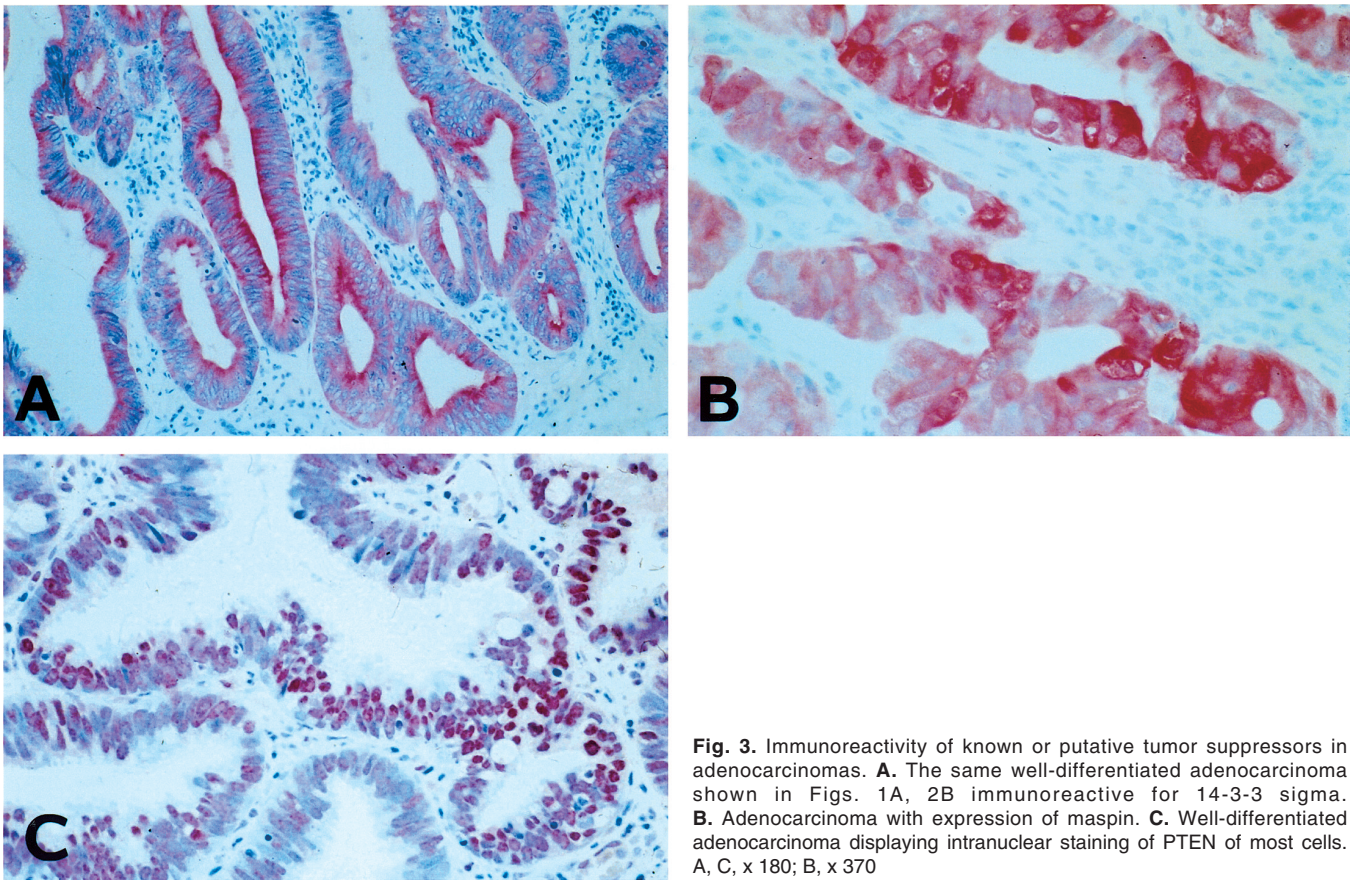

Fig. 3. Immunoreactivity of known or putative tumor suppressors in adenocarcinomas. **A.** The same well-differentiated adenocarcinoma shown in Figs. 1A, 2B immunoreactive for 14-3-3 sigma. **B.** Adenocarcinoma with expression of maspin. **C.** Well-differentiated adenocarcinoma displaying intranuclear staining of PTEN of most cells. A, C, x 180; B, x 370

Immunohistochemistry of signet ring cell carcinomas

Three of the five signet ring cell carcinomas represented pure forms with an abundant extracellular mucus production and two had developed within urothelial carcinomas (Table 2). Most of them expressed

Table 3. Immunohistochemical expression patterns of proteins associated with cell differentiation or known/putative tumor suppressor activity in coexistent urothelial carcinomas (UC; 12 cases), UC with an extracellular mucus production (4 cases) and UC with a glandular differentiation (6 cases).

	Coexistent UC			UC with mucus production			UC with glandular differentiation		
Type of protein	No staining	Focal	Homogeneous	No staining	Focal	Homogeneous	No staining	Focal	Homogeneous
pS2	10	1	1	1	2	1	3	2	1
MUC5AC	10	1	1	1	3	-	4	2	-
hSP	10	2	-	4	-	-	4	2	-
Sialosyl-TN	6	5	1	1	-	3	2	3	1
COX-1	-	-	12	-	2	2	1	-	5
COX-2	2	2	8	1	2	1	-	1	5
CAV-1	11	1	-	4	-	-	5	1	-
14-3-3 sigma	4	6	2	-	2	2	-	5	1
SYK	1	1	10	-	-	4	-	-	6
PTEN	5	1	6	3	-	1	3	1	2
Maspin	2	3	7	-	2	2	-	1	5

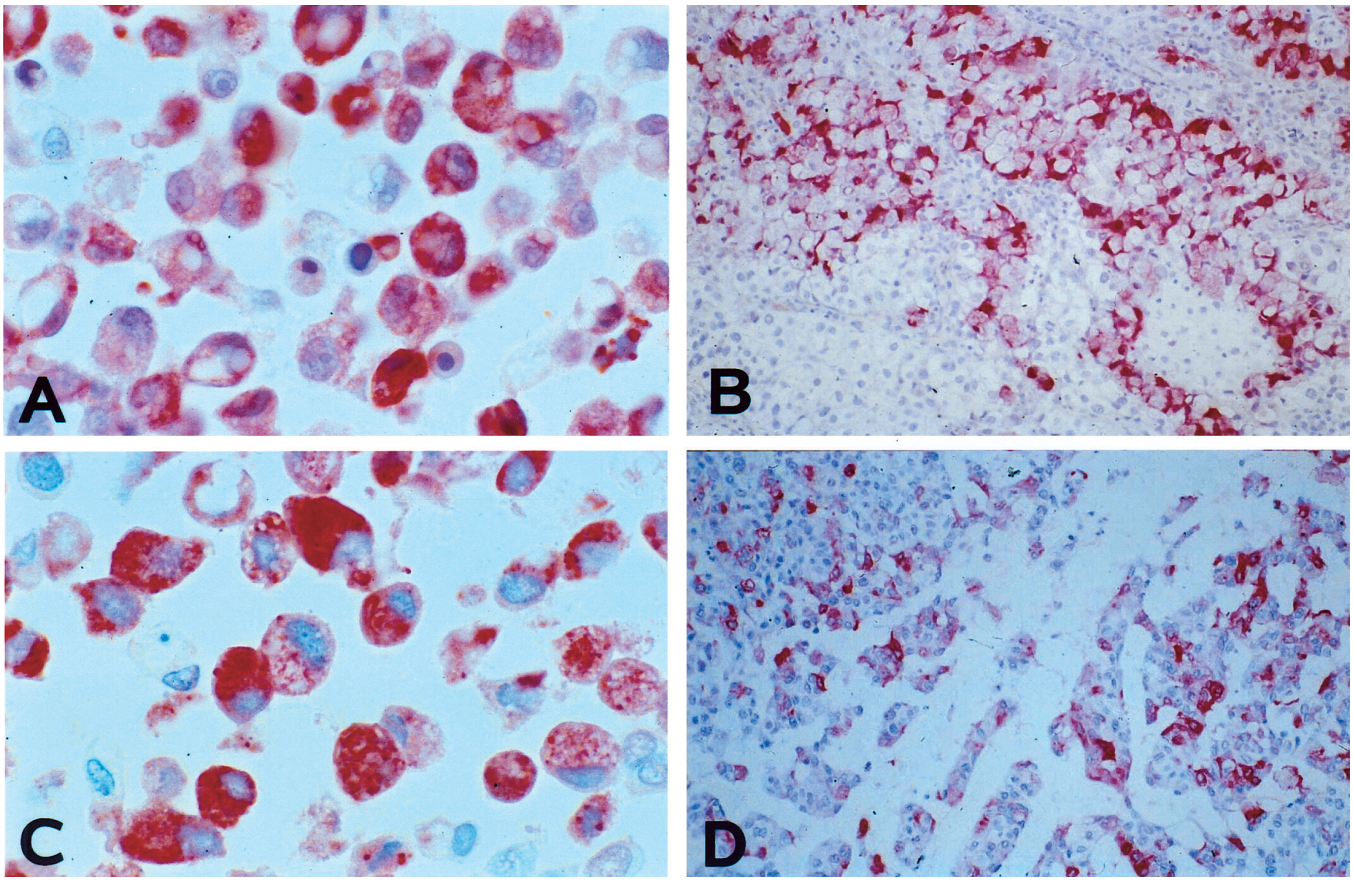

Fig. 4. Expression of the pS2 peptide and the sialosyl-Tn antigen in signet ring cell carcinomas and in mucus producing urothelial carcinomas. **A.** Pure signet ring cell carcinoma with a strong secretion of pS2. **B.** Large groups of signet ring cells immunoreactive for pS2 within a urothelial carcinoma lacking an expression. **C.** Pure signet ring cell carcinoma with expression of the sialosyl-Tn antigen. **D.** Extracellular mucus secreting (pale areas in the center) urothelial carcinoma containing numerous interspersed tumor cells positive for pS2. A, C, x 900; B, D, x 180

the pS2 protein (Fig. 4A,B), the MUC5AC mucin and the sialosyl-Tn antigen (Fig. 4C). In contrast to adenocarcinomas, signet ring cell carcinomas did not express the spasmolytic polypeptide and a single case was immunopositive for COX-1 and for COX-2. Among the tumor suppressor genes, the PTEN protein product was only expressed in two carcinomas.

Immunohistochemistry of urothelial carcinomas with a glandular differentiation and an extracellular mucus production

Included in the current study were six urothelial carcinomas with a focal glandular differentiation, characterized by tubular formations lined by cuboidal to slightly columnar cells, surrounded by urothelial carcinoma cells, as distinguished from true adenocarcinomas, the glands of which showed a back-to-back arrangement. Four solid urothelial carcinomas lacking glands and signet ring cells exhibited a focal production of extracellular epithelial mucus, strongly positive in the Alcian blue stain. Both variants expressed the pS2 peptide (Fig. 4D), the MUC5A mucin and the sialosyl-Tn antigen in a higher percentage than the not otherwise differentiated urothelial carcinomas coexisting with adenocarcinomas (Table 3), largely similar to the expression patterns of adenocarcinomas and signet ring cell carcinomas (Table 2). As distinguished from adenocarcinomas and signet ring cell carcinomas, they always showed an immunoreactivity for the 14-3-3 sigma and SYK proteins (Table 3).

Discussion

A large body of immunohistochemical studies exists addressing the activity of a wide variety particularly of tumor suppressor genes in urothelial carcinomas, but little is known about the protein expression profiles of adenocarcinomas and signet ring cell carcinomas of the urinary bladder. To gain insight into the phenomena underlying the formation of these nonurothelial cancer types, we explored a broad panel of proteins, associated with phenotypical cell differentiation and tumor suppressor functions.

Expression of proteins related to cell differentiation

As a main finding, we detected for the first time that the vast majority of adenocarcinomas and signet ring cell carcinomas expressed the pS2 peptide (TFF1) in contrast to the normal urothelium, which was immunonegative. The pS2 protein belongs to the trefoil factor family peptides and plays a role in terminal differentiation of mucus-producing cells (for review of the literature see Poulsom and Wright, 1993; Sands and Podolsky 1996; Tomasetto et al., 2000; Mathelin et al., 2005). The pS2 peptide mapped together with the spasmolytic polypeptide (TFF2) and the intestinal trefoil factor (ITF) on chromosome 21q 22.3. It is normally secreted by the epithelia of the gastrointestinal tract, particularly by the surface foveolar epithelium of the stomach, and coexpressed with the MUC2 and MUC5AC mucin glycoproteins (Tomasetto et al., 2000; van de Bovenkamp et al., 2005). The pS2 protein protects and maintains the integrity of the mucosal layer, stimulates mucosal regeneration following injury and promotes healing of ulcerative lesions of the small and large bowel and the stomach (for review of the literature see Poulsom and Wright, 1993; Taupin et al., 2001; Mathelin et al., 2005). The significance of pS2 in tumor cell differentiation, particularly in the formation of adenocarcinomas, is supported by its expression in gastric, colorectal, pancreatic, endometrial and ovarian adenocarcinomas (for review of the literature see Henry et al., 1991; Poulsom and Wright, 1993; Sands and Podolsky, 1996; Mathelin et al., 2005).

As a further new observation, we were able to document that nearly half of the adenocarcinomas and most signet ring cell carcinomas secreted the MUC5AC apomucin, the vast majority of the adenocarcinomas (9 of 10 MUC5AC positive tumors) and all positive signet ring cell carcinomas coexpressed the pS2 peptide, whereas the normal urothelium lacked this expression. However, pS2 more frequently showed a homogeneous expression profile than did MUC5AC. Among normal tissues, MUC5AC is exclusively expressed in the columnar surface (foveolar) epithelium of the stomach (for review of the literature see Pinto-de-Sousa et al. 2004). Regarding cancers, all ovarian mucinous cystadenocarcinomas (Albarracin et al., 2000) as well as a high percentage of gastric (Reis et al., 1997; Pinto-de-Sousa et al., 2004), proximal colon (Bara et al., 1984) and pancreatic carcinomas (Ho et al., 2003) were reported to secrete the MUC5AC mucin.

The absence of the pS2 peptide and of the MUC5AC glycoprotein in the normal urothelium, but their expression in adenocarcinomas and signet ring cell carcinomas point to a tumor-associated de novo expression or reexpression, respectively, of normally cryptic antigenic determinants. The appearance of both proteins obviously plays a crucial role in the phenotypical development of adenocarcinomas in the urinary bladder, as already proposed in our earlier reports (Kunze et al., 2001; Kunze and Francksen, 2002). The tumor-associated resurgence or neoexpression of pS2 and MUC5AC might be the result of the embryologic derivation of the urinary bladder from the multipotent tissues of the cloacal endoderm and the Wolffian ducts, capable of undergoing several pathways of phenotypical cellular and structural differentiations. The expression of the pS2 peptide and the MUC5AC glycoprotein in approximately half of urothelial carcinomas with a glandular differentiation and in most of those with a secretion of extracellular mucin argues in favor of the concept that adenocarcinomas may develop from preexistent urothelial carcinomas, mediated by the concurrent synthesis of both these proteins. This view is in line with

our previous results yielding a secretion of MUC5AC in 54% of TCC with a true and in 36.8% with a pseudoglandular differentiation (Kunze et al., 2001; Kunze and Francksen, 2002). However, since approximately only half of the admixed adenocarcinomas expressed pS2 and MUC5AC, the secretion of these proteins appears not to be a prerequisite for the morphogenesis of adenocarcinomas from preexistent urothelial carcinomas. Whether the secretion of the pS2 peptide and the MUC5AC apomucin represents merely a phenotypical epiphenomenon or reflects an underlying pathogenetic mechanism, remains to be elucidated by further, particularly moleculargenetic, studies. The differential expression profiles allow us to separate two subtypes of adenocarcinomas. Those with secretion of pS2 and MUC5AC share common expression characteristics with colorectal and gastric cancers and are, therefore, recommended to be referred to as adenocarcinomas of the intestinal type. The second type lacked an expression and largely resembled common adenocarcinomas arising in organs other than the intestine. A possible different prognostic significance of the two cancer types remains to be settled by clinico-pathological follow-up studies.

In contrast to pS2, the spasmolytic polypeptide, also a member of the trefoil family, appears not to play a role in the development of adenocarcinomas and signet ring cell carcinomas, because it was expressed only weakly and heterogeneously in a few pure adenocarcinomas. This suggests different signalling pathways for the two trefoil factors, although both genes are located on the same chromosome. The spasmolytic polypeptide is in normal tissues predominantly synthesized by the mucous neck cells of the gastric mucosa (Taupin et al., 2001; van de Bovenkamp et al., 2003). Among cancers, the polypeptide was expressed in advanced gastric cancers of the diffuse, but not intestinal type (Theisinger et al., 1991) and in a high incidence of mucin-producing adenocarcinomas of the pancreas (Ohshio et al., 2000).

Expression of the cyclooxygenase-2 was absent in one-third of the adenocarcinomas and in most signet ring cell carcinomas. The cyclooxygenase-1 was present in all adenocarcinomas - with one exception -, while the majority of signet ring cell carcinomas showed a loss of expression. The two isoforms of cyclooxygenases represent key rate-limiting enzymes, converting arachidonic acid to prostaglandin, which is metabolised to various biologically active prostanoids by prostaglandin E synthase (for review of the literature see Williams et al., 1999; Müller, 2004). COX-1, as a so-called housekeeping enzyme, is constitutively expressed in most normal tissues, whereas COX-2 is absent or only weakly expressed in normal samples and can rapidly be induced by a wide variety of stimuli (Williams et al., 1999). Our findings indicate that a down-regulation of COX-2 activity is related to the development of adenocarcinomas and loss of both COX-1 and COX-2 to the formation of signet ring cell carcinomas. A variety of other human cancers such as, for example, gastric (Ristimäki et al., 1997; Saukkonen et al., 2001) and colorectal (Sano et al., 1995; Soslow et al., 2000) carcinomas, were reported to overexpress the protein product and the mRNA of the COX-2 gene.

The protein of the caveolin-1 gene was neither expressed in the normal urothelium nor in adenocarcinomas and signet ring cell carcinomas. Caveolin-1 is a major integral and structural component of vesicular invaginations of the plasma membrane, called caveolae (Razani et al., 2002). The findings that the caveolin-1 protein was absent in adenocarcinomas as well as in undifferentiated small cell carcinomas, but was strongly expressed in squamous cell carcinomas and in approximately half of urothelial carcinomas as recently reported (Kunze et al., 2006a) point to a differential role of the gene in bladder carcinogenesis and during the histogenesis of the various phenotypical tumor subtypes. The significance of caveolin-1 in phenotypical cell differentiation is supported by results obtained in some other carcinomas. Thus, undifferentiated (anaplastic) carcinomas, follicular carcinomas and adenomas of the thyroid failed to express the caveolin-1 protein as opposed to papillary cancers which revealed a positive immunoreactivity (Ito et al., 2002). Ovarian serous adenomas showed a positive immunostaining, mucinous adenomas as well as mucinous cancers were negative, and serous and endometrioid cancers were characterized by reduced expression levels (Wiechen et al., 2001).

Expression of tumor suppressor genes

An exploration of the significance of some of the known or candidate tumor suppressors revealed that the 14-3-3 sigma protein product was absent in half of the adenocarcinomas and in all signet ring cell carcinomas. The 14-3-3 gene is activated by the tumor suppressor p53 in response to DNA damage, particularly following radiation, and has been demonstrated to be involved in cell cycle regulation by arresting proliferating cells at the G2 checkpoint, allowing repair of altered DNA (Hermeking et al., 1997). In a previous immuno-histochemical study, we found a total loss of expression in undifferentiated small cell carcinomas and a down-regulation in high-grade, high-stage urothelial carcinomas, whereas all squamous cell cancers proved to be intensely immunoreactive (Kunze et al., 2006b). These results are compatible with the view that the 14-3-3 sigma gene is implicated in the morphogenesis of nonurothelial bladder cancers, but mediated by differential signalling molecular pathways. This is supported by a different frequency of promoter methylation of the gene in the various phenotypical tumor subtypes (Kunze et al., 2006b), adenocarcinomas revealing the highest rate (Kunze and Schlott, 2007), indicating that epigenetic mechanisms are possibly responsible for transcriptional deregulation or even silencing.

Expression of the SYK protein was lacking in one-

fourth of the adenocarcinomas and in all signet ring cell carcinomas, in contrast to the normal urothelium which stained strongly immunopositive. The nonreceptor type of SYK protein-tyrosine kinase is involved in the development and activation of lymphocytes as well as in several signal transduction pathways of nonhematopoietic cells (for review of the literature see Sada et al., 2001). Its significance as a tumor suppressor in bladder carcinogenesis is supported by results recently obtained, yielding a loss of immunoreactivity in 40% of squamous cell carcinomas, in one-third of undifferentiated small cell carcinomas and in half of high-grade, high-stage urothelial carcinomas (Kunze et al., 2006b).

Maspin was either not expressed or was underexpressed, evidenced by only a focal immunoreactivity, in an overall incidence of 41% of the adenocarcinomas and was absent in all signet ring cell carcinomas. A similar underexpression in the coexistent urothelial carcinomas suggests a tumor suppressor effect of the protein in bladder carcinogenesis. Maspin is a member of the serpin superfamily of serine proteinase inhibitors (Potempa et al., 1994; Sager et al., 1996) and is synthesized by many normal tissues in association with secretory vesides and the cell surface (Pemberton et al., 1997). A tumor suppressor activity of maspin was also detected in human breast cancer with a stepwise down-regulation with progression from ductal carcinoma in situ to invasive carcinomas in conjunction with a shorter disease-free survival (Maass et al., 2001). In contrast, most pancreatic ductal adenocarcinomas (Lyun et al., 2002) and colorectal adenomas and adenocarcinomas overexpressed the maspin protein, but with a sequential decrease in the incidence from adenomas to metastatic carcinomas (Song et al., 2002). Loss of protein expression of the PTEN gene in one-third of the adenocarcinomas and in all signet ring cell carcinomas as well as in 41.7% of the admixed urothelial cancers in contrast to a strong immunostaining of the normal urothelium indicates a tumor suppressor activity during bladder cancer development as also reported for various other cancers (for review of the literature see Simpson and Parsons, 2001).

All together, underexpression of the known or putative tumor suppressor genes studied herein appears to be implicated in bladder carcinogenesis, in general, but not specifically associated with the morphogenesis of adenocarcinomas.

Conclusions

Exploring the significance of a broad spectrum of proteins involved in various signalling pathways of cell differentiation, we were able to detect the expression of the pS2 peptide and the MUC5AC apomucin in primary adenocarcinomas and signet ring cell carcinomas of the urinary bladder. The absence of both proteins in the normal urothelium points to a tumor-associated reexpression or de novo expression, probably as a result of the embryologic derivation of the bladder from the omnipotent cloacogenic tissues. The expression of pS2 and MUC5AC in urothelial carcinomas with a glandular differentiation support the view that adenocarcinomas may develop histogenetically from preexistent urothelial carcinomas. Those which secrete the pS2 peptide and the MUC5AC glycoprotein are proposed to be referred to as adenocarcinomas of the intestinal type, as distinguished from common adenocarcinomas without any expression.

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