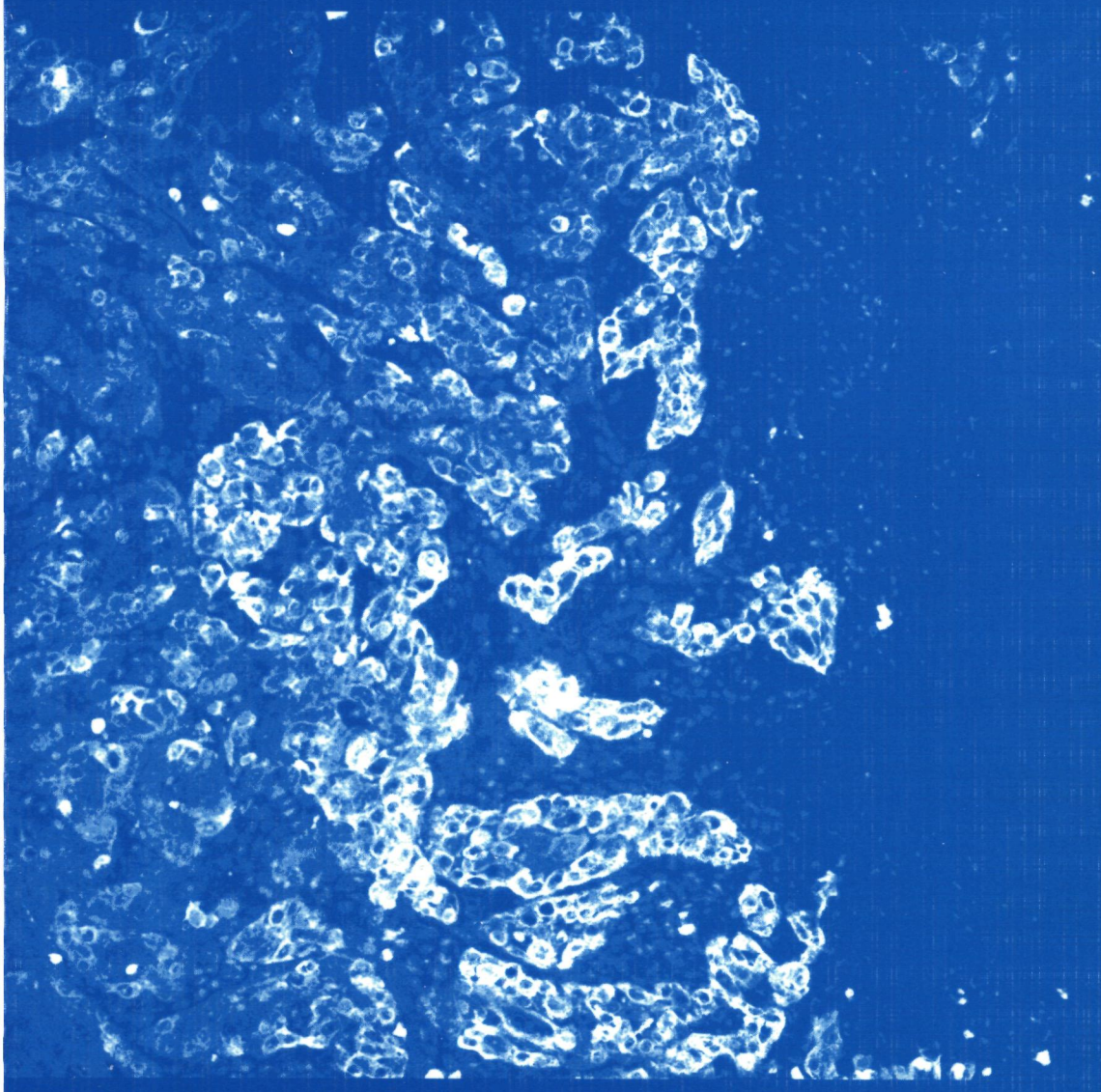
# CYTOKERATINS IN BLADDER CANCER

## AN IMMUNOHISTOCHEMICAL STUDY ON FEATURES OF TUMOR PROGRESSION



H.E.SCHAAFSMA

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## AN IMMUNOHISTOCHEMICAL STUDY ON FEATURES OF TUMOR PROGRESSION

Een wetenschappelijke proeve
op het gebied van de Medische Wetenschappen

**PROEFSCHRIFT**

ter verkrijging van de graad van doctor
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volgens besluit van het College van Decanen
in het openbaar te verdedigen
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door

**HENDRIK EWOUT SCHAAFSMA**

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Prof. Dr. D.J. Ruiter

Aan mijn ouders

Aan Eugénie, Bas en Bob

Cover: "interface phenomenon" in oral squamous cell carcinoma (immunostained with monoclonal antibody CK18-2 - anticytokeratin 18).

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# CONTENTS

# CHAPTER I

# General Introduction

The use of molecular markers in the study of biological processes, and in particular in the study of neoplasms, is increasing at a steady pace. This increase is stimulated by the insight that the results may be translated into clinical practice, including surgical pathology.

The markers are detectable by antibodies and nucleotide probes. Antibodies recognizing differentiation related molecules are a group of particular interest. Amongst the differentiation markers, intermediate filament proteins (IFPs) hold an important position, based on the fact that they were claimed to exhibit a cell lineage-specific expression pattern [1-4]. Although many reports by now have shown that the expression of certain IFPs may be lost during malignant transformation, or that aberrant or ectopic expression can occur, the use of IFPs as differentiation markers still deserves much attention in tumor biology and diagnostic pathology (see reviews [5-8]).

IFPs are major components of the mammalian cytoskeleton. They have an average diameter of approximately 10 nm, a size between that of microfilaments (6 nm) and microtubules (25 nm). They have been tentatively assigned a specific role in the maintenance of cell shape, in intracellular trafficking of cytoplasmic organelles, and in the constitution of certain intercellular junctional complexes [9-11].

Six types of IFPs (types I to VI, see Table I) are now distinguished, based on amino acid sequence homology and patterns of tissue distribution [9,12,13]. The largest group of IFPs is formed by the cytokeratin (CK) family, which is mainly found in epithelia. CKs consist of **type I** (small, acidic; including CK9-CK20) and **type II** (large, neutral to basic; including CK1-CK8) subtypes [14,15]. **Type III** IFPs comprise the mesenchymal protein vimentin, the protein desmin specific for muscle tissue, glial fibrillary acidic protein expressed in glial cells and astrocytes [16] and peripherin, specific for neuronal cells [17]. **Type IV** IFPs represent the neurofilament protein triplet expressed in nerve cells [18], **type V** the nuclear A and B-type lamin proteins which constitute the nuclear lamina [19-21] and the **type VI** IFP compises nestin, associated with CNS stem cells [22].

**Table I.** Types of intermediate filament proteins and their tissue type specificity

| TYPE of INTERMEDIATE FILAMENT PROTEINS | | TYPES of TISSUE or CELLS |
|---|---|---|
| I | CYTOKERATINS numbers 9-20 | EPITHELIUM |
| II | CYTOKERATINS numbers 1-8 | EPITHELIUM |
| III | VIMENTIN | MESENCHYMAL TISSUE |
| | DESMIN | MUSCLE TISSUE |
| | GLIAL FIBRILLARY ACIDIC PROTEIN | ASTROCYTES |
| | PERIPHERIN | NEURONAL CELLS |
| IV | NEUROFILAMENTS | NERVE CELLS |
| V | LAMINS | NUCLEAR LAMINA of ALL CELLS |
| VI | NESTIN | CNS* STEM CELLS |

* central nervous system

The initial studies in the IFP field were performed by biochemical analysis (i.e. gel electrophoresis) of cultured cells and tissues. However, this technique is quite laborous and does not allow direct morphological correlation to the object under study. Therefore, immunohistological procedures have been used increasingly after the development of monoclonal antibodies (mAbs), specifically reacting with individual CKs [13]. These techniques have improved the sensitivity of the studies, allowing more detailed information. At present, the applicability of most mAbs on frozen tissue sections only poses an important limitation for a wide use in surgical pathology. This is caused by the routine tissue processing techniques that change the antigenic sites of the proteins, resulting in loss of many antigenic epitopes [7,23]. Recently, however, epitope retrieval techniques seem to be very promising in this respect.

CKs form obligatory pairs, resulting in heteropolymers of type I and type II IFPs, and combinations of at least two CKs occur in a differentiation related pattern in the diverse epithelia according to certain rules [13,24]. Initially these CK combinations appeared to be fully retained in the corresponding carcinomas as a sort of "fingerprint" [14,25-27]. These diverse CK combination were therefore thought to be useful in tracing the site of origin of a carcinoma, in determining the exact cellular origin of carcinoma types, or more generally in detecting the direction of differentiation. After more CK-subtype antibodies have become available, rather

complicated patterns and exceptions to the fingerprint concept emerge, such as loss of the originally CKs. Also, re-expression of CKs normally only present during embryogenesis have been observed [28-30]. Although these aberrant expression patterns may complicate the identification of the precise type of cellular differentiation, they may shed new light on the abnormal organization of the cytoskeleton in tumors. We therefore wondered if the process of tumor progression would be associated with particular changes in CK expression.

The process of tumor progression in epithelial cancer can morphologically be followed by a spectrum of preneoplastic changes, ranging from hyperplasia, to dysplasia, and subsequently to invasive cancer with the potential for metastatic spread. Several cancers seem to follow this sequence of events, e.g. squamous cell carcinoma of the uterine cervix and colonic adenocarcinoma. Transitional cell carcinoma of the urinary tract is also representative in this respect. Transitional cell carcinoma forms a spectrum, from noninvasive tumors with low cellular anaplasia and favourable prognosis at one end, and highly anaplastic, deeply invasive carcinomas with a poor prognosis at the other end [31-38].

The underlying studies are an extension on the observation describing that the expression of CK18 increased during malignant progression of bladder carcinoma [39]. The study was limited to CK18, as it dealt with one of the first mAbs to recognize an individual CK. This observation was important because it might result in additional prognostic parameters applicable to patients with a transitional cell carcinoma of the urinary tract.

Subsequently, the number of monospecific anti-CK mAbs has increased and allowed a more extensive study of a possible role of other CKs in the progression of transitional cell carcinoma. Based on our interest for urological pathology in particular and tumor progression in general, and the availability of the specific reagents, we decided to embark on a CK immunophenotyping study of the normal and neoplastic transitional epithelium of the urinary tract.

In this thesis we have adressed the following *specific questions*:

1. Does the CK expression pattern of normal urothelium differ from that in transitional cell carcinoma?

2. Can heterogeneity of the CK expression patterns in the various grades and stages of transitional cell carcinoma be found? If so, what are possible explanations?
3. Can aberrant CK expression or loss of expression be detected during progression of transitional cell carcinoma. If so, are there any physiological or clinical implications?
4. Can the observations on CK expression in transitional cell carcinoma be adopted to other types of carcinoma?
5. What is the clinical relevance of CK subtyping in tumor lesions?

We approached these questions in a series of studies on human normal tissues and tumor lesions, mainly employing immunohistochemical techniques. Special attention was given to the correlation of the staining patterns with the pathomorphological findings, such as grade of differentiation and level of invasion of the tumors.

Normal urothelium was studied first *(Chapter 2)* to obtain a basis for the comparison with transitional cell carcinoma *(Chapter 3)*. As it appeared that certain CKs were aberrantly expressed in the invasive component of these neoplasms, we extended the study to autologous metastases in *Chapter 4*. The extent of the aberrant expression was further explored in another type of carcinoma, i.e. squamous cell carcinoma *(Chapter 5)*. Finally, we reviewed the literature on the clinical relevance of CK subtyping with emphasis on its use in surgical pathology *(Chapter 6)*.

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# CHAPTER II

# Distribution of cytokeratin polypeptides in epithelia of the adult human urinary tract

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## SUMMARY

Cytokeratin expression was studied in the epithelia lining the normal human urine conduction system using immunohistochemistry on frozen sections employing a panel of 14 monoclonal antibodies. Eleven of these anti-cytokeratin antibodies reacted specifically with one of the 19 human cytokeratin polypeptides. Profound differences were found in the cytokeratin expression patterns between the different types of epithelium in the male and female urinary tract. In the areas showing morphological transitions of transitional epithelium to columnar epithelium and of nonkeratinizing squamous epithelium to keratinizing squamous epithelium gradual shifts of cytokeratin expression patterns were observed, often anticipating the morphological changes. However, also within one type of epithelium, i.e. the transitional epithelium, two different patterns of cytokeratin expression were found. Expression of cytokeratin 7 was homogeneous in the transitional epithelium of renal pelvis and ureter but heterogeneous in the transitional epithelium of the bladder. Furthermore, intraepithelial differences in cytokeratin expression could be shown to be differentiation related. Using a panel of chain-specific monoclonal antibodies to cytokeratins 8 and 18 conformational and/or biochemical changes in the organization of these intermediate filaments were demonstrated upon differentiation in columnar and transitional epithelium.

## INTRODUCTION

Normal human epithelial cells and their tumors contain cytokeratins (CKs) as their intermediate filament constituents. It has been shown that CKs consist of a family of 19 diferent polypeptides (Moll et al. 1982) while subsets of 2 to 10 of these CK polypeptides are expressed in the different epithelia depending on their type of differentiation (Tseng et al. 1982; Quinlan et al. 1985). For many of these tissues data concerning CK distribution are available, as based on (two-dimensional) gel electrophoretic analyses of total epithelium or of isolated areas, as well as cell cultures (Moll et al. 1982; Moll et al. 1983a; Moll et al. 1983b;

Achtstätter et al. 1985; Rheinwald et al. 1985). Monoclonal anti-CK antibodies, and especially chain-specific antibodies recognizing only one CK polypeptide, enable the study of expression of this type of intermediate filament proteins in more detail, even at the single cell level, using immunohistochemical methods (Ramaekers et al. 1987a; Cooper et al. 1985).

Achtstätter et al. (1985) reported the presence of 11 different CK polypeptides in the different epithelia of the male urinary tract using two-dimensional gel electrophoresis. Differences in CK patterns were related to known morphological differences in the different areas studied.

In the current study normal adult human male and female epithelia, lining the urine conducting system were examined immunohistochemically using a large panel of monoclonal anti-intermediate filament antibodies. As a result, we were able to identify and localize separately 7 of 11 CKs reported by Achtstätter et al. (1985) in these tissues. The advantages of this approach over gel electrophoretic analysis of tissues became clear by our observation that certain cytokeratins may be distributed heterogeneously throughout morphologically homogeneous epithelial layers.

## MATERIALS AND METHODS

*Tissues.* The tissue specimens used in this study were obtained from the human urinary tract at autopsy, which was performed within 5.5 h after death. Tissues from six males and three females were snapfrozen and stored in liquid nitrogen. The age of the patients ranged from 61 to 85 years with an average of 74 years. In each case tissue samples were taken from at least 11 different sites, which included renal calyx, renal pelvis, ureteropelvic junction, ureter, bladder (dome, lateral wall, trigone), urethra (proximal, middle, distal) and external urethral orifice (see Fig. 1). Transitional epithelium of one male and urethral epithelium of two males appeared to be unsuitable for examination because of severe mechanical or autolytic damage. Of all nine cases at least six samples per site were examined. Examination of the tissues in H&E stained sections revealed no significant abnormalities.

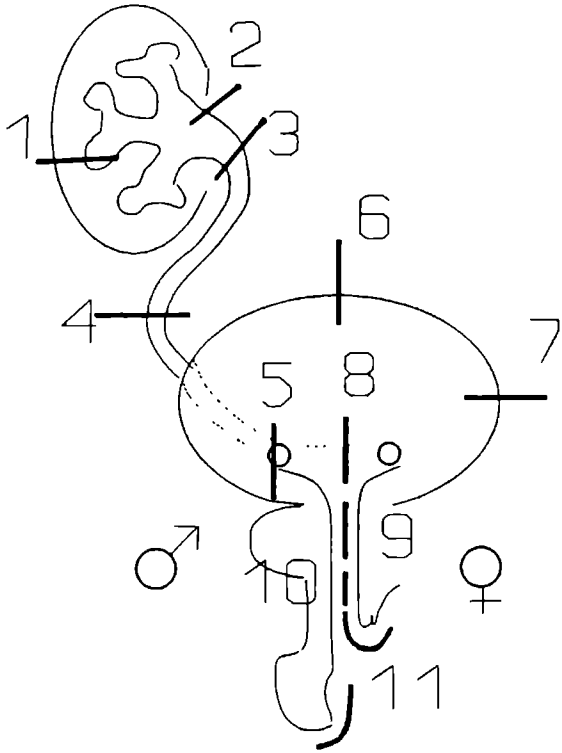



**Figure 1.** Schematic representation of the urinary tract showing the sites from where the tissue samples were obtained. **1** renal calyx (in one sample also containing a renal papilla); **2** renal pelvis; **3** ureteropelvic junction; **4** ureter; **5** near ureterovesical junction; **6** bladder dome; **7** lateral bladder wall; **8** trigone; **9** proximal urethra; **10** mid urethra; **11** distal urethra and urethral orifice, in male containing distal urethra, fossa navicularis and glans penis.
The main type of epithelium in **1-9** is transitional epithelium, while the main type of epithelium in **10-11** in female is nonkeratinizing squamous epithelium. In male these are (pseudo)stratified columnar epithelium in the urethra, nonkeratinizing squamous epithelium in the fossa navicularis and keratinizing squamous epithelium in the glans penis

*Antibodies.* Fourteen monoclonal anti-CK antibodies, two monoclonal anti-vimentin antibodies and one anti-desmin antibody were used in this study.

The specificity of these different antibodies has been summarized in Table 1,

and has been described previously (for references see Table 1). Recent investigations have indicated that M20 antibody does not recognize CK18 but reacts exclusively with CK8, which may show breakdown products in the CK18 region (van Muijen et al. 1987a). Figure 2 shows the immunoblotting results of the M20 antibody on some carcinoma cell lines (T24, RT4 and A431), expressing amongst others CK8. The results in lanes 1, 3 and 5 demonstrate that this antibody reacts with a protein band at 52kDa, corresponding to CK8. However, in the RT4 cell line preparation also some faint protein bands, representing CK8 breakdown products are seen (lane 3). The position of the 52kDa protein band is further confirmed by subsequent incubations with the other monoclonal CK antibodies recognizing CK7 and CK18 (lanes 2, 4 and 6). Immunoblotting was performed as described before (Broers et al. 1986).

**Table 1.** Specificity of monoclonal intermediate antibodies used in this study

| Antibody | Protein(s) recognized | Reference |
|---|---|---|
| clone 80 | most CKs | van Muijen et al. 1984 |
| RCK102 | CKs 5+8 | Broers et al. 1986 |
| RCK103 | * | Ramaekers et al. 1987a |
| 6B10 | CK 4 | van Muijen et al. 1986 |
| RCK105 | CK 7 | Ramaekers et al. 1987a |
| LE41 | CK 8 | Lane, 1982 |
| M20 | CK 8 | van Muijen et al. 1987a |
| RKSE60 | CK 10 | Ramaekers et al. 1983; 1987b |
| 1C7 | CK 13 | van Muijen et al. 1986 |
| RGE53 | CK 18 | Ramaekers et al. 1983 |
| 2C8 | CK 18 | unpublished |
| RCK106 | CK 18 | Ramaekers et al. 1987a |
| CK18-2 | CK 18 | Broers et al. 1986 |
| LP2K | CK 19 | Lane et al. 1985; Broers et al. 1986 |
| V9 | vimentin | van Muijen et al. 1987a |
| RV202 | vimentin | Ramaekers et al. 1987a |
| D33 | desmin | van Muijen et al. 1987a |

* Not yet fully characterized
Abbreviation: CK = cytokeratin

*Immunohistochemistry.* The indirect immunoperoxidase staining procedure was performed on frozen sections as described previously (van Muijen et al. 1986).

Staining patterns were designated as homogeneously positive when all cells in a particular cell layer were positive, or heterogeneously positive when both negative and positive cells were observed in the same cell layer(s).

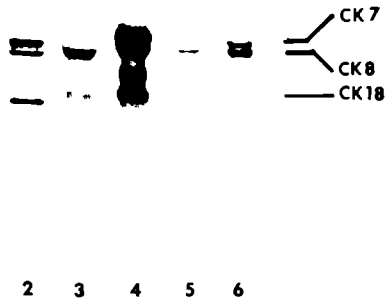



**Figure 2.** Immunoblotting study on cytoskeletal preparations from the two bladder carcinoma cell lines T24 (lanes **1** and **2**) and RT24 (lanes **3** and **4**), and the cell line A431, derived from an epidermoid carcinoma of the vulva (lanes **5** and **6**). The nitrocellulose strips were incubated with the antibody M20 (lanes **1**, **3** and **5**) and subsequently with the antibodies RCK105, staining the CK7 protein band, and RCK106, staining the CK18 band (lanes **2**, **4** and **6**)

## RESULTS

In Fig. 3 the CK phenotype of the epithelial lining of the urinary tract at different sites, as recognized by the monoclonal anti-cytokeratin antibodies, is summarized per type of epithelium. All epithelial cells were stained by the antibodies clone 80 and RCK103 (not included in Fig. 3). RCK102 stained all epithelial cells with the exception of certain cell layers in squamous epithelium (see below).
The staining patterns of the other anti-CK antibodies varied and will be described in detail. Vimentin positive epithelial cells were not observed. Throughout the different epithelia scattered vimentin positive cells were seen, most probably

representing Langerhans or inflammatory cells. Desmin positivity was only observed in muscle cells (results not shown).

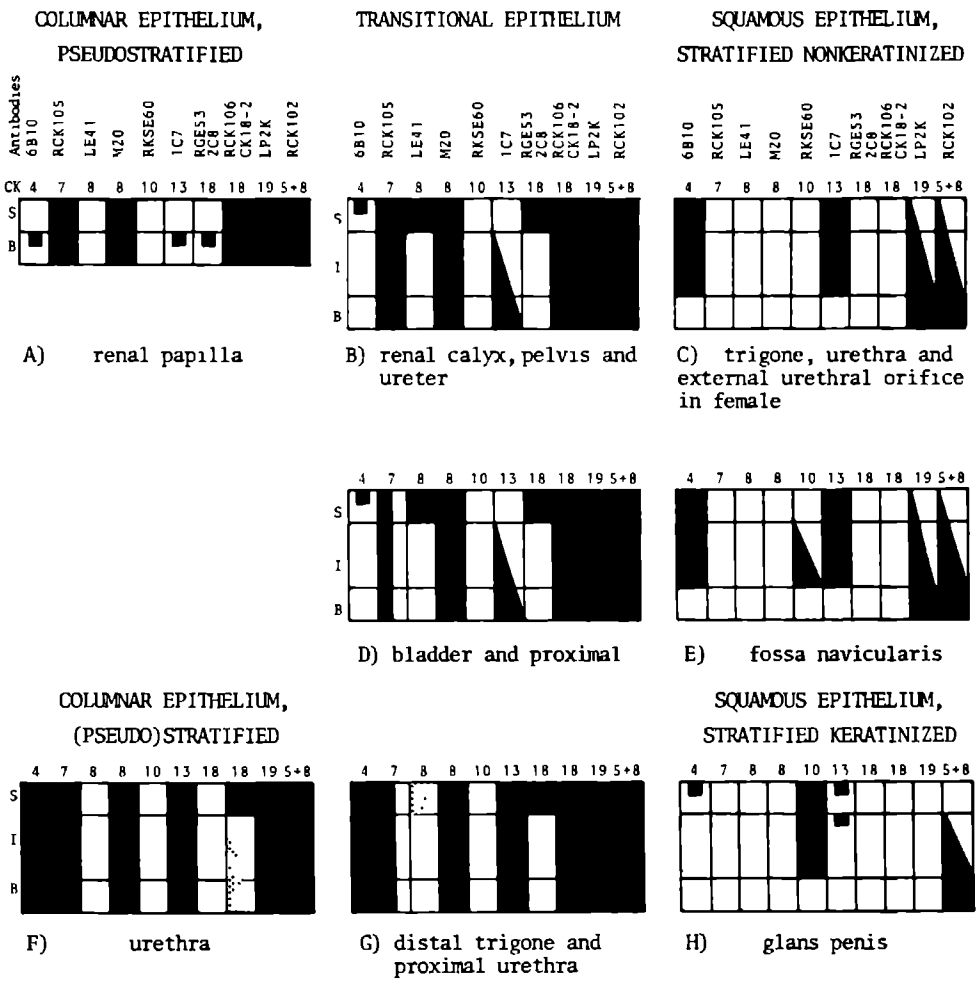



**Figure 3A-H.** Schematic representation of the cytokeratin distribution patterns as detected by the different monoclonal antibodies. **Filled bars** indicate a positive reaction, **open bars** indicate a negative reaction, while a **little square** indicates that only a few cells are positive. A **partly filled bar** means that only part of the cells in the respective layers is positive: a **vertically divided bar** means that both positive and negative areas were seen, an **obliquely divided bar** means that the expression pattern differs in the the individual layers and a **stippled bar means that a weakly positive reaction was observed.**
**Abbreviations:** CK = cytokeratin(s); S = superficial (cell) layer; I = intermediate cell layers; B = basal cell layer

*Renal calyx up to trigone*

In one of the specimens of the renal calyx we observed the presence of pseudostratified columnar epithelium lining a renal papilla (Fig. 3A), adjacent to the transitional epithelium of the calyx. All cell layers of this columnar epithelium were positive for RCK105 (CK7), M20 (CK8), LP2K (CK19) and only two of the CK18 antibodies, i.e. RCK106 and CK18-2. A few basal cells were positive for 6B10 (CK4; Fig. 4A, upper part) and to an even lesser degree with 1C7 (CK13; Fig. 4B, upper part) and 2C8 and RGE53 (CK18; Fig. 4C, upper part). No reactivity was observed for LE41 (CK8).

In transitional epithelium (Fig. 3B and D) all cell layers were positive for M20 (CK8, Fig. 4D), RCK106 and CK18-2 (CK18) and LP2K (CK19). The other two CK18 monoclonal antibodies (RGE53 and 2C8) stained only umbrella cells (Fig. 4E) with cell extensions reaching down between intermediate cell layers or staining sporadically intermediate cells lying directly beneath the umbrella cells. All umbrella cells were also positive for LE41 (CK8; Fig. 4F), although cell extensions were not found to be stained. Only sporadically umbrella cells were positive for 6B10 (CK4; Fig.4G), while almost all baal and intermediate cells were heterogeneously positive for 1C7 (CK13; Fig. 4H), especially the lowerintermediate cells. Only sporadically all layers except the umbrella cells were stained with this antibody (Fig. 4I). Up to the bladder all epithelial layers of the transitional epithelium were positive for RCK105 (CK7), but in the bladder areas of varying size were negative and alternated with CK7 positive areas (Fig. 4J).

*Trigone*

In the trigone the major part of the transitional epithelium reacted similar to bladder transitional epithelium. In the distal part of the trigone (Fig. 3G), i.e. the bladder neck, transitions were observed in the staining patterns of antibodies 6B10 (CK4), 1C7 (CK13) and LE41 (CK8). CK4, which detected only sporadically in umbrella cells of the tissues described above, was now found in an increasing number of cells in this umbrella cell layer (Fig. 5A). More distally in the trigone also the intermediate and basal cell layers became increasingly

positive, although they were stained weaker than the umbrella cell layer (Fig. 5B). In the area were the umbrella cells became positive for 6B10 (CK4), most cells, in all layers of the transitional epithelium, became positive for 1C7 (CK13; Figs. 5C and D). On the contrary, the expression of CK8 in the umbrella cells, as monitored by LE41, diminished.

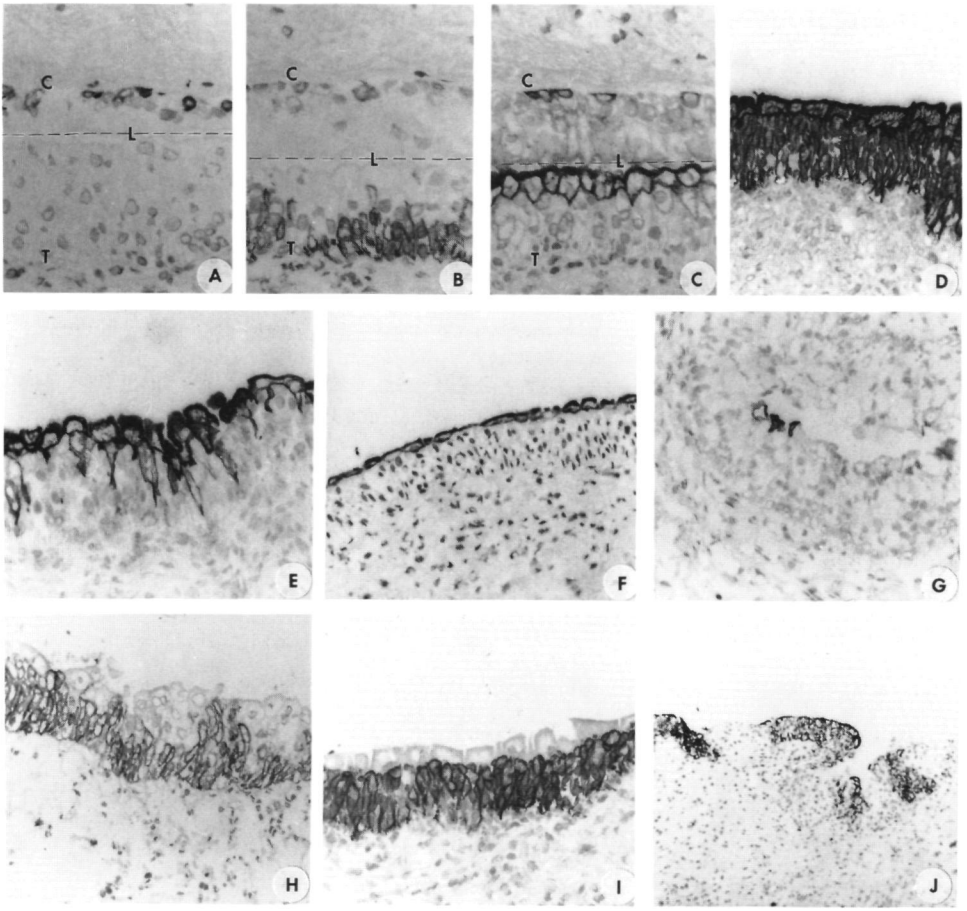
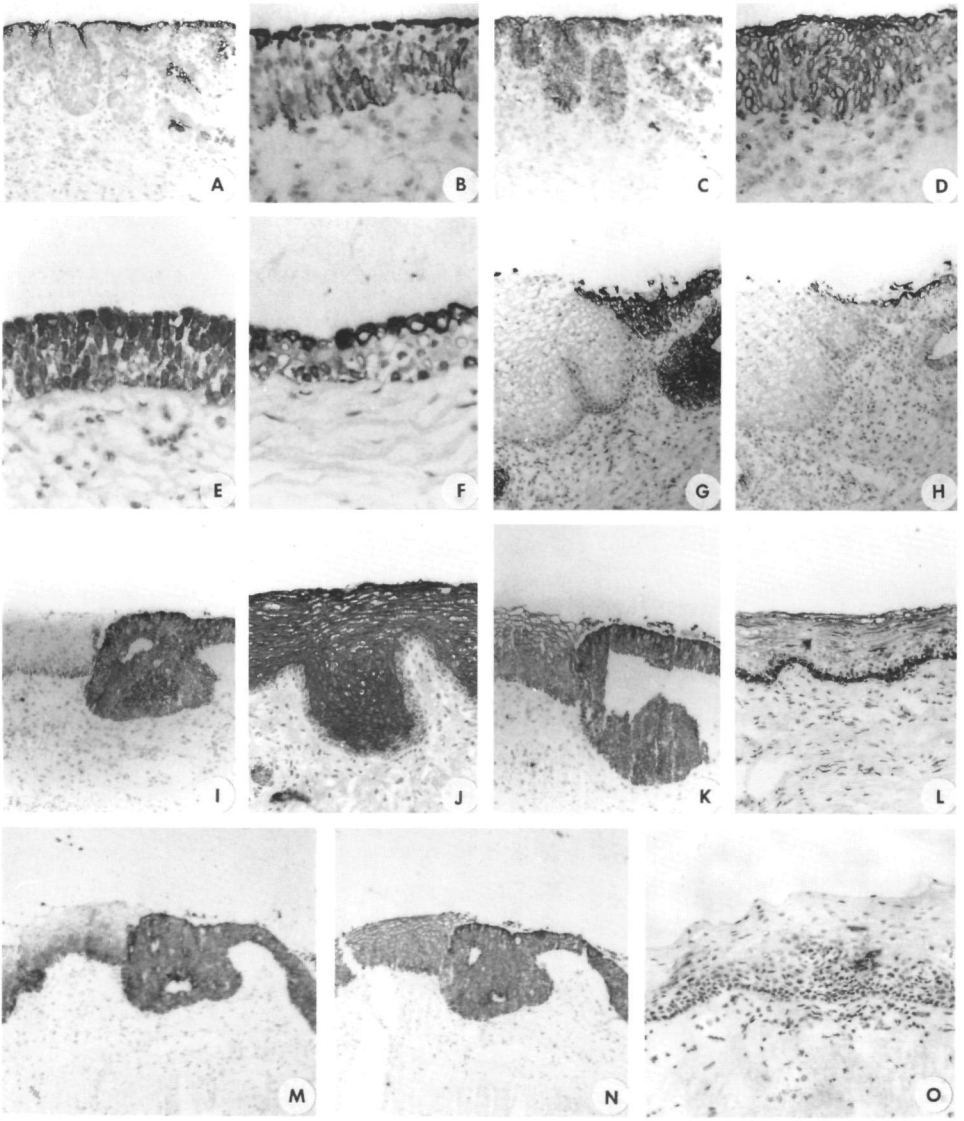



**Figure 4A-J.** Immunoperoxidase staining patterns of frozen sections from: renal papilla and renal calyx for 6B10 (**A**), 1C7 (**B**) and RGE53 (**C**), showing pseudostratified columnar epithelium (C - basal cell layer) lining the renal papilla in the **upper part** and the transitional epithelium (T - basal layer) of the renal calyx in the **lower part** of **A-C** (L marks the collapsed lumen); transitional epithelium of the bladder for M20 (**D**), RGE53 (**E**), LE41 (**F**), 1C7 (**H and I**) and RCK105 (**J**); transitional epithelium of the ureter for 6B10 (**G**). A-D x280, E-I x200, J x80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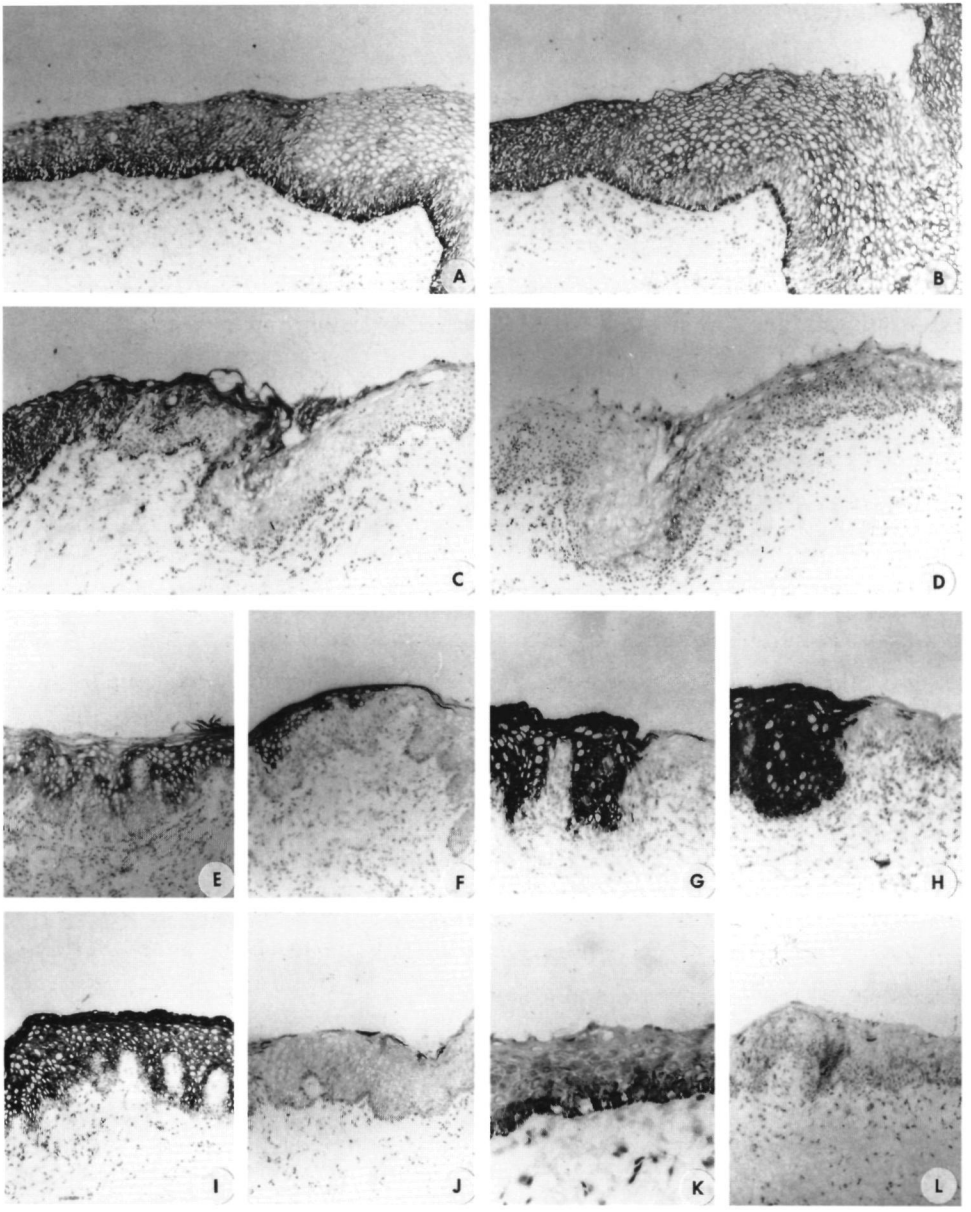
(Pseudo)stratified columnar urethral epithelium (Fig. 3F) was homogeneously positive for CK4 (Fig. 5E), CK7, CK8 (M20) and CK13. Positive staining with anti-CK18 antibodies was only observed with CK18-2 and RCK106, with a strong signal in the superficial cells and a variable to negative staining reaction in the other epithelial cell layers. Cells in the basal layers were found to be more intensely stained by RCK106 (Fig. 5F), than for CK18-2.

The female external urethral orifice was lined by nonkeratinizing squamous epithelium with CK distribution similar to those observed in an area of nonkeratinizing squamous epithelium in one sample of a female trigone (Fig. 3C). Such squamous epithelium showed an abrupt change in the CK expression pattern as compared to the columnar or transitional epithelium. CKs7, 8 (Fig. 5G), 10 and 18 (Figs. 5H and I) could not be detected in this type of squamous epithelium, while CK4 (Fig. 5J) and CK13 (not shown) were present in all except the basal cell layer. In a minority of these squamous areas LP2K (CK19) reacted homogeneously positive (Fig. 5K). In most squamous areas this antibody showed a homogeneously positive reaction only in basal cell layers, while its reactivity was heterogeneous in the higher cell layers (Fig. 5L). RCK 102 showed a distinct reaction in basal and lower suprabasal cell layers, while the other layers were predominantly negative (Fig. 5M). RCK103 showed a strong staining reaction in the basal cells, with a slightly less extense staining reaction in the other cell layers (Fig. 5N). Reactivity for (CK10) was found only once in a few suprabasal cells (Fig. 5O).

**Figure 5A-O.** Immunoperoxidase staining patterns of frozen sections from: transitional epithelium in distal trigone for 6B10 (**A and B**); **B** is further distally than **A** and for 1C7 (**C and D**); urethral columnar epithelium for 6B10 (**E**) and RCK106 (**F**); adjacent areas of nonkeratinizing squamous epithelium (at the left side of the figures) for M20 (**G**), RGE53 (**H**), RCK106 (**I**), LP2K (**K**), RCK102 (**M**) and RCK103 (**N**); nonkeratinizing squamous urethral epithelium for 6B10 (**J**), LP2K (**L**) and RKSE60 (**O**). Note both homogeneous (**K**) and heterogenous (**L**) reactions for LP2K in this latter type of epithelium. A, C, G, H, I, K, M, N x80; J and L x100; O x150; B and D x200; E and F x280

Between urethral columnar epithelium and the nonkeratinizing squamous epithelium lining the fossa navicularis (Fig. 3E) an abrupt change in CK expression pattern was observed. Proximally in the fossa CK19 was extensively but heterogeneously expressed (Fig. 6B). Up to the external urethral orifice reactivity for CK19 decreased considerably (Fig. 6C). Cells in the intermediate layers were heterogeneously positive for RKSE60 (CK10) with an increasing number of RKSE60 posotive cells in the distal portion of the fossa (Figs. 6D-E). The positive reaction for RCK102 seen in the fossa decreased rather abruptly in the upper cell layers when going from the proximal to the distal part (Fig. 6A). The expression pattern of the other CKs was similar to that seen in nonkeratinizing epithelia in the urethra as described above. However, with respect to the CK4 expression pattern it should be noted that in most cases 6B10 staining deminished before the transition into keratinizing squamous glanular epithelium became morphologically evident. Initially only basal cells were unstained, while subsequently also the intermediate cell layers and finally the superficial layer became negative (Figs. 6F and G). CK13 expression showed in most cases an abrupt change (Fig. 6H). The keratinizing glanular epithelium (Fig. 3H) expressed CK10 in all excepted the basal cell layer (Fig. 6I). CK4 was found to be focally distributed in the stratum corneum (Fig. 6J). RCK102 showed a positive reaction in the basal and lower suprabasal cells (Fig. 6K), while CK13 was found occasionally in only a few cells (Fig. 6L).

**Figure 6A-L.** Immunoperoxidase staining patterns of frozen sections from: nonkeratininzing squamous epithelium at proximal fossa navicularis (**left** a part of the urethra) for RCK102 (**A**) and LP2K (**B**); distal fossa navicularis for LP2K (**C**) and RKSE60 (**D**), the transition between fossa navicularis and glans penis lined by keratinizing squamous epithelium (**right** side) for RKSE60 (**E**), 6B10 (**F and G**) and 1C7 (**H**); the glans penis for RKSE60 (**I**), 6B10 (**J**), RCK102 (**K**) and 1C7 (**L**). A-L x80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## DISCUSSION

The distribution of cytokeratin (CK) polypeptides was studied immunohistochemically in the epithelial lining of the normal adult human urinary tract, using a panel of monoclonal antibodies. This technique allows light microscopic interpretation at the single cell level.

The panel of monoclonal antibodies recognized separately seven of the 11 CKs reported by Achtstätter et al. (1985) to occur in the male urinary tract epithelia.

A limitation of the immunohistochemical approach is that the antigenic epitope recognized by a certain monoclonal antibody can vary in protein structure as aresult of of biological activity and malignant transformation. This may result in an alteration of the detectability or accessibility of the comopnent under investigation, i.e. the antigenic epitope is masked or unmasked. This phenomenon is well illustrated by the fact that four different CK18 antibodies showed two completely different expression patterns in the urinary tract epithelia. RGE53 and 2C8 mainly stained the superficial umbrella cells, while all cell layers were positive with RCK106 and CK18-2 (compare Ramaekers et al. 1985; Nadakavukaren et al. 1984; Achtstätter et al. 1985; Feitz et al. 1986). Also in the (pseudo)stratified columnar epithelium of the urethra and the renal papilla these two subgroups of CK18 antibodies could be distinguished on basis of their reaction patterns. A similar observation was made for the anti-CK8 antibodies LE41 and M20. These results may possibly be explained by a phenomenon recently described by Franke and coworkers (1987), who showed that a CK18 dependent antibody Ks18.18 interacted only with its antigen when this was present in heterotopic coiled-coil complexes, notably with CK8. It can also not be excluded that (de)phosphorylation may play a role in the phenomenon (Sternberger et al. 1983).

Apparently the epitopes recognized in CK18 by RGE53 and 2C8, but also by the monoclonal antibody CK1, described by Achtstätter et al. (1985), and in CK8 by LE41, are structuraly or biochemically different in the basal and intermediate cell layers as compared to the umbrella cell layer.

In transitional epithelium other CKs studied showed differences in expression depending on the site. This indicates that differences in CK distribution between

morphologically identical transitional epithelium. It was striking to note that CK7 was found heterogeneously distributed in the epithelium of the bladder, including the trigone, whereas it was homogeneously expressed in the renal pelvis and ureter. This observation may be the results of epitope masking sa described above for CKs8 and 18. It remains to be examined wether or not these differences in CK7 structure or content are related to functional differences between the bladder on one hand and the higher urinary tract on the other hand. Epithelia of other hollow organs with a reservoir function, such as the digestive tract, seem to lack CK7 completely, while ductal structures (for example bile ducts) frequently show the presence of CK7 (Osborn et al. 1986; Ramaekers et al. 1987a).

CK4 expression in transitional epithelium was most pronounced in the distal trigone and was sporadically found in the more proximal transitional epithelium. Gel-electrophoretic data published by Moll et al. (1982) and Achtstätter et al. (1985) showed that CK4 was found occasionally only at low levels in transitional epithelium. In the same distal trigonal area all cell layers including umbrella cells were CK13 positive, while more proximally only basal and intermediate cells were stained heterogenously. Also in this area CK8 expression in umbrella cells, as detected by LE41, diminished. We considered that here the CK expression in transitional epithelium partially anticipates the morphological transition into the columnar urethral epithelium. Our observations for CK4 and CK13 differed from those reported previously (van Muijen et al. 1986) in that these authers found CK4 and CK13 in suprabasal cells. Most probably squamous metaplasia has been studied for this study. Our observations of CK13 expression are partly in accordance with those of Huszar et al. (1986), who reported that their antibody Ks8.12, recognizing both CK13 and CK16, in some sections did not react well with certain luminal cells, most probably due to the fact that the epitope recognized by Ks8.12 was less accessible in these cells. We prefer to consider the umbrella cells as a separate cell type with a CK expression pattern only partly related to that of the underlying cells.

A second possible transition zone is observed in the epithelium of the renal papilla, consisting of two or three layers of columnar cells without umbrella cells. This epithelium lies between the epithelium of the renal collecting tubules

(negative for CK4 and CK13, unpublished data) and the transitional epithelium of the renal calices (heterogeneously positive for CK4 and CK13). The CK distribution in the epithelium of the renal papilla is different from both adjacent types of epithelium in the expression of CK4 and CK18 (as recognized by RGE53 and 2C8) in several basal cells, contrasting with the expression of these two CKs only in umbrella cells for regular transitional epithelium.

In the columnar epithelium of the urethra, which is histologically classified as a pseudostratified or stratified type of epithelium (Ham, 1965; Bloom et al. 1975), we observed staining of CK18 only with RCK106 and CK18-2 and of CK8 only with M20. These results are in contrast to the gel electrophoretic data of Achtstätter et al. (1985), who could not find CKs8 and 18 in the urethra, and show the additional value of cytokeratin immunohistochemistry. The presence of CK4 and CK13 in this type of epithelium supports its stratified rather than pseudostratified nature (see Sun et al. 1985). The female urethra is mainly lined by nonkeratinizing squamous epithelium, while in male only foci of squamous epithelium can be found (Bloom et al. 1975). Neither could we demonstrate CKs7, 8 or 18 in this type of epithelium, nor significant levels of CK10 expression in urethral squamous epithelium, with the exception of one sample in which a few RKSE60 positive cells were detected. CK4 and CK13 were expressed in all suprabasal cells as reported previously (van Muijen et al. 1986). The heterogenous CK19 pattern seen in the former nonkeratinizing squamous epithelium was also found in the proximal fossa naviculari. In the distal fossa an increase of CK10 positive cells was observed and a decrease of CK19 and CK4 reactive cells. Normally the glans penis is lined by a keratinizing squamous epithelium (Ham, 1965) as observed also in all six male patients examined by us. The CK distribution pattern in this type of epithelium was, however, not identical to that in epidermis (Huszar et al. 1986; van Muijen et al. 1986; van Muijen et al. 1987b; Moll et al. 1982). For example, CK4 and CK13 are normally not found in adult epidermis, while they are detected in the keratinizing glanular epithelium. Achtstätter et al. (1985) reported that apparently nonkeratinizing squamous glanular epithelium contained CK13 and CK19, but also CK1, which is a marker for keratinization. When comparing the staining patterns of the CK8 antibodies LE41 and M20 with those of RCK102, recognizing CK5 and CK8, one may

conclude that the positive reaction for RCK102 in stratified squamous epithelia represents the distribution of CK5 in these tissues.

*In summary,* we can conclude that monoclonal antibodies to individual cytokeratin polypeptides are valuable markers for the detection and characterization of the different morphological types of epithelia occurring in human male and female urinary tract. Furthermore, within one type of epithelium cytokeratin patterns can be related to stage of differentiation. Future studies with these antibodies in neoplasms of these epithelia will have to reveal whether cytokeratin expression is related to morphology, site of origin or degree of tumor progression.

Acknowledgements.

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# CHAPTER III

# Distribution of Cytokeratin Polypeptides in Human Transitional Cell Carcinomas, with Special Emphasis on Changing Expression Patterns During Tumor Progression

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## ABSTRACT

The expression of cytokeratin (CK) polypeptides was studied in 59 transitional cell carcinomas (TCCs) of the urinary tract of different grade and stage. Using a panel of 14 chain-specific monoclonal CK-antibodies we identified immunohistochemically 8 different CKs separately, ie, CKs 4, 7, 8, 10, 13, 14, 18,and 19, while in immunoblotting studies CK5 expression was detected indirectly by using the antibody RCK102, recognizing CK5 + 8.

In the low grade TCCs (G1-G2) the CK distribution was comparable to that in normal urothelium, however with a variable expression of CK13 in the different tumors and a uniform distribution of CK7. In higher grade TCCs (G3) a decrease in CK13 expression was observed, particularly in the areas of muscle invasion. Furthermore, the appearance and increasing expression of CK14 (not present in normal urothelium or G1 TCCs) with higher grade and stage was striking. With tumor progression changes in epitope configurations of CK8 and CK18 were detected, as concluded from immunohistochemical assays with the panel of monoclonal antibodies for each of these two CKs. In extreme cases this resulted in differential staining patterns of the invasive and non-invasive components within one tumor. In 7 out 32 of TCCs, some of which showed areas with evident squamous differentiation, a decrease in the expression of CK7 and/or CK8 was seen.

We conclude that tumor progression in TCCs is associated with discrete changes of CK expression, which can be detected using monoclonal antibodies.

## INTRODUCTION

Normal epithelia and carcinomas contain cytokeratins (CK) as their main intermediate filament constituents. Specific subsets of different CK polypeptides are expressed in different epithelia depending on their origin or type of differentiation [1-3].

With respect to the different types of urothelium and the tumors derived from them some reports have already described intermediate filament expression

patterns, mainly based on electrophoretic studies or using immunohistochemical assays employing a limited number of chain-specific CK antibodies [4-7]. Recently, however, Moll et al [8] have published an extended study on the expression in normal and malignant urothelium, while we [9] examined the CK distribution in a large series of samples from different localizations in the normal urinary tract. A profound heterogenity within these epithelia and within tumors derived therefrom became obvious from these studies.

Because satisfactory morphological detail can be resolved down to the single cell level using immunohistochemical procedures, these are preferable to gel electrophoretic analyses, especially in the study of tissues with variable morphology. The latter certainly applies to the different grades of transitional cell carcinoma (TCC): the malignant potential of TCCs is related to the degree of architectural and cytologic atypia (grade) and the level of infiltration (stage) [10-15]. As a result TCCs seem very suitable to use in the study of expression patterns of CKs in relation to tumor behavior. To provide a solid basis for such a study we have recently performed an extensive study of the normal epithelia lining the surface of the urinary tract using a large panel of chain-specific monoclonal CK antibodies (anti-CKs 4, 7, 8, 10, 13, 18 and 19) [9]. In the different types of normal urothelia, ie, transitional epithelia and squamous epithelia, different CK expression patterns showed a relation to tissue morphology, the specific localization of a certain epithelium within this tract, the cell type, or partly to the state of differentiation within the epithelium. In the present study the panel of polypeptide-specific antibodies was essentially extended and applied to a series of 59 TCCs from 48 patients and selected for their differences in malignant potential, ie, grade and stage. In this way we intended to examine a possible relationship between the cytokeratin expression patterns and the biological behavior of the tumor, with special attention to tumor progression, ie, increasing grade and stage. The results were compared to CK expression patterns in the normal epithelia of the urinary tract.

## MATERIALS AND METHODS

*Tissues.* The different types of normal epithelium of the urinary tract used for

screening antibody LL002 ( directed against CK14) were obtained from autopsies as described in a previous report [9]. Seventeen tissue samples, derived from six different patients, included transitional epithelium from renal pelvis and ureter (n = 2), bladder (trigone excluded; n = 3), trigone (n = 5; in one specimen also an area of nonkeratinizing squamous epithelium was seen) and male proximal urethra (n = 2). The other types of epithelia included columnar urethral epithelium (females, n = 2; male, n = 1), non-keratinizing squamous urethral epithelium (female, n = 1, same specimen as for columnar epithelium) and two specimens with keratinizing squamous glanular epithelium. One of these latter specimens also contained nonkeratinizing squamous epithelium of the fossa navicularis.

Tumor samples of TCCs were obtained immediately after transurethral resection or other surgical procedures. Fifty-five TCCs were resected from the urinary bladder, while in four cases TCCs were derived from the renal pelvis. Parts of the fresh tissue samples were snap-frozen and stored in liquid nitrogen until use. The main part was used for conventional pathological examination. The vast majority of the tumors had not been exposed to medical treatment other than transurethral resection of possible prior lesions.

*Grading and Staging of the Transitional Cell Carcinomas.* Grading and staging was performed on conventional paraffin sections. The tumors were graded according to the method described by Ooms et al [16-17] to obtain reproducible grading results. This method involves the classical WHO light microscopic criteria [18] followed by a morphometrical assay, in which the mean nuclear areas of 50 basal cells, 50 superficial cells and 50 of the largest nuclei are calculated on a graphic tablet (Kontron-MOP videoplan). This morphometrical grade is compared with that made by the pathologist. In cases of disagreement the slides were reviewed by the pathologist for a final conclusion.

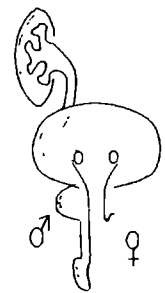
Staging was performed according to the TNM classification of the International Union Against Cancer [19]. In biopsy material the maximal degree of muscle or perivesicle infiltration could not be assessed exactly, so that in those cases pT2 must be interpretated as "at least pT2".

*Antibodies.* The panel of monoclonal chain-specific CK antibodies used in this study has been described before [9]. Table 1 summarizes specifications and references of the individual antibodies [9, 20-26]. The antibody LL002 was not described in our previous study [9], but has been shown to react specifically with CK14 in immunoblotting [26]. The specificity of LL002 for CK14 is further illustrated in the Results section. Furthermore, we have now performed immunoblotting studies with antibody 2C8 on bladder cancer samples, showing a specific reaction of this antibody only with CK18, as well as 2D-immunoblotting studies with antibody M20, proving its specificity for CK8 (see Results). For immunoblotting assays the mouse monoclonal anti-vimentin antibody RV202 [21], as well as the CK17 antibody E3 [27] (provided by Dr. S. Troyanovski, Moscow) were used.

*Immunohistochemistry and Immunoblotting.* The indirect immunoperoxidase technique was applied to 4 micron thick cryostat sections in which the peroxidase activity was detected with 3-amino-9-ethylcarbazole [28].
For the immunoblotting studies both cytoskeletal preparations from RT4 cells [8], TR146 cells, and TCCs were used. RT4 cells were extracted with 1% Triton X-100 in phosphate buffered saline, containing 0.4 mM phenylmethyl sulphonyl fluoride (PMSF), 1 mM EGTA and 1 mM EDTA. Cytoskeletal preparations from TCC specimens were made as follows: 20 micron thick frozen sections (about 15 to 30 sections needed for one two-dimensional gel) were immediately after cryosectioning extracted for 15 minutes on ice with buffer containing 1.5 M KCl, 0.5% Triton X-100, 5 mM EDTA, 0.4 mM PMSF, and 10 mM Tris.HCl, pH 7.2. The 2000 rpm pellet (Minifuge; 10 min. at 0° C) was washed several times in cold buffer 5 mM EDTA, o.4 mM PMSF and 10 mM Tris.HCl, pH 7.2 by 4 min. centrifugation steps at 3000 rpm. The final pellet was dissolved in SDS-sample buffer and stored at -20° C until use. One- and two-dimensional gel electrophoresis and immunoblotting were performed essentially as described before [28].

**Table 1.** Specificity of the monoclonal cytokeratin (CK) antibodies used in this study and their immunohistochemical reactivity with the different types of epithelium in the normal urinary tract *.

| Antibody | Cytokeratin(s) recognized | Reference | Reactivity pattern: Transitional epithelium (TE) | Reactivity pattern: Nonkeratinizing squamous epithelium (NKSE) | Reactivity pattern: Keratinizing squamous epithelium (KSE) |
|---|---|---|---|---|---|
| 6B10 | CK4 | 20 | S[1] | +[2] | S[3] |
| RCK105 | CK7 | 21 | -/+[4] | - | - |
| RCK102 | CK5+8 | 23 | + | B | B |
| LE41 | CK8 | 22 | U | - | - |
| M20 | CK8 | 9, this study | + | - | - |
| RKSE60 | CK10 | 24 | - | -[5] | +[2] |
| 1C7, 2D7 | CK13 | 20 | B | +[2] | F |
| LL002 | CK14 | 26 | - | F[6] | +[7] |
| 2C8 | CK18 | 9, this study | U | - | - |
| RGE53 | CK18 | 24 | U | - | - |
| RCK106 | CK18 | 21 | + | - | - |
| CK18-2 | CK18 | 23 | + | - | - |
| LP2K | CK19 | 23, 25 | + | B | B |



| Types of epithelium in the urinary tract | |
|---|---|
| Kidney | |
| Renal pelvis | -TE |
| Ureter | -TE |
| Urinary bladder | -TE |
| Trigone | -TE,NKSE |
| Urethra | -TE,NKSE |
| Male | |
| Fossa navicularis | -NKSE |
| Glans penis | -KSE |

* For a detailed description see reference 9 (the urethral columnar epithelium is not included in this summary).
**S** Sporadically positive (< 5%)
**F** Focally positive ( 5% - 50%)
**+** Homogeneously positive
**-/+** Negative areas in a positive epithelium
**B** Basal cells positive, as well as several parabasal cell layers
**U** Umbrella cells positive

**1** Only positive in umbrella cells
**2** All cell layers positive except for the basal cell layer
**3** Only positive in the stratum corneum
**4** Homogeneously positive in the renal pelvis and ureter
**5** Focally positive in the NKSE of the fossa navicularis
**6** Focally positive in basal cells and sporadically positive in intermediate cells in trigone and urethra, extensively positive in fossa navicularis
**7** all cell layers positive except for the stratum corneum

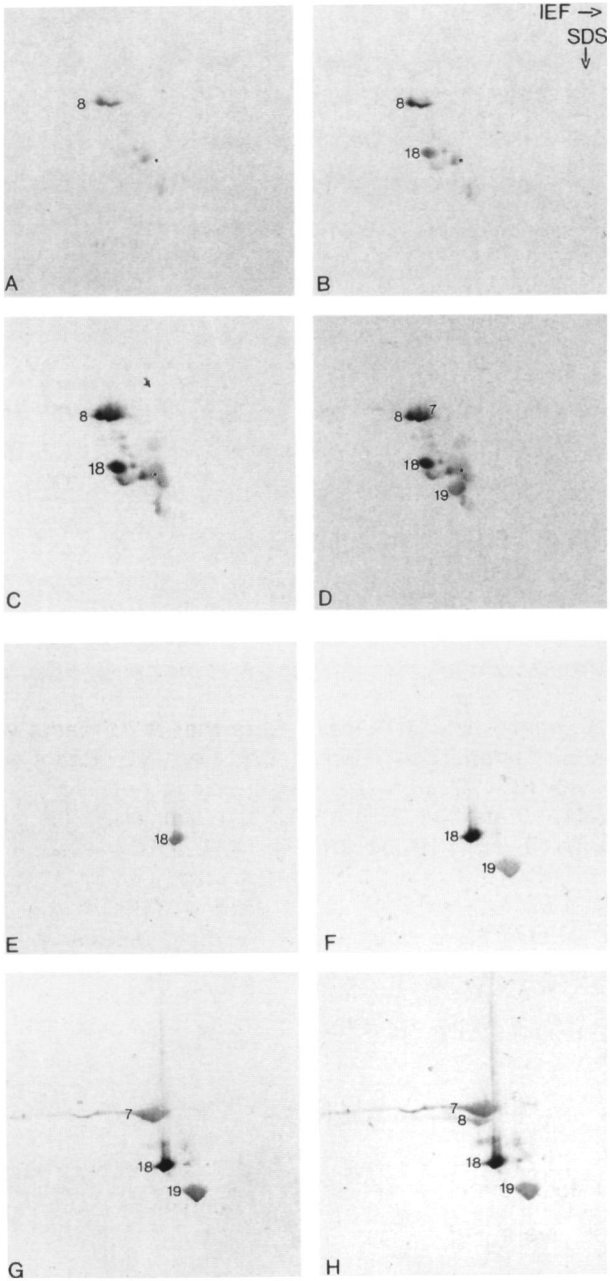
## RESULTS

*Characterisation of antibodies M20, 2C8 and LL002*

In an earlier study [9] we have characterized antibody M20 by one-dimensional immunoblotting assays, which indicated its specificity for CK8. However, in these studies we occasionally observed a faint reaction in the CK18-region on the immunoblots. To exclude cross-reactivity of M20 with CK18 two-dimensional immunoblotting was performed on RT4 cytoskeletal preparations (Figures. 1A to D). Our results (Figure 1A) show that M20 reacts with CK8 in two-dimensional blots and that the faint cross-reactivity in the CK18-region represents breakdown products of CK8. Reincubation of the two-dimensional blots with the antibodies to CK18 confirm this conclusion (Figure 1B). Figures 1C and 1D, which show the results of reincubations of this blot with antibodies to CK7 and CK19, further substantiate the specificity of M20. Also 2C8 was further characterized in this way (Figures. 1E to H), and was shown to react only with CK18 in RT4 cell extracts (Figure 1E). Again, reincubation of the immunoblot with antibodies specific for CK19, CK7 and CK8 support this interpretation. To illustrate the specificity of antibody LL002 for CK14, we have performed an immunoblotting study on CKs of TR146 cells. The results shown in Figures 1I to L clearly demonstrate that LL002 reacts with a CK protein migrating at a position slightly below CK8 and above CK17. Furthermore, the fact that the isoelectric pH of this protein is in between that of CK8 and CK17 proves that LL002 recognizes only CK14 in these cells.

*Expression of CK14 in normal epithelia of the urinary tract.*

Antibody LL002 did not stain the transitional epithelium including the prostatic part of the urethra (Table 1 and Figure 2A). In columnar urethral epithelium several positive areas were found in which especially the basal cells and some parabasal cells were stained (Figure 2B). Nonkeratinizing squamous epithelium of the trigone and urethra showed some areas with positive basal cell layers and sporadically positive intermediate cells (Figure 2C). Nonkeratinizing squamous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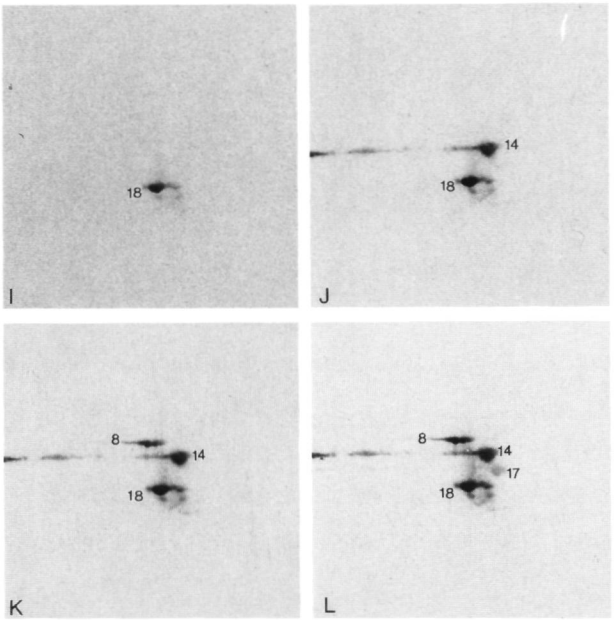


**Figure 1.** Characterization of monoclonal antibodies M20 (panels **A-D**), 2C8 (panels **E-H**) and LL002 (panels **I-L**) by two-dimensional immunoblotting assays on cytoskeletal prepararions of RT4 cells. Note that M20 reacts with CK8 and a breakdown product (asterisk, **A**), which does not comigrate with CK18 (**B**; CK18 detected with RCK106). In **C** and **D** the positions of CK7 (detected with RCK105) and CK19 (detected with LP2K) are indicated. Also note that 2C8 reacts only with CK18 (**E**). The positions of CK19 (**F**; detected with LP2K), CK7 (**G**; detected with RCK105) and CK8 (**H**; detected with M20) are indicated as such. **B**, **C** and **D**, as well as **F**, **G** and **H** show results of reincubation of the blots shown in **A** and **B**, respectively. For the characterization of antibody LL002, TR146 blots were incubated first with RCK106 (detecting CK18; **I**), then with LL002 (**J**), and subsequently with M20 (detecting CK8; **K**) and E3 (detecting CK17; **L**). Note that the CK spot recognized by LL002 migrates in the position of CK14.

epithelium in the fossa navicularis was almost completely positive in the basal and parabasal cell layers (Figure 2D), while the keratinizing squamous epithelium of the glans penis was completely positive, except for the stratum corneum (Figure 2E).

The results obtained with the immunoperoxidase technique on bladder cancer frozen sections are described in the following paragraphs, summarized in Table 2 and depicted in Figures 2 to 4.

Of the 59 TCCs examined, 52 showed similar CK expression patterns, while in the remaining 7 cases (all G3) a remarkable deviation from these expression patterns was observed. Table 2 summarizes our clinicopathologic and immunohistochemical data for all TCCs, with these 7 tumors grouped separately at the end. This group will be described in a separate paragraph after the prevailing CK expression patterns. Frequently within one tumor quantitative differences in staining patterns for the individual CKs were observed. These were scored as major and minor expression patterns.

From Table 2 it is obvious that TCCs of all grades and stages show a homogeneous expression of CKs detected by RCK105 (CK7; Figure 2F), RCK102 (CKs5+8), M20 (CK8), RCK106 and CK18-2 (both CK18) and LP2K (CK19), except for one case with RCK102 (case 27; Figure 2G) and two exceptions for RCK106 (Figures 2H,I) and CK18-2 (cases 26 and 27). RKSE60 (CK10) showed no staining reaction except for one case (case 35a) where focally RKSE60 positive cells were seen.

*Expression of cytokeratins 4 and 13*

The CK4 expression did not appear to be related to that of other CKs, especially not to CK13. 6B10 (CK4) showed a focal staining reaction in the minority of TCCs (Figure 2J). In three cases 6B10 stained superficial cells more extensively (Figure 2K) than in the other cases. In two of these cases the superficial cells were morphologically identical to umbrella cells, while in the third case the superficial cells were part of adenomatoid structures (Figure 2L). Two TCCs were almost homogeneously positive for 6B10 (Figure 2M).

The CK13 expression could be divided into three patterns with similar staining for both CK13 antibodies (see Table 3). Firstly, an extensive to almost homoge-

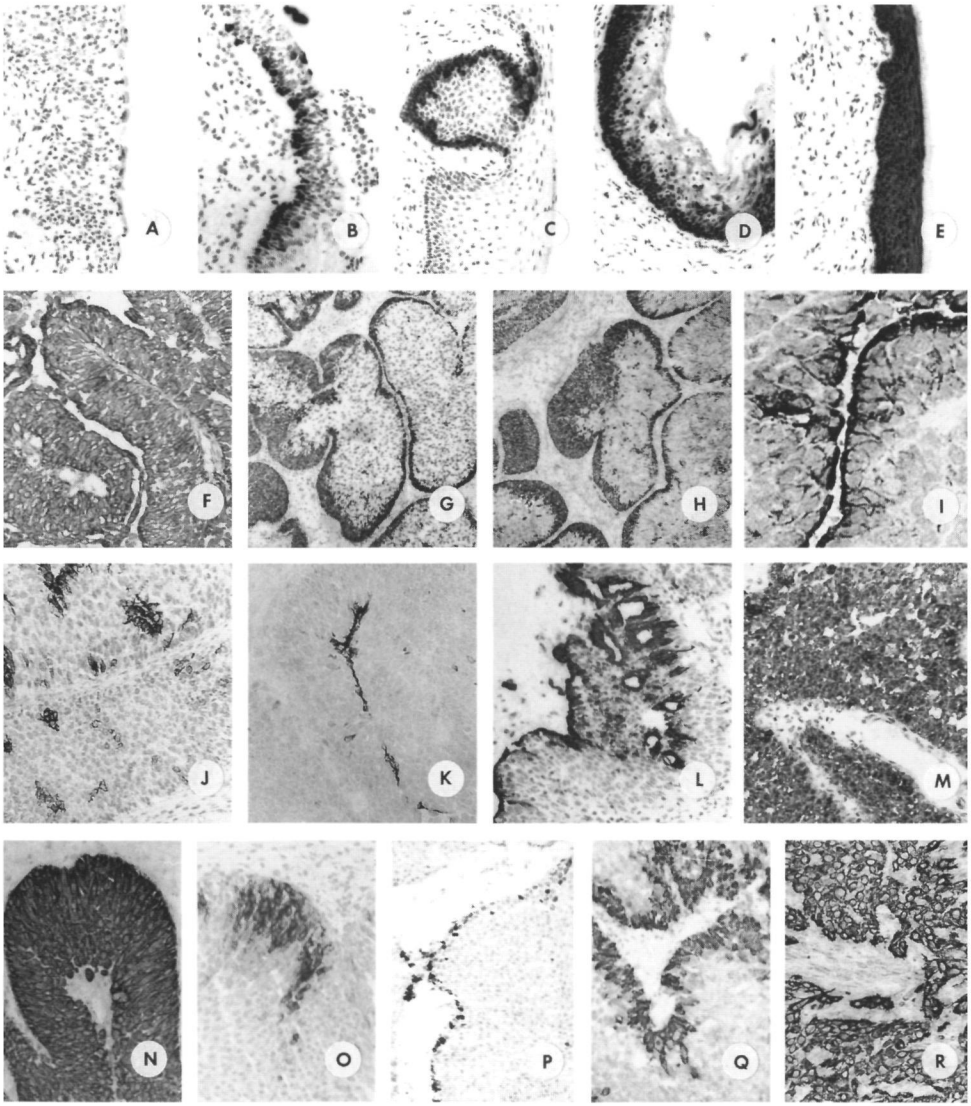


**Figure 2. A-E:** Immunoperoxidase staining patterns of frozen sections from normal epithelia of the adult human urinary tract, incubated with the cytokeratin 14-antibody LL002 and including urinary bladder transitional epithelium (**A**), columnar epithelium of the urethra (**B**), nonkeratinizing squamous epithelium of the female urethra (**C**) and of the male fossa navicularis (**D**), and the keratinizing squamous epithelium of the glans penis (**E**).
**F-R:** Immunoperoxidase staining patterns of human transitional cell carcinomas showing the typical pattern of RCK105 (**F**, case 26). Furthermore are shown the occasionally occurring patterns of RCK102 (**G**) and RCK106 (**H,I**), from case 27 (**G,H**), and case 26 (**I**), variable patterns of 6B10 (**J-M**, cases 26, 32, 18 and 33), two typical patterns of 2D7 (**N,O**, cases 32 and 8, respectively) and typical patterns of LL002 (**P,R**, cases 15, 28a and 37, respectively).
Magnifications: A-F and K-R, X 100-120; G and H, X 40; I X 350.

**Table 2.** Clinical and histopathologic data and results of cytokeratin immunohistochemistry of 59 transitional cell carcinomas.

| Clinical Data | | | Cytokeratin subtype / monoclonal antibody | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 4 | 7 RCK | 5+8 RCK | 8 | 8 | 10 RKSE | 13 2D7 | 14 | 18 | 18 | 18 RCK106 | 19 | |
| Case | Grade | Stage | 6B10 | 105 | 102 | LE41 | M20 | 60 | 1C7 | LL002 | 2C9 | RGE53 | CK18-2 | LP2K | Remarks |
| 1a | 1 | Ta | S | + | + | S | + | - | S | - | +/- | + | + | | LP2K stains all 48 cases homogeneously (+) positive |
| 1b | 1 | Ta | S[1] | + | + | U | + | - | F | - | U | + | + | | |
| 2 | 1 | Ta | - | + | + | U[8] | + | - | + | - | U | + | + | | |
| 3 | 1 | Ta | - | + | + | U | + | - | + | - | U | U[5] | + | | |
| 4 | 1 | Ta | - | + | + | U/+ | + | - | + | - | U | + | + | | |
| 5 | 1 | Ta | - | + | + | U/F | + | - | B[6]/F | - | U | U[5] | + | | |
| 6 | 1 | Ta | - | + | + | U/F | + | - | +[3] | - | U | + | + | | |
| 7 | 1 | Ta | S | + | + | F | + | - | + | - | -/± | + | + | | |
| 8 | 1 | Ta | - | + | + | U[8] | + | - | F | - | U[8] | + | + | | |
| 9 | 1 | Ta | - | + | + | U/F | + | - | S | - | U5/- | + | + | | |
| 10 | 1 | Ta | F | + | + | U[8] | + | - | + | - | U[8] | + | + | | |
| 11 | 2 | Ta | F | + | + | U/U[8] | + | - | -/B[7] | -/B | U/U[8] | U[8] | + | | progression into G3T2 |
| 12a | 2 | Ta | F | + | + | U/U[8] | + | - | + | - | U/U[8] | U[8] | + | | progression into G3T2 |
| 13 | 2 | Ta | F | + | + | -/+ | + | - | +/B[7] | - | +/- | U[5]/U[8] | + | | |
| 14 | 2 | Ta | - | + | + | F | + | - | B[6]/+ | - | U | U[8] | + | | renal pelvis |
| 15 | 2 | Ta | - | + | + | +/- | + | - | B[6]/+ | -/B | U/U[8] | +/U[5] | + | | renal pelvis |
| 16 | 2 | Ta | S | + | + | +/± | + | - | + | - | -/U[8] | + | + | | renal pelvis |
| 17a | 2 | Ta | - | + | + | F | + | - | F | - | U | ±/+ | + | | |
| 17b | 2 | Ta | S | + | + | U/F | + | - | + | - | U | U[8] | + | | |
| 18 | 2 | Ta | U/S | + | + | +/- | + | - | + | -/B[7] | U/F | +/F | + | | |
| 19 | 2 | Ta | - | + | + | U[8] | + | - | + | - | U | + | + | | |
| 20a | 2 | Ta | - | + | + | F[1] | + | - | + | - | B/U[8] | U[8] | + | | |
| 20b | 2 | Ta | - | + | + | F[1] | + | - | + | - | B/U[8] | U[8] | + | | |
| 21 | 2 | Ta | - | + | + | U/F | + | - | B[6] | - | U[5]/+ | +/U[5] | + | | |
| 22 | 2 | Ta | - | + | + | U | + | - | S | - | U/U[8] | U[8]/+ | + | | |
| 23 | 2 | T1 | S | + | + | F[2] | + | - | -/B | F2 | F[2] | F/+[2] | + | | |
| 24 | 2 | T1 | S | + | + | U5/F[2] | + | - | B[6]/- | F[2] | U[5]/F[2] | + | + | | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 25a | 3 | Ta(1) | - | + | + | U[5]/F | + | - | -/B[6] | S | +/± | + | + | |
| 25b | 3 | Ta(1) | - | + | + | U[5]/F | + | - | B[6] | - | +/U[5] | + | + | |
| 25c | 3 | Ta(1) | S | + | + | F/U[5] | + | - | -/B[6] | S | +/F | + | + | |
| 26 | 3 | Ta(1) | U/F | + | + | F[1] | + | - | -/B[6] | - | -/F[4] | U[4] | U[9]/U | |
| 27 | 3 | Ta(1) | - | + | B[7]/+ | -/B | + | - | F | -/B[6] | B[7]/+ | B[7]/+ | B[9]/+ | |
| 28a | 3 | Ta(1) | - | + | + | U/F | + | - | F | -/B[7] | +/U[5] | + | + | in follow-up metastases |
| 28b | 3 | Ta(1) | - | + | + | U[5]/F | + | - | F | -/B[6] | + | + | + | |
| 29a | 3 | Ta(1) | - | + | + | -/B | + | - | F | - | ±/B[6] | B[6]/± | + | in follow-up metastases |
| 29b | 3 | Ta(1) | F | + | + | S | + | - | -/B[7] | - | F | F/± | + | |
| 30 | 3 | Ta(1) | - | + | + | U5/F | + | - | B[7]/+ | -/B[7] | +/- | + | + | |
| 31a | 3 | T1 | - | + | + | -/B[2] | + | - | +/B | -/B[7] | ±/B[2] | + | + | |
| 31b | 3 | T1 | - | + | + | -/B[2] | + | - | +/± | -/B[7] | ±/B[2] | + | + | |
| 32 | 3 | Ta(2) | S/U | + | + | U[6]/U[5] | + | - | +[3] | -/B | U/U[9] | U[6] | + | |
| 33 | 3 | Ta(2) | + | + | + | -/F | + | - | S | - | -/± | +/± | + | |
| 34 | 3 | T1(2) | F | + | + | F[2] | + | - | +/- | F[2] | F[2] | + | + | |
| 35a | 3 | Ta(2) | -/B[7] | + | + | S | + | F | + | -/B[6] | -/U | + | + | |
| 35b | 3 | T2 | -/B[7] | + | + | F | + | - | + | -/B[9] | F | + | + | |
| 36a | 3 | T2 | S | + | + | F | + | - | +/- | -/+ | +/F | +/B | + | |
| 36b | 3 | T2 | - | + | + | F | + | - | F | F | +/F | + | + | after irradiation |
| 37 | 3 | T2 | - | + | + | - | + | - | - | + | F | + | + | |
| 38 | 3 | T2 | - | + | + | - | + | - | - | - | +/- | F | + | renal pelvis, metastases |
| 39 | 3 | T2 | +/- | + | + | F | + | - | +/- | F | F | +/- | + | |
| 40 | 3 | T2 | - | + | + | F | + | - | S | F | +/- | +/- | + | |
| 41 | 3 | T2 | - | + | + | S | + | - | - | - | F | +/- | + | |
| 42 | 3 | T2 | F | + | + | S | + | - | F | + | F | +/- | + | |
| 43 | 3 | Ta | F | S | + | ±/- | +/U[5] | -/+ | B[6]/+ | -/B[6] | S | +/U[5] | + | |
| 44 | 3 | Ta(1) | +/- | -/+ | + | U[5]/± | + | F | B[6]/- | -/B[6] | U[5]/F | U[5]/+ | + | squamous metaplasia |
| 45 | 3 | Ta(2) | -/B[7] | S | + | - | F | - | +[3] | +[3] | - | - | F | squamous metaplasia |
| 46 | 3 | Ta(2) | S | ±/F | + | - | -/+ | S | + | + | -/+ | -/+ | + | squamous metaplasia |
| 47 | 3 | T1(4) | - | + | + | - | F | - | + | + | S[2] | F | +/- | after irradiation |
| 48 | 3 | T2 | - | - | + | +/- | + | - | -/F | -/F | -/+ | +/- | + | |
| 12b | 3 | T2 | - | F | + | F | + | - | - | - | F | +/- | + | |

( ) Stage based on the routine paraffin sections
/ Two staining patterns (dominating pattern, area > 50% / minor pattern, area < 50%)
S Sporadically positive cells (< 5%)
F Focally dispersed positive cells (5%- 50%)
\+ Homogeneously strong positive
± Weakly positive
\- No staining observed
U Umbrella cell (or umbrella-like cell) layer positive
B Basal cells positive

1 Only positive in umbrella cells
2 Strongly and heterogeneously positive in infiltrating cells
3 All cell layers positive except for (several) umbrella (like) cells
4 Staining limited to the luminal part of thesuperficial cells
5 Also staining in several underlying cell layers
6 Also staining in several parabasal cell layers
7 Also staining focally in other cell types
8 All other cell types than umbrella cells are weakly stained
9 All other cell types than basal cell are weakly stained

neous staining reaction, with exception of umbrella cells (Figure 2N), and continuous staining of the basal and parabasal cell layers, dominating in most G1 and G2 tumors; second, a positive staining reaction limited to small focal areas with basal and parabasal cells positive (Figure 2O), dominating in superficial G3 tumors; third, no staining at all, occurring in deeply infiltrating G3 TCCs (not shown). In the noninfiltrating part of G3pT2 TCCs we observed relatively frequent extensive CK13 expression in contrast to the superficial, ie, pTa and pT1, G3 TCCs (Table 3).

In case 17, two asynchronous G2 samples (interval, 3.5 months) showed differences in their staining pattern, with one sample extensively positive and the other focally positive. A similar phenomenon was observed in case 36, although the second sample (interval, 19 months) was obtained after radiotherapy.

**Table 3.** Degree of immunohistichemical expression of CK13 in different steps of tumor progression of transitional cell carcinoma (n = 52)

| Steps of tumor progression | | Degree of immunohistochemical expression | | |
|---|---|---|---|---|
| Grade and Stage | Number of TCCs studied | Extensively positive | Focally positive | Negative |
| G1pTa | 11 | 7 | 4 | 0 |
| G2pTa | 14 | 11 | 3 | 0 |
| pT1 | 2 | 1 | 1 | 0 |
| G3pTa/1 | 12 | 4 | 8 | 0 |
| pTa/1(2)* | 4 | 3 | 1 | 0 |
| pT2 | 9 | 3 | 3 | 3 |

* In frozen sections of these four cases only the pTa/1 component was observed, while the whole neoplasm was staged as pT2 on basis of paraffin embedded material.

*Expression of cytokeratin expression 14*

Table 2 indicates that with increasing degree of malignancy, an increasing

number of TCCs showed LL002 reactivity. In the lower grade tumors the staining was confined to basal cells (Figure 2P). In higher grade tumors a tendency of suprabasal cell staining was seen (Figure 2Q) and in two cases of G3pT2 TCCs all tumor cells were positive for LL002 (Figure 2R). In three TCCs CK14 positive cells were only found in the infiltrating tumor component, while the noninfiltrating part was negative (not shown).

*Expression of different CK8 and CK18 epitopes*

As mentioned before, two antibodies to CK18 (RCK106 and CK18-2) and one antibody to CK8 (M20) showed a homogeneous staining in the 52 TCCs independent of stage and grade (Figures 3A,B). Fewer cells were positive with antibody RGE53 (CK18), which gave variable intense staining with a preference for the superficial cell layers in G1 and G2 tumors (Figures 3C,D). With antibody 2C8 (CK18), as well as with LE41 (CK8), a significantly different staining pattern was observed as compared to the other CK18 and CK8 antibodies. Both antibodies strongly stained umbrella cells or superficial (umbrellalike) cells in all noninfiltrating areas of TCCs, with 2C8 showing reactivity in more umbrella cells than LE41 (Figure 3E). Next to this pattern both antibodies showed localized-to-extensive staining reactions in the other cell layers of a number of TCCs. These staining reactions were generally weak and only in a few cases was a predominantly positive pattern seen (Figures 3F,G). However, in G3 TCCs antibody 2C8 showed extensive positivity in the majority of these tumors (Figure 3H). As seen in Figures 3I to P, the invasive parts, whenever present in the TCCs, showed variable numbers of strongly positive cells with both antibodies. This was also the case in those tumors in which the noninfiltrative part was predominantly negative (Figures 3I to K). Again, also in these infiltrating areas, 2C8 stained more cells than LE41; LE41 was completely negative staining on two deeply infiltrating tumors (cases 37 and 38). The infiltrating tumor cells bordering the stroma were found to be strongest positive (Figure 3N); occasionally this was seen also in the noninfiltrating part (arrows, in Figures 3O,P) especially in the vicinity of infiltrating cells.

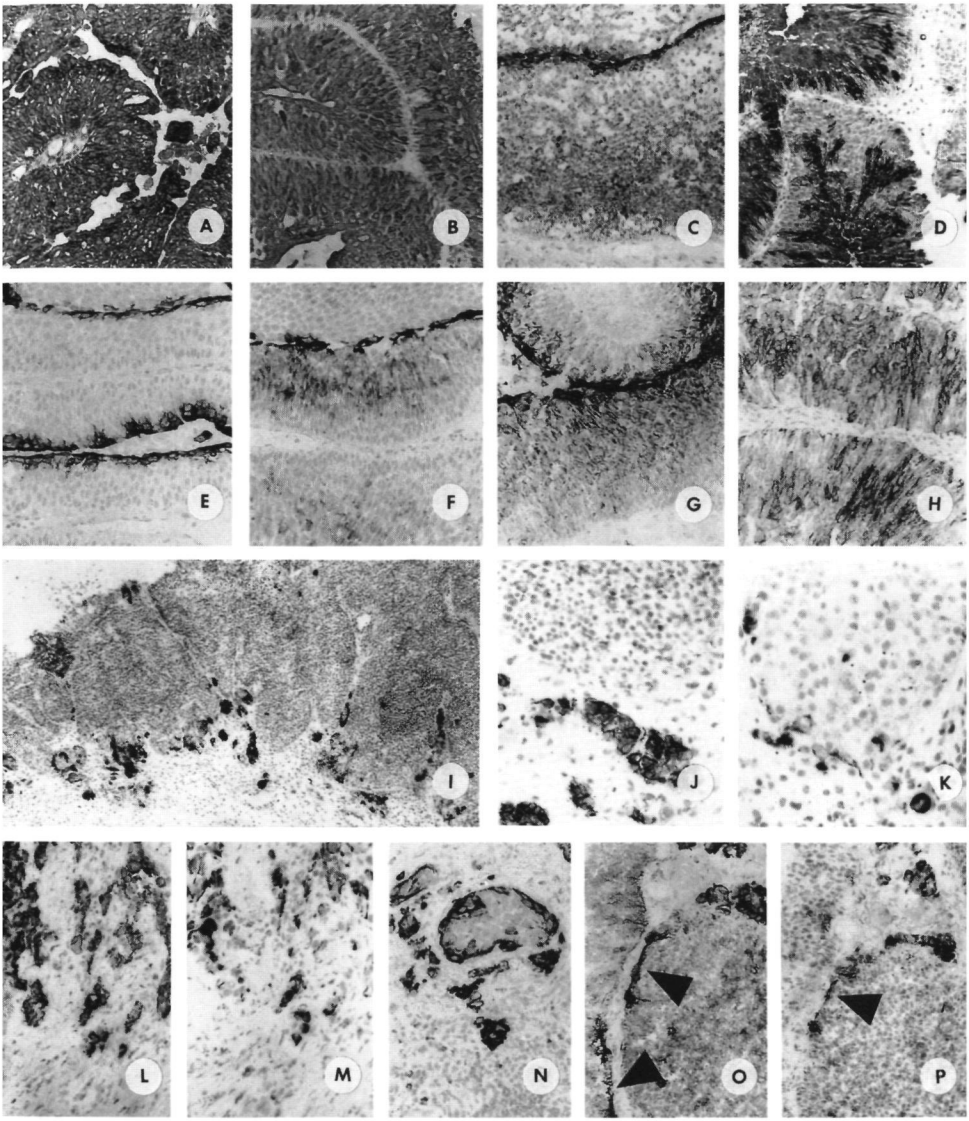


**Figure 3.** Immunoperoxidase staining patterns of frozen sections of transitional cell carcinomas showing typical staining patterns for the different cytokeratin 8- and 18-antibodies, including M20 (**A**, case 26), RCK106 (**B**, case 6), RGE53 (**C**, case 6; **D**, case 5), 2C8 (**E**, case 6; **G**, case 20b; **H**, case 25c; **I** and **J**, both case 23; **L**, case 36b; **O**, case 31a) and LE41 (**F**, case 6; **K**, case 23; **M**, case 36b; **N**, case 34; **P**, case 31a). Note in **I** and **J** positive staining in the invasive tumor compartment for 2C8, while the noninvasive part is predominantly negative. A similar observation for LE41 is depicted in **K**. Note also focal positive staining for 2C8 and LE41 in basal cells of the noninvasive part in **O** and **P**, respectively.
Magnifications: A-H and L-P, X 100-120; I X 35; J X 150, K X 175.

*G3 carcinomas with a "deviating" CK expression pattern.*

Seven G3 carcinomas differed considerably in their CK expression pattern from the
other 52, especially characterized by a reduced expression or absence of CK7 and CK8 (as detected by M20). Hematoxylin and eosin-stained paraffin sections of these tumors revealed large tumor cells that were often strongly eosinophilic. In three carcinomas resemblance to nonkeratinizing squamous cells was evident (Figure 4A). Although no consistent relationship between morphology and the varying expression patterns of the other CKs (Table 2) was observed htere was a proportional increase in the incidence of staining with RKSE60 (CK10) within this group (3 of 7 tumors; Figure 4D). In two tumors several scattered CK10 positive cells were found and in the third tumor the expression was patchy. In six tumors CK7 expression (RCK105) was significantly decreased (Figure 4B) or lost, and in four cases CK8 (as detected by M20) was diminished. In three of the latter TCCs the other CK8 antibody did not stain at all. CK18 (as detected by RCK106 and CK18-2) was diminished in two cases (Figure 4C). CK10 expression was increased and present in three tumors (Figure 4D). The expression of CK4 and CK14 (Figure 4E) seemed to be increased, while expression of CK13 (Figure 4F) and CK19 appeared unchanged as compared to the other 52 TCCs.

*Immunoblotting studies*

Immunoblotting studies were performed on a selected series of bladder cancers, including one G1, two G2s and four G3 TCCs. One of the G3 tumors showed a squamoid differentiation. The results of the assays are depicted in Figure 5 and do, in general, confirm our immunohistochemical studies. They add biochemical support for the presence of CK5 in some TCCs (lanes 2 and 7 of Figure 5C). This individual cytokeratin polypeptide could not be demonstrated to occur in certain samples on basis of immunohistochemistry, because RCK102 recognizes both CK5 and CK8.
These immunoblotting studies detected CKs 7 (Figure 5F), 8 (Figure 5C), 18

(Figure 5E) and 19 (not shown) in all seven cases, independent of grade and stage, while CK13 (Figure 5D) was not detected in significant amounts in two G3 TCCs. CK4 (Figure 5A) was only found in one G3 tumor. CK14 was detected next to CK5 in the TCC with squamoid differentiation (Figures 5B,C; lane 7) and in one of the other G3 tumors (Figure 5B, lane 6). In addition CK5 was detected in one of the G2 TCCs. The immunoblotted protein bands, migrating below the main band in lane 7 of Figure 5B and in Figure 5F represent degradation products of CK14 and CK7, respectively.

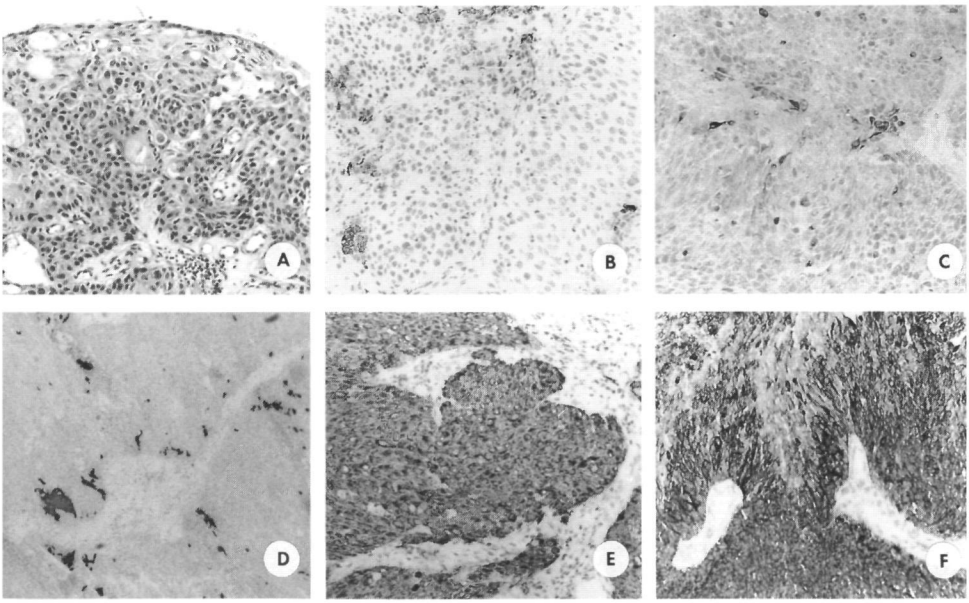


**Figure 4. A**: Transitional cell carcinoma (case 44) with extensive squamous metaplasia, shown in an H&E stained routine paraffin section.
**B-F**: Immunoperoxidase staining patterns of frozen sections of grade 3 transitional cell carcinomas showing deviating keratin expression patterns with antibodies RCK105 (**B**, case 45), RCK106 (**C**, case 45), RKSE60 (**D**, case 43), LL002 (**E**, case 45) and 2D7 (**F**, case 43).
Magnifications: A-C, E and F X 100-120 ; D X 40.

Two-dimensional immunoblotting studies confirmed the identity of the cytokeratin polypeptides detected in the one-dimensional immunoblotting assays using

polypeptide-specific antibodies. Figures 5G to I show the identification of cytokeratins 18 and 19 in a G1 TCC, and show their position in relation to vimentin. These results do also confirm our findings described above that antibody 2C8 is specific for CK18.

## DISCUSSION

The expression of different cytokeratins was studied in relation to tumor progression in TCC. In the low grade carcinomas the CK distribution pattern is in agreement with that in normal transitional epithelium [9] (Table 1), with the exception that CK13 was nonuniformly distributed in the basal compartment of the tumors and that CK7 was homogeneously positive in in TCCs independent of localization in either the upper or lower urinary tract. In the higher grade malignancies the following observations were most striking: 1) a decrease in CK13 expression with increasing grade and stage, 2) the appearance of CK14, 3) changes of CK8 and CK18 epitope configurations related to tumor stage and (to a lesser degree) to grade, and 4) decrease of expression of CK7 and CK8 in relation to squamous metaplastic changes.

In a considerable number of G1 TCCs and G2 TCCs expression of CK13 was reduced as compared to normal urothelium, resulting in tumors that were only focally positive. This phenomenon was neither associated with site, rate of recurrence or other follow-up data, nor with the expression of other CKs. For example, some recurrent tumors showed different expression patterns of CK13 as compared to the previous lesions. In G3 carcinomas a pronounced decrease of CK13 expression was found in the part infiltrating the muscle. Moll et al [8] also have described a decrease of CK13 expression with increasing grade and in metastases. However, in contrast to our findings all low grade carcinomas, that these authors examined, were found to have high CK13 levels.

Although CK4 has been proposed to occur in CK filaments in combination with CK13 [20], the expression pattern of CK4 is not related to that observed for CK13.

The expression of CK14 in 5 of the 17 G2 carcinomas suggests a relation of

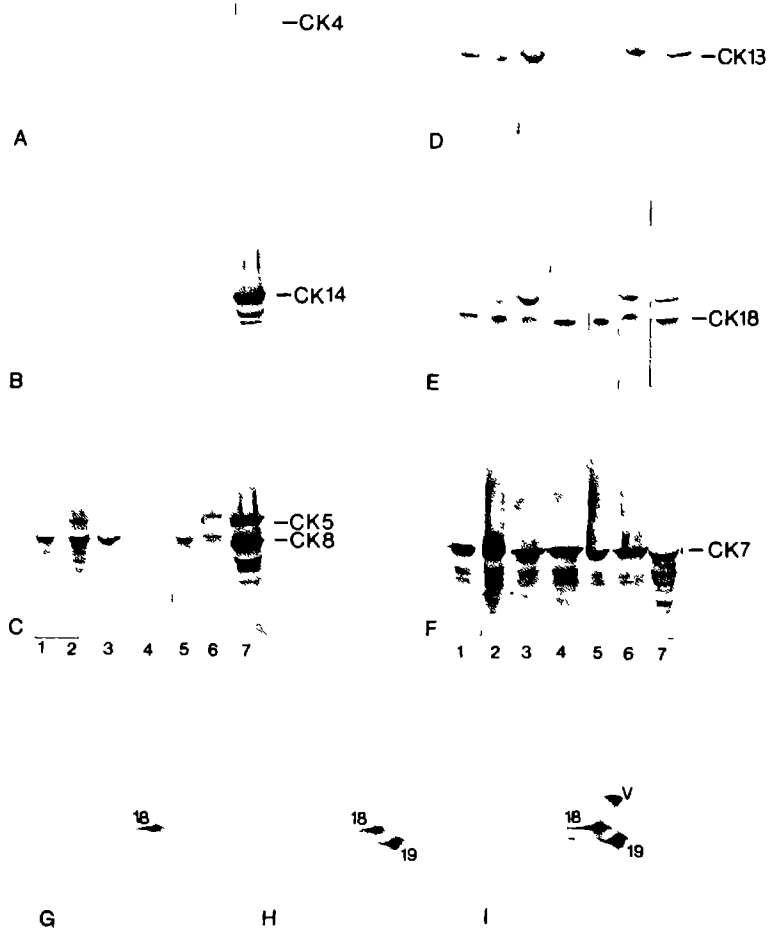


**Figure 5. A-F:** One-dimensional immunoblotting study on a G1 TCC (lane 1; case 1a), two G2 TCCs (lanes 2 and 3; cases 22 and 21, respectively) and four G3 TCCs (lanes 4-7; cases 27, 29a, 26 and 46, respectively) incubated with 6B10 (**A**), and subsequently with LL002 (**B**) and RCK102 (**C**), or 1C7 (**D**) and subsequently with 2C8 (**E**), or only RCK105 (**F**). The individual cytokeratin polypeptides, which are recognized by the diverse monoclonal antibodies are indicated.
**G-I:** Two-dimensional immunoblotting study on a TCC (case 1a), subsequently incubated with antibodies 2C8 (**G**), LP2K (**H**) and RV202 (**I**), detecting CK18, CK19 and vimentin, respectivily, which are indicated as such.

expression of this protein with tumor progression in TCC. It was not detected in normal transitional epithelium, either by our immunohistochemical studies or according to gel electrophoretic data of Achtstätter et al [4] and Moll et al [8]. In this study we have shown that it is also not detected in G1 TCCs by antibody LLOO2. Of the deeply infiltrating tumors some were extensively CK14 positive, while most of the other G3 tumors showed focal positivity. Our data are, however, not readily interpreted by a simple relation between CK14 expression and the clinicopathological parameters of tumor progression. For example, 7 of 25 G3 TCCs were negative and of the 10 TCCs showing muscle infiltration 3 were completely negative in these areas.

CK14 reactivity was confined to the basal and parabasal cells in the nonkeratinizing squamous epithelium of the normal adult urinary tract. CK14 was homogeneously expressed in all cells of the keratinizing squamous epithelium of the glans penis, although the stratum corneum was negative. These observations are in line with CK14 expression observed in TCCs having large eosinophilic cells and squamous differentiation. Cintorino et al [7] have reported that antibody SK2-27, recognizing CKs14, 16, and 17 reacted focally in normal transitional epithelium and in G1 and G2 TCCs. In squamous epithelium and in G3 TCCs, this antibody reacted homogeneously with all cells. Moll et al [8] reported that the antibody KA1, considered by Jarasch et al [30] to be specific for CKs5 and 14, and antibody IVD3A9, most likely directed against CK14, both react with basal cells in normal urothelium and more heterogeneously in G1 and G2 TCCs. Surprisingly, G3 TCCs were described to be CK14 negative. Future studies will have to reveal whether or not epitope-masking phenomena may explain the discrepancy between these findings and our findings reported above. That epitope masking may play a role in CK14 immunohistochemistry is illustrated by our findings in case 26 in which immunoblotting shows the presence of CK14 (Figure 5B, lane 6), while in immunohistochemistry no staining is observed with LLOO2.

In our studies several different antibodies to CK8 and CK18 were used. Since these antibodies show two different staining patterns in normal urothelium [9], we were interested in their reactions with TCCs. On one hand an extensive reaction pattern in all cell layers is obtained with antibodies M20 (CK8), RCK106 and

CK18-2 (CK18), and on the other hand a more restricted umbrella-cell-related pattern is seen with LE41 (CK8), RGE53 and 2C8 (CK18) [6,9]. Cintorino et al [7] have described the umbrella-cell-related staining pattern for their antibody SK60-61, which they consider to be specific for CKs8 and 18, while Achtstätter et al [4] showed a similar reaction for antibody CK1, specific for keratin 18. In G1 TCCs LE41 and 2C8 preferentially stained superficial, ie, umbrella-like cells, while RGE53 reacted more extensively in our recent immunoperoxidase studies than reported in previous immunofluorescence studies [6], probably as a result of the use of a more sensitive technique. The intensity and extent of the staining reaction of RGE53 in the intermediate and basal cell layers was, however, still less than that of the two other CK18 antibodies, RCK106 and CK18-2, which stain virtually all TCC cells, independent of stage and grade. An increase of the number of cells positive for 2C8 and even for LE41, although to a lesser extent, in superficial G3 TCCs is observed as compared to the G1 and G2 carcinomas. It is striking that the exposure of the 2C8 epitope is observed to a lesser degree in the superficial part of the deeply (pT2) infiltrating tumors. A prominently positive reaction for 2C8 is observed in the infiltrating component of G2 and G3 tumors. Frequently the major part of the 2C8 and LE41 positive cells were those bordering at the stroma. In the noninfiltrating part of three TCCs, expression of the CK8 and CK18 epitopes as detected by LE41 and 2C8, respectively, was mainly observed in a few basally located cells clusters. Similary, Cintorino et al [7] reported on one G2 tumor in which their antibody SK60-61 stained the deeper invasive portions with higher intensity, while in three infiltrating G3 TCCs these authors found an extensive reaction for SK60-61. The biological significance of this phenomenon of CK8 and CK18 epitope unmasking or epitope formation is unknown. One explanation may be that the three-dimensional organization of the heteropolymer filaments formed by CK8 and CK18 changes during tumor progression, as it does during the process of normal differentiation in the urothelium. It is interesting to note that the umbrella cells, which need a flexible cytoplasmic architecture to cope with the morphologic deformation during urinary bladder extension, show a reaction pattern with the CK8 and CK18 antibodies, which also recognize the invasive TCC cells. One might speculate that a flexible cytoskeletal organization is a prerequisite for the invasive proper-

ties of these latter cells. One might further speculate that the noninvasive but strongly 2C8- and LE41-positive basal cells may be ultimately capable of stromal infiltration.

Another explanation of these antibody discrepancies may be that they show different affinities for their respective epitopes. Indications for such an interpretation of apparent cell specificity of CK8 and CK18 antibodies are obtained from immuno(histo)chemical studies in other tissues. These experiments rank the antibodies in order of strength to a degree: LE41 << M20 and 2C8 < RGE53 < CK18-2 < RCK106. As a result, a higher concentration of CK8 and CK18 in umbrella cells than in other cell types alone might explain the observed staining differences. This argues that weaker antibodies are sometimes more useful than stronger antibodies because the allow the detection of quantitative differences. Studies on normal epithelia suggest a close correlation between morphologic characteristics and CK-expression patterns. In cancers CK expression patterns are also related to the type of differentiation rather than to that of the cell origin [28]. In the present study we find support for this view in our results with seven TCCs showing a deviating CK composition. Morphologically the tumor cells of most of these cases appeared to have a large and compact eosinophilic cytoplasm, suggesting early squamous differentiation. Areas of squamous differentiation were evident in three cases. Related to these observations is the fact that six of the seven TCCs showed a loss or absence of CK7 and in four cases a diminshed expression of CK8. We expected also CK18 to be diminished in all these cases as for example, was reported for squamous areas in a Brenner tumor [31], but by immunohistochemistry this was only obvious in two cases. Interestingly, these two particular cases showed extensive CK14 positivity in the tumor cells, a further indication of potential squamous differentiation because CK14 is one of the fundamental hallmarks of keratinocytelike cells and is absent from simple epithelial cells. CK10, a marker for keratinization was expressed relatively frequently in the deviating TCCs. Recently other authors [7, 8, 32-34] also described differences of CK expression patterns in urothelial cancers between transitional cell differentiation and other types of (frequently squamous) differentiation. Most of these immunohistochemical data were obtained with nonmonospecific anticytokeratin antibodies [7, 32-34].

We can state that CK expression in transitional cell carcinomas of the human urinary tract shows to a certain extent, correlations with morpologic features (squamous differentiation), the malignant potential (grade/ stage) and with the specific tumor compartment ( infiltrating or noninfitrating part), ie, aspects of tumor progression. As a result, a selected panel of CK antibodies may become a useful tool in future biologic studies of bladder cancer, for example, in defining genetic aberrations in specific tumor cell populations.

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# CHAPTER IV

# Cytokeratin expression patterns in metastatic transitional cell carcinoma of the urinary tract: an immunohistochemical study comparing local tumor and autologous metastases

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## ABSTRACT

The cytokeratin (CK) expression patterns of local, ie, primary or recurrent, high-grade-malignant transitional cell carcinomas (TCCs) of the human urinary tract and autologous lymphogenic and hematogenic metastases (n=33) were compared. Special attention was paid to CK expression in the tumor invasion front and other areas where tumor-stroma interaction occurred to visualize cell populations with a metastatic phenotype. For this purpose polypeptide-specific monoclonal antibodies to CKs4, 7, 8, 10, 13, 14, 16, 17, 18 and 19 were used, employing the immunoperoxidase method.

Results show that:

-1- An increased expression of CK8 and CK18 is seen in the TCC tumor cells at the interface with peritumoral stroma in the tumor invasion front and with intratumoral stroma ("interface phenomenon"). Other than reflecting a quantitative change, this phenomenon might be explained by unmasking of CK8 and CK18 epitopes occurring in these regions.

-2- Although in general the expression of CK13 in local TCC is decreased with increase of histopathologic parameters for progression, ie, grade and stage, an extensive proportion of CK13 positive tumor cells can still be found in some TCCs, even in metastases.

-3- Morphologically recognizable types of aberrant differentiation in TCC, ie, pseudosarcomatous or squamous differentiation and marked loss of differentiation, show altered expression of many of the CKs studied.

## INTRODUCTION

The cytokeratin (CK) protein family, which composes the main type of intermediate filaments in normal and neoplastic epithelial cells, consists of at least 20 different polypeptides (numbered 1 to 20[1-3]) not including the hair cytokeratins[4]. Certain combinations of these CK polypeptides are synthesized in specific types of epithelium. Under certain circumstances, such as metaplasia[5], hyperproliferation[6], or neoplastic growth and progression, the CK expression pattern of a

certain epithelium can change. Although initial studies showed that neoplasms retain the CK pattern of the normal epithelium from which they are derived[1,3] and CK typing could therefore be useful in determining the site of origin of a metastasis, deviating CK expression patterns in preneoplastic lesions[7] and cancers of different organ systems have recently been reported[8-11].

Cytokeratins not expressed in the normal state of a certain epithelium can appear in cancers. Also a correlation between altered CK expression patterns and the degree of tumor cell anaplasia has been reported[8-12]. Recently we[8] and others[10] showed that during tumor progression of local transitional cell carcinomas (TCCs) a decrease of CK13 can be seen in the tumor cells, whereas in contrast CK14 became detectable using certain antibodies. Furthermore mainly the invasive TCC compartment showed an apparently altered CK cytoskeleton structure, as detected with monoclonal antibodies to different epitopes of CK8 and CK18. We interpreted these findings as an adaptation of the cytoskeleton to a more flexible and motile cell phenotype. This assumption was supported by our observations that umbrella cells in the urinary bladder, which have to be flexible during expansion of the organ, showed the same reaction pattern as compared to the invasive TCC cells with the different CK8 and CK18 antibodies.

In the present study, we examined the CK expression patterns in lymphogenic and hematogenic metastases of TCC of the urinary tract from 17 patients as the ultimate end point of TCC progression. In 10 out of the 17 cases, the CK staining reaction of local tumor and metastasis could be compared, allowing us to recognize possible alterations in CK expression between these stages. We also considered the possibility that metastatic cells might be selected from a pool of primary tumor cells with a particular CK expression pattern; for example, those cells that exhibited specific CK8 and CK18 epitopes in the primary tumor, could be studied. Particular attention was paid to the specific location of the tumor cells, especially in the tumor invasion front and other areas where tumor-stroma interactions occur.

## MATERIALS AND METHODS

*Tissues.* The 33 tumor samples (Table 1) were snap frozen and stored in liquid nitrogen immediately after surgery or during autopsy. No overlap between this series of tumor samples and those described previously[8] exists. Approximatlely 5-$\mu$ thick cryostat sections were made and stained by the indirect immunoperoxidase procedure, essentially as described previously[8], but using 3-3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen for the detection of peroxidase activity.

A more extensive panel of monoclonal CK antibodies was used than in our previous study[8], most of which are monospecific for individual CKs. To obtain a reference for the staining patterns of these additional antibodies, normal urothelium and a series of at least 15 local TCCs of different stage and grade were stained. These specimens were taken from the tissue bank described in two previous reports[8,13].

Metastatic and local high-grade malignant TCC specimens (n=33) from the bladder or the upper urinary tract were examined. These included 23 TCC metastases from 17 different patients and 10 corresponding local TCCs, which were primaries or recurrences in the urinary tract. Samples from four patients were obtained at autopsy (see Table 1).

*Antibodies.* The panel of monoclonal CK antibodies used in this study includes most of the reagents described before[8] and reacting specifically with CKs4, 7, 8, 10, 13, 14, 18 or 19. Their reaction patterns in normal transitional epithelium are included in Table 2. In addition the following antibodies were used:

- The mouse monoclonal antibody **LL001**[14] specific for CK14, reacts similar to antibody **LL002** in normal urothelium and local TCC[8].
- The mouse monoclonal antibody **RCK107**, specific for CK14[15] reacts immunohistochemically more extensively than LL001 and LL002, by staining basal cells heterogeneously both in normal transitional epithelium and in G1 TCCs. In squamous metaplastic urothelium, all cells are positive except for basal and some parabasal cells.
- The mouse monoclonal antibody **KA1**, reacting with the CK5/14 complex[16],

**Table 1.** Clinical data and morphological characteristics of TCCs used in this study (local TCCs and metastases).

| Case - Site | Interval between the samples (months) | THERAPY | Special morphologic aspects | Invasion front present in the sample Y/N |
|---|---|---|---|---|
| *"Classical" TCC* | | | | |
| 1- BLADDER<br>LUNG | <br>2 | - | | Y<br>N |
| 2- BLADDER<br>LIVER | <br>½ | Bladder radio-therapy | | Y<br>N |
| 3- BLADDER<br>LIVER | <br>1 ½ | - | | N<br>Y |
| 4- BLADDER<br>LYMPH NODE | -<br>Cystectomy | Radiotherapy & chemotherapy | | Y<br>Y |
| 5- BLADDER<br>LYMPH NODE | <br>1 ½ | Chemotherapy | Necrosis (bladder)<br>Severe polymorphism and tumor giant cells | N<br>Y |
| 6- RENAL PELVIS<br>LYMPH NODE | -<br>Nephrectomy | - | | Y<br>N |
| 7- RENAL PELVIS<br>LYMPH NODE | -<br>Nephrectomy | - | Large eosinophylic cyto-plasm | N<br>Y |
| 8- LYMPH NODE[1] | | - | | N |
| 9- LYMPH NODE[1] | | - | | Y |
| 10- LYMPH NODE[1] | | Chemotherapy | | Y |
| 11- LIVER[2]<br>LUNG<br>2 LYMPH NODES | -<br>Autopsy | - | Basaloid aspect of the cells | Y<br>N<br>Y/Y |
| 12- LYMPH NODE[3] | | - | | Y |
| *Carcinomas "deviating" morphologically from classical TCC* | | | | |
| 13- LYMPH NODE[1] | | - | Pseudosarcomatous carci-noma | N |
| 14- BLADDER<br>2 LYMPH NODES<br>LIVER | 3<br>Autopsy | Bladder radiotherapy | Undifferentiated carcinoma with giant cells | N<br>Y/Y<br>Y |
| 15- BLADDER NECK<br>LIVER<br>PITUITARY GLAND | -<br>Autopsy | - | Oat cell-like, but neuro-endocrine marker N-CAM and electronmicroscopy are negative | Y<br>Y<br>Y |
| 16- LUNG[1] | Autopsy | Chemotherapy | Extensive squamous me-taplasia | N |
| 17- BLADDER<br>ABDOMINAL WALL | 26 | Both after chemotherapy | Extensive squamous me-taplasia | N<br>N |

Footnotes: 1) primary in ureter
2) primary in renal pelvis
3) primary in bladder

stains virtually all basal and several suprabasal cells of normal transitional epithelium, whereas squamous metaplastic urothelium is homogeneously positive. In most G1 and G2 TCCs in a series of 55 tumors (predominantly the same series as described earlier[8]), basal and several parabasal cells are positive, whereas a considerable part of the G3 TCCs is negative.

- The mouse monoclonal antibody **Ks8.12**, described to be reactive with CKs13 and 16[17] as well as with CK15[15,18], was purchased from Sigma Chemical Co. through Brunschwig Chemie, Amsterdam, the Netherlands. In normal transitional epithelium, all cells, except for the umbrella cells, are positive. In a series of 59 TCCs[8], this antibody reacted similarly to, although slightly more extensively than, our CK13 antibodies 1C7 and 2D7.

- The mouse monoclonal antibody **LL025**, specific for CK16[15], does not react with normal transitional epithelium and only weakly in squamous metaplastic urothelium, with negative reaction in basal and some parabasal layers. In a series (a part of the series described previously[8]) of 15 TCCs (G1-3) sporadic positive tumor cells were observed only in three out of nine G3 TCCs.

- The mouse monoclonal antibody **E3**, reacting with CK17[19,20], stains normal transitional epithelium heterogeneously, usually reacting with a minority of the basal cells, although in some samples all cell layers were reactive, with the most intense reaction on sporadic umbrella cells. In squamous metaplasia, extensive staining is observed in all but the basal and some parabasal cell layers. In 15 local TCCs (a part of the series described previously[8]), basal cells were stained most intensely, whereas suprabasal cells were stained with variable intensity. In all grades, but especially in G3 TCC, homogeneously positive cases were found as well as less extensively stained tumors. One TCC of the "deviating" tumors, described in our previous report[8], was negative with E3.

- The mouse monoclonal antibody **KA5**, recognizing CKs1, 9 and 10[21], does not stain normal transitional epithelium, whereas squamous metaplasia shows extensive staining except for basal and some parabasal cell layers. In a series of 55 TCCs[8] (G1-3) most of the cancers are negative and only a few cases show sporadic positive cells.

- The mouse monoclonal antibody **CAM5.2**, specific for CK8[5], reacts similarly to M20, another anti-CK8 antibody[8]. With this antibody both normal transitional

epithelium and the 15 TCCs mentioned above were homogeneously positive. Squamous metaplastic urothelium is negative. With respect to CAM5.2, it should be kept in mind that this antibody reacted weakly with purified CK7[22] (unpublished results from CM Alexander, PC Stasiak and EB Lane[5]).

- The mouse monoclonal antibody **RV202**, is directed against vimentin[13].

## RESULTS

The CK expression patterns seen in the series of TCC metastases from 17 patients, including 10 cases with corresponding local tumors, are summarized in Table 2 and depicted in Figures 1 to 4. Morphologically the TCCs could be subdivided into two groups, one with features of classical TCCs and the other group with a morphology deviating from classical TCC.

### *CK EXPRESSION IN "CLASSICAL" TCCs AND THEIR METASTASES.*

In 12 cases ( with 22 specimens), the lesions were morphologically typical transitional cell carcinomas.

No consistent differences in the expression patterns of the different CKs were found when the local TCC and hematogenic or lymphogenic metastasis were compared. The vimentin antibody did not stain tumor cells, and the broad-spectrum epithelial marker RCK102 stained all cases extensively, as did LP2K (to CK19). The reactivity patterns of the other antibodies reacting with individual CKs can be summarized as follows:

### *Cytokeratin 7.*

Homogeneous positive reaction was observed in the 22 specimens of both local and metastatic TCC, indicating a complete conservation of CK7 during malignant progression.

**Table 2.**

| Antibody | 6B10 | 1C7 2D7 | Ks8.12 | LL025 | RKSE60 10 KA5 | E3 | KA1 | LL001 LL002 | RCK107 |
|---|---|---|---|---|---|---|---|---|---|
| Cytokeratin subtype | 4 | 13 | 13, 15,16 | 16 | 1,9,10 | 17 | 5,14 | 14 | 14 |
| Normal** urothelium | $S^0$ | $B^2$ | $B^2$ | - | - | -/$B^2$ to + | $B^2$ | - | B/- |
| Invasive G3 TCC | - to + | - to + | - to + | - to S | - to -/+ | -/B to + | - to $B^2$ and + | - to $B^2$ and + | - to $B^2$ and + |
| "Classical" TCC | | | | | | | | | |
| 1-Local | - | - | - | - | - | + | $B^2$ | - | ±1/± |
| Meta | - | - | S | - | - | $B^3$/- | $B^2$/- | - | $B^2$/- |
| 2-Local | S | S | S | S | S | -/$B^2$ | -/B | -/$B^3$ | -/$B^3$ |
| Meta | S | - | F | - | - | ±[1]/- | +/$B^4$ | -/$B^3$ | -/$B^{3,6}$ |
| 3-Local | S | -/B | -/$B^3$ | -/+ | S | ±/+[1] | ±[1]/+ | -/B | +/±[1] |
| Meta | S | -/+ | S | S | - | + | + | S | + |
| 4-Local | - | - | - | - | - | -/$B^3$ | $S^1$ | S | -/B |
| Meta | S | S | $S^6$ | - | - | +/±[4] | - | - | -/$B^2$ |
| 5-Local | S | - | -/B | S | S | $B^3$/- | -/+[1] | +/- | F |
| Meta | - | - | -/$B^3$ | - | $S^6$ | S | +[1]/F | +/$B^2$ | - |
| 6-Local | - | - | -/± | F | - | B/± | S | B/$B^2$ | B/$B^2$ |
| Meta | S | S | S | S | - | F | S | - | S |
| 7-Local | F | ±[1,5] | ±[1,5] | - | - | + | B | +/- | +/±[1] |
| Meta | F | +/- | +/- | +/- | - | + | +[1]/$B^4$ | +/- | + |
| 8-Meta | - | S | S | - | - | -/B | - | - | - |
| 9-Meta | S | S | $B^2$/- | - | - | $B^2$/+[4] | S | - | $B^2$/- |
| 10-Meta | - | - | S | S | - | +/- | S | S | +[4]/$B^2$ |
| 11-Meta | F | + | + | S | - | +/- | +/- | S | $B^2$/- |
| Meta | F | + | + | - | - | -/$B^2$ | B/+[4] | -/B | -/B |
| Meta | -/+[1] | + | + | F | S | -/B | + | -/B | $B^2$/- |
| Meta | F | + | + | - | S | ±[1]/- | $B^3$/- | -/B | $B^2$/- |
| 12-Meta | S | F | $S^1$ | - | - | + | $S^1$ | $B^2$/- | +/±[1] |

Carcinomas, morphologically "deviating" from classical TCC

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 13-Meta | - | - | - | - | - | - | - | - | - |
| 14-Local | - | - | - | - | - | B[6]/- | + | S | S |
| Meta | - | - | F | - | - | +/- | + | S | +/- |
| Meta | - | - | +/- | - | +/-[5] | +/- | + | S | +/- |
| Meta | - | - | +/- | - | - | +/- | + | - | +[5]/- |
| 15-Local | F | +/B[4] | +/- | S | - | B/- | +/- | S | B/- |
| Meta | S | S | +/- | - | - | B[3]/- | S | - | B/- |
| Meta | S | S | S | - | - | -/B | S[1] | S[1] | F[1] |
| 16-Meta | - | - | +/- | -/+ | - | +/- | +[4] | -/+[1] | + |
| 17-Local | F | +/+[7] | + | +/- | S[8] | S | + | +/B[2] | B[2]/- |
| Meta [9] | S | + | + | B[2]/- | - | F[1] | + | B[2]/- | B[2]/- |

Immunohistochemical staining results of cytokeratin markers for stratified squamous and transitional epithelium and basal cell components in TCC metastases and several autologous local high-grade malignant TCCs, showing heterogeneous relations between the results with the different cytokeratins, between antibodies recognizing the same cytokeratins, and sometimes between samples from one case.

./. Two staining patterns are observed: dominating pattern of the tumor (>50%) / minor pattern (<50%).
S Sporadically positive cells (<5%).
F Focally dispersed positive cells (5% to 30%)
+ Homogeneously strongly positive
± Weakly positive
- No staining observed
B Basal cells positive

0) only positive in superficial (umbrella) cells.
1) (several) cells, which are adjacent to the stroma, i.e. "basal" cells, are more intensely stained.
2) also staining in several parabasal cell layers; in normal transitional epithelium are all cells except the umbrella cells positive with Ks8.12.
3) in small areas also staining of all epithelial cells.
4) small totally negative areas are present.
5) the most intense staining reaction is situated at the periphery of the whole tumor lesion.
6) in several cells the most intense staining is present at the stromal side of basal cells.
7) basal cells are negative.
8) negative with antibody KA5.
9) tumor localisation in the abdominal wall, 26 month after cystectomy.
**) data described in references 13 and 8

The different CK8 and CK18 antibodies showed different staining patterns. LE41 stained fewer tumor cells than the other two CK8 antibodies CAM5.2 and M20. The CK18 antibody 2C8 stained fewer tumor cells than RGE53, which in its turn stained less extensively than the other CK18 antibodies CK18-2 and RCK106. As a result of the different degree of staining (both qualitatively and quantitatively), particular staining patterns emerged, which can be described as follows (Figures 1 and 2a-h):

1) Peripherally located carcinoma cell clusters within a tumor lesion were more intensely and extensively stained than the more centrally located cell clusters (Figures 2a-c). This staining pattern was most prominent with LE41 and 2C8 in cases 3 (liver), 4 (lymph node), 5 (lymph node), 6 (renal pelvis), 8, 9, 14 (lymph node) and 15 (pititary).

2) Peripheral individual tumor cells within a given tumor cell cluster ("basal" cells) reacted more frequently and more strongly than those located more centrally

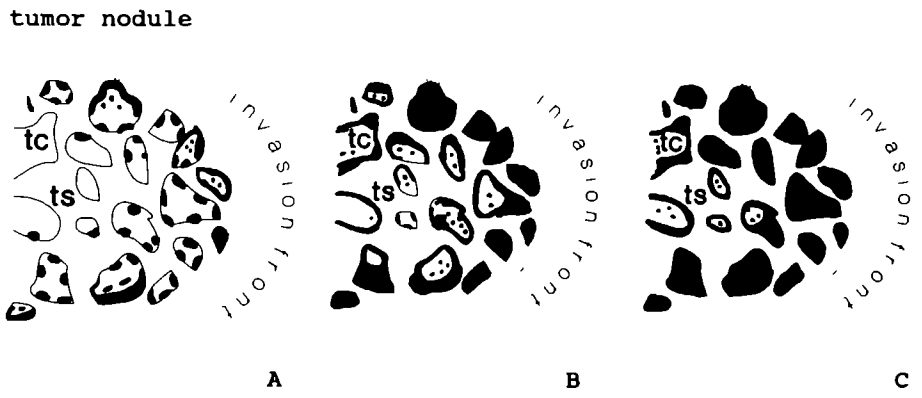


**Figure 1.** Schematic representation of the "interface phenomenon" in TCC based on CK8 and/or CK18 staining. It is characterized by preferential staining of carcinoma cells located at the interface with stroma, especially in the invasion front. This results in a gradient in expression from the invasion front to the center of the tumor. The degree of staining (**A** local expression, **B** moderate extensive expression, **C** extensive expression) depends on the antibody used as well as tumor qualities.

within the cluster, depending on which antibody was used (Figures 2d,e) and on the localisation of the cluster in the whole tumor lesion (see above). This staining pattern was most prominent with LE41 and 2C8 and in a lesser degree with RGE53 and was observed only a few times with the other, more extensive staining antibodies (cases 3 [bladder], 9 and 11 [lung and lymph node]). In cell clusters with minimal CK8 and CK18 expression, staining was often observed to be polarised within the cell, located at the stromal side of individual basal cells (Figures 2f,g).

3) In small tumor clusters, relatively more cells seemed to be positive than in larger tumor cell groups (Figure 2h).

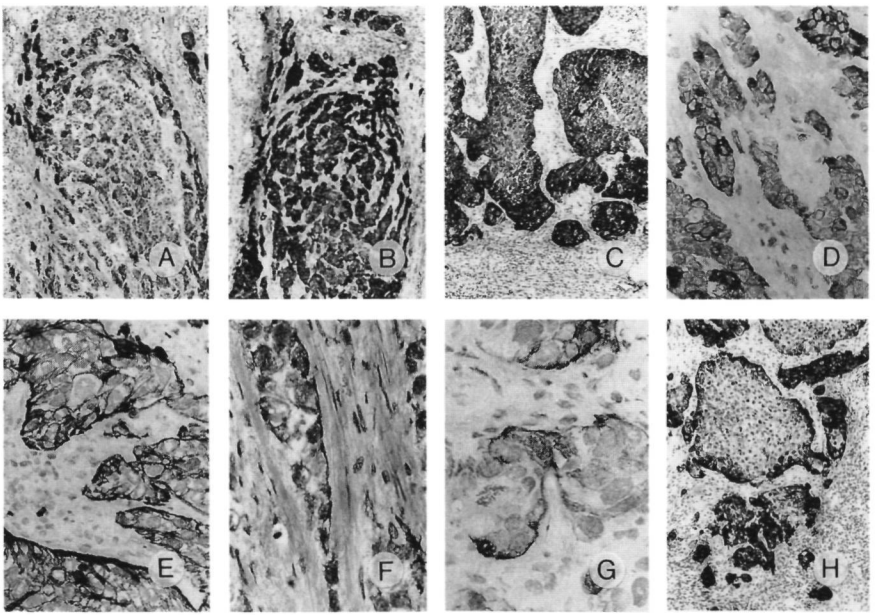


**Figure 2.** Immunoperoxidase staining patterns of frozen sections of local transitional cell carcinomas or metastases, showing typical staining patterns for the different CK8- and CK18-antibodies, illustrated for 2C8 (**A**, case 4 - lymph node metastasis), CK18-2 (**B**, same case as in A), RCK106 (**C**, case 9), RGE53 (**D**, case 3 - bladder), LE41 (**E**, case 6 - lymph node), RGE53 (**F**, case 3 - bladder), 2C8 (**G**, case 3 - bladder) and RCK106 (**H**, case 9). Magnifications A-C,H x50; D-F x200; G x300.

These distribution patterns were observed consistently, and they hold true for the local TCC as well as the metastases. In some cases the phenomenon described above was less prominent when a diffuse strong staining reaction was observed even with the generally "less extensive" staining antibodies (LE41, 2C8 and RGE53). In fact, the expression of CK8 and CK18 appeared to be conserved throughout the final steps of malignant progression.

*Cytokeratin 4 and 13.*

Expression of CK13, as recognized by the antibodies 1C7 and 2D7, was low in 10 out of the 12 cases and only sporadically positive cells were found in most of these local and metastatic tumors (Figure 3a). In the remaining two cases, numbers 7 and 11, all 6 specimens, including one local TCC were extensively positive for both CK13 antibodies (Figures 3b,c).
Expression of CK4 (Table 2) was also very low in most cases and was found to be associated with the degree of CK13 expression. The two cases that were extensively positive for CK13 showed a slightly higher expression level for CK4 as compared to the other 10 cases (Figure 3d).

*Cytokeratin 16.*

CK16 (Table 2), as recognized by LL025, was mostly found to be focally expressed in a few dispersed cells in a minority of the local and metastatic tumors (Figure 3e). The antibody Ks8.12, which recognizes CK13, CK16 and CK15, stained the TCCs more extensively than the monospecific CK13 and CK16 antibodies taken together, suggesting the presence of some CK15. In 3 cases that were all negative with the two specific CK13 antibodies, and of which only one showed some positive reaction with the monospecific CK16 antibody LL025, there was positive staining with Ks8.12.

*Cytokeratin 17.*

The expression of CK17 (Table 2) was variable between the samples, even within

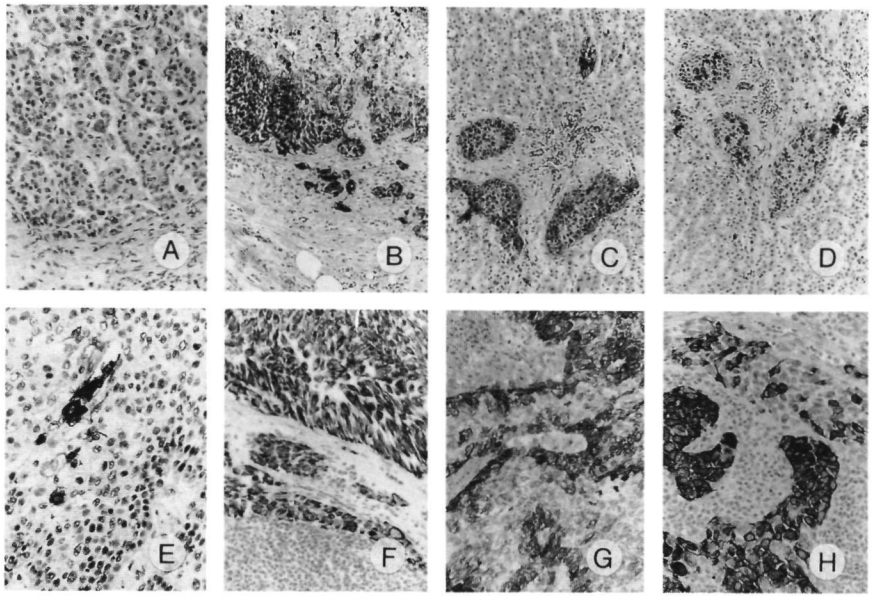


**Figure 3.** Immunoperoxidase staining patterns of frozen sections of local transitional cell carcinomas or metastases, including 2D7 (**A, B, C**, respectively case 4 - lymph node, case 7 - lymph node, case 11 - liver), 6B10 (**D**, case 11 - liver), LL025 (**E**, case 6 - kidney), E3 (**F, G, H**, case 7 -lymph node, case 6 - kidney and case 10, respectively). Magnifications B-D,F,H x50; A,G x75; E x100.

individual cases (Figures 3f-h). Extensively positive cases were found next to cases that showed sporadic positivity in the high-grade malignant local TCC as well as in metastases.

*Cytokeratins 1, 9 and 10.*

Only sporadic staining was observed in some tumor cells with antibodies RKSE60 and KA5, used to recognize cytokeratins expressed in keratinizing differentiation. Their expression appeared unrelated to expression of other CKs (Table 2).

*Cytokeratin 14.*

The three monoclonal antibodies that specifically react with CK14 (Table 2) all gave similar staining patterns when the local and metastatic TCCs were compared. They preferentially stained the basal and parabasal cells (ie, cells at the interface with the stroma) with occasional staining in virtually all tumor cells. Antibodies LL001 and LL002 reacted similarly. The antibody RCK107 stained more cases than the former two, and its reaction pattern was more extensive, in that it stained more cells. Antibody KA1, reacting with both CK14 and CK5, reacted in a pattern that was neither related to the CK14-specific antibodies nor to any of the other CK antibodies.

*CK EXPRESSION IN CARCINOMAS "DEVIATING" MORPHOLOGICALLY FROM CLASSICAL TCC.*

The 5 nonclassical TCCs included 1) a (pseudosarcomatous) spindle cell carcinoma (case 13), 2) an undifferentiated TCC with tumor giant cells and local spindle cell differentiation (case 14), 3) a poorly differentiated carcinoma with small basophylic cells (case 15) and 4) two cases of TCC with varying degrees of squamous differentiation (cases 16 and 17).

In this heterogeneous group of tumors striking differences are observed in CK expression in comparison to the classical TCCs. In the pseudosarcoma (case 13), only vimentin was homogeneously positive expressed. CK8 and CK18 were heterogeneously extensive expressed with a gradient pattern for the different antibodies, whereas only LE41 gave a negative staining result. The CK8 and CK18 did not show zonal differences in staining intensities. A limited expression was found for CK19. Antibody RCK102 reacted extensively positive.

In case 14 (Figure 4a) the bulk of the tumor showed a comparable reaction pattern to the classical TCCs (for example, Figure 4b), with the exception of a variable but mostly extensive vimentin staining reaction (Figure 4c). The giant cells showed a staining pattern similar to that of the other tumor cells. In only one sample from this case was a relationship seen between CK8 expression (with

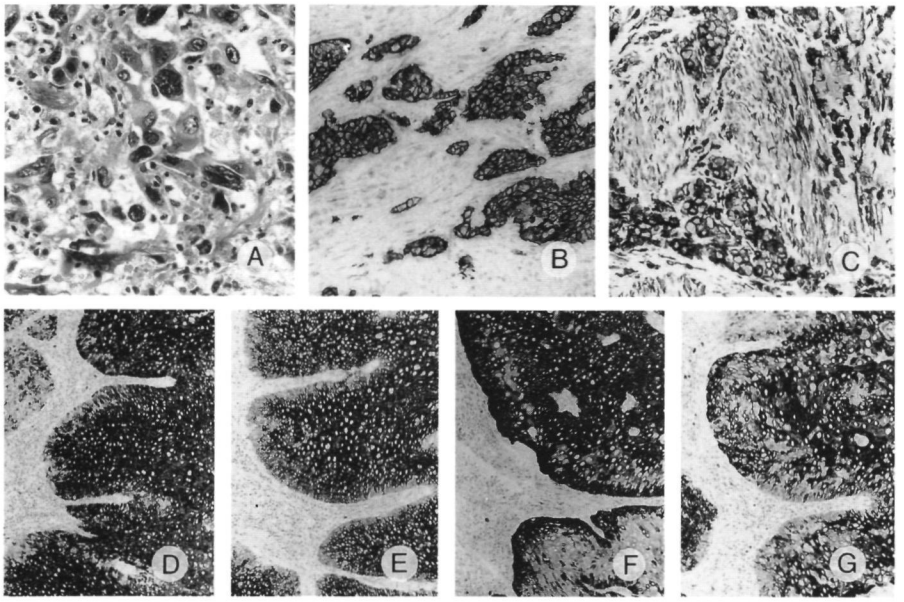


**Figure 4. (A)** "Undifferentiated" carcinoma of the bladder, shown in a H&E-stained section (case 14). **(B-G)** Immunoperoxidase staining patterns of frozen sections of local and metastatic urinary tract carcinomas, morphologically deviating from the typical transitional cell carcinoma. The sections were incubated with antibodies LP2K (**B**, case 14 - bladder), RV202 (**C**, case 14 - bladder), and LP2K (**D**), 2D7 (**E**), LL002 (**F**) and LL025 (**G**) from case 17 (bladder). Magnifications D-G x50; A-C x230.

LE41 in a lymph node) and the peripheral location of tumor cells (see above). The expression pattern of case 15 did not substantially differ from that of the classical TCCs, except for a decreased expression of CK18.

Cases 16 and 17, both with squamous differentiation, showed almost complete absence of CK7 expression, an extensive decrease of CK18 and a slight decrease in CK8, predominantly detected with LE41, as compared to the classical TCCs. CK19 was focally less extensively expressed (Figure 4d). In case 16, extensive expression of CK4 and CK13 (Figure 4e) was observed, and in both cases 16 and 17, extensive expression of CK14 (Figure 4f) and CK16 (Figure 4g) also were found, whereas CK10 and CK17 antibodies did not stain significantly more cells than in all other TCCs.

## DISCUSSION

Several rules for the distribution of the various cytokeratins and their combinations have been described for adult human epithelial tissues, both in the normal tissues and benign as well as malignant tumors[23]. For example, CKs 4 and 13 are related to epithelial stratification, whereas CKs7, 8, 18 and 19 are described to be merely associated with glandular differentiation[1,3,24]. After examination of extensive series of normal and malignant epthelial tissues, however, several exceptions to these general rules were reported[9,12]. For example, our immunohistochemical studies on urothelium[13] and cancers derived there from[8] showed alterations in cytokeratin expression related to tumor progression. Another complicating factor is introduced by a diversity in reaction patterns of individual monoclonal antibodies all reactive with the same CK protein. Furthermore different tumor compartments showed differences in their cytokeratin expression patterns, whereas the morphologic phenotype also influences the intermediate filament composition of cells. In the present study, we compared the cytokeratin expression patterns in local high-grade malignant TCCs, ie, a primary tumor or a local recurrence, to those in TCC metastases, with special emphasis on aberrations as described above.

*Increased expression of cytokeratins 8 and 18 in areas of tumor-stroma interaction.*

From the results presented here, the increased detectability of CK8 and CK18, reported earlier by us to occur during malignant progression of TCC[8,25] can be placed in a new perspective. By the use of our panel of specific CK8 and CK18 antibodies, which show different reaction patterns in normal and malignant urothelium[8,13], given tumor areas were positive for a set of CK8 and CK18 antibodies, whereas on the same area another set of such antibodies gave negative results. The use of this panel of antibodies showing different degrees of reactivity with CK8 and CK18 allows a crude histochemical titration of antigen accessibility, if not protein quantity. Quantitation at the cell level is generally not possible with the immunoperoxidase detection because the method depends for

its sensitivity upon a saturation amplification reaction. Furthermore we observed regional differences in the staining intensity of TCCs with the antibodies. We often noticed the most intense reaction in tumor cells in close proximity to stroma and in the periphery of the tumor nodule, ie, the invasion front. At one extreme, in several cases antibodies LE41 and 2C8 showed exclusive reactivity with cells in these peripheral areas. Similar observations were made in the invasion front of otherwise CK8- and CK18-negative non-urological squamous cell carcinomas (Schaafsma, to be published) and in epidermal neoplasia[28]. Cintorino et al[27] also commented upon more intense staining reaction at the front of a deeply infiltrating TCC. We interpret this staining pattern as an "interface phenomenon", as we think that the expression in the tumor cells is influenced by their micro-environment, ie, the stroma. Therefore the tumor cells at the interface with the stroma show the most prominent changes in expression.

We would suggest that either these different antibodies recognize different configurations of the CK8 and CK18 antigens or that these areas contain higher amounts of these proteins. The suggestion made in our previous report[8] that reactivity with LE41 or 2C8 of high-grade malignant TCC cells in local tumors might detect a potentially metastatic clone, is not supported by our current findings in TCC metastases. The fact that the metastatic lesions showed similar regional distribution patterns of CK8 and CK18 epitope configurations is more in line with the idea of stromal components influencing the phenotype of adjacent tumor cells. The reason for not appreciating this phenomenon in our earlier study lies in the fact that in the previous study, small fragments of transurethral tumor resections were used, making orientation very difficult. We did, however, observe and report the more extensive reaction in the infiltrative tumor compartment as compared to the exophytic growing high-grade malignant component[8]. Extensive CK8 and CK18 expression, as observed in umbrella cells of normal transitional epithelium, as well in tumor areas in close proximity to stromal cells could be a result of related phenomena. In both situations the CK8/18 positive cells "seal off" the epithelium from its environment. Further studies are in progress to examine the cell biological background of these observations. In our experiments we will explore two possible explanations. One is the possible relationship between CK8/18 expression, characteristic of secretory or glandular cells, and a

possible secretory function of the TCC cells at the stroma border, ie, specialization of tumor cells. The second possibility is that a relation exists between the quantitative and qualitative changes in the cytoskeleton of these cells and the loss of differentiation in neoplasms[11] or immaturity in embryological tissues[28], resulting in increased flexibility of the cytoplasm, as might be needed for cell migration.

*Cytokeratin 13 can be expressed extensively in TCC metastases.*

Our earlier observations[8,13] and those of Moll et al[10] showed that with progression of TCC (G1pTa to G3pT2), CK13 expression decreased in the tumor cells. Therefore we assumed to find low CK13 expression levels in TCC metastases. In an extended series of metastatic TCCs, this hypothesis was only partly confirmed by the present study. The 23 metastases examined showed CK13 expression similar to that described for high-grade malignant local TCCs[8]. Of the 10 cases in which the reaction patterns could be directly compared to those in the matching local cancer, nine cases showed a similar staining pattern in both tumor stages. For example, in three cases (cases 7, 11 and 17), where the local TCC showed an extensive CK13 expression, this was also found in the metastases. In other cases (except one), where the local tumor showed low CK13 levels, the metastasis also showed a very limited reaction with the CK13 antibodies. Case 15 showed extensive CK13 reactivity in the local tumor but loss of this reactivity in the metastases. Only in one case (no. 17) could squamous metaplasia explain the extensive CK13 expression in this otherwise typical high-grade malignant TCC. Moll et al[10] descibed absence of CK13 in their series of five TCC metastases except for one case, which also showed squamous metaplasia. In our series the extensive CK13 expression of two cases (cases 7 and 11) remained unexplained, although in one case the cytoplasm of the tumor cells was relatively large and eosinophilic as seen by hematoxylin and eosin (H&E) staining, which might indicate early stages of squamous differentiation. It should be noted, however, that carcinomas of the squamous phenotype do not necessarily contain CK13. For example, Broers et al[11] reported a decrease in CK13 expression upon dedifferentiation of squamous cell carcinomas of the lung, resulting in 50% CK13

negative cases in poorly differentiated squamous carcinomas. Kuruc et al[29] showed that CK13 is not expressed in squamous cell carcinomas of the skin, although this had been expected on basis of results of Nischt et al[30] and because of its transient expression in certain stages of fetal skin development[31]. Our preliminary studies in head and neck mucosal squamous cell carcinomas are in line with these observations, in that CK13 is not a major component in these tumors (to be published).

*Cytokeratin 16 as an indicator of squamous differentiation*

CK16 has been reported to be related to hyperproliferation in stratified epithelial cells[6,32,33], but it has also been correlated with a basal cell phenotype in certain malignancies[15]. As expected, we did not find a significant reaction in normal transitional epithelium for CK16. In squamous metaplasia in the bladder, however, CK16 expression was found in most suprabasal cells. In both local bladder cancers as well as in the metastases, CK16 (as recognized by LLO25) was only found sporadically in scatterd cells, except for the two cases with squamous metaplasia, which displayed more extensive positive reactivity. Our immunohistochemical observations concerning CK16 are generally in line with gel electrophoretic data[10,34]. The results suggest a relation of CK16 with squamous differentiation, rather than with proliferative state, at least in the bladder. This assumption may be supported by the observation that the metastasis of case 7 also showed a relatively high CK16 level. In this case, CK16 expression might be related to the expression of CK13, a marker for stratified, squamous epithelium which is not keratinizing. In this particular case, however, morphological characteristics for such a type of differentiation were not found, although a suggestion was present in the extensive eosinophilic cytoplasm of some cells.

Antibody Ks8.12 showed a more extensive staining pattern than we would expect from the combined results of antibodies 1C7, 2D7 and LLO25. Recent reports now suggest that this antibody reacts with CK15 [15,18] as well as the earlier described CK13 and CK16 reactivity. Data presented here therefore point to the expression of CK15 particularly in TCCs with "deviating" morphology, but not or to a much lesser extent in the typical TCCs. This might also explain why

expression of CK15 in TCC was not reported earlier[10,34].

*Cytokeratin 7 as marker for transitional cell differentiation*

In normal transitional epithelium, CK7 is expressed homogeneously in the upper urinary tract but heterogeneously in the lower part[13]. This is observed with two independent monoclonal antibodies to CK7[12]. In TCCs, also those in the bladder, the expression of CK7 is in general homogeneous. In the differential diagnosis of carcinomas, CK7 can be used as an indicator for urothelial differentiation[12], especially in male, where it can distinguish urothelial malignancies from, for example prostate cancer, renal cell carcinomas etc.. We must keep in mind, however, that the expression of CK7 can be found to be decreased, particularly when squamous differentiation is present[8].

*Cytokeratin 17*

Using our immunohistochemical procedure, the expression of CK17 appears to be less extensive in normal urothelium than in local TCC and metastases. No relation was observed between advanced stage and increased CK17 expression, because a number of advanced cancers with only limited expression of CK17 were also found. No morphological explanation or relation to expression patterns of other CKs could be found in these TCCs with limited CK17 expression. It was striking that, in the two TCC cases with squamous metaplasia, no increase of CK17 expression could be seen, although extensive CK17 expression was found in normal squamous metaplastic urothelium. Our immunohistochemical results appeared to be in line with the gel electrophoretic data of Moll et al[10].

*Cytokeratin 14*

In this study, the panel of CK14 antibodies was extended from our earlier study. Two different sets of results were obtained. LL001 and LL002 were both negative in normal transitional epithelium; conversely, RCK107 was reactive with several basal cells in normal urothelium. This difference also was present in the

cancers, frequently with more staining by the latter antibody. In general, LL001 and LL002 reacted similarly. In our previous study[9], an increase of CK14 expression was noted in parts of the tumors during tumor progression. We reported that this increase was not consistently present in tumors infiltrating muscle. From the results of the present study, we could even state that there is no relation of CK14 expression and the metastatic potential of TCC. Extensive CK14 expression was found only in one case of the two TCCs with squamous metaplasia. No constant relation between increase of CK14 and CK13 expression was detected. The antibody KA1, described as recognizing complexes of CK5 and CK14, reacted extensively in basal cells of normal transitional epithelium and in the majority of G1 and G2 TCCs studied in our series. These results are in accord with data of Moll et al[10]. No consistent pattern of CK expression was noted with increasing degree of malignancy for this antibody.

Independent of morphological characteristics, the expression patterns of individual CKs observed in this series of TCCs showed interrelationships only for CK8 and CK18 and to a minor degree between CK4 and CK13; some crude quantititative relation appeared to exist between CK17 and CK14 if CK14 expression was determined with antibody RCK107.

*Conclusions.* We conclude from the present and previous studies that for human urothelium, CK expression is not fully conservative during neoplastic progression. Expression patterns in TCC depend on morphological characteristics and type of differentiation, the degree of anaplasia, and on interactions with the microenvironment.

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# CHAPTER V

# Increased expression of cytokeratins 8, 18 and vimentin in the invasion front of mucosal squamous cell carcinoma

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## ABSTRACT

The immunohistochemical expression patterns of cytokeratins 8 and 18 and of vimentin were examined in frozen sections of 120 human mucosal squamous cell carcinomas with special emphasis to the topological distribution in the tumour. This was done in order to evaluate in squamous cell carcinoma a particular expression pattern observed recently by us in transitional cell carcinoma of the urinary tract and designated as "interface phenomenon". This phenomenon implying maximal expression of cytokeratins 8 and 18 at the tumour front and to a lesser extent also in areas of intratumorous stroma contact, was also found in about 50% of the squamous cell carcinomas examined. It was also found for vimentin, which contrasted with transitional cell carcinoma. The percentages of occurrence of the phenomenon varied for the different sites of origin of the tumour. Tumour grade did not influence the results. These findings further supported the idea that invasive carcinoma cells interacting with the stromal micro-environment display a characteristic intermediate filament phenotype that deviated from the pattern expected on the basis of their direction of differentiation. These changes might reflect a phenotype involved in invasive, migrating, and proliferating activities.

## INTRODUCTION

In early studies of intermediate filament protein expression, cytokeratin and vimentin were considered as typical for epithelial and mesenchymal differentiation, respectively [1]. However, an increasing number of reports indicated coexpression of vimentin and cytokeratins in nonneoplastic and neoplastic epithelium (for reviews see [2,3]) thus complicating these initial expectations. Even coexpression of cytokeratin in mesenchymal tissue was observed [3,4]. With respect to the different cytokeratin polypeptides occurring in specific combinations within the different epithelia [6], the early reports also suggested a strong preservation of the original combination of cytokeratins in a specific epithelium during malignant transformation [5]. However, when comparing the normal situation to a diversity

of biological in vivo and in vitro situations such as hyperproliferation [6], premalignancy [7], neoplastic growth [8-10] or tissue culture [11], changes in cytokeratin expression patterns were also observed.

The original "rules" of vimentin and cytokeratin expression are still helpfull, although new insights have shown the situation to be more complex. For example, cytokeratin 7 is, as a rule, expressed in many simple epithelia and remains predominantly restricted to adenocarcinomas [12]. It is only sporadically and focally expressed in squamous cell carcinomas [12,13]. On the other hand, cytokeratins 8 and 18, also considered as specific for simple epithelia in earlier studies [5,14], are found variably in squamous cell carcinomas [5,9,13,15]. A positive correlation between increased expression of cytokeratin 8 and 18 and an increase in the histological grade of anaplasia [9] had been reported. Only recently, a relationship of cytokeratins 8 and 18 expression with location of carcinoma cells at the tumour invasion front or in other areas of stroma interaction [16,17] had been noticed. An increased expression of vimentin in carcinomas was found to be related to parameters of malignant potential such as grade, growth fraction, hormone independency and survival rate [18-22]. Encouraged by these data we have studied such topographical expression patterns in carcinomas, in particular those with expected low amounts of cytokeratin 8, 18 and vimentin, i.e. squamous cell carcinomas [5].

## MATERIALS AND METHODS

*Tissues.* One hundred and twenty fresh tumour samples of squamous cell carcinomas from different mucosal sites were obtained after surgery (Tables 1 and 2). A small sample from the specimen was snap frozen in liquid nitrogen and stored until further use. From the head and neck region we collected 80 samples of non-irradiated, squamous cell carcinoma lesions (from 61 different patients) including 65 primary tumours and 15 samples from lymph node metastases.

Forty squamous cell carcinoma samples were derived from the vulva, oesophagus and lung.

Spindle cell carcinomas were not included in this series.
Samples were preferentially taken from the transition zone between tumour and surrounding tissue. The pathology diagnosis was made on routinely processed tissue samples. The carcinomas were graded according to WHO criteria [23].

*Immunohistochemistry.* The indirect immunoperoxidase technique was applied to approximately 5 micron thick parallel cryostat sections essentially as described before [8]. The panel of monoclonal antibodies consisted of the chain-specific anti-cytokeratin 8 antibodies Cam5.2 [15,24,25] and M20 [4,8], the anti-cytokeratin 18 antibodies 2C8 [8], RGE53 [14], CK18-2 [8] and RCK106 [8], and the anti-vimentin antibody RV202 [8]. The slides were assessed by one experienced pathologist. The degree of cytokeratin staining was scored as - (negative), ± (limited staining, i.e. less than 50% of the cells were positive) or + (extensive staining, i.e. more than 50% of were positive). In some occasions a sporadical positivity was mentioned separately representing less than 1% positive cells. Vimentin staining was scored in only two divergent groups, one showing extensive positivity (more than 75% of the carcinoma cells were positive) and the other showing focal positivity (less than 25% of the cells were positive).

## RESULTS

*Expression of vimentin.* (Table 1 and Figure 1)

In each carcinoma several scattered positive cells could be detected, which we considered to represent lymphoid cells, and were therefore left out of consideration. Most of the carcinomas examined were predominantly negative for vimentin. In 58 (48%) samples vimentin positive carcinoma cells were found. Two types of vimentin staining patterns of the tumour cells could be observed (see Table 1). The first pattern consisted of a randomly extensive staining of (large) areas of the carcinoma (Figures 1a-d). The second pattern was formed by (small) positive cells clusters of basal and parabasal cells, located preferentially in extensions protruding from the tumour cell clusters (Figures 1e-g). The

**Table 1:** Vimentin staining patterns in mucosal squamous cell carcinomas.

| tumour site, grade and number of cases | | EXTENSIVE staining * | LIMITED staining ** | total number of positive cases (percentage) |
|---|---|---|---|---|
| FLOOR of MOUTH | G1 n=3 | | 2 | 8 (57%) |
| | G2 n=7 | - | 3 | |
| | G3 n=4 | | 3 | |
| TONGUE | G1 n=5 | - | 3 | 13 (72%) |
| | G2 n=10 | 4 | 4 | |
| | G3 n=3 | 2 | - | |
| TONSIL | G1 n=4 | - | 3 | 7 (64%) |
| | G2 n=5 | 1 | 2 | |
| | G3 n=2 | - | 1 | |
| LARYNX | G1 n=10 | | 2 | 11 (30%) |
| | G2 n=8 | - | 3 | |
| | G3 n=19 | | 6 | |
| LUNG | G1 n=9 | 1 | 1 | 9 (33%) |
| | G2 n=10 | - | 3 | |
| | G3 n=8 | - | 4 | |
| OESOPHAGUS | G2 n=3 | - | 3 | 4 (66%) |
| | G3 n=3 | | 1 | |
| VULVA | G1 n=2 | - | 1 | 6 (86%) |
| | G2 n=5 | | 5 | |

* Extensive positivity in more than 75% of the tumour cells
** Focal positivity in less than 25% of the tumour cells, especially in which cells located in small tumour cell clusters and protrusions were stained.

number of vimentin positive cases was approximately the same in samples containing the most peripheral part of a tumour as compared to samples containing centrally located portions. The smallest cell clusters that showed strong vimentin staining were most frequently found in the outer rim of the conglomerate. The number of vimentin positive epithelial cells in the protrusions varied, ranging from a few cells to many cells in most of the buddings. The frequency of vimentin positive cases differed between carcinomas from different sites of origin (Table 1). The 15 metastases from the head and neck carcinomas showed equal vimentin expression levels as their primary lesions. No relation

was found between the presence of vimentin expression and the degree of keratinization or other parameters of tumour grade.

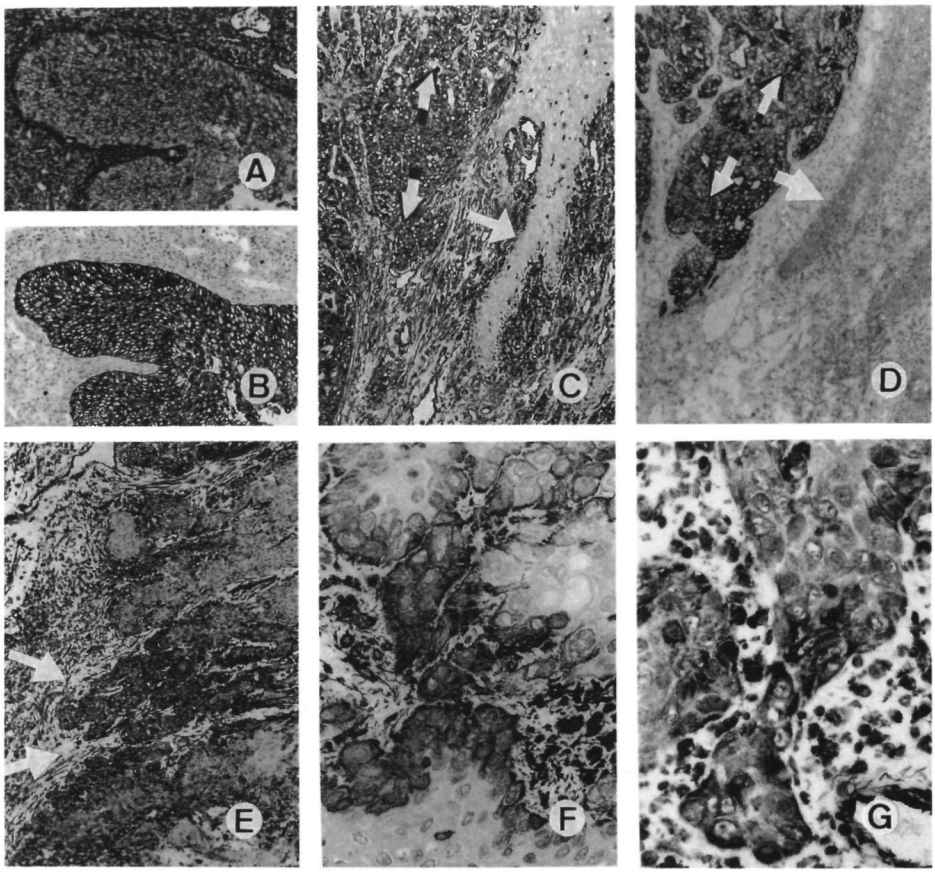


**Figure 1.** Immunoperoxidase staining in frozen sections of mucosal squamous cell carcinomas. Extensive vimentin staining is shown in **A** and **C**, while in parallel sections (**B** stained with M20; **D** stained with CAM5.2) the carcinomatous area (in **C** and **D** small arrows are placed inside a cluster of carcinoma cells) also shows expression of cytokeratin 8. Note in parallel sections **C** and **D** that adjacent squamous epithelium (large arrow) is negative for cytokeratin 8 (CAM5.2) and vimentin.
**E-G**: Preferential vimentin expression in the tumour front (**E**; front at the left side, see small arrows) and tumour buds (**F** and **G**)
Magnifications: A,B 75X; C,D 150X; E 125X; F 250X; G 500X. Tumour origin: tongue (A-D), tonsil (E), lung (F), vulva (G).

*Expression of cytokeratins 8 and 18* (Tables 2 and 3; Figure 2)

**Expression According to Cell Types**

Both cytokeratins 8 and 18 were frequently (in 90% of the cases) expressed in the squamous cell carcinomas examined (Table 2). Their distribution pattern was heterogeneous, while the number of positive cells varied considerably between different cases and between different regions within the same tumour. Cytokeratins 8 and 18 were found to be preferentially expressed in the peripheral (basal) cells of the tumour cell clusters, often extending into the parabasal region. In some cases all cells of a tumour cell cluster were found to be positive. Occasionally a rim of cytokeratin staining was found only at the stromal side of basal cells (Figures 2a,b).

In sporadic cases (6 out of 120 samples) cytokeratin 8 and 18 staining was found focally in the suprabasal cell layers (Figure 2c) or only in the layers bordering cornifying cells (Figure 2d), while the other layers were predominantly negative.

**Expression According to Tumour Area**

When surveying the architecture of the tumour it became evident that cytokeratin 8 and 18 expression was often found in the area of the tumour front with the surrounding tissue. This phenomenon was observed in about half of the 74 samples, in which the outer rim of the tumour was present (see Table 3 and Figures 2e-g). The basal and parabasal cells at the tip of the protruded cell clusters often showed the highest expression levels. This phenomenon was observed in tumours of different grade and degree of keratinization.

**Cytokeratin Gradients as Detected by Different Antibodies**

The 2 anti-cytokeratin 8 antibodies Cam5.2 and M20, produced similar staining patterns, although Cam5.2 staining patterns were generally slightly stronger and/or more extensive than those for M20. In 8 head and neck carcinomas, however a significantly lower staining level or even a negative result was seen with M20 (Figure 3), while Cam5.2 stained more extensively.

The four different anti-cytokeratin 18 monoclonal antibodies also showed quantitative differences with respect to the proportion of positive tumour cells (Figures 2h-j and Figure 3). Firstly, the antibodies RCK106 and CK18-2 stained

**Table 2:** Cytokeratin 8 and 18 levels as detected by antibodies CAM5.2 and RCK106, respectively, in mucosal squamous cell carcinomas and scored to tumour grade, irrespective of tumour area.

| tumour site, grade and number of cases | | EXTENSIVE staining | | | LIMITED staining | | | NEGATIVE |
|---|---|---|---|---|---|---|---|---|
| | | 8*+ 18+ | 8+ 18± | 8± 18+ | 8± 18± | 8± 18- | 8- 18± | |
| FLOOR of MOUTH | G1 n=3 | | 1 | 1 | 1 | | | |
| | G2 n=7 | | 2 | | 2 | 1 | 1 | 1 |
| | G3 n=4 | 1 | | | 1 | 1 | 1 | |
| TONGUE | G1 n=5 | | | | 2 | | 1 | 2 |
| | G2 n=10 | 4 | | | 3 | 1 | 2 | |
| | G3 n=3 | 2 | | | 1 | | | |
| TONSIL | G1 n=4 | 1 | | 1 | 1 | | | 1 |
| | G2 n=5 | | | | 4 | | 1 | |
| | G3 n=2 | | | | 1 | | | 1 |
| LARYNX | G1 n=10 | 2 | 1 | | 4 | 1 | | 2 |
| | G2 n=8 | 2 | 1 | | 4 | | 1 | |
| | G3 n=19 | 10 | 3 | 3 | 2 | | 1 | |
| LUNG | G1 n=9 | 6 | | | 1 | | | 2 |
| | G2 n=10 | 7 | 1 | | 1 | | 1 | |
| | G3 n=8 | 4 | 2 | 1 | | | 1 | |
| OESOPHAGUS | G2 n=3 | | | | 2 | | 1 | |
| | G3 n=3 | 1 | | | 2 | | | |
| VULVA | G1 n=2 | | | | | | | 2 |
| | G2 n=5 | | | 1 | 1 | 1 | 1 | 1 |

* cytokeratin number.
Extensive staining and + indicate that more than 50% of the (basal and parabasal) cells are positive.
Limited staining and ± indicate that less than 50% of the (basal and parabasal) cells are positive.
Negative and - indicate that no specifically stained cells are found.

similarly and most extensively, although RCK106 reacted slightly more extensively than CK18-2. Secondly, RGE53 showed less extensive staining patterns and lower numbers of positive cases as compared to RCK106 and CK18-2.

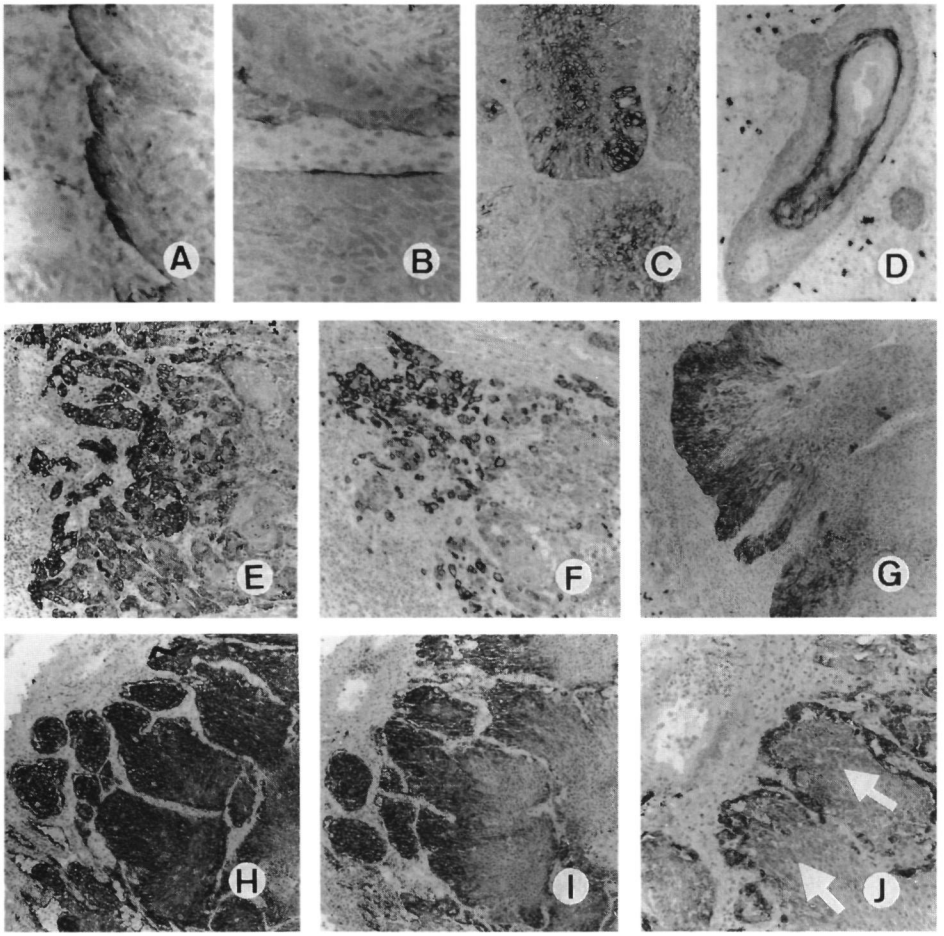


**Figure 2.** Immunoperoxidase staining in mucosal squamous cell carcinomas for cytokeratins 8 and 18, employing different monoclonal antibodies. Note the linear staining at the stromal side of peripherally located carcinoma cells as shown in **A** and **B** with antibody CAM5.2 . **C** shows a random staining pattern with M20, while in **D** only the cell layer directly beneath the keratinized cells is stained with CAM5.2.
**E-G**: Most staining is shown in the tumour front (at the left side of the figures) with antibodies CK18-2, RCK106 and M20, respectively.
**H-J**: Gradient pattern for cytokeratin 18 in a pulmonary squamous cell carcinoma displaying most staining with RCK106 (**H**), slightly less staining with RGE53 (**I**), while in a detail of the same area as shown in **H** and **I** only weak staining is seen with 2C8 (**J**, arrows). Note strong staining of residual alveolar cells in **J**.
Magnifications: A,B 200 X; C,D 100 X; E-I 60 X; J 120 X.
Origin of tumours: larynx (A,B,C,G), floor of mouth (D,E), tonsil (F), lung (H-J).

Finally, the least staining was observed with 2C8. As a result the 2C8 positive areas were increasingly overlapped by RGE53, CK18-2 and RCK106 positive areas. Basically, the results taken together indicate that a so-called "gradient-pattern" exsists for the extent of staining by the anti-cytokeratin 8 [8,16] and 18 antibodies.

**Cytokeratin 8 versus 18**

The tumour staining patterns for cytokeratin 8 and 18 were generally related to each other. Overlap of the staining patterns was, however, not complete. Frequently the positive areas were distributed differently or their staining levels varied (Table 2).

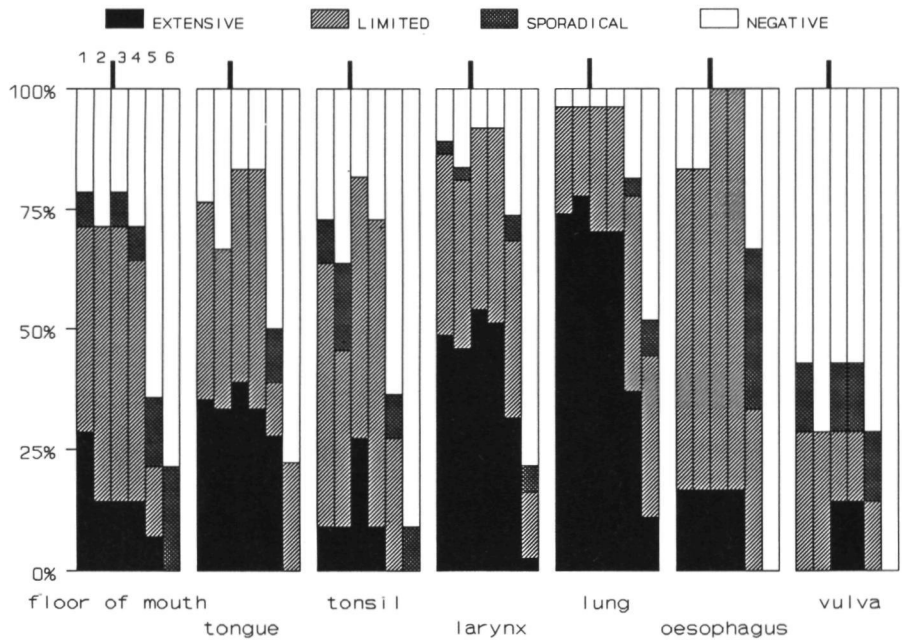


**Figure 3.** Levels of cytokeratin 8 and 18 expression in mucosal squamous cell carcinomas (G1-3) of different sites of origin, as monitored by the 6 different monoclonal antibodies used.
Extensive positivity: more than 50% of the cells are stained.
Limited positivity: less than 50% of the cells are stained.
Sporadical positivity: only very few (less than 1%) cells are positive.
Negative: no specific staining of tumour cells is seen.
The 6 individual bars per tumour type represent the results of the 6 antibodies, i.e. 1: CAM5.2, 2: M20, 3: RCK106, 4: CK18-2, 5: RGE53 and 6: 2C8.

**Tumour Staining Levels According to Site of Origin**

The expression levels of both cytokeratins 8 and 18 as well as vimentin varied between the tumours from different sites of origin (Table 1 and Figure 3). Pulmonary squamous cell carcinomas showed a rather extensive cytokeratin 8 and 18 staining reaction, while for example, vulvar carcinomas showed low levels of cytokeratin expression. In this series no consistent differences in the staining patterns between the primary tumour and metastasis were noticed.

Although vimentin was also positive in these same types of budding tumour areas, no consistent topological correlation with the cytokeratin expression was seen (Table 3).

## DISCUSSION

Although little is known about the function of the different types of intermediate filaments, new perspectives have been opened up by, as yet incompletely understood patterns of cytokeratin subtype and vimentin expression in the course of neoplastic disease [21,22,26]. Recently, we described the influence of the position of tumour cell clusters within transitional cell carcinoma on cytokeratin 8 and 18 expression [16]. In such tumours increased cytokeratin 8 and 18 expression was found especially in the invasion front and to a lesser degree in areas of intratumorous stroma contact. In the present study we examined squamous cell carcinomas of diverse sites of origin for this so-called "interface phenomenon". The normal squamous epithelium did not in general express cytokeratin 8 or 18 when tested with our antibodies. Squamous cell carcinomas were chosen because of their supposed low expression levels of cytokeratins 8 and 18, in contrast to adenocarcinomas which normally show high levels of these intermediate filament protein subtypes [5]. Although in our initial study [16] vimentin did not exhibit the interface phenomenon in transitional cell carcinoma, we included this intermediate filament protein in the present study, because of reported vimentin expression in squamous cell carcinoma [18,27,28].

**Table 3:** The number of cases showing cytokeratin 8 and 18 expression in the tumour invasion front (middle column) or with simultaneous vimentin expression in the invasion front area (right column)*.

| tumour site, grade and number of cases | | increased expression of cytokeratin 8 and/or 18 in the tumour invasion front | | simultaneous expression of vimentin and cytokeratin 8 and/or 18 in the tumour invasion front |
|---|---|---|---|---|
| FLOOR of MOUTH | G1 n=3 | 2 | | 1 |
| | G2 n=5 | 3 | 63% | 2 |
| | G3 n=3 | 2 | | 1 |
| TONGUE | G1 n=3 | 2 | | 2 |
| | G2 n=7 | 4 | 69% | 3 |
| | G3 n=3 | 3 | | 3 |
| TONSIL | G1 n=1 | - | | - |
| | G2 n=3 | 1 | 20% | - |
| | G3 n=1 | - | | - |
| LARYNX | G1 n=7 | 3 | | - |
| | G2 n=5 | 2 | 36% | - |
| | G3 n=13 | 4 | | - |
| LUNG | G1 n=4 | 1 | | - |
| | G2 n=5 | 1 | 41% | - |
| | G3 n=3 | 3 | | - |
| OESOPHAGUS | G2 n=2 | - | 40% | - |
| | G3 n=3 | 2 | | 1 |
| VULVA | G1 n=1 | - | 66% | - |
| | G2 n=2 | 2 | | 1 |
| totals | n=74 | 35 | 48% | 14 out of 35 |

* Only those samples that actually contain a tumour margin (74 of the 120 samples) are scored.

*Simple Cytokeratins in Squamous Cell Carcinomas*

Our present panel of cytokeratin subtype specific antibodies indicated gradients of expression of these constituents, especially for cytokeratin 18 in transitional

cell carcinomas and squamous cell carcinomas [8,16]. Such reaction patterns for cytokeratin subtype antibodies have also been described by others and related to antigen quantity or the epitope masking phenomenon [29]. Bosch et al. [29] demonstrated cytokeratins 8 and 18 in basal cells of normal mucosal squamous epithelium, although in very low concentrations. These authors related the presence of these cytokeratins to the proliferative potential of the cells in these layers. In their view it was therefore understandable that cytokeratins 8 and 18 were expressed in growing squamous cell carcinoma.

As expected and in line with the results of others [9,15] we found most squamous cell carcinomas examined to express cytokeratins 8 and 18 rather commonly, although in variable degrees. In our study the expression level was not found to be related to tumour grade, as reported by others [9,18]. However, in our view an additional important aspect was the topographical distribution of the cells expressing cytokeratins 8 and 18 [16]. In the squamous cell carcinomas that we had examined these cytokeratins were predominantly expressed in the peripherally located (basal) tumour cell. It is even more worthy of note, that the most likely area of detection is in the outer rim of the whole tumour, i.e. the invasion front, a phenomenon not observed for other cytokeratins (unpublished observations). This interface phenomenon was not found at every tumour margin. Similar observations of cytokeratin 8 and 18 expression in the stroma-tumour interface had been reported for squamous cell carcinoma derived from the keratinizing squamous epithelium of the skin [17]. These findings showed that squamous cell carcinomas originating from normally keratinizing squamous epithelium might also express cytokeratins 8 and 18 and that this phenomenon was not only restricted to squamous cell carcinoma derived from mucosal epithelia.

From Figure 3 it can be seen that expression of both cytokeratins 8 and 18 occurs to a higher extent in the squamous cell carcinomas that originate from simple epithelia through squamous metaplasia (for example lung) as compared to those derived from pre-existing squamous epithelium. In this respect, the recent observations by Smedts et al. [13] in cervical squamous cell carcinoma support this finding.

The observation that vimentin could be expressed in basal and parabasal cells of squamous cell carcinomas, and especially in cells budding from tumour cell clusters, will for practical reasons be evaluated together with the peripheral expression of cytokeratins 8 and 18.

These zonal phenomena have not yet been fully appreciated. For interpretation of these phenomena we have to consider firstly that the expression of a certain cytokeratin subtype is not necessarily monofunctional. This might especially be the case for cytokeratins 8 and 18. On the one hand they represent "primitive" cytokeratins expressed in early tissue development during embryogenesis [4,30,31], and on the other hand they make up intermediate filaments in highly differentiated and specialized tissues [5]. At first sight this indicates diverse function. The expression of cytokeratins 8 and 18, and also of vimentin, in the embryo might be interpretated as being related to the undifferentiated state of cells or a transient type of differentiation, cell migration, proliferation, poor cell cohesion, etc. These conditions could be correlated in experimental situations with an increase in the appearance of vimentin and to a lesser extent also with an increased expression of cytokeratins 8 and 18 in tissue culture of squamous cell carcinoma and ascites or pleural effusion [11,32-34]. The above mentioned cellular processes may also play a role in solid cancer.

Few reports on the relation between intermediate filament expression patterns and the biological behaviour of a tumour are available [26]. In this respect, recent publications on the presence of vimentin in breast carcinoma in relation to expression of the proliferation-associated marker Ki67, metastatic potential and prognosis are important [21,22]. Coexpression of vimentin and cytokeratins in several other types of cancer has been reported to be correlated with a poor prognosis, but frequently this was only related to tumour grade [18,20].

Although in this study maximal expression of cytokeratins 8 and 18, as well as vimentin, was found in the proliferating front of squamous cell carcinomas, we were not satisfied with the hypothesis that the expression of these constituents was fully due to supposed proliferative activity of the tumour cells in this region. Recent data on cytokeratin 8 neo-expression in mouse skin squamous

cell carcinoma and absence of cytokeratin 8 in papilloma have been described as being important in relation to invasive growth [35]. Furthermore, an essential role of cytokeratin 18 in the invasive and metastatic behaviour of a melanoma cell line was suggested by Chu et al. [36]. We must keep in mind that at the stroma-tumour interface, and especially in the tumour front several biological events take place. Possibly, differences in the physiological environment or status of cells in this area, for example, the presence of resting cells, might explain local differences in intermediate filament expression patterns. A morphological argument against the proliferation hypothesis and in favour of a correlation between cytokeratin 8 and 18 expression on the one hand and an interaction with the neighbouring cells on the other hand, was the preferential presence of these proteins at the stromal side of the tumour cells (Figures 2a,b and similarly 1g).

*Environmental influences on cytokeratin expression*

Data on all sorts of environmental influences on cytokeratin expression must be taken into account when interpreting the cytokeratin 8 and 18 expression patterns as described above [37,38].

Treatment of the squamous cancers by means of radiotherapy and/or chemotherapy was shown to influence intermediate filament expression patterns in these tumours. Fischer et al. [28] correlated vimentin expression in head and neck cancers to previous non-surgical treatment. They found expression of vimentin in a lower percentage of cases compared to our study. This was remarkable as in our study only non-previously treated cases were included. This difference might be explained by the use of different antibody panels and other technical factors.

Recent reports have shown an influence of underlying mesenchyme or basement membrane component on cytokeratin expression in epithelial cells [39,40]. Collagen VII, which constitutes to the anchoring filaments of the basement membrane, was reported to be absent around simple epithelia which were cytokeratin 8 and 18 positive, and to be present at the border of stratified (squamous) epithelia [41], which are normally negative for cytokeratins 8 and 18

[29]. This basement membrane constituent was also abundantly present in squamous cell carcinomas of different origin, but might be absent in the invasion front [41,42]. It was therefore tempting to test the hypothesis of the coincidence of cytokeratin 8 and 18 or vimentin positive cell buds on the one hand, and the absence of collagen VII around these structures on the other hand. Our preliminary studies have not so far shown such a correlation (unpublished data).

*Cytokeratins 8 and 18 are not always associated with Vimentin*

In this study, we found co-localisation of vimentin and cytokeratins 8 and 18 in only a minority of cases. In most squamous cell carcinomas examined these intermediate filament proteins showed apparently independent expression patterns. Coexpression of vimentin and cytokeratins has been reported before in head and neck squamous cell carcinoma [18,28]. Wallner et al. [27] have noted that vimentin and cytokeratin are coexpressed mainly in the tumour invasion front. In these studies, however, broad-spectrum cytokeratin antibodies, often recognizing several of the cytokeratin subtypes, were used. In our study we concentrated deliberately on two cytokeratin subtypes normally not present in squamous epithelium, i.e. cytokeratins 8 and 18. Henzen-Logmans et al. [18] have described a relation in squamous cell carcinoma between tumour grade and vimentin expression, while others have not found such a relationship [27,28]. In our study the small cell clusters positive for cytokeratin 8, 18 or vimentin could be also found in well-differentiated carcinomas.

*Conclusions.* Aberrant expression of cytokeratins 8 and 18 in squamous cell carcinoma has previously been related to tumour progression, emphasizing the role of grade of anaplasia [9,18]. In the present immunohistochemical study of squamous cell carcinoma lesions, the topographical localization of distinct tumor cells, i.e. at the stroma-tumor interface, was shown to be of special importance for expression of cytokeratins 8 and 18 and vimentin. This suggested a particular phenotype of the carcinoma cells involved in an environmental interaction that is expected to be most prominent during invasive growth. Further exploration of the biological basis of this phenomenon and its possible prognostic

significance is needed.

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# CHAPTER VI

# Cytokeratin subtyping in normal and neoplastic human epithelium.
## Basic principles and diagnostic applications.

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## INTRODUCTION

Conventional pathology diagnosis is estimated to offer problems and to remain inconclusive in 4-10% of the neoplasms examined [1]. Part of these difficulties is a consequence of inadequate sampling and poor preservation of biopsies. In the more optimally sampled cases, additional techniques can help to reach a more precise diagnosis. In particular this is the case in those lesions where the type of differentiation or primary site of a certain cancer is unclear on basis of routine histology, using conventional staining techniques. Immunocytochemical procedures play a major role in obtaining extra information on the type of cells, in particular since many tissue markers have been identified and antibodies to these antigens are available. In this respect, reagents the to cell lineage dependent proteins that constitute the intermediate filament proteins (IFPs) play a central part. This family of cytoskeletal proteins has been described to consist of 6 types, classifying different tissues. The largest group of IFPs is formed by the cytokeratins (CKs), which are mainly found in epithelia. CKs are divided into **type I** (small, acidic; including CK9-CK20) and **type II** (large, neutral to basic; including CK1-CK8) [2,3]. **Type III** IFPs comprise the mesenchymal protein vimentin, the protein desmin specific for muscle tissue, glial fibrillary acidic protein expressed in glial cells and astrocytes [4] and peripherin specific for neuronal cells [5]. **Type IV** IFPs consist of the neurofilament protein triplet expressed in nerve cells [6], **type V** the nuclear A and B-type lamin proteins which constitute the nuclear lamina [7-9] and the **type VI** IFP compises nestin, specific for CNS stem cells [10]. In pathological situations and during neoplastic growth these cell lineage markers are generally retained, although switches in their expression patterns have been found (for recent reviews see [1,11-18]).

## CYTOKERATINS

In human tissues, cytokeratins form a subfamily of at least 20 different proteins, not including the hair keratins. The latter comprise a group of approximately 10 additional primary gene products [19]. Our current understanding of CK

biology is mainly based on the following two issues. Firstly, Moll et al. [2,3], have catalogued the human CKs and listed them numerically as CK 1 to 20. Heid et al. [19] have made an inventory of the hair or "hard" trichocytic keratins, which occur also in certain papillary appendages of the tongue and Hassal's bodies in the thymus [20]. The widely adopted numerical classification system facilitates discussions significantly, while it easily correlates the molecular weight and isoelectric pH of the individual CK estimated by different laboratories [18,21]. Secondly, a cell lineage dependent pattern of CK expression has emerged from biochemical and immunohistochemical studies, showing that specific combinations can occur in different directions of epithelial differentiation [22]. In general, an acidic CK protein has to combine into a pair with a neutral to basic CK protein to form intermediate filaments. Usually, a subset of 2 to 10 of the 20 CKs is expressed by any epithelium in a composition that is dependent on cell type, stage of embryonic development, cellular growth and environment, pathological status, and the degree of cellular differentiation. These characteristics make CKs suitable as differentiation markers. In this overview we summarize their role in the study of biological processes and their potential for application in surgical pathology.

*Basic cytokeratin expression patterns in adult human tissues*

Many monoclonal antibodies (mAbs) have been developed against the various CKs. In addition to broadly cross-reacting mAbs, which stain virtually all epithelial tissue types, mAbs and some polyclonal antibodies have been developed which react with only one of the 20 CKs. The large majority of CKs can now be recognized individually by such reagents [18,23].
The CK composition of epithelia appears to follow basic principles and can be summarized as follows [13,18] (see also Table 1).

**Simple epithelia** express primarily CK8 and CK18 and often CK7 and CK19. The recently catalogued CK20 is less frequently found in these types of epithelia than the former CKs [3].

**Table 1.** Major patterns of cytokeratin expression in relation to epithelial type of differentiation (simplification).

| CELL/TISSUE TYPE | CKs | EXAMPLES |
|---|---|---|
| SECRETORY cells | 8,18 | acinar cells (prostate, pancreas, etc.), liver parenchyma, endocrine cells, proximal renal tubules |
| LUMINAL cells (mixed epithelia included) | 8,18,19,20 | gastric foveolar and intestinal cells |
| | 8,18,19 | tubular area of prostate |
| | 7,8,18,19 | mamma, endometrium, distal renal tubules |
| BASAL, RESERVE, MYOEPITHELIAL cells in mixed epithelia | 5,14,(15),17 | glands (salivary glands, mamma, prostate, etc.) bronchus, uterine endocervix |
| BASAL cells of stratified epithelia | 5,14,15 | squamous epithelium |
| SUPRABASAL cells of stratified epithelium | 4,13 | nonkeratinizing squamous epithelium |
| | 1,2,10,11 | epidermis |
| | 1,2,9 | plantar epidermis |
| | 3,12 | cornea |
| | 6,16 | "reactive and (hyper)proliferative" squamous epithelium |
| UROTHELIUM | (4),5,7,8,13, 17,18,19,20 | urinary tract |
| SEROSA, MESOTHELIUM | 5,7,8,18,19 | peritoneum, pleura |

**Stratified epithelia** express CK5, 14 and 15 [24-28] in the basal cell layer. The suprabasal cells express additionally at least one other pair of CKs, depending

on their morphologic type of differentiation. The expression of these differentiation-specific CK pairs correlates to four distinguished differentiation pathways: a) Nonkeratinized squamous epithelium shows suprabasal expression of CK4 and CK13 [27]. b) Keratinized squamous epithelium expresses CK1 and CK10 suprabasally [13]. c) The corneal epithelium contains CK3 and CK12 [28]. d) CK6 and CK16 may be expressed transiently in regenerating and hyperproliferative statified epithelium, as an alternative suprabasal pair under conditions where the usual CKs as described above, are not expressed normally [13,28]. Further specialized functions of squamous epithelium are reflected by the expression of CK2 and CK11, in particular in hard-worn epidermal sites and CK9 in palm and sole skin areas [2,17,29,30].

CK19 occupies an ambiguous position within these classifications, since next to its presence in simple epithelia, it can also be expressed in statified epithelia, generally in basal cells, but also weakly in scattered suprabasal cells of nonkeratinized squamous epithelium [31]. Also low levels of simple epithelial CK8 and CK18, can be demonstrated in nonkeratinized squamous epithelium [32].

CK15 has been demonstrated in all cell layers of nonkeratinized squamous epithelium [26,33,34], while in the skin this CK subtype is expressed in a topically restricted pattern [34].

**Complex or mixed epithelia** show a combination of simple and stratified CK profiles. They comprise pseudostratified epithelia, which have frequently a typical basal or myoepithelial cell layer, such as eccrine glands and respiratory tract epithelium [34-38]. Myoepithelial and other basal or reserve cell types such as those in mamma, prostate, bronchus and endocervix express CK5 [24] and CK14 as well as CK15 [23,34,36,38] and CK17, especially in the larger ducts or bronchi [34,39,40]. CK8 and CK18, and more variably CK7 and CK19, are expressed in the suprabasal or luminal compartment.

Recently basal cells [41] of prostatic tubules and acini have been shown to display a complex pattern with unexpected CKs [40,42], comprizing CKs 5, 6, 8, 10, 13, 14, 17 and 18, while CK19, was found to be only focally present in acinar basal cells. CK10 and CK13 have not been noticed by others in these ducts, using the same or similar mAbs [27,43]. Salivary ducts show a similar, mixed CK expression pattern, although CK10 expression is found inconsistently [38,44] and

CK6 has not been examined as yet in this epithelium [24,38,39,44,45]. Extensive study for the CK expression patterns in mammary glandular ducts [46-48] show as additional features of basal cells weak expression of CK7 and CK19, at least in larger ducts. CK6 is not observed in normal breast, in contrast to its being reported in prostate. However, in larger ducts of the mamma some CK14 positive luminal cells are observed [49].

The **transitional epithelium** of the urinary tract is another complex epithelium with CK13 in basal and parabasal layers, while CKs5, 14 and 17 are additionally, but heterogeneously expressed in basal cells and sporadically found in suprabasal cells. CK4 is sometimes found, while heterogeneous CK20 expression is observed in the superficial or so-called umbrella cells. CKs7, 8, 18 and 19 are found in all cell layers of the urothelium [3,24,50-54], CK15, however, is not observed [26].

*Cytokeratin expression patterns during embryonic and fetal development*

Knowledge concerning CK expression in prenatal developing tissues is still largely incomplete, but certain patterns of expression are emerging. These patterns will be discussed here because of their relevance in understanding CK dynamics in pathology especially in neoplasms, showing an analogy with the above mentioned differentiation pathways. CK8 and CK18 are the first IFPs to be detected during embryogenesis and have been shown to occur in early mesenchymal tissues and developing organs, such as the early stages of the heart [55]. In fetal skin maturation [56,57] the simple epithelial CKs8, 18 and 19 are expressed first, followed by CK4 and CK13, with CK4 also in the basal cell layers from which it disappears later on. The next step of maturation involves the onset of CK10 expression, while CKs4, 8, 13, 18 and 19 vanish.

In the developing liver [58-60] and pancreas [61] a transient expression of CK7 and CK19 occurs in the parenchym. Transient CK7 expression is also described in fetal and neonatal stomach, where the expression is the strongest in the superficial foveolar area [62]. Most relevant for fetal kidney is that the proximal tubules display permanent CK8 and CK18 expression, and transiently CK19 and vimentin expression. The latter is not found in the distal tubules [63].

In fetal lung, basal cells become positive for CK14, only in the third trimester, although they can be detected morphologically in earlier stages [64].

*Basic cytokeratin expression patterns in squamous metaplasia, preneoplasia and carcinomas*

The pathways of CK expression described above for the different types of epithelia, as well as the fact that a considerable part of CKs can be detected separately by immunocytochemical techniques, has created expectations with respect to their applicability as markers in pathology diagnosis especially in distinguishing between different carcinoma types. Initially, the set of CKs, which is present in the normal epithelium, was presumed to remain present in a virtually unaltered combination in the neoplasm originating from that epithelium. Later on many exceptions to this "fingerprint" concept were found, most of which are related to loss of tumor differentiation, metaplastic changes or transdifferentiation and intrinsic changes inherent to neoplasia. Many of these exceptions that have been reported may be incorporated in the interpretation of CK expression patterns of malignancies. With regards to squamous metaplasia and dysplasia, important steps in the carcinogenesis of epithelia, these lesions will be discussed, preceding a general survey of CK expression in the major types of carcinoma.

**Squamous metaplasia** generally shows loss or decrease of most simple epithelium CKs, i.e. CK7, CK8 and CK18 [23,34,50,65], while CK19 may be homogeneously retained in all cell layers of metaplastic epithelium [34,50] (for exceptions see [65]). Extensive CK19 expression in squamous metaplasia contrasts to the more basal cell layer related pattern of CK19 in preexistent squamous epithelium, while other stratification-related CKs, also appear in the lesions [34]. Expression of CK1 and CK10/11 may precede morphological keratinization, while it is accompanied by a decrease in CK4 and CK13 expression [34].

Studies on **preneoplasia** using extensive panels of chain-specific CK mAbs are scarse and are mainly restricted to the squamoid lineage. In hyperplasia no signi-

ficant alterations in CK expression are found [66-68], while an increase of CK19 expression has in particular been correlated with early preneoplastic changes of nonkeratinized squamous epithelium [66,69,70]. Especially in severe dysplasia an increased expression of other simple epithelial CKs, mostly CK8 and CK18, becomes perceptible [67,71,72]. Similar to other pathological situations, the levels of the specific squamous differentiation related CK pairs, i.e. CK4 and CK13 or CK 1 and CK10 decrease in dysplasia [68,70,73-75]. Expression of other stratification related CKs, such as CK5 and CK14, may persist depending on the degree of anaplasia [71,73], with CK16 expression levels often clearly increase with increasing grade of dysplasia. The complex situation in the uterine cervix has been extensively examined and will be described separately below.

Early biochemical studies [2] show that the CK expression patterns of **squamous cell carcinoma** display significant levels of CKs 5, 6, 13, 14, 17, and 19 and lower or variable levels of CKs 4, 8, 10/11, 15, 16 and 18. In the more sensitive immunohistochemical studies using mAbs, these data are largely confirmed [36,66,73,76,77]. With increasing tumor grade an increased expression of the simple epithelial CKs [69,76,78] and a decrease of squamous differentiation related CKs, i.e. CK1, CK10, CK13 and CK16 occurs [36,66,69,70,74]. In addition, keratinization of a squamous cell carcinoma may co-incide with changes in CK expression patterns [23,77]. Different levels of CK8 and CK18 in squamous cell carcinoma (Figure 1) are reported for the different primary tumor sites, showing higher levels of these two simple CKs in carcinomas evolving through a "metaplastic" pathway as compared to carcinoma directly originating from squamous epithelium [79]. In squamous cell carcinoma frequently minor vimentin positive areas are described to be related to stroma interaction [79,80], to tumor grade [81] or previous irradiation [82].

All **adenocarcinomas** express CK8 and CK18, and frequently CK19. CK7 shows a distinctly organ related expression and is present in carcinomas of the mamma, lung, pancreas, biliary tract, ovary, endometrium and endocervix, while gastric carcinoma is heterogeneous in this respect and colonic and prostatic carcinoma are predominantly negative [78,83]. CK20 is present in adenocarcinomas

arising from CK20-producing epithelia, such as colonic carcinoma [3,23,54]. Stratified epithelium related CKs may occur in certain types of adenocarcinoma, for example pancreatic and pulmonary adenocarcinoma, as well as "Müllerian" epithelium derived carcinomas [36,76,84-86].

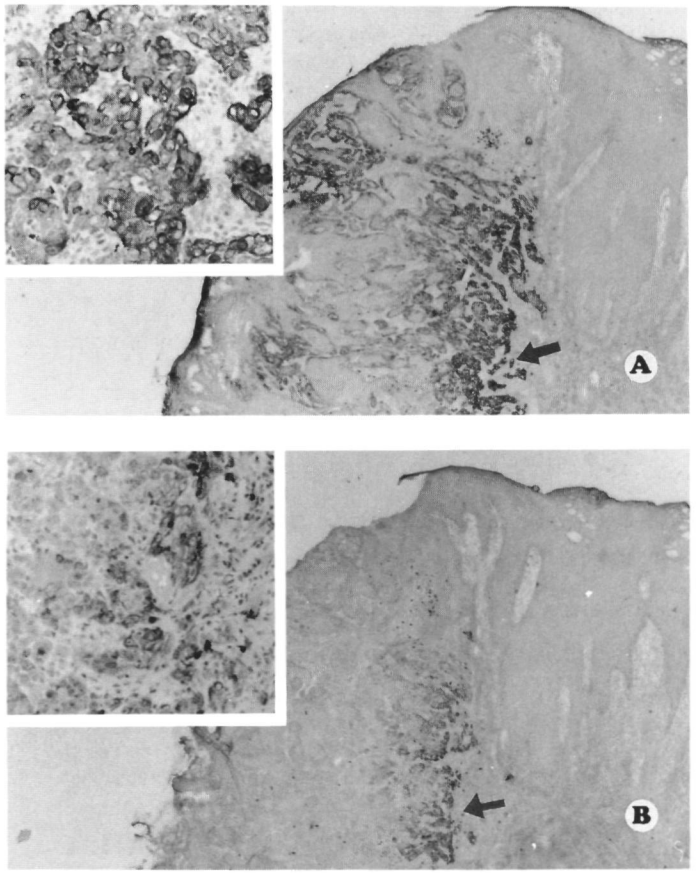


**Figure 1.** Squamous cell carcinoma of the floor of mouth immunostained with two different CK18 mAbs. Note the absence of expression of CK18 in the hyperplastic squamous epithelium (right side of the section) and presence of a heterogeneous expression in the carcinomatous area (left side). Different levels of immunostainig are seen as a result the use of two different mAbs (**A** is stained with the mAb CK18-2 and **B** with RGE53), known for their differences in immunoaffinity [51,52,79]. Most and/or preferential expression of CK18 was seen at the tumor margin, i.e. in the tumor invasion front, which is postulated to represent an "interface phenomenon"[51,52,79]. (Insert: detail of the invasion front - see arrow - showing a more extensive staining in **A** as compared to **B**). Magnification 25X, insert 140X.

**Table 2.** Nonepithelial human tissues which have been reported to stain with CK antibodies.

| FETAL TISSUES | NEOPLASTIC TISSUE |
|---|---|
| smooth muscle cells (vascular wall)[55,89],<br>stroma of umbilical cord[55],<br>myocardium[55],<br>spinal arachnoid cell[246],<br>Sertoli cells[201] | lymphoma[224,256,257],<br>epithelioid sarcoma[258-260],<br>malignant fibrous histiocytoma[261,262],<br>synovial sarcoma[88,263],<br>leiomyo(sarco)ma[249,250,264],<br>rhabdomyosarcoma[12,265],<br>liposarcoma[260],<br>Ewing's sarcoma[266-268],<br>chondrosarcoma [269],<br>chordoma[270-272],<br>melanoma**[224,273-276],<br>vascular neoplasms[250,277-279],<br>chondroblastoma[280],<br>rhabdoid tumor[12,281],<br>granulosa cell tumor[86,173,177],<br>fibro-thecoma[173],<br>(Sertoli-)Leydig cell tumor[173],<br>germ cell tumors (see in text),<br>desmoplastic round-cell tumor[282,283],<br>ependymal tumors [284],<br>myxopapillary ependymoma[255],<br>meningeoma[285],<br>primitive neuroectodermal tumors[286],<br>astrocytoma[287-289],<br>oligodendroglioma[288],<br>infantile fibromatosis[290],<br>tumor of arterioventricular nodal region[291],<br>(malignant) peripheral nerve sheath tumor[292-294]. |
| ---------- | |
| **ADULT TISSUES** | |
| myofibroblasts in atherosclerotic plaques[247],<br>submesothelial cells[56,92,93,248],<br>smooth muscle cells[249,250],<br>endothelial cells near synovia[251,252],<br>reticulum cells in lymph node* and spleen[12,253,254],<br>spinal arachnoidal cells[246],<br>spinal ependymal cells[246],<br>plexus choroid[255] | |

FOOTNOTES TO TABLE 2.

* Increase of CK positive reticulum cells is observed in lymph nodes of fetus and neonate, in case of sarcoidosis, toxoplasmosis, Hodgkin's disease and metastases[253,275]

** CK expression may prevail in melanoma metastases, also seen in in vitro studies[274,295].

**Neuroendocrine carcinoma**, in its spectrum from small cell undifferentiated carcinoma to carcinoid, has been shown to express CK8 and CK18 consistently. CK19 can be found in a more restricted fashion [2,36,76], while CK4, CK7 [76,78], CK16 or CK17 are reported to occur sporadically [36]. CK20 is not expressed in these tumors, with Merkel cell tumor as clear exception [54]. Vimentin is reported to be expressed more frequently in rectal than in non-rectal carcinoids [87].

*Cytokeratins in nonepithelial cells*

In human tissues, CKs have also been demonstrated in non-epithelial tissues (see Table 2 and for review [11]), while on the other hand vimentin can be found in several epithelia (see for review [15]). The percentages of positive cases or the extent of CK expression vary widely depending on tissue type, reagents used and technical procedures. Gradually, it is becoming clear that apparently ectopic expression of CKs follows certain concepts. CKs expressed in nonepithelial tissues are generally the simple CKs [88-90], especially CK8 and CK18, but not CK20. Sporadically the myoepithelial CK17 has been found in tumor stroma [46,91]. This "ectopic" CK expression can possibly be related to their transient expression in developing fetal tissues or in proliferating cells such as myofibroblasts and (sub)mesothelial cells [55,90,92,93]. Otherwise CKs can be found under normal physiological situations in smooth muscle. The expression of CK8 and/or CK18 can thus no longer be considered as typical of epithelium. In order to prevent diagnostic misinterpretation in the distinction between mesenchymal and epithelial differentiation, we suggest to use (cocktails of) broadly cross-reacting CK-reagents which do not react with CK8 or CK18, but for example with CKs7, 19, 5 and 14. To be aware of CK expression in mesenchymal tumors may be invaluable, as it can help in the diagnostic process, for example in the case of epitheloid sarcoma and sarcomatoid mesothelioma. Unawareness of aberrant expression may lead to inproper interpretations and diagnostic errors.

A major problem in the utilization of mAbs, and IFP antibodies in particular, is the loss of immunogenicety upon fixation with cross-linking reagents. This has hampered the application of these antibodies in the past and is at present slowly being overcome by the fact that mAbs, reactive after formalin fixation and paraffin embedding, become available [18,54,83,94].

The **fixation problem** is easily overcome by using frozen sections, but routine practice mostly employs paraffin embedded, formalin fixed tissue. As a result the most common causes of poor reproducibility of immunohistochemical assays are the unpredictable alterations that are introduced to the antigenic sites by the tissue processing procedures. In contrast to the cross-linking fixatives, such as formaldehyde, the coagulating, often alcohol based fixatives, show more frequently a better preservation and/or presentation of the antigenic epitopes [15]. Excessive heat during fixation or processing is also deleterious to the antigenic sites. Pretreatment of sections with proteases can make antigenic sites, masked by formalin, more accessible. The conditions of effective digestion are related to the fixation time [95], type of fixative or even to tumor type [96]. Antigen retrieval in formalin fixed tissue can be enhanced by microwave oven heating in the presence of heavy metal ions [97]. Microwave-irradiation in saline as an alternative fixation procedure, preceding embedding, has shown to give good antigen preservation [98,99]. Battifora and co-workers [15,100] have suggested that antigen destruction can be best monitored by surveying vimentin staining results.

Also with respect to antigen preservation it should be realized that autolysis can render tissue suboptimal for immunostaining procedures. These effects have recently been discussed [101] for vimentin and CK reagents, showing wide differences in antigen preservation. However, morphological decline does not necessarily mean a complete immunodeterioration of antigens. With respect to interpretation of antigen staining at the cellular level, one should keep in mind that the conditions (such as developing time and concentration) of enzyme-precipitate reactions, such as peroxidase-DAB or alkaline phosphatase-Fast Red TR, influence the staining pattern [102,103].

One of the problems arising during the interpretation of the immunostaining results may be caused by structural alterations of CK proteins, rendering some, in particular conformational, CK epitopes undetectable in immunohistological assays. This phenomenon is called **epitope masking** and may in part be overcome by staining tissues and cells with more than one mAb against a single CK. For instance, CK18 can hardly be detected in certain tissues with mAb 2C8 or RGE53, while RCK106 resulted in more extensive staining [50-52,85,79]. Antigen quantity may also play a crucial role [50-52,79]. Similarly, this phenomenon is observed with CK14 mAbs (RCK107 versus LL001 and LL002) and CK19 mAbs [31,61,104]. These differences in reactivity patterns of the individual CK mAbs may be due to a different structural organisation of the CK filaments under different circumstances.

***Cytokeratin expression in specific tissues and their malignancies***

In the following paragraphs we will review the relevant CK expression patterns in normal and pathological situations arranged in alphabetical order according to the major organ systems. The basic rules of CK expression as discussed above, will not be emphasized again for the individual tissue and tumor types, unless relevant for the case.

## ADRENAL GLAND AND EXTRA-ADRENAL PARAGANGLIA

In routinely fixed normal adrenal cortex, especially in the zona glomerulosa, CKs can be found after immunostaining, employing mAbs with strong CK8 and CK18 reactivity. Focal CK19 positive cells may also be found [105]. Data on the percentage of positive cells diverge, partly depending on the cortical zones examined and the technique applied [105-108]. A decrease in the detectability of CKs is reported in cortical tumors in the transition from adenoma to carcinoma, of which the latter are frequently negative [105-108]. Vimentin staining, however, increased upon malignant progression.

Several authors found that CKs were not expressed in ganglion cells in

normal adrenal medulla [107-109], intestine or urinary bladder [109,110]. Others, however, report CKs in adrenal medulla [111]. In pheochromocytoma absence of CKs has been reported [107,108], but CK positive cells are also sparsely found in some cases [109]. Paragangliomas are reported to be CK negative in most studies [15,109,112,113], although positive cases have been noted [114]. Relatively extensive vimentin staining was found particularly in metatastic adrenal pheochromocytomas [109].

## ENDOMETRIUM

CKs8, 18 and 19 are uniformely distributed in normal endometrium, while CK7 is widely distributed in the secretory phase, although restricted to smaller and variable proportions of glandular cells in proliferative endometrium [86]. Endometrial adenocarcinoma cells [86] are all positive for CK8, CK18, almost all for CK19, while CK7 shows pronounced quantitative variability [83,86]. Small traces of CK20 are found [86], while also stratification related CK4, 5 and 13 have been detected mainly localized in squamous areas but not strictly confined to this type of diffentiation. It is remarkable that CK7 and CK18 have been reported to remain present in these squamous metaplastic areas [86].

The epithelial component of normal endometrium and also that of endometriosis, is shown to coexpress vimentin and CKs. The pattern and the extent of this phenomenon is different when pre-ovulatory and post-ovulatory conditions are compared [86,115,116]. Most extensive vimentin coexpression occurs in the proliferative phase. Vimentin is frequently retained and in general extensively expressed in endometrial carcinoma [15,86,117]. In endometrial clear cell carcinoma vimentin was only expressed to a limited extent, while the simple epithelial CKs are extensively present [86]. For mixed Müllerian tumors and stromal cell sarcomas see the section "ovary".

## ESOPHAGUS

The CK expression pattern of the esophageal mucosa is that of a typical nonkeratinized squamous epithelium as outlined above. CK19 is strongly expressed in basal cells and heterogeneously weakly expressed in the suprabasal layers [32]. CK4 and CK13 are present in all suprabasal cells [27]. Moreover, very

low levels of CK8 and CK18 can also be detected in the basal cell compartment [32]. An increased suprabasal expression of CK19 is described with advancing grades of dysplasia [118], while, as expected CK13 expression decreases [118]. Also with respect to dysplasia, the morphological phenotype influences the CK expression patterns [71]. The more undifferentiated adenoid pattern shows an increase of CK8, while an increase of squamous cell related CKs, as detected by the mAb 34ßE12 [21], is seen in typical squamoid dysplasia [71]. In squamous cell carcinoma a similar relation was found for these characteristic stratification related CKs which are strongly expressed in typical squamous carcinoma but weakly or not at all in undifferentiated and spindle cell carcinoma.

## GALL BLADDER

Adenocarcinomas of the gall bladder frequently express CK20, next to CKs7, 8, 18 and 19 [3,54]. Surprisingly, normal gall bladder epithelium contains only sporadic CK20 positive cells [3].

## GASTROINTESTINAL TRACT

From stomach to rectum the mucosal lining contains CKs8, 18 and 19. Recently the previously mentioned IT protein, has been characterized as CK20 [3] and is a member of the group of simple epithelium CKs. The gastric foveolar epithelium is uniformely and strongly positive for this latter CK subtype. The specific glands of the corpus mucosa as well as most mucus cells of the pyloric glands, however, remained unstained. In crypts of the small intestine CK20 is expressed in a patchy fashion, but stains the lining cells of the villi strongly and uniformly. In the colonic mucosa a similar pattern is found. Also a variable number of endocrine cells in the mucosa is CK20 positive.

In the normal stomach, the presence of CK7 is still a matter of discussion. Several CK7 mAbs remain negative upon immunohistochemistry of stomach epithelium, while at least one CK7 mAb (OVTL12/30) shows significant positivity in frozen sections of this tissue [83]. Metaplastic intestinal mucosa in the stomach shows CK7 positivity [119]. This is peculiar, since CK7 is normally not found in the small or large intestine [43,120] (for exceptional staining in duodenum, see [83]). In gastric adenocarcinomas, CK7 is found to be heterogeneously

expressed in some cases and is absent in others. This is dependent on the CK7 mAb used [2,78,83,120,121]. CK20 is frequently found in diverse types of gastric adenocarcinoma [54].

Intestinal adenocarcinomas are CK7 negative, while the expression of the other four simple epithelium CKs, i.e. CK8, CK18, CK19 and CK20, is maintained [54]. The observation of increased CK staining in normal mucosa and adenomatoid polyps of patients with familiairy polyposis coli [122] is possibly of biological importance. This phenomenon might be related to increased levels of intermediate filament proteins upon malignant transformation of the colonic mucosa [122-124]

## KIDNEY

In the normal glomerulus CKs7, 8, 17, 18 and 19 and some vimentin are present. In the tubules CK8 and CK18 are the major constituents, while CK7 and CK19 are variably expressed depending on the cell type and segment of the tubule under investigation [43,63,83]. In pathologically altered epithelium of the proximal tubule (i.e. atrophy) vimentin and some CK17 become expressed, while also in the distal tubule expression of CK7 and CK19 increases. This abnormal expression pattern partly resembles the earlier mentioned fetal situation [63]. The recently described simple epithelium related CK20 is not found in the kidney [3].

In renal cell tumors some authors have discriminated two "classes", i.e. those tumors with and those without vimentin coexpression [125]. To this latter group belong the relatively rare types of chromophobe renal cell carcinoma and the oncocytoma [125]. The CKs expressed in the clear cell type of renal cell carcinoma are predominantly CK8 and CK18 and rather frequently also heterogeneous expression of CK19 is seen. CK7 can be found only in a minority of cases, but when present the tumor is usually high grade malignant. The eosinophylic-granular and basophylic type of renal cell carcinoma additionally expresses CK19 and fequently also CK7 [125]. In other studies, presence of CK7 has only exceptionally been reported [78,83]. The data on vimentin and CK coexpression vary mostly depending on the type of tissue fixation, the antibodies used and tumor grade [106,125-130], vimentin expression has, however, been related to poor prognosis [128].

## LIVER

Different CK expression patterns have been reported for the various cell types which compose the liver. The liver parenchyma expresses CK8 and CK18 and the intrahepatic bile ducts additionally express CK7 and CK19 [131]. In the parenchyma a CK8 and CK18 expression gradient has been described depending on the location of the cell in the lobulus [60,131]. In pathological situations, such as cirrhosis [132], nodular hyperplasia [133], cholestatic liver disease [134], alcoholic liver disease [135], hepatocytes may express CK7 and CK19. These two CKs are also transiently present in the fetal liver [59,60]. Initially hepatocellular carcinoma was considered to express CK8 and CK18 only. This was thought to distinguish these tumors from cholangiolar carcinoma and many metastatic carcinomas [136]. More recent studies show that hepatocellular carcinoma is able to express additionally CK7 and CK19 or even sporadically vimentin [15,132,137,138]. The fibrolamellar type of hepatocellular carcinoma is conspicuous by abundant CK7 expression [139]. Hepatoblastoma express CKs8, 18 and 19 and in some cases CK7, while cells embedded in osteoid-like material coexpressed vimentin [140].

## LUNG

The CK patterns of developing and adult brocheal mucosa have been described [84] and CKs typical of a mixed type of epithelium are expressed, i.e. CKs7, 8, 18, 19 and scarsely CK20 in columnar cells and CKs5, 14, 15 and 17 in basal cells [3,26,36,84].

At first glance, the four major types of lung carcinoma show the typical differentiation lineage related CK expression patterns. Squamous cell carcinoma may moderately express CKs4, 13, 16 and extensively CKs14, 15 and 17. The expression of stratification related CKs4, 13 and 16 diminishes with increasing histological grade of malignancy, which does not influence the expression of the basal cell CKs14, 15 and 17 [26,36,76]. However, the expression of simple epithelial CK8 and CK18, which are already significantly present in well-differentiated squamous cell carcinoma [76], was shown to increase upon progression [76]. Adenocarcinomas of the lung are characterized by the typical simple CKs7, 8, 18 and 19, while focal expression of CK14 and CK17 can be found in a minority of cases [36]. The extensive CK7 expression in adenocarcinoma and large

cell undifferentiated carcinoma therefore, distinguishes these malignancies from squamous cell carcinoma. The stable expression of CK14 in poorly differentiated squamous cell carcinoma and its absence in adenocarcinoma and the neuroendocrine tumor types, including small cell undifferentiated carcinoma, is another important discriminating feature.

Most neuroendocrine carcinomas, i.e small cell undifferentiated carcinoma and carcinoid, express only CK8 and CK18 [36,76]. Unexpected extensive expression of CK7 in sporadic squamous cell carcinoma is reported [76,78]. This finding may be due to mixed differentiation, often occuring in lung cancer. Extensive expression of vimentin is notorius in part of the large cell undifferentiated carcinomas, and to some degree also in the adenocarcinomas [141,142].

The clear cell ("sugar") tumor of the lung is reported to express vimentin [143], but not CKs. When a tumor with this type of morphology occurs in the lung but expresses CKs it is reported most likely a metastasis of renal cell carcinoma.

## MAMMARY GLAND

In the mammary gland the terminal ductal lobular units are of special interest, because of their proliferation in pregnancy and the concept that from the cells in these segments breast cancer develops. The luminal cells in this area extensively express CKs7, 8 and 18, while CK19 is found in the majority of cells, while basal cells express CKs5, 14 and 17. Occasionally luminal cells expressing CK5 and CK17 has been reported [49,144-146]. CK19 negative luminal cells appear at puberty [147] and are found in significant numbers in benign tumors. In general, the invasive component of breast cancers exists of CK19 positive cells [47]. In the heterogeneous group of benign tumors both luminal and basal cell CKs remain present in the respective cell types [46]. In some cases of sclerosing adenosis all cells may stain for CK14 [148]. Similarly, also in epitheliosis the basal cell CK immunoreactivity is detected in the streaming sheetlike intraluminal proliferations [146,149]. In carcinoma CK8 and CK18 are uniformly expressed, as are CK7 and CK19 in the large majority of tumors [47,78] (compare however for CK7 refs [150,151]). Most of the carcinomas are negative for basal cell CKs. CK17 is expressed in up to 30% of carcinomas [46,49], while in an extended series CK14 is found in about 10% of the invasive cancers [49,148,152]. These cancers can not

be distinguished morphologically from CK14/17 negative invasive carcinomas, but this CK14 expression has been related to poor prognosis [17,153]. A relatively high concordance is found between CK14/17 expression and CK16 expression, but not between these markers and Ki-67 [49]. The overlapping expression of basal CKs and CK16, formerly designated as hyperproliferation marker, may be explained by the fact that all three CKs are related to squamous differentiation [23,28,36,65,77,79,154]. Even CK15 positive carcinomas are found, especially in a subgroup of high grade malignant ductal carcinomas [26].

Also vimentin expression is related to poor prognosis and correlated with high grade, low estrogen receptor content, a high Ki-67 defined growth fraction, p53 expression and EGF receptor content [99,155-158]. Vimentin is extensively found in medullary breast carcinoma, but generally not in lobular carcinoma [145,155,158]. Vimentin, however, has also been described in some cases of fibrocystic disease [46,158] and is expressed to low degree in normal basal cells [46,145].

## MERKEL CELL

Merkel cells and Merkel cell tumors are described to express high levels of CK8 and CK20, as well as CKs7, 18 and 19 [3,54,159-161]. In Merkel cell tumors coexpression with neurofilament proteins which occur in perinuclear aggregates, has been reported [159,160,162]. A positive staining is reported with a mAb (34ßE12) recognizing stratification related CKs, but the authors speculated on cross-reactivity as an explanation for this unexpected finding [163].

## MESOTHELIUM

The IFP make-up of mesothelial or serosal cells is in part dependent on their morphological presentation. Normal mesothelium expresses CKs7, 8, 18 and 19, and CK5 heterogeneously. Vimentin is also found and in reactive processes its expression increases [24,92]. Proliferating, reactive (sub)mesothelial cells, showing myofibroblastic characteristics, coexpress CKs and vimentin [24,92]. The expression of simple epithelium CKs is largely retained in mesothelioma [78,83,142,164,165]. Furthermore, the epithelial structures of mesothelioma may show an enhanced number of CK5 positive cells, which may even occur in the sarcomatoid components. CK is however not expressed in pure sarcomatoid

mesothelioma [24]. In some mesotheliomas even CKs4, 6, 14 and 17 have been reported [166].

## ORAL CAVITY

The oral nonkeratinized epithelium (soft palate, buccal and vestibular mucosa, tongue, floor of mouth, oropharynx) and the keratinized epithelium (hard palate, gingiva, papillae of the tongue), including the unique junctional epithelium, have been described extensively with respect to their CK expression patterns [30,66,161,167]. The oral squamous epithelia deviate in the CK content from squamous epithelia occurring in other organs by expression of CK16, especially in the keratinized areas [2,161]. The junctional epithelium is unique because it is thought to originate from reduced enamel epithelium and it is attached to the cementum of the tooth with an external as well as internal basal lamina.

During development, the enamel epithelium is shown to coexpress vimentin and a complex CK pattern, i.e. CKs5, 14 and 17, which are typical of basal cells, as well as smaller amounts or traces of CKs7, 8, 18 and 19 [168]. Furthermore, IFP antibodies are used in studies of several odontogenic tumors [169,170] and have shown relatively complex CK patterns. The mAb LP34 (recognizing CK5, CK6 and CK18 and possibly others [171]) has been shown to differentiate dentigerous cysts from odontogenic keratocysts [172].

## OVARY

The ovarian surface epithelium expresses CKs7, 8, 18 and 19 in all cells and vimentin in most, while CK20 is not found [66,173]. Rete ovarii cells and granulosa cells of primary as well as cystic follicles express CK8 and CK18 and vimentin, while oocytes appeared to be devoid of CKs or vimentin [66,173,174]. Sporadic cells positive for CK7 are observed in the ovarian stroma [173]. The most common ovarian neoplasms are the epithelial neoplasms classified into benign, borderline and malignant variants, partly coexpressing CK and vimentin. In serous tumors, clear cell and endometrioid carcinoma frequent coexpression is present, with decreasing vimentin expression only in the poorly differentiated cases [66,115,173]. In mucinous tumors, minimal coexpression is found, similar to the endocervical mucinous epithelium, although acetone fixation of frozen

sections increased the number of vimentin coexpressing cases [173]. The CK expression pattern of the above mentioned types of ovarian cancer is more or less similar to that of the endometrial neoplasms (see above). A discrepance involves the more extended CK7 expression in the ovarian tumors, although a few mucinous tumors may show low expression levels of CK7 [86]. Significant CK20 staining, furthermore, was only found in the mucinous carcinoma type [54]. Stratifcation related CKs were not found in the mucinous and clear cell type [86], although others detected CKs4, 5, 10, 13 and 14 in mucinous tumors [173]. Stratified CKs could be found in serous and endometrioid types [86,173].

In Brenner tumor the epithelial component does not express vimentin and displays CKs typical of transitional epithelium (see section "urinary bladder").

The malignant mixed Müllerian tumors show CK and vimentin coexpression in both tumor components, although to a variable degree related to cell type [173,175,176]. Fibromas, (Sertoli-)Leydig cell tumors, granulosa cells, granulosa cell tumors [173,177] and endometrial stromal sarcoma [178,179] can also express CKs next to vimentin, which concerns mainly CK8 and CK18, although in a Leydig cell tumor only CK7 is mentioned [173]. For germ cell tumors see the section "testis".

## PANCREAS

The pancreatic ducts have been shown to express extensively CKs7, 8, 18 and 19 and a small number of cells also contain CKs4, 5, 14 and 17 [33,84,85]. The adult **exocrine** pancreas [61,85] shows CK8 and CK18. Adenocarcinomas are sofar shown to express CKs7, 8, 18 and 19 [15,24,78,84,85,120]. In typical adenocarcinoma, CK4 varies between absence, and limited to extensive expression. Additionally, heterogeneous staining is described for CKs5, 10, 13, 14 and 17, while their expression is related to morphologically recognizable squamous differentiation only in some cases [85]. Furthermore CK20 is often found to a significant degree [54]. In the adult **endocrine** compartment of the pancreas CKs are less extensively expressed, manifesting as some weakly CK8 and CK18 positive cells [84,85], with an occasional report mentioning a subpopulation expressing CK7 or CK19 [61]. In islet cell tumors CK expression is reported to be higher in non-insulinoma then in insulinoma [109].

## PITUITARY

In the normal pituitary gland, the endocrine cells express only CK8 and CK18, while in the folliculo-stellate cells also CK7 and CK19, as well as vimentin and GFAP are detected [180,181]. The squamous epithelial cells of the pars tuberalis and Rathke's cyst, which is considered as the origin of the craniopharyngioma, express next to the simple epithelial CKs, vimentin and GFAP also some stratified CKs [181]. In adenomas distinguishable intracytoplasmic CK patterns, as detected with mAb CAM5.2, are suggested to be related the production of growth hormone[182]. Also in adenomas, Crooke's hyalin is shown to contain CK8 and CK18 [183].

## PROSTATE

As mentioned earlier, the basal cells of the prostate contain CKs5, 6, 8, 10, 13, 14, 17, 18 and partly CK19 [40,42]. Luminal cells of the most distal acinar structures, which becomes apparent during puberty, express CK8 and CK18, while in the more proximally located luminal cells also CK19 is found [42,184]. In the pseudostratified and transitional epithelium-like lining of most proximal tubules CKs5, 7, 8, 13, 18 and 19 are found [42]. CK20 is expressed in sporadic glandular cells [3]. Vimentin is extensively coexpressed with CKs in alveolar luminal cells [42], is retained in hyperplasia and in a small part of cases of prostatic intraepithelial neoplasia (PIN). In PIN, furthermore, an increased expression of CK19 is found [42]. In basal cell hyperplasia, which may morphologically resemble PIN, these cells additionally express CK5 and CK13, thus distinguishing them from PIN cells. Adenocarcinoma of the prostate follows the CK expression of the PIN, with a variable number of CK19 positive cells [42]. CK7 is shown to be almost absent in luminal acinar cells and in adenocarcinoma [43,78,184], although certain antibodies may detect expression of CK7 in prostatic cancer [83]. Vimentin expression in prostatic carcinoma is reported inconsistently [42,184,185]. The rare basaloid carcinoma of the prostate, which is suggested to be of limited malignant potential, is reported to stain extensively with a mAb, reactive with basaloid or stratification related CKs [186].

## SALIVARY GLAND

Salivary glands and their tumors have been studied mainly with respect to their myoepithial component, which is extensively reactive with CKs 5, 14, 17 [24,38,39,44,187] (see above). Luminal acinar cells show CK8 and CK18, while CK7 and CK19 appear in the ductal structures. In pleiomorphic adenoma the diverse cell types retain their CK expression pattern, while most unstructured solid and trabecular regions showed staining variability, with most consistent staining for CK14 and inconsistent staining with the CKs of normal luminal cells, i.e. CKs7, 8, 18 and 19 [38,44]. In epidermoid areas CKs10, 13 and 14 can be found [38]. Basal cell adenoma and adenolymphoma (Whartin tumor) have been examined with broad spectrum mAbs [187-189].

## SKIN

The CK patterns in this typical keratinized squamous epithelium is outlined earlier. Basal cell heterogeneity is shown in rete pegs and in dermal papillae or flat epidermis [25]. In the skin of the adult nipple CK19 is expressed by some basal cells [147], while it is normally absent in the basal cell layer of skin [73]. In pathological conditions of keratinized squamous epithelium, the expected CK reaction patterns can be percepted and correlated with tissue morphological parameters, such as anaplasia and proliferation [73].

In squamous cell carcinoma [73,190] variable expression of simple epithelial CKs may be a common feature with CK7 limited and CK20 absent. With decrease of the differentiation level a down regulation of the stratified epithelial CKs1, 5, 10 and 14 is noted.

In basal cell carcinoma CK8 is commonly found, while CKs4, 18 and 19 are only expressed in a low number of cells and cases [190]. Habets et al. [191] could demonstrate significant levels of CK19, and CK7, but not CK8 in basal cell carcinoma. In immunoblotting studies CKs5, 14, 17 and low levels of CKs6, 8 and 15 have been found, but CKs7, 18 and 19 are not listed [2]. CKs can also discriminate cell types in skin appendages and their tumors [73,192]. Also in nonneoplastic skin disorders CK-antibodies can be diagnostically useful or can clarify cell biological aspects of the skin differentiation process. For example, in basal cells of epidermolysis bullosa simplex [193] clumped tonofilaments, labeled

with CK5 and CK14 mAbs, are described to occur in basal and parabasal cells. In hyperproliferative skin disorders and after (experimental) trauma an increase of CK16 expression anticipates epidermal proliferation, followed by a decrease of CK10 [194]. It is concluded that CK16 expression is not a consequence of hyperproliferation but indicates that hyperproliferation has been triggered [195,196]. In psoriasis, CK17, which is not present in normal epidermis, has been shown to be expressed in the upper part of the skin and a decrease of CK17 expression parallels succesful therapy [197]. Also a decrease of CK16 levels have been shown to correlate with succes of treatment [198]. In anogenital condylomata accuminata the alteration of CK1 and CK10, expressed in the normal squamous epithelium, into expression of CK4 and CK13 is described [199].

## TESTIS AND GERM CELL TUMORS

Differences in the pattern and level of CK and vimentin expression have been described for the developing and adult rete testis as well as epididymis, depending on age and anatomical region [200]. In developing, prepuberal, senile and atrophic testis, the Sertoli cell can coexpress CK8 and CK18 along with vimentin [201,202], while in the normal mature testis these cells express vimentin only. In case of intratubular germ cell neoplasia, Soosay et al. [203] found the atypical cells to lack CKs, while the Sertoli cells in these atypical and adjacent atrophic tubules could show CK expression. Niehans et al. [204], however, interpret the atypical cell as CK positive. Initially, seminomas and dysgerminoma were decribed to express vimentin but not CKs, other than in sporadic cells, possibly representing syncytiotrophoblastic cells [202,205]. More recently these germ cell tumors were found to react with CK antibodies showing limited tumor areas expressing CK8, CK18 and scanty CK19 [91,173,206,207], while in dysgerminoma CK7 is also observed [173]. The occasional syncytiotrophoblastic cells in typical seminoma also express CK7 [208]. Even sporadic CK4 or CK17 positive cells have been observed in seminoma. Moreover, vimentin negative seminomas, and occasionally seminomas coexpressing several IFP types have recently been reported [91,206,209]. These data are considered as suggestive for divergent types or transitional forms of seminoma.

Recently, spermatocytic seminoma with scattered CK18 positive tumor cells,

was reported [81].

In the group of nonseminoma, the embryonal cell carcinoma, choriocarcinoma and endodermal sinus tumor have been reported to stain extensively for CK8 and CK18. However, staining in embryonal cell carcinoma was weakly and formalin fixed tumors of this type easily loose their CK immunoreactivity [80,173,203,208]. Additionally, CK19 is expressed scarsely in embryonal cell carcinoma and strongly expressed in the latter two tumor types and CK7 is reported inconsistently [80,173,209]. Even some CKs4, 17 and some CK10 expression may be found in embryonal carcinoma and endodermal sinus tumor, indicating a potential for squamous epithelial differentiation [80,173,209]. Individual scattered syncytiotrophoblastic cells can be found to be CK7 positive [80,208]. It is therefore surprising that CK7 is absent in a choriocarcinoma component [80]. Choriocarcinoma cell lines were, however, described to be CK7 positive [208]. Also of the placental syncytiotrophoblast only a typical subset of cells, i.e. at the main stem villi, stain for CK7 [208]. The more mature epithelial component in teratoma expresses CKs according to their morphological type of differentiation [210,211].

## THYMUS

Epithelial cells of the normal thymus contain next to simple epithelium CKs, along with CKs characteristic of stratified epithelia [2,20,212], while in immunohistochemical studies the stratification related CK5 is found in reticulum cells [24], and also the simple epithelium CK20 is found in sporadic cells, mostly in the medulla [3]. In Hassal's bodies CK18 has been immunostained by strongly reacting mAbs (DA7 and DC10) [213] next to immunoreactivity for CK10. Different CK expression patterns are described in relation to the anatomic areas or in relation to hyperplasia of the thymus, such as in myasthenia gravis [214-216]. In thymoma, even in those that are predominantly lymphocytic, a relatively high number of CK positive cells can be found [14,217]. In contrast, lymphomas involving the thymus will give negative results, except for foci of residual thymic tissue which may occasionally be found.

## THYROID

The normal, nodular and neoplastic thyroid all three express CK8 and

CK18 to a similar extent. CK19, however is less extensively expressed, except in papillary and mixed papillary-follicular carcinoma, which both express CK19 extensively [218]. In squamous areas CKs4, 13 and 10 can be found [218,219]. Vimentin coexpression is well known in the thyroid gland. This holds true for the neoplasms of which medullary carcinoma exhibits most limited vimentin expression [108,220].

## URINARY BLADDER

In the early fetal renal pelvis the epithelial lining expresses CKs8, 18, 19 and some CK7, but also some vimentin. In later developmental stages, vimentin expression decreases while CK7 levels increase [63]. Additionally, in the basal cell compartment of developing transitional epithelium CKs4, 5, 13 and 17 can be found [40,63]. Juvenile ureter transitional epithelium has been mentioned to be uniformely CK14 positive [25], which is in contrast to the adult situation [52]. The superficial umbrella cells are strongly stained by CKs8, 18 and 20 reagents, while CK8 and CK18 can furthermore also be demonstrated in basal and parabasal cells, although only by a subgroup of mostly strongly staining mAbs [3,50,53]. In adult transitional epithelium, CK7 is homogeneously expressed in renal pelvis, ureter, and heterogeneously in the bladder lining [50], even after use of strongly staining CK7 mAbs.

In all grades of transitional cell carcinoma CK7, however, is homogeneously expressed (Figure 2) as long as squamoid differentiation is absent [51-53]. During and tumor progression from noninvasive grade 1 to invasive grade 3 or metastasis, CK13 expression, typical for mature transitional epithelium, decreases considerably and most high-grade cases are CK13 negative. In this respect transitional cell carcinoma resembles squamous cell carcinoma, which also looses CK13 upon dedifferentiation. In a minority of cases of grade 3 transitional cell carcinoma CK13 can remain present [51-53,74]. Surprisingly, in high grade transitional cell carcinoma CK20, which in normal urothelium is only present in the umbrella cells, is extensively expressed [54]. During tumor progression several other CKs, such as CK14 may become expressed, but these characteristics do not appear to have prognostic consequences [51-53]. Squamous metaplasia in nonneoplastic

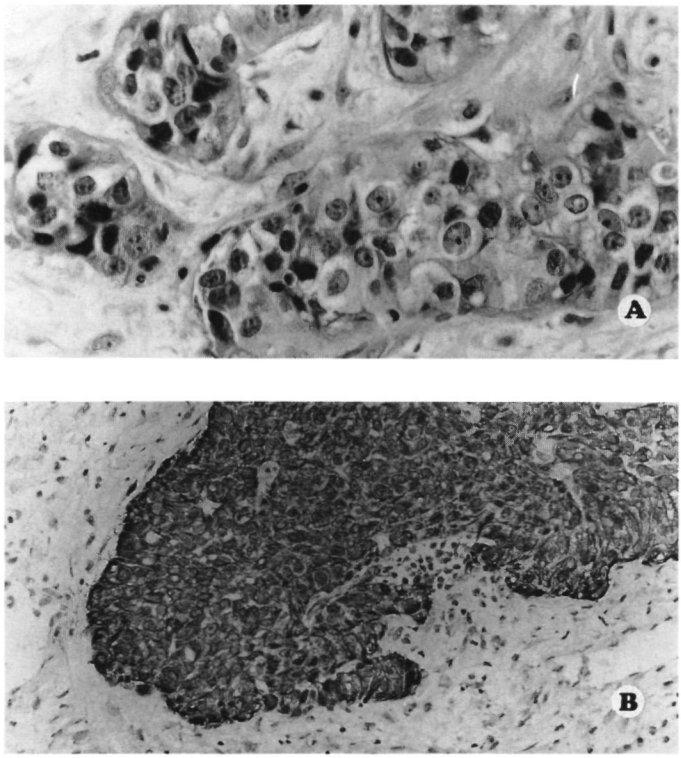


**Figure 2.** Metastasis of an unknown primary carcinoma in a axillary lymph node of a 47 year old male. (**A**) H&E stained paraffin section; (**B**) frozen section immunostained with a CK7 mAb (RCK105). Based on morphology, and a homogeneous and extensive positivity for CK7 we suggested the urinary bladder as the primary tumor site, which was confirmed in subsequent clinical analyses. Magnification (**A**) 400X, (**B**) 200X.

urothelium and in transitional cell carcinoma displays CK expression patterns deviating from normal urothelium and from the classical transitional cell carcinoma pattern. This type of differentiation displays regular squamous characteristics [51-54,221], such as decreased expression of CK7, CK8, CK18 and CK20 and increased levels of CK4, CK13, CK14 and CK17.

## UTERINE CERVIX

The CK expression patterns of the diverse cell types at the cervical squamo-columnar junction and of these cell types during changes from basal cell hyperplasia, through (immature) squamous metaplasia and different stages of cervical intra-epithelial neoplasia (CIN), upto squamous cell carcinoma and adenocarcinoma, are extensively described [23,65,77,171]. The most important features are described in relation to the endocervical reserve cells, which are shown to express a complex mixture of basal cell or stratification related CKs and several simple epithelial CKs. These reserve cells express extensively CKs8, 14, 15, 16, 17, 18 and 19, and to a lesser degree CKs5, 6 and 7 (Figure 3).

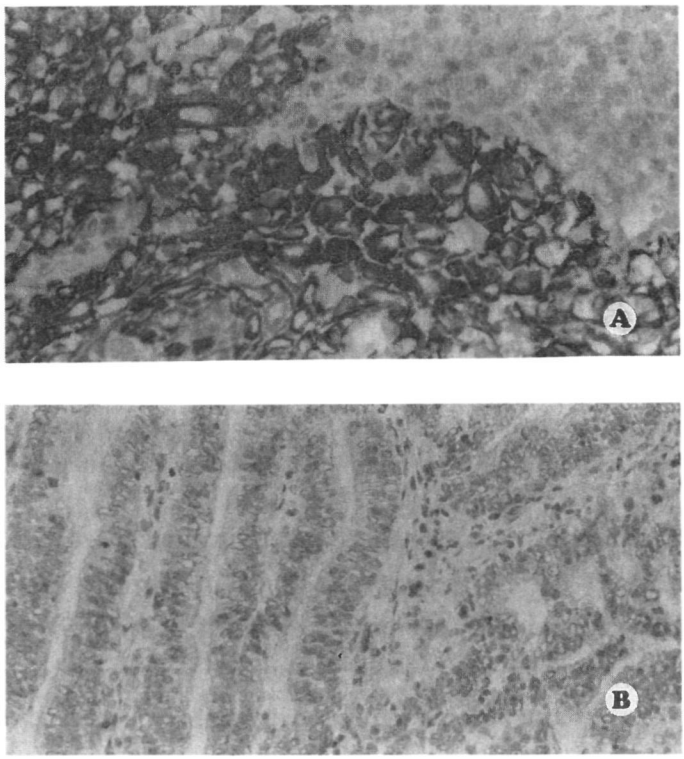


**Figure 3.** Frozen sections of a squamous cell carcinoma (**A**) and an adenocarcinoma (**B**) of the uterine cervix immunostained using a CK5 mAb (AE14) showing positivity in (**A**) and no reaction in (**B**). Magnification (**A**) 400X, (**B**) 200X (courtesy: Dr. Frank Smedts, Delft, The Netherlands)

Some of the endocervical columnar cells may express basal cell or stratification related CKs, along with the simple epithelial CKs. The CK pattern in squamous metaplasia is more or less as can be expected from its type of morphological differentiation, but CK16 which is mostly not expressed, may in some cases be extensively present. In dysplasia or CIN, CK4 and CK13 expression decreases, in particular in CIN III, but some expression remains. Other stratification related CKs remain extensively present, except for CK17. The expression of simple epithelial CK8 and CK18 and of CK17 increases considerably with increased severity of CIN. Approximately 10% of CIN I and CIN II lesions display this combination, while in CIN III, 50% of lesions contain this CK set. This has induced the hypothesis that expression of CK17 along with CK8 and CK18 may be a prognosticator of progression. In CIN III even some CK7 can be found in basal and parabasal cells. This pattern is continued in squamous cell carcinoma with an additional variable expression of CK17 depending on tumor type. In squamous cell carcinoma of the cervix the keratinized variant displays lower levels of CK8 and CK18 than the nonkeratinized type. The absence of CK15 in half of the nonkeratinized squamous cell carcinomas is remarkable since this CK has been related to a squamoid type of epithelial differentiation [26,34]. In cervical adenocarcinoma significant levels of CKs7, 8, 14, 17, 18 and 19 and sporadic expression of CKs4, 5, 6, 10, 13, 16 and even CK20 are found.

## VULVA

In early stages of vulvar squamous cell carcinoma CK10 is reported to be rarely detectable, regardless of tumor grade, while it is demonstrated in many advanced stage tumors [222]. A CK10 negative reaction was suggested to be related to tumor relapse of vulvar squamous cell carcinoma [223]. Relatively little CK8 and CK18 is reported in these carcinomas, as compared to the mucosal squamous cell carcinomas [67,70]. At the stroma-tumor interface significant vimentin expression can be observed in the carcinoma cells [70].

CK reagents are used most frequently to distinguish epithelial from nonepithelial cells, despite the awareness that CK expression may occur also in nonepithelial tissues (see Table 2). In general, CK immunohistochemistry will be used in diverse areas which will demand different types of CK reagents. Some general remarks in this respect are useful for a better understanding of the practical applications.

1/- Strongly reacting CK antibodies with broad specificity are useful in the distinction of epithelial and mesenchymal cells or to detect sporadic epithelial tumor cells in a mesenchymal background.

2/- MAbs, recognizing formalin fixed and paraffin embedded CK epitopes or CKs subjected to other types of tissue processing techniques, are a prerequesite for full employment of CKs as markers in routine surgical pathology.

3/- Monospecific CK mAbs recognize one type of CK, some of which mark a certain epithelial differentiation pathway, and may therefore reveal the primary site of a carcinoma.

4/- Strongly immunoreacting, but differentiation type specific mAbs are useful to distinguish different types of epithelial cells. For example, basal or stratified epithelial cells can be recognized separately from luminal or glandular type of differentiation in the differential diagnosis of adenocarcinoma versus nonmalignant ductal structures.

5/- Certain CK mAbs detect only specific CK configurations. Conformation dependent reactivity of such mAbs has been shown to be useful in the detection of apparent structural changes in the epithelial cytoskeleton. These may then correlate to the functional status of the cell [50-52,78].

It is stressed that certain monospecific CK mAbs still show broad tissue reactivity patterns because of the presence of the target CK in many tissues, which is for example the case for CK8. Of the broadly cross-reacting mAbs, which are shown to interact with more than one CK in immunoblotting, several are known to stain strongly only with a few of these CKs when applied to tissue sections, and react only weakly or not at all with the others. This limits the use of these mAbs as pan-epithelial reagents. A cocktail of different mAbs

can, however, help to increase CK detection or staining levels [14,224,225]. Thus, it will be important to specify the different reactivity levels of mAbs or cocktails of mAbs with the individual CKs. This should not only be the case in scientific publications, but also in data sheets or manifacturers instructions of commercially available mAbs. When comparing CK reagents to other "epithelium specific" markers, the former show in general a relatively high sensitivity as well as high specificity [14,224,226,227].

*Applications in histopathology*

Consequent to the considerations described above, several applications of CK subtype specific mAbs in diagnostic histopathology can be anticipated. The main field of interest is in the differential diagnosis of tumors, with use in distinction between epithelial and non-epithelial type of differentiation. Moreover, some monospecific CK antibodies have already proven their usefulness in the distinction of different types of carcinoma, and it is to be expected that several of the newly developed CK reagents will further expand these possibilities.

In the differential diagnosis of adenocarcinoma, detection of CK subtypes can be most helpful. For example, "Müllerian" derived carcinomas of the female genital tract show CK7 expression, while colonic carcinoma does not express significant levels of this protein. Gastric cancer may show CK7 only in some cases. On the contrary, CK20 can be detected in both gastro-intestinal tumors (albeit only in a part of gastric cancers) and is virtually absent in the non-mucinous ovarian carcinomas and in endometrium carcinoma [3,54,86]. In male, the main CK7 positive carcinomas are transitional cell carcinoma (Figure 2) and non-squamous, nonsmall cell pulmonary carcinomas [78]. CK20 is frequently found in transitional cell carcinoma (without squamous differentiation) but is not significantly present in pulmonary carcinoma [54].
The presence of basal cell or stratification related CKs may be helpful in the differential diagnosis of adenocarcinoma. Especially CK5 is consistently, although limited, expressed in endometrial carcinoma (glandular structures included), distinguishing this "Müllerian" derived carcinoma from carcinoma of colon,

stomach, breast and kidney [24,88]. CK4 in "Müllerian" carcinoma may also add some weight in discriminating these tumors from others although certain adenocarcinomas, such as pancreas and lung, occasionally express CK4 and or CK5, corresponding to their generally known potential for focal squamous differentiation [24,84,85]. Clear cell carcinoma of ovary and endometrium can not be discriminated from renal cell carcinoma on basis on intermediate filament expression. Another basal cell marker, CK14, has been shown to be consistently expressed in pulmonary squamous cell carcinoma, also in the poorly differentiated subtype. Taken together with the observation that adenocarcinoma and large cell undifferentiated carcinoma of the lung express CK7 in significant amounts, the data presented sofar [30,76] indicate that a clear distinction between small cell anaplastic carcinoma of the lung, which contains almost exclusively CK8 and CK18 and nonsmall cell carcinoma, can be made.

Next to these major areas of application these antibodies are applicable to specific cases. For example, CK reagents can facilitate and increase the detection rate of (micro)metastases in lymph nodes and bone marrow, but the clinical significance of the detection of micrometastases is disputed [228-230]. Furthermore, in the absence of chorionic villi one can make a diagnosis of intrauterine pregnancy in abortion material or curettage by demonstration the CK positive intermediate trophoblast lying in-between the CK negative decidual cells [231]. In prostatic tissue, basal cell related or stratified CK mAbs are used in the differential diagnosis between adenocarcinoma versus atypical nonmalignant lesions, also after radiotherapy. The group of benign lesions more or less contain the basal cell layer, which can be stained with these CK mAbs [232-234]. Especially in needle biopsies caution is requested in the interpretation of this application of CK immunohistochemistry, because of sampling errors or rare deviating carcinoma subtypes [186].

*Application in cytopathology*

The use of immunocytochemical procedures in cytopathology is slowly becoming more widely acccepted. Different preparation techniques can be employed, including cytocentrifugation and fixation techniques [16], or even paraffin-embed-

ding of cell pellets [225,235], special sedimentation and fixation techniques [16], and application of immmunocytochemistry to routinely-fixed and Papanicolaou stained smears [236,237].

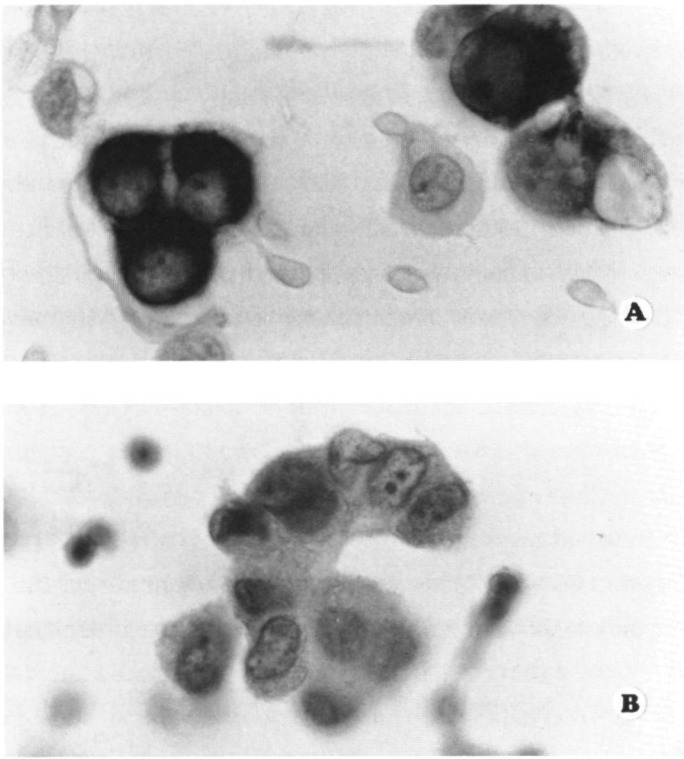


**Figure 4.** Cytological smears of ascites, previously Papanicolaou stained, and thereafter immunostained for CK7 using mAb OVTL12/30. The ovarian carcinoma cells are positive (**A**, clusters), while mesothelial cells (**A**, single cells) are negative in this specific procedure. Colonic carcinoma cells are CK7 negative (**B**). Magnificatinon 1000X (courtesy: Dr. Frank Smedts, Delft, The Netherlands)

Typical differential diagnostic problems in cytopathology concern the distinction of (atypical) mesothelial cells from malignant cells, in most cases adenocarcinoma on the one hand, and on the other the classification of the primary location of the adenocarcinoma. In this respect, CK7 is a useful marker. Recently, a mAb

to CK7, reactive in paraffin embedded tissues [83], has also been shown to immunoreact in Papanicolaou stained slides [237]. As in histopathology [78,83], this reagent (OVTL12/30) discriminates between different types of adenocarcinoma, but additionally separates mesothelial cells and mesothelioma (which do not stain with this mAb in this technique) from several CK7 positive adenocarcinomas (Figure 4) [237]. This is surprising, since in fresh frozen sections the mAb reacts with mesothelial cells, including mesothelioma. Apparently, fixation and other steps in the staining process destroy or mask their epitope for CK7, recognized by this mAb.

For most of the questions in cytopathology, the rules described above for histopathology can be in general be applied [16,238,239]. It should be kept in mind when evaluating IFP in cytological preparations that vimentin coexpression may be an even more common phenomenon in effusions than expected on the basis of histopathological data [16].

*Other applications of cytokeratin antibodies*

Other possible applications of antibodies to CKs in medical diagnosis include the detection of extracellular CK in serum or body fluids. Such assays have been described for CK8 or CK18 in serum or urine, monitoring transitional cell carcinoma or different types of adenocarcinoma [240-242]. Antibodies to tissue polypeptide antigen (TPA) have been used extensively in serum assays for monitoring tumors. The antigens recognized by these antibodies have now been identified as CK8 and possibly also CK18 and CK19 [243,244]

Flow cytometry of cell suspensions of tumors has proven to be a useful technique in the estimation and quantification of various cell components. These estimations may however, be severely impaired because of mixed cell populations found in such tumor cell suspensions. By staining cells for CK the epithelial and mesenchymal cell populations may be distinguished, enhancing specificity or sensitivity for the target cell population. The application of CK and vimentin reagents in the flow cytometric analysis of body cavity effusions reveals that this type of assay significantly increase the level of tumor cell detection [16,245].

*Conclusions*: Insight in CK expression patterns of diverse types of normal and malignant epithelial tissue is still increasing. The number of monospecific CK mAbs is gradually increasing, as is the availability of such mAbs suitable for application in formalin fixed tissue. Further exploration of their usefulness on large panels of cancer lesions is, however, still needed in order to improve their reliability for clinicopathological purposes. A possible prognostic significance of certain CK expression patterns now emerges [79,99,153,156,223] and may in the future turn out to be relevant. Studies on biological processes in which CKs are involved will enhance our understanding and applicability of these intriguing cytoskeletal proteins.

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# CHAPTER VII

## Summary

## Samenvatting

## Dankwoord

## Curriculum vitae

## Summary

This thesis focusses on the exploration of immunohistologically determined expression patterns of cytokeratin (CK) subtypes in human transitional cell tumors of the urinary tract. Transitional cell tumors cover a spectrum from a noninvasive transitional cell papilloma, without anaplastic cell changes and with a favourable prognosis on the one hand, to invasive, highly anaplastic and metastasizing carcinoma with a poor prognosis on the other hand.
Our main interest was to explore the CK expression patterns in relation to the progression stage of transitional cell carcinoma lesions and to evaluate the biological or clinical significance of aberrating CK expression patterns found in the neoplasm as compared to the normal transitional epithelium.

In Chapter 1 a short introduction is given concerning the general properties of the cytoskeletal proteins of which CKs form a subgroup. The aims, rationale and global approaches of the studies are also mentioned briefly.

The CK expression patterns in normal urothelium are described in Chapter 2, as a basis for the subsequent studies of the tumor lesions. One of our observation is that staining patterns of CK8 and CK18 are antibody dependent, related to masking of certain epitopes. The difference in CK7 expression between the transitional epithelium of the upper and the lower urinary tract was also striking. For this phenomenon no plausible explanation could be provided sofar. Certain differentiation specific CKs, such as CK13, showed a cell type restricted expression pattern.

In Chapter 3 the CK expression patterns are described in relation to progression of transitional cell carcinoma. CK8 and CK18 detectability was increased in the invasive tumor component and especially in the cells at the periphery of the invasive cell clusters. This phenomenon was observed using certain antibodies of the anti-CK8 and CK18 panel. We speculate on the biological significance of this phenomenon in relation to invasive growth and about the phenotype of potentially metatastic cells. Expression of the differentiation related CK13 decreased in high grade and stage transitional cell carcinoma. Variable, aberrant expression of CK14 appeared in these carcinomas. In

high grade tumors squamoid differentiation was accompanied by loss of CKs normally present in transitional cell carcinoma. Especially the finding of loss of CK7 has practical consequences, because it is used in surgical pathology as a marker for transitional cell differentiation.

The concept that increased detectability of CK8 and CK18 might indicate a potentially metastatic phenotype was challenged in the study (Chapter 4) on transitional cell carcinomas and autologous metastases. It appeared that the metastases showed the same CK staining patterns as the original high grade bladder tumors, without a predominance of cells with increased CK8 and CK18 staining. However, the possibility of a transiently increased exposure of CK8 and CK18 epitopes during the actual proces of tumor cell spread could not be explored in our studies. In the invasion front of metastases we observed, otherwise, a similarly increased detectability of these two CKs as seen in the original bladder tumors. These findings are suggestive for a role of the CK cytoskeleton in tumor invasion of transitional cell carcinoma.

In Chapter 5 we tested whether this phenomenon was restricted to transitional cell carcinoma or could be found also in another type of carcinoma. Similar staining patterns for CK8 and CK18 in the invasion front and/or in the interface between tumor cells and stroma were observed in mucosal squamous cell carcinomas. In squamous cell carcinoma, also an increased vimentin expression was observed, although a direct topological relation between vimentin and the two CKs was not found. Our immunohistochemical findings, suggesting on a role of CK8 and CK18 in invasion or metastatic potential of tumor cells are in line with those obtained by other groups [1,2].

In Chapter 6 we reviewed the most relevant literature on CK immunohistochemistry in general and CK subtyping in human tissues in particular. Emphasis was put on the use of CK antibodies in surgical pathology. In addition, a detailed survey on the CK distribution in normal tissues and carcinomas was given. This survey showed particular patterns of CK expression that, according to the present views, are mainly differentiation related. However, CK expression in tumors may quite often not represent the pattern expected on basis of the distribution pattern seen in the corresponding normal tissue. For some types of carcinomas, such as breast cancer and Grawitz tumor, consistent relations have

been found between the prognosis of the disease and the type of IFP expression.

In *conclusion*, our immunohistochemical studies on CK subtyping demonstrate that:

1- In normal urothelium, CK expression is generally related to morphologically observed differentiation patterns, although in anatomical transition zones between different types of epithelium changes in the CK expression pattern can preceed morphological alteration. Furthermore, heterogeneity without a morphological substrate can be found, such as for CK7 expression, which is patchy in the bladder and homogeneous in the upper urinary tract.

2- In the low grade malignant transitional cell neoplasms, the CK expression seen in the normal transitional epithelium is largely retained. However, expression of the differentiation related CK13 may be decreased.

3- With progression of transitional cell carcinoma CK expression diverges from the nonneoplastic transitional epithelium by loss of some original CKs, e.g. especially of CK13 and to a lesser extent of CK7, 8 or 18. Also an increased expression or neo-expression of several other CKs, e.g. CK4, 10, 14, 16, and 17 is notable. Changes in CK8 and CK18 detectability show a topological relation to areas of tumor invasion and changes in the expression of the other CKs are mainly related to differentiation type or grade of anaplasia.

4- Invasiveness of transitional cell carcinoma coincides with changes in the immunohistological detectability of CK8 and CK18. A basically similar phenomenon is present in mucosal squamous cell carcinoma.

5- Monoclonal antibodies, recognizing different epitopes of the same CK subtype, can yield different immunohistochemical staining patterns, which may confuse the interpretation of staining patterns. However, the underlying process of epitope masking/unmasking has probably biological significance.

6- Bearing in mind the limitations due to aberrant expression patterns, CK subtypes can be used in surgical pathology as an aid in tracing the original site of carcinomas or in determining the precise direction of differentiation. Examples include the differential diagnosis of types of pulmonary neoplasms and the discrimination of intestinal adenocarcinoma from other types of adenocarcinoma.

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Dit proefschrift richt zich op het onderzoeken van immuunhistologisch bepaalde expressiepatronen van cytokeratine (CK) subtypes in humane overgangscelcarcinomen van de urinewegen. Overgangsceltumoren beslaan een spectrum waarvan de ene zijde begrensd wordt door het niet-invasieve overgangscelpapilloom, zonder anaplastische celveranderingen en met een gunstige prognose, en de andere zijde door het invasieve, sterk anaplastische en metastaserende carcinoom met een slechte prognose.
Ons hoofddoel is het zoeken naar CK expressiepatronen in relatie tot stadia van progressie van overgangscelcarcinoom alsmede het biologische of klinische belang schatten van CK expressiepatronen in het neoplasma, die afwijken van het normale overgangsepitheel.

In Hoofdstuk 1 wordt een korte inleiding gegeven over de algemene eigenschappen van cytoskeleteiwitten waarvan CKs een subgroep zijn. De doelstellingen, overwegingen en globale benadering worden ook kort genoemd.

De CK expressiepatronen in normaal urotheel worden beschreven in Hoofdstuk 2 en dienen als basis voor de vervolgstudies betreffende de tumoren. Een van onze waarnemingen is dat aankleuringspatronen van CK8 en CK18 antilichaam afhankelijk zijn, hetgeen gerelateerd is aan maskering van bepaalde epitopen. Het verschil in expressie van CK7 tussen het overgangsepitheel van de hogere urinewegen en dat van de lagere is ook opmerkelijk. Wij kunnen tot nog toe hier geen plausibele verklaring voor geven. Bepaalde differentiatie specifieke CKs, zoals CK13, tonen een celtype bepaald expressiepatroon.

In Hoofdstuk 3 worden de CK-expressiepatronen beschreven gerelateerd aan progressie van het overgangscelcarcinoom. In het tumorinvasiefront en vooral in de cellen die aan de rand van de invaderende celclusters liggen, kunnen wij immuunhistologisch CK8 en CK18 gemakkelijker en/of vaker vinden. Dit fenomeen wordt waargenomen doordat wij gebruik maken van bepaalde antilichamen behorend tot het anti-CK8 en CK18 panel. Wij speculeren over het biologische belang van dit fenomeen in relatie tot invasieve groei en over het fenotype van potentieel metastaserende cellen. Expressie van het differentiatie

gerelateerde CK13 neemt af in het overgangscelcarcinoom van hoge graad en stadium. In deze carcinomen blijkt sprake te zijn van variabele, onverwachte expressie van CK14. In hooggradige tumoren gaat plaveiselcellige differentiatie gepaard met een verlies aan CKs, die normaliter aanwezig zijn in overgangscelcarcinoom. Vooral het verlies van CK7 heeft practische gevolgen, omdat dit CK in de diagnostiek als een marker voor overgangsceldifferentiatie gebruikt wordt door de patholoog.

Het concept dat een verhoogde mogelijkheid om CK8 en CK18 te signaleren zou kunnen duiden op een in potentie metastaserend fenotype, wordt beproefd in de studie van overgangscelcarcinomen en autologe metastasen (Hoofdstuk 4). Het blijkt dat de metastasen hetzelfde CK expressiepatroon hebben als de oorspronkelijke blaastumoren, zonder dat het aantal CK8 en CK18 aankleurende cellen is toegenomen. De mogelijkheid van voorbijgaande verhoogde expressie van CK8 en CK18 epitopen tijdens het ware moment van tumorceluitzaaiing kan echter niet bekeken worden in onze studies. Aan de andere kant zien wij in het invasiefront van metastasen een gelijksoortige verhoogde herkenning van deze twee CKs, net als in de blaastumoren zelf. Deze bevindingen zijn suggestief voor een rol van het CK cytoskelet bij tumorinvasie van overgangscelcarcinoom.

In Hoofdstuk 5 testen wij uit of dit fenomeen beperkt blijft tot overgangscelcarcinoom of dat het ook in een ander type carcinoom gevonden kan worden. Gelijksoortige kleuringspatronen voor CK8 en CK18 in het invasiefront en/of in het raakvlak tussen tumorcellen en stroma worden gezien in plaveiselcelcarcinoom van slijmvliezen. Bij plaveiselcelcarcinoom wordt ook nog verhoogde expressie van vimentine gevonden, hoewel een direct topologisch verband tussen vimentine en de beide CKs niet wordt gevonden. Onze immuunhistochemische bevindingen, die suggestief zijn voor een rol van CK8 en CK18 bij invasie of metastaserend vermogen van tumorcellen, passen bij die welke verkregen zijn door anderen [1,2].

In Hoofdstuk 6 geven we een overzicht van de meest relevante literatuur over CK immuunhistochemie in het algemeen en CK subtypering in humane weefsels in het bijzonder. Nadruk wordt gelegd op het gebruik van CK antilichamen in diagnostische pathologie. Voorts wordt een gedetailleerd overzicht

gegeven van de CK distributie in normale weefsels en carcinomen. Het blijkt dat CK expressie patronen, volgens de huidige opvattingen, voornamelijk gerelateerd zijn aan differentiatie. Het kan echter nog al eens voorkomen dat de CK expressie in tumoren niet overeenkomt met het patroon zoals dat verwacht wordt op basis van de verdeling in het corresponderende normale weefsel. Voor enkele typen carcinoom, zoals bijvoorbeels borstkanker en de Grawitz tumor, wordt een relatie gevonden tussen prognose en het type IFP expressie.

Concluderend, tonen onze immuunhistochemische studies het volgende aan:
1- In normaal urotheel, is de CK expressie in het algemeen gerelateerd aan morfologisch herkenbare differentiatiepatronen, hoewel in anatomische overgangsgebieden tussen verschillende typen epitheel reeds verschuivingen optreden voorafgaand aan de morfologische veranderingen. Bovendien kan heterogeniteit gevonden worden zonder morfologisch substraat, zoals bijvoorbeeld het geval is voor CK7 dat vlekkerige expressie heeft in de blaas en homogene expressie in de hogere urinewegen.
2- In laaggradige overgangsceltumoren, komt de expressie van CKs overeen met die in normaal overgangsepitheel. De expressie van het aan differentiatie gerelateerde CK13 kan echter reeds afgenomen zijn.
3- Bij progressie van overgangscelcarcinoom wijkt de CK expressie verder af van die in niet-neoplastisch overgangsepitheel door het verloren gaan van oorspronkelijke CKs, met name CK13 en in mindere mate CK7, 8 of 18. Toegenomen expressie of neo-expressie van verscheidene andere CKs, zoals CK4, 10, 14, 16 en 17 is vermeldenswaardig. Veranderingen in de traceerbaarheid van CK8 en CK18 tonen een topologische relatie met gebieden van invasieve tumorgroei. Veranderingen in de expressie van andere CKs zijn merendeels gerelateerd aan de differentiatierichting of mate van anaplasie.
4- Monoclonale antilichamen, die verschillende epitopen herkennen van hetzelfde CK subtype, kunnen verschillende immuunhistochemische kleuringspatronen opleveren, waardoor verwarring kan optreden bij de interpretatie van aankleuringspatronen. Het onderliggende mechanisme van epitope maskering/demaskering is waarschijnlijk echter biologisch gezien van waarde.
6- CK subtypes kunnen gebruikt worden in diagnostische pathologie als hulp-

middelen bij het vinden van de primaire haard van carcinomen of in het bepalen van de differentiatierichting, maar men moet beducht blijven voor de beperkingen als gevolg van afwijkende expressie. Voorbeelden zijn de differentiaal diagnose bij de hoofdtypen van longtumoren en het onderscheiden van intestinaal adenocarcinoom van andere typen adenocarcinoom.

**Referenties**


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In de laatste maanden was Rosi Hansen op het Biomedisch Centrum van de Universiteit van Limburg een gezellige en behulpzame gastvrouw.

De auteur van dit proefschrift werd op 5 augustus 1954 geboren te Oranjestad (Aruba). Zijn schooltijd werd overwegend in Den Haag doorgebracht en in 1972 in Leeuwarden afgesloten met het eindexamen Gymnasium ß (Stedelijk Gymnasium). In 1973 ving hij in Leiden aan met de studie geneeskunde, nadat het voorafgaande jaar wegens uitloting voor geneeskunde, de studie rechten was ingezet. Tijdens de studie geneeskunde was hij enige jaren student-assistent bij de neuropatholoog Professor Dr. G.Th.A.M. Bots, alwaar hij de eerste boeiende aspecten van de pathologie gewaar werd. In 1980 werd het arts-examen afgelegd. Hierop aansluitend heeft hij tot half 1982 de dienstplicht vervuld als scheepsarts bij de marine, waarbij Curaçao meestentijds de thuishaven was. Na enige maanden ondersteunend onderzoek op de afdeling Neurologie van het Academisch Ziekenhuis te Leiden verricht te hebben, werd in 1983 begonnen met de opleiding tot patholoog-anatoom in het Laboratorium voor Pathologie van hetzelfde ziekenhuis (opleider Professor Dr. Ph.J. Hoedemaeker). Een belangrijk jaar van deze opleiding kon plaats vinden in het Westeinde Ziekenhuis te Den Haag (opleider A.P.R. Blok). Vanaf 1987 tot 1992 was hij werkzaam als patholoog in het Instituut voor Pathologie van het Radboudziekenhuis te Nijmegen. De laatste 3 jaren hiervan werd gewerkt in een half-time aanstelling, terwijl een andere half-time aanstelling bestond in het Canisius-Wilhelmina Ziekenhuis te Nijmegen. Sinds 1992 is hij full-time in dienst bij het Canisius-Wilhelmina Ziekenhuis, waarbij met het Instituut voor Pathologie en de afdeling Urologie van het Radboud Ziekenhuis nog enige banden onderhouden worden.

## STELLINGEN

1- De gelokaliseerde expressie van cytokeratines 8 en 18 speelt een rol bij het proces van invasieve groei van het overgangscelcarcinoom van de urinewegen en van het plaveiselcelcarcinoom.
(dit proefschrift)

2- Bij de interpretatie van immuunhistochemisch bepaalde expressie van cytokeratines dient rekening gehouden te worden met verandering in epitoop-configuratie. Dit maskeringsfenomeen kan ondervangen of onderkend worden door toepassing van meerdere monoclonale antistoffen tegen hetzelfde antigeen.
(dit proefschrift)

3- Voor het verschil in expressie van cytokeratine 7 tussen het urotheel van de urineblaas en dat van de hogere urinewegen bestaat geen afdoende wetenschappelijke verklaring.
(dit proefschrift)

4- Morfologische waarneming aan ziekteprocessen behoort niet zelfstandig door goedwillende medisch-biologische onderzoekers te worden verricht, maar behoort door een professional, in casu de patholoog, te worden gesuperviseerd.

5- Cytokeratine-subtypes dienen primair aangeduid te worden volgens de door Moll beschreven nummering.
(Moll et al., Cell 1982, 31:11-24)

6- Met het oog op efficiente bedrijfsvoering, opleiding en kwaliteitseisen is het opportuun om te streven naar een combinatie van analist/obductie-assistent en niet voort te borduren op de oude combinatie van mortuariummedewerker/obductie-assistent of zelfs op de full-time obductie-assistent.

7- De WHO-gradering voor het oppervlakkige blaascarcinoom kan niet zonder meer worden toegepast in gevallen van diep-invasieve blaascarcinoom.

8- De patholoog die prostaatweefsel beoordeelt dient op de hoogte te zijn van de morfologische beelden van prostaatcarcinoom, die het gevolg zijn van voorafgaande behandeling d.m.v. medicamenteuze "totale androgene deprivatie", te weten een sterke tumorcelatrofie. Bovendien dient deze er bij vriescoupe-onderzoek tijdens prostatectomie door de chirurg nadrukkelijk op attent gemaakt te worden of deze behandeling heeft plaats gevonden.

9- In de opleiding tot klinisch werkzaam patholoog dient binnen de bestaande opleidingsduur meer tijd ingeruimd te worden voor de cytologie. Deze wordt verkregen door een reductie in de tijd die nu door de arts-assistent in opleiding aan de obducties wordt besteed.

10- De positie van de wedstrijdroeier in het schip, te weten met zijn gezicht in de richting van het zog, versterkt de satisfactie bij een winnende race, zeker wanneer reeds kort na de start een voorsprong is genomen.

11- Om hinder veroorzaakt door honden en hun begeleiders in te perken, verdient het overweging de viervoeters op de openbare weg aan de voorzijde te voorzien van een muilkorf en aan de achterzijde van een vuilkorf.

Stellingen behorend bij het proefschrift "Cytokeratins in bladder cancer: an immunohistochemical study on features of tumor progression"

Nijmegen, 27 mei 1993, H.E.Schaafsma