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CLINICAL STUDY

Immunohistochemical characterization of urothelial carcinoma

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Abstract: From the archive of BB Biocyt company, 32 urinary bladder carcinomas (urothelium carcinomas, UC) and 7 cases of chronic cystitis were selected and examined in semiserial sections for the following antigens: 1) cell proliferation marker Ki-67 (expressed in the nuclei), 2) cell cycle regulator p16/INK4a polypeptide (expressed in the cytoplasm and nuclei), 3) urothelium marker p63 (expressed in the nuclei), 4) cytokeratin 7 (CK7). 5) cytokeratin 20 (CK20) and 6) high molecular weight cytokeratin (HMWCK). Invasive urothelium carcinomas showing a high grade dysplasia (invasive HG UC) comprised over the half (20 out of 32) of the investigated tumours. Microinvasion to lamina propria (seen in three HG papillary carcinomas) was regarded as an early infiltration even when the position of muscular layer could not be determined. Classical invasion across the urinary bladder wall and/or to surrounding tissues was found in 17 cases of low-differentiated HG UCs. The rest (9 out of 32 neoplasms) were either non-invasive papillary carcinomas of high (non-invasive HG UC, 5 cases) or low malignant potential (noninvasive LG UC, 4 cases). Finally, 3 cases were papillary urothelium neoplasms of low malignant potential (PUNLMP). HMWCK was present in all invasive tumours, whereas the frequency of other urothelium markers ranged from 65 to 88 %. Nevertheless, at least two markers were expressed in each invasive tumour. Staining for Ki-67 antigen was positive in over 50 % of the nuclei of HG UCs, while in the LG UCs, the frequency of positive Ki-67 staining did not exceed 25 %. In PUNLMP, the positive rate of Ki-67 stained dysplastic cells was below 10 %. The staining for p16 antigen did not correlate with the degree of dysplasia within urothelium tumours. For routine diagnostic, we recommend to combine the Ki-67 staining with detection of HMWCK. In cases of chronic cystitis, which developed urothelial hyperplasia and/or squamous metaplasia, the presence of p63 antigen was a relevant marker confirming the urothelial origin of the altered transitional cells *(Tab. 6, Fig. 4, Ref. 69)*. Full Text in PDF *www.elis.sk*.
Key words: urothelium carcinoma (UC), low grade (LG) dysplasia, high grade (HG) dysplasia, invasive growth, Ki-67, p16 and p63 antigens, cytokeratin 7, cytokeratin 20, high molecular weight cytokeratin (HMWCK).

During last years, the classification of urothelium carcinomas (UC) has undergone several improvements (43). The division into the non-invasive grade l (G1) neoplasms showing a low grade dysplasia, non-invasive papillary carcinomas of a high grade dysplasia (G2) and invasive low-differentiated carcinomas (G3) was revised. The G1 neoplasms were grouped to benign papillomas and papillary UCs of low malignant potential (LG UC). The distinction between papillary urothelium neoplasms of low malignant potential designated PUNLMP (19) as compared to papillary carcinomas of low malignant potential (LG UC) was highlighted by WHO classification (1998) and finally accepted the by International Society of Urology Pathologists (ISUP) in 2004 (17, 56). The difference between PUNLMP, LG UC and papillary carcinomas of high malignant potential (HG UC) was based on the symmetrical versus variable thickness of papillary structures and on cytological criteria reflecting the degree of the urothelium dysplasia (Tab. 1). These criteria of dysplasia have been more precisely defined (41, 42, 46, 68), since PUNLMP should be distinguished from LG UC on one hand, as well as from urothelium hyperplasia on other hand.

PUNLMP as well as LG UC may appear as papillary tumours showing exophytic growth confined to the urothelial epithelium without disrupting its basement membrane (clinically pTa). A classical criterion for invasive growth is the infiltration of *lamina muscularis* (1, 2) of urinary bladder wall, typically found by pT2 tumours (18). In contrast, the pT1 tumours invade the connective tissue just adjacent to the basement membrane (i.e. the *lamina propria*), but does not grow into the smooth muscle layer. At histological examination, the pT1 tumours are mainly non-invasive (and/or microinvasive) papillary carcinomas of high malignant potential (HG UCs). The most progressed UCs grow not only across the urinary bladder wall, but also into surrounding adipose tissue (pT3) and/or to neighbour organs including regional lymph nodes (pT4). In men, prostata gland is most frequently involved. At histological examination, the invasive HG UCs (former G3) correspond mainly to non-differentiated UCs consisting of pleomorphic and/or anaplastic cells.

As mentioned, the pT1 tumours are high grade (HG) papillary carcinomas showing exophytic as well as endophytic growth. Their papillae reveal irregular thickness due to extensive dysplasia. The HG UCs may be regarded as non-invasive even when showing mi-

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Tab. 1. Characterization of PUNLMP and papillary carcinomas as notified by the WHO/ISUP classification (2004).*

PUNLMP	Non-invasive LG UC	Non-invasive HG UC
Regular and symmetric papillary structure, papillary fusion extremely rare	Less symmetric papillary structure, occasionally variable thickness of papillae as well as their exophytic growth along with occasional fusion	Papillary structures upmost irregular, papillary fusion and endophytic growth frequent, papillae show variable thickness. Microinvasion to lamina propria possible (a sign of transition to invasive growth)
Dysplastic cells seen rarely, the Ki-67 positive rate below 10 %.	Basal cell dysplasia frequent, their polarity and orientation disrupted, the positive rate of Ki-67 cells close to 25 %	The frequency of dysplastic and/or pleomorphic cells high, the Ki-67 positive rate close to or even over 50 %
Great majority of cell nuclei shows regular size and shape, some oval cells may be seen	The oval and/or elongated cell nuclei, more frequent, the chromatin shows a fine appearance	The enlarged, polymorphic nuclei are abundant showing coarse chromatin granules and darker stain (hyperchromasia)
Mitoses hardly found	Mitoses present but not frequent	Mitosis relatively frequent
Surface urothelium cells always present	Surface "umbrella cells" can be still found	Surface urothelium cell are rarely seen
Recurrences possible, but metastasis never occurs	Recurrences very frequent, invasive growth is an exception, metastases are rare	Progression to invasive growth frequent, metastases possible, the surrounding epithelium shows carcinoma in situ

*Notice: modified from Montironi et al (42) and Miyamoto et al (39)

Tab. 2. Examples of cell cycle related proteins over expressed in urothelial tumours.

Protein	Function	Diagnostic role	References
Cyclin D	Regulates G1/S phase transition	Differentiation between LG UC and HG UC	17, 66, 67
p53	Transcription factor (TF) for cyclin expression, cell division and apoptosis regulator	Differentiation between PUNLMP and LG UC; its mutations important for UC pathogenesis	26, 60
pRb	Regulation of TF release for expression of enzymes involved in DNA synthesis	Low grade expression might indicate repeated tumor growth	20
Survivin	Apoptosis inhibitor	Over expression in the nuclei correlates with proliferation and dysplasia in HG UC	11, 57, 58
p16	Cyclin inhibitor	Correlation with cervical but not with urothelial dysplasia	29, 67
Ki-67	Cofactor for cell DNA synthesis	Cell proliferation indicator, excellent correlation with urothelial dysplasia	35, 48, 53, 62

croinvasive growth into the lymphatic spaces of *lamina propria* (i.e. pseudovascular growth) just below the preserved basement membrane [39]. However, some HG UCs (former G2 carcinomas), which show more prominent spread across *lamina propria,* might be classified as potentially invasive, even when their relationship to *lamina muscularis* is not clear (23, 51).

In order to recognize of the urothelial origin of non-differentiated invasive HG UCs especially within the prostatic area, staining for cytokeratins (such as CK7 and/or CK20 antigens) was recommended along with the p63 antigen staining (5, 63). Within normal urothelium, the CK7 antigen is expressed in many cells, while the CK20 antigen is confined surface "umbrella cells" only. The HMW-CK and p63 antigens can be found in basal urothelium cells mainly (10). In contrast, the dysplasic urothelium cells express CK20 as seen in papillary HG UC, where many cells are positive for this antigen. The CK7 and CK20 antigen staining was recommended for differential diagnostic of low-differentiated carcinomas in order to distinguish their adenomatous versus squamous cell origin (40). The CK7 and p63 antigen staining along with the detection of high molecular cytokeratin (HMWCK/clone 34βE12) were frequently applied to recognize the invasively growing HG UCs in males from their non-differentiated prostatic counterparts (20, 31, 50).

The correct judgment of cytological signs of urothelium dysplasia needs relatively much experience. For easier orientation,

Tab. 3. Grading of expression for antigens detected by immunohistochemical staining.

Grading scale	Estimated number of positive cells	Verbal estimate
4	90 %	very frequent
3	50–89 %	frequent
2	11–49 %	not frequent
1	<10 %	very few
0	<0.1 %	negative

several immunohistochemical markers of cell proliferation (such as for Ki-67 antigen) were applied to achieve precise grading. While the Ki-67/MIB-7 antigen correlates well with the cellular DNA synthesis, the other cell division regulator proteins listed in Table 2 are less reliable in this respect. As markers for proliferation of dysplastic urothelium cells, mainly p53 and Ki-67 proteins were recommended (55, 64). Estimation of the proportion of Ki-67 positive cells helps to differentiate between PUNLMP and LG UC on one hand, and especially between LG UC versus HG UC on other hand (28, 53). As stressed by Kunjin et al (30), the combination of CK20 and Ki-67 markers for the assessment of urothelium dysplasia is superior to other staining combinations. In this study, we included the p16 staining, which appeared highly relevant for the grading of cervical dysplasia (54), however, here with some disappointing results.

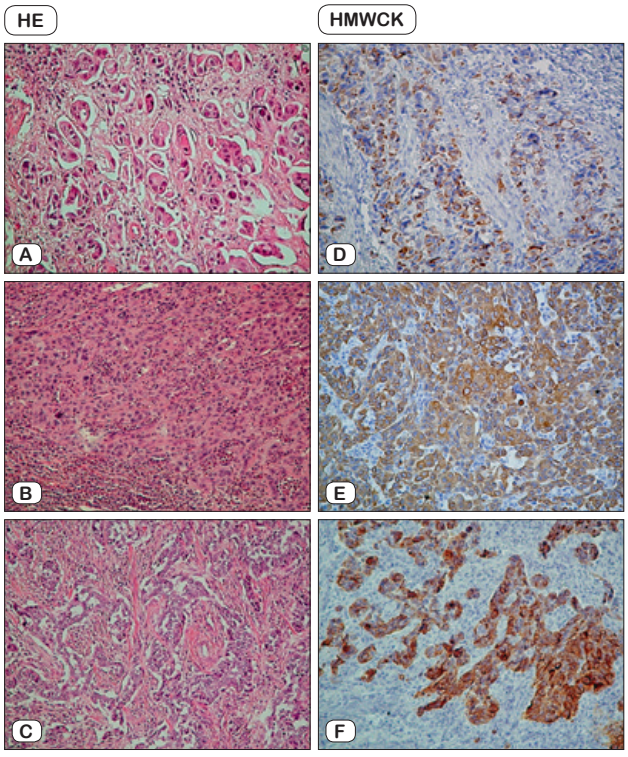

Fig. 1. Examples of low-differentiated invasive urothelium carcinomas (invasive HG UC). HE staining (in the left), HMWCK staining (in the right). A and D – micropapillary carcinoma; B and E – solid carcinoma; C and F – pseudoglandular structures.

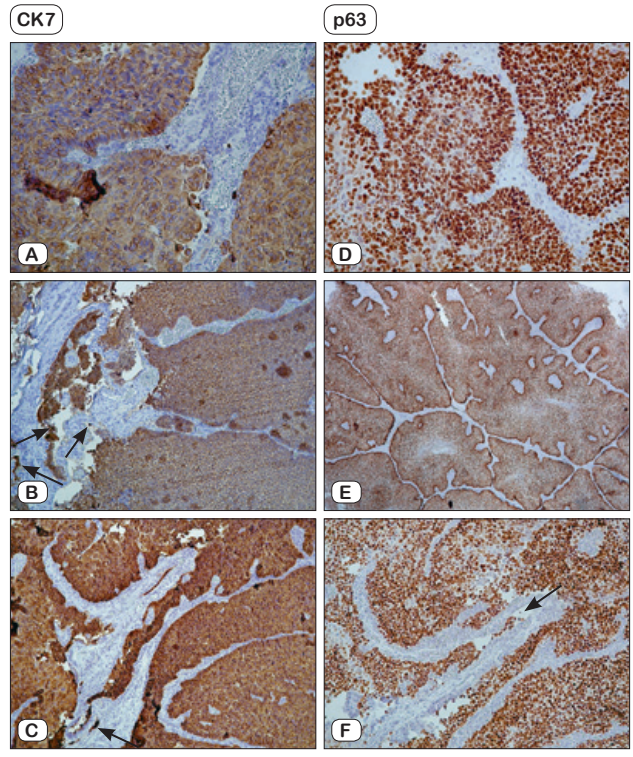

Fig. 2. Examples of microinvasive growth of papillary HG UCs. 2A and 2D: non-invasive HG UC (tumour number 1 from Table 5). A: CK 7 antigen staining (in the left) shows pseudovascular growth; D: p63 antigen staining (in the right), shows endophytic growth with budding of dysplastic urothelium cells into *lamina propria.* 2B and 2E (tumour number 19 in Table 4): microinvasive growth of the papillary HG UC showing scattered dysplastic cells within *lamina propria* (arrows) following disruption of the basement membrane. 2C and 2F (tumour number 20 in Table 4): endophytic growth of potentially invasive papillary HG UC showing disseminated dysplastic cells within the *lamina propria* following disruption of basement membrane (arrow). B and C stained for CK7 antigen; E and F stained for p63 antigen.

Materials and methods

We examined 32 urothelium neoplasms and 7 cases of chronic cystitis from the archive of BB Biocyt as registered in 2009. From the given neoplasms, non-differentiated carcinomas formed more than half (55 %) of the cases (17/32), while the rest were papillary carcinomas showing various degree of dysplasia, originally classified as non-invasive. At least 20 parallel sections (4 µm thick) were prepared and attached to 10 precoated slides (FLEX Dako). At least a single slide was stained with hematoxylin eosin (HE), the rest were handled by immunohistochemical methods. All the slides were deparaffinized, revitalized and rehydrated within the

Tab. 4. Frequency of antigen expression in invasive HG UC tumours.*

Number		Antigen					
	Diagnosis	p16	Ki-67	p63	CK7	CK20	HMWCK
17	low-differentiated HG UC	0/1***	0/0	0/5	0/2	0/6	0/0
		1/1	1/0	1/0	1/1	1/4	1/1
		2/4	2/2	2/0	2/1	2/6	2/8
		3/4	3/15	3/5	3/5	3/1	3/8
		4/7	4/0	4/7	4/8	4/0	4/0
Total		17	17	17	17	17	17
3	papillary HG UC**	0/0	0/0	0/0	0/0	0/1	0/0
		1/1	1/0	1/0	1/0	1/0	1/0
		2/2	2/0	2/0	2/0	2/2	2/0
		3/0	3/3	3/0	3/1	3/0	3/2
		4/0	4/0	4/3	4/2	4/0	4/1
Total		3	3	3	3	3	3

* grading scale described in Table 3, ** papillary carcinomas showing microinvasive growth, *** grade scale/number of tumors

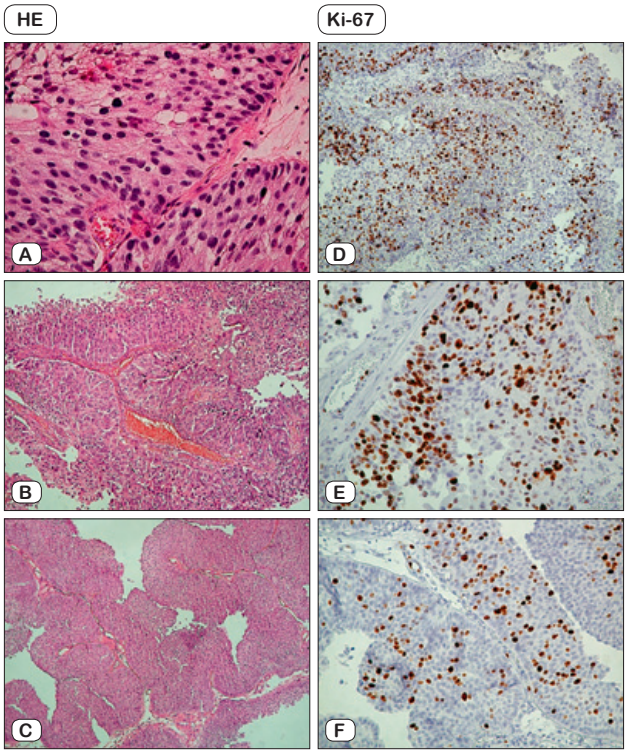

Fig. 3. Different grade of dysplasia by non-invasive HG UC versus LG UC in Ki-67 antigen staining. 3A to 3E: Non-invasive HG UC of high grade malignant potential. In the left: HE staining (3B and 3C) shows the polymorphism of the nuclei of dysplastic cells. In the right: Ki-67 staining (3D and 3E) points at the nuclear polymorphism of dysplastic cells. 3C and 3F: Non-invasive LG UC revealing relatively symmetric papillary structure (HE stain, 3C). By Ki-67 staining (in the right, 3F) the nuclei are smaller and more uniform, the positive rate being below 25 %.

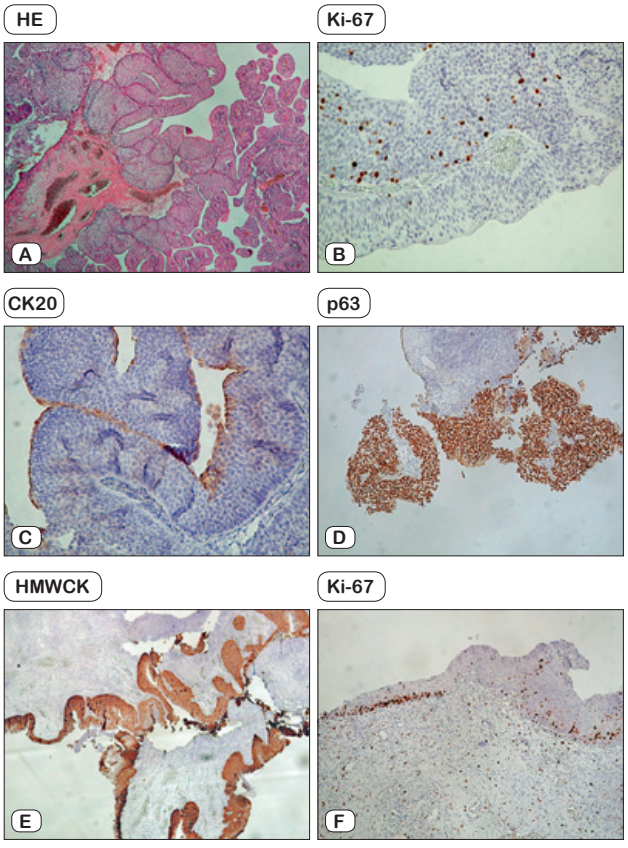

Fig. 4. PUNLMP and the reactive urothelium in chronic cystitis. 4A to 4C: papillary urothelial neoplasm of low malignant potential (PUNLMP) at HE staining (A), Ki-67 staining (B) and CK20 antigen staining (C). 4 D: urothelium hyperplasia (p63 antigen staining) . 4E: polypoid urothelium hyperplasia (HMWCK antigen staining). Fig. 4F: reactive focal urothelium hyperplasia (at the right side of the Figure the Ki-67 antigen staining shows irregular distribution of proliferating cells, whereas the normal urothelium reveals the Ki-67 cells located at basal layer).

PT Link equipment (Dako). Before staining, they were treated with 3 % H_2O_2 (to remove the endogenous peroxidase). In the first trial, the sections were treated with monoclonal antibodies (MoAb) against CK7, CK20, HMWCK, p63 and Ki-67 antigens (Dako), while one control slide was treated with the washing buffer (phosphate buffered saline, PBS) only. The MoAbs were used in dilutions provided by the manufacturer (being a part of corresponding kits). After washing 3 times, the slides were overlaid with the second labelled antibody (anti-mouse/Px) for 45 minutes, washed again three times and finally, treated with the chromogen solution (DAB, provided by each kit). The p16 antibody kit was purchased from the CINTec. One slide was treated with p16 MoAb after the same pretreatment procedure as described above, while another slide was first treated with the 'blocking reagent" from Dako (to prevent nonspecific staining). One negative control slide was overlaid with PBS only. Then, the immunostaining procedure was continued with the second Px-labeled antibody as described above. The parallel slides (numbered 1–10) were viewed in the microscope Nikon Eclipse 200 (objectives 4x, 10x, 20x and 40x) and photographed. For watching more details, the microscope was equipped with a glycerin immersion objective 60x. The frequency

Tab 5. Frequency of antigen expression in non-invasive HG UC tumours.*

Number		Antigen					
	Diagnosis	p16	Ki-67	p63	CK7	CK20	HMWCK
5	Non-invasive HG UC**	0/0	0/0	0/0	0/0	0/0	0/0
		1/2	1/0	1/0	1/1	1/2	1/1
		2/1	2/0	2/2	2/0	2/0	2/1
		3/2	3/5	3/2	3/1	3/3	3/3
		4/0	4/0	4/2	4/3	4/0	4/0
Total		5	5	5	5	5	5

* grading scale described in Table 3

Tab. 6 Frequency of antigen expression in non-invasive LG UC and PUNLMP tumors.*

Number		Antigen					
	Diagnosis	p16	Ki-67	p63	CK7	CK20	HMWCK
4	Non-invasive LG UC	0/0	0/0	0/0	0/0	0/1	0/0
		1/0	1/0	1/0	1/0	1/2	1/0
		2/2	2/4	2/0	2/0	2/1	2/2
		3/1	3/0	3/3	3/1	3/0	3/2
		4/1	4/0	4/1	4/3	4/0	4/0
Total		4	4	4	4	4	4
3	PUNLMP	0/0	0/0	0/0	0/0	0/0	0/0
		1/0	1/3	1/0	1/0	1/3	1/0
		2/3	2/0	2/2	2/1	2/0	2/3
		3/0	3/0	3/1	3/0	3/0	3/0
		4/0	4/0	4/0	4/2	4/0	4/0
Total		3	3	3	3	3	3

* grading scale described in Table 3

of positive cells per 1000 tumour cells was estimated for each antigen staining procedure in each tumour and the values were registered as presented in the Table 3.

Results

Low-differentiated urothelial carcinomas

The great majority of invasive UCs (also referred to as invasive HG UC), were non-differentiated neoplasms consisting of pleomorphic cells with large nuclei and abundant cytoplasm. Low-differentiated UCs clearly infiltrated the muscular layer of urinary bladder. Unfortunately, no clinical data describing the extent of their invasive growth outside of urinary bladder wall were available. Histologically, a proportion of invasive HG UCs could be classified as of micropapillary type (Figs 1A and 1D). Other invasive HG UCs were referred to as nested type (Figs 1B and 1E), since they consisted of solid strands and/or globular nests of pleomorphic cells. Further invasive HG UCs consisted of irregularly distributed pleomorphic cells grouped into the pseudoglandular formations (Figs 1C and 1F), while additional ones were composed of anaplastic cells showing sarcoma like growth. All low-differentiated UCs expressed the HMWCK antigen, while the other urothelium markers were less regularly found. As shown in the Table 4, the CK7 antigen was missing in 2 cases (10 %), the CK20 and p63 antigens were not seen in 6 or 5 cases (30 %). From differential diagnostic point of view, at least two urothelium markers were always present distinguishing the tumours in question from low-differentiated prostata carcinomas. When the CK7 and CK20 antigens were absent, the p63 and HMWCK markers were positive; if the p63 antigen was not found, the HMWCK and CK7 markers (occasionally also CK20) were present (Tab. 4). The cell proliferation marker Ki-67, as a rule, was positive in many nuclei (over 50 %) in each low-differentiated carcinoma at a frequency similar to the papillary non-invasive HG UCs. Noteworthy, three relatively differentiated papillary HG UCs, which showed microinvasive growth into the *lamina propria* (as detected by p63 and/or CK7 staining) (Figs 2B to 2C), were classified as potentially invasive and therefore included into Table 4.

Non-invasive papillary urothelial carcinomas

The cardinal sign of papillary HG UCs is the irregular structure and uneven distribution of dysplatic urothelium cells forming clusters of variable thickness. In non invasive UCs revealing a high grade of malignant potential (HG UC), the enlarged nuclei of dysplastic cells were uneven in size, showed oval or elongated shape and dark basophilia due to hyperchromatic appearance of large granular chromatin (Figs 3A and 3B). The variable size and shape of the nuclei in dysplastic cells could be highlighted by positive Ki-67 staining. The Ki-67 positive nuclei dominated in the majority of dysplastic cells covering the papillae, comprising over the half (50 %) of the tumour cells (Figs 3C and 3D). As summarized in the Table 5, the typical findings in non-invasive HG UCs were as follows: 1. The staining for Ki-67 antigen, which was positive in over 50 % of tumour cells and correlated well with the degree of dysplasia. 2. In contrast, the p16 antigen positive rate did not correlate with the degree of malignant potential. In at least five HG UC tumours (regardless whether invasive or non-invasive) only a few single cells were positive for this antigen. 3. The staining for p63, CK7 and HMWCK antigens was regularly positive in all non-invasive HG UC tumours.

The papillary carcinomas of low malignant potential (non-invasive LG UC) showed a more regular papillary structure. The dysplastic cells occupied mainly a slim strip along the basement membrane being less abundant and showing nuclei just slightly increased in size (Fig. 3C). Their proportion, as judged according to the Ki-67 positive staining, was clearly lower as compared with the above mentioned non-invasive HG UC tumours; in general, the Ki-67 positive rate did not exceed the value of 25 % (Tab. 6). However, LG UCs still showed some degree of variability in the shape and thickness of their papillae, which occasionally even fused with each other. The cell proliferation marker Ki-67 pointed at a clear decrease from HG UC tumours (>50 %) through LG UC tumours (about 25 % or less) to PUNLMPs (see below).

Papillary urothelium neoplasms of low malignant potential (PUNLMP) and reactive hyperplasia

Some papillary tumours cannot be regarded for carcinoma, since they show a quite regular differentiated structure (Fig. 4A).

The number of cell layers covering the papillae is slightly over 7, which number is regarded for the maximal thickness of urothelium. The PUNLMP cells nearly uniform in size form quite regular lines and possess slightly enlarged oval shaped nuclei distributed perpendicularly to the basement membrane. The positive rate of Ki-67 stained cells in the PUNLMP is relatively low, as a rule, it does not exceed 10 % (Tab. 6). Occasionally, the CK20 antigen can be found at apical location similarly to typical surface "umbrella" cells, while the HMWCK antigen is distributed rather at basal localization.

The criterion of different positive rate of Ki-67 antigen could not be used to distinguish between PUNLMP and urothelium hyperplasia. The main criterion for the latter was the positive p63 antigen staining of basal and parabasal urothelium cells along with the normal localization of the CK7 and HMWCK positive cells. In the case of polypoid hyperplasia, the urothelium covered finger-like protrusions of urinary mucosa (Fig. 4D). The hyperplastic mucosa might have shown a relatively abundant number of Ki-67 positive cells at the rate ranging slightly over 10 %, however, neither the signs of dysplasia nor the growth or a real papillary neoplasm could be noticed in such cases. Noteworthy, in the so called miropapillary hyperplasia, the papillae-like structures lack their own capillary supply (Fig. 4E). When areas of denudation were covered with a single line of dysplastic Ki-67 positive cells, this finding could be interpreted as carcinoma *in situ*, as found in one out of our 7 chronic cystitis cases. Taken together, for the diagnosis of hyperplasia or metaplasia, the p63 staining was useful, while for the recognition of any kind of dysplasia, the Ki-67 antigen could highlight the altered nucleus to cytoplasm ratio.

Discussion

The CK 7 staining was positive in all papillary carcinomas and PUNLMP papillomas, the only exception were 2 out 17 low-differentiated carcinomas. These results are in accord with the findings that this antigen used to be expressed in tumours of epithelial origin with the exception of cervical carcinomas, renal carcinomas, prostate and colon carcinomas (8, 9). Cytokeratin 20 was reported to occur in 70–80 % of transitional cell carcinomas (6). In accord with this, we have not found this antigen in 6 out 17 low differentiated carcinomas and in 2 out 12 papillary carcinomas (regardless of the grade of dysplasia), which means 27.5 % of negative UCs. In cases of urothelial hyperplasia as well as in PUNLMP, the presence of CK20 antigen within surface "umbrella" cells was of diagnostic importance (3). The p63 antigen was a helpful diagnostic marker in HG UC neoplasms to demonstrate the microinvasive growth into the *lamina propria* (Figs 2A–2F). In invasive low-differentiated UC, the p63 expression declines (63), this phenomenon was also noticed in our material (Tab. 4).

As described by Margolis et al (35, 36) in a large cohort of 713 patients, the Ki-67 antigen was probably the most reliable proliferation marker documenting an aggressive growth, tumour recurrence and a worse prognosis. The Ki-67 protein is expressed in nuclei of cells, which synthesize DNA (in the S phase). Its expression correlates precisely with the extent of ^{3}H-thymidine and/or 5-bromodeoxyuridine (BrdU) incorporation into the newly copied chromosomal DNA (22, 32). In cases on non-invasive HG UC and LG UC, the Ki-67 positive rate (index) helps to discriminate between their high grade versus low grade malignant potential based on the estimated number of dysplastic tumour cells. Before the acceptance of the WHO/ISUP classification, some authors described the Ki-67 stain as helpful in recognizing the difference between G1 and G2 papillary carcinomas (13) and pointed out that the Ki-67 was a better marker than the p53 stain (52). The Ki-67 stain represents a useful diagnostic tool, especially in cases, when small tumour fragments only are at disposal. Tables 4, 5 and 6 clearly show that the Ki-67 positive rate decreased from papillary HG UC through LG UCs to PUNLMP. In addition to this, the Ki-67 staining is useful for identification of dysplastic cells, which can replace the denuded urothelium in flat *in situ* carcinoma (pTis). Either the Ki-67 positive and/or the p53 antigen positive cells may form a single line of dysplastic or even pleomorphic cells with irregular shaped nuclei showing pagetoid spread (33, 37, 49). If such cells form more than a single layer, the CK20 antigen might be also expressed (46). Both, Ki-67 as well as CK20 staining were useful markers for differentiation between dysplasia on one hand, non-neoplastic hyperplasia and/or reactive urothelium atypia on other hand (42).

Another histological sign of cardinal importance is the invasive growth clearly present by all low-differentiated UCs (Figs lA–1F). In these clearly invasive low-differentiated carcinomas as well as in their metastases, the expression of metallothionein was demonstrated as a highly reliable marker (59). The matter of controversy is the essentially non-invasive HG UCs, clinically pT1 grade, which shows endophytic growth of various extent into the *lamina propria.* Some authors claimed that the depth of endophytic growth into the subepithelial connective tissue might be of prognostic importance; they suggested to classify the pT1 tumours into the stages pT1a and pT1b (12, 61). Nevertheless, this subdivision has not been widely accepted. Certain forms of microinvasion (such as pseudovascular growth) can be classified as non-invasive provided that the basement membrane remains preserved (Figs 2A and 2D). Another kind of subepithelial growth of HG UCs with the basement membrane disrupted (Figs 2B – 2F) might be regarded as invasive, since such tumours would continue their growth into deeper structures with a probability of 25–29 % (27, 34). At histological examination, the adipose tissue may be seen just adjacent to the urothelium, and not even the position of the muscle layer is always clear-cut [51]. Regarding these considerations, we list in the Table 4 (but still separately) the 3 papillary HG UCs, because they showed more extensive microinvasion into the *lamina propria.*

Our results showed that the p16 antigen staining was not a relevant marker for the detection of dysplastic urothelium cells in contrast to cervical and/or anal squanous cell dysplasia. Some authors recommend the p16 antigen demonstration in small cell UCs, especially if they are negative for p63 and/or CK20 proteins. Nevertheless, the p16 polypeptide was seen in many UCs, pointing out the possibility that the p16 inhibitor kinase was over expressed not only in association with the HPV-coded E7 oncogen. Clearly, HPV was found very rarely causing UC (7). The virus possible related to papillary UCs is the human papovavirus called BKV (24, 65). The recently developed DNA tests can distinguish the sequence

of JC virus as compared to the BK virus (44) and seem promising for the elucidation of the possible role of certain BKV variants in the pathogenesis of UCs. During human papovavirus latency, a portion of the genome encoding a part of T antigen (called small *t*) could become integrated into the nuclei of infected urothelium cells. The expression of corresponding protein could play some role in the transformation process of the basal urothelium cells.

More than the exogenous BKV, endogenous mutations of p53, pRb and/or other regulatory proteins listed in the Table 2, might be of importance for UC pathogenesis. The most frequently discussed mutations concern the intron P2RY5 of the pRb gene (14, 15, 16). PUNLMP seems to appear in response to benign and/or reactive hyperplasia, while the carcinoma in situ might be the origin for HG UC, which finally may result into invasive and later on low-differentiated HG UCs (25). In addition to single mutations, foreign DNA fragments and/or endogenous repetitive DNA motifs could accumulate in the nuclei of pluripotent (precursor) urothelium cells causing what is called a microsatellite instability (MSI). These changes along with the deletions in p53 protein genome may lead to a precancerous state, which could change into real carcinoma, if they occur in both chromosome alleles.

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