

## Four Tumour Markers for Urinary Bladder Cancer - Tissue Polypeptide Antigen (TPA), HER-2/neu (ERB B2), Urokinase-type Plasminogen Activator Receptor (uPAR) and TP53 Mutation

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**Abstract.** *Purpose:* Tissue polypeptide antigen (TPA) is present in the proteolytic fragments of cytokeratins 8, 18 and 19 as a component of the cytoskeleton of nonsquamous epithelia. HER-2/neu protein is a transmembrane tyrosine kinase cell surface growth factor receptor that is expressed on normal epithelial and some cancer cells. The urokinase-type plasminogen activator receptor (uPAR) is a GPI-linked single-chain glycoprotein. Mutations of the tumour suppressor gene P53 (TP53) are frequently correlated with tumour development and progression. We compared TPA, HER-2/neu and uPAR, and TP53 mutation in tumour-free and bladder cancer patients. *Materials and Methods:* Clinical samples were used from 60 patients with tumours of the urinary bladder and from 9 patients with benign urological diseases. TPA was analyzed by the immunoluminometric assay LIA-mat® TPA-M Prolifigen®. HER-2/neu was measured using the Bayer Oncoprotein test. uPAR was measured with the IMUBIND® Total uPAR ELISA Kit. Mutation status in TP53 exons 5, 6, 7 and 8 was analyzed by temperature gradient gel electrophoresis of exon-specific PCR products and by sequence analysis. Statistical analysis included ROC, Mann-Whitney U-test and Pearson's correlation. *Results:* Pathological concentrations of TPA, HER-2/neu and uPAR are detectable in the serum and in urine of bladder cancer patients. The calculated diagnostic sensitivity for TPA in serum was 68.3%, for TPA in urine 33.3%, for HER-2/neu 86.7% and for uPAR 79.5%. Pathological levels of TPA in serum ( $p=0.001$ )

and HER-2/neu ( $p=0.001$ ) were significantly higher in patients with bladder cancer in comparison to the control group. For superficial bladder cancer, the mutation frequency in TP53 was 50%, while for invasive bladder cancer the mutation frequency in TP53 was 100%. Elevated TPA, HER-2/neu and uPAR levels were associated with all grades and stages of bladder cancer. *Conclusion:* TPA, HER-2/neu or uPAR can differ between bladder cancer patients and the control group, but not between superficial and invasive bladder cancer. TP53 mutation frequently occurs in higher stages of bladder tumours.

Tissue polypeptide antigen (TPA) belongs as an epithelial marker to the tumour-associated antigens. TPA was identified by Björklund and Björklund as a potential tumour marker in 1957 (1, 2). It has been reported that the main subunit is a 43 kD, single-chain polypeptide with an isoelectric point at pH 4.5 (3). It is a tumour-related protein originally isolated from extracts of pooled tumours. TPA is present in the proteolytic fragments of cytokeratins 8, 18 and 19, which are released into body fluids as a sign of cell death (2, 4). In normal tissue, TPA was found, too (4, 5). Higher values of TPA have been described for liver diseases and inflammatory processes (4, 6, 7). Some studies reported an increase of TPA serum concentration in tumour patients (6, 8-12). The reason may be increased cell destruction accompanied by invading tumour cells (7, 13). At present, it can be considered as a possible differentiation and / or proliferation marker of epithelial tissue (6).

The oncoprotein HER-2/neu (also known as c-erbB-2) encodes a transmembrane glycoprotein that is similar to the epidermal growth factor (EGF) receptor. Structural homology with the EGF receptor and possession of tyrosine kinase activity and its ability to generate mitogenetic responses all point to the c-erbB-2 receptor (14, 15). The HER-2/neu protein is a transmembrane tyrosine kinase cell

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Table I. Staging and grading of bladder cancer patients.

Staging / Grading	G1	G2	G3	Total
Ta	11	12	1	24 (2 Tis)
T1	4	14	4	22 (4 Tis)
T2	0	2	5	7 (1 Tis)
T3	0	2	3	5
T4	0	0	2	2
Total	15	30	15	60

surface growth factor receptor that is expressed on normal epithelial cells and overexpressed in some cancer cells. The full length HER-2/neu oncoprotein has a molecular weight of 185000 D and is composed of three parts: the internal tyrosine kinase portions, responsible for intracellular signalling, a short transmembrane portion and the extracellular domain (ECD), which is the portion that interacts with growth factors. The ECD of HER-2/neu has been shown to be shed from the cell surface as a glycoprotein between 97,000 and 115,000 D. The shed ECD circulates in normal individuals and has been found to circulate in abnormally high levels in cancer patients (26).

The urokinase-type plasminogen activator receptor (uPAR) is a GPI-linked single-chain glycoprotein having a molecular weight between 50 kD and 60 kD. uPAR is composed of three domains: Domain I is involved with uPA binding, while Domains II and III aid in orienting the uPAR molecule on the cell membrane (17). The presence of a cellular receptor for uPA was first demonstrated by Vassalli *et al.* (18), who observed a saturable specific binding of uPA to the surface of monocytes. uPAR bind both the enzymatically inactive single-chain pro-uPA and the enzymatically active two-chain HMw-uPA with high affinity (17).

Mutations of the tumour suppressor gene *TP53* are frequently correlated with tumour development and progression in bladder cancer (19). *TP53* is localized on the short arm of chromosome 17 (20). *TP53* has an influence on cell cycle regulation, gene transcription, DNA repair, genome stability, chromosome segregation, angiogenesis and apoptosis. Genetic alteration is the most frequent reason for a change of function of *TP53*. Loss of function may also be due to binding with viral oncoproteins or cellular gene products or may be caused by dislocation of the protein to cytoplasm (21, 22). A very important function of wild-type *TP53* is the induction of apoptosis (23).

In this study, we compared the results of measuring four different tumour markers for bladder cancer: TPA, HER-2/neu, uPAR and *TP53* mutations. We examined the following questions: Which marker has the highest sensitivity / specificity? Which marker correlates with superficial and invasive bladder cancer?

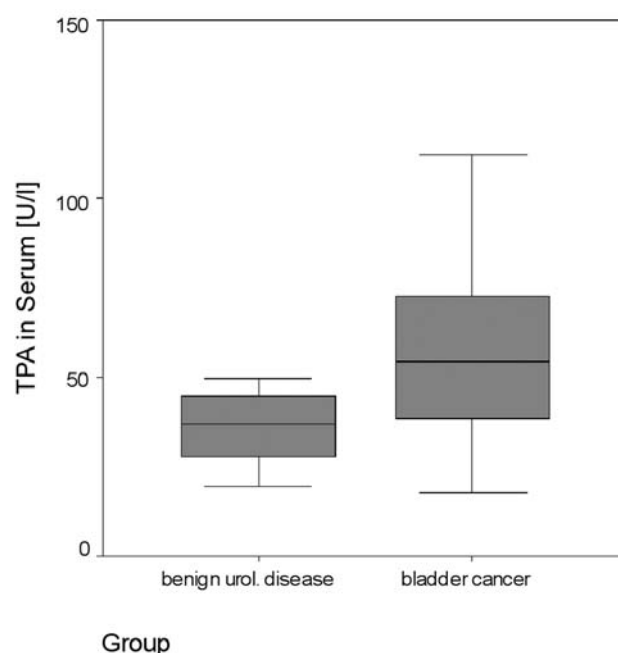


Figure 1. Boxplot for TPA in serum.

## Materials and Methods

We used material from 60 patients with primary diagnosed tumours of the urinary bladder and from 9 patients with benign urological diseases. Fifty-seven male patients and 12 female patients (average age 63.8 years) were included. The staging and grading of the bladder cancer patients is shown in Table I. Genetic mutation analysis was carried out in urothelial samples and in urine. The TPA concentration was analyzed in serum and in urine. HER-2/neu and uPAR were analyzed in urine.

**Measurement of TPA in serum and urine.** Ten ml urine were centrifuged at 3000 rpm for 15 minutes. Fifty µl of Tween 20 were added per 10 ml of urine. For the assay, 50 µl of the pre-treated urine and 150 µl of kit diluent were added to the test tube (24). The TPA concentration was measured by immunoluminometric assay with LIA-mat® TPA-M Prolifigen® (AB Sangtec Medical, Bromma, Sweden). TPA-M LIA is a one-step luminometric immunoassay based on reaction tubes coated with monoclonal antibodies against cytokeratins 8, 18, 19 and soluble tracer antibodies conjugated with isoluminol (25).

**Measurement of HER-2/neu.** The currently most widely used method to analyse the HER-2/neu gene and protein are the Fluorescent-In-Situ-Hybridization (FISH) for DNA. Immunohistochemistry (IHC) detects the full-length HER-2 molecule and an immunoassay technique enables a quantitative estimate to be made of the circulating ECD. FISH and IHC are performed primarily on the tissue biopsy taken at the time of primary diagnosis and reflect the status of the gene or protein at that time point, whereas the ELISA method enables

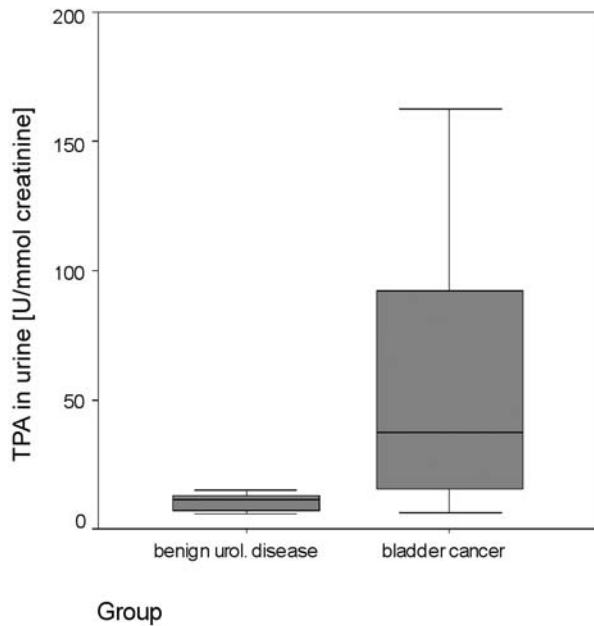


Figure 2. Boxplot for TPA in urine.

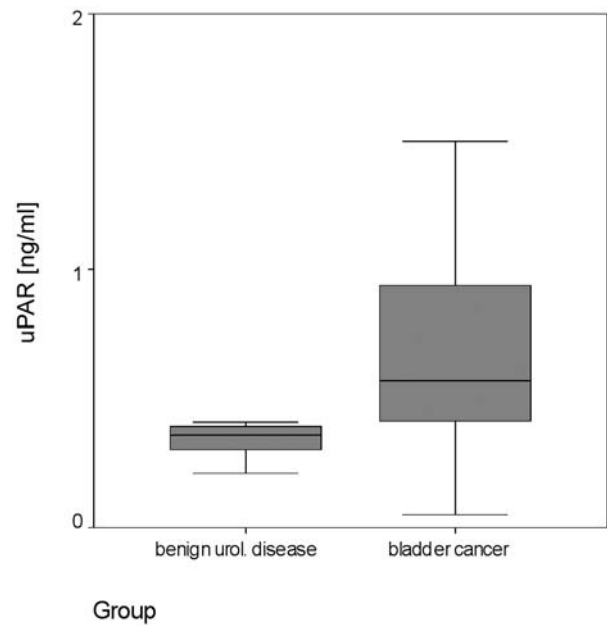


Figure 4. Boxplot for uPAR.

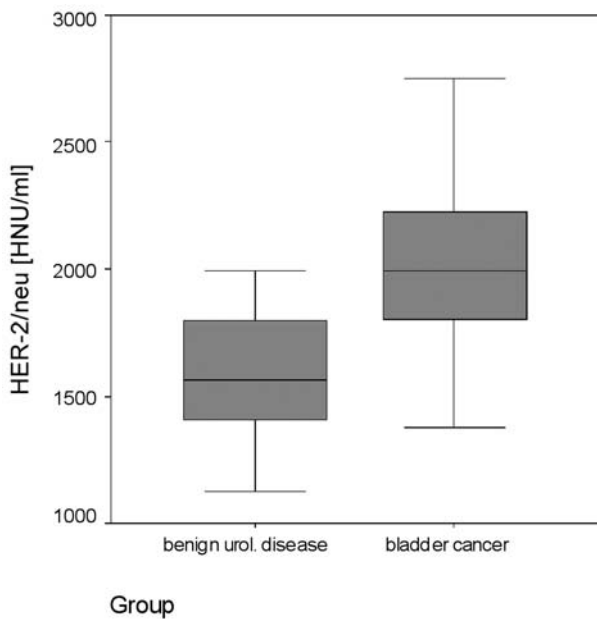


Figure 3. Boxplot for HER-2/neu.

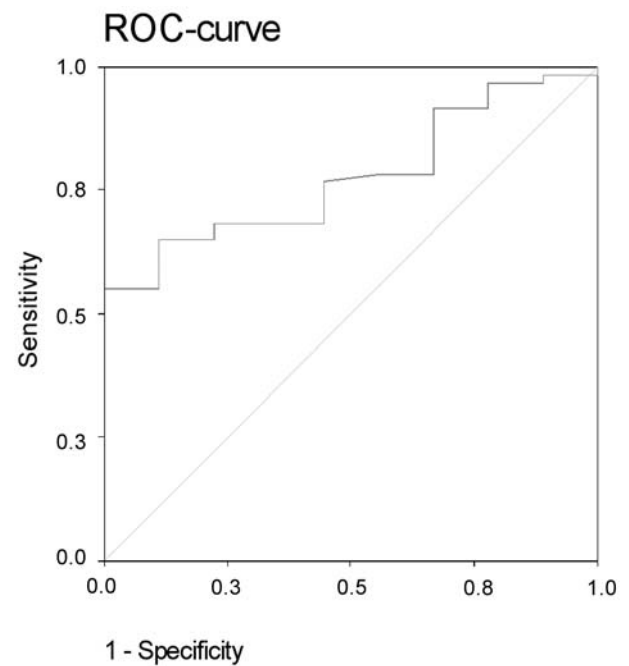


Figure 5. ROC-curve for TPA in serum.

subsequent measurement to be made, thereby allowing oncologists to follow the course of the disease. The HER-2/neu ELISA is a sandwich enzyme immunoassay that utilizes two monoclonal antibodies to quantitate the ECD of the HER-2/neu protein in serum (26).

*Screening of uPAR.* The IMUBIND® Total uPAR ELISA kit (American Diagnostica Inc., Greenwich, USA) is an enzyme-linked immunoassay for the quantitation of human urokinase-type plasminogen activator receptor (uPAR) in tissue extracts, human plasma and cell culture supernatants. The lower detection limit of

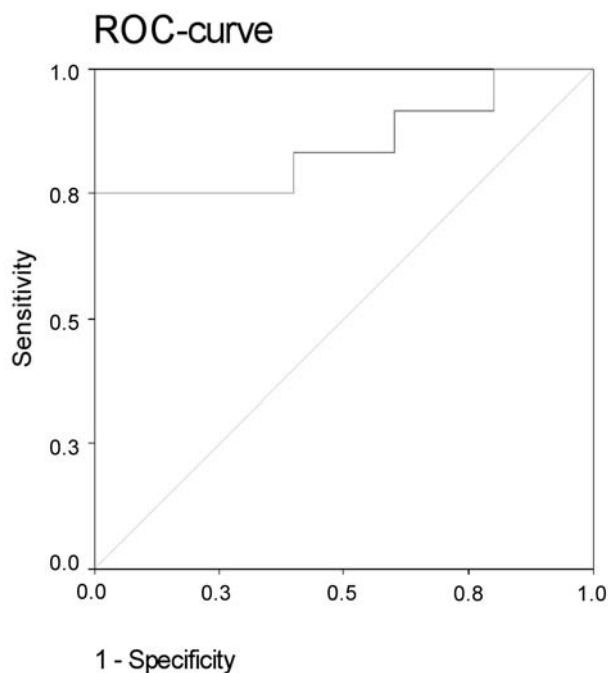


Figure 6. ROC-curve for TPA in urine.

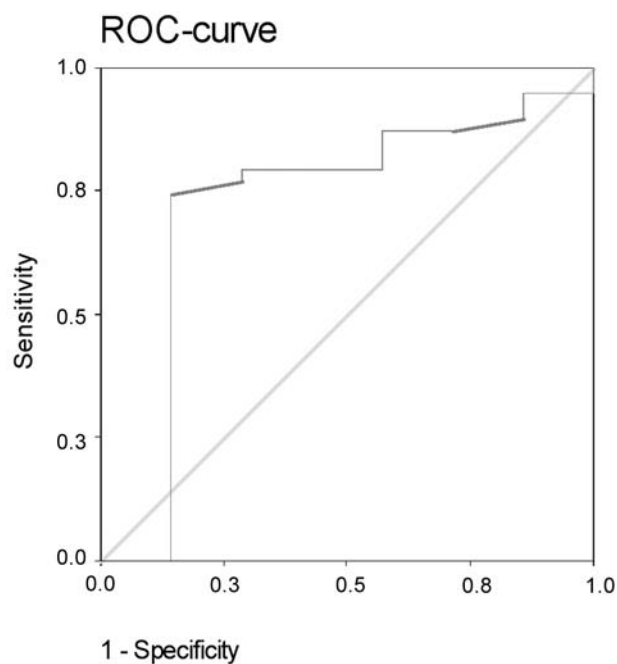


Figure 8. ROC-curve for uPAR.

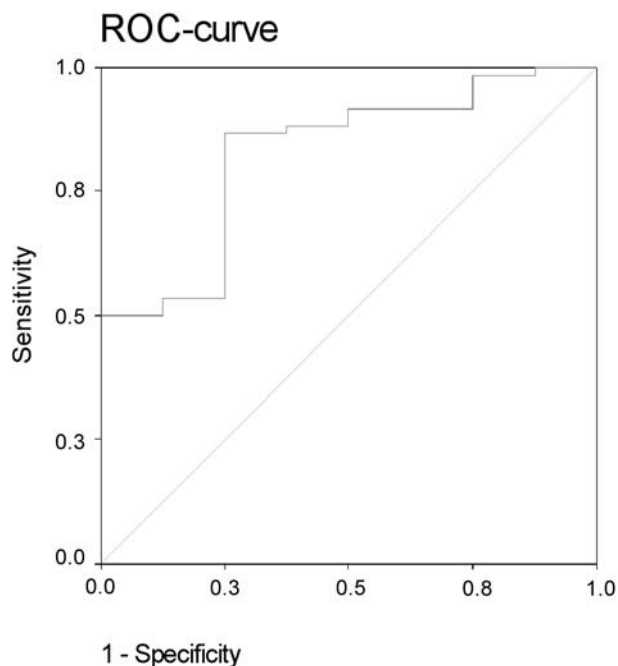


Figure 7. ROC-curve for HER-2/neu.

Table II. Sensitivity and specificity for measured tumour markers.

	TPA in serum	TPA in urine	HER-2/neu	uPAR
Sensitivity	68.3%	33.3%	88.9%	79.5%
Specificity	88.9%	100%	62.5%	71.4%

uPAR as the capture antibody. Samples incubate in precoated microtest wells and a second, biotinylated antibody is used to recognize the bound uPAR molecules. Adding streptavidin conjugated horseradish peroxidase (HRP) completes the formation of the antibody-enzyme detection complex. The addition of a perborate / 3,3', 5,5' - tetramethylbenzidine (TMB) substrate, and its subsequent reaction with the HRP, creates a blue-coloured solution. Sensitivity is increased by addition of a sulfuric acid stop solution, yielding a yellow colour. Total uPAR levels are quantified by measuring solution absorbance at 450 nm and comparing the values with those of a standard curve.

*Screening of TP53 mutations.* DNA was isolated by phenol extraction from cellular urine sediments and tumour tissue. Amplification in separate reactions by polymerase chain reaction (PCR) of TP53 exons 5, 6, 7 and 8 was performed with so-called GC-clamped primers (16). Mutation screening was performed by temperature gradient gel electrophoresis (TGGE). Technical details of the methods used are described in previous reports (27). Each TGGE experiment was carried out with a DNA-negative PCR-control sample and with a positive mutation control to rule

this assay is 0.1 ng total uPAR/mL of sample. Soluble, native and recombinant uPAR, as well as uPAR/uPA and uPAR/uPA/PAI-1 complexes, are all recognized by this assay. The IMUBIND® Total uPAR ELISA employs a rabbit polyclonal antibody against human

Table III. Elevated levels of tumour markers in superficial and invasive bladder cancer.

	TPA in serum	TPA in urine	HER-2/neu	uPAR
Elevated levels for pTa, pT1	31 out of 46 [67.4%]	4 out of 11 [36.4%]	41 out of 46 [89.1%]	24 out of 31 [77.4%]
Elevated levels for pT2-4	10 out of 14 [71.4%]	0 out of 1 [0%]	11 out of 14 [78.6%]	7 out of 8 [87.5%]

out potential contamination. A 4-band-pattern of silver staining in the TGGE was estimated as indicative of a mutation. Sequencing was done for either mutant and, for control, wild-type bands, excised from the silver-stained TGGE-gel, or directly on the isolated DNA: the specific bands or exons were reamplified with the same PCR primers as before, but instead of a GC-clamp, a biotinylated primer at the 5'-end was used (28).

Statistical analysis included ROC, Mann-Whitney *U*-test and Pearson's correlation.

## Results

*Tumour marker levels in bladder cancer patients and tumour-free patients.* In serum of bladder cancer patients, the median TPA concentration was 54.5 U/l, while in the urine of bladder cancer patients the median TPA was 37.4 U/mmol creatinine. In serum of patients with benign urological diseases, the median TPA was 37.2 U/l, while in the urine of these patients the median TPA was 10.8 U/mmol creatinine (Figures 1 and 2). The median HER-2/neu concentration in the urine of bladder cancer patients was 1988.5 HNU/ml, and the median uPAR concentration was 0.57 ng/ml. In the urine of patients with benign urological diseases, the median HER-2/neu concentration was 1563 HNU/ml, and the median uPAR concentration in this group was 0.36 ng/ml (Figures 3 and 4).

After ROC-curve analysis the following optimal cut-offs were found: TPA in serum: 47 U/l (Figure 5), TPA in urine: 60 U/mmol creatinine (Figure 6), HER-2/neu: 1610 HNU/ml (Figure 7), uPAR: 0.4 ng/ml (Figure 8)

The calculated diagnostic sensitivity for TPA in serum was 68.3%, for TPA in urine 33.3%, for HER-2/neu 86.7% and for uPAR 79.5%. The calculated diagnostic specificity for TPA in serum was 88.9%, for TPA in urine 100%, for HER-2/neu 62.5% and for uPAR 71.4% (Table II).

Pearson's significance with the Chi-Quadrat-test showed that pathological levels of TPA in serum ( $p=0.001$ ) and HER-2/neu ( $p=0.001$ ) were significantly higher in patients with bladder cancer in comparison to the control group. Pathological levels of TPA in urine ( $p=0.140$ ) and for uPAR ( $p=0.006$ ) could not give such a high statistical significance.

Table IV. TP53 mutation in superficial and invasive bladder cancer.

	pTa, pT1	pT2-4	Total
TP53 - Mutation	23 [50%]	13 [100%]	36 [61%]
TP53 - Wild-type	23 [50%]	0 [0%]	23 [39%]
Total	46	13	59

For all analysed tumour markers the Mann-Whitney *U*-test was calculated. The significance for TPA in serum was  $p=0.008$ , for TPA in urine  $p=0.027$ , for HER-2/neu  $p=0.003$  and for uPAR  $p=0.064$ . HER-2/neu had the highest sensitivity, and it could make the best statistically significant differentiation between patients with bladder cancer and tumour-free patients.

*Which marker correlates with superficial and invasive bladder cancer?* We measured pathological values of TPA in serum for invasive bladder cancer in 10 out of 14 cases (71.4%); in urine, pathological TPA levels were detected in 0 out of 1 case. Pathological levels for HER-2/neu for invasive bladder cancer were found in 11 out of 14 cases (78.6%), and for uPAR in 7 out of 8 cases (87.5%). For superficial bladder cancer, we found elevated TPA levels in 31 out of 46 cases (67.4%) in serum, and in 4 out of 11 cases (36.4%) in urine. Pathological levels for HER-2/neu for superficial bladder cancer were found in 41 out of 46 cases (89.1%), and for uPAR in 24 out of 31 cases (77.4%). These results are presented in Table III. The Chi-Quadrat-Test of Pearson's significance between the analysed tumour markers and invasive bladder cancer found no result  $p<0.005$ .

TP53 mutation in patients with bladder cancer is significantly correlated with tumour staging. We detected mutations of TP53 in invasive bladder cancer in 13 out of 13 cases (100%); in superficial bladder cancer, we found 23 out of 46 cases (50%) with mutation of TP53. Pearson's significance with the Chi-Quadrat-Test between mutation of TP53 and invasive bladder cancer was calculated with  $p=0.001$  (Table IV).

Thus, mutation in TP53 is the best marker to differentiate between invasive and superficial bladder cancer.

## Discussion

In the reported experiments, TPA showed only a slight sensitivity (68.6% for TPA in serum and 33.3% for TPA in urine), but a very good specificity (88.9% for TPA in serum and 100% for TPA in urine) for bladder cancer patients. This is comparable with the 54.7 % sensitivity and 100 %

specificity as published by Maulard *et al.* (6) for TPA in serum. Stieber *et al.* (29) found 16% sensitivity and 95% specificity for TPA in serum. In the study published by Sanchez-Carbayo *et al.* (12), an 80.2% sensitivity and a 95% specificity was reported for TPA in urine. Menendez Lopez *et al.* (11) found a specificity of 73% and a sensitivity of 45%. All these reported results correlate with our results.

Comparative cytokeratin analyses have shown cytokeratin 19 to be an essential part of TPA in bladder cancer (29). The analysis in non-invasive tumours seemed to be difficult. Pathological TPA serum concentration was found in 31 out of 46 patients (67.4%) with superficial bladder cancer; elevated TPA levels in urine were found in 4 out of 11 patients (36.4%). In invasive tumour stages (pT2 – pT4), the pathological TPA concentrations were elevated in serum (10 out of 14 = 71.4%). A missing correlation of TPA level with tumour grading in bladder cancer, confirming our results, has already been reported (8, 10). We found no statistically significant difference between superficial / invasive bladder cancer and elevated levels of TPA in serum / urine. This has already been reported by Filella *et al.* (9) and by Casetta *et al.* (30).

It is known that TPA is not specific for tumours and that an inflammation can influence the TPA levels (4, 6, 7). The number of patients in the present study was insufficient to reach a conclusion regarding the influence of inflammation.

In this study, HER-2/neu showed a very high sensitivity (88.9%) and a good specificity (62.5%). HER-2/neu was the best of all the analysed tumour markers of this study. However, HER-2/neu could not differentiate between superficial and invasive bladder cancer, since we found elevated HER-2/neu levels in 89.1% of superficial and in 78.6% of invasive bladder cancer patients. The results of Lonn *et al.* (31) show that elevated erb-b2 levels correlate with tumour grade of superficial bladder cancer. HER-2/neu and the other analysed tumour markers were not specific for bladder cancer. Neumann *et al.* (26) measured elevated levels of HER-2/neu in breast cancer.

The sensitivity for uPAR was high at 79.5%, while the specificity was 71.4%. Our results do not differ from other studies (17). Shariat *et al.* (32) found that elevated uPAR levels are significantly higher in bladder cancer patients than in healthy individuals. Casella *et al.* (33) tested uPAR and uPA before cystoscopy and showed that uPAR could help to find high-risk patients for bladder cancer.

The mutation frequency in the so-called high-risk exons 5 - 8 of the *TP53* gene is approximately 40 % in bladder cancer tissue (34, 35). Mutation of *TP53* might accelerate carcinogenesis, especially by enhancement of cell proliferation, loss of apoptosis and by insufficient DNA repair (36). It has now been widely accepted that the p53 state plays a role in the progression of bladder tumours (37, 38). In our study, we found a mutation frequency of 100% for invasive bladder cancer and of 50% for superficial

bladder cancer, confirming already published results (35). Mutations in the *TP53* gene are correlated with infiltrating bladder cancer and qualify as a marker.

## Conclusion

1. For all analysed tumour markers, pathological concentrations were detectable in serum and / or in urine of bladder cancer patients.
2. Pathological levels of TPA in serum ( $p=0.001$ ) and HER-2/neu ( $p=0.001$ ) were significantly higher in patients with bladder cancer in comparison to the control group. Elevated TPA levels in serum and elevated HER-2/neu levels give a reference to malignant diseases.
3. HER-2/neu had the highest sensitivity of all analysed tumour markers.
4. Elevated levels of TPA, HER-2/neu and uPAR had approximately the same frequency in invasive and superficial bladder cancer. The mutation frequency of *TP53* in invasive bladder cancer was significantly higher than in superficial bladder cancer ( $p=0.001$ ).

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