

Altered p53/p16 expression is linked to urothelial carcinoma progression but largely unrelated to prognosis in muscle-invasive tumors

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ABSTRACT

Background: Most inactivating p53 mutations result in a nuclear p53 accumulation – detectable by immunohistochemistry (IHC). p53 alterations leading to a complete lack of p53 protein and absence of immunostaining do also occur – not easily detectable by IHC. p16 is upregulated in p53 inactivated cells. We hypothesized that a positive p16 IHC may help to distinguish p53 inactivation in IHC negative cases.

Material and methods: We investigated p53 and p16 immunostaining on 2710 urothelial bladder carcinomas in a tissue microarray format to understand their impact in relation to clinicopathological parameters of disease progression and patient outcome.

Results: p16 immunostaining was absent in normal urothelium but occurred in 63.5% (30.4% strong) of cancers. p16 strongly positive cases increased from pTaG2 low-grade (9.6%) to pTaG3 high-grade tumors (46.5%, $p < .0001$) but decreased from pTaG3 to pT4 (33.3%; $p = .0030$). Among pT2–4 carcinomas, p16 positivity was linked to high-grade ($p = .0005$) but unrelated to overall survival. p53 staining was negative in 8.4%, very weak in 15.4%, weak in 55.3%, strong in 4.7%, and very strong in 16.2% cancers. p53 negative (potentially p53 null phenotype), strong, and very strong p53 positivity increased from pTaG2 low-grade to pTaG3 high-grade tumors ($p < .0001$) and from pTaG3 to pT2–4 cancers ($p = .0007$). p53 staining was largely unrelated to histopathological parameters or patient prognosis among pT2–4 carcinomas, except of p53 strong/very strong immunostaining. p16 expression predominated in tumors with very strong, strong, and negative p53 staining and the combination of p53 negative/p16 strongly positive cancers was linked to features of tumor aggressiveness.

Conclusion: Aberrant p53 and p16 immunostaining increases during grade and stage progression although p53 negative and p16 positive immunostaining lack prognostic significance in pT2–4 carcinomas. Potential diagnostic features are that high level p16 expression is limited to neoplastic urothelium and p53 null phenotype to aggressive cancers (grade 3 and invasive).

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Background

Urinary bladder cancer is the tenth most common malignant tumor type worldwide [1]. About 80% of patients present with low-grade non-invasive (pTa) or minimally invasive (pT1) cancers, which are characterized by a good prognosis and can be removed by transurethral resection. However, more than 60% of these tumors recur and about 20% will further progress to life threatening muscle-invasive disease requiring surgical removal of the bladder [2]. In patients with muscle-invasive bladder cancer, the clinical outcome is highly variable, but

almost 50% of patients develop early metastasis and eventually die from their disease [3]. A better understanding of the molecular features underlying disease progression will eventually enable a better prediction of the individual patient prognosis and thus optimize treatment decisions and allow an early aggressive treatment in curable patients at high risk.

Alterations of the p53 tumor suppressor belong to the most frequent genetic alterations in malignant tumors. Reduced p53 function compromises critical cellular programs including the induction of apoptosis in DNA damaged cells, which

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consequently enables tumor progression through acquisition of additional genetic changes (summarized in [4]). Mechanisms for p53 inactivation include functionally relevant gene mutations (in particular missense mutation) as well as structural chromosomal alterations, mostly 17p deletions. These alterations can result in a reduced or completely lost p53 expression or function (summarized in [5,6]). Many inactivating p53 mutations result in a nuclear accumulation of the defective p53 protein, which subsequently inactivates normal p53 proteins expressed by the unaffected second p53 allele through direct binding (summarized in [4]). Various p53 mutations not only deplete the normal p53 functions but also provide additional oncogenic functions ('gain of function mutations') (summarized in [7]). More than 1000 studies have analyzed p53 alterations in bladder cancer but their prognostic role – especially in pT2–4 carcinomas – is still disputed [8–10]. Most studies have used immunohistochemistry (IHC) to compare p53 positive (considered p53 mutated) and p53 negative tumors (considered p53 normal) and disregarded the potential of complete p53 inactivation by a combination of mechanisms that result in a complete lack of p53 protein in tumor cells. Such a p53 null phenotype has been described to occur in approximately 80% of p53 IHC completely negative female genital tract and breast tumors [11]. Hodgson et al. [12,13] have recently suggested that such a 'p53 IHC negative null phenotype' may also occur in 20–26% of bladder carcinomas. In addition, Sangoi et al. found *TP53* mutations in all of 21 p53 negative urothelial carcinomas *in situ* [14]. However, absence of p53 immunostaining can also result from a very low p53 protein level below the detection limit or by poor stainability of over-fixed tissues by IHC. p16 is a tumor suppressor protein that inhibits cell cycle progression from G1 to S phase through binding and inactivating cyclin dependent kinases 4 and 6 (CDK4 and CDK6) [15]. Expression of p16 is intimately connected with p53 and retinoblastoma (pRb) expression. Loss of p53 and/or pRb function yields an oncogenic stress, which induces p16 expression in cancer cells (summarized in [16]). We therefore hypothesized that p16 expression analysis may be useful for the identification of cancers with a 'p53 IHC negative null phenotype' based on a genetic alteration.

To better understand the clinical relevance of p53 and p16 alterations – including a 'p53 IHC negative null phenotype' – in urothelial carcinomas, we studied the relationship between p53/p16 immunostaining and clinicopathological parameters of disease progression as well as patient outcome in more than 2700 urothelial carcinomas in a tissue microarray (TMA) format.

Material and methods

Tissue microarrays

Our set of TMAs contained one sample each from 2710 urothelial tumors of the bladder archived at the Institute of Pathology, University Hospital Hamburg, Hamburg, Germany, Institute of Pathology, Charité Berlin, Germany, Department of Pathology, Academic Hospital Fuerth, Fuerth, Germany, or Department of Pathology, Helios Hospital Bad Saarow, Bad Saarow, Germany, and/or treated at Department of Urology,

Table 1. Patient cohort.

	Study cohort on TMA (n = 2710)
Follow up	636
Months	
Mean	26.7
Median	15.0
Pathological tumor stage	
pTa	887 (38.4%)
pT1	49 (2.1%)
pT2	462 (20.0%)
pT3	615 (26.6%)
pT4	298 (12.9%)
Tumor grade	
G2	820 (30.6%)
G3	1858 (69.4%)
Pathological lymph node status	
pN0	734 (62.0%)
pN+	449 (38.0%)
Resection margin status	
R0	595 (80.6%)
R1	143 (19.4%)
Lymphatic vessel infiltration	
L0	275 (49.5%)
L1	281 (50.5%)
Blood vessel infiltration	
V0	450 (74.4%)
V1	155 (25.6%)

University Hospital Hamburg, Hamburg, Germany, Department of Urology, Charité Berlin, Berlin, Germany, Department of Urology, Helios Hospital Bad Saarow, Bad Saarow, Germany, Department of Urology, Albertinen Hospital, Hamburg, Germany, and Department of Urology and Urological Oncology, Pomeranian Medical University, Szczecin, Poland. Patients at each center were treated according to the guidelines at the time. In brief, patients with pTa/pT1 disease underwent a transurethral resection of the bladder tumor with or without postoperative or adjuvant instillation therapy, while most patients with pT2–4 disease were treated by radical cystectomy. Available histopathological data including grade, tumor stage (pT), lymph node status (pN), and status of blood vessel (V) and lymphatic vessel (L) infiltration are shown in Table 1. The grading of pTa tumors included both a classification according to WHO [17] and Mostofi [18], which were valid at the time of the respective diagnoses. A centralized review of the cases was not done. Clinical follow-up data (overall survival, OS) were available from 636 patients with pT2–4 carcinomas treated by cystectomy. The tissues were fixed in 4% buffered formalin and then embedded in paraffin. The TMA manufacturing process has previously been described [19,20]. In brief, one tissue spot (diameter: 0.6 mm) was transmitted from a cancer containing donor block into an empty recipient paraffin block. The use of archived remnants of diagnostic tissues for TMA manufacturing, their analysis for research purposes, and patient data were according to local laws (HmbKHG, §12) and analysis had been approved by the local ethics committee (Ethics Commission Hamburg, WF-049/09). All work has been carried out in compliance with the Helsinki Declaration.

Immunohistochemistry

Freshly cut TMA sections were immunostained on one day and in one experiment. Slides were deparaffinized with xylol,

rehydrated through graded alcohol series, and exposed to heat-induced antigen retrieval for 5 min in an autoclave at 121 °C in a DakoTarget Retrieval SolutionTM (Agilent, Santa Clara, CA; pH 9 for p16 and pH 7.8 for p53). Endogenous peroxidase activity was blocked with Dako Peroxidase Blocking SolutionTM (Agilent, Santa Clara, CA; #52023) for 10 min. Primary antibodies specific for p53 (rabbit recombinant, MSVA-053R, MS Validated Antibodies GmbH, Hamburg, Germany; #5358-053R) and for p16 (rabbit recombinant, MSVA-016R, MS Validated Antibodies GmbH, Hamburg, Germany; #3626-016R-01) were applied at 37 °C for 60 min at a dilution of 1:150 each. Bound antibody was then visualized using the EnVision Kit (Agilent, Santa Clara, CA; #K5007) according to the manufacturer's directions. The sections were counterstained with hemalaun. The percentage of positive neoplastic cells was estimated, and the staining intensity was recorded as 0, 1+, 2+, and 3+. For statistical analyses, tumor staining results were categorized into four groups for p16 as previously described [21]. Tumors without any staining were considered negative. Tumors with 1+ staining intensity in $\leq 70\%$ of cells or 2+ intensity in $\leq 30\%$ of cells were considered weakly positive. Tumors with 1+ staining intensity in $> 70\%$ of cells, or 2+ intensity in 31–70%, or 3+ intensity in $\leq 30\%$ were considered moderately positive. Tumors with 2+ intensity in $> 70\%$ or 3+ intensity in $> 30\%$ of cells were considered strongly positive. Tumor categorization was different for p53 in order to potentially identify p53 null phenotypes and tumors with physiological p53 expression. p53 was categorized into five groups. Tumors without any staining were considered 'p53 negative' (potentially p53 null phenotype). Tumors with staining of any intensity in $\leq 5\%$ of cancer cells were considered 'p53 very low'. Tumors with 1+ staining in $> 5\%$ of cancer cells, or tumors with staining of any intensity in $> 5\%$ but $\leq 50\%$ of cancer cells were considered 'p53 low'. Tumors with 2+ or 3+ staining in $> 50\%$ but $< 80\%$ of cancer cells were considered 'p53 strong' (potentially p53 mutated). Tumors with 2+ or 3+ staining in $\geq 80\%$ of cancer cells were considered 'p53 very strong' (potentially p53 mutated).

Statistics

Statistical calculations were performed with JMP 16 software (SAS Institute Inc., Cary, NC). Contingency tables and the Chi²-test were performed to search for associations between molecular features and tumor phenotype. Survival curves were calculated according to Kaplan–Meier's method. The Log-Rank test was applied to detect significant differences between groups. A *p* value of $\leq .05$ was considered as statistically significant.

Results

Technical issues

Of our 2710 urothelial carcinomas, 2425 (89.5%) were interpretable for p53 and 2481 (91.5%) for p16. Non-interpretable

tumors were caused by a lack of unequivocal tumor cells on the TMA spots or absence of entire tissue spots on the TMA.

p16 in urothelial carcinomas

p16 staining was not seen in normal urothelium. A nuclear and cytoplasmic p16 staining occurred, however, in 63.5% of urothelial carcinomas. The pattern of staining was heterogeneous and included cases with a predominantly basal location of p16 positive cells, cases with a mosaic pattern showing intermingled positive and negative cells as well as tumors with a diffuse staining of most cells. The latter pattern was particularly common in pTaG3 and in invasive carcinomas. Based on our classification scheme, p16 staining was considered weak in 558 (22.5%), moderate in 263 (10.6%), and strong in 755 (30.4%) cancers. Representative images are shown in Figure 1. The relationship between p16 staining and tumor phenotype is shown in Table 2. The fraction of p16 positive and especially of strongly positive cases increased markedly from pTaG2 low-grade (9.6%) to pTaG3 high-grade cancers (46.5% strongly positive, $p < .0001$ for pTaG2 low vs. pTaG3) but then continuously decreased from pTaG3 to pT2 (41.3% strongly positive), pT3 (36.5%) and pT4 (33.3%; $p = .0030$). Within pT2–4 carcinomas, p16 positivity was associated with higher grade ($p = .0005$) but unrelated to clinical outcome (Figure 2), and marginal with neoadjuvant chemotherapy ($p = .0555$; supplementary figure 1).

p53 in urothelial carcinomas

The p53 staining was always nuclear and both the fraction of positive cells and the staining intensity were variable. p53 staining was recorded as negative in 203 (8.4%), very weak in 373 (15.4%), weak in 1341 (55.3%), strong in 115 (4.7%), and very strong in 393 (16.2%) cancers. Representative images are shown in Figure 3. The relationship between p53 staining and tumor phenotype is shown in Table 3. Within pTa tumors, both the fraction of p53 negative (potentially p53 null phenotype) and the p53 very strong tumors (potentially mutated) increased markedly from pTaG2 low-grade to pTaG3 high-grade tumors ($p < .0001$). The fraction of tumors with both negative (potentially p53 null phenotype) and very strong staining further increased from pTaG3 to muscle-invasive (pT2–4) cancers ($p = .0007$). However, within pT2–4 carcinomas, the p53 status was largely unrelated to parameters of malignancy or patient prognosis (Figure 2, Table 3). Although OS was longest in 'p53 very low' cancers – supposed to be 'p53 normal' – this difference reaches statistical significance only in comparison to tumors with p53 strong or very strong immunostaining ($p = .0325$). In addition, p53 strong and very strong immunostaining was associated with neoadjuvant chemotherapy ($p = .0002$; supplementary figure 1).

p53 and p16

A comparison of p53 and p16 revealed a significant association between both parameters (Figure 4; $p < .0001$).

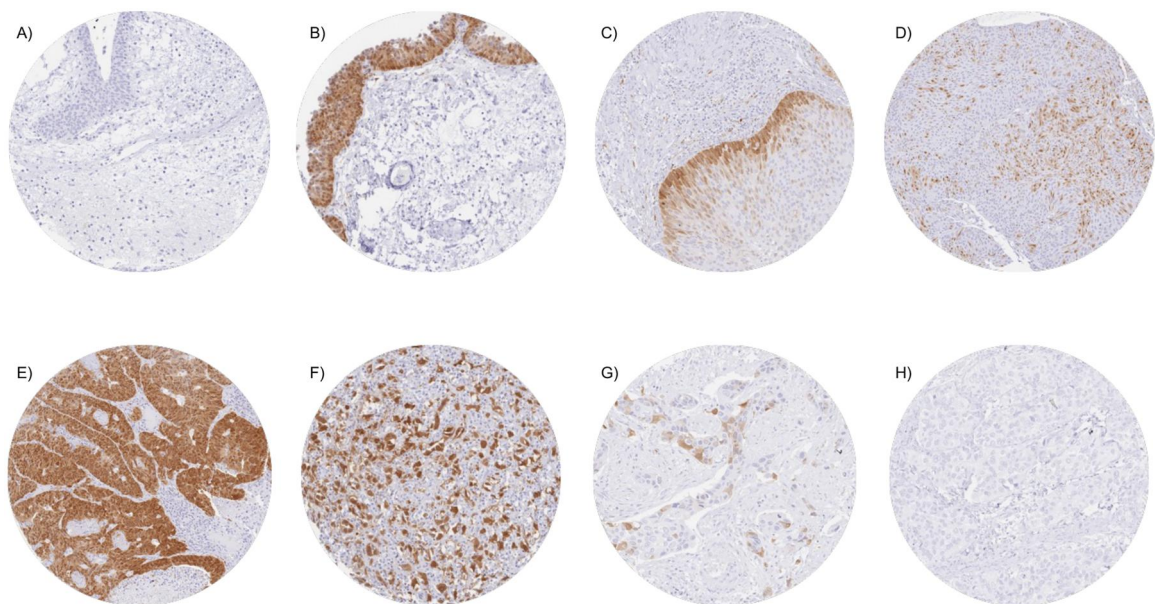


Figure 1. p16 immunostaining in normal and neoplastic urothelium. The panels show a lack of p16 staining in normal urothelium (A), a moderate to strong p16 positivity in an urothelial dysplasia (B), a predominantly basal p16 staining in a pTaG2 urothelial tumor (C), a moderate p16 staining of intermingled individual cells in a pTaG2 urothelial tumor (D), a strong diffuse nuclear and cytoplasmic p16 staining in a pTaG3 (E), and a muscle-invasive urothelial carcinoma (F). Other muscle-invasive urothelial carcinomas showed a weak to moderate p16 focal positivity (G) or a complete absence of p16 immunostaining (H).

Table 2. p16 immunostaining and cancer phenotype.

	n	p16 immunostaining result				p
		Negative (%)	Weak (%)	Moderate (%)	Strong (%)	
All cancers	2481	36.5	22.5	10.6	30.4	<.0001
pTa G2 low	437	15.1	54.2	21.1	9.6	
pTa G2 high	209	35.9	39.7	13.4	11.0	
pTa G3	127	20.5	20.5	12.6	46.5	
pT2	424	38.0	12.7	8.0	41.3	.0030*
pT3	564	49.8	8.9	4.8	36.5	.0005*
pT4	279	46.2	12.9	7.5	33.3	
G2	95	63.2	12.6	4.2	20.0	
G3	1145	43.6	10.8	6.8	38.8	.1751*
pN0	610	46.9	8.7	5.2	39.2	
pN+	420	46.0	12.4	6.4	35.2	.1707*
R0	503	44.7	9.7	4.4	41.2	
R1	130	47.7	10.8	8.5	33.1	.0284*
L0	223	49.3	8.1	3.1	39.5	
L1	249	41.4	11.6	8.4	38.6	
V0	379	44.6	8.2	5.3	42.0	.0985*
V1	139	48.9	13.7	5.8	31.7	

*Only in pT2–4 carcinomas.

Remarkably, the fraction of p16 strongly positive cases was highest in tumors with either very strong or negative p53 staining (Figure 5). Further analyses to assess the effects of p16 staining in p53 negative (potential null phenotype) cases revealed that p53 negative/p16 strong cases were linked to high-grade in pTa tumors (supplementary table 1). Moreover, OS of patients with p53 negative/p16 strong tumors tended to be worse than in patients with p53 negative/p16 negative cancers, although this difference did not reach statistical significance (Figure 2).

Discussion

The results of this study delineate a group of bladder tumors with complete absence of p53 immunostaining. Because

unfavorable tumor phenotypes and p16 overexpression were common in these tumors, we hypothesize that they may carry a p53 null phenotype.

Molecular analyses of >2000 cancers represent an ideal application of the TMA technology developed by members of our group more than 20 years ago [20]. The method enables a highly standardized high throughput analysis of tissues [22]. The random selection of one cancer containing tissue sample per patient measuring 0.6 mm in diameter even assures identical tissue quantities analyzed by patient and avoids a selection bias potentially induced by pathologists selecting tumor areas that are ‘representative’ based on their perception. The only study comparing molecular info obtained on a TMA versus whole sections with clinical outcome information found that TMA data provided markedly better prognostic information than obtained from corresponding whole sections despite of a lower 53 positivity rate in TMA spots [23]. Because the use of multiple samples per patient always results in an unequal number of interpretable spots per patient and an unequal representation of heterogeneity due to variable distances between the punches, we strongly advocate the use of one sample per patient. Others and we have earlier shown that one 0.6 mm core per cancer is sufficient to find clinically relevant associations between molecular markers and tumor phenotype (reviewed in [24]).

The successful p16 IHC analysis of more than 2400 urothelial carcinomas revealed a marked increase of p16 expression with increasing grade in pTa tumors, a continuous reduction of p16 expression from pTaG3 to pT4 carcinomas, and a lack of prognostic impact in 595 muscle-invasive carcinomas. At least 35 studies have previously analyzed p16 immunostaining in urothelial bladder cancer, but information about the relationship between p16 immunostaining and tumor phenotype or prognosis in important subgroups of bladder cancers is rare. Among at least 10 studies analyzing p16

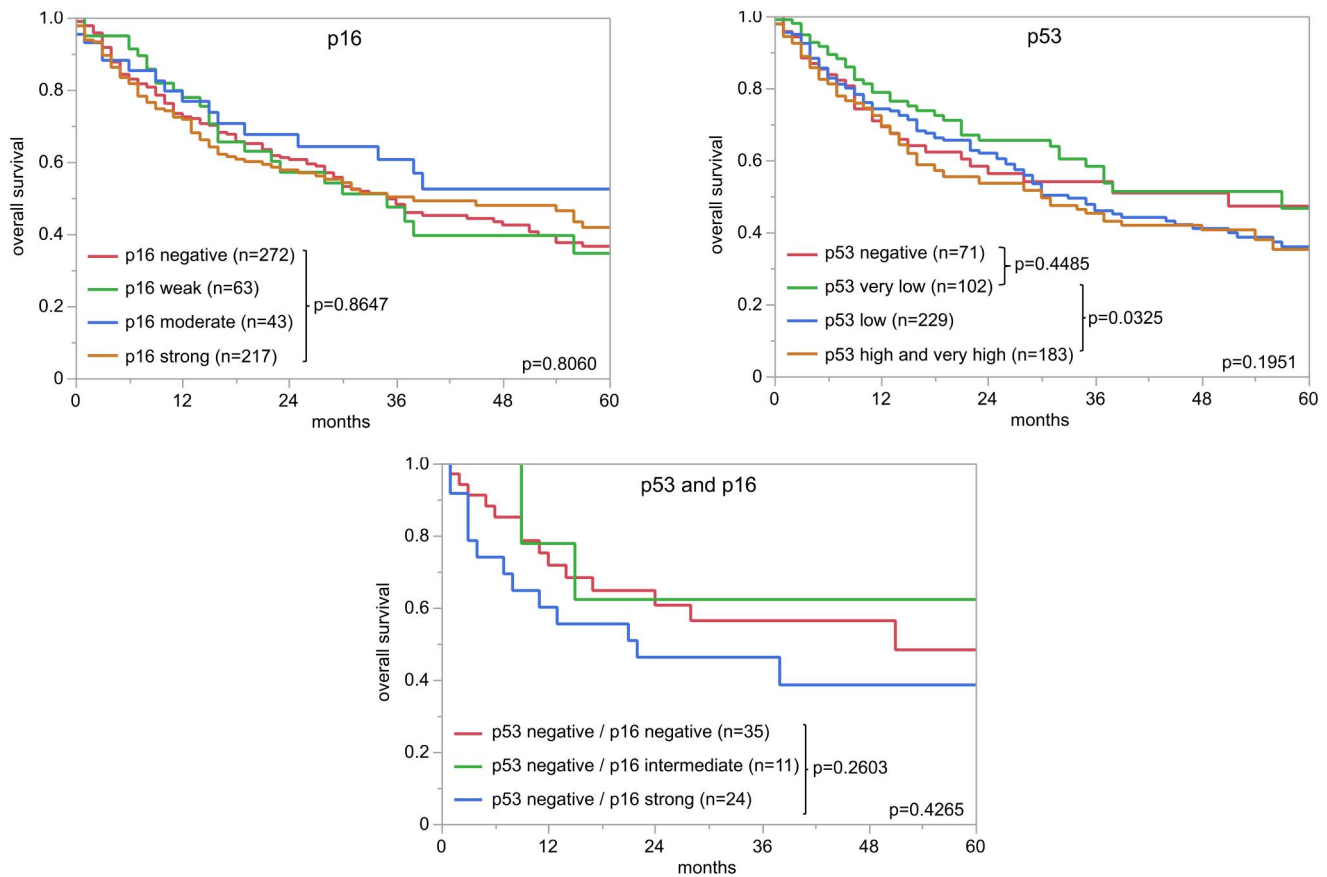


Figure 2. p53, p16, and p53/p16 combined immunostaining and prognosis in muscle-invasive urothelial carcinomas.

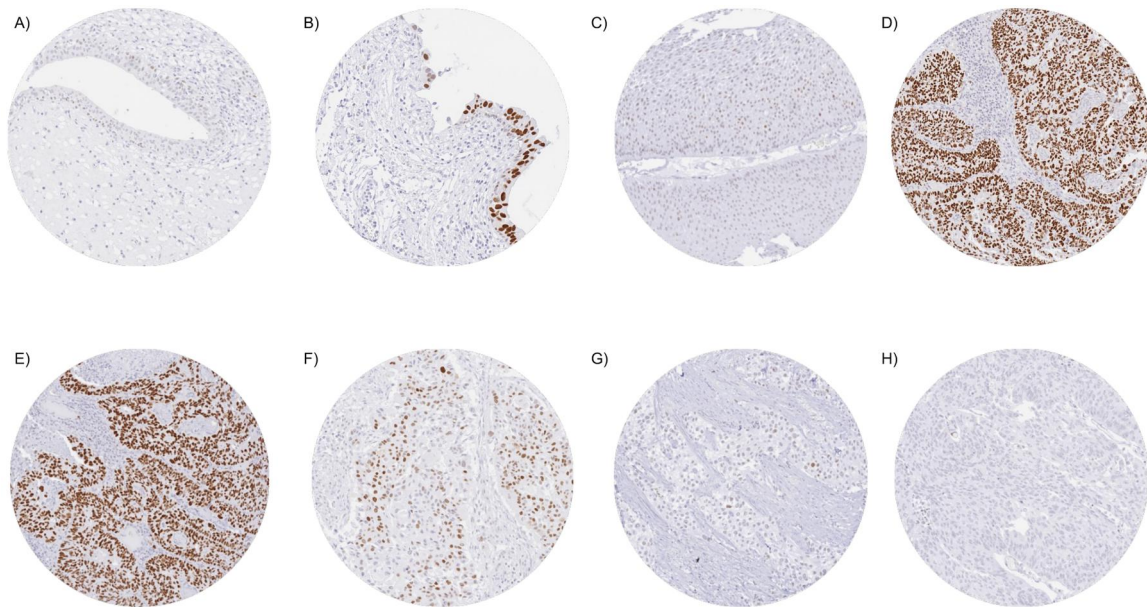


Figure 3. p53 immunostaining in normal and neoplastic urothelium. The panels show a faint p53 staining in only a few cells (wild type) of normal urothelium (A), a strong p53 positivity in a urothelial carcinoma *in situ* (B), a weak p53 in up to 20% of cells of a pTaG2 urothelial tumor (C) as well as a strong nuclear p53 staining in all cells of a pTaG3 urothelial tumor (D) and a muscle-invasive urothelial carcinoma (E). Other muscle-invasive urothelial carcinomas showed a weak to moderate p53 staining in 50–80% of cells (F), a weak to moderate p53 staining of only a few cells (wild type) (G) or a complete absence of p53 immunostaining (probably p53 null type) (H).

immunostaining in pTa tumors, only five studies looked at different grades in 56–121 tumors. One study had reported higher positivity rates in high-grade (51%) than in low-grade pTa (32%) tumors [25], while four studies could not find significant differences between low-grade and high-grade

tumors [26–29]. Among five studies analyzing the clinical relevance of p16 immunostaining in muscle-invasive pT2–4 cancers, only one had earlier suggested a link of p16 loss to reduced progression-free survival [30], while four studies found no association between p16 immunostaining and

Table 3. p53 immunostaining and cancer phenotype.

	n	p53 immunostaining result					p
		Negative (%)	Very low (%)	Low (%)	Strong (%)	Very strong (%)	
All cancers	2425	8.4	15.4	55.3	4.7	16.2	<.0001
pTa G2 low	431	0.5	12.1	87.0	0.2	0.2	
pTa G2 high	207	0.5	14.0	82.6	1.9	1.0	
pTa G3	130	5.4	11.5	62.3	3.8	16.9	
pT2	400	10.8	13.3	43.5	6.5	26.0	.1815*
pT3	550	11.6	19.6	40.7	6.2	21.8	
pT4	270	15.2	14.8	41.9	5.6	22.6	.1341*
G2	92	12.0	19.6	47.8	7.6	13.0	
G3	1103	12.2	16.3	41.3	6.0	24.2	
pN0	596	12.6	18.6	39.8	6.5	22.5	.4386*
pN+	407	14.0	16.7	42.3	4.2	22.9	
R0	489	14.5	18.8	38.7	5.7	22.3	.8626*
R1	123	14.6	14.6	40.7	5.7	24.4	
L0	218	11.9	17.9	45.0	5.0	20.2	.4784*
L1	241	15.8	17.8	38.2	5.8	22.4	
V0	365	12.9	16.4	43.3	6.6	20.8	.4013*
V1	135	17.8	18.5	36.3	4.4	23.0	

pT: pathological stage; G: grade; pN: pathological lymph node status; pN0: no lymph node infiltration; pN+: lymph node infiltration; R: resection margin status; R0: clear; R1: residual tumor; L: lymphatic vessel infiltration; L0: absent; L1: present; V: blood vessel infiltration; V0 = absent; V1 = present.

*Only in pT2–4 carcinomas.

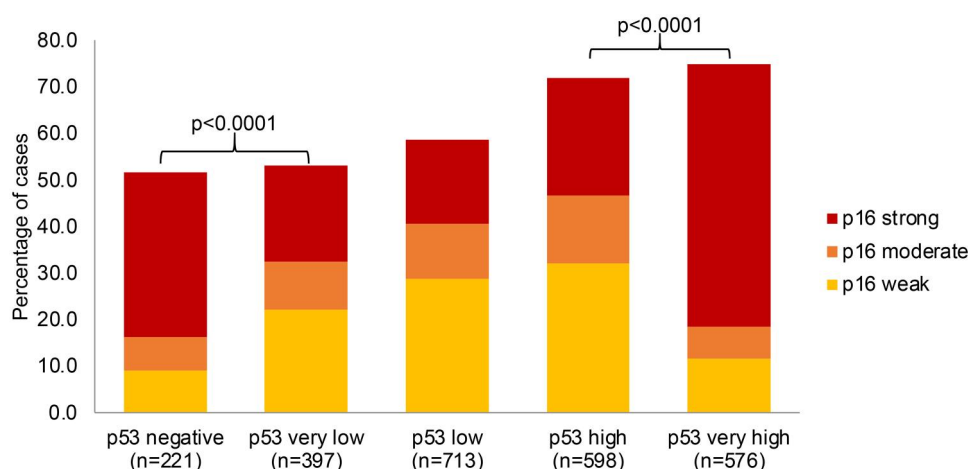


Figure 4. p53 versus p16 immunostaining.

patient prognosis or tumor phenotype [31–34]. Overall, it appears highly likely that the p16 status – if at all – is not sufficiently prognostic to assist in the clinical decision making. It is noteworthy that some studies had interrogated their data for cases with reduced p16 expression based on the assumption that p16 is a tumor suppressor gene for which reduced expression is the expected pathogenic mechanism [35]. However, given the virtual absence of p16 staining in normal urothelial cells, p16 positivity unequivocally represents p16 overexpression in our experimental set-up. Functional studies have demonstrated that a marked upregulation of p16 can occur in cells with impaired pRb or p53 function as an attempt to compensate the oncogenic effects of pRb and/or p53 loss [36,37]. Based on our findings, p16 IHC might be diagnostically useful for detecting urothelial cancer cells. Several authors have suggested the use of p16 IHC for a safer detection of neoplastic cells in flat urothelium [38] or in urine cytology (34) since the diagnosis of dysplasia and even carcinoma *in situ* is susceptible for significant inter-observer variability [39,40].

For p53, our data showed a continuous increase of the fraction of negative and of very strongly positive cases with grade in pTa tumors although the p53 staining patterns were unrelated to stage and clinical outcome in invasive cancers. These data are in agreement with the majority of at least more than 100 earlier studies analyzing cohorts of 26–588 bladder tumors by p53 IHC to estimate the clinical relevance of p53 (PubMed® search (09/22: ‘p53 cancer immunohisto*’)). These authors often described markedly higher p53 positivity rates in pTa high-grade tumors than in pTa low-grade tumors [41–46] and a further increase in p53 positivity from pTa high-grade to more advanced stages [8,47–50]. Previous data on the prognostic role of p53 staining are more controversial [8,9]. At least eight studies described a strong link between p53 positivity and poor prognosis in muscle-invasive urothelial cancers [48,49,51–56]. However, at least six other studies on 40–100 patients have been unable to verify a prognostic impact of p53 staining [57–62] and even meta-analyses of p53 data have failed to find a relevant link to prognosis [8,9]. Considering these data

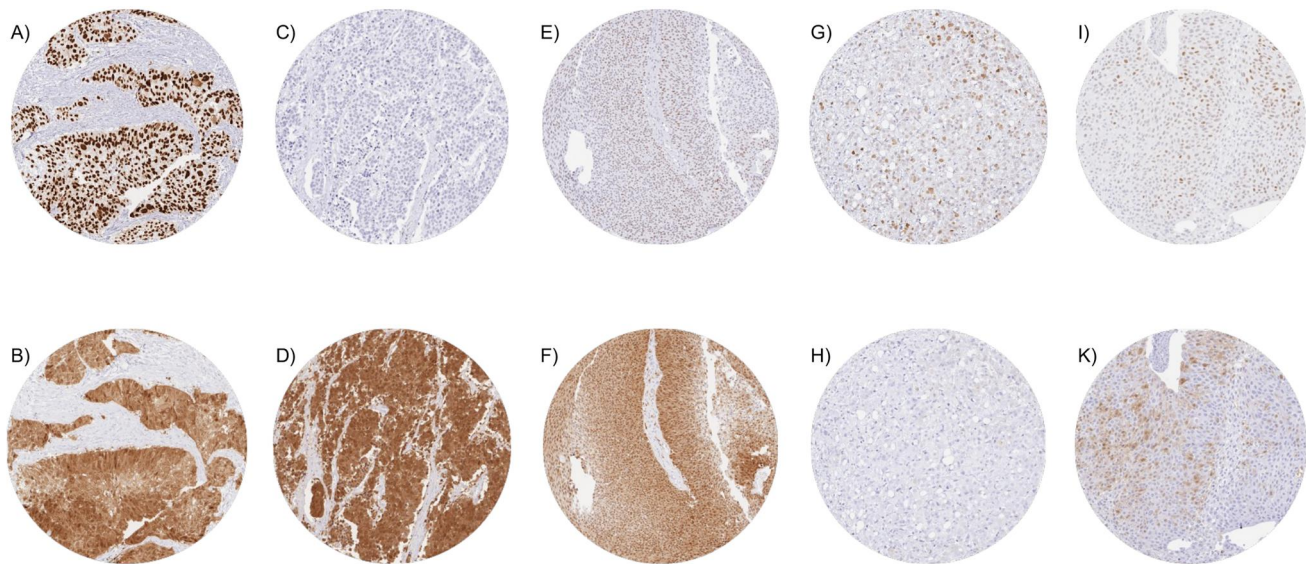


Figure 5. Pattern of p53 and p16 immunostaining in urothelial carcinomas. The panels show combinations of p53 and p16 staining in five muscle-invasive urothelial carcinomas. Tumor 1 shows strong p53 (A) and strong p16 (B) staining (probably p53 mutated). Tumor 2 shows a complete lack of p53 staining (C) and a strong p16 staining in all cells (D; p53 null type; probably p53 inactivated). Tumor 3 contains few p53 positive cells (E) and shows strong p16 positivity of tumor cells (F). This tumor is probably p53 wildtype and has p16 dysregulation because of another, unknown reason. Tumor 4 contains few p53 positive cells (G) and lacks detectable p16 staining (H; probably p53 wildtype). Tumor 5 contains few p53 positive cells (I; probably p53 wildtype) and shows p16 staining of a fraction of cells (K; mosaic pattern; mechanism unknown).

in combination with our results, it is concluded that the prognostic impact of p53 alterations that are detectable by IHC cannot be massive in pT2–4 urothelial carcinomas.

Far more than 95% of studies analyzing p53 by IHC have used cut-offs based on the percentage of p53 positive cells to define p53 negative and p53 positive cases (most often $\geq 10\%$ or $\geq 20\%$ stained tumor cells, PubMed® search (09/22: 'p53 cancer immunohisto*')). It is of note, however, that p53 protein is physiologically expressed in normal cells and that the fraction of p53 positive cells depends on the functional status of individual tissues [63]. The level of physiological p53 expression for example increases markedly in the epidermis after prolonged sun exposure, probably because more DNA repair is needed due to UV light driven cell damage [64]. In cancers, normal p53 protein levels are increased in case of rapid proliferation, genomic instability induced by p53 independent mechanisms, or after chemo- or radiotherapy (summarized in [65–67]). Percentage based cut-offs may therefore not ideally be suited for the distinction of p53 mutated tumors. This is also because tumors that have completely lost p53 expression such as for example by the combination of a deletion of one allele and a nonsense-mutation of the second would be considered p53 negative, which means 'p53 normal'. In order to attempt to distinguish tumors with normal and abnormal p53 function, we selected a five-tier classification of p53 IHC data including the following categories: 'p53 negative' tumors did not show the slightest p53 staining in any cell, a finding that is also consistent with complete loss of p53 expression. 'p53 very low' tumors showed $<5\%$ p53 positive cells of any intensity, a finding that would be expected in tissues with normal p53 function. 'p53 low' tumors showed 5% to $<50\%$ p53 positive cells of any intensity or a weak positivity in 5–100% of cells. Such findings would be consistent with a normal p53 function in tissues suffering from elevated cellular stress but

could also occur in cases with p53 mutation but suboptimal immunostaining due to preanalytical tissue damage. 'p53 strong' and 'p53 very strong' tumors showed an at least moderate p53 staining in 50–80% and >80 –100% of tumor cells. Both findings are likely linked to p53 mutations, especially in cases with $>80\%$ p53 positive cells. According to Nguyen et al. [68], p53 negative and p53 strong/very strong tumors were considered as *TP53* abnormal, whereas p53 very low and low tumors were considered as *TP53* wildtype.

In the absence of p53 sequencing data for our cohort, we cannot precisely specify which of our tumors were p53 mutated. However, validity of our assumptions is supported by the significant increase of all three p53 categories considered to be linked to p53 deficiency (p53 negative, p53 strong, p53 very strong) from pTaG2 low-grade to pTaG3 and from pTaG3 to pT2–4. Moreover, all three potentially p53 mutated groups were statistically linked to strong p16 expression, a feature that may reflect compensatory p16 upregulation in case of p53 inactivation. In combination, these findings provide strong evidence for the existence of a 'p53 IHC negative null phenotype' in urothelial carcinoma. Given that only about half of our p53 negative cases showed strong p16 expression, we would estimate that about 4–5% of pTaG3 tumors and about 7–10% of muscle-invasive carcinomas share a p53 null phenotype with complete loss of normal p53 protein in tumor cells. Considering that at least a focal reduction of immunoreactivity caused by preanalytical tissue damage is often seen in tumor tissues, IHC studies on TMAs will regularly result in a fraction of false negative cases due to the sampling of non-immunoreactive tissue [69]. That such a 'false negativity' applies for at least a subset of our p53 negative pT2–3 carcinomas is supported by the somewhat better prognosis of p53 negative/p16 negative cancers as compared to p53 negative/p16 strongly positive tumors.

Despite of a tendency towards a slightly shorter OS in patients with p53 alterations, our p53 and p16 stainings provided very little prognostic information. This contrasts with many other tumor entities for which at least p53 alterations represent an accepted hallmark of aggressive disease [70–73]. One might speculate, that molecular parameters like p53 alterations that enable cellular dedifferentiation, may exert an unusually mild impact on the clinical outcome in muscle-invasive urothelial cancer. The limited prognostic impact of urothelial tumor cell differentiation is also demonstrated by the poor prognosis of well-differentiated invasive urothelial carcinomas of ‘nested type’ [74,75] and the general recommendation to not grade pT2–4 urothelial carcinoma because of a lack of prognostic relevance of the histologic grade in this tumor entity.

Conclusion

Our data show that detectable p53 and p16 immunostaining increases during grade and stage progression of urothelial neoplasms but that these alterations have very limited, and clinically non-relevant prognostic significance in pT2–4 carcinomas. That high level p16 expression is limited to neoplastic urothelium and that the ‘p53 IHC negative null phenotype’ is largely limited to grade 3 and invasive urothelial carcinomas are features that could potentially be exploited in diagnostic pathology/cytology.

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Ethical approval

The Ethics Committee of the Hamburger Ärztekammer approved the study (WF-049/09).

Consent form

In compliance with the local law (HmbKHG, §12a), informed consent was not obtained.

Disclosure statement

The rabbit recombinant p53 antibody, MSVA-053R, and the rabbit recombinant p16 antibody, MSVA-016R were purchased from MS Validated Antibodies GmbH (owned by a family member of GS).

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Data availability statement

Raw data are available upon reasonable request. All data relevant to the study are included in the article.

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