Connexin 43 expression predicts poor progression-free survival in patients with non-muscle invasive urothelial bladder cancer

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ABSTRACT

Objectives To evaluate the protein expression of connexin 43 (Cx43) in primary urothelial bladder cancer and test its association with the histopathological characteristics and clinical outcome.

Methods A tissue microarray containing 348 tissue samples from 174 patients with primary urothelial carcinomas of the bladder was immunohistochemically stained for Cx43. The intensity of staining was semiquantitatively evaluated (score 0, 1+, 2+, and the association with clinicopathological features was assessed. Univariable and multivariable analyses were performed to identify predictors for progression-free survival (PFS).

Results Membranous Cx43 immunoreactivity was detected in 118 (67.8%) of 174 analysable urothelial carcinomas, of which 31 (17.8%) showed even a strong (score 2+) and mainly homogeneous staining. Strong expression levels of Cx43 (score 2+) were associated with higher tumour grade, multiplicity and increased proliferation (all p<0.05). In the subgroup of patients with stage pTa and pT1 bladder tumours (n=158), strong Cx43 expression (p<0.001), solid growth pattern (p<0.001) and increased Ki-67 proliferation fraction (p<0.05) were significantly associated with shorter PFS in an univariable Cox regression analysis. In multivariable Cox regression models, Cx43 immunoreactivity and histological growth pattern remained highly significant and adverse risk factors for PFS.

Conclusions The expression levels of Cx43 are frequent in non-muscle invasive bladder cancer (NMIBC), with high expression levels being associated with poor prognosis. Routine assessment of Cx43 expression may improve the identification of high-risk NMIBC.

INTRODUCTION

Despite improvements in diagnosis and management of urothelial bladder cancer (BC), the risk of tumour progression and recurrence remains a relevant problem. Patients with primary BC initially present mostly with non-muscle invasive bladder cancer (NMIBC) with either papillary non-invasive (pTa) or early invasive (pT1) urothelial carcinoma (70–80%), whereas the remaining 20–25% of primary tumours are already muscle invasive (≥pT2) at first diagnosis.1 2 Among NMIBC, almost 70% recur after initial transurethral resection and up to 25% show progression into muscle-invasive disease.3 Currently, the risk of recurrence and progression is assessed by clinicopathological factors.4 However, clinical and pathological parameters cannot accurately predict individual disease courses. Therefore, patients still need to be closely monitored to detect tumour recurrence and progression, leading to high healthcare costs of this disease.5 Markers that can diagnose NMIBC with a high risk of progression are needed for better and more specific surveillance strategies.6 Despite decades of research for biomarkers that allow a valid prediction of NMIBC progression,7–10 none are routinely used in clinical practice.

Cell migration is a fundamental process and is essential for many physiological functions of the organism. In addition, cell migration has an active role in pathophysiological processes such as tumour growth and the ability to metastasise.11 12 Several proteins are involved in cell migration or its modulation; these include connexins (Cxs), which are considered to compose gap junctions to form intercellular channels.13 14 The channels composed by Cxs serve as selective gates for the transport of small molecules such as growth factors, second messenger molecules or ions between cells.15 Cxs are therefore essential for cell homoeostasis and play an important role in the regulation of proliferation, cell growth and apoptosis.16 17 Cxs comprises a family of more than 20 proteins. One of the most studied Cx proteins is Cx 43 (Cx43).18 19 Cx43 is expressed in most epithelial tissues. Previous studies showed reduced Cx43 expression in cancerogenesis and therefore Cx43 was initially thought to have only a tumour suppressor role.20–22 However, there is growing evidence that Cx43 is involved in cancer development and metastatic processes and overexpressed in invasive lesions of some solid tumours such as breast or colon cancer.23–25

A broad expression analysis of Cx43 in urothelial carcinomas has not been conducted so far and there are no studies available on the prognostic relevance of Cx43 in BC. We aimed to analyse the expression patterns of Cx43 in a fairly large cohort of primary BCs and correlated these to clinicopathological parameters including tumour stage, grade, multifocality, adjacent carcinoma in situ, growth pattern and finally, disease course.

METHODS

BC tissue microarray

Tissue microarrays (TMAs) contained 348 formalin-fixed, paraffin-embedded urothelial BC tissues from 174 patients and were constructed as
previously described. All tumour samples were represented in duplicate tissue cores (diameter 1 mm). Specimens were collected between 1990 and 2006 by the Institute of Surgical Pathology, University Hospital Zurich, Switzerland. TMA includes a series of 174 consecutive (non-selected) primary urothelial bladder tumours. Finally, TMA contained 90 pTa, 68 pT1 and 16≥pT2 tumours. H&E-stained slides of all specimens were re-evaluated by a board-certified pathologist (PJW). Tumour stage and grade were assigned according to Union for International Cancer Control (UICC) and WHO criteria.

Retrospective clinical follow-up data were available for all the 174 patients (100%). The median follow-up period for the entire cohort was 110.6 months (range 32.4–266.8 months). Adjuvant bladder installation therapy (BCG or chemotherapy) could not be evaluated properly due to missing data in about 50% of the patients. Clinicopathological data are summarised in table 1. TMA and associated clinicopathological data have been previously published.

### Immunohistochemistry

TMA was freshly cut and was used on 3 μm paraffin sections, as described previously. Additionally, to analyse the immunoreactivity of Cx43 in non-dysplastic urothelium, eight slides were cut from formalin-fixed, paraffin-embedded urothelium of the bladder neck of patients without any history of urothelial dysplasia or BC. For immunohistochemical detection of Cx43 on tissue samples, antihuman Cx43 antibody from Sigma (C6219, dilution 1:200) was used. Ki-67 was detected with clone Molecular Immunology Borstel-1 (MIB-1) (dilution 1:50; Dako, Glostrup, Denmark).

Immunohistochemical studies used an avidin-biotin peroxidase method with a diaminobenzidine chromagen. After antigen retrieval (microwave oven for 30 min at 250 W), immunohistochemistry was conducted using an autostainer (Ventana, Tucson, Arizona, USA) following the manufacturer’s instructions.

### Evaluation of immunohistochemistry

Slides were evaluated by two experienced pathologists (LB, PJW). Immunoreactions for Cx43 were evaluated using a semi-quantitative three-scale scoring system by considering scores 0–2+, in which score 0: no staining; score 1+: weak staining; score 2+: strong staining. For statistical analysis, cases exhibiting a score of 0 or 1+ were pooled in a Cx43 low-expression group, whereas cases with a score of 2+ were categorised in a Cx43 high-expression group. The percentage of Ki-67 positive cells of each specimen was determined as described previously. High Ki-67 labelling index was defined as more than 10% of positive tumour cells. If different staining intensity was observed between the duplicate tissue cores, the core with more representative tumour tissue was chosen. If both duplicate cores showed equal amounts of representative tumour tissue, the intensity of the core with more homogenous staining intensity was selected.

### Statistical analysis

Statistical analyses were performed with the survival package in R V3.0.3 (http://www.r-project.org) and SPSS V22.0 (SPSS, Chicago, Illinois, USA). Differences were considered statistically significant if p<0.05. To study statistical associations between clinicopathological and immunohistochemical data, contingency table analysis and two-sided Fisher’s exact tests were used. Univariable and multivariable Cox regression analyses were used to evaluate statistical association between clinicopathological/ immunohistochemical data and progression-free survival (PFS) and recurrence-free survival (RFS). The assumptions of proportional hazards were satisfied in the Cox regression model and were graphically assessed by looking at the log-minus-log plot. All variables in the multivariable model were added in one single step (enter method). Harrell’s concordance index (c-index) was calculated to assess predictive accuracy.

PFS and RFS curves were calculated using the Kaplan-Meier method with significance evaluated by two-sided log-rank statistics and were plotted with pointwise bands at a confidence level of 0.95. For the analysis of PFS in NMIBC, patients were censored at the date when there was a stage-shift (from pTa to pT1–T4 or from pT1 to pT2–T4), or if a distant metastasis was detected. Grade progression (low grade (or G1–2) to high grade (G3)) without stage-shift was not considered as progression. For the analysis of RFS, patients were censored at the date when there was a recurrent and histologically proven tumour in the bladder. Any positive resection or another transurethral resection of the bladder (TUR-B) within 3 months after primary TUR-B was not considered as recurrence but residual tumour.
RESULTS
Staining patterns of Cx43
Cx43 protein expression in BC tissue samples was investigated by immunohistochemical analysis of TMA containing 174 specimens from patients with primary urothelial carcinoma of the bladder. All 174 (100%) patients could be evaluated for Cx43 immunostaining.

Cx43 immunoreactivity mainly showed a homogeneous membranous staining in 170 of 348 TMA spots (48.9%). A relatively small proportion of the investigated tumours showed Cx43 immunoreactivity only in the intermediate and upper layers (n=52, 14.9%), whereas a few cases were found to have Cx43 expression only in the basal layer (n=9, 2.6%). Figure 1A represents a tumour tissue example of Cx43 negative staining (score 0). Figure 1B shows an example of weak Cx43 staining (score 1+), whereas figure 1C depicts a typical example of strong Cx43 staining (score 2+) in the plasma membrane of the tumour cells.

Strong expression levels of Cx43 (score 2+) were found in 31 of 174 patients (17.8%). Eighty-seven (50%) of the other tumours showed weak expression levels (score 1+) of Cx43, and 56 (32.2%) were found to have no staining (score 0) for Cx43. All other clinicopathological data are summarised in table 1.

In non-dysplastic urothelium of the bladder neck of individuals without any previous history of bladder dysplasia or cancer, seven out of eight samples showed a weak but consistent Cx43 protein expression in the basal and intermediate layers (see online supplementary figure S1A–F) as well as in Brunn’s nests (see online supplementary figure S1G). Another single sample showed a strong Cx43 staining intensity (see online supplementary figure S1H). Urothelial umbrella cells at the surface were negative for Cx43 in all normal cases.

Correlation of Cx43 expression with clinicopathological parameters
Cx43 was correlated with clinicopathological characteristics (stage, grade, adjacent carcinoma in situ, multiplicity, growth pattern and Ki-67) of the tumours (table 2). Strong staining of Cx43 was associated with higher grade (p=0.026) and multiplicity (p=0.004). Moreover, strong staining of Cx43 was significantly associated with high Ki-67 labelling index (p=0.014). High Ki-67 labelling index showed a significant correlation with all clinicopathological characteristics (p<0.05), except for tumour multiplicity (data not shown). Results of

| Variable                      | Categorisation | Cx43 expression | p Value |
|-------------------------------|----------------|-----------------|---------|
| Tumour stage                  |                |                 |         |
| pTa                           | 76 (84)        | 14 (16)         | 0.815   |
| pT1                           | 54 (79)        | 14 (21)         |         |
| pT2                           | 10 (77)        | 3 (23)          |         |
| pT3                           | 2 (100)        | 0 (0)           |         |
| pT4                           | 1 (100)        | 0 (0)           |         |
| Histological grade            |                |                 |         |
| G1 low grade                  | 40 (91)        | 4 (9)           | 0.195   |
| G2 high grade                 | 68 (78)        | 19 (22)         |         |
| G3 low grade                  | 35 (81)        | 8 (19)          |         |
| Adjacent carcinoma in situ    |                |                 |         |
| No                            | 128 (81)       | 30 (19)         | 0.311   |
| Yes                           | 15 (94)        | 1 (6)           |         |
| Multiplicity                  |                |                 |         |
| Solitary                      | 109 (88)       | 15 (12)         | 0.004   |
| Multipolar                    | 34 (68)        | 16 (32)         |         |
| Growth pattern                |                |                 |         |
| Papillary                     | 130 (82)       | 29 (18)         | 1.000   |
| Solid                         | 13 (87)        | 2 (13)          |         |
| Immunohistochemistry           |                |                 |         |
| Ki-67 labelling index ≤10%    | 95 (88)        | 13 (12)         | 0.014   |
| Ki-67 labelling index >10%    | 48 (73)        | 18 (27)         |         |

Data are presented as numbers (percent). \(*\) denotes Pearson (two-sided); bold face representing p values <0.05. \(\dagger\) denotes Fisher’s exact test (two-sided); bold face representing p values <0.05.

Prognostic potential of Cx43 in primary pTa and pT1 urothelial tumours
A total of 158 patients underwent TUR for a primary pTa or pT1 urothelial carcinoma of the bladder and were followed for a median of 110.7 months (range: 32.4–245.9 months). From the 158 NMIBC cases, 22 (13.9%) cases showed progression

Figure 1  Immunohistochemical staining with Cx43: An example of negative staining Score 0 (A) and weak (score 1+) staining pattern (B) of the Cx43 protein; Strong staining (score 2+) pattern (C) of Cx43.
In this group, high expression levels (score 2+) of Cx43 were found to be an independent predictor of shorter PFS (table 4). Kaplan-Meier analyses for PFS and RFS depicted in figures 2A, B, respectively, show that patients with strong Cx43 immunoreactivity have a significantly shorter PFS and RFS than patients with weak or negative staining for Cx43. The clinical benefit was moreover confirmed by analysing the C-index in the multivariable Cox regression model. The C-index was higher using the multivariable model with Cx43 than without (C-index with Cx43: 0.813; C-index without Cx43: 0.723).

In a descriptive subgroup analysis for patients with pT1 high grade tumours (n=40) strong Cx43 expression (p=0.024) was significantly associated with reduced PFS (see online supplementary figure S2A). Strong Cx43 immunoreactivity (p=0.257) was not associated with shorter RFS in this subgroup analysis (see online supplementary figure S2B).

**DISCUSSION**

This is the first study analysing immunohistochemical Cx43 expression in primary BC and its association with prognosis. In our study, we found that Cx43 was highly expressed in 31 out of 174 investigated urothelial tumours. Strong Cx43 staining was associated with higher grade, multifocal tumours and increased Ki-67 labelling index. Furthermore, high expression levels of Cx43 were found to be an independent predictor of PFS in NMIBC. Our findings suggest that Cx43 may play a role in the prognosis of patients with non-muscle invasive urothelial tumours.

So far, only two studies have investigated the role of Cx26 in BC and both have found an overexpression of Cx26 in human BC and an association with adverse pathological features. Other study, by Corteggio et al., examined the expression levels of Cx43 in bovine urothelium and bovine urothelial cancer. The authors found that Cx43 was expressed in normal urothelium, papillary neoplasms and carcinoma in situ, but not in invasive urothelial cancer. In addition, Cx43 has been found to be altered in the detrusor muscle of the bladder in patients with overactive bladder syndrome or interstitial cystitis.

Cx43 expression is upregulated in various other tumour entities, including glioma, colon cancer and invasive breast cancer. In contrast, several studies have reported the downregulation of Cx43 expression in bladder cancer cells. This finding may be related to the fact that Cx43 expression is associated with a better prognosis in BC patients.

**Table 3** Analysis of factors for tumour progression

| Variable                  | Categorisation | Tumour progression (TP) n* | Events | p Value† |
|---------------------------|----------------|---------------------------|--------|---------|
| **Pathological data**     |                |                           |        |         |
| Tumour stage              |                |                           |        |         |
| pTa                       | 90             | 10                        | 0.360  |         |
| pT1                       | 68             | 12                        |        |         |
| Histological grade        |                |                           |        |         |
| G1                        | 44             | 3                         | 0.085  |         |
| G2                        | 86             | 12                        |        |         |
| G3                        | 28             | 7                         |        |         |
| Histological grade        |                |                           |        |         |
| Low grade                 | 99             | 10                        | 0.083  |         |
| High grade                | 59             | 12                        |        |         |
| Adjacent carcinoma in situ|                |                           |        |         |
| No                        | 146            | 20                        | 0.545  |         |
| Yes                       | 12             | 2                         |        |         |
| Multiplicity              |                |                           |        |         |
| Unifocal tumour           | 115            | 15                        | 0.465  |         |
| Multifocal tumour         | 43             | 7                         |        |         |
| Growth pattern            |                |                           |        |         |
| Papillary                 | 151            | 17                        | <0.0001|         |
| Solid                     | 7              | 5                         |        |         |
| **Immunohistochemistry**  |                |                           |        |         |
| Cx43                      |                |                           |        |         |
| Score 0 or 1+             | 130            | 12                        | <0.0001|         |
| Score 2+                  | 28             | 10                        |        |         |
| Ki-67 labelling index     |                |                           |        |         |
| ≤10%                      | 106            | 9                         | 0.003  |         |
| >10%                      | 52             | 13                        |        |         |

*Only primary pTa and pT1 tumours are included.
†Log-rank test (two-sided); bold face representing p values <0.05.
‡Staging and grading according to the 1973 WHO classification system.
§Staging and grading according to the 2004 WHO classification system.

**Table 4** Univariable and multivariable Cox regression analyses for tumour progression

| Variable                  | Categorisation | Univariable analysis | Multivariable analysis |
|---------------------------|----------------|----------------------|-----------------------|
|                           |                | HR                   | 95% CI                | p Value†      | HR                   | 95% CI                | p Value†      |
| Tumour stage (pTa vs pT1) |                | 1.481                | 0.635 to 3.454        | 0.363        | 0.706                | 0.258 to 1.933        | 0.499        |
| Histological grade        |                | 2.072                | 0.894 to 4.804        | 0.09         |                      |                       |              |
| Adjacent carcinoma in situ|                | 1.563                | 0.363 to 6.723        | 0.549        |                      |                       |              |
| Multifocality              |                | 1.401                | 0.565 to 3.472        | 0.467        |                      |                       |              |
| Growth pattern (papillary vs solid) |    | 7.653                | 2.715 to 21.568       | <0.001       | 13.377               | 3.314 to 53.99        | <0.001       |
| Cx43                      |                | 5.266                | 2.224 to 12.467       | <0.001       | 7.754                | 2.763 to 21.76        | <0.001       |
| Ki-67                      |                | 3.36                 | 1.428 to 7.904        | 0.006        | 1.76                 | 0.627 to 4.941        | 0.283        |

*Staging and grading according to the 2004 WHO classification system.
Cx43 in solid tumours; for example, in ovarian cancer, non-melanotic skin tumours and lung cancer.\textsuperscript{38–40}

The role of Cxs in tumours and in cancer progression seems to be controversial. This may be due to cellular heterogeneity of the analysed tumours and the complex multilevel process of tumorigenesis and progression. However, more reports suggest that Cx43 is overexpressed in some solid tumours and is involved in late metastatic steps. This could be demonstrated for breast cancer, melanoma and oral squamous cell carcinoma.\textsuperscript{25 41 42}

As previously published, increased proliferation as assessed by Ki-67 was highly associated with tumour grade, stage and shorter PFS in this cohort.\textsuperscript{27} The prognostic role of Ki-67 in urothelial cancer and its association with pathological parameters and prognosis has already been shown in several studies.\textsuperscript{43 44} Here, we observed a significant correlation between the proliferation index (Ki-67) and Cx43 (p=0.014).

As the proportion of muscle-invasive BC samples was limited, we cannot draw any conclusions for this group of tumours. The prognostic role in locally advanced invasive BC has to be evaluated in further studies with higher sample sizes.

Our results demonstrate the potential diagnostic value of Cx43 for the assessment of progressive primary NMIBC. Our findings suggest that the overexpression of Cx43 in BC facilitates tumour cell survival and progression by enhanced gap junction activity, which has previously been shown to induce tumour growth and tumour cell survival.\textsuperscript{45–47} In addition to its prognostic significance, specific pharmaceutical or genetic approaches to inhibit Cx43 activity may provide ways to reduce progression in NMIBC.

CONCLUSION
Positive immunoreactivity of Cx43 is frequently observed in BC and showed a significant correlation with different clinicopathological variables in BC.

High expression levels of Cx43 were associated with poor prognosis in NMIBC. Routine assessment of Cx43 expression may improve the identification of high-risk NMIBC. Further prospective studies and larger cohorts are needed to confirm the prognostic impact of Cx43 in BC.

Figure 2 Kaplan-Meier analyses for progression-free survival (A) and for recurrence-free survival (B) for Cx43 staining (n=158). For statistical analysis for survival curves log-rank test was used. Pointwise bands at a confidence level of 0.95 were computed. N-values represent the number of patients in each group.

Take home messages
\begin{itemize}
    \item Besides clinicopathological parameters no biomarkers are established to assess prognosis in primary bladder cancer (BC).
    \item Connexin 43 (Cx43) has been proposed to be involved in cancer development in solid tumours such as breast cancer or colon cancer.
    \item This is the first study demonstrating the prognostic value of immunohistochemical Cx43 expression in primary non-muscle invasive BC.
    \item Strong Cx43 protein expression was associated with worse clinicopathological features and was furthermore an independent risk factor for shorter progression-free survival.
    \item Routine assessment of Cx43 expression may improve the identification of high-risk non-muscle invasive BC.
\end{itemize}

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REFERENCES
1 Epstein JI, Amin MB, Reuter VR, et al. The World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder. Bladder Consensus Conference Committee. \textit{Am J Surg Pathol} 1998;22:1435–48.
2. Sanchez-Carbayo M, Socci ND, Lozano J, et al. Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays. J Clin Oncol 2006;24:778–89.

3. Lapham RL, Ro JY, Staeckel GA, et al. Pathology of transitional cell carcinoma of the bladder and its clinical implications. Semin Surg Oncol 1997;13:307–18.

4. Sylvester RJ, van der Meijden AP, Oosterlinck W, et al. Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials. Eur Urol 2006;49:466–5; discussion 75–7.

5. Svatek RS, Hollenbeck BK, Holden S, et al. The economics of bladder cancer: costs and considerations of caring for this disease. J Urol 2014;191:623–62.

6. Juffs HG, Moore MJ, Tannock IF. The role of systemic chemotherapy in the management of muscle-invasive bladder cancer. Lancet Oncol 2002;3:73–87.

7. Wild PJ, Herr A, Wisssmann C, et al. Gene expression profiling of progressive papillary noninvasive carcinomas of the urinary bladder. Clin Cancer Res 2005;11:14415–29.

8. Fristrup N, Ulhoi BP, Birkenkamp-Demtroder K, et al. Connexin 43 in urothelial cell carcinoma with fibroblast growth factor receptor 3 and MIB-1 is superior to pathologic grade for the prediction of clinical outcome. J Clin Oncol 2003;21:1912–21.

9. van Rhijn BW, Vis AN, van der Kwast TH, et al. Molecular grading of urothelial cell carcinoma with fibroblast growth factor receptor 3 and MIB-1 is superior to pathologic grade for the prediction of clinical outcome. J Clin Oncol 2003;21:1912–21.

10. Cordes I, Kluth M, Zygis D, et al. Expression and function of gap junction proteins in non-muscle invasive bladder cancer. Am J Pathol 2012;180:1824–34.

11. Friedl P, Gilmour D. Collective cell migration in morphogenesis, regeneration and development. Nat Rev Mol Cell Biol 2007;8:1043–55.

12. Kumar NM, Gilula NB. The gap junction communication channel. J Natl Cancer Inst 1993;85:53–9.

13. Condes I, Kuhl M, Zügß D, et al. FTEN deletions are related to disease progression and unfavourable prognosis in early bladder cancer. Histopathology 2013;63:670–7.

14. Friedl P, Gilmour D. Collective cell migration in morphogenesis, regeneration and cancer. Nat Rev Mol Cell Biol 2009;10:445–57.

15. O’Hayre M, Salanga CL, Handel TM, et al. Chemokines and cancer: migration, intracellular signalling and intercellular communication in the microenvironment. Biochem J 2008;409:635–49.

16. Sarkis AS, Dalbagni G, Cordon-Cardo C, et al. Nuclear overexpression of p53 protein in transitional cell bladder carcinoma: a marker for disease progression. J Natl Cancer Inst 1993;85:53–9.

17. Langlois S, Cowan KN, Shao Q, et al. The tumor-suppressive function of connexin43 phosphorylation: structural changes and biological effects. Biochem J 2009;419:621–72.

18. Wei C, Xu X, Lo CW. Connexin and cell signalling in development and disease. Annu Rev Cell Dev Biol 2004;20:811–38.

19. Su V, Liu AF. Connexins: mechanisms regulating protein levels and intercellular communication. FEBS Lett 2014;588:1212–20.

20. Solan JL, Lampe PD. Connexin 43 phosphorylation: structural changes and biological effects. Biochem J 2009;419:621–72.

21. Zhao W, Han HB, Zhang ZQ. Suppression of lung cancer cell invasion and metastasis by connexin43 involves the secretion of follistatin-like 1 mediated via membrane connexin. FEBS Lett 2014;588:1212–20.

22. Han Y, Zhang PJ, Chen T, et al. Connexin43 Expression Increases in the Epithelium and Stroma along the Colonic Neoplastic Progression Pathway: Implications for Its Oncogenic Role. Gastroenterol Res Pract 2011;2011:56179.

23. Stoeletov K, Strnadl J, Zardozian E, et al. Role of connexins in metastatic breast cancer and melanoma brain colonization. J Cell Sci 2013;126(Pt 4):304–13.

24. Kononen J, Bubendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4:844–7.

25. Poyet C, Jentsch B, Herrmans T, et al. Expression of histone deacetylases 1, 2 and 3 in uterine bladder cancer. BMC Clin Pathol 2014;14:10.

26. Fritzsche FR, Weichert W, Roske A, et al. Class I histone deacetylases 1, 2 and 3 are highly expressed in renal cell cancer. BMC Cancer 2008;8:381.

27. Nozato A, Bubendorf L, Tinner EM, et al. Microarrays of bladder cancer tissue are highly representative of proliferation index and histological grade. J Pathol 2001;194:349–57.

28. van Rhijn BW, Vis AN, van der Kwast TH, et al. Molecular grading of urothelial cell carcinoma with fibroblast growth factor receptor 3 and MIB-1 is superior to pathologic grade for the prediction of clinical outcome. J Clin Oncol 2003;21:1912–21.

29. Tang Z, Han HB, Zhang ZQ. Suppression of lung cancer cell invasion and metastasis by connexin43 involves the secretion of follistatin-like 1 mediated via membrane connexin expression in human detrusor muscle from stable and unstable bladders. BJU Int 2003;92:297–305.

30. Neuhaus J, Pfeiffer F, Wolburg H, et al. Alterations in connexin expression in the bladder of patients with urge symptoms. BJU Int 2005;96:760–7.

31. Kanczuga-Koda L, Sulkowski S, Laczenowski A, et al. Increased expression of connexins 26 and 43 in lymph node metastases of breast cancer. J Clin Pathol 2006;59:429–33.

32. Zhang W, Nwagwu C, Le DM, et al. Increased invasive capacity of connexin43-overexpressing malignant glioma cells. J Neurosurg 2003;99:1039–46.

33. Uhmhauer S, Ruch RJ, Fanning J. Gap junctional intercellular communication and connexin 43 expression in ovarian carcinoma. Ann Oncol 2000;11:999–1000.

34. Wilgenbus KK, Kirkpatrick CJ, Knueckel R, et al. Expression of Cx26, Cx32 and Cx43 gap junction proteins in normal and neoplastic human tissues. Int J Cancer 1992;51:522–9.

35. Jinn Y, Ichikoa M, Marumo F. Expression of connexin32 and connexin43 gap junction proteins and E-cadherin in human lung cancer. Cancer Lett 1998;127:161–9.

36. Haass NK, Rippenger D, Władykowsi E, et al. Melanoma progression exhibits a significant impact on connexin expression patterns in the epidermal tumor microenvironment. Histochim Clin Biol 2010;133:113–24.

37. Brockmeyer P, Jung K, Perske C, et al. Membrane connexin 43 acts as an independent prognostic marker in oral squamous cell carcinoma. Int J Oncol 2014;45:273–81.

38. Lopez-Beltran A, Luque RJ, Alvarez-Kindelan J, et al. prognostic factors in stage T1 grade 3 bladder cancer survival: the role of G1-S modulators (p53, p21Waf1, p27kip1, cyclin D1, and cyclin D3) and proliferation index (ki67-MIB1). Eur Urol 2004;45:606–12.

39. Quintero A, Alvarez-Kindelan J, Luque RJ, et al. Ki-67 MIB1 labelling index and the prognosis of primary TaT1 urothelial cell carcinoma of the bladder. J Clin Pathol 2005;58:93–8.

40. Mendosa-Naranjo A, Saez PJ, Johannsson CC, et al. Functional gap junctions facilitate melanoma antigen transfer and cross-presentation between human dendritic cells. J Immunol 2007;178:6949–57.

41. Katakowski M, Buller B, Wang X, et al. Functional microRNA is transferred between glioma cells. Cancer Res 2010;70:8259–63.

42. Lane DW. Life cycle of connexins in health and disease. Biochem J 2006;394 (Pt 3):527–43.

43. Mostofi FK, Sobin LH, Torloni H. Histological typing of urinary bladder tumours. International Classification of Tumours, No. 10. Geneva: World Health Organization, 1973:21–31.

44. Ebet I, Sautier G, Epstein J, et al. Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs. Lyon: IARC Press, 2014.
Supplemental Figure 1: Immunohistochemical staining with Cx43 in non-dysplastic urothelium, weak but consistent staining was observed in 7 out of 8 samples in the basal and intermediate layers (A-F) as well as in Brunn’s nests (G). One single sample showed a strong Cx43 staining intensity (H). Urothelial umbrella cells at the surface were negative for Cx43 for all cases.
Supplemental Figure 2: Kaplan-Meier analyses for progression-free survival (A) and for recurrence-free survival (B) for Cx43 staining ($n=40$) in patients with pT1 high grade tumours. For statistical analysis for survival curves Log-Rank test was used.
**ABSTRACT (native language German)**

**Ziele:** Evaluation der Proteinexpression von Connexin 43 (Cx43) beim primären Urothelkarzinom der Harnblase bezüglich möglicher Assoziationen mit histopathologischen Eigenschaften als auch mit klinischen Verlauf.

**Methoden:** Erstellung eines Gewebemikroarray (TMA) von 348 Gewebeproben von insgesamt 174 Patienten mit primären Urothelkarzinom der Harnblase. Der TMA wurde immunhistochemisch auf Cx43 untersucht und die Intensität der Färbung semiquantitativ evaluiert (Score 0, 1+, 2+). Die statistischen Assoziationen von Cx43 mit klinischen und pathologischen Eigenschaften wurden untersucht. Zur Bestimmung von Prediktoren des progressionsfreien Überlebens (PFS) wurden univariabile und multivariabile Cox-Regressionsanalysen durchgeführt.

**Resultate:** Cx43 wurde in 118 (67.8%) der insgesamt 174 Urothelkarzinome exprimiert, von welchen 31 Tumore (17.8%) eine starke (score 2+) und mehrheitlich homogene Exprimierung zeigten. Eine starke Exprimierung von Cx43 (score 2+) war mit höherem Tumorgrad, Multifokalität und mit einer gesteigerten Ki-67-Proliferationsfraktion assoziiert (alle p < 0.05). In der Subgruppe von pTa und pT1 Blasentumore (n = 158), zeigten sich eine starke Cx43 Immunexpression (p < 0.001), ein solider Tumorwachstum (p < 0.001) und eine gesteigerte Ki-67-Proliferationsfraktion (p < 0.05) als signifikante Prediktoren für ein kürzeres PFS in der univariablen Cox-Regressionsanalyse. In der multivariablen Cox Regressionsanalyse blieben die starke Cx43 Proteinexpression und der solide Tumorwachstum signifikante Risikofaktoren für ein kürzeres PFS.

**Schlussfolgerung:** Die Cx43 Proteinexpression beim nicht-muskelinvasiven Urothelkarzinom der Harnblase (NMIBC) ist ein häufiges Ereignis, wobei die starke Proteinexpression mit einer schlechten Prognose assoziiert ist. Die routinemäßige immunhistochemische Färbung von Cx43 könnte möglicherweise Hochrisiko-NMIBC identifizieren.