Loss of AQP3 protein expression is associated with worse progression-free and cancer-specific survival in patients with muscle-invasive bladder cancer

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Author contributions

Peter C. Rubenwolf, Wolfgang Otto and Nikolaos Georgopoulos conceptualised the study.

Peter C. Rubenwolf, Wolfgang Otto and Arndt Hartmann prosecuted the study.

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Abstract

Purpose: Urothelial carcinoma has recently been shown to express several aquaporins (AQP), with AQP3 being of particular interest as its expression is reduced or lost in tumours of higher grade and stage. Loss of AQP3 expression was associated with worse progression-free survival (PFS) in patients with pT1 bladder cancer. The objective of this study was to investigate the prognostic value of AQP3 expression in patients with muscle-invasive bladder carcinoma (MIBC).

Methods: Retrospective single-center analysis of the oncological outcome of patients following radical cystectomy (Cx) due to MIBC was performed. Immunohistochemistry was used to assess AQP3 protein expression in 100 Cx specimens. The clinical value of the marker was analyzed in relation to progression, cancer-specific and overall survival using Kaplan-Meier and multivariate Cox regression analysis (CRA).

Results: Loss of or weak AQP3 expression was associated with a statistically significantly worse PFS (19% vs. 75%, p=0.043) and CSS (18% vs. 75%, p=0.030) and, alongside lymph node involvement, was an independent predictor of PFS (HR 2.872, CI: 1.058 – 7.796, p=0.038) and CSS (HR 3.332, CI: 1.221 – 9.089, p=0.019) in CRA. Conclusions may be limited by the size of the patient cohort and the restricted statistical power of the study.

Conclusions: Although the results of the study would be strengthened by a larger, more appropriately powered, prospective, multi-institutional study, our findings strongly suggest that AQP3 expression status may represent an independent predictor of PFS and CSS in MIBC and may help select patients in need for (neo-)adjuvant chemotherapy.

Introduction

Urothelial carcinoma of the bladder (UBC) is the 4th most common malignancy in men and the 8th most common cause of male cancer death in the United States[1]. Pathological staging and grading continue to be the most powerful criteria in relation to therapy of choice and prognosis of the disease. Whereas molecular diagnostics have become an integral part of routine clinical management for patients with breast, colon and lung cancer, biomarkers play only a minor role in current management strategies for UBC. Therefore there is still a need for prognostic molecular markers that can help clinicians select patients in need of early surgical and/or neoadjuvant management.

The prognostic significance of aquaporin (AQP) water channel expression in UBC has not been investigated in larger patient cohorts to date. AQPs are a family of trans-membrane channel-forming proteins that selectively allow water and other small, uncharged molecules to pass across cell membranes in response to osmotic or pressure gradients. We have previously demonstrated that normal, i.e. non-diseased, human urothelium expresses several AQPs, suggesting a potential role in water and urea transport across the urothelial layer[2]. Predominant expression of AQP3 is supportive of the hypothesis that the urothelium may be able to modify volume and final composition of the urine[3]. Apart from its crucial significance in human physiology, there is strong presumptive evidence that AQPs play a role in carcinogenesis, such as in tumour angiogenesis and cell migration[4].

On the basis of our findings in normal human urothelium, we have previously investigated the expression and potential significance of AQPs in UBC and showed that there is a significant correlation between AQP3 protein expression and tumour stage and grade, with AQP3 expression being reduced or lost in tumours of higher grade and stage. We concluded that AQPs may play a role in the progression of UBC and, in particular, that this could be of prognostic value[5]. In support of this, we have recently demonstrated that loss of AQP3 protein expression was an independent

marker associated with worse progression-free survival in patients with pT1 UBC, a finding that was confirmed by multivariate Cox regression analysis[6].

To date, the clinical significance of AQP3 protein expression in muscle-invasive bladder cancer (MIBC) remains unexplored. The aim of the present study was to investigate the prognostic value of AQP3 in terms of progression and survival of patients diagnosed with MIBC and to discuss the potential usefulness of the marker with regards to the design of therapeutic strategies.

Material and Methods

Patients with MIBC (pT2-T4), who were subjected to transurethral resection of the bladder followed by radical cystectomy, lymph-node dissection and urinary diversion between 2001 and 2006 were included in the present study. Cases in which the histological evaluation of the cystectomy specimen did not confirm MIBC, patients with distant metastases at initial diagnosis and those who received neoadjuvant chemotherapy prior to surgery were not included in the study.

Postoperatively, adjuvant chemotherapy (CTx) was recommended to patients with lymph-node involvement (pN+). All patients underwent regular follow-up visits consisting of physical examination, ultrasound and laboratory tests. CT and skeletal scans were restricted to patients with suspicion of recurrent and/or metastatic disease. All surgical procedures, CTx and follow-up visits were carried out at the Department of Urology, Regensburg University Medical Center. Collection of tissue specimens had the approval of the local research ethics committee and full informed patient consent.

We retrospectively reviewed the histopathological and clinical data of all patients in relation to tumour recurrence, disease progression, and cancer-specific survival. Mean follow-up was 33 ± 3.1 months. Range, median

Immunohistochemistry

Cystectomy specimens were processed, fixed in 10% formalin, dehydrated and embedded in paraffin wax. Dewaxed 4µm tissue sections were subjected to antigen retrieval by boiling for 10 min in tris-ethylenediaminetetraacetic acid before labeling with titrated primary antibody (polyclonal anti-AQP3, host: rabbit, antigen: human AQP3, dilution 1:2000, Abcam, USA) for 16h at 4°C, as described[5]. Positive control tissues of normal human urothelium known to express the antigen and negative controls in which the primary antibody was omitted were included in all experiments. All slides were examined in a blind fashion by two of the authors (P.R. and A.H., an expert uropathologist) using a Primo Star™ microscope (Carl Zeiss Microimaging, Jena, Germany).

Assessment of AQP3 protein expression

Labelled photomicrographs were ranked according to immunohistochemical patterns relating to the expression and distribution of AQP3 within the tumour. The ranking criteria used to grade the antigen expression were as follows:

0 - no expression

1 - cytoplasmatic expression

2 - weak cell membrane-associated expression

3 - intense expression restricted to the cell borders

Each sample was assigned a numerical value according to its rank. Representative examples for each rank are shown in Figure 1.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics, version 21.0 (SPSS Inc, Chicago, IL). Different patient characteristics and clinicopathological parameters were evaluated in relation to the AQP3 protein expression status of the patients using Fisher's exact test. Multivariate Cox regression analysis was performed to explore the prognostic value of AQP3 expression and other clinicopathological parameters in terms of progression, cancer-specific and overall survival. Progression-free, cancer-specific and overall survival rates were calculated by Kaplan-Meier analysis and Log rank test. P-values <0.05 were considered statistically significant.

Results

Patient characteristics

100 patients met the inclusion criteria. Patient characteristics, clinical and histopathological data are summarized in Figure 1.

Oncological outcome

10% of patients were diagnosed with local tumour recurrence in the course of follow-up. Systemic progression, i.e. distant metastases, occurred in 30% of patients. At the end of the follow-up period a total of 40% of patients were alive, while 60% had died (38% cancer-specific mortality and 22% from other causes than UBC).

Expression of AQP3

Overall, 65% of tumours were classified as AQP3-negative (no expression, rank 0). Expression of AQP3 was shown to be present in 35% of all tumour specimens. Intense membranous expression, as seen in normal human urothelium, was found in 23% (rank 3), whereas weak (rank 2) or cytoplasmic (rank 1) labeling was present in 11 and 1 tumours, respectively. Interobserver variability was 6%. These cases were re-evaluated and the most appropriate rank was agreed upon.

Having primarily classified the tumours into AQP3-positive and -negative, the proportion of AQP3-positive tumour areas relative to the whole tumour specimen was subsequently examined, to provide an idea of AQP3 expression heterogeneity. Overall, 20% of specimens exhibited AQP3-positivity in more than 75% of the total area of the tumour, whereas expression between 26-75% and < 25% of the tumour was observed 49% and 21%, respectively. However, further stratification of the cohort according to AQP3 expression heterogeneity did not show relevant differences in survival analysis (results not shown).

Representative immunohistochemistry findings are shown in Figure 1.

In multivariate regression analysis, loss of or reduced AQP3 expression (rank 0-2 vs. 3) was shown to be independent of clinical and histopathological parameters, such as gender, age, pT stage, nodal stage, surgical margin status, associated carcinoma in situ and adjuvant chemotherapy.

Kaplan-Meier analysis of AQP3 expression in relation to progression-free and cancer-specific survival

Intense, cell membrane-associated AQP3 expression (rank 3) in the cystectomy specimen was associated with a statistically significantly better 5-year progression-free survival (PFS 75% vs. 19%, p=0.043) and cancer-specific survival (CSS 75% vs. 18%, p=0.030) compared with loss of or reduced/abnormal expression (rank 0, 1 and 2). Despite better overall survival (OS) in patients with intense AQP3 expression (rank 3), the difference did not reach statistical significance (51% vs. 11%, p=0.070). Representative results are shown in Figure 2A and B.

Multivariate Cox regression analysis of the AQP3 expression status and clinicopathological parameters in relation to PFS, CSS and OS

Multivariate Cox regression analysis revealed lymph node (LN) involvement (HR 2.07, CI 1.26 – 3.40, p=0.004), positive surgical margins (HR 2.15, CI 1.07 – 4.30, p=0.031) and AQP3 expression status (HR 2.87, CI 1.06 – 7.80, p=0.038) to be independent predictors of PFS. LN involvement (HR 2.09, CI 1.31 – 3.33, p=0.002) and AQP3 expression (HR 3.33, CI 1.22 – 9.09, p=0.019) were found to be independent predictors of CSS, whereas pT stage (HR 1.67, CI 1.05 – 2.67, p=0.032) and LN status (HR 1.54, CI: 1.07 – 2.21, p=0.019) were independent predictors for OS. By contrast, the AQP3 expression status did not yield statistical significance with regard to OS (HR 1.96, CI 0.99 – 3.90, p=0.055) Results are shown in Supplementary Table 2.

Discussion

In the past 2 decades, a plethora of urothelial carcinoma-associated biomarkers have been identified, including growth factors and their cognate receptors, oncogenes, oncoproteins,

tumor suppressor genes, hormone receptors, proliferation and apoptosis markers, and cell adhesion molecules [7]. However, the diagnosis and therapeutic management of patients with bladder cancer continues to be based primarily on conventional clinical and pathological tumour staging and grading.

Despite efforts in establishing tissue biomarkers that may assist urological surgeons in selecting patients in need for early radical surgery and (neo-)adjuvant chemotherapy, the major findings in this field have had little clinical and translational impact so far [7, 8]. To date, lymph node status (pN+/-) is the most widely accepted clinical predictor of progression-free and cancer-specific survival in patients with MIBC. Hautmann et al provided compelling data of a large cohort of 1100 patients with MIBC having undergone radical Cx without (neo-) adjuvant chemotherapy. Involvement of only a single lymph node (pN1) was associated with cancer-specific death in over 80% of patients. None of the patients with pN2/3 survived. The authors suggested that adjuvant CTx should be recommended to patients with histologically-proven lymph node involvement. They hypothesized that all patients with MIBC potentially benefit from neoadjuvant CTx [9]. However, Burger et al only recently presented the results of a survey revealing strong misgivings against neoadjuvant CTx amongst European urologists, presumably due to the lack of clearly defined parameters or markers that help select patients who could benefit from the treatment [10].

We have previously demonstrated that several AQPs are expressed both in well-established urothelial cancer cell lines and in human bladder carcinoma specimens. Our results indicated that there is a correlation between AQP3 protein expression and tumour stage and grade, with APQ3 expression being reduced or lost in tumours of higher grade and stage. Previous investigations into the significance of AQPs in non-urological tumours have almost invariably demonstrated over-expression of AQP3 and it has been hypothesized that that AQP3 may be a promising drug target in the treatment of various epithelial tumours [11, 12]. The contrasting expression pattern of AQP3 between UBC and carcinoma types of non-urothelial origin is striking and may be explained by the hypothesis that in human urothelium expression and function of AQP3 is associated with the phenotype (proliferative or differentiated) of the cells. Thus, it is conceivable

that loss of differentiation may be paralleled by loss of AQP3 expression in tumour cells. Moreover, it is tempting to speculate that loss of AQP3 in UBC may be associated with resistance to apoptotic stimuli, as previously shown for AQP 8 and 9 in hepatocellular carcinoma [13]. Based on these findings, we concluded that AQPs may play a role in the progression of UBC and, in particular, that this could be of prognostic value [5]. In support of this, we have provided strong presumptive evidence that loss of AQP3 protein expression is associated with worse progression-free survival in patients with pT1G2/G3 carcinomas and we hypothesized that this could also apply to muscle-invasive tumours [6]. This hypothesis seems to be confirmed by the results of the present study. Intense, cell membrane-restricted AQP3 expression in the cystectomy specimen was associated with both a statistically significantly better 5-year progression-free and cancer-specific survival compared with loss of or reduced and abnormal expression, respectively. Moreover, expression of AQP3 and lymph node involvement were shown to be independent predictors of progression and cancer-specific survival in multivariate Cox regression analysis. It is noteworthy in this context that the AQP3 expression status was shown to be independent of clinicopathological parameters such as pathological stage and lymph node involvement.

Collectively, our previous and current findings suggest that loss of AQP3 expression may be implicated in a molecular program associated with loss of differentiation, progression to muscle-invasive disease and, ultimately, with mechanisms resulting in metastatic spread, such as lymphovascular invasion and resistance to apoptotic stimuli. Future studies, using our relevant *in vitro* models, will aim to address these aspects in order to understand the importance of loss of AQP3 expression in UBC and its underlying molecular mechanisms.

In relation to the clinical significance of our findings, we propose that the immunohistochemical assessment of AQP3 in UBC specimens may constitute a clinically relevant supplementary parameter that could, alongside conventional grading and staging, be implemented in future diagnostic and clinical management strategies. Assuming that

AQP3 is an independent marker of disease progression, its expression status in the primary transurethral resection specimen could serve as a novel marker that enables urological surgeons to select candidates for early cystectomy (AQP3-negative pT1G3 tumours) versus patients who may benefit from conservative treatment (AQP3-positive tumours). Similarly, loss of AQP3 in muscle-invasive UC of the bladder could serve as a criterion for the selection of patients who are candidates for neo-adjuvant chemotherapy prior to Cx.

Nevertheless, the strength of the conclusions from our results may be limited, as we performed a retrospective, single-center study on a relatively limited number of Cx specimens. Selection bias is likely and has to be considered in the interpretation of the results. Moreover, the large confidence intervals found in multivariate analysis indicate restricted statistical power of the study. Considering the low proportion (23%) of cases with intense AQP3 expression and the low mortality in this group, 100 specimens may be low for a reliable multivariate analysis. Thus, a prospective, appropriately powered, multi-institutional center study comprising a large number of patients would be required to draw more clinically relevant conclusions from our preliminary observations. Of note also, UC is well-known for its extraordinary tumour heterogeneity reflected by diverse morphological manifestations and various molecular alterations associated with these different tumour phenotypes. Hence, a single marker such as AQP3 is unlikely to be adequate to sufficiently characterize the progression risk and probability of recurrence. Clinical nomograms combining the results from selected biomarkers as well as clinicopatholgical parameters need to be established to increase the accuracy of clinical predictions.

Conclusions

Biomarkers that prospectively evaluate tumour aggressiveness, probability of recurrence, progression risk and overall prognosis would improve the care and prognosis of patients with bladder cancer. The present study provides *prima facie* evidence that the expression of AQP3 by the tumours is an independent predictor of progression-free and cancer-specific survival in patient with muscle-invasive bladder cancer. Integration of AQP3 with conventional clinical and pathologic staging may refine clinical decision making for the selection of candidates benefiting from (neo-)adjuvant chemotherapy.

Conflict of interest: The authors declare that they have no conflict of interest.

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