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ORIGINAL ARTICLE

Expression of aquaporin water channels in human urothelial carcinoma: correlation of AQP3 expression with tumour grade and stage

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Abstract
Purpose To study the expression, localization and potential clinical significance of aquaporin water channels both in well-established urothelial cancer (UC) cell lines and in human bladder carcinoma specimens of different stages and grades and to discuss the clinical relevance of the findings.
Methods AQP transcript and protein expression by RT4, RT112 and T24 UC cell lines was investigated using reverse transcriptase polymerase chain reaction and immunofluorescence labelling. Immunohistochemistry (IHC) was used to assess AQP protein expression in 94 UC specimens of various grades and stages.
Results AQP3 and 9 transcripts were expressed in low-grade RT4 and RT112, but not in high-grade T24 cells. By contrast, AQP4 mRNA was absent in RT4, but expressed by RT112 and T24. Transcripts for AQP7 and 11 were detected in all three UC cell lines. Immunofluorescence microscopy confirmed the expression of AQP3, 4 and 7 at the protein level. By IHC, AQP3 was shown to be intensely expressed by 86 %, 66 % and 33 % of specimens of stage pTa, pT1 and pT2 tumours, respectively ($p < 0.001$). Whereas 100 % of G1 tumours were positive, only 73 % and 55 % of G2 and G3 tumours were found to express AQP3 ($p = 0.004$).
Conclusions This is the first study to demonstrate that several AQPs are expressed in UC. Our results indicate that there is a correlation between AQP3 protein expression and tumour stage and grade, with AQP3 expression being reduced or lost in tumours of higher grade and stage. Taken together with the available evidence from other studies, we conclude that AQPs may play a role in the progression of UC and, in particular, that this could be of prognostic value.

Keywords Aquaporins · Carcinogenesis · Urothelial carcinoma · Bladder cancer · Cell lines · Biomarker

Introduction

AQPs are transmembrane pore-forming proteins that have been previously shown to play a fundamental role in numerous physiological processes, most notably in fluid absorption and secretion. To date, 13 different mammalian AQPs have been identified at the molecular level and localized to specific tissues [1].

Rubenwolf et al. [2] have previously provided molecular evidence that human urothelium expresses several AQPs, suggesting a potential role in water and urea transport across the urothelial layer. Predominant expression of AQP3 is

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supportive of the hypothesis that the urothelium may be able to regulate osmolality and composition of the urine [3].

Analysis of several human diseases has confirmed that AQPs are involved in various pathological conditions and provide promising drug targets [4]. Moreover, there is strong presumptive evidence that AQPs play a role in carcinogenesis, specifically in tumour angiogenesis and cell migration [5]. In the overwhelming majority of high-grade tumour cells of different tissue origins, AQPs were shown to be overexpressed, suggesting AQP-facilitated tumour cell migration and spread [6–8].

To date, studies on the expression and significance of AQPs in urothelial carcinoma (UC) are scarce, and we were the first to suggest a correlation between AQP3 protein expression and tumour stage and grade [9]. In particular, we have recently demonstrated that the loss of AQP3 protein expression appears to be associated with worse progression-free survival in a subset of patients with pT1 carcinomas [10]. However, the underlying molecular mechanisms and the prognostic value of AQPs in muscle-invasive tumours have not been explored.

Moreover, human UC has not been systematically investigated with regard to all members of the AQP family and any correlation with different stages and grades of UC. The principal aim of this study was to characterize the expression pattern of AQP channels in UC cells in vitro as well as in surgical samples of UC, using a large panel of clinical specimens, ranging from superficial to muscle-invasive UC. The potential biological and clinical significance of our findings is discussed.

Materials and methods

Tissue recovery

Collection of tissue specimens was approved by the local research ethics committee, and full informed patient consent was obtained. Tumour biopsies were obtained from 94 non-consecutive patients who underwent primary transurethral resection or radical cystectomy for UC in the Department of Urology, Regensburg University Medical Center, between 2002 and 2009. Clinicopathological data are summarized in Table 1 of the Supplementary Methods section. All surgical specimens were assessed histopathologically by a single expert uropathologist (FH) for grading and staging based on the criteria of the 1973 WHO classification and 2010 TNM system [11, 12].

Cell culture

Three established UC-derived cell lines, RT4, RT112 and T24, were obtained from the Health Protection Agency Culture Collection (HPACC, Porton Down). RT4, RT112 and T24 cell lines were derived from UC of grades 1, 2 and 3, respectively, and have been well characterized [13]. These cell lines were specifically chosen as they cover phenotypes ranging from well differentiated to highly malignant, thus representing a wide spectrum of tumour grades and stages. Characteristics of the cell lines are explained in detail in the Supplementary Methods section.

RNA isolation and transcript analysis

Human AQP0 to 12 primers were designed (Supplementary Methods, Table 2), and total RNA from UC cell cultures was isolated, reverse-transcribed to cDNA and used for RT-PCR, exactly as previously described [2]. PCR products were analysed by 2 % agarose gel electrophoresis. RT-positive and RT-negative controls were included in all PCR reactions.

Immunofluorescence microscopy

UC cells were seeded at 1×10^5 cells/ml onto multiwell glass slides, fixed and incubated sequentially in primary and secondary antibodies, as described elsewhere [2]. Normal human urothelial cell cultures known to express AQPs were used as positive controls [2], and secondary antibody-only negative controls were included. Immunolabelling was visualized by epifluorescence on an Olympus B×60 microscope.

Immunohistochemistry

Surgical samples of UC were processed and dewaxed, and 5-μm tissue sections were subjected to antigen retrieval, before labelling with titrated primary antibodies for 16 h at 4 °C, as described elsewhere [2]. The rationale for selecting a subset of AQPs (AQP3, 4, 7, 9) for immunohistochemistry was based on the gene expression profile shown in urothelial carcinoma cell lines (current study) and in several independent normal human urothelial cell lines [2]. Positive control tissues from 15 independent samples of normal human urothelium and negative controls in which the primary antibody was omitted were included in all experiments (see Supplementary Figure S1). All slides were examined blind by P.R. and an expert uropathologist (F.H.). Labelled photomicrographs were evaluated according to immunohistochemical patterns related to the expression and distribution of AQPs within the tumour.

Statistical analysis

SPSS, version 19.0 (SPSS Inc., Chicago, IL, USA), was used for statistical analysis. AQP3, 4, 7 and 9 protein

expression was analysed in relation to tumour stage and grade, and results were compared using the chi-squared test for comparative analysis of percentage positivity, combined with the Goodman and Kruskal's test as a measure of rank correlation, to illustrate the significance of percentage AQP3 expression. p values <0.05 were considered statistically significant.

Results

AQP expression by UC cell cultures in vitro

To determine which AQP genes are expressed by human UC cell lines, RT-PCR was used to detect transcripts present in RT4, RT112 and T24 cells.

Expression of AQP7 and 11 transcripts was detected consistently in cultures of all three cancer cell lines, whereas AQP3, 4 and 9 exhibited differential expression patterns in each cell line. AQP3 and 9 were shown to be present in RT4 and RT112, but not in T24 cells. By contrast, expression of AQP4 was detected in RT112 and T24 cells, but not in RT4 cells. AQP0, 1, 2, 5, 6, 8, 10 and 12 were not expressed. Representative results are shown in Fig. 1. These findings provide *prima facie* evidence that human UC cell lines express AQPs, at least at the transcript level.

Having detected AQP expression at the mRNA level, the expression and cellular localization of AQP protein in cultured UC cell lines was examined by immunofluorescence microscopy. AQP3, 4 and 7 were expressed in UC cell lines, in agreement with the RT-PCR results. AQP9 expression, however, was not detectable. AQP3 revealed strong immunoreactivity at the cell borders of RT4 and RT112 cells, but not in T24 cell lines, where the antibody exhibited weak cytoplasmic labelling in a subpopulation of cells (Fig. 1).

Immunohistochemical expression of AQP3, 4, 7 and 9 in UC specimen

To examine AQP expression in surgical specimens of UC, representative biopsies from 94 histopathologically proven UC were included in the study.

AQP3 expression

Overall, AQP3 was found to be expressed by 68 % of all UC specimens, whereas 32 % of the tumours were AQP3 negative. AQP3 was expressed by 86 %, 66 % and 33 % of stage pTa, pT1 and pT2 tumours, respectively. Whereas 100 % of G1 tumours were positive, only 73 % and 55 % of G2 and G3 tumours were found to express AQP3. The differences in AQP3 expression between grade and stage were statistically significant ($p < 0.001$ and 0.004). Results are summarized and representative immunohistochemistry findings shown in Fig. 2.

AQP3 was shown to be intensely expressed at cell borders in basal, intermediate and partly superficial cell layers by 77 % of non-muscle-invasive UC. No immunoreactivity was detected in the lamina propria, smooth muscle or endothelium (see Supplementary Figure S2).

By contrast, in muscle-invasive high-grade UC, expression of AQP3 was found to be markedly more heterogeneous, with most tumours exhibiting large AQP3-negative areas and scattered clusters of positive cells. The abrupt transition from strongly labelled to unlabelled cells, shown in Fig. 2g, was particularly striking.

Having primarily classified the tumours into AQP3 positive and AQP3 negative, the proportion of AQP3-positive tumour areas relative to the whole tumour specimen was examined, to provide an idea on AQP3 expression heterogeneity. Overall, only 33 % of all specimens included in the study exhibited AQP3-positivity in more than 75 % of the total area of the tumour, which is comparable to normal, i.e. non-diseased human urothelium. In fact, AQP3 expression detectable in >75 % of the specimen was observed solely in pTa tumours.

AQP4, 7 and 9 expression

Whereas there was intense expression in 15 independent samples of normal human urothelium (positive control), there was no immunoreactivity in tumour tissues irrespective of grade and stage (see Supplementary Figure S3). This suggests that AQP4 is not detected in human UC, at least by IHC.

AQP7 was found to be expressed in the cytoplasm and nuclei of all tumour samples, regardless of grade and stage, in agreement with the transcript findings (see Supplementary Figure S4).

No immunoreactivity was found with the antibody against AQP9 (Supplementary Figure S5).

Discussion

The principal objective of this study was to characterize AQP expression in UC cell lines in vitro using established cell lines and in surgical specimens of UC of various grades and stages and to discuss the potential biological and clinical significance of the findings.

Our results demonstrate the expression of several members of the AQP family by three well-characterized UC cell lines of different malignant potentials. These three cell lines have previously been extensively

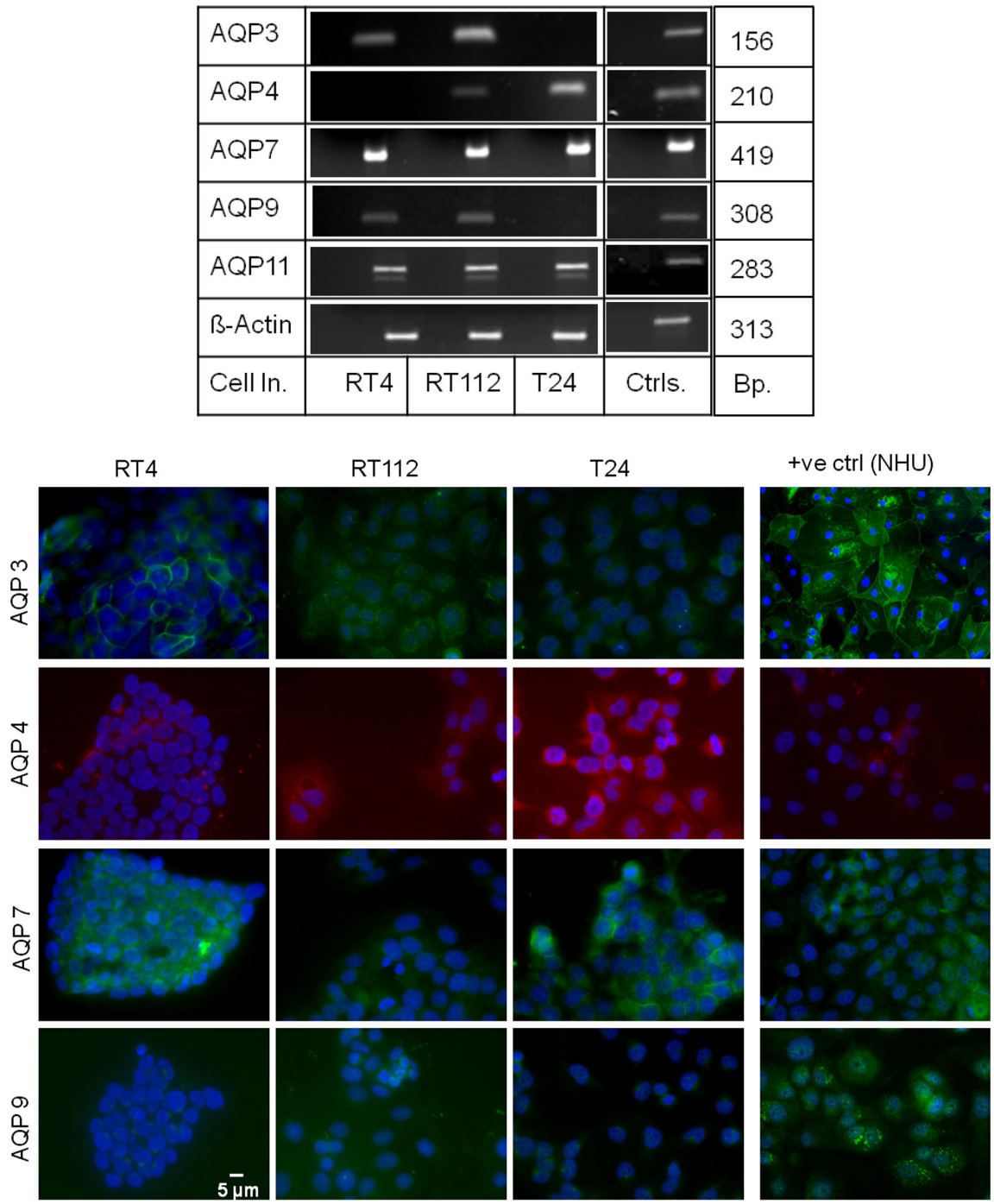
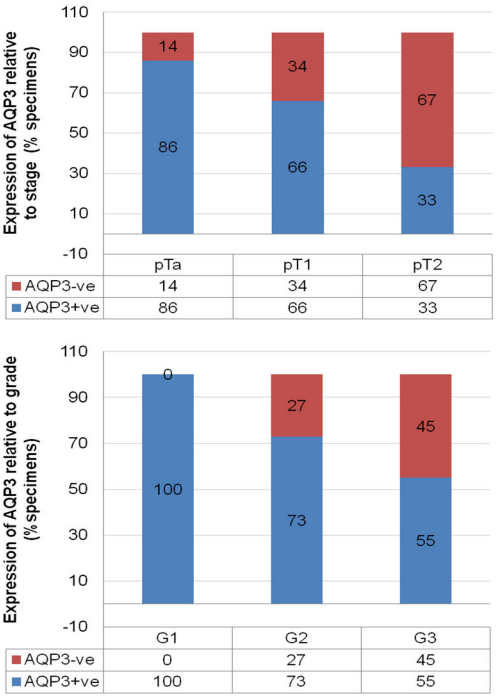


Fig. 1 AQP transcript and protein expression in three established urothelial carcinoma cell lines. *Upper panel* shows RT-PCR results. Transcripts for AQP7 and 11 were expressed by all three cell lines. AQP3 and 9 mRNA was detected in RT4 and RT112, whereas it was absent in T24 cells. AQP4 was expressed by RT112 and T24, but not by RT4. ß-actin, a no-template control, and genomic DNA served as negative and positive controls, respectively. RT(−), reverse-transcriptase-negative samples; RT(+), reverse-transcriptase-positive samples. *Lower panel* shows immunofluorescence labelling. AQP3, 4 and 7 were detected by immunofluorescence microscopy in all three cell lines, in agreement with the PCR results. AQP9 was found to be not expressed. Normal human urothelial (NHU) cell cultures known to express AQP3, 4, 7 and 9 were included as positive controls

characterized and have been shown to recapitulate the differentiation (grade) and invasion (stage) characteristics of the tumours of origin using in vitro tissue reconstruction experiments [13].

The expression found in UC cell lines is in agreement with a previous study on normal human urothelial (NHU) cell cultures, which showed the expression of AQP3, 4, 7 and 9 on the transcript and on the protein level [2].

	AQP 3 protein expression						Σ	
Grade	G1		G2		G3			
Stage	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
pTa	15	0	14	1	8	5	**37**	**6**
pT1	0	0	8	7	12	3	**20**	**10**
pT2	0	0	0	0	7	14	**7**	**14**
Σ	**15**	**0**	**22**	**8**	**27**	**22**	**64**	**30**



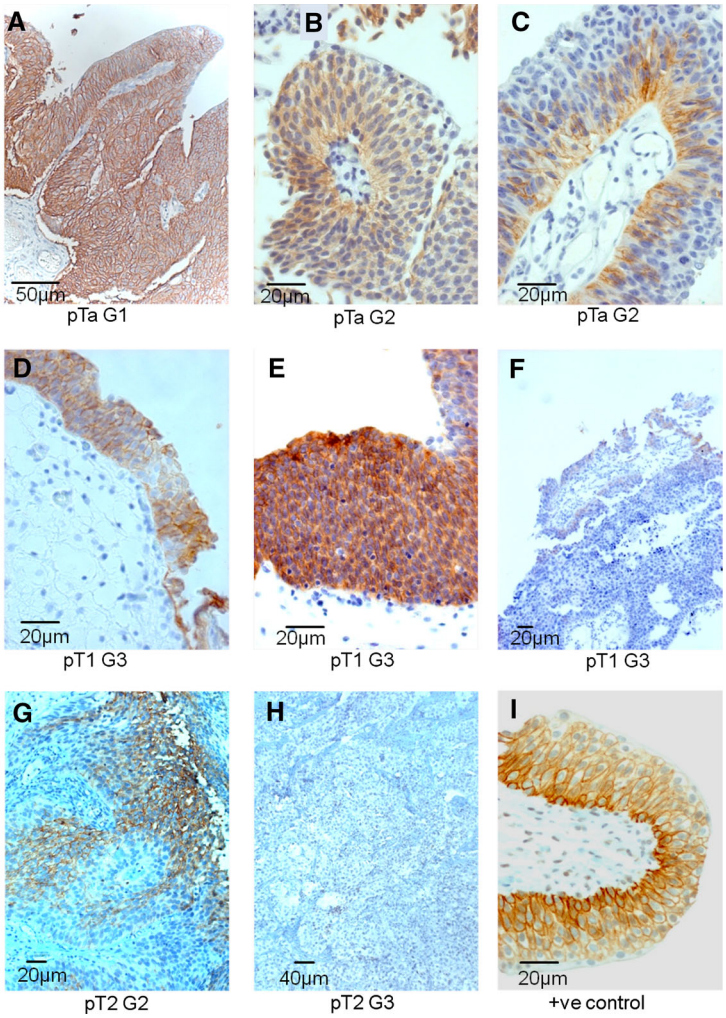


Fig. 2 *Left panel* Expression of AQP3 in 94 bladder cancer specimens relative to stage and grade. Tumour characteristics are shown in the table. Graphs illustrate immunohistochemistry findings. The results suggest that there is a correlation between loss of AQP3 protein expression and increasing tumour stage and grade. *Right panel* AQP3 immunoperoxidase labelling of UC tumour samples of various stages and grades. Intense expression of AQP3 throughout a specimen of a non-muscle-invasive low-grade tumour with distinct labelling of the cell borders (**a**). Less intense expression of AQP3 in the basal and suprabasal cell layers of a pTaG2 tumour specimen (**b**, **c**). Different expression patterns in three independent samples of pT1 high-grade tumours (**d**–**f**). Irregular basal and intermediate expression with expanded AQP3-negative compartments, as shown in **d**. Intense expression of AQP3 throughout the specimen (**e**). No expression of AQP3 by another, independent, sample of a pT1G3 tumour (**f**). Heterogeneous expression of AQP3 with abrupt transition from strongly labelled to unlabelled cells in a muscle-invasive high-grade tumour (**g**). By contrast, no immunoreactivity was found in another, independent tumour biopsy of the same stage and grade (**h**). A section of normal human ureter serves as positive control. Intense expression of AQP3 in basal and intermediate layers of the urothelium with distinct labelling of the cell borders (**i**)

In the present study, transcripts for AQP7 and 11 were consistently detected in all three cancer cell lines, whereas there was differential expression for AQP3, 4 and 9. It is of note that AQP3 and 9 transcripts were shown to be present in well-differentiated RT4 and RT112, but not in poorly differentiated T24 cells. By contrast, AQP4 was found to be not expressed by RT4 cells, whereas it was present in RT112 and T24 cells. Our transcript findings could be supported by immunofluorescence microscopy on the protein level.

Having characterized AQP expression in human UC cell lines, we next investigated AQP protein expression in UC specimens by immunohistochemistry. Our results provide strong presumptive evidence that there is a correlation between loss of AQP3 protein expression and increasing tumour stage and grade.

To our knowledge, our study is the first report of loss of expression of AQP3 in high-grade bladder tumour cells. Previous investigations into the significance of AQPs in non-urological tumours have almost invariably

demonstrated the overexpression of AQP3 and it has been hypothesized that AQP3 may be a promising drug target in the treatment for various epithelial tumours [14, 15]. The contrasting expression pattern of AQP3 between UC and carcinoma types of non-urothelial origin is striking. The finding of intense expression of AQP3 in most well-differentiated UC, but not by two-thirds of invasive high-grade tumours, may be explained by the hypothesis that in human urothelium, expression and function of AQP3 are associated with the phenotype (proliferative or differentiated) of the cells. We have previously found induction of AQP3 protein expression in response to differentiation of NHU cells [2]. Thus, it is conceivable that the loss of differentiation may be paralleled by the loss of AQP3 expression in tumour cells. Therefore, collectively, our previous and current findings reveal a unique pattern of AQP3 expression during normal human urothelial cyto-differentiation and deregulation during urothelial carcinogenesis which is different to the changes observed in most other tumour tissues.

Another aspect to be considered in this regard is that the loss of heterozygosity of chromosome 9, where the AQP3 gene is located, is frequently present in UC [16]. This also suggests that the loss of AQP3 expression may be implicated in a molecular programme associated with the loss of differentiation and progression to muscle-invasive disease.

From a biological point of view, the results of the present study raise several questions: first, whether the loss of AQP3 is a driving change in malignancy or occurs rather as a secondary change; second, whether the loss of AQP3 contributes to the malignant phenotype of UC and, assuming it does, whether it provides a means to differentially target tumour versus normal tissues; third, given that AQP3 is inducible, to what extent its absence in late-stage tumours reflects the tumour microenvironment; fourth, why the transcript results for AQP4 are not in agreement with the protein data from tumour specimens.

With regard to the clinical significance of our findings, we have previously provided strong presumptive evidence that the loss of AQP3 protein expression is associated with worse progression-free survival in patients with pT1G2/G3 carcinomas, and we hypothesize that this could also apply to muscle-invasive tumours [10]. Even if the loss of AQP3 was due to dedifferentiation only, it is noteworthy that in the present series, 55 % of grade 3 carcinomas expressed the marker. Assuming that AQP3 is an independent marker of disease progression, the expression status in the primary transurethral resection specimen could serve as a novel marker that helps urological surgeons select candidates for early cystectomy (AQP3-negative T1G3) versus patients who may benefit from BCG treatment (AQP3-positive immunohistochemistry). Similarly, if the loss of AQP3 in pT2G3 patients turns out in current studies to be associated with lymph node micrometastases, this could help select patients who are candidates for neoadjuvant chemotherapy. However, this is hypothetical since the prognostic value of AQP3 in terms of recurrence, progression and survival of patients diagnosed with UC needs to be confirmed. These issues are the subject of current studies using the clinico-pathological parameters of a large cohort of patients with UC of different grades and stages.

Conclusions from our results may be limited as we performed an observational study on a limited number of selected UC specimens. Regarding our in vitro results, the effects of siRNA-mediated AQP3 silencing on biological endpoints relevant to tumour progression (proliferation, migration, invasion, resistance to apoptotic stimuli, etc.) in one or more cell lines would strengthen the dataset and add some mechanistic insight into how changes in aquaporin expression may regulate tumour biology. Future studies, using our relevant in vitro models, will aim to address these aspects in order to understand the importance of the loss of AQP3 expression in UC and its underlying molecular mechanisms.

Conclusion

This is the first study to demonstrate that several AQPs are expressed in human UC cell lines and tumour biopsies of UC. Our results indicate that there is a significant correlation between AQP3 protein expression and tumour stage and grade, with a progressive loss of AQP3 expression in more malignant tumours. Whether AQPs play any biological role in the carcinogenesis and progression of UC and, in particular, whether this could be of prognostic or therapeutic value have yet to be determined. However, our observation of a unique AQP3 deregulation pattern in UC may be of clinical importance and implications, which merit further future investigations.

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

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