Exploring the Human Protein Atlas (HPA) Portal for New Biomarkers in Urinary Bladder Carcinoma

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Summary

Urinary bladder cancer is the fifth most common cancer form in industrial countries. The primary risk factors for bladder cancer are tobacco smoking, exposure to aromatic amines from occupational sources, cancer drugs and Schistosomal infection. Vegetables, fruit and a high intake of fluid are considered as risk reducing factors for developing bladder cancer. Diagnosis and management of bladder cancer includes cystoscopy, an invasive method causing patients pain and discomfort. Hence, there is great need for non-invasive methods, such as biomarkers predicting tumour recurrence or progression.

The aim of this study was to finding new potential biomarkers for urinary bladder carcinoma by exploration of the Human Protein Atlas (HPA) database portal, an antibody-based protein atlas comprising histological images which are a resource for many areas of biomedical research, including biomarker discovery. Initially, 35 selected proteins were evaluated in 42 tissue scores, representing bladder cancer biopsies from 24 individual cancer patients of which 7 had low-grade and 17 had high-grade tumours. The protein expression in tumour cells was scored and related to high and low tumour grade.

Expression pattern for two proteins, α4, also known as immunoglobulin binding protein 1 (IGBP1) and UPF0500 protein C1orf216, were significantly altered (p < 0.5) with tumour grades. The function of C1orf216 is so far unknown. α4 is a regulator of protein phosphatase 2 (PP2A) and thereby controls dephosphorylation of substrates important in cell survival, apoptosis and cell migration. None of the proteins are characterized in bladder cancer or in any other cancer form. The proteins identified in this histological/pathological study need further investigation to confirm their potential as biomarkers for urinary bladder cancer. Future studies involve more expanded clinically based TMAs linked to patient follow up data, giving the opportunity to study whether protein expression might be related to factors such as sex, age, smoking habits, treatment, tumour progression, relapse frequency and patient survival, in addition to tumour stage and grade. These studies will resolve whether α4 and C1orf216 can be part of a future set of biomarkers helping patients with urinary bladder cancer.
Introduction

Bladder cancer is a potentially lethal disease that causes people, mainly men, suffering all over the world. Except age, the primary risk factors for bladder cancer are tobacco smoking and exposure to aromatic amines from occupational sources. Cigarette smoking increases bladder cancer risk 2- to 4-fold, and 30 to 50 % of all bladder cancers are caused by smoking. The relative risk of developing cancer in the bladder is also believed to depend on genetic variants of important enzymes which metabolize hazardous chemicals of cigarette smoke, like glutathione S-transferases (GSTs) and N-acetyltransferase 2 (NAT2). Other factors that can contribute to the development of bladder cancer include drugs such as the cancer drug cyclophosphamide and the analgesic phenacetin (Kirkali Z *et al.*, 2005) and chronic inflammation due to the infectious parasite *Schistosoma*. In the latter case, the risk is probably related to bacterial and viral infections associated with *Schistosomal* infestation rather than the parasite itself (Shokeir AA *et al.*, 2004).

Factors that have shown to reduce the risk of bladder cancer are cessation of smoking and a diet that is high in vegetables and fruit and low in fat (Hirao Y *et al.*, 2009). It is also shown that high fluid intake is associated with a reduced risk of bladder cancer in men. One theory for this is that less concentrated urine and more frequent urination reduce the exposure of the bladder urothelium to urinary carcinogens (Michaud DS *et al.*, 1999).

In the western world bladder cancer is, following prostate, lung, and colon cancers, the fourth most common malignancy among men (Kirkali Z *et al.*, 2005) and the fifth most common cancer form in the total population. Globally bladder cancer is the seventh most common cancer form, but the prevalence shows great variation among the continents. In North Africa, where infection with the parasite *Schistosoma mansoni* is still endemic, bladder cancer is the second most prevalent cancer type, with 12.1 % of the cancer cases (American Cancer Society 2007).

There are some changes in proliferation and homeostasis of the normal cell that are required to develop tumour growth. These include alterations in responding to growth signals, avoiding of apoptosis, unlimited replication, capability of maintaining angiogenesis, tissue invasion and metastasis (Hanahan D *et al.*, 2000). Bladder cancer is associated with several oncogenic mutations which cause disturbances of important signal pathways. These alterations are slightly diverse for the different kinds of tumours (figure 1). For example, low-grade papillary tumours are associated with activated MAPK (mitogen-activated protein kinase) pathway,
because of FGFR3 (fibroblast growth factor receptor 3), PI3K (phosphatidylinositol 3-kinases) or Ras mutations, which influence gene expression, mitosis, differentiation and cell survival/apoptosis. Invasive tumours, on the other hand, show much lower FGFR3 mutation frequency but rather dysfunction of Rb (retinoblastoma) and p53 (protein 53) pathways involved in tumour suppression (Díaz De Ståhl T et al., 2008). P53 is mutated in up to 50 % of bladder cancers with increased mutation frequency with tumour stage and grade. The mutation is enhanced specifically in high-risk early stage cancers such as carcinoma in situ and poorly differentiated papillary and early stage invasive tumours. P53 mutations are also associated with an increased risk of progression (Schulz WA, 2006). Other molecular alterations in invasive bladder cancer are deletions in chromosome 9 (rare in superficial tumours), amplification of HER2 (human epidermal growth factor receptor 2), over expression of the EGF (epidermal growth factor) receptor and homozygous deletion of the tumour suppressor gene PTEN (phosphatase and tensin homolog) leading to over activated mTOR (mammalian target of rapamycin) and PI3K, and thus, dysregulation of cell growth and proliferation respectively (Knowles MA, 2006).

**Figure 1.** The integration of signal pathways affected by molecular alterations in bladder cancer. Low-grade papillary tumours are associated with activated MAPK pathway, because of FGFR3, PI3K or Ras mutations, leading to increased cell proliferation rate. Muscle infiltrating bladder tumours are associated with dysfunction of Rb and p53 pathways, leading to increased cell survival and cell proliferation. Green text corresponds to changes in low-grade tumours and blue text changes in high-grade tumours.
Diagnosis of bladder cancer initially includes physical examination, cystoscopy, computed tomographic urography, and urine cytology. (Patel AR et al., 2009). Later a transurethral resection of the bladder (TURB) is performed which has both the intention to diagnose, stage and to treat Ta and T1 bladder tumours and removal of visible high-grade tumours TURB is a surgical procedure that involves insertion of a cystoscope into the bladder, removal of cancer tissue for biopsy and burning of any remaining cancer cells of with a resectoscope tool passed through the cystoscope. Investigating the inside of the bladder makes it possible to see if there are any cancer cells present to set a diagnosis (Adiyat KT et al., 2010). With the TURB biopsies a characterization of the pathological stage (pT-stage) of the tumour is performed. This is done according to the Tumour-Node-Metastasis (TNM) classification-system (figure 2), an important prognostic factor of bladder carcinoma. TNM is divided into different states of tumours (Tis, Ta, T1, T2, T3 and T4) in relation to the bladder wall and surrounding tissue and organs (Wester K, 1999).

**Figure 2.** Stages according to the 1997/2002 TNM classification. Picture by Jana Howe of the Uppsala Regional Oncological Centre.

Most of bladder tumours are transitional cell carcinomas (TCCs), characterized by malignant proliferation of the urothelium. TCCs are subdivided into non-invasive papillary and non-papillary invasive carcinoma types. The majority of bladder carcinomas (70-80 %) are classified as Ta, a non-invasive papillary carcinoma. These low-grade tumours are well-differentiated and superficial and do not penetrate the epithelial basement membrane. In the opposite to Ta, T1 tumours penetrate the basement membrane and invade sub-epithelial connective tissue, but not the inner muscle layer. T2a and T2b tumours invade superficial and deep muscle respectively. T3 tumours extend beyond the bladder wall but do not invade
adjacent structures such as prostate, uterus, vagina, pelvic wall or abdominal wall, like T4 tumours do. T2 and T3 are classified as invasive bladder cancer and T4 is called advanced bladder cancer. Carcinoma in situ (CIS), which correspond to Tis in the TNM classification system, are strictly superficial, “flat tumours”, with a poorly differentiated epithelium. CIS is always high-grade and often concurrent with other high-grade tumours (T2-T3) elsewhere in the bladder (Knowles MA, 2006).

Management of non-muscle invasive disease includes TUR followed by adjuvant intravesical chemotherapy or immunotherapy to reduce tumour recurrence or progression. Invasive disease, which is potentially lethal, has to be treated with neoadjuvant chemotherapy to reduce the size of the tumour followed by radical cystectomy, lymph node dissection and adjuvant chemotherapy to decrease the risk of relapse (Patel AR et al., 2009).

The low-grade Ta tumours rarely progress, but the majority of these (in 30–80 % of patients) will recur within a 5-year period (Smith's General Urology). Patients with this tumour type therefore require periodically performed cystoscopy, which is an invasive method causing patients pain and discomfort. Hence, there is great need for non-invasive and less expensive supplement methods, such as highly sensitive and highly specific biological markers predicting tumour recurrence or progression to higher stage and metastasis, and markers to screen among individuals at a higher risk for bladder cancer (e.g. smokers, occupational exposure to aromatic amines, schistosomiasis) (Lokeshwar VB et al., 2005).

An ultimate biomarker is sensitive, specific and is able to diagnose, prognose, and predict therapeutic responses (Diamandis EP, 2004) but it is not likely that a single marker can be prognostic for bladder cancer patients but rather a combination of several independent markers (Bryan RT et al., 2010). The best of the currently available non-invasive test are voided urine cytology (VUC) and assaying of urinary constituents with tests like bladder tumour antigen (BTA), nuclear matrix protein (NMP)-22 and immunocyst. These tests are complex (Bailey MJ et al., 2003) and suffer from low sensitivity and specificity. Thus there is great need for new better urinary bladder markers that can complement and be a less expensive alternative to urine cytology and cystoscopic evaluation to detect recurrence (Diamandis EP, 2004).
**Human Protein Atlas**

The Human Protein Atlas (HPA) (www.proteinatlas.org), funded by the Knut and Alice Wallenberg Foundation, is an antibody-based protein atlas comprising histological images of 48 normal human tissues and 20 different cancer types as well as 47 different human cell lines (Uhlén et al., 2005). The latest version (6) of the atlas, released March 2010, includes nine million images which are based on 11274 antibodies and represent 8489 human protein-coding genes (www.proteinatlas.org).

The images in HPA are stained by immunohistochemistry (IHC) on tissue microarrays (TMAs) using both affinity-purified monospecific antibodies (msAbs) generated by The HPA program, and antibodies from commercial sources (Uhlén et al., 2005). Generation of antibodies involves the production of Protein Epitope Signature Tags (PrESTs) that is used as antigens for production of polyclonal antibodies, including the affinity-purification step, thus generating msAbs. The affinity-purified antibodies are then validated on protein microarrays which provide information about the specificity and purity of the msAbs and Western blot that give important data about protein size and expression patterns (Nilson P et al., 2005). HPA provides expression and localization profiles of proteins representing various protein classes, such as enzymes, protein receptors, kinases, peptidases, transcription factors and candidate genes for cancer or cardiovascular diseases (Uhlén et al., 2005). Thus, HPA is a resource for many areas of biomedical research, including protein science and biomarker discovery (Berglund et al., 2008)

The aim of the present study was to find candidate biomarkers for urinary bladder carcinoma by staining bladder tumour TMAs with selected antibodies from the HPA database portal.

**MATERIALS AND METHODS**

Based on previous observations and screening of antibodies by the Human Protein Atlas (HPA) program, possible key proteins and potential biomarkers in bladder cancer were selected to be further investigated in more expanded tissue micro arrays (TMAs). The antibodies are produced by the HPA program except for two commercial antibodies Glycophorin A from Lab Vision/Neomarkers (Fremont, CA, USA) and TCP10L (A-12) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All antibodies used were rabbit polyclonals except for Glycophorin A which was mouse monoclonal.
A literature study on each of the proteins of interest were done in UniProt (www.uniprot.org) and PubMed (www.ncbi.nlm.nih.gov/pubmed) to see if any of the antibody targets had any known function in normal urinary bladder, bladder cancer or any other cancer form.

In this study two TMA blocks was used, with in total 42 tissue scores, representing bladder cancer biopsies from 24 individual cancer patients which 7 had low-grade and 17 had high-grade tumours. The biopsy samples are from patients at Uppsala university hospital between 1993 and 2002 with both high and low cancer grade, including stages 2A, 2B and 3.4. The study was approved by the regional ethical review board of Uppsala (reference number 2005:339). The TMA blocks were cut at 4 µm sections and baked over night at 50°C.

**Immunohistochemistry**

The staining was done with UltraVision LP detection system (Laboratory Vision, Fremont, USA), a polymer-based immunohistochemical method which is based on a polymer backbone to which multiple antibodies and horseradish peroxidise (HRP) molecules (figure 3). The specific antibody, bound to an antigen in tissue sections, is located by a secondary antibody conjugated to the HRP-labelled polymer that recognizes rabbit immunoglobulins. For mouse antibodies a rabbit anti-mouse link is used. The polymer complex is then visualized with chromogen.

![Figure 3. Two-step Polymer Method (Envision™).](image)
Slides were deparaffinised in xylen, hydrated in ethanol to distilled water (dH$_2$O) and blocked for endogenous peroxidase to reduce nonspecific background staining for 5 minutes in 0.3 % H$_2$O$_2$ in 95 % ethanol in a Autostainer (Leica Microsystems, Wetzlar, Germany). This was followed by 2 times washing in TBS Tween Buffer®, 10x concentrate. Working solution originally contains 0.05 % (v/v) Tween 20® (Laboratory vision).

Heat Induced Epitope Retrieval (HIER) was performed in pre-treatment (PT) module buffer pH6® (Laboratory Vision) with a pressure boiler (Decloaking chamber, Biocare Medical, Walnut Creek, CA). After sections were washed in dH$_2$O until they reached room temperature (RT) and then placed in TBS Tween Buffer®.

The slides were incubated in UltraVision LP Ultra V Block (Laboratory Vision) for 5 minutes at RT to block nonspecific background followed by incubation with primary antibodies (table 1) for 30 minutes and two washing steps in TBS Tween Buffer®.

An incubation step for 30 minutes at RT in UltraVision LP Primary Antibody Enhancer (Laboratory Vision) with anti-mouse Ig specificity was performed for the mouse antibodies.

UltraVision LP HRP Polymer (Laboratory Vision) was then applied and slides were incubated for 30 minutes at RT. Sections were then incubated for 10 minutes in RT in UltraVision LP DAB Plus Chromogen, which contains diaminobenzidine (DAB) in buffer, followed by two times wash in dH$_2$O.

Counterstained in Mayer’s haematoxylin (Histolab, Gothenburg, Sweden), dehydration in graded ethanol and xylene and coverslipped was performed using an Autostainer (Leica).

**Scoring**

The IHC was scored according to the HPA scoring system, which includes the intensity and quantity of tumour cell staining. The intensity was scored as negative (score 0), weak (score 1), medium (score 2) or strong (score 3) and the quantity was scored as < 25 % (score 1), 25-75 % (score 2) or > 75 % (score 3). Final score was calculated as the product of the intensity- and quantity score and then categorized as negative (score 0), medium expression (1-4) and high expression (5-9).
Statistical analyses

To statistically analyse if there was any correlation between final score and tumour grade, the Fisher’s exact probability test (two-sided) was used. Due to small tumour material, antibodies with p < 0.08 were considered interesting for continuing studies.

Interesting antibodies were further investigated with 3 larger scale TMAs. They represented low-grade tumours (Ta, T1), high-grade tumours (2b, T2-4) and primary tumours, metastasis and normal tissue from bladder respectively. In all, the TMAs comprised 75 low-grade tissue scores (33 Ta and 42 T1) and 39 grade tissue scores (30 T2, 6 T3 and 3 T4) and four morphologically normal urothelium samples. High- and low-grade tumours and normal tissue were scored and analysed statistically as described previously.

RESULTS

Five of the 35 selected antibodies showed tendency (p < 0.08) towards different expression pattern in high-grade (T2-T4) and low-grade (Ta and T1) tumours. The targets for the five antibodies included protein α4, also known as, immunoglobulin binding protein 1 (IGBP1) kruppel-like factor 4 (KLF4), laminin subunit alpha-3 (LAMA3), serum paraoxonase (PON3) and UPF0500 protein C1orf216 (table 1).

Table 1. Proteins and their localization, function (UniProt) and expression differences between tumour grades.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Localization</th>
<th>Function</th>
<th>Expression</th>
</tr>
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<tbody>
<tr>
<td>Protein α4</td>
<td>Cytoplasmic/membrane</td>
<td>Regulation of protein phosphatase 2 (PP2A)</td>
<td>↑ High-grade compared to low-grade (p &lt; 0.08)</td>
</tr>
<tr>
<td>C1orf216</td>
<td>Cytoplasmic/membrane</td>
<td>Unknown</td>
<td>↓ High-grade compared to low-grade (p &lt; 0.01)</td>
</tr>
<tr>
<td>KLF4</td>
<td>Nucleus</td>
<td>Transcription factor Epithelial cell differentiation</td>
<td>↑ High-grade compared to low-grade (p &lt; 0.05)</td>
</tr>
<tr>
<td>LAMA3</td>
<td>Cytoplasmic</td>
<td>Cell adhesion, Signal transduction, Differentiation of keratinocytes</td>
<td>↑ High-grade compared to low-grade (p &lt; 0.01)</td>
</tr>
<tr>
<td>PON3</td>
<td>Membrane</td>
<td>Hydrolyzes aromatic lactones such as statin prodrugs (by similarity)</td>
<td>↓ High-grade compared to low-grade (p &lt; 0.01)</td>
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C1orf216=UPF0500 protein C1orf216, KLF4=Kruppel-like factor 4, LAMA3=Laminin subunit alpha-3, PON3=Serum paraoxonase
Staining of \(\alpha 4\) in bladder cancer displayed a tendency (\(p < 0.08\)) to be more negative (scorecat = 0) in low-grade than high-grade tumours. The positive tumours showed cytoplasmic and membranous staining. The nuclear KLF4 expression was significantly increased in high-grade tumours compared to low-grade (\(p < 0.05\)). The cytoplasmic LAMA3 expression, in tumour cells, was significantly more common (\(p < 0.01\), scorecat = 1-3) among high-grade than low-grade tumours. In addition, there was a tendency that LAMA3 was up-regulated in high-grade compared low-grade tumours (\(p < 0.08\), scorecat = 0). PON3 was mainly membranous expressed and showed a significantly higher (\(p < 0.01\)) expression among low-grade tumour cells.

For C1orf216, there was a clear difference in the frequency of negative tumours comparing different malignancy grades, with more negative scores among high-grade than low-grade tumours (\(p < 0.01\)). Low-grade tumours displayed a significantly (\(p < 0.01\)), higher expression than high-grade tumours. The staining was moderate with a cytoplasmic and membranous distribution in 42 \%, whereas only four (24 \%) the high-grade tumours were positive. C1orf216 had different staining patterns in basement membranes of normal compared to cancerous tissue. This is obvious in the staining presented on the human protein atlas (HPA) website (www.proteinatlas.com), where basement membranes show more intense staining in normal urothelium compared to corresponding tumours. The tissue microarray (TMA) with 42 tissue scores did not contain any normal tissues, but none of the tumours showed any clearly stained basement membranes. On the other hand, there was very intense staining in stroma and vascular tissue.

Of these five antibodies, four were further stained in three extended scale TMAs. Out of these, two antibodies, LAMA3 and PON 3, showed too weak staining and were not pursued any further, while the expression of \(\alpha 4\) and C1orf216 were significantly correlated with tumour grade (table 2).

Table 2. Protein expression patterns altered with grade.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Expression differences grade</th>
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<tbody>
<tr>
<td>(\alpha 4)</td>
<td>(\downarrow) High-grade compared to low-grade ((p &lt; 0.01))</td>
</tr>
<tr>
<td>C1orf216</td>
<td>(\downarrow) High-grade compared to low-grade ((p &lt; 0.05))</td>
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In the extended TMA, α4 showed significantly higher (p < 0.01) expression in low-grade tumours than high-grade tumours (figure 4, 6A). Of the 75 low-grade tumours, 58 % scored high (4-9) and only 8 % of the 39 high-grade tumours samples. Consequently, high-grade tumours were significantly more often negative and moderately expressed than low-grade tumours (p < 0.05, p < 0.01 respectively), which means that the protein α4 expression was decreased in more advanced tumours. There was also significantly different expression in T1 than in Ta tumours (p < 0.05) (figure 5).

**Figure 4.** Score distribution difference in α4 staining. There was significantly higher (p < 0.01) α4 expression in low-grade tumours than high-grade tumours.

**Figure 5.** Score distribution difference in α4 staining. There was significantly higher (p < 0.05) α4 expression in T1 tumours compared to Ta tumours.
Of the five normal tissue samples, four showed distinct moderate to high expression in basement membranes staining for C1orf216 (figure 6 B). In addition to the expression in basement membranes and some stromal staining, the expression in normal urothelial cells were negative or < 25% moderately stained. Neither low-grade tumours nor high-grade tumours showed any significant clear basement membrane expression, besides one low-grade tumour which was very weak. Staining for C1orf216 was significantly more often negative in high-grade than low-grade tumour cells (p < 0.05). There was also a tendency towards higher expression in low-grade compared to high-grade tumour tissues (p < 0.08).

A. Protein α4

B. C1orf216

Figure 6. Expression of α4 and C1orf216 in bladder cancer presented by IHC (x20): A. Protein α4 staining revealed significantly higher (p < 0.01) expression in low-grade tumours (Ta-T1) than in high-grade tumours (T2-T4). The α4 expression in T1 tumours were significantly higher (p < 0.05) than in Ta tumours. B. C1orf216 displayed moderate to strong expression in basement membranes in normal urothelium while basement membranes in low-grade Ta tumours were negative. There was also a tendency towards higher C1orf216 expression in low-grade compared to high-grade tumour tissues (p < 0.08).
DISCUSSION

Of the 35 proteins initially investigated in this search for candidate biomarkers in urinary bladder cancer, two proteins gave interesting results. These two were α4 (also known as immunoglobulin binding protein 1 (IGBP1)) and UPF0500 protein C1orf216. Protein α4 did not only display higher expression (scorecat = 5-9) in low-grade (Ta and T1) tumours but also showed higher expression in T1 than Ta tumours. According to the Human Protein Atlas (HPA) the α4 expression is > 75% moderate (final score = 6) in normal urothelium. This corresponds to the fact that α4 expression is higher in low-grade tumours and supports α4 down regulation with more malignant tumours (high-grade), except for the higher α4 expression in T1 than in Ta tumours.

Almost all of the C1orf216 protein stainings were negative (scorecat = 0), with more negative staining in high-grade tumours. There was also a tendency towards more moderate staining in low-grade compared to high-grade tumour tissues. The C1orf216 expression in tumour cells were similar to the expression in normal bladder tissue, except for urothelial basement membranes which were strong or moderate expressed in normal tissue. This specific expression pattern was unfortunately not seen in low-grade Ta tumours with intact basement membranes. The basement membranes function as a barrier that tumour cells have to penetrate to disseminate and metastasize (Patarroyo M et al., 2002). Expression of C1orf216 in Ta tumour basement membrane could implicate that C1orf216 has a function in retaining basement membranes intact. Disappearance of expression in these low-grade non-invasive papillary carcinoma means that the tumour cell growth in some way has affected the basement membrane homeostasis. Maybe it could be one of several losses needed to finally break the barrier for invasion of underlying connective tissue.

There is no information about C1orf216 function in the literature and there is just evidence of C1orf216 at transcription level (UniProt). Protein α4, on the other hand, is described at protein level and is more studied than C1orf216. Neither C1orf216 nor α4 is characterized in bladder cancer or any other malignancy.

Protein α4 is a key component in the ability of cells to maintain/modulate the activities of the protein phosphatase 2A (PP2A) family of phosphatases. PP2A is a trimeric protein composed by a scaffolding (A) subunit, a regulatory (B) subunit, and a catalytic (C) subunit. The B subunit determines substrate specificity and subcellular localization of PP2A, and the C subunit binds to α4. Binding of α4 to the C subunit stabilize it in an catalytically inactive
conformation until it can be partnered with relevant A and B subunits to build active PP2A phosphatase complexes. In this way α4 maintains proper PP2A function and assembly. Protein α4 promotes cell survival and recovery from DNA damage under times of stress by providing functional PP2A that can dephosphorylate substrates required to resolve DNA damage and promote cell survival. Without α4, C subunits rapidly decline and PP2A can no longer perform substrate dephosphorylation and therefore not modulate phosphorylation of stress response proteins, which eventually leads to apoptosis. (Kong M et al., 2009). By regulating PP2A, α4 is also involved in regulation of cell spreading, migration and cytoskeletal architecture by influencing the cellular GTPase Rac (Kong M et al., 2007). Rac is a subfamily of the Rho family of GTPases widely known to play an important role in cancer development (Ellenbroek SI et al., 2007).

Other studies have revealed that the loss of specific PP2A enzymes is a critical determinant towards cellular transformation and cancer. This can result in upregulation of protein kinases involved in mitogenic and survival signalling (e.g. AKT and MAPK), stabilization of protoncogenes (e.g. MYC), destabilization tumour suppressors (e.g. p53 and Rb), or the elimination of proapoptotic signalling pathways (e.g. BAD) (Eichhorn PJ et al., 2009).

It is possible that α4, through the regulation of PP2A, has a role in cellular transformation and origin of bladder tumour cells by promoting cell survival, inhibit apoptosis and influence cell spreading and migration. The normal bladder urothelium samples are collected from patients with current tumour growth in the bladder. Even though these cells are considered normal, they are still from patients suffering of disease. This means that they can possess ongoing processes as a reaction to disturbed cell environment, like inflammation, DNA-damage or stress. Considering that α4 has a function during cellular stress, this could explain the moderate expression in normal urothelium. Why the expression is down regulated in more advanced tumours is harder to explain.

In the present study, proteins were screened for being potential biomarkers in urinary bladder cancer. In the initial screening, 35 proteins were stained by immunohistochemistry (IHC) in a rather small 42 tissue score tissue micro array (TMA). In subsequent screening, two interesting proteins were further investigated in an expanded 75 tissue score TMAs. The expanded TMA revealed clearly different result for protein α4 expression compared to initial screening, which indicate poor reproducibility. In the 42 tissue score TMA, α4 expression was significantly downregulated in lower grade tumours. In the 75 tissue score TMA there
was significantly downregulation of $\alpha_4$ in high-grade tumours compared to low-grade tumours. The explanation for this could possibly partially be explained by the differences in sample size and indicates that the TMA with fewer samples may not be suited for this kind of screening. If there was no limitation in time and tumour samples, the more expanded TMA should have been more optimal for selecting interesting protein in the beginning.

There are many confounders that may be responsible for a poor reproducibility in IHC staining. This could be variation in tissue preparation (e.g. the time and temperature for fixation, histoprocessing and section storage), laboratory analysis (e.g. retrieval and IHC-protocol) and quantitation of the biopsy specimen (e.g. subjective scoring). New biomarkers therefore need standardisation of laboratory techniques and subjective judgement of the results (Wester K, 1999).

In this histological/pathological study we found that protein $\alpha_4$ and UPF0500 protein C1orf216 may be interesting as biomarkers in urinary bladder carcinoma. Further investigation is needed to confirm their potential. Future studies involve more expanded clinically based TMAs linked to patient follow up data, giving the opportunity to study whether protein expression might be related to factors such as sex, age, smoking habits, treatment, tumour progression, relapse frequency and patient survival, in addition to tumour stage and grade. These studies will resolve whether $\alpha_4$ and C1orf216 can be part of a future set of biomarkers helping patients with urinary bladder cancer.

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