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Optimizing search algorithms in biomarker exploration for urinary bladder carcinoma using the Human Protein Atlas as platform

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Summary

The Human Protein Atlas is a database (www.protein.org) providing protein expression images in varied human tissues as well as cancers. Expression of specific proteins can be viewed in a histological manner which is achieved by combining immunohistochemistry and tissue micro-array. Antibodies are used to analyse tissue samples and thereby showing the protein expression level and position in different human tissues. Recently an advanced search tool was added to the database, which provides the possibility to search for proteins with specific expression patterns in normal and/or cancer tissues. Here, I used the advanced search to find potential biomarkers for characterizing bladder cancer. Additionally, lists of proteins previously linked to bladder cancer were investigated.

A list of 60 specific antibodies derived from different search results was investigated closer. Only 21 antibodies were used to analyse a wide-range tissue microarray (TMA) with samples from bladder cancer patients with different tumor stages of bladder cancer. The different stages are called Ta and T1-T4, where Ta is benign and T4 is the most malign. The wide-range tissue microarray included 37 Ta, 20 T1, 36 T2, 6 T3, 3 T4 and 19 matched primary and metastasis tumors as well as 7 normal tissue samples. Immunohistochemistry was performed to test the protein expression pattern for tumor stage correlation. Out of 21 antibodies, the binding of 15 to the tissue array was more or less correlated to tumor stage. An antibody binding to aldo-keto reductase family 1 member C3 (AKR1C3) and one antibody binding to Cytokeratin 18 (KRT18) indicated a significant different expression pattern of the proteins in Ta tumors compared to other stages. The expression was down-regulated in high-stage tumors. A specific antibody binding to Stathmin (STMN1) and an antibody binding to membrane-spanning 4-domains subfamily a member 10 (MS4A10) demonstrated gradual up-regulation of the proteins expression in high-stage tumors. Additional interesting results were shown by an antibody against Stefin B (CSTB) and an antibody binding to polymeric immunoglobulin receptor (PIGR), both displayed down-regulation of these proteins in high-stage tumors. Stefin B was highly expressed in normal tissue as well as Ta and had lower expression in T1 and T2-4. PIGR was down-regulated in all tumor stages.

The human protein atlas proved to be a useful tool in identifying key proteins or candidate biomarkers characterizing bladder cancer. The 12 patients representing bladder cancer was prepresented in the database turned out to be enough to find proteins with heterogeneous expression pattern in bladder cancer.

Abbreviations

HPA Human Protein Atlas
HPR Human Protein Resource
IHC immunohistiochemitry
SCC squamous cell carcinoma
TMA tissue microarray

Introduction

Bladder cancer is the seventh most common cancer among males globally. It is the fifth most common cancer cause for men in developed countries, and the eighth most common in the third world [American Cancer Society 2007]. The high incidence in developed countries is probably due to smoking. The highest incidence of bladder cancer cases is found in Egypt, and it is the most common cancer causing death in northern Africa for both sexes. In no other area in the world is bladder cancer among the top five death-causing cancers [Parkin *et al.* 2005]. The bladder cancer in northern Africa is mostly squamous cell carcinoma (SCC) and related to the infectious nematode *Schistosoma hematobium*. It is verified that a history of urinary schistosomiasis is associated with increased risk of bladder cancer. *Schistosoma* accounts for approximately 16% of bladder cancer cases in Egypt [Badwani *et al* 1998].

The problem with bladder cancer is the difficulties in predicting the outcome. Superficial tumors can progress into invasive and malignant tumors, but most superficial tumors are benign. Knowles (2006) discusses a question that still is unresolved whether there is a progression of the superficial tumor to an invasive tumor or if the two are two separate tumors with different genotype. Either way, it is important to distinguish between the two groups, e.g. by reliable biomarkers. The two superficial stages are usually called Ta and T1 and the malignant muscle invasive tumor stages are called T2, T3 and T4, representing different depths of invasion (shown in figure 1). Superficial tumors are a heterogeneous group; some can be completely benign papillary tumors that rarely progress. They can also be papillary or flat lesions that progress to a greater degree, e.g. by invasion of the lamina propria (loose connective tissue beneath epithelium) and further [Cookson *et al.* 1997, Cheng *el al.* 1999]. The most common bladder cancers (70-80%) are well-differentiated low-grade papillary tumors (Ta). Ta and T1 are both benign tumors. T1 penetrates the basement membrane into lamina propria, T2 invades the superficial muscle and/or the deep muscle of the bladder, while T3 extends beyond the bladder wall. T4, the last stage, invades nearby organs such as the prostate [Montironi and Lopez-Beltran 2005] (see figure 1).

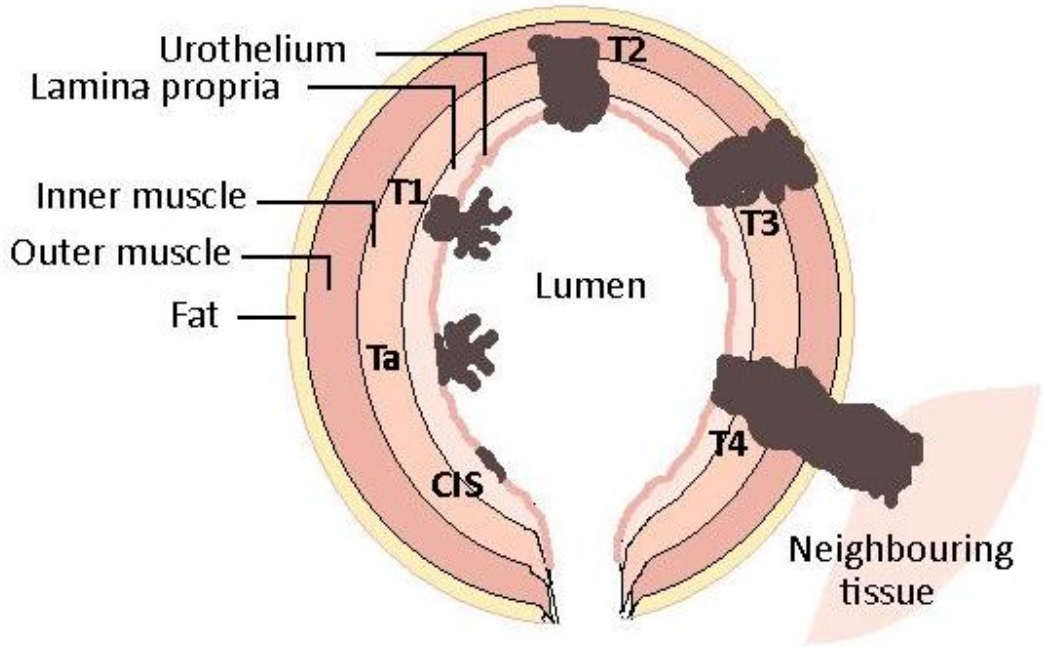

Figure 1. The different stages of bladder cancer shown in a schematic figure of the bladder. The different layers from the outside and in are: fat, outer muscle, inner muscle, lamina propria (loose connective tissue) and urothelium (the epithelial layer in urinary bladder). In the centre of the bladder is lumen, the cavity of the bladder. CIS are carcinoma in situ, the early form of carcinoma. Ta and T1 are benign tumor stages, T1 invades lamina propria. T2 invades muscle layers and T3 grows beyond the bladder wall. T4 invades nearby organs.

Human Protein Atlas - Organization

The Swedish Human Protein Atlas (HPA) program is funded by the Knut and Alice Wallenberg Foundation. It is a cooperation between the Royal Institute of Technology (KTH, Stockhom) and the Rudbeck Laboratory (Uppsala University, Uppsala). The Stockholm site handles production and quality assurance of antibodies. The Uppsala site is responsible for large-scale protein profiling in tissues and cells, generation of digital images and annotation of each image [Uhlén *et al.* 2005b].

Human Protein Atlas – An image database

HPA is a histological image database available on the internet (www.proteinatlas.org). HPA provides digital images of protein expression shown by immunohistochemistry, which show where and how the target protein is expressed in tissue samples. Normal tissues (48 different types) and several different cancer forms (20 different) are used to profile the expression of each protein. This is possible by combining large-scale production of antibodies and construction of tissue microarrays (TMA). Most antibodies used in the atlas are generated by the human protein resource group (HPR) in Stockholm, where protein epitope signature tags (PrESTs) are used as both antigen and affinity ligand in the antibody purification. Commercially available antibodies are also included in the atlas [Uhlén *et al.* 2005a].

TMA is a technique that makes it possible to analyze hundreds of separate tissue samples simultaneously. Paraffin-embedded tissues are used. A hollow needle moves a specific part of the tumor and puts it into an empty paraffin block, resulting in an array pattern of several samples.

The new paraffin block can then be sectioned and used in histological analyzes. All samples are surgical material. “Normal tissue” is defined as non-neoplastic and morphologically normal. This is necessary since it cannot be guaranteed that the tissue is perfectly normal, since most normal tissue collected was localized near tumor tissue.

After analyzing the tissue array with antibodies, each image is scored and annotated to provide an overview of the protein expression pattern. Every tissue sample included in the array is evaluated manually by specialized personnel. Intensity, fraction stained and sub-cellular position are noted. Terms like negative, weak, moderate and strong are used in the scoring of the protein expression. These images are then added to the online database together with the scores as an overview of the protein expression in several different tissues at the same time, a protein atlas of the human body.

Western blot analyses are also included in the atlas. The Western blots are performed using a standardized protocol and are conducted in a single experiments. Human plasma, two different lysates from cancer cell lines and two different lysates from human tissues are used for analyzing commercial antibodies that passed the immunohistochemistry and all HPR antibodies. Western results are scored into eight different categories representing for instance: supportive, non-supportive or uncertain. One single band at the expected position in relation to the size ladder is a supportive result, a strong band at the wrong position is non-supportive and uncertain can e.g. refer to a western without any bands.

The potential in using the atlas to investigate cancer has been evaluated previously [Kampf *et al.* 2004, Björling *et al.* 2007]. The result indicated that tissue profiling with antibodies could be a valuable tool in clinical proteomics.

In October 2007, a new feature was added to the atlas; an advanced search tool, making it possible to search in the atlas for proteins with specific expression patterns in normal and/or cancer tissues. In the advanced search it is possible to choose unlimited number of criteria for the protein expression pattern, both proportion and score for each tissue can be defined. Search strategies have previously been investigated previously [Björling *et al.* 2007, Sjöstedt, E. 2007 unpublished].

A previous Human Protein Atlas study

In my previous study [Sjöstedt, E. 2007 unpublished] I investigated the potential of using the advanced search in the atlas to search for potential biomarkers for bladder cancer. A good biomarker would be a protein that can reveal the tumor malignancy or predict the outcome (be prognostic) for the patient. The question was whether it was possible to find potential biomarkers by searching for specific expression patterns in the atlas. The investigation was performed by evaluation of hit lists from the advanced search. Basic search criteria were used to find up-regulated, down- regulated and heterogeneously expressed proteins. Expression in normal tissue was included in the criteria, which later turned out to be problematic. The antibodies in the search hit list (106 antibodies of 5438 present in the database) were first examined one by one. Immunohistochemical images were visually evaluated to establish the quality and specificity of the antibody to make sure that its protein binding was specific and the location of the protein was connected to cancer cells. A literature search (PubMed database by NCBI) was performed for each protein on the hit list with the most specific antibodies (corresponding to 49 found

antibodies). The proteins were divided into three categories; well-characterized, unknown (never associated with cancer) and semi-well characterized (associated with bladder cancer but not verified). Proteins characterized in cancer, but not mentioned in connection with bladder cancer, were removed from the list. A few proteins were investigated further. This showed that the advanced search tool potentially can find possible biomarkers for bladder cancer but further development is needed.

Immunohistochemistry

Immunohistochemistry is a technique used to localize an antigen (protein) in a tissue sample using antibodies. It is a widely used strategy for diagnosis of e.g. cancer, since molecular changes can be found by using this technique. An antibody is added to a tissue specimen and binds to its antigen. A secondary antibody is then added to bind the primary antibody. Different detection systems can be used, e.g. fluorescence. In a commonly used method, horseradish peroxidase (HRP) is conjugated to the secondary antibody (figure 2). When a chromomeric substrate (e.g. 3,3'-diaminobenzidine, DAB) is added, HRP converts it into intensely colored molecules [Veitch *et al.* 2004]. As result of the HRP-reaction the position of the antigen in a tissue sample is shown as a brown-black spot. In addition, samples are analyzed with hematoxylin, showing basophilic structures such as nucleus in a cell in blue color.

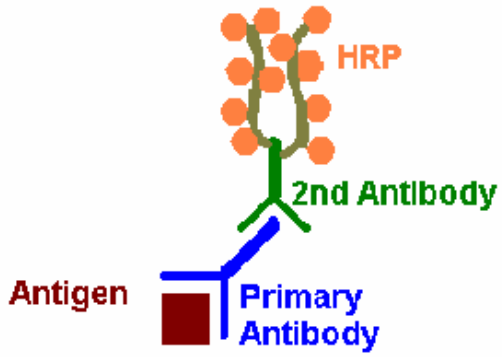

Figure 2. Immunohistochemistry (IHC). The primary antibody binds to the antigen and the secondary antibody binds to the primary antibody. Conjugated to the secondary antibody is horseradish peroxidase (HRP), necessary for the visualization. HRP converts a chromomeric substrate into an intensely colored molecule which give rise to the brown/black color that indicates the position of the antigen.

Aims

In this study, the HPA was evaluated for identification of biomarkers for urinary bladder cancer, to improve the search criteria. A group of 27 antibodies from my recent study [Sjöstedt, E. 2007 unpublished] that were new to bladder cancer association was included in this study for further investigation.

The questions were:

- Could an advanced search include well-established and already found markers?
- Could an optimization improve the result of an advanced search?
- Were the 12 patients in the atlas enough to find potential markers for bladder cancer?

The final aim was to find the most promising protein in the atlas and verify its differential expression among tumors in a wide-range bladder TMA. This was a rough test procedure performed as a preliminary test to find potential antibodies that may be tested further. A phase 2 TMA is under construction, where correlations to other clinical parameters can be investigated.

Results

Search for biomarkers for bladder cancer

First I searched for proteins that had been associated to bladder cancer previously for any reason. This search was not specialized with respect to any particular function or relation to the cancer. Well-established proteins linked to bladder cancer were found by literature search in PubMed (NCBI database) and among commercially available antibodies for research on and diagnosis of bladder cancer. Antibodies found previously in the HPA with unique patterns that might be of interest in urinary bladder cancer are referred to here as "visually found antibodies". Additionally, gene expression analysis studies [Dyrskøt *et al* 2004 and 2005] have suggested a number of genes associated to bladder cancer. A list of antibodies corresponding to the proteins of these genes was produced.

The next step was to match the list of interesting proteins to the protein atlas and find antibodies that were linked to these proteins. This was not needed for the visually found since they were based on antibodies available in the atlas. The list of antibodies based on established proteins whose expression was related to urinary bladder cancer corresponded to 15 genes (proteins) from review articles (PubMed) and 28 from reviews of bladder cancer markers from the commercial antibody producer Geneway Biotech. Genes associated with urinary bladder cancer suggested from gene-expression array studies had very low representation in the atlas. Only 19 from a list of 115 genes were available in the database. Visually found antibodies resulted in a list of 81 antibodies.

All three groups of antibodies were examined. Expression patterns of the target proteins and the number of low and high grade tumors were noted for the 12 patients in the atlas. Most tumors were high grade. The number of patients with low grade tumors varied from 0 to 5, but most commonly was 3 patients out of 12 in the atlas. Since the visually found antibodies were based on the atlas, they were all represented. The list of antibodies to proteins from established genes was based on gene names which were matched to an antibody in the atlas. Some of the antibodies did not bind to any of the 12 tumor samples. Of the antibodies to the 42 established gene, 8 failed to bind. Among the 19 genes identified in the gene expression study [Dyrskøt *et al.* 2005], no antibody binding was found to the bladder cancer tissue for 3.

Searches in Uniprot and PubMed were performed for each protein to categorize the proteins functionally. However, among the functional groups, no group had a predominant position. Thus, no obvious trends were found.

Search-criteria optimization had no actual effect

By studying the lists of antibodies mentioned above, search criteria were improved to increase the quality of the search result (table 1). Each criterion was based on the specific expression pattern of the proteins, i.e. 3=strong means that at least 3 out of the 12 patients in the atlas should show a strong expression of the protein. The quality of the search result was analyzed by matching the hit list with the lists of established proteins, "visually found antibodies" as well as the 19 genes from the gene expression study. The more matches to the different lists the better quality of the search. After the optimization where different criteria was tried out, the search result increased with another four antibodies matching the "visually found" (from 5 to 9 antibodies out of 81). The

improvement of the search was defined by comparing to a previous study [Sjöstedt, E. 2007 unpublished] that used very basic search criteria.

Table 1, The list of optimized criteria.

	Criteria[1]		Number hits[2]	Number matches[3]
1	3= strong	8 <moderate	12	4
2	8=strong	3<moderate	7	2
3	2=strong	10=negative	4	2
4	5=strong	5<moderate	21	2
5	12≠strong	4=moderate		
	2=weak	4=negative	55	2
Total			99	12

[1]The criteria were the definition of protein expression in a total number of 12 cancer patient shown in the atlas. Criterion number 1 was proteins being strongly expressed in at least 3 out of 12 patients, and lower expression level than moderate in 8 out of 12 patients (this means no or weak expression).
[2]The number of hits was the number of proteins found in the specific search.
[3]Results from each search that matched the lists of visually found antibodies as well as established proteins and proteins from the gene-expression research.

25 antibodies were selected for further evaluation

Comparison of the result of Sjöstedt (2007 unpublished) with the new optimized search, resulted in 20 antibodies in common. Six of these antibodies had already been analyzed previously in the wide-range TMA. Nine antibodies from the visually found list matched the new search result. However, one of these antibodies was already represented in the comparison between former and new searches mentioned above. In addition, 27 antibodies remaining from the former study were examined and 5 of these were already represented in the matching above. The total number of antibodies was 43. A literature search was performed for all proteins against which these 43 antibodies were directed, to determine to what extent these proteins had been characterized in bladder and other cancer. Proteins well-characterized in bladder cancer were discarded. Secondly, Western blot results for all the 43 antibodies were examined. Western blots with supportive score in the atlas were prioritized. After the Western blot evaluation, 18 proteins remained for further investigation. Five from the search comparison (former search and new search), 10 from the previous study (which were not investigated further) and three antibodies matching the visually found list and the new search. In addition, 17 antibodies from the new optimized search result were also investigated in line with above which resulted in seven additional proteins to analyse. Altogether, 25 proteins and their antibodies were investigated further in this study.

Three antibodies failed the pre-test

Each antibody was used in immunohistochemical analysis of bladder cancer with dilutions according to the atlas to test its quality and to verify the dilution. The result was then compared to images in the database to verify the expression pattern of the protein and dilution. This was performed with all 25 antibodies. Seven of them did not match and were retested in new dilutions. For most of the antibodies the pattern was equal to the differential pattern showed in the atlas. However, three antibodies did not match the atlas well enough to continue with in this

study (PTPN1, HPRK670013 (LAMB1) and HPRK140369 (COX8C)). Additionally, a commercial CCR6 was removed, since the HPR antibody for the same protein was included as well. After the pre-test procedure the number of antibodies was 21.

Expression of 15 of 21 proteins was correlated to stage in the urinary bladder TMA

I used an extensive specific wide-range bladder cancer TMA to verify the protein expression in the few-sample urinary bladder TMA presented in the atlas. This TMA is used to investigate potential correlations to tumor stage. The urinary bladder TMA was analyzed with the 21 antibodies finally selected. The staining results were scored and analyzed. In figures 3-8 results with six different antibodies are shown that illustrate the most interesting results. The remaining antibodies with correlations showed less significant differences. Antibodies without correlation to stage (6 out of 15) bound randomly to samples from all cancer stages or bound similarly to samples from all stages. The results are summarized in table 2.

Table 2, Binding of antibodies to urinary bladder cancer TMA.

Gene name	Expression in normal tissue	Change of expression compared to increased tumor stage.	Comparison between stages.	Significance
AKR1C3	Strong	↓	Ta differ	Ta> T1-T4, p<0.001
ARHGEF1	Weak	↑	T2-4 differ	Ta-T1 <T2-4, p=0.007
ASAH1	Weak	↓	Ta differ	Ta>T1-4, p<0.001
ASB8	Mod. or strong	Ta differ	N	
CCR6	Weak or mod.	N	N	
CHD8	Mod.	T1 differ	N	
CLIC3	Weak	Small ↓	N	
CSTB	Neg or mod.	↓	Ta differ	Ta>T1, p=0.04; Ta>T2-4, p=0.005
DLG4	Weak	N	N	
GSTK1	Weak	N	N	
IDH2	Weak	↑	Ta differ	Ta< T1-4, p=0.003
KRT18	Weak or mod.	↓	Ta differ	Ta>T1, p=0.016; Ta>T2-T4, p<0.001
LMTK2	Weak	N	N	
MAOA	Weak	↓	Ta differ	Ta> T1-4, p<0.001
MLRM	Weak	↓	G	Ta>T1>T2-4, p<0.013
MS4A10	Most neg.	↑	G	Ta<T1, p=0.016
MTMR10	Weak	Small ↓	N	
PIGR	Mixed pos.	↓	Between Ta and T2-4	Ta> T2-4, p<0.001
Rad17	Weak	N	N	
RBP1	Weak	N	N	
STMN1	Neg.	↑	G	Ta<T1<T2-T4, p<0.001

Each protein is represented by its gene name. Expression in normal tissue is noted as well as the change of expression in correlation to the stages Ta, T1 and T2-4. The significant differences between stages are listed in addition to the calculated p-values. “G” means gradual change that means a significant difference between all stages; between Ta and T1 as well as between T1 and T2-4. “N” means nothing that means non-significant correlation of the expression pattern. Up- and down regulation is marked as arrows, indicating the relation to tumor stages from low- to high grade.

One type of correlation found was that the expression in Ta differed substantially from the other stages. Proteins with this behavior were AKR1C3 and KRT18 (figures 3 and 4). The expression of AKR1C3 in normal tissue was strong and there was a significant down-regulation in T1 or more advanced stages, compared to Ta. The KRT18 antibody showed similar pattern, indicating impaired expression in tumors at a low-differentiated stage. The expression in normal tissue of KRT18 was weak or moderate. The significant differences appeared when comparing negative and positive categories between tumor stages.

One other form of correlation was a gradual change of expression pattern going from lower to higher tumor stages, as shown by STMN1 expression. There were significant differences between all stages. STMN1 showed a similar expression in T2-4, primary tumors and matched metastases (see figure 5). MS4A10 showed a similar expression pattern except when comparing Ta to T1 ($p= 0.016$). These proteins were not expressed in normal tissue samples and were up-regulated in advanced tumor stages.

As shown in figure 7, the expression of CSTB seemed to be decreased in higher tumor stages. Negative and weak expression indicated a pattern similar to KRT18, where Ta was significantly different from more advanced stages. A gradual change of expression was found when the strong expression of the protein was compared to the different stages, Ta compares to T1 ($p=0.001$), T1 to T2-4 (0.042) as well as Ta and T2-4 ($p<0.001$).

Finally, one more interesting expression distribution is presented in figure 8. The protein is PIGR and was expressed in all normal tissue samples, whereas the expression was reduced in almost all tumor cells. All samples of normal tissue (7 out of 7) analyzed with PIGR antibody were positive. The figure indicates that PIGR was down regulated in almost all tumors. Small percentages in all stages were positive, which seems to be down regulated in high stage tumors. Significance between the stages were investigated, by comparing Ta to T1, T1 to T2-4 and Ta to T2-4. There was only a significant difference between Ta and T2-4.

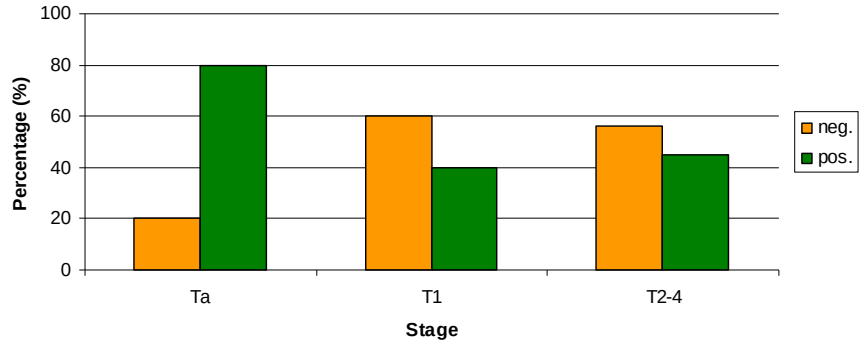

Figure 3, AKR1C3. Immunohistochemical analysis of aldo-keto reductase family 1 member C3 (AKR1C3) expression in bladder specific TMA. The percent of negative (a tumor without expression of the protein) and positive (expression shown by antibody binding) samples were calculated. The number of patient analyzed for each stage are shown in table 4.

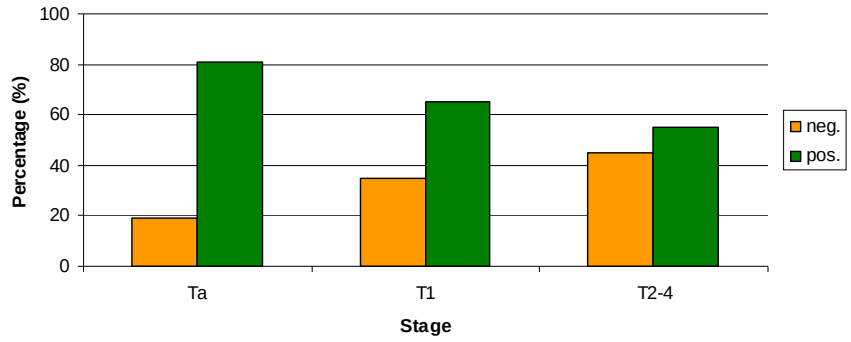

Figure 4, KRT18. Cytokeratin 18 (KRT18) expressed in bladder cancer specimen with specific tumor stage. Expression was analyzed as for figure 3.

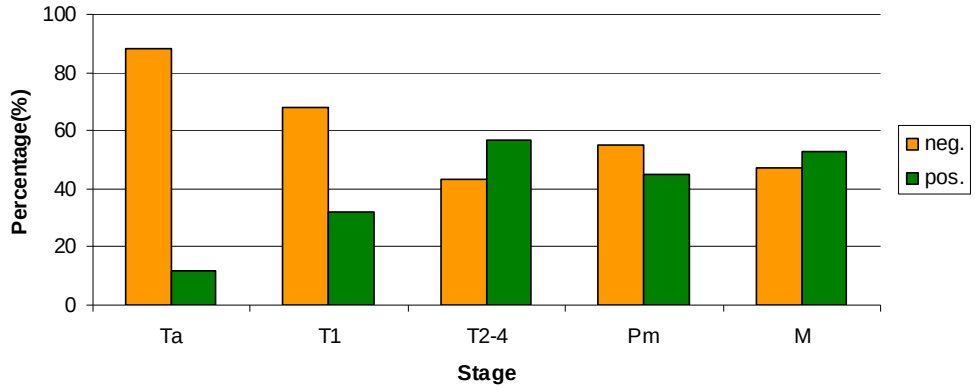

Figure 5, STMN1. The expression of stathmin (STMN1) in different stages of bladder cancer analyzed as for figure 3. This figure also includes the protein expression in Pm, primary tumors (T2-4), and coupled metastasis tumors (M).

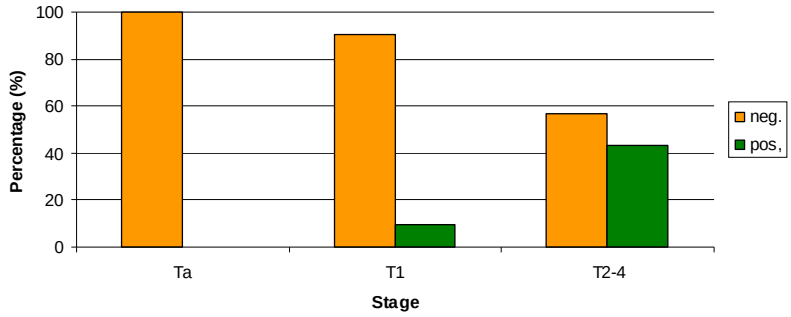

Figure 6, MS4A10. Membrane-spanning 4-domains subfamily A member 10 (MS4A10) analyzed as for figure 3.

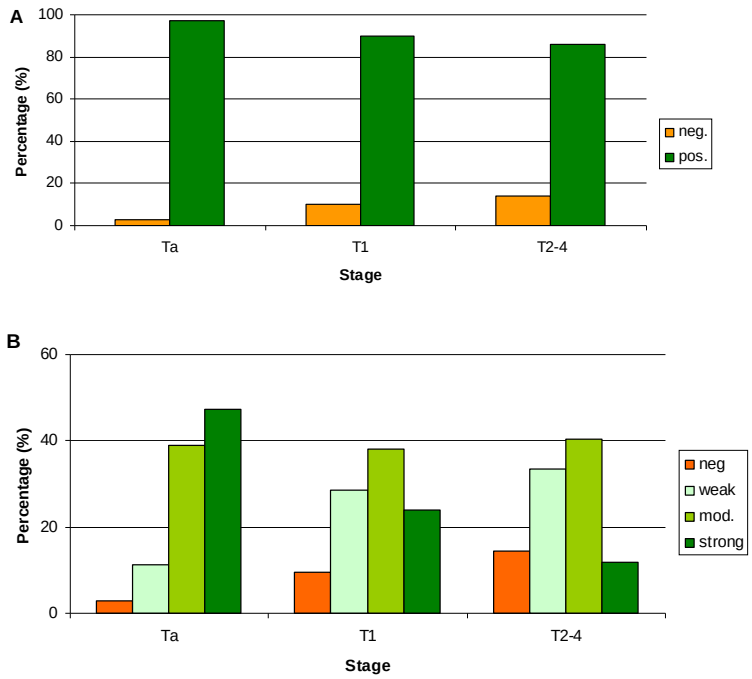

Figure 7, Expression of CSTB. The figures show the expression of the cystein protease inhibitor stefin B (CSTB) in different stages of bladder tumor analyzed as for figure 3 (A). The positive scoring was originally divided into groups; weak, moderate and strong which refers to different expression levels of the protein (B).

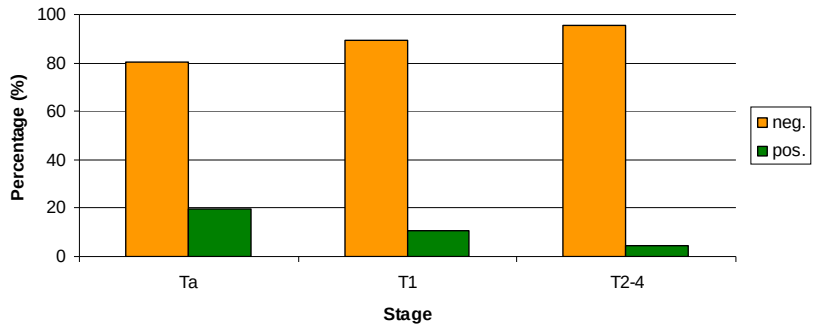

Figure 8 Expression of PIGR. The expression pattern of PIGR (polymeric immunoglobulin receptor) was analyzed as for figure 3.

Position of the antigen matched the UniProt data

Each protein was investigated in the UniProt knowledge-database. Location data from UniProt and the pattern observed after staining matched well. Proteins such as glutathione S-transferase kappa 1 (GSTK1) located in the peroxisome had a clear granular expression position indicating cell organelle location. Proteins located in the membrane were most problematic. About a fifth (4 of 21) of the antibodies did not show the same subcellular location of the protein as UniProt. All four of them were claimed to be located in the membrane [UniProt].

Discussion

The improved criteria were mostly designed by looking at the visually found list, since that list had the largest number of antibodies. Few antibodies to proteins identified by gene-expression studies in bladder cancer were found in the atlas. Additionally, several antibodies did not bind to tumor tissue or bound to all tissues and thus were not useful when designing optimum criteria. The well-established list of markers had similar problems. Another problem with the improvement was that searches without a specific number of strong or negative as criteria resulted mostly in a too large number of protein hits. The more specific the criteria were, the fewer hits there were in the result. The aim was to optimize the specificity of different criteria to find as few hits as possible and match as many proteins and antibodies as possible to the different lists. In this study, one search was performed where a criterion said that none of 12 samples showed strong expression which resulted in 55 protein hits. The improvement of the criterion did not affect as much as expected. No clear result or guideline was found when optimizing search strategies.

Since high grade tumors are over-represented in the atlas only expression in high stages such as T1 or T2-4 are well represented. Expression in the 12 patients of the atlas mostly reflected the expression pattern in T1 or T2-4. This was probably the reason why few established biomarkers were included even after the optimization. Expression pattern in the 12 patients in the atlas showed only a fraction of the protein expression in high and low stage tumors. Proteins up- or down-regulated at high stages can thereby show a different pattern in the atlas and become more difficult to find by search criteria.

The high number of high-grade tumors in the atlas does not affect the possibilities to find heterogeneously expressed proteins. As mentioned in the result; finding and verifying heterogeneous patterns was successful and indicated that 12 patients are enough.

The expression in normal bladder tissue in the atlas was noted for each antibody, but it was not used as criteria. The previous study [Sjöstedt, E. 2007 unpublished] showed problems with normal tissue. In most cases analyse of protein expression in normal tissue did not match the expression images in the atlas. The optimal would be strong or negative expression in normal tissue that easily could be compared to tumor expression. Analyses with antibodies that showed a strong protein expression in normal tissue in the atlas resulted in several cases with weak expression instead. This might be caused by the low number of samples normal tissue in the atlas, three. One other explanation might be the age of the antibody. Antibodies usually loose some of the activity after some time, especially if they are thawed repeated times. In this study I disregarded normal tissue and only noted it at the end to compare expression, this way exclusions due to miss-staining in normal tissues were avoided. For some proteins (e.g. CSTB in figure 7), normal tissue expression constituted a background in tumors of all stages, indicating a normal expression level of the protein in all stages of bladder tumors.

Antibodies correlating to stage

Binding of the majority of the 21 investigated antibodies was more or less correlated to tumor stage. For some of the 21 antibodies, no correlation was found, as listed in table 2. Binding of six antibodies demonstrated an interesting and quite clear correlation to stage (figure 3-8). All these antibodies, as mentioned in materials and methods, were evaluated in a literature search. They were chosen since the Western blot was either blank or supportive and no previous research in

bladder cancer was found, regardless of the amount of research performed on these proteins in other cancer types.

Aldo-ketoreductase
Aldo-ketoreductase family 1 member C3 (AKR1C3) is an enzyme catalyzing the conversion of aldehydes and ketones to alcohols. AKR1C3 is involved in progesterone signaling and metabolism [UniProt]. Progesterone is important in breast development and studies from 2004 showed a correlation to breast cancer [Lewis *et al.* 2004, Ji *et al.* 2004]. AKR1C expression was found to be reduced in cancer tissue compared to normal breast tissue [Lewis *et al* 2004]. Ji *et al* (2004) noticed only reduction in AKR1C1 and 2 and not AKR1C3 in breast cancer cell lines.

The results in this study indicated a reduced expression of the protein in tumor tissues, decreasing with increasing tumor stage (figure 3). AKR1C3 has not been associated with bladder cancer before, though its sibling, AKR1C2, has been linked to bladder cancer. In contrast to Lewis *et al.*(2004), Tai *et al.*(2007) reported that up-regulation of AKR1C2 correlated to progression in bladder cancer. By performing immunohistochemistry on 347 specimens, they claimed that AKR1C2 was associated with bladder cancer invasiveness [Tai *et al.* 2007]. AKR1C2 and 3 share 84% sequence identity, and still very differently expressed in bladder cancer.

Cytokeratin 18
Cytokeratin 18 (KRT18) is the intermediate filament keratin, involved in the cytoskeleton, structure and cell adhesion etc. [UniProt]. The cytoskeleton in epithelial cells consists mainly of cytokeratins [Hesse *et al.* 2001]. Most cytokeratins are missing in bladder epithelium, which instead has large amounts of specific keratins, such as cytokeratin 18 [Moll *et al.* 1982]. In two reports from 2004 and 2005 respectively, KRT18 was linked to breast cancer [Woelfle *et al.* 2004 and Bühler *et al.* 2005]. Both reports showed that cytokeratin 18 was down-regulated in advanced stage and high-grade breast cancer. A similar trend was found in this study when examining expression in bladder cancer. Cytokeratin was expressed in normal tissue and in 80% of cancer patients with Ta stage tumors. The expression decreased and the percentage of negative tumors increased in T1 and T2-4 (see figure 4).

Stathmin
Stathmin (STMN1), also called oncoprotein 18, is a phosphoprotein and a key regulator in the microtubule (MT) filament system. It destabilizes microtubules and promotes disassembly of microtubules [UniProt]. Stathmin is strongly expressed in several human cancers and has been investigated in both prostate and breast cancer. Mistry *et al.*(2006) carried out several studies investigating consequences of the introduction of an anti-stathmin ribozyme. They found that anti-stathmin could suppress a malignant phenotype in prostate cancer cells in vitro. In a recent study, they investigated the influence of the anti-stathimin ribozyme on chemotherapy and their result indicated that introduction of an anti-stathmin ribozyme increased the positive effect of the chemotherapy [Mistry *et al.* 2006]. Alli *et al.* (2007) studied stathmin in breast cancer and stathmin effects on specific chemotherapy treatments. Chemotherapy drugs are commonly based on anti-microtubule agents. Thereby stathmin suppression can enhance the effect of specific chemotherapy. My result matches the previous observations, that protein expression was correlated to the malignancy of the cancer. Normal tissue showed no expression in the bladder. Almost 90% of the Ta-tumors patients were negative as well. The protein was expressed in more

then 50% of patients with T2-4 staged tumors (figure 5). Stathmin is definitely an interesting biomarker candidate, primarily due to its possibilities as a chemotherapy target.

Membrane spanning protein
Accordning to UniProt is membrane-spanning 4-domains subfamily A member 10 (MS4A10) a probable component of a multimeric receptor complex and involved in signal transduction. Ishibashia *et al.* (2001) discovered and investigated the family MS4A and discussed similarities to e.g. CD20 and the b subunit of the high-affinity IgE receptor. They mentioned that investigation in cancer might be interesting. I found no previous research on cancer expression of this protein. Investigations of MS4A10 in bladder cancer in this study showed an up-regulation in T1 and T2-4 compared to Ta. There was no expression in normal tissue or in Ta tumors (figure 6).

Cystein protease inhibitor
Stefin B (CSTB) is a cystein protease inhibitor acting as inhibitor for e.g. cathepsins L, H and B [UniProt]. Stefin B expression was suggested to correlate to progression and shorter survival in patients with colorectal cancer according to Kos *et al.* 2000. A study of prognostics in squamous cell carcinoma in the head and neck (SCCHN), investigated the risk of recurrence. SCCHN-related death was higher in patients with low Stefin B expression [Strojan *er al.* 2000]. In my study, Stefin B expression was correlated to decreased malignancy of bladder cancer. Expression decreased in bladder tumors at higher stages.

Polymeric immunoglobulin receptor
Polymeric immunoglobulin receptor (PIGR) binds polymeric IgA and IgM at the basolateral surface of epithelial cells and transports it to the apical surface. During the transportation the secretory component is cleaved from the transmembrane segment and can be secreted [UniProt]. Hirunsatit *et al.* (2003) investigated single nucleotide polymorphisms (SNPs) in PIGR associated with nasopharyngeal cancer (NPC). Epstein-Barr virus (EBV)associated NPC is an important SCC common in Southeast Asia. It is a multifactoral genetic disease. Specific SNPs led to problems when the IgA-EBV complex bound to the receptor. If the efficiency of PIGR to release the IgA-EBV complex was altered, the risk of NPC was higher. A Chinese article points in the same direction but identified a different SNP [Fan *et al.* 2005]. These previous investigations of PIGR have not dealt with its expression level in cancer, only the correlation between SNPs and increased risk of NPC. PIGR expression in bladder cancer was significantly down-regulated in all tumor stages. Normal tissue expressed the protein very heterogeneously, which also was found in some cases of low-stages, such as Ta and T1.

Samples in the atlas are enough for finding heterogeneous expression patterns
All criteria used for searching the atlas were based on heterogeneous expression patterns among the 12 bladder tumors presented in the atlas, which is defined as a expression including all four score groups (strong, moderate, weak and negative). All selected 21 proteins displayed a heterogeneous expression in the wide-range bladder TMA, for some correlated to stage and for some not. These results imply that the expression pattern of the different proteins in the atlas is reliable: a heterogeneous pattern in the 12 patients of the atlas was correlated with a differential expression pattern in 140 patients.

Some of the TMA (140 patients) expression patterns were less heterogeneous than in the atlas (12 patients), but overall the results were convincing. Cases where the expression pattern in the TMA differed mostly from the atlas were proteins that showed strong expression in a third (4/12) of the patients in the atlas and no expression in the rest. These antibodies displayed a more frequent miss-match between expression pattern in the wide-range TMA and the 12 patients in the atlas. Tumors with weak staining (low protein expression) were over-represented when the wide-range TMA were analyzed, compared to the atlas. Also, tumors scored in the strong-score category were more common in the atlas, than in the TMA-scoring.

Materials and methods

Identification of proteins differentially expressed in bladder cancer

A list of proteins with well-established correlation to bladder cancer was created by literature search in PubMed (NCBI), with the search words "review", "bladder cancer" and "biomarker". Proteins found were evaluated by an additional literature search to verify the result of the first search. Gene names for each protein were matched to the protein atlas to find corresponding antibodies in the database. The same procedure was performed for markers from a commercial antibody producer (Geneway Biotech). Gene names from the gene expression project [Dyrskjott et al 2005] were matched to the atlas as well. The category "visually found antibodies" was found either by employees working with the atlas or by browsing the atlas images in previous attempts to find potential markers in urinary bladder cancer. A proteins of interest is a protein with unique expression pattern in bladder cancer patients, it can be up- or downregulation compared to normal tissue, or it can be a strange pattern within the differ tumor cases.

The subcellular location and function of the proteins (UniProt) was noted as well as the pattern of protein expression in the 12 bladder cancer patients in the atlas. The expression pattern (number of weak, moderate, strong or negative patient samples) was noted for low grade and high grade tumors separately. Criteria for the improved search was designed, inspired by the different expression patterns in the lists (used criteria is shown in the result table 1). The criteria were formed by determining the number out of 12 patients that should be strong, moderate, weak or negative. All criteria were based on the 12 bladder cancer patients represented in the atlas. The improved search was made by investigating the most common expression patterns for the proteins and antibodies in the lists. Different criteria were tested to find as many matches to the lists as possible in relation to the number of total hits of the search (table 1). The new improved search was performed in the internal atlas consisting of 6077 different antibodies at that time.

Matching of lists

The three lists (well-established, visually found and found by gene-expression arrays) were matched to the list from the recent study [Sjöstedt, E. 2007 unpublished], as well as the new improved search. The well-established list and gene-expression lists were compared by the gene names, while the visually found list was compared with the antibody ID. The result of the improved search was compared to the list of antibodies from the previous study, in which very basic search criteria were used, to see if the optimization was successful.

To find interesting antibodies for further investigation in bladder cancer a match was made between the former search (list of genes from Sjöstedt, E. 2007 unpublished) and new search (that was optimized in this study) to find which proteins turned up in both studies.

The most interesting proteins to analyze were selected by evaluating the literature search and Western blot results available in the protein atlas performed by the human protein resource (HPR) group. Each protein was examined in PubMed to determine the level of characterization for each protein. Proteins not mentioned in bladder cancer were chosen. Only Western blots with supportive bands at the right position or negative results without any bands were accepted. Negative result without band is accepted since they are uncertain while several unspecific bands are definitely poor quality. Antibodies can sometimes not be suited for western and therefore not

bind in that technique, but can still bind specific in immunohistochemistry. All proteins that passed these criteria were analyzed by immunohistochemistry (see below). A small tissue microarray (TMA) with 12 random patient samples of bladder cancer was used for a pre-test of each chosen antibody. Each antibody was diluted according to the atlas data (table 3). The result was compared to the images in the atlas to verify the dilution. In cases of a mismatch, where the concentration or location of the protein did not match the atlas, a re-testing was performed with a new dilution.

Wide-range tissue microarray for urinary bladder cancer

Only antibodies that passed the pre-test were used to analyse the wide-range TMA. The bladder cancer TMA included 3 different slides and altogether 140 different tumors. The number of normal tissue samples in the array was 7. The different tumors were distributed as follow; 37 Ta, 20 T1, 36 T2, 6 T3, 3 T4 and 19 primary tumors with matched metastases (the exact number is shown in figure 4). The primary tumors were T2 or higher stage.

Table 4, Number of specific tumor stages tested with each antibody.

Gene name	Ta	T1	T2	T3	T4	Pm[1]	M[2]	total	Normal tissue
AKR1C3	40	20	39	6	3	19	19	146	7
ARHGEF1	38	19	36	6	3	18	18	138	7
ASAH1	37	20	42	7	3	20	18	147	7
ASB8	36	19	32	6	3	19	19	134	7
CCR6	34	22	29	6	3	19	19	132	7
CHD8	37	19	32	6	3	20	19	136	8
CLIC3	35	20	36	6	3	18	17	135	7
CSTB	36	21	33	7	2	20	19	138	7
DLG4	39	20	36	7	3	19	19	143	7
GSTK1	36	18	38	7	3	19	18	139	7
IDH2	37	19	36	6	3	18	19	138	8
KRT18	37	20	41	7	3	20	19	147	7
LMTK2	32	22	34	6	3	20	19	136	7
MAOA	36	19	37	7	3	19	19	140	8
MLRM	37	19	38	6	3	19	19	141	7
MS4A10	39	21	35	7	2	20	19	143	7
MTMR10	37	22	37	7	3	19	19	144	6
PIGR	36	19	38	6	3	19	19	140	7
Rad17	39	17	36	6	3	20	18	139	7
RBP1	34	20	30	6	2	19	18	129	7
STMN1	41	19	39	6	3	20	19	147	7
sum	773	415	754	134	60	404	392	2932	149
Average[3]	37	20	36	6	3	19	19	140	7

[1]The primary tumor T2-4 causing metastases.
[2]The metastases tumor from other location then urinary bladder, taken from same patients represented in Pm.
[3]The number variation was due to technical issues

Paraffin sections of TMA were first deparaffinized supervised by an automatic machine (LEICA autostainer XL from Leica microsystems) that moved the rack of slides in time; 11 minutes in xylene followed by 6 min in absolute ethanol, 3 min in 95% ethanol containing 0.03% hydrogen peroxide. The hydrogen peroxide was added to block endogenous peroxidase. This was then followed by 3 min in 95% ethanol, 3 min in 80% ethanol and finally a wash in distilled water for 30 seconds. The slides were then baked in 250 ml TRS pH 6 buffer from LabVision in a pressure boiler (DC2002 Decloaking chamber), at 125°C for 4 minutes at a pressure 90-130 kPa and then allowed to cool for 20 in the pressure boiler and then by rinsing with distilled water.

The following steps were performed automatically by an automated immunohistochemistry apparatuses (Autostainer 480®, DakoCytomation, Glostrup, Denmark) at room temperature, and each reagents were applied in a volume of 300 µl per slide. After incubation of each reagent each slide was rinsed by wash buffer from LabVision named Tris Buffered Saline & Tween 20 (20x) which was prepares by adding 0.5 l stock to 9.5 l distilled water. In addition, an extra volume of 15 ml Large Volume Tween 20 (polyoxyethylenesorbitan monolaureate) was added to every 10 l buffer. The glass slides were placed into the Autostainer machine and the diluted antibodies in each specific vial was placed to its position. The machine then performed the following steps: each glass slide was rinsed with wash buffer. After that primary antibodies was applied, after incubation for 30 minutes another rinse was performed, optional enhancer from LabVision was then added (only used for mouse antibodies). After an additional rinse, HRP was added (horse radish peroxidase linked to the secondary non-specific antibody) and after a double rinse was DAB (3,3'-Diaminobenzidine, substrate for HRP) was added. This resulted in a brown color where the antibody was bound. After rinsing hematoxylin (from Histolab) was added, which provided the blue color of the cell structures. The program was finished after a final rinse.

The stained glass slides were then put back to the LEICA. The slides were treated with saturated lithium carbonate for one minute, 5 minutes of rinsing in distilled water was followed by 3 minutes in 80% ethanol, 6 minutes in 95% ethanol, 6 minutes in absolute ethanol and after 6 minutes in xylen each slide was covered automatically with pertex and glass covers. The cover glass was then fixed by incubation 10 minutes in 50°C.

Table 3, Dilution for each antibody according to the atlas**.**

Gene name	Antibody ID	Atlas dilution	Dilution for re-testing [1]	Species	Source
AKR1C3	CAB010874	1/2500		Mouse	Sigma
ARHGEF1	HPA012924	1/600		Rabbit	HPR[2]
ASAH1	HPA005468	1/300		Rabbit	HPR[2]
ASB8	HPA003299	1/50	1/100	Rabbit	HPR[2]
CCR6	HPA014488	1/40	1/20	Rabbit	HPR[2]
CHD8	HPA000849	1/50		Rabbit	HPR[2]
CLIC3	HPA005963	1/50		Rabbit	HPR[2]
COX8C	HPA003127	1/20	1/15	Rabbit	HPR[2]
CSTB	CAB000470	1/20	1/25	Mouse	Serotec
DLG4	CAB002000	1/250		Mouse	Upstate
GSTK1	HPA006311	1/50		Rabbit	HPR[2]
IDH2	HPA007831	1/50		Rabbit	HPR[2]
KRT18	CAB000030	1/1000		Mouse	Sigma
LAMB1	HPA004056	1/100	1/50	Rabbit	HPR[2]
LMTK2	HPA010657	1/250		Rabbit	HPR[2]
MAOA	CAB009437	1/500		Rabbit	SCB[3]
MLRM	CAB004503	1/100		Mouse	SCB[3]
MS4A10	HPA014778	1/200		Rabbit	HPR[2]
MTMR10	HPA006081	1/75		Rabbit	HPR[2]
PIGR	HPA012012	1/150		Rabbit	HPR[2]
PTPN1	CAB015217	1/500	1/200	Rabbit	SCB[3]
Rad17	CAB004551	1/20		Mouse	SCB[3]
RBP1	HPA007338	1/150	1/250	Rabbit	HPR[2]
STMN1	CAB010107	1/250		Mouse	SCB[3]

[1]In 7 cases a re-testing was made, new dilutions are noted as well.
[2]Human protein resource produced antibody.
[3]Santa cruse biothec produced antibody.

Annotation

The subcellular location of antibody binding was noted and the staining pattern was scored for each antibody in the wide-range bladder TMA. The staining intensity was scored 1-3 (1 = weak, 2 = medium and 3 = strong). The distribution was also scored 1-3 where 1 corresponds to <25%, 2 to 25 - 75% and 3 to >75% of the total number of cancer cells in each tissue sample. The two numbers were multiplied and resulted in a final score 1-9 (1-2 was weak, 3-4 was moderate and 6-9 was strong expression). The expression pattern for each antibody in the bladder TMA was analyzed in Excel.

Statistical analysis

Matlab was used to calculate Fisher exact probability test (two-tailed). $P<0.05$ was considered significant.

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