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IMAC fractionation in combination to LC/MS reveals H2B and NIF-1 peptides as potential bladder cancer biomarkers

Maria Frantzi[1,3] , Jerome Zoidakis[1], Theofilos Papadopoulos[1], Petra Zürbig[3], Ioannis Katafigiotis[4] , Konstantinos Stravodimos[4] , Andreas Lazaris[5] , Ioanna Giannopoulou[5], Achilles Ploumidis[6], Harald Mischak[2,3], William Mullen[2] and Antonia Vlahou[1*]

[1]Biomedical Research Foundation Academy of Athens, Greece

[2] Institute of Cardiovascular and Medical Sciences, University of Glasgow, United Kingdom

[3] Mosaiques diagnostics GmbH, Hannover, Germany

[4] 1st Department of Urology, 'Laiko' General Hospital, University of Athens, School of Medicine

[5] 1st Department of Pathology, University of Athens, School of Medicine, Athens, Greece

[6] Department of Urology, Athens Medical Centre, Athens, Greece

ABSTRACT

Improvement in bladder cancer (BC) management requires more effective diagnosis and/or prognosis of disease recurrence and progression. Urinary biomarkers attract special interest due to the non-invasive means of urine collection. Proteomic analysis of urine entails the adoption of a fractionation methodology to reduce sample complexity. In this study, we applied immobilized metal affinity chromatography in combination with high resolution LC-MS/MS for the discovery of native urinary peptides potentially associated with BC aggressiveness. This approach was employed towards urine samples from patients with invasive BC, non-invasive BC and benign urogenital diseases. 1845 peptides were identified,

corresponding to a total of 638 precursor proteins. Specific enrichment for proteins involved in nucleosome assembly and for zinc-finger transcription factors was observed. The differential expression of two candidate biomarkers, histone H2B and NIF-1 (zinc finger 335) in BC, was verified in independent sets of urine samples by ELISA and by immunohistochemical analysis of BC tissue. The results collectively support changes in the expression of both of these proteins with tumour progression, suggesting their potential role as markers for discriminating BC stages. Also, the data indicate a possible involvement of NIF-1 in BC progression, likely as a suppressor and through interactions with Sox9 and HoxA1.

KEYWORDS: urine, bladder cancer, peptidomics, NIF-1, H2B

SIGNIFICANCE / NOVELTY

This study provides an in-depth analysis of the urinary peptidome in association with bladder cancer (BC). IMAC fractionation combined with LC-MS/MS was applied. Native urinary peptides originating from histones and zinc-finger proteins, not yet detected in urine, were enriched. Differential expression of H2B and NIF-1 with BC stage was further verified in urine by ELISA and tissue by immunohistochemistry. We hypothesize an involvement of NIF-1 in BC progression likely through interactions with Sox9.

INTRODUCTION

Bladder cancer (BC) is the second most frequent genitourinary malignancy after prostate cancer[1]. According to TNM criteria, urothelial cancers are classified into different stages: Ta for non-invasive papillary carcinoma, Tis for carcinoma *in situ*, T1 for tumours that have invaded the subepithelial connective tissue and stages T2-4 for muscle invasive cancers[2, 3]. Disease prognosis and BC treatment strategies are related to tumour stage and other clinico-pathological characteristics[4].

Currently the golden standard in BC initial diagnosis and surveillance is the histopathologic analysis of biopsy specimens obtained by cystoscopy. Of the non-invasive methods, urinary cytology is being applied, as adjunct to the cystoscopic evaluation, even though it is associated with low sensitivity particularly in the detection of low grade disease[5]. Other urinary molecular BC tests have been recently introduced into clinical practice, like NMP22, UroVysion and Immunocyt. However, none of them could successfully replace cystoscopy as a stand-alone test[6]. The main clinical needs for BC currently include development of non-invasive, more accurate tests for initial diagnosis and surveillance. Main areas of applications are the early detection/prognosis of disease recurrence and progression as well as prediction of response to neoadjuvant and adjuvant therapies[7, 8].

Urine serves as a biomarker source for BC, not only because it is in direct contact with the urinary tract, but also because it is easily and non-invasively accessible [9]. However, due to its high complexity and high concentration of salts and interfering compounds, adoption of a protein separation or enrichment step is required when targeting the discovery of new biomarkers. We recently described the application of IMAC fractionation in combination to "bottom-up" proteomics (e.g. tryptic digestion followed by LC/MS analysis) for the enrichment of low abundance proteins in urine[10]. This approach proved to be an effective tool

for the isolation of matrix metalloproteinases and other proteases and proteins involved in cytoskeletal rearrangement[10]. Among the latter, Profilin-1 was found to provide prognostic information[10]. In this study, we focus on the naturally occurring urinary peptides in association with BC stages, following enrichment with IMAC fractionation and a "top-down" (e.g. lack of tryptic digestion) MS analysis. The clinical objective of the current study is, thus, diagnostic with the ultimate goal to improve management of patients with BC. A clear enrichment for peptides corresponding to Zn-finger proteins may be observed as well as for nucleosomal proteins, -not yet included in existing urine peptidome databases, selectively from cancer samples. The differential expression of histone H2B and Zn finger 335 (NIF-1) in urine was further confirmed by ELISA analysis and initial evidence for the functional relevance of these proteins was provided by immunohistochemistry of BC tissue sections.

EXPERIMENTAL PROCEDURES

Urine sample collection, handling and storage

Urine samples (random-catch) were collected in two centres: Laikon Hospital and Asklepieion Voulas General Hospital both in Athens, Greece. Proper informed consent procedures under Institutional Review Board approved protocols were followed. Samples were collected according to the standard protocol for urine collection defined by the European Kidney and Urine Proteomics (EuroKUP) and Human Kidney and Urine Proteome Project (HKUPP) networks (http://www.eurokup.org/node/138) including freezing at -80^{o}C within 3h of collection and a brief centrifugation step prior to proteome analysis[11]. Samples were collected in between 2003-2012, therefore storage time differed per sample nevertheless this was evenly distributed among groups.

Immobilized Metal Affinity Chromatography (IMAC)

IMAC chromatography was performed, as previously described[10]. IMAC uncharged resin (Profinity-BioRad) was used to generate a 10mL chromatography column (BioRad) according to the manufacturer's instructions. In brief, 0.5mL of resin was applied in the column, washed with 8mL of deionized water, followed by a wash with 8mL of binding buffer (50mM NaH_2PO_4, 300mM NaCl, 1mM Imidazole, pH 8). Prior to metal ion loading (Ni^{2+}), the column was equilibrated with 8mL of 50mM CH_3COONa, 300mM NaCl, pH 4. A volume of 8mL 200mM $NiSO_4$, pH 6.8 was added and the column was washed again with 10mL of deionized water, followed by a wash with 8mL binding buffer (50mM NaH_2PO_4, 300mM NaCl, 1mM Imidazole, pH 8). One mg of total protein corresponding to 210 to 770 µl per sample) was mixed with binding buffer at a ratio of 1:4 respectively and applied to the column. Several fractions of flow through were collected (2mL/fraction). The column was then washed with 10mL of wash buffer (50mM NaH_2PO_4, 300mM NaCl, 5mM Imidazole, pH 8), during which 5 fractions were collected (2mL/fraction) to monitor washing efficiency. Elution was performed by the addition of 4mL elution buffer (50mM NaH_2PO_4, 300mM NaCl, 500mM Imidazole, pH 8). Elution fractions were dialyzed (700Da cut-off) against 3L of water. Following this dialysis step, eluates were dried by speedvac and resuspended in 100µl of HPLC grade H_2O. An aliquot was taken for LC-MS/MS analysis, as described below. The remaining sample was stored at -80^{o}C. IMAC was performed on individual urines from patients with non-invasive (5 Ta and 5 T1) and invasive (5 T2+) BC and benign urothelial diseases (n=5). Detailed clinical data per sample are provided in **Supplementary Table 1.**

LC-MS Analysis for Peptide Sequencing

LC-MS analysis was performed using an Ultimate3000 nano-LC pump (Dionex, Camberley UK) coupled to an Orbitrap LTQ-Velos FTMS via Proxeon nano spray source (Thermo Fisher, Hemel, UK). 5 µL were loaded onto a Dionex 100µm x 2 cm 5 µm C18 nano trap column and separated on a Dionex C18 reverse phase column (75 µm ID × 150 mm, 100 Å). Peptides were eluted using a gradient of 1-5-40% B (80% ACN, 0.1% formic acid) in 0-5-60 min at a flow rate of 300 nL / min. The eluate from the column was directed to the Thermo Proxeon nano spray ESI source operating in positive ion mode then into the Orbitrap LTQ Velos FTMS. The ionisation voltage was 2.5 kV and the capillary temperature was 200 ºC. The mass spectrometer was operated in MS/MS mode scanning from 380 to 2000 amu. The top 20 multiply charged ions were selected for MS/MS analysis using data dependant dynamic exclusion. The repeat count was 1 and repeat duration 15 seconds, exclusion mass widths were ± 5 ppm. The fragmentation method was HCD at 35% collision energy. The resolution of ions in MS1 was 60000 and 7.500 for HCD MS2.

Data analysis

Raw spectral data from LC-MS/MS analysis were uploaded to Thermo Proteome Discoverer 1.2. Peptides were considered if signal to noise ratio was higher than 1.5 and belonged to precursor ions of 700 – 8000 Da. Peptide and protein identification was performed with SEQUEST algorithm using Uniprot version 01052012 of human taxonomy. No enzyme cleavage was selected and oxidation of methionine and hydroxylation of proline were chosen as variable modifications. Precursor tolerance was set at 10 ppm and 0.1 Da for MS/MS fragment ions. Resulting peptides and protein hits were further screened by accepting only those hits listed as high confidence by the Proteome Discoverer software. FDR was estimated to be 0.4%.

ELISA

For the investigation of H2B and NIF-1 levels in individual urine samples, commercially available ELISA Kits from USCN LIFE, INC. were employed. A total of 166 individual urine samples from patients with benign diseases of the urogenital tract (BPH, inflammation, lithiasis, hematuria), patients with Ta BC, T1 BC and invasive T2+ urothelial carcinoma were employed (demographics and clinical information per sample are available in **Supplementary Table 1)**. H2B levels in urine were determined in a total of 147 urine samples: 39 benign, 40 Ta, 36 T1 and 32 T2+. NIF-1 was quantified in a total of 158 urine samples: 47 benign, 44 Ta, 36 T1 and 31 T2+ (all raw data-measurements per sample are provided in **Supplementary Table 3).** The assays were performed according to the manufacturer's instructions using 100μl of urine per well. The NIF-1 kit was approved and validated for use in urine by the manufacturer. In the case of the H2B kit, a negative urine sample was spiked with all the H2B standards and in all cases the H2B measurements were very close to the expected ones (R^2= 0.9931, standards used: 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5 ng/ml; data not shown). For all samples with concentration <LOQ, "0" values were used for statistical analysis.

Immunohistochemistry

Formalin-fixed tissue specimens from a total of thirty two patients [mean age at diagnosis 67.1 years (age range: 50–86 years); 27-male, 5-female; **Supplementary Table 1**] with BC were employed for immunohistochemical analysis. These samples were obtained from first transurethral resection of the bladder or through radical cysto-prostatectomies. Sections were evaluated with hematoxylin-eosin stain and graded according to the WHO 1973 and WHO/ISUP (2004) as grade 1 (5 cases), grade 2 (13 cases), grade 3 (14 cases) and as low-

grade (17 cases) and high-grade papillary urothelial carcinomas (15 cases), respectively. Staging was performed according to TNM/UICC(2009) classification[3], as follows: pTa (11 cases), pT1 (12 cases), pT2 (5 cases), pT3 (2 cases) and pT4 (2 cases). In situ carcinoma co-existed in 6 cases.

Immunohistochemical staining for Histone2B and NIF-1 was performed on 4μm thick formalin-fixed paraffin sections. To enhance antigen retrieval, sections were microwave-treated in 10 mM citrate buffer, pH 6.0 at 750W for three cycles of 5min each. This protocol was selected following initial optimization experiments, during which antigen retrieval with different buffers (10 mM citrate buffer pH 6.0 or 1 mM EDTA pH 8.0), or without antigen retrieval were performed. As positive controls for both H2B and NIF-1, immunohistochemistry of paraffin-embedded human breast carcinoma tissues were used. Negative controls had the primary antibody omitted and replaced by non-immune normal serum from the same species. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in Tris-buffered saline (TBS). After rinsing with TBS, normal horse serum was applied for 30 minutes to block non-specific antibody binding. Subsequently, sections were incubated overnight at 4 ^{0}C with the following antibodies: a rabbit monoclonal antibody against Histone2B (clone EP957Y, Millipore, Temecula, CA, USA), diluted 1:500 and a rabbit polyclonal antibody against NIF-1 (cat.No.IHC-00194, Bethyl Laboratories, Montgomery, TX, USA) diluted 1:80.

A three-step technique (Avidin-Biotin-Peroxidase, Vector Laboratories, Burlingame, CA, USA) was used for visualization, with diaminobenzidine as a chromogen according to the kit instructions. Finally, sections were counterstained with hematoxylin and mounted.

Evaluation of Immunohistochemistry

Evaluation of the immunohistochemical staining was performed independently by two pathologists (AL, IG) without knowledge of the clinical data for each patient. Through this initial manual analysis no spurious staining and a homogeneous pattern of expression for the two proteins throughout the epithelial cells in the section were in general observed. A score per section was assigned according to the number of positive cells and staining intensity. Specimens were scored as 0: negative, 1+: low, 2+: moderate, 3+: intense expression (data not shown). Nuclear immunoreactivity for H2B and nuclear-cytoplasmic immunoreactivity for NIF-1 were observed.

To assess the samples independent from the visual inspection, which may be subject to observer bias, an image analysis approach was implemented using the Image J software that enables analysis of a RGB image in separate channels, as described by Ruifrok and collaborators[12]. Briefly, every tissue section was scanned under the microscope using X20 magnification. Depending on tissue size, 5-10 images were acquired per section. For each image, 10-50 fields of identical area dimensions (16200nm^2) were selected to be measured. Optical density was normalized over the tissue substrate which was employed as negative background. Mean values of intensity were calculated per image and subsequently for each section (tissue sample). All raw data-measurements per sample are provided in **Supplementary Table 3.**

Statistical Analysis

Statistical analysis was performed using the SPSS statistical software (SPSS 17.0: IBM). The Kolmogorov – Smirnov test (K-S test) was employed to test for normal data distribution. In

case that data normality could not be demonstrated, the Mann-Whitney test was applied, whereas in cases of normal distribution the t-test was used. In all cases, $p<0.05$ was considered statistically significant. The boxplots were created by the use of the Origin software (OriginPro v.8: OriginLab Corporations).Correlations between biomarker levels and urinary protein content were investigated using the Spearman's rho and Pearson correlation tests.

RESULTS

Workflow for biomarker discovery

To enrich for potential BC urinary biomarkers, a proteomics approach was utilized combining a fractionation methodology coupled to LC-MS/MS. IMAC–Ni was used to reduce complexity, while allowing for the selective enrichment of metal binding proteins. Five samples per category (benign, non-invasive Ta and T1, invasive T2+) were fractionated. As this methodology is cumbersome and of low reproducibility, the received protein identifications per sample were compiled to form a list of identifications per category e.g. benign, non invasive, invasive, representing the sum of identifications of all samples in the respective group). Comparison of different groups was then conducted in a qualitative manner and findings were prioritized based on the following parameters: a) strength of evidence for differential expression (number of samples where the peptide was present); b) (parental) protein functional annotation; c) potential relevance to bladder cancer invasion.

Following this procedure the presented identifications and respective differences (based on presence-absence) between groups cannot be considered as "significant changes". However, this list in combination with a thorough protein functional annotation and literature mining,

becomes *suggestive* of potential disease related biomarkers, meriting further investigation. Among the latter, two proteins, Zinc-Finger 335 (NIF-1) and H2B were selected for further validation by antibody –based assays in independent sets of urine and tissue samples. A schematic representation of this study design and workflow is depicted in **Figure 1**.

IMAC fractionation provides enrichment of naturally occurring urinary peptides

The received 1845 peptide identifications from LC-MS/MS analysis of the eluted fractions incorporating stringent identification criteria, corresponded to 638 distinct precursor proteins in total (**Supplementary Table 2**). Common identifications (46 precursor proteins) with the existing urine peptidome database[13] involved mainly serum proteins like Haemoglobin subunits, Immunoglobulin chains, Albumin, Plasma protease inhibitors, Apolipoprotein isoforms, Complement components, and Transthyretin, as well as Collagen and Fibrinogen fragments and others (e.g. Osteopontin, Gelsolin, Protein S100-A9, etc). The enrichment for many of these proteins was expected due to their affinity for metal ions (e.g. Albumin, Serotransferrin etc). Interestingly, 592/638 proteins or 92.8% of our identifications had not been previously detected in unfractionated urine[13]. This is likely due to their low abundance, and may indicate good efficiency of the enrichment strategy**.** Metal affinity chromatography using Ni^{+2} as an immobilized ligand is an efficient strategy for the purification of proteins with C_2H_2 zinc-finger domains[14] and/or being phosphorylated[15]**.** Nevertheless, as shown (**Supplementary Table 2**) the affinity of the identified peptides for the IMAC Ni column cannot be readily attributed to these factors (e.g. metal binding and phosphorylation). Since Histidine (H), Arginine (R) or Lysine (K) are amino acids that show affinity for Ni^{2+} [16], peptides containing these amino acids may also bind to the IMAC column. In fact, 94% of the identified peptides contain at least one of Histidine (H), Arginine (R) or Lysine (K).

Functional Annotation of the peptidomics data reveals a high number of nucleosome assembly and Zn finger transcription factors in bladder cancer urine samples.

"In silico" evaluation of the findings for their biological significance was conducted using a combination of GO analysis, text mining, as well as search through expression databases such as the Human Protein Atlas database (www.proteinatlas.org). **Figure 2** illustrates the distribution of received identifications, according to the protein biological function (similar distribution was received within each individual group, e.g. invasive, non-invasive and benign- data not shown).

As shown, various identified proteins are immunological components, such as proteins involved in complement activation, cytokine signalling and inflammation. A substantial part of identifications consist of proteins involved in signalling pathways: such as Ras-related proteins, G-protein signalling modulators and Rho-related GTP-binding proteins (Puratrophin-1, Kalirin and Tuberin). Other peptide identifications correspond to proteins with demonstrated function in cytoskeleton remodelling and cell adhesion rearrangements: e.g. Gelsolin, Slit homolog 2, Slit homolog 3, and its receptor Roundabout 4, all known to regulate actin polymerization. Proteins involved in oxidative stress response were also enriched, such as, Peroxiredoxin-2 (Natural killer cell-enhancing factor B) and Glutathione S-Transferase P as well as proteins involved in apoptosis, (Anamorsin or Cytokine-induced apoptosis inhibitor 1 etc.).

Interestingly, an over-representation in the cancer samples of peptides related to nuclear-nucleosomal proteins was observed (such as many peptides deriving from Histones) as well as of Zn finger transcription factors (**Supplementary Table 2**). Taking this into account and in combination with the novelty of these initial findings, as well as antibody availability, we

opted to further investigate the value of histone H2B and Zinc finger 335 (NIF-1) as potential biomarkers for bladder cancer.

ELISA analysis in urine samples confirms mass spectrometric results

The urinary protein levels for NIF-1 and H2B were quantified by ELISA in independent sets of individual urine samples (detailed data per sample are provided in **Supplementary Table 3)**.

A total of 158 urine samples were analysed for NIF-1: 47 samples from patients with benign urothelial diseases, 44 from patients with Ta, 36 from T1 and 31 from T2+ BC. In the vast majority of urine samples, NIF-1 could be detected, as shown by the number of positive samples: In total, 106/111 (or 95.9%) cancer cases were positive for NIF-1. The mean values and standard deviation of NIF-1 levels are summarized in **Figure 3A**. Progressively increasing levels of urinary NIF-1 are observed with increasing tumour stage. In general, the levels of NIF-1 did not follow normal distribution (according to the K-S test, data not shown), therefore, the Mann- Witney test was applied to estimate statistical significance. This analysis showed significant differences between the NIF-1 levels in the benign samples compared to the cancer groups (B vs. Ta $p=0.043$), (B vs. T1 $p=0.018$) (B vs. T2+ $p=0.0000062$). In addition, the T2+ exhibited a significant increase compared to non-invasive Ta (Ta vs. T2+ $p= 0.014$), although the difference between T1 and T2+ was not statistically significant. This trend of expression was also observed when the ELISA data were normalized according to creatinine **(Supplementary Table 3).**

For H2B, 147 urine samples were analysed by ELISA. In this case, larger variations (compared to NIF-1) in the number of positive samples per group could be observed: Detectable levels for H2B were observed in 15 out of 39 (38.5%) benign samples, 35 out of 40 (87.5%) Ta samples, 19 out of 36 (52.8%) T1 and 23 out of 32 (71.9%) T2+ samples.

When considering the levels of expression (summarized in **Figure 3B**), H2B was found at significantly higher levels in the cancers compared to the benign samples (Benign vs. Ta p=0.0000046, Benign vs. T1 p=0.042, Benign vs. T2+ p=0.001 according to Mann Whitney; **Figure 3B**). Between the cancer categories, statistically significant difference was found only for Ta vs. T1 pair sets (Ta vs. T1 p=0.033-Mann Whitney). As in the case of NIF-1, this observed trend of expression was not affected by data normalization according to creatinine. However, when the normalization was calculated over the total protein, these trends of expression with tumour stage could no longer be detected for neither H2B nor NIF-1 **(Supplementary Table 3).** This attenuation of urinary biomarker changes when normalized according to total protein in bladder cancer is in line to recent reports[17].

Differential expression of NIF-1 and H2B in BC tissue sections

The results from the urine analysis prompted a further investigation of the association of NIF-1 and H2B with BC at the tissue level. Towards that end, immunohistochemical analysis of BC tissue specimens was performed. A colour deconvolution image analysis method (Image J software) was implemented to allow for a more accurate and objective signal quantification. The results of this analysis were overall in accordance to the scoring provided by the pathologists (data not shown). Twenty-eight sections were stained for NIF-1 divided in 3 BC groups, 11 Ta, 10 T1 and 8 T2+. Five to ten (depending on the tissue section size) representative images were selected from each section (average number of images per sample was 7 for Ta, 7 for T1 and 5 for T2+; raw data are provided in **Supplementary Table 3**). Nuclear, as well as, cytosolic NIF-1 expression was observed in a relative homogeneous pattern among epithelial cells per section (**Supplementary Table 3**). Deviations from the mean corresponded to images containing lower number of epithelial cells and/or occasionally epithelial cells of different morphology (**Supplementary Figure**) suggesting that these may

represent different stages in the disease evolution. Quantification of the IHC intensity revealed a clear overall reduction of NIF-1 expression as cancer stage progresses (**Figure 4A**). Statistical analysis of the received optical density (OD) values supported a significant difference between non-invasive (Ta, T1) and invasive (T2+) cancers (Ta vs. T1 p=0.191 for Ta vs. T2+ p=4.5E-6 and T1 vs. T2+ p=0.001 according to t-test (normality was predicted based on the K-S test). Mean value (SD) per stage and respective raw data are provided in **Supplementary Table 3.**

In the case of H2B, the same quantification strategy was applied. Nuclear staining was observed which, similarly to NIF-1, exhibited a relative homogeneous staining throughout the section in areas of similar tissue and cell morphology. Occasionally, epithelial cells were found to differ in their intensity measurements within the same section (representative images are also presented in **Supplementary Figure**) likely attributed to differences in the degree of aggressiveness. A difference in the overall staining intensity between the three cancer categories could be observed (**Figure 4B**). However, in contrast to NIF-1, the OD increases with increasing cancer stage. A statistically significant difference was observed between the non-invasive (Ta, T1) and T2+ invasive BC (Ta vs. T2+ p=0.001 and T1 vs. T2+ p=0.009) whereas no significant difference was observed between the non-invasive Ta and T1 cancers (Ta vs. T1 p=0.998; in all cases, the t –test was applied as OD data followed normal distribution according to the K-S test). Mean value (SD) per stage and respective raw data are provided in **Supplementary Table 3.**

Collectively, these results supported an association of NIF-1 and H2B tissue levels with tumour stage (even though with opposing trends of expression: decreased levels for NIF-1 and increased for H2B in the invasive versus non-invasive groups).

No further associations of H2B to histological grade were observed. In the case of NIF-1, immunoreactivity was inversely associated with tumour grade (WHO 2004 classification p=0.013). Using the WHO 1973 grading system, NIF-1 expression displayed a significant negative correlation between grade 2 and grade 3 carcinomas (p=0.003) (**Supplementary Table 3**).

H2B and NIF-1 have been found to interact with each other[18]. The existence of possible correlation between the OD values of the two proteins was also investigated by the performance of a curve estimation (**Table 1**). A statistically significant correlation (p= 0.036) following an inversely proportional with a Pearson Correlation value -0.391 was received, suggesting that as H2B levels increase with tumour stage, and NIF-1 levels decrease.

DISCUSSION

Bladder cancer is a malignancy of particular interest for clinical proteomics approaches due to increased tumour heterogeneity, high recurrence and progression rates, and lack of effective non-invasive diagnostic and prognostic tools. Urine as a biological fluid has the advantage of being easily and non-invasively obtained. To reduce sample complexity allowing for efficient biomarker discovery, the application of enrichment strategies is a frequently and successfully applied approach[19]. IMAC has been used to enrich for intact proteins and peptides with high affinity to metal ions[20] and in phosphoproteomic studies [21, 22]. We previously applied this strategy to study urinary proteins associated with BC[10]. In that case, the enriched proteins were analysed following SDS-PAGE of the IMAC eluted fractions and tryptic digestion of the excised bands. In the present study, we focused on the low molecular weight proteome using a gel-free approach that did not involve tryptic digestion prior to the MS analysis.

We were able to detect peptides from 638 different proteins. In reference to the existing peptide database[13] which has been generated from >9,000 datasets, 46 out of the previously reported 115 precursor proteins were also represented in the IMAC peptide eluates. As aforementioned (results), these included many plasma and matrix structural proteins. Interestingly, many of the 592 proteins not reported in the peptide database and representing a wide variety of cellular pathways have been reported to be involved in BC and/or have already been evaluated as BC biomarkers:

Urinary Fibrinogen fragments [23], Peroxiredoxin [24, 25], Tenascin [26], have all been associated with specific BC phenotypes. Similarly, urinary Apolipoprotein A-II, Ceruloplasmin and Complement C4 were found to be over-represented in BC[27]. Hyaluronidase activity[28] and TGF-beta[29] have also been investigated in association with BC. Moreover, Ceruloplasmin, Dipeptidyl peptidase, Aminopeptidase isoforms, Leukine-rich alpha-2 glycoprotein are some of the proteins that were also selectively enriched from BC samples in our previous IMAC study involving analysis of the urinary (higher molecular mass) proteome[10].

Of the urinary proteins, selectively enriched in BC samples, several have also been associated with BC at the cell and/or tissue level: GST isoforms were suggested as BC immunocytochemical markers to increase diagnostic accuracy of urine cytology[30] . S100A9 has been suggested as a prognostic marker for invasive BC following microarray analysis[31], and Thymosin beta -4 was found up-regulated in BC by tissue microarrays[32]. In addition, Tumour protein 63 identified in T1 BC samples in our series, has been reported to negatively regulate EMT and metastasis in BC[33]; Y-box-binding protein 2 identified in T2+ BC samples promotes BC progression[34] and NKG2D ligand 1 is presumably involved in the inhibition of tumour growth during BC treatment with BCG[35]. Collectively, these results support the efficiency of the proteomics strategy to enrich for proteins of relevance to BC.

Among the most prominent peptides detected were fragments from histone isoforms and histone regulators: Multiple peptides of Histone H4, H2A and H2B were identified in BC urines. Additionally, proteins involved in histone modifications and regulation were also enriched, such as Histone-lysine N-methyltransferase SETD1A, POU domain-class 2 involved in histone H2B transcription[36] and Histone acetyltransferase p300. The latter is associated with DNA damage response, resulting in alterations of H2A.X, mainly through Bromodomain adjacent to zinc finger domain protein-2B[37], a protein which was also identified in our study. Another related protein is NIF-1 (NRC-interacting factor; Zn finger 335). NIF-1 seems to act as a key molecule between two sub-complexes: one that displays histone methyltransferase activity and another that is involved in nuclear receptor-mediated transcription[18, 38]. This dual action promotes the expression of several transcription factors[18, 39] among which HoxA1 and Sox9[18, 40], as supported by siRNA studies.

Based on this evidence supporting a functional association of H2B and NIF-1, in combination with a lack of prior studies of these proteins in BC, H2B and NIF-1 were selected to be further evaluated using immunological-based assays. Increased urinary levels for these proteins were observed in the presence of urothelial carcinoma. The most aggressive T1 and T2+ groups exhibit the highest levels of NIF-1 and Histone H2B. Nevertheless, we should note that no direct (per sample) correlation between the discovery set (LC-MS data) and verification set (ELISA data) could be observed as the two proteins were not detected in the benign and non-invasive samples in the former (discovery) whereas the ELISA assays demonstrated that some of these samples did contain detectable amounts of the respective proteins (**Supplementary Table 3**). Given the low number of samples in the discovery set and the high technical variability associated with IMAC fractionation, statistically meaningful comparison between the LC-MS and the ELISA data is not possible. The high inter-individual variability, particularly in the case of H2B, should also be noted, supporting

the presence of co-founders affecting the levels of these urinary proteins. Hematuria does not appear to affect NIF-1 and H2B measurements based on the samples analysed, even though analysis of a larger number of samples will be required to confirm this initial observation. In addition, no correlations between the NIF-1/H2B levels and total urinary protein could be observed ($p>0.3$ in all cases according to Pearson and Spearman's rho correlation tests).

The fact that normalization to total protein was found to affect the value of H2B and NIF-1 as biomarkers may relate to the association of bladder cancer with increased protein in urine[41, 42], rendering normalization according to protein inappropriate for this disease, as also supported by recent publications[17]. Moreover, we should note that even though an initial characterization of the performed assays was conducted (as described in the materials and methods), the need for further characterization of the assays including an accurate determination of LOQ, linear range for quantification, matrix effects etc. according to regulatory requirements is required for clinical implementation[43, 44]. In addition, even though recognition of the expected protein bands is confirmed by the manufacturer, further investigation of the antibody specificity including a detailed characterization of potential isoforms of the two proteins in urine is required.

At the tissue level, a clearer pattern was received, strongly suggesting the association of these proteins with BC stage. In this case, NIF-1 expression decreases, whereas H2B increases with progressing tumour stage. The tissue H2B data are in good agreement to the urine H2B expression pattern. This observation can probably be explained in part by the polyploidy frequently observed in neoplastic cells, resulting in increased histone levels.

In the case of NIF-1, opposing trends of expression between the tissue (decrease in T2 cancers) and urine (increase in T2 cancers) are observed. The reason for this observation is unknown at present. One possible explanation could be that with progression to advanced stages the number of shed cancer cells increases substantially in the urine of patients.

Therefore, although NIF-1 production per cell is reduced, the urinary concentration of the protein increases. Further experiments are required to test this hypothesis, including an investigationn of urine and tissue samples from the same patient.

Our current knowledge on NIF-1 is limited; in addition, no NIF-1 correlations to any type of cancer have been reported. However, it should be noted that our observations appear to match well with reported expression patterns of the NIF-1 direct targets, Sox9 and HoxA1: both factors show negative or weak expression levels in urothelial cancer according to the Human Protein Atlas (www.Proteinatlas.org), in which appears to be a tissue-specific pattern. This preliminary evidence suggests a functional involvement of NIF-1 in combination with Sox9 and HOXA1 in BC tumour progression. Specifically, we may hypothesize that loss of NIF-1 expression leads to BC progression through silencing of Sox9, a transcription factor that binds CDKN1A (cyclin-dependent kinase inhibitor 1A) promoter and inhibits cell growth in vivo[40]. Alternative mechanisms may include interactions of NIF-1 with other tumour suppressors like DBC-1[18]. In these aggressive states where polyploidy is observed, in contrast to NIF-1, H2B levels are elevated, reflected in the observed NIF-1/ H2B inverse association. NIF-1 could also be associated with H2B regulation at the epigenetic level, either by its integral methyltransferase activity or through interactions with ASCOM (NRC-methyltransferase) complex[18]. Under hormone induction, alterations of H3 methylation status could cause H2B/H2A rearrangements and nucleosome displacement[45].

Collectively, the presented study has enriched our knowledge on the peptidome of human urine and more importantly, has revealed novel potential biomarkers such as NIF-1, possibly of functional relevance in BC progression. This study thus opens up many new avenues of research targeting, validation of the differential expression of these proteins in BC and investigation of their specific context of use. **Table 2** highlights additional promising candidates from our findings, selected based on their potential functional relevance and

existing knowledge in the field. Moreover the confirmation of the built hypothesis on NIF-1/Sox9 involvement in BC progression as well as investigation of the proteolytic mechanisms leading to the generation of the reported urinary peptides from their precursor proteins in relation to disease progression warrant further investigation.

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AUTHOR INFORMATION

Corresponding Author

* Telephone: 30 210 65 97 506, fax: 30 210 65 97 545

Email: vlahoua@bioacademy.gr

NOTE

H. Mischak is founder and co-owner of Mosaiques Diagnostics, Germany and P. Zürbig and M. Frantzi are employed by Mosaiques Diagnostics. All other authors declare that they have no competing interests.

ABBREVIATIONS

Ab: Antibody, ACN: Acetonitrile, amu: Atomic Mass Unit, ASCOM: Activating signal cointegrator-2 complex, BC: Bladder Cancer, BCG: Bacillus Calmette-Guerin, BPH: Benign prostatic hyperplasia, BTA: Bladder Tumour Antigen, CDKN1A: Cyclin-dependent kinase inhibitor-1A, DBC: Deleted in Breast Cancer, ECM: Extracellular Matrix, ELISA: Enzyme-linked immunosorbent assay, EMT:Epithelial to Mesenchymal Transition, ESI: Electrospray Ionization, EuroKUP: European Kidney and Urine Proteomics, FTMS: Fourier Transform Mass Spectrometry, GO: Gene Ontology, GTP: Guanosine Tri-Phosphate, H2B: Histone2B, HCD: High energy Collision Dissociation, HKUPP: Human Kidney and Urine Proteome Project, HOXA1: Homeobox A1, HPLC: High Performance Liquid Chromatography, IHC: Immunohistochemistry, IMAC: Immobilized Metal Affinity Chromatography, IPI: International Protein Index, LC: Liquid Chromatography, LTQ: linear trap quadrupole, MRM: Multiple Reaction Monitoring, MS: Mass Spectrometry, NIF-1: NRC interacting factor-1, NKG2D: Natural-killer group 2, member D, NMP22: Nuclear Matrix Protein 22, NRC: Nuclear receptor coactivator, RGB: Red Green Blue, Robo: Roundabout homolog, SDS PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis, Sox9: Sex determining region Y-box 9, SPSS Statistical Package For Social Sciences, TBS: Tris-buffered saline, TNM: Tumour Nodes Metastases, WHO: World Health Organization, Zn: Zinc.

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Table 1. Pearson Correlation analysis for H2B and Zn finger protein 335/NIF-1 based on the respective OD values from the same tissue specimens. An inverse correlation of the tissue levels of the two proteins is predicted.

Correlation : NIF-1 & H2B mean optical density

		H2B Optical Density	NIF-1 Optical Density
H2B Optical Density	Pearson Correlation	1	-,391*
	Sig. (2-tailed)		,036
	N	29	29
NIF-1 Optical Density	Pearson Correlation	-,391*	1
	Sig. (2-tailed)	,036	
	N	29	29

*. Correlation is significant at the 0.05 level (2-tailed).

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Table 2. Short-list of promising candidates among the peptidomics findings meriting further investigation. Selection was based on strength of evidence for differential expression in the discovery phase (e.g. selective presence in specific stages, number of samples expressing the peptide etc.) but also presumed functional relevance to BC in combination to lack of existing association with BC, based on existing knowledge.

	Protein	Stage Identified	Reported Function
1	Bromodomain adjacent to zinc finger domain protein 2B	T2+	Transcription regulation, DNA damage response, Regulation of H2A.X [46]
2	Histone acetyltransferase p300	B	Chromatin remodeling –Removal of H2A-H2B dimers DNA damage response [47]
3	Zinc finger protein 703	T2+	Transcription regulation, Breast cancer oncogene [48]
4	Transcription factor Sp2	T1	Zinc associated transcription factor, Oncogene [49]
5	POU domain, class 2, transcription factor 1	T1	H2B regulation, Somatic and cancer stem cell identity [50, 51]
6	Roundabout homolog 4	T1,T2+	Slit protein Receptor – Angiogenesis, Tumour vascular targeting [52]
7	Slit homolog 2	T1	Inhibition of cell migration [53]
8	A disintegrin and metalloproteinase with thrombospondin motifs 12	T1	Zinc Metalloprotease, Tissue remodelling & Angiogenesis [54]
9	Meprin A subunit alpha	T1	Zinc Metalloprotease. Pro-angiogenic & Pro-migratory activity [55]

Figure legends

Figure 1. Schematic represantation of the study design.

Figure 2. (Precursor))Protein classification of the received identifications based on protein biological function. Classification in 13 categories was conducted (based on GO and Medline search) that include: immune response, cytoskeleton arrangement, ECM and cell adhesion associated proteins, metabolic processes, transport, stress response, proliferation and/or apoptosis, differentiation, transcription regulation, chromatin remodelling, proteolysis and signal transduction.

Figure 3. **(A)** Zn finger protein 335/ NIF-1 and **(B)** H2B ELISA data, following analysis of a total number of 158 (NIF-1) and 147 (H2B) urine samples. Mean values, standard deviation and number of positive samples for the different categories and respective box plots are shown (raw data per sample are provided in **Supplementary Table 3**).

Figure 4. Representative images from immunohistochemical analysis of BC tissue sections and respective quantification of **(A)** Zn finger protein 335/NIF-1 and **(B)** H2B expression. A colour deconvolution plugin from Image J software enabled the quantitation of signal intensity between the different BC stages .

* : statistical significant difference

Supplementary Table 1. Clinical information for urine and tissue samples used in the study. Three xls sheets are included, one for each of the different studies: "Urine samples employed in LCMS" (discovery set); "Urine samples employed in Elisa" (verification set) ; "BC Tissue specimens" (IHC set)

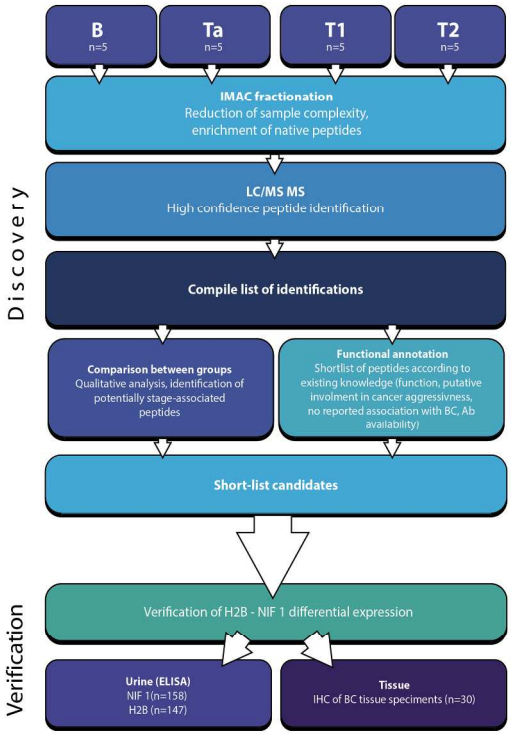
Supplementary Table 2. List of urinary peptides and precursor proteins identified following IMAC fractionation and LC-MS/MS. Two xls sheets are included: "Peptides" showing the MS identifiers for each peptide and "Proteins" summarizing list if precurson proteins and respective functional annotation analysis.

Supplementary Table 3. Urinary ELISA data and immunohistochemical measurements for NIF-1 and H2B per sample. Three xls sheets are included: "Elisa data" showing values per sample and graphs following different normalization means ; "IHC values and graphs" showing mean measurements in reference to different grading systems; and "IHC-Image J calculations" showing intensity measurements per image and section.

Supplementary Figure legend

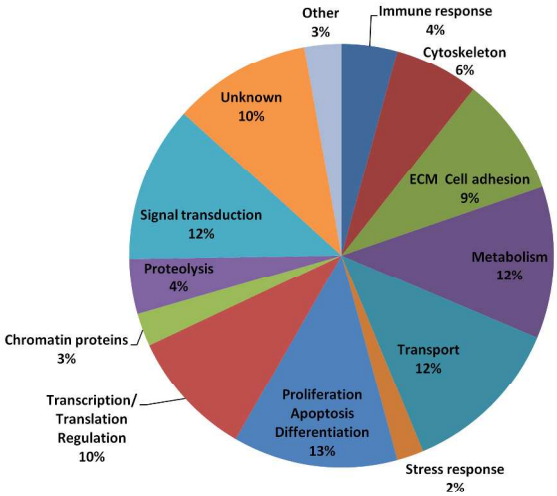
Supplementary Figure 1. Images acquired following staining of bladder cancer tissue specimens for H2B and NIF-1, and representing cases of intensity deviations (from the mean throughout the section). Variations in intensity measurements are usually associated with biological variability within the same section, (e.g. different nuclei morphology, staining intensity per cell) reflecting more likely differences in the degree of aggressiveness of the cancer cells.

Figure1

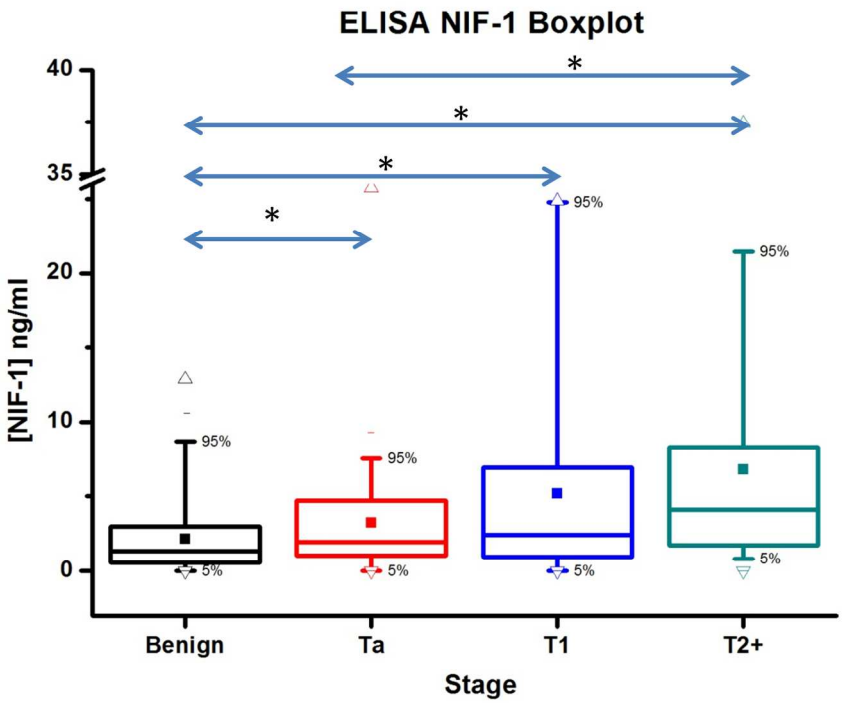


254x190mm (300 x 300 DPI)

Figure 2

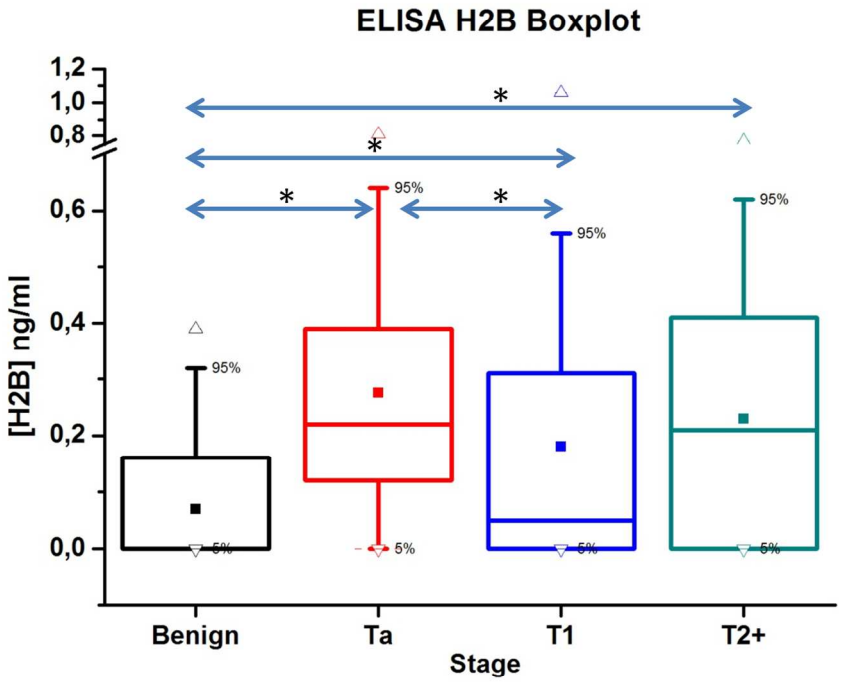


254x190mm (300 x 300 DPI)



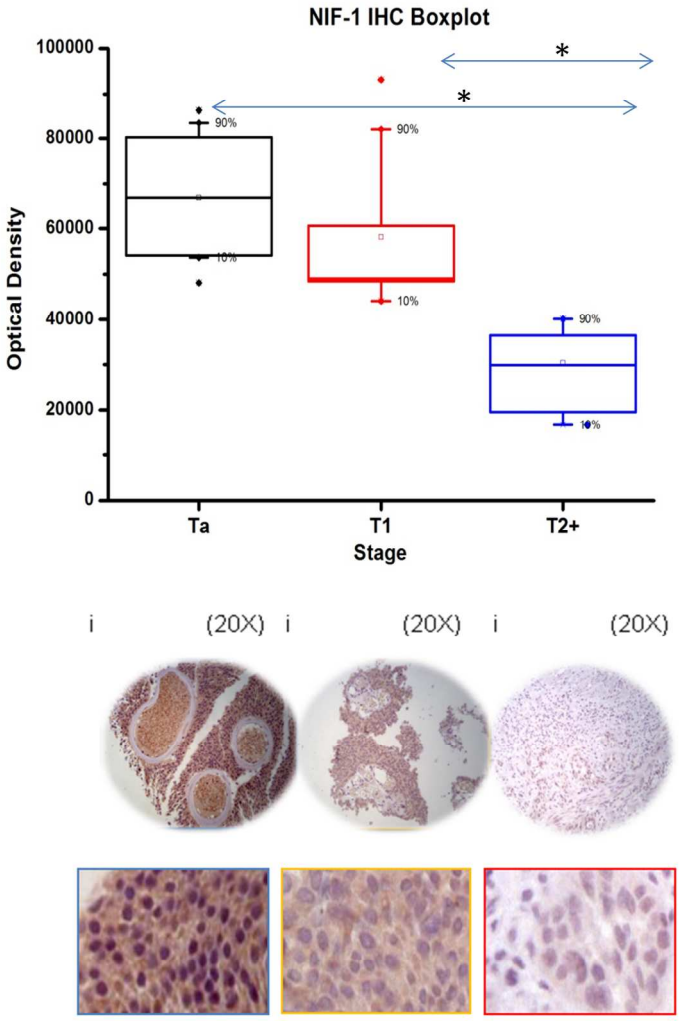
	n	[NIF-1] ng/ml	st.dev	Positive
Benign	47	2,19	2,73	38/47
Ta	44	3,26	4,11	42/44
T1	36	5,19	6,48	34/36
T2+	31	6,80	7,97	30/31

154x190mm (300 x 300 DPI)

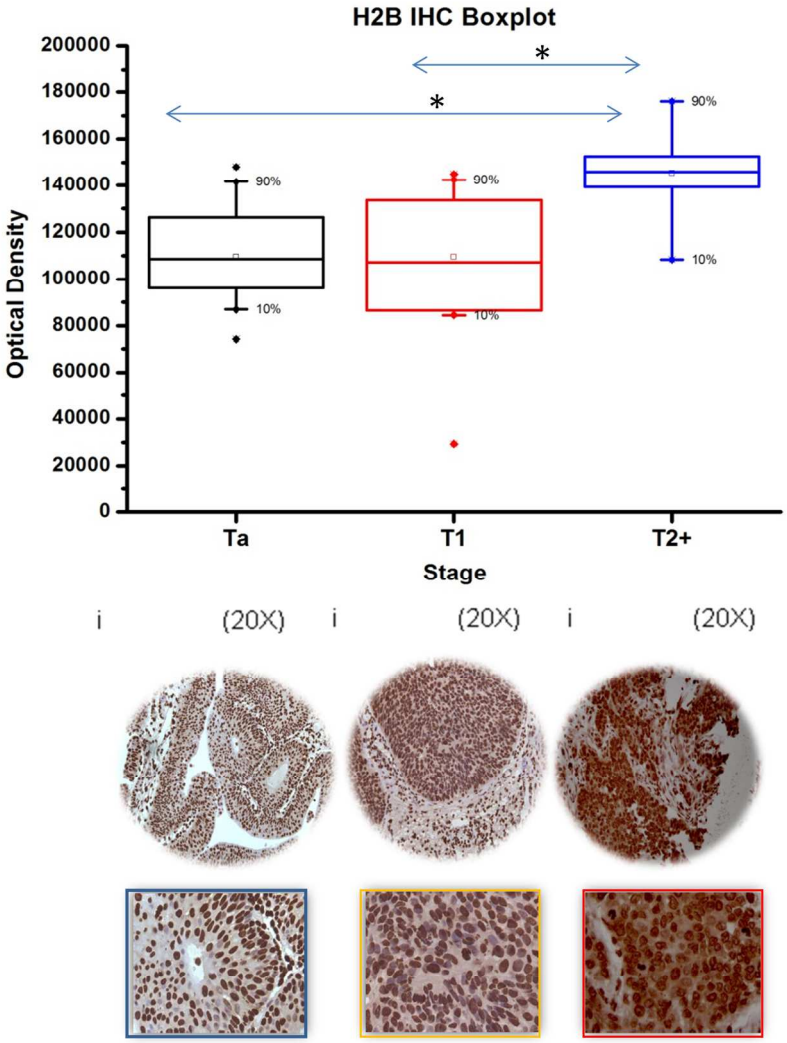


	n	[H2B] ng/ml	st.dev	Positive
Benign	39	0,069	0,111	15/39
Ta	40	0,262	0,212	35/40
T1	36	0,181	0,235	19/36
T2+	32	0,230	0,223	23/32

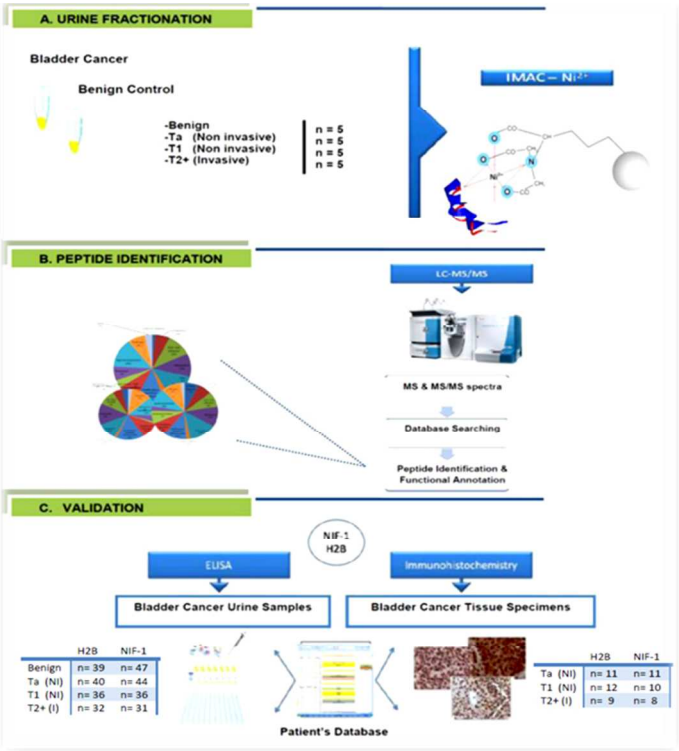
149x190mm (300 x 300 DPI)



173x186mm (300 x 300 DPI)



161x176mm (300 x 300 DPI)



81x60mm (300 x 300 DPI)