EUROPEAN UROLOGICAL SCHOLARSHIP PROGRAMME (EUSP)

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1. Background

Bladder cancer (BC) represents a serious healthcare concern worldwide. At any given time, more than 500,000 people in the United States have had or are currently living with a diagnosis of BC. BC is the most expensive solid tumour to treat due to its high recurrence rate and need for continued surveillance. BC is estimated to cost USD 3.7 billion/per annum in the U.S. with the majority of this cost spent on diagnosis and surveillance of the disease. Despite these startling figures, little progress has been made to improve BC early diagnosis, disease survival and reduction of costs related to lifelong follow-up in the previous decades.

Treatment and staging are carried out by transurethral endoscopic resection of the tumour, but over 50% of patients will present with at least one recurrence within five years and 5-30% will progress to muscle-invasive BC (MIBC). After tumour resection, patients are monitored with an intensive surveillance protocol including frequent cystoscopies for the first two years, followed by less frequent observations if patients remain recurrence-free. With age and smoking being the two most common risk factors, the combination of our ageing population and these life-long invasive and costly cystoscopic monitoring procedures will only negatively impact our healthcare system.

BC prognosis and survival is directly related to the stage (Ta and T1, non-muscle-invasive tumours, T2-T3-T4, muscle-invasive cancers) and grade of disease presentation with low progression rates in low-grade (LG) tumours and high progression and low survivals rates in high-grade (HG) tumours.

Cystoscopy and cytology are currently the gold standards for BC detection in symptomatic patients. Although useful, cystoscopy is an invasive and costly test. While cytology is highly specific (90-100%), it has a low sensitivity of 60-90% in HGBC, only 35-50% in LGBC and as low as 15% during BC follow-up of recurrent tumours.

Intense work is being done in the field of bladder tumour markers with the goal of early BC diagnosis (initial diagnosis and at tumour recurrence) without the need for invasive cystoscopic evaluation. Voided urine provides an excellent source of exfoliated cells from the bladder and is the ideal medium for non-invasive BC biomarker research. Numerous urine-based markers have been investigated and some have been found to have better sensitivity than cytology, but frequently with lower specificity. For most methods, the sensitivity is lowest for early stage disease; therefore a negative result cannot fully rule out BC. Since the ideal biomarker should be urine-based (due to its non-invasive nature), stable, sensitive, specific and cost-effective, the currently available markers are
all lacking one or more of these requirements. The development of new non-invasive BC markers, used either alone or in combination with the current markers, should therefore be a high-priority research goal in order to help diagnose BC at an early stage and distinguish between LG and HG disease.

2. Epigenetic and bladder cancer
Epigenetics represent heritable traits in cells that do not involve permanent alterations of DNA that can lead to the development and progression of cancer. These include DNA methylation, histone modifications and miRNA–mediated regulation. DNA hypermethylation, the most studied epigenetic mechanism, occurs when DNA becomes methylated at CpG-rich regions commonly located in the gene promoter regions, leading to gene inactivation. Methylated genes have been linked to BC, many related to pathways involved in genetically unstable BC. Several groups have studied the development of urine-based DNA methylation assays with high sensitivity, specificity, and reproducibility while using the fewest markers possible for cost effective, diagnostic utility. The detection of methylated biomarkers has several advantages over conventional genetic markers. CpG island hypermethylation constitutes a positively detectable signal as opposed to a loss of signal. Aberrant CpG hypermethylation usually does not occur in normal cells.

3. Brief critical assessment of recently discovered BC urinary biomarkers
Recent technological advances have allowed increased breadth to the field with the identification of the number of newer markers. Overall, while these urinary markers achieved high sensitivity and specificity, there are still a number of limitations preventing widespread clinical applicability which are as follows:

a. Few, if any, of these discovered urinary markers have been validated on a large sample set.

b. There is a lack of standardisation of markers and need for simpler detection methods.

c. These markers were identified by mining the data obtained by only one technique/biologic approach (genetics or epigenetics) often without mechanistic rationale for the biomarker choice.

d. Many biomarkers have failed as most are unable to detect both LG and HG BC characterized by distinct molecular pathways and share few molecular alterations.
Specific applications for primary diagnosis vs. detection of recurrent disease need to be clarified. The performance of these markers is likely to be different in these two different settings.

4. Study design
Case control study evaluating gene expression of DNA methylation- and histone modification-related genes as epigenetic urinary biomarker panel of BC.

4.1 Study cohort
We accrued 167 controls and 202 BC cases. All study participants provided written informed consent for use of urine for research and the study protocol was approved by the ethical committee of our institution. Urine sample was collected before the endoscopic resection or radical cystectomy. Patients diagnosed with a urologic disorder other than BC constitute the control group. Exclusion criteria for control cases were: history of BC, prostate (PCa) or kidney cancer, suspicion of concomitant PCa (defined by an abnormal DRE), concomitant kidney cancer (defined by the detection of an unknown renal mass by ultrasound or CT scan), carcinoma of the upper urinary tract, urinary infection and macrohematuria. Smoking habits were captured in both cases and controls.

4.2 Study sample preparation
Before endoscopic resection or radical cystectomy, freshly voided urine (50 mL) was collected from each patient. RNA was then extracted using the ZR Urine RNA Isolation Kit™ (Life Technologies) within 30 minutes and converted immediately to cDNA. Urinary cDNA was then stored at -20°C until use.

4.3 Arrays Analysis
The Applied Biosystems® TaqMan® Array Human DNA Methylation And Transcriptional Repression 96-well Plate (Life Technologies) was used for the arrays analysis. It contained 24 assays as DNA methylation and histone modification associated genes and 8 assays as candidate endogenous control genes (see Table 1). All assays were plated in triplicate. Of the total cohort, 23 patients (9 controls and 14 BC) were selected for the arrays analysis.

4.4 Assays Analysis
The most stable endogenous control genes and the most interesting genes of interest were selected from the arrays analysis and tested on the entire biobank. QuantStudio™ 6-7 Flex Real-Time PCR
System (Life Technologies) was used for the assays analysis. For qPCR, 5μl of TaqMan Universal PCR Master Mix (Applied Biosystems), 0.125μl of Taqman Gene Expression Assay and 1μl of cDNA were brought to a total volume of 10μl adding DNAse- and RNAse-free water. The PCR conditions were: 90°C for 10’, 95°C for 15” repeated for 40 cycles and 60°C for 1’. Each sample was assayed in duplicate. Amplification plots were assessed using the detection software SDS v2.0.1 (Applied Biosystems) to confirm that the threshold cycle (Ct) value corresponded with the midpoint of the logarithmic amplification.

5. Results

5.1 Arrays analysis
Of the 8 endogenous control genes tested with the arrays analysis, GUSB and HPRT1 were those that were found with the highest stability value (average expression stability of 90%) and therefore selected for the final analysis.

Of the 24 genes of interest, TRDMT1 and HDAC9 were significantly different expressed between control and BC patients. Among BC patients, DNMT1 and HDAC3 were significantly different expressed between HG and LG patients (p=0.03 and p=0.008, respectively).

Finally, GUSB, HPRT1, TRDMT1, HDAC9, HDAC3 and DNMT1 were all selected for the final analysis.

5.2 Assays analysis (preliminary results)
Around 20% of samples were excluded because of less than 10µg of RNA was obtained or there was low RNA quality. All four genes (TRDMT1, HDAC9, HDAC3 and DNMT1) were significantly less expressed in the control group (p<0.001, p=0.004, p=0.002 and p<0.001, respectively). Further analyses are ongoing investigating the different gene expressions between LG and HG BC, muscle invasive BC and non-muscle invasive BC, female and male patients and smokers and non-smokers.

6. The team
The study was conducted under the supervision of Prof. Shariat, Prof. Klatte and Prof. De Martino.

Prof. Shariat is a world-renowned academic uro-oncologic surgeon with extended research career and a particular expertise in BC. He has a proven track record in translational research and of multi-institutional studies bringing together groups working in different countries. He has several multi-institutional and international research projects under way.
Prof. Klatte is an academic uro-oncologic surgeon and a closed collaborator of Prof. Shariat in the Department of Urology.

Prof. de Martino is a biologist with a deep knowledge of oncologic urology and a large amount of experience in BC gene expression investigations.

**Table 1**

DNA methylation and histone-modification associated genes and candidate endogenous control genes for the arrays analysis.

<table>
<thead>
<tr>
<th>18S</th>
<th>GAPDH</th>
<th>HPRT1</th>
<th>GUSB</th>
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**Published articles (with acknowledgment to the EUSP)**


*These authors contributed equally as co-first authors


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