Abstract. The aim of the present study was to identify and test a urine marker panel of genes involved in DNA methylation and histone modification for the detection of urothelial carcinoma of the bladder (UCB). RNA samples obtained from the voided urine of 227 patients with asymptomatic microscopic haematuria (AMH) were analysed. Gene array analysis was performed on 18 randomly selected cDNA samples, which revealed that histone deacetylase 9 (HDAC9), HDAC3, tRNA (cytosine-5′)-methyltransferase1 and DNA methyltransferase 1 were differentially expressed between patients with UCB and control subjects. Subsequently, reverse transcription-quantitative polymerase chain reaction analysis was employed to test the performance of the identified four-gene panel on the remaining 209 cDNA samples. In this targeted discovery cohort, all four genes were significantly associated with UCB on univariable analyses [each odds ratio (OR) >2, P<0.05], but only HDAC3 was significant following multivariable analysis (OR=2.8, P=0.011). The addition of HDAC3 to a base risk factor model improved its accuracy by 1.4%. These data suggest that urinary HDAC3 is associated with the presence of UCB in patients with AMH; however, HDAC3 improved the accuracy of the established risk factors only to a marginal extent.

Introduction

Urothelial carcinoma of the bladder (UCB) is the second most common urological cancer with 430,000 new cases diagnosed worldwide annually (1). A total of 80% of the patients present with non-muscle invasive bladder cancer (NMIBC) (2), which has a recurrence rate of ~50% (3). NMIBC requires intensive, invasive follow-up and repeat interventions, making it one of the most costly malignancies per patient (4).

Cystoscopy is the established standard tool for the workup of patients with asymptomatic microscopic haematuria (AMH) (5), but it is invasive, costly and imperfect. AMH is a major challenge for health care providers due to its high prevalence, limited healthcare resources and low adherence to unpractical algorithms in current guidelines (6). Ultimately, <5% of patients investigated are subsequently diagnosed with UCB (7). Intense research has therefore been undertaken to identify easier, better, faster, more cost-effective urinary markers for UCB detection, aiming to single out the patients who may truly benefit from cystoscopy, and avoiding unnecessary testing and possible iatrogenic complications (8).

Indeed, several urine-based markers have been shown to have a potential use as a non-invasive adjunct to cystoscopy for the detection of UCB (9-15), but none has yet fulfilled the criteria to be recommended for routine care (6).

Epigenetic changes play a key role in UCB pathogenesis and progression. Indeed, UCB is defined by multiple epigenetic events that inactivate known tumour suppressors through DNA methylation and histone modification (16). In human DNA, methylation is a molecular process targeting CpG dinucleotides. Most genes have unmethylated CpGs in their 5′ regulatory region. DNA methyltransferases (DNMTs) catalyse the methylation of these CpGs, thereby inducing transcriptional silencing, leading to suppressed tumour growth and cancer cell invasion (17).

Given the unmet need for urinary biomarkers in the diagnostic setting and the pivotal role of epigenetic modifications in
UCB carcinogenesis, we hypothesised that urinary epigenetic biomarkers are associated with the presence of UCB. To test this hypothesis, we identified and validated urinary markers involved in DNA methylation and histone modification for UCB detection in patients with AMH.

Materials and methods

Study cohort. We prospectively collected voided urine samples from 300 patients with AMH between 2013 and 2016. All individuals were evaluated by urinalysis, cystoscopy, ultrasound or computed tomography scan of the urinary tract and bladder biopsies, when appropriate. None had suspected urinary tract infection, known IgA nephropathy, indwelling catheters, visible haematuria, known UCB, concurrent or previous upper tract urothelial carcinoma, renal cell carcinoma or prostate cancer. The patients provided written informed consent and all the procedures were carried out under the Good Scientific Practice Standards. The Ethics Committee of the Medical University of Vienna approved the present study (Study ID 1162/2013).

Pathological evaluation. Two expert genitourinary pathologists processed and reviewed all the pathological specimens according to standard protocols. Pathological stage was assigned according to the 2009 TNM classification (18), and grade according to the 2004 WHO/ISUP classification (19).

Urine processing and RNA quality control. All analysed urine samples were negative for leukocytes and nitrite, but positive for erythrocytes. Midstream-voided urine samples (50 ml) were collected in a sterile cup prior to instrumentation or contrast studies. For each sample, a unique identification number was assigned prior to laboratory processing, and the laboratory staff were blinded to the suspected diagnosis. Total RNA was purified using the ZR Urine RNA Isolation Kit (Zymo Research Corporation) and RNA quantity and quality were assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) according to the manufacturer’s instructions. Of the 300 RNA samples, 73 were excluded from further studies due to low quantity and/or quality. Total RNA (0.5 µg) from the 227 remaining samples was used to synthesise complementary DNA (cDNA) using Taqman Reverse Transcription Reagents (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Gene discovery by qPCR. The four target genes [DNMT1, histone deacetylase 3 (HDAC3), HDAC9, tRNA (cytosine-5′)-methyltransferase 1 (TRDMT1)] exhibiting differential expression were subsequently analysed by standard RT-qPCR on cDNA synthesised from RNA of our independent cohort constituted by the 209 remaining urine samples. Among the eight candidate endogenous reference genes, ACTB, HPRT1 and GUSB exhibited the highest expression stability and were therefore used in subsequent validation experiments.

The TaqMan® Gene Expression Assays (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used for DNMT1 (assay ID Hs00154749_m1), HDAC3 (Hs00187320_m1), HDAC9 (Hs00206843_m1), TRDMT1 (Hs00189402_m1), GUSB (Hs99999908_m1), HPRT1 (Hs99999909_m1) and ACTB (Hs99999903_m1). The RT-qPCR mixture was prepared for each sample as follows: 10 µl TaqMan™ Universal PCR Master Mix, no AmpEraser™ UNG (Applied Biosystems; Thermo Fisher Scientific, Inc.), 1 µl TaqMan Gene Expression Assay and 3 µl cDNA were brought to a total volume of 20 µl by adding DNase and RNase-free water. Each sample was assayed in triplicate. The RT-qPCR reactions were analysed on the system used for arrays analysis with the following conditions: 90°C for 10 min and 40 cycles at 95°C for 15 sec followed by 60°C for 1 min. Amplification plots were assessed using the detection software SDS v2.0.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.) to confirm that the quantification cycle (Cq) value corresponded with the midpoint of the logarithmic amplification. Data were then processed using DataAssist v3.0.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.). Cq values not detected in the measurable range (Cq>40) were considered as not determinable.

Statistical analysis. Categorical variables are presented as numbers and percentages, and continuous variables as median and interquartile range (IQR). Group differences in categorical variables and continuous variables were analyzed
with chi-square tests and Mann-Whitney U tests, respectively. The proportions of patients with detectable HDAC3, HDAC9, TRDMT1 and DNMT1 were compared using Chi-squared tests. For quantitative analysis, the relative expression of HDAC3, HDAC9, TRDMT1 and DNMT1 was determined by normalising against their levels for the three reference genes (ΔCq method). Inter-class fold changes were calculated using the 2^−ΔΔCq method (21). Odds ratios (OR) and 95% confidence intervals (95% CI) were obtained from univariable and multivariable logistic regression models. The full multivariable model combined all significant variables of the univariable analyses, and the reduced model was obtained after including variables that had a P-value of <0.1. The area under the curve (AUC) quantified the predictive accuracy. Statistical testing was two-sided and a P-value <0.05 was considered statistically significant. Analyses were all conducted with R 3.5 (The R Project for Statistical Computing).

Results

Discovery experiments. The gene array included 24 target genes associated with DNA methylation and transcriptional repression, as well as 8 reference genes. It was run on 18 randomly selected patients (11 UCB cases and 7 controls). HDAC9 and TRDMT1 were found to be differentially expressed between cases and controls (fold change 3.5 and 2.6, respectively) (Table S1).

Among UCB cases, 7 had low-grade and 4 had high-grade disease. DNMT1 and HDAC3 exhibited a higher expression rate in high-grade vs. low-grade UCB (fold change 2.1 and 2.2, respectively). Tumour stage, smoking status and sex did not exhibit relevant differences between cases and controls or between high- and low-grade tumours.

Targeted discovery experiments/validation. The expression of HDAC3, HDAC9, TRDMT1 and DNMT1 was analysed in cDNA samples derived from the 209 remaining urine samples. The characteristics of the 209 patients in this targeted discovery cohort are shown in Table I.

The controls were younger (P=0.001), were more likely to be female (P=0.02) and less likely to be smokers compared with the UCB group (P=0.008). In the UCB group (n=117), 113 patients (97%) were diagnosed with NMIBC and 4 (3%) with muscle-invasive UCB. A total of 78 cases had low-grade UCB (67%) and 39 had high-grade UCB (33%).

DNMT1 and HDAC3 exhibited a higher expression rate in high-grade vs. low-grade UCB (fold change 2.1 and 2.2, respectively). Tumour stage, smoking status and sex did not exhibit relevant differences between cases and controls or between high- and low-grade tumours.

Using a backward selection multivariable analysis based on the full model, age (P<0.001), smoking status (P=0.042) and HDAC3 (P=0.011) remained independently associated with the presence of UCB. Addition of HDAC3 to a base model for prediction of UCB with age, sex and smoking status improved the AUC by 1.4% (79.8 vs. 81.2%, Fig. 1), with a sensitivity and specificity of 89 and 63%, respectively, at the optimal cut-off. Combination analysis of various genes did not achieve a greater improvement in AUC (data not shown). The sensitivities, specificities, positive and negative predictive values of all four markers are reported in Table SII. Presence of HDAC3 was not associated with any other tumour characteristics (Table SIII).

Discussion

DNA methylation and histone modification genes play a key role in UCB development and progression (16), but only limited data are available on their expression in urine samples (22). Specifically, HDACs deacetylase the amino acid lysine on histone proteins, thereby rendering the chromatin structure inactive (23). Expression of HDACs is altered in multiple cancers (24). In UCB, the expression of HDACs was shown to be deregulated, and expression quantity has been associated with higher grade and a higher risk of progression (25). Overall, however, data on the urinary expression of DNMTs and HDACs as diagnostic markers in UCB are scarce.
In the present study, we used pre-made gene arrays to analyse the expression of candidate genes associated with DNA methylation and transcriptional repression, and subsequently investigated those with differential expression in an independent set of patients. Among all genes, *HDAC3* was found to be independently associated with UCB. Furthermore, it improved the accuracy of a multivariable base model, albeit only to a marginal extent of 1.4%. However, there is no generally accepted cut-off in AUC increase that is considered clinically significant. *TRDMT1* and *HDAC9* were found to be upregulated in UCB patients compared with controls. To the best of our knowledge, *TRDMT1* (syn. *DNMT2*) has not yet been described as a potential marker for UCB. *TRDMT1* upregulation was detected in several tumour samples, and it may be involved in gene silencing and RNA-mediated epigenetic
heredity (26,27). Similarly, altered expression of HDAC9 has not been associated with the presence of UCB; however, its overexpression has been described in multiples cancers, and appears to promote tumour cell growth and cell cycle progression (28-30), all of which are considered key steps in UCB development and progression. In the present study, both genes were found to be overexpressed in UCB samples, although they failed to demonstrate an independent association on the multivariable analysis. This may be due to the small sample of patients analysed, limiting the statistical power.

DNMT1, conversely, has been previously shown to be overexpressed in UCB. In an immunohistochemical study on 130 UCB samples, DNMT1 immunoreactivity was higher in UCB samples compared with controls (31). This is in agreement with our observation that DNMT1 is more frequently detected in urine samples from UCB patients. Moreover, DNMT1 has been found to be associated with more advanced tumour stage (24% expression in ≤T1 vs. 55% in T2-4 tumours) (31). While we did not observe an association of urinary DNMT1 with stage, it is important to note that only 4 patients had muscle-invasive UCB. However, differential expression according to tumour grade was observed. Finally, recent findings support a possible promising role of DNMT1 as early biomarker for UCB (32).

The genes differentially expressed in the first part of our study were further analysed in an independent group of 209 UCB patients. While all four genes were found to be significantly associated with the detection of UCB on univariable analyses, only HDAC3 remained significantly associated on the multivariable analysis. HDAC3 is part of the class I HDAC family, which also includes HDAC1, HDAC2 and HDAC8. Deregulated expression of class I HDACs has been described in UCB, and HDAC inhibitors reduce UCB cell proliferation in vitro (33). Data on the role of HDACs in UCB are inconsistent. While the majority of the studies report overexpression of HDACs in cancers cells, some data suggest that elevated class I HDAC expression may improve prognosis in selected tumours (34). While Junqueira-Neto et al (35) observed higher HDAC3 levels in low-grade tumours compared with controls, others found no differences when data were analysed at the protein level (36). In the present study, the quantity of urinary HDAC3 expression was lower in low-stage, low-grade, unifocal and small-sized UCB, although the differences were not statistically significant (data not shown). These data suggest a possible positive prognostic role of HDAC3 overexpressed in UCB, which must be evaluated in further studies.

No significant association was found between overexpression of the four genes and any of the clinical variables. This implies that the urinary markers are able to detect UCB irrespective of stage and grade, which may be an important prerequisite for a routinely applied urine test.

There were certain limitations to the present study. Following RNA purification, ~25% of RNA samples were excluded due to low quantity/poor quality. This may represent a problem in routine clinical practice, although constant improvements in RNA purification are achieved. Another limitation was patient selection. Indeed, although the cohort was well-selected, the overall number of patients was limited. Moreover, the majority of patients included in this study were affected by UCB, making this patient sample not representative of a typical AMH population. Of the 4 genes, only HDAC3 was associated with UCB on the multivariable reduced analysis. However, the other markers may improve their role in UCB detection if more patients are analysed.

Finally, adding HDAC3 to the base model achieved only a small improvement in UCB detection, minimising its potential impact on routine clinical practice. Further external validation studies with a larger population are required before HDAC3 can be considered as a potential diagnostic marker in patients with AMH. The methylation status of specific substrates of each of these 4 genes may also be measured in order to support our data.

In conclusion, the data presented herein suggest that urinary HDAC3 is associated with the presence of UCB in patients with AMH following adjustment for the effects of standard variables. However, HDAC3 expression improves the accuracy of established risk factors only to a marginal extent.

Acknowledgements
Not applicable.

Funding
The present study was supported by the development fund of the CHUV-University Hospital, the European Urological Scholarship Program and the Anniversary Fund of the Oesterreichische Nationalbank (project no. 15362).

Availability of data and materials
The datasets used and analysed during the present study are available from the corresponding author on reasonable request.

Authors' contributions
IL, MDM and TK conceived and designed the study, analysed and interpreted the results, conducted the statistical analyses and drafted the manuscript. IL, SLH, AH, MS, SFS, MDM and TK acquired the data. TK, MDM, SLH, AH, MS and SFS critically revised the manuscript for important intellectual content. MDM and TK supervised the study.

Ethics approval and consent to participate
Patients provided written informed consent and all procedures were carried out under the Good Scientific Practice Standards. The Ethics Committee of the Medical University of Vienna approved the present study (Study ID 1162/2013).

Patient consent for publication
Patients provided informed consent.

Competing interests
The authors declare that they have no competing interests.
References


