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Urologic Oncology: Seminars and Original Investigations ▮ (2018) ▮▮▮–▮▮▮

UROLOGIC ONCOLOGY

Original article

Detection of urothelial carcinoma, upper tract urothelial carcinoma, bladder carcinoma, and urothelial carcinoma with gross hematuria using selected urine-DNA methylation biomarkers: A prospective, single-center study

Run-Qi Guo, M.D.[a,b,c,1], Geng-Yan Xiong, M.D.[a,b,c,1], Kai-Wei Yang, M.D.[a,b,c], Lei Zhang, M.D.[a,b,c], Shi-Ming He, Ph.D.[a,b,c], Yan-Qing Gong, Ph.D.[a,b,c], Qun He, M.D.[a,b,c], Xue-Ying Li, M.P.H.[d], Zi-Cheng Wang, Ph.D.[a,b,c], Zhen-Qing Bao, M.D.[a,b,c], Xue-Song Li, M.D.[a,b,c,*], Kai Zhang, M.D.[a,b,c,*], Li-Qun Zhou, M.D.[a,b,c,*]

[a] *Department of Urology, Peking University First Hospital, Beijing, China*
[b] *Institute of Urology, Peking University, Beijing, China*
[c] *National Research Center for Genitourinary Oncology, Beijing, China*
[d] *Department of Medical Statistics, Peking University First Hospital, Beijing, China*

Abstract

Introduction: Hematuria is the most common symptom of urothelial carcinomas (UC) but is often idiopathic. Cystoscopy is expensive which involves considerable patient discomfort, and conventional urine cytology for noninvasive UC detection and disease monitoring suffers from poor sensitivity. We aim to evaluate the performance of genes selected from a previous study in detecting UC, especially among patients with gross hematuria, as well as upper tract urothelial carcinoma (UTUC) and bladder carcinoma separately, in voided urine samples.

Methods: Using methylation-specific polymerase chain reaction, we examined the promoter methylation status of 10 genes in voided urine samples among 473 patients at our institution, including 217 UC patients and 256 control subjects.

Results: The final combination of *VIM*, *CDH1*, *SALL3*, *TMEFF2*, *RASSF1A*, *BRCA1*, *GDF15*, and *ABCC6* identified UC with a sensitivity of 0.83 and a specificity of 0.60. Additionally, a panel of selected genes (*CDH1*, *HSPA2*, *RASSF1A*, *TMEFF2*, *VIM*, and *GDF15*) identified UTUC with a sensitivity of 0.82 and a specificity of 0.68, while a panel of selected genes (*VIM*, *RASSF1A*, *GDF15*, and *TMEFF2*) identified bladder carcinoma with a sensitivity of 0.82 and a specificity of 0.53. Remarkably, a different panel (*CDH1*, *SALL3*, *THBS1*, *TMEFF2*, *VIM*, and *GDF15*) identified UC in patients with gross hematuria with 0.89 sensitivity and 0.74 specificity, and sensitivity (0.91) and specificity (0.92) could be achieved when cytology was included.

Conclusions: The selected urine-DNA methylation biomarkers are reliable, noninvasive, and cost-effective diagnostic tools for bladder carcinoma and UTUC, especially among patients with gross hematuria.

Keywords: DNA methylation; Noninvasive detection; Transitional cell carcinoma; Urinary biomarkers; Urothelial carcinoma

1. Introduction

Hematuria is the most common symptom of urothelial carcinomas (UC) but is often idiopathic. Cystoscopy, urinary cytology, and computed tomography urography remain the gold standards in diagnosing UC, including bladder cancer and upper tract urinary carcinoma (UTUC) [1,2]. However, cystoscopy is an expensive test that involves considerable patient discomfort and exhibits

This work was supported by Grants from the Collaborative Research Foundation of Peking University Health Science Center and National Taiwan University, College of Medicine (BMU20120318), the Natural Science Foundation of Beijing Municipality (7152146), the Clinical Features Research of Capital (No. Z151100004015173) and the Capital Health Research and Development Special Fund (2016-1-4077).

[1]Guo Run-Qi and Xiong Geng-Yan contributed equally to this paper.

[*] Corresponding authors.
E-mail addresses: pineneedle@sina.com (X.-S. Li), kaizhangpku@163.com (K. Zhang), zhoulqmail@sina.com (L.-Q. Zhou).

https://doi.org/10.1016/j.urolonc.2018.04.001
1078-1439/

variable sensitivity depending on many factors [3], and conventional urine cytology for noninvasive UC detection and disease monitoring suffers from poor sensitivity, particularly for low-grade carcinoma [4].

Epigenetic changes are common events in cancer, and DNA methylation, which is usually associated with gene expression loss, is well characterized [5,6]. Several reports indicate that bodily fluids, including urine [7–11], gastric juice [12], plasma/serum [13], and sputum [14,15], can be used for the noninvasive detection of cancer through the hypermethylation of DNA. In previous work, we identified that the methylation status of *CDH1*, *HSPA2*, *RASSF1A*, *GDF15*, *BRCA1*, *THBS1*, and *TMEFF2* in tissue samples was significantly associated with tumor stage, tumor grade, and lymph node status in UTUCs [16]. Because bladder cancer and UTUC display clinical and genomic similarities [17], we hypothesized that the same panel of biomarkers in urine sediments might be useful for the noninvasive, early detection of UC for patients with gross hematuria. Thus, we first evaluated the performance of the selected 10 genes (*ABCC6*, *BRCA1*, *CDH1*, *GDF15*, *HSPA2*, *RASSF1A*, *SALL3*, *THBS1*, *TMEFF2*, and *VIM)* from the previous study in detecting UC in voided urine samples, especially among patients with gross hematuria.

2. Patients and methods

2.1. Patients

After the study protocol received approval from the Institutional Review Board of Peking University First Hospital (experimental protocol U20161201, IRB approval number 2016[1253]), a consecutive group of patients with diagnosed urothelial carcinoma at our institution between July 2016 and January 2017 were enrolled in this study. Exclusion criteria were a second previous or concomitant genitourinary malignancy, recurrent or concomitant urinary tract infections, and urolithiasis. All patients underwent standard urological evaluation including cystoscopy and computed tomography urography. UC diagnosis was made by the attending physicians and confirmed by histological evaluation of the resected tissue samples. Controls consisted of patients with benign urologic disorder with no cystoscopically visible or pathologically diagnosed urothelial carcinoma, and no history of urothelial carcinoma or other genitourinary malignancies. All patients in the control group underwent surgery due to benign disease. Clinical and pathological data for the population studied are shown in Table 1.

2.2. Urine sample collection and processing

Voided urine samples (1 per patient) in both groups were collected at Peking University First Hospital during the morning before any surgical procedures. Urine storage and processing conditions were standardized: each approximately 50 ml urine sample was immediately centrifuged at 4,000*g* for 10 minutes, and then the pelleted sediment was washed twice with phosphate-buffered saline and stored at –80°C.

2.3. DNA extraction and sodium bisulfite treatment

All the DNA samples were extracted from cell fragments in urine sediment using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The isolated nucleic acids were stored at −80°C for further analysis. For the bisulfite transformation, approximately 1.5 μg tumor DNA was treated with sodium bisulfite using the EpiTect Fast Bisulfite Conversion Kits (Qiagen, Hilden, Germany).

2.4. Methylation-specific polymerase chain reaction

The methylation status of the 10 gene promoters was analyzed by methylation-specific polymerase chain reaction (PCR) (MSP). The PCR primers were designed to specifically amplify the promoter regions of the gene of interest. If the sample DNA was originally unmethylated, an MSP reaction product will be detectable when using the primer set (labeled as "U") designed to be complementary to the unmethylated DNA sequence. No product will be generated using a primer set (labeled as "M") designed to be complementary to the derivative methylated DNA sequence. Conversely, an MSP product will be generated only with the M primer set if the sample was originally methylated, and the U primers will not amplify such a template. In all, 38 PCR cycles were carried out for every reaction, and primers and reaction conditions are described in Supplementary Table 1. Commercially available methylated human genomic DNA (Qiagen, Hilden, Germany) was used as a positive control. Water blanks and PCR mixtures were used as a negative control. The MSP results were analyzed on 3% agarose gels stained with ethidium bromide viewed under UV light.

2.5. Statistical analysis

Statistical analyses were performed using chi-square tests for categorical variables. Univariate logistic regression analyses were performed using epigenetic biomarkers to predict carcinoma. Multivariate logistic regression models including only predictors in the univariate analyses ($P < 0.05$) were then generated, and an index was established according to the regression coefficient and the status of selected genes. The odds ratios were calculated with 95% CI. Two-tailed tests were considered statistically significant if $P < 0.05$. The predictive accuracy of biomarkers was evaluated by calculating the area under the receiver operating characteristic (ROC) curve (AUC). Each biomarker was added according to the multivariate analysis and AUC values in the order from high to low. The ROC curve of both specificity and sensitivity of single or combined