Urine Cell-Free DNA integrity as a marker for early bladder cancer diagnosis: Preliminary data

Valentina Casadio, B.S.ᵃ,*, Daniele Calistri, Ph.D.ᵃ, Michela Tebaldi, B.S.ᵃ, Sara Bravaccini, B.S.ᵃ, Roberta Gunelli, M.D.ᵇ, Giuseppe Martorana, M.D.ᶜ, Alessandro Bertaccini, M.D.ᶜ, Luigi Serra, M.D.ᵈ, Emanuela Scarpi, M.S.ᵉ, Dino Amadori, M.D.ᶠ, Rosella Silvestrini, Ph.D.ᵃ, Wainer Zoli, Ph.D.ᵃ

ᵃ Biosciences Laboratory, IRCCS Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST), Meldola, Italy
ᵇ Department of Urology, Morgagni Pierantoni Hospital, Forlì, Italy
ᶜ Department of Urology, University of Bologna, S. Orsola-Malpighi Hospital, Bologna, Italy
ᵈ Pathology Unit, Morgagni Pierantoni Hospital, Forlì, Italy
ᵉ Unit of Biostatistics and Clinical Trials, IRCCS Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST), Meldola, Italy
ᶠ Department of Medical Oncology, IRCCS Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST), Meldola, Italy

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Abstract

Objectives: Urine cell-free (UCF) DNA has recently been proposed as a potential marker for early bladder cancer diagnosis. It is known that normal apoptotic cells produce highly fragmented DNA while cancer cells release longer DNA. Therefore, we verified the potential role of UCF DNA integrity in early bladder cancer diagnosis.

Materials and methods: UCF DNA was isolated from 51 bladder cancer patients, 46 symptomatic patients, and 32 healthy volunteers. To verify UCF DNA integrity, sequences longer than 250 bp, c-Myc, BCAS1, and HER2, were quantified by real time PCR.

Results: At the best cutoff value of 0.1 ng/μl, UCF DNA integrity analysis showed a sensitivity of 0.73 (95% CI 0.61–0.85), and a specificity of 0.84 (95% CI 0.71–0.97) in healthy individuals and 0.83 (95% CI 0.72–0.94) in symptomatic patients. Receiver operating characteristic (ROC) curve analysis revealed an area under the curve of 0.834 (95% CI 0.739 – 0.930) for healthy individuals and 0.796 (95% CI 0.707– 0.885) for symptomatic patients.

Conclusions: These preliminary data suggest that UCF DNA integrity is a potentially good marker for early noninvasive diagnosis of bladder cancer. Its diagnostic performance does not seem to vary significantly, even in an “at risk” population of symptomatic individuals. © 2013 Elsevier Inc. All rights reserved.

Keywords: Urine cell-free DNA integrity; Bladder cancer; Early diagnosis

1. Introduction

Early diagnosis has an important impact in increasing disease-free survival and reducing mortality in patients with different tumor types. With regard to bladder cancer, an early diagnosis increases the probability of finding a non-invasive tumor, and therapy has a better chance of being successful [1,2]. Early cancer detection is more acceptable to patients when tumor cells can be sought in body fluids, without using invasive diagnostic approaches. In the case of bladder cancer, cystoscopy is still considered the diagnostic gold standard. However, for reasons of invasiveness and cost, it cannot be used for the screening of low incidence tumors, such as those of the bladder. The only noninvasive approach currently used is voided cytology, but this method has low sensitivity in detecting low grade and stage tumors [3]. Numerous new molecular markers have been proposed to increase the accuracy of cytology, such as gene alterations [4,5], NMP 22 [6], telomerase activity [4,7], urine dipstick [8], BTA-stat and BTA-trak [9]. Although some of these markers have FDA approval, none has entered into
clinical practice, probably due to high costs (e.g., FISH UroVysion; Abbott/Vysis, Abbott Park, IL), insufficient accuracy or analytical performance [10,11]. Therefore, despite the large number of studies and results on new biomarkers, there is still a need for a low-cost test with adequate accuracy to implement or replace cytology and cytoscopy.

Recent efforts by our group have shown, through pilot and confirmatory case-control studies, the ability of urine telomerase activity determined by the telomeric repeat amplification protocol (TRAP) to accurately detect the presence of bladder cancer [12,13]. We recently confirmed an improved diagnostic accuracy of combined cytology, TRAP assay and FISH on a series of symptomatic patients [4]. As expected, the sensitivity of cytology and TRAP assay in combination was greater than that of the single approaches, while combined TRAP assay and FISH provided the best trade-off between gain in sensitivity and loss in specificity [4]. It would also be interesting to consider other approaches to see whether diagnostic accuracy and cost-effectiveness can be further improved.

The presence of circulating cell-free DNA in plasma or serum has been reported to be a suitable marker of prostate [14] and lung cancer [15,16], but few studies have focused on the potential of urine cell-free (UCF) DNA to detect bladder cancer [17–19].

In our study, we assessed whether DNA integrity in urine supernatant is capable of distinguishing cancer patients from healthy individuals or from those with benign urologic diseases. This hypothesis is based on the well-known fact that DNA from normal cells in apoptosis is highly fragmented, while DNA from necrotic cancer cells maintains its integrity [20]. To this end, we analyzed urine cell-free DNA fragments longer than 250 bp of 3 biomarkers frequently amplified in bladder cancer: c-Myc (8q24.21), HER2 (17q12.1), and BCAS1 (20q13.2) [21–23].

2. Materials and methods

2.1. Case series

A total of 129 individuals (51 bladder cancer patients at first diagnosis, 46 symptomatic patients with benign urologic diseases, and 32 healthy individuals; Table 1) were recruited from the Urology Department of Morgagni-Pierantoni Hospital (Forlì). All participants provided written informed consent to take part in the study and the local Ethics Committee reviewed and approved the study protocol. Patients with bladder cancer or benign urogenital diseases were submitted to cystoscopy, after which those with malignancies or suspicious lesions underwent transurethral resection of the bladder (TURB). The final diagnosis of cancer was based on histologic results from TURB. Tumor type and differentiation were based on 2004 World Health Organization criteria. Symptomatic patients were diagnosed with different benign diseases: prostatic adenomas or benign hyperplasia (14 cases), kidney stones (3), cysts (3), lithiasis (3), prostatitis (3), bladder edema (1), and bladder diverticula (1). Eight patients did not have any disease. Twenty-eight percent of symptomatic patients had hematuria at the time of urine collection. The control group of healthy individuals with no previous urogeneric diseases or cancer was matched to patients by gender and age. UCF DNA integrity analysis was performed blindly in all individuals and was evaluable for the entire case series. Cytologic results were available for 76 patients (41 cancer cases and 35 symptomatic individuals).

2.2. Urine collection

First morning-voided urine samples for UCF DNA analysis were collected from healthy individuals about 1 week after cytologic examination, and from symptomatic individuals and bladder cancer patients immediately before cystoscopy. All patients and controls were instructed to give clean-catch urine samples, maintained at 4°C for a maximum of 3 hours. Thirty milliliter aliquots of urine were centrifuged at 850 g for 10 minutes and the supernatants were transferred to cryovials and immediately stored at −80°C until use.

2.3. UCF DNA analysis

DNA was extracted and purified from 2 ml of supernatant by Qiamp DNA minikit (Qiagen, Milan, Italy) according to the manufacturer’s instructions. At the same time, DNA was extracted from a human bladder cancer cell line (MCR) using the same minikit. DNA was quantified by spectrophotometry (NanoDrop ND-1000; Celbio, Milan, Italy).

Real Time PCR reactions were carried out by Rotor Gene 6000 detection system (Corbett Research, St.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Male</th>
<th>Female</th>
<th>Age (years)</th>
<th>Grade</th>
<th>Stage</th>
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<td>32</td>
<td>21</td>
<td>11</td>
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</tr>
<tr>
<td>Symptomatic patients</td>
<td>46</td>
<td>33</td>
<td>13</td>
<td>27, 19</td>
<td>—</td>
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<td>51</td>
<td>36</td>
<td>15</td>
<td>25, 26</td>
<td>22, 21</td>
<td>36, 4</td>
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Neots, UK) using IQ SYBR Green (Bio-Rad, Milan, Italy). Sequences longer than 250 bp, corresponding to 3 oncogenes were analyzed: c-Myc (locus 8q24 and 21, amplification product 264 bp), BCAS1 (locus 20q.13.2, amplification product 266 bp), HER2 (locus 17q12 and 1, amplification product 295 bp). A short 125 bp fragment of STOX1 (locus 10q21 and 3) was analyzed to check for potential PCR inhibition. Primer sequences were as follows: c-Myc fw TGGAGTACACCGCATATC, rev AC-CCACCTCTCTCTAAC; BCAS1 fw GGGTCA-GAGTTCTTGTGAG, rev CGTTGTCCTGAAACAGACA; HER2 fw CCAGGTGTCTCCTCAGTTGT, rev TCAGTATGCCCTACCCCTTC; STOX1 fw GAAA-CAGGGCAGCAAGAAG, rev CAGACAGCATGGAGGTGAGA. PCR conditions for the oncogenes were as follows: 95°C for 3 minutes, then 45 cycles at 94°C for 40 seconds, 56°C for 40 seconds, and 72°C for 1 minute. PCR conditions for the short STOX1 sequence were as follows: 95°C for 90 seconds, then 45 cycles at 94°C for 40 seconds, and 54°C for 1 minute. All real-time PCR amplifications were performed in duplicate on 10 ng of each UCF DNA sample. Various amounts of DNA from the MCR cell line (0.01, 0.1, 1, 5, 10 and 20 ng) were also analyzed to construct a standard curve. The UCF DNA value for each sample was obtained by Rotor Gene 6000 detection system software using standard curve interpolation. The analysis was repeated if the difference between duplicate samples was greater than 1 cycle threshold. The final UCF DNA integrity value was obtained by the sum of the 3 oncogene values. Real-time experiments were performed independently in duplicate on the same 8 samples to test assay imprecision. The coefficients of variation (CV) were then calculated for c-Myc, HER2, BCAS1, and STOX1.

2.4. Statistical analysis

The relationship between UCF DNA integrity value and the different subgroups (healthy individuals, symptomatic individuals, and cancer patients) was analyzed using a non-parametric ranking statistic test. The most discriminating cutoff values between healthy individuals, symptomatic individuals, and cancer patients were identified using receiver operating characteristic (ROC) curve analysis. The true positive rates (sensitivity) were plotted against the false positive rates (1-specificity) for all classification points. Accuracy was measured by the area under the ROC curve (AUC), which represents an average probability of correctly classifying a case chosen at random. Study endpoints were sensitivity (the proportion of cancer patients who were correctly identified by the test or procedures) and specificity (the proportion of healthy individuals who were correctly identified), with their 95% confidence intervals (CIs) [24]. Spearman’s nonparametric test was used to evaluate the correlation between total DNA value and DNA integrity. The McNemar test was used to compare sensitivity and specificity rates. P values < 0.05 were considered statistically significant. Statistical analyses were performed using SPSS statistical software (ver. 12.0; SPSS GmbH Software, Bologna, Italy).

3. Results

Total free DNA was quantifiable by spectrophotometry, showing a median value of 6 ng/µl (range 2–138 ng/µl). UCF DNA integrity analysis was feasible and results were evaluable for all 129 individuals. The 125 bp STOX1 sequence was amplified in all samples, excluding the presence of PCR inhibitors. CVs were calculated, considering 2 measurements of each gene in a series of 8 samples, to test the interim imprecision of each assay. CVs were ≤0.12 for HER2, BCAS1, and STOX1, and ≤0.23 for c-Myc.

No significant correlation was found between total free DNA and DNA integrity, thus suggesting that they are independent variables. Median values of UCF DNA integrity were significantly different in the subgroups; the value was significantly (P < 0.0001) lower (0.004 ng/µl, range 0–1.883) in healthy individuals, more than 3-fold higher in symptomatic individuals (0.014 ng/µl, range 0–4.460) in cancer patients (P < 0.0001). The AUC for UCF DNA integrity was 0.834 (95% CI 0.739–0.930) for cancer patients and healthy individuals, and 0.796 (95% CI 0.707–0.885) for cancer patients and symptomatic individuals (Fig. 1 and Fig. 2). Analysis of sensitivity and specificity at different UCF DNA integrity cutoff values (0.04–0.14 ng/µl) showed a sensitivity ranging from 0.78 (95% CI 0.67–0.89) to 0.53 (0.39–0.67); specificity varied from 0.81 (95% CI 0.67–0.95) to 0.91 (95% CI 0.81–1.00) in healthy individuals and from 0.63 (95% CI 0.49–0.77) to 0.87 (95% CI 0.77–0.97) in symptomatic patients (Table 2). The best cutoff value was 0.1 ng/µl, providing the best trade-off between loss in sensitivity (0.73, 95% CI 0.61–0.85) and gain in specificity.

Fig. 1. ROC curve of UCF DNA integrity for cancer patients and healthy individuals. (Color version of figure is available online.)
in both healthy individuals (0.84, 95% CI 0.71–0.97) and symptomatic patients (0.83, 95% CI 0.72–0.94). A comparison of diagnostic performance between conventional cytology and UCF DNA integrity showed an increase in sensitivity from 0.53 (95% CI 0.35–0.71) to 0.70 (95% CI 0.62–0.68) \( (P = 0.132) \), while specificity was slightly but not significantly reduced from 0.92 (95% CI 0.81–1.03) to 0.75 (95% CI 0.72–0.94) \( (P = 0.102) \) (Table 3). In a breakdown analysis of the difference in clinical pathologic subgroups, we observed an improved performance of UCF DNA integrity compared with that of cytology, more evident in males and independent of age. More importantly, sensitivity of UCF DNA integrity increased for low grade tumors, from 0.15 (0–0.34) to 0.72 (0.53–0.91) \( (P = 0.008) \), and for early stage Ta-T1 tumors, from 0.50 (0.30–0.70) to 0.72 (0.57–0.87) \( (P = 0.058) \). There were too few patients with advanced tumors to permit such analysis. Specificity of UCF DNA integrity did not vary significantly with respect to cytology as a function of gender, age, or any other clinical pathologic variables. To improve diagnostic performance, we constructed a flow diagram for a combined analysis of cytology and UCF DNA integrity (Fig. 3). In the diagram we assumed that all cytology-positive individuals would not be subjected to further diagnostic evaluations and would therefore be considered as “positives.” Conversely, all individuals with dubious or negative cytology would be submitted to UCF DNA integrity analysis. With this combined analysis, we obtained 0.81 sensitivity (95% CI 0.69–0.93), higher than that obtained with cytology (0.53) or UCF DNA integrity alone (0.73), and 0.77 specificity (95% CI 0.63–0.91).

To verify the role of each single gene in determining test accuracy, we also performed ROC curve analysis for each gene and obtained the following AUCs: 0.762 for c-Myc, 0.861 for BCAS1, and 0.757 for HER2. In symptomatic patients vs. cancer patients, AUCs were as follows: 0.763 for c-Myc, 0.764 for BCAS1, and 0.708 for HER2.

We also analyzed c-Myc and HER2 amplification in a subgroup of 36 patients. FISH analysis showed a high correlation between the quantity of urinary DNA fragments and amplification in tissue for HER2 gene \( (P = 0.004) \), but no correlation for c-MYC \( (P = 0.140) \) (data not shown). As far as we know there is no commercially available probe for FISH analysis of BCAS1, we thus used an immunohistochemical approach which highlighted high expression of the gene in all tumor samples, confirming the potential role of this marker in bladder carcinogenesis.
4. Discussion

Numerous articles have been published on the role of DNA alterations, such as genetic and epigenetic modifications in urine sediment, as biomarkers for early bladder cancer diagnosis [25], but very few studies have investigated the potential role of DNA in urine supernatant [17–19] (see supplementary table at www.urologicconology.org). Cell-free DNA can easily be detected in very small volumes of urine and, unlike RNA or enzymes, has good stability.

Table 3

Sensitivity and specificity of cytology and UCF DNA integrity in detecting bladder cancer

<table>
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<tr>
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<th>Sensitivity</th>
<th>Specificity</th>
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<tr>
<td></td>
<td>Healthy individuals</td>
<td>Symptomatic patients</td>
</tr>
<tr>
<td>Cytology*</td>
<td>n=16/30</td>
<td>nd</td>
</tr>
<tr>
<td>Rate (95% CI)</td>
<td>0.53 (0.35–0.71)</td>
<td>0.92 (0.81–1.00)</td>
</tr>
<tr>
<td>UCF DNA integrity†</td>
<td>n=21/30</td>
<td>27/32</td>
</tr>
<tr>
<td>Rate (95% CI)</td>
<td>0.70 (0.62–0.78)</td>
<td>0.84 (0.71–0.97)</td>
</tr>
<tr>
<td>P‡</td>
<td>0.132</td>
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</table>

Sensitivity and specificity were evaluated in samples for which the results of both cytology and UCF DNA integrity analysis were available (30 cancer patients and 24 symptomatic individuals).

nd = not done; UCF = urine cell-free.

* Suspicious results were not included.
† Cutoff 0.1 ng/μl.
‡ McNemar test.

Fig. 3. Flow diagram of sequential cytology and UCF DNA analysis.
Secondly, cell-free DNA in body fluids is suitable for multiple analyses, such as mutations, microsatellite loss of heterozygosity (LOH), and epigenetic alterations [26]. In our study, we evaluated DNA integrity, a specific marker of nonapoptotic cancer cells.

As demonstrated by Chang and coworkers in 2007 [18] and confirmed by Zancan et al. in 2009 [19], cell-free DNA is not sufficiently accurate to correctly distinguish between bladder cancer patients and healthy individuals. We thus decided to concentrate on DNA integrity, a specific marker of nonapoptotic cancer cells, and to analyze only long DNA fragments. Moreover, to increase the diagnostic accuracy of the test, we performed the analysis on 3 specific sequences longer than 250 bp located at loci: 8q24.21 (c-Myc), 17q12.1 (HER2), and 20q13.2 (BCAS1). These regions were selected because they are known to be amplified during bladder cancer development and progression [27,28]. Our results confirmed that UCF DNA integrity in urine is a determining feature for bladder cancer detection, with a higher sensitivity than conventional noninvasive cytology, especially for low-grade tumors. Such a characteristic is important for a new marker aimed at early bladder cancer diagnosis [29]. This line of research has already been pursued by our group in healthy individuals and cancer patients [12,13] and in a clinical setting of symptomatic patients [4,7]. The symptomatic subgroup and individuals exposed to industrial chemicals are the main candidates for a screening program of low prevalence tumor histotypes [2,8]. Interestingly, in the present study, we found similar specificity in healthy individuals and symptomatic patients, suggesting that the test can also accurately identify tumors among benign disease with a low number of false positives. The high percentage of true positives is probably ascribable to the analysis of DNA integrity at 3 genomic regions specifically amplified in bladder cancer.

We also analyzed the DNA status index (DSI) i.e., the ratio between DNA integrity of the 3 genes (c-Myc, HER2, BCAS1) and the short fragment STOX1. As expected, a positive correlation was found between the DSI and the presence of bladder cancer (data not shown). These results seem to indicate that in cancer samples, DNA predominantly exists as long fragments, suggesting that its origin is mainly from nonapoptotic cells.

It has recently been demonstrated that the combination of 2 or more urine assays improves the detection of urothelial cancers [30]. Therefore, in an attempt to increase sensitivity, we constructed a diagnostic algorithm in which cytology and UCF DNA integrity were tested sequentially. In our series of symptomatic individuals, this combination demonstrated improved sensitivity compared with that of each test alone. Moreover, sensitivity and specificity were similar to those recently reported using the combination of urine telomerase activity, cytology, and FISH [4]. We believe that our results have an important additional value in terms of the cost/benefit ratio. In fact, in the present study, we combined only 2 tests rather than 3; secondly, UCF DNA integrity analysis is easier to perform and to interpret than FISH UroVysion, which requires the consensus of 2 experts. Results for UCF DNA integrity are obtainable in a relatively short time (about 2 working days) and, most importantly, this approach costs less than one-third of the cost of FISH UroVysion. In addition, only small volumes (2 ml) of urine are required for the evaluation of cell-free DNA integrity and this analysis, unlike TRAP assay [7], is not affected by the presence of contaminants, such as inflammatory cells, as shown by the high specificity in symptomatic patients.

Important limitations of the present work are the relatively small case series and the fact that both cytology and UCF DNA results were not available for all patients. However, such limitations are acceptable in a pilot study of a new diagnostic approach, such as ours. A confirmatory study on a larger case series is ongoing to validate our results.

In conclusion, these preliminary results are promising and pave the way towards a new molecular approach for the early noninvasive diagnosis of bladder cancer, which could be used in combination with or as an alternative to voided cytology.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.urolonc.2012.07.013.

References


