

# Urinary Cell-Free DNA Quantification as Non-Invasive Biomarker in Patients with Bladder Cancer

Antonin Brisuda<sup>a</sup> Eva Pazourkova<sup>b</sup> Viktor Soukup<sup>c</sup> Ales Horinek<sup>d, e</sup>  
Jan Hrbáček<sup>a</sup> Otakar Capoun<sup>c</sup> Iveta Svobodova<sup>d</sup> Sarka Pospisilova<sup>d</sup>  
Marie Korabecna<sup>d</sup> Jaroslav Mares<sup>f</sup> Tomáš Hanuš<sup>c</sup> Marek Babjuk<sup>a</sup>

<sup>a</sup>Department of Urology, 2nd Faculty of Medicine, Charles University and University Hospital Motol, Departments of <sup>b</sup>Nephrology and <sup>c</sup>Urology, <sup>d</sup>Institute of Biology and Medical Genetics and <sup>e</sup>3rd Medical Department, 1st Faculty of Medicine, Charles University and General Faculty Hospital, and <sup>f</sup>Department of Biology and Medical Genetics, Charles University in Prague, 2nd Faculty of Medicine, Prague, Czech Republic

## Key Words

Cell-free DNA · Urinary marker · Bladder cancer · Real-time PCR

## Abstract

**Introduction:** Concentration of urinary cell-free DNA (ucfDNA) belongs to potential bladder cancer markers, but the reported results are inconsistent due to the use of various non-standardised methodologies. The aim of the study was to standardise the methodology for ucfDNA quantification as a potential non-invasive tumour biomarker. **Material and Methods:** In total, 66 patients and 34 controls were enrolled into the study. Volumes of each urine portion (V) were recorded and ucfDNA concentrations (c) were measured using real-time PCR. Total amounts (TA) of ucfDNA were calculated and compared between patients and controls. Diagnostic accuracy of the TA of ucfDNA was determined. **Results:** The calculation of TA of ucfDNA in the second urine portion was the most appropriate approach to ucfDNA quantification, as there was logarithmic dependence between the volume and the concentration of a urine portion ( $p = 0.0001$ ). Using this methodology, we were able to dis-

criminate between bladder cancer patients and subjects without bladder tumours ( $p = 0.0002$ ) with area under the ROC curve of 0.725. Positive and negative predictive value of the test was 90 and 45%, respectively. **Conclusion:** Quantification of ucfDNA according to our modified method could provide a potential non-invasive biomarker for diagnosis of patients with bladder cancer.

© 2015 S. Karger AG, Basel

## Background

Bladder cancer is the fourth most prevalent malignancy in men and the ninth in women [1]. There are 2 clinically important forms of bladder cancer: the non-muscle invasive form and the muscle invasive one. As the muscle invasive cancer represents potentially life-threatening disease with capability to form metastases, its early diagnosis and treatments is of utmost importance. The non-muscle invasive cancer comprises about 80% of cases of which nearly 70% recur within 3 years of follow-up [2]. Therefore, patients with this cancer must be thoroughly and regularly checked by performing an endoscopy of the urinary blad-

**Table 1.** Study population characteristics

	n	Age, median (95% CI)	Male	Female	Subgroups	n
Controls	34	62.5 (57.7–66.2)	22	12	HEALTHY group BENIGN group	23 11
Patients	66	67.5 (64.0–69.0)	44	22	pTa	21 LG 4 HG 25
					pT1	8 LG 12 HG 20
					pT2	0 LG 9 HG 9
					pT3	0 LG 6 HG 6
					pT4	0 LG 5 HG 5
					CIS	1

der. In this context, the need for simple and non-invasive marker becomes even more essential. Especially combinations of markers seem to be promising [3]. Besides urine cytology, none of the investigated markers became widely accepted and commonly used in the clinical practise [4].

Cell-free nucleic acids circulating in blood were first discovered as early as the 1940s [5]. Their diagnostic and prognostic potential for detection of different cancers was identified later and it is widely explored till today [6]. Moreover, other conditions may be monitored using circulating cell-free nucleic acids, for example, acute myocardial infarction [7] or renal transplant rejection [8]. Several studies investigated serum DNA hypermethylation in patients with bladder cancer [9, 10] while other studies used DNA integrity index as a diagnostic marker [11]. Cell-free DNA (cfDNA) originates during apoptosis and necrosis. Fragments of cfDNA are also released from normally functioning living cells [12]. Apoptotic cells produce shorter uniform fragments of cfDNA, whereas cancer cells, often undergoing necrosis, release longer fragments of cfDNA with higher DNA intactness. DNA integrity is then defined as the ratio of longer to shorter DNA fragments, which ought to be higher in patients with cancer [13]. Such an approach was used in the study by Casadio et al. [14] who analysed DNA sequences specific for bladder cancer in urine supernatant. The results were promising for the early non-invasive diagnosis of bladder cancer and the method was recommended to be used in combination with voided cytology.

Few studies [14–17] investigated levels of cfDNA in urine supernatant as a diagnostic and prognostic marker in bladder cancer patients. One of these studies compared 4 different methods for DNA quantification [15]. With regards to urinary cell-free DNA (ucfDNA) concentrations among patients with cancer and controls, no differences were found. In another study [16], the authors measured ucfDNA concentrations adjusted to urine creati-

nine. Using the amplification of 400-bp fragment of  $\beta$ -actin gene by real-time PCR, the authors were able to distinguish patients and controls.

In this study, we focused on proper standardization of the methodology for ucfDNA quantification as a potential tool for bladder cancer diagnosis. We tested the ability of this biomarker to distinguish bladder cancer patients with different tumour stages from control patients.

## Material and Methods

### Study Group

In the period February 2012–September 2014, naturally voided urine samples were collected from 119 individuals recruited at the departments of urology from 2 different academic institutions. The study was approved by institutional review boards in both institutions. Informed consent was signed by each subject. Nineteen patients with positive urine culture and/or leukocyturia were excluded from the study [15]. First, 2 urine samples (the first morning urine and the second morning urine voided approximately 2 h after the first one) were collected from 27 individuals (11 healthy volunteers, 6 control patients with various benign urological disorders and 10 bladder cancer patients). The second morning urine proved to be more appropriate for ucfDNA analyses. Furthermore, a method for ucfDNA quantification was established (see details in the Results and Discussion section). Accordingly, the second morning urine was collected from each of the remaining 73 individuals. In total, the study group consisted of 34 controls (23 healthy volunteers (= HEALTHY group) and 11 patients with benign urological disorders (= BENIGN group)) and 66 bladder cancer patients (= CANCER group). The groups were not different in terms of age and sex ( $p > 0.05$ ). Study population characteristics are summarized in table 1. Neither patients nor controls presented with malignancies other than bladder cancer. Patients with indwelling catheter were not included in the study. In the controls, absence of a malignancy was confirmed by ultrasound of the kidneys, full bladder and/or by cystoscopy. Urine cytology, urinalysis and urine cultures were performed. Patients with known or suspected bladder cancer underwent transurethral resection of the bladder (TURB; 45 patients) or radical cystectomy (21 patients) and stages with grades were verified by pathologists (table 1). The WHO 2004 grading

system was used. All tumours were urothelial carcinomas. Out of the patients undergoing TURB, recurrent disease formed 17.8% (one pTa LG, 7 × pT1 HG). The rest were primary carcinomas. Urine cytology was assessed by 2 independent uropathologists and a consensus was arrived at in case of discrepancy. PAP I + II were considered negative finding and PAP III–V as positive finding. In 7 controls and 3 LG tumours, the urine cytology was inconclusive.

#### *Urine Collection, ucfDNA Extraction and Quantification*

Each urine portion was collected in a sterile container and the exact volume of the portion was recorded. Samples needed for urine cytology, urinalysis and urine culture were retrieved from this container immediately. Regarding ucfDNA analysis, 50 ml of urine was stabilized with 535 µl of 0.5 M EDTA, centrifuged immediately at 4,000 g for 10 min at 10°C and the supernatant was stored at –20°C until assayed.

ucfDNA was isolated from 2 ml of urine with QIAamp Circulating Nucleic Acid Kit (Qiagen, Germany) according to the manufacturer's instructions and eluted into 50 µl of Elution buffer.

ucfDNA quantification was performed by the real-time PCR method (ABI PRISM 7900HT, Applied Biosystems, USA) using *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) gene (GenBank: NG\_007073.2). The standard curve (10 ng/µl – 3 pg/µl) was generated by serial dilution of a standard genomic DNA (TaqMan Control Genomic DNA (Human), 10 ng/µl, Applied Biosystems, USA). The PCR was performed with 5 µl of extracted DNA in a total reaction volume of 20 µl. All samples were run in triplicates. The cost of the assay is approximately 35 Euros per 1 sample. From a practical point of view, the whole laboratory work-up for one person takes minimally 5 h of the lab staff. During one assay, up to 10 samples can be run.

#### *Statistical Analysis*

Non-parametric tests were used for statistical evaluation. ucfDNA concentrations, urine volumes and total amounts (TA) of ucfDNA were tested by Wilcoxon test for paired samples. Regression analysis was used for evaluation of relation between concentrations and volumes. The differences between the TA of ucfDNA in the control and patient groups were tested using the Mann–Whitney test for independent samples. The Kruskal–Wallis test was used for the evaluation of differences among the HEALTHY group, the BENIGN group and the CANCER group (or cancer subgroups). A test for pairwise comparison of subgroups was performed by Mann–Whitney tests with Bonferroni correction.  $p < 0.05$  was considered statistically significant. All statistical analyses were performed by MedCalc (MedCalc Software, Broekstraat 52, 9030 Mariakerke, Belgium).

## **Results**

#### *Relation between Concentration and Volume*

The first morning urine portion and consecutive second urine portion from 27 individuals were collected and analysed. Urinary cfDNA concentrations ( $c_1$ ) (ng/ml) and volumes ( $V_1$ ) (ml) of the first voided urine were measured. The same data ( $c_2$  and  $V_2$ , respectively) were obtained for the second voided urine. Second morning portions yielded

higher values of ucfDNA concentrations ( $p = 0.044$ ). However, volumes of the second portions were significantly lower ( $p = 0.002$ ). In contrast, TA of ucfDNA (ng), calculated as  $c$  multiplied by  $V$ , were similar in both portions ( $p = 1.0$ ). Using regression analysis, the relation between volumes and concentrations was tested. Regression was performed by equation  $y = a + b \times \log(x)$ , where  $x = V_2/V_1$  and  $y = c_1/c_2$  (based on a premise:  $c_1 \times V_1 \approx c_2 \times V_2$ ). There was a logarithmic dependence between volume and concentration ( $p = 0.0001$ ). Therefore, the TA of ucfDNA was incorporated into the ucfDNA quantification:

$$TA = c \times V.$$

For practical reasons, second voided portion collected 2–3 h after the first urination was selected as the most appropriate for ucfDNA analyses.

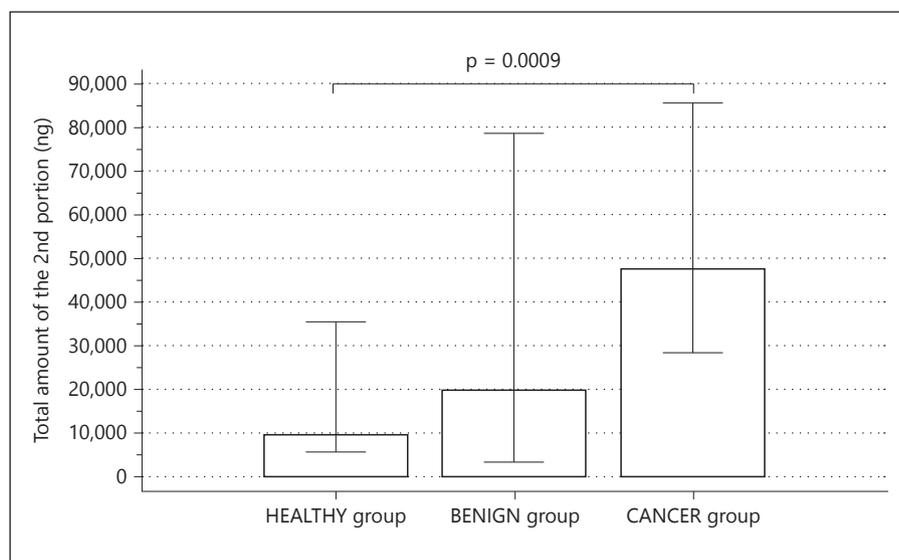
#### *Comparison of TA of ucfDNA in Patients and Controls*

When comparing all controls ( $n = 34$ ) with all bladder cancer patients ( $n = 66$ ), the Mann–Whitney test provided significant difference in favour of the bladder cancer group, that is, their urine contained significantly higher TA of ucfDNA ( $p = 0.0002$ ).

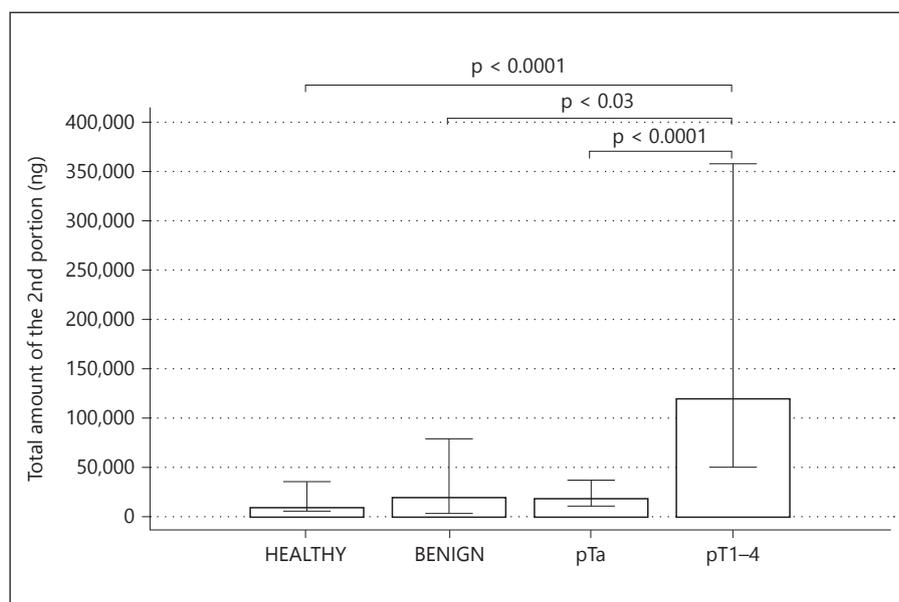
The controls were thereafter subgrouped into the HEALTHY group and the BENIGN group and separate analysis was performed. The comparison, made by Kruskal–Wallis ANOVA ( $p = 0.00085$ ) with post-hoc analysis, showed significant difference between the HEALTHY group and the CANCER group ( $p = 0.0009$ ). Surprisingly, there was no difference between the BENIGN group and the CANCER group ( $p = 0.3$ ; fig. 1). In view of this finding, the CANCER group was subgrouped into a pTa group and a higher stages group (pT1–4) and Kruskal–Wallis test with post-hoc analysis was performed ( $p = 0.000001$ ). Significant differences were found between each of the control subgroups and the higher stages group. Interestingly, the subgroup pTa did not differ from either of the control subgroups, but it did differ from the more advanced tumours group (fig. 2).

Furthermore, the sensitivities and specificities with particular cut-off values were calculated (table 2). The receiver operating characteristic (ROC) curve was constructed to clearly demonstrate the potential of TA of ucfDNA to distinguish bladder cancer patients and persons without a cancer. The area under the ROC curve was 0.725 with the best sensitivity and specificity of 42.4 and 91.2%, respectively (fig. 3). On the selected cut-off value of 65,055 ng, positive predictive value and negative predictive value was 90 and 45%, respectively.

**Fig. 1.** Distribution of TA of ucfdNA in the second morning portion in the HEALTHY group, the BENIGN group and the CANCER group; connectors indicate classes with significant difference ( $p < 0.05$ ).



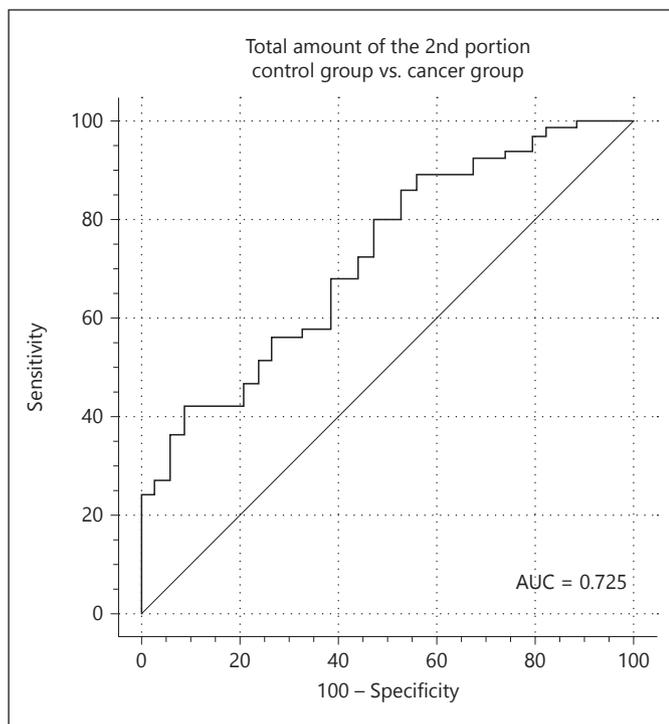
**Fig. 2.** Distribution of TA of ucfdNA in the second morning portion in the HEALTHY group, BENIGN group, tumour stage pTa group and stages pT1–4 group; connectors indicate classes with significant difference ( $p < 0.05$ ).



**Table 2.** Several examples of cut-off points and related values of sensitivity and specificity

Cut-off, ng	Sensitivity, %	Specificity, %
87,991	36.4	94.1
52,800	47.0	79.4
39,124	56.1	73.5
19,866	68.2	61.8
13,601	80.3	52.9
8,257	89.4	44.1

The diagnostic performance to detect pTa patients resulted in sensitivity and specificity of 12 and 91.2%, respectively. The higher stages (pT1–4) operated on the values of 62.5 and 91.2%, respectively. Concerning the grades, LG tumours were detected with sensitivity and specificity of 20.7 and 91.2%, respectively. On the other hand, in HG tumours cfDNA provided values of 59.5 and 91.2%, respectively. Urine cytology resulted in sensitivity of 38.5% and specificity of 92.6% in diagnosis of LG tumours. Corresponding figures in diagnosis of HG tumours were 86.5 and 92.6%, respectively. Using the TA of



**Fig. 3.** ROC for the control and the cancer group (Youden index  $J = 0.336$ , associated criterion (cut-off) = 65,055 ng; sensitivity = 42.4%; specificity = 91.2%).

cfDNA as a test to increase the sensitivity in detecting LG tumours did not show any significant gain. Only one extra LG patient was detected by the test for a cost of 3 false positive cases (rise of the sensitivity to 42.3% and drop of the specificity to 81.5%). Additionally, 2 extra HG cases, which were missed by cytology, were also detected (rise of the sensitivity to 91.9%).

## Discussion

Urine-derived cfDNA seems to be a promising marker for non-invasive bladder cancer diagnostics. Urine is in direct contact with bladder mucosa. As such, it seems to be the proper body fluid for detection of ucfDNA originating from bladder tumours. Urine sampling is feasible, non-invasive, with no demands on patients and personnel, without any potential risks and repeatable.

To date, several studies have been published investigating new bladder cancer markers present in urine, but only a few of them dealt with cfDNA in supernatant and its quantification. After a promising pilot study by Zancan et al. [17], the predictive value of ucfDNA was

confirmed by Chang et al. [16] who adjusted the ucfDNA concentrations to urine creatinine. In a subsequent study, Zancan et al. [15] found no differences in ucfDNA concentrations between patients with cancer and controls.

The aim of this study was to deepen our knowledge about cfDNA in urine across a variety of bladder cancer stages and grades, to optimize the methodology of ucfDNA quantification and to test ucfDNA as a potential diagnostic marker for non-invasive bladder cancer detection. Three main questions had to be answered:

- (1) Which portion of urine is the appropriate one for the ucfDNA quantification?
- (2) How to quantify the ucfDNA?
- (3) Can ucfDNA be used as a potential bladder cancer marker?

From the methodological point of view, it was important to decide which portion of urine should be preferentially used for ucfDNA analysis. Cannas et al. [18] demonstrated good DNA stability in the urine under various experimental conditions (urine from 2 different populations, stored under various temperatures, with/without EDTA). However, he did not refer to ucfDNA, the stability of which may be challenged in urine concentrated overnight. In a theory, concerning the ucfDNA quantity, there might be differences between the first and the second voided urine of a day. To test this theory, the first and the second morning urine voided 2–3 h after the first one was compared in our study. We found statistically significant differences in the levels of ucfDNA concentrations (higher in the second urine portion) and in the voided volumes (lower in the second portion). On the other hand, TA of ucfDNA were similar in both portions. For practical and methodological reasons, second morning urine was selected as the referential portion. First, by use of the second voided urine, the dwell time of urine in the bladder was standardized. Second, the second morning urine is less concentrated with a lower level of cytolysis and therefore more appropriate for laboratory workflow including urine cytology.

In previous studies [15, 16], 2 approaches to ucfDNA quantification were used. Zancan et al. [15] measured only the ucfDNA concentrations. Chang et al. [16] adjusted ucfDNA concentrations to urine creatinine. The concentration of urinary creatinine depends on the level of glomerular filtration, fluid intake (i.e. on total urine volume) and on individual metabolism. Creatinine excretion is higher in men than in women, it varies by ethnicity and declines with age [19]. According to our theory, the amount of ucfDNA discharged into the urine from the tumour cells is dependent on the duration of physical

contact of urine with the tumour surface. Therefore, no adjustment to creatinine was considered necessary. The given amount of ucfDNA becomes diluted in a certain volume of urine. We have demonstrated that the highest ucfDNA concentrations were detected in patients with the smallest urine volumes. Using regression analysis, we confirmed the logarithmic relation between the concentration and the volume in a particular portion of urine. Finally, the TA of ucfDNA was used for ucfDNA quantification.

Using TA of ucfDNA, we were able to discriminate between patients with bladder cancer and controls ( $p = 0.0002$ ). When analysing the subgroups of controls, no difference was found between the BENIGN group and the CANCER group. In view of this finding, a detailed analysis of subgroups with low stage (pTa) and higher stages (pT1–4) was performed. The BENIGN group did differ from the pT1–4 cancer group, but did not differ from the pTa group. This finding indicates some level of similarity between the pTa patients and the patients with benign urological diagnoses with regard to the levels of ucfDNA. This observation can be supported by the results of both previously mentioned studies. The study by Zancan et al. [14] could not distinguish between patients and controls. It preferentially analysed patients with the stage pTa. Looking at the study population characteristics in our study, pTa patients represented the largest subgroup of bladder cancer patients, with LG being the highly predominating grade inside this stage. Still, pTa LG tumours predominate in an unselected bladder cancer population. From this reason, the calculated overall sensitivity is expected to be even worse. Missing out on these patients at the time of primary diagnosis is a problem. On the other hand, keeping in mind that pTa LG patients hold a low potential for developing a progression, the possible clinical impact of missing those patients during follow-up would not be so serious and the intervals between cystoscopies could be potentially prolonged when the test is negative. Chang et al. [16] included patients with all cancer stages and therefore they were able to discriminate between patients and healthy subjects. Interestingly, they did not find any differences among cancer stages of pTa, pT1 and pT2 + 3. In contrast, we found significant difference between pTa tumours and higher stages. For the sake of small numbers, to perform statistical subanalysis inside the CANCER cohort of pT1–4 was not possible. Additionally, neither of the 2 mentioned studies declared if the status of controls was healthy or with benign urological disease or both. In fact, every patient presenting before an urologist has some kind of urological problem and when the bladder cancer is ruled out, the patient most

likely becomes a patient with a benign disorder. Most of our healthy volunteers were recruited from the elderly patients being hospitalized at different departments such as cardiology and the like. Given the fact that healthy volunteers formed the majority of our controls, the overall specificity is probably somewhat overestimated.

ROC analysis resulting from our study provided only moderate prediction accuracy with fairly good specificity of 91.2% and poor sensitivity of 42.4% on the selected cut-off value with the limitations mentioned earlier. The poor sensitivity results from the effect of the predominating stage pTa where there was no difference between the levels of cfDNA in controls and pTa patients (fig. 2). Apparently, there was a trend toward higher levels of ucfDNA in the urine collected from the patients with higher stages and grades as showed by the sensitivity of 62.5% for diagnosis of pT1–4 stage disease and sensitivity of 59.5% for diagnosis of HG lesions. Those patients represent the most clinically serious cases with HG tumours slightly prevailing in our cohort. With regards to urine cytology, its diagnostic performance was similar to the results published elsewhere. In an effort made to increase the sensitivity of cytology by use of cfDNA, only negligible benefit was gained for a cost of decrease in specificity. It may be concluded that urine cytology and TA of cfDNA have similar diagnostic performance thus not helping each other.

Our study had certain limitations. Many aspects such as the number and size of the tumours (i.e. tumour surface), their grades and stages combined, presence of urinary tract infection, leukocyturia, and so on may affect the levels of ucfDNA. Especially tumours' surface or volume are difficult to measure or assess since the subjective nature of cystoscopy during TURB and we were not able to evaluate them. Urinary cfDNA levels (albeit adjusted to urine volume) can fluctuate not only in oncological, but also in some benign urological diseases. This has been clearly demonstrated. From the statistical point of view, there were relatively small numbers of subjects in the subanalysis comparing the first and the second urine portion. In the same way, small numbers were a possible issue in the subgroup analyses, even though all post-hoc corrective analyses gave significant results.

## Conclusion

It can be concluded that urinary cfDNA quantification could be included into a new diagnostic strategy leading to early non-invasive diagnosis of patients with bladder

cancer, in particular of higher stages. This could be potentially achieved by the combination of ucfDNA analysis with other clinical assays. To confirm this, further studies with a larger number of subjects and subsequent validation will be necessary.

On the basis of our experience with quantitative ucfDNA analyses, we suggest the following:

(1) The second morning urine sample should be used for analyses;

(2) The TA of ucfDNA should be calculated instead of determining the use of ucfDNA concentration alone.

### Acknowledgement

The study was supported by the grant No. NT12417 of the Internal Grant Agency of the Ministry of Health, the Czech Republic.

### References

- 1 Larré S, et al: Screening for bladder cancer: rationale, limitations, whom to target, and perspectives. *Eur Urol* 2013;63:1049–1058.
- 2 Saad A, et al: A study comparing various non-invasive methods of detecting bladder cancer in urine. *BJU Int* 2002;89:369–373.
- 3 Soukup V: Panel of urinary diagnostic markers for non-invasive detection of primary and recurrent urothelial urinary bladder carcinoma. *Urol Int* 2015, Epub ahead of print.
- 4 Schmitz-Dräger BJ, et al: Molecular markers for bladder cancer screening, early diagnosis, and surveillance: the WHO/ICUD consensus. *Urol Int* 2015;94:1–24.
- 5 Mandel P, Metais P: Les acides du plasma sanguin chez l'homme. *C R Seances Soc Biol Fil* 1948;142:241–243.
- 6 Esposito A, et al: Monitoring tumor-derived cell-free DNA in patients with solid tumors: clinical perspectives and research opportunities. *Cancer Treat Rev* 2014;40:648–655.
- 7 Antonatos D, et al: Cell-free DNA levels as a prognostic marker in acute myocardial infarction. *Ann N Y Acad Sci* 2006;1075:278–281.
- 8 García Moreira V, et al: Cell-free DNA as a noninvasive acute rejection marker in renal transplantation. *Clin Chem* 2009;55:1958–1966.
- 9 Hauser S, et al: Serum DNA hypermethylation in patients with kidney cancer: results of a prospective study. *Anticancer Res* 2013;33:4651–4656.
- 10 Ellinger J, et al: Hypermethylation of cell-free serum DNA indicates worse outcome in patients with bladder cancer. *J Urol* 2008;179:346–352.
- 11 Hauser S, et al: Cell-free serum DNA in patients with bladder cancer: results of a prospective multicenter study. *Anticancer Res* 2012;32:3119–3124.
- 12 Jung K, Fleischhacker M, Rabien A: Cell-free DNA in the blood as a solid tumor biomarker – a critical appraisal of the literature. *Clin Chim Acta* 2010;411:1611–1624.
- 13 Wang BG, et al: Increased plasma DNA integrity in cancer patients. *Cancer Res* 2003;63:3966–3968.
- 14 Casadio V, et al: Urine cell-free DNA integrity as a marker for early bladder cancer diagnosis: preliminary data. *Urol Oncol* 2013;31:1744–1750.
- 15 Zancan M, et al: Evaluation of cell-free DNA in urine as a marker for bladder cancer diagnosis. *Int J Biol Markers* 2009;24:147–155.
- 16 Chang HW, et al: Urinary cell-free DNA as a potential tumor marker for bladder cancer. *Int J Biol Markers* 2007;22:287–294.
- 17 Zancan M, et al: Free DNA in urine: a new marker for bladder cancer? Preliminary data. *Int J Biol Markers* 2005;20:134–136.
- 18 Cannas A, et al: Implications of storing urinary DNA from different populations for molecular analyses. *PLoS One* 2009;4:e6985.
- 19 Dyer AR, et al: Evaluation of measures of urinary albumin excretion in epidemiologic studies. *Am J Epidemiol* 2004;160:1122–1131.