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RESEARCH ARTICLE

CYFRA 21-1: A potential molecular marker for noninvasive differential diagnosis of urothelial carcinoma of bladder

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Abstract

Establishing CYFRA 21-1 detection for noninvasive differential diagnosis of urothelial carcinoma (UC) of bladder would help to improve assessment and follow-up of patients, as well as to improve screening of high-risk groups. The study group comprised of 147 subjects including 72 patients with UC of bladder, 75 controls and 17 follow-up cases. The levels of CYFRA 21-1 in serum, urine and urinary cell lysate were estimated by high sensitivity ELISA. Our results indicate that urinary CYFRA 21-1 provides a high value of overall sensitivity for UC of bladder and is also useful even for detection of low grade tumors that might indicate possible earlier detection and treatment administration.

Keywords: Urinary bladder neoplasms, urogenital cancer, diagnostic biomarker, early detection, cytokeratins, noninvasive diagnosis

Introduction

Bladder cancer is a common tumor of the urinary tract, accounting for 6–8% of all male malignancies and 2–3% of all female malignancies (MacVicar, 2000). Urothelial carcinoma (UC) of bladder accounting for about 90% of all bladder tumors (Metts et al. 2000) and is the second most common urological malignancy after prostate cancer (Lamm & Griffith 1992). Despite treatment, more than 50% of papillary tumors recur or progress. Mortality from bladder cancer has steadily increased. Therefore, long-term follow-up is required (Droller 1998). The gold standard for bladder carcinoma detection and post treatment surveillance protocols is cystoscopy, which is expensive and invasive (Chao et al. 2001). Urine cytology has also been used for many years, but its sensitivity for the diagnosis of low grade tumors is low (Nisman et al. 2002). The detection of tumour associated markers, such as nuclear matrix protein (NMP22), fibrin/fibrinogen degradation product (FDP), and bladder tumour antigen (BTA), in the urine has been evaluated extensively. However, the consensus is that these tests are not sufficiently sensitive for routine clinical use as a substitute for cytoscopy (Konety & Getzenberg 2001). Use of urinary peptides proteomics (Schiffer et al. 2009), microsatellite analysis (Wild et al. 2009), microRNAs (Dyrskjøt et al. 2009) and DNA microarray (Wang et al. 2009) might be useful for diagnosis, but these are very expensive and need scientific expertise. Therefore, the evaluation of inexpensive easier technique and other markers in voided urine samples is imperative. Ideally, this marker would be noninvasive, inexpensive, simple to use, unaffected by interpreter variability, has near perfect sensitivity, is specific for all stages and grades of tumor, and has the ability to reflect the severity and aggressiveness of the disease. A noninvasive method for the detection of UCs of the urinary bladder would help to improve assessment and the follow-up of patients with bladder carcinoma, as well as improve screening of high-risk, for the development of these malignancies (Chao et al. 2001). In recent years, many investigators have outlined a variety of applications of cytokeratins (CK), a major component of intermediate filaments, as diagnostic markers for differential diagnosis of carcinomas at the histologic level (Moll et al. 1982, Cooper et al.

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1985). During neoplastic transformations, CKs remain unchanged and they may be found intratumorally as well as in serum as partly degraded soluble fragments. Soluble proteolytic fragments can be measured in serum and other body fluids (Moll et al. 1982). Theoretically, the serum concentration of CK degradation product could reflect the neoplastic load and degree of malignant cell lysis. Thus, blood or any body fluid titre of such CK fragment might be a useful tumor marker in patients with neoplastic diseases (Andreadis et al. 2005).

CK-19 has the lowest molecular weight of human CKs (40 kDa) with known expression in normal urothelium as well as in urothelial tumors (Southgate et al. 1999). The proteolytic part of CK-19, referred to as CYFRA 21-1, is detected as a soluble molecule in serum and other body fluids, and it has been measured as a tumor marker in several neoplastic diseases (Andreadis et al. 2005). High levels of CYFRA 21-1 have been recorded in patients with solid tumors, including lung (Stieber et al. 1994), gastric (Nakata et al. 1996), ovarian (Gadducci et al. 2001), breast (Nakata et al. 2000) and prostate (Theyer et al. 1999) cancers. CYFRA 21-1 has been routinely used for 10 years as a serum tumor marker for diagnosing non-small cell lung cancer and a recurrence marker in this case (Niklinski et al. 1995). Early studies of bladder cancer showed the importance of CYFRA 21-1 in the diagnosis, prognosis, follow-up and prompt recognition of disease recurrence (Pariente et al. 1997). Dittadi et al. suggested that cells and cell debris contain a large amount of CYFRA 21-1 (Dittadi et al. 1996). The cell pellet so obtained is likely to have malignant cells that are shed during the process of micturition.

CYFRA 21-1 is a very promising tumor marker for the noninvasive diagnosis of UC of the bladder. It may also have the potential to detect premalignant disease and to predict the likelihood of recurrence. Soluble CK-19 fragments (CYFRA 21-1) are detectable using enzyme linked immunosorbent assay (ELISA) with the aid of two specific monoclonal antibodies (BM 19.21 and KS 19.1) (Pariente et al. 1997). In the present study, we aim to investigate the levels of soluble fragments of CK-19 in circulation, as well as in voided urine samples and in the lysates of urinary exfoliated cells by CYFRA 21-1 specific ELISA in bladder cancer (UC) patients, urolithiasis patients and in normal healthy individuals and, finally, to compare the sensitivity and specificity in each case with respect to grade and severity of disease.

Subjects and methods

Subjects

The sample collection for this study was conducted from March 2007 to July 2010 at All India Institute of Medical Science, New Delhi, India. The study included a total of 147 subjects in which 72 patients with UC of bladder, 20 patients of urolithiasis who were admitted to Department of Urology at All India Institute of Medical Sciences and 55 healthy age and sex-matched controls. There were 45 patients with non-muscle invasive (superficial) bladder UC and 27 patients with muscle-invasive UC of bladder. All 20 urolithiasis patients (urinary calculi) free from any malignant disorder were included in the study as controls. The non-muscle invasive urothelial tumors were graded according to the 2004 WHO grading system into low grade and high grade papillary neoplasms. There were 28 patients with low grade UC and 17 patients with high grade UC. The study protocol was approved by the Institute's ethical committee, and patients and controls gave informed consent. The patient and control groups were of similar socio-economic status. All the study subjects had normal serum albumin levels (>3.5 gm/dl) and were not anemic (hemoglobin > 12 gm/dl). None of the patients had any other significant disease or malignancies except bladder cancer and only the newly diagnosed patients with no prior chemotherapeutic treatment were included in this study. The patients with other histological subtypes (squamous cell, small cell, etc.), adenomatous carcinoma, second synchronous or metachronous malignancy, chronic infections of the urinary tract (prostatitis, cystitis or tuberculosis) were excluded from the study and only the patients with UC of bladder were included.

Methods

Sample collection

We collected venous blood and urine samples from each of the patients before surgery. A total of 4 mL venipuncture blood was collected from study subjects in plain endotoxin free vials. The tubes were centrifuged for 10 min at 3000 rpm, and serum was separated and stored at –20°C for further use.

First void urine specimens (50 mL) were collected in morning from the patients and the control groups in sterile plastic tubes and stored at –20°C for further use. Just before the analysis, urine was centrifuged at 10,000 rpm to separate exfoliated cells from urine. One milliliter of urine supernatant was collected for the test, and the cell pellet was further processed for urinary cell lysate preparation.

Urinary cell lysate preparation and protein estimation

The cell pellet was washed twice with phosphate buffer saline. The viable cells were stained with trypan blue and counted on Hemocytometer. Approximately, 106 cells in 1 mL of homogenization buffer were sonicated at 2 hz. The homogenization buffer contained PBS, lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100), PMSF (50 mM PMSF in isopropanol), protease inhibitors (100×) (1 ug/mL of leupeptin, aprotinin, and pepstatin). After sonication, the sample was centrifuged at –40 C and 10,000 rpm. The supernatant was stored at –200 C for further experiments. For equal concentration loading in all ELISA wells, the protein concentration of urinary cell lysate was estimated by Bradford's method and adjusted at 100 μg/mL.

CYFRA 21-1 ELISA

The level of CYFRA 21-1 in serum, in urine supernatant and in cell lysate was measured by high sensitivity ELISA by using commercially available kits supplied by DRG Diagnostics (Frauenbergstr, Marburg, Germany) which is based on the sandwich principle. The DRG CYFRA 21-1 ELISA uses two monoclonal antibodies KS 19.1 and BM 19.21 to determine CK-19 fragments. According to the manufacturer, the analytical sensitivity was found to be <0.266 ng/mL, intra-assay coefficient of variation was between 1.9 and 2.3% and inter-assay coefficient of variation was between 4.8–7.6%.

Statistical analysis

Statistical assessment was carried out with the SPSS 10.0 for Windows statistical software. Data are expressed as median (range) and differences in median values of the CYFRA 21-1 level between bladder cancer patients of various groups and control subjects were assessed for statistical significance using Kruskal Wallis non-parametric test with Bonferroni corrections for multiple comparisons. A p-value <0.003 was considered statistically significant. Moreover, diagnostic sensitivity, diagnostic specificity and receiver operating characteristics (ROC) curves and area under the ROC curve (AUC) were computed vs pathological grading and cut-off values were chosen. The positive predictive value (PPV) and negative predictive value (NPV) were calculated as follows:

$$\text{PPV} = \frac{\text{number of true positive}}{\text{number of true positive} + \text{number of false positive}}$$

$$\text{NPV} = \frac{\text{number of true negatives}}{\text{number of true negatives} + \text{number of false negative}}$$

Results

The study groups

The study group comprised of 147 subjects including 72 patients with UC of bladder (50 men, 22 women) and 55 healthy controls (38 men, 17 women) and 20 patients with urolithiasis (12 men, 8 women) taken as controls. The age range was 35–72 years (mean 53.9 ± 10.48) for the patients and 32–67 years (mean 49 ± 6.45) for the control subjects. According to 2004 WHO grading of superficial carcinoma of bladder, 28 patients were found to have low grade non-muscle invasive tumors, 17 patients were found to have high grade non-muscle invasive tumors and 27 patients were having muscle-invasive UC of bladder (Table 1). Out of 27 patients with muscle-invasive UC of bladder, 17 patients (11 without recurrence and 6 with recurrence) were followed-up after 6 months of cystectomy (Table 2B).

CYFRA levels in serum, urine and urinary cell lysate

The median levels of CYFRA 21-1 in serum were found to be 6.14, 0.98 and 1.02 ng/mL in bladder cancer patients (all), healthy controls and urolithiasis patients, respectively. The value was significantly higher in patients than both, healthy as well as urolithiasis controls ($p < 0.0001$) (Table 3A). Median urinary CYFRA in bladder cancer patients, healthy controls and urolithiasis patients was 8.80, 1.01 and 1.45 ng/mL, respectively. The values of CYFRA were not significant for bladder cancer patients (all), healthy controls and urolithiasis patients when compared between urine and serum (Table 3A). As similar to the values for serum, the urinary concentration in patients was shown significantly higher CYFRA levels than both of the controls ($p < 0.0001$).

Moreover, when cell lysate was used to evaluate CYFRA 21-1 levels, the median values were 26.38, 1.61 and 8.92 ng/mL, respectively for bladder cancer patients, healthy controls and urolithiasis patients. As in urine and serum, the concentration of CYFRA 21-1 was significantly higher in the bladder UC patients than controls

Table 1. Demographic and clinical data for all the subjects included in the study.

	Patients	Controls I (healthy individuals)	Controls II (urolithiasis patients)
Total No. (n)	72	55	20
Male/Female	50/22	38/17	12/8
Age: range (years) (mean ± SD)	36–71 (53.9 ± 10.38)	32–65 (49 ± 6.2)	29–69 (54.3 ± 14.3)

Table 2A. Level of CYFRA 21-1 in bladder cancer patients and controls.

Subjects	Serum median (range) (ng/mL)	Urine supernatant median (range) (ng/mL)	Urinary cell lysate median (range) (ng/mL)
Bladder cancer			
All patients (LG + HG + MI) (n = 72)	6.14 (2.23–20.50)	8.80 (3.26–19.30)	26.38 (11.0–48.7)
Non-muscle invasive low grade (LG) (n = 28)	3.63 (2.23–7.91)	5.78 (3.26–8.40)	16.95 (11.0–22.0)
Non-muscle invasive high grade (HG) (n = 17)	6.18 (4.2–9.2)	7.98 (4.41–10.40)	26.40 (22.1–34.5)
Muscle invasive (MI) (n = 27)	14.60 (10.75–20.5)	14.71 (10.4–19.3)	38.64 (31.2–48.7)
Controls			
Urolithiasis controls (n = 20)	1.22 (0.78–1.61)	1.45 (1.2–3.0)	8.92 (4.53–11.7)
Healthy controls (n = 55)	0.98 (0.38–1.98)	1.21 (0.38–3.20)	1.61 (0.4–5.4)

($p < 0.0001$). The level of CYFRA 21-1 in cell lysate was significantly higher than urine and serum both. ($p < 0.0001$) (Table 3A).

CYFRA 21-1 distribution by grade and stage of bladder carcinoma

The analysis of CYFRA 21-1 concentration among patients with low grade, high grade non-muscle invasive tumors, muscle-invasive tumors, urolithiasis patients, healthy controls and follow-up cases are shown in Table 2A and2B. The variance by grade and stage was highly significant. Comparisons between different grades showed significantly higher CYFRA 21-1 values for high grade non-muscle invasive tumors as compared with low grade non-muscle invasive tumors in serum, urine and urinary cell lysate ($p < 0.0001$). The difference between high grade non-muscle invasive and muscle invasive tumors was also significant ($p < 0.0001$) for serum urine and urinary cell lysate. The median CYFRA 21-1 levels in serum were 3.7-, 6.3-, and 14.89-fold greater and in urine 4.77-, 6.59- and 12.15-fold greater in patients with low grade, high grade and muscle invasive tumors than the levels in normal control participants. In urinary cell lysate, this difference was even more pronounced with median CYFRA 21-1 levels in patients with low grade, high grade and muscle-invasive tumors being 10.52-, 16.39- and 24-fold greater than healthy controls. Patients with invasive bladder carcinoma had significantly higher levels of urine CYFRA 21-1 compared with patients with non-muscle invasive disease ($p < 0.0001$) in all the three cases, i.e. in serum, urine and urinary cell lysate (Table 3A and 3C). The statistical significance of results for follow-up study subjects (with recurrence and without recurrence) was also compared (Table 3B and 3C).

Analysis of ROC curves

The efficacy of CYFRA 21-1 as a diagnostic test for bladder UC was assessed by ROC curve analysis. The results of CYFRA 21-1 levels in patients from low grade, high grade and invasive tumors were used to construct the ROC model for each type of sample analyzed, i.e. serum, urine supernatant and urinary cell lysate. Using the cut-off level of 1.91 ng/mL defined from this model, the sensitivity of the assay for detection of low grade bladder tumors in

Table 2B. Follow-up (muscle invasive): after 6 months of cystectomy.

Subjects		Serum median (range) (ng/mL)	Urine supernatant median (range) (ng/mL)	Urinary cell lysate median (range) (ng/mL)
Before cystectomy ($n = 17$)		14.18 (11.43–19.27)	14.56 (10.66–19.24)	38.31 (31.2–46.28)
Follow-up (after 6 months of cystectomy)	Without recurrence ($n = 11$)	1.03 (0.45–1.98)	1.59 (1.42–2.39)	3.80 (2.46–6.11)
	With recurrence ($n = 6$)	5.48 (3.21–8.4)	7.56 (5.71–9.43)	29.86 (26.4–34.5)

Table 3A. Statistical significance of results in all study groups.

Significance	Serum	Urine supernatant	Urinary cell lysate
All bladder cancer patients vs Non-muscle invasive low grade	0.0001	0.0032	0.0001
All bladder cancer patients vs Non-muscle invasive high grade	0.8184	0.0987	0.8592
All bladder cancer patients vs Muscle invasive	0.0001	0.0001	0.0001
All bladder cancer patients vs. Healthy controls	0.0001	0.0001	0.0001
All bladder cancer patients vs Urolithiasis controls	0.0001	0.0001	0.0001
Non-muscle invasive low grade vs. Non-muscle invasive high grade	0.0001	0.0001	0.0001
Non-muscle invasive low grade vs Muscle invasive	0.0001	0.0001	0.0001
Non-muscle invasive low grade vs. Healthy controls	0.0001	0.0001	0.0001
Non-muscle invasive low grade vs Urolithiasis controls	0.0021	0.0025	0.0001
Non-muscle invasive high grade vs Muscle invasive	0.0001	0.0001	0.0001
Non-muscle invasive high grade vs Healthy controls	0.0001	0.0001	0.0001
Non-muscle invasive high grade vs Urolithiasis controls	0.0001	0.0001	0.0001
Muscle invasive vs Healthy controls	0.0001	0.0001	0.0001
Muscle Invasive vs Urolithiasis controls	0.0001	0.0001	0.0001
Healthy controls vs Urolithiasis controls	0.6023	0.0030	0.0024

$P < 0.003$ is statistically significant, Bonferroni correction.

Table 3B. Statistical significance of results in follow-up study subjects.

Significance	Serum	Urine supernatant	Urinary cell lysate
Muscle invasive vs Follow-up without recurrence	0.0001	0.0001	0.0001
Muscle invasive vs Follow-up with recurrence	0.0001	0.0001	0.0001
All bladder cancer patients vs Follow-up without recurrence	0.0001	0.0001	0.0001
All bladder cancer patients vs Follow-up with recurrence	1.000	1.000	0.0023
Healthy controls vs Follow-up without recurrence	0.095	0.375	0.0001
Healthy controls vs Follow-up with recurrence	0.0001	0.0001	0.0001

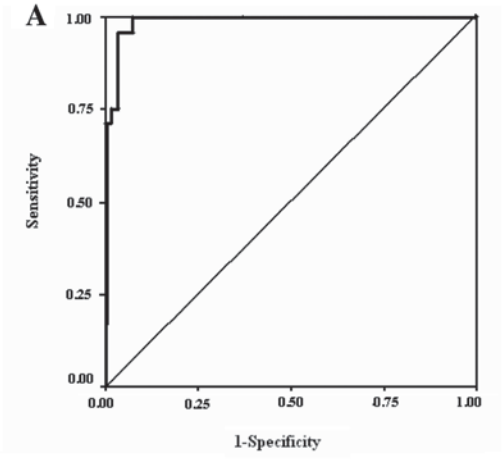
$P < 0.003$ is statistically significant, Bonferroni correction.

serum was 100%, and the specificity was 98.2%. The results from the patients in high grade are shown in the second ROC model, which was focused on the detection of high grade tumors (Figures 1A, 1B, and 1C). An optimal cut-off level of 4.36 ng/mL resulted in a sensitivity of 94% and a specificity of 98%. In addition, the results derived from patients from invasive tumors were analyzed by another ROC model focused on differential diagnosis of invasive tumors from superficial tumors. Cut-off at 10.97 ng/mL gave 96.3% sensitivity and 100% specificity. The AUC for serum (Figure 1A) in bladder UC patients and healthy controls at 95% confidence interval (0.973–1.00) was 0.989. AUC for urinary supernatant in bladder UC patients and healthy controls (Figure 1B) at 95% confidence interval

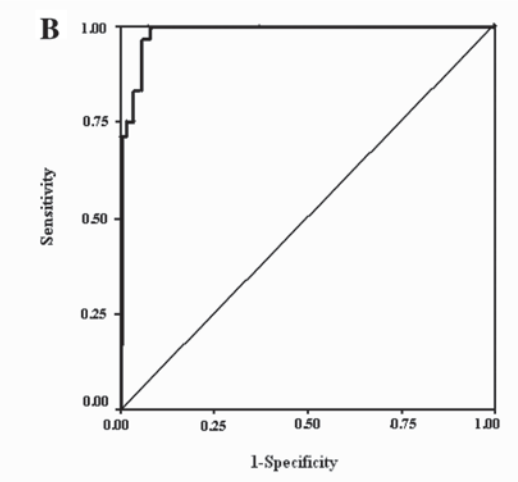
Table 3C. Statistical significance of results in different samples.

Significance	Serum vs urine supernatant	Serum vs urinary cell lysate	Urine supernatant vs urinary cell lysate
All bladder cancer	1.000	0.0001	0.0001
Non-muscle invasive low grade	0.040	0.0001	0.0001
Non-muscle invasive high grade	0.383	0.0001	0.0001
Muscle invasive	1.000	0.0001	0.0001
Urolithiasis controls	0.095	0.0001	0.0001
Healthy controls	1.000	1.0000	1.0000
Follow-up without recurrence	0.0022	0.0001	0.0032
Follow-up with recurrence	0.0016	0.0001	0.0001

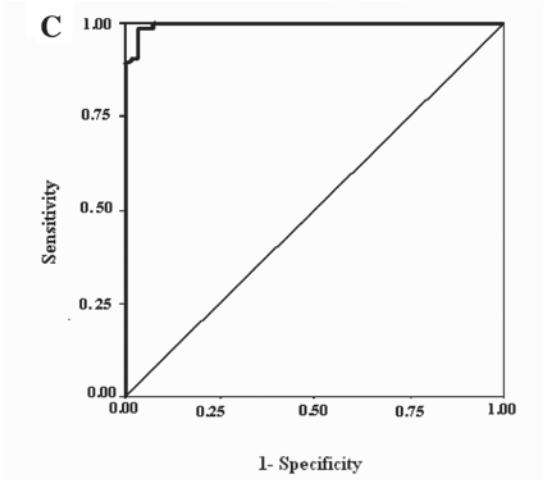
$P < 0.003$ is statistically significant, Bonferroni correction.



AUC: 0.989 (0.973-1.00) *Value in bracket is 95% confidence interval



AUC : 0.979 (0.968-1.00) *Value in bracket is 95% confidence interval



AUC 0.966 (0.989-1.00) *Value in bracket is 95% confidence interval

Figure 1. (A) Receiver operating characteristics (ROC) curves for serum concentration of CYFRA 21-1 in total bladder UC patients and healthy controls. (B) Receiver operating characteristics (ROC) curves for urinary supernatant concentration of CYFRA 21-1 in total bladder UC patients and healthy controls. (C) Receiver operating characteristics (ROC) curves for urinary cell lysate concentration of CYFRA 21-1 in total bladder UC patients and healthy controls.

(0.968–1.00) was 0.979. In the ROC analysis of urinary cell lysate in bladder UC patients and healthy controls (Figure 1C) at 95% confidence interval (0.989–1.00), it was 0.966.

Similarly the cut-offs for the diagnosis of low grade tumors, high grade tumors and muscle-invasive tumors were obtained from urinary CYFRA levels as 2.6, 4.9 and 10.53 ng/mL, respectively using ROC models. Moreover, we also evaluated the optimal cut-off values for detection of low grade, high grade and muscle-invasive disease by analysis of CYFRA 21-1 in urinary cell lysate; these were found to be 8.2, 22.25 and 31.46 ng/mL, respectively. The sensitivity, specificity, NPV, PPV etc. are elaborated in Table 4.

Discussion

Urinary tumor markers are of interest when repeated cystoscopies are demanded. At such times, alternative noninvasive diagnostic methods might be helpful to reduce or guide the use of cystoscopy. The greatest challenge in the surveillance of non-muscle invasive bladder carcinoma is the early detection of recurrence before progression of the tumor. Monitoring of the disease could accelerate diagnosis before the scheduled cystoscopies, leading to an early detection of recurrence. Moreover, disease monitoring could detect persistence of the disease in complicated bladder tumors. Bladder cancer patients would greatly benefit from serial urinary tumor marker measurement, and the number of cystoscopies could be substantially reduced in long-term surveillance. Based on these results, urinary tumor markers could be seriously considered as adjuncts providing guidance for individualizing intercystocopic periods in this situation (Sánchez-Carbayo et al. 2001).

Although no marker has yet replaced the need to perform cystoscopy and cytology, a new sensitive and noninvasive method for the detection of bladder cancer can minimize the cost and difficulty of screening and long-term surveillance of patients who have or are at risk for bladder cancer (Chao et al. 2001). In the current study, in an attempt to improve the sensitivity and specificity for diagnosis of bladder carcinoma, we evaluated the efficiency of specific ELISA test to detect molecular marker CYFRA 21-1 in serum, urine supernatant and cells lysate from voided urine samples.

An ideal urine test for the detection of bladder carcinoma should be rapid and technically easy to perform and interpret. Tests like NMP22, BTA stat, FDP, hyaluronic acid/hyaluronidase, and immunocyt have been evaluated for the detection of bladder tumors and have shown sensitivity ranging between 52 and 91.2% and specificity ranging between 60 and 91% (Stampfer et al. 1998; Ramakumar et al. 1999; Mian et al. 1999; Lokeshwar et al. 2000). Bjorklund and Bjorklund isolated the tissue polypeptide antigen (Bjorklund & Bjorklund 1957) which was defined later as the complex of CK-8, -18, and -19 (Weber et al. 1984). Various assays identifying different CK from this triad have been developed. The urinary bladder carcinoma antigen (UBC) assay identifies CK-8 and -18. The tissue polypeptide specific antigen (TPS) assay is specific for CK-18. These tests resulted in a sensitivity of 64–80.2% and a specificity of 84–95% when used for detection of bladder carcinoma (Sánchez-Carbayo et al. 1999; Mian et al., 2000; Sánchez-Carbayo et al. 2000). The concentration of soluble fragments of CK-19 can be determined by the CYFRA 21-1 assay using two mouse monoclonal antibodies. The epitopes for these antibodies are located in the *rod* domain of the polypeptide chain within amino acids 311–367 (Bodenmüller 1995). This assay when applied to urine samples for detection of bladder tumors the sensitivity ranged between 64.8 and 96.9%, and the specificity was between 67.2 and 97.2% (Pariente et al. 2000; Nisman et al. 2002). The variability of the results can be attributed to different patient populations.

The different steps leading to the development of urothelial tumors, first involve urothelial cell damage and the exophytic growth of the tumors into the bladder which may exfoliate lysed cells in urine and thus their CK-19 content. The onset of invasion occurs with the disruption of the basement membrane and a probably concomitant discharge of intracellular components (including CK-19) in the bladder. On this basis, it is rational to assume that the presence of these components might be detected and monitored in urine if a reliable marker is available (Pariente et al. 1997). Moreover, we also collected exfoliated cells from urine by means of centrifugation. The cell pellet so obtained is likely to have malignant cells that are shed during the process of micturition. When these cells were sonicated, they got ruptured and the intracellular contents got released in the solution. We thereby removed the cellular debris using centrifugation and the

Table 4. Cut-off (ng/mL), diagnostic sensitivity, diagnostic specificity, PPV, NPV of different patient groups.

	Serum			Urine supernatant			Urinary cell lysate		
	LG	HG	MI	LG	HG	MI	LG	HG	MI
Cut-off (ng/mL)	1.91	4.36	10.97	2.6	4.9	10.53	11.05	22.25	31.46
Diagnostic sensitivity	97%	94%	96.3%	92.9%	94.1%	96.3%	96.4%	94.1%	96.3%
Diagnostic specificity	98.2%	98%	100%	96.4%	100%	100%	100%	100%	100%
PPV	94.12%	88.36%	99.64%	88.49%	99.35%	99.64%	99.65%	99.35%	99.64%
NPV	99.10%	99.02%	98.96%	97.85%	99.06%	98.96%	98.94%	99.06%	98.96%

LG, non-muscle invasive low grade; HG, non-muscle invasive high grade; MI, muscle invasive; PPV, positive predictive value; NPV, negative predictive value.

supernatant was used to assay CYFRA 21-1 fragments using sandwich ELISA.

In addition, previous study has revealed instability and differences in CYFRA 21-1 concentration in uncentrifuged/centrifuged urine. It was also elucidated that the concentration of CYFRA 21-1 did not change if the urine sample was frozen immediately and stored at –20°C before assay (Nisman et al. 2002). Dittadi et al. suggested that cells and cell debris contain a large amount of CYFRA 21-1 (Dittadi et al. 1996) and thus, must be eliminated by centrifugation. Therefore, we froze collected urine samples immediately after collection at –20°C and centrifuged the samples just before the assay to collect urine supernatant and cellular pellet. For equal concentration loading in all ELISA wells, we estimated the protein concentration of urinary cell lysate by Bradford's method.

In a study by Pariente et al., when a cut-off of 4 ng/mL of urinary CYFRA 21-1 was used, 96% sensitivity and 74% specificity was obtained. Moreover, with the same cut-off value, sensitivity was 80% for grade 1, 92% for grade 2 and 100% for grade 3 tumors (Pariente et al. 1997). Also in a study by Sánchez-Carbayo et al., Urinary CYFRA 21-1 had the highest sensitivity of 83.8% and 95% specificity at the cut-off of 5.4 ng/mL when compared to other markers like NMP22, UBC and TPS (Sánchez-Carbayo et al. 1999). In another study by Nisman et al., it was determined that an optimal cut-off value of 4.9 ng/mL resulted in an overall sensitivity of 79.3% and a specificity of 88.6% for the diagnosis of primary tumors (Nisman et al. 2002). In yet another study by Morsi et al., the cut-off of 8.1 ng/mL sensitivity was 85.7% but specificity was only 46% (Morsi et al. 2006). On the other hand, Dittadi et al. reported a much lower sensitivity of 75% but a much higher specificity of 95% for the same assay (Dittadi et al. 1996). We have found a considerably higher sensitivity of 92.9% and specificity of 96.4% at the cut-off of 2.6 ng/mL for urinary CYFRA 21-1.

The current study demonstrated a highly significant correlation between the tumor grade and stage as well as the levels of CYFRA 21-1. This is in accordance with other previous studies by Nisman et al. (Nisman et al. 2002), Pariente et al. (Pariente et al. 1997) and Sánchez-Carbayo et al. (Sánchez-Carbayo et al. 2000), all of whom reported a global statistical difference for this marker according to tumor grade. The implication of this correlation seems to be related to bladder tumor biology. Cells from high-grade bladder carcinoma are less cohesive and shed more readily into urine than cells from well-differentiated tumors (Messing & Catalona 1998). Muscle-invasive tumors resulted in significantly higher levels of urinary CYFRA 21-1 than non-muscle invasive tumors (pTa or pT1). These results are similar to the results of previous studies (Pariente et al. 1997; Nisman et al. 2002; Sánchez-Carbayo et al. 2000). Our results demonstrate a considerably high sensitivity and specificity at various cut-offs, selected for detection of bladder tumors. Instead of defining a single cut-off for detection of bladder malignancy, we determined different cut-offs for low and high grade and invasive tumors thus enabling us to detect not only malignancy but also allowing a differential diagnosis that can be extremely helpful as an adjunct to cystoscopy/cytology in determining the grade of the tumors. Moreover, according to a study by Andreadis et al., CYFRA 21-1 is a remarkable indicator of chemotherapy response, thus it can also play a major role in prognosis of bladder tumors (Andreadis et al. 2005).

Apart from this, we also compared in a single study the differences in the levels of CYFRA 21-1 in three different types of samples: serum, urine supernatant and urinary cell lysate in the same patients. This is the first attempt in which urinary cell lysate was used to measure CYFRA 21-1 levels. This was to determine whether lysate serves as a better tumor marker for the differential diagnosis of bladder tumors. Our results show that in all bladder cancer patients, the cell lysate contains significantly higher concentration (median, 26.38 ng/mL) of CYFRA 21-1 as compared to serum (median, 6.14 ng/mL) and urine (median, 8.80 ng/mL). For further confirmation of CYFRA 21-1 values, we have collected samples from follow-up patients after cystectomy. Interestingly, CYFRA 21-1 levels have shown significant decline in non-recurred cases as compared to samples with recurrences. Although the sample size is small, the results are important which further reassures the CYFRA 21-1 as a noninvasive and potential marker for the diagnosis of bladder cancer.

Our previous works have shown that oxidative stress and Th1/Th2 cytokine imbalance play an important role in pathogenesis of bladder cancer (Badjatia et al. 2010; Satyam et al. 2011). In the present study, our results confirm the relevance of CYFRA 21-1 for noninvasive diagnosis of UC bladder tumors. Especially by using urinary cell lysate, we got very high sensitivity and specificity of 96.4 and 100%, respectively, at the cut-off of 11.05 ng/mL for overall detection of UC bladder tumors from healthy controls. We also got 100% specificity each in the case of low grade, high grade and muscle-invasive tumors for differential diagnosis of the tumors. Our results indicate that urinary CYFRA 21-1 provides a high value of overall sensitivity for UC of bladder and is also useful even for detection of low grade tumors that makes both early detection and treatment possible. Our study suggests diagnostic improvement of urine-based molecular markers for the detection of bladder cancer in the urine and could improve the sensitivity for noninvasive diagnosis of urinary bladder tumors and reducing the need of a cystoscopy.

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Declaration of interest

The authors report no conflict of interest.

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