The clinical relevance of urinary soluble fas (sFas) for diagnosis of bilharzial bladder cancer

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Background: Fas/CD95, a membrane-bound type I protein, plays a key role in induction of apoptosis and in tumorigenesis.

Objective: We adapted and evaluated measurement of urinary sFas using enzyme-linked immunosorbent assay and compared the results with voided urine cytology.

Materials and Methods: Voided urine samples were provided from 203 individuals (120 bladder cancer [112 bilharzial]; 43 benign urologic disorders [20 bilharzial dysplastic lesions]; and 40 healthy volunteers). Urine sediment was used for cytology and the supernatant for estimation of sFas by ELISA.

Results: A receiver operating characteristic curve (ROC) was used to determine the best cutoff value for urinary sFas. Positivity rates and mean rank levels for sFas showed significant difference among the three investigated groups (p < 0.0001), and was related to bilharzial infection, pathological type, clinical stages, and histological grades (p < 0.01). The sensitivity of sFas for early detection of bladder cancer, especially those with superficial and low grades tumors, was superior to urine cytology; moreover, results from sensitivity of urine cytology were improved when combined with sFas.

Conclusion: Urinary sFas may be used as a novel noninvasive diagnostic marker for bilharzial bladder cancer patients. Further multicentric studies are warranted to corroborate these findings and to establish an optimal sFas cut-point.

Keywords: Bilharzial bladder cancer, sFas, voided urine cytology

Bladder cancer is a global problem, however the highest frequency is observed in Egypt. This high incidence is believed to be the result of endemic infestation by Schistosoma hematobium, which contributes to defining the characteristic pathology

Abbreviations
sFas = soluble Fas
ELISA = enzyme linked immune assay
ROC curve = receiver operating characteristic curve

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“Schistosoma-associated bladder cancer (SABC)” [1] or simply bilharzial-associated bladder cancer (BBC). According to the registry of the National Cancer Institute, Cairo, 2002[2], bilharzial-associated bladder cancer represents the commonest malignancy among all diagnosed cancer cases in Egypt.

To date, the criterion standard for bladder cancer detection remains cystoscopic biopsy of suspicious lesions. Not only this technique is invasive, but 10% to 40% of malignancies may be undetected by this procedure [3]. On the other hand, urine cytology is known for its good specificity, but poor sensitivity for early stages and low grades of cancer [4]. Thus, an array of urine markers that take advantage of exfoliated cells in the urine for detection of cell surface antigens, nuclear morphology, or gene expression have
been studied in bladder cancer [5-13] to improve the diagnostic ability of urine cytology and perhaps to reduce the need for frequent cystoscopies during follow-up. This is especially true in those with low-risk disease [14]. While few markers have undergone clinical trials and have been approved for clinical use, most remain investigational and are undergoing further development and preclinical evaluation.

Derailment of apoptosis plays an important role in the development, growth and therapy resistance of malignant tumors, and influences the prognosis. As a member of TNF-family receptors, Fas (Apo-1/CD95) is a cell surface protein that can induce apoptosis through its cytosolic tail after binding to its specific ligand, Fas Ligand (FasL). Fas is found in two forms, transmembrane and soluble. The soluble form of Fas (sFas), which has five variants produced via alternative mRNA splicing, inhibits Fas-mediated apoptosis by neutralizing FasL or anti-Fas antibody [15]. Increased concentration of serum sFas has been reported in various neoplastic diseases, such as leukemia, lymphoma, and breast cancer. In bladder cancer patients, the association between increase in serum sFas and poor prognosis was evident [16]. These findings suggest that cancer cells upregulate or stimulate sFas production to protect themselves from Fas-mediated apoptosis [17].

This study was conducted to assess the diagnostic suitability of sFas in voided urine samples especially those from subjects with bilharzial infestation in comparison to urine cytology. Moreover, we endeavored to investigate its correlation with clinicopathological features.

Materials and methods

Study population

A total of 203 subjects were enrolled. After obtaining informed consent, patients provided a single voided urine sample and a cytologic test for urine sediment was performed before cystoscopy. All patients underwent cystoscopy as the reference standard for detection of bladder carcinoma. Accordingly, 163 patients included in the study were diagnosed into malignant and benign groups. The malignant group included 120 patients (mean age, 62 ± 11 y; range, 25–83 y). Of those patients, 44 were diagnosed by histopathology as transitional cell carcinoma (TCC), and 76 as squamous cell carcinoma (SCC). Tumor staging and grading were determined according to TNM and World Health Organization classification [18,19]. The benign group included 43 patients with benign urological diseases (mean age, 43 ± 15 y; range, 21–75 y). A group of 40 healthy volunteers (mean age, 39 ± 8 y; range, 25–57 y) were recruited from the hospital laboratory staff as control subjects.

Collection of samples

Sera (5ml) and voided urine (30–60 mL) samples were obtained from all individuals before they received any treatment and before they underwent surgery. Each urine sample was collected into an approved Urine Collection Cup that measures volume and was sealed immediately and placed on ice then centrifuged for 15–20 min at 2500–4000×g. The urinary sediment was washed twice with phosphate-buffered saline at pH 7.0. A portion of the pellets was used for cytologic and microscopic examinations [20].

Detection of schistosomiasis antibodies in serum

The sera were used for detection of schistosomiasis antibodies by the indirect hemagglutination test, using a Cellognost Schistosomiasis H kit (Dade Behring Marburg GmbH, Marburg, Germany) [21].

Assessment of human urinary sFas

sFas level was quantified by the ELISA sandwich method according to manufacturer’s guidelines (Quantikine). Assay diluent was added to microtiter plates coated with a monoclonal antibody specific for sFas. Then, 100 µl of urine supernatants and standards were added to the wells and incubated for 2 hours at room temperature. After washing, sFas conjugant (polyclonal antibody against Fas conjugated to horseradish peroxidase) was added to wells and further incubation for 2 hours at room temperature was made. Second wash was done, then tetramethyl benzidine substrate was added and the plate was incubated for 30 min at room temperature in a dark place for chromogenic reaction. The reaction was then stopped by H₂SO₄. The optical density was measured at 450 nm/620 nm dual wavelength using a spectrophotometer plate reader. A standard curve was created using computer software to determine sFas concentrations that are expressed in ng/mL. To control for the differences in urine concentration, the protein in urine was determined by Bradford’s method [22] using bovine serum albumin as a standard. The sFas concentration was then expressed as ng/mg protein.
Statistical analysis
The threshold value for optimal sensitivity and specificity of sFas was determined by a receiver operating characteristic (ROC) curve, which was constructed by calculating the true-positive fraction (sensitivity %) and false-positive fraction (100 – specificity %) of the above-mentioned markers at several cut-off points. The ROC curve can be used to select the best cutoff for the diagnostic test that maximizes the sensitivity and minimizes the false-positive rate [23]. Univariate analyses were performed using a Chi-square test; the level of significance was determined to be less than 0.05. All analyses were performed using software in the Statistical Package for the Social Sciences (SPSS, Chicago, IL).

Results
A total of 203 subjects were included in this study. One hundred and twenty were diagnosed with bladder cancer. One hundred twelve had bilharzial bladder cancer and the remaining (n = 8) had nonbilharzial bladder cancer. Among the 43 patients diagnosed with benign urological diseases, 20 showed benign bilharzial lesions [12 with bilharzial dysplasia, 8 with bilharzial cystitis], and the rest (n = 23) had nonbilharzial lesions (11 with renal stones, 7 with bladder polyp, and 5 with pyelonephritis). All healthy volunteers (n = 40) were nonbilharzial.

ELISA performance characteristics
Pooled voided urine samples were used only for the validity experiments and to assure the reproducibility of the assays as follows:

(A) We tested the precision of the urinary sFas by measuring the same sample pool for (a) six times in the same assay (intra-assay variation) and (b) for a five times in different times of the day or in different days (inter-assay variation). The results were reproducible as shown in (Table 1).

(B) In an experiment on the analytical recovery of the investigated urinary sFas we used three pools from urine supernatant 0.157, 0.428, and 1.448 ng/mg protein for sFas, each sample was assayed in duplicates after addition of three different amounts of manufacturer-supplied kit standards (62.5, 250, 1000). The calculated recovery range is shown in (Table 1).

Table 1. Performance of investigated urinary angiogenic markers

<table>
<thead>
<tr>
<th>Performance</th>
<th>Urinary sFas</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Pool 1</td>
</tr>
<tr>
<td>• Assay Precision (Code repetition)</td>
<td></td>
</tr>
<tr>
<td>Intra-assay (6)</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.157</td>
</tr>
<tr>
<td>SD</td>
<td>0.14</td>
</tr>
<tr>
<td>CV%</td>
<td>8.9</td>
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</table>

| Inter-assay (5)  |              |              |
| Mean         | 0.157        | 0.428        | 1.448        |
| SD           | 0.015        | 0.038        | 0.038        |
| CV%          | 9.5          | 9.1          | 9.5          |

• Analytical recovery

| Basal | 0.157 | 0.428 | 1.448 |
| Added | 62.5  | 62.5  | 62.5  |
| Recovered | 62.5 | 61    | 62.5  |
| Recovery% | 100  | 97.6  | 100   |

| Basal | 0.157 | 0.428 | 1.448 |
| Added | 250   | 250   | 250   |
| Recovered | 249 | 248   | 248   |
| Recovery% | 99.6 | 99.2  | 99.2  |

| Basal | 0.157 | 0.428 | 1.448 |
| Added | 1000  | 1000  | 1000  |
| Recovered | 999 | 1000  | 1000  |
| Recovery% | 99.9 | 100   | 100   |
**Cutoff point for urinary sFas**

The benign and healthy normal groups were combined in a nonmalignant group and the best cutoff value for urinary sFas for the discrimination between the nonmalignant from malignant groups was calculated using ROC as 993.4 ng/mg protein which maximized the sum of the sensitivity and specificity at which highest predictive values are reached protein (Figure 1).

**The positivity of urinary sFAS among the different groups**

As shown in Table 2, mean rank level of urinary sFas was increased 2.55 and 4.73 fold in the malignant group compared with benign and control groups ($P<0.0001$), respectively. While mean rank level of urinary sFas was increased 4.7 fold in benign group as compared with the control group ($p < 0.0001$).

![ROC curve analysis for sFas to calculate the best cut-off point to discriminate between malignant and nonmalignant groups. Open circle denotes best cut-off point of sFas (solid line) as 993.4 ng/mg protein (sensitivity = 97.5% and specificity = 96.34%; AUC [SE] = 0.998 [0.007], 95% confidence limits range = 0.962–0.998, $p < 0.0001$).](image)

**Table 2.** Positivity rates of sFas and urine cytology among different investigated groups

<table>
<thead>
<tr>
<th>Control (n = 40)</th>
<th>Benign (n = 43)</th>
<th>Malignant (n = 120)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Non-Bilh. (n = 23)</td>
</tr>
<tr>
<td>sFas (ng/mg protein)</td>
<td>30.12</td>
<td>55.81</td>
</tr>
<tr>
<td>≥993.4 ng/mg protein</td>
<td>0 (0%)</td>
<td>9 (20.9%)</td>
</tr>
<tr>
<td>Positive urine Cytology</td>
<td>0 (0%)</td>
<td>2 (4.7%)</td>
</tr>
</tbody>
</table>

* Significant $p < 0.0001$ using nonparametric test. *Pearson Chi-square test.

"Statistical significance was found between the 3 studied groups (control, benign, and malignant) regarding sFas and cytology ($\chi^2 = 157$, and 75.4, at $p < 0.0001$, respectively).

Statistical significance was detected between bilharzial benign group (bilharzial dysplasia and bilharzial cystitis) versus bilharzial malignant group ($\chi^2 = 82.4$, at $p < 0.0001$). Statistical significance was detected between malignant bilharzial and nonbilharzial group ($\chi^2 = 20.7$, at $p < 0.0001$). No statistical difference between bilharzial (bilharzial dysplasia, and bilharzial cystitis) and nonbilharzial benign groups.
Relation between positivity rate of urinary sFas and clinicopathological factors

The relation between urinary sFas among the different clinicopathological factors in the malignant group revealed a significant difference to bilharziasis as 111 out of 117 (94%) bilharzial bladder cancer patients showed positive sFas (\( \chi^2 = 17.8, p < 0.0001 \)), pathological types as all the SCC cases showed positive sFas (\( \chi^2 = 5.31, p = 0.021 \)), clinical stages as 103 of early stage bladder cancer patients showed positive sFas (\( \chi^2 = 7.7, p = 0.005 \)), and histological grades were all of low grade bladder cancer patients were positive sFas (\( \chi^2 = 7.4, p = 0.006 \)).

Combined sensitivity and specificity of urinary sFAS and urine cytology

Sensitivity and specificity for urinary sFAS and urine cytology as well as their combination were tested for detection of bladder cancer, superficial and low-grade tumors as shown in Table 3. The sensitivity of urinary sFas was higher than urine cytology for detection of bladder cancer, superficial and low-grade tumors. Moreover, the sensitivity of urine cytology was improved when both were combined.

Discussion

To our knowledge, this is the first study to quantify the basal concentration of urinary sFas in bilharzial and nonbilharzial bladder cancers and to establish a cutoff value, which may be of clinical importance in detecting bladder cancer and in evaluating its stage and grade in comparison to urine cytology. The urinary sFas was assessed in 203 individuals using ELISA technique as a quantitative method. Urinary levels of these factors were found to be influenced by hydration status and urine output, so their levels were normalized to urinary protein content (mg) for the accuracy of the test. The normalization to total protein was found to be better than to creatinine because total protein is less influenced by hematuria, a condition commonly found in bladder cancer patients [24]. The performance of the urinary sFas by ELISA has been evaluated and was reliable for their quantization in minimal amount of voided urine samples. Authors found it is more interesting to evaluate the functional sensitivity, which is preferable for the evaluation of all low-range urinary marker concentrations of clinical relevance, either at initial diagnosis or at follow-up. Our functional sensitivity study was performed in the actual conditions of a routine clinical laboratory: we measured the urine pools using three different lots of calibration standards. The intra- and interassay CVs and the analytical recovery percentage for the investigated urinary marker ranged from 8.9–9.5, and 97.6%–100% as recommended for tumor markers [25]. The diagnostic profile of urinary sFas was evaluated using a ROC curve. Both the positivity rates of sFas were significantly high in bladder cancer patients compared with the benign cases while it was not detected in controls. Similarly, the level of sFas in bladder cancer cases was 86.7- and 172.63-fold higher than benign and control individuals, respectively. For benign cases, urinary sFas levels were 25.69 folds higher than control individuals, indicating the usefulness of sFas as powerful urinary diagnostic marker for bladder cancer. This result confirms the hypothesis that apoptosis-regulating genes play a critical role in carcinogenesis. Fas/FasL system exerts a central role in the apoptosis process and its alterations are noticeable in bladder cancer [14]. Soluble Fas, is expressed and shedded by human transitional bladder carcinoma cell lines. It is generated by alternative mRNA splicing [26, 27]. sFas has been identified in the supernatants of several tumour cell lines [28-30]. It does prevent recognition of malignant cells by the immune system [28, 31-33].

Table 3. Combined sensitivity and specificity for investigated parameters in detection of bladder cancer, especially superficial, low-grade cancer

<table>
<thead>
<tr>
<th>Investigated parameters</th>
<th>Bladder cancer</th>
<th>Superficial bladder cancer</th>
<th>Low grade bladder cancer</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Sens.%</td>
<td>Spec.%</td>
<td>Sens.%</td>
</tr>
<tr>
<td>Urinary sFas</td>
<td>97.5</td>
<td>88</td>
<td>97.2</td>
</tr>
<tr>
<td>Urine cytology</td>
<td>62.5</td>
<td>97.6</td>
<td>67</td>
</tr>
<tr>
<td>Urinary sFas + Urine cytology</td>
<td>99.2</td>
<td>88</td>
<td>99.1</td>
</tr>
</tbody>
</table>
In the present study bilharzial was detected in 133 patients. Of them, 113 were diagnosed as having bladder cancer while the remaining had benign urological diseases (n = 20). Among the enrolled patients collectively, positive sFas was significantly higher in patients (benign versus malignant) with bilharzial infection compared with those with no bilharzial infection. Bilharziasis was associating at 30% (6/20) and 99.1% (111/112) positive sFas (≥993.4 ng/mg protein) among the benign cases versus the bladder cancer cases, respectively. For the all the groups, significant increment of sFas was reported in bilharzial bladder cancer patients compared with nonbilharzial patients (χ² = 20.7, p < 0.0001); however, no significant differences were reported among benign bilharzial and nonbilharzial patients. Moreover, the current study detected 2 false-positive smears in benign cases with bilharzial dysplastic lesions. The presence of atypical cells in these benign cases may be attributed to morphologic alterations in premalignant cells or reactive atypia to bilharzial infestation. In the future, cystoscopies and biopsies will determine whether the positive cytology was false-positive or an indication of early tumor detection.

The relationship between a positivity rate of sFas and urine cytology with different clinicopathological factors among bladder cancer patients was investigated. The sFas positivity rate was significantly increased with bilharzial infection. This significant association reflects the more aggressive phenotype of this bladder cancer subtype. Similarly, sFas positivity rate increment towards SCC versus TCC was significant. The incidence of bladder neoplasm secondary to bilharziasis, which is frequently associated with the development of SCC, is particularly high in the Nile River Valley. Our results confirm the usefulness of using urinary sFas for diagnosing bilharzial bladder carcinoma either as TCC or SCC. Moreover, sFas positivity rates were increased in cancer patients in a manner directly related to tumor stage and burden, suggesting a potential role for sFas in the biology of malignant disease. When a urine molecular marker is used for diagnostic work-up of bladder cancer, it should have a high sensitivity [34]. As reported in Table 3, the sensitivity of urinary sFas was superior over urine cytology for early detection of bladder cancer patients with superficial and low grade tumors. Although further multicentric studies will be required to define the impact of urinary sFas on early detection and disease monitoring before clinical application, longitudinal follow-up of bilharzial cases is needed to understand its association in cancer development. Our data predict a causal role for sFas in early detection of bilharzial bladder cancer, and this antagonistic, antiapoptotic protein may well become a novel target in both detection and intervention.

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