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Original article

Elevated urinary total sialic acid and increased oxidative stress in patients with bladder cancer

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Background: Increased production and release of sialic acid have been reported in many malignant conditions including bladder cancer. 8-hydroxydeoxyguanosine (8-OHdG) and malondialdehyde (MDA) have been widely used as oxidative stress biomarkers.

Objective: Determine urinary levels of total sialic acid (TSA), 8-OHdG, and MDA in patients with urinary bladder cancer, and evaluate their clinical relevance.

Patients and methods: Forty-five patients with histologically proven bladder cancer and 41 healthy subjects were recruited for the study. Morning urine samples were collected from all participants for measurements of TSA, 8-OHdG and MDA using thiobarbituric assay, competitive ELISA and spectrophotometry methods, respectively. Histological examination was performed for all patients.

Results: Bladder cancer patients excreted urinary TSA, 8-OHdG, and MDA significantly higher than healthy controls. Based on receiver operating characteristic curve analysis, urinary TSA had adequate diagnostic potential to distinguish patients from healthy populations, and its cutoff value was chosen at 95.26 μg/g creatinine. Sensitivity, specificity, and accuracy of urinary TSA determination were 75.6%, 75.6%, and 75.6%, respectively. Both in patient and healthy groups, urinary TSA was linearly correlated with urinary 8-OHdG. Patients with high-severity grade (n=27) excreted urinary TSA significantly greater than those with low-severity grade (n=18).

Conclusion: Urinary TSA, 8-OHdG, and MDA increased in patients with bladder cancer. The elevated urinary TSA was associated with enhanced oxidative stress. In addition, urinary TSA increased with progressiveness of the tumor.

Keywords: Bladder cancer, 8-OHdG, oxidative stress, transitional cell carcinoma, urinary sialic acid

Urinary bladder malignancy is a significant public health problem in the world. It is the fourth most prevalent cancer in men and the second most common genitourinary cancers [1, 2]. In Thailand, estimated incidence of bladder cancer is reported at 4.2 and 1.3 per 100,000 populations in males and females, respectively [3]. The most common type of bladder tumor is transitional cell carcinoma (TCC) accounting more than 90% of all cases. Due to wide spectrum of the disease, TCC is categorized into three main types, i.e., superficial (Ta-Tis-T1), muscle-invasive (T2-T4), and disseminated (N+/M+) types. TCC frequently recurs posing a difficulty of management. Approximately 50-70% of the superficial TCC recurs, and 10-30% usually progresses to muscle-invasive type and eventually metastasis. To date, cystoscopy combined with urine cytology is used as a gold standard technique for bladder cancer diagnosis. Although a great effort has been put into biomarker discovery during the past decade, none of the reported biomarkers has a potential for use in clinics [4]. Discovery of new clinically useful biomarkers for diagnosis and prognosis of TCC remains challenging.

Sialic acid or neuraminic acid, a nine-carbon negatively charged amino sugar is commonly found

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as a terminal sugar residue in oligosaccharide chains of glycoconjugates. Altered glycosylation, particularly sialylation is a main feature of cancer cell surface, which has been suggested to play a critical role in tumor invasion and metastasis [5]. Increased sialylation of glycoprotein on cancer cell surface is frequently found. It is associated with increase in invasiveness and metastatic potential [5, 6]. Determination of sialic acid in body fluid samples has been widely investigated to establish as potential tumor marker in malignancies [7-9]. Increased total sialic acid (TSA) in blood circulation has been reported in many cancers such as breast cancer [10], colorectal cancer [11], cholangiocarcinoma [12], and prostate cancer [13]. In bladder cancer, elevated plasma lipid-bound sialic acid (LSA) is observed in both the early and advanced stages [14]. Although an increased serum LSA in bladder cancer patients is documented, it is questionable to use as a screening marker [15]. No significant difference of serum TSA between bladder cancer patients and healthy controls is also observed [16]. Akcay et al. [17] demonstrated that urinary excretion of TSA in patients with superficial bladder cancer was significantly greater than that in healthy volunteers. It was reduced after a successful treatment holding a promise as monitoring marker for drug response. However, they investigated in 24-hour urine, which is much less practical in collection than the spot urine. Urinary excretion of TSA in spot-morning urine has not been explored.

Oxidative stress mediates carcinogenesis. It is capable of inducing DNA damage and mutation [18]. Oxidative stress in the body is frequently characterized by either increases in reactive oxygen species (ROS) and oxidative damage products or decrease in antioxidants. 8-hydroxydeoxyguanosine (8-OHdG) and malondialdehyde (MDA), oxidative damage products of DNA, and membrane lipids have been widely used as oxidative stress biomarkers. Formation of 8-OHdG increases in circulating leukocytes of patients with superficial TCC, compared to the healthy controls [19]. In addition, elevated urinary 8-OHdG has been demonstrated in patients with bladder cancer [20]. Increased serum MDA and decreased circulating antioxidants (vitamin E and C, superoxide dismutase and glutathione peroxidase) have been found in patients with bladder carcinoma, and the extent of oxidative stress correlates with disease severity [21]. Although enhanced oxidative stress in bladder cancer patients is well recognized, urinary excretion of MDA in these patients has not been investigated. In this study, we investigated urinary excretion of TSA, 8-OHdG, and MDA in patients with bladder cancer and evaluated their association with the disease severity.

Patients and methods

Participants and specimen collection

Forty-five patients with superficial bladder tumors (31 males, 14 females) admitted to the Division of Urology, King Chulalongkorn Memorial Hospital, Bangkok, and 41 healthy subjects (20 males, 21 females) were recruited for the study. Flexible cystoscope was employed to detect masses in the patients' bladders. The spot morning urine samples were collected from all subjects and kept at -20^{O}C until analysis. Written informed consents were obtained from all participants prior to specimen collection. Research protocol was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

Bladder biopsied tissues from the patients were histologically examined to define severity of the tumors. The tumors were classified into low- and high-severity grades by a pathologist (P.S.). Low-severity grade was defined as low-grade superficial TCC, whereas the high-severity grade included high-grade superficial and muscle-invasive TCC.

Biochemical measurements

Urinary creatinine was measured by the Jaffe method [22]. TSA in urine samples was determined by the modified periodate-thiobarbituric acid method [23]. Urinary MDA was determined by thiobarbituric acid method [24]. Urinary level of 8-OHdG was measured using enzyme-linked immunosorbent assay (ELISA) kit (JAICA, Tokyo, Japan).

Statistical analysis

Continuous data were presented as mean ± standard deviation (SD) or median (interquartile range, IQR). Two independent groups were compared by two-sample t-test or Mann-Whitney test where appropriate. Spearman's rank correlation test was performed to determine the association between two variables. Receiver operating characteristic (ROC) curve analysis was performed, and appropriate cutoff values were chosen to calculate diagnostic values of urinary TSA determination. A two-sided p-values <0.05 was considered as statistically significant. Statistical

analyses were performed using STATA version 8.0 (Stata Corp, College Station, USA).

Results

Forty-five patients with urinary bladder cancer aged 63.6 ± 11.2 years, and 41 healthy volunteers aged 62.0 ± 10.7 years were recruited. The patient group consisted of 31 (68.9%) men and 14 (31.1%) women. In healthy group, there were 20 (48.8%) men and 21 (51.2%) women. Age (p = 0.482) and gender distribution (p = 0.093) between the patients and controls were not significantly different (**Table 1**). Urinary levels of TSA, 8-OHdG, and MDA in bladder cancer patients were significantly higher than that in healthy controls.

To evaluate the diagnostic power of urinary TSA, 8-OHdG, and MDA, ROC curves were generated. Area under ROC curve (AUC) of urinary TSA was 0.789 (95%CI=0.691-0.887) (**Fig. 1**). AUC of urinary 8-OHdG and MDA were 0.647 (95%CI=0.528-0.767) and 0.641 (95%CI=0.523-0.759), respectively (**Fig. 1**). Based on the ROC analysis, only urinary TSA had adequate diagnostic potential, and we chose the cutoff at 95.26 μg/g creatinine. Determination of urinary TSA provided sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of 75.6%, 75.6%, 77.2%, 73.8%, and 75.6%, respectively.

Table 1. Urinary TSA, 8-OHdG, and MDA in bladder cancer patients and healthy controls

Variables	Patients	Healthy	P-value
Number	45	41	
Age (years)	63.6 ± 11.2	62.0 ± 10.7	0.482
Gender			
Male (%)	30 (66.7)	20 (48.8)	0.093
Female (%)	15 (33.3)	21 (51.2)	
TSA (μg/g creatinine)	126.25 (68.90)	68.33 (52.01)	<0.001
8-OHdG (ng/g creatinine)	8.12 (7.01)	4.13 (4.85)	0.019
MDA (μmol/g creatinine)	9.54 (8.65)	6.76 (5.83)	0.024

Age is presented as mean ± SD. TSA, 8-OHdG and MDA are presented as median (IQR).

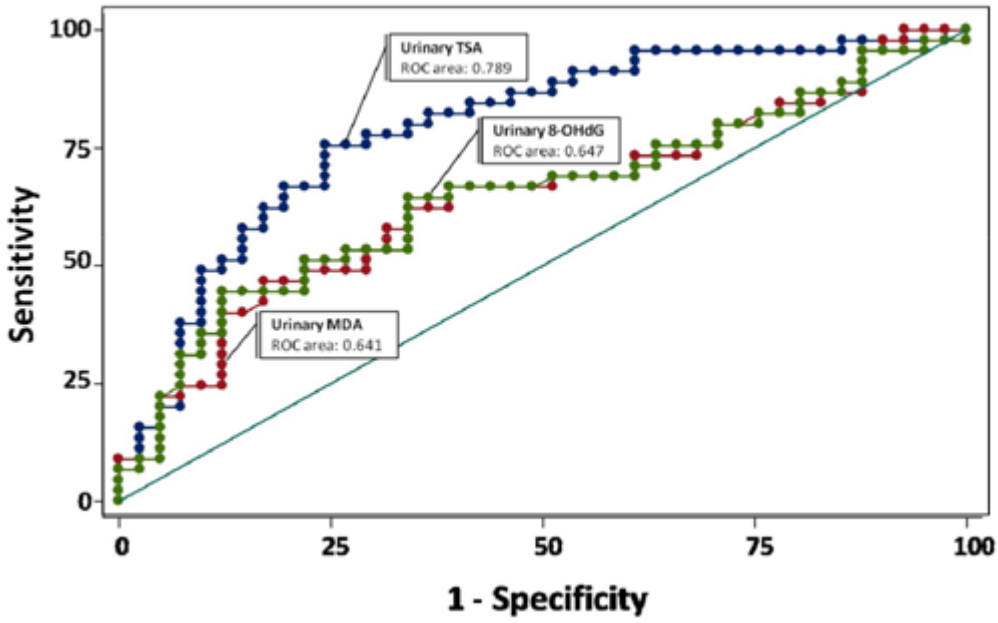

Fig. 1 ROC curves of urinary TSA, 8-OHdG and MDA to assess how well the tests can distinguish patients with bladder cancer from healthy subjects. Area under ROC curve of urinary TSA, 8-OHdG and MDA were 0.789 (95%CI=0.691-0.887), 0.647 (95%CI=0.528-0.765) and 0.641(95%CI=0.523-0.759), respectively. Area under curve of urinary TSA was significantly greater than urinary 8-OHdG (p = 0.028) and MDA (p = 0.041), which indicated a better diagnostic potential.

To find the association between urinary TSA excretion and oxidative stress status, scatter plot and Spearman's rank correlation test were carried out. Urinary TSA was significantly correlated with urinary 8-OHdG in both patients (Spearman's rho = 0.311, p = 0.038) and healthy subjects (Spearman's rho = 0.475, p = 0.002) groups (**Fig. 2**). In the healthy group, a significantly positive correlation between urinary TSA and MDA was observed (Spearman's rho = 0.575, p = <0.001). However, significant correlation of urinary TSA and MDA in the patient group was not revealed (Spearman's rho = -0.201, p = 0.186).

The patients were histologically classified into two groups, those with high- (n=18, 40%) and low-severity grades (n=27, 60%). We examined if urinary excretions of TSA, 8-OHdG, and MDA were increased with tumor progressivity. Patients with high-severity grade excreted urinary TSA significantly greater than those with low-severity grade as shown in **Fig. 3**. A trend of increased urinary 8-OHdG excretion in high-severity grade patients was also observed, although it was not statistically significant. However, there was no significant difference of urinary MDA compared between patients with high- and low-severity grades.

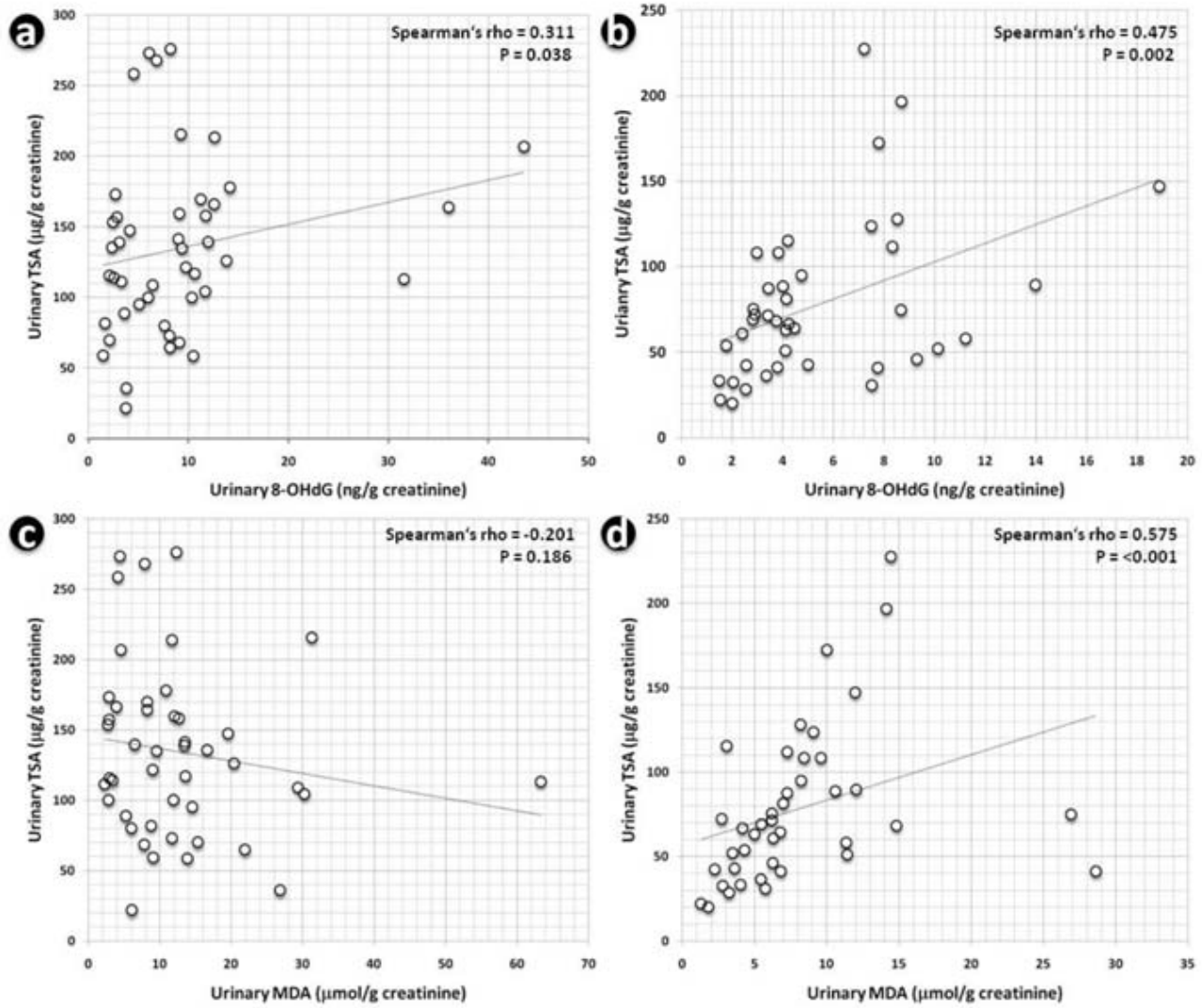

Fig. 2 Bivariate analysis to assess associations of urinary TSA excretion with urinary 8-OHdG and MDA in bladder cancer patients (**a, c**) and healthy subjects (**b, d**). Urinary TSA was linearly correlated with urinary 8-OHdG both in the patient (**a**) and healthy (**b**) groups with Spearman's rho of 0.311 (p = 0.038) and 0.475 (p = 0.002), respectively. Significantly positive correlation of urinary TSA and urinary MDA was found in the healthy controls (**d**) but not in the patients (**c**).

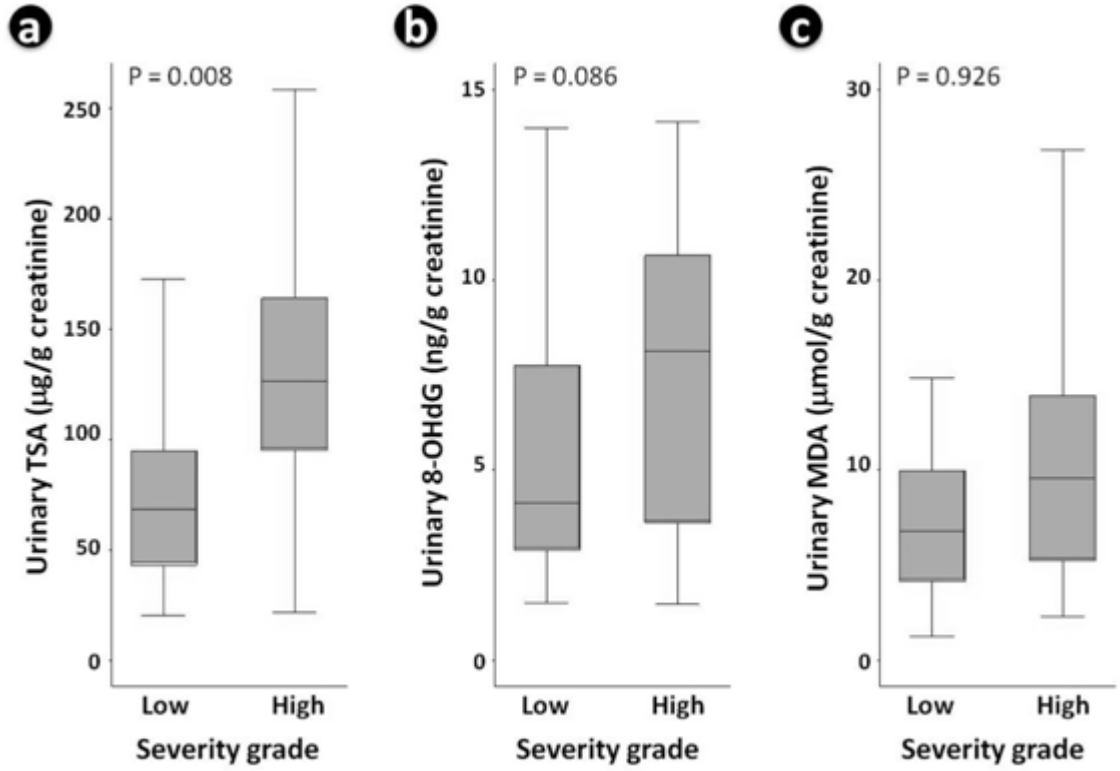

Fig. 3 Comparisons of urinary TSA, 8-OHdG, and MDA among patients with urinary bladder tumor with different histological severity grades. Patients with high-severity grade (n=27) had significantly higher level of urinary TSA compared to those with low-severity grade (n=18) (**a**). Trends of increases in urinary 8-OHdG (**b**) and MDA (**c**) were observed in patients with more advanced stage of tumors.

Discussion

The number of new bladder cancer cases is increasing annually, and bladder cancer is the fourth and eighth most common malignancy in men and women, respectively [25]. The important risk factors of bladder cancer are smoking and arsenic (in drinking water) exposures [26] involved in ROS production [26, 27]. It is known that ROS-induced damage to lipids, proteins and DNA plays a critical role in carcinogenesis [28]. The present study investigated urinary excretions of TSA, 8-OHdG, and MDA in patients with bladder cancer. These urinary parameters were excreted significantly higher than the healthy controls. Studies suggested that sialic acid and 8-OHdG could be biomarkers of bladder cancer [20, 29]. Moreover, clinical utility of sialic acid has been suggested in various diseases such as breast cancer [30], colorectal cancer [31], lung cancer [32], idiopathic deep vein thrombosis [33], and type 2 diabetes mellitus [34]. Our study confirmed that urinary excretion of TSA in bladder malignancy was greater than the healthy condition. Elevated urinary excretion of sialic acid in patients with bladder tumor was first demonstrated by Konukoglu et al. [29], but it was measured in 24-hour urine samples. To our knowledge, the present study is the first report that showed an increased TSA level in spot-morning urine of bladder cancer patients compared to the healthy controls. Instead of 24-hour urine, we suggest to use spot urine for sialic acid determination, as it is much more practical for collection in the clinics.

We also found that urinary TSA excretion was associated with severity of the tumors. Muscle-invasive bladder tumors are much more likely to progress to metastasize tumors than the low-grade ones, and the 5-year survival rates in muscle-invasive tumors patients are considerably low [25]. The urinary excretion of TSA in the patients with high-severity grade was significantly higher than those with low-severity grade as shown in **Fig. 3**. This agrees with the study by Konukoglu et al. [29]. We suggested that urinary TSA level could be used to distinguish the patients with high-severity grade from those with low-severity grade. In addition, it may be clinically useful

for post-therapeutic monitoring, as a significant reduction of urinary sialic acid in bladder tumor patients after a successful treatment has been demonstrated [29].

As patients with bladder cancer excreted urinary TSA, 8-OHdG, and MDA significantly higher than healthy controls, their determinations could provide some diagnostic meaning. Based on the present ROC curve analysis, determination of urinary TSA was adequate for diagnostic purposes (AUC=0.789, 95%CI=0.691-0.887). Appropriate cutoff for urinary TSA was chosen at 95.26 μg/g creatinine, because it provided the highest accuracy (minimal false-negative and false-positive results) and imparted adequate sensitivity and specificity. We suggested that urinary TSA could be used as adjuvant marker (combined with other diagnosing means such as urine cytology) for bladder cancer diagnosis. However, increased circulating and urinary TSA have been found in many diseased conditions, using TSA level alone for diagnosing purpose or as a specific tumor marker is not recommended.

In our study, significant associations of urinary TSA with urinary 8-OHdG both in bladder cancer patients and controls were found. This might imply that increased ROS generation in malignant cells, indicated by increased urinary 8-OHdG lesion, was involved in the synthesis of sialylated molecules. However, this hypothesis needs further investigation. Association of oxidative stress parameters (lipid peroxide and protein carbonyl) with sialoconjugates (prostate specific antigen and protein-bound sialic acid) was demonstrated in prostate cancer. The authors hypothesized that oxidative stress might be associated with the degree of sialylation of proteins and these changes gradually contributed to the prostatic carcinogenesis [35]. To our knowledge, the present study is the first report of association of high urinary TSA and enhanced oxidative stress in bladder cancer patients.

In agreement with previous studies, our findings supported that there were high oxidative stress in bladder cancer patients [21, 36]. Depleted antioxidant enzymes in patients with bladder cancer have been reported [37], and vitamin E supplement has been suggested to reduce the risk of bladder cancer [38, 39]. Furthermore, curcumin has been shown to inhibit progression of bladder tumor [40, 41]. Therefore, supplementation of antioxidants such as vitamin E [38], selenium [42], and curcumin [41] to increase the antioxidative magnitude in the body may be beneficial for prevention and treatment of bladder cancer. However, further clinical trial is needed to validate the hypothesis.

Increased serum TSA has been reported in cigarette smokers [43], suggesting that tobacco smoking is associated with increase TSA production. We compared the urinary TSA among non-smoking, quitted smoking (more than 10 years) and current smoking bladder cancer patients. Although it was not statistically significant (maybe due to small sample size), levels of urinary TSA in non-smoking and quitted smoking patients were lower than that in the current smoking patients. Cigarette smoking may associate with an increased urinary TSA in bladder cancer patients. Further studies in larger populations are required to validate this relationship.

In conclusion, urinary levels of TSA, 8-OHdG and MDA were elevated in bladder tumor patients. This indicated that there were increases in oxidative stress, and synthesis and shedding of sialic acids in the patients. Elevated urinary TSA concentration was associated with increased oxidative stress. Determination of urinary TSA is a non-invasive mean that provides an adequate diagnostic potential. Therefore, it may be useful for detecting bladder cancer, evaluating its severity, and monitoring recurrence to reduce the number of surveillance cytoscopies performed each year.

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