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Serum-based protein biomarkers of bladder cancer: A pre- and post-operative evaluation

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ABSTRACT

Urinary bladder cancer (BC) is the fifth most common cancer worldwide with alarming mortality. Shortcomings of urine cytology and cystoscopy and sparse improvements in the survival rate prompt us to evolve surrogate serum based protein biomarkers to identify BC at an early stage. Previously, we showed that aberrant expression of S100A4, S100A8, S100A9, carbonic anhydrase I (CA I) and Annexin V proteins in pre-operative BC serum compared to healthy controls (HC) (Clin Chim Acta, 2014; 36: 97–103). Here we further evaluate and validate these findings with follow-up post-operative BC patients. This study was conducted on 160 sera samples comprising healthy controls (HC, n = 52), pre-operative (n = 55) and post-operative (n = 53) BC patients. Enzyme-linked immunosorbent assay (ELISA) was used to appraise the aberrantly expressed proteins. ELISA results revealed that the expression levels of S100A8, S100A9, S100A4, and CA I were gradually and significantly reduced; concomitantly, Annexin V was progressively and significantly increased in post-operative compared to pre-operative BC sera samples. Serum protein biomarkers appear to be an encouraging and least-invasive approach for BC identification and prognosticating patient outcomes.

1. Introduction

Bladder cancer (BC) is one of the most common cancers and rated highest in the Europe followed by United States (US) and Asia. The annual cost of BC treatment in the US alone is estimated to increase up to US $ 5 billion by 2020. [1,2]. Cytologically BC can be classified into low-grade (LG) and high grade (HG) tumors. Cytological well-differentiated LG BC has a high recurrence rate as superficial noninvasive papillary protuberances HG BC is barely differentiated and may metastasize to local lymph nodes and distant anatomic sites [3]. Only few studies have investigated the role of serum proteins in BC identification. Minami et al. proteomic study showed significantly reduced post-operative expression levels of S100A8 and S100A9 serum proteins compared to pre-operative BC cases only in two patients [4]. Other two different studies revealed contradictory findings one showed overexpression of S100A4 and Annexin V BC; [5] the other study reported down-regulation of Annexin V in BC [6]. Lee et al. observed Carbonic anhydrase I (CA I) altered pattern in plasma of BC [7]. In our previous study we also identified that above mentioned protein are being differentially expressed in BC serum samples compared to healthy controls (HC) [8,9]. To further evaluate and validate these finding we designed the present study. In this study, we followed up post-operative BC patients till 90 days and appraised the level of these differentially expressed proteins compared to the pre-operative BC condition as well as HC sera samples to lend support to data pertaining to the clinical efficacy of these proteins.

2. Materials and methods

2.1. Patients and sample collection

The institutional review board and ethical committee of King George's Medical University (KGMU), and the Centre of Biomedical Research, Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS) Campus, Lucknow, India, approved this study. All

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subjects were registered from the Department of Urology, KGMU. The study included males >40 years of age with presenting frequent urination, dysuria, and hematuria. Patients registered in this study had not been administered any treatment or suffered any co-morbid conditions. Age-comparable males were comprised as healthy controls. Exclusion criterion comprising as follows: tuberculosis, diabetes, urinary tract infections, arthritis, renal pathology, other malignancies, endocrine disorders and drug abuse. All subjects gave written informed consent. The occurrence of bladder tumors in patients was appraised using urine cytology and cystoscopy. Consequently, transurethral resected tissue samples were collected to perform histopathological evaluation to define LG and HG BC. Total participated subjects in this study are as follows; pre-operative BC cases (LG, n = 35; HG, n = 20), post-operative BC cases (LG, n = 33; HG, n = 20), and HC (n = 52).

The pre- and post-operative samples were collected from the same patients. Serum samples were collected 24 h before operation followed by 30, 60, and 90 days of post-operative visit to appraise the level of differentially expressed proteins (S100A8, S100A9, S100A4, Annexin V, and CA I). For pre-operative condition, serum-samples were collected before administration of any therapy.

Venous blood specimens were collected in vacutainer tubes. The collected blood samples were endorsed to clot at room temperature (RT) for 30 min. The clotted blood samples were centrifuged at 3000 g at 4 °C for 10 min; the supernatant was collected as serum. These sera samples were promptly stored at −80 °C till proteomics experiments were performed.

2.2. Abundant protein depletion

The proteomic analysis was performed using a multiple affinity removal system (MARS) spin column (LCGC Life Sciences LLP, New Delhi, India obtained from Agilent Technologies, Santa Clara, CA, USA) to reduce high abundant proteins (albumin, alpha-1 antitrypsin, haptoglobin, transferrin, IgA, and IgG) in the collected serum samples per the manufacturer's instructions and protocol. The processed serum samples were investigated for altered proteins expression using ELISA.

2.3. Enzyme-linked immunosorbent assay

Nunc-immuno 96 micro-well plates (P.D. Scientific, Lucknow, India obtained from Fisher Scientific Ireland Ltd, Ballycoolin, Dublin) were used to perform ELISA. The plates were coated with antibodies at 4 °C by incubating overnight. The wells were thoroughly washed and 100 μl of serum from HC, pre- and post-operative BC patients was added to the ELISA plate in dilution 1:20 in 5% BSA. The plates were incubated at RT for 2 h followed by incubation with 100 μl of secondary antibodies conjugated to peroxidase at RT for an hour. Next 100 μl HRP-avidin working solution was added, incubated for 1 h at RT and washed again. Afterwards, 100 μl of TMB (3,3′,5,5′-tetra methyl benzidine) (TCI chemicals India Pvt. Ltd.) substrate was added and the plates were incubated in the dark at RT for 30 min. Then 50 μl of stop solution was added to wells of the plates and the optical density (OD) was measured at micro-plate reader (iMARK Bio-Rad, Bio-Rad Laboratories, India) absorbance set to 450 nm.

2.4. Statistical analysis

Data were tested for normality, and parametric testing was used when appropriate. Results are presented as box plots indicating the 25th to 75th percentile for the expression of various proteins in serum samples from HC, pre-operative, and post-operative LG and HG BC subjects. The median, the 50th percentile, is shown between the boxes of 25th and 75th percentiles. The multiple groups- HC, pre-operative LG and HG, post-operative LG and HG at the 30, 60, and 90 days of interval were compared by one-way ANOVA followed by Tukey-Kramer test using GraphPad InStat 3.0 to identify differentially expressed proteins. Accuracy, sensitivity, specificity, and receiver operating characteristic (ROC) curve analysis of individual serum proteins biomarkers and urine cytology were also executed to verify the robustness and clinical utility for discriminating specific cohorts. For statistical analysis of urine cytology, when cells in urine appears normal; denoted as 1, and cells in urine appears abnormal; denoted as 2. C-indexes statistics between HC and pre-op BC of various protein biomarkers were also calculated.

Table 1
Summary of clinico-pathological information of BC patients and healthy controls.

Characteristics	BC patients	Healthy controls
Race	Indian	Indian
No. of subjects	108	52
Age (mean, range)	51, 40–70	53, 41–68
Gender		
Male	108	52
Female	0	0
Cancer grade		
Preoperative		
Low grade (LG)	35	0
High grade (HG)	20	0
Postoperative		
Low grade (LG)	33	
High grade (HG)	20	
BMI (median, range)	22.2, (15.3–26.4)	21.4 (16.4–28.0)
Hematuria	25	0
Medications	0	0
Smoking habit		
Nonsmokers	50	13
Ex-smokers	32	15
Smokers	26	24

3. Results and discussion

The clinico-pathological data of all participants in this study are summarized in Table 1.

To evaluate the expression level of proteins as determined in our previous study, we performed the comparative HC, pre- and post-operative protein expression appraisal using ELISA.

ELISA test revealed that the expression levels of S100A8, S100A9, S100A4, and CA I proteins were significantly higher ($p < 0.001$) and annexin V was significantly lessen ($p < 0.001$) in pre-operative LG and HG BC compared to HC (Fig. 1). These findings are concurred to our previous observations [13,14]. The new observations with post-operative follow up at the interval of 30, 60, and 90 days reveals that the levels of S100A8, S100A9, S100A4, and CA I proteins were gradually and significantly ($p < 0.001$) decreased and annexin V was progressively and significantly ($p < 0.001$) increased compared to pre-operative LG and HG BC (Fig. 1). The expression levels of S100A8, S100A9, S100A4, CA I, and annexin V proteins at 90 days of post-operative LG and HG BC are comparable to HC ($p > 0.05$) (Fig. 1).

Detailed statistical outcome among various groups are presented in Supplementary information.

C-indexes statistics between HC and pre-operative BC of S100A8, S100A9, S100A4, CA I, and annexin V proteins presented 0.914, 0.8503, 0.9583, 0.9108, and 0.9124, respectively.

ELISA based findings are compared with conventional used urine cytology and detailed statistical analyses are explained in Table 2.

Urine cytology is one of the commonly used modality for BC identification. Its attribute to identify shed intact cells in urine