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Low- and high-grade bladder cancer appraisal via serum-based proteomics approach

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ABSTRACT

Objective: To address the shortcomings of urine cytology and cystoscopy for screening and grading of urinary bladder cancer (BC) we applied a serum-based proteomics approach as a surrogate tactic for rapid BC probing.
Methods: This study was performed on 90 sera samples comprising of low-grade (LG, n = 33) and high-grade (HG, n = 32) BC, and healthy controls (HC, n = 25). Two-dimensional gel electrophoresis (2DE) tactic was executed to describe serum proteome. MALDI-TOF-MS (MS) was used to identify the characteristics of aberrantly expressed proteins in 2DE and validated using Western blot (WB) and ELISA approach. Receiver operating characteristics (ROC) curve analysis was also performed to determine the clinical usefulness of these proteins to discriminate among LG, HG and HC cohorts.
Results: This comprehensive approach of 2DE, MS, WB and ELISA reveals five differentially expressed proteins. Among them two biomarkers (S100A8 and S100A9) were able to accurately (ROC, 0.946) distinguish 81% of BC (LG + HG) cases compared to HC with highest sensitivity and specificity. With a comparable tactic, two biomarkers (S100A8 and S100A4) were able to precisely (ROC, 0.941) discriminate 92% of LG cases from HG with utmost sensitivity and specificity.
Conclusions: Serum proteomics probing appears to be an encouraging and least-invasive tactic for screening and grading of BC.

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1. Introduction

Despite the remarkable progress in diagnosis and treatment, cancer is still a serious jeopardy to the society. Urinary bladder cancer (BC) is the fifth most common cancer worldwide. In the United States alone approximately 71,000 new cases of BC with 14,000 deaths were identified in 2009 [1]. A recent study in the European Union reported 19.5% cases of BC per 100,000 populations with a mortality of 7.9%. The incidence of BC diverges globally with the highest rates in Europe and America and lowest rates in Asia [2]. BC can be broadly divided into low grade (LG) and high grade (HG). Since the recurrence rate of BC is very high and more than half recur within 5 years, therefore, early or preliminary stage screening of BC is highly desired.

The gold standard clinical method to diagnose BC is currently cystoscopy, although it is an invasive, unpleasant, and expensive approach. Sometimes it may miss a flat lesion – especially carcinoma in situ (CIS) – which is considered HG BC. Voided urine cytology is a non-invasive routine method for BC identification, but it has limited sensitivity for LG BC [3,4]. Various urine-based biomarkers (UroVysion®, ImmunoCyt™, BTA-TRAK™, BTA-stat™, telomerase, nuclear matrix protein 22 (NMP-22), fibrin degradation product (FDP), BLCA-4, and cytokeratin) have been developed for BC appraisal and exhibited limited sensitivity for LG BC detection [3,4].

Blood serum has recently received remarkable attention as an excellent tool to develop BC biomarkers because it continuously perfuses the tissues and hence presumably picks up the aberrant proteins secreted and shed from tissues. A recent study reveals that 2 to 6 kDa serum proteins were differentially expressed in BC [5]. Expression levels of S100A8 and S100A9 serum proteins were significantly reduced in post-operative compared to pre-operative BC cases [6]. The expression level of S100A8 protein was significantly higher in BC tissue compared to controls [7]. Recent studies revealed that the overexpression of S100A4 and annexin V [8–10] and under-expression of S100C (also known as S100A11) [11] can induce the metastasis in BC. In contrast, another study reveals that the down-regulation of annexin V causes BC heterogeneity [12]. Moreover, a recent study revealed that carbonic anhydrase 1 (CA 1) and leucine-rich α-2-glycoprotein 1 (LRG 1) in plasma may be a possible biomarker of BC [13]. Collectively these studies have not specified whether these protein biomarkers are strong enough to differentiate LG and HG BC. Hence, further validation is required to evaluate the role of these protein biomarkers for differentiation between LG and HG BC, which remains unexplored to date. Moreover,

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re-evaluation of the expression pattern of annexin V is essential to reduce the ambiguity, as controversial results mentioned in the two studies [10,12]. Therefore the present study was designed to epitomize that gap and to lend support to the existing data pertaining to the role of various proteins to differentiate LG and HG BC which may help in the clinical management and treatment of BC.

2. Materials and methods

2.1. Patients and sample collection

Study was approved by the institutional review board and ethical committee of King George's Medical University (KGMU), and Centre of Biomedical Research, Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS) Campus, Lucknow, India. All subjects were enrolled from the Department of Urology, KGMU. The study comprised males >40 years of age with symptoms of frequent urination, dysuria, and hematuria. Patients involved in this study had not been administered any treatment or endured any co-morbid situations. Age-comparable males were included as healthy controls (HC). Exclusion criteria consisted of the following: urinary tract infections, renal pathology, diabetes, other malignancies, arthritis, tuberculosis, endocrine disorders and drug abuse. Written informed consent was obtained from all subjects.

The occurrence of bladder tumors in patients was first evaluated using cystoscopy. Consequently, transurethral resected tissue specimens were gleaned to execute histopathological appraisal to define LG and HG BC. Adjacent, non-involved tissue samples were also gleaned from a few patients and used as controls. All tissue samples were snap-frozen using liquid nitrogen and stowed in a −80 °C freezer until histopathological evaluation. Tumors were categorized using World Health Organization (WHO)/International Society of Urological Pathology (ISUP) guidelines.

2.2. Histopathological examinations

Within 3 to 5 days of stowage, all tissue specimens were settled in 10% buffered formalin and entrenched in paraffin wax for histopathological appraisal. Tissue specimens were sliced at a thickness of 5 to 6 μm exerting a microtome followed by hematoxylin and eosin (H & E) staining. An average of 3 to 6 slices was evaluated for each tissue specimen.

2.3. Serum sample

Human venous blood specimens were collected between 8:00 a.m. and 10:00 a.m. in vacutainer tube. The blood was allowed to clot at room temperature for 30 min. Clotted blood samples were centrifuged at 3000*g* at 4 °C for 10 min to collect the supernatant serum. Collected serum samples were rapidly stored at −80 °C until proteomics experiments were performed. A total of 90 sera were collected from 33 patients with LG BC, 32 patients with HG BC, and 25 HC.

2.4. Abundant protein depletion

For effective proteomic analysis, collected serum samples were processed using a multiple affinity removal system (MARS) spin column (Agilent Technologies, Santa Clara, CA) to reduce the highly abundant proteins (albumin, alpha-1 antitrypsin, haptoglobin, transferrin, IgA, and IgG) according to the manufacturer's instruction and protocol. The obtained low-abundance proteins and peptides were analyzed using two-dimensional gel electrophoresis. In the discovery phase five samples in each category were evaluated.

2.5. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2DE) was performed using standard protocol. In brief, 30 μg of proteins were mixed with 200 μl of the rehydration buffer. The Ready Strip™ IPG Strips (Bio-Rad) pH 3–10 nonlinear, 11 cm was rehydrated with the above mix for overnight at 20 °C. The rehydrated strips were electrophoresed and equilibrated. The equilibrated IPG strip was then transferred onto the top of 12% polyacrylamide gel, sealed with 1% agarose containing a trace of bromophenol blue solution and a second dimensional electrophoresis was carried out until the dye front reached to the bottom of the gel. Altered-expressed proteins were determined using MALDI-TOF-MS (MS) and protein database.

2.6. Gel digestion

Stained pieces of gels were excised and cleaned in 50% acetonitrile three times, allowed to dry for 20 min in a Speed-Vac. For further reduction, gel pieces were treated with dithiothreitol (10 mM) in ammonium bicarbonate (5 mM) at pH 8.0 and 50 °C for 45 min, subsequently alkylated with iodoacetamide (50 mM) in ammonium bicarbonate (5 mM) in the dark at 25 °C for 1 hour. Again the gel pieces were cleaned three times with the help of 50% acetonitrile and vacuum dried; subsequently gel pieces were re-swelled with modified trypsin (Promega, 50 ng) in ammonium bicarbonate (5 mM). The gel pieces were immersed in 10 μl of ammonium bicarbonate (5 mM) and then digest with trypsin at 37 °C for 16 hours. Supernatants were collected and treated with 5% trifluoroacetic acid in 50% acetonitrile to extract the peptides. This step was done twice and the supernatants were pooled. Extracted peptides were vacuum-dried, re-dissolve in ddH_2O (5 μl), and stowed at −80 °C until MALDI-TOF-MS analysis was performed.

2.7. Determination of protein by MALDI-TOF-MS

Excised proteins were digested with a proteolytic enzyme to engender peptides. Identification of proteins was executed using a peptide mass fingerprinting (PMF) data bank search and MALDI-TOF-MS analysis. In brief, trypsin digested protein sample (0.5 μl) was mixed with matrix solution (0.5 μl). Matrix solution comprises *R*-cyano-4-hydroxycinammic acid at a concentration of 1 mg/ml of 50% acetonitrile (v/v) and 0.1% trifluoroacetic acid (v/v). The complete mixture was added onto an anchor-chip target (Bruker Daltonics) and allowed to dry. To avoid sample preparation bias, triplicates were used. Auto-flex III mass spectrometer (Bruker Daltonics) in reflector mode was used to acquire PMF data. Sophisticated numerical annotation procedure (SNAP) was applied for interpretation of spectrum. This procedure applied the following metrics: Peak detection algorithm, signal-to-noise (S/N) ratios, ≤25; upper-limit number of peaks, 10; lowest intensity threshold, 0; comparative intensity threshold, 0%; quality factor, ≤1000; SNAP normal composition, normalizing; baseline subtraction, median; flatness, 0.8; median level, 0.5. A peptide calibration standard (Bruker Daltonics) was applied for calibration of spectrometer. Trypsin autolysis peaks at m/z 842.51 and m/z 2211.10 were used for internal calibration. Peaks comprising mass range of m/z 800–3000 were exerted to engender a PMF that was explored against the Swiss-Prot/TrEMBL databank utilizing Mascot software v 2.3.02 (Matrix Science, London, U.K.). The search parameters comprises: *Homo sapiens;* partial protein N-terminal acetylation, partial methionine oxidation, carbamidomethylation of cysteine, partial modification of glutamine to pyroglutamate, and tryptic digest with ≤1 missed cleavage; and a mass threshold of 50 ppm. Determination of protein was established on the basis of considerable MASCOT scores ($p < 0.05$), interpretation of spectrum, p*I* value on 2DE and experimental versus theoretical molecular mass.

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