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Differential regulation of urine proteins in urothelial neoplasm☆

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ABSTRACT

Urothelial neoplasm of the urinary bladder has a high rate of multifocality and recurrence. To understand this we first need to understand the changes in the molecular level that distinguishes a normal individual from a patient and also a low grade neoplasm from a high grade. In this work we aim to study the urine proteome of Indian patients with urothelial neoplasm categorised on the basis of their p53 immunohistochemistry. The urine samples of pre-operative patients were subjected to two dimensional gel electrophoresis followed by densitometric analysis and spot identification using MALDI mass spectrometry. Our study shows that few proteins such as albumin, alpha 1 antitrypsin, apolipoprotein A1, transferrin, transthyretin, haptoglobin and haemoglobin β chain were upregulated and inter alpha trypsin inhibitor heavy chain was downregulated in the disease samples. Further we have reported that some of these proteins show an association with disease severity. The present study marks the first step in the identification of new diagnostic markers as well as therapeutic targets.

Biological significance
Bladder carcinoma is the ninth most common cancer worldwide. It has gained attention within both clinicians and cancer biologists because of its recurrence and mortality rate. Identifying the prognostic factors of progression is a challenge, so that high risk patients who may be a candidate for a radical cystectomy may be identified. In this study we have attempted to study the changes observed in the urinary protein levels of urothelial neoplasm patients. The samples were graded based on p53 immunohistochemistry staining. We have reported eight (8) proteins, mostly highly abundant; those have exhibited differential regulation in case of diseased samples. This study is first of its kind that associates the changes in the urinary protein levels to that of the severity of the disease. We believe that the findings can be used as a stepping stone in the development of a noninvasive prognostic tool for the disease.
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1. Introduction

Urothelial neoplasms of the upper urinary tract account for approximately 5% of all epithelial tumours of the urinary tract only, whereas urinary bladder tumours are the most common and are the fourth most common form of cancer. This disease is mainly diagnosed in the elders, approximately at the age of 65 years and is more common in men than in women [1]. Roychowdhury and co-workers showed that, p53 protein expression is associated with high grade urothelial neoplasm and advanced stage of the disease [2].

Mutations of p53 are found to be present in 40–45% of cancers, including all sites combined. Indeed, p53 mutation is the most frequent genetic event demonstrated to date [3]. These point mutations lead to the loss of its tumour suppressing function. The wild-type p53 protein has a short half-life of 15 to 30 min, whereas the mutated p53 gene results in a protein with a prolonged half-life, which is the basis of its nuclear accumulation that is detectable by immunohistochemistry (IHC). The accumulated p53 has been associated with the progression of bladder cancer and might play a role in the evolution of the tumours to a higher grade, shown in earlier studies [2,4].

Urothelial neoplasm occurs at different sites in the urinary bladder with varying frequency. Vigilant monitoring of patients after definitive treatment for urothelial neoplasm is essential owing to the high rate of multifocality and recurrence. However, there are no clear cut ways of predicting which urothelial carcinomas would subsequently recur or progress or which muscle invasive tumours would progress following treatment. In this work, for the first time, we have tried to establish a correlation between different tumour grades and changes in the protein profile of urine samples of urothelial neoplasm patients. Urine being the body waste is easily obtainable for monitoring at various stages of the disease and hence will help in establishing a non-invasive disease and post-treatment monitoring method. Results indicate differential expression of proteins like inter alpha trypsin inhibitor heavy chain, apolipoprotein A1, haptoglobin and transferrin which could be easily monitored at various stages of the disease in a non invasive method.

2. Materials and methods

2.1. Material

Amicon ultra centrifugal filter units with 5 kDa cut off membrane and PVDF membrane were obtained from Millipore. Ethanol from Merck, 2D rehydration buffer, 17 cm pH 3–10 IPG strips, Isoelectric focussing system, two dimensional gel electrophoresis (2-DE) system and western blot transfer setup were purchased from Bio-Rad. Colloidal Coomassie from Sigma and sypro ruby stain from Invitrogen. Sequence grade trypsin was purchased from Promega, in-gel tryptic digestion kit from Pierce Biotechnologies and α-cyano-4-hydroxycinnamic acid (CHCA) was purchased from Waters. Primary antibodies for the two proteins and anti-mouse HRP-conjugated IgGs used as secondary antibodies were purchased from abcam. All other reagents, if not mentioned otherwise, were purchased locally and were of analytical grade.

2.2. Samples

Urine samples were taken from pre-operative, non-azotemic patients suffering from urothelial neoplasm (n = 12). These samples were further categorised into low grade (n = 6) and high grade (n = 6) depending on p53 immunohistochemistry [2]. These patients also suffered from various age related and lifestyle related disorders such as bronchial asthma and hypertension (Table 1). We collected urine samples from non-cancer individuals who are under medication for bronchial asthma or hypertension and are above the age of 50 years as our control samples (n = 3). Normal samples (n = 4) were collected from healthy volunteers within our institute. Urine samples were obtained from RG Kar Medical College, Kolkata with informed written consent following the guidelines of the Institutional Ethical Committee of RG Kar Medical College, Kolkata 700037, India and Institutional Animal & Bio ethics committee of Saha Institute of Nuclear Physics. The Institutional Ethical Committee R. G. Kar Medical College, Kolkata 700037, India and Institutional Animal & Bio ethics committee of Saha Institute of Nuclear have also specifically approved the current study.

2.3. Sample preparation

Urine samples (~30 ml) were centrifuged to remove cellular debris [5]. The supernatant was then centrifuged in Amicon ultra centrifugal filter units with 5 kDa cut off membrane concentrated to a volume of 2 ml and then proteins were precipitated using 75% ethanol. The precipitated proteins were directly solubilised in 2D rehydration buffer.

2.4. 2-DE and image analysis

The solubilised samples were separated first on the basis on pI on 17 cm pH 3–10 IPG strips and then on the basis of molecular weight by 2-DE. Gels were stained either with colloidal Coomassie [6] or sypro Ruby according to manufacturer's instructions. Each sample was processed in duplicates. Images of the stained gels were taken and densitometry analyses were done on Versa Doc series 3000 imaging system using PDQuest software (version 7.1, Bio-Rad). Spot volumes (intensity) of the desired spots were normalised as parts per million (ppm) of the total spot volume using the spots that were present in all gels, to calculate the relative density of a spot in a sample. Student t-test was performed to find the significance of the densitometric changes [7,8].

2.5. In-gel tryptic digestion and mass spectrometry

Protein spots from gels, stained with Coomassie and Sypro-Ruby from normal and diseased samples, were excised. Spots were destained followed by in-gel tryptic digestion. Peptides were then extracted and run on a MALDI ToF/ToF (AB 4700, Applied Biosystems) following published protocol [9]. MS of the digested peptides was done in positive ion mode. Autotryptic and common keratin peaks were excluded from the MS/MS analysis. 12 most intense peptides from each spot were subjected to MS/Ms analysis. GPS explorer V3.6 was used to generate the peak list using MS and MS/MS data. The data was searched