Identification and Characterization of Urinary Proteins as Post Surgery Surveillance Markers in Urothelial Carcinoma

THESIS

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By

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Under the Supervision of

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CERTIFICATE

This is to certify that the thesis entitled **Identification and Characterization of Urinary Proteins as Post Surgery Surveillance Markers in Urothelial Carcinoma** and submitted by **NITU KUMARI** ID No **2013PHXF109P** for award of Ph.D. of the Institute embodies original work done by her under my supervision.

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Date:	Date:	Date:

Dedication

Dedicated to my beloved Parents and Husband

ACKNOWLEDGEMENT

"Plan out your work and work out your plan"

--Kauṭilya Chanakya

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Finally, I would like to say myself: Outstanding! This is truly above and beyond!

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No.52-11-EC(5/17) Date : 01.03.12

To
Dr.Sunita Saxena,
Director, National Inst. of Pathology,
ICMR, Safdarjang Hospital Campus
New Delhi – 110 029

Reference : "Identification and characterization of urinary proteins as post surgery surveillance markers in urothelial carcinoma."

Subject: Ethics committee approval of the study document.

Dear Dr.Sunita Saxena,

We have received from you the following study documents of the above referenced ongoing study in your department :

i. Study Protocol

ii. Patient Information Sheet & Consent Form (Hindi & English)

At the Ethics Committee meeting held on **1st March 2012**, the above submitted documents were examined and discussed. After consideration, the Committee has cleared the above documents and gives its approval for the same.

The members who attended this meeting held on **01.03.12** at which your submitted documents were discussed, are listed below:

1. Dr.C.D.Tripathi, Member & HOD Pharmacology
2. Dr.R.P.Narayan, Member & Prof. Burns & Plastic
3. Dr.B.C.Kabi, Member & HOD Biochemistry
4. Dr.Deepthi Nair, Member & Sr.Microbiologist
5. Dr.Sumathi Muralidhar, Member & Sr.Microbiologist
6. Mr.S.N.Makwana, Social Scientist & PRO
7. Mr.Tilak Raj Talwar, Public Representative
8. Mr.Ankur Chibbar, Legal Expert
9. Dr.N.K.Mohanty, Member Secretary & Prof.Urology

It is to be noted that neither you nor any of your proposed study team members were present during the decision-making procedures of the Ethics Committee.

Yours Sincerely

(Dr.N.K.Mohanty)

Member Secretary, Ethical Committee,
VM Medical College and Safdarjung Hospital

Abstract

Abstract

Urothelial bladder is the sixth most commonly diagnosed malignancy in the India. Urothelial cancer is morphologically classified into PUNLMP (Papillary Urothelial Neoplasm of Low Malignant Potential), Low Grade (LG) and High Grade (HG). Urothelial bladder cancer is classified on the basis of muscle invasion, among them 75% are non-muscle invasive bladder cancer (NMIBC) with stage pTa and pT1 and the rest are muscle invasive bladder cancer (MIBC) with stage pT2-pT4. Treatment of bladder cancer is based on the stage and grade of bladder cancer. The recurrence rate for NMIBC after tumor resection is high, with estimates ranging from 35 to 80%. Diagnosis of bladder cancer, in those patients with hematuria, involves cystoscopy along with imaging, cytology and biopsy. Cystoscopy and cytology are the current standards for initial diagnosis of recurrence, but limitations exist. Extensive efforts have been made for identifying urinary biomarkers with high specificity and sensitivity to improve patients' survival, reduce costs and frequency of cystoscopies.

Quantitative proteomics approach has been used to develop a biomarker which is helpful in patient monitoring and it may replace current diagnostic invasive tools. More recent, 8-plex iTRAQ LC-MS/MS (Isobaric tag for relative and absolute quantitation Liquid chromatography- Mass spectrometry) has been used for proteome-profiling of bladder cancer. The present study includes Low grade non muscle invasive (LGpT1, n=2), high grade non muscle invasive (HGpT1, n=2), High grade muscle invasive (HGpT2, n=2; one recurrent on follow-up (after 3 months), other non-recurrent cases) and their paired adjacent normal mucosa (n=6). The experiment was designed with two sets which includes technical, experimental and biological replicates to ensure quality control. Protein Pilot™ (Version 5.0,

Sciex) was used for peptide matching, protein identification, and relative protein quantitation. Data are available via ProteomeXchange with identifier **PXD007070**. Bioinformatics tools such as Ingenuity pathway analysis (IPA), ontotool-pathway express and Cytoscape has been used for protein-protein interaction, pathway and function involved by differentially expressed protein. PANTHER (Protein ANalysis THrough Evolutionary Relationships) and Exocarta databases have been also used for determination of Gene ontology terms and presence of exosomal urinary protein in bladder cancer respectively. Differentially expressed tumor proteins of bladder cancer were further verified in formalin-fixed-paraffin-embedded tissue (FFPE), n=119 cases using immunohistochemistry. The verified tumor proteins were validated in the urine sample for their presence using Western blot (n=36). Urinary marker having sensitivity and specificity more than 80% was further validated on large cohort of urine sample of patients with follow up data and non-malignant urine sample using ELISA (n=150). Specificity and sensitivity were calculated using histopathology as a gold standard. Statistical analysis such as Mann Whitney test and Kaplan Meier analysis was performed using Statistical Package for the Social Sciences (SPSS) software version 19.

Findings revealed that total 1137 proteins with at least two peptides were differentially expressed in bladder cancer compared to normal mucosa. Deregulated proteins were further analyzed and 64 proteins were found deregulated in all groups of bladder cancer. Among these proteins, 9 were commonly upregulated and 19 were commonly downregulated.

Enrichment classifications of the deregulated proteins in different categories (Cellular component, biological process and molecular function) were determined using PANTHER 9.0. It identified 40.9% deregulated protein involves on cell part as a Cellular component, 27.5% in metabolic process as a biological process and 42% deregulated proteins having

catalytic activity as a molecular process. The Ingenuity Pathway Analysis (IPA) Core Analysis generated the top 11 Network and revealed top three interactions between 24, 14 and 11 of deregulated proteins. These proteins are involved in Cellular Movement, Hematological system, Immune cell Trafficking, Nucleic Acid Metabolism, Small Molecule biochemistry, Cell Death and Survival, Cellular Development, Cellular Growth and Proliferation.

Differentially expressed tumor tissue proteins may be involved in exosomal protein formation, hence a comparative analysis was performed between differentially expressed proteins (n=1137) discovered by iTRAQ LC-MS/MS and urinary exosomal proteins reported to be specific to bladder cancer (n=248) on Exocarta database. The common and unique proteins lists were identified and 120 were found common among them. Among these, SERPING1 was upregulated in all stage and grade of bladder cancer and further validated in FFPE tissue and urine sample. Further, enriched pathways included cellular architecture, motility, cell to cell adhesion, tumorigenesis and metastasis. Proteins in the 9 top-ranked pathways included CTNNA1 (Catenin Alpha 1), CTNNB1(Catenin Beta 1), VSAP (Vasodilator-stimulated phosphoprotein), ITGA4 (Integrin Subunit Alpha 4), PAK1(P21 Activated Kinase 1), DDR1 (Discoidin Domain Receptor Tyrosine Kinase 1), CDC42 (Cell Division Cycle 42), RHOA (Ras Homolog Family Member A), NRAS (Neuroblastoma RAS Viral Oncogene Homolog), RHO (Ras family), PIK3AR1/MLCP (Myosin light-chain phosphatase), MLC1 (Myosin light-chain protein), MMRN1 (Multimerin 1), and CTTNBP2 (Cortactin-binding protein 2) and network analysis revealed 10 important hub proteins and identified inferred interactor NF2 (Neurofibromin 2). The importance of identifying interactors is that they can be used as targets for therapy, which inhibit protein-protein interactions and suppress tumor growth and progression by hindering the exosome biogenesis.

Abstract

In silico analysis provides a deep understanding of differentially expressed bladder tumor protein with available database using bioinformatics tools. Along with this an experimental verification and validation are important procedure for the discovery of biomarker. Three commonly upregulated, SERPING1 (Plasma protease C1 inhibitor), SOD2 (Superoxide dismutase 2) and HSPB6 (Heat shock protein beta-6) proteins found involved in top three ingenuity network and three variably deregulated proteins, PRDX1 (Peroxiredoxin 1), PRDX2 (Peroxiredoxin 1) and Tenascin C were selected for validation in urine of bladder cancer for discovery of a non-invasive biomarker. Immunohistochemistry on FFPE tissue showed 90-100% positivity of SOD2, SERPING1, HSPB6, PRDX1 and PRDX2 while Tenascin C was expressed in only 40% of cases. Adjacent bladder mucosa was also tested for all markers and expression of the marker was found in normal mucosa only for SERPING1 (40%). SOD2, SERPING1, HSPB6, PRDX1 and PRDX2 were further validated by Western blot on urine samples. Markers which showed more than 80% sensitivity and specificity were evaluated by ELISA in both patients and control urine sample. A significant elevation in concentration of urinary SOD2, PRDX1 and PRDX2 in bladder cancer patients compared to non-malignant urine was found ($p<0.001$). Median concentration of urinary marker was taken as cut off value for Kaplan Meier analysis. Survival analysis showed urinary SOD2 concentration higher than 2100 pg/ml was significantly associated with recurrence or poorer survival and log-rank test was <0.025.

In conclusion, the use of iTRAQ labeling of bladder cancers combined with LC-MS/MS has led to the discovery of several novel, differentially expressed proteins in these tumors. A panel of three best performing biomarkers (SOD2, PRDX1 and PRDX2) achieved a sensitivity of more than 80 and a specificity of 100. Quantitation of urinary SOD2 may help to

extend the period between cystoscopies in the surveillance of patients with recurrent bladder cancer and can also be used as non-invasive biomarker.

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♣Abbreviation ♣

2DE	Two-dimensional gel electrophoresis
ASRs	Age-standardised rate
BC	Bladder cancer
BCG	Bacille Calmette-Guérin
BTA	Bladder tumor antigen
BTA	Bladder tumor antigen
CDC42	Cell Division Cycle 42
CE-MS	Capillary electrophoresis–mass spectrometry
CIS	Carcinoma *in situ*
CTNNA1	Catenin Alpha 1
CTNNB1	Catenin Beta 1
CTTNBP2	Cortactin-binding protein 2
DDR1	Discoidin Domain Receptor Tyrosine Kinase 1
DIGE	Difference gel electrophoresis
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FFPE	Formalin fix paraffin embedded
FISH`	Fluorescence *in situ* hybridization
GO	Gene ontology
HA	Hyaluronic acid
HG	High Grade
HGPT1NR/HGT1NR	High grade non-invasive non-recurrent
HGPT1R/HGT1R	High grade non-invasive recurrent
HGT1/HGPT1	Non-muscle-invasive bladder cancer high grade
HGT2/HGPT2	Muscle-invasive bladder cancer High grade
HPLC	High Performance Liquid Chromatography
HSPB6	Heat shock protein beta-6

ICAT	Isotope-coded affinity tag
IHC	Iummunohistochemistry
ILVs	Intraluminal vesicles
IPA	Ingenuity pathway analysis
ITGA4	Integrin Subunit Alpha 4
iTRAQ	Isobaric tag relative and absolute quantitation
LC	Liquid chromatography
LC-MS	Liquid chromatography–mass spectrometry
LG	Low Grade
LGT1/LGPT1	Non-muscle-invasive bladder cancer low grade
LGT2/LGPT2	Muscle-invasive bladder cancer low grade
MIBC	Muscle-invasive bladder cancer
MLC1	Myosin light-chain protein
MLCP	Myosin light-chain phosphatase
MMRN1	Multimerin 1
MS	Masspectrometry
MVBs	Multivesicular bodies
MW	Molecular weight
NF2	Neurofibromin 2
NMIBC	Non-muscle-invasive bladder cancer
NMP22	Nuclear matrix protein 22
NMP22	Nuclear Matrix Protein Number 22
NRAS	Neuroblastoma RAS Viral Oncogene Homolog
PAK1	P21 Activated Kinase 1
PANTHER	Protein ANalysis THrough Evolutionary Relationships
PCR	Polymerase chain reaction
PRDX1	Peroxiredoxin 1
PRDX2	Peroxiredoxin 2
PUNLMP	Papillary Urothelial Neoplasm of Low Malignant Potential
RHO	Ras Family Protein
RHOA	Ras Homolog Family Member A

RNA	Rribonucleic acid
SEER	Surveillance, Epidemiology and End Results
SELDI-MS	Surface-enhanced laser desorption/ionization
SERPING1	Plasma protease C1 inhibitor
SILAC	Stable isotope labeling with amino acids in cell culture
SOD2	Superoxide dismutase 2
SPSS	Statistical Package for the Social Sciences
TNC	Tenascin C
UBC	Urinary Bladder Cancer
UC	Urothelial Cancer
UEs	Urinary exosomes
VSAP	Vasodilator-stimulated phosphoprotein
WHO	World Health Organization

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Chapter 1

INTRODUCTION

Chapter 1

1.1 Introduction

Bladder cancer (BC) is the most prevalent type of urothelial cancer and is associated with the highest rate of recurrence among all cancer (Chavan *et al*., 2014). Hence, patients with bladder tumors need surveillance and follow-ups to detect recurrences. BC is a male dominating cancer and the incidence ratio of males to females is 3:1 (Ferlay *et al*., 2012). Incidence of BC increases with age and the highest frequency is between the ages of 60-70 years (Chavan *et al*., 2014). Smoking is a common risk factor and increases the level of urinary amines which is associated with BC and other urinary tract cancer (Martini *et al*., 2013).

Histological subtypes of BC include urothelial cell carcinoma (90%), squamous cell carcinoma (6-8%) and adenocarcinoma (1-2%). Urothelial cell carcinoma originates from the epithelial layer of the bladder called Urothelium. About 80% of urothelium carcinoma are carcinoma *in situ* (CIS) or non-muscle-invasive bladder cancer (NMIBC) and labeled on the extent of infiltration into the urothelium or invasion of the muscularis propria (NMIBC-pTa/pT1/pTis); the remaining 20% present are muscle-invasive bladder cancer (MIBC) and showing muscle invasion deep into the bladder wall (MIBC-pT3 to pT4). Grades of bladder cancer are a measurement of the dedifferentiation of the tumour cells and classified into low and high grade tumors (MacLennan *et al*., 2007).

The pathologist's assessment of stage and grade is used together with other clinical parameters when a decision about treatment is made. Patients with MIBC generally have a poor prognosis compared to NMIBC since more than 50% of MIBC patients succumb to their disease within 5 years of diagnosis (Van *et al*., 2010). Among NMIBC patients more

than 70% will have at least one recurrence after successful treatment. The highest rate of first recurrence is observed in patients with high-grade T1 tumours (pT1HG), having approximately 29% probability of progression within 5 years of initial diagnosis (Pan *et al*., 2010). Hence, the patients should be on follow-up every 3-6 months for 3 years and thenceforth yearly.

Painless micro- or macroscopic hematuria is an important symptom of bladder cancer. Other symptoms include dysuria (pain during urination) and urgency (sudden urge to urinate), but these symptoms are not specifically associated with bladder tumors and are also found in cystitis, prostatitis or bladder stones. When patients present at the urologist with these complaints, the presence of hematuria is first investigated (Oosterlinck *et al*., 2002).

Urine cytology and cystoscopy are commonly used techniques for the diagnosis and surveillance of bladder cancer. The definitive diagnosis is made through endoscopic investigation of the bladder by using a cystoscope. Cystoscopy can identify most papillary and solid lesions, but it is invasive to the patients, whereas urine cytology is limited by the low sensitivity (only 4–31%) for low grade bladder cancer (Lotan *et al*., 2003).

For these reasons, some tumor markers have been investigated for early diagnosis and surveillance however their sensitivity and specificity are limited. Also, they were found unable to predict the clinical outcome of BC patients. Therefore, one of the most important clinical challenges is the identification of novel biomarkers which can predict recurrence in patient having NMIBC. Proteins secreted from tumor cells reflect the various states of the tumor in real time and under specific conditions, and their expression patterns are expected to be different from normal cell components. Thus, proteins secreted into several body fluids, such as the serum, urine, cerebrospinal fluid, tears, and saliva from tumor cells and

conditioned media of cultured tumor cells have been investigated. Approximately 20-25% of cellular proteins from tumors are secreted into body fluids and it has been suggested that secreted proteins from tumor detected in non-invasive or minimally invasive samples like blood, urine, saliva are a promising source of diagnostic and surveillance biomarkers (Chou *et al*., 2015).

Urine contains major water soluble and cellular excreted material from body and its examination gives information to the clinician about the state of the patient's health. Urine is produced in the kidney by filtration of the blood in the glomeruli. Urine contains proteins that originate from blood proteins that have passed through the kidney and proteins from the urinary tract epithelium. These epithelial proteins are of great interest when searching for urinary biomarkers for urinary bladder cancer (Budman *et al*., 2008).

Tumor cells secrete proteins and various extracellular vesicles, including exosomes, into their tumor microenvironment. Exosomes are small vesicles that contain miRNA, mRNA, and proteins with the potential to alter signaling pathways in recipient cells (Jeppesen *et al*., 2014). Exosome research has flourished and found the involvement of exosomes in progression of cancer (Silvers *et al*., 2016). Nowadays various exosome databases such as Exocarta, Vesiclepedia and Urinary Exosome Protein Database are available and have disease-specific information. Analysis of these databases gives an idea of the involvement of differentially expressed proteins in the formation or secretion of urinary exosome.

1.2 Gaps in existing Research:

The follow-up of patients diagnosed with urinary bladder cancer requires urine cytologic examination by trained pathologists and cystoscopy by trained urologists. The peripheral medical centers in our country do not always have a resident urologist thus decreasing the compliance because it entails travel to a specialist center for follow-up. Hence, it is important to identify markers for surveillance of the population and monitoring for recurrence. The identification of tumour specific proteins that can be developed as non-invasive diagnostic tests for routine screening will result in improving surveillance with regular follow-up.

The quest for urinary bladder cancer biomarkers is ongoing, but the specificity and sensitivity of markers (BTA Stat, BTA Trak, NMP 22, Bladder Chek, Immunocyt and UroVysion) found so far are not sufficient to replace urinary cytology as a screening tool (Frantzi *et al*., 2015). Although these biomarker candidates have been extensively studied, they do not seem to have a direct connection with the disease pathophysiology. As a consequence, discordant findings might be a result of identified proteins being indicative of chronic inflammation and microvascular or macrovascular damage rather than the presence of cancer. A major concern is that most of these proteins (or peptides derived from them) have also been described as biomarkers of kidney and/or other lower abdominal diseases; thus, these proteins might not be specific biomarkers of bladder cancer. The decreased sensitivity could also be attributed to urine proteomics approach. As urine proteins reflect the excretion of body metabolites, the proteins found are not specific for cancer but may indicate a disease status. Nonetheless, the observed agreement between the various studies in this area supports the validity of proteomics approaches to biomarker discovery (Mitra *et al*., 2010 and Herman *et al*., 2008).

The proposed study is planned to identify proteins/peptides which will differentiate between bladder cancer and normal bladder mucosa. The secretion of these proteins in urine would be detected and those which either singly or in combination, is found in detectable quantities in urine would be used to monitor patients on follow-up to establish their role as markers of recurrence. Antibodies of the detected proteins can be developed for use as routine markers of recurrence and screening with urine as a sample.

1.3 Hypothesis and research question

This study proposed to identify tumor specific protein in urine of urothelial cancer patients to serve as post surgery surveillance markers. The null and alternative hypothesis is as follows:-

Hypothesis: There is secretion of cancer specific proteins in urine of urothelial bladder cancer patients which can serve as protein predictive biomarkers for recurrence

The search for urinary biomarkers is a difficult task with many pitfalls. The journey from identifying a candidate biomarker to becoming a true biomarker in clinical use is long. Hence, validation for use in clinical practice has to be done on large cohorts. It is also noticed that instead of using one biomarker, identification of a combined range of different biomarkers as a panel to achieve the required level of specificity and sensitivity is a better approach. The clinical utility of newly discovered biomarkers depends upon some added values beyond currently available standards of care. The clinical potential and challenges in implementing biomarkers in the management of patients who have bladder cancer may be

addressing different aspects according to the intended purpose: diagnosis, prognosis or prediction of treatment response.

The main rationale behind this study is to identify highly specific and sensitive tumor specific biomarkers in urine which can be used for surveillance of patients replacing frequent cystoscopy. To achieve this aim, tumour samples from each grade and stage were collected; proteins were isolated and subjected for iTRAQ LC-MS/MS. The deregulated proteins of all samples were analysed for pathways involved and Gene Ontology annotations. The commonly upregulated proteins were then verified for their presence in tissue by immunohistochemistry. The secretions of these tumor specific proteins in urine sample were verified by Western blot. ELISA was performed to quantitate the protein and find its association with clinicopathological parameters and recurrence.

Chapter 2

Aim and Objectives

Chapter 2

Aim and Objectives

Aim

To identify tumor specific protein in urine of urothelial cancer patients to serve as post surgery surveillance markers.

The formulated key questions include

Do urothelial cancer cells release cancer specific proteins in urine which can serve as biomarker for early diagnosis, recurrence and surveillance.

To answer these questions, objectives were designed as

Objective

1. **To identify the differentially expressed tumour tissue proteins in urinary bladder cancers compared to normal bladder mucosa**

   High resolution quantitative proteomics approach (8-plex-iTRAQ-LC-MS/MS) was assessed for global protein profiling of bladder cancer.Differentially expressed proteins were further analyzed to determine Gene Ontology (GO) annotations and of their function, pathways and protein-protein interaction in the Ingenuity Pathways Analysis (IPA) system.

2. **To identify differentially expressed tumor protein secreted in the urinary exosomes using *in-silico* analysis.**

   A comparative analysis was performed between differientally deregulated proteins and urinary exosomal protein reported in bladder cancer on Exocarta database. The Urinary bladder cancer specific exosomal proteins were further analysed to identify enriched pathways by Onto-tool Pathway Express (http://vortex.cs.wayne.edu/ontoexpress) and protein-protein interaction visualized using Cytoscape.

3. **To detect the presence of the differentially expressed tumour specific proteins in urine and evaluate the role of urinary biomarkers for recurrence of Urinary Bladder Carcinoma**

   Three commonly upregulated (SERPING1, SOD2 and HSPB6) proteins and three variably deregulated proteins (PRDX1, PRDX2 and Tenascin C) were selected for verification in FFPE tissue of bladder cancer and adjacent mucosa was selected as a control using immunohistochemistry. Furthermore, these markers were validated in urine of bladder cancer and urine of non-malignant cases as a control by Western blot (for the confirmation of their presence) and ELISA (was used in large cohort of urine sample).

Chapter 3

Review of Literature

Chapter 3

Review of literature

3.1 Normal cells, Cancer cells and etiology of cancer

The human body is made of trillions of live cells or normal cells, they are under strict growth control while cancer cells grow and divide more rapidly than normal. Cancer cells are mainly two types, benign and malignant tumors. Benign tumors refer to tumor cells and may closely resemble normal cells but do not grow into adjacent tissues, whereas malignant tumors are less well differentiated than normal cells or benign tumor cells, more aggressive and have the ability to metastasize by invading and destroying nearby tissue (Aguirre *et al*., 2007). During premalignant changes cells start to lose their normal appearance, and are termed as atypical cells. The state when cells start to proliferate too much, in a dysregulated way, is called hyperplasia. Neoplasia is defined as an abnormal growth of cells that produce new tissue and dysplasia is when a tissue develops abnormally.

Malignant cells usually have the characteristics of rapidly growing cells, having a high nucleus-to-cytoplasm ratio, prominent nucleoli, many mitoses, and relatively little differentiated structure (Blackadar *et al*., 2016). The tumor cells do not remain in their original site; instead, they are able to detach from the primary tumor, invade surrounding tissues, survive in body's circulation, adhere to capillaries and set up areas of proliferation away from the site of their original appearance. The spread of tumor cells and establishment of secondary growth is called metastasis and causes 90% of all cancer deaths. Cancer is a disease that develops over time (Moreno *et al*., 2008). For a cell to transform into a cancer cell it has to accumulate the previously stated characteristics.

The etiology of cancer includes genome instability, mutation and tumor-promoting inflammation. It broadly includes i) change in the DNA sequence due to chromosomal instability (chromosomes are rearranged); ii) aneuploid cells (a product of failed mitosis resulting in an abnormal number and composition of chromosomes); iii) Synonymous point mutations (single changes in a base pair and codon is changed, but not translated into amino acid) and non-synonymous point mutation (changes the DNA sequence in an exon and get translated sequence changed amino acid) (Geiger *et al*., 2010); iv) Nonsense mutations are point mutations that change a codon from an amino acid coding codon into a stop codon and resulting in an incomplete protein; v) copy number changes (not always reflected in the amount of gene product); vi) epigenetic changes in cancer (hypermethylation of CpG islands in promoter regions can silence tumor suppressor genes and hypomethylation can activate expression of oncogenes) (Razin *et al*., 1991); vii) Modifications of histones can either increase the expression of oncogenes or silence tumor suppressor genes; viii) angiogenesis (formation of new blood vessels for development of tumor since the tumor needs a supply of nutrients and oxygen to be able to grow) and ix) evading apoptosis (should go into apoptosis but cancer cells can avoid this). Tumours usually acquire at least one of the hallmarks of cancer (Figure 3.1). The tumor cells can also be stimulated by the stroma and stromal cells (consisting of fibroblasts, lymphocytes, pericytes, and vascular cells), which all influence tumor growth (Hanahan *et al*., 2000; Lengauer *et al*., 1998; Aguirre *et al*., 2007; Folkman *et al*., 2006 and Jackson *et al*., 2009)

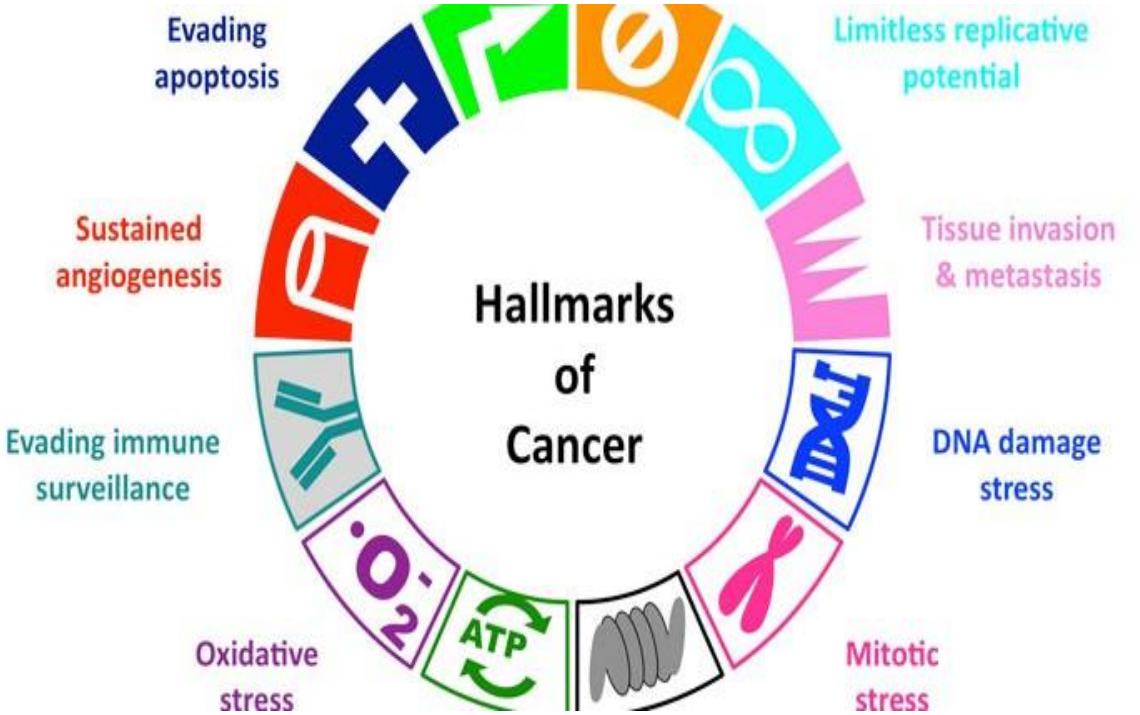


Figure 3.1: The top 10 Hallmarks of Cancer (Hanahan *et al*., 2000)

These are the general hallmarks of cancer that provide a logical framework for understanding the remarkable diversity of neoplastic diseases. The heterogeneity of tumor is very random and unpredictable and the mechanism of tumorigenesis among different organs varies and is not well known. Incidence, prevalence and mortality rate of cancer varies among population, sex, age, lifestyle and types of cancer.

3.2 Epidemiology of Bladder cancer: World wide

Mortality rate has increased by 6% between 2012 and 2014. In 2012, there were 4,78,180 deaths out of 29,34,314 cases, in 2013 there were 4,65,169 death out of 30,16,628 cases

and 4,91,598 people died out of 28,20,179 cases in 2014. Mortality (3%) and occurence rate (7%) of bladder cancer were estimated among all types of cancer (Figure 3.2).

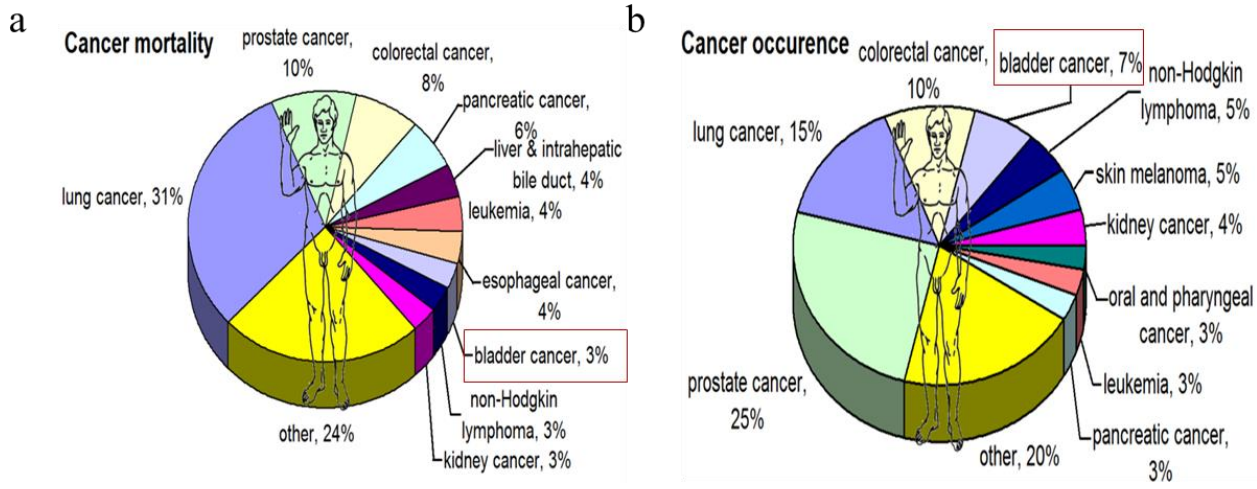


Figure 3.2: Pie chart showing a) cancer mortality rate and b) Occurrence rate (%) all over the world (Source GLOBOCAN 2012).

An estimated 429,000 bladder cancer cases occurred in 2012, making the disease the ninth most common cause of cancer for both sexes combined. Bladder cancer is relatively common in more developed regions, where 60% of all incident cases occur, and it occurs among men more than in women (sex ratio worldwide of 3.5:1). Rates of bladder cancer in males are high in Southern and Western Europe [the age-standardised rate (ASRs) 21.8 and 19.7 per 100,000, respectively] and in Western Asia (19.0) and Northern Africa (15.1) where bladder cancer is linked to chronic schistosomal infection. Female rates are much lower, with the highest in Northern America (5.1) and Western Europe (4.3). In both sexes, low incidence rates are seen in South-Eastern and South-Central Asia, and in sub-Saharan Africa. There were an estimated 165,000 deaths from bladder cancer worldwide, with similar numbers in less (85,000) and

more (80,000) developed regions. Mortality rates are much lower than the incidence rates, with the highest ASRs estimated in Western Asia in men (8.4 per 100,000) and in Northern Africa (1.6) in women (Figure 3.3a and b) (Ferlay *et al*., 2012; Bray *et al*., 2013).

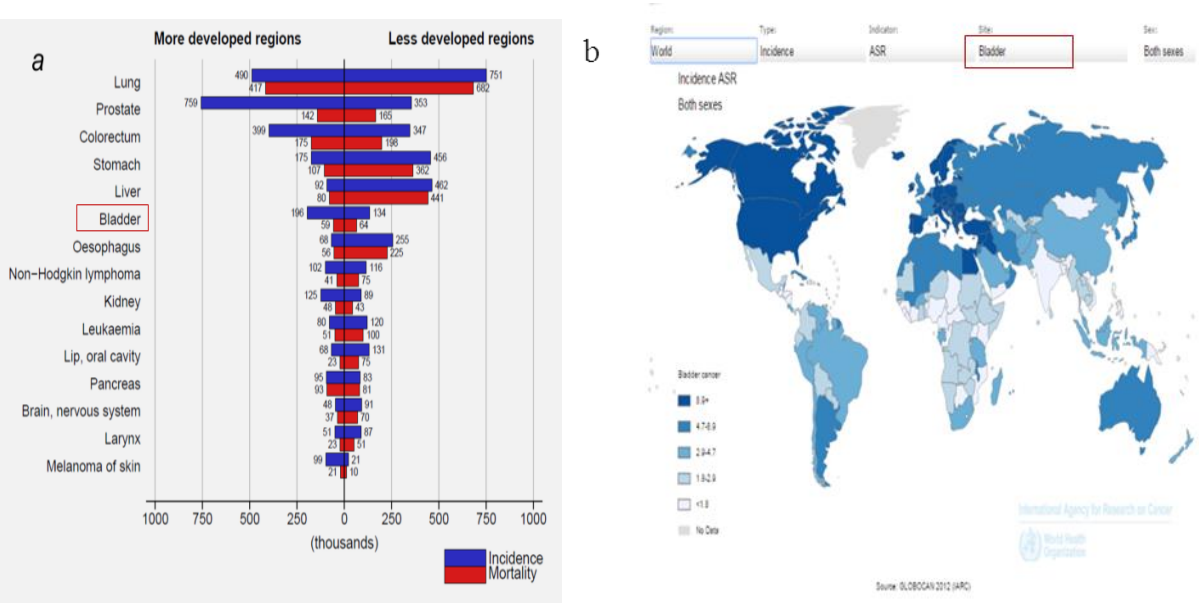


Figure 3.3: a) Estimated numbers (thousands) of new bladder cancer cases (incidence) and deaths (mortality) is more in developed regions compared to less developed regions of the world in 2012 and b) The World map showing the incidence of bladder cancer worldwide.

As per SEER (Surveillance, Epidemiology and End Results) program, the estimated numbers of new cases of bladder cancer were 20.1 per 100,000 men and women per year. The number of deaths was 4.4 per 100,000 men and women per year. These rates are age-adjusted and based on 2009-2013 cases and deaths. In 2013, there were an estimated 587,426 people living with bladder cancer in the United States. The lifetime Risk of Developing Cancer showed that approximately 2.4 percent of men and women will be diagnosed with bladder cancer at some point during their lifetime, based on 2010-2012 data (Horner *et al*., 2013).

3.3 Epidemiology of Bladder cancer: India

In India, more than 1300 Indians die every day due to cancer according to National Cancer Registry Program India Council of Medical Research (ICMR). The total number of cancer cases in India is 10,14,934 and bladder cancer constitutes 1.61% of the total (Figure 3.4).

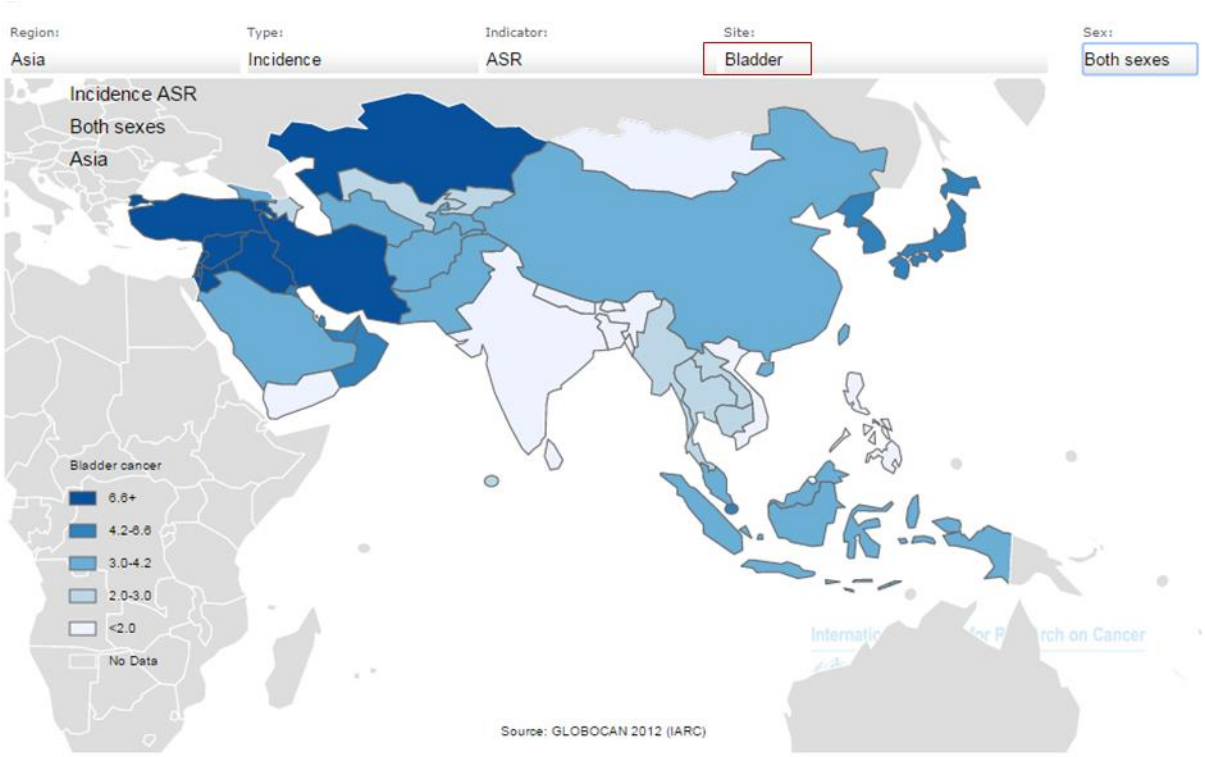


Figure 3.4: Incidence of bladder cancer in India and worldwide.

About 90% of the cases of bladder cancer reported in India are the Transitional cell type, now known as Urothelial carcinoma. The rest is constituted by Squamous cell carcinoma (5%), Adenocarcinoma (2%), small cell and sarcomatoid carcinoma. The overall incidence of Bladder cancer is 7% and increasing in the developing country day by day, hence it is important to address the issue of bladder cancer (Ferlay *et al*., 2008 and Bray *et al*., 2013).

3.4 Morphology of Normal urinary bladder

The urinary bladder is a hollow elastic organ located in the pelvic cavity that functions as storage chamber of urine. Urine produced by the kidneys flows through the ureters to the urinary bladder and flows out from the body through urethra. The innermost layer of the urinary bladder is made up of 3-4 layers of mucosa that lines the hollow lumen. The urinary bladder is lined with 5-6 layers of transitional epithelium and provides protection to the underlying tissues from acidic or alkaline urine. Cells of the transitional epithelium has a thicker than normal plasma membrane (Junqueira *et al*., 1992).

Under the mucosal layer is the submucosa, a layer of connective tissue with blood vessels and nervous tissue that supports and controls the surrounding tissue layers.The visceral muscles of the muscularis layer surround the submucosa and provide the urinary bladder with its ability to expand and contract. The muscularis is commonly referred to as the detrusor muscle and contracts during urination to expel urine from the body. The muscularis also forms the internal urethral sphincter, a ring of muscle that surrounds the urethral opening and holds urine in the urinary bladder (Wheater *et al*., 1979). The cells in the urothelium are also connected in a way that allows it to distend to a great extent. The basal membrane of the urothelium is very thin. The phenotype of a tumor is different from healthy tissue. The phenotypic difference is the result of a genotype difference that manifests itself as a difference in protein expression. The structure of the tissue gets more disorganized with higher tumor grade. In the case of bladder cancer the difference in protein expression should in theory, result in a difference in the protein composition in the urine that the bladder encloses (Lukacz *et al*., 2011).

3.5 Morphology of Urothelial Bladder

The Urinary bladder is lined by Urothelium or Transitional cell epithelium. The transitional epithelium accommodates variations in the quantity of the fluid in an organ or tube. It comprises of multiple layers of epithelial cells, which can contract and expand and are positioned in the urinary bladder. The cells become flattened on stretching due to dilatation/filling of the bladder.

The normal urothelial mucosa averages five to seven cell layers and three types of cells: basal, intermediate and umbrella cells. The surface epithelial cells are rounded and bulge out and are called “umbrella cells”. The intermediate cell layer is about six cells thick in the contracted bladder, where they are oriented with their long axis perpendicular to the basement membrane. The nuclei are oval with nuclear grooves and have finely granular chromatin. The cytoplasm is relatively abundant and amphophilic and may be vacuolated.

The cell membranes are distinct with desmosomes connecting them. The basal layer is one cell thick, composed of cuboidal cells. The lamina propria is a compact layer of fibrovascular tissue with a variable number of smooth muscle fibres, the muscularis mucosae. This layer is usually situated midway between the epithelium and the muscularis proper (detrusor muscle). The smooth muscle fibres may be found in three distribution patterns, 1) continuous layer, 2) discontinuous or interrupted layer and 3) scattered thin bundles. The detrusor muscle consists of haphazardly arranged thick bundles of smooth muscle. Adipose tissue may be present in the lamina propria as well as in muscularis propria. The muscularis is surrounded by the adventitia which is a coat of fibroelastic tissue and perivesical fat (Mostofi, 1960).

3.6 Risk factors for urothelial bladder cancer

The etiology of bladder cancer isn't entirely clear. Certain inherited metabolic factors may play a role. People whose bodies metabolize toxic chemicals quickly may be less susceptible to bladder cancer than people who metabolize the same chemicals more slowly. Significant among these chemicals are polycyclic hydrocarbons found in cigarettes and in some industrial chemicals (Jiang *et al*., 2012; Rasool *et al.*, 2014). Excretion of cancer-causing agents (carcinogens) in the urine may lead to the development of bladder cancer.

The International Agency for Research on Cancer (IARC) and The World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) had evaluated evidence for various environmental and chemical carcinogens risk for bladder cancer are shown in Table 3.1.

Table.3.1: IARC and WCRF/AICR Evaluations of Bladder Cancer Risk Factors

Increases risk of BC (sufficient evidence)	May increase risk of BC (limited evidence)
• Aluminium production • 4-Aminobiphenyl • Arsenic and inorganic arsenic compounds[a] • Auramine production • Benzidine • Chlornaphazine • Cyclophosphamide • Magenta production • 2-Naphthylamine • Painting • Rubber production industry • *Schistosoma haematobium* • Tobacco smoking • Ortho-Toluidine • X-radiation, gamma-radiation	• 4-Chloro-ortho-toluidine • Coal-tar pitch • Coffee • Dry cleaning • Engine exhaust, diesel • Hairdressers and barbers (occupational exposure) • Printing processes • Textile manufacturing • Tetrachloroethylene

[a] Arsenic in drinking water is classified by WCRF/AICR as a possible cause of bladder cancer based on sufficient evidence (reproduced from http://www.cancerresearchuk.org/)

The risk factors may have a different impact on the incidence and pathophysiology of Urothelial bladder cancer. The common risk factors are as follows:

Age: The incidence of bladder cancer increases, as people grow older and is particularly higher after the age of 60 years. The marked increase in the incidence of bladder cancer in elderly correlates with the long latency period required for carcinogenic induction by the environmental factors. The average age at diagnosis is 68 or 69. People younger than 40

rarely get the disease and in these patients, it is mostly low grade papillary TCC with low risk of recurrence (Jemal *et al*., 2011).

Sex: TCC Bladder is two to ten times more frequent in men than in women, a difference that is not entirely explained by differences in cigarette smoking or occupational exposure. The male to female ratios are 3:1 in U.S, 7:1 in Italy and 10:1 among American Indians. The male preponderance appears less striking in Squamous cell carcinoma, which suggests a sex linked genetic susceptibility for TCC (Yee *et al*., 2011).

Race: Caucasians are twice as likely to develop bladder cancer as blacks and Hispanics. Asians have the lowest rates of the disease. Whites get bladder cancer twice as often as African, Americans and Hispanics. The lowest rates are among Asians (Yee *et al*., 2011). The lifetime risk of developing bladder cancer has been estimated to be 2.8% for women, 0.9% for black men, 1% for white women and 0.6% for black women (Schatte *et al*., 2006). The striking difference in the gender and the ethnic incidence provide some evidence for some distinct genetic susceptibility to bladder cancers.

Smoking: Smoking cigarettes is the major risk factor for bladder cancer in both men and women. Parkin *et al* had estimated that there is four times higher bladder cancer risk in smoker compared with non-smokers. Risk increases with the increasing number of cigarettes smoking each day (Parkin *et al*., 2010). Cigarette smoking contributes to more than 50% of cases, beside smoking cigars or pipes also increases the risk. Cancer-causing chemicals in tobacco smoke are absorbed into the blood, filtered out by the kidneys and then, as a part of the urine, stored in the bladder. In the long term, this appears to cause damage to the bladder

lining. The risk is even greater for women (Mostafa *et al.*, 2003). Cancer risk varies with types of tobacco, higher risk for black 'air-cured' than blond 'flue-cured' tobacco. Black tobacco has higher concentrations of N-nitrosamine and 2-napthylamine. Smokers of black tobacco have higher levels of aromatic amines in their urine than smokers of blond tobacco. These aromatic amines are known urothelial carcinogens and the ability to detoxify them is compromised in people who are 'slow acetylators' and it is suggested that these people are at higher risk than 'fast acetylators'. Tobacco tars have been shown to induce bladder papillomas and carcinomas in mice. Prolonged exposure of the bladder to such urinary carcinogens during the excretory process may lead to the development of bladder cancer. Exposure to environmental tobacco smoke (ETS) during childhood increases the risk of bladder cancer by 40% (Gupta *et al*., 2016).

Infections: Infection with certain parasites increases the risk of bladder cancer. In some parts of the developing world, especially Egypt, a chronic parasitic infection (*schistosomiasis-Bilharzia haematobia*) can lead to squamous cell carcinoma of the bladder (Mustacchi, 2003). Human papilloma virus (HPV) is also doubling the risk of bladder cancer in patients with *condylomata acuminata* (genital warts).

Occupational exposure: Exposure to carcinogens in the workplace also increases the risk for bladder cancer. Often, these cancers develop many years later (latency period of 15-40 yrs). In the year 1895, the surgeon Howe *et al* was first to establish a relation between bladder cancer and exposure to chemical dyes and demonstrated in 1938 that the industrial chemicals arylamine-2 –naphthylamine could induce bladder cancer (Hueper *et al*, 1938). Medical

workers exposed during the preparation, storage, administration, or disposal of antineoplastic drugs (used in chemotherapy) are at increased risk. Occupational risk factors include recurrent and early exposure to hair dye, and exposure to dye containing aniline, a chemical used in medical and industrial dyes. Most bladder cancer carcinogens are aromatic amines. The most investigated compounds are 2 naphthylamine, benzidine and 4–aminobiphenyl (Rota *et al*., 2014). Other carcinogens are derived from aromatic amines. Smokers who work with toxic chemicals are at especially high risk of bladder cancer (Cancer Research UK).

People living in areas where pesticides are widely used and the drinking water contains high levels of arsenic are more likely to develop bladder cancer. Workers at increased risk include hair dressers, machinists, printers, painters, truck drivers, workers in rubber, textile, leather industry (Aben *et al*., 2002).

A number of **other factors** have been suggested as possible causes of bladder cancer (Aben *et al*., 2002) including:

- **Diet.** High intake of cholesterol, fatty meals, and fried food is associated with increased risk of bladder cancer.
- **Chlorine by-products.**
- **Cyclamate.** Early studies linking this artificial sweetener to bladder cancer in mice caused the Food and Drug Administration (FDA) to ban cyclamate in 1969. Subsequent studies haven't found clear association,
- **Saccharin.** Animal studies have shown a relationship between this artificial sweetener and bladder cancer. But the association between saccharin and cancer in humans isn't clear. However the products containing saccharin carry a warning

about a possible link to bladder cancer.

- Consumption of *Aristolochia fangchi* (herb used in some weight-loss formulas)
- External beam radiation.
- Family history of bladder cancer (several genetic risk factors identified)
- Abnormalities of chromosome 9 and also p53 mutations
- Treatment with certain drugs (e.g. cyclophosphamide, ifosfamide (IFEX) - used to treat cancer)

3.7 Morphology of Urothelial cancer

As already discussed, the Urothelial cancer (Transitional cell cancer) constitutes 90% of the Urinary bladder malignancies. Other cancer types are the adenocarcinomas and squamous cell cancer. In cancer of the Urinary bladder the epithelium undergoes changes and according to the severity of change the malignancy is graded into Papillary Urothelial Neoplasia of Low Malignant Potential (PUNLMP), Low Grade (LG) and High Grade (HG) Urothelial cancers (Amin, 2009).

3.7.1 PUNLMP

PUNLMP is a papillary low grade urothelial neoplasm which does not have the capacity to invade or metastasize. The histological architecture is akin to normal urothelium and the nuclear features are only slightly abnormal. They show multiple layers of cells with preservation of superficial cell layer and delicate fibro vascular stalks. Cells have moderately

distinct border and homogeneous amphophilic to acidophilic cytoplasm. Cytoplasmic clearing is reduced. Nuclei may be round or elongated and maintain their normal perpendicular orientation of the surface and the basal lamina (Figure 3.5). Chromatin is evenly dispersed and finely granular and nucleoli are small or absent. Mitosis is occasional (McDougal *et al*., 2011).

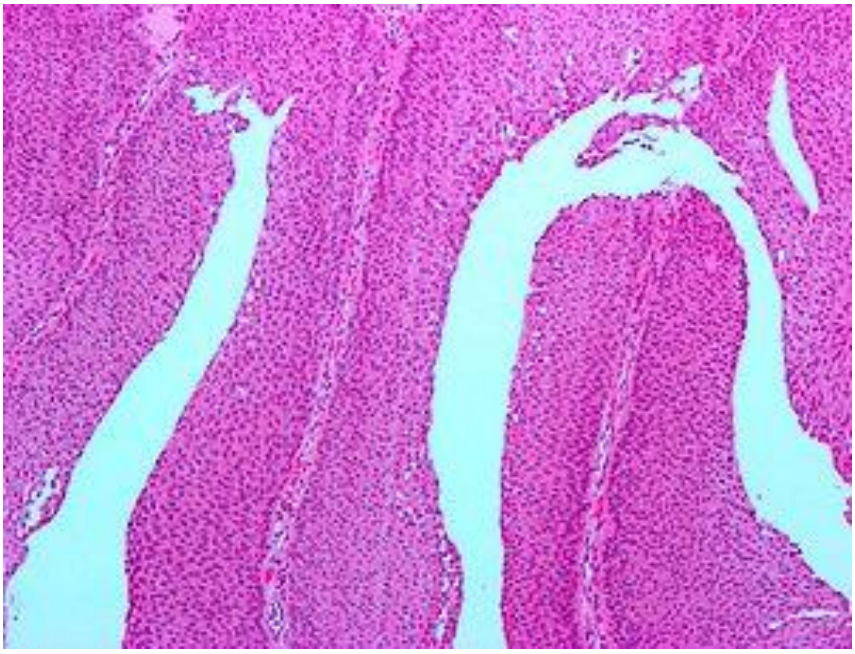


Figure 3.5: Hematoxylin and Eosin stained section of papillary low grade urothelial neoplasm

3.7.2 Low Grade Urothelial Carcinoma

Cells are arranged covering papillary stalks and resemble PUNLMP architecturally and cytologically. These tumours have the capacity to invade and metastasize (rarely). The superficial layer is partially preserved. Cells are uniform in size and evenly distributed with indistinct borders and little or no cytoplasmic clearing. Nuclei are rounded and slightly pleomorphic with irregular nuclear borders (Figure 3.6). Chromatin is evenly dispersed and

finely granular. Mitosis is seen scattered throughout the epithelium and not just at the basal layer (Amin *et al*., 2009 and McDougal *et al*., 2011).

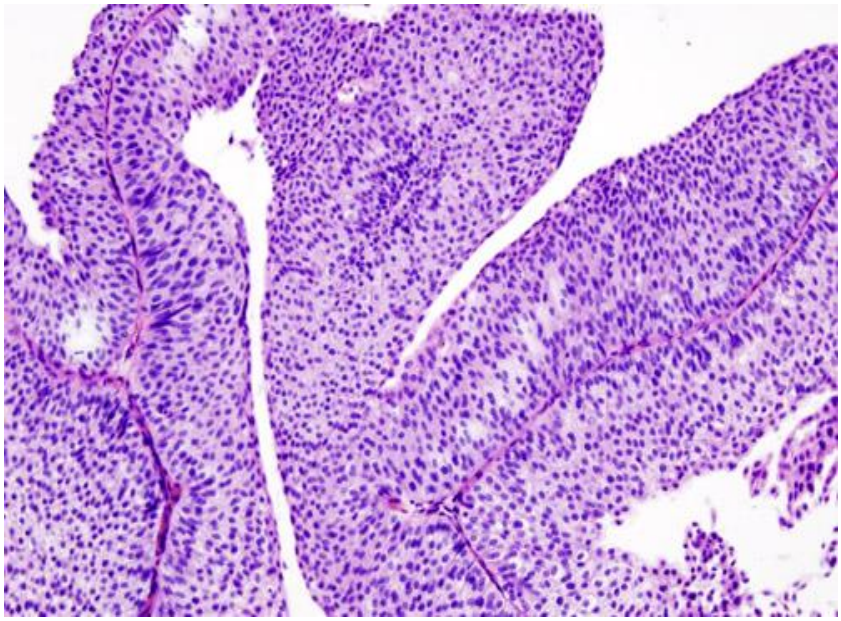


Figure 3.6: Histopathology of Low grade urothelial carcinoma of the urinary bladder showing papillary cores covered by stratified epithelium of transitional type.

3.7.3 High Grade Urothelial Carcinoma

High grade tumors may be papillary or nodular and are often invasive. At least 50% of all urothelial neoplasms and 60-80% of all the carcinomas are high grade. They are usually infiltrating and have cells arranged in sheets, nests and cords. Papillary component, if present, shows loss of polarity of the cells covering the stalk. Cells have indistinct borders. Cytoplasm is homogeneous and may be vacuolated. Nuclei tend to cluster and vary considerably in shape. Nuclear chromatin is coarsely granular and unevenly dispersed (Figure 3.7). Mitoses are common and may be abnormal. Intracellular and extracellular mucins may be present with

foci of malignant glandular and squamous differentiation (Amin *et al*., 2009 and McDougal *et al*., 2011).

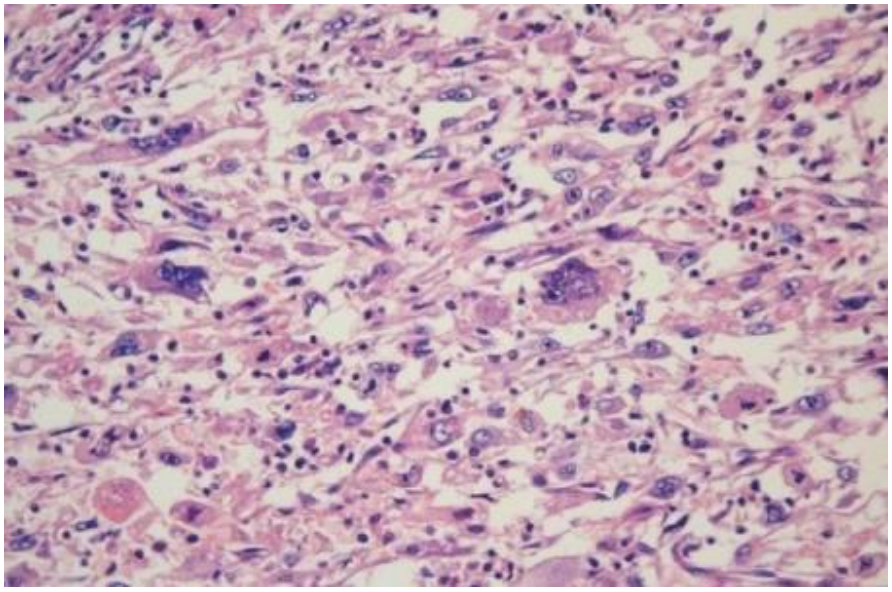


Figure 3.7: Hematoxylin and Eosin stained section showing high grade urothelial bladder cancer with the tumour cells showing features of nuclear pleomorphism multinucleation.

3.7.4 Adenocarcinoma

Primary adenocarcinoma of the bladder comprises less than 2% of all bladder tumors and is categorized into metastatic, primary, and urachal adenocarcinomas. Metastasis occurs commonly from breast, prostate, colon, and tumors of the female reproductive tract. Morphologically, these tumors are of enteric type, signet ring cell type or mucinous, and are muscle-invasive at the time of initial diagnosis. Primary adenocarcinoma of the bladder is

curable with radical cystectomy or pelvic exenteration (Figure 3.8). Diagnosis of adenocarcinoma is based on the morphology (Kumari *et al*., 2015).

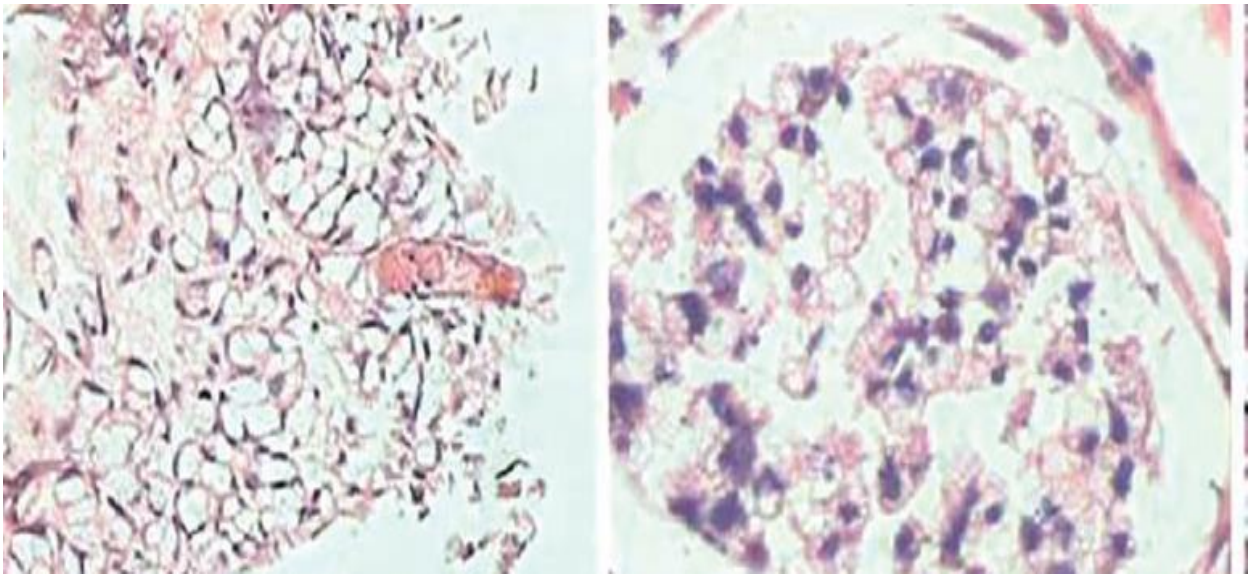


Figure 3.8: Adenocarcinoma of bladder. (a) Hematoxylin and eosin (H and E) stained section shows signet ring cells in nests, (b) sheet of signet ring cells in pool of extracellular mucin.

3.7.5 Squamous cell carcinoma

Squamous cell carcinoma is a malignant neoplasm derived from bladder urothelium with pure squamous phenotype of urinary bladder cancer. Squamous cell carcinoma of the bladder is essentially similar to the tumors arising in other organs. Because many urothelial carcinomas contain a minor squamous cell component, a diagnosis of squamous cell carcinoma of the bladder should be rendered only when the tumor is solely composed of a squamous cell component in the absence of a conventional urothelial carcinoma component. In the bladder,

the deposition of Schistosoma eggs commonly provokes a severe inflammatory response and fibrosis.The eggs are found embedded in the lamina propria and muscularis propria of the bladder wall; many of the eggs are destroyed by host reaction and become calcified (Figure 3.9) (Felix *et al*., 2008).

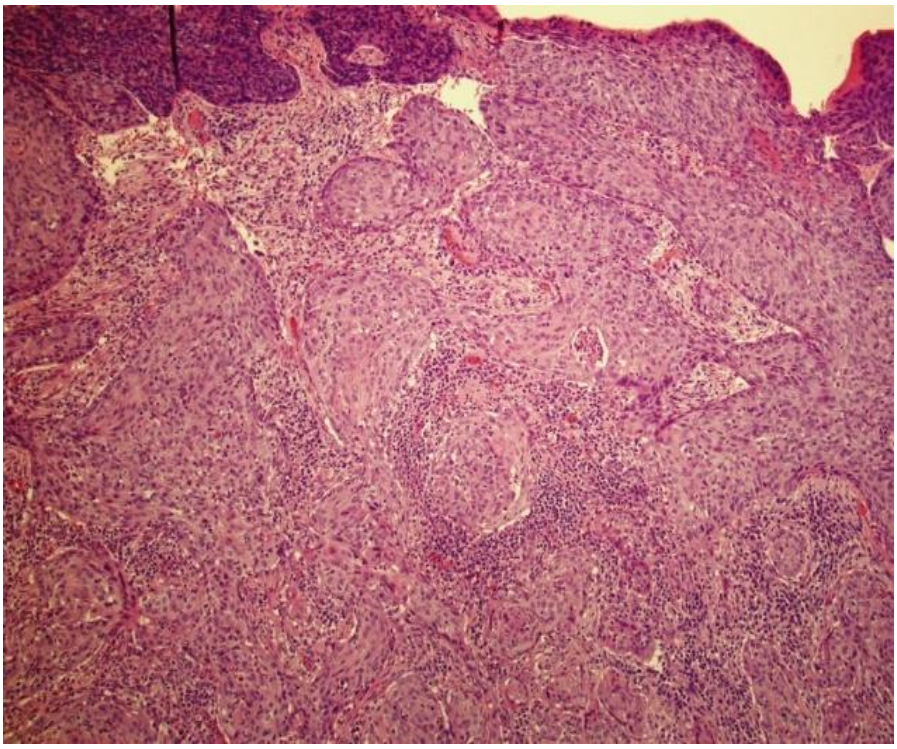


Figure 3.9: Deeply infiltrated moderately differentiated squamous cell carcinoma of the bladder.

3.8 Evolution of Classification of Bladder (Urothelial) Cancer (Kumari *et al*., 2015).

Bladder cancer is classified on the basis of morphological appearance, pattern of growth and depth of invasion. As it has been seen that the microscopic appearance i.e., the grade, does not always conform to the clinical behavior, the classification incorporated the depth of invasion i.e., stage to show behaviour and guide the treatment. Wallace (1956) proposed staging into

mucosal (T1), muscular (T2), perivesical (T3) and pelvi-fixation (T4). It is only after 1956 that the pTa stage i.e., confined to the urothelium was recognized. While superficial and deep muscle invasion was proposed, it is not practical as it cannot be decided on biopsy specimens and can be staged only on radical cystectomy specimens. The staging system is given in Table 3.2 and 3.3 and histopathological staging in Figure 3.10.

Table 3.2: Clinical staging of Bladder carcinoma

Stage	**T**	**N**	**M**
Stage 0a	Ta	N0	M0
Stage 0is	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2a	N0	M0
	T2b	N0	M0
Stage III	T3a	N0	M0
	T3b	N0	M0
	T4a	N0	M0
Stage IV	T4b	N0	M0
	Any T	N1-3	M0
	Any T	Any N	M1

Table 3.3: TNM stage of Bladder carcinoma

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ta	Noninvasive papillary carcinoma
Tis	Carcinoma *in situ*: "flat tumor"
T1	Tumor invades subepithelial connective tissue
T2	Tumor invades muscularis propria
pT2a	Tumor invades superficial muscularis propria (inner half)
pT2b	Tumor invades deep muscularis propria (outer half)
T3	Tumor invades perivesical tissue
pT3a	Microscopically
pT3b	Macroscopically (extravesical mass)
T4	Tumor invades any of the following: prostatic stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall
T4a	Tumor invades prostatic stroma, uterus, vagina
T4b	Tumor invades pelvic wall, abdominal wall
Regional lymph nodes (N)	
Regional lymph nodes include both primary and secondary drainage regions. All other nodes above the aortic bifurcation are considered distant lymph nodes.	
NX	Lymph nodes cannot be assessed
N0	No lymph node metastasis

N1	Single regional lymph node metastasis in the true pelvis (hypogastric, obturator, external iliac, or presacral lymph node)
N2	Multiple regional lymph node metastasis in the true pelvis (hypogastric, obturator, external iliac, or presacral lymph node metastasis)
N3	Lymph node metastasis to the common iliac lymph nodes
Distant metastasis (M)	
MO	No distant metastasis
M1	Distant metastasis

UICC led by Mostofi for the first time attempted to formulate a morphologic classification acceptable to an international group. Though a number of variants are noted, Urothelial tumours account for 90% of the bladder cancers and 5% are Squamous cell carcinomas in non-endemic regions with 2% being adenocarcinomas and the rest of the variants fit into the remaining 3% (McDougal *et al*., 2011).

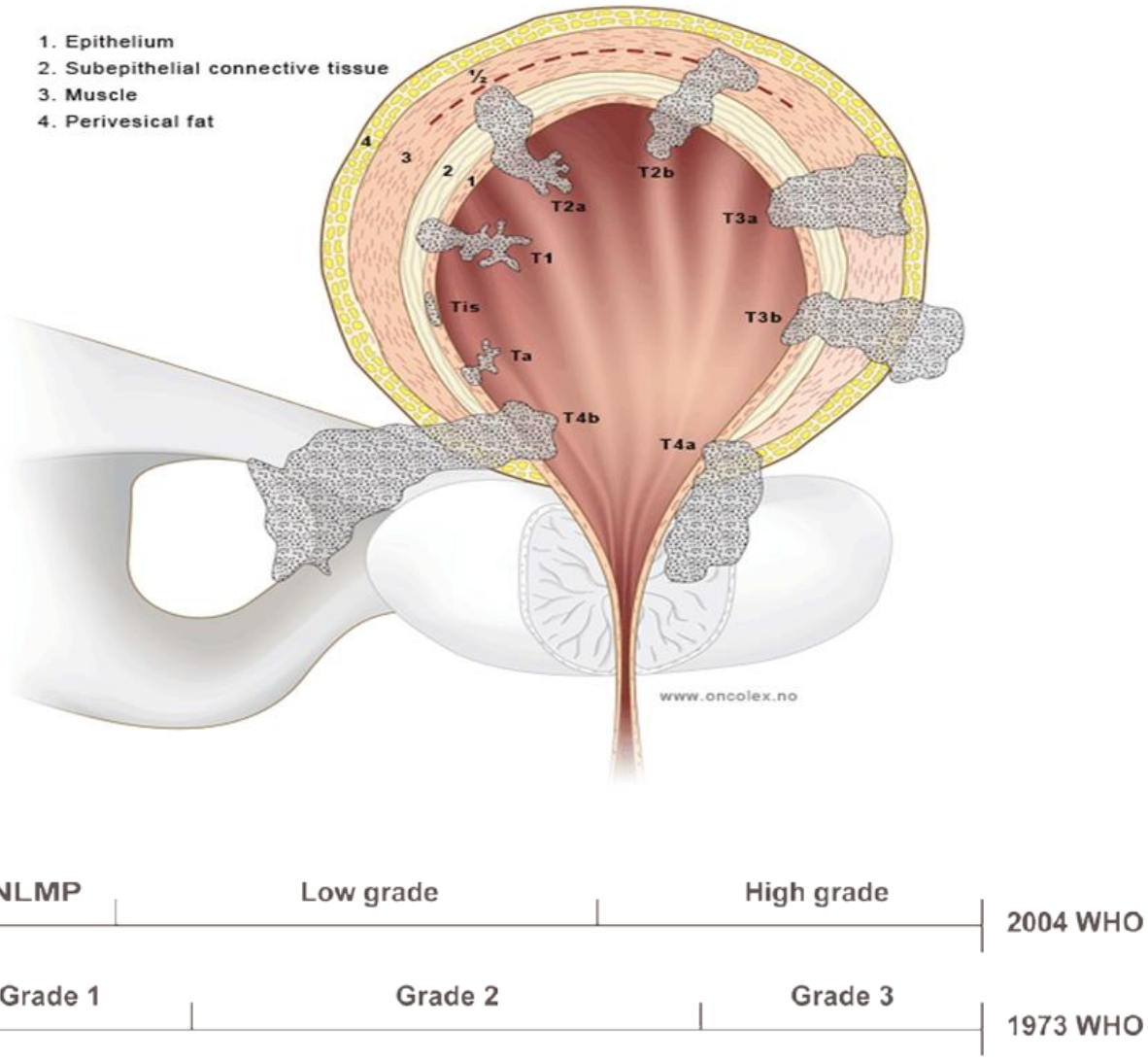


Figure 3.10: Histopathological staging of bladder cancer of 1973, 2004 and 2009 (Source: Cancer Registry of Norway; http://oncolex.org)

3.9 WHO / ISUP classification of tumors of urinary tract (Amin *et al*., 2004)

Urothelial tumors

Infiltrating urothelial carcinoma

- with squamous differentiation
- with glandular differentiation
- with trophoblastic differentiation
- Nested
- Microcystic
- Micropapillary
- Lympho epithelioma-like
- Lymphoma-like
- Plasmacytoid
- Sarcomatoid
- Giant cell
- Undifferentiated

Non-invasive urothelial neoplasias

- Urothelial carcinoma *in situ*
- Non-invasive papillary urothelial carcinoma, high grade
- Non-invasive papillary urothelial carcinoma, low grade
- Non-invasive papillary urothelial neoplasm of low malignant potential

- Urothelial papilloma
- Inverted urothelial papilloma

Squamous neoplasms

- Squamous cell carcinoma
- Verrucous carcinoma
- Squamous cell papilloma

Glandular neoplasms

- Adenocarcinoma
- Enteric
- Mucinous
- Signet-ring cell
- Clear cell
- Villous adenoma

Neuroendocrine tumours

- Small cell carcinoma
- Carcinoid
- Paraganglioma

Melanocytic tumours

- Malignant melanoma
- Nevus

Mesenchymaltumours

- Rhabdomyosarcoma
- Leiomyosarcoma
- Angiosarcoma
- Osteosarcoma
- Malignant fibrous histiocytoma
- Leiomyoma
- Haemangioma
- Other

Haematopoietic and lymphoid tumours

- Lymphoma
- Plasmacytoma

Miscellaneous tumours

- Carcinoma of Skene, Cowper and Littre glands
- Metastatic tumours and tumours extending from other organs

Diagnosis of most of these lesions is based on morphology and there is little role for ancillary techniques for either diagnostic or prognostic decisions.

Most cancers have a molecular classification or immune phenotyping or cytogenetic classification to further subclassify tumours with worse prognosis, better chemotherapy response or aggressive behavior within the same morphologic grade. Best practice recommendations by ISUP in 2014 do not advocate the role of Immunohistochemistry (IHC)

in either the distinction of dysplasia versus carcinoma *in situ* and in the grading of papillary urothelial carcinoma. IHC may have a limited but distinct role in staging of bladder cancer. In a subset of cases, depending on the clinical and histologic context, broad-spectrum cytokeratins (to identify early or obscured invasion) and desmin (to distinguish muscle from desmoplasia) may be helpful.

Though not in routine use, recent molecular classifications of non-muscle invasive bladder carcinoma by methylation of tumour suppressor genes identified 3 subgroups: pTa Low-Grade, pT1 Low-Grade, and pT1 High-Grade. TSG methylation also predicted recurrence in non-muscle invasive subgroups (Sacristan *et al*, 2014). Moreover molecular classification of bladder carcinoma by workers of MD Anderson suggested that it was similar to breast carcinoma. (Choi *et al*, 2014). Three subtypes have been identified.

The ***basal subtype*** of muscle-invasive bladder cancer expresses CD44, KRT5, KRT6 and CDH3. It is biologically aggressive and sensitive to chemotherapy. In breast cancer, basal cancers are known as triple-negative disease (ER-/PR-/Her2neu-) and some of them are also highly sensitive to chemotherapy.

The ***luminal subtype*** expresses biomarkers shared by the luminal A and B subtypes of breast cancer (CD24, FOXA1, GATA3, ERBB2). The drugs used in these subgroups of breast cancer may be effective for some bladder cancer patients. These patients have a better prognosis than those with basal subtype.

The ***p53-like subtype*** resembles luminal A breast cancers, which are estrogen-receptor positive and these tumors resist chemotherapy.

While recent advances suggest that these molecular patterns can be used for treatment modification and prognostication, the profile predictive for recurrence has not yet been identified and at present treatment is based on the grade and stage of tumour (Hall *et al*., 2007; Prasad *et al*., 2011).

3.10 Management of bladder cancer

The urothelial (transitional cell) carcinoma is mainly managed by cystoscopy for diagnosis and surveillance. Fluorescence cystoscopy offers improvement in the detection of flat neoplastic lesions, such as carcinoma *in situ*. Non-muscle-invasive bladder cancer is typically managed with transurethral resection of bladder tumor (TURBT) and perioperative intravesical chemotherapy. Intravesical BCG therapy is preferred over mitomycin for those at high risk of disease progression. For muscle-invasive disease, standard management is radical cystectomy (Table 3.4). In these patients, neoadjuvant chemotherapy or postoperative adjuvant chemotherapy should be considered based on pathologic risks, such as positive lymph nodes or pathologic T stage (Kresowik *et al*., 2009). Multidrug systemic chemotherapy involving cisplatin is commonly used.

Table 3.4: Treatment of Urothelial Bladder Carcinoma (Hall *et al*., 2007 and Sharma *et al*., 2009)

TUMOR	***TREATMENT***
Low-grade Ta	Transurethral resection of bladder tumor (TURBT) without intravesical chemotherapy
	A single dose of intravesical chemotherapy (not immunotherapy) within 24 hours of resection to prevent recurrence
High-grade Ta	Repeat TURBT (if lymphovascular invasion, incomplete resection, or no muscle in the specimen), consider intravesical BCG or mitomycin
Carcinoma *in situ*/tumor *in situ*	TURBT followed by intravesical BCG once in a week for six weeks
Low-grade T1	Repeat TURBT followed by intravesical BCG or mitomycin
High-grade T1	Repeat TURBT, followed by intravesical BCG or mitomycin, or cystectomy
T2a or T2b (organ confined)	Radical cystectomy followed by chemotherapy in high-risk patients (e.g., those with nodal involvement, high-grade histology, transmural or vascular invasion, pathologic T3 lesion)
T3a or T3b	Radical cystectomy followed by adjuvant chemotherapy, consider neoadjuvant chemotherapy
	Two trials have shown survival benefit with neoadjuvant chemotherapy (three cycles of methotrexate, vinblastine, doxorubicin [Adriamycin], and cisplatin [Platinol]) in T2 or T3 disease
T4a, T4b, or metastatic disease	Chemotherapy alone or in combination with radiation therapy, except in high-risk patients (e.g., those with poor performance status, visceral [lung or liver] disease, bone disease, poor cardiac status)

3.11 Diagnosis of bladder lesions

A cystoscope consists of a light source and a tube containing either lenses or optical fibers to transport an image recorded at the tip of the instrument to a monitor. A rigid or flexible scope can be used depending on the purpose of the investigation. The cystoscope is placed into the urethra until the bladder is reached. The bladder is filled with water and investigated. Originally only white-light was used during cystoscopy, but over the years, some disadvantages became apparent and lead to the development of new techniques. It is not possible to determine the histologic stage and grade of a tumor with classic white-light cystoscopy and this necessitates a second investigation. Additionally, white-light cystoscopy has a low sensitivity for the detection of carcinoma *in situ* (CIS).

More recently, photodynamic diagnosis (PDD) was developed to improve cystoscopic detection of bladder tumors. During PDD fluorescence (blue light) is used. Prior to investigation, a photosensitizer [(hexi)-5-aminolaevulinic acid, 5-HAL or 5-ALA)] is instilled into the bladder for one hour and the cystoscopy is carried out within two hours after emptying the bladder (Zuiverloon *et al*., 2013). Multiple studies demonstrated a higher sensitivity of PDD than white-light cystoscopy for the detection of papillary lesions and CIS, but the low specificity still remains a problem (Figure 3.11).

Narrow band imaging (NBI) is a new technique that does not require administration of an exogenous contrast agent. The technique uses different wavelengths (narrow band widths) strongly absorbed by haemoglobin, thus enhancing the contrast between bladder mucosa and vascular structures. In the same line, more vascularized malignant tissue is distinguished easily from normal urothelial tissue (Figure 3.12).

Studies demonstrated a higher sensitivity for NBI compared to classic white-light cystoscopy, but NBI is still in the developing phase and more research is needed. As seen with PDD, the specificity of NBI is also negatively influenced by factors, like prior use of BCG, bleeding during the procedure and the presence of inflammation. Till date, non-invasive techniques for identification of bladder lesions and confirmation of diagnosis are not available.

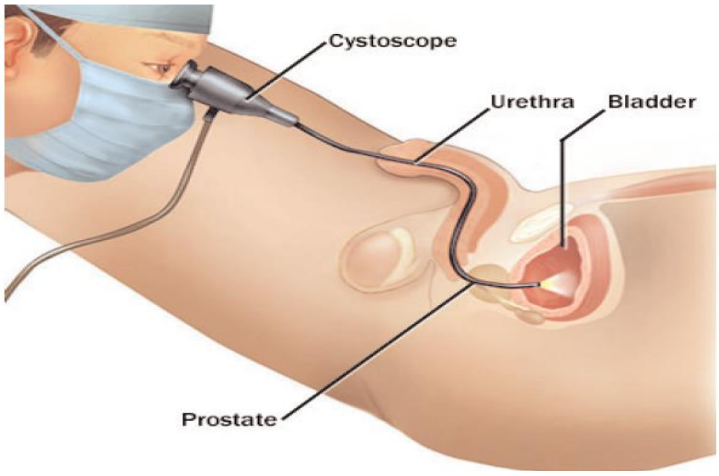


Figure 3.11: Visual view of Cystectomy of bladder cancer (Mayo foundation for medical education and research, MFMER)

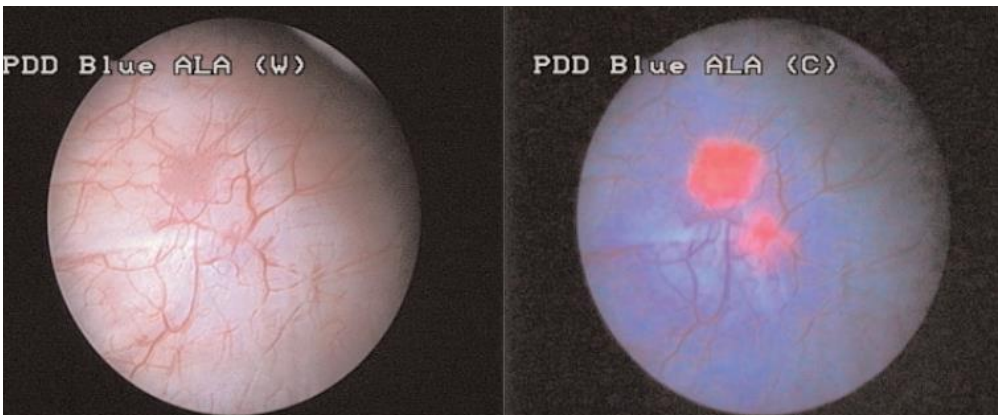


Figure 3.12: Photodynamic diagnosis of papillary lesion with white light on the left and PDD on the right. (www.robourology.co.uk)

3.12 Recurrent bladder cancer

The outlook and treatment of recurrent bladder cancer depends on the location and extent of the recurrent cancer and the type of prior treatment. Non-invasive bladder cancers often recur locally in the bladder. The recurrence may be either in the same site as the original cancer or at other sites in the bladder (Kamat *et al*., 2015).The diagnosis of recurrence is again based on the cystoscopic examination and biopsy. These tumors are often treated with a repeat TURBT. In case of frequent recurrences, the patient is managed with a cystectomy.

3.13 Urine cytology

Urine cytology coupled with cystoscopic examination remains the standard in the initial evaluation of urinary bladder cancer. Microscopic examination of exfoliated urothelial cells from urothelium into voided urine (Figure 3.13) is most widely used non-invasive diagnostic method but it has low sensitivity. The specificity of urine cytology is greater than 90%, while the sensitivity for carcinoma-*in-situ* (CIS) and high-grade disease can be as high as 80 to 90%. As indicated before, however, the main limitation of voided cytology is the low sensitivity (approximately 20-50%) for detecting low grade non-invasive bladder cancer and PUNLMP (papillary urothelial neoplasm with low malignant potential). Urinalysis is performed before urine cytology to detect hematuria (blood in urine) and urine culture is performed to rule out infection (Koss *et al*., 2006 and Larsen *et al*., 1990).

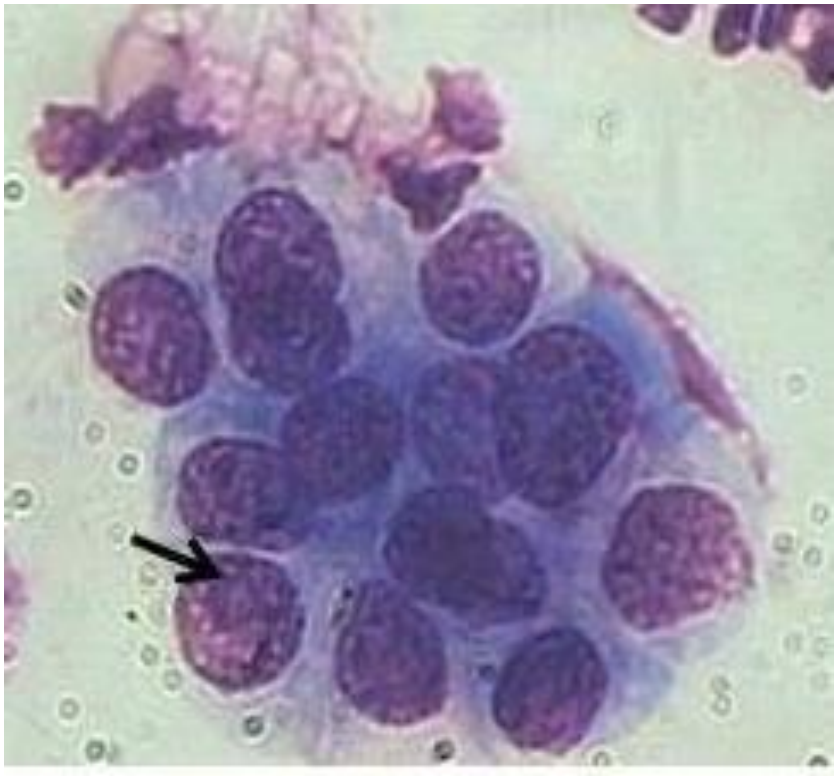


Figure 3.13: Urine cytology showed atypical malignant cells in urine of urothelial bladder cancer.

3.14 Proteomics biomarkers

The term "proteOME" was given by the groups of Wilkins and Wasinger as the complement of the genOME" describing the analysis of all the proteins in living systems (Gooley *et al*., 1996). Proteomics highlights molecular changes in disease-related proteins which can be applied for early diagnosis, molecular targeting or monitoring of therapeutic success (Etzioni *et al*., 2003). The genomic landscape gives the information and understanding of genomic alterations and provided a large number of target molecules but their validation at protein level is still needed. While, proteomics can discover, identify and validate the protein markers

directly. The rapid progress in proteomics would improve the usefulness and accuracy of diagnostic, prognostic and predictive markers. Hence, proteomics provides a step ahead in the field of biomarker development (Apweiler *et al*., 2009).

Griffths *et al* summarizes the recommended tools for bladder cancer diagnosis as cystoscopy (invasive) and urine cytology (non-invasive) till 2013. However, Cheung *et al* has developed fluorescence cystoscopy as well as narrow-band imaging (NBI) cystoscopy for small papillary tumors or carcinoma *in situ* (CIS) for those cases that could be missed by standard white-light cystoscopy (2013). Consequently, bladder cancer has the highest cost from diagnosis to death among all cancers (Smith and Guzzo, 2013). Hence, there is a necessity for an appropriate cancer marker for screening with high risk people is also needed early diagnosis as well as lifelong surveillance of patients with history of bladder cancer.

Potential applications of protein biomarkers in patients with bladder cancer are:

1. **Monitoring and Diagnosis**
    - Early and noninvasive detection
    - Increased sensitivity for low grade and CIS
    - Surveillance of recurrent tumour
    - Follow-up monitoring
2. **Prognosis**
    - Progression of NMIBC (including CIS) to MIBC
    - Disease-specific survival and overall survival
3. **Recurrence**
    - Prediction of treatment response

- Response to intravesical chemotherapy for low to intermediate-risk NMIBC
- Response to intravesical BCG treatment for intermediate to high-risk NMIBC or CIS
- Selection of highest-risk patients for earlier or more aggressive interventions
- Responses of patients with MIBC to (neo)adjuvant chemotherapy
- Responses of patients with metastatic bladder cancer to chemotherapy

4. **Therapeutic interventions**
    - Identification of new biological targets for drug development

3.15 Proteomic platforms

The development of biomarkers for routine clinical applications has 3 different phases that can be described generically as discovery, verification and validation. Preferably the discovery or preclinical phase is performed in an unbiased fashion with a sufficient number of well matched samples to power the analysis. Proteomics provides a technical platform which addresses all these phases and helps in development of biomarkers. Common techniques for proteome analysis are gel based two dimensional gel electrophoresis followed by mass spectrometry (2DE-MS or DIGE-MS) and 1-dimensional electrophoresis–liquid chromatography with tandem mass spectrometry detection. The gel-free approaches include liquid chromatography coupled to mass spectrometry (LC-MS), surface enhanced laser desorption/ionization coupled to mass spectrometry (SELDI-TOF), and capillary electrophoresis coupled to mass spectrometry (CE-MS) combined with isobaric tags for relative and absolute quantitation (iTRAQ), label-free or shotgun proteomics and MRM, and protein microarrays as well as a bioinformatics-based approach (Figure 3.14). Most

commonly used validation methods are western blot, immunohistochemistry, immunofluorence and ELISA.

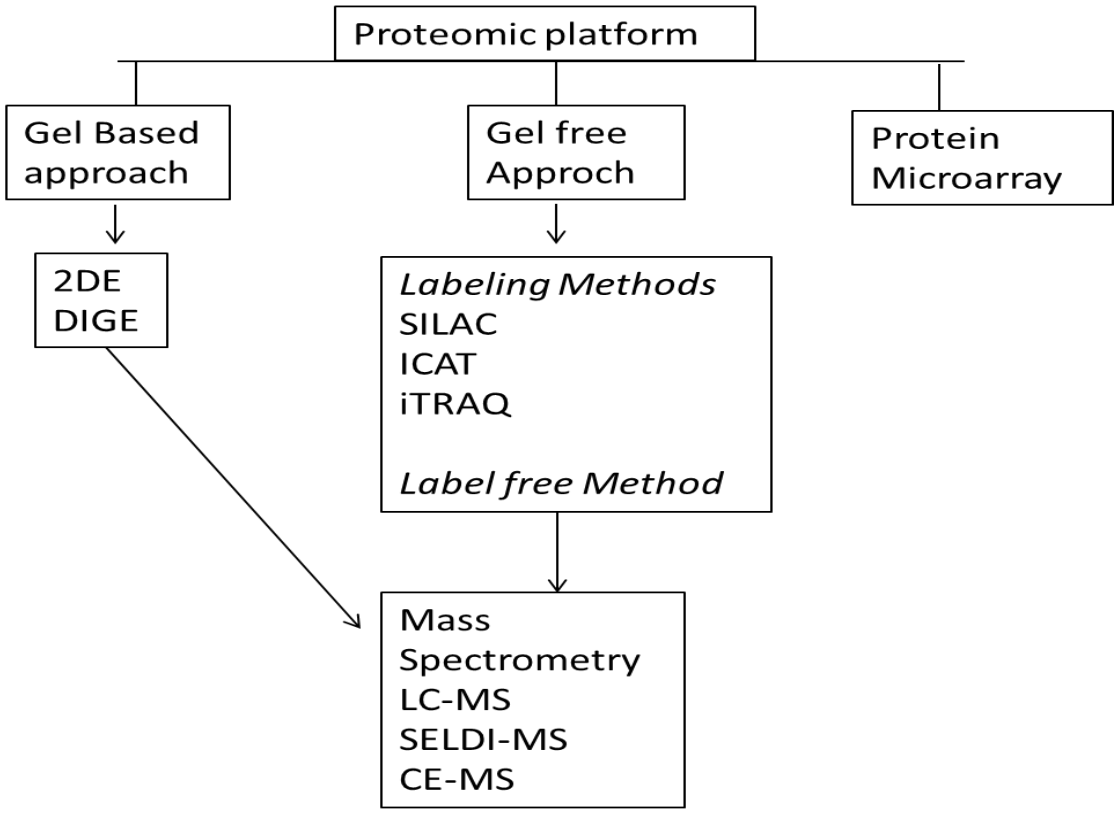


Figure 3.14: The proteomic platform

3.15.1 Two-dimensional gel electrophoresis coupled to mass spectrometry (2DE-MS)

2DE was first reported for protein separation by O′Farrell and J. Klose (1975) and is still widely used today. The basic principle of 2-DE consists mainly of two steps of separation; first dimension and second dimension. In the first dimension, protein molecules are separated depending on their isoelectric point (pI). In the second dimension, protein resolution is performed based on molecular weight using SDS-PAGE. Every protein has different

isoelectric point and molecular weight and using these properties the proteins are separated by 2DE more efficiently rather than 1D-SDS PAGE. A prominent advantage of 2- DE is that the resolution acquired during the first dimensional separation is not missed in the second electrophoresis when IEF (isoelectric focusing) gel strip is connected to the SDS- PAGE gel. Once the separation is finished, the proteins are stained and identified by MS and further analyzed by computer-assisted programs. 2-DE is a powerful and widely used method for analysis of complex protein mixtures with exceptional ability to separate the thousands of proteins at once. It provides direct visual confirmation of changes in protein/post-translational modifications (PTMs) abundance, detecting post- and co-translational modifications, which cannot be predicted from the genomic sequence. Other applications of 2-DE include whole proteome analysis, cell differentiation, detection of biomarkers and disease markers, drug discovery, cancer research, microscale protein purification, and characterization (Dudhe *et al*., 2014 and Klose *et al*.,1995).

Advantages of 2-DE is robustness (higher number of spots with very less coefficient of variation (CVs), visualized mapping analysis [unique features to resolve intact full-length proteins (up to 5000 protein) in a single gel and also give the information about physico-chemical properties such as MW (molecular weight) and pI (Isoelectric point) with possible quantification based on the spot intensity] and provides a compatible platform for further analysis (Dudhe *et al*., 2014).

Limitations of 2-DE is low reproducibility, difficulty in separating hydrophobic and extremely acidic or basic proteins, narrow dynamic range of 2-DE (highly abundant peptides

mask low abundant ones, which may be reflective of low abundant proteins) and Low throughput and labor- intensiveness (Kannan *et al*., 2012).

The development of image technology has introduced differential imaging gel electrophoresis (DIGE) technique. This method was designed in an attempt to increase sensitivity and reproducibility of 2-DE using multiplexed fluorescent dyes- labeled protein samples. 2D-DIGE is based mainly on running more than one sample (maximum 3 samples) on a single gel at once to address the issue of gel-to gel variability. In this technique, different fluorescent cyanine (Cy) dyes are used for labeling proteins from different samples (Cellulaire *et al*., 2002). After mixing these samples in equal ratio and running them together as one sample, same protein from different samples migrates to the same position on the 2D gel where it could be easily explored and differentiated by the different fluorophore-labeled dye and imaged to calculate its abundance (Magdeldin *et al*., 2014). 2D-DIGE is an important tool, especially for clinical laboratories involved in the determination of protein expression levels and disease biomarker discovery. When absolute biological variation between samples is the main objective, as in biomarker discovery, 2D-DIGE is one of the methods of choice (Zhang *et al*., 2009).

3.15.2 Labelling and label-free methods for quantitative proteomics

The mass spectrometry based quantitative proteomics is powerful to discover biomarkers that can provide diagnostic, prognostic and therapeutic targets and it also have an application in translational medical research. Currently, except 2-D gel-based methods for MS-based quantification strategy, several advanced techniques are available for quantitative assays having high throughout values. The methods are stable isotope labeling with amino acids in cell culture (SILAC), isotope-coded affinity tag (ICAT), the isobaric tags for relative and

absolute quantification (iTRAQ) and label-free quantitation. At present, several stable isotope labeling quantitative techniques, including SILAC, ICAT and iTRAQ etc, have been widely applied in identification of differential expression of proteins, post-translational modifications and protein-protein interactions in order to look for novel candidate diseases biomarkers from different physiological states of cells, body fluids or tissue samples (Wu *et al*., 2006).

3.15.3 Stable isotope labeling with amino acids in cell culture (SILAC)

SILAC is a quantitative technique which detects differences in protein abundance between samples in cell culture using non-radioactive stable isotopic labeling and allowing superior quantitative analysis of the cellular proteome compared to other labeling methods.

Two populations of cells are grown in growth medium, among them one is fed with media containing normal amino acids and other is fed with growth medium containing amino acids labeled with stable (non-radioactive) heavy isotopes. For example, the medium can contain arginine labeled with six carbon-13 atoms (^{13}C) instead of the normal carbon-12 (^{12}C). When the cells are growing in this medium, they incorporate the heavy arginine into all of their proteins. The approach is that the proteins from both cell populations can be combined and analyzed together by mass spectrometry. The ratio of peak intensities can be differentiated in a mass spectrometer for such peptide pairs reflects the abundance ratio for the two proteins (Gevaert *et al*., 2008).

The great advantages of SILAC are quantitative accuracy and reproducibility over chemical labeling or label-free quantification strategies and widely applied to characterize the proteomic changes between biological samples. A limitation with SILAC is that it cannot be used to compare more than three samples at one time. Chemical labeling approaches (such as

iTRAQ and TMT) currently offer higher multiplexing capability than SILAC, but can suffer from problems of quantitative accuracy (Chen *et al*., 2015).

3.15.4 Isotope-coded affinity tag (ICAT)

ICAT is an isotopic labeling method that uses chemical labeling reagents followed by tandem mass spectrometry allows sequence identification and accurate quantification of proteins in complex mixtures, and has been applied to the analysis of global protein expression changes. These chemical probes consist of three elements: a reactive group for labeling an amino acid side chain (e.g., iodoacetamide to modify cysteine residues), an isotopically coded linker, and a tag (e.g., biotin) for the affinity isolation of labeled proteins/peptides. For the quantitative comparison of two proteomes, one sample is labeled with the isotopically light (d0) probe and the other with the isotopically heavy (d8) version (Shiio *et al*., 2006). To minimize error, both samples are then combined, digested with a protease (i.e., trypsin), and subjected to avidin affinity chromatography to isolate peptides labeled with isotope-coded tagging reagents. These peptides are then analyzed by liquid chromatography-mass spectrometry (LC-MS). The ratios of signal intensities of differentially mass-tagged peptide pairs are quantified to determine the relative levels of proteins in the two samples. The original tags were developed using deuterium, but later the same group redesigned the tags using 13C instead to circumvent issues of peak separation during liquid chromatography due to the deuterium interacting with the stationary phase of the column (Tao *et al*., 2003).

3.15.5 iTRAQ (isobaric tags for relative and absolute quantification)

iTRAQ was developed to overcome limitations of isotope tagging (Ross *et al*., 2004). This reagent were designed for the multiplexed analysis of up to 4 samples and further designed as 8-plex kit (Choe *et al*., 2007) and now available as 10-plex Isobaric labeling kit. The iTRAQ

tags react with primary amines of peptides and label them. The isobaric nature of the tags means that the same peptide from each of the samples being compared appears as a single peak in the mass spectrum. This isotopic labeling strategy minimizes the complexity of the data where "light" and "heavy" versions of each peptide are detected and compared in each mass spectrum.

The iTRAQ^{TM} tags are isobaric labels that react with primary amines of peptides including the N-terminus and ε-amino group of the lysine side-chain. Each label has a unique charged reporter group, a peptide reactive group, and a neutral balance group to maintain an overall mass of 145Da (Figure 3.15). When a peptide is fragmented by MS/MS fragmentation, the iTRAQ^{TM} reporter groups break off and produce distinct ions at m/z 114, 115, 116, 117, 118, 119, 121 and 122. The relative intensities of the reporter ions are directly proportional to the relative abundances of each peptide in the samples that are being compared. In addition to producing strong reporter ion signals for quantification, MS/MS fragmentation of iTRAQ^{TM}-tagged peptides also produces strong y- and b-ion signals for more confident identification. During the design of the iTRAQ^{TM} tags, the reporter ion masses were carefully selected in order to minimize interference from noise in the low mass region such as matrix ions, immonium and fragment ions. This is the reason that the 8-plex reagents skip from 119 to 121, since the phenylalanine immonium ion appears at m/z 120. Each isobaric tag has a unique charged reporter group, a peptide reactive group, and a neutral balance group to maintain an overall mass of 145Da. The general workflow for an iTRAQ^{TM} experiment with 8 tags is shown in Figure 3.15. Each sample is reduced, alkylated, and digested with trypsin. Each set of peptides is then labeled with a different one of the 8 iTRAQ^{TM} tags, pooled,

separated by liquid chromatography (LC), and the resulting fractions are analysed using mass spectrometry (MS).

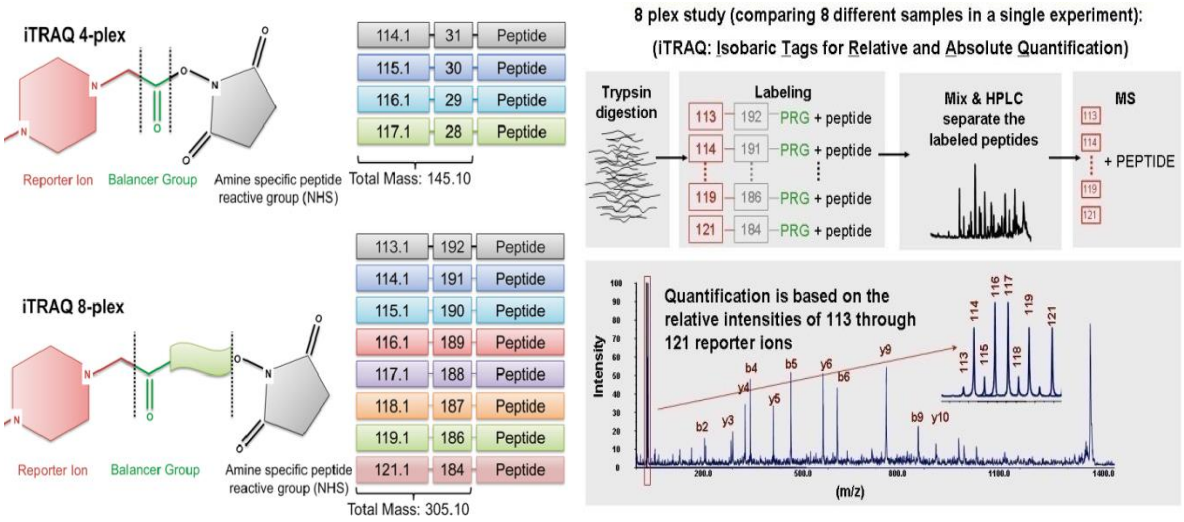


Figure 3.15: Structure of the iTRAQ™ reagents and iTRAQ experiment work flow (Source SCIEX™)

Fractionation of labelled peptides will be needed in order to detect relatively-low abundance components before MS. Strong cation exchange chromatography is able to fraction in different fractions from the pooled peptides. Multidimensional protein identification technology (MudPIT) is a common technique for whole proteomic analysis such as iTRAQ™ comparisons, and can be performed off-line or coupled directly to the mass spectrometer (Washburn *et al*., 2001). There are many options of chromatography techniques, including affinity chromatography, ion exchange chromatography, size-exclusion chromatography and reversed-phase chromatography.

Matrix-assisted laser desorption ionisation (MALDI) MS/MS and electrospray ionization (ESI) MS/MS are the most common types of Mass spectrometer used for iTRAQ™ analysis,

and there have been several comparisons of the two types of instrument for accuracy and performance of iTRAQ^{TM} quantification. Under standard MS/MS fragmentation [collision-induced dissociation (CID)], an ion trap is unable to analyze small product ions because of their low mass cut-off limitation. This meant that traditionally, iTRAQ^{TM}-based quantification was not possible using an ion trap or hybrid instrument containing an ion trap such as the LTQ-Orbitrap. Recently developed fragmentation methods now make it possible to perform iTRAQ^{TM} based quantification on an LTQ-Orbitrap and include Pulsed Q Dissociation (PQD) (Bantscheff *et al*., 2007) and higher energy C-trap dissociation (HCD) (Zhang *et al*., 2009). Both fragmentation methods are less suited for protein identification at a proteomic scale than CID fragmentation, but when combined with CID, HCD allows sensitive and accurate iTRAQ^{TM} quantification of whole proteomes (Köcher *et al.*, 2009).

There are several different software packages for performing database searches with iTRAQ^{TM} data and many utilize MASCOT as the search engine. Software that supports iTRAQ^{TM} quantification will have several particular features: the ability to exclude the iTRAQ^{TM} reporter ion masses from the search, identify spectra with fixed iTRAQ^{TM} modifications [N-term (iTRAQ^{TM}), lysine (iTRAQ^{TM}) and methyl methane thiosulfonate (MMTS) modification of cysteine residues] and to apply correction factors to the peak areas of the iTRAQ^{TM} reporter peaks in peptide spectra identified. Although it is possible to manually calculate relative quantification, many software packages will also be able to perform this function automatically. The ratio is calculated by selecting one tag as the reference mass and applying the following calculation: ratio = $^{\text{fragment corrected area}}$ / $_{\text{reference corrected area}}$. A normalization factor is usually also applied, and can be useful to normalize any deviances in iTRAQ^{TM} ratios due to unequal total protein in each sample set and impurities in

the iTRAQ™ tags themselves (normalized iTRAQ™ Ratio = $^{\text{Ratio}}$ / $_{\text{median iTRAQ}^{TM}\text{ Ratio of all found pairs}}$).

iTRAQ™LC-MS/MS data can be simplified by first applying a cut-off to remove proteins that are detected with less than a certain number of peptides, and also with less than a certain total ion score confidence interval. A good starting point is to apply a very stringent filter and reduce if necessary (i.e. discard proteins detected with less than 95% total ion score confidence interval or unused score ~ 1.3 and less than 2 peptides). The data may then be filtered on the basis of fold change and grouped into up- or down-regulation.

Quantitative proteomic experiments such as iTRAQ™ are performed to understand disease status in an unbiased fashion which provides clues for further study, rather than to provide definitive answers. It is important to validate the Mass spectrometry data that are produced in the iTRAQ™ experiments.

3.15.6 Label-free proteomics

This method can be used for both relative and absolute quantification, which is a rapid and low-cost alternative to other quantitative proteomic approaches. Label-free quantification is a method in mass spectrometry that aims to determine the relative amount of proteins in two or more biological samples. Unlike other methods for protein quantification, label-free quantification does not use a stable isotope containing compound to chemically bind to and thus label the protein. Label-free quantification may be based on precursor signal intensity or on spectral counting. The first method is useful when applied to high precision mass spectra, such as those obtained using the new generation of time-of-flight (ToF), fourier transform ion cyclotron resonance (FTICR), or Orbitrap mass analyzers. The high-resolution power facilitates the extraction of peptide signals on the MS1 level and thus uncouples the

quantification from the identification process (Zhu *et al*., 2009). In contrast, spectral counting simply counts the number of spectra identified for a given peptide in different biological samples and then integrates the results for all measured peptides of the protein(s) that are quantified. Typically, peptide signals are detected at the MS1 level and distinguished from chemical noise through their characteristic isotopic pattern. These patterns are then tracked across the retention time dimension and used to reconstruct a chromatographic elution profile of the mono-isotopic peptide mass. The total ion current of the peptide signal is then integrated and used as a quantitative measurement of the original peptide concentration. In contrast to differential labeling, every biological specimen needs to be measured separately in a label-free experiment. The extracted peptide signals are then mapped across few or multiple LC-MS measurements using their coordinates on the mass-to-charge and retention-time dimensions (Bantscheff *et al*., 2007).

"Quanti" is recently developed software which is capable of relatively accurate label-free quantification of proteins with correction of responses to instrumental fluctuation (Tang *et al*., 2006). This software has been applied in several studies on urine proteomics. MaxLFQ is also newly developed label-free software that can handle very large experiments, uses delayed normalization which makes it compatible with different separation procedures, and extracts the maximum ratio information from peptide signals (Cox *et al*., 2014).

3.15.7 Surface-enhanced laser desorption/ionization coupled to mass spectrometry (SELDI-MS)

Surface-enhanced laser desorption/ionization time of flight (SELDI-TOF) technology reduces the complexity of a biological sample by selective interactions of polypeptides with different properties on the surface (hydrophilic or hydrophobic materials, cationic or anionic matrices,

lectin, or antibody affinity reagents) coupled with a TOF mass spectrometer. After the interaction phase, only proteins or peptides of interest can bind to the surface of the SELDI chip, depending on the concentration, pH, salt content, presence of interfering compounds like lipids, etc., while the unbound samples are washed away (Issaq *et al*., 2003).

A matrix is added to the sample surface to absorb energy and to allow vaporization and ionization by laser for further MS detection. SELDI-MS can detect different protein expression patterns of body fluid and tissue specimens between patients and healthy subjects and widely used method in clinical proteomics and biomarkers discovery (Huang *et al*., 2009). SELDI-TOF is an easy to use technique and a low sample volume (<10 µL) without prior concentration or precipitation of proteins is required. However, there are several limitations including low reproducibility and comparability of datasets due to different chip surfaces and conditions, restriction to selected proteins, as well as low resolution of the mass spectrometer. Recently, material-enhanced laser desorption/ionization (MELDI) with broad active binding surfaces and more appropriate mass spectrometers, such as MALDI-TOF/TOF instruments has been introduced to solve the low reproducibility of binding to SELDI surfaces (Najam-ul-Haq *et al*., 2007) and to improve the low resolution of the mass spectrometer (Orvisky *et al*., 2006) respectively.

3.15.8 Liquid Chromatography-Mass spectrometery (LC-MS)

LC provides a powerful fractionation method that employs one or more inherent characteristics of a protein, its mass, isoelectric point, hydrophobicity, or biospecificity. This method separates large amounts of analytes on HPLC (High Performance Liquid Chromatography) column or small amount of analytes (peptides) on a capillary LC column

with high sensitivity and can be automated. LC/MS can identify low-abundance and hydrophobic proteins not seen by 2DE and thus is considered a complementary method for 2DE in proteomics (Nägele *et al*., 2004).

The column contains the sorbent materials with various physical, chemical, and immunological properties. When the sample dissolves in a solvent and subsequently moves through the column, the peptides in the sample can be separated by elution at different time points depending on their separation characteristics. A sequential separation using different matrices in two independent steps provides a multidimensional fractionation that can generate large amount of information. Strong cation exchange column (SCX) is a good choice for separate urinary peptides before injection to reverse phase columns coupled to MS.

Recently, weak anion exchange columns are used for fractionation and enrichment of low abundant proteins excreted in any biological fluid (urine). Affinity chromatography columns before LC-MS runs are the other alternative methods for capturing subproteomes from the biological fluids such as glycoproteome and phosphoproteome. LC technique can be coupled with diverse types of mass spectrometry instruments that affect the accuracy and confidence of identification and quantification. The high resolution instruments such as the Fourier transform ion cyclotron resonance (FTICR) and orbitrap or hybrid and tribrid instruments such as Q-exactive (hybrid of quadrupole and orbitrap) and orbitrap Fusion (tribrid of quadrupole, orbitrap, and linear ion trap) may couple to LC for clinical sample analysis. Proteins separated by LC could be quantified with the labeling (e.g., stable isotope affinity tag and isobaric tags) and label-free techniques. Quantification of proteins or peptides in large scale is possible only by gel-free MS based methods, which is considered an advantage for these techniques. As LC-MS is time-consuming and sensitive towards interfering compounds

(e.g., salts) and precipitation of analytes on LC-column materials, it is not yet suitable for routine clinical diagnostic tests.

3.15.9 Capillary electrophoresis coupled to mass spectrometry (CE-MS)

CE-MS is a widely-used MS-based approach for the proteomic analysis of body fluids such as urine that provides high resolution protein separation based on differential migration through a buffer-filled capillary column in an electrical field (300 to 500 V/cm). CE can be coupled either with MALDI (off-line) or ESI (on-line). CE is the most applicable approach for identification of proteins and peptides as disease biomarkers.

CE-MS offers several advantages such as 1) fast and robust separation using inexpensive capillaries, 2) compatible with most buffers and analytes, 3) provides a stable constant flow, which may avoid interfering subsequent MS detection by buffer gradients, 4) CE interfaced with almost any mass spectrometer, and 5) ability to recondition fast with NaOH after each run. A limitation of CE-MS is especially applicable for analysis of the low molecular weight (<20 kDa) molecules. Its disadvantages are limited capacity to separate high molecular weight proteins (>20 kDa) and low-abundance proteins, lack of reproducibility and robustness. Another limitation of CE-MS is that only small sample volume can be loaded onto the capillary, leading to a lower selectivity compared with LC. Improved methods of ionization by micro- and nano-ion sprays, as well as improvements of the detection limits of mass spectrometers, can resolve the problem to a large extent. Advantages and disadvantages of CE-MS in regard to biomarker discovery and clinical applications have been described in a recent review (Mischak *et al*., 2009).

3.15.10 Protein microarrays

As a non-MS-based approach, protein microarrays can be used to discover proteomic biomarkers in biofluid samples, including serum, plasma, and urine. Protein microarrays (or protein chips) are designed like a solid-phase ligand-binding assay systems using immobilized antibodies or antigens on a support surface, generally a slide or membrane. A single sample is hybridized to the array. The captured antigens or antibodies are subsequently detected. Wide advantages of protein microarrays include high-throughput; sensitivity and discovery of low molecular weight markers make them an ideal approach for clinical proteomics. However, microarrays have limitations such as requirement for a highly specific probe for each analyte, low density coverage that allows detection of only a few proteins, variable specificity, and lack of detection of posttranslational modifications.

3.16 Proteome Database

Biological databases are essential for research and medical studies. They organize and integrate biologically related information in a required format and provide a variety of relevant data with easy access for researchers (Zhang *et al*., 2011). The public database repository for proteomics data is PRIDE (PRoteomicsIDEntifications) that includes protein and peptide identifications, post-translational modifications and supporting spectral evidence (Vizcaíno *et al*., 2016). Other proteome data repository such as PeptideAtlas, global proteome machine database, Tranche, NIST libraries and Spectra ST libraries are available for future proteomics publications (Riffle *et al*., 2009). Human proteome organisation (HUPO) is providing a data repository specific for disease, urinary protein and exosomal proteins (Mayer *et al*., 2013). Other than that few database are also made such as urinary database (Urine

proteomics and urine database) and exosome database (Urinary Exosome Protein Database, Exocarta database and Vesiclepedia).

3.17 Proteomics in bodily fluids

Several promising potential biomarkers have been discovered in bodily fluids including blood and urine, using high-throughput proteomics approaches. These have the advantage of enabling noninvasive clinical measurements in patients. Proteomic biomarkers of bladder cancer in plasma or serum are yielding several candidate biomarkers, such as protein S100-A8 (also known as calgranulin-A), protein S100-A9 (also known as calgranulin-B), α-1-acid glycoprotein 1 (AGP1), carbonic anhydrase 1, haptoglobin and leucine-rich α-2-glycoprotein (LRG) (Frantzi *et al*., 2015). Urine is a valuable source of molecules capable of being diagnostic markers especially for bladder diseases. The strength of urine in comparison to plasma and tissue samples is the noninvasive collection procedure and less complex protein content. The complications in biopsy based diagnosis (i.e., invasiveness and dependence of diagnosis on pathologist expertise and observation) make urinary biomarkers a safe reliable alternate for diagnosis. In addition, lack of limitation in amount of specimen at the time of collection and relatively stable content of peptides and proteins because of complete proteolytic process by endogenous proteases during the storage in the bladder make urine an ideal specimen for biomarker research (Shao *et al*., 2011). However, molecular biomarkers have not become practical in clinics yet, and extensive attempts have been devoted to validate these molecular markers. Proteomic techniques as well as advanced statistical analysis and bioinformatics knowledge are versatile tools in urinary biomarker discovery. It is expected

that advances in analytical tools and software programs as well as accurate study design in the near future will improve sensitivity and specificity of available biomarkers.

3.18 Currently available urine tests

In an effort to overcome the need for cystoscopy, several urine-based tests have received FDA approval: immunoassays to detect urinary proteins, such as bladder-cancer-associated antigens (BTA TRAK®, BTA stat®, Polymedco, NY, USA) and nuclear matrix protein NMP22® (Alere, MA, USA); an immunocyto fluorescence-based test (ImmunoCyt™/uCyt+™, Scimedx, NJ, USA); and a fluorescence *in situ* hybridization-based assay (UroVysion®, Abbott laboratories, IL, USA). The initial overall performance of these FDA approved tests was encouraging. Unfortunately this performance has not always been reproduced when the assays are evaluated in independent patient populations. Thus, the clinical utility of the FDA-approved biomarkers for diagnosis of bladder cancer remains questionable and a substantial need for implementation of better biomarkers into routine clinical practice remains (Table 3.5).

Table 3.5: Characteristics of Urine-Based Bladder Tumor Markers

Test	Marker Detected	Assay Type	Testing Situation	Sensitivity (%)	Specificity (%)	Limitation
BLCA-4 (Van *et al*., 2004)	BLCA-4 transcriptio n factor	ELISA	Specialized laboratory	89–96	100	Randomized trials are needed to further study their usefulness on a larger scale
BTA stat®(Polyme dco, Cortlandt Manor, NY) ((Guo *et al.*, 2014 and Pode *et al*., 1999)	Compleme nt factor H-related protein	Colorimetric immunoreacti on	Point-of-care	57–83	68–72	Showed high false-positive results, needs to cross-validate on other genitourinary diseases
BTA TRAK®(Poly medco, Cortlandt Manor, NY) ((Yafi *et al.*, 2015 and Ellis *et al*., 1997)	Compleme nt factor H	Sandwich immunoassay	Specialized laboratory	66–72	51–75	
Hyaluronic acid, hyaluronidase (Lokeshwar et al., 1997)	Hyaluronic acid, hyaluronida se	Immunoassay	Specialized laboratory	92–100	89–93	Refinement in the assay technique and evaluation in larger clinical trials

ImmunoCyt™ (DiagnoCure, Quebec City, Quebec, Canada) (Dimashkieh *et al.,* 2013 and Vriesema *et al*., 2001)	Mucins, high-molecular-weight Carcinoembryonic antigen	Immunofluorescence, cytology	Specialized laboratory	50–100	69–79	Showed false-positive results needs to cross-validate on other genitourinary diseases limitation of the test is the need of trained personnel to perform and interpret
Lewis X antigen (Pode *et al*., 1998)	Lewis X blood group antigen	Immunocytology with P12 monoclonal antibody	Specialized laboratory	80	86	Testing on more heterogeneous populations of patients, to determine the true specificity.
Microsatellite markers (Van *et al*., 2001)	Highly polymorphic DNA repeats	PCR	Specialized laboratory	72–97	80–100	There is a need for expensive equipment and trained personnel.
NMP22 (Yafi *et al.,* 2015; Del *et al*., 1999 and Serretta *et al*., 1998)	Nuclear mitotic apparatus	Sandwich immunoassay	Specialized laboratory	47–100	60–70	10% to 20% rate of false-positive results and does not gives clear specific diagnostic information
Quanticyt™ (Gentian Scientific Software, Niawer, The Netherlands) (Witjes *et al*., 1998)	Nuclear shape, DNA content	Feulgen stained specimen image analysis by dual parameter morphometry	Specialized laboratory	45–59	71–93	It requires technical expertise and expensive equipment

Survivin (Altieri *et al*., 2003)	Survivinantiapoptotic protein	BioDot system	Specialized laboratory	64–100	87–93	This assay remains experimental, requiring further validation of its sensitivity, sensibility, and accuracy
Telomerase (Yoshida *et al*., 1997)	Human telomerase messenger RNA	PCR	Specialized laboratory	62–81	80–96	The clinical applicability of the telomerase assay could be limited because of high range of specificity.
UBC™ test(ID L Biotech, Bromma, Sweden) (Sumi *et al*., 2000)	Cytokeratins 8 and 18	1-step immunoassay	Specialized laboratory	66–82	83–90	This test requires investigation in multicenter trials.
UroVysion™ (Vysis, Downers Grove, IL) (Sarosdy *et at*., 2002)	Aneuploidy chromosome 3, 7 and 17 and loss of 9p21 locus	Multitarget FISH	Specialized laboratory	36–100	89–96	High cost and is not a point-of-care, the poor positive predictive value leads to false-positive results.

Levels of many of the potential biomarker candidates have been found, by various research groups using different proteomics platforms, to undergo significant changes in patients with bladder cancer, in a consistent manner. These listed biomarkers are not FDA approved (Table 3.6).

Table 3.6: Candidate urinary proteomic biomarkers of bladder cancer

Biomarker	**Change in urinary concentration**	**References**
Apolipoprotein A-I	Increased	Lei *et al*., 2013; Li *et al*., 2014; Li *et al*., 2011; Li *et al*., 2012; Chen *et al*., 2013; Lindén *et al.,* 2012; Chen *et al*., 2012
Fibrinogen β chain	Increased	Li *et al*., 2012; Chen *et al*., 2013; Lindén *et al* 2012; Chen *et al*., 2012
α-1-antitrypsin	Increased	Chen *et al*., 2013; Lindén *et al*., 2012; Chen *et al*., 2012
Apolipoprotein A-II	Increased	Chen *et al*., 2013; Chen *et al*., 2012
Fibrinogen γ chain	Increased	Li *et al*., 2011; Lindén *et al.,* 2012; Chen *et al*., 2012
Haptoglobin	Increased	Chen *et al*., 2013; Chen *et al*., 2012
α-2-macroglobulin	Increased	Li *et al*., 2011; Lindén *et al*., 2012; Chen *et al*., 2012
Uromodulin	Decreased	Li *et al*., 2011; Chen *et al*., 2010
Vitamin D-binding protein	Increased	Li *et al*., 2011; Lindén *et al*., 2012; Chen *et al*., 2012
ADAM 28	Increased	Tyan *et al*., 2011; Yang *et al*., 2011
Afamin	Increased	Chen *et al*., 2010; Chen *et al*., 2012
Apolipoprotein A-IV	Increased	Chen *et al*., 2010; Lindén *et al*., 2012

Apolipoprotein B-100	Increased	Chen *et al*., 2012; Chen *et al*., 2013
Carbonic anhydrase 1	Increased	Chen *et al*., 2010; Lindén *et al.*, 2012
Heparin cofactor 2	Increased	Chen *et al*., 2010; Lindén *et al*., 2012
Profilin-1	Increased	Zoidakis *et al*., 2012; Lindén *et al*., 2012
Protein S100-A8	Increased	Chen *et al*., 2010; Lindén *et al*., 2012
Retinoic acid receptor responder protein 1	Increased	Chen *et al*., 2010; Lindén *et al*., 2012
Semenogelin-1	Increased	Chen *et al*., 2010; Lindén *et al*., 2012
Fibrinogen α chain	Increased	Lei *et al*., 2013; Lindén *et al*., 2012
Serotransferrin	Increased	Yang *et al*., 2011; Chen *et al.*, 2010
Serum amyloid A-4 protein	Increased	Chen *et al*., 2010; Lindén *et al*., 2012
Thrombospondins 1 and 2	Increased	Lindén *et al.*, 2012; Chen *et al*., 2013

A number of markers that take advantage of exfoliated cells in the urine for detection of cell-surface antigens, nuclear morphology, or gene expression or proteome profiles have been studied in bladder cancer. Although most of them remain investigational and are undergoing preclinical evaluation, few have undergone clinical trials and have been approved for clinical use. None of these tests, however, meet all of the criteria of an ideal tumor marker. Some of the newer tests are close, but require automation of the testing process to decrease the time and expense, or need additional testing on heterogeneous populations of patients to determine their accuracy.

It has been reported in various types of malignancy including bladder cancer that exosomes are secreted in the tumor microenviroment and biological fluids. The secretion of exosomes in urine of bladder cancer is also known (Franzen *et al*., 2014). Many studies have revealed the roles of exosome in cell-cell communication and material transfer in immune cells and tumor cells (Akers *et al*., 2013; Gabriel *et al*., 2013; Théry *et al*., 2002). Tumor utilizes the extracellular vesicles-mediated cargo mechanism to recruit normal epithelial cells to act as malignant and communicate with the environment (Roma *et al*., 2014).

Taylor et al have demonstrated that the tumor cells produce higher proportions of exosomes than normal cells and establishment of a premetastatic niche (Taylor *et al*., 2002). Exosomes have been isolated in a variety of biofluid such as blood, urine, semen, saliva, ascites, breast milk, etc (Raposo *et al*., 2013; Henderson *et al*., 2012 and Mathivanan *et al*., 2010), and the stability of their contents in numerous bodily fluids. Urine remains in contact of tumor in the bladder cancer. Urine is a potential source of exosome in bladder cancer patients and it is readily available fluid that can be collected in large quantities in a non-invasive fashion (Li *et al*., 2011 and Zhou *et al*., 2006). Urinary exosomes constitute approximately 3% of the total protein content in the urine (Zhou *et al*., 2006 and Théry *et al*., 2002). Exosomes are of particular interest in biomarker identification and potential therapeutic application.

In the present study, Quantitative proteomic approach (iTRAQ labelling, LC-MS/MS) was used for the identification of differentially deregulated protein in urothelial bladder tumors compared to normal mucosa. To understand the robust data, the gene ontology term identification and enrichment analysis was done by ingenuity pathway analysis (IPA) software. Differentially expressed proteins were analysed for the identification of exosomal urine with urinary exosome using database (Exocarta).The deregulated proteins were verified

for their universal presence in paraffin embedded tissue of bladder tumor biopsy and adjacent normal mucosa. The presence of identified deregulated tumor specific proteins was further validated in urine samples of bladder cancer compared to non-malignant urine samples.

Chapter 4

Material and Methods

Chapter 4

Materials and Methods

This study included patients of bladder cancer presenting to the Outpatient Department of Urology, Safdarjung Hospital, New Delhi over a period of three years. The study was conducted at the National Institute of Pathology, ICMR, New Delhi.

4.1 Sample Selection

Samples used for this study were obtained with informed consent and with the approval of the Safdarjung hospital Ethics Committee (EC/SJH/VMMC/Project/I4/07-325).

Selection of Patients or Inclusion criteria

Patients presenting with hematuria

CT scan, abdominal ultrasound and urine cytology suggesting malignancy

Exclusion criteria

Metastatic disease

Concurrent tumours

Associated upper tract transitional cell carcinoma

Control groups selection

Paired adjacent normal mucosa (tissue sample)

Age-sex matched non-malignant control urine

Stones- kidney or ureteric,

Glomerulo-Nephritis

Neurogenic bladder

Benign prostatic hyperplasia

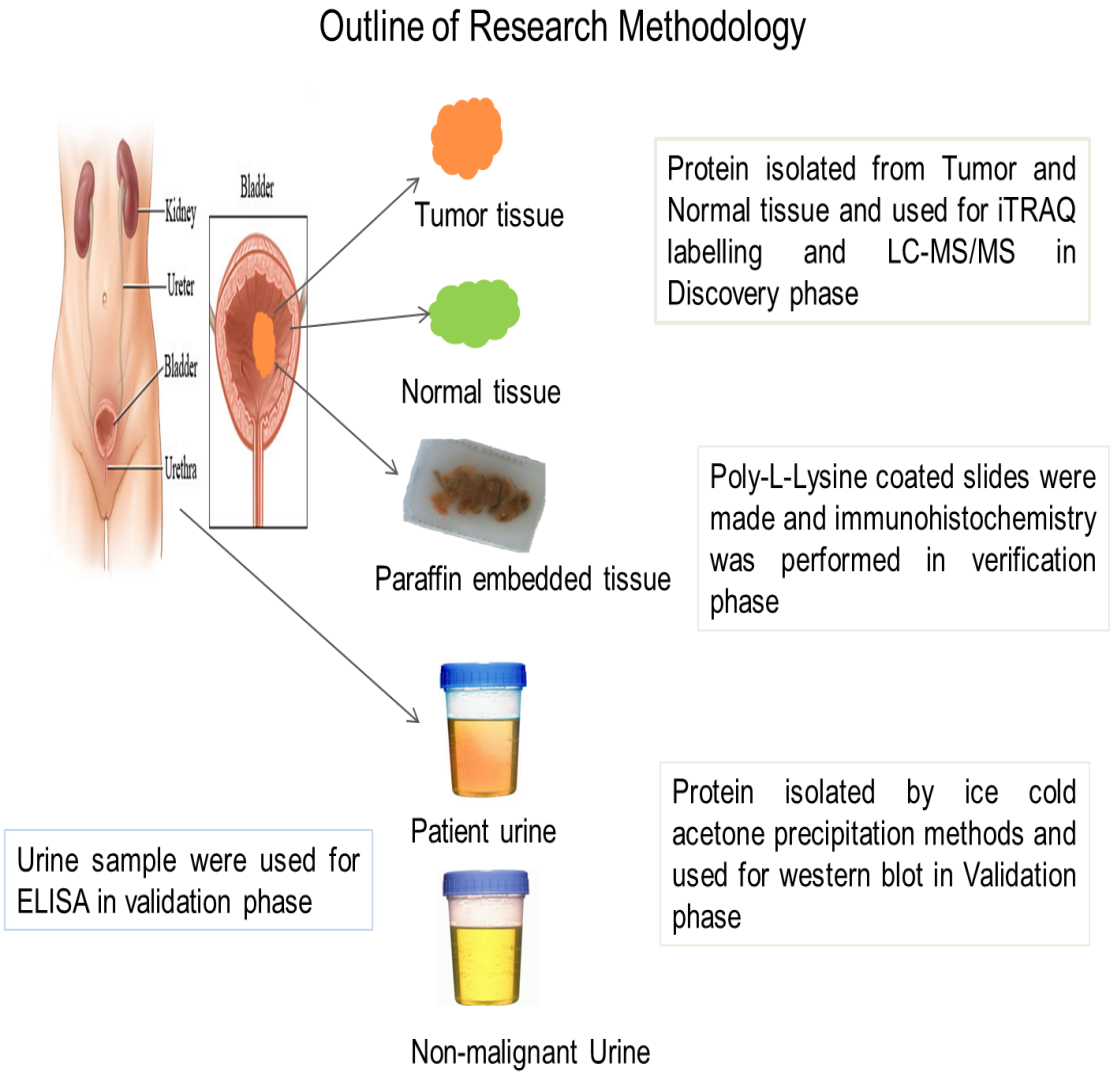


Figure 4.1: Study plan of material and methods used in discovery and validation phase.

Paired tissue samples from tumour and adjacent normal mucosa were collected during surgery (Transurethral resection of bladder tumour or Radical Cystectomy) from bladder cancer patients presenting with hematuria and radiologically diagnosed with urinary bladder cancer. The clinical and pathological data were recorded. These included clinical tumor, grade, and staging (followed 2004 ISUP bladder cancer classification); site of the lesion, histopathological differentiation, age and gender of the patients. The histologic diagnosis for

each sample was reconfirmed using microscopic examination of a hematoxylin and eosin stained paraffin section of each research tissue block.Demographic details of samples used in each phase of experiment has been summaries in Table 4.1. Independent set of samples was used in each phase such as discovery (tissue sample, n= 12 using iTRAQ LC-MS/MS), verification (FFPE tissue sample, n=119 using IHC) and validation (urine sample, n=36 using Western blot and urine sample, n=150 using ELISA).

Table 4.1: Demographic details of study cohort

Total Number (n=322)	**Discovery phase (iTRAQ) Tissue**		**Verification phase (IHC) FFPE tissue**		**Validation phase (WB) Urine**		**Validation phase (ELISA) Urine**	
	Tumor tissue, n=6 (%)	**Normal mucosa, n=6 (%)**	**Patients, n=119 (%)**	**Normal mucosa, n=5 (%)**	**Patients n=26 (%)**	**Control n=10 (%)**	**Patients n=100 (%)**	**Control n=50 (%)**
Median Age	61	61	58	54	58	53	58	55
1st to 3rd IQR	56 to 62	56 to 62	53 to 68	51 to 60	52 to 64	48 to 60	49 to 56	45 to 61
Sex								
F	0(0)	0(0)	16 (14)	0	4 (15)	1 (10)	14 (14)	12 (24)
M	6(100)	6(100)	103 (86)	5 (100)	22 (85)	9 (90)	86 (86)	38 (76)
Grade LG	2 (33)	--	57 (48)	--	9(35)	--	39 (39)	--
HG	4 (67)	--	62 (52)	--	17(65)	--	61 (61)	--
Stage PT1	3 (75)	--	94 (78)	--	17(65)	--	61(61)	--
PT2	1 (25)	--	25 (22)	--	9(65)	--	39 (39)	--
Recurrence	1 (25)	--	--	--	9 (35)	--	41 (27)	--
Urine cytology	--	--	--	--	12 (46)	--	35 (53)	--

IQR-interquartile range F-Female; M-Male; LG-low grade; HG-High grade; pT1-Non-muscle invasive; pT2-Muscle invasive

4.2 Sample processing

Tissue sample

After the excision and collection of tissue from bladder cancer patients, samples were transported from operation theater to laboratory on ice, all samples were washed with 1X PBS (phosphate buffer saline, pH 7.4) and stored at -80°C until further use. Tissue was divided into two pieces; one tissue piece was collected in 10% formalin and embedded in paraffin for histopathological analysis and the other was used for this study. All samples were properly labeled with their ID, weight, size and date on sample tags.

Protein extraction protocol

Dissected tissues (25 mg per samples) from the stored sample was thawed on ice and washed with 1X PBS before protein extraction. The weighed and washed tissue was homogenized in liquid nitrogen and crushed sample was mixed in 1x RIPA buffer with protease inhibitors cocktails (P8340, Sigma Aldrich). Protease Inhibitor Mix was added during the extraction procedure to block the possible protein degradation.The homogenates were then subjected to sonication 75Hz, 1min for 1 cycle on ice followed by centrifugation at 13,000 for 20 min, 4^{o}C. Total protein in the supernatant was collected and stored at -80°C until further use.

Crude lysates contain a number of endogenous enzymes, such as proteases and phosphatases, which are capable of degrading proteins in the extracts. Protease inhibitor was added at the time of collection to minimize or prevent protein degradation. This protease inhibitor cocktail has been optimized and tested for mammalian tissue extracts. It contains inhibitors with a broad specificity for serine, cysteine, and acid proteases, and amino peptidases. This Protease

Inhibitor cocktail is used as 0.1µl per 1µl of 1X RIPA lysis buffer (Merck Millipore) during the extraction procedure of protein to block the possible protein degradation.

4.3 Quantitation of Protein

Protein concentration was determined by a BCA (bicinchoninic acid) assay (Thermo Scientific™ Pierce™). The BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid for the colorimetric detection and quantitation of total protein. The BCA assay relies on two reactions. First, the peptide bonds in protein reduce Cu^{2+} ions from the copper (II) sulfate (cuprous ion) to Cu^{+}(cupric ion) using a unique reagent containing bicinchoninic acid. The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution. Next, two molecules of bicinchoninic acid chelate with each Cu^{+} ion, forming a purple-colored complex that strongly absorbs light at a wavelength of 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000µg/mL).

Accordingly, protein concentrations generally are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA). A series of dilutions of known concentration were prepared and concentration of each unknown protein is determined based on the standard curve.

Preparation of Standards and Working Reagent

Standard stock solution of 2mg/mL Albumin Standard was used to prepare a set of diluted standards.

Table 4.2: Preparation of Diluted Albumin (BSA) Standards

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0= Blank

Preparation of the BCA Working Reagent (WR)

The following formula was used to determine the total volume of WR required: (# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required

Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of the BCA Reagent B (50:1, Reagent A: B). For the above example, combine 50mL of Reagent A with 1mL of Reagent B. Note: When Reagent B is first added to Reagent A, turbidity is observed that quickly disappears upon mixing to yield a clear, green color. The WR is stable for several days when stored in a closed container at room temperature (RT).

4.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Table 4.3: Components of SDS-PAGE gel

Chemicals (for 2 gels)	**Resolving gel (12.5%)**	**Resolving gel (10%)**	**Stacking gel (4%)**
Milli-Q	4.7ml	3.9ml	3.0ml
1.5mM Tris(pH 8.8)	3.75ml	2.5ml	---
0.5mM Tris (pH 6.6)	--	---	1.25ml
29:1 acrylamide/Bis-acrylamide	6.25ml	3.4ml	0.6ml
10% SDS	150µl	100µl	50µl
10% APS	150µl	100µl	50µl
TEMED	15µl	10µl	10µl

Gel casting chamber (Bio-Rad) was arranged and filled with isopropanol for checking the leakage of gel, if there was no leakage isopropanol was removed.SDS resolving gel was poured in gel casting plates at left for solidification (Approximately 1 hour). After solidification of resolving gel, stacking gel was poured over it and the comb was inserted properly and left for solidification (Approximately 30 minutes). Protein samples mixed with sample loading buffer (4:1) were mixed thoroughly and incubated in water bath (Julabo) for 10 minutes at 100°C for the denaturation of proteins. After incubation, samples were cooled to room temperature. Then, casted gel was placed into gel running chamber containing running buffer and comb was removed slowly without disturbing the wells formed. Protein

samples and 5µl of protein ladder was loaded into wells. Then, gel was run at 60V initially for proper stacking of protein in stacking gel and then run at 90V for 3 hours. Run was stopped when the tracking dye reached the bottom of gel.

4.5 Staining of the polyacryamide gel

Coomassie Brilliant Blue R250 stain

The gel was washed 3 times for 5 minutes each in distilled water to remove SDS present in the gel. Added an adequate amount of Coomassie Brilliant Blue R250 stain to cover the gel. Gently shake the gel on rotor for overnight. Rinsed the stained gel in a large volume of distilled water for 3 times for 5 minutes each. Gel was destained in Coomassie Brilliant Blue, De-Staining Solution, 30% Methanol or 30% Acetic acid until desired resolution is attained. Stored the stained gel in 1% Acetic acid.

Coomassie Brilliant Blue G250 stain

12.5g of $Al_2(SO_4)_3$ was dissolved in 50ml of distilled water and mixed with 25ml of 96% Ethanol and mixed for 30 minutes on magnetic stirrer.Then added 0.05g of CBB stain and mixed well on magnetic stirrer for 1 hour. After forming homogenous solution, 5.8ml of 85% o-Phosphoric acid was added to form colloidal solution and mixed well.Finally volume of stain was madeup to 250ml with distilled water.

Gel was washed gel 3 times for 5 minutes each in distilled water to remove SDS present in this.Added an adequate amount of colloidal Coomassie Brilliant Blue G-250 stain to cover the gel. Gently shake the gel on the rotor for overnight. Rinsed the stained gel in a large volume of distilled water for 2 times for 5 minutes each. Destained gel in Coomassie Brilliant Blue

Destaining Solution until desired resolution is attained. Stored the stained gel in Distilled water.

Silver staining

Protein samples (5µg) were separated on 12% SDS-PAGE at 90V for 2.30hrs. After the separation, SDS-PAGE, gel was washed with MilliQ water 3 times. The gel was further subjeted for fixing solution for atleast 1 hr or overnight. Followed by incubation in 5% ethanol for 15 min. Washed three times with MilliQ water. Gel was further incubated in sensitizing solution for 1 min in dark.Washed 3 times with MilliQ water. Incubation in silver nitrate solution for 20 mins in dark. Washed 3 times with MilliQ water for 5 min each. Gel transferred in developing solution incubation till bands are visible. Washed with MilliQ water for 5min. Add stopping solution for atleast 20min. Washed with water and image was captured. All steps were done on the shaker except gel in developing solution.

4.6 Protein Digestion and Labeling with iTRAQ Reagents

Proteins (100 µg of each) were taken and precipitated with pre-chilled acetone (six volume of acetone) kept it overnight at -80^{o}C. Precipitated samples were centrifuged at 10,000 rpm for 30 min, 4^{o}C and pellet reconstituted in 1X PBS. Each protein sample was mixed in 20 µl of dissolution buffer (0.5 M triethylammonium bicarbonate, pH 8.5) and denaturant buffer (0.05 % w/v SDS) were further subjected to reducing agent [reduction of cysteine S-S bridges by the addition of 2µl of 50 mM tris-2-carboxyethyl phosphine (TCEP)], followed by incubation for 1 h at 60^{o}C. Cysteines were blocked by adding 1 µl of 200mM methyl-methanethiosulfonate in isopropanol and 10 min incubation at room temperature. Protein were

further digested with 10µl freshly prepared trypsin (manufacturer name) solution in milliQ water and incubated at 37^{o}C overnight. After digestion all tryptic digest was kept for drying in a SpeedVac (Labconco) and further reconstituted into dissolution buffer and maintain the pH ~8.0. Labeling with iTRAQ 8-plex (ABSCIEX, Darmstadt, Germany) was performed at RT for 2 h. The samples in each 8-plex were then pooled and evaporated in a centrifugal vacuum concentrator (not completely dry or upto final 50µl volume).

4.7 Strong Cation Exchange (SCX) peptide fractionation

Fractions were dissolved in ammonium formate (5mM) in 30% acetonitrile (ACN) with 0.1% formic acid and fractionated using ICAT^{TM} (Sciex) cartridge. The cartridge was conditioned with 500mM ammonium format in 30% ACN with 0.1% formic acid and sample were eluted from lower to higher concentration (30mM, 80mM, 120mM, 180mM, 250mM, 300mM, 400mM and 500mM) with the rate of 1drop/sec.This resulted in a total of 8 SCX fractions per sample set. These fractions were dried by speed vacuuming (Labconco).

4.8 Nano Reverse-phase-LC-MS/MS analysis

After SCX fraction, each fraction was reconstitutedin 0.1% formic acid.Samples were loaded into propylene glass and 3-5µg of the sample was injected into trapping column (for more purification or desalting) before introduced into MS via a C18 column of LC system (ABSciex). All SCX fractions were analyzed in duplicates.

4.9 Protein Identification

The Raw MS spectra data were processed with Analyst (version) to extract mass spec files (.wiff). These files were analyzed using ProteinPilot (version 5.0, ABSciex) which employs the Paragon™ search algorithm. To minimize the false positive rate, a strict cutoff for protein identification was used. Proteins were identified on the basis of having a minimum unused score of 1.3 and 2 or more than 2 peptides identified. For quantitative analysis, a protein must have minimum two unique peptide matches with iTRAQ ratios and at least one of them with an expectation <0.01. The peptide and protein were identified on the basis of having atleast two peptide with an ion score above 99% confidence and considered for relative quantitation. Only peptides unique for a given protein were, excluding those common to other isoforms or proteins of the same family. The detected protein threshold (unused score (confidence)) in the software was set to 2.0 to achieve 99% confidence, and identified proteins were grouped by the ProGroup algorithm (Applied Biosystems, Foster City, CA) to minimize redundancy. The bias correction and background correction options were executed. Protein relative expression ratios were based on the peak area ratios of the peptides from the same protein and the resulting dataset was auto bias-corrected to eliminate any variability. Fold changes in protein expression greater than +1 and less than -1 were determined as upregulated and downregulated respectively. Using of paired samples and multiplexing (8-plex iTRAQ) minimizing confounding factors or measured analytes simultaneously in a single MS run of various samples to avoid inter-run variations of the protein present in different clinical samples. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier **PXD007070**.

4.10 Preprocessing of tissue for Immunohistochemistry

Grossing

A part of Tissue sample was processed in histopathology department of National Institute of Pathology. Tissue section was collected from this department along with pathology report and images and further used for immunohistochemistry.

Tissue processing for Immunohistochemistry (IHC)

1. **Fixation**

   The tissue was fixed in buffered (10% neutral Buffered formalin) solution. In this process, the tissue structure was fixed by the formation of cross-links between proteins. Thus, it will be in a stable state to assess accurately the normality or abnormality of the tissue.

2. **Dehydration**

   The specimens were processed through graded alcohol (70%, 95% and 100%) for 20 mins and which replace the water in the samples by absolute solution of alcohol.

3. **Clearing**

   The alcohol in the samples was removed and cleared in chloroform.

4. **Tissue processing**

   Tissue was processed by using automatic tissue processing (Microm STP 120 spin tissue processor). The protocol was used is for overnight processing of tissues for 18 hours. It contains Formalin, Alcohol, Xylene, and Paraffin Wax.

5. **Embedding**

   Tissue samples were embedded in paraffin wax to make impregnated tissue blocks. It was paraffin embedding in plastic chuck blocks.

6. **Cutting**

   This prepared paraffin block was used for section cutting. It was done by using Microtome (Leica). They were cut at 3µm thickness. Then, the sections were floated on water and transferred to the PLL slides or albumin coated slides.

7. **Poly-L-Lysine solution**

   10% Poly-L-Lysine (Sigma), working solution was prepared.

   Slides were put into the Poly-L-Lysine solution for overnight.

   Then PLL coated slides were allowed to air dry and after that slides with tissue sections was used for IHC.

4.11 Immunohistochemistry (IHC)

Tissue blocks were obtained from archives at The National Institute of Pathology, ICMR, New Delhi.

Protocol:

The FFPE sections were placed in incubator set at 60°C for 15 to 20 minutes. Slides were immersed in xylene twice for 20 minutes each for deparaffinization. Slides were placed in absolute ethanol twice for 20 minutes each and there after placed in 90%, 70% and 50% respectively for 10 minutes for rehydration. Then, they were placed in distilled water for 5

minutes. After that, slides were placed in TE buffer (Tris-EDTA buffer) and kept in water bath at 95°C for 20 minutes for Antigen Retrieval.They were then allowed to cool at room temperature.Slides were washed with TBST (Tris Buffer Saline- Tween20) for 5 minutes. Then, they were placed in power blocker (Biogenex) for 30 minutes. Then slides were again washed with TBST for 5 minutes. Endoperoxide activities were blocked by incubating with 3% H_2O_2 for 10 minutes at room temperature.Then slides were again washed with TBST for 5 minutes. Slides were incubated with primary antibody (Table 4.4) for overnight at 4°C.

Table 4.4: Primary antibody dilution and incubation time used in IHC

Antibody	Dilution	Incubation Time
SERPING1 (ThermoScientific)	1:200	Overnight
HSP20/HSPB6 (SantaCruz))	1:100	
TENASCINC (ThermoScientific)	1:100	
SOD2 (SantaCruz)	1:200	
PRDX1 (ThermoScientific)	1:100	
PRDX2 (ThermoScientific)	1:200	

After overnight incubation with primary antibody, slides were washed twice with TBST for 5 minutes each.Then slides were incubated with Super Enhancer (Biogenx) for 30 minutes at room temperature for proper binding of the Secondary Antibody (Biogenx) to the Primary Antibody. Slides were again washed twice with TBST for 5 minutes each.Then slides were incubated with HRP conjugated Secondary Antibody (dilution 1:20000) for 2 hours at room

temperature. After incubation, slides were washed twice with TBST for 5 minutes each.Then 2-3 drops of DAB was added and when brown colour appeared, slides were washed with distilled water immediately.After that, counter stained with Haematoxylin for 30 seconds and then differentiated with 1% acid alcohol (1% hydrochloric acid in 70% alcohol) and then washed with distilled water. Slides were mounted with DPX and observed under light microscope.

4.12 Imaging/Digital Scanning Microscope

The slides were placed on the slide scanning microscope (Carl-Zeiss). The setup was set to scan a tissue section at 10X magnification. In the meanwhile, V-Slide software was started. The area to be scanned was selected manually and then it was searched. Subsequently, the scanned slide images were viewed using Metaclient software.

4.13 Isolation of urinary protein

The Sample was initially centrifuged at 4000xg for 20 minutes and supernatant was transferred into new tube and added sodium azide and protease inhibitor (1mg/ml) and then stored at -80°C. Stored sample was thawed in ice (4°C) and then centrifuged at 4000xg for 5 minutes at 4°C. 10ml of supernatant was taken into 50ml tube and 10ml of ice cold acetone was added and then incubated at -20°C for overnight. After the overnight incubation, sample was centrifuged at 13000xg for 20 minutes at 4°C. Then supernatant was discarded and pellet was washed with ice cold acetone and centrifuged at 8000xg for 10 minutes at 4°C. Supernatant was discarded and pellets were dried in air at 4°C. Dried pellets were dissolved into 1X Solubilization buffer and 1µg/µl of protease inhibitor and stored at -20°C.

4.14 Western blotting

After completion of SDS-PAGE, the stacking gel was removed away and one corner from the lower part of the resolving gel was cut (Note: This cut corner part would enable correct orientation of the gel if it "flips over" during equilibration). The gel was equilibrated in transfer buffer for 1 to 5 min. Pre-wetted and equilibrated the nitrocellulose membrane in transfer buffer for 10-15 min. Placed the electro-transfer cassette in a tray filled to a depth of 3 cm with chilled transferbuffer. Assemble the transfer stack so that proteins migrate toward the membrane. For negatively charged proteins, build the stack on the half of the cassette that will face the anode (+). Prewet a sponge and placed it on the submerged part of the cassette. Press gently to remove any air bubbles. Place two prewetted blotting papers on to the sponge. Place the membrane on top of the blotting papers.Placed the gel on top of the membrane. Placed two additional prewetted blotting papers on the gel. Finally placed a prewetted sponge on top of the stack and close the cassette, after gently. Pressing to remove air bubbles. Added prechilled transfer buffer to the transfer tank. Placed the cassette in the transfer tank. Connected the transfer tank to the power supply and run at 150V for 2 hours. After transfer, blocked the membrane in 5% BSA (in TBS) for overnight at 4°C.

Incubated with primary antibody (Table 4.3) for 3 hours at room temperature. Washed the membrane 5 times for 5 min per wash with TBST on the shaker. Incubated with HRP conjugated secondary antibody (Table 4.4) for 2 hours at room temperature. Washed the membrane 5 times for 5 min per wash with TBST on rotor and one time with TBS to remove present Tween20.

Visualization of western blot

Equal volume of H_2O_2 and chromogen was mixed and the membrane was visualized using Chemiluminescent ECL agent (Millipore).Imaging was done in chemidoc MP (Bio-Rad).

Table 4.5: Dilution and incubation of primary and secondary antibodies used in western blot.

Antibody dilution and incubation time					
Primary Antibody	**Dilution**	**Incubation Time**	**Secondary antibody**	**Dilution**	**Incubation Time**
Mouse-anti-SOD2(SantaCruz)	1:10000	3 hours	Anti-mouse-HRP (SOD2)	1:20,000	2 hours
Mouse-anti-HSP20/HSPB6 (SantaCruz)	1:5000		Anti-mouse-HRP (HSP20/HSPB6)	1:10,000	
Rabbit-anti-SERPING1 (ThermoScientific)	1:1000		Anti-rabbit-HRP (SERPING1)	1:10,000	
Rabbit-anti-PRDX1 (ThermoScientific)	1:10000		Anti-rabbit-HRP (PRDX1)	1: 20,000	
Rabbit-anti-PRDX2(ThermoScientific)	1:10000		Anti-rabbit-HRP (PRDX2)	1: 20,000	

4.15 ELISA (Enzyme-linked immunosorbent assay)

Preparing urine samples

Collect urine and centrifuge the samples at 4,000x g for 5 min and aliquot, quick freeze in dry ice and store at -80°C until use.

A sandwich ELISA measures antigen between two layers of antibodies (capture and detection antibody). The target antigen must contain at least two antigenic sites capable of binding to antibodies. Sandwich ELISAs remove the sample purification step before analysis and enhance sensitivity (2–5 times more sensitive than direct or indirect).

Enzyme-linked immunosorbent assay (ELISA) was used to quantify SOD2, PRDX1 and PRDX2 concentrations in urine. ELISA kit for detection of SOD2 (R&D Systems, DYC3419), PRDX2 (R&D Systems, DY3489) and PRDX1 (Abcam, ab185983) were obtained. The assays were performed in duplicate with 100 µl of urine sample added to each well. The protocol given in the manufacturers' instructions was followed. The readings were taken in the ELISA plate reader at 450nm. Protein concentrations were normalized to the creatinine concentration in urine and expressed as amount of proteins in picograms (pg) excreted per mg of creatinine for SOD2 and in micrograms (µg) excreted per mg of creatinine for PRDX1 and PRDX2.

4.16 Bioinformatic Analysis

To identify under- and over-represented functional categories we used Protein ANalysisTHrough Evolutionary Relationships (PANTHER) database v 6.1

(www.pantherdb.org).Pathway and network analysis were performed using Ingenuity Pathway Analysis of Differentially regulated proteins (Ingenuity Systems, Inc., Redwood City, CA; www.ingenuity.com). IPA is widely used to infer upregulated proteins in terms of an interaction network and predominant canonical pathways and functional annotations created by manual curation of the scientific literature.By running the core analysis, these proteins were overlaid onto a global molecular network developed from the information in the ingenuity knowledge base (IKB), a regularly updated and curated database that consists of interactions between different proteins gathered from scientific literature.To calculate statistical significance, IPA uses a hypergeometric distribution (Fisher's Exact Test), which calculates the probability (p) of finding a given number of proteins (n) from the input data in each of the pathways. Ingenuity queries a proprietary database of Canonical Pathways; a pathway was considered as significantly enriched if both the false discovery rate (FDR) for the pathway was less than 0.01, and the pathway included at least 4 of the identified proteins of our dataset.Networks of these focus proteins were then algorithmically generated by including as many primary proteins as possible and other nonfocus proteins from the IKB that are needed to generate the network based on connectivity.

4.17 Comparative analysis with Database using web-based tool and Cytoscape software

Exosomal proteins present in the urine were identified from the Exocarta database (www.exocarta.org). A seed list was prepared by selecting exosomal proteins specific only for bladder cancer. The ‘seed list’ was imported to the Pathway-Express module of Onto-tool (http://vortex.cs.wayne.edu/projects.htm) and the pathways in which exosomal proteins were involved were identified. Impact factor was calculated by the web-based software (Pathway-

Express module) using both a statistically significant number of differentially expressed genes and biologically meaningful changes on a given pathway. Pathway-Express provides two types of p-values for each pathway: (i) p-value obtained using the classical statistics (referred to as classical p-value) and (ii) p-value obtained using the impact analysis (referred to as gamma pvalue). The classical p-value is calculated as an over-representation analysis using hypergeometric test. The gamma p-value is the p-value provided by the impact analysis.

Exosomal proteins involved in pathways with the high impact factor (cutoff >10), were selected for studying protein–protein interactions (PPIs) network and hubs. Protein–protein interactions (PPIs) were commonly understood as physical contacts with molecular docking between proteins that occur in a cell or in a living organism in vivo. Each of these interactions is specifically adapted to carry out certain biological functions. A PPI network is represented with proteins as nodes and interactions between nodes as the edges. The common proteins among top 9 pathways were further studied by web-based software, POINeT (poinet.bioinformatics/tw). The number of iterations used was one i.e., only first degree neighbors of queried nodes were searched. The protein–protein interaction (PPI) information collected is integrated by various protein databases to provide PPI filtering and network topology analysis capability. PPI was filtered for a number of literatures (>1), shared GO (Gene Ontology) terms (>1) and for interactors within the same species (Human). Graph centrality values average distance, index of aggregation, connectivity, cluster coefficient and degree of each subnetwork were computed. The PPI network was visualized using the Cytoscape software (2.8.2 version) as an undirected graph in a force-directed layout. It evaluated node essentiality by topological characters. The degree of a node was the number of links incident to this node in a network and identified hub nodes which are highly connected

nodes in a network and vital for the proper function of a network. Top 10 nodes ranked by degree scores were identified as hubs. Node centrality values were calculated in Cytoscape using the Network Analysis and cytoHubba plugin. In network analysis, node color was based on Degree and intensity on Betweenness.

4.18 Statistical analysis

Chi-square test was performed to find the significant association between categorical variables in immunohistochemistry analysis. The Mann Whitney test was used to evaluate the significance of differences in marker concentration between each group. Median concentration of urinary marker was taken as a cut-off for survival analysis. Kaplan Meier analysis for recurrence-free survival was done and significance was computed by log-rank. A probability less than 0.05 was considered significant. All statistical analyses were performed using the statistical package for the Statistical Package for social sciences (SPSS) software version 19 (SPSS, Chicago, IL, USA).

Chapter 5

To identify the differentially expressed tumour tissue proteins in urinary bladder cancers compared to normal bladder mucosa

Chapter 5

5.1 Introduction

Quantitative proteomics offer considerable opportunities for understanding or discovering biomarkers from tumor milieu and it may enhance diagnosis and treatment strategy. During the last few years, a number of promising biomarkers have been discovered, but they did not reach the clinic because they were less specific and less sensitive. Efforts have been undertaken to discover biomarker for bladder cancer using tissue biopsies or urine to help in diagnosis and treatment of patients. These studies have provided diagnostic/prognostic markers, which are having low sensitivity and specificity, as urinary proteins may represent a composite picture of all the diseases in an individual, both transient and chronic. A few of these biomarkers are FDA (Food and Drug Administration) approved, but their clinical application is still not established (Frantzi *et al*., 2015). Hence, there is need of exploratory study to discovery a panel of biomarkers for early diagnosis, prognosis and surveillance marker of patients.

Several proteomic studies have also been reported providing a huge list of differentially deregulated proteins involved in pathophysiology of bladder tumors. Ruppen *et al* has reported a differential protein profiling of human bladder cancer cell line by iTRAQ-two-dimensional LC-MS/MS and identified Kiss-1 gene to be a metastatic suppressor of human bladder cancer. In 2010, Chen *et al* discovered novel biomarkers (apolipoprotein A-I, apolipoprotein A-II, peroxiredoxin-2 and heparin cofactor 2 precursor) in urine of bladder cancer patients but tumor specificity was not demonstrated. Similarly, they have validated 63 urine proteins by multiple reaction monitoring-based mass spectrometry but urinary protein secreted by bladder tumor has

not been shown (Chen *et al*., 2012). TAGLN2 as a urinary biomarker was discovered by 4-plex iTRAQ-LC-MS/MS (Chen *et al*., 2015).

The aim of the current study was to discover a tumor specific biomarker which is also secreted in urine of patients with bladder cancer. Herein, we applied iTRAQ based proteomic method to screen protein expression changes in different stages of Urothelial bladder cancer. We report relative quantification information of 1137 proteins with identification of atleast two peptides by iTRAQ. Six potential biomarkers (SERPING1, SOD2, HSPB6, PRDX1, PRDX2 and Tenascin C) were evaluated on FFPE for confirmation and validated on urine sample by Western blotting and ELISA.

5.2 Results

5.2.1 Study design

Primary tumour tissue samples and adjacent mucosa, histopathologically confirmed cases of LGPT1 (Low grade non muscle invasive, n=2), HGPT1 recurrent (n=1), HGPT1 non-recurrent (n=1), and 2 cases of HGPT2 were used for discovery phase proteomics. All patients were male with median age of 61 (interquartile range; 53 to 68). The schematic workflow of study design is given in Figure 5.1.

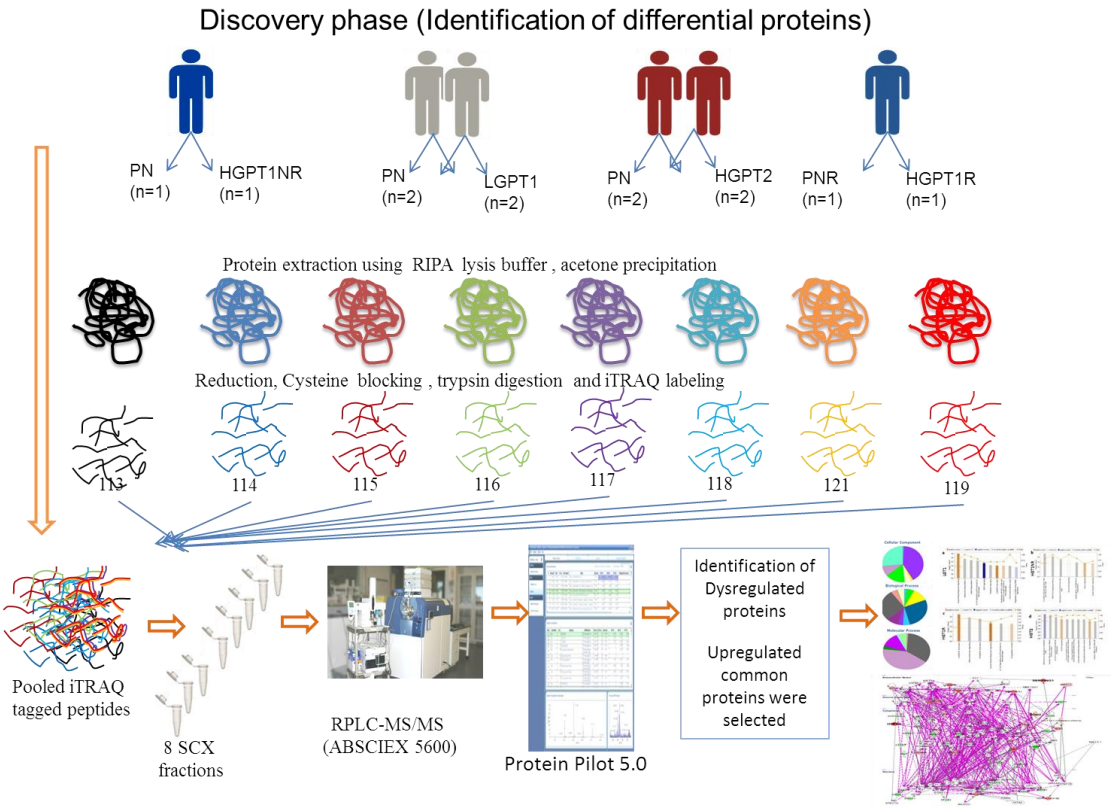


Figure 5.1: Schematic representation of workflow of chapter 5. Paired samples including low grade stage 1 (LGPT1), High grade stage1 non recurrent tumor tissue (HGPT1NR), High grade stage 1 recurrent tumor tissue (HGPT1R), High grade stage 2 (HGPT2) and adjacent mucosa (PN) were confirmed by histopathology. Precipitated proteins were digested with trypsin and labelled with iTRAQ reagent (SCIEX). Eight fractions were collected from pooled labelled samples after strong cation exchange chromatography (SCX) and subjected for reverse phase liquid chromatography (RPLC-MS/MS). Initial discovery phase by 8-plex iTRAQ MS-based approaches resulted in the identification of a large number of potential biomarkers. The raw data was processed by using Protein Pilot 5. Deregulated proteins were further analysised by Panther database and Ingenuity pathway analysis.

5.2.2 Samples for iTRAQ labelling and LC-MS/MS

Tissue samples (including 6 tumor and 6 normal tissue) were selected for discovery phase and 2 sets of iTRAQ LC-MS/MS was performed.

SDS-PAGE stained with silver stain was performed for all the samples to examine the quality and the intact protein bands were seen ranging from low molecular to high molecular weight in both tumor and adjacent non tumor samples (Figure 5.2a and 5.2b).

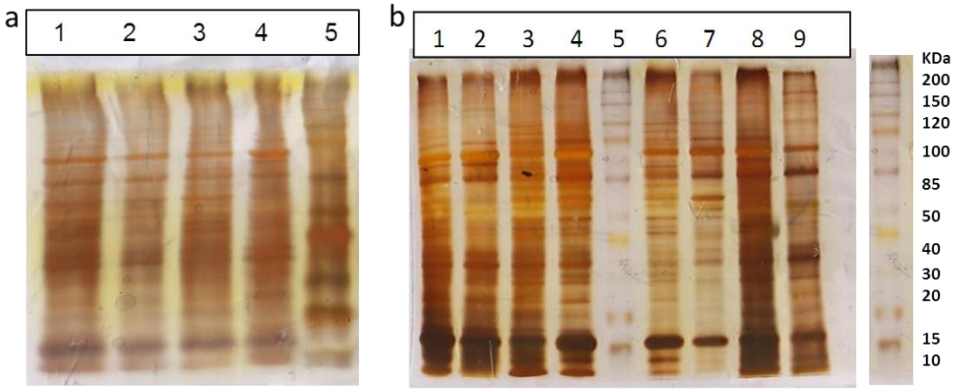


Figure 5.2: SDS-PAGE (12.5%) image stained with silver staining of 12 cases included tumor and paired adjacent normal mucosa having 4µgm sample per well showed protein distribution of a) well no 1=011 LGPN1; 2=011PN; 3=010PN; 4=010HGPT2; 5=protein marker and b) well no 1=014 LGPN1; 2=014PN; 3=017PN; 4=017LGPT1; 5=protein marker; 6=020HGPT2; 7=020PN; 8=013HGPT1; 9=013PN) used in 1st set and 2nd set of iTRAQ experiment.

Samples labelled with iTRAQ reagent were as listed in table for both sets (Table 5.1). Correlation plot was generated between 1st and 2nd iTRAQ set of experimental and technical replicates used in this phase (Figure 5.3). Data analysis was performed and iTRAQ ratio was calculated using Protein Pilot (version 5.0). Proteins tagged with iTRAQ reagent could be seen in mass spectra as a relative quantitation of those proteins in each sample (Figure 5.4).

Table. 5.1: Tissue sample selected for discovery phase for iTRAQ in 1st and 2nd set

iTRAQ 1st Set			iTRAQ 2nd Set		
Sample code	Sample Id	iTRAQ tagging	Sample code	Sample Id	iTRAQ tagging
011PN	12	113	014PN	8	113
011LGPT1	3TR1	114	014HGPT1	5	114
010PN	11TR1	115	017PN	18	115
011LGPT1	3TR2	116	017LGPT1	20	116
010HGPT2	2	117	020PN	23	117
010PN	11TR2	118	020HGPT2	24	118
013HGPT1	4ER1	119	013HGPT1	4ER2	119
013PN	14ER1	121	013PN	14ER2	121

PN= patient adjacent normal; LGPT1= Non-muscle invasive low grade; HGPT1= Non-muscle invasive high grade; HGPT2= Muscle invasive high grade; TR= Technical repliate; ER= Experimental replicate

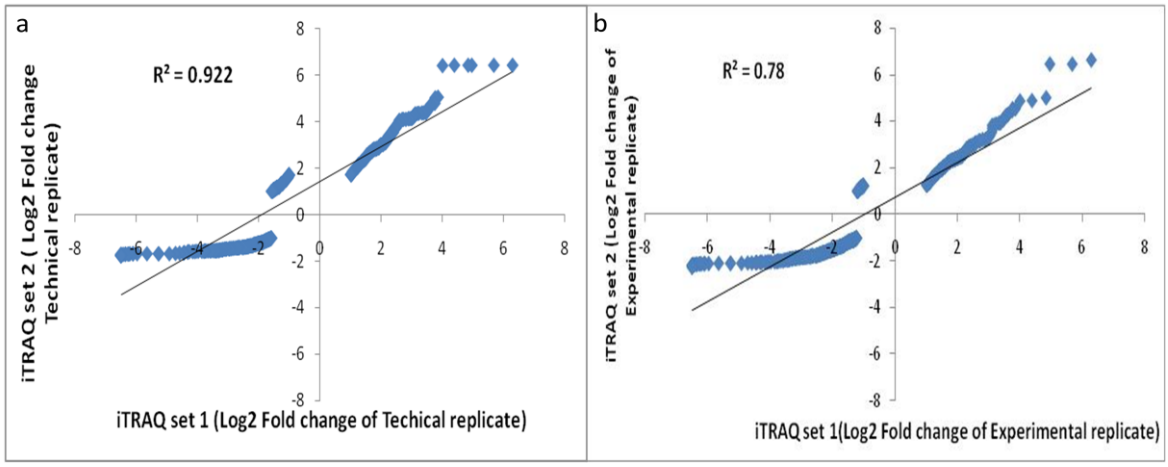


Figure. 5.3: Correlation plot of log2 fold change a)between technical replicates and b) experimental replicates of iTRAQ set1 and iTRAQ set2.

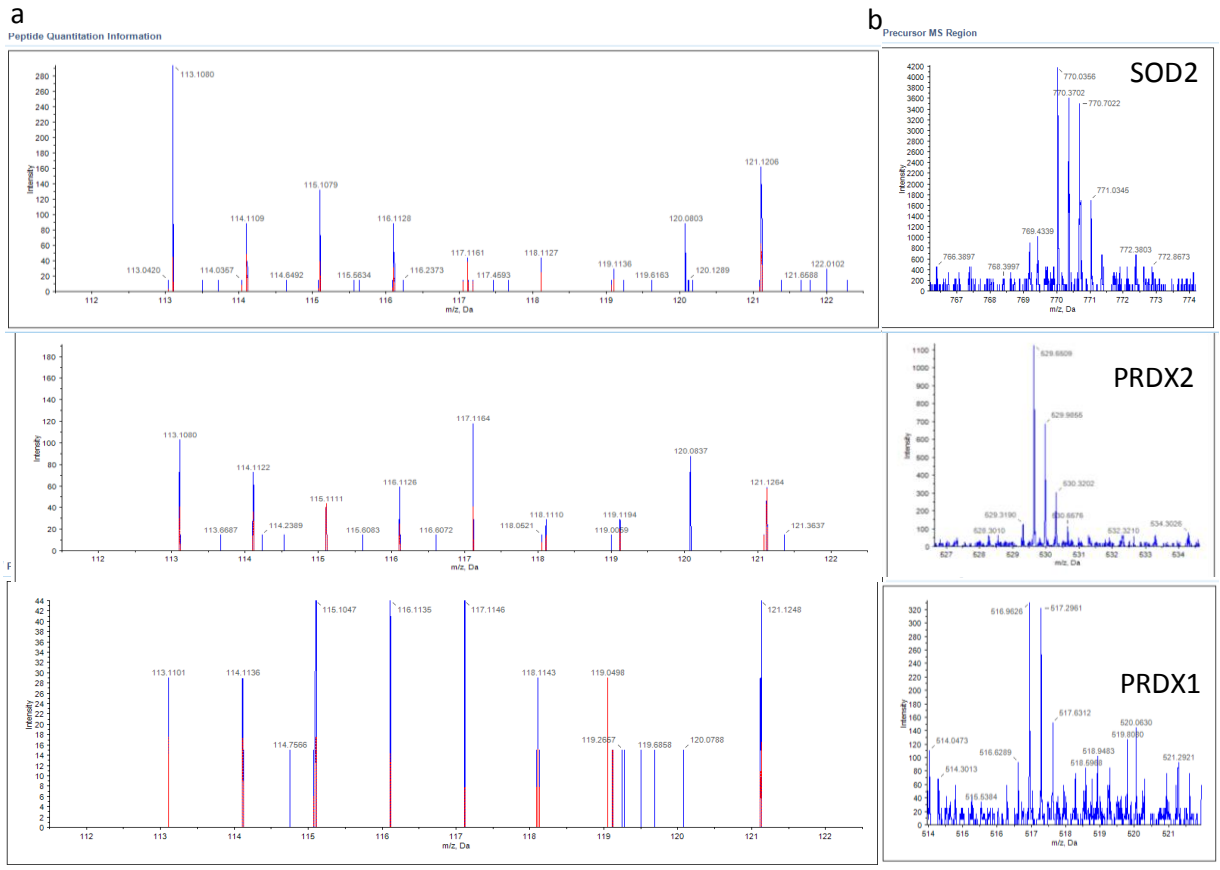


Figure 5.4: A representative mass spectral images showed a) iTRAQ tagging and b) MS region spectral of deregulated protein/peptide (SOD2, PRDX2 and PRDX1) of each sample derived from ProteinPilot (version 5.0).

5.2.3 iTRAQ labeling, Identification and Quantitation of protein

A total of 1795 proteins with ≥1peptide (15385peptides) and 1137proteins with ≥2 peptide (15005 peptides) were identified in urothelial bladder cancer compared to normal mucosa. The average difference of number of proteins with 1 or 2 peptide identified is 144.5 in each group. The total tissue proteins identified with at least 1 peptide in LGT1 were 1795 (278 upregulated and 456 downregulated), in HGT1NR were 1795 (285 upregulated and 307 downregulated), in HGT1R were 1530 (217 upregulated and 369 downregulated) and in HGT2 were 1795 (366

upregulated and 545 downregulated).The tissue proteins identified with at least 2 peptides in LGT1 were 1455 (205 upregulated and 376 downregulated), in HGT1R were 1398 (230 upregulated and 252 downregulated), in HGT1NR were 1137 (159 upregulated and 285 downregulated) and in HGT2 were 1399 (277 upregulated and 457 downregulated). The remaining proteins were not dysregulated.

Table 5.2: Distribution of identified and deregulated proteins with one and two peptide in subgroups of bladder cancer

	1 peptide		2 peptide	
	Protein identified	Deregulated protein (up/down)	Protein identified	Deregulated protein (up/down)
LGT1	1795	278/456	1455	205/376
HGT1NR	1795	285/307	1137	159/285
HGT1R	1530	217/369	1398	230/252
HGT2	1795	366/545	1399	277/457

Proteins with at least two peptides were further analyzed for deregulated proteins unique and common in two; three and four groups were identified and are shown in the scatter plot and Venn diagram (Figure 5.5a and 5.5b). Among all subgroups, high grade muscle invasive group showed the highest number of proteins (n=178). Common proteins (n=75) between low grade non-invasive (LGT1) and high grade non-invasive, non-recurrent (HGT1NR) bladder cancer, were highest whereas only 39 proteins were common between low grade non-invasive (LGT1) and high grade non-invasive recurrent (HGT1R) bladder cancer. The recurrent tumour showed 74

unique proteins (Figure 5.5b). The 64 deregulated proteins common to all cases were seen to be showing varied regulation as seen in the heat map (Figure 5.5c).

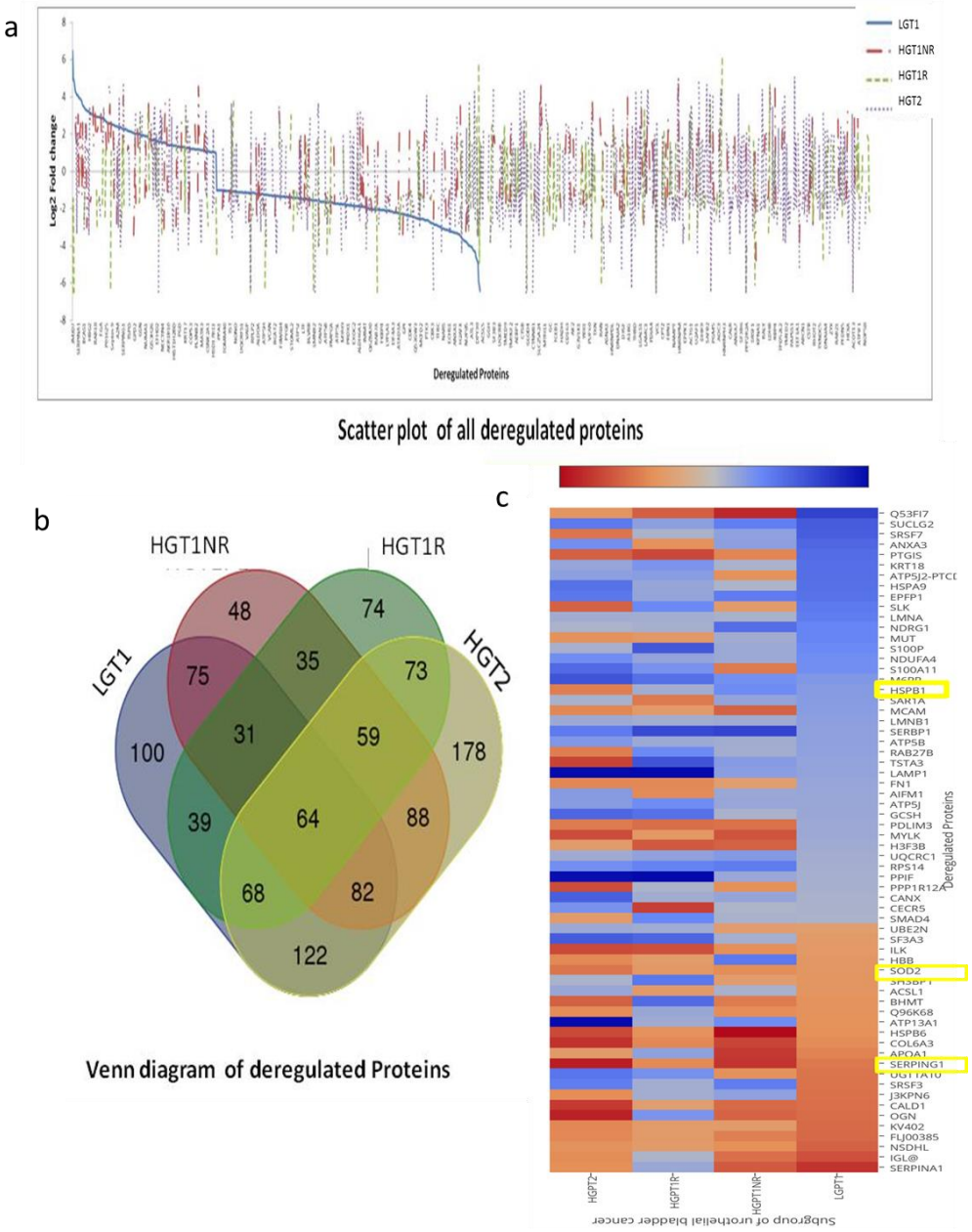


Figure 5.5: Deregulated proteins identified in the discovery phase

a) Scatter plot showed fold change of all deregulated proteins of LGT1, HGT1R, HGT1NR and HGT2 of bladder cancer. b) Venn diagram showed unique, common between two, three and four

sub groups of urothelial bladder cancer patients. c) Heat map showing deregulated profile of 64 common proteins in all subgroups (LGT1, HGT1NR, HG1R and HGT2) of urothelial cancer. All common proteins were on y-axis and subgroups of urothelial cancer on x-axis. Increasing blue color gradient showed downregulation and increasing red color gradient showed upregulation of proteins.

Among the common deregulated proteins, only 9 were upregulated in all groups and 19 were downregulated in all (Table 5.3). Rest 36 proteins were variably regulated in subgroups of bladder cancer.

Table 5.3: Fold change of common up and downregulated proteins in subgroups of bladder cancer

Name	Protein code	LGT1	HGT1NR	HGT1R	HGT2
Upregulated common proteins					
Sterol-4-alpha-carboxylate 3-dehydrogenase	NSDHL	2.83	1.65	1.18	1.57
FLJ00385 protein	Q8NF17	2.70	2.10	1.22	1.87
Ig kappa chain V-IV	KV402	2.64	1.17	1.18	1.89
Caldesmon	CALD1	2.51	2.64	1.05	3.87
Plasma protease C1 inhibitor	H9KV48	2.27	4.05	1.81	4.62
Collagen type VI, alpha 3	D9ZGF2	1.75	3.61	1.85	4.12
Heat shock protein beta-6	K7EP04	1.66	5.14	1.58	3.52
Superoxide dismutase	Q7Z7M4	1.36	1.73	1.34	2.38
Integrin-linked protein kinase	ILK	1.25	1.66	3.32	3.49
Downregulated common proteins					
Calnexin	B4DGP8	-1.09	-1.51	-1.24	-3.77
Peptidyl-prolylcis-trans isomerase	PPIB	-1.17	-1.44	-6.50	-6.34
40S ribosomal protein S14	RS14	-1.20	-2.82	-2.62	-2.22
Cytochrome b-c1 complex	QCR1	-1.22	-1.86	-1.61	-1.29
Glycine cleavage system H-protein	Q6QN92	-1.42	-1.06	-3.18	-3.55
ATP synthase-coupling factor 6	Q6IB54	-1.45	-1.46	-2.30	-1.79
Lysosome-associated membrane glycoprotein 1	LAMP1	-1.57	-1.74	-6.50	-6.46
Putative uncharacterized protein DKFZp686P17171	Q63HR1	-1.66	-4.62	-4.45	-2.88
ATP synthase subunit beta	ATPB	-1.66	-1.26	-1.38	-1.95
Lamin-B1	LMNB1	-1.69	-1.29	-1.24	-1.34
Cation-dependent mannose-6-phosphate receptor	Q53GY9	-1.94	-2.21	-3.23	-4.13
NADH dehydrogenase ubiquinone 1 alpha 4	NDUA4	-2.27	-1.37	-1.55	-2.26
Protein S100-P	S100P	-2.39	-1.37	-3.97	-1.14
Transformation-related protein 14	Q597H1	-2.54	-3.28	-1.17	-1.05
Lamin A/C transcript variant 1	Q5I6Y6	-2.78	-1.09	-1.24	-1.26
Chaperonin 10-related protein	Q9UNM1	-3.14	-2.83	-1.51	-2.98
cDNA FLJ51907, highly similar to Stress-70 protein	B7Z4V2	-3.23	-1.01	-1.49	-3.20
cDNA, FLJ94640, highly similar to Homo sapiens keratin 18	B2RA03	-3.26	-1.06	-2.05	-1.49

Among the 9 upregulated proteins, Sterol-4-alpha-carboxylate 3-dehydrogenaseis involved in cholesterol and steroid biosynthesis and Ig kappa chain V-IV region Len activates the complement pathway. Information about the FLJ00385 protein is minimal. The protein lysate was obtained from the whole tumour tissue and hence also included certain stromal proteins like Caldesmon (present in smooth muscle tissue), Collagen, type VI, alpha 3 (found in connective tissue) and Integrin-linked protein kinase (an extracellular matrix protein) which were found upregulated.

SOD2 (Superoxide dismutase), HSP20 (a stress protein) and SerpinG1 (C1 Plasma protease inhibitor) were upregulated (more than 1.5 fold) in low grade and all high grade subtypes of urothelial bladder cancer and may be secreted from tumor epithelial cells. SerpinG1 expression is 4 fold upregulated in HGPT1NR and HGPT2, 2 fold highly express in LGPT1 and 1.8 fold upregulated in HGPT1R of urothelial bladder cancer. Heat shock beta 6/HSP20 protein is 5 fold in HGT1NR and 3.5 folds upregulated in HGPT2 whereas expression of HSPb6 is less than 2 fold upregulated in HGPT1R and LGPT1. Superoxide dismutase was 2 fold upregulated in HGPT2 and less than 2 fold higher expressions HGT1NR, HGPT1R and LGPT1.

Calnexin (a chaperon protein) and Chaperonin 10-related protein was downregulated in bladder cancer compared to adjacent mucosa. Calnexin is 1.5 fold downregulation in LGPT1, HGPT1 but 3 fold downregulation in HGPT2 of bladder cancer. Expression of Chaperonin 10 was -2.5 fold in all except in HGPT1R (-1.5 fold change).

Highest downregulation of Lysosome-associated membrane glycoprotein 1 was -6.5 and -6.3 in HGT1R and HGPT2 of bladder cancer respectively whereas -1.4 in HGT1NR and -1.1 in LGT1

of bladder tumor compared to adjacent normal. Glycine cleavage system H-protein, Lamin-B1, Cation-dependent mannose-6-phosphate receptor, Protein S100-P, Transformation-related protein 14 and Lamin A/C transcript was downregulated (less than -4 fold) in bladder tumor. Downregulation in expression of 40S ribosomal protein S14, Putative uncharacterized protein DKFZp686P17171, cDNA FLJ51907, Peptidyl-prolylcis-trans isomerase, highly similar to Stress-70 protein and cDNA, FLJ94640 highly similar to keratin 18 were seen in bladder tumor compared to normal mucosa. ATP synthase subunit beta, ATP synthase-coupling factor 6, NADH dehydrogenase ubiquinone 1 alpha 4 and Cytochrome b-c1 complex were less than 1.5 fold downregulation.

5.2.4 Gene ontology

Gene Ontology (GO) terms determine the structure, localization and function. The GO consists of three parts: the cellular component, biological process and molecular function. Cellular component termed as localization in parts of the cell or its extracellular environment. The GO term divided the differential proteins into 8 categories: cell part (GO:0044464) - 40.90%, organelle (GO:0043226)-26.50%, macromolecular complex (GO:0032991)- 15.60%, membrane (GO:0016020)- 9.20%, extracellular region (GO:0005576)- 5.40%, extracellular matrix (GO:0031012)- 1.50%, cell junction (GO:0030054)- 0.80% and synapse (GO:0045202)- 0.20% (Figure 5.6a). The categories Biological process represents a molecular event with defined beginning and end. Biological process of GO uses 13 terms: cellular process (GO:0009987)- 27.50%, metabolic process (GO:0008152)- 27.50%, cellular component organization or biogenesis (GO:0071840)- 11.40%, localization (GO:0051179)- 8.30%, biological regulation (GO:0065007)-5.10%, response to stimulus (GO:0050896)- 4.80%, developmental process (GO:0032502)- 4.80%, multicellular organismal process (GO:0032501)- 4.40%, immune system

process (GO:0002376)- 3.60%, biological adhesion (GO:0022610)- 2.00%, reproduction (GO:0000003)- 0.40%, locomotion (GO:0040011)- 0.20% and growth (GO:0040007)- 0.10% (Figure 5.6b). Molecular function represents the fundamental function of an analyte at the molecular level. It includes 9 molecular function; catalytic activity (GO:0003824)-42.10%, binding (GO:0005488)- 33.80%, structural molecule activity (GO:0005198)- 12.90%, transporter activity (GO:0005215)- 5.20%, receptor activity (GO:0004872)- 2.50%, translation regulator activity (GO:0045182)- 1.80%, antioxidant activity (GO:0016209)- 1.10%, signal transducer activity (GO:0004871)- 0.40% and channel regulator activity (GO:0016247)- 0.10% (Figure 5.6c).

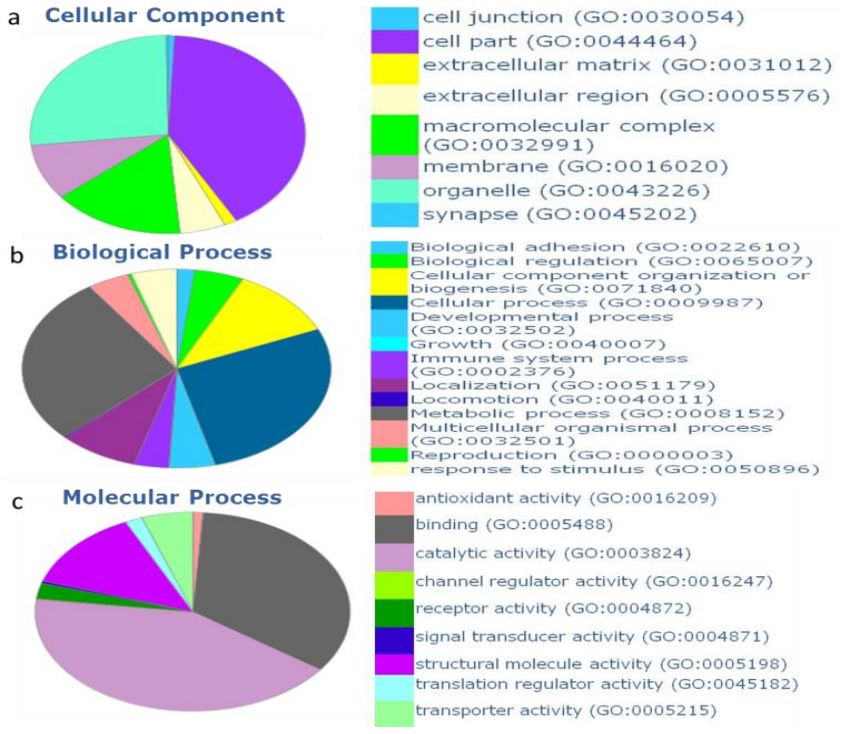


Figure 5.6: Determination of Gene ontology (GO) terms of deregulated proteins of subgroups of urotheilal bladder cancer using PANTHER 9.0. Pie chart is showing a) Cellular

component (40.9% cell part), b) Biological Process (27.5% cellular and 27.5% metabolic process), c) molecular Process (42% catalytic activity).

5.2.5 Ingenuity pathway analysis

Bioinformatics knowledge-based analysis of the quantitative data of four different subgroups of bladder cancer (LGPT1, HGPT1R, HGPT1NR and HGPT2) by the Ingenuity Pathway Analysis system indicated log 2-fold altered expression in various canonical pathway (Figure 5.7 a, b, c & d). The top canonical pathways that were significantly associated with our datasets having positive Z-score included acute phase response signaling, FXR/RXR Activation, LXR/RXR Activation, Production of Nitric oxide and Reactive oxygen species in Macrophages, IL12 signaling and production in macrophages, RhoA Signaling, Death Receptor signaling, eNOS signaling and ILK signaling in all subgroups of bladder cancer. Significantly enriched altered molecular pathway with negative z-score includes EIF2 Signaling, Regulation of eIF4 and p7056K Signaling and Aldosterone signaling in Epithelial cells in all subgroups of bladder cancer. Common pathways were acute phase response signaling, LXR/RXR Activation, IL12 signaling and production in macrophages and FXR/RXR activation found only in LGpT1 and HGpT1R (Figure 5.7 a & c).

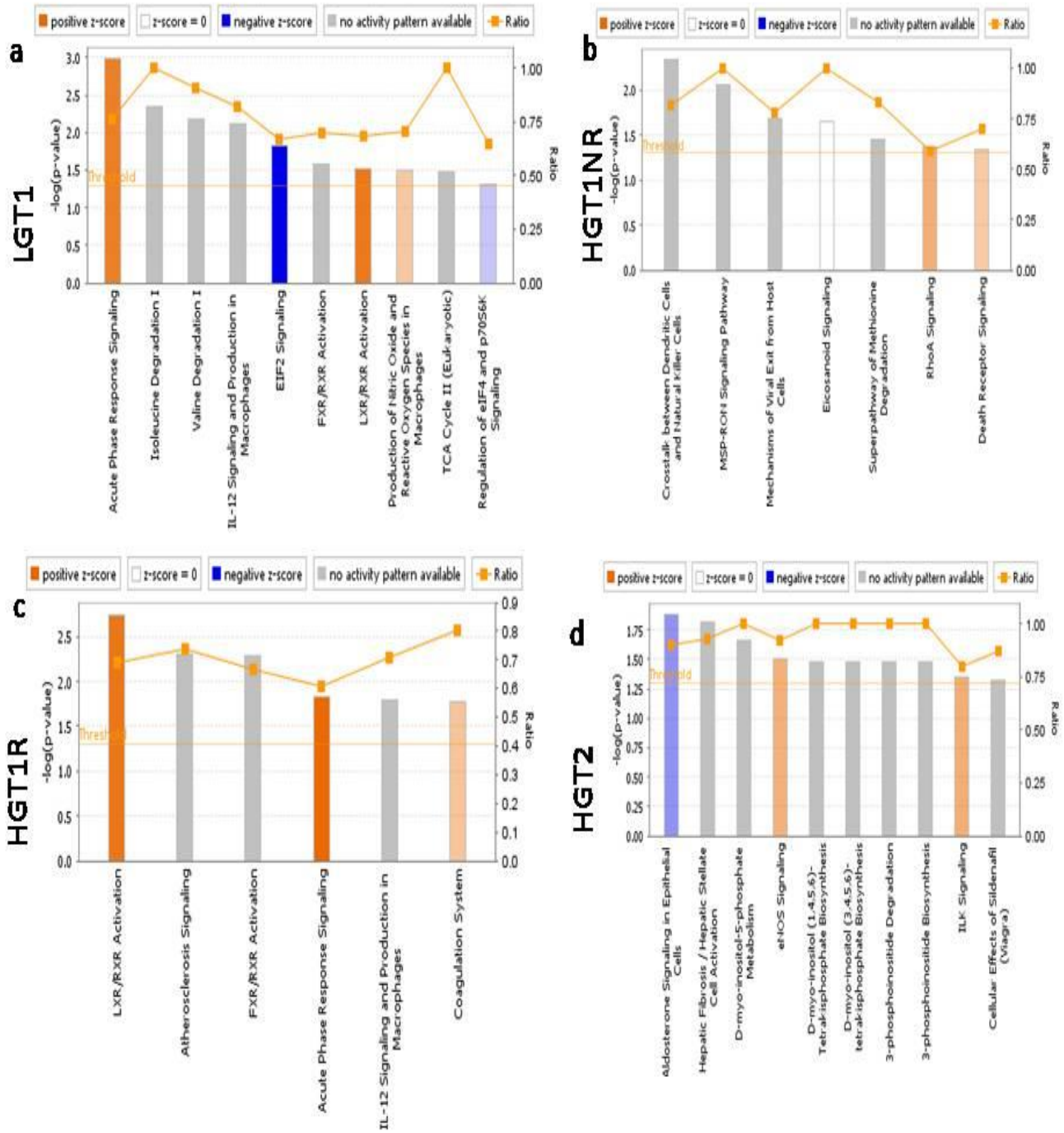


Figure 5.7: Ingenuity canonical pathway

Deregulated protein list analysis on Ingenuity™ platform showed proteomic alterations in various predominant canonical pathways and the bars display the significance of these

associations (the −log of P value calculated by Fisher's exact test right-tailed), and the orange squares connected by a thin line represent the ratio of the number of proteins in our dataset of a given pathway to the total number of proteins in this canonical pathway a) LGT1, b) HGT1R, c) HGT1NR and d) HGT2 of bladder cancer patient with threshold 1.3 and p-value <0.05.

The Ingenuity Pathway Analysis (IPA) Core Analysis generated the top 11 Network and revealed top three interactions between 24, 14 and 11 of deregulated proteins. These proteins are involved in Cellular Movement, Hematological system, Immune cell Trafficking, Nucleic Acid Metabolism, Small Molecule biochemistry, Development Disorder, Cell Death and Survival, Cellular Development, Cellular Growth and Proliferation (Table 5.4).

Table 5.4: Ingenuity network summarizes top diseases and function associated with significantly up/down regulated proteins

	Molecules in Network	Score	Focus Molecules	Top Diseases and Function
1	26s Proteasome, AIFM1, AKT, APOA1, CALD1, CANX, ERK1/2, estrogen receptor, FN1, HSPA9, HSPB1, IgG, IGHG1, ILK, Jnk, KRT18, LAMP1, LDL, LMNA, MCAM, MUT, MYLK, NDRG1, NFκB(complex), P38 MAPK, p85 (pik3r), PPIF, PPP1R12A, SAR1A, SERPINA1, SMAD4,Smad2/3**, SOD2**, SRSF3, UBE2N	51	24	Cellular Movement, Hematological system Immune cell Trafficking
2	ANGPTL4, ANXA3, ATP5B, ATP5J, CASP4, CHK4, COL12A1, COL6A3, CST5, CXCL8, EPAS1, FBN1, FHL1, Fibrinogen, FSH, GLS, GRB2, HIF1A, **HSPB6**, HSPE1, Lh, LMNB1, LONP1,MAP1LC3B, NRG1,PDLIM3,PRKCZ,PTGIS, S100A11,SERBP1, SF3A3, SFPQ, STK17A, TCR, TGFB1	25	14	Nucleic Acid Metabolism, Small Molecule biochemistry, Development Disorder
3	ACSL1, BRCA1, CDH4, DDB2, F11, FKBP5,G6PD, GF11, H3F3A/H3F3B, HBB, Histone h3, HNF1A, HNF1A, HNF1B, ICAM1, IDH1, IGFBP7, JMJD1C, MTA2, MYC, NDRG1, NR5A2, PGR, RAB27B, REL, RNU7-1, RPS14, S100P, **SERPING1**, SMO, SOD2, SRSF7, TP63, UGT1A7 (includes others), WNT5A, XRCC6	18	11	Cell Death and Survival, Cellular Development, Cellular Growth and Proliferation
4	MiR-124-3P (and other miRNAs w/seed AAGGCAC), SUCLG2	2	1	Cellular Function and Maintenance, Lipid Metabolism, Nucleic Acid Metabolism
5	ERM, SLK	2	1	Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance
6	LY6D, TSTA3	2	1	Carbohydrate Metabolism, Cell-to-cell Signalling and interaction, Post-Translational Modification

7	RAC1, SH3BP1	2	1	Cell Morphology, Carbohydrate Metabolism Cardiovascular system development and function
8	KAT5, OGN	2	1	Cancer, Cell Death and Survival, Cellular Development
9	MGEA5, NSDHL	2	1	Developmental Disorder, Hereditary Disorder, Neurological Diseases
10	miR-145-5p (and other miRNAs w/seed UCCAGUU), NDUFA4	2	1	Cancer, Cell Morphology, Cellular Response to therapeutics
11	RTN4, UQCRC1	2	1	Cell Death and Survival, cell morphology, Cellular Assembly and Organization

Ingenuity network was derived by merging top 3 networks and includes 49 deregulated proteins. Top three networks revealed 3 upregulated proteins (SERPING1, SOD2 and HSPB6) of all patients of urothelial bladder cancer and showed similar network interaction. Subcellular view of the network showed SERPING1 in plasma membrane and SOD2 and HSPB6 in cytoplasmic localization (Figure 5.8).

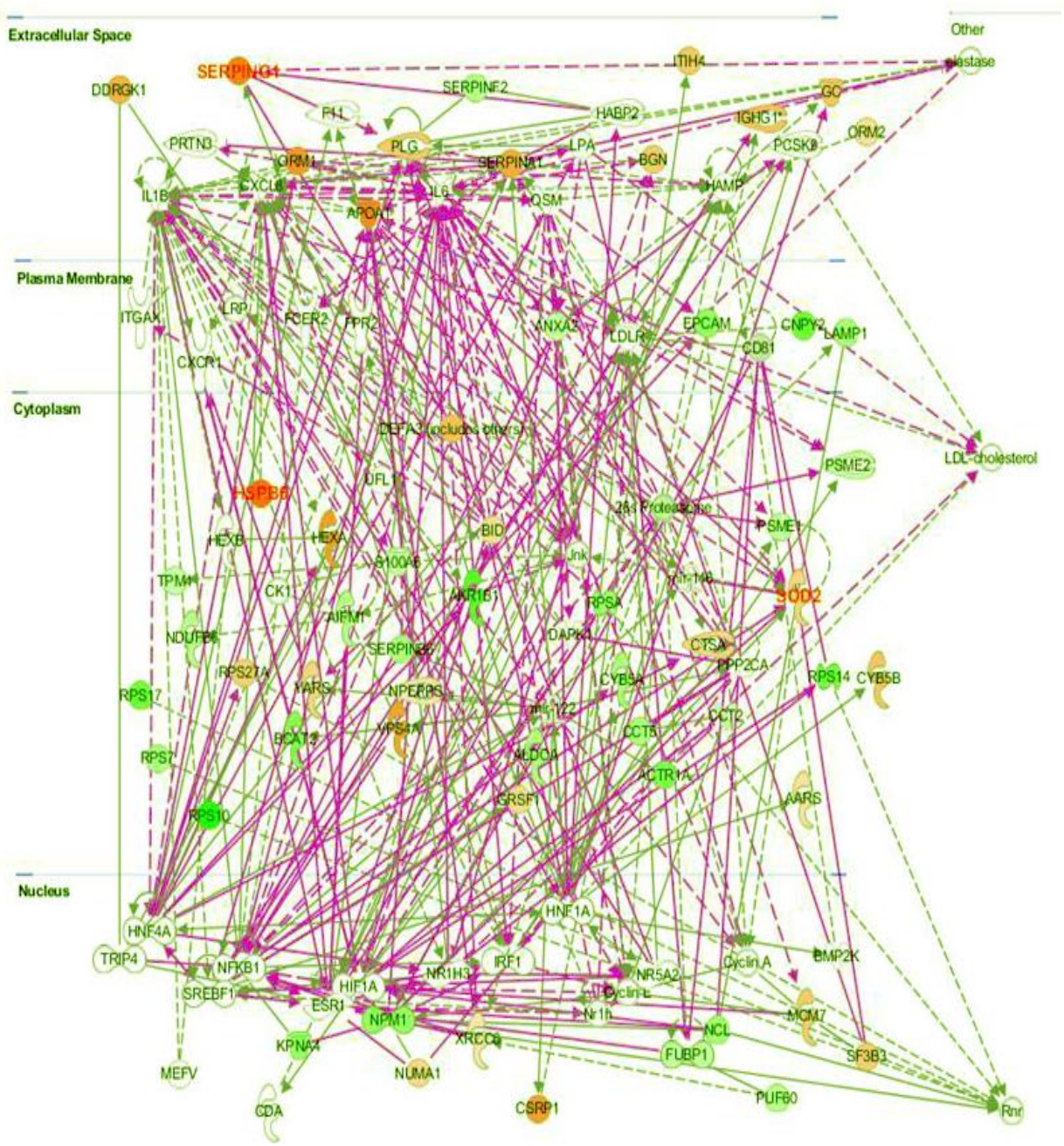


Figure 5.8: Ingenuity merged network of altered expressed proteins of Urothelial patients in three top most network and bolded proteins (SERPING1, SOD2 and HSPB6) were common in these topmost network.

A biological interaction network of deregulated proteins analysis on Ingenuity™ platform and subcellular view of protein-protein interaction showed different shapes (proteins), lines (biological relationship), solid lines indicate direct interaction, and dotted lines indicate indirect interactions between the proteins. Molecule with red color are upregulated and molecule with green color are downregulated.The color intensity of the node color indicated the degree of up or down regulation. Upregulated proteins (SERPING1, HSPB6 and SOD2) found in all patients in top three network, are boldly highlighted in network and these are potential candidates chosen for further validation.

5.3 Discussion

The bladder cancer cells produce and secrete proteins or shed exfoliated cells into the urine and detection of these analytes may serve as biomarkers with higher specificity and may also help in discovery of therapeutic molecules. To address this issue, we applied isobaric peptide tags (8-plex-iTRAQ) and LC-MS/MS to identify proteins that are differentially expressed in paired bladder cancer tissue (tumor and adjacent normal mucosa) specimens. Advantages of multiplexing in the iTRAQ (8-plex) are the cost and time of experiment (reduction in MS run by 8-fold) as well as reduced inter-run variability. The pre-fractionation steps before MS may improve the identification rate in comparison to other quantitative proteomic methods. Experimental and technical replicate may also reduce the inter run variability and enhance the identification of proteins (Jain *et al*., 2015; Sharma et al., 2014 and Latosinska *et al*., 2015). For the discovery of biomarker, the tissue biopsies from Low grade non-muscle invasive (LGPT1),

high grade non-muscle invasive (HGPT1) and high grade muscle invasive (HGPT2) were selected for proteome profiling and bladder tumor specific proteins were identified.

Among the 1137 differentially expressed proteins, 64 proteins were deregulated in all groups and 9 proteins were found upregulated and 19 proteins were found downregulated in all 4 groups. The other 36 proteins showed variable deregulation.

Proteins that were found up-regulated in all stages of bladder cancer are Sterol-4-alpha-carboxylate 3-dehydrogenase (NSDHL), Integrin-linked protein kinase, Heat shock protein beta-6, Superoxide dismutase 2, Caldesmon, Immunoglobulin kappa variable 4-1, Collagen alpha-3(VI) and SERPING1. Among them only Collagen alpha-3(VI) chain is an extracellular matrix protein, while the rest are localized in the cell cytoplasm. Further, these upregulated proteins have been found to be involved in the different pathways including, NSDHL and Superoxide dismutase 2 in Oxidoreductase pathway, HSPB6 and Caldesmon in muscle contraction pathway, Immunoglobulin kappa and SERPING1 in activation of complement pathway. Among them, few proteins were reported as secretory protein such as SerpinG1, immunoglobulin and Collagen alpha-3 (VI) on Uniprot database. Tumor proteins of bladder cancer with higher expression also have more chances to be secreted into the urine; hence they can also be used as a potential biomarker.

Of the upregulated proteins, Sterol-4-alpha-carboxylate 3-dehydrogenase (NSDHL) is NAD(P) dependent steroid dehydrogenase-like, belongs to family of oxidoreductases and participates in biosynthesis of steroids. It was reported to be expressed in tissue of bladder carcinoma along with breast, colorectal, renal and other cancer in Gene-to-System Breast Cancer (G2SBC) database (Mosca *et al*., 2010). NSDHL was found upregulated in human cancer cell line

(lymphoma U-937 cells) by iTRAQ- LC-ESI-MS/MS and associated with lysosomal membrane fluidity and dynamics, particularly cholesterol, sphingolipid and glycosphingolipid metabolism (Parent *et al*., 2009).

FLJ00385 protein is an integral component of membrane. Only basic information like sequence, structure and principal function were documented on Uniprot database but not reported in any tumors. Gao *et al* identified Ig kappa chain V-IV region Len in serum of hepatocellular carcinoma patients (Gao *et al*., 2015). Caldesmon is a calmodulin binding protein and inhibits the ATPase activity of myosin in smooth muscle. It has been used to distinguish between myofibroblasts and smooth muscle cells of the bladder, and also between the smooth muscle cells of the muscularis mucosae and those of the muscularis propria. Overexpression of caldesmon was associated with tumor progression with non-muscle-invasive bladder carcinoma (Council *et al*., 2015).

Collagen, type VI, alpha 3 (COL6A3) binds to extracellular matrix proteins is involved in organizing matrix components is highly expressed in a variety of cancers and causes tumor progression. COL6A3 directly affects tumor cells by acting on the Akt–GSK-3β–β-catenin–TCF/LEF and promoting the protumorigenic factors and inducing epithelial–mesenchymal transition (Chen *et al*., 2008).

Cancer-specific alternative splicing of COL6A3 in colon, pancreatic, bladder and prostate cancer were reported using genome exon array (Thorsen *et al*., 2013). Overexpression of stromal expression of COL6A3 was reported in pancreatic (Arafat *et al*., 2011) and ovarian cancer (Ismail et al., 2000) and colorectal cancer, which was associated with the poor differentiation of tumor cells (Qiao *et al*., 2014). Integrin-linked kinase (ILK) are protein kinases that induces

extracellular signals regulating cell migration, focal adhesion, actin cytoskeletal organization and tumor progression (Persad *et al*., 2014).Overexpression of ILK was reported in human lung cancer and promotes cell migration and invasion, prostate cancer and breast cancer metastasis and tumourigenesis (Zhao *et al*., 2000 and Hinton *et al*., 2008). Deregulation of ILK I was well documented and its inhibition induces the apoptosis in human bladder cancer cells (Hinton *et al*., 2008 and Zhu *et al*., 2012.)

Plasma protease C1 inhibitor (SERPING1) or C1-inhibitor is a protease inhibitor and involved in the regulation of the complement cascade. It inhibits the activated C1r and C1s of the first component of complement system. Deficiency of SERPING1 is well associated with hereditary angio neuroticoedema. Differential expression of this protein was identified in an exploratory study of various cancers (Hummerich *et al*., 2006 and Mosca *et al*., 2010) but not proven as biomarker. Lower level of expression of SERPING1 in breast cancer compared to stable mammary intraepithelial neoplasia was found by microarray analysis (Namba *et al*., 2004.) but similar association has not been reported in bladder cancer.

Heat shock protein beta-6 (HSPB6 or hsp20) is a small heat shock family of proteins and most highly expressed in different types of muscle including bladder, uterine smooth muscle, skeletal muscle, vascular, colonic and cardiac muscle (Salinthone *et al*.). HSPB6 involves in many pathological processes such asstress response, vasodilation, hyperplasia, insulin resistanceand regulation of muscle contraction (Dreiza *et al*., 2010 and Ju *et al*., 2015).However, the function of HSPB6 in tumor development is not well understood. Down-regulation of HSP20 was identified in colorectal cancer and its overexpression in a human colorectal cancer cell line enhanced caspase-3/7 activity and down-regulation of the anti-apoptotic proteins (Bcl-xL and Bcl-2) and causes lymph node metastasis, and tumor recurrence (Ju *et al*., 2015).

Superoxide dismutase 2 (SOD2) binds to the superoxide, byproduct of the mitochondrial electron transport chain or oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen (Becuwe *et al*., 2014). This protein clears mitochondrial reactive oxygen species (ROS) and protects against cell death. It plays an anti-apoptotic role against oxidative stress, inflammatory cytokines and ionizing radiation. SOD2 has cytoprotective effects and its linked to increased invasiveness and tumor metastasis (Pias *et al*., 2003).

Several studies have shown upregulation of SOD2 protein in penile, cervical, gastric/esophageal, colorectal, lung cancer cells when compared to normal tissues (Termini *et al*., 2015; Holley *et al*.,2012;Johnson *et al*., 2005 and Kinnula*et al*., 2004). Overexpression of SOD2 was reported in metastatic bladder cancer cell line compared to non-meastatic bladder cancer cell line (Jin *et al*., 2015). Deregulation of SOD2 causes the alteration in redox pathway and plays a role in tumor progression and metastasis (Hempel *et al*., 2007 and Connor *et al*., 2009).

Calnexin is a chaperone and continuously delivered to the plasma membrane of cell and then internalized for lysosomal degradation. Calnexin was found downregulated in tumor tissue in the present study and it is also reported to be present in exosomes released by bladder cancer (Okazaki *et al*., 2000 and Welton *et al*., 2010).

S100 is a calcium-binding protein P (S100P)and involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation (Bresnick *et al*.,2015). Shiota*et al*., 2011proved that overexpression of S100P was involved in cisplatin sensitivity of bladder cancer cell line.In the present study. We found the downregulation of S100P in bladder cancer compared to normal adjacent mucosa. S100P was well studied as

diagnostic marker in pancreatic cancer, prostate, breast cancer and colorectal cancer (Shiota *et al*., 2011; Zhang *et al*., 2014; Parkkila *et al*., 2008 and Chiang *et al*., 2015).

Lamin was discovered as components of the nuclear matrix, it gives structural support of the nucleus to facilitating chromatin organization, DNA repair and gene regulation. (Dechat *et al*., 2010; Dittmer and Misteli, 2011). The role of lamin B1 in tumor development and progression is not well known. The expression of lamin B1 is reduced in colon cancer, lung cancer, and gastric cancer (Broers *et al*., 1993 and Moss *et al*.,1999), whereas the expression of lamin B1 is increased in pancreatic cancer, prostate cancer and hepatocellular carcinoma (Coradeghini *et al*., 2006; Lim *et al*., 2002 and Sun *et al*., 2010). Lamin A/C transcript variant 1 was found downregulated in bladder tumor as reported in some tumors types, such as breast, ovarian and colon cancers (Capo-Chichi *et al*., 2011; Capo-chichi *et al*., 2011 and Belt *et al*., 2011).

Some proteins such as DKFZp686P17171 (putative uncharacterized protein), S14 (40S ribosomal protein) and FLJ94640 (highly similar to Homo sapiens keratin 18) were found downregulated in bladder tumor in our study, whereas role of these proteins are still not studied in cancer.

Cytochrome b-c1 complex subunit 1 is a component of the ubiquinol-cytochrome c reductase complex, which involved in the mitochondrial respiratory chain (Blakely *et al*., 2005). Downregulation of Cytochrome b-c1 complex subunit was estimated in bladder cancer and similarly found listed in gastric cancer by iTRAQ (Wang *et al*., 2016). ATP synthase (ATP synthase-coupling factor 6 and subunit beta) down-regulation was associated with bladder tumor and similarly found in 5-Fluorouracil treated colorectal carcinomas and causes drug resistance in such patients (Shin *et al*., 2004).

The glycine cleavage system (GCS or glycine decarboxylase complex or GDC) is a series of enzymes that are triggered in response to high concentrations of the amino acid glycine (Kikuchi *et al*., 1973). This complex system is composed of four proteins: the T-protein, P-protein, L-protein, and H-protein. Mitochondrial glycine cleavage system H-protein was found downregulated in our study. The H-protein is responsible for interacting with the three other proteins and acts as a shuttle for some of the intermediate products in glycine decarboxylation (Kikuchi *et al*., 2008). Glycines are essential metabolites for cancer cells and provide precursors for macromolecules (Amelio *et al*., 2014).

Function of transformation-related protein 14 has not been reported in association with cancer. Chaperonin 10-related protein is also known as HSP10 and found downregulated in bladder cancer and also detected in tumors of germ cell origin (Mehta *et al*., 1987).

Lysosome-associated membrane glycoprotein 1 (LAMP-1) also known as CD107a (Cluster of Differentiation 107a) plays a role in tumor cell differentiation and metastasis (Laferte *et al*., 1989). Downregulation of LAMP1 was found in primary urothelial bladder cancer in our study but literature reports its overexpression is seen in metastatic cancers such as melanoma, pancreatic cancer, lung cancer and colon cancer (Castronovo *et al*., 1998; Agarwal *et al*., 2015; Jensen *et al*., 2013 and Künzli *et al*., 2002). Cation-dependent mannose-6-phosphate receptor variant (CD-MPR) is synthesizing Lysosomal enzymes and loss of heterozygosity (LOH) at the CD-MPR locus has been displayed in various cancer types including breast and liver and also seen downregulated in present study (Ghosh *et al*., 2003 and De Souza *et al*., 1995).

NADH dehydrogenase [ubiquinone] 1 alpha 4 transfers electrons from NADH to ubiquinone and is associated with disorder of the mitochondrial respiratory chain (Loeffen *et al*., 1998). Association with cancer has not been reported.

Among the commonly deregulated proteins, 38 proteins were variably deregulated in subgroups of bladder cancer. Some of them have already been reported in bladder cancer as biomarkers. Memon AA *et al* found low expression of S100A11 in association with poor survival in patients with bladder cancer (Memon *et al*., 2005). Similarly we found downregulation in all subgroups of bladder cancer except high grade recurrent bladder tumor. Deregulation of Ras like protein Rab27 was seen at transcriptional level in bladder cancer and also seen in our present study except advanced stage of bladder cancer (Ho *et al*., 2012). Involucrin is a component of the keratinocyte and was found expressed in early and advanced bladder cancer and downregulated in high grade recurrent and non-recurrent groups of bladder cancer. Irregular expression of involucrin by IHC was reported in different types of bladder cancer (Asamoto *et al*., 1989). Fibronectin 1 (FN1) is extracellular matix protein and involved in focal adhesion and upregulated expression was reported in bladder cancer (Ewald *et al*., 2013)

Annexin proteins are well-known Ca(2+) regulated phospholipid- and membrane-binding proteins and also play important roles in tumor development, metastasis and drug resistance. Annexin A3 was found upregulated in high grade non-recurrent tumor whereas A3 has already proven as a biomarker with potential value for Upper tract urothelial carcinoma diagnosis (Lu *et al*., 2014). Apolipoprotein A-I has been proven as a potential biomarker for the diagnosis of bladder cancer and also found upregulated in all subgroups of bladder cancer included in this study except high grade non-recurrent group (Li *et al*., 2014).

Some differentially deregulated proteins such as Tenascin C (TNC) was upregulated in low grade non-muscle invasive and high grade muscle invasive bladder cancer. TNC is an extracellular matrix protein and is involved in cancer prognosis and tumor progression. It has been well studied in tumor tissue of bladder cancer and reported to be of prognostic significance in superficial and invasive bladder cancer (Brunner *et al*., 2004) but its secretion in urine of bladder cancer patients is not studied.

Peroxiredoxin 1 (PRDX1) is a thiol-dependent antioxidant Prx family of enzymes (Rhee *et al*., 2001) that is over-expressed by multiple cancers (kim *et al*., 2007). Elevated Prx1 expression at mRNA in bladder cancer is associated with diminished overall survival and poor clinical outcome (Quan *et al*., 2006) but not discovered as non-invasive biomarkers for bladder cancer. Peroxiredoxin 2 (PRDX2) is also a member of Prx family and discovered as a urinary biomarker for bladder cancer (Chen *et al*., 2010). These antioxidant proteins participate actively in redox regulation and may play a role in recurrence of tumor. Other antioxidant protein SOD2 was upregulated in all subtypes of bladder cancer whereas perioxiredoxin showed an irregular expression pattern. Hence, these antioxidants were selected for further validation in urine sample of bladder cancer to understand their role in recurrent bladder cancer.

Cancer is a disease known to involve dysfunction of multiple biological processes. GO analysis offers the predominance characterization of proteome data into specific subcellular categories and function which gives a direction for further research. Our GO enrichment analysis showed that deregulated proteins are significantly involved in cell part, have catalytic activity and into cellular process.

The ingenuity pathway analysis provides the interaction and fundamental pathways of the input analytes. Interestingly, analysis showed similar network, number of deregulated proteins in each network, pathways in each groups of bladder cancer. Our results show that the top three network of each sub groups of bladder cancer involve common mechanisms and fundamental pathways, such as Cellular Movement, Hematological system, Immune cell Trafficking, Nucleic Acid Metabolism, Small Molecule biochemistry, Development Disorder, Cell Death and Survival, Cellular Development, Cellular Growth and Proliferation, which are implicated in various cancers. These provide a strategy for selection of candidate markers. Hence the common proteins (bold in network table), HSPB6, SOD2 and SERPING1 and three deregulated proteins (Tenascin C, PRDX1 and PRDX2) are further selected for verification and validation. Bladder cancer is common in males and discovery of a candidate biomarker may help to develop a new monitoring strategy. Identifying the PPI interface to discover other molecules has become a new direction for targeted cancer therapy.

Furthermore, these leads from high-throughput data require verification and validation on a large cohort of bladder cancer patients with complete clinical annotation. Urine is the best sample for bladder cancer diagnosis because tumors are in direct contact; cancer cells can directly exfoliate and may also secrete proteins into the urine sample. The areas of further development should include testing of tumor specific biomarker in urine sample and measurement of sensitivity and specificity of individual markers and their association with survival of patients. This will eventually develop a non-invasive biomarker. Such signatures will eventually lead to the development of biomarker with diagnostic and prognostic values which can be applied in clinical practice.

5.4 Conclusion

Large portfolio of differentially expressed proteins of bladder cancer was generated and we believe our analysis provides leads for future study, which may be useful in diagnostic and therapeutic perspectives.

Chapter 6

To identify differentially expressed tumor protein secreted in the urinary exosomes using *in-silico* analysis

Chapter 6

6.1 Introduction

Exosomes are microvesicles shed by vesiculation events from various living cells secreted by most cell types, both cancerous and normal and secreted in biological fluids (Pisitkun *et al*., 2004) and are thought to play important roles in intercellular communications. They are generated via diverse biological mechanisms triggered by pathways involved in oncogenic transformation, microenvironmental stimulation, cellular activation, stress, or death (Welton *et al*., 2010). Biogenesis of the exosome begins with the secretion of signal peptide proteins in the cytoplasm. Intraluminal vesicles (ILVs) are formed through inward budding of endosomes to form multivesicular bodies (MVBs) or multivesicular endosomes (MVE). The MVB fuses with the cellular membrane, and the exosomes are released. MVBs can also be either degraded (by fusion with lysosomes) or recycled (Sascha *et al*., 2006). Although exosomes were originally described in 1983, interest in these vesicles has increased dramatically in the last few years, after the finding that they contain RNA, protein, lipid and other biomolecules (Clotilde *et al*., 2011). It is clear that exosomes have specialized functions and play a key role in intercellular signaling and waste management. It is also well accepted that urinary exosomes (UEs) may be used as a novel biomarker source for early disease detection, classification, prediction severity,outcome and response to treatment (Irena *et al*., 2009). Consequently, there is a growing interest in the clinical application of exosomes as it can potentially be used for prognosis, therapy, and as biomarkers for health and disease. Cell-derived vesicles are released in many and perhaps all biological fluids, including blood (Caby *et al*., 2005), urine (Pisitkun *et al*., 2004), breast milk (Admyre *et al*., 2007), malignant ascites

(Andre *et al*., 2002 and bard *et al*., 2004), amniotic fluid (Asea *et al*., 2008), bronchoalveolar lavage fluid (Admyre *et al*., 2003) and culture medium supernatant in cell cultures (Elham *et al*., 2012 and Munoz *et al*., 2013). The content and secretion of these vesicles are dependent on cellular origin. It may be hypothesized that exosomes present in the urine may be secreted from any of the abdominal and pelvic organs such as the kidney, prostate, ovary, and urinary bladder. Studies were done on identification of exosomal proteins, since the first publication on proteomic profiling of UEs by Pisitkun *et al*., while Mathivanan *et al*., created a database, named ‘‘Exocarta’’ specifically for exosomes (Mathivanan *et al*., 2009). Vesiclepedia database and urinary exosome protein database also contain information about exosomes, but these databases include those proteins which are also in the Exocarta database.

Urinary exosomes may originate from the lining of the urinary tract, including glomerular podocytes, renal tubule cells, and urinary bladder. The number and content of UEs may change over time in association with disorders that affect the urinary system. Most of the membrane and cytoplasmic proteins of tumor cells will be excreted into urine by the process of endo and exocytosis making the microvesicles and exosomes a rich source of cancer-specific proteins (Pisitkun *et al*., 2004; Welton *et al*., 2010 and Anastasiadis *et al*., 2012). Exosomes specifically secreted by the bladder lining or urinary bladder cancer cells into the urine may be the result of the interaction of some specific pathways. Identification of the interacting molecules of these pathways will identify targets of therapeutic interest and prevent tumor progression.

Bladder cancer specific urinary exosomal proteins available on Exocarta database were analyzed using computational tools. Pathway based studies and system biology approach were

performed to identify exosomal proteins specific to urinary bladder cancer. Diffrentially expressed proteins obtained by iTRAQ LC-MS/MS were compared with urinary exosomal proteins of bladder cancer on exocarta database. The present study identifies urinary bladder cancer-specific urinary exosomal proteins and analysis of their interactions revealed proteins which can be used as therapeutic targets.

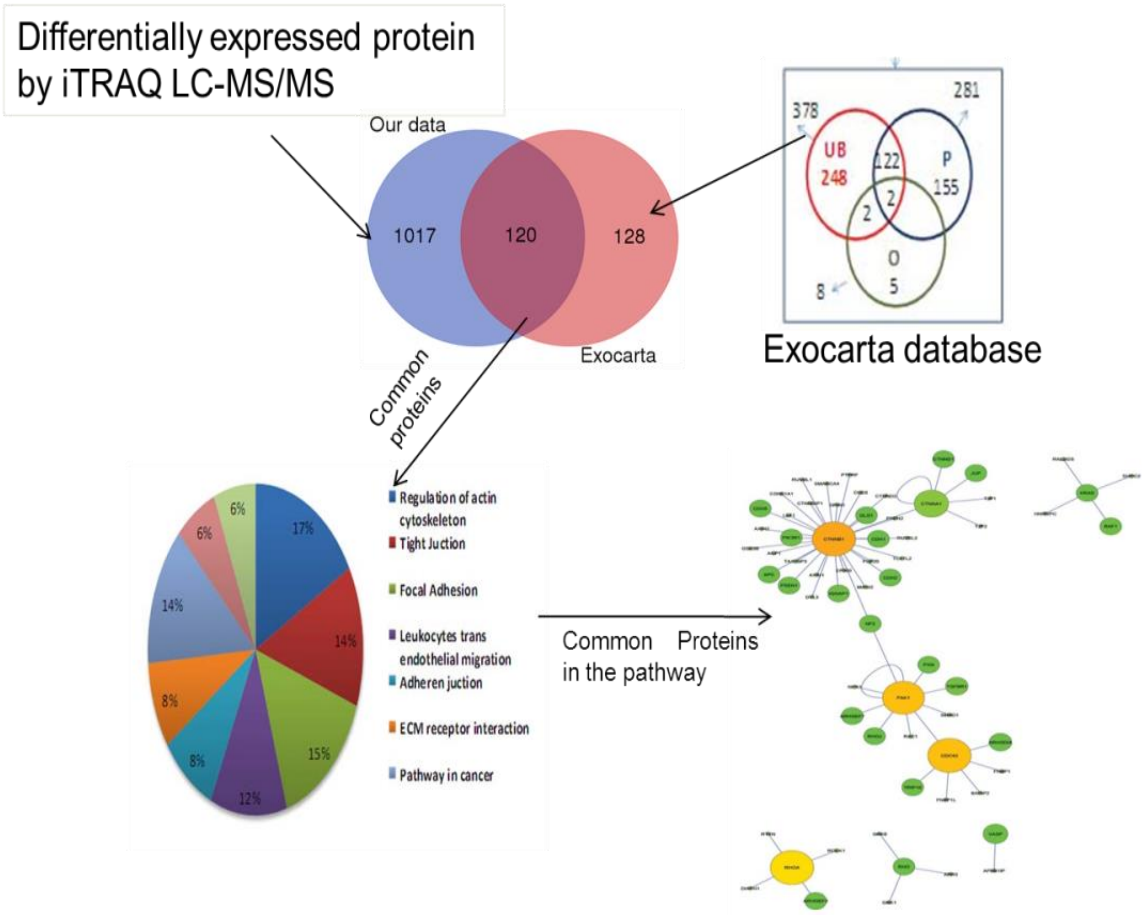


Figure 6.1: Overview Chapter 6

The graphical image showed 120 common protein between iTRAQ LC-MS/MS and Exocarta database. These common proteins were involved into various pathway and protein-protein interaction and inferred proteins.

6.2 Result

A total of 4505 exosomal proteins are reported in the Exocarta database, of which 1152 were reported in the urine and 248 were reported specific to bladder cancer.

The urinary bladder cancer specific exosomal proteins were taken as the seed protein list for identification of signaling pathways involved in exosome formation and secretion. Pathway-Express module from Onto-tools (http://vortex.cs.wayne.edu/ontoexpress) was used to identify the pathways which were most significantly involved. Impact factor was calculated by the Pathway-Express module and 9 Pathways having impact factor >10 were found to be significantly involved (Figure 6.2 and Table 6.1).

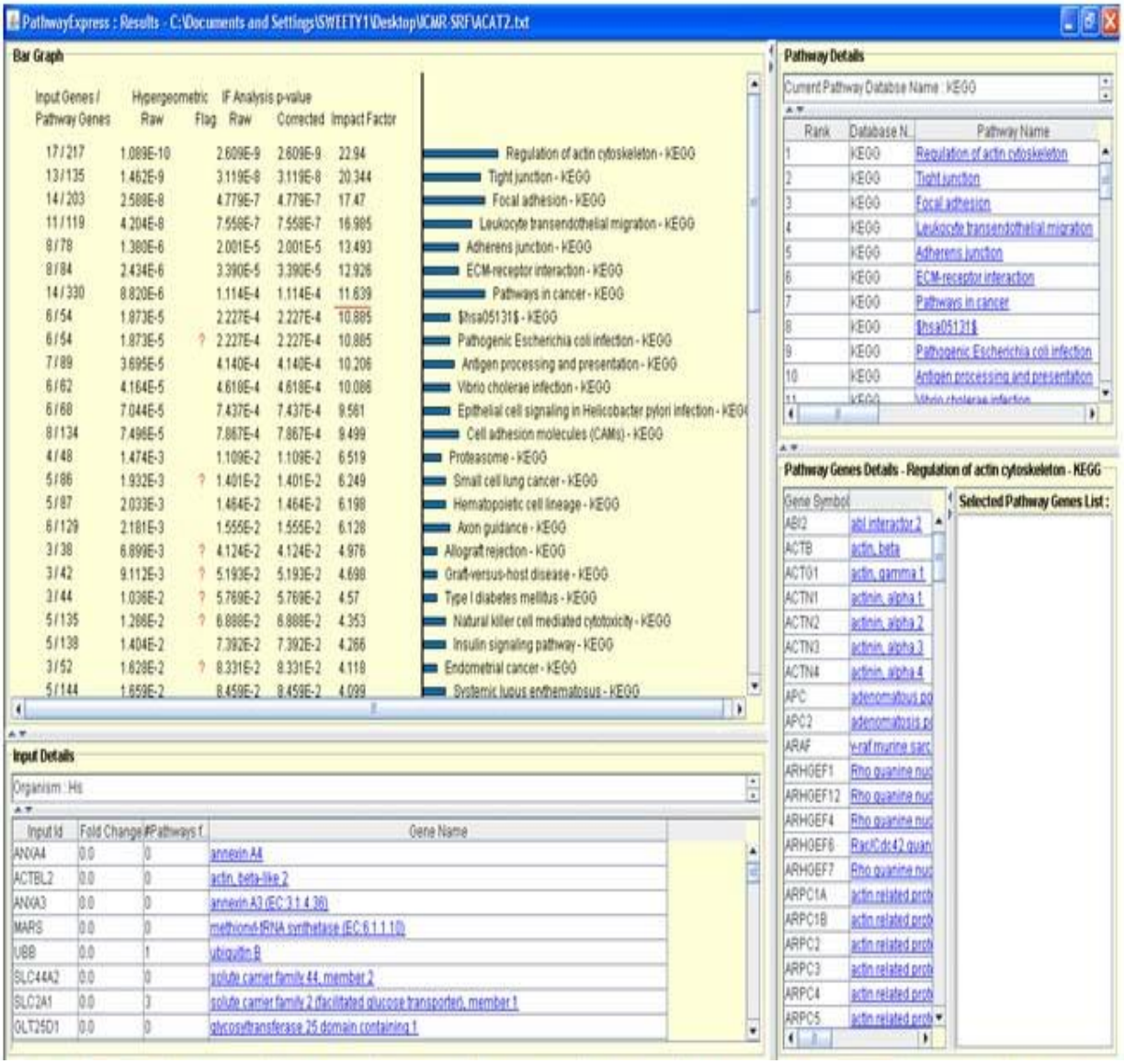


Figure 6.2: Pathway analysis by using the web tool Pathway Express shows the impact factor of involved pathways and the proteins involved in each pathway.

Table 6.1: Pathway enriched by urinary bladder cancer specific exosomal proteins (n=248).

S.No	Pathways	Input protein / Total proteins in pathway	Impact factor	P-value
1	Regulation of actin cytoskeleton	16/217	20.75	2.11E-8
2	Tight junction	13/135	20.34	3.11E-8
3	Focal adhesion	14/203	17.47	4.77E-7
4	Leukocytes endothelial migration	11/119	16.98	7.55E-7
5	Adherens junction	8/78	13.49	2.01E-7
6	ECM receptor	8/84	12.92	3.39E-5
7	Pathway in cancer	14/330	11.63	1.11E-4
8	Shigellosis	6/54	10.88	2.22E-4
9	Pathogenic E coil interaction	6/54	10.88	2.22E-4

Data of iTRAQ LC-MS/MS showed 1137 differentially expressed proteins in bladder tumor compared to adjacent normal mucosa. The common and unique proteins list were identified between iTRAQ LC-MS/MS data (our data; n=1137) and urinary exosomal bladder cancer proteins (exocarta; n=248) and found 120 were common proteins among them (figure 6.3). Among 120 common proteins, we found SERPING1 was upregulated in all stage and grade of

bladder cancer was further validated in FFPE (formalin fixed paraffin embedded) tissue and urine sample in validation phase (chapter 7).

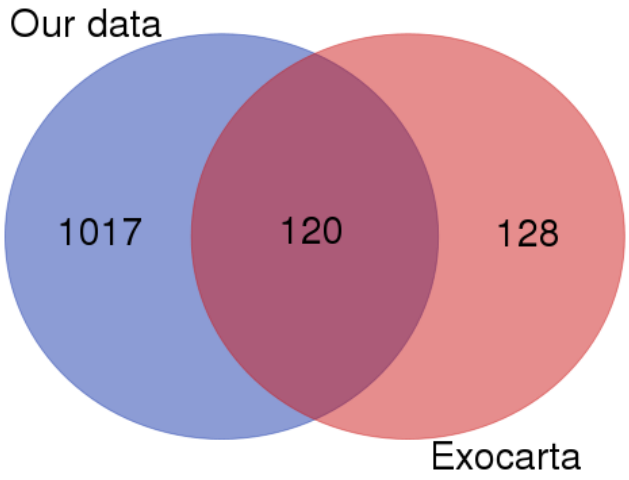


Figure 6.3: Venn diagram showed common and unique proteins of tumor tissue proteome obtained by iTRAQ-LC-MS/MS (our data) and urinary exosomal proteins reported only in bladder cancer (Exocarta database).

Out of 248 bladder cancer specific exosomal proteins, 16 are involved in regulation of the actin cytoskeleton, 13 in tight junction, 14 in focal adhesion, 11 in leukocytes transendothelial migration, 8 in adherens junction, 8 in ECM receptor interaction, 13 in pathways in cancer, 6 in Shigellosis and 6 in Pathogenic Escherichia coli interaction pathways (Figure 6.4). These 9 pathways had a total number of 88 proteins, of which 14 were proteins interacting in more than one of the pathways and included A-catenin/CTNNA1, B-catenin/CTNNB1, Rho,RhoA, cdc42, ECM/MMRN1, Ras/N-Ras, Rac/21/PAK1,RTK/DDR1, VASP MLC/MLC1, ITGA4, PIK3C2A/MLCP and CTTNBP2/Cortactin (Table 6.2).

Among 14 exosomal protein involved in topmost pathway, 9 (CTNNA1,CTNNB1, Rho, RhoA, cdc42, Ras/N-Ras, Rac, VASP and ITGA4) proteins were also found in our differentially expressed bladder tumor tissue protein list.

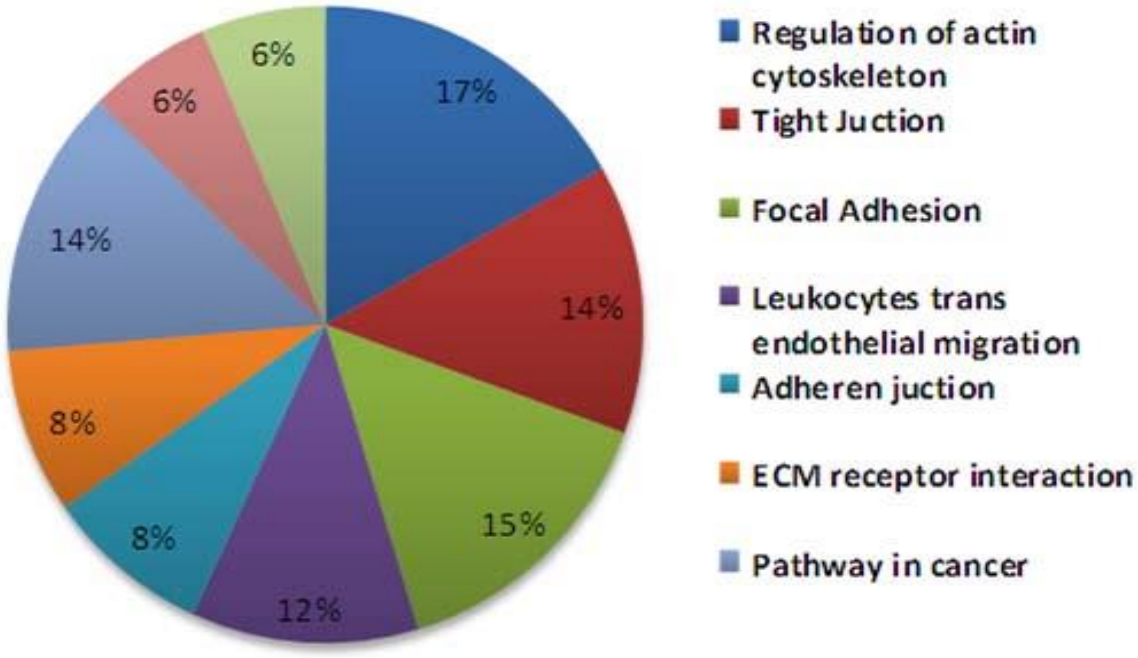


Figure 6.4: Pie diagram showing the percentage of proteins in each pathway involved in bladder cancer.

Table 6.2: List of 14 common urinary bladder cancer specific proteins involved in 9 topmost pathways

Name of Exosomal Proteins (% of most commonly involved)	Regulation of actin cytoskeleton (16/217)	Tight Junction (13/135)	Focal Adhesion (14/203)	Leukocytes trans endothelial migration (11/119)	Adherens junction (8/78)	ECM receptor interaction (8/84)	Pathway in cancer (13/330)	Shigellosis (6/54)	Pathogenic E coil interaction (6/54)
CTNNA1				yes	yes		yes		
3/9= 33.4%									
CTNNB1		yes	yes	yes	yes		yes		yes
6/9=66.7%									
CDC42	yes	yes	yes	yes	yes		yes	yes	yes
8/9=88.9%									
ECM			yes				yes		
2/9=22.3%									
MLC	yes			yes					
2/9=22.3%									
Rac (PAK21)			yes		yes		yes		
3/9=33.4%									
RAS	yes	yes							
2/9=22.3%									
RHO	yes	yes					yes		yes
4/9=44.5%									
RHOA			yes	yes	yes				
3/9=33.3%									
RTK/DDR1	yes		yes						

2/9=22.3%								
VASP		yes	yes					
2/9=22.3%								
MLCP/PIK3C2A	yes		yes					
2/9=22.3%								
ITGA/ITGA4		yes			yes			
2/9=22.3%								
Cortactin/CTTNBP2						yes	yes	
2/9=22.3%								

6.2.1 Protein–protein interaction network analysis and visualization in Cytoscape

The 14 common proteins were used for graphic visualization of the network in Cytoscape. All protein–protein interactions showed one large network by CTNNA1, CTNNB1, VSAP, ITGA4, PAK1, DDR1, CDC42, RHOA, NRAS, RHO and PIK3AR1 and 3 small networks by MLC1, MMRN1, and CTTNBP2. In the large network, CDC42, CTNNB1, RHOA and PAK1 were hub proteins, showed maximum interaction with other proteins (Figure 6.5). Small hubs were formed by proteins VASP, KRAS, ITGA4, DDR1 and RHO, whereas PIK3C2A interacted only with PAK1 and CTNNB1 in a large network through EGFR. Hub proteins of the large network were connected by a number of inferred proteins. Feedback loops were formed by CTNNA1, DDR1, ITGA4, VASP, PAK1, RHOA and CDC42. All protein–protein interactions were complex due to a large number of interactions; hence the network was revisualized as filtered protein–protein interactions (filtered-PPI) (Figure 6.6). It showed 8

proteins in the network among 14 proteins. Among these MLC1, CTTNBP2 and MMRN1 were found to form small individual networks. In filtered- PPI, only 4 hub proteins were present (CTNNA1,CTNNB1, CDC42 and PAK1), in which direct interactions were found between CTNNB1 and CTNNA1; CDC42 and PAK1 whereas PAK1 and B-catenin interacted through inferred protein NF2. A-catenin and PAK1 formed a self loop in the large network. KRAS, RHOA, RHO and VASP formed smaller networks individually.

Cyto Hubba plugin was used to rank the proteins on the basis of four different centrality values – Degree, Betweenness, Closeness and Bottleneck (Table 6.3). B-catenin, CDC42 and PAK1 were ranked as the important proteins on the basis of Degree, Betweenness, Closeness, Bottleneck and Sum rank. Filtered-PPI and centrality value showed B-catenin-PAK1-CDC42 had the highest sum of Degree, Betweenness, Closeness and Bottleneck. All three also interacted both with inferred protein NF2 and A-catenin. Hence, it was found that A-catenin, B-catenin, NF2, Pak1 and CDC42 are the most important proteins.

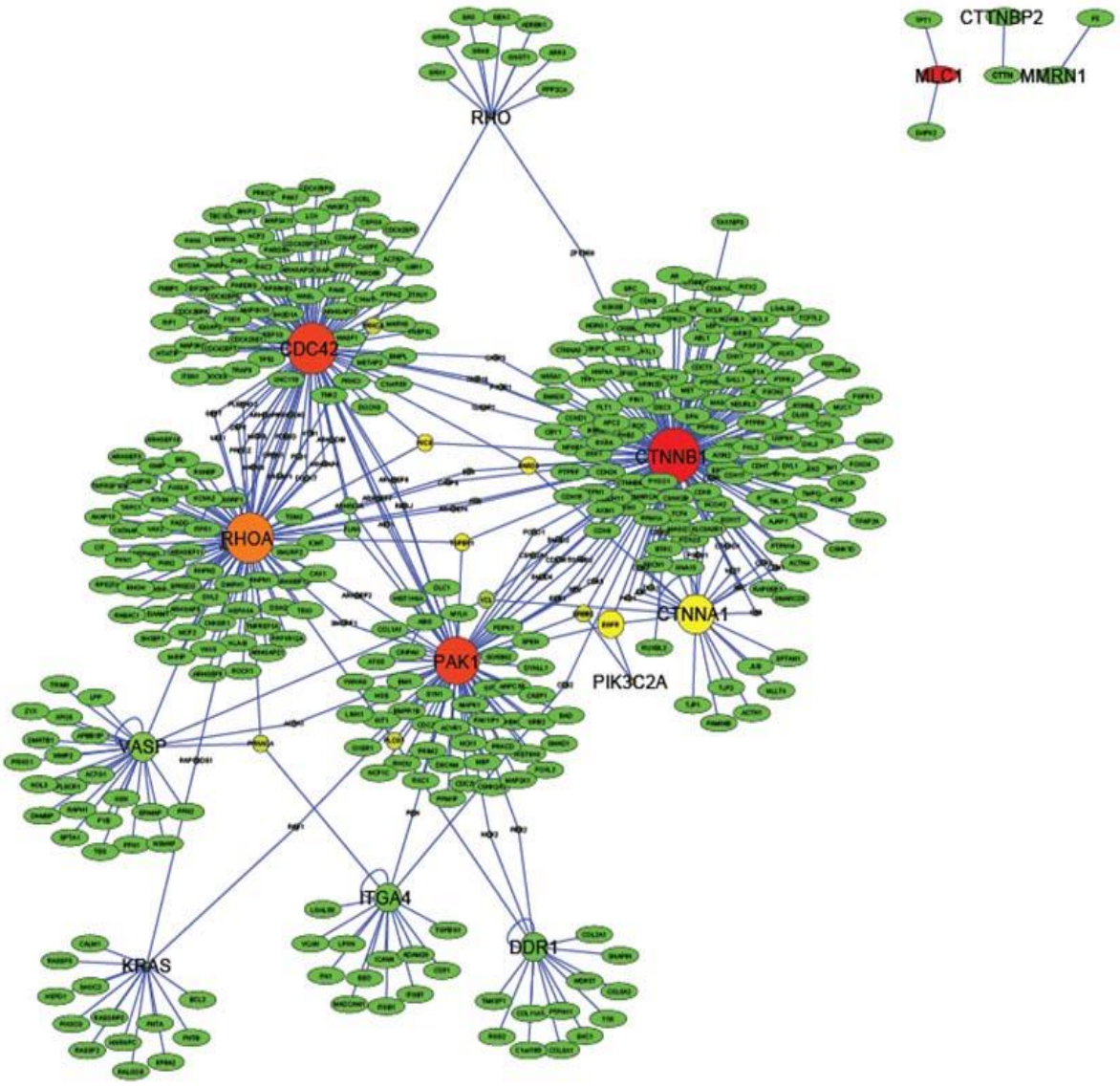


Figure 6.5: Network diagram showing all the interactions of the common exosomal proteins in the involved pathways and their interaction.

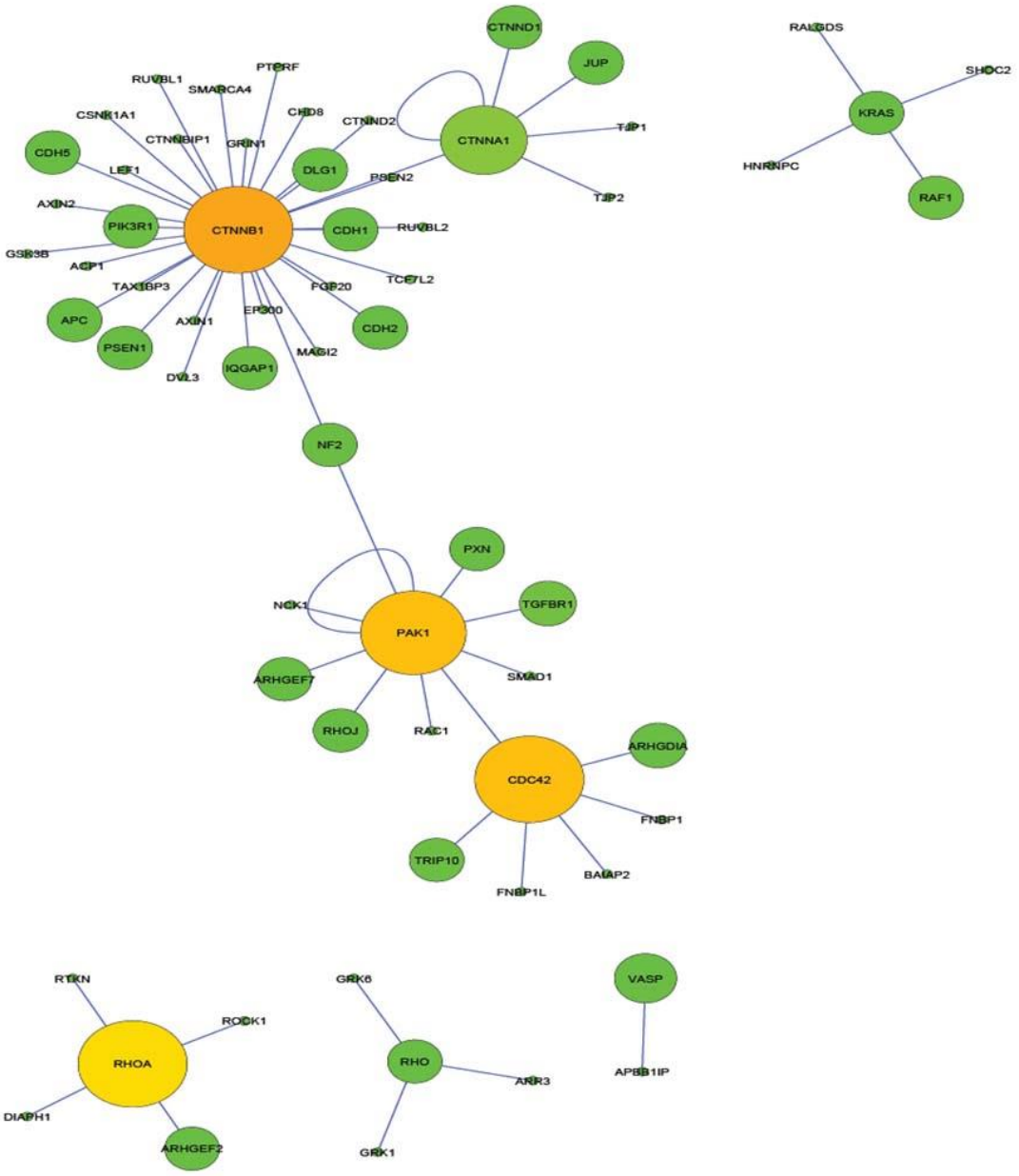


Figure 6.6: Network diagram of the filtered PPI showing hub proteins CTNNB1, PAK1 and CDC42. Inferred protein NF2 is seen between CTNNB1 and PAK1.

Table 6.3: Centrality values of topped 10 proteins ranked by Degree, Closeness, Betweeness, Bottle neck and Sum of centrality score.

Degree	Betweeness	Closeness	Bottle neck	Sum
CTNNB1	CTNNB1	CTNNB1	CTNNB1	CTNNB1
CDC42	CDC42	CDC42	CDC42	CDC42
RHOA	PAK1	PAK1	PAK1	PAK1
PAK1	RHOA	RHOA	RICS	RHOA
VASP	VASP	RICS	ERBB2	VASP
CTNNA1	KRAS	TGFBR1	FAS	CTNNA1
DDR1	ITGA4	PARD3	RHOA	DDR1
ITGA4	DDR1	IQGAP1	FLNA	ITGA4
KRAS	RHO	TRIP10	VCL	KRAS
RHO	RICS	PIK3R1	VASP	RHO

6.3 Discussion

Exosomes contain various molecular constituents of their cell of origin, including proteins and RNA. Exosomes can be take up by tumor cells and it also can transfer molecules from one cell to another. Hence exosome can be used as a source of potential biomarkers for that specific tumor.

Various databases assemble a list of molecules which are reported to be present in exosomes. The analysis of present iTRAQ LC-MS/MS data or differentially deregulated proteins with available exosomal molecules provides an origin of exosome formation and may facilitate development of novel strategies for diagnostics, monitoring, and therapeutics.

Exosomes are highly complex nanometer-sized vesicles that are ubiquitous in biological systems. There is considerable interest to understand the physiological functions of exosomes in various cancers. Few studies of exosomes in various malignancies like prostate, breast, and colorectal cancers, melanoma, pleural mesothelioma, malignancies of the central nervous system, and others have been reported. The proteins associated with bladder cancer diseases could be detected on exosomes isolated from urine, indicating a possible use for urine exosomes as biomarkers (Caby *et al*., 2005; Munoz *et al*., 2013 and Mathivanan *et al*., 2009). For instance, Pisitkun *et al*. demonstrated the excretion of exosomes containing aquaporin-2 protein in autosomal dominant and autosomal recessive nephrogenic diabetes insipidus patients (Pisitkun *et al*., 2004). Similar proteomic studies performed on urinary exosomes generated molecular signatures, illustrating valuable potential for diagnostic, prognostic and pathophysiological discovery. Exosomes secreted by tumor cells are present in the urine and make a microenvironment in the bladder and may come in contact with the normal epithelial lining. While the mechanism of interaction of tumor and normal cells with exosomes is still

not clear, it is possible that exosomes bind with specific ligands to cell surfaces. Upon binding, exosomes can enter target cells in one of two ways: by being taken up by the target cell's endocytic pathway or by fusing to the target cell's membrane and releasing its contents directly into the cytoplasm. Exosomes are increasingly recognized as mediators of intercellular communication due to their capacity to merge with and transfer a repertoire of bioactive molecular content (cargo) to recipient cells (Keller *et al*., 2006 and Inge *et al*., 2008).

Exosomal proteins and gene expression have been reported to be increased in cancers and may be due to the secretion of exosomes at an increased rate by tumor cells undergoing active proliferation and invasion. The acquisition of a motile and invasive phenotype is an important step in the development of tumors and ultimately metastasis. This step requires the abrogation of cell–cell contacts, the remodeling of the extracellular matrix and of cell-matrix interactions, and finally the movement of the cell mediated by the regulation of actin cytoskeleton. The 9 pathways enriched included motility, cell to cell contact, metastasis receptor interaction and tumorigenesis. A-catenin and B-catenin are described to be associated with enhanced cell motility and cancer invasion, especially in patients with a cytoplasmic localization of this protein (Meng *et al*., 2009). These cytoskeleton proteins may provide infrastructure for exosome formation and their cell to cell movement. The tight junction is an intracellular junctional structure that mediates adhesion between epithelial cells and permeability across epithelial cell sheets. Microparticles or exosomes originated from leukocytes can induce differential effects on endothelial function and promote angiogenesis. The adherens junction is an element of the cell–cell junction in which cadherin receptors bridge the neighboring plasma membranes via their homophilic interactions. Cadherins associate with cytoplasmic

proteins, called catenins, which in turn bind to cytoskeletal components, such as actin filaments and microtubules (Yonemura *et al*., 1995). These molecular complexes further interact with other proteins, including signaling molecules. The regulatory mechanism of adherens junction may contribute to normal morphogenetic cell behavior as well as to the pathogenic one, such as cancer invasion and metastasis (Bosman *et al*., 2003). The extracellular matrix (ECM) consists of a complex mixture of structural and functional macromolecules and serves an important role in adhesion, migration, differentiation, proliferation, and apoptosis. 'Pathway in cancer' explains that cell growth and survival are regulated by complex signaling pathways that can be disrupted to cause cancer (Liotta *et al*., 1986). The pathway in cancer regulates cell proliferation, migration, apoptosis, and differentiation and any aberration in the pathway may cause uncontrolled cell growth. Almost 7 pathways explain the exosomal biogenesis, their specific secretion from tumorigenic environment. Proteins involved in Shigellosis and Pathogenic E. coli interaction pathways may explain their presence in urinary exosomes caused by bacterial infection found to occur frequently in these patients (Chairoungdua *et al*., 2010). Network analysis identified interacting proteins and subnetworks. The four proteins involved in the large network included B-catenin which is a component of the cadherin-based adherens junction complexes that form at cell–cell adhesion sites. Intracellular proteins like CD8 and CD9 (a protein present in exosome in almost all lower abdominal cancers), inhibit Wnt signaling and its inhibition reduces the cellular pool of B-catenin by enhancing the exosome-associated export of B-catenin from the cell (Benninger *et al*., 2006). A-catenin is a linking protein between cadherins and actin-containing filaments of the cytoskeleton are involved in the formation of exosomes. These proteins play a major role in the actin cytoskeleton, cell–cell adhesion and

pathways in cancer. A-catenin and B-catenin interact with each other and form a cadherin–catenin complex which further activates and interacts with the known oncoproteins cdc42-rac1-pak1. cdc42 (a Cell division control protein 42 homolog) has a role in cell division in normal conditions, but in adverse conditions it is involved in exosomal complex formation by various pathways. Target molecules for Cdc42 have been identified to be p21-activated kinase. This gene encodes a family member of serine/threonine p21-activating kinases, known as PAK proteins. These proteins are critical effectors that link RhoGTPases to cytoskeleton reorganization and nuclear signaling, and they serve as targets for the small GTP binding proteins Cdc42 (Francis *et al*., and Klose *et al*., 2010). A-catenin regulates cell proliferation by reducing B-catenin transcriptional activity, cdc42 and other oncogenes. It is also reported that loss of B-catenin may cause prostate cancer (Miller *et al*., 2005) and bladder cancer. NF2, interacting between B-catenin and PAK1, has been shown to act as a tumor suppressor primarily through its functions as a cytoskeletal scaffold. It also associates with a downstream effector of Rho small G proteins, which is associated with the formation of stress fibers and cytokinesis (Miller *et al*., 2005). The inferred protein NF2 was not reported in urine and may not be present in exosomes but their presence in cells may be necessary for the formation of exosomes or for cellular proliferation. NF2 causes a genetic disorder called neurofibromatoses that cause tumors to grow along nerves and produce other abnormalities such as skin changes and bone deformities (Miller *et al*., 2005). Exact role of NF2 in bladder cancer patients is still not known but network analysis suggests a crucial role for this protein in the uncontrolled function of oncogene and cell cycle regulators. Targeted therapy at the check points by using drugs or monoclonal antibodies may block the growth and spread of cancer by interfering with specific molecules involved in tumor growth and progression. Bevacizumab (avastin) is

an angiogenesis inhibitor used as a tumor growth inhibitor in colorectal cancer, rectal cancer, metastatic breast and lung cancer in combination with Capecitabine (Cohen *et al*., 2007 and Ferrara *et al*., 2005). The presence of proteins in urinary exosomes likely to act with NF2 may suggest a role for Bevacizumab therapy in bladder cancer. Hence, we can use Bevacizumab therapy that might be useful as a targeted therapy against NF2 in urothelial cancer. However, further functional study to establish the role of this protein in bladder cancer should be accompanied by the role of Bevacizumab in suppressing tumor growth.

6.4 Conclusion

This analysis showed the presence of exosomal proteins alpha-catenin, beta-catenin, PAK1, CDC42 and inferred protein NF2 in the urine to be of importance in the biology of the tumor. However, the importance of these proteins in monitoring and therapy has to be elucidated on a large cohort of clinical samples. Targeted therapy of NF2 with monoclonal antibodies should also be explored for clinical application.

Chapter 7

To detect the presence of the differentially expressed tumour proteins in urine and evaluate the role of urinary biomarkers for recurrence of Urinary Bladder Carcinoma

Chapter 7

7.1 Introduction

Current quantitative proteomics tools (iTRAQ LC-MS/MS) provides large-scale and huge list of proteins. Although these high end techniques provide robust data, but have limited application because of high cost and need for technical expertise. Discovery of an ideal biomarker/s that will be of use in accurate monitoring for recurrence remains challenging. Urinary markers that could be useful alternative in place of or as an adjunct to current diagnosis of bladder cancer and surveillance techniques, or biomarkers that could predict recurrence of disease or used to stratify risk in patients may be beneficial to clinicians for determining surveillance. At present there is no non-invasive urinary marker in clinical practice, which will predict the progression or recurrence of urothelial cancer.

More than 15 known urinary biomarkers with high specificity and/or sensitivity have been identified in literature, and among them few have been approved by the U.S. Food and Drug Administration (FDA) for diagnosis or surveillance of bladder cancer. They are nuclear matrix protein 22 (NMP22) (Yafi *et al*., 2015), bladder tumor antigen (BTA-stat) (Guo *et al*., 2014), BTA TRAK (Yafi *et al*., 2014), fluorescence in situ hybridization- FISH (UroVysion, Sarosdy *et al*., 2002), and fluorescent immunohistochemistry (ImmunoCyt, Dimashkieh *et al*., 2013).

Although none of these biomarker have been able to replace cystoscopy, some are found to be more sensitive than cytology (Yafi *et al*., 2015; Guo *et al*., 2014; Yafi *et al*., 2014). Despite this, urologists are slow to take up these biomarkers replacing cystoscopy or existing detection strategies. The reasons for lack of acceptability are: 1) less specific than gold standard histopathology and gives more false positive result; 2) inablity to differentiate between urinary

bladder cancer and inflammation or other benign (such as BPH) or malignant urologic conditions (such as renal and prostate cancer) that also cause false positives; 3) there are no cut-off values or scoring criteria for developed test. Hence there is a need for the discovery of a biomarker to predict recurrence with more precision. The present study identified six proteins (SOD2, SERPING1, HSPB6, Tenascin C, PRDX1 and PRDX2) in the discovery phase which appeared as potential biomarkers. Their expression and localization was verified in formalin fixed paraffin embebded tissue by immunohistochemistry.

The secretion of these proteins into the urine would make these markers useful urinary biomarkers and hence western blot was performed to detect the presence of these proteins in urine. The proteins found in the urine were then validated by ELISA on larger patient cohort. The identified urinary biomarkers and their cut off concentration was determined to find their association with survival of bladder cancer patients.

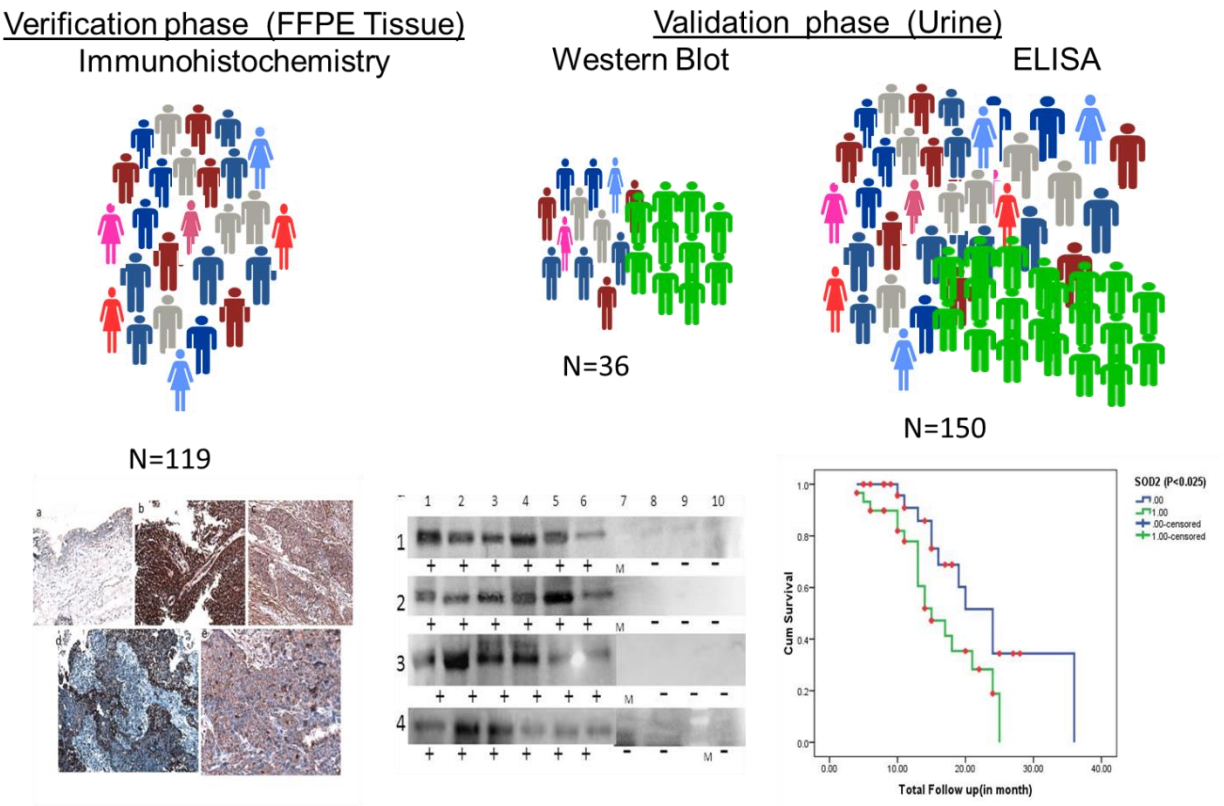


Figure 7.1: Overview of Chapter 7

Identified candidate proteins were verified on FFPE (Formalin fixed paraffin embeded) tissue using immunohistochemistry (IHC). Secretion of these tumor specific proteins in urine were validated by Western blot and ELISA. A representative image of SOD2 localization in FFPE tissue, Western blot and survival plot.

7.2 Result

7.2.1 Immunohistochemistry

Formalin fixed, paraffin embedded (FFPE) tissue from 119 cases of urothelial cancer were used for verification of expression of protein (SOD2, SERPING1, HSPB6, Tenascin C, PRDX1 and PRDX2) in tumor by immunohistochemistry. These cases included LGPT1 (n=53), LGPT2 (n=4), HGPT1 (n=41) and HGPT2 (n=21) and adjacent normal mucosa as non-malignant control from 5 cases for the expression and localization of markers. Hematoxylin and eosin staining were performed on each tissue section to confirm the presence of tumor in the section before immunohistochemistry.

The Immunohistochemical analysis showed expression of SOD2 (98%), SERPING1 (98%), HSPB6 (97%), PRDX1(96%), PRDX2 (96%) and Tenascin C (40%) in urothelial cancer cases and absence of all these protein proteins in normal mucosa except SERPING1 in 40% (2/5) cases (Figure 7.2).

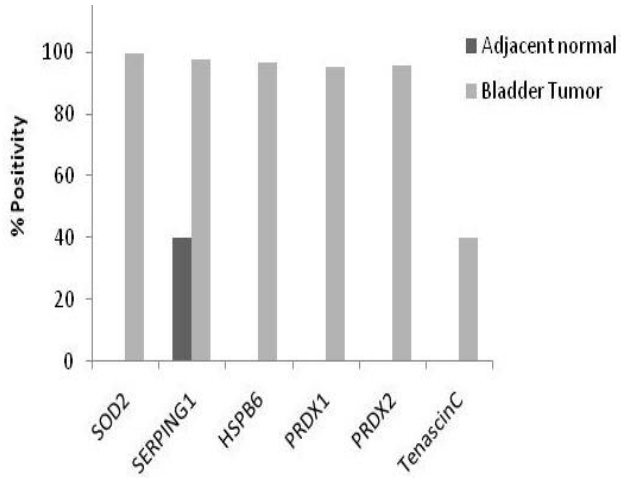


Figure 7.2: Bar graph showing % positivity of SOD2, SERPING1, HSPB6, PRDX1, PRDX2 and Tenascin C in adjacent normal mucosa and bladder tumor

7.2.1.1 SERPING1 (Plasma protease C1 inhibitor)

SerpinG1showed positivity or cytoplasmic and membranous expression in tumor cells in all subgroup of bladder cancer. No significant difference seen between different grade and stage. SerpinG1 was also found positive in lymphocytes along with expression in tumor cells (Figure 7.3). LGPT2 is a rare subgroup of bladder cancer and SerpinG1 was expressed in more than 70% tumor cells in these cases. In other subgroups of bladder cancer most of the cases were scored as 2 (30-70% +ve tumor cells) for SerpinG1 (Figure 7.4).

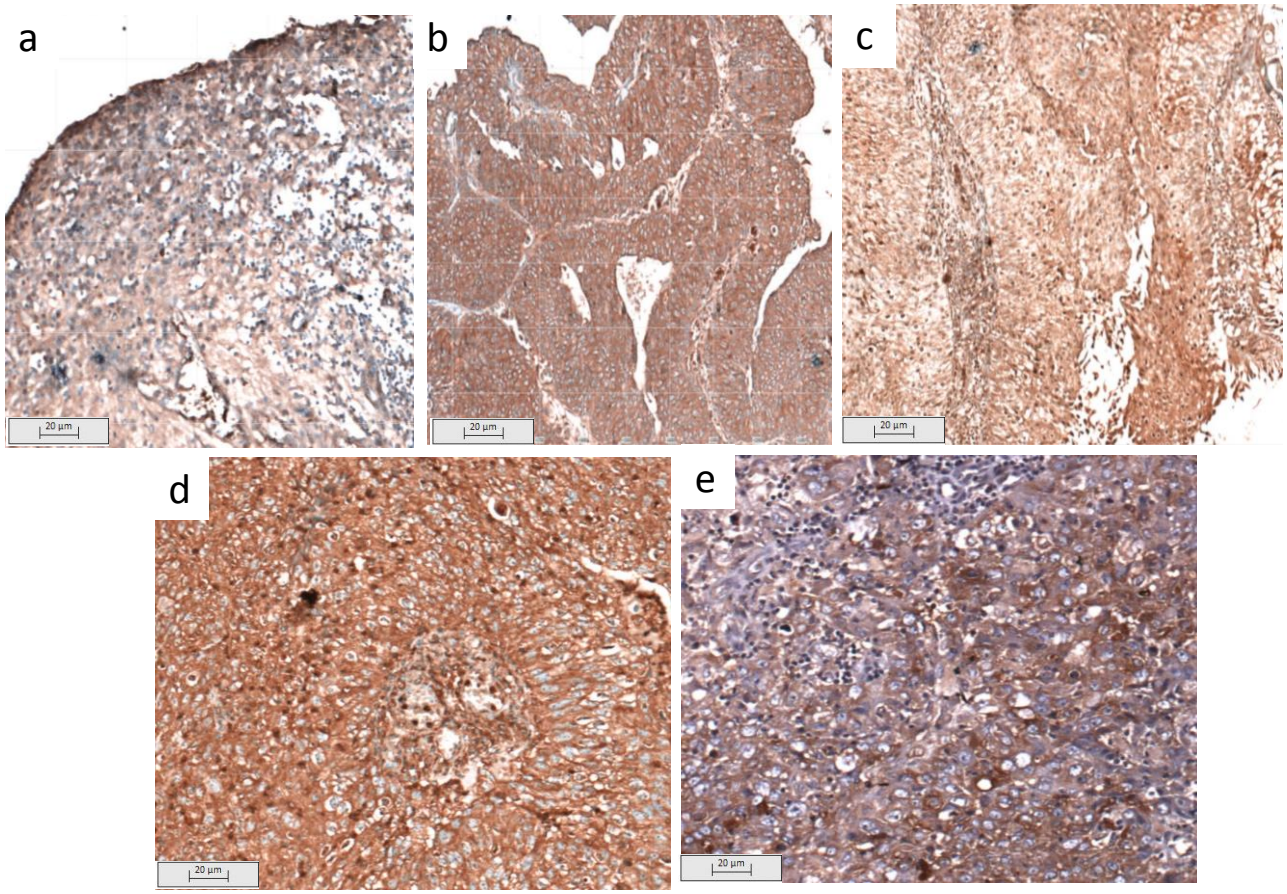


Figure 7.3: SPERPING1 showed positive expression in cytoplasm of a) Normal mucosa, b) LGPT1, c) LGPT2 d) HGPT1 and e) HGPT2 of urothelial bladder cancer captured at 20X using digital imaging.

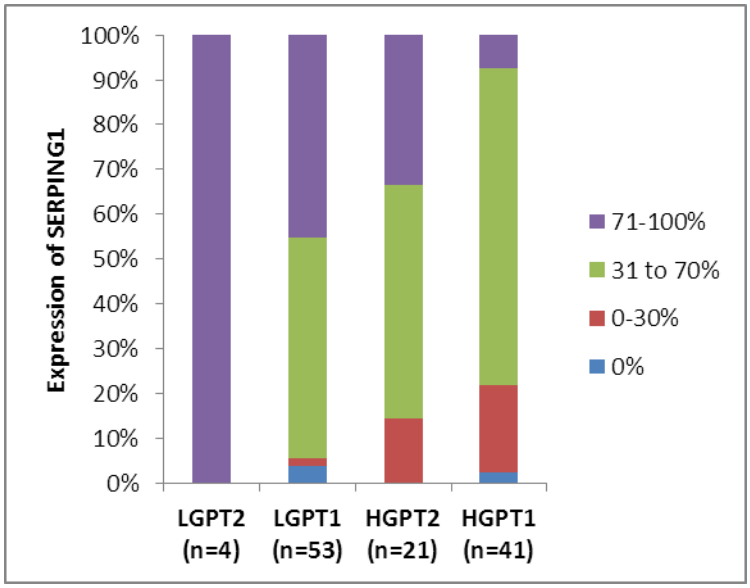


Figure 7.4: Stacked column showed % positive expression of SERPING1 in tumor cells of LGPT2, LGPT1, HGPT2 and HGPT1 of bladder cancer.

7.2.1.2 Heat Shock protein Beta 6 or Heat shock protein 20 (HSPB6 or HSP20)

Heat shock protein beta6 (HSP20/HSPb6), a stress protein showed cytoplasmic and membranous expression in tumor cells (Figure 7.5). Expression of HSPb6 was found in more then 50% tumor cells were positive in all stage and grades of bladder cancer. Approximately 10% cases showed almost 100% positivity (scored as 3) in HGPT1 while 30% positivity (scored as 1) was found in LGPT1 cases of bladder cancer (Figure 7.6). The expression was not found significantly different among the 4 groups. No expression of HSP20 was found in normal mucosal cells.

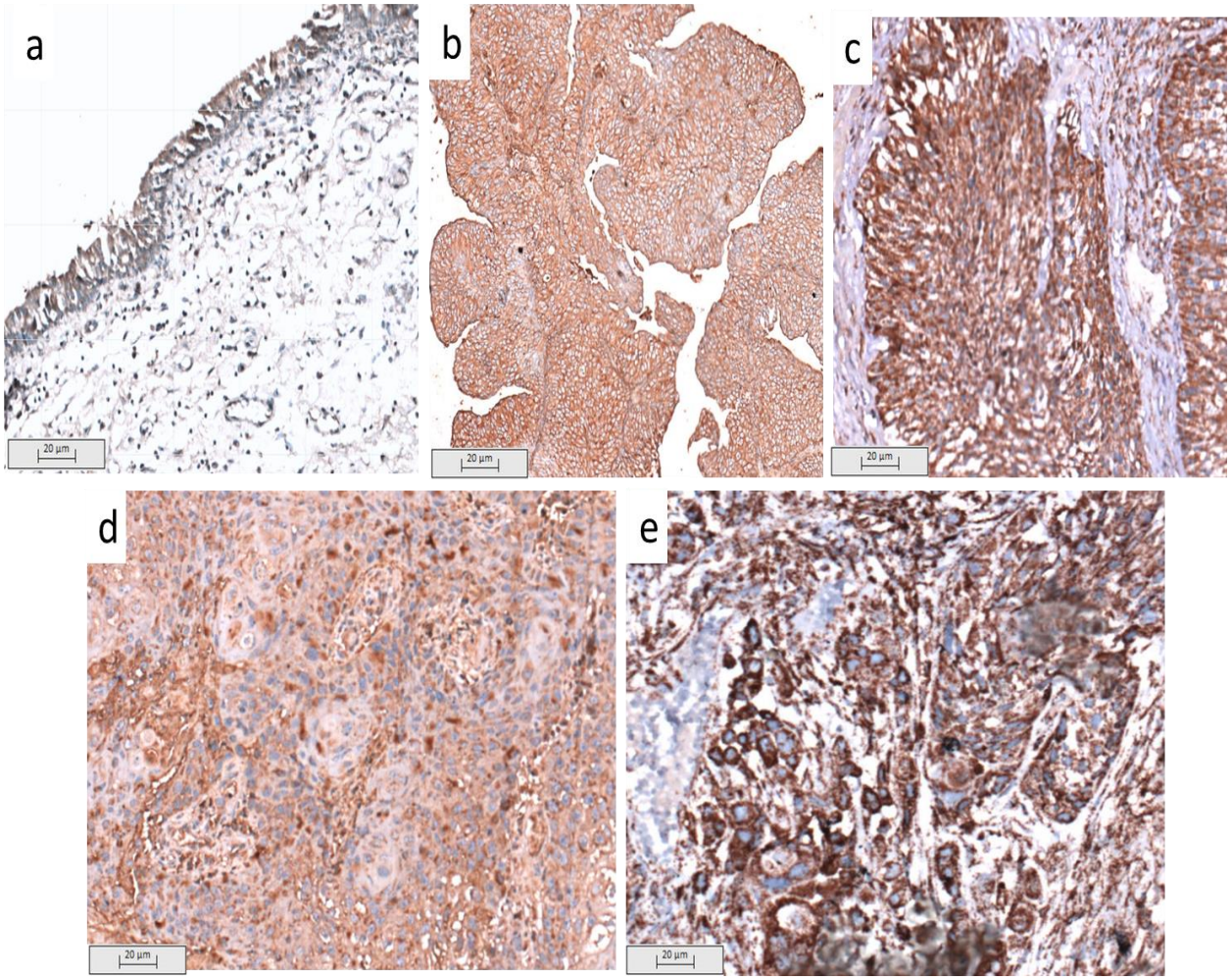


Figure 7.5: Immunohistochemistry of HSPB6 showed a) negative expression in adjacent mucosa and positivity in b) LGPT1 c) LGPT2, d) HGPT1 and e) HGPT2 of urothelial bladder cancer captured at 20X using digital imaging.

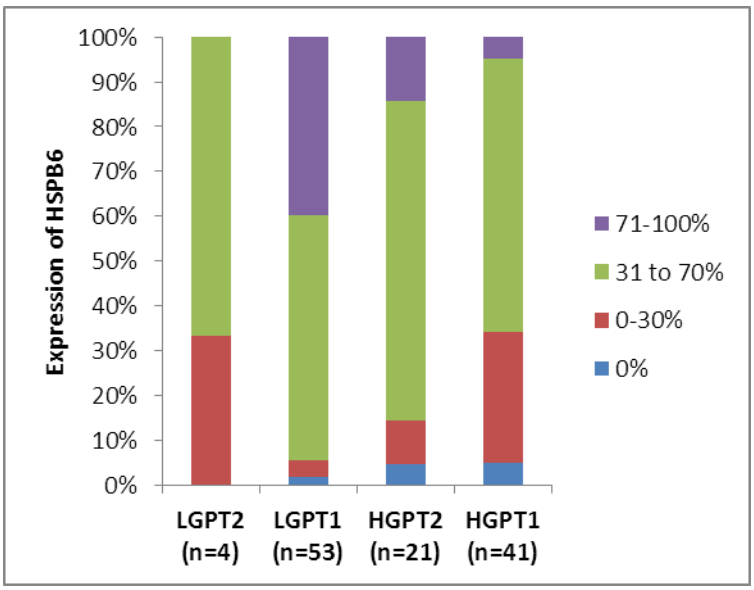


Figure 7.6: Stacked column showed percentage positivity of HSPB6 in LGPT2, LGPT1, HGPT2 and HGPT1 of bladder cancer.

7.2.1.3 Tenascin C (TNC)

Tenascin C expression was not seen in the tumor cells or in normal epithelium but stroma was found positive in Low grade tumors with very high intensity, whereas dispersed and faint intensity was seen in high grade tumor (Figure 7.7). Significantly higher intensity of expression of tenascin C was seen in stroma of high grade tumors ($p<0.001$). Expression of tenascin C was scored 1 in low and high grade muscle invasive tumors whereas the score was 2 in low and high grade non-muscle invasive bladder cancer (Figure 7.8).

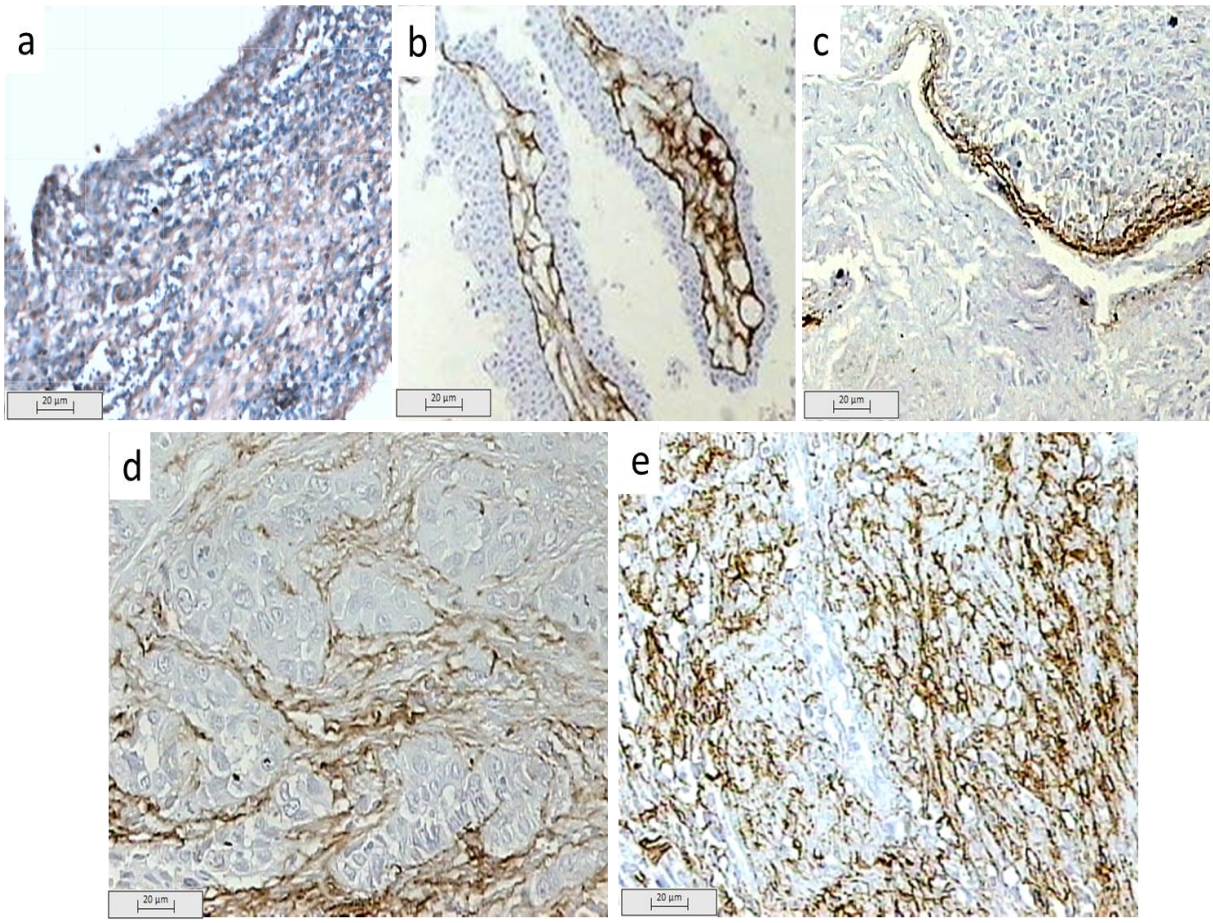


Figure 7.7: A representative image of Tenascin C showed a) no expression in normal mucosa b) stromal staining in low grade tumor with tumor cells negative for TNC c) stromal expression in HGPT1 d) and e) fragmented TNC positive fibers in extracellular matrix positivity in HGPT1 and HGPT2 of bladder cancer respectively.

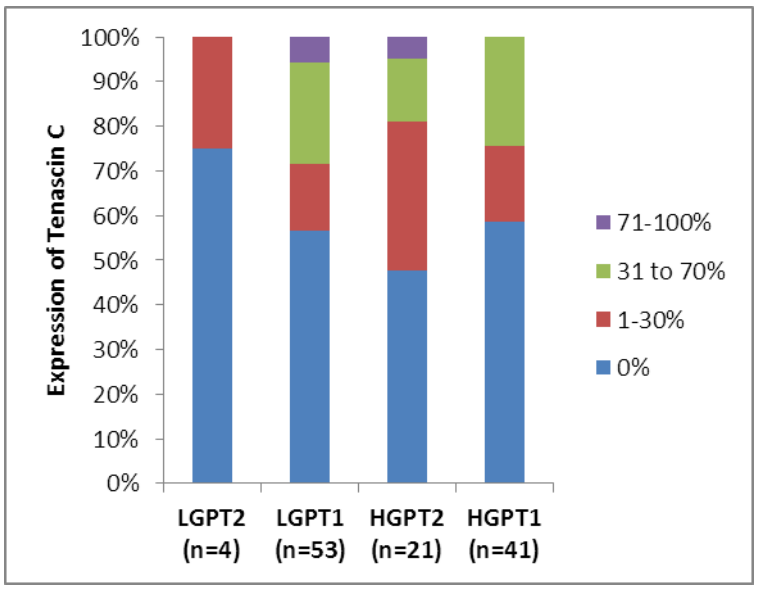


Figure 7.8: Stacked column showed percentage cases positive for Tenascin C in stroma of LGPT2, LGPT1, HGPT2 and HGPT1 of bladder cancer.

7.2.1.4 Peroxiredoxin 1 (PRDX1)

Expression of peroxiredoxin1 was cytoplasmic and membranous in all subgroups of bladder tumor, but no expression was seen in normal mucosa (Figure 7.9). Distribution of percentage positivity in each group of bladder cancer did not show any statistically significant difference. Immunoscoring analysis showed more than 50% of urothelial bladder cancer cases of all stages and grades showed positivity between 50 to 70% and scored as 2 (Figure 7.10).

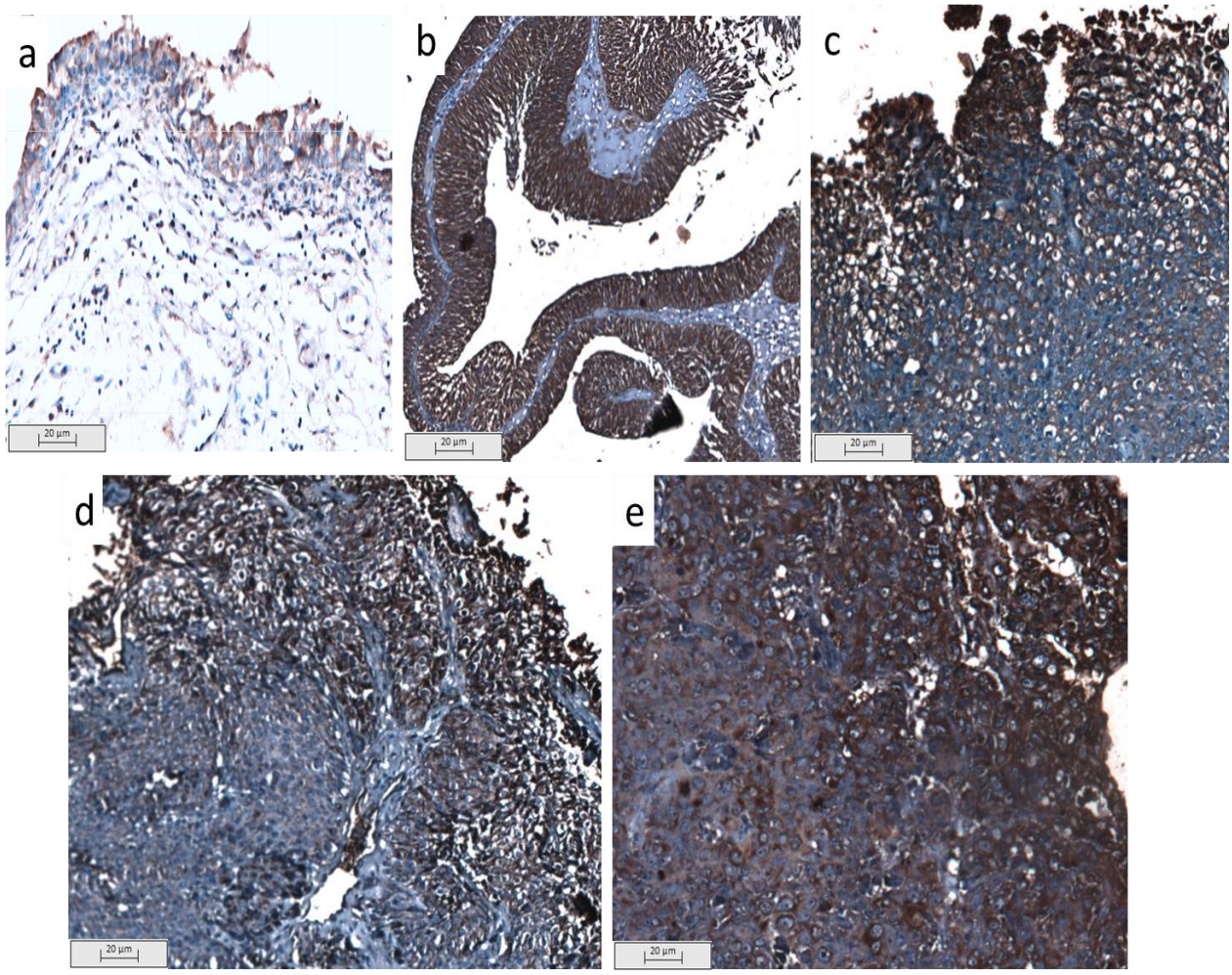


Figure 7.9: A representative image of PRDX1 showed no expression in a) adjacent mucosa and b) cytoplasmic and membranous expression in LGPT1 c) LGPT2, d) HGPT1 and e) HGPT2 of urothelial bladder cancer.

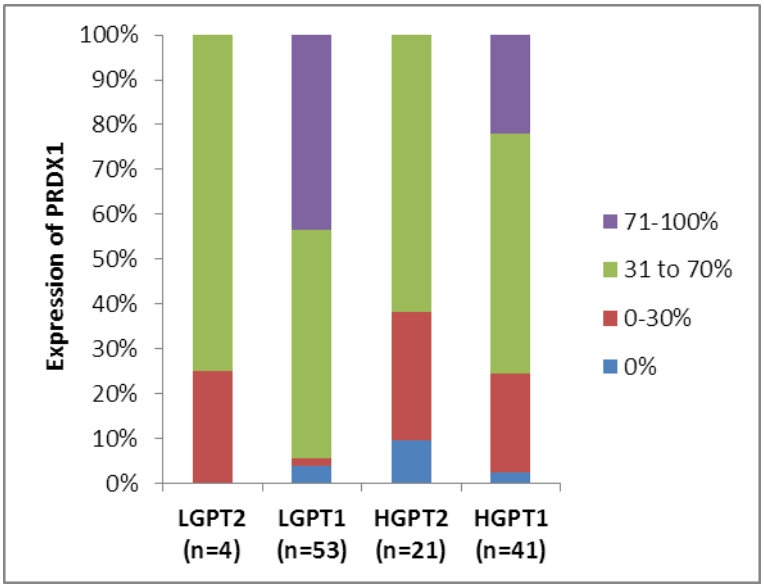


Figure 7.10: Stacked column showed percentage positivity of PRDX1 in LGPT2, LGPT1, HGPT2 and HGPT1 of bladder cancer.

7.2.1.5 Perioxiredoxin 2 (PRDX2)

PRDX2 staining also showed membranous and cytoplasmic expression in tumor cells and no expression in normal mucosa (Figure 7.11). Immunoscoring analysis showed mix distribution of percentage positive tumor cells in subgroups of bladder cancer. Statistical test showed no significance difference between the groups (Figure 7.12).

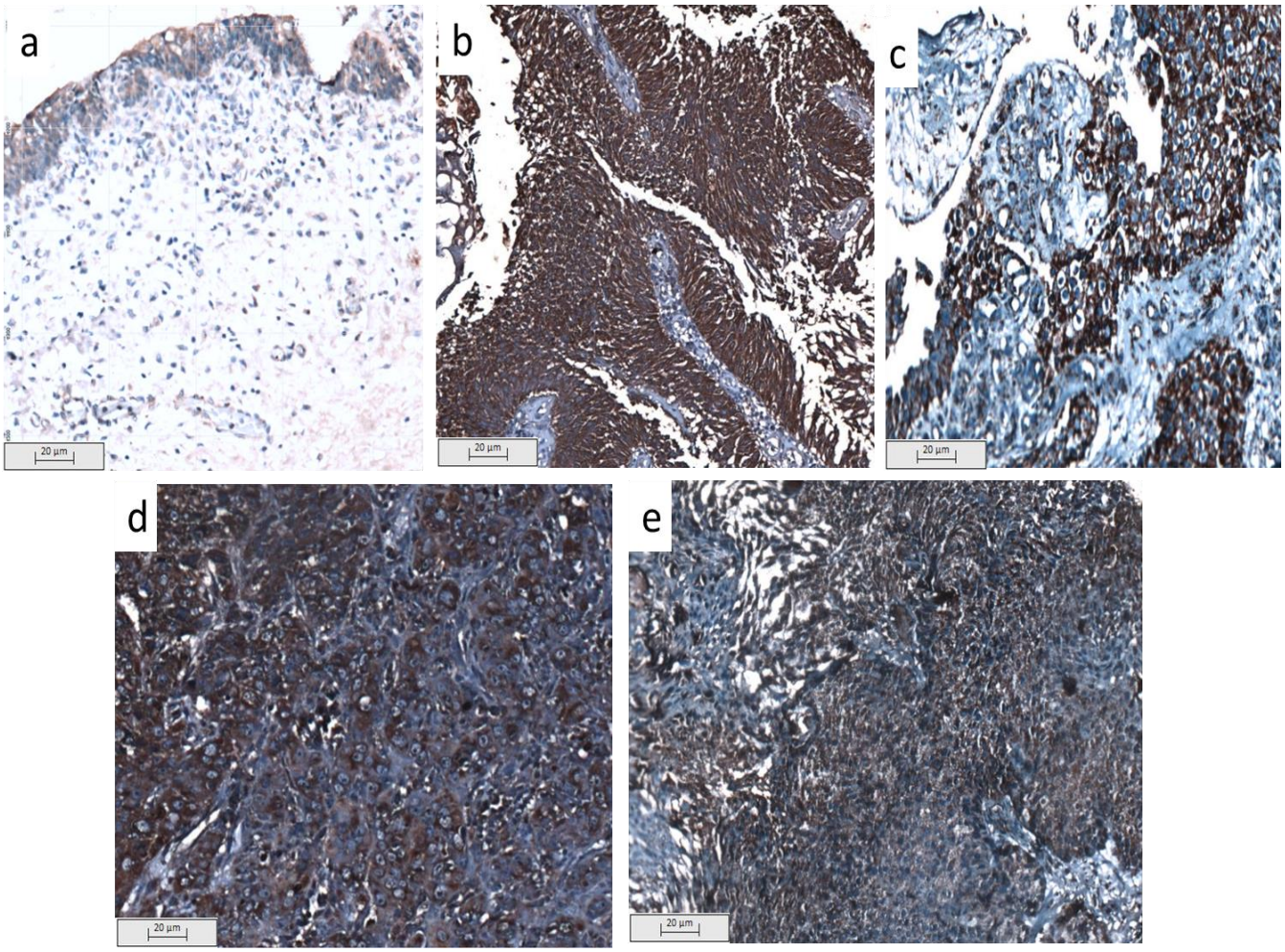


Figure 7.11: A representative image of PRDX2 showed cytoplasmic expression in a) adjacent normal mucosa, b) tumor papillae are positive (LGPT1), c) LGPT2, d) HGPT1 and e) HGPT2 of urothelial bladder cancer.

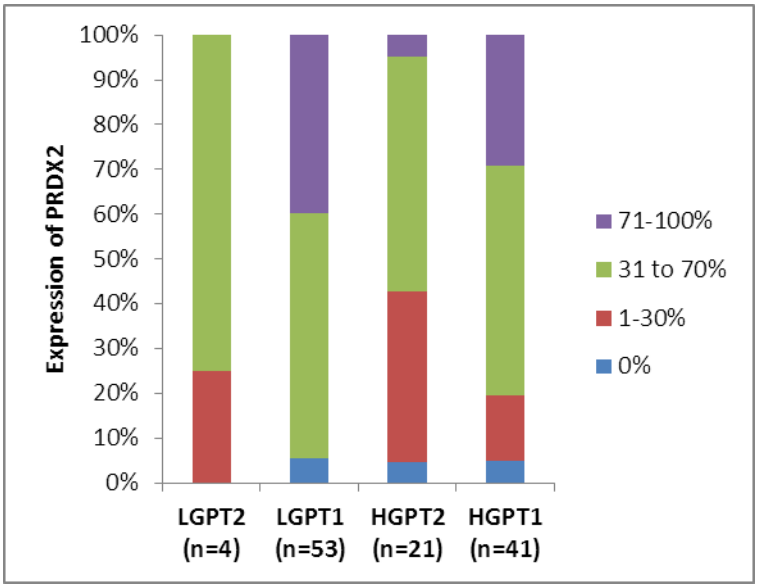


Figure 7.12: Stacked column showed percentage cases positivity for PRDX2 in LGPT2, LGPT1, HGPT2 and HGPT1 of bladder cancer.

7.2.1.6 Superoxide dismutase 2 (SOD2)

Cytoplasmic and membranous expression of Superoxide dismutase 2 (SOD2) was seen in all grades and stage of bladder cancer. It showed expression in the invasive front of tumor in HGpT2 but there is no difference in localization in low grade tumor (Figure 7.13). Immunoscoring showed highest percentage positive cells (30-70% groups) and scored as 2 of all subgroups of bladder cases. Most of the cases showed more than 30% positive tumor cells in all grades and stages of bladder cancer but no statistical significance were found between them (Figure 7.14).

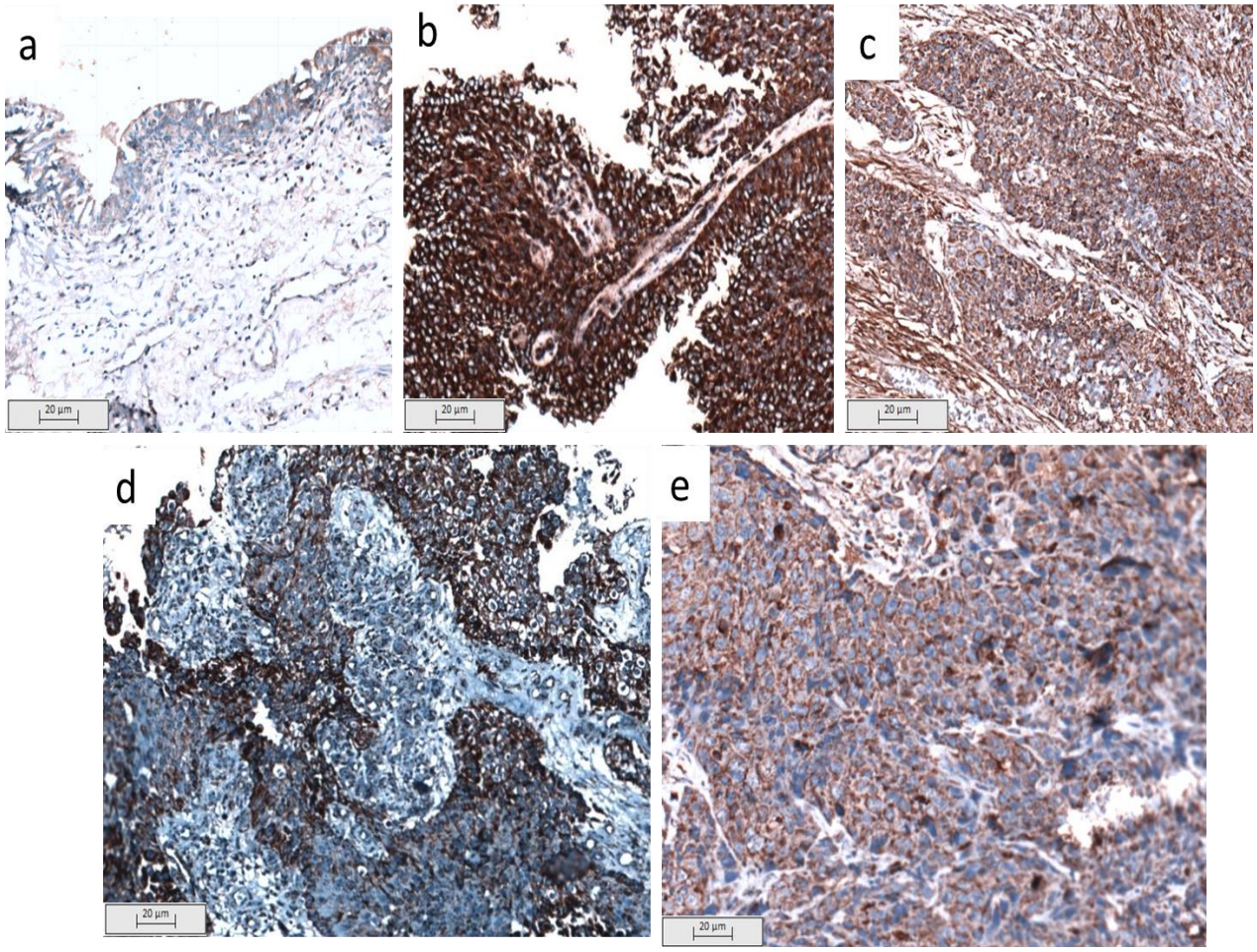


Figure 7.13: Immunohistochemistry for SOD2 showed negative expression in a) adjacent normal mucosa, b) tumor papillae are positive (LGPT1), c) LGPT2, d) HGPT1 and e) HGPT2 of urothelial bladder cancer.

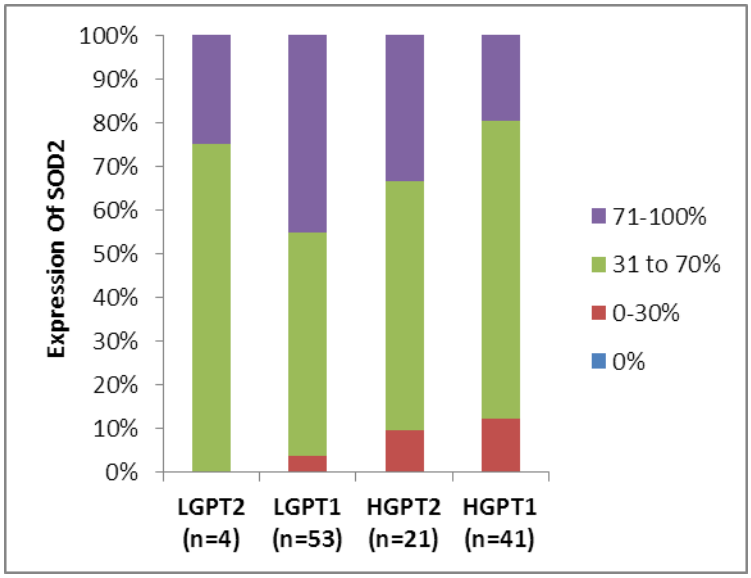


Figure 7.14: Stacked column showed % positivity of SOD2 scored as 1, 2 and 3 (increasing order of % of tumor cells) in LGPT2, LGPT1, HGPT2 and HGPT1 of urothelial bladder cancer

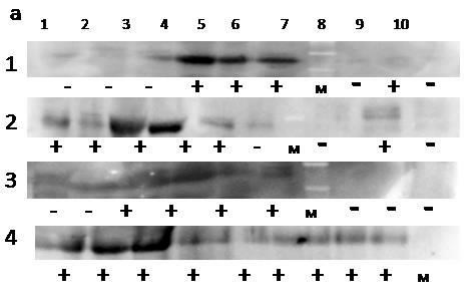
7.2.2 Western blot of urine samples

Urine sample from 26 cases of urothelial cancer and 10 non-malignant urine were used for confirming the presence of proteins in urine by Western blot.

Western blotting for all markers was done in 4 blot images where blot no 1 to 3 include bladder cancer patient urinary protein from well 1 to 6, protein marker (M) in well 7 and non-malignant urinary protein in wells 8 and 9. Blot no 4 includes 8 patients urinary protein (well 1 to 8), protein marker in well 9 and protein of non-malignant subject in well 10. Positive bands of western blot are shown as symbol "+" and negative band as symbol "-" for all the proteins tested.

7.2.2.1 Urinary SERPING1

Western blot analysis showed a band of 55kD molecular weight in 21 out of 26 bladder cancer urine samples and faint band in 2 out of 10 non-malignant urine sample, as depicted in Figure 7.15 Both sensitivity and specificity of SerpinG1 was calculated to be 80%, with histopathology taken as gold standard.



b

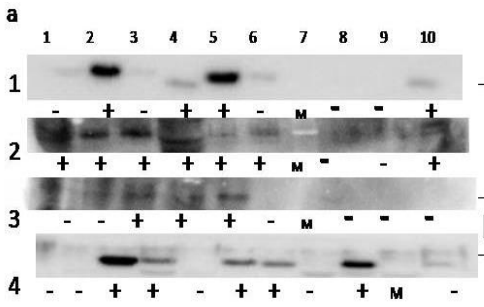
	Sensitivity (True positive) %	Specificity (True negative) %
SERPING1	80	80

Figure 7.15: Urinary SerpinG1 western blot analysis and Sensitivity and specificity

a) Western blot analysis showed in 4 blot images whereas blot no 1 to 3 have bladder cancer patient urine from well 1 to 6, 7 having protein marker (M) and 8 to 9 wells have non-malignant urinary protein. Blot no 4 had 8 patients urinary protein (well 1 to 8), protein marker in well 9 and protein from non-malignant subject in well 10 were tested for SerpinG1 proteins. It showed 21 positive bands (+) out of 26 total urinary patients sample and 2 positive and 8 negative (-) non-malignant urine samples for SerpinG1 urinary protein and b) Bladder cancer patients showed 80 % sensitivity and 80% specificity for urinary SerpinG1 protein as histopathology was gold standard.

7.2.2.2 Urinary HSPB6

Western blot analysis of urinary 20KD HSPB6 proteins showed positivity in 17 bladder cancer patients out of 26 and 2 non-malignant urine sample showed faint positive among 10 non-malignant subjects. Sensitivity and specificity values for HSPB6 were 65 % and 80 % respectively (Figure 7.16).



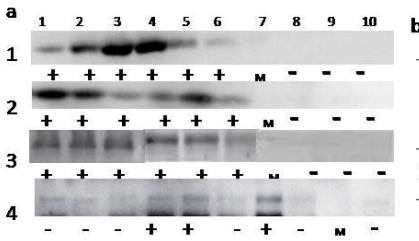
	Sensitivity (True positive) %	Specificity (True negative) %
HSPB6	65	80

Figure 7.16: Urinary HSPB6 western blot analysis and Sensitivity and specificity

a) Western blot showed 17 positive in bladder cancer patient urine and 2 in non-malignant urine sample, respectively and b) found only 65% sensitivity and 80% specificity urinary HSPB6 biomarker.

7.2.2.3 Urinary PRDX1

Western blot analysis showed a band of 21kD molecular weight in 21 out of 26 bladder cancer urine samples and negative band was seen in all non-malignant urine samples, as depicted in Figure 7.17. Sensitivity and specificity of PRDX1 was 80% and 100% respectively.



	Sensitivity (True positive) %	Specificity (True negative) %
PRDX1	80	100

Figure 7.17: Estimation of urinary PRDX1 by Western blot

a) Urinary proteins were found positive in 21cases out of 26 bladder cancer patients and all 10 non-malignant urine samples were negative by western blot analysis, **b)** found 80% sensitivity and 100% specificity for urinary PRDX1.

7.2.2.4 Urinary PRDX2

Western blot analysis showed a band of 22 kD molecular weight in 21 out of 26 bladder cancer urine samples and negative band was seen in all non-malignant urine sample, as depicted in Figure 7.18. Sensitivity and Specificity of PRDX2 was 80% and 100% respectively.

	Sensitivity (True positive) %	Specificity (True negative) %
PRDX2	80	100

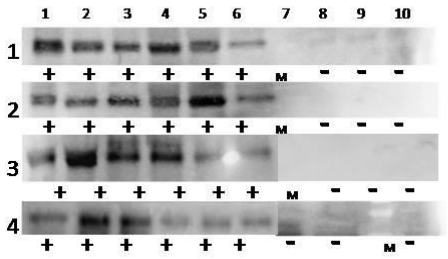
Figure 7.18: Estimation of urinary PRDX2 by Western blot

Urinary proteins were found positive in 21 cases out of 26 bladder cancer patients and 10 cases were negative out of 10 non-malignant urine sample by western blot analysis, b) found 80 % sensitivity and 100% specific for urinary PRDX2

7.2.2.5 Urinary Superoxide dismutase (SOD2)

Western blot detected SOD2 protein (25KD) in 24 out of 26 urine samples of bladder cancer patients. The SOD2 protein is detected in the urine of patients but was not present in non-malignant urine. Urine sample of all histopathologically positive bladder cancer patients showed 92.3 % sensitivity and 100% specificity of urinary SOD2 (Figure 7.19).

a



b

	Sensitivity (True positive) %	Specificity (True negative) %
SOD2	92.3	100

Figure 7.19: Estimation of urinary SOD2 by Western blot

a) Urinary proteins were found positive in 24 cases out of 26 bladder cancer patients and 10 cases were negative out of 10 non-malignant urine sample by western blot analysis, b) found 92.3 % sensitivity and 100% specific for urinary SOD2.

7.2.3 Estimation of Urinary protein by ELISA

The proteins which were detected to be high sensitivity and specificity by Western blot in urine, was further estimated in urine of bladder cancer and non-malignant control by ELISA. Urine samples of 100 urothelial bladder cancer (41 cases were recurrent) and 50 non-malignant urine sample were included in this phase. SERPING1 was positive in both tumors and inflammatory cells and hence was deemed nonspecific as a marker. Tenascin C was positive only in the stroma and not in the tumor cells. HSPB6 showed a low sensitivity and specificity and hence was not considered a good marker. PRDX1, PRDX2 and SOD2 markers showed high sensitivity and specificity, hence was considered for ELISA.

7.2.3.1 Urinary PRDX1

Levels of PRDX1 protein was quantified in urine of 100 cancer patients and 50 non-malignant controls by ELISA. Concentration of urinary PRDX1 were quantified and median concentration is 29.4 ng/ml and found significant elevation of urinary PRDX1 in bladder cancer patients compared to non-malignant urine using a commercial ELISA kit ($p < 0.001$). Median concentration of urinary PRDX1 was higher in urine sample of recurrent bladder cancer compared to primary bladder cancer patient but there is no significant difference (Figure 7.20 a and b)

Median concentration (29.4 ng/ml) of urinary PRDX1 was taken as cut off value and Kaplan Meier survival analysis was done. The concentration of urinary PRDX1was not associated with survival (Figure 7.20c).

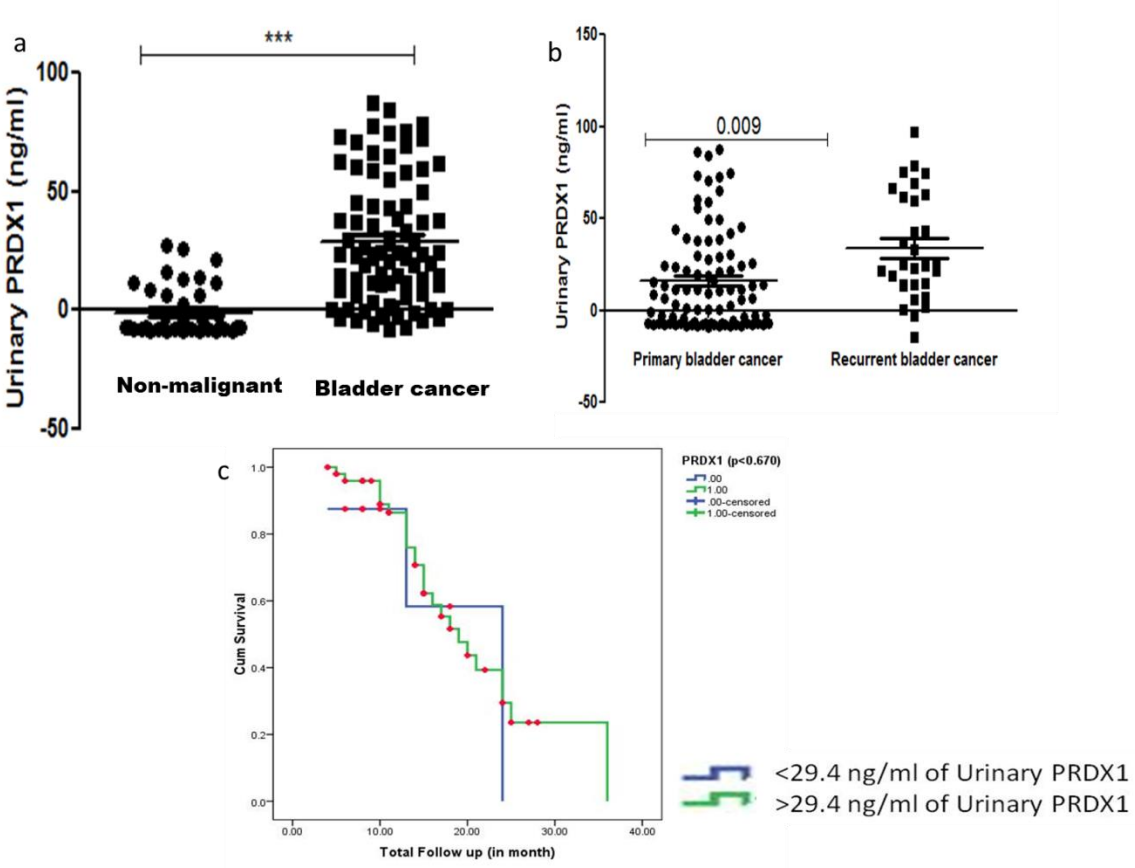


Figure 7.20: Estimation of urinary PRDX1 by ELISA

a) Urinary concentration of PRDX1 was calculated in urine sample and found significantly elevated urinary conc. of PRDX1 in urine of bladder cancer patient compared to non-malignant control (p-value< 0.001, calculated by Mann Whitney U test) b) Elevated urinary concentration of PRDX1 was found in urine sample recurrent bladder compared to primary bladder cancer and b) Kaplan Meier analysis showed concentration of PRDX1 (29.4 ng/ml) was not associated with survival.

7.2.3.2 Urinary PRDX2

Levels of PRDX2 protein was quantified in urine of 100 cancer patients and 50 non-malignant controls by ELISA. Concentration of urinary PRDX2 were quantified and found significant elevation of urinary PRDX2 in bladder cancer patients compared to non-malignant urine using a commercial ELISA kit ($p < 0.001$). Significant elevation of urinary PRDX2 in recurrent bladder cancer compared to primary bladder cancer patients ($p=0.003$) (Figure 7.21 a and b).

Median concentration (27.94 ng/ml) of urinary PRDX2 was taken as cut off value and Kaplan Meier survival analysis showed lower concentration (<27.94 ng/ml) of urinary PRDX2 was associated with recurrence and poorer survival of bladder cancer patients though it was not statistically significant (Figure 7.21c).

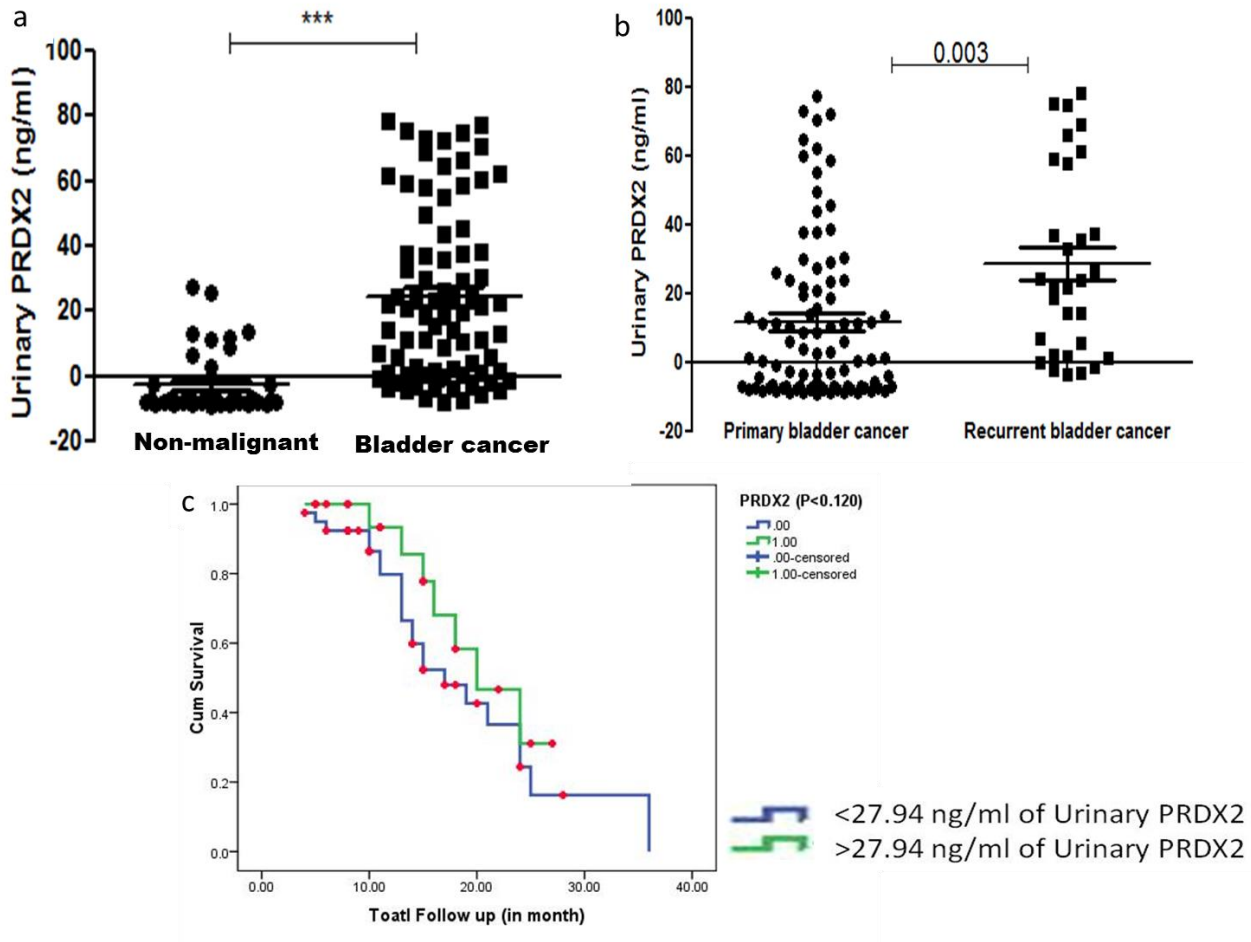


Figure 7.21: Estimation of urinary PRDX2 by ELISA

a) Urinary concentration of PRDX2 was found significantly elevated bladder cancer patient compared to non-malignant control (p-value < 0.001, calculated by Mann Whitney U test) b) Significant elevation in concentration of urinary PRDX2 in recurrent bladder cancer compared to primary bladder cancer (p-value 0.003) and c) Kaplan Meier analysis showed lower concentration (<27.94 ng/ml) of PRDX2 associated with recurrence and poorer survival of bladder cancer patients (log-rank t-test, P<0.125).

7.2.3.3 Urinary Superoxide dismutase (SOD2)

Quantification of SOD2 protein levels in urine from patients and controls was performed by ELISA. Significant elevation of Urinary SOD2 was found in bladder cancer patients compared to non-malignant controls ($p < 0.001$). Median concentration of urinary SOD2 was significantly higher in urine sample of recurrent bladder cancer compared to primary bladder cancer patient (Figure 7.22 a and b).

Urinary SOD2 (Median concentration of bladder cancer patient; 2100pg/ml) was taken as cut off value and Kaplan Meier survival analysis showed that higher than 2100pg/ml of urinary SOD2 was significantly associated with recurrence of patient or poorer survival and p-value was calculated by log-rank t-test, $p<0.025$ (Figure 7.22 c).

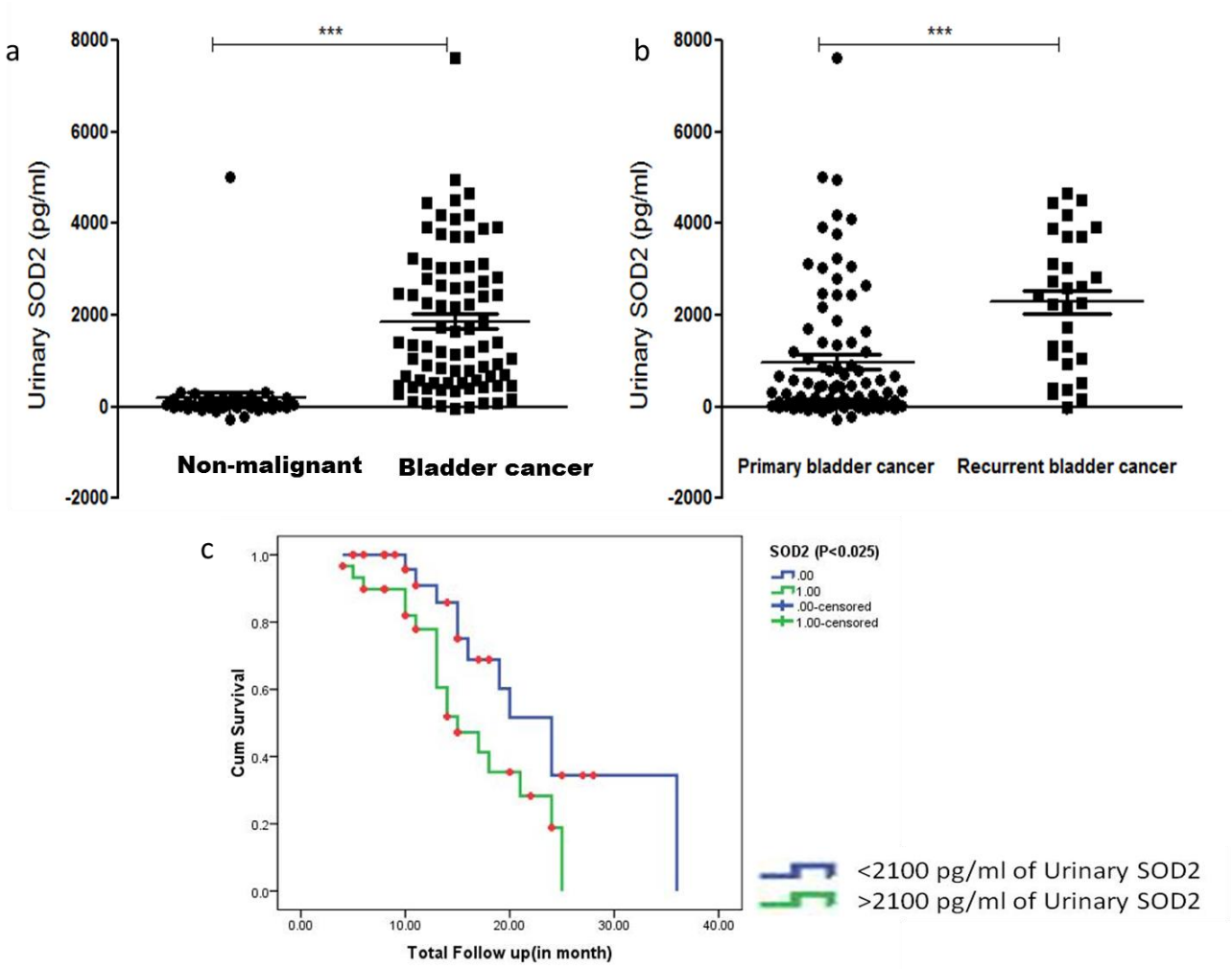


Figure 7.22: Estimation of urinary SOD2 by ELISA

a) Urinary concentration of SOD2 was calculated in urine sample and found significantly elevated urinary concentration of SOD2 in urine of bladder cancer patient compared to non-malignant control (p-value < 0.001, calculated by Mann Whitney U test) b) Significant elevation in concentration of urinary SOD2 in recurrent bladder cancer compared to primary bladder cancer (p-value < 0.001) and c) Kaplan Meier analysis showed higher urinary SOD2 (>2100pg/ml) was significantly associated with recurrence and poorer survival of bladder cancer patients (log-rank t-test, p<0.025).

Altogether, six marker (SOD2, PRDX1, PRDX2, SERPING1, HSPB6 and TNC) were expressed in tumor tissue (both by MS and IHC methods), five markers (SOD2, PRDX1, PRDX2, SERPING1 and HSPB6) were secreted in urine of bladder cancer (Table 7.1) of these high urinary level (>) of SOD2 may be used as recurrence surveillance marker.

Table 7.1: Summary of investigated biomarker

Protein code	**Protein name**	**Methods of identification in tissue**	**Localization**	**Methods of identification in Urine**
SOD2	Superoxide dismutase2	MS, IHC	C, M	WB, ELISA
PRDX1	Peroxiredoxin-1	MS, IHC	C, M	WB, ELISA
PRDX2	Peroxiredoxin-2	MS, IHC	C, M	WB, ELISA
SERPING1	Plasma protease C1 inhibitor	MS, IHC	C, M	WB
HSPB6	Heat shock protein beta6	MS, IHC	C, M	WB
TNC	Tenascin	MS, IHC	E	WB

MS-Mass spectrometry; IHC-Immunohistochemistry; WB- Western blot; ELISA-Enzyme linked immunosorbent assay; C-Cytoplasm; M-membrane and E-Extracellular matrix

7.3 Discussion

Biomarkers discovery begins with preclinical studies by comparing tumor and non-tumor tissue. The exploratory studies identify a unique profile that appears to be over expressed or under expressed in tumor tissue relative to control tissue using proteomics tools based on mass spectroscopy and provides ideas for detection of cancer. Fresh organ specific tumor tissue is usually used for clinical screening purposes, but its procurement is too invasive. Hence, a non-invasive or minimally invasive sample (urine or blood) and unused archival sample (FFPE) can be used in validation phase and for development of a clinical assay or for discovery of biomarkers. Immunohistochemistry (for FFPE), ELISA and Western blots (for urine samples) have been extensively used for this purpose.

It is not necessary that differential proteins identified in exploratory and validation phase will progress consecutively through all clinical phases, but it gives a potential direction. Taken together, we have identified secretion of cancer specific markers in urine which may serve as non-invasive biomarkers for bladder cancer. Biomarkers that represent highly sensitive and specific indicators of disease pathways are often used as substitutes for outcomes in clinical trials where they can be used to predict and evaluate the clinical risk and/or benefit of a treatment, which is the optimal objective of all therapeutic interventions.

SERPING1 showed 90 to 100 % positive expression in all sub groups of bladder tumor either in early or advance stage cancer. SERPING1 is a Serine proteinase inhibitor which regulates complement activation by inhibition of activated C1r and C1s of complement component. Serping1 is also identified in exosomes derived from the bladder cancer reported in the exocarta database. SERPING1 was listed as response to stress, defense and immune regulator molecules in squamous cell carcinoma development (Hummerich *et al*., 2006 and Chi *et al*., 2006).

SERPING1 had also been reported to have higher expression in mammary intra epithelial neoplasia (MIN) and MIN-O (MIN-outgrowth) compared with Ductal carcinoma in situ of breast cancer (Namba *et al*., 2004).

Our immunohistochemistry result of SERPING1showed positivity in normal mucosa (2 cases out of 5) of bladder cancer. SERPING1 also showed tumor stroma-specific expression in cancers of the breast or colorectum (Kiflemariam *et al*., 2015).

HSPB6 is a small heat shock protein showing 90 to 100 % positive expression in bladder cancer and also reported to be expressed in non-small cell lung carcinoma (Chen *et al*., 2014). HSPB6 and SERPING1 were secreted into urine of bladder cancer but sensitivity was not more than 80%. Hence they were not further studied on large cohort. Evidence of urinary HSPB6 and SERPING1 were not well documented.

Tenascin C (TNC) is a major extracellular matrix protein in solid tumor tissues, and showed 40% positivity compared to expression in normal mucosa. Variation in expression pattern differ between low and high grade bladder tumor.Similarly, expression of TNC was reported in bladder cancer and difference in expression pattern in the tumour stroma or cytoplasm of the tumour cells may suggest its role as prognostic biomarker (Brunner *et al*., 2004). Tenascin C did not show any positivity in urine sample.

Urine is a source of high abundant protein and low abundant protein and the best non-invasive sample of bladder cancer because tumor outgrowth may come in contact with urine or tumor cells exfoliate into the urine or tumor cells secrete proteins into urine. For development of a diagnostic and prognostic biomarker, no depletion or enrichment methods had been used during discovery, for the success of its clinical application. Urinary proteome are not well studied yet, hence there is no internal control for urinary protein sample used in Western blot experiment and

presence and absence of protein band was taken as criteria for Western blot analysis. Urinary proteins having more than 80% sensitivity and 100% specificity were further validated in large cohort using ELISA. To the best our knowledge, this is the first proteomic study detecting the secretion of tumor specific protein (SerpinG1, HspB6 and SOD2) in urine of human bladder cancer patients.

Peroxiredoxins (PRDXs) are a member of glutathione peroxidases family which destroys peroxides, organic hydroperoxides and peroxynitrite (Rhee *et al*., 2005 and Rhee *et al*., 2006). Peroxiredoxin 1 (PRDX1) is an antioxidant enzyme and plays an important role in H2O2-mediated cell signaling. PRDX1 inhibits the activation of oncogenes (c-Abl and c-myc, and PTEN) which is essential for its tumor-suppressive function (Cao *et al*., 2009). We found expression of PRDX1 was significantly increased in bladder cancer tumor when compared to paired adjacent normal tissues and similarly reported in esophagus squamous cell carcinoma tissues (Hoshino *et al*., 2007 and Ren *et al* 2013) and in colon carcinoma (Rho *et al*., 2008). We found the sensitivity and specificity of PRDX1 was 80 and 100% respectively and elevated level of urinary PRDX1 (29.4µg/ml) in bladder cancer patients compared to non-malignant urine. Urinary concentration of PRDX1 was also not associated with diseases free survival of patient. Similarly, Quan *et al* found enhanced PRDX1 expression in bladder cancer tissue is strongly associated with development and progression but its expression did not correlate with disease-free survival in patients with bladder cancer (Quan *et al*., 2006). We found higher expression of PRDX2 was in tissue and urine of bladder cancer and similarly Yi *et al* also showed elevated urinary PRDX2 in bladder cancer urine sample (Chen *et al*., 2010). We also found lower level of urinary PRDX2 (< 21.4 µg/ml) to be associated with recurrence, whereas

either Chen *et al* or any other available literature did not show any association with survival of patients.

Expression of SOD2 was more than 90% in tissue and urinary SOD2 showed 93% sensitivity and 100% specificity. SOD2 having cytoprotective effects and involves in Detoxification of Reactive Oxygen Species and Cellular responses to stress. It has been reported that overexpression of SOD2 is linked to increased invasiveness of tumor metastasis (Pias *et al*., 2003). Overexpression of SOD2 in lung, penile cancer, colorectal, breast cancer, cervical and gastric cancer malignant tumors has also been reported (Holley *et al*., 2012; Johnson *et al*., 2005; Kinnula *et al*., 2005 and Termini *et al*., 2011). Reduced expression of SOD2 causes DNA damage and increases cell proliferation (Hurt *et al*., 2007). SOD2 has a role in free radical regulation and its main function is to convert superoxide anion into hydrogen peroxide (H2O2), which is subsequently converted to water by catalases (Liu *et al*, 2004). Hence, levels of SOD2 should be maintained to fight against the tumor. Here, we report higher concentration (>2100pg/ml) of urinary SOD2 was associated with recurrence of bladder cancer patients or poorer survival of patients. SOD2 is well studied in cancer but there is no evidence of urinary SOD2 or also not association with survival of cancer patients.

7.4 Conclusion

Our results indicate that urine is a useful non-invasive source of biomarkers and the tumor specific urinary biomarker, SOD2, SERPING1, HSPB6, PRDX2 and PRDX1 were identified and may be used as diagnostic biomarker. Urinary SOD2 may be used for the surveillance of bladder cancer patient and may be useful as a marker for assessing the recurrence or progression of human bladder cancer.

Chapter 8

Conclusion and

Future Scope of the study

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8.1 Conclusion

The present study was undertaken to identify the proteome profile of urothelial carcinoma using iTRAQ labeling combined with LC-MS/MS and has led to the discovery of several novel, differentially expressed proteins in these tumors. Further, it was aimed to find a tumor specific marker in urine to develop a non-invasive biomarker for surveillance of patient as well as associated with survival of patients. Differentially expressed proteins of tumour compared with adjacent normal mucosa was analysed and grouped into unique and common proteins. Further, various bioinformatics tools such as Ingenuity pathway analysis (IPA), ontotool-pathway express and Cytoscape has been used for identifying protein-protein interaction, pathway and function involved by differentially expressed protein. Exocarta databases have been also used for identification of presence of exosomal urinary protein in bladder cancer deregulated proteins. Differentially expressed tumor proteins of bladder cancer were further verified in formalin-fixed-paraffin-embedded tissue using immunohistochemistry. The verified tumor proteins were validated in the urine sample for their presence using Western blot. Urinary marker having sensitivity and specificity more than 80% was further validated on large cohort of urine sample of patients with follow up data and urine of non-malignant sample using ELISA. The major outcome of the same is given below:-

1. Identification of total of 1795 proteins with ≥1peptide (15385 peptides) and 1137 proteins with ≥2 peptide (15005 peptides) were identified in urothelial bladder cancer compared to normal mucosa.

2. The tissue proteins identified with at least 2 peptides in LGT1 were 1399 (205 upregulated and 376 downregulated), in HGT1R were 1399 (230 upregulated and 252 downregulated), in HGT1NR were 1137 (159 upregulated and 285 downregulated) and in HGT2 were 1399 (277 upregulated and 457 downregulated). The remaining proteins were not dysregulated.
3. Among all subgroups, high grade muscle invasive group showed the highest number of deregulated proteins (n=178). Common proteins (n=75) between low grade non-invasive (LGpT1) and high grade non-invasive, non-recurrent (HGpT1NR) bladder cancer, were highest.
4. The 64 deregulated proteins were found common to all groups of bladder cancer. Among these proteins, 9 were commonly upregulated and 19 were commonly downregulated.
5. We found 40.9% deregulated protein involved as a Cellular component, 27.5% metabolic process and 42% deregulated proteins having catalytic activity as a molecular Process using PANTHER 9.0.
6. The Ingenuity Pathway Analysis (IPA) Core Analysis generated the top 11 Network and revealed top three interactions between 24, 14 and 11 of deregulated proteins. These proteins are involved in Cellular Movement, Hematological system, Immune cell Trafficking, Nucleic Acid Metabolism, Small Molecule biochemistry, Cell Death and Survival, Cellular Development, Cellular Growth and Proliferation.
7. Differentially expressed tumor tissue proteins may involve in exosomal protein formation, hence a comparison was performed with urinary exosomal proteins reported specific to bladder cancer on Exocarta database.
8. A comparative analysis between differentially expressed bladder tumor tissue proteins and urinary exosomal proteins reported on exocarta database showed 120

common proteins. These 120 differentially expressed protein may forms exosomes and among them SERPING1 was further validated. SERPING1 showed upregulation in all stages and grades of bladder cancer and was also found in urine of bladder cancer patients.

9. Enriched pathways of urinary exosomes included cellular architecture, motility, cell to cell adhesion, tumorigenesis and metastasis.
10. Proteins in the 9 top-ranked pathways included CTNNA1 (alpha-catenin), CTNNB1 (beta-catenin), VSAP, ITGA4, PAK1, DDR1, CDC42, RHOA, NRAS, RHO, PIK3AR1, MLC1, MMRN1, and CTTNBP2 and network analysis revealed 10 important hub proteins and identified inferred interactor NF2. The importance of identifying interactors is that they can be used as targets for therapy, which inhibit protein-protein interactions and suppress tumor growth and progression by hindering the exosome biogenesis.
11. The result of immunohistochemistry of SERPING1, SOD2, HSPB6, PRDX1 and PRDX2 showed cytoplasmic and membranous expression of tumor cells while Tenascin C was found expressed in stroma.
12. Immunohistochemistry analysis showed no significant differences in expression of SERPING1, SOD2, HSPB6, PRDX1 and PRDX2 between different grade and stage of bladder cancer while Tenascin C showed significantly higher intensity of expression in high grade tumors in the superficial luminal areas ($p<0.001$). Adjacent bladder mucosa was also tested for all markers and no expression of the marker was found in normal mucosa except for SERPING1 (40%).
13. Urinary SERPING1 and HSPB6 showed equal to or less than 80% sensitivity and specificity while urinary SOD2, PRDX1 and PRDX2 showed more than 80 % sensitivity and specificity.

14. Significant elevation in urinary concentration of SOD2, PRDX1 and PRDX2 was found in urothelial bladder cancer patients compared to urine of non-malignant cases (p-value < 0.001).
15. Significant elevation in urinary concentration of SOD2 and PRDX2 was found in recurrent urothelial bladder cancer patients compared to urine of primary bladder cancer (p-value < 0.001).
16. Median concentration (29.4 ng/ml) of urinary PRDX1 was taken as cut off value but was not associated with recurrence of bladder cancer patients.
17. Kaplan Meier survival analysis of urinary PRDX2 showed lower concentration (<27.94 ng/ml) was associated with recurrence and poorer survival though it was not statistically significant.
18. Higher than median concentration (2100pg/ml) of urinary SOD2 was significantly associated with recurrence or poorer survival of bladder cancer patients (p<0.025).

8.2 Future Scope of the study

High urinary SOD2 and lower PRDX2 concentrations were associated with poorer survival of bladder cancer patients individually in the present study. More number of tumor markers may be tested in urine and the urinary marker profile also can be tested, by multiplex ELISA, on patients for long term monitoring to detect recurrence. It is expected that combined test and the compliance will be high compared to cystoscopy and more sensitive compared to urine cytology for detecting recurrences.

The control group for urine samples included renal stones, glomerulonephritis and BPH but future work could include the cases with cystitis and of lower urinary tract infection so as to check specificity of these markers. Future study may also include the analysis of association of these markers with grade and invasion of urothelial cancers of bladder to decide on the efficacy of these markers in all grades and stages. Multiple urine samples collected at different time points in both recurrent and non-recurrent patients can identify concentration for increased sensitivity to be used as the clinical cut-off for monitoring of patient.

Hence it is suggested that a customized multiplex ELISA kit may be prepared for validation on a larger cohort including infectious, inflammatory and malignant conditions. Analysis of this cohort can give cut-off values for various conditions, thus making the analysis of value to the clinician. The threshold of a marker can be useful only after subgroup validation at various time points during follow-up. Future studies could compare samples from the same patient before, during and after treatment, and in patients with and without recurrence on surveillance.

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~ References ~

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Appendix

Apendix

Running buffer recipe

1X Running buffer (1 liter)	
250mM Tris	3.03g
1.92M Glycine	14.4g
SDS	1g

Tris Buffer saline tween20 (TBS.T) buffer recipe

1X TBS.T (1 liter)	
Tris-base	0.97g
Tris-HCl	6.6g
NaCl	8.8g
Tween-20	500µl

Coomassie Brilliant Blue G250 stain

Coomassie blue stain (G-250)	
0.02% CBB G-250	0.05g
5% Al2(SO4)3	12.5g
10% Ethanol (96%)	25ml
2% o-Phosphoric acid (85%)	5.8ml
45% milli-Q	219ml

Destainer solution (G-250)	
10% Ethanol (96%)	25ml
2% o-Phosphoric acid (85%)	5.8ml
70% milli-Q	219.2ml

Silver staning

Fixing Solution (50ml)

10% Acetic acid-5ml

75µl Formaldehyde

50% methnol-25ml

Milli Q water- volume make upto 50ml

Sensitizing solution

0.02% sensitizer $Na_2S_2O_3$ (Sodium thiosulphate)- 0.01gm

MilliQ water- volume make upto 50ml

Silver solution

0.2% Silver Nitrate ($AgNO_3$)-0.1gm

75µl Formaldehyde- 37.5 µl

MilliQ water- volume make upto 50ml

Developing Solution (50ml)

3% Sodiumbicarbonate (Na_2CO_3)- 1.5gm

25 µl formaldehyde

100 µl of sensitizing solution

MilliQ water volume upto 50ml

Stop Solution

1.4% EDTA- 0.7gm

MilliQ water- volume make upto 50ml

1X TBS.T (1 liter) (pH-7.4)	
Tris-base	0.97g
Tris-HCl	6.6g
NaCl	8.8g
Tween-20	500µl

Harris's Hematoxylin solution	
Hematoxylin	5.0g
Alcohol (95%)	50.0ml
Ammonium alum or Potassium alum	100g
Distilled water	1000ml

1X TE buffer (for 1 liter) (pH-9.8)	
Tris base	1.21g
EDTA	0.372g

Solubilization buffer	
Urea	7 M
Thiourea	2 M
Tris	20 mM
CHAPS	2%

1X Transfer buffer(1 liter)	
Glycine	14.4g
Tris	3.04g
Methanol	100ml

List of Publications

~List of Publications~

1. Agrawal, U[1]., **Kumari, N**[1]., Mishra, A. K., Vasudeva, P., Kumar, A., Mohanty, N. K., & Saxena, S. (2016). Immune signature of urothelial cancer associated with grade, recurrence, and invasion. In *Urologic Oncology: Seminars and Original Investigations* (Vol. 34, No. 9, pp. 418-e17). Elsevier. (Equal first author)

2. **Kumari, N**., Saxena, S., & Agrawal, U. (2015). Exosomal protein interactors as emerging therapeutic targets in urothelial bladder cancer. *Journal of the Egyptian National Cancer Institute*, *27*(2), 51-58.

3. **Kumari, N**., Dubey, U.S. and Agrawal, U. (2015). Evolution Of Classification Of Bladder (Urothelial) Cancer. *National Journal of Integrated Research in Medicine*, *6*(6), 89-94.

4. **Kumari, N**., Vasudeva, P., Kumar, A. and Agrawal, U. (2015). Adenocarcinoma of urinary bladder: A report of two patients. *Journal of cancer research and therapeutics*, *11*(4), 1033-1036.

5. **Kumari, N**., Jha, A., Vasudeva, P. and Agrawal, U. (2016). High-Grade Urothelial Carcinoma of Bladder Transforming to Micropapillary Variant on Follow-Up. *Iranian Journal of Medical Sciences*, *41*(2).

6. **Kumari, N**., Malarkar, D. Dubey, U.S. Vasudeva, P., Kumar, A. Nanda, R. Agrawal, U. Saxena, S. Urinary SOD2 as survellaince marker for Urothelial bladder cancer using quantitative Proteomics approach (8-plex iTRAQ LC-MS/MS) *(Manuscript submitted)*

~Conference attended and presentation~

1. Poster presented entitled "**Association of Th1 cytokine response with disease progression in urothelial bladder carcinoma"** in "IMMUNOCON-2013" (40th annual Conference on Indian Immunological Society) on 17 November 2013.
2. Poster presented under award categories entitled "**Role of cytokines in recurrence-free survival of Bladder cancer patients**" in 32nd Annual Convention of Indian Association for Cancer Research on 13-16 February 2013.
3. Oral presentation on "**Host Immune Responses in Urinary Bladder Cancer**" for *Dr Sriramachari Young Scientist Award*at National Institute of Pathology, 1st May 2012

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UROLOGIC ONCOLOGY

Original article

Immune signature of urothelial cancer associated with grade, recurrence, and invasion

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Abstract

Background: Urothelial carcinoma (UC) is one of most common genitourinary malignancy and the spectrum of disease ranges from in situ lesions to muscle-invasive cancers. The non–muscle-invasive lesions have tendency to recur or progress to muscle-invasive disease. The study of the immune profile may identify immune determinants associated with high-grade, recurrence, and invasion in patients with UC.

Methods: Pathway-focused RT^2 profiler arrays were used to screen patients with UC for dysregulation of candidate genes of Th1-Th2-Th3 and NFκB pathways, which were then validated by real-time polymerase chain reaction on tumor samples and correlated with grade, recurrence, and invasion of tumors to identify their role in predicting behavior of the tumor. The cytokines found associated with recurrence were then validated in urine of patients with UC.

Results: IFNγ, IL2, IL4, IL10, IL17, CCL7, CTLA4, and SPP1 of the cytokine pathway and TLR4, TLR3, RELA, NFκB1, and MYD88 of the NFκB pathway were found differentially expressed in patients with urothelial cancer by array and quantative real-time polymerase chain reaction. Among these, IL10 and SPP1 were found consistently up-regulated in high-grade, invasive, and recurrent cases and up-regulated IL10 and CTLA4 were found associated with a short recurrence-free survival time ($P = 0.001$ and $P = 0.065$). Urinary IL10 concentration was significantly higher in both patients with cancer and cystitis compared with healthy controls, but the difference in concentration between patients with cancer and cystitis patients was not statistically significant. However, urinary CTLA4 concentrations were found to be significantly higher in urothelial cancer patients compared with healthy controls and cystitis cases and found to be associated with poor recurrence-free survival.

Conclusion: The study indicates that high urinary CTLA4 concentration raises the index of suspicion of recurrence in a known case of urothelial cancer and may be used as a surveillance marker.

Keywords: Urothelial cancer; Cytokines; NFκB; CTLA4

1. Introduction

The biological behavior of the tumor is a result of alterations in the tumor and its microenvironment and the factors they release. Chief among these are the inflammatory cytokines and chemokines and the genes of the upstream and downstream signaling pathways affecting

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Full Length Article

Exosomal protein interactors as emerging therapeutic targets in urothelial bladder cancer

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KEYWORDS

Exosome;
Urinary bladder cancer

Abstract *Background:* Exosomes are rich sources of biological material (proteins and nucleic acids) secreted by both tumor and normal cells, and found in urine of urinary bladder cancer patients.
Objective: The objective of the study was to identify interacting exosomal proteins in bladder cancer for future use in targeted therapy.
Methods: The Exocarta database (www.exocarta.org) was mined for urinary bladder cancer specific exosomal proteins. The urinary bladder cancer specific exosomal proteins (n = 248) were analyzed to identify enriched pathways by Onto-tool Pathway Express (http://vortex.cs.wayne.edu/ontoexpress).
Results: Enriched pathways included cellular architecture, motility, cell to cell adhesion, tumorigenesis and metastasis. Proteins in the 9 top-ranked pathways included CTNNA1 (alpha-catenin), CTNNB1 (beta-catenin), VSAP, ITGA4, PAK1, DDR1, CDC42, RHOA, NRAS, RHO, PIK3AR1, MLC1, MMRN1, and CTTNBP2 and network analysis revealed 10 important hub proteins and identified inferred interactor NF2.
Conclusions: The importance of identifying interactors is that that they can be used as targets for therapy, for example, using Bevacizumab (avastin – an angiogenesis inhibitor) against NF2 to inhibit protein–protein interactions will inhibit tumor growth and progression by hindering the exosome biogenesis.

Introduction

Exosomes are microvesicles shed by vesiculation events from various living cells secreted by most cell types, both cancerous and normal and secreted in biological fluids [1] and are thought to play important roles in intercellular communications. They are generated via diverse biological mechanisms triggered by pathways involved in oncogenic transformation, microenvironmental stimulation, cellular activation, stress, or

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Evolution Of Classification Of Bladder (Urothelial) Cancer

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Abstracts: The classification of bladder tumors has undergone a change over the years but still has not achieved success in predicting the behavior. The correct cellular classification of a tumor helps initiate appropriate treatment. Recently functional, genomic and proteomic data have been of help in aiding prognosis and modifying the treatment in many cancers. However, this data is not routinely integrated into the classification, and treatment protocols in bladder carcinoma hinge on grade and depth of invasion. An in depth understanding of the implication of grade, stage, molecular features on survival is necessary to understand the behavior of the tumor. The classification of Urothelial cancer has undergone a lot of change in terminology over the past century but we have still not identified markers (both morphologic and molecular) for preventing recurrences. It is believed that the treatment protocols should be based on a combination of these and we still have to conduct large-scale follow-up studies to identify these parameters. We present here the changes in bladder cancer classifications over the past century and the implications thereof in this review. [Agrawal U NJIRM 2015; 6(6):89-94]

Key Words: Urothelial cancer, classification.

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Introduction: Urothelial (Transitional cell) cancer of Urinary Bladder encompasses the spectrum of non-muscle-invasive (NMIUC), muscle-invasive (MIUC), and metastatic disease with an age-adjusted incidence rate of 21.1 per 100,000 population per year[1]. The extent of disease determines clinical behavior, treatment, and prognosis. The high-grade non-invasive cancers may progress to muscle-invasive tumors or have recurrences in about 30% cases[1]. The standard of care is different in various stages. Classification systems of tumors give an idea of the aggressiveness and prognosis in the patients and are thus useful indicators for clinical management of the patient. As far back as 1921, Broders' classified epitheliomas of the genito-urinary regions including cervix, labia, vagina, urethra, penis, bladder, pelvis of the kidney and ovary as Grades1-4[2]. In his classification he based the grades on the proportion of differentiated epithelium (3/4ths differentiated and 1/4th undifferentiated in Grade 1 to fully undifferentiated in Grade 4). As this classification was universal for all epithelium, be it squamous, columnar or transitional and all organs with epithelial tumors it was not taking into account the depth of invasion of the tumor or the pattern of growth. However, Broder reported that the increasing size of the tumor and grade was found to be associated with poor survival. The microscopic appearance i.e., the grade, does not always conform to the clinical behavior. Hence, a composite reporting including pattern of growth, depth of invasion and morphologic appearance were proposed by pathologists with some advocating the incorporation of clinical staging. Subsequent classifications specifically for Urothelial cancer included the pattern of growth, depth of invasion and grade of tumor. Almost all classifications to date include papillary, solid/infiltrating and mixed patterns of growth and almost invariably the solid/infiltrating pattern of growth was reported to have a worse prognosis[1].

Material and Methods: Extensive literature search was done using various internet search engines to identify review manuscripts as well as guidelines provided by WHO (World Health Organisation), UICC (Union for International Cancer Control) and ISUP (International Society of Urologic Pathologists) on urothelial carcinoma classifications from the earliest classification of Broders' who described epithelial malignancies as epitheliomas. The literature was thoroughly examined to understand the presentation, diagnostic features, tumor stage, management, and outcome of various stages and grades of urothelial carcinoma. The review does not include the various comparative studies for interobserver and intraobserver concordance for the 1972 and 2004 classifications though the conclusions of various observers has been summed up.

Urothelial cancer classifications

The earliest recorded classification of Urinary bladder tumors, proposed by Ash in 1940 classified the most benign appearing papillary tumor as carcinoma because of their great tendency to recur locally[3]. Dukes and Masina classified these tumors into low, average and high grades in 1949[4] and took account of pathological staging which included the depth of tumor invasion into the lamina propria and muscularis

Adenocarcinoma of urinary bladder: A report of two patients

ABSTRACT

Adenocarcinoma of the bladder is a rare tumor. Primary and metastatic adenocarcinomas of urinary bladder are morphologically similar, but histogenetically different. We present two cases, a signet ring cell adenocarcinoma with follow-up and another of glandular adenocarcinoma of urinary bladder. Pathological evaluation and immunohistochemical panel of eight markers (E-cadherin, CK20, CK7, CDX2, estrogen receptor (ER), gross cystic disease fluid protein 15 (GCDFP15), 34bE12, and prostate specific antigen (PSA) provides a diagnostic confirmation of primary adenocarcinoma with the positive expression of E-cadherin and CK20 in case 1 and metastatic adenocarcinoma of prostate with profile of E-cadherin+, CK20–, GCDFP15+, 34bE12+, and PSA+ in case 2.

KEY WORDS: Adenocarcinoma, immunohistochemistry, urinary bladder cancer

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INTRODUCTION

Urinary bladder cancer is more common in males with the predominant (90%) morphologic type being urothelial carcinoma (previously known as transitional cell carcinoma). The second common subtype is the squamous cell carcinoma which is more common in geographic locales where schistosomiasis is endemic. Primary adenocarcinoma of the bladder comprises less than 2% of all bladder tumors and is categorized into metastatic, primary, and urachal adenocarcinomas.[1] Metastasis occurs commonly from prostate, breast colon, and tumors of the female reproductive tract. Morphologically, these tumors are of enteric type, signet ring cell type or mucinous, and are muscle-invasive at the time of initial diagnosis. Primary adenocarcinoma of the bladder is curable with radical cystectomy or pelvic exenteration. The diagnostic dilemma lies in the differentiation of the tumor as primary or metastatic.[2,3] Diagnosis of adenocarcinoma is based on the morphology and here we present two cases of adenocarcinoma of bladder with the demonstration of a panel of immunostains, including CK20, CK7, E-cadherin, CDX2, estrogen receptor (ER), gross cystic disease fluid protein 15 (GCDFP15), 34bE12, and prostate-specific antigen (PSA).[4-7] This panel is of diagnostic value in differentiating primary bladder adenocarcinoma from metastatic lesions of secondary adenocarcinoma.

CASE HISTORY

Case 1

A 55-year-old man was admitted with complaints of hematuria, pyuria, and burning sensation in lower urinary tract for last 3 months. There was no prior history of hypertension, diabetes, or coronary artery disease. The patient was diagnosed and operated for bladder stone in 1989 and renal stones in 1999 and was stable after surgery. The physical examination of patient was normal. On ultrasonography, bladder mass measuring 3.8 cm × 2.3 cm was seen on right lateral wall. Transurethral resection of bladder tumor (TURBT) was performed and the biopsy specimen was sent for histopathological examination. Morphologic examination revealed signet ring cells in clusters with vacuolated cytoplasm and eccentric nucleus [Figure 1a] and the patient was diagnosed to have signet ring cell adenocarcinoma. Immunohistochemical profile showed cytoplasmic localization of CK20 and membranous expression of E-Cadherin [Figure 2a, b, g, and h], while signet ring cells were negative for all other markers [Figures 2d and e and 3a, b, d, e, g, h, j, k, m, and h]. Two cycles of intravesical Bacillus Calmette-Guérin (BCG) and interferon-alpha2B (IFN-α2b) were given to the patient and patient was on follow-up; inspite of treatment the patient reported 10 months after initial diagnosis with the symptoms of hematuria, pyuria, and burning sensation in lower urinary tract. On ultrasonography, recurrent bladder mass measuring 4.6 cm × 3.1 cm was seen on right lateral wall and patient was now operated by radical cystectomy. The histopathologic examination revealed signet ring cell carcinoma with tumor cells present scattered singly and in sheets as well as in small acinar formations and clusters lying in lakes of mucin. Tumor cells were seen infiltrating the detrusor muscle.

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Case Report

High-Grade Urothelial Carcinoma of Bladder Transforming to Micropapillary Variant on Follow-Up

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Abstract

Micropapillary variant of urothelial carcinoma (UC) of the bladder is an aggressive tumour, comprising 0.6-6% of all UC. It generally presents with high-grade and stage, and has been reported as having a worse prognosis when compared to traditional UC. We report the case of a 58-year-old man who presented with macroscopic haematuria. The patient was diagnosed with high-grade urothelial carcinoma and returned with recurrence after 16 months. Histopathology after transurethral biopsy revealed a non-muscle invasive high-grade bladder tumour at first presentation, whereas tumour recurrence was reported after 1.5years. The histopathology at recurrence revealed a high-grade, muscle invasive, micropapillary variant of urothelial carcinoma with focal adenomatous morphology. Immunohistochemical expression of $CK7^{+}$/$CK20^{+}$ in tumour cells and negativity for PSA, AMACR, and CDX2 in paraffin section helped in identifying the tumour as primary in the urinary bladder. Radical cystectomy was performed and the patient has no distant metastases on follow-up. The specific morphology even within the high-grade urothelial cancer cases is important to discern for proper treatment.

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Keywords ● Transitional cell carcinoma ● Micropapillary variant ● Bladder

What's Known

- Micropapillary variant is a less common presentation of urothelial cancer.

What's New

- Transformation of a known high-grade urothelial carcinoma case into micropapillary morphology.
- Multiple sections should be examined for any focus of transformation as the management changes with this morphology.

Introduction

Urothelial cancers (UC) are common and worldwide incidence is very high. Several histologic subtypes of bladder cancer such as microcystic, micropapillary, and nested variant are seen. Micropapillary urothelial carcinoma is a rare aggressive subtype of transitional cell carcinoma.[1] The presence of micropapillary component (MPC) in urothelial carcinoma was found to be associated with high-grade and advanced stage of tumour, though low-grade and non-invasive cases have been reported. Micropapillary variant was first described in 1994, though fewer than 300 cases have been reported. Micropapillary variant of bladder cancer (MPBC) occurs in only 0.6-6% of bladder cancer cases and shows a strong male predominance.[2,3] The histology of MPBC resembles that of micropapillary subtypes of breast, lung, stomach and colon, as well as serous ovarian carcinoma.[1] The micropapillary component of these tumours may be encountered on the surface of non-invasive component, the invasive

Biographies

Brief Biography of Candidate

Nitu Kumari did her Masters in Biotechnology from Bansathali University. During this tenure, she did her dissertation from National Intitute of Plant and Genomic Research (NIPGR), New Delhi. After that she did 6 months Biotech Industrial Training Programme (Sponsored by Department of Biotechnology, Government of India) at Auroprobe Laboratories Ltd. NBE complex Ghaziabad. She qualified GATE and joined the research team at the National Institute of Pathology (NIP) in 2010. Her research interest are in the area of Oncology, Proteomics, Biomarker discovery and Immunology. She worked as Project-JRF on Cancer in North East India Understanding the role of Tobacco under the supervision of Dr. Sunita Saxena at NIP, New Delhi. After that she joined a project entitled 'Characterisation of Host Immune Profile Associated with Biologic Behavior of Superficial Transitional Cell Carcinoma of Urinary Bladder' under the supervision of Dr. Sunita Saxena and Dr Usha Agrawal. Further, she was awarded as ICMR Senior Research Fellow (SRF) entitled 'Differential Protein Profile for Identification of Markers in Recurrent Urothelial Cancer'. While doing SRF, she registered for PhD at Birla Institute of Science and Technology, Pilani. She has presented her research work in national and international conferences. She has published 2 original research articles, 1 review and 2 case reports in peer reviewed national and international journals.

Brief Biography of the Supervisor

Dr. Sunita Saxena is a Consultant and Ex-Director of National Institute of Pathology, one of the premier institute of Indian Council of Medical Research in India. A pathologist by training, she has evolved beyond diagnostic pathology to using trans disciplinary approaches in understanding important cancers in India. She is one of the few molecular pathologists in country with keen interest in Oncopathology and Molecular oncology. She started her career in research working on medical diseases of the kidney and she has now diverted her specific research interest to tumor biology, specifically Breast Tumors, Tobacco Associated cancers and Genito urinary cancers. She has been trained at International Agency for Research in Cancer, Lyon, France for various genetic and molecular biology based technologies. Her current research interest is identification of genetic risk factors, prognostic and predictive biomarkers and novel drug targets for Breast cancer and Tobacco associated cancers in North east region using genome-wide approaches. Dr Sunita Saxena is recipient of Yamigawa-Yoshida fellowship of UICC, WHO fellowship, Novartis Oration and Dr. P.N.Wahi awards of ICMR and K.C.Basu Mullick award for best research work from Indian Association of Pathologists and Microbiologists. She is also Fellow of National Academy of Medical Sciences, Indian Association of Cancer Research, Proteomic Society of India, Human Genomic Organization and Indian College of Pathologists. She is also a life time International Member of Union against Cancer. She is a member of Member of Scientific Advisory committee and Project Review Committee of various reputed ICMR institute and funding body. She has more than 96 research articles in reputed journal and 2 book chapters.

Brief Biographgy of Co-Supervisor

Dr Usha Agrawal is presently a medical scientist working at National Institute of Pathology, ICMR, New Delhi. Dr Usha is a medical graduate from VSS Medical College, Burla, Odisha who did her postgraduation in Pathology at the prestigious Dr A L Mudaliar Post-graduate Institute of Basic Medical Sciences, Taramani, Madras. Her interest in research started at this Institute where she worked on proliferation markers in lymphomas. She was an ICMR SRF and after completion of her studies she joined as faculty in Annamalai University, Chidambaram, Tamilnadu and served there for 4 years. Subsequently she moved to Pravara Rural University, Loni, Maharashtra for 2 years. She joined the ICMR family in 2002 and has been working both as a diagnostic pathologist and a medical researcher since then. She is a Faculty and Course Coordinator for DNB Pathology, Guide for DNB Pathology, Co-guide for MS Surgery, Radiology and ENT students and MD Pathology students. During her tenure in NIP, ICMR she has worked towards her doctorate. Her area of work is on oncopathology and cancer immunology. Her research interest is Oncopathology, Oncoimmunology, Biomarker discovery and genitourinary cancer. She has 41 publications in national and international journals. She is a reviewer for both national and international journals of repute. Dr Usha Agrawal is at present working in the areas of Genitourinary cancers and biobanking. She is also the recipient of HRD fellowship for short-term foreign fellowship in “Quality control and Quality assessment” in Biobanking at BC Cancer Agency, Victoria, BC, Canada.

Brief Biography of the Co-Supervisor

Dr. Uma S. Dubey is presently working as an Associate Professor in the Department of Biological Sciences at BITS Pilani-Pilani Campus. She has served this department for last 14 years at various teaching and research related posts. Earlier, she has conducted teaching and program development in the departments of Biotechnology, Microbiology and Environmental Sciences at the Institute of Life Sciences, Kanpur University. She also has a research experience of an year at the department of Plant Sciences, University of Alberta, Canada. She has done her Ph.D. in Immunology (under the Supervision of Prof. S.S. Agarwal) from Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow.

Teaching: She has taught more than 15 different courses in the department of Biological Sciences. She has been involved in the course planning and development of many of these. Besides this, she has initiated 2 new courses in Immunology and Cancer Biology. She has coordinated the course restructuring of M.Sc. and ME programs of the Biological Science Department as DCA Convener from 2012 -2014 and is continuing as a member of the same. She has coordinated the Science, Imagination and Discovery (SID) workshop at BITS Pilani. She has been Judge of various events of APOGEE from 2006-onwards in Biological Sciences and Medical Sciences categories and is a Life member of Indian Immunological Society and Member of My India Team, BITS Pilani.

Research: Her research interest is in both, theoretical and practical aspects of Immunology and Cancer Biology. Specifically in (i) Cellular Immune Responses: She has been involved in comparative studying on Lymphocyte proliferation, Natural Killer cell function and Antibody dependent cellular cytotoxicity at normal and febrile temperatures. Also the cell cycle proliferation kinetics of lymphocytes and cell lines is of interest. (ii) Mathematical Modelling of Immune system: She has been involved in studying the interaction of various components of the immune system with each other, with cancer cells and with infectious agents in the presence and absence of environmental toxicants. The analysis requires theoretic, mathematically and computational input. (iii) Cancer Biology: She is interested in studying the anticancer properties attributed to Camel milk and its associated mechanisms of action. She is also interested in alpha lactalbumin sequence analysis in various species. It is the primary component of HAMLET a recently discovered anticancer molecule (derived from human milk) devoid of any side effect. She has 12 original research articles, 3 book chapters and 1 lab manual to her credit.