Identification of prostate cancer specific biomarkers and protein targets associated with androgen independent cancer progression

Introduction:

In western countries, prostate cancer (PCa) is the most common carcinoma in men and the second leading cause of cancer related mortality in men. In the year 2016 in the United States, an estimated 180,890 new cases diagnosed and 26,730 men died of PCa. About 1 man in 7 will be diagnosed with PCa throughout his lifetime. In the past the incidence rate of PCa in India was lower as compared to the developed countries, however recently more number of cases are diagnosed due to regular screening with increased health awareness and improved diagnostic technologies. It is indicating that rate of PCa incidence in Indians is not very far behind the western countries. PCa is often curable when diagnosed in the early stages. However, none of the currently used prostate cancer biomarkers, including PSA are satisfactory. Hence, the attempt of omics approaches including, genomics, transcriptomics, proteomics and metabolomics may better diagnostic tools for prostate cancer. These studies have reported a number of potential biomarkers for diagnostic purposes. Their diagnostic application in clinics is still limited which indicates the need for development of new biomarkers for screening of PCa. Non-invasive diagnostic tests are easy to perform routine screening for cancer diagnosis. Therefore, serum profiling is always an attractive approach for novel biomarkers identification. Numerous studies have applied proteomic and genomic approaches to analyze the serum of cancer patients and identified gene based and protein based biomarkers. Very limited information is available about serum metabolome particularly lipid alterations from PCa patients. Alternatively, human immune system will produce autoantibodies against tumor-derived factors that appear during the tumorigenesis. Therefore, identification of altered lipids and tumor-associated autoantibodies in PCa patient's serum may have utility in early diagnosis of cancer.

Initially PCa responds to androgen deprivation therapy, eventually most of the patients will relapse to castration resistant PCa (CRPC) due to the growth of androgen insensitive cancer

cells, rendering the effective therapy useless. Therefore, progression of androgen independent prostate cancer (AIPC) is a crucial step during the course of treatment. It has been reported that the non-steroidal signaling pathways activates androgen receptor (AR) in androgen deprivated conditions and promote the androgen independent PCa. However, the precise molecular mechanism in hormone dependent switch is poorly understood. Based on these limitations for diagnosis and treatment options for PCa, the present thesis work is focused on the following objectives.

Objectives of the study:

1. To identify serum lipid signatures associated with prostate cancer and their evaluation as potential biomarkers
2. To identify potential serum autoantibodies as biomarkers for early diagnosis of prostate cancer
3. To understand novel mechanisms involved in progression of androgen independent PCa and ascertaining their potential as new therapeutic targets

Methodologies used and results:

Identification and validation of prostate cancer associated lipids signatures by lipidomics approach as potential new biomarkers:

Lipids are complex biomolecules have been involved in several biological functions and alterations in lipids are implicated in several diseases such as inflammation, diabetes, renal and heart failures as well as many cancers. In our study, we aim to identify serum lipid signatures as prostate tumor specific biomarkers by investigating complete lipid profiles including different classes of lipids: FAs, TGs, DGs and PLs. Total lipids from serum samples (prostate cancer patients and healthy individuals) were extracted using Bligh and Dyer method with minor modifications. We have applied ESI-MS/MS and GC-MS to identify significantly altered lipids in serum from PCa patients compared to healthy controls. This lipidomic approach revealed 24 lipids significantly different in cancer patient's serum (n = 18) compared to normal (n = 18) with no history of PCa. Hierarchical clustering and

principal component analysis (PCA) were used to analyze differentially regulated lipids and the results have shown promising information that could clearly differentiate cancer patients and control groups. Correlation and partition analysis along with Formal Concept Analysis (FCA) have identified that PC (39:6) and FA (22:3) could classify samples with higher certainty and cataloging patients with 100% sensitivity (all 18 control samples are classified correctly) as well as 77.7% specificity, with p-value of 1.612×10^{-6} in Fischer's exact test. Further, we employed GC-MS to denote fatty acids differentially regulated in PCa patients and observed that alpha-linolenic acid (ALA) levels are altered in PCa. To determine the effect of ALA in survival of human PCa cell lines LNCaP and PC3, we performed an *in vitro* proliferation assay. ALA induces the proliferation of LNCaP and PC3 cells significantly both in dose and time dependent manner. Interestingly, in contrast to its pro proliferative role, higher concentration of ALA at 100 µM suppresses cell proliferation and causing the cell death. However, detailed investigation to understand its role in PCa progression needs to be pursued. Taken together, we proposed that the altered lipids PC (39:6) and FA (22:3) might offer a new set of possible serum biomarkers for diagnosis of prostate tumors. Therefore, validation of these signatures in large cohort of samples with diverse prostate pathology needs to be performed.

Identification and evaluation of prostate cancer associated autoantibodies as new biomarkers:

Tumor-associated antigens released into the blood could induce humoral immune response leading to generation of autoantibodies. Many tumor-associated antibodies have been identified in various cancers and their potential as novel biomarkers for cancer diagnosis are validated. The present study deals with the antibody profiling of PCa patients serum to identify potential new biomarkers. In order to fulfil our aim, we have resolved PCa tissue proteins by 2-DE and immunoblotted using either pooled serum from PCa patients or from normal controls as a primary antibody source. The antigens recognized by antibodies in PCa patient's serum were identified by LC-MS/MS. Eighteen antigens from 21 different spots associated with PCa were identified by mass spectroscopy approach. Further to confirm the specificity of newly identified autoantibodies, we have cloned PRDX2, PRDX6 and

ANXA11genes into pexp5-ct/topo® vector. This system produces His's-tag fusion proteins which were purified with Ni-NTA column chromatography. Using these recombinant antigens, we have confirmed the autoantibodies response to PRDX2, PRDX6 and ANXA11 in both normal and PCa patient's sera. Further, autoantibody response for PRDX6 and ANXA11 were validated in an independent set of PCa patient's and healthy individuals sera. We found high abundance of antibodies against PRDX6 and ANXA11 in PCa patients compared to the controls. Formal concept analysis method was applied to analyze whether the abundance of these autoantibodies could persuade the classification of patients. However, single antibody alone can distinguish prostate tumor and healthy controls with 70% to 80% of sensitivity whereas combination of both PRDX6 and ANXA11 antibodies increased the sensitivity to 90% for tumors and 100% for healthy controls.

Identification of novel mechanisms regulating androgen independent phenotype and their role in the treatment of prostate cancer:

Earlier reports suggest that IL-6 induces growth of PCa cells by promoting expression of AR regulated genes even in the absence of androgens. Thus we aim to investigate the precise molecular mechanism involved in the development of IL6 mediated castration resistant prostate cancer. Proteomic analysis of LNCaP cells stimulated with IL6 was performed using 2-DE coupled MALDI-TOF-MS/MS analysis. We found differential expression of 27 proteins in LNCaP cells upon stimulation with IL6. A global proteome network for differentially regulated proteins were constructed using STRING software and it highlights that VCP (Valosin-containing protein) plays a major role in regulating expression of altered proteins. VCP (p97) is a member of the AAA ATPases, regulates the several ATP-dependent cellular processes such as ubiquitin-mediated proteolysis, DNA repair, membrane fusion and dynamics of sub cellular compartments, gene expression, and cell growth. These noteworthy observations encouraged us to study role of VCP in androgen independent growth of LNCaP cells under IL6 stimulation. In our study we found that IL6 regulates the VCP expression via STAT3 activation through pim-1. VCP is induces AR regulated gene PSA expression in androgen independent manner. Further this observation was confirmed by treating the VCP positive LNCaP cells with AR inhibitor Bicalutamide. The elevated PSA levels remain

unaffected even with AR inhibition in VCP positive cells compared to control EGFP expressing LNCaP cells AR inhibitor attenuated PSA expression. This result clearly indicates that VCP has a novel role in development of CRPC. Elevated expression of VCP promotes cell proliferation, migration and invasion of PCa cells in vitro. Inhibition of VCP by its allosteric inhibitor reverses its pro survival effect leading to PCa cell death. Mechanistic studies demonstrate that cell death occurs due to apoptosis by caspase activation, increased expression of cell cycle inhibitors p21 and p27kip1 along with active PARP and reduced anti- apoptotic protein and Bcl-2. VCP regulates the invasion and migration of cancer cells by altering expression of cell adhesion proteins E-cadherin and Vimentin. Furthermore, we performed immunostaining for VCP expression in human PCa tissue using prostate tissue micro arrays (TMAs). Immunohistochemistry results revealed weak staining in benign prostate hyperplasia and abundant staining in PCa. Thus, from this objective of the study we report VCP as a promising target for the androgen independent prostate cancer treatment.