Dissertation

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Antibody Microarray as a proteomic tool for effective diagnosis and prediction of prognosis in cancer

This work was carried out in Division of Functional Genome Analysis

at the German Cancer Research Centre (DKFZ)

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Thesis Declaration

I hereby declare that I have written the submitted dissertation myself and in this process, I have used no other sources or materials that those listed in the references.

Place and date:

……

Harish Srinivasan.

Dedicated to the

Thousands of Tamil lives

Lost during

The last stages of

War in Sri Lanka.

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Table of contents

Abbreviations

APC	Adenomatous Polyposis Coli protein
APS	Ammonium peroxydisulfate
AUC	Area Under the Curve
BCL2	Apoptosis regulator BCL-2
BSA	Bovine Serum Albumin
CASP3	Caspase-3
CCD	Charge-Coupled Device
CCL18	C-C motif Chemokine 18
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A
DKFZ	German Cancer Research Centre
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	De-oxy Ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
ERBB2	Proto-oncogene c-erbB2
FBS	Fetal Bovine Serum
HCl	Hydrochloric acid
HPV	Human Palliloma Virus
IHC	Immuno-histochemistry
JUN	proto-oncogene c-JUN
KEGG	Kyoto Encyclopedia of Genes and Genomes
LIMMA	Linear Models for Microarray Data

LMNA	Lamin A
LYAM1	L-Selectin
mRNA	MessengerRNA
OCLN	Occludin
PBS	Phosphate-buffered Saline
RNA	Ribonucleic acid
ROC	Receiver Operating Characteristic
rpm	rotations per minute
RPMI1640	Rosewell Park Memorial Institute Medium
S100A9	Calgranulin B
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
siRNA	small interfering RNA
TBS	Tris-buffered Saline
TCA	Trichloro acetic acid
TGFB	Transforming Growth Factor β
TIA1	Nucleolysin TIA1 isoform-p40
TMA	Tissue Microarrays
TP53	Tumor Protein p53
UV	Ultra-Violet
v/v	volume per volume
VEGFA	Vascular Endothelial Growth Factor A
w/v	weight per volume
WHO	World Health Organization
WMA	World Medical Association

Summary

Effective prediction of diagnosis and prognosis in cancer is an important step for selection of a suitable treatment regimen. In this dissertation, the importance of using antibody microarray for effective diagnosis and prognosis of cancer was studied using bladder and gastric cancers.

In the first part of study, establishment of a protein signature to predict recurrence of non-muscle invasive bladder cancer was aimed at. Antibodies against cancer-related proteins were spotted and proteins from recurrent and non-recurrent non-muscle invasive bladder cancer tissues incubated. The protein profiles of the samples were analyzed for statistical significance and differential expression of proteins among the cancer groups. After a series of analysis using bioinformatic tools, a 20 protein-signature predicting recurrence of non-muscle invasive bladder cancer was identified along with important molecular mechanisms underlying recurrence.

High grade gastric adenocarcinomas are often lethal with metastasis and frequent recurrence. The second study concentrated on more personalized cancer medicine by direct comparison of healthy controls and gastric adenocarcinoma tissues from the same patient. Antibody microarray was used to study the protein profiles of gastric cancer by incubating protein samples from healthy controls in tandem with the cancer protein from the same patient. Statistically and clinically significant proteins were identified including a 16 protein signature for betterment of individual-based cancer treatment regimen. Identified biomarkers included known therapeutic targets such as VEGFA, S100A9 and newly identified markers like OCLN and TIA1.

The analyses on two cancer types revealed two different protein signatures with high specificity and sensitivity. Moreover, our findings were clinically relevant and superior to many other approved available methods for diagnosis and prognosis.

Zusammenfassung

Die effektive Diagnose und Vorhesage der Prognose einer Krebserkrankung sind wichtige Schritte für die Auswahl eines geeigneten Behandlungsschemas. In dieser Dissertation wurde die Bedeutung von Antikörper-Microarrays für die effektive Diagnose und Prognose von Krebserkrankungen am Beispiel von Blasen- und Magenkrebs untersucht.

Das Ziel des ersten Teils der Arbeit war die Etablierung einer Proteinsignatur zur Vorhersage eines Rezidivs bei nicht-muskulärem invasivem Blasenkrebs. Antikörper gegen krebsrelevante Proteine wurden auf Microarrays aufgebracht und mit Proteinen aus Tumorgeweben von rezidivierendem und nicht-rezidivierendem, nicht-muskulärem invasivem Blasenkrebs inkubiert. Die Proteinprofile der Proben wurden auf statistische signifikante Unterschiede geprüft und die differentielle Expression der Proteine zwischen den unterschiedlichen Krebsarten analysiert. Mit Hilfe von bioinformatischen Auswertungsmethoden konnte eine Proteinsignatur bestehend aus 20 Proteinen zur Vorhersage eines Rezidivs bei nicht-muskulärem invasivem Blasenkrebs sowie wichtige, dem Rezidiv zugrundeliegende molekulare Mechanismen identifiziert werden.

Magen-Adenokarzinome in fortgeschrittenen Stadien verlaufen häufig tödlich, mit Metastasierung und hoher Rezidivrate. Der zweite Teil der Arbeit konzentrierte sich stärker auf personalisierte Krebsmedizin. Hierzu wurden Kontrollen aus gesundem Gewebe und Gewebeproben aus dem Magen-Adenokarzinom eines Patienten direkt miteinander verglichen. Unter Verwendung eines Antikörper-Microarrays wurden die Proteinprofile von Magenkrebs untersucht, indem Proteinproben aus den gesunden Kontrollproben und Tumorproben des gleichen Patienten zusammen inkubiert wurden. Statistisch und klinisch signifikant unterschiedlich experimente Proteine einschließlich einer Proteinsignatur aus 16 Proteinen zur

Verbesserung eines individualisierten Krebs-Behandlungsschemas wurden identifiziert. Die identifizierten Biomarker umfassten sowohl bekannte therapeutische Targets wie VEGFA und S100A9 als auch neu entdeckte Marker wie OCLN und TIA1.

Die Analyse zweier Krebsarten ergab zwei unterschiedliche Proteinsignaturen mit hoher Spezifität und Sensitivität. Darüber hinaus waren unsere Ergebnisse klinisch relevant und vielen anderen anerkannten und verfügbaren Methoden zur Diagnose und Prognose überlegen.

1 Introduction

1.1 Cancer

Cancer is a non-communicable disease with high complexity and heterogeneity. It is the second leading cause of death after cardio-vascular diseases (Lozano et al., 2012). 13% of all death occurrences in world are caused by cancers. Approximately, five hundred out of a hundred thousand people are at high risk of cancer occurrence in their life time (WHO, 2009). Cancer nomenclature depends on the part of the body they originate from. All types of cancers are clinically distinct to each other but they all develop from uncontrolled cell growth resulting in formation of tissue mass of unstructured cells called tumor. Cancer is classified into two general types based on their aggressiveness in growth. Benign tumors are those that grow without invading other adjacent tissues. Tumors that invade nearby tissues and often organs are called malignant tumors. Malignant tumors often infiltrate into the blood stream, travel to other distant areas from their organ of origin and form colonies of tumors there. This process is called metastasis and this potential of tumors best describes the aggressiveness of the corresponding cancer (Weinberg, 2007). The metastatic cancers more lethal that they account of over 90% of all cancer related deaths (WHO, 2009).

Cancer is a disease of malfunctioning cells because the native tissue is both structurally and functionally disorganized in such a way that the immune responses are often evaded or suppressed. A transformation of a single cell within an organism can lead to formation of a tumor. This transformation can be facilitated by cancer causing agents like free radicals, toxins, UV exposure and viruses for example human papilloma virus (HPV). Most of these agents cause functional mutations in DNA (Weinberg, 2007). Mutations are known to be spontaneous but

with high rarity in human body. Various safeguard mechanisms facilitate human body to protect it from serious cellular defects and mutations. Mechanisms like DNA damage repair, cell cycle arrest, deregulation of cellular energetics are normally active in cells. For example, DNA repair system removes a part of mutated DNA that might happen during the DNA replication process. Cell division process, highly controlled normally is arrested when the cells undergo severe damage due to mutations or stress. In certain cases when a fundamental damage happen to DNA or cell itself that cannot be repaired, then cells undergo a process of programmed cell death also called as apoptosis (Alberts B, 2007).

Most types of cancers arise when the above mentioned mechanisms do not work properly, especially under constant dysregulation of the genes involved in these key processes. Based on their activities, these self-protective genes are classified into two types: oncogenes and tumor suppressor genes. Growth-promoting genes that contribute to carcinogenesis by favoring the tumor cells to evade various growth control signaling mechanisms are called oncogenes. Tumor suppressor genes, often down-regulated in cancers are generally necessary for cells to maintain the important growth control barriers and balancing proliferating ability and death of the cells (Weinberg, 2007). Apart from these genes, several other mechanisms like angiogenesis, promoting inflammation and self signaling for proliferation of cells also facilitate cancer development (Hanahan and Weinberg, 2011). All the above mentioned processes account for the multistep process of carcinogenesis which makes treating the disease a challenge for modern biologists (Hanahan and Weinberg, 2011).

1.1.1 Bladder cancer

Bladder cancer is the fourth most common cancer in men and ninth most common cancer in women. It accounts for more than 3% of total cancer related deaths in a year (Siegel et al., 2012). According to estimations by world health organization (WHO), hundred and fifty thousand people die every year of bladder cancer and it belongs to highly monitored post-operative cancers (Jacobs et al., 2010; WHO, 2009). Though the cancer is less lethal than many other major cancer types, the cost of treatment and periodical surveillance of the patients are rather expensive and often result in disappointing results (Sanchez-Carbayo and Cordon-Cardo, 2007). Cancers in bladder are clinically classified into two major types based on the types of cells cancer develop from, urothelial cell carcinomas which constitute of 95% of malignant bladder cancers and squamous cell carcinoma that constitute 5% of bladder cancers (Luis et al., 2007).

1.1.1.1 Urothelial cell bladder cancer

Pathologically, urothelial cell cancers are classified into superficial muscle or non-muscle invasive cancers and muscle invasive cancers based on their invasiveness. About 75% to 85% newly diagnosed bladder cancers are non-muscle invasive cancers (grades Ta and T1) and 15% to 25% are muscle invasive bladder cancers (grades T2 and T3) (figure 1). Even after an initially successful treatment by complete surgical resection of the tumors, 60% to 70% of the tumors will recur and 10% to 30% will progress to become muscle invasive cancers. Most of the muscle invasive cancers are also metastatic (Jacobs et al., 2010). Most of the non-muscle invasive tumors recur within five year after the surgical resection of the primary tumors. Regular surveillance cystoscopy and urine cytology is employed every three months during the first two years after resection, at longer intervals over the next two years, and annually thereafter (Montie et al., 2009). The lifetime surveillance costs right from pre-operative phases ranges from US$

99,000 to US$ 121,000 (Avritscher et al., 2006). Above the financial burden, the physical and psychological stress, the patients are under is more important. Patients live in a state of uncertainty and are confronted with the possibility of yet another cancer diagnosis every three months of continuous surveillance (Jacobs et al., 2010; Luis et al., 2007).

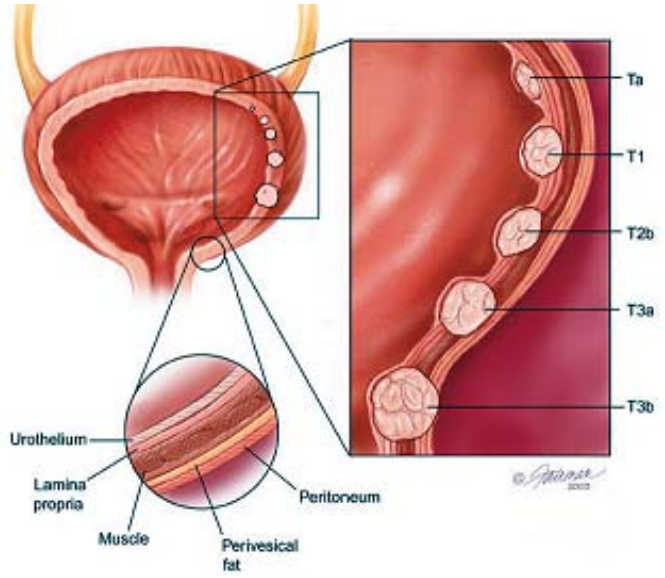

Figure 1. Anatomy of bladder- grading of urothelial cell carcinoma based on their depth of invasion (grades Ta to T3b). Ta tumors do not invade basal muscle or lamina propria and T1 do not invade muscle (Adapted from department of urology at Michigan urology centre, University of Michigan).

1.1.1.2 Prognostic assessment of non-muscle invasive bladder cancer

Most prognostic assessments of non-muscle invasive bladder cancer usually employ several features of tumors like multifocality, clinical grading, size and stage (Dubosq et al., 2012). Many immuno-histochemical markers have been proposed but in clinical settings, the practice of these markers has very less impact (Matsushita et al., 2011). Molecules like proliferation activators, proto-oncogenes and caveolins at mRNA level are also being used with not much correlation to the recurrence of the cancer (Dubosq et al., 2012; Mitra et al., 2009). Epigenetical modifications

in promoter sequences are being implemented as predictors of tumor progression (Jeong et al., 2012). Lower levels of galectin-8 in tumor cells compared to healthy controls are known to predict recurrence of non-muscle invasive cancer but without great specificity (Kramer et al., 2011). Mutations in TP53 are also studied as predictors of high risk for recurrence (Andreasson et al., 2008; Dexlin et al., 2008). Apoptosis regulators like CASP3, BCL2 and TP53 in combination with other genes predict the outcome of bladder cancer in non-muscle invasive patients (Korkolopoulou et al., 2000). However, no dependable biomarker or a set of markers presently exist to predict recurrence with clinically significant accuracy in non-muscle invasive bladder cancer (Babjuk et al., 2008; Babjuk et al., 2011; Dubosq et al., 2012). Thus, biomarkers for effective prediction of recurrence in non-muscle invasive bladder cancer can dramatically reduce the burden caused by the disease on patients and extremely high financial need for health care.

1.1.2 Gastric cancer

Gastric cancer is second most common in mortality among cancers worldwide, mostly occurring in developing nations (Dicken et al., 2005). According to WHO estimations, gastric cancer leads to seven hundred and thirty seven thousand deaths every year which is 10% of overall cancer related deaths worldwide (Dicken et al., 2005; WHO, 2009). The mortality rate has remained unchanged over the past three decades despite advancement of surgical techniques and therapeutics. Gastric cancers are generally highly incident in patients over forty five years old, hence the mortality rate is quite high despite curative therapy most commonly involving surgical resection of whole stomach or a part of it. The treatment strategy and response are quite poor such that over all five year survival rates rage about 10% to 25% (Takeno et al., 2008).

Besides the high number of incidence and mortality, gastric cancer is a heterogeneous cancer in terms of clinical and pathological classification, genomic and proteomic background of the tumor mass and clinical outcomes (Zheng et al., 2004). Gastric cancer types are named after their cells they originate from. Most of the diagnosed gastric cancers are adenocarcinomas (95%), other types include squamous cell carcinoma, neuroendocrine tumors, stromal tumors and gastric lymphoma (Dicken et al., 2005; Zheng et al., 2004).

1.1.2.1 Gastric adenocarcinoma grading and characteristics

Gastric adenocarcinoma arising from secretory epithelia in the inner lining of gastric mucosa is the most aggressive form of gastric cancers. Based on the invasiveness of the tumor and differentiation of the cells, adenocarcinoma is clinically and pathologically graded into four types (figure 2) (Dicken et al., 2005). Most of the adenocarcinomas diagnosed belong to advanced stages (T3/T4) of the cancer accompanied by lymph node metastasis. The association of numerous lymph nodes morphologically with stomach tissues enables irresistible metastasis of tumor cells in advanced stages of the cancer (Senapati et al., 2008). Furthermore, advanced stages of gastric adenocarcinoma recur at relative higher rates of 40% to 70% after the curative surgical resection of primary tumor. Routine surveillance by means of endoscopy followed by adjuvant chemotherapy after primary resection leads to a median survival of nineteen months. In case of limited surveillance it drops to just eight months. Advanced stages of carcinoma without curative resection of tumors have a median survival rate of merely five months (Dicken et al., 2005). Such low survival rates combined lymph node metastasis make treatment options not so much responsive and in turn lead to increased mortality (Dicken et al., 2005; Pietrantonio et al., 2013; Zheng et al., 2004).

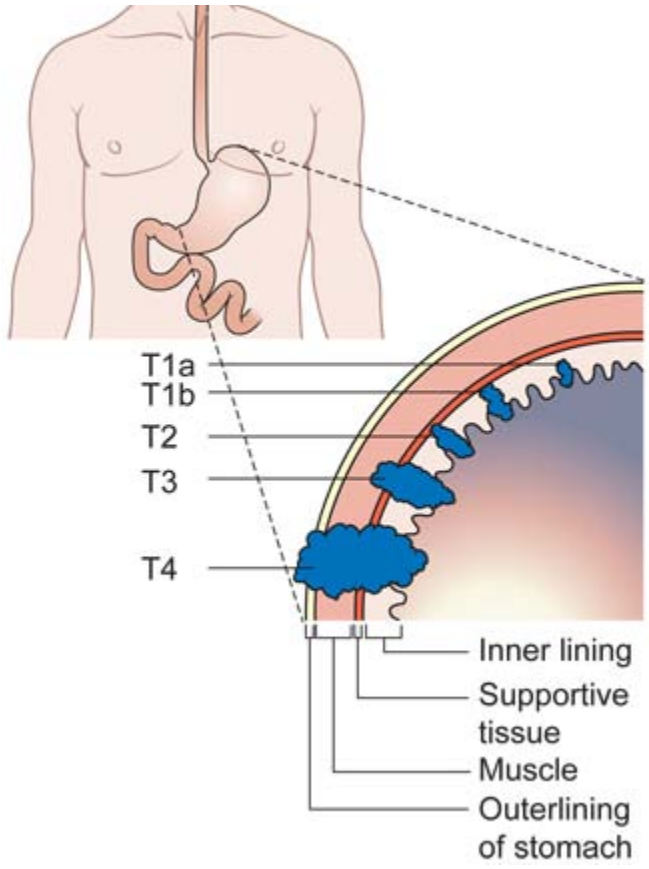

Figure 2. Anatomy of stomach- staging of gastric adenocarcinoma based on their extent of invasiveness (grades Ta to T4). Ta tumors originate at the inner lining and slowly protrude out invading other layers of stomach. T4 tumors are highly metastatic (Adapted from cancer research UK-homepage for gastric cancers).

1.1.2.2 Biomarkers and treatment in gastric adenocarcinoma

Gastric adenocarcniomas are often diagnosed merely by the localization of the tumor cells and their invasiveness. There are very few molecular biomarkers available to grade them according to their invasiveness (Pietrantonio et al., 2013). Diagnosis based on the transcript levels of p53 and mutations in APC genes predicted gastric cancer but failed to differentiate the type of tumor (Zheng et al., 2004). Growth factor receptors like EGFR, c-met and ERBB2 have been used as predictors of later stages of malignancy but with much instability. CCL18 combined with certain T-cell receptors are employed as prognostic indicators in lower stages of gastric cancer (Leung et

al., 2004). Epithelial-mesenchymal transition marker proteins like E-cadherin are also used as predictors for higher stages of gastric adenocarcinomas. Certain cytokines are used as predictors of prognosis in gastric cancers associated with Helicobacter pylori infection but they do not have much significance in prediction of other types of adenocarcinoma (Ellmark et al., 2006).

Many promising molecular biomarkers like S100A9, VEGFA and mucin family of proteins are also tested as potential therapeutic targets. Despite recent developments in gene sequencing and molecular diagnostics, many of these biomarkers are inconsistent in predicting a unanimous treatment regimen. Few treatment strategies have been introduced but molecular complexity and drug resisting capability of the cancers make the treatment of adenocarcinomas nearly impossible. Nevertheless, many biomarkers are being evaluated by various clinical trials to identify individual-based criteria and establish customized personal therapeutic approaches (Pietrantonio et al., 2013). The best treatment regimen available so far for higher stages of gastric cancer is curative surgical resection of the tumor followed by adjuvant chemotherapy with oxaliplatin and capecitabine. These two agents are reportedly highly efficient combined with less toxic effects and side-effects (Cunningham et al., 2010). Unfortunately, clinical trials on different biomarkers are experiencing critical ignorance such that most fail rather than considered as potential targets. Thus, introducing new therapeutic targets and predictive markers along with immuno-based screening tools will enable developing patient-specific chemotherapy in turn optimal drug efficiency and minimal adverse effects.

1.2 Antibody microarrays in cancer

Omic technologies are excellent tools for both diagnostic and prognostic biomarker identification in cancers, a disease with high molecular and metabolic complexity. DNA microarrays are widely employed for initial screening studies on biomarkers at transcript levels in a wide variety of diseases. Inconsistency in validation of biomarkers identified by various genomic technologies led to the emergence of proteomic applications in biomarker discovery. Antibody-based technologies are extremely advantageous in biomarker discovery as they use biological end product which indulge in vital cellular activities (Michaud et al., 2003). NHS–based protein labeling methods provide a platform for large scale analysis of antibodies. Optimizing various factors including sensitivity and specificity for targeting analytes in low quantities brought in a new method of proteomic analysis (Wingren et al., 2007). Thus antibody microarrays are relatively a new tool for an analysis of protein abundance in parallel and highly multiplexed manner (Brennan et al., 2010) (figure 3).

Antibody microarrays are extremely different tissue microarrays where the interpretation and validation of enriched targets are often subjected to differing opinions among individuals. Another advantage antibody array has over tissue microarray is the use of non-invasive body fluids like urine, saliva and plasma (Alhamdani et al., 2009). Solid supports like glass slides coated with chemical binders are used to immobilize antibodies on the glass surface. Cancer-related antibodies are used for the production of antibody arrays in large scale (Kusnezow et al., 2003). The number of targets used in the arrays varied from mere hundred to as high as thousand in fact with higher specificity and sensitivity (Sanchez-Carbayo et al., 2006; Schroder et al., 2010). Studies involving protein extracts from tissues and cells are rarely done using antibody microarrays due to technical aspects (Hoheisel et al., 2013). However, optimized protocols and

detailed assessment are being established which permit antibody microarray with high accuracy and reproducibility for effective cancer diagnosis and prognosis (Alhamdani et al., 2010; Schroder et al., 2010). Recently, higher sensitivity detecting single molecules using antibody microarrays have been reported (Schmidt et al., 2011). Thus, antibody microarray being an immuno-based assay, can bridge the gap between effective diagnosis and personalized cancer medicine.

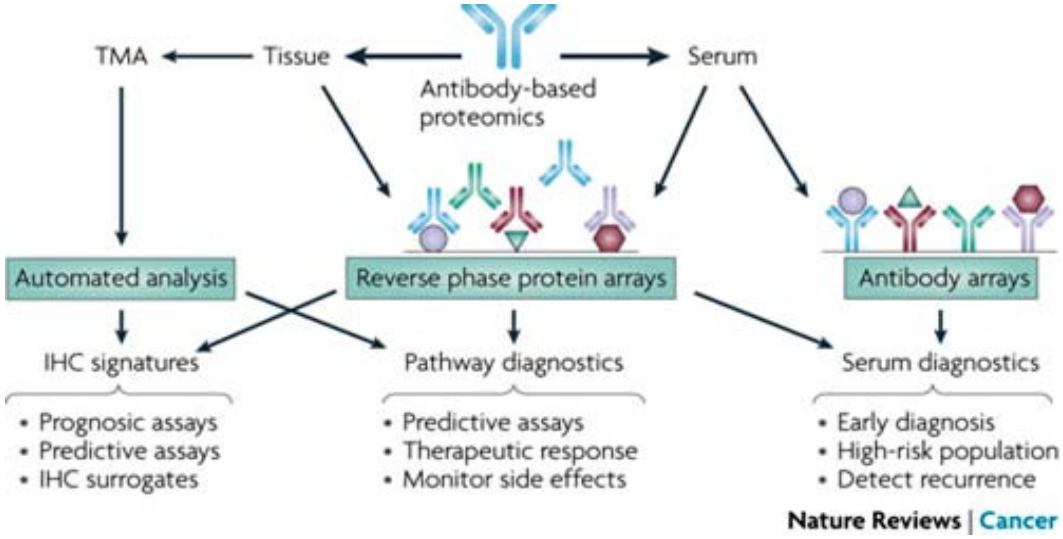

Figure 3. Proteomic technologies being employed for effective cancer diagnosis and personalized cancer medicine. (Adapted from Brennan et al 2010 Nature reviews cancer).

1.3 Aim

In this dissertation, potentiality in using antibody microarray as a tool for effective cancer diagnosis and prediction of prognosis is studied.

The first part of the study is aimed at predicting recurrence of non-muscle invasive bladder cancer. For this purpose, twenty five non-muscle invasive bladder cancer samples with a minimum of five year follow-up time after the initial surgical resection were analyzed using antibody microarray. After the analysis, the identified protein signature would be helpful for the prediction of recurrence in non-muscle invasive bladder cancer and to establish a signature-based treatment regimen for bladder cancer patients.

The second part of the study is aimed at identifying more personalized biomarkers for effective diagnosis and treatment of gastric adenocarcinomas. Thirty cancer tissues and healthy control tissues from the same patient were analyzed using antibody microarray to identify a significant protein profile. Identified protein profile will provide valuable insights on tumor development in gastric adenocarcinoma. The identified protein profile will not only help in an individual-based treatment regimen for gastric cancer treatment but also will identify more therapeutically important targets.

2 Material and Methods

2.1 Materials

2.1.1 Instruments

Instrument	Manufacturer
12-channel-pipette Biohit proline	Biohit, Helsinki, Finland
96-well flat-bottom block	Qiagen, Hilden, Germany
96-well reaction plates	Steinbrenner, Wiesenbach, Germany
6-well culture plates	Steinbrenner, Wiesenbach, Germany
24-well culture plates	Steinbrenner, Wiesenbach, Germany
Automatic developing machine	Amersham, Freiburg, Germany
Beckmann GS-6KR centrifuge	Beckmann, Wiesloch, Germany
Centrifuge 5810 R	Eppendorf, Hamburg, Germany
Centrifuge 580 R	Eppendorf, Hamburg, Germany
Cell culture incubator	Koettermann, Haenigsen, Germany
Cell culture microscope	Carl Zeiss, Jena, Germany
Cell viability analyzer	Beckmann, Wiesloch, Germany
Dismembrator	B.Braun Biotech, Melsungen, Germany
Dry block heating system	Grant instruments, Cambridge, UK
Electrophoresis power supply	E-C apparatus corporation, USA
Epoxysilane-coated slides Nexterion-E	Schott, Jena, Germany
Infinite m200 multimode reader	Tecan Grp Ltd, Maennedorf, Switzerland
MicroGrid II Array-Roboter	Biorobotics, Cambridge, UK
Mini-protein electrophoresis system	Bio-Rad Laboratories, Munich, Germany
Power supply E835	Hoefer, CA, USA
QuadriPERM plates	Vivascience, Hannover, Germany
ScanArray 4000XL	Perkin Elmer, Massachusetts, USA
Sigma 2k15 centrifuge	M&S laborgeraete gmbh, Wiesloch, Germany

SlideBooster hybridization station	Advalytix, Munich, Germany
SMP3B stealth pins	Telechem, CA, USA
TE70 PWR semidry transfer unit	Amersham, San Fransisco, USA
Vortex	Scientific industries Genie-2, New York,USA
Water bath SW22	Julabo Labortechnik, Seelbach, Germany

2.1.2 Chemical reagents, enzymes and general materials

Reagent	Supplier
2-Mercaptoethanol	Roche Diagnostics, Mannheim, Germany
6-Aminocaproic acid	Sigma-Aldrich, Munich, Germany
12-Maltoside	Sigma-Aldrich, Munich, Germany
Acetic acid	Mallinckrodt Baker, Greisheim, Germany
Acrylamide (30%w/v)/Bisacrylamide (29/10.8%)	Bio-Rad Laboratories, Munich, Germany
Ammonium peroxydisulfate (APS)	Sigma-Aldrich, Munich, Germany
ASB-14	Sigma-Aldrich, Munich, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, Munich, Germany
Bromphenol blue	Sigma-Aldrich, Munich, Germany
Chloroform	Merck, Darmstadt, Germany
Cleanascite™ lipid removal agent	Biotech support group, New Jersey, USA
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Munich, Germany
ECL hyperfilm	GE Healthcare Europe, Freiburg, Germany
Ethylenediaminetetraacetic acid (EDTA)	Merck, Darmstadt, Germany
Ethanol	Merck, Darmstadt, Germany
Glycerin	Roth, Karlsruhe, Germany
Glycine	Roth, Karlsruhe, Germany
Hydrogen chloride (HCl)	Merck, Darmstadt, Germany
Isopropanol (2-propanol)	Mallinckrodt Baker, Greisheim, Germany
Magnesium chloride	Merck, Darmstadt, Germany

Membran Protran NCBA85	Whatman gmbh, Hassel, Germany
Methanol	Merck, Darmstadt, Germany
Milk powder (Skimmed)	Sigma-Aldrich, Munich, Germany
Na_2HPO_4	Merck, Darmstadt, Germany
NaH_2PO_4	Merck, Darmstadt, Germany
NaOH	Merck, Darmstadt, Germany
Natriumacetat	Merck, Darmstadt, Germany
Natriumazide	Merck, Darmstadt, Germany
Natriumchloride	Merck, Darmstadt, Germany
Natriumcitrate	Merck, Darmstadt, Germany
Na-cholate	Sigma-Aldrich, Munich, Germany
NP-40 substitute	Sigma-Aldrich, Munich, Germany
Nuclease free water	Ambion, Austin, USA
PMSF	Sigma-Aldrich, Munich, Germany
Potassium Chloride	Sigma-Aldrich, Munich, Germany
Potassium dihydrogen phosphate	Sigma-Aldrich, Munich, Germany
Sodium Carbonate	Sigma-Aldrich, Munich, Germany
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, Munich, Germany
Spectra multicolor broad range protein ladder	Thermo Scientific, Rockford, USA
Sulphorhodamine B (SRB)	Sigma-Aldrich, Munich, Germany
T-PER tissue protein extraction reagent	Thermo Scientific, Rockford, USA
TEMED	Bio-Rad Laboratories, Munich, Germany
Trichloro acetic acid (TCA)	Fisher Chemicals, Reading, UK
Tris-Base	Sigma-Aldrich, Munich, Germany
Tris-HCl	Sigma-Aldrich, Munich, Germany
Triton-X100	Sigma-Aldrich, Munich, Germany
Tween-20	Sigma-Aldrich, Munich, Germany
Tween-80	Sigma-Aldrich, Munich, Germany

2.1.3 Cell culture

Reagent	Supplier
DMEM	Gibco/Invitrogen, Karlsruhe, Germany
DMEM/F12 (Ham)	Gibco/Invitrogen, Karlsruhe, Germany
Fetal Bovine Serum (FBS)	Gibco/Invitrogen, Karlsruhe, Germany
L-Glutamine	Gibco/Invitrogen, Karlsruhe, Germany
Phosphate buffered saline (PBS)	Gibco/Invitrogen, Karlsruhe, Germany
Penicillin 1000u/ml-Streptomycin 100µg/ml	Gibco/Invitrogen, Karlsruhe, Germany
RPMI	Gibco/Invitrogen, Karlsruhe, Germany
Trypsin/EDTA solution	Gibco/Invitrogen, Karlsruhe, Germany

2.1.4 Dyes and Kits

Item	Supplier
The blocking solution	CANDOR Biosci. gmbh, Wangen, Germany
Dy-549-NHS	Dyomics, Dresden, Germany
Dy-649-NHS	Dyomics, Dresden, Germany
ECL prime western blot detection kit	GE Healthcare Europe, Freiburg, Germany
Novagen BCA protein assay kit	Merck, Darmstadt, Germany
siPORT™ *NeoFX*™ Reverse transfection kit	Ambion, Austin, USA

2.1.5 Antibodies and synthetic RNAs

Item	Supplier	Dilutions
Anti.TIA-1	Santa Cruz, Biotech. Inc., Texas, USA	1:1000
Anti.S100A9	Santa Cruz, Biotech. Inc., Texas, USA	1:500
Sec. antibody-Goat	Santa Cruz, Biotech. Inc., Texas, USA	1:5000
GAPDH-HRP conjugated	Sigma-Aldrich, Munich, Germany	1:25000
TIA1 siRNA-1	Qiagen, Hilden, Germany (SI00133098)	

TIA1 siRNA-2	Qiagen, Hilden, Germany (SI00133105)
TIA1 siRNA-3	Qiagen, Hilden, Germany (SI00133112)
TIA1 siRNA-5	Qiagen, Hilden, Germany (SI00301917)
TIA1 siRNA SMARTpool	Dharmacon, Rockford, USA (M-013042-02-005)
Allstars Negative control siRNA	Qiagen, Hilden, Germany (1027280)

2.1.6 Cell lines

Cell lines	Cellular origin	Tumorigenic	Source
AGS	Stomach	Yes	Cell lines service (300408)
HGC-27	Stomach	Yes	Cell lines service (300436)
MKN-45	Stomach	Yes	Dr. Christiane Dinsart, DKFZ

2.1.7 Buffers and Solutions

Solutions and Buffers	Components
APS	10% APS w/v in ddH_2O
10X PBS	137 mM NaCl 27 mM KCl 100 mM NaH_2PO_4 17 mM KH_2PO_2 dissolved in ddH_2O
PBST	10X PBS with 0.1% (v/v) Tween 20
PBSTT	10X PBS with 0.1% (v/v) Tween 20 and 0.1% (v/v) Triton-X 100
10X TBS	500 mM Tris.HCl 1500 mM NaCl dissolve in ddH_2O and pH 7.6
TBST	10X TBS with 0.1% (v/v) Tween 20
10% SDS	10% (w/v) SDS dissolve in ddH_2O

Stacking gel buffer	1.5 M Tris.HCl dissolve in ddH_2O and pH 6.8
Resolving gel buffer	1.5 M Tris.HCl dissolve in ddH_2O and pH 8.8
10X SDS gel Tank buffer	50 mM Tris-Base 400 mM glycine 10% (w/v) SDS dissolve in ddH_2O
Lysis buffer for protein isolation	500 µl of NP-40 1000 µl of Na-Cholate 1000 µl of ASB-14 1000 µl of 12-maltoside 2000 µl of glycerol (99%) 1000 µl of sodium carbonate 167 µl of magnesium chloride 500 µl of EDTA.2Na 50 µl of PMSF 100 µl of protease and phosphotase inhibitor 0.4 µl of Benzonase (100U/µl) 2683 µl of ddH_2O
Western blot anode buffer I	30 mM Tris-Base 20% (v/v) methanol dissolve in ddH_2O
Western blot anode buffer II	5 mM Tris-Base 20% (v/v) methanol dissolve in ddH_2O
Western blot cathode buffer	20 mM 6-aminocaproic acid 20% (v/v) methanol dissolve in ddH_2O
Milk-blocking buffer	5% milk powder in 1XTBST
Milk blocking buffer for microarray	10% milk powder in 1XPBSTT

2.2 Methods

2.2.1 Selection and collection of samples for the study

2.2.1.1 Bladder cancer samples

Primary tumors were resurrected from patients with low-grade (stages Ta and T1) non-muscle-invasive bladder cancer at the Department of Urology and Pathology of Henri Mondor Hospital in Paris, France. All subjects were informed with a written consent and the same was obtained from all subjects. The analysis was approved by the local ethics committee and all the experiments conformed to the principles set out in the WMA Declaration of Helsinki. Only samples were considered with a patient follow-up of at least five years post the surgical resection of the primary tumour. Nineteen patients experienced recurrence within the five years of follow-up while six patients did not. In addition, three normal bladder tissue samples were available as controls. The age and gender of all the samples studied are equally distributed and none of the patients did receive any cancer-related therapy before sampling. The tissue samples were snap-frozen in liquid nitrogen soon after resection to protect the tissue and cellular components. Then, the tissue samples were pulverized using dismembrator under deep freeze conditions (-50ºC) and protein isolation was carried out.

2.2.1.2 Gastric cancer samples

Primary tumors from patients diagnosed with high-grade and stage invasive gastric adenocarcinoma along with a part of the normal tissue surrounding the tumors were resurrected at the Department of Gastroenterology, University medical centre, Ljubljana, Slovenia. All subjects were informed with a written consent and the same was obtained from all subjects. The analysis was approved by the local ethics committee and all the experiments conformed to the

principles set out in the WMA Declaration of Helsinki. Twenty five pairs (normal and tumour) of tissue samples were collected from twenty five patients and the samples were snap-frozen in liquid nitrogen until further use to protect the tissue and cellular components. Then, the tissue samples were pulverized using dismembrator under deep freeze conditions (-50ºC) and protein isolation was carried out.

2.2.2 General methods in molecular biology

2.2.2.1 Protein isolation

Homemade lysis buffer was used for the isolation of whole protein from tissues and cells.

From Tissues

Pulverized tissue was collected in eppendorf tubes and ice cold lysis buffer was added at a volume of 10 µl/1 µg of tissue. A disposable syringe with needle was used to break and tear the tissue and the solution was aspirated and suspended continuously. The eppendorf tubes were shaken gently for 10 minutes on ice and centrifuged for 5 minutes at 13,000 rpm in a table top centrifuge at 4ºC. The supernatant was collected in a fresh eppendorf tube and stored at -20ºC.

From Cells

Medium from the 6-well plates was aspirated and 60 µl of lysis buffer was added to each well of the plate. A cell scraper was used to spread the lysis buffer over the cells and to detach them. Lysates were transferred to eppendorf tubes and the tubes were gently shaken on ice for 10 minutes. Then the tubes were centrifuged for 5 minutes at 13,000 rpm in a table top centrifuge at 4ºC. The supernatant was collected in a fresh eppendorf tube and stored at -20ºC.

2.2.2.2 Lipid removal from protein samples

Protein samples were often contaminated with lipids that made quantification and labeling of the samples difficult. Cleanascite™ was added to the samples to remove lipids. Cleanascite solution was added to the sample at a ratio of 1:2 and incubated for an hour at 4ºC with constant shaking at a very low speed. After centrifugation at 13,000 rpm for 5 minutes, the supernatant free from lipids was collected in a fresh eppendorf tube and the concentration was measured by BCA assay. Then the tubes were stored at -20ºC. The precipitate containing lipids was discarded.

2.2.2.3 Protein quantification by BCA assay

Total protein from both tissue samples and cells were quantified using Novagen BCA protein assay kit. This method involves the colorimetric detection of cuprous cation using bicinchoninic acid (BCA) which is formed by the reduction of Cu+2 to Cu+1 by the protein in an alkaline medium. BCA reagent A and reagent B were mixed at a ratio of 50:1 to make the BCA working reagent. 10 µl of BSA dissolved in lysis buffer and samples were pipette into the wells of a fresh 96-well microtiter plate. 200 µl of the BCA working reagent was added to each well and the plate was incubated at 37ºC for 30 minutes. The absorbance was then measured at 562 nm and a standard curve of absorbance versus standard proteins (in µg) was prepared to determine the concentration of the samples.

2.2.2.4 Western blot analysis

10% SDS gels were used for resolving protein. 10% and 5% acrylamide/bisarcylamide were used respectively for resolving and stacking part of the gel. 0.06% (w/v) ammoniumpersulphate and 0.1% (v/v) N, N, N', N' – tetramethylethylenediamine (TEMED) were used to induce the polymerization of the gel. 10 µg of protein with rotiload loading dye were boiled together for 5 minutes and loaded into the respective slots in the gel. A prestained- protein ladder was also

loaded referring to the molecular weight. Electrophoresis of the gel was carried out for 90 minutes at 135 V and 500 mA in 1X SDS-gel tank buffer. The transfer of polypeptides from the gel to a nitrocellulose membrane was carried out by TE70 PWR semidry transfer apparatus. A sandwich model was prepared by soaking Whatman filter papers in anode buffers I, II and cathode buffer. The membrane was activated in anode buffer II.

The stacking part of the gel was cut and the sandwich was assembled with the filter papers, membrane and the gel after which the semidry electrophoretic transfer was carried out for 60 minutes at 35 V and 500 mA. To detect the transferred protein, after the transfer the membrane was blocked for 1h at room temperature with the milk blocking buffer. After blocking, the membrane was incubated with the diluted primary antibody over night at 4ºC. After incubation, the membrane was washed 3 times with 1XTBST and was incubated with secondary antibody conjugated with horse radish peroxidase for 1h at room temperature. Then, the membrane was washed for 3 times with 1XTBST and protein was detected by enhanced chemiluminescence (ECL) using the ECL prime western blot detection kit. The ECL substrate was prepared according to the manufacturer's instructions and incubated on the membrane for 1 minute and the solution was drained off. Now, the membrane was kept on a clean plate inside the LAS Fujifilm 5000 machine and images were captured using a CCD camera on exposing the membrane gradually to the X- rays. Similarly, the procedure was repeated for the detection of the house keeping protein in the same membrane.

2.2.3 Methods in cell culture and related assays

2.2.3.1 Routine maintenance of cells

Cell lines were maintained at 37ºC and 5% CO_2. MKN-45 cells were maintained in RPMI with 4.5 g/L D-glucose supplemented with 10% fetal bovine serum (FBS), 1000 u/ml penicillin and 100 µg/ml streptomycin and 2 mM glutamine. AGS cells were maintained in DMEM with 4.5 g/L D-glucose supplemented with 10% fetal bovine serum (FBS), 1000 u/ml penicillin and 100 µg/ml streptomycin and 2 mM glutamine. HGC-27 cells were maintained in DMEM/F12 (ham) with 4.5 g/L D-glucose supplemented with 5% fetal bovine serum (FBS), 1000 u/ml penicillin and 100 µg/ml streptomycin and 2 mM glutamine. All cells were passaged every 2 to 3 days and sub cultured in fresh medium.

2.2.3.2 Transfection of cells

All the RNA transfections in this study were carried out in 6-well or 96-well plates using siPORT^{TM} *NeoFX*^{TM} (Ambion) reagent. Reverse transfection by means of siPORT^{TM} *NeoFX*^{TM} involves simultaneous tranfecting and plating of cells. siPORT^{TM} *NeoFX*^{TM} transfection agent and the RNA molecules are mixed and distributed on the culture plates over which the cells are overlaid. The final transfection volume in a 6-well plate is 2.5 ml of medium containing 2 x 10^5 cells per well and in a 96-well plate is 100 µl of medium containing 5 x 10^3 cells per well. As the transfection complexes are stable in presence of serum, no change of medium or other precautionary measures taken in case of traditional transfections methods are needed. The final concentration of the RNA molecules transfected ranges from 5 nM to 50 nM. After this procedure, the plates were maintained at 37ºC and 5% CO_2.

2.2.3.3 Cell viability using Sulphorhodamine B (SRB)

After 48 hours of incubation of cells at 37ºC, the plates were taken out and medium is discarded. 10% (w/v) TCA was added to the wells and the plates were incubated at 4ºC for 2 hours to fix the cells. TCA is then discarded and the plates were rinsed with water and dried at 37ºC for 20 minutes. 0.05% (w/v) of sulphorhodamine B (SRB) reagent was added to the wells and the plates were incubated for 30 minutes at room temperature in dark. The plates were washed for 3 to 4 times with 1% (v/v) acetic acid to remove SRB reagent and then dried for 20 minutes at 37ºC. 100 mM Tris was added to the plates and the plates were shaken for 10 minutes after which, the absorbance was measured at 570 nm from the stained cells and at 650 nm from blank after which, the results were tabulated for calculating the percentage of viable cells after transfection.

2.2.4 Antibody microarray

2.2.4.1 Selection of antibodies and generation of microarray

In-house developed antibody microarray had 813 antibodies directed against 724 cancer related proteins. The antibodies were selected based on previous studies of transcription profiling involving many cancer entities and strong literature search on cancer related proteins. The list of antibodies is found in annexure A. The antibodies were printed on epoxysilane-coated Nexitron-E slides using the contact printer MicroGrid-2 and SMP3B pins at a humidity of 40% to 45%. The buffer composed of 0.1 M carbonate buffer of pH 8.5 with 0.01% tween-20, 0.05% sodium azide, 0.5% dextran, 5 mM magnesium chloride and 1 mg/ml of antibody. Streptavidin molecules labeled with Cy3 or Cy5 were spotted as dye controls and the slides were allowed to quilibrate at humidity of 40% to 45% overnight and then stored at 4ºC in dry, dark conditions until further use.

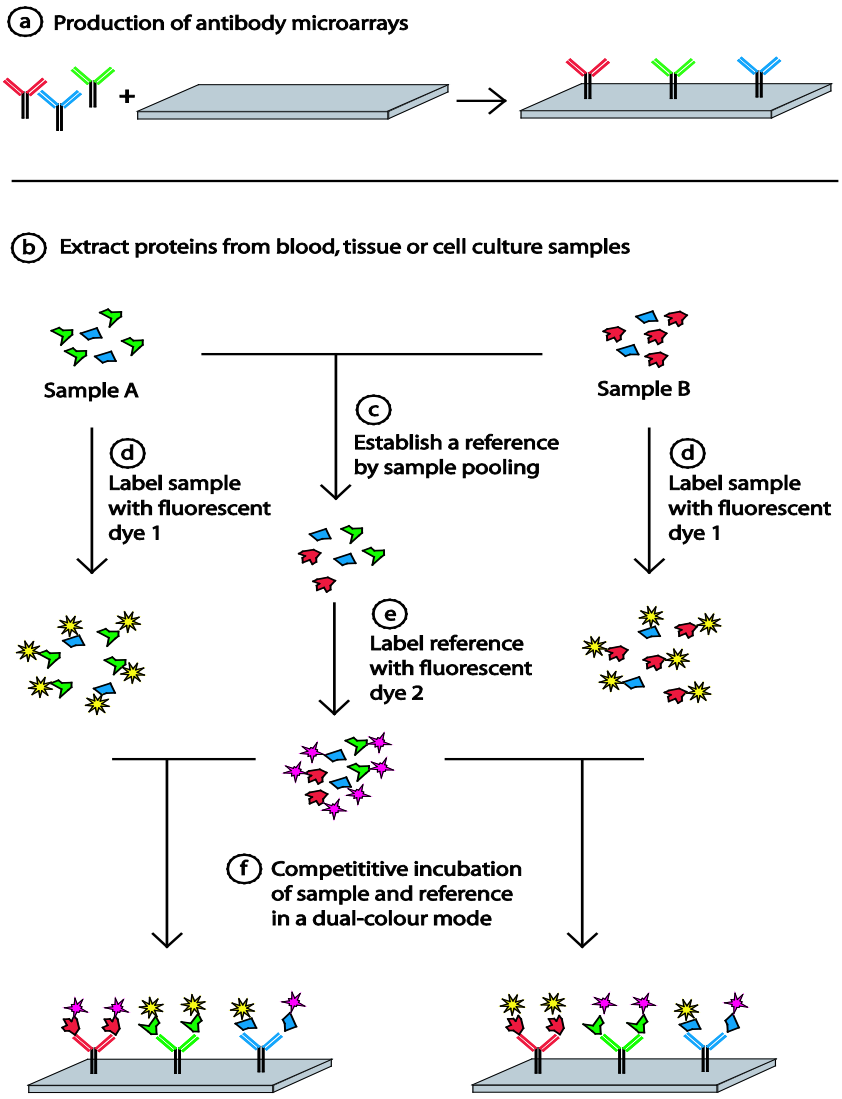

Figure 4. overview of production of antibody microarrays. Steps a to f constitute the proteome profiling of cancers using antibody microarrays.

2.2.4.2 Labeling of protein samples

Extracted and estimated protein samples were labeled at a dye/protein molar ratio of 1:18, assuming that the average weight of protein is 60 KDa. The NHS-esters of dye paris DY-549 (Cy3) and DY-649 (Cy5) were used to label the protein samples. The protein samples were all adjusted to a concentration of 1 mg/ml and labeling was carried on in the dark in 0.1 M

carbonate buffer, pH 8.5 at 4ºC for 2 hrs. Later, the unreacted dye was quenched by adding 10% glycine in the dark at 4ºC for 30 minutes. The non-incorporated and inactivated dye molecules were removed from the samples by using zeba spin-desalting columns with a cutoff of 3.5 KDa and a buffer change to PBS according to the manufacturer's recommendations. After dye removal, the labeled samples were stored at -20ºC until further use.

2.2.4.3 Blocking of slides and Incubation of samples

The slides were taken out and allowed to settle at room temperature for about 10 minutes. Then they were washed with 1X PBST in continuous agitation at room temperature for 4 times at regular time interval. Then the slides were blocked with 1 ml of the blocking solution from candor buffer on the slide booster instrument for 4 hrs at room temperature. The blocking solution was then removed and 10 µl of each of the labeled samples (Cy3 and Cy5) were added to 580 µl of the blocking solution from candor buffer such that the total incubation volume of 600 µl kept ready for incubation. 600 µl of incubation solution was added to each slide and the incubation was carried on the slide booster instrument for 16 hrs at room temperature. After the incubation, the slides were washed with 1X PBST on the slide booster and then washed 4 times in continuous agitation at room temperature. The slides were quickly air dried using a strong clean air blower.

2.2.4.4 Scanning and signal detection of slides

Slides were scanned and the fluorescence of Cy3 and Cy5 were measured using ScanArray 4000XL instrument. The slides were scanned at a resolution of 10 µm, maintaining constant laser power and photomultiplier (PMT). The excitation of Cy5 was achieved by a laser emission at a wavelength of 635 nm while Cy3 excitation was achieved by a laser emission at a wavelength of 532 nm. Two separate images were made for each dye and were then quantified.

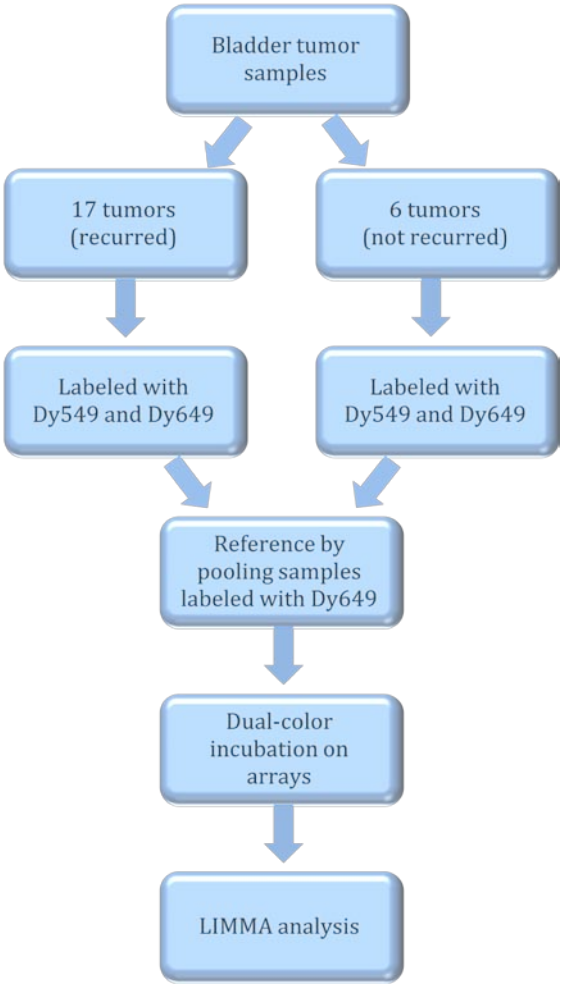

Figure 5. Flowchart of the experimental procedure to analyse non-muscle invasive bladder cancer samples.

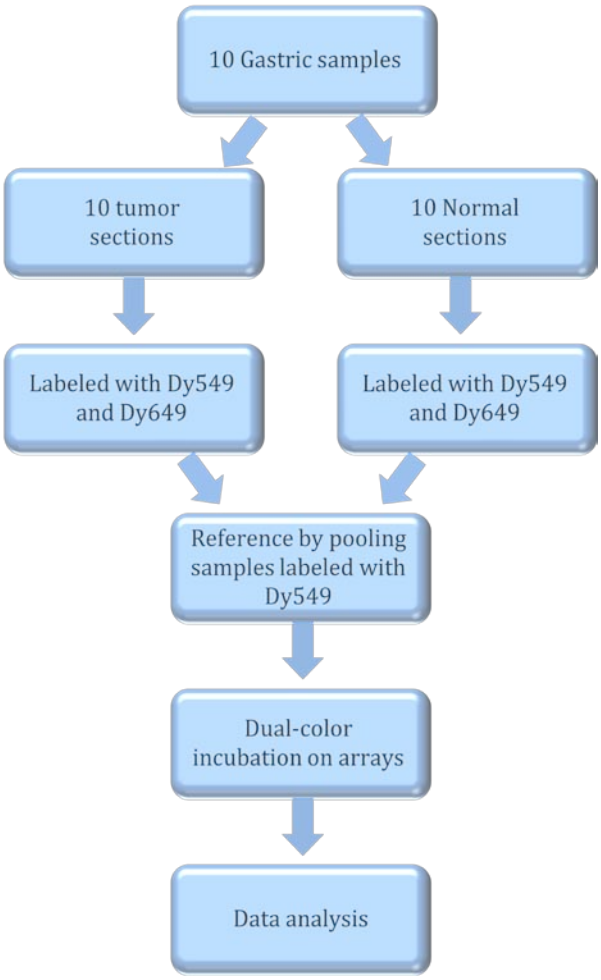

Figure 6. Flowchart of the experimental procedure to analyse gastric cancer samples.

2.2.4.5 Software used in image, data analysis and interpretation

Software	Manufacturer
GenePix Pro 6.0	Axon Instruments, CA, USA
LIMMA Package R-bioconductors	Fred Hutchinson cancer research centre, USA
Chipster package (v1.4.6)	CSC-IT centre for science, Espoo, Finland
STRING 9.1 database for known and predicted protein-protein interaction.	
DAVID bioinformatics resources 6.7- functional annotation and microarray analysis	
KEGG (Kyoto Encyclopedia of Genes and Genomes) Kanehisa Labs, Kyoto, Japan	

2.2.4.6 Quantification of signal intensities from slides

Image analysis was carried out using GenePix Pro 6.0 software where the images were uploaded into the software and overlaid one over the other. The spots in the merged image were analyzed primarily where by the yellow spots represented equal expression of the target protein in both channels and red, green colors representing the differentially expressed target proteins in either of the channels. Further quantification was done by .gal files which were placed over the merged image spots. The gal files were made as a source to carry information on the spots and corresponding protein targets. Thus the signal intensity from a spot in the image is transferred as information on a target protein. Similarly the raw signal intensities form each spot were calculated and stored as a valued table (.gpr files). Apart from the intensities of the spots the gal files also transfer the information on intensity of the background, number of pixels standard deviation and median etc. of both spot and background intensities.

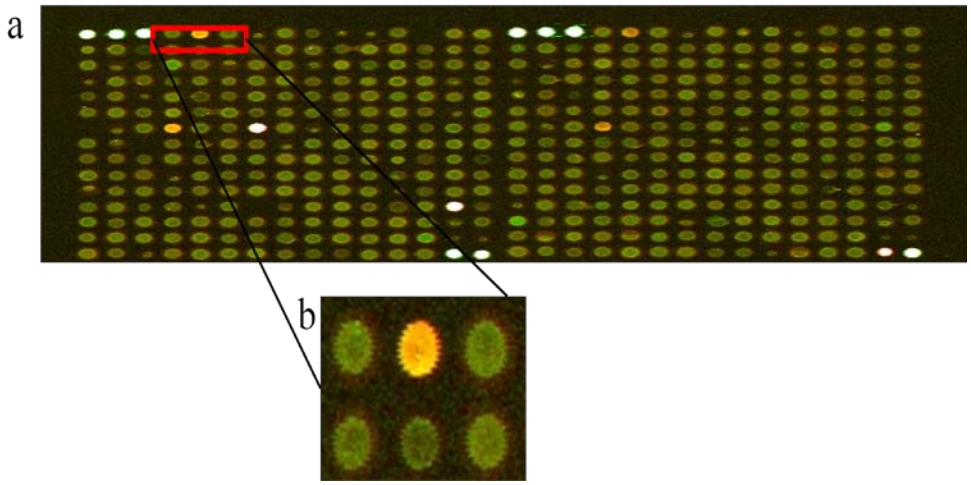

Figure 7. (a) A dual color picture of an antibody microarray slide (only a part) incubated with the bladder cancer samples. (b) spots showing the differential expression (colors yellow and green)

2.2.5 Bioinformatics and statistical methods

2.2.5.1 Data analysis by LIMMA bioconductors

The tables with values from spot intensities (.gpr files) were analyzed using the LIMMA package of R-bioconductors (Smyth et al., 2005). The resulting data were normalized by the application of an invariant Loess method developed and described by the usefulness of the available reference samples (Sill et al., 2010). The sample pools and the individual samples were analyzed using a one-factorial linear model fitted with LIMMA which resulted in a t-test based on moderated statistics. Then the resulting p-values were adjusted for multiple testing by controlled discovery of false proteins according to the Benjamini and Hochberg methods (Smyth, 2004). The differential abundance of proteins in the samples were presented in log2 fold changes where highly abundant proteins had log2 values above 0(positive values) and less abundant proteins had values below 0(negative values). Adjusted p-values were also presented in log form and the proteins with p-values less than 0.05 were considered significantly abundant between two test classes. LIMMA bioconductors was also used for further analysis on the differentially abundant proteins which resulted in volcano plots, box plots and dot plots. Bladder cancer samples and gastric cancer samples (test set only) were analyzed using LIMMA bioconductors.

2.2.5.2 Methods used in sample classification

After normalization of the raw data, an unsupervised hierarchical cluster of samples was created to check the various technical and handling artifacts in the data. Then the highly significantly differentially abundant proteins from the LIMMA analysis were used to build a random forest classifier (Breiman, 2001). The samples were analyzed by building different test and training sets by the use of leave-one-out algorithm in a reiterated outer-loop. This was performed by the application of framework of R called comprehensive package for supervised classification

(CMA) (Slawski et al., 2008). After a serious of leave-one-out loops, the classification results were plot in appropriate ROC-curve and AUC plots.

2.2.5.3 Data analysis by Chipster package

A training set of Gastric cancer samples were analyzed using Chipster software package (v1.4.6. CSC, Finland). After image analysis using GenePix pro 6.0, the tables with values from spot intensities (.gpr) files were loaded in the Chipster software and analyzed. The intensity ratios were generated using the median signal intensities with local background of each spot in both red (DY-649) and green (DY-549) channels. By the application of a Loess method with a background correction offset [0, 50] for the normexp method, the intensity ratios were normalized . An unsupervised hierarchical cluster of samples was made with the normalized data to study and neglect (if any) technical and handling artifacts. The significance in differential abundance of proteins was tabulated by the application of empirical Bayes test with the p-values adjusted as per Benjamini-Hochberg method (Smyth, 2004). The empirical Bayes make use of a moderated t-statistic in which posterior residual standard deviations were applied rather than of ordinary standard deviations, which gave a far more stable inference when the number of arrays were small (Smyth, 2004). The proteins with an adjusted p-value of 0.05 and less than that were considered significantly differentially abundant in the samples. An unsupervised hierarchical clustering coupled with a boot-strap method was followed to get an optimized signature to classify the normal and tumor groups. The resulting list consisting signature proteins were then tabulated with their log fold change and adjusted p-values respectively. The total number of samples analyzed and classified based on the signature of proteins were then tabulated.

2.2.5.4 Interaction and pathway analysis

The functions and interactions between the proteins were studied and evaluated using STRING 9.1, open-source software (Szklarczyk et al., 2011). The list of significantly differentially abundant proteins were loaded into the software online and results reported interactions among the proteins were evaluated and compared to the pattern of abundance of the proteins in the sample sets. The functional significance of the various interactions were also evaluated and compared to the abundance pattern of the proteins. The desired results were then tabulated and separate figures interpreting the interactions were made. The list of significantly differentially abundant proteins and their pattern of abundance was loaded into KEGG pathway (Kyoto Encyclopedia of Genes and Genomes) and the resulting pathways were evaluated (Kanehisa et al., 2012). The resulting pathways were also used as a reference for detailed analysis of the abundant proteins.

3 Results

3.1 Recurrence prediction in non-muscle invasive bladder cancer

3.1.1 Hierarchical clustering of samples to study incubation batch effect

In the present study, the protein samples from non-muscle invasive bladder cancer were compared for the identification of a protein signature to predict recurrence. The samples were analyzed using antibody microarrays constructed as mentioned in sections 2.2.4(1, 2 and 3) in methods. To also study the possible technical artifacts that may result, four samples were repeatedly incubated in different batches. As the incubations of the samples on the antibody microarrays were carried out on different time points, an unsupervised clustering of the samples was done after normalization of the data. In the hierarchical cluster, the samples were found equally distributed irrespective of the batch they were incubated on the arrays (figure 8). Most of the duplicates for example samples 1868, 4131 and sometimes even the triplicates for example samples 677-1, 2552 were clustered together in the hierarchical cluster (figure 8) referring to the presence of no technical artifacts such as handling, day of incubation, position of the slides in slidebooster and incubation of samples on different arrays itself.

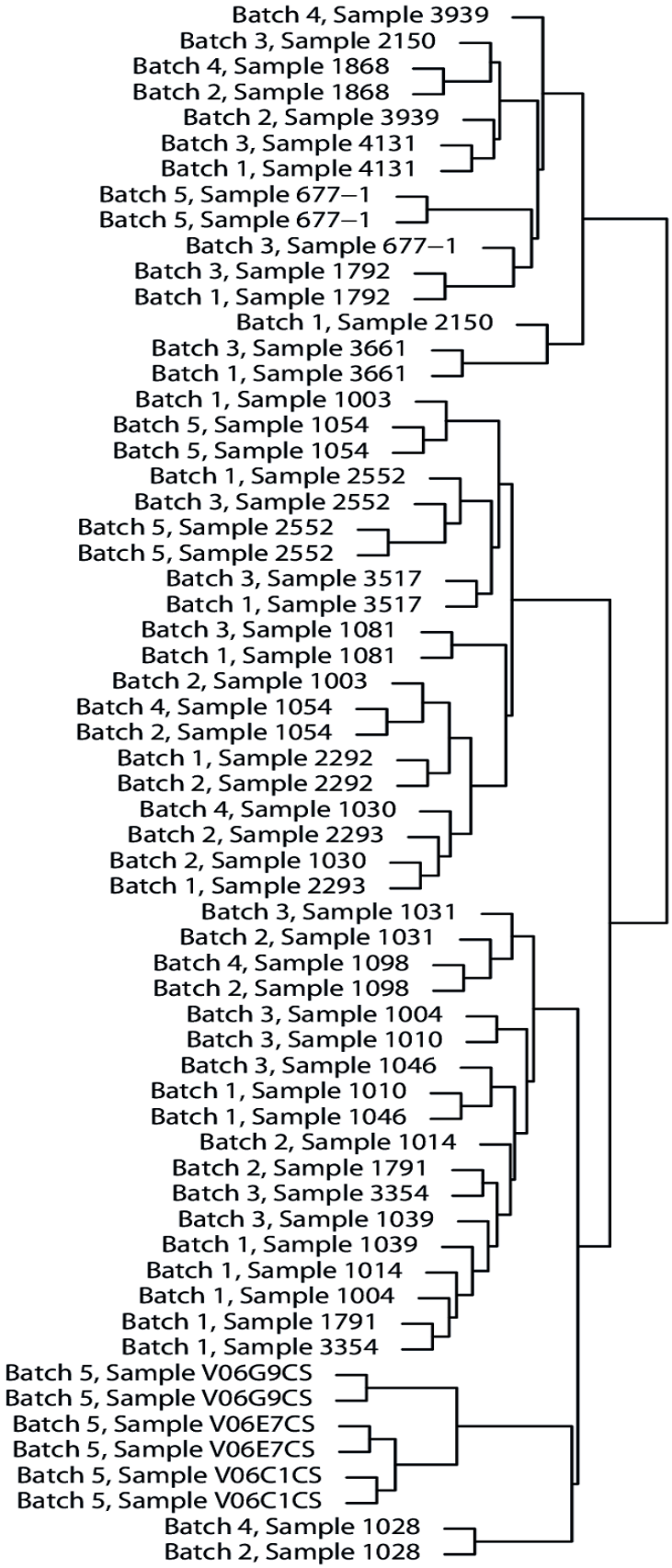

Figure 8. Hierarchical cluster analysis of the sample set. The Euclidean distance and the average linkage method were used. For each sample, the identifier and the incubation batch are given. The incubation batches do not cluster. Repeated samples, however, are located next or very close to each other. Samples whose names start with the letter V represent healthy bladder and cluster next to each other.

3.1.2 Differential expression of proteins between normal bladder and non-muscle invasive bladder tumors

Protein samples isolated from three normal bladder tissues (healthy), labeled and incubated in duplicates was compared with protein samples from twenty five non-muscle invasive bladder tumor tissues to check the differential abundance of proteins. Overall, sixteen proteins (adjusted p-value less than 0.05) were found to be significantly differentially abundant between tumor and normal tissues.

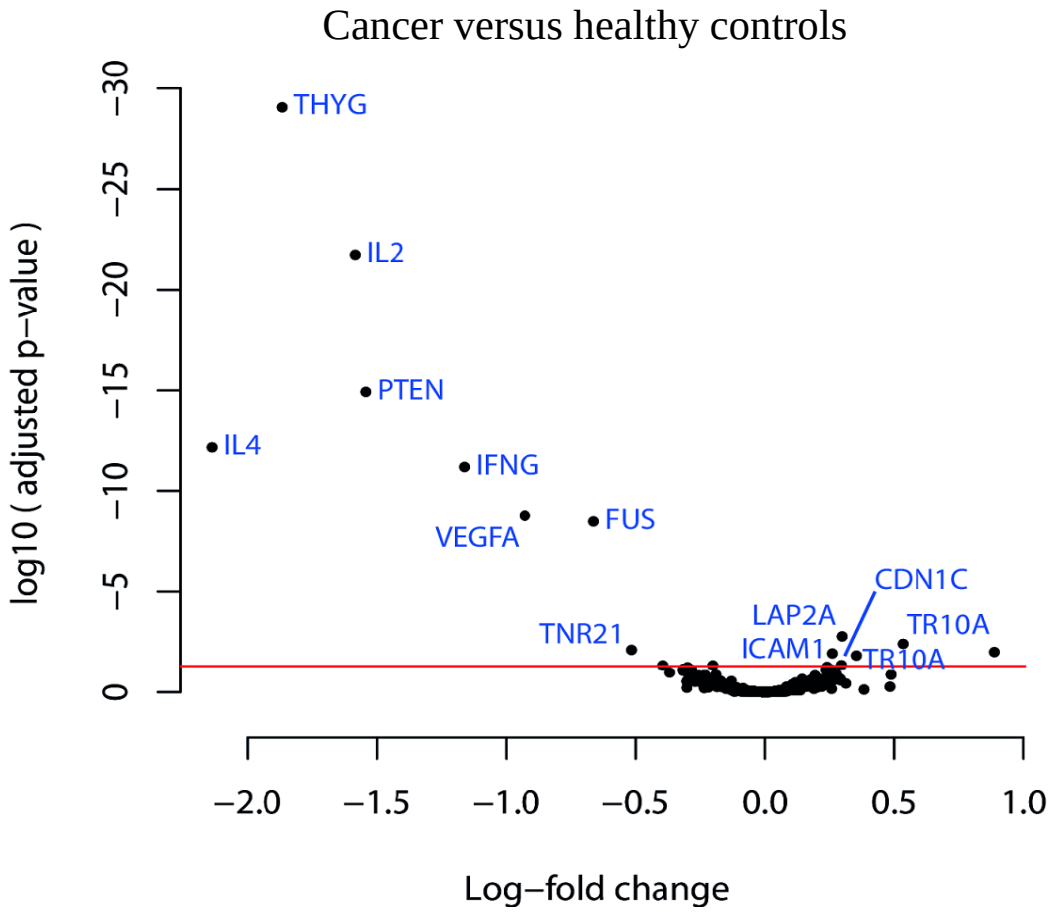

Figure 9. Volcano plot summarising expression differences between samples from cancer and healthy patients. The Log-fold change and respective significance level as an adjusted p-value are given. The black dots above the red line indicate proteins with significantly ($p<0.05$) different expression.

A volcano plot containing the details of the differentially abundant proteins is shown in figure 9. Among the significantly abundant proteins, Tumor necrosis factor receptor superfamily member 10A (TNFRSF10A), Cyclin-dependent kinase inhibitor 1C (CDKN1C) and Lamina-associated polypeptide 2, Thymopoietin isoform alpha (LAP2A) were significantly very highly abundant in tumor samples with log2 fold change and adjusted p-value of (0.54, 0.0002), (0.32, 0.007) and (0.30, 0.0005) respectively. Similarly proteins, Interleukin-4 (IL4), Thyroglobulin (THYG) and Interleukin-2 (IL2) were significantly less abundant in tumor samples with log2 fold change and adjusted p-value of (-2.15, 2.87e-13), (-1.87, 2.91e-33) and (-1.58, 2.61e-24) respectively. Two different antibodies representing TNFRSF10A were found significantly more abundant in the tumor samples which explain the high specificity and robustness of the antibody microarray. A table with the log2 fold changes and adjusted p-values of all the twenty significantly differentially abundant proteins is shown in the supplementary section (table S1).

3.1.3 Differential expression of proteins between recurrent and non-recurrent non-muscle invasive bladder tumors

To understand the proteins involved in recurrence of non-muscle invasive bladder cancers, twenty five tumor samples were analyzed with complex antibody microarrays. All the samples considered for analysis belonged to low grade and stage (Ta and T1) of tumor. Those patient samples only with a follow-up period of five years after the resection of primary tumors were considered for analysis. Tumors that recurred within five years post primary resection were considered recurrent group while those that did not recur within five years were considered to be cured of bladder cancer. Among the twenty five samples used in the analysis, nineteen samples had recurred within five years and six samples did not recur and were considered as a group of non-recurrent tumors or cured. Protein samples were isolated from all the patient tissues along

with three normal bladder tissues and were labeled in two fluorescent dyes Cy3 (DY-549) and Cy5 (DY-649). A common reference pool was made by pooling equal amount of protein from all the samples for normalization of the data and incubations of the individual samples were performed in presence of the reference pool for the same purpose. All the samples were incubated on the antibody microarray consisting eight hundred and thirteen antibodies directed against seven hundred and twenty four cancer related proteins. Incubations of random samples were performed in different days and time but without any technical and handling variations (figure 8).

Among the seven hundred and twenty four antibodies detected in the array, two hundred and fifty five proteins were significantly differentially abundant among the recurrent and non-recurrent group (table S2). A hundred and five proteins among the significantly differentially abundant were more abundant in recurrent tumors while remaining hundred and fifty were found lesser in abundance in the group. A hundred and two proteins among the two hundred and fifty five were very highly significantly differentially abundant with an adjusted p-value less than 0.003. The results from the whole analysis were exhibited as a volcano plot most of the proteins named with the log fold change and adjusted p-values (figure 10). The individual protein expression variations were studied to assess the significance of findings from individual patients. A box plot was made for some of the proteins and is represented in figure 11. Lamin-A (LMNA) and transcription factor AP-1 (JUN) were the most abundant proteins in recurrent tumors while L-Selectin (LYAM1) and Cyclin-Dependent Kinase Inhibitor 1A (CDKN1A) were found with very less abundance in recurrent tumors (figure 11).

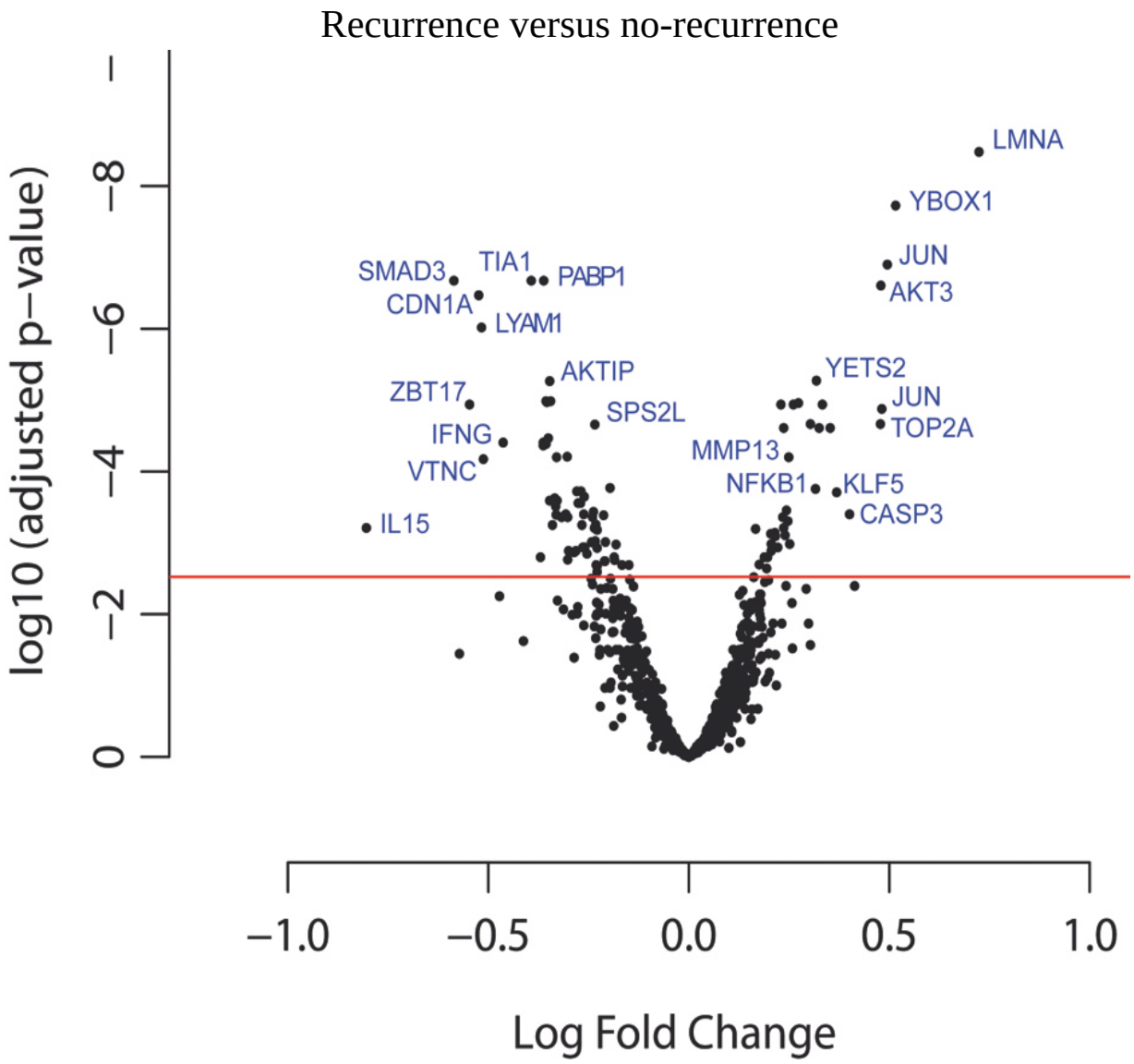

Figure 10. Volcano plot of differentially abundant proteins. Horizontally, the degree of expression variation is shown; the vertical axis indicates the significance level. The black dots represent the proteins that were analysed. The red line stands for an adjusted p-value of 0.003. All proteins located above this threshold are highly significantly informative about the recurrence status of a patient. Some particular proteins are named.

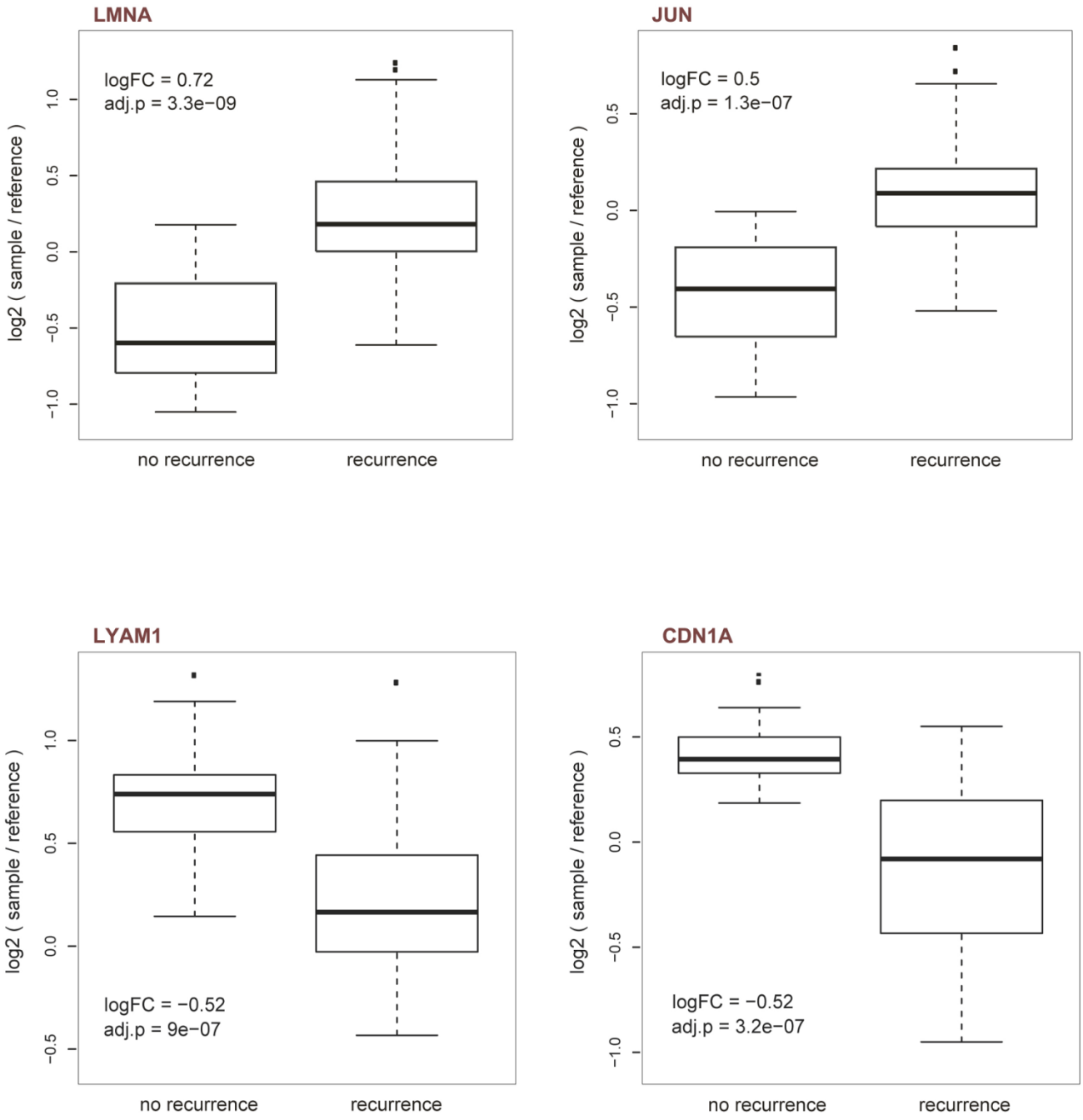

Figure 11. Distribution of protein expression in all patient samples. Data are shown for proteins LMNA and JUN (up-regulated in non-recurrent tumours) as well as LYAM1 and CDN1A (down-regulated in non-recurrent tumours).

3.1.4 Accurate prediction of recurrence in non-muscle invasive bladder cancer by protein signature

A multivariate analysis was performed to assess the prognostic capabilities of the highly significantly differentially abundant proteins. A rule for accurate classification of the samples was established based on random forest method (Breiman, 2001) combined with the twenty proteins that were most significantly differentially abundant. The training set was classified with 100% sensitivity and specificity. Leave-one-out cross validations were performed to assess the efficient transferability of the classification to other test sets. The classification results for the different test sets in the cross validation steps were summarized as a receiver operating characteristics (ROC) curve (figure 12). The miss-classification rate for the cross-validation was 20% (SD 0.08). Also the area under the curve (AUC) value of 0.91 exhibits the extremely high accuracy of discriminating between the patients with and with-out recurrence (figure 12). That corresponds to the overall sensitivity of 80% at 100% specificity. Twenty proteins that were highly significantly differentially abundant based on which the samples were classified is shown in table 1 along with their corresponding log fold change and adjusted p-value. A strong study on already published peer reviews connecting these twenty abundant proteins is also shown in the table 1.

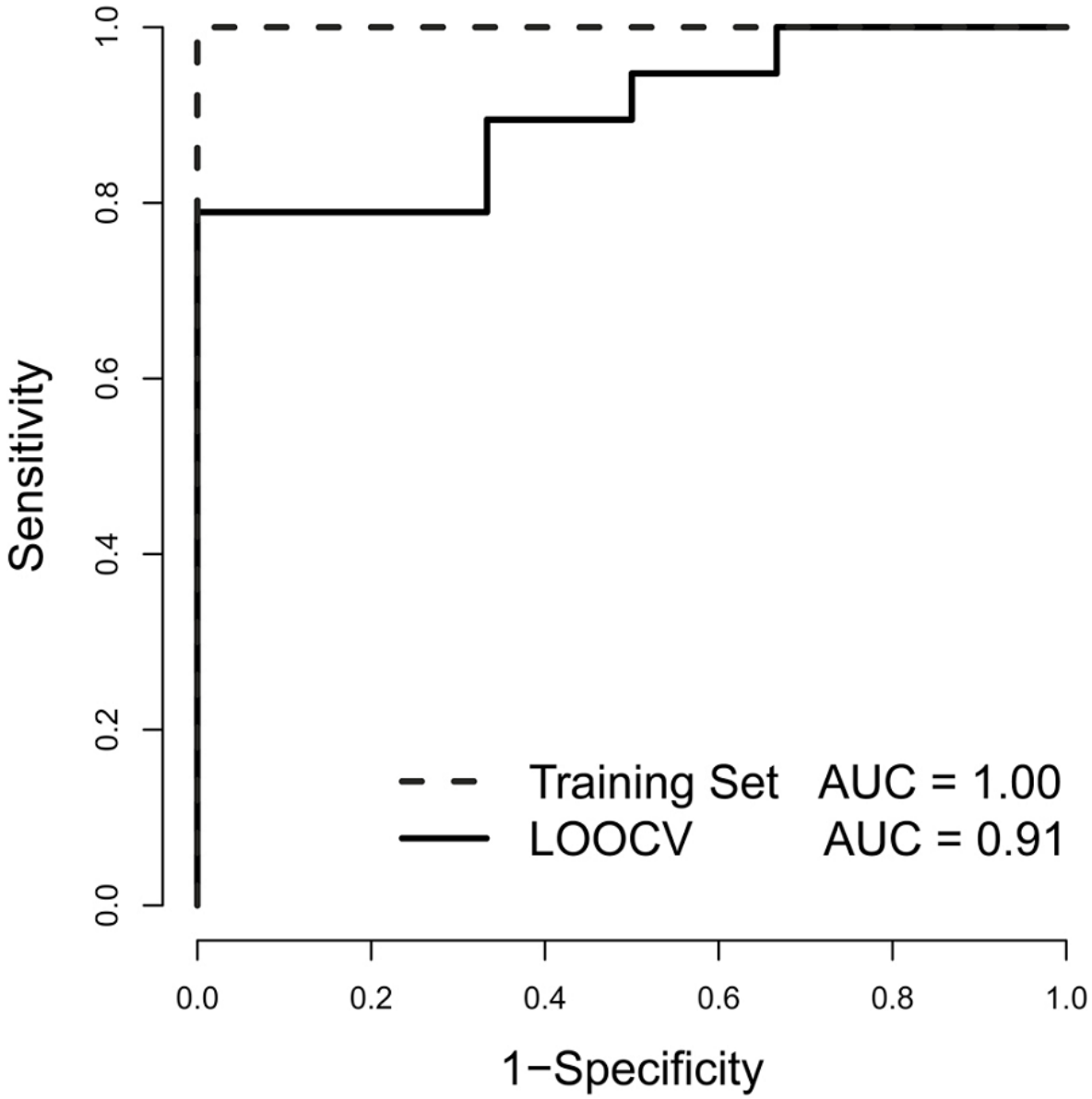

Figure 12. Receiver operating characteristic (ROC) curves resulting from random forest classification. Discrimination within the trainings set was 100% (dotted line). Stringent leave-one-out cross validation yielded an AUC value of 0.91 (black line).

Protein Uniprot entry name	Uniprot accession	Adjusted p-value	Log fold change	Reported studies	
LMNA_HUMAN	P02545	3.3e-09	0.72	BC	(Konstantakou et al., 2009; Stravopodis et al., 2009)
YBOX1_HUMAN	P67809	1.9e-08	0.52	RC C	(To et al., 2010) (Gluz et al., 2009)
JUN_HUMAN	P05412	1.3e-07	0.50	RBC BC RC	(Mitra et al., 2009) (Ling et al., 2011) (Ouyang et al., 2008)
AKT3_HUMAN	Q9Y243	2.5e-07	0.48	BC RC	(Ching and Hansel, 2010) (Bonin et al., 2008)
YETS2_HUMAN	Q9ULM3	5.2e-06	0.32	-	No cancer related reference
CADH1_HUMAN	P12830	1.2e-05	0.33	RBC	(Gallagher et al., 2008; Negraes et al., 2008)
TIA1_HUMAN	P31483	2.1e-07	-0.39	C	(Alvaro et al., 2005)
SMAD3_HUMAN	P84022	2.1e-07	-0.58	C	(Penuelas et al., 2009; Poncelet and Schnaper, 2001)
PABP1_HUMAN	P11940	2.1e-07	-0.36	C	(van Duin et al., 2005)
CDN1A_HUMAN	P38936	3.4e-07	-0.52	RBC BC	(Shariat et al., 2008) (Shariat et al., 2007)
LYAM1_HUMAN	P14151	9.6e-07	-0.51	C	(St Hill, 2011)
AKTIP_HUMAN	Q9H8T0	5.4e-06	-0.34	C	(Cinghu et al., 2011)
PRI1_HUMAN	P49642	1.0e-05	-0.35	C	(Yotov et al., 1999)
HSP7C_HUMAN	P11142	1.0e-05	-0.34	-	No cancer related reference
RSSA_HUMAN	P08865	1.1e-05	-0.35	C	(Qiu et al., 2008)
GRM1A_HUMAN	Q96CP6	1.1e-05	0.28	C	(Martino et al., 2012) (Speyer et al., 2012)
TPA_HUMAN	P00750	1.1e-05	0.23	BC	(Louhelainen et al., 2006) (Knowles et al., 1993)
ZBT17_HUMAN	Q13105	1.1e-05	-0.54	C	(Ikegaki et al., 2007) (Iraci et al., 2011)
LAMP2_HUMAN	P13473	1.1e-05	0.26	C	(Lee et al., 2012) (Tung et al., 2010)
JUN_HUMAN	P05412	1.3e-05	0.48	RBC BC RC	(Mitra et al., 2009) (Ling et al., 2011) (Ouyang et al., 2008)

Table 1. List of the twenty proteins with the most significant expression variations between recurrent and non-recurrent tumours. Log fold changes in expression and the related adjusted p-values are shown. Also, literature is listed in which the protein or respective gene was reported in connection with bladder cancer (BC), recurrence of bladder cancer (RBC), recurrence of other cancer forms (RC), or cancer overall (C).

3.1.5 Functional annotation of highly significantly abundant proteins

The possible functional aspects between recurrence and non-recurrence of the samples were studied based on the functional studies on the significantly abundant proteins using a web-based analysis platform DAVID (Huang da et al., 2009). About 56% proteins among the highly abundant in recurrent tumors were expressed inside the cells while 61% of the proteins among those that were less abundant were secreted by the cells or expressed in the extracellular space. Web-based pathway analysis program KEGG (Kanehisa et al., 2012) showed a strong suppression in the TGF-beta signaling pathway in the recurrent cancer when the significant proteins were tested using the program (figure 13). The expression of important factors like INFG, TNFA, TGFB and THBS1 was less in recurrent samples while inhibitor of MAPK3 (known as ERK1) was highly abundant and again Mothers against Decapentaplegic Homolog proteins like SMAD1, 2 and SMAD3 were significantly less abundant in recurrent samples. Transcription factor SP1, an important downstream signaling protein was also less abundant in recurrent samples. Along with TGFB pathway, proteins involved in apoptosis were also strong regulated. Among those more abundant proteins in recurrent samples were effectors Caspase 3 (CASP3) and 9 (CASP9), both in their active form and the regulator Bcl2 associated –X (BAX) (table S2). Interestingly, the Trail-receptor which could active all the above proteins was also high in abundance in recurrent samples. On the other hand, FAS was less abundant in recurrent samples. Similarly some of the transcription factors previously studied in carcinogenesis and more widely in other diseases were also differentially abundant in recurrent samples. Variations in regulation of most of the cancer related proteins in cancer pathways were colored and given as a supplementary (figure S1, S2).

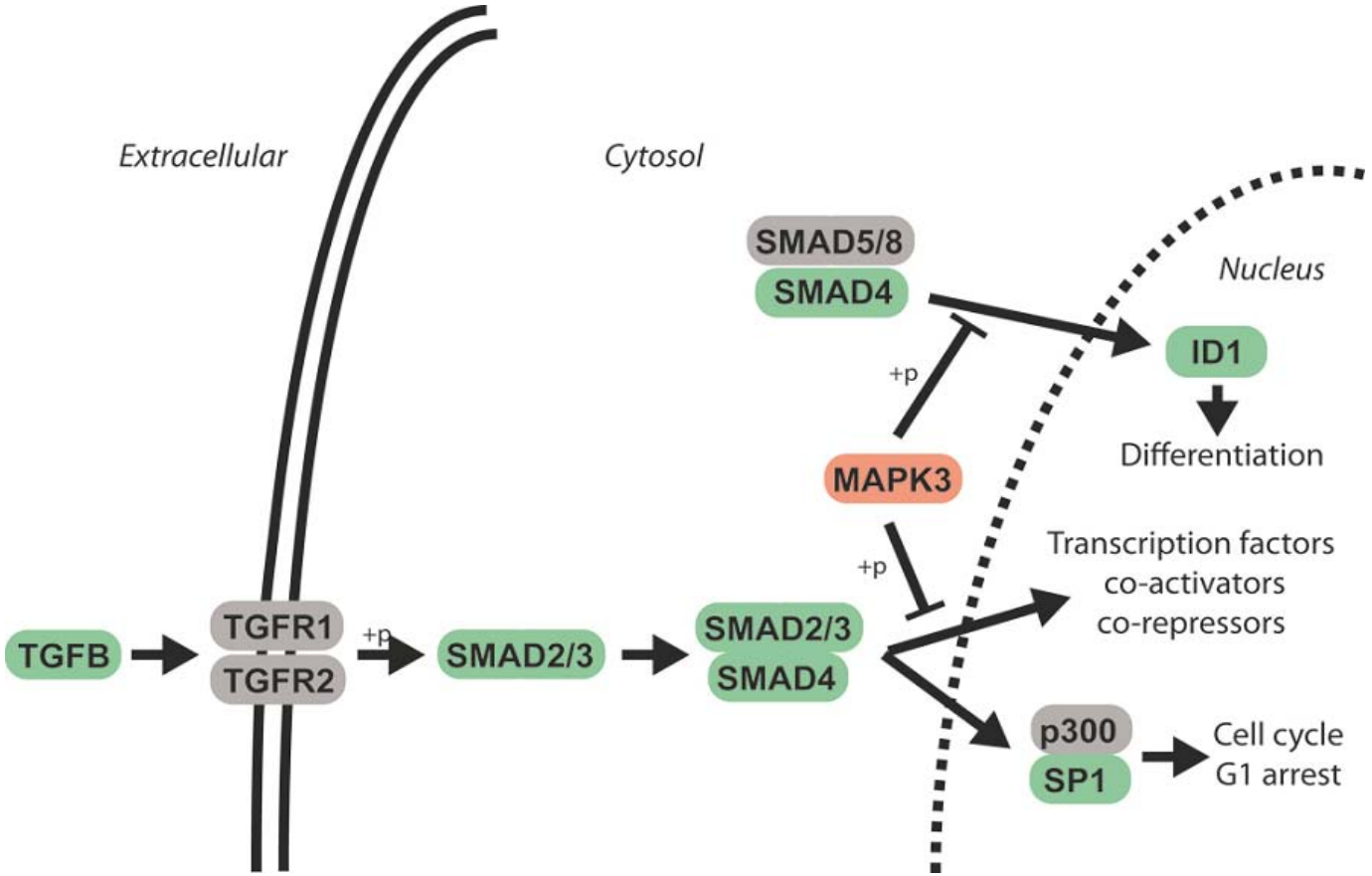

Figure 13. KEGG analysis of the TGF-beta signalling pathway. Affected proteins are labelled in green or red if their expression was lower or higher, respectively, in recurrent rather than non-recurrent cancer.

The pathways in signaling and cancer from KEGG analysis most often highlighted those proteins involved in apoptosis and cell proliferation. This fact was also identified by the studies on gene ontology as functions of many abundant proteins in recurrent samples were representing both pro-apoptotic and anti-proliferative effects. In addition to pathways and analysis of proteins based on their ontology, important proteins like transcription factors, cell cycle regulators, those among the abundant proteins were also analyzed by web-based protein-protein interaction software STRING 9.1 (Szklarczyk et al., 2011). Among the highly abundant proteins in recurrent tumors, transcription factor AP-1 (JUN), Nuclear factor NF-kappa B1 (NFκB1), DNA Topo isomerase-2 alpha (TOP2A), Krueppel-like factor (KLF5) and Cadherin-1 (CDH1) were interacting among each other by protein-protein binding and activation of expression (figure 14). The less abundant proteins in recurrent tumors like Mothers against Decapentaplegic Homolog-3 (SMAD3), Matrix Metalloproteinase-1 (MMP1), Tissue Inhibitor of Metalloproteinases 1 (TIMP1), Vascular Endothelial Growth Factor A (VEGFA) and transcription factor SP1 were reported to interact among each other by activation, inhibition and binding (figure 14). Among the interactions, JUN a highly abundant protein was studied earlier to inhibit TIMP1, a less abundant protein in recurrent tumors. Similarly, many such interactions were reported from the analysis and studied vastly through peer reviewed publications.

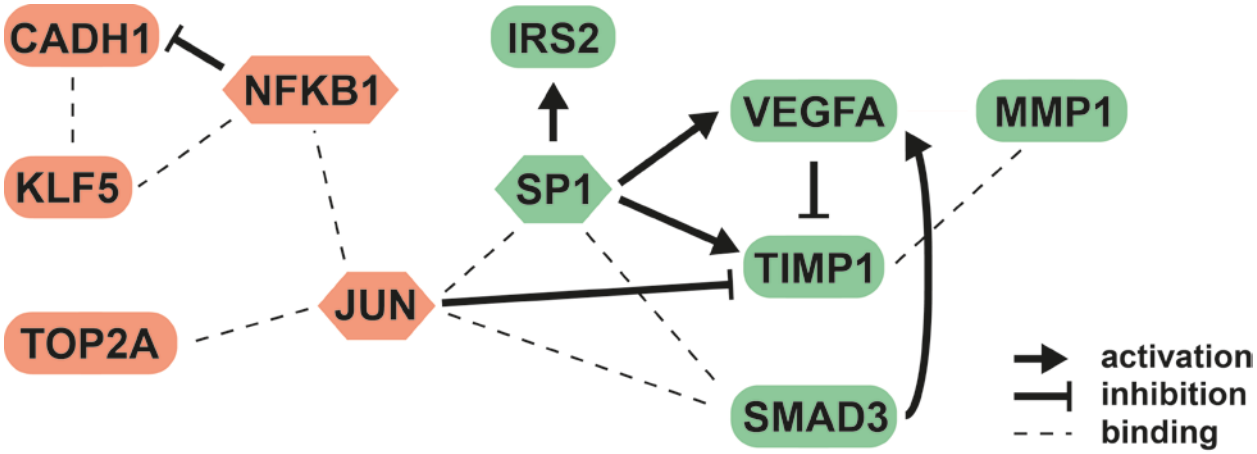

Figure 14. Protein interactions of strongly regulated proteins. Analysis of the protein expression data with the pathway analysis software STRING 9.1 revealed several protein interactions. Proteins coloured in red were higher expressed in recurrent than non-recurrent tumours; green-coloured proteins exhibited lower expression.

3.2 Protein biomarker identification in Gastric adenocarcinoma

3.2.1 Hierarchical clustering of samples after normalization

In the current study, the protein samples from normal gastric tissues and high stage and grade gastric adenocarcinoma tissues were compared for the identification of protein biomarkers for effective diagnosis and prognosis. The samples were analyzed using antibody microarrays constructed as mentioned in sections 2.2.4(1, 2 and 3) in methods. To also study the consistency of the expression, all samples were incubated in duplicates on the arrays. The samples were incubated on the antibody microarrays in a single batch to minimize the technical artifacts that may arise. An unsupervised clustering of the samples was done after normalization of the data. In the hierarchical cluster, the samples were found clustered according to the type of the tissue (figure 15). All the duplicates N,DN and T,DT were clustered together in the hierarchical cluster (figure 15) referring to the presence of no technical artifacts such as handling, position of the slides in slidebooster and incubation of samples on different arrays itself. These results suggested that the clustering and segregation of normal and tumor samples were only based on the differential abundance of proteins in them and not any other artifacts. After normalization the data were analyzed for differential abundance of proteins.

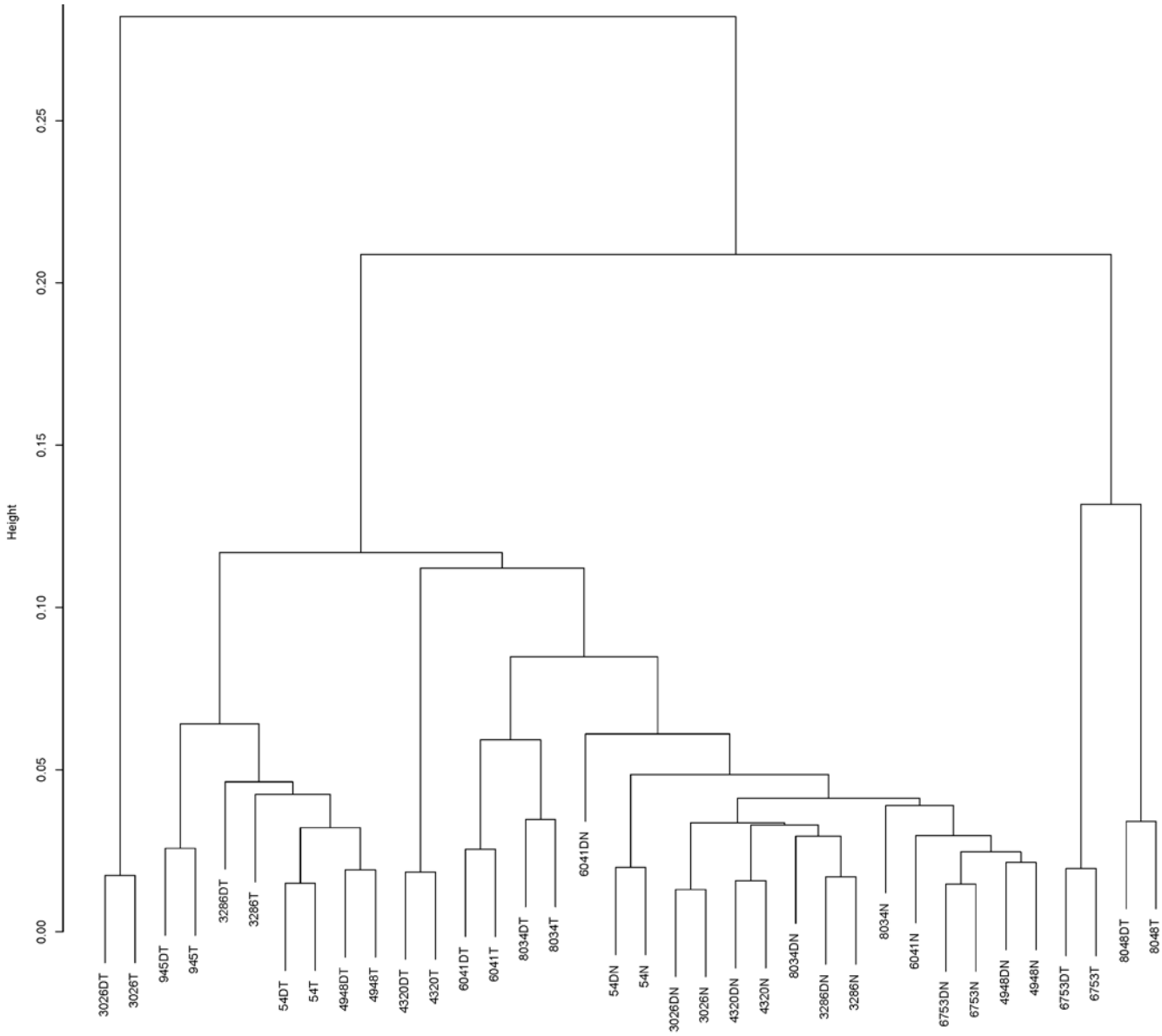

Figure 15. Hierarchical cluster analysis of the sample set. The Euclidean distance and the average linkage method were used. The samples names are given, normal (N, DN) and tumor (T, DT). The incubation was done on the same day. The duplicates of the samples were clustered apart from the cluster groups of normal and tumor groups.

3.2.2 Differential expression of proteins between normal gastric tissues and gastric adenocarcinoma tissues

To identify protein biomarkers for effective diagnosis of gastric adenocarcinoma, twenty five surgically removed tumor tissue samples along with adjacent normal tissue samples from patients diagnosed with gastric adenocarcinoma were analyzed with complex antibody microarrays. All the samples considered for analysis belonged to high grade and stage (T3 and T4) of tumor. Those patient samples only without prior treatment for gastric cancer were considered for analysis. Among the twenty five pair (normal and tumor) of tissues used in the analysis, ten pairs were considered as training set and fifteen pairs were considered as test set. Protein samples were isolated from all the tumor and normal tissues and were labeled in two fluorescent dyes Cy3 (DY-549) and Cy5 (DY-649). For incubations of the training set of samples, a common reference pool was made by pooling equal amount of protein from all the samples for normalization of the data and incubations of the individual samples were performed in presence of the reference pool for the same purpose. The test sets were incubated separately by swapping the dyes labeled and nature of the samples and vice versa. All the samples were incubated on the antibody microarray consisting eight hundred and thirteen antibodies directed against seven hundred and twenty four cancer related proteins.

3.2.2.1 Differential expression of proteins- training set of samples

Among the seven hundred and twenty four antibodies detected in the array, eight proteins were significantly differentially abundant between normal and tumor samples (table 2). Three proteins were significantly differentially more abundant in tumor samples while remaining five proteins were found lesser in abundance. These proteins were very highly significantly differentially abundant with an adjusted p-value less than 0.04 (table 2). A heat-map featuring the eight highly

differentially abundant proteins is represented in figure 16. Nucleolysin-TIA1 (TIA1) was found highly significantly differentially more abundant in the tumor samples with a log fold change of 1.19 and an adjusted p-value of 0.04. While, Mucin-6 (MUC6) was found very less in abundance in tumor samples with a very high significance (log fold change -2.45 and adjusted p-value 9e-06). Gamma-Enolase (ENOG), Epidermal growth factor (EGF) and Folate receptor alpha (FOLR1) were also among the highly regulated proteins (figure 16).

Protein Uniprot entry name	Uniprot accession	Adjusted p-value	Log fold change
EGF_HUMAN	P01133	0.04	-0.30
ENOG_HUMAN	P09104	0.02	0.28
IRS2_HUMAN	Q9Y4H2	0.03	-0.26
MPIP2_HUMAN	P30305	0.04	-0.24
FOLR1_HUMAN	P15328	0.04	0.53
GBRB1_HUMAN	P18505	0.04	-0.28
MUC6_HUMAN	Q6W4X9	9e-06	-2.45
TIA1_HUMAN	P31483	0.04	1.19

Table 2. List of the eight proteins with the most significant abundance between normal and tumor samples in the training set. Log fold changes in expression and the related adjusted p-values are shown.

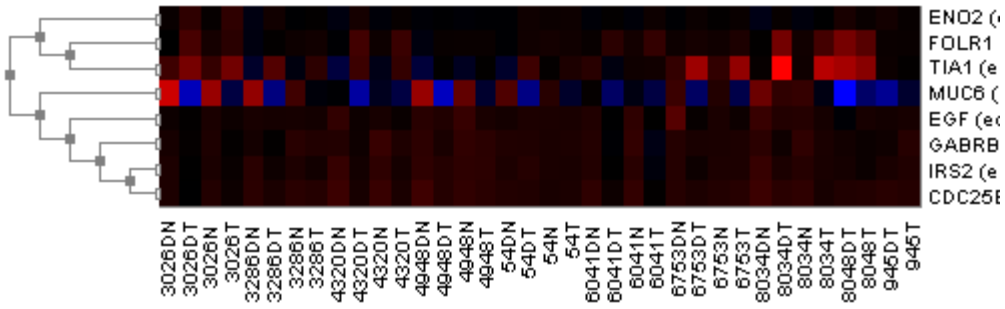

Figure 16. Heat map of proteins with significant differential abundance in normal and tumor samples (also in duplicates). Highly abundant proteins were represented in red while proteins with less abundance were represented in blue. Only proteins with an adjusted p-value less than 0.05 were represented in the heat map. The sample set here is considered to be the training set.

3.2.2.2 Differential expression of proteins- test set of samples

Incubations of fifteen pairs (normal and tumor) samples were carried out by dye swap method. Normal sample labeled with Cy3 from a patient is incubated with tumor sample labeled with Cy5 from the same patient. Alternatively, duplicates from the same samples were incubated in the opposite manner by labeling tumor samples in Cy3 and normal samples in Cy5. Similar steps were followed for incubations of all the thirty samples. Among the seven hundred and twenty four proteins detected in the array, ninety one proteins were found significantly differentially abundant between normal and tumor samples, out of forty two proteins were more abundant in tumor samples and forty nine proteins were less abundant in them. About sixteen proteins were highly significantly regulated with an adjusted p-value less than 0.03. Many differentially abundant proteins among the training set samples were also found differentially abundant in the test set samples. Among the most significantly abundant proteins, Calgranulin B (S100A9), Insulin-like growth factor-binding protein 7 (IGFBP7) and Calponin-2 (CNN2) were found more abundant in tumor samples while Mucin 6 (MUC6) was less in abundance in tumor samples.

Protein Uniprot entry name	Uniprot accession	Adjusted p-value	Log fold change
AQP_HUMAN	P29972	0.002	1.74
CD27_HUMAN	P26842	0.02	3.45
CNN2_HUMAN	Q99439	1.4e-07	1.84
ENOG_HUMAN	P09104	0.03	1.04
IBP7_HUMAN	Q16270	6.13e-06	2.02
IFNG_HUMAN	P01579	0.004	1.74
IL10_HUMAN	P22301	0.002	2.77
ITA5_HUMAN	P08648	0.002	2.21
OCLN_HUMAN	Q16625	0.03	0.57
S10A9_HUMAN	P06702	2.31e-05	2.01
TFPI2_HUMAN	P48307	0.01	2.51
TIA1_HUMAN	P31483	0.001	0.67
VEGFA_HUMAN	P15692	0.002	2.88
CKS2_HUMAN	P33552	0.004	-1.83
DKK3_HUMAN	Q9UBP4	0.03	-0.85
MUC6_HUMAN	Q6W4X9	1.88e-07	-3.71

Table 3. List of the top sixteen proteins with the most significant abundance between normal and tumor samples in the test set. Log fold changes in expression and the related adjusted p-values are shown. Three proteins (colored green), ENOG, TIA1 and MUC6 were found significantly differentially abundant in both the training and test sets of samples.

Three proteins found highly regulated in the training set samples were also found among the highly differentially abundant proteins in tumor samples of the test set. ENOG and TIA1were found more abundant among the highly regulated proteins in the test set samples while MUC6 was found less abundant in them. Most significantly differentially abundant proteins were listed

along with their log fold change and adjusted p-values in table 3. The results from the whole analysis were exhibited as a volcano plot most of the proteins named with the log fold change and adjusted p-values (figure 17).

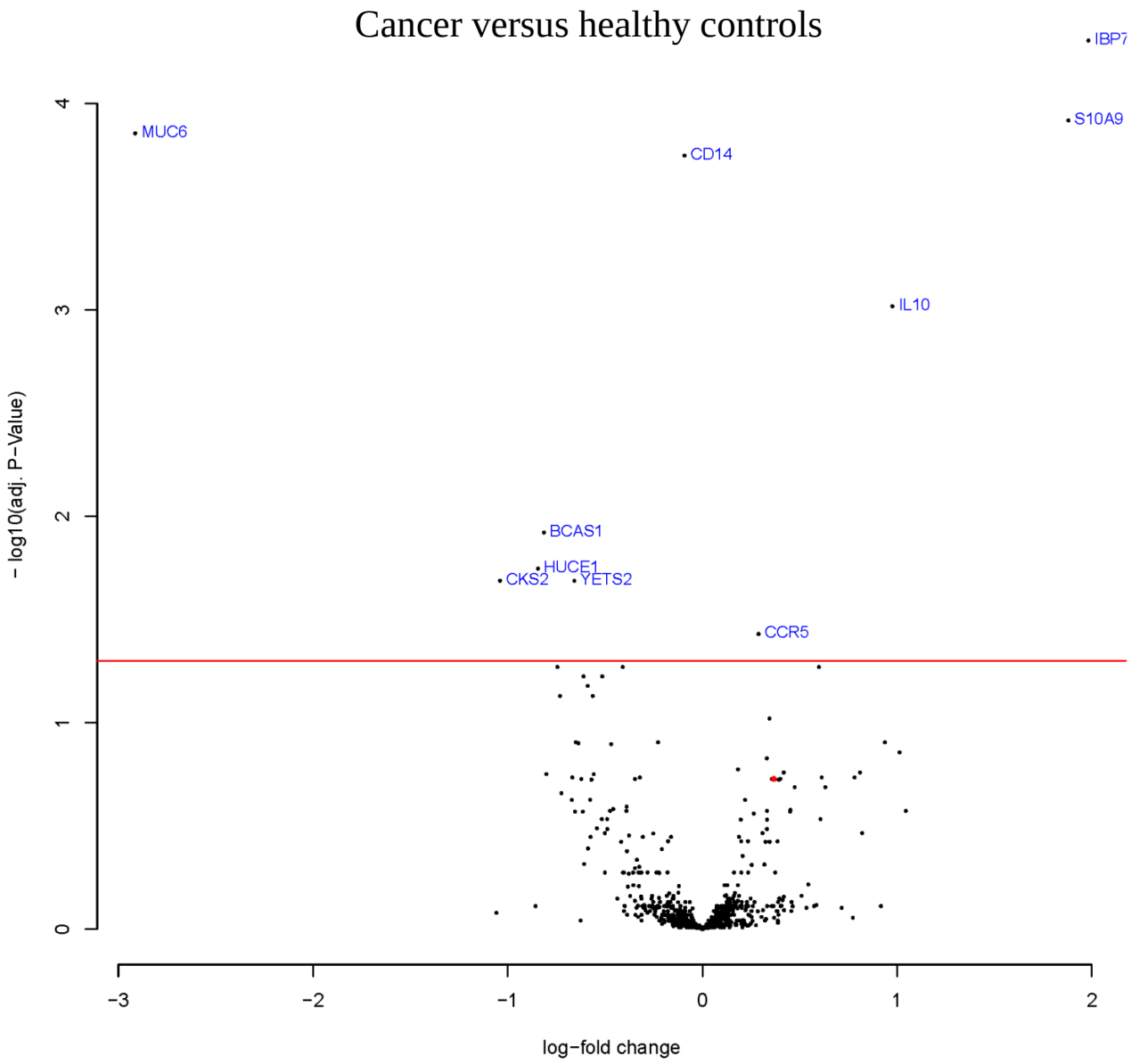

Figure 17. Volcano plot of differentially abundant proteins. Horizontally, the degree of expression variation is shown; the vertical axis indicates the significance level. The black dots represent the proteins that were analysed. The pink dot represents TIA1 which was also found differentially abundant in the training set. Some other particular proteins are named.

The individual protein expression variations were studied to assess the significance of findings from individual patients. To test differential abundance of proteins and specificity of their regulation in normal and tumor tissues, a western blot analysis was carried out. Two protein pools were made by random selection of normal and tumor samples. These two pools of normal (Normal 1 and 2) and tumor protein (Tumor1 and 2) were resolved in 10% and 15% SDS-poly acrylamide gels and transferred to nitrocellulose membranes as mentioned early in methods section 2.2.2.4. Antibodies against human TIA1 and S100A9 were incubated on the membranes to detect the expression of respective proteins in normal and tumor samples. Antibody against human glyceraldehyde-3-phosphate dehyrogenase (GAPDH) was used as a loading control. The differential abundance of these proteins is exhibited visually in figure 18a and 18b.

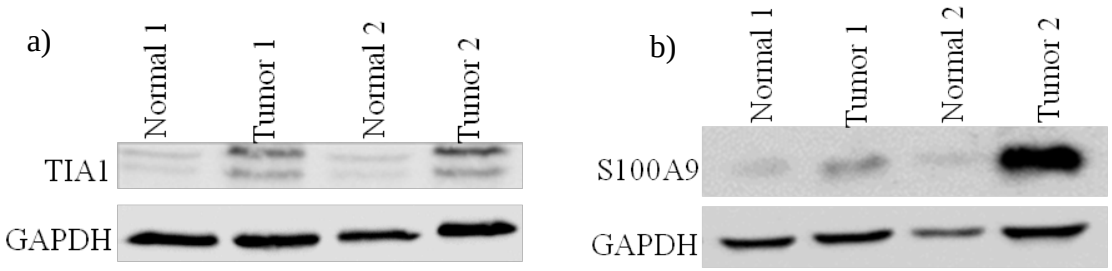

Figure 18. Immunoblot analysis of expression of proteins TIA1 and S100A9. Normal 1 and 2 are pools of proteins made by pooling random normal samples. Tumor 1 and 2 are pools of proteins made by pooling random tumor samples. Anti-GAPDH conjugated with horse-radish peroxidase against human is used as a loading control. a) TIA1 found more abundant in tumor samples as compared to normal. b) S100A9 was also found in more abundance in tumor samples as compared to normal.

4 Discussion

4.1 Cancer biomarkers identification by antibody microarrays

Cancer as a disease is too difficult to manage as it is life-threatening when it affects many vital organs in humans. There is a need for early and non-invasive diagnosis of the disease in one hand and to minimize the patient's psychological and financial stress on the other. An effective diagnosis will provide ways to not only reduce the false positive cases but also the strategies for effective therapy (Hartwell et al., 2006). Gold standard diagnostic methods using both genomic and proteomic methods were studied and developed extensively in the past decade. Proteomics as it deals with proteins, the biological end-product and acting-product in a complex system provided further more details in the mechanics of disease occurrence and recurrence (Alhamdani et al., 2009). Two-dimensional gel electrophoresis and Mass-spectrometry were two proteomic methods, extensively used with vast limitations on sensitivity and reproducibility and optimization.

The use of complex affinity protein-array technologies improved the limitations with other proteomic methods and increased the relevance of underlying molecular mechanisms such as protein-protein interactions, binding and activation of expression etc. (Alhamdani et al., 2009). These systems not only improve the specificity, sensitivity and robustness but also operate with minute probe and sample requirements mostly in picolitre and femtolitre (Alhamdani et al., 2009). Like DNA microarrays, the antibody microarrays were also printed on a solid support (often microscopic slides) coated with chemical binders on the surface (Wingren et al., 2007). Antibody microarrays being a novel technology have evolved from very less number of probes being used (between twenty and two hundred previously) to large numbers (eight hundred in our

case) (Schroder et al., 2010; Wingren et al., 2007). Experimental procedures like sample preparation, labeling and incubation procedures were optimized regularly (Alhamdani et al., 2009; Alhamdani et al., 2010). The selection of antibodies is based on the previous results from other high-throughput techniques with a vast coverage of previous studies on cancer biomarkers identification. Cancers often studied are pancreatic, breast and colon in comparison to their respective healthy controls. Eight hundred and ten antibodies raised against seven hundred and forty one cancer-related proteins are spotted on glass slides coated with epoxysaline. Six hundred and sixty eight affinity-purified polyclonal antibodies from rabbit were provided by Eurogentec and other hundred and forty two were purchased commercially and provided by collaborating partners. All these antibodies are immobilized on the slide surface in duplicates for consistency of the results. 10 µg of antibody is used for producing 1000 microarrays which is much lesser than the amount spent on various ELISA experiments. In case of two dimensional gel electrophoresis, samples are denatured and iso-electrically separated for an effective analysis. Furthermore, samples are depleted in mass-spectrometry experiments. Samples incubated on the antibody microarrays are maintained in their native state (Alhamdani et al., 2009; Schroder et al., 2010). All these advantages provide more native and specific at the same time sensitive analysis of samples (Alhamdani et al., 2009; Schroder et al., 2010).

Above all, as mentioned earlier, the whole method deals with proteins, the biological end-product. Therefore, it seems that antibody microarrays can be implemented in effective biomarker identification for diagnosis and therapy of various cancers at proteome level. In reference to the above claims, two cancers (bladder and gastric) were analyzed using above developed antibody microarray.

4.2 Non-muscle invasive bladder cancer

Non-muscle invasive bladder cancer, being a low grade, low stage tumor with high probability of recurrence is very important to study as the prognosis and prediction of recurrence can have several clinical consequences. The current study uses complex antibody microarrays to look out for molecular variations to predict recurrence at protein level since they are responsible for most of the cellular activities and thus representing the best functionality relevant differences. Many of the earlier studies used body fluids from both low and high grade tumors for an effective diagnosis and prognosis of the disease in patients with bladder cancers. Some of them even implemented proteomic methods. Antibody microarrays were implemented to profile serum samples from bladder cancer patients (Sanchez-Carbayo et al., 2006). The study revealed not only the differentiation of healthy and cancer patients but also a four protein signature to separate patients into high and low risk groups. The study used mere hundred and fifty antibodies on the array which were previously studied in bladder cancers. A mass-spectrometry based study was done by analyzing the urine samples of patients diagnosed with muscle invasive bladder cancer (Schiffer et al., 2009). The analysis could predict muscle invasive bladder cancer with a sensitivity of 92% and specificity of 68%.

Most of the proteomic-based studies in bladder cancers were concerned with both low and high grade cancers, while we focus only on the low grade and low stage cancers. Another interesting aspect of our study is the follow-up time. Although a five-year follow-up limited the sample size, we insisted on it as the recurrence history of most of the studied non-muscle invasive bladder tumors were within five years of the follow up. The proteome analysis is carried on the proteins from the tissue extracts of primary tumors diagnosed with non-muscle invasive disease since patient prognosis depends strongly on the likelihood of local recurrence. As regular transurethral

resections of tissues are performed upon during initial diagnosis of the cancer, enough patient material was available for the whole analysis.

4.2.1 Differential expression of proteins between recurrent and non-recurrent non-muscle invasive bladder tumors

The current study on non-muscle invasive bladder cancers with long term follow-up on recurrence identifies two hundred and fifty five proteins (figure 10) with significant differential abundance which is surprising as most of the target proteins were selected on the basis of studies from different cancers in comparison to their related healthy tissue. High number of proteins regulated between two tumor types is very interesting to study along with their functional annotation. These very high numbers of variations in proteins levels indicate massive differences in the biology of recurrent and non-recurrent tumors. As proteins prominently studied in bladder cancers are also differentially regulated, the results are easy to compare with the available literature and to identify new biomarkers along with the already studied ones (table 1). More importantly, many such proteins those are significantly differentially more abundant in recurrent tumors are known key players in vital functions in oncogenesis. At the same time, many abundant proteins were either studied vastly in high grade bladder cancers or other cancers as potential therapeutic targets. These previous studies strongly encouraged carrying on further bioinformatics analysis on pathways involved, interactions and disease related modifications. Proteins, JUN, TOP2A and NFκB1 were often studied in various types of cancers. The top twenty significantly differentially abundant proteins were tabulated with some of their respectively studied literature (table 1).

Among the significantly highly abundant proteins in recurrent tumors, JUN, a well known transcription factor and a proto-oncogene was extensively studied previously. Elevated levels of JUN had been identified as a prognostic marker for high risk and recurrence of prostate cancer (Ouyang et al., 2008). Few studies in transcriptional level in different cancers also identified JUN as a prognostic factor for high risk and disease progression. A transcriptional analysis to predict survival and recurrence in bladder cancer samples identified a four-transcript signature including JUN. The analysis also predicted bad outcome in correlation with high abundance of JUN (Mitra et al., 2009). The knockdown of phospholipase Cε affected the expression of JUN, in turn negatively regulating the proliferation of BIU-87 bladder cancer cells (Ling et al., 2011). Among the significantly less abundant proteins in recurrent tumors, SP1, a transcription factor that binds to gc-rich motifs with high affinity to regulate the expression of vital genes involved in cell growth, apoptosis and differentiation was well studied in high grade cancer including muscle invasive bladder cancer. SP1 was identified as a potential therapeutic target in high grade high stage muscle invasive bladder cancers. Small drug molecules targeting specificity proteins (SP1, SP2 and SP3) decreased their expression, inhibited the cell proliferation and tumor cell growth in muscle invasive bladder cancers (Chadalapaka et al., 2010; Chadalapaka et al., 2008; Jutooru et al., 2010). It is quite fascinating that the abundance of two transcription factors already studied in bladder cancers appeared differently in the current study. The studies from literature correlate with their abundance in recurrent non-muscle invasive tumors. From the above studies, one can hypothese that different mechanisms by transcription factors JUN and SP1 account for recurrence and progression of non-muscle invasive bladder respectively. The current study any way is not detailing the functional aspects of the differentially abundant proteins.

4.2.2 Apoptotic proteins and recurrent non-muscle invasive bladder cancers

Interestingly, pro-apoptotic proteins like active or cleaved CASP3, CASP9 and BAX are found more abundant in the recurrent non-muscle invasive bladder cancers (figure 10). CASP3 levels were known to be low in high grade muscle invasive bladder cancers (Karam et al., 2007). In other cancer entities, CASP3 over-expression was associated with recurrence and high cancer mortality rates (Huang et al., 2011; Jonges et al., 2001; Konstantinidou et al., 2007). CASP3, a cysteine-aspartic acid protease that activates a cascade of pro-apoptotic proteins and in turn apoptosis, is also known for its non-apoptotic roles such as differentiation, dedifferentiation and activation of immune cells (Fujita et al., 2008; Kennedy et al., 1999; Szymczyk et al., 2006). Active CASP3 had been reported as a prognostic marker in colorectal cancers with combined activation and over- expression of CD57 (Jonges et al., 2001). The increase in recurrence of apoptotic tumors after radio or chemotherapy was illustrated by the ability of active CASP3 to repopulate tumor cells through prostaglandin E2 (Huang et al., 2011). Apoptotic tumor cells leads to an increase in the levels of arachidonic acid in the extracellular space through CASP3-mediated calcium-dependent phospholipase A2 (iPLA2) activation. This activates of prostaglandin E2 through prostaglandin synthase H (PGH2), a downstream product of arachidonic acid and is known to be a key regulator of tumor growth. Apart from the above observations, Huang et al could also correlate higher levels of active CASP3 with high occurrence of tumor recurrence in head and neck squamous cell carcinoma and breast carcinoma which also supports our observations. The current study identifies both active form of CASP3 and CASP9 along with PGH2 more in abundance in the recurrent non-muscle invasive tumors. As the pattern of abundance correlate with the above observations, it can be proposed that a

similar mechanism of active CASP3 apoptosis-mediated tumor cell repopulation via PGH2 is seen also in recurrence of non-muscle invasive bladder cancers.

4.2.3 Functional annotations by pathway analysis and interactive studies

Apart from the apoptotic-regulatory proteins, a detailed functional annotation on the significantly differentially abundant proteins reveals a strong inhibition in the TGFβ signaling pathway in the recurrent non-muscle invasive tumors. The activators of the signaling pathway like IFNγ, TNFα, TGFβ and SMAD family of proteins along with transcription factor SP1 are found less abundant in recurrent tumor samples, while on the other hand MAPK3 (also known as ERK1) is high in abundance (figure 13). Similar repression of TGFβ/SMAD signaling were previously observed in different cancer entities and the repression was strongly associated with either a high risk for recurrence of the disease or a poor outcome (Alazzouzi et al., 2005; Xie et al., 2003). Further detailed analysis using online bioinformatics tools like KEGG, DAVIDGO and STRING 9.1 provide more insights on protein interactions and activation or repression of expression (figure 14). Among the highly significantly more abundant proteins in recurrent tumors, the interactions among JUN, NFκB1, KLF5, TOP2A and CDH1 are extensively reported in studies on oncogenesis and other disease conditions. TOP2A, a nuclear enzyme involved in processes such as chromosome decondensation, chromatid separation and relief of torsional stress that occurs during transcription and replication of DNA was observed to interact directly with JUN which increases the decatenation activity of TOP2A on DNA (Kroll et al., 1993). JUN was also observed to interact with NFκB1, a transcriptional regulator that is activated by various intra- and extra-cellular stimuli like cytokines, oxidant-free radicals and viral products. When activated, NFκB1 is translocated into the nucleus to stimulate the expression of genes involved in a wide variety of biological functions. The interaction between JUN and NFκB1 in endothelial

cells was exhibited to regulate the expression of vascular cell adhesion molecule-1 (VCAM1). This activation is an important feature of the initial steps of pathogenesis in atherosclerosis (Ahmad et al., 1998). KLF5, a transcription factor that binds to gc box promoter elements, localized to the nucleus binding the EGF response element was observed interacting with inflammatory response element NFκB1. Platelet derived growth factor-A (PDGFA) gene expression was exhibited to be activated by the interaction of p50 subunit of NFκB1 and KLF5. The interaction forms a protein complex on the binding site of KLF5 on the promoter chains of PDGFA (Aizawa et al., 2004). CDH1, a calcium dependent cell-cell adhesion glycoprotein whose loss of function is associated with cell proliferation and invasion is inhibited by NFκB1. NFκB1 is observed to suppress the expression of CDH1and induce the expression of vimentin, a mesenchymal specific gene. This induction leading to the epithelial to mesechymal transition of mammary cells is exhibited in breast cancers (Chua et al., 2007).

Significantly less abundant proteins in recurrent non-muscle invasive tumors are observed interacting among themselves, especially proteins such as SP1, SMAD3, VEGFA and TIMP1 are often reported in previous studies on cancer entities (figure 14). VEGFA, a growth factor activating angiogenesis and endothelial cell proliferation interacted with SP1 in pancreatic adenocarcinoma cells through IRS2, a downstream molecule of IGF 1 receptor signaling pathway. This activation of expression of VEGFA by SP1 causes pancreatic cancer cell proliferation (Neid et al., 2004). Induction of VEGFA expression by SP1 when associated with P38 kinase was also observed in cardiomyocytes (Lin et al., 2011). TIMP1 expression was effected by SP1 in human embryonic kidney cells which leads to uncontrolled proliferation of them (Lee et al., 2004). Combined over-expression of SMAD3 and SP1 in glomerular cell cancers induced the promoter activity of α2 (I) collagen gene (COL1A2) that lead to the

activation of TGFβ signaling pathway (Poncelet and Schnaper, 2001). These interactions reported and observed in various disease conditions explain their biological significance and vitality in not only onceogenesis but also general growth regulating mechanisms. It can be easily conceivable that interactions among both highly abundant and less abundant proteins in recurrent non-muscle invasive tumors are directly or indirectly responsible for disease recurrence or progression.

4.2.4 Recurrence prediction in non-muscle invasive bladder cancers

The current study reveals a 20 protein signature to predict the recurrence of cancer with very high accuracy (figure 12). The available methods for prediction of recurrence appear to be very inferior to the high accuracy in sensitivity and specificity of this 20 protein signature. The protein signature could predict recurrence with 80% sensitivity and 100% specificity as compared to the available approved NMP22 assay for bladder cancer diagnosis (Grossman et al., 2005) which exhibits a sensitivity of merely 55.7% and a specificity of 85.7%. Though, the number of samples analyzed appears to be very less for such a claim, the five year follow-up time appears clinically significant. The sample limitations can also be rectified by some non-invasive analysis on body fluids as most of the proteins in the signature are known to be secreted. The gold-standard diagnostic methods in clinical settings including immuno-histo chemistry are all immuno-based assays. Antibody microarray, as itself is an immuno-based assay has the great potential to be used in clinical situations. Furthermore, these marker proteins can be translated easily to standard immuno-based assay platforms that are routine in use in clinical settings. On a broader outlook, such a test for prediction of recurrence can adjust the treatment regimen and rigidity of surveillance, especially in bladder cancers where patients were followed up for years

using cytoscopy methods. This can not only reduce surveillance costs but also improve the patients' outcome substantially.

4.3 Biomarker identification for gastric adenocarcinoma by antibody microarrays

Far too many studies emphasize the role of different regulators of cell cycle, cell growth, proliferation, angiogenesis and apoptosis in gastric cancer formation and evaluate their potential as a valuable therapeutic target. Various methods are being employed to study the molecular basis of gastric adenocarcinoma, but only few evolve till the treatment strategies. Despite recent developments in high-throughput methods like signature based molecular diagnostics, mass spectrometry (MS) based proteomic methods and next generation sequencing, availability of suitable biomarkers at protein levels often, is disappointing. Either occurrence of high false positive cases or controversial published data on the role of molecular markers restricts, not only the implementation of many markers but also fails to address the response due to inter-individual variability (Pietrantonio et al., 2013). DNA microarray, as a powerful high-throughput technique only identifies novel genes which as biomarkers, fail at protein level when conventional immuno-based assays such as immuno-histochemistry (IHC) and ELISA were used (Zheng et al., 2004). Advances in large-scale gene expression profiling combined with network analysis identified several biomarkers without much clinical relevance on the stage dependent expression of the genes. Moreover, these markers do not predict the progression and patient outcome which is again important for an optimal treatment strategy (Takeno et al., 2008). Apart from DNA and RNA microarrays, tissue microarrays are good tools for protein level biomarker identification with much limitations in number of molecules studied (Senapati et al., 2008). Individual analysis

on cytotoxic molecules predicts acute gastric mucosal lesions (Suzuki et al., 2003). Similar analysis on other molecules involved in key biological processes, showed their significance as prognostic indicators in gastric adenocarcinomas but with much inconsistence disallowing them from being recognized as standard biomarkers (Kodama et al., 2008; Leung et al., 2004). So far, only antibody microarray based study on gastric adenocarcinomas deals with cancers associated with Helicobacter pylori infection. The study composed of mere hundred and twenty seven antibodies against immunoregulatory antigens. Apart from identifying plenty of previously reported proteins, the analysis also identified protein signatures associated with tumors and bacterial infection (Ellmark et al., 2006).

We used complex antibody microarrays with eight hundred and ten antibodies described earlier (section 4.1) to analyze high grade, high stage (most even had lymph node metastasis) gastric adenocarcinoma samples. To get more insight on a personalized treatment strategy point of view, gastric adenocarcinoma tissues along with healthy tissue controls from the same patients are analyzed. The analysis comparing twenty five healthy controls with same number of gastric adenocarcinomas (both training and test sets) reveals several interesting candidates.

4.3.1 Differentially regulated proteins in gastric adenocarcinoma

Two different analyses on different sample sets identify ninety eight significantly differentially abundant proteins between cancer and healthy controls (table 2, 3 and figure 16, 17). Along with a lot of newly observed proteins, few proteins already described in gastric oncogenesis are identified from the analysis. Interestingly, five proteins are differentially abundant in both the training and test set of samples among which three are in the most significantly abundant proteins (table 3). Strikingly, proteins involved among highly invasive cancers including gastric adenocarcinomas are also present among the significantly abundant proteins. These results

triggered our interest for further analyses on the proteins with literatures. Proteins like AQP1, CNN2, TIA1, OCLN, S100A9, VEGFA and DKK3 best described oncogenesis.

AQP1

Aquaporin 1 (AQP1), a water channel protein facilitates water flux across cell membranes is found highly significantly more abundant in gastric adenocarcinoma tissues. Belonging to a family of small integral membrane proteins, they physically resemble channel proteins and highly abundant in erythrocytes and renal tubes. Also found in abundance in epithelial and endothelial cells, they are associated with cell migration, metastasis and angiogenesis. It is previously reported to be over-expressed in various human malignancies (Hu and Verkman, 2006). AQP1 is proposed as a biomarker of early diagnosis of renal cancer on the analysis of urine samples from patients with renal cancer (Morrissey et al., 2010). AQP1 in protein level is a poor prognostic factor in basal-like breast carcinomas as occurrence of death in patients suffering from breast cancers directly correlated with the high abundance of AQP1 (Otterbach et al., 2010). To our interest, this protein is not previously reported in gastric adenocarcinomas.

CNN2

Calponin 2 (CNN2) plays a role in smooth muscle contraction and cell-cell adhesion. It is found highly significantly more abundant in gastric cancer tissues. CNN2 is known to bind to actin and other channel proteins; for example, a calcium channel protein called calmodulin and involves in the structural organization of actin filaments. Other known functions of this protein include wound healing and positive regulation of cell proliferation. CNN2 being highly abundant in rectal cancers is proposed as a diagnostic biomarker at transcript level (Choi et al., 2011). CNN2 is only reported in transcript levels in gastric cancers.

TIA1

Nucelolysin TIA1 (TIA1), also called as T-cell intracellular antigen is an mRNA binding protein is expressed when cells undergo specific stress. They involve in both transcriptional and post-transcriptional gene expression in eukaryotic cells. They activate nucleolysis against cells that target cytotoxic lymphocytes. Over expression of TIA1 under various stress conditions is studied in different human cells (Dinh et al., 2013; Gottschald et al., 2010). An important function of TIA1 is to regulate alternative pre-mRNA splicing of approximately 15% of the cassette human exons. High lethality rates in mice lacking TIA1 also shows their importance in regulation of important genes. Interestingly, TIA1 is not studied well in gastric cancers except inconsistent reports on abundance in protein level but in cancers like, colon, ovary and lymphoma TIA1 is found high in abundance (Izquierdo et al., 2011).

OCLN

Occludin (OCLN) found high in abundance in gastric adenocarcinoma is a protein known for its localization in tight junctions of both epithelial and endothelial cells. Over-expressing OCLN in cells lacking tight junctions induces cell adhesion. Mutations involved in OCLN gene leads to a rare neurological disorder called pseudo-TORCH syndrome. Belonging to the claudin family of proteins, OCLN is associated with epithelial-mesenchymal transition in colorectal cancers, which is one of the initial mechanisms in oncogenesis. It if found that OCLN is regulated by transcription factor AP4 which is a strong prognostic factor in colorectal cancers (Jackstadt et al., 2013). In skin cancers, it is observed that OCLN is involved in Calcium ion-dependent homeostasis and other important cellular processes in oncogenesis like apoptosis, adhesion and differentiation (Rachow et al., 2013). One of the earlier studies on the expression of OCLN in esophageal, colon and gastric tissues found OCLN's localization in the tight junctions of the

polarized epithelial cells. Further investigation with immuno-histochemical methods exhibited the expression of two tight junction proteins, OCLN and Zonula occludens (ZO1) in the highly differentiated tumor epithelia of colon and gastric cancers (Kimura et al., 1997). Gastric cancer cells in response to epidermal growth factor (EGF) treatment translocated OCLN from cytoplasm to the tight junctions which explain the role of it in cell-cell adhesion (Yoshida et al., 2005). From these results we suggest that OCLN is involved by similar mechanisms of oncogenesis in gastric adeonocarcinomas.

S100A9

S100A9 also known as calgranulin-B belongs to S100 family of calcium binding proteins localized mostly in the cytoplasm and nucleus. S100A9 is often studied in cancers for its properties other than calcium binding like apoptosis, cell cycle progression and differentiation. It also plays a role as a pro-inflammatory mediator in acute chronic inflammation. S100A9 is highly abundant in gastric cancer cells in the transcript level (El-Rifai et al., 2002). Apart from gastric cancers S100A9 is reported to be highly abundant at protein level in ovarian, hepato-cellular and breast cancer. Interestingly, S100A9 is highly abundant in both malignant epithelial and stromal cells in high grade gastric adenocarcinoma leading to their detection in plasma samples of gastric cancer patients (Wang et al., 2013). It is not so surprising that S100A9 triggers cascade of signaling molecules including p38 mitogen-activated protein kinase and NFκB leading to the migration of gastric cancer cells and in turn invasiveness of the gastric adenocarcinoma cells (Kwon et al., 2013).

VEGFA

Vascular endothelial growth factor A (VEGFA) is a protein with all great potential functions involved in oncogenesis such as enhancing endothelial cell proliferation, promoting cell migration and inhibition of apoptotic signals. It is not only a growth factor with great abilities but also an active protein in vasculogenesis, endothelial cell growth, angiogenesis and promotes permeability of blood vessels. Such a protein is often a therapeutic target in many cancers and other diseases also. High abundance of VEGFA combined with SP1 at protein levels is directly correlated with the grade and stage of gastric adenocarcinomas. This co-expression resulted in poor prognosis of the disease (Yao et al., 2004). Metastasis and migration abilities of gastric adenocarcinoma cells are inhibited strongly when integrin alpha v beta 6 (IAVB6) function is lost. VEGFA is known to activate the integrin molecule IAVB6 which strongly suggests the therapeutic importance of drugs inhibiting VEGFA (Zhao et al., 2010). Another interesting study involving protein kinase D2 (PKD2) and VEGFA in gastric cancer cells finds the importance of the role of VEGFA in endothelial cell proliferation and migration. Invivo knockdown of VEGFA and PKD2 in mice results in inhibition of angiogenesis and tumor growth (Azoitei et al., 2010). Strikingly, VEGFA is very highly abundant in our analysis on gastric adenocarcinoma samples.

DKK3

Dickkopf related-protein 3 (DKK3) is found less abundant in the gastric adenocarcinomas. Often referred as one of the vital tumor suppressor genes, DKK3 is often missing in various cancer cells. Wnt signaling which is widely studied in biology of cancers can be modulated by varying concentrations of other soluble including DKK3. DKK3 at protein level is very less in abundance in gastric and colon cancer cells (Byun et al., 2005). More detailed molecular analysis of gastric cancers illustrated epigenetic down-regulation leading to the inactivation in the translation of

DKK3 genes in gastric cancers (Sato et al., 2007). More recently, plasmid-mediated functional resurrection of DKK3 in gastric cancer cells decreased the aggressiveness and migration of the cells indicating the possible tumor suppressor role of DKK3 in gastric cancer. Moreover, DKK3 expression in gastric cancers negatively correlated with the tumor size, metastasis and grade of the tumor indicating the prognostic significance of it in gastric cancer (Xu et al., 2012).

From the above exhibited significantly abundant proteins in gastric adenocarcinomas, we suggest that similar mechanisms of oncogenesis may be the causes of cancer in sets of samples in analyses. The stability and consistency of some of the markers can be a boon to available molecular diagnostics as all of them are studied at the protein level. A signature of top sixteen proteins from table 3 will help not only to predict prognosis but also to establish individual-based treatment strategy as the comparisons here are made matching the healthy controls and the gastric adenocarcinoma tissues after surgical resection from the same patient.

5 Conclusions

The current study presented here focused on the application of complex antibody microarrays as a diagnostic and prognostic tool in human cancers. In the first part of the study, protein profiles of non-muscle invasive bladder cancer samples were studied and a signature of 20 proteins was identified to predict the recurrence of the cancer. Apart from the prediction of recurrence with high specificity and sensitivity, the important molecular mechanisms that may lead to recurrence of cancer were also identified. The results from this analysis will prove to be efficient in predicting the recurrence of cancer by the application of the protein signature in large sets of non-muscle bladder cancer samples with appropriate follow-up time. Moreover, this effective prediction will aid the clinicians to develop an optimal strategy to follow-up and treat the patients.

In the second part of the study, analysis of gastric adenocarcinoma samples by two different methods of incubations on the antibody microarrays identified a set of 16 proteins highly differentially regulated between healthy controls and cancer tissues. Proteins identified included many validated and well studied therapeutic targets which are under clinical trials. A strong literature search on the identified proteins provided more insights on the molecular mechanisms of oncogenesis and metastasis in gastric tissues. The results from this analysis will help clinicians to identify patients with higher risk of metastasis and death occurrence. Validation of the protein profile in large sets of tumors will favor individual-based treatment regimen for gastric cancer. More functional validations on the identified targets will help in the development of therapeutics to treat gastric adenocarcinoma.

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7. Appendix

7.1 Supplementary table S1

Supplementary table S1. List of the sixteen proteins with the significant expression variations between healthy controls and tumours. Log fold changes in expression and the related adjusted p-values are shown.

Sl.No.	Uniprot Entryname	Uniprot accession	adjusted P Value	log Fold change
1	THYG_HUMAN	P01266	8.65E-30	-1.86
2	IL2_HUMAN	P60568	1.90E-22	-1.58
3	PTEN_HUMAN	P60484	1.20E-15	-1.54
4	IL4_HUMAN	P05112	7.06E-13	-2.14
5	IFNG_HUMAN	P01579	6.67E-12	-1.16
6	VEGFA_HUMAN	P15692	1.68E-09	-0.93
7	FUS_HUMAN	P35637	3.25E-09	-0.66
8	LAP2A_HUMAN	P42166	0.002	0.30
9	TR10A_HUMAN	O00220	0.004	0.53
10	TNR21_HUMAN	O75509	0.008	-0.52
11	ALBU_HUMAN	P02768	0.010	0.89
12	ICAM1_HUMAN	P05362	0.012	0.26
13	TR10A_HUMAN	O00220	0.015	0.36
14	CDN1C_HUMAN	P49918	0.045	0.30
15	IL6_HUMAN	P05231	0.046	-0.20
16	ETS1_HUMAN	P14921	0.046	-0.39

7.2 Supplementary table S2

Supplementary table S2. List of the two hundred and fifty five proteins with the significant expression variations between recurrent and non-recurrent non-muscle invasive bladder cancer. Log fold changes in expression and the related adjusted p-values are shown.

Sl.No.	Uniprot Entryname	Uniprot accession	adjusted P Value	log Fold change
1	LMNA_HUMAN	P02545	3.32E-09	0.72
2	YBOX1_HUMAN	P67809	1.87E-08	0.52
3	JUN_HUMAN	P05412	1.27E-07	0.50
4	TIA1_HUMAN	P31483	2.13E-07	-0.39
5	SMAD3_HUMAN	P84022	2.13E-07	-0.59
6	PABP1_HUMAN	P11940	2.13E-07	-0.36
7	AKT3_HUMAN	Q9Y243	2.47E-07	0.48
8	CDN1A_HUMAN	P38936	3.38E-07	-0.52
9	LYAM1_HUMAN	P14151	9.62E-07	-0.52
10	YETS2_HUMAN	Q9ULM3	5.28E-06	0.32
11	AKTIP_HUMAN	Q9H8T0	5.38E-06	-0.35
12	PRI1_HUMAN	P49642	1.04E-05	-0.36
13	HSP7C_HUMAN	P11142	1.04E-05	-0.34
14	RSSA_HUMAN	P08865	1.07E-05	-0.35
15	GRM1A_HUMAN	Q96CP6	1.10E-05	0.27
16	TPA_HUMAN	P00750	1.14E-05	0.23
17	ZBT17_HUMAN	Q13105	1.14E-05	-0.55
18	CADH1_HUMAN	P12830	1.15E-05	0.33
19	LAMP2_HUMAN	P13473	1.15E-05	0.26
20	JUN_HUMAN	P05412	1.34E-05	0.48
21	LIFR_HUMAN	P42702	2.17E-05	0.30
22	TOP2A_HUMAN	P11388	2.17E-05	0.48
23	SPS2L_HUMAN	Q9NUQ6	2.20E-05	-0.23
24	UBIQ_HUMAN	P62988	2.47E-05	0.24
25	NFAC4_HUMAN	Q14934	2.47E-05	0.35
26	SF3B3_HUMAN	Q15393	2.47E-05	0.32

27	2DMB_HUMAN	P28068	3.41E-05	-0.35
28	FAK1_HUMAN	Q05397	3.93E-05	-0.36
29	IFNG_HUMAN	P01579	3.95E-05	-0.46
30	SP1_HUMAN	P08047	4.14E-05	-0.36
31	ACTN1_HUMAN	P12814	4.31E-05	-0.36
32	TIE1_HUMAN	P35590	6.17E-05	-0.30
33	TIMP1_HUMAN	P01033	6.32E-05	-0.33
34	MMP13_HUMAN	P45452	6.32E-05	0.25
35	VTNC_HUMAN	P04004	6.76E-05	-0.51
36	K1C17_HUMAN	Q04695	0.0002	-0.20
37	NFKB1_HUMAN	P19838	0.0002	0.32
38	KLF5_HUMAN	Q13887	0.0002	0.37
39	NAP1_HUMAN	Q9BU70	0.0002	-0.27
40	RL10_HUMAN	P27635	0.0002	-0.28
41	MMP1_HUMAN	P03956	0.0002	-0.26
42	CDKN3_HUMAN	Q16667	0.0002	-0.33
43	CD59_HUMAN	P13987	0.0003	-0.33
44	PO2F2_HUMAN	P09086	0.0003	-0.35
45	MPIP2_HUMAN	P30305	0.0003	-0.28
46	FRAP_HUMAN	P42345	0.0003	-0.27
47	IRS2_HUMAN	Q9Y4H2	0.0003	-0.33
48	B2LA1_HUMAN	Q16548	0.0003	0.24
49	ERBB2_HUMAN	P04626	0.0004	-0.24
50	CASP3_HUMAN	P42574	0.0004	0.40
51	FINC_HUMAN	P02751	0.0004	-0.31
52	LAC_HUMAN	P01842	0.0004	-0.26
53	AURKB_HUMAN	Q96GD4	0.0004	-0.33
54	MPP3_HUMAN	Q13368	0.0004	-0.21
55	CD2A2_HUMAN	Q8N726	0.0004	-0.32
56	SOX9_HUMAN	P48436	0.0004	0.23
57	EPCAM_HUMAN	P16422	0.0004	-0.30
58	TSP3_HUMAN	P49746	0.0004	-0.24
59	O00446_HUMAN	O00446	0.0005	0.25
60	CP3A7_HUMAN	P24462	0.0006	-0.23

61	THYG_HUMAN	P01266	0.0006	-0.34
62	NMDE3_HUMAN	Q14957	0.0006	-0.27
63	IL15_HUMAN	P40933	0.0006	-0.80
64	AQP1_HUMAN	P29972	0.0006	0.24
65	LAT1_HUMAN	Q01650	0.0006	-0.24
66	GSHB_HUMAN	P48637	0.0006	0.17
67	RPB3_HUMAN	P19387	0.0007	-0.23
68	K1C19_HUMAN	P08727	0.0007	0.21
69	PAK2_HUMAN	Q13177	0.0007	0.20
70	ZN593_HUMAN	O00488	0.0008	0.24
71	MYD88_HUMAN	Q99836	0.0008	0.22
72	IL8_HUMAN	P10145	0.0009	-0.23
73	SEP15_HUMAN	O60613	0.0010	-0.21
74	CUL2_HUMAN	Q13617	0.0010	-0.24
75	TNF13_HUMAN	O75888	0.0010	-0.24
76	EPHB3_HUMAN	P54753	0.001	0.21
77	APBA1_HUMAN	Q02410	0.001	0.25
78	MK10_HUMAN	P53779	0.001	-0.18
79	IL10_HUMAN	P22301	0.001	0.22
80	GDN_HUMAN	P07093	0.001	-0.26
81	HMMR_HUMAN	O75330	0.001	-0.26
82	OLFM4_HUMAN	Q6UX06	0.001	-0.23
83	LYAM1_HUMAN	P14151	0.001	0.21
84	CISY_HUMAN	O75390	0.001	-0.30
85	ID2_HUMAN	Q02363	0.001	-0.28
86	MUTED_HUMAN	Q8TDH9	0.001	-0.29
87	SEPR_HUMAN	Q12884	0.001	-0.25
88	TR10A_HUMAN	O00220	0.002	-0.37
89	K2C8_HUMAN	P05787	0.002	-0.19
90	TNFB_HUMAN	P01374	0.002	0.20
91	ANFB_HUMAN	P16860	0.002	0.19
92	CP1B1_HUMAN	Q16678	0.002	-0.30
93	BRPF3_HUMAN	Q9ULD4	0.002	-0.18
94	GBRB1_HUMAN	P18505	0.002	-0.21

95	AP4B1_HUMAN	Q9Y6B7	0.002	-0.21
96	SIA7F_HUMAN	Q969X2	0.002	0.18
97	HXC11_HUMAN	O43248	0.002	-0.17
98	PIGC_HUMAN	Q92535	0.002	-0.15
99	TRI22_HUMAN	Q8IYM9	0.002	-0.23
100	OSTP_HUMAN	P10451	0.002	-0.23
101	ZO2_HUMAN	Q9UDY2	0.002	0.19
102	PO2F1_HUMAN	P14859	0.003	-0.23
103	ARI4A_HUMAN	P29374	0.003	0.16
104	KPYM_HUMAN	P14618	0.003	-0.20
105	SHIP1_HUMAN	Q92835	0.003	-0.24
106	K1C18_HUMAN	P05783	0.003	-0.15
107	LYAM1_HUMAN	P14151	0.003	0.20
108	ENOG_HUMAN	P09104	0.004	0.19
109	IFNG_HUMAN	P01579	0.004	-0.24
110	RL29_HUMAN	P47914	0.004	0.24
111	EWS_HUMAN	Q01844	0.004	0.41
112	HSF1_HUMAN	Q00613	0.004	-0.14
113	KLF8_HUMAN	O95600	0.004	0.29
114	TR10B_HUMAN	O14763	0.004	-0.20
115	NFKB2_HUMAN	Q00653	0.004	-0.22
116	NEP_HUMAN	P08473	0.004	-0.19
117	IFM1_HUMAN	P13164	0.005	0.14
118	RPN2_HUMAN	P04844	0.005	0.18
119	TGFA_HUMAN	P01135	0.005	0.18
120	CP3A5_HUMAN	P20815	0.005	0.13
121	CATA_HUMAN	P04040	0.006	-0.47
122	MLH1_HUMAN	P40692	0.006	-0.17
123	MAGA8_HUMAN	P43361	0.006	-0.16
124	P53_HUMAN	P04637	0.006	-0.19
125	IFNA1_HUMAN	P01562	0.006	-0.33
126	KSYK_HUMAN	P43405	0.006	0.18
127	K1C14_HUMAN	P02533	0.007	0.17
128	CCL4_HUMAN	P13236	0.007	-0.23

129	TR13C_HUMAN	Q96RJ3	0.007	0.26
130	STK19_HUMAN	P49842	0.007	0.15
131	FLOT1_HUMAN	O75955	0.007	0.18
132	FANCC_HUMAN	Q00597	0.007	-0.17
133	Q16590_HUMAN	Q16590	0.007	-0.22
134	EF1G_HUMAN	P26641	0.007	0.14
135	PIR_HUMAN	O00625	0.008	-0.28
136	EDN1_HUMAN	P05305	0.008	-0.18
137	PCNA_HUMAN	P12004	0.008	-0.17
138	S100P_HUMAN	P25815	0.008	-0.15
139	CDC5L_HUMAN	Q99459	0.008	-0.19
140	MUC6_HUMAN	Q6W4X9	0.009	-0.31
141	KLH12_HUMAN	Q53G59	0.009	-0.17
142	DNLI3_HUMAN	P49916	0.009	0.16
143	MAD1_HUMAN	Q05195	0.009	0.18
144	CTRB1_HUMAN	P17538	0.009	-0.14
145	SRGEF_HUMAN	Q9UGK8	0.009	-0.15
146	OSTP_HUMAN	P10451	0.009	-0.15
147	TIMP2_HUMAN	P16035	0.010	-0.22
148	GRPR_HUMAN	P30550	0.010	-0.28
149	VIME_HUMAN	P08670	0.010	-0.21
150	TOP2A_HUMAN	P11388	0.010	-0.23
151	MUC2_HUMAN	Q02817	0.010	0.15
152	MLH3_HUMAN	Q9UHC1	0.010	0.18
153	MOT4_HUMAN	O15427	0.010	-0.19
154	VEGFA_HUMAN	P15692	0.010	-0.29
155	ID1_HUMAN	P41134	0.010	-0.23
156	EI2BA_HUMAN	Q14232	0.011	-0.17
157	HNRPC_HUMAN	P07910	0.011	-0.15
158	CASP9_HUMAN	P55211	0.014	0.30
159	ST14_HUMAN	Q9Y5Y6	0.011	0.18
160	VAS1_HUMAN	Q15904	0.011	-0.19
161	SMAD4_HUMAN	Q13485	0.013	-0.13
162	MTA1_HUMAN	Q13330	0.013	0.14

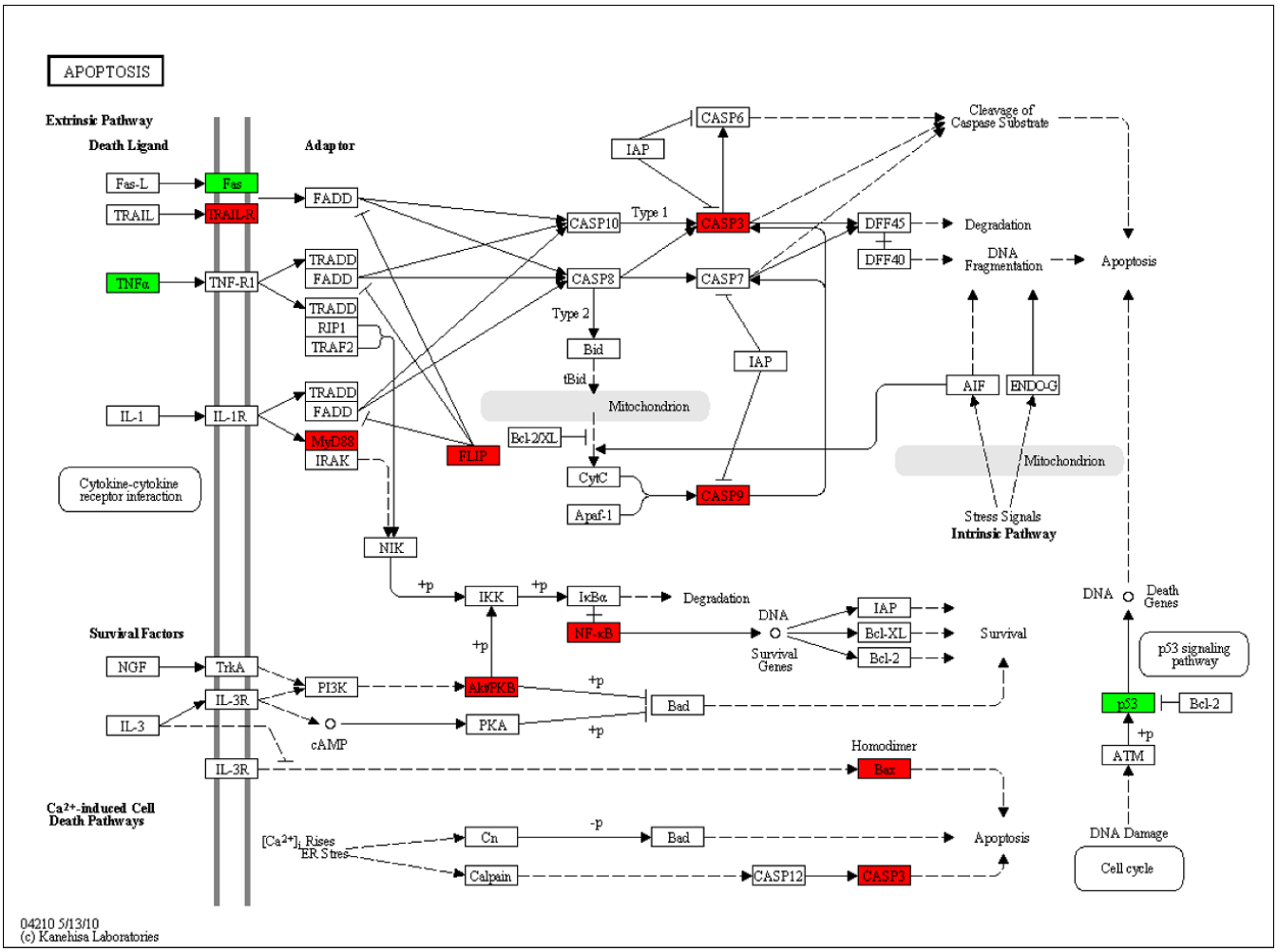
163	CAV3_HUMAN	P56539	0.013	0.15
164	PTEN_HUMAN	P60484	0.014	-0.26
165	LUM_HUMAN	P51884	0.014	-0.15
166	TMED2_HUMAN	Q15363	0.014	0.23
167	CD81_HUMAN	P60033	0.014	0.21
168	MD2L1_HUMAN	Q13257	0.014	0.14
169	DC12_HUMAN	Q96FZ2	0.015	0.17
170	ICAM1_HUMAN	P05362	0.015	-0.16
171	TMM54_HUMAN	Q969K7	0.015	-0.14
172	IL1RA_HUMAN	P18510	0.015	0.16
173	TNFA_HUMAN	P01375	0.015	-0.24
174	RBM42_HUMAN	Q9BTD8	0.015	-0.13
175	TNF13_HUMAN	O75888	0.015	0.18
176	RARB_HUMAN	P10826	0.015	-0.14
177	IL6RA_HUMAN	P08887	0.015	0.13
178	TR10A_HUMAN	O00220	0.016	-0.22
179	PHB_HUMAN	P35232	0.017	-0.13
180	CAH2_HUMAN	P00918	0.017	0.15
181	NPT1_HUMAN	Q14916	0.018	-0.19
182	IGF2_HUMAN	P01344	0.018	-0.19
183	MMP1_HUMAN	P03956	0.018	-0.19
184	PSB4_HUMAN	P28070	0.018	-0.14
185	MK03_HUMAN	P27361	0.018	0.20
186	SODC_HUMAN	P00441	0.018	-0.16
187	MCP_HUMAN	P15529	0.019	0.13
188	MTHR_HUMAN	P42898	0.020	-0.12
189	BEX3_HUMAN	Q00994	0.021	0.13
190	GPX4_HUMAN	P36969	0.021	0.18
191	MTA2_HUMAN	O94776	0.021	-0.13
192	IL2_HUMAN	P60568	0.021	-0.23
193	FGF7_HUMAN	P21781	0.021	-0.14
194	ICAM1_HUMAN	P05362	0.022	-0.14
195	1C01_HUMAN	P30499	0.023	0.15
196	HSBP1_HUMAN	O75506	0.024	0.17

197	TRFE_HUMAN	P02787	0.024	-0.41
198	P5CR1_HUMAN	P32322	0.025	0.16
199	PGH2_HUMAN	P35354	0.025	0.17
200	CATD_HUMAN	P07339	0.027	0.30
201	CCR7_HUMAN	P32248	0.025	0.17
202	SORL_HUMAN	Q92673	0.028	0.15
203	AIFM1_HUMAN	O95831	0.030	0.26
204	CBX3_HUMAN	Q13185	0.029	-0.13
205	L1CAM_HUMAN	P32004	0.031	-0.20
206	BAX_HUMAN	Q07812	0.031	0.16
207	IBP3_HUMAN	P17936	0.031	0.14
208	MK04_HUMAN	P31152	0.031	0.11
209	CFLAR_HUMAN	O15519	0.031	0.15
210	SYSC_HUMAN	P49591	0.031	-0.17
211	IL4_HUMAN	P05112	0.031	-0.14
212	ETS2_HUMAN	P15036	0.031	-0.18
213	ZG16_HUMAN	O60844	0.032	0.11
214	MMP10_HUMAN	P09238	0.032	0.13
215	T4S4_HUMAN	P48230	0.032	0.13
216	P63_HUMAN	Q9H3D4	0.032	0.11
217	BRAF1_HUMAN	P15056	0.032	-0.22
218	RL27A_HUMAN	P46776	0.032	-0.15
219	WNT2B_HUMAN	Q93097	0.033	0.13
220	CAD13_HUMAN	P55290	0.033	-0.14
221	IL1R2_HUMAN	P27930	0.033	0.13
222	HXA9_HUMAN	P31269	0.033	-0.11
223	O14997_HUMAN	O14997	0.034	0.15
224	HERP1_HUMAN	Q15011	0.034	-0.20
225	TIP_HUMAN	Q8TB96	0.034	-0.16
226	NEK2_HUMAN	P51955	0.034	0.12
227	DHRS2_HUMAN	Q13268	0.036	-0.15
228	HMGB1_HUMAN	P09429	0.035	0.14
229	CENPF_HUMAN	P49454	0.035	-0.11
230	PO5F1_HUMAN	Q01860	0.036	0.20

231	ECHM_HUMAN	P30084	0.036	-0.57
232	TR10B_HUMAN	O14763	0.037	0.22
233	S19A1_HUMAN	P41440	0.036	-0.12
234	FGF2_HUMAN	P09038	0.037	-0.22
235	ABCG2_HUMAN	Q9UNQ0	0.037	-0.12
236	RAB1A_HUMAN	P62820	0.037	0.12
237	TBB5_HUMAN	P07437	0.037	0.15
238	LEUK_HUMAN	P16150	0.039	0.18
239	CKAP2_HUMAN	Q8WWK9	0.039	0.13
240	NUPR1_HUMAN	O60356	0.039	-0.13
241	TNR6_HUMAN	P25445	0.041	-0.29
242	VINEX_HUMAN	O60504	0.040	-0.12
243	PIGT_HUMAN	Q969N2	0.040	0.13
244	CAV2_HUMAN	P51636	0.041	0.18
245	UN93B_HUMAN	Q9H1C4	0.042	-0.16
246	FASTK_HUMAN	Q14296	0.042	0.13
247	VGFR2_HUMAN	P35968	0.042	-0.14
248	SYG_HUMAN	P41250	0.042	0.14
249	TGFB2_HUMAN	P61812	0.042	-0.12
250	IL13_HUMAN	P35225	0.043	0.18
251	S1PR1_HUMAN	P21453	0.043	0.12
252	CCNB1_HUMAN	P14635	0.043	-0.15
253	CD44_HUMAN	P16070	0.043	-0.16
254	ENC1_HUMAN	O14682	0.047	-0.16
255	PLSL_HUMAN	P13796	0.047	-0.11

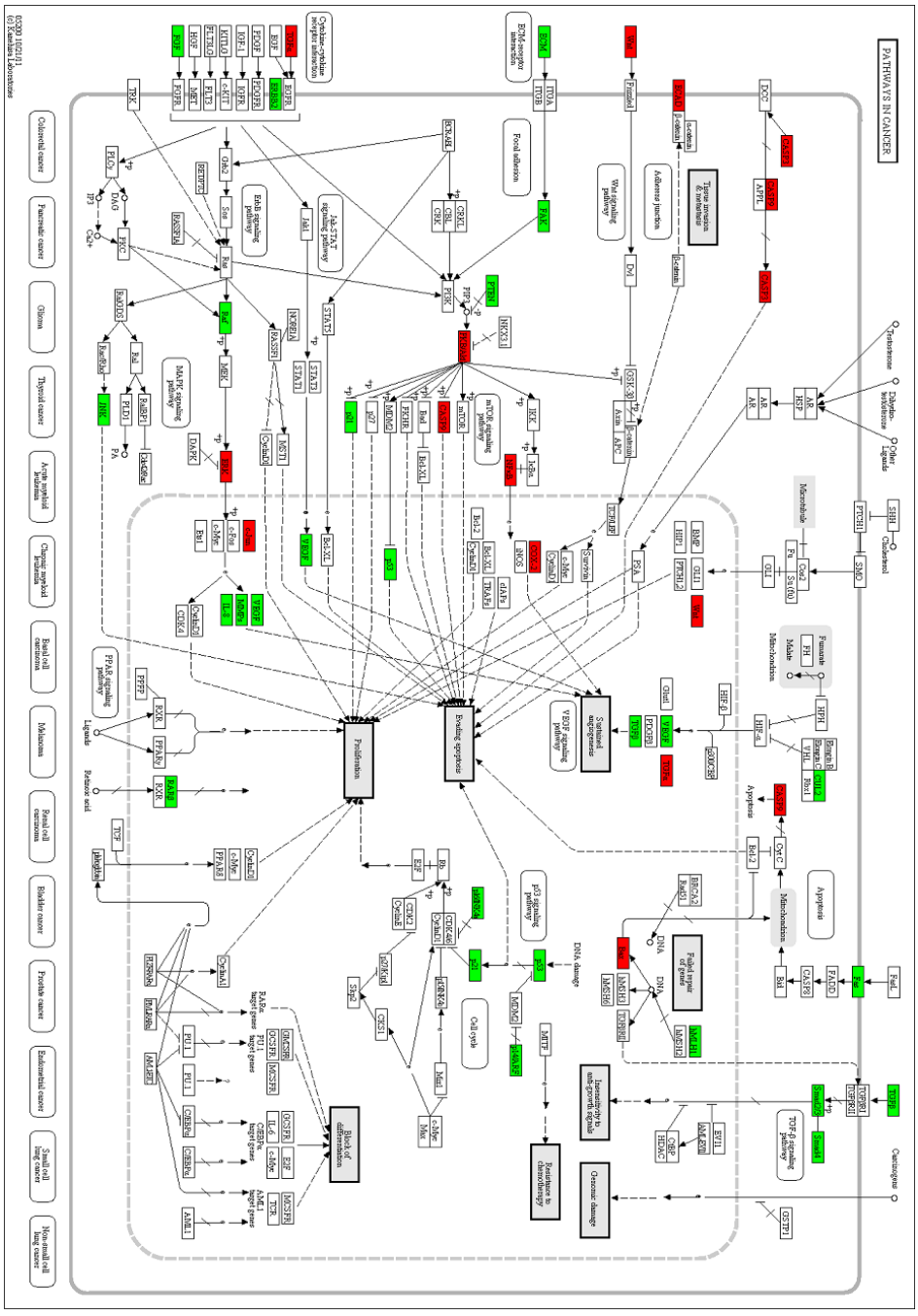
7.3 Supplementary figure S1

Supplementary figure S1. Differential abundance of apoptotic-related proteins between recurrent and non-recurrent non-muscle invasive bladder cancer. Highly abundant proteins were coloured red and less abundant proteins were coloured green. KEGG pathway analysis software was used to identify the proteins.



7.4 Supplementary figure S2

Supplementary figure S2. Differential abundance of proteins between recurrent and non-recurrent non-muscle invasive bladder cancer in various cancer pathways. Highly abundant proteins were coloured red and less abundant proteins were coloured green. KEGG pathway analysis software was used to identify the proteins.



7.5 Publications based on the thesis

1. Harish Srinivasan, Yves Allory, Martin Sill, Mohamed Saiel Saeed Alhamdani, Francois Radvanyi, Jörg D. Hoheisel and Christoph Schröder (2013). Prediction of recurrence of non muscle-invasive bladder cancer by means of a protein signature identified by antibody microarray analyses. (*Submitted- In revision*).

2. Harish Srinivasan, Damjana Kastelic, Radovan Komel, Christoph Schröder and Jörg D. Hoheisel (2013). Immuno-based biomarker identification for effective diagnosis and prediction of prognosis in gastric adenocarcinomas- an antibody microarray study. (*In preparation*).