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PROTEIN EXPRESSION IN PROSTATE CANCER

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Cover designed by Helena Lexander. 2-DE gel in artificial image colors. A magnification of my favorite proteins.

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The agony of cancer. It is awesome. It is inspiring. It is terrible. It is wonderful.

Charles Huggins (1901-1997)

Nobel laureate in Physiology and Medicine 1966

ABSTRACT

The molecular pathology of prostate cancer (PCa) is complex and the pathways and the acquired molecular defects responsible for PCa initiation, development and progression are still largely unknown. Tumors of the prostate have an unpredictable behavior and in clinically detected cancers it is presently not possible to assess tumor aggressiveness. PSA is currently the only clinically used biomarker for detection of PCa, but its specificity is low and there is a need for additional tissue and serum biomarkers for early PCa detection and for prediction of prognosis.

In this thesis two-dimensional gel electrophoresis (2-DE) was used to study the protein expression in benign and malignant prostate tissue. 2-DE is a powerful method that can visualize the protein phenotype of a cell and downstream effects of specific gene regulations that cannot be detected on a genetic level. Proteins are separated according to their size and charge and 2-DE has therefore the potential to separate post-translational modifications (PTMs), including truncated protein variants.

In PCa, tumor heterogeneity and the small size of the tumors make it difficult to sample representative cells for 2-DE analysis. We have developed and evaluated a modified non-enzymatic sample preparation (NESP) scraping technique to extract cells from fresh prostate tissue.

The human prostate is composed of three anatomical zones: the peripheral (PZ), central (CZ) and transition (TZ) zone. The functional roles of the zones remain largely unknown. A majority of clinically diagnosed cancers arise in the PZ. We found 18 protein spots in 2-DE gels with significantly different expression levels between the three anatomical zones. The identified proteins suggest functional differences between the zones, and also support the hypothesis that CZ may be of different embryonic origin than PZ and TZ.

We analyzed the protein expression profile of PCa in order to identify proteins with decreased or increased expression in malignant cells, possibly contributing to the understanding of carcinogenesis in the prostate. We detected 63 polypeptides with differential expression in benign prostatic tissue and PCa. By correlating the protein expression with the differentiation markers Gleason grade and DNA ploidy we could distinguish 39 polypeptides which expression levels associated with tumor dedifferentiation. Some of the findings may have the potential to become diagnostic or prognostic biomarkers for PCa. We also showed that multivariate analysis may be applied to discriminate potentially high malignant samples within a group of samples with unpredictable outcome.

Key words: two-dimensional gel electrophoresis, protein expression, biomarker, Gleason grade, DNA-ploidy

PUBLICATIONS AND MANUSCRIPTS

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LIST OF ABBREVIATIONS

2-DE	Two-Dimensional Gel Electrophoresis
DHT	Dihydrotestosterone
DRE	Digital Rectal Examination
DTT	Dithiothreitol
ESI	Electrospray Ionization
FPC	Familial Prostate Cancer
fPSA	Free PSA
GCAT	Glyco-Capture Affinity Tags
GST	Glutathione-S-Transferase
HPC	Hereditary Prostate Cancer
HPR	Swedish Human Proteome Resource
HSP	Heat Shock Protein
HUGO	Human Genome Organization
HUPO	Human Proteome Organization
ICAT	Isotope Coded Affinity Tags
IEF	Isoelectric Focusing
IPG	Immobilized pH-Gradient
m/z	mass/charge ratio
MALDI	Matrix assisted Laser Desorption/Ionization
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MudPIT	Multidimensional Protein Identification Technology
Mw	Molecular weight
NESP	Nonenzymatic Sample Preparation
NMP	Nuclear Matrix Proteins
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered saline
pI	Isoelectric point
PIN	Prostatic Intraepithelial Neoplasia
PMSF	Phenylmethyle sulphonyle fluorid
PTM	Posttranslational modifications
SDS	Sodium Dodecyl Sulphate
SELDI	Surface Enhanced Laser Desorption/Ionization
SPC	Sporadic Prostate Cancer
SS	Scraped Sample
TB	Tissue Block
TEMED	N, N, N´, N´- tetramethylethylenediamine
TNM	Tumor/Lymph node/Metastasis
TOF	Time of Flight
tPSA	Total PSA
TRIS	Tris(hydoxymethyl)aminomethane
WW	Wet Weight

INTRODUCTION

Cancer

Cancer is derived from a single benign cell which has undergone mutation (Figure 1). The initial mutation is carried on to a genetically homogeneous clone when the transformed cell divides. In normal cells proliferation occurs only when required. With additional genomic alterations a cell population that can escape normal controls of proliferation may ultimately evolve into cancer. This multistep process can occur in any of the mutated subclones that initially was derived. Natural selection of clones with the most advantageous properties may result in tumor heterogeneity. Hanahan and Weinberg suggest six essential features of the cancer cell phenotype: self-sufficiency in growth signals, disregard of signals to stop proliferation, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis [3]. The probability of these multiple mutational events to occur during an average human lifetime is relatively low.

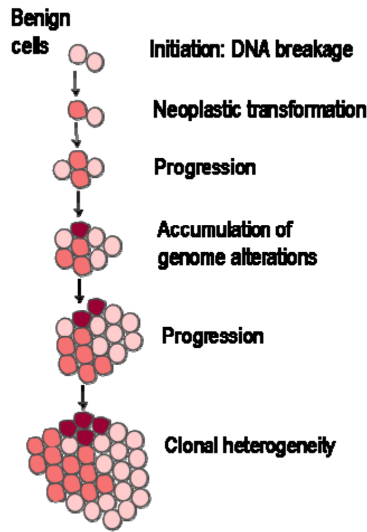

Figure 1. Carcinogenesis progression

History of prostate cancer

Prostate cancer (PCa) was first described in the medical literature in 1817 by the London physician George Langstaff [4]. The first time a prostate was surgically removed (by radical perineal prostatectomy) was in 1904 at the Johns Hopkins hospital by Young. This technique was used as the standard method for prostatectomy for 40 years and only minor modifications were made in order to reduce the morbidity of the operation. However, almost all patients became impotent after surgery. In 1945 Millin introduced the radical retropubic prostatectomy and this method was further modified in 1983 by Walsh in order to maintain sexual potency. In 1941 Huggins

reported that androgen removal greatly aided patients with advanced PCa and also that oral estrogens had the same effect on PCa patients as castration. He was awarded the Nobel Prize in Physiology and Medicine in 1966 for his discoveries concerning hormonal treatment of PCa. Hormone therapy, or androgen ablation, for advanced PCa is still common practice.

Epidemiology

Incidence and Mortality

PCa is the second most common cancer among men world wide [5] and thus, a leading health care concern in many countries. In Sweden the annual incidence of Pca is currently approximately 9000 (2003) [6]. It has been estimated that in 2005, more than 230 000 men will be diagnosed with PCa in the United States and almost 30 000 will die of the disease [7]. According to the World Health Organization there were 679,023 new cases and 221,002 deaths from PCa world wide in 2002 [8]. In general, PCa is a slow-growing tumor, with time to clinical progression often between 10-20 years. Only about 11% of all prostate cancers become clinically apparent, and 3% of them kill the patient [9].

Etiology

The underlying causes of the development of PCa remains largely unknown. The disease is heterogeneous, probably reflecting a complex interaction between environmental and genetic factors. The only known risk factors for PCa are age, family history of PCa (genetic predisposition) and ethnicity.

Racial and Ethnic Differences

The incidence of PCa varies between countries and ethnic groups. The highest rates of PCa are found in Scandinavia, the United States and Canada, while China and other parts of Asia have the lowest rates [10,11]. Mortality also varies worldwide, with the highest rates reported in Scandinavia and the Caribbean and the lowest in China, Japan, and countries of the former Soviet Union [12]. According to a Multiethnic Cohort Study the incidence of PCa is highest in African-American men, with a more

than doubled risk compared to Caucasians. [13]. However, it is unclear whether these differences are a result of exposure to unknown risk factors or to actual differences in the biology of the tumor. It has been shown that the prevalence of PCa in Japanese immigrants in USA is increased compared to Japanese people living in Japan [14,15], strongly suggesting the contribution of environmental risk factors.

Age

PCa is critically related to age. More than 70% of all patients with PCa are diagnosed after the age of 65 [12]. Overall, the lifetime risk of developing clinical PCa is 1 in 6 men. However, it has been suggested from an autopsy study in men dying accidentally in Detroit, that as many as 8 % of men have PCa in their twenties and that this figure would rise to 80 % when they reach their seventies [16]. This study supports the hypothesis that most men will get PCa if they live long enough and they are more likely to die with PCa than of it. The average age at diagnosis is 71 years and the average age at death is 78 years [17]. It has been estimated that a 50-year-old man has a 2.9% risk of dying of PCa [18].

Genetics

There are three epidemiological forms of PCa; Sporadic (SPC), Familial (FPC) and Hereditary (HPC). Sporadic form of PCa means that the disease occurs randomly in the population (i.e., the patient reports no family history of PCa) and this form constitutes 80-90% of all PCa cases [19]. The genomic alterations in PCa cancer that are responsible for sporadic cancer are mostly somatic changes. A number of genes have been identified for their role in sporadic PCa.

The familial aggregation of PCa was observed as early as in the 1950s [20]. FPC is defined as a clustering of PCa cases within members of a family, whereas HPC is a subtype of FPC with a Mendelian pattern of inheritance linked to a single gene that is transmitted as an autosomal dominant of high penetrance. The definition of HPC is a clinical definition based on distribution and age at onset of the disease in a given family. HPC requires any of the following three criteria: a family with three generations affected, three first-degree (brother(s) or father) relatives affected, or three relatives with early onset disease (affected before the age of 55 years) [21]. FPC and HPC account for 10-20% of PCa [21,22]. In 1992, the first segregation analysis was

performed and it was estimated that HPC accounts for approximately 5-10% of all PCa and 43% of cases with early onset disease [23]. Later segregation analyses have reached similar conclusions [24,25]. Men with HPC are diagnosed an average of 6-7 years earlier than sporadic PCa cases [26]. For men with one first-degree relative with PCa, the relative risk (RR) of developing PCa is approximately 2 and this risk increased to 5 and 11 for men with two or three affected first-degree relatives, respectively [27]. An increased familial risk may be due to genetic factors but could also be the result of shared environmental factors, such as correlation in lifestyle and dietary habits. Twin studies can provide information whether familial aggregation of cancer is due to hereditary or environmental factors. A Scandinavian twin study, where an increased risk for PCa was found among monozygotic as compared to dizygotic twins, estimated the total effects of heritable factors to 42% and lifestyle/environmental factors to 58% [28].

PCa susceptible loci

Genome wide scans and linkage analysis have been performed to identify PCa susceptibility loci and several candidate genes have been reported. At least eight putative PCa susceptibility loci have been found; HPC1 (1q24-25) [29], HPC2 (17p11) [30], PCAP (1q42.2-43) [31], HPCX (Xq27-28) [32], CAPB (1q36) [33], HPC20 (20q13) [34], (16q23.2) [35], MSR1 (8p22-23) [36]. The most frequently discussed genes are HPC2/ELAC2 [30], HPC1/RNASEL [37], MSR1 [38], and BRCA2 [39]. Several loci associated with aggressive forms of PCa has also been reported [40-44].

Polymorphisms

Familial PCa, not caused by Mendelian high-penetrance genes, are thought to be associated with shared environmental factors or more common genetic variants, multiple low-penetrant genes or polymorphisms, possibly involved in some of the genes and pathways that determine HPC incidence [45-47]. These polymorphisms are thought to occur at high frequencies in the general population and may therefore have a high impact on the risk of PCa. Several candidate genes have been examined for polymorphisms that were thought to be predictive for PCa; androgen receptor, 5 alpha-reductase type II (SRD5A2), CYP17, CYP3A4, the vitamin D receptor, PSA, GST-T1, GST-M1, GST-P1, IGF-I, and IGF binding protein 3. However, only GST-T1 and IGF-I polymorphisms were found to be modestly associated with PCa risk [48].

Epigenetic modifications

Epigenetic changes are defined as heritable changes in gene expression that occur without changing the order of bases in the DNA sequence [49]. Examples of epigenetic changes are DNA methylation (hypo- or hypermethylation) and histone modification (acetylation or deacetylation). These changes have been shown to be involved in the malignant transformation and progression of PCa [50]. Many gene promoters contain GC-rich regions also known as CpG islands. Abnormal methylation in these regions can lead to chromosomal instability and transcriptional gene silencing [51]. Loss of expression of the glutathione-S-transferase P1 enzyme (GSTP1) is a frequent finding in PCa and GSTP1 silencing is directly associated with promoter hypermethylation [52-55]. There have been many reports on hypermethylated genes in PCa but GSTP1 is the only gene that has shown sufficient specificity and sensitivity for detection of PCa to be a potentially useful marker.

Diet

The Western lifestyle, with higher intake of fat, meat and dairy products, may be responsible for the increased PCa risk [56-58]. The lower mortality in PCa in Japan compared to USA may be related to the difference in intake of soybean products that are rich in isoflavones [59]. In many studies high intake of tomato products has been shown to correlate with lower risk of developing PCa [60]. The explanation to this could be that tomatoes contain high levels of lycopene, a carotenoid that has been shown to have anti-oxidative capacity. Other protective dietary factors that have been discussed are vitamin E and D, selenium and zinc [61].

Smoking

The role of smoking in PCa is unclear. Most studies have been unable to show any association between smoking and development of PCa [62]. However, one Swedish [63] and two American cohorts [64,65] showed a significant risk for developing PCa among smokers.

Physiology & Histopathology

The normal prostate gland of a young man measures approximately 4x3x3 cm and is located immediately below the bladder where it surrounds the urethra. The human prostate is composed of three anatomical zones – the peripheral (PZ), central (CZ) and transition (TZ) zone (Figure 2) [66]. The peripheral zone is the largest region, comprising almost 70% of the prostate [67]. It is the region most susceptible to inflammation [68] and the majority of prostatic carcinomas originate from this zone [69]. TZ gives rise to the majority of benign hyperplastic nodules of the prostate [70] and a minority of carcinomas [69]. CZ differs histologically from PZ and TZ and is less often affected by disease [71].

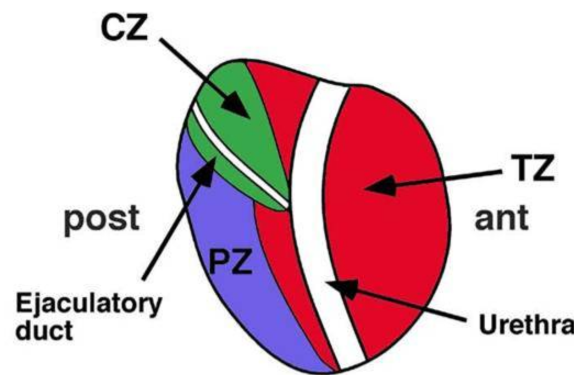

Figure 2. The anatomical zones of the prostate. Sagittal section.

Together with the other accessory sex glands, the seminal vesicles and the bulbourethral glands, the prostate produce the male seminal fluid that consitutes the ejaculate. The fluid is composed of sugars, proteins and minerals such as acid phosphatase and citric acid. It contains the proteases fibrinolysin and prostate specific antigen (PSA), the enzyme amylase, kallikreins, semenogelin, fibronectin, phospholipids, cholesterol, zinc, calcium and many other proteins of unknown function. The purpose of a majority of these chemicals is to facilitate sperm mobility and survival. The secretions are also thought to protect the urinary and reproductive systems from pathogens.

The prostate is composed of a fibromuscular stroma and of numerous branching glands terminating in ducts that ultimately empty into the urethra. Each glandular acinus is lined with secretory luminal epithelial cells (typically cuboidal to columnar), basal cells and scattered neuroendocrine cells. Glandular acini and ducts with basal and secretory cells are found in all three zones of the human prostate. The secretory cells produce and secrete the seminal fluid into the prostatic duct. The basal cell layer is believed to contain a stem cell population for the epithelial prostate cells [72]. The

stromal compartment includes smooth muscle cells, fibroblasts and endothelial cells. Stromal-epithelial interactions remain poorly understood, but the stroma is clearly the major inducer of prostatic epithelial cell growth and differentiation in the development of normal prostate as well as PCa [73]. Over 98% of all PCa are adenocarcinomas that arise from epithelial cells in the acini or ducts of the prostate glands [74].

Hormonal regulation

The growth, development and function of the prostate gland are dependent of the presence of male hormones, i.e. androgens. The most important androgen is testosterone, a steroid hormone that is produced mainly in the testes. Testosterone in the blood is converted to dihydrotestosterone (DHT) in the prostate by the enzyme 5α-reductase. Prostate cells, as well as PCa cells, require the presence of androgens to survive. Therefore, removal of androgens kills a large majority of PCa cells.

Prostate cancer development

The molecular pathology of PCa is complex and multiple genes are thought to be involved. Dietary, lifestyle-related and environmental factors, such as chronic or recurrent prostate inflammation [75], have also been recognized to initiate and promote cancer development. Studies have provided clues as to how PCa arise and progress. But the molecular pathways and the acquired molecular defects that are responsible for PCa initiation, development and progression are still largely unknown. An important model of carcinogenesis in colon cancer was established by Vogelstein and colleagues in 1988 [76]. It describes the non-random accumulation of genetic aberrations that are responsible for colon cancer progression. Such a linear progression model is more difficult to establish for PCa due to its more heterogeneous pathology. However, a model of prostatic carcinogenesis has been proposed based on the morphologic continuum of PIN and the multi-step theory of carcinogenesis [2,77,78] (Figure 3).

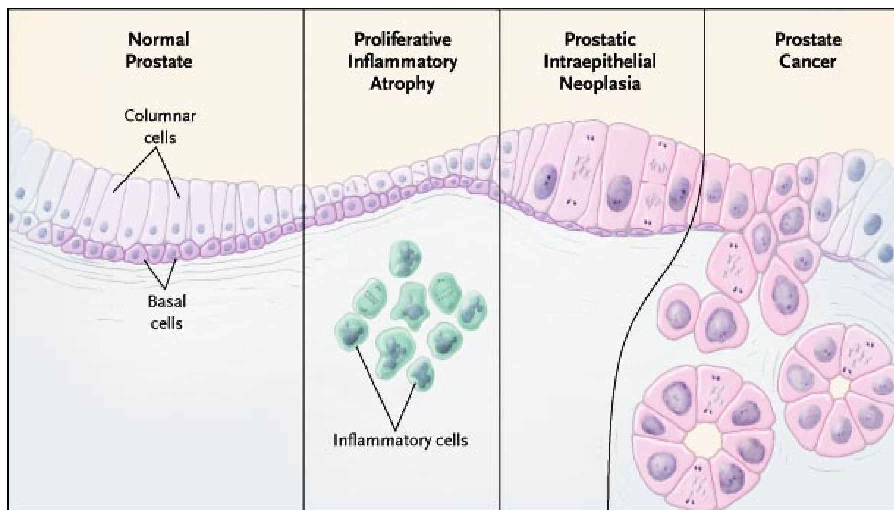

Figure 3. Tumor development in prostate cancer [2].

Prostatic Intraepithelial Neoplasia

Prostatic Intraepithelial Neoplasia (PIN) is considered to be the most likely precursor of PCa. PIN is defined as non-invasive, preneoplastic growth of the cells lining prostatic ducts and acini. PIN is associated with progressive abnormalities of phenotype and genotype which are intermediate between normal prostatic epithelium and cancer. PIN is commonly divided into low grade (PIN I) and high-grade (PIN II and PIN III) lesions [79]. At least four distinct architectural patterns can be distinguished in high grade PIN: flat, tufting, micropapillary and cribriform [79]. Disruption of the basal cell layer increases with increasing grades of PIN, whereas in prostate carcinoma, there is a complete loss of the basal cell layer [80]. PIN is most commonly found in the PZ and coexists with cancer in more than 85% of cases [81,82]. It has been suggested that most patients with PIN will develop carcinoma within ten years.

Methods for diagnosis and prediction of prognosis of prostate cancer

Standard methods for diagnosis and assessment of prognosis in PCa include digital rectal examination (DRE), serum prostate specific antigen (PSA) and ultrasound-directed biopsies. The DRE and PSA tests cannot alone diagnose PCa but they indicate that further testing is needed. A biopsy is currently the only method to make a definitive diagnosis of PCa. Most commonly, transrectal needle biopsies are taken

according to a standardized schedule. In order to improve the PCa detection rate multiple biopsy cores are taken [83] and recent studies suggests that 8 to 12-cores are optimal [84].

Gleason grading

For histopathological grading of PCa, the Gleason grading system is now universally acknowledged [85]. The Gleason grading separates the architectural features of the cancerous glands into 5 histologic patterns of decreasing differentiation, pattern 1 being most differentiated and pattern 5 being least differentiated. The Gleason sum (score) is obtained by adding the dominating (primary) grade and the next most common (secondary) grade. The Gleason grade is one of the strongest predictors of outcome [86]. However, a limitation of this grading system is that a majority of newly diagnosed cancers are Gleason score 6 tumors, which can be either aggressive or indolent.

Staging

For accurate treatment of PCa, it is necessary to determine the stage of the disease. If the tumor is no longer confined to the prostate, the cancer is defined as non-curable and the treatment will be palliative. The most widely used system for staging PCa is called the TNM system [87]. It describes the extent of the primary tumor (T stage), the absence or presence of spread to nearby lymph nodes (N stage) and the absence or presence of distant metastasis (M stage).

DNA ploidy

DNA ploidy patterns are classified as diploid, tetraploid or aneuploid. Some prefer to categorize the ploidy pattern as diploid or non-diploid. Both flow cytometry and static image analysis can be used to determine ploidy. DNA ploidy in PCa has been extensively studied and has been found to provide prognostic information independent of histologic grade and tumor stage [88-90]. In most studies a correlation between DNA ploidy and tumor grade and stage has been found [91].

Treatment

In the management of patients with PCa, the uncertainty about the tumor aggressiveness remains a dilemma. The main treatment options for localized, organ-confined PCa include surgery (radical prostatectomy), radiotherapy, and watchful waiting. All methods have their risks and benefits and the treatment choice is largely based on the patient’s preference. In metastatic or advanced Pca, hormone deprivation is used as palliative therapy, i.e. for symptom relief.

Borttaget:

Biomarkers in prostate cancer

Numerous candidate PCa molecular markers have been reported over the years [92], but only a few of them are currently used in clinical practice. The College of American Pathologists has classified prognostic factors in three categories [93]. Category I prognostic factors are supported by the literature and generally used in patient management. Category II are factors that have been extensively studied biologically and clinically but whose importance remains to be validated in statistically robust studies. Category III factors have insufficient data to support their role in prediction of prognosis. The majority of new tissue-based biomarkers fit within category III. DNA ploidy is currently the only ancillary method for prognostication of PCa that has reached Category II [93,94]. PSA is a category I prognostic factor.

PSA

Prostate-specific antigen (PSA) is a 30-33 kDa serine protease [95] that belongs to the kallikrein family of proteases and is also called human kallikrein 3 (hK3) [96]. PSA is produced exclusively by the epithelial cells of the prostatic glands and ducts. Under normal circumstances. PSA is secreted into the seminal fluid where its function is to digest the gel formed by semenogelins and fibronectin after ejaculation [97]. Small amounts of PSA naturally leak into the bloodstream but in cancer there is an increased leakage, because of a deficient basement membrane and an increased number of epithelial cells and also because the epithelial cells loose their contact with the excretory ducts [98]. The PSA test measures the level of PSA in serum and is used as a tool to detect PCa. The higher the PSA level, the more likely it is that cancer is present. A cut-off level of 4υg/l is traditionally used. However, about 70% of men with an elevated PSA do not have detectable PCa at biopsy [99]. Furthermore, 20% of

all men with clinically significant PCa have a normal PSA [100]. It has been shown that PSA is tissue specific but not tumor specific since also benign prostatic disease such as BPH and prostatitis can cause an elevation of serum PSA [101]. There are several forms and derivatives of PSA that have been discussed as biomarkers for PCa; PSA density, free PSA, complex PSA and PSA-specific membrane antigen (PSMA). Most of the circulating PSA is complexed with the protease inhibitor α1-antichymotrypsin and only minor fractions are complexed with α1-antitrypsin and α2-macroglobulin [102]. The unbound (free) form of PSA constitutes approximately 5-40% of serum PSA [103]. Especially the ratio of fPSA to tPSA has shown to be of clinical value. The proportion of fPSA is significantly lower in patients with PCa than in those with BPH or prostatitis [104] and it has been shown that the ratio between free (fPSA) and total PSA (tPSA) in serum can help to differentiate between patients with benign disease and those with cancer [105-107].

In order to reduce the mortality of PCa, screening for serum PSA has been proposed. Screening is controversial and has been debated for many years. Two large clinical trials are under way to establish whether PCa screening is effective and reduces mortality [108,109]. The increasing use of PSA testing has led to a rapid increase in the incidence of PCa. More cancers are identified at an earlier stage, when they can be treated effectively. However, there is also a risk of overdiagnosis and overtreatment of clinically harmless cancers.

Cancer Proteomics

The proteome is defined as the complete set of proteins encoded by the genome, including splice variants and post translational modifications, for a particular organism, tissue, cell or subcellular compartment [110]. Proteomics represents technologies for analysis of the proteome under a given set of physiological or developmental conditions.

Proteomics in cancer research can be used to identify tissue and serum biomarkers for early cancer detection and to follow treatment effects and disease progression.

There has been enormous progress in the field of proteomics since the human genome project, HUGO, published the complete human DNA sequences in 2001[111,112] The international Human Proteome Organisation (HUPO) was formed the same year [113].

No complete proteome map of any organism has been presented yet but there are presently seven HUPO initiatives, each based in different countries. The HUPO projects for brain, liver and plasma are a few examples. The Swedish Human Proteome Resource (HPR) program aims to systematically explore the human proteome with Affinity (Antibody) Proteomics. HPR has created a Protein Atlas, where the affinity-purified antibodies are used for immunohistochemical staining to show the expression and localization of proteins in a large variety of normal human tissues and cancer cells. The Protein Atlas is part of the HUPO Human Antibody Initiative (HPI) [114,115].

An enormous amount of data have been produced in the field of proteomics but a problem has been that the information has been scattered over many resources. In 2002, the HUPO Proteomics Standard Initiative (PSI) was created with the aim to define community standards for proteomics data in order to make the information more accessible [116].

The number of protein-coding genes is far fewer than the number of different proteins. There is only a moderate correlation between mRNA transcript profiles and corresponding protein abundance in the same cells or tumors for most cellular gene products [117,118]. There are several modifying steps that has to be completed before a newly synthesized polypeptide sequence is converted into a functional protein. Many genes can be variously spliced [119] and mRNA editing is also quite common[120]. Post-translational modifications (PTMs) can be either non-covalent or covalent and may change the proteins behavior significantly. Examples of non-covalent modifications include incorporation of cofactors such as heme, protein folding, and the association of subunits to form an oligomeric protein. The most common covalent PTMs that are discussed in proteomics contexts are cleavage of signal peptides and modifications on the amino acid residues, including phosphorylation, glycosylation and ubiquitination [121].

Protein glycosylation is known to be involved in cell adhesion, protein targeting, and protection from proteolytic attack and many reports describe alterations to the normal cellular glycosylation in cancer [122,123].

The importance of phosphorylations has been stated by Cohen [124], “Protein phosphorylation regulates most aspects of cell life, whereas abnormal

phosphorylation is a cause or consequence of disease." Phosphoproteins, especially tyrosine kinases and their substrates, have been discussed as possible cancer markers [125].

Abnormal processing of proteins may also be significant during disease. E.g. complement C3 [126]. The increase or decrease of a given protein may in fact be a truncated protein variant which in turn may result in a very different biological interpretation.

In addition to qualitative alterations, the quantity of proteins and/or modified proteins varies significantly between individuals, cell types, and even within the same cell under different stimuli or different disease. Proteomics has the advantage that it can give us information at a "global" level about many of these postgenomic issues.

Advances in two-dimensional gel electrophoresis (2-DE)

2-DE is currently the most powerful high resolution technique for the separation and quantitative analysis of complex mixtures of proteins. Proteins are separated according to their size and charge and 2-DE has therefore the potential to separate PTMs, including truncated protein variants. The number of different proteins present in a particular cell type has been estimated to about 5,000-10,000 [127]. The number of individual polypeptide spots detected with the 2-DE method is variable and depends on a number of parameters such as sample type, prefractionation, sample load, pH range, gel size and detection technology. Up to 10,000 and on average about 2,000 protein spots can be detected in one gel. However, these protein spots represents a lower number of protein identites due to PTMs. Recognition of PTMs in 2-DE gels requires that one of the separation parameters (pI or Mw) must be altered in the modified form. Since the mass alteration generally is too small to be detected, the pI shift is the parameter most often recognized. Phosphorylated or glycosylated proteins may often be observed as a train like pattern, either horizontally or vertically, or both.

However, the 2-DE technique has some limitations. Proteins expressed at low levels, so called low-abundance proteins (transcription factors and some cell-signalling proteins), hydrophobic membrane and nuclear proteins, proteins with extreme pI as

well as very large or very small proteins can be difficult to separate and/or to detect in 2-DE gels.

In silver-stained 2-DE gels, the detection sensitivity is approximately 1 ng of protein, corresponding to proteins present at 10,000 to 20,000 molecules per cell. This is just above the copy number limit of most proteins with important regulatory functions in cells. However, this in not necessarily a drawback of the technology since we do see more of the downstream effects of these regulatory proteins.

Sample preparation

Proteomic studies and 2-DE can be performed on almost all kinds of tissues, cells or body fluids (serum, microfluids, urine, ejaculate, cerebrospinal fluid, saliva). Fresh tissue or cell samples have been shown to give better resolution of 2-DE gels than frozen samples and are therefore preferable [128]. Formalin fixed paraffin embedded (FFPE) samples are generally not suitable for 2-DE based proteomics [129]. However one group claimed that they extracted and recovered proteins of cells from FFPE PCa tissue and analyzed them with nanoflow reversed-phase liquid chromatography (nanoRPLC)-MS/MS [130].

Preparation of cells from tissue

Purification of epithelial cells from tumor tissue can be made by various techniques, such as scraping, squeezing, fine needle aspiration (FNA), laser capture microdissection (LCM) and dynabeads. For tumor samples, the most important is to get representative cells. There is no standard sampling technique that is suitable for all kinds of samples. Tumors have different characteristics and it is important to optimize the sampling technique according to tissue type [128]. In PCa, tumor heterogeneity and the small size of the tumors make it difficult to sample representative cells. Also, the high abundance of proteolytic enzymes in the prostate with rapid protein degradation after the prostate has been surgically removed requires a fast harvesting technique. We have used a scraping method on fresh prostate tissue to avoid over sampling and to shorten the preparation time. Cells are collected in medium with protease inhibitor within seconds after scraping and the cell pellets are stored in a freezer within 15 min.

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Sub-cellular pre-fractionation and immuno depletion

The quantity of proteins in tumor tissue and body fluids varies greatly. When analyzing protein content, low-abundance proteins may be obscured by high-abundance proteins, i.e. cytoskeletal proteins, albumin and immunoglobulins. Protein prefractionation and enrichment strategies, such as subcellular fractionation (for cells or tissues), and immunodepletion (mainly for serum) may concentrate low-abundance proteins for analysis. Subfractionation of cell components can be performed with numerous techniques [131,132]. Depletion is most often done by the use of antibody columns [133,134]. Specific removal of high-abundance proteins, such as albumin, immunoglobulins (IgG and IgA), antitrypsin, transferrin and haptoglobulin, can delete approximately 85-90% of the total protein mass from human plasma or serum [132]. Membrane and nuclear proteins are known to be difficult to extract and solubilize. There have been advances in new detergents [135,136] and organic solvents that have been applied to improve the solubilization of hydrophobic proteins [137,138]. However, most fractionation and depletion techniques require large amounts of sample. PCa samples are often not sufficiently large for pretreatment with these techniques. It has been argued that fractionation techniques are difficult to standardize and therefore might reduce the reproducibility. It has also been discussed that potentially interesting smaller proteins might adhere to the depleted serum proteins and then accidentally be removed from the samples. In the case of subcellullar fractionation, samples are often diluted and require concentration steps after the fractionation procedure.

The sample preparation procedure is extremely important to enable extraction of as many protein species as possible. The proteins have to be denatured, disaggregagated, reduced and solubilized to be separated properly in 2-DE gels. The most important steps are cell disruption, protease inactivation and solubilization. Unfortunately there is no single method that suits all kinds of samples and 2-DE experiments. The sampling technique has to be optimized for each particular sample type. However, some general recommendations can be made. Fresh tissue should preferably be used, protease inhibitors should be added and the sample should always be prepared on ice. The protocol should be as simple as possible in order to avoid reproducibility problems. Additional sample preparation steps may improve the quality of the final 2-DE result, but at the possible expense of selective protein loss.

First dimension

During the 1980s, discussions regarding poor reproducibility of the 2-DE technique were in focus. These problems have been considerably overcome by recent advances in 2-DE technology, such as the use of immobilized pH gradients (IPGs) [139,140]. One way to increase the resolution of low abundance proteins is to use multiple overlapping narrow pH-range IPG strips [131,141]. However, the limited amount of protein in most PCa samples makes it difficult to run multiple 2-DE gels. Basic proteins, with a pI above 7 are known to be difficult to separate in the first dimension. The combined use of anodic cup-loading and hydroxyethyldisulphide containing solution (DeStreak™ Amersham Biosciences, Uppsala, Sweden) has been shown to increase the resolution of basic proteins [142]. Cup-loading has also proved to be better than in-gel rehydration for samples containing large amounts of salt ions [131]. It has been shown that there are significant losses of protein (up to 80%) during the 2-DE procedure [143,144]. A majority of the protein losses seems to occur when the sample is loaded onto the IPG strips, but there are also losses during the IEF and the equilibration steps. Cup-loading was associated with 25% better overall uptake than in-gel rehydration [144]

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Second dimension

There have been many improvements in almost all steps of the 2-DE procedure the past years. However, this is not the case for the second dimension, the SDS-PAGE, where little has changed since Laemmli developed the technique [145] and the multiphasic zone electrophoresis theory was introduced [146,147] in the 1970s. The Mw range of detectable polypeptides in a standard Tris-glycine 2-DE system is about 15-150 kDa, but the proteins in a cell can range between 5-500 kDa. The introduction of a Tris-tricine buffer system [148] and improvements in that system [149] have made it possible to separate also low Mw proteins at 3-30 kDa. One way of getting around the problem is to combine several gels in different Mw ranges, but this is laborious and demands large sample quantities. The recent introduction of neutral pH gels (NuPAGE® Novex BisTris system, Invitrogen, Criterion™ XT, Bio-Rad) has also led to improvements in terms of better separation and resolution of small to medium sized proteins by utilizing a neutral pH environment which minimizes protein modifications.

Protein detection techniques

To be able to visualize and quantify proteins after separation in 2-DE gels it is necessary to use an adequate staining method. The enormeous variation in characteristics and abundance of the individual proteins put high demands on the staining technique. The most important requirements are high sensitivity, high linear dynamic range, high reproducibility and compatibility with mass spectrometry. Major developments of protein staining methods have occurred during the the past five years. Fluorescence dyes such as Sypro® Ruby (Invitrogen, Molecular probes, Bio-Rad) and Deep Purple™ (Amersham Biosciences) are some of the commercially available and more commonly used products presently. Fluorescence stains enables quantitation of protein spots down to 0.1 ng per spot, with a dynamic range of four orders of magnitude [150]. The fluorescence techniques are also highly reproducible and completely compatible with mass-spectrometric analysis and have therefore surpassed the more conventional staining methods such as silver staining, Coomassie Blue, and colloidal Coomassie Blue. However, since silver staining is the most sensitive non-radioactive detection technique and the costs for reagents are relatively low, it is still widely used. There are presently more than 100 different modifications of the original silver staining protocol that was introduced by Merril *et al.* in 1979 [151]. Some silver stains are more quantitative than others but silver staining methods generally show a nonlinear relationship between spot volume and protein amount. For this project, we have chosen a silver staining technique for detection of potential biomarkers. The ratio factor limit we used to select proteins with differential expression was 1.5 fold change. We believe that even a 50% increase or decrease of a protein may result in significant effects on biological and pathological processes and we did not want to exclude any potential biomarkers due to a too strict fold change limit. The semi quantitative feature of silver staining was not considered to be a problem since accurate quantification of selected candidate biomarkers will be performed during the validation process.

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It is known that silver nitrate in combination with formaldehyde in the fixation and development step can interfere with mass spectrometric analysis [152]. Alternative silver staining methods that omit the aldehydes have been developed to improve MS compatibility [152,153]. However, such modified techniques often results in increased

background and reduced staining homogeneity. Another way of dealing with the problem is to improve the destaining protocols [154]. We have stained preparative gels with Sypro® Ruby. Analytical silver stained gels have also been analyzed by MALDI using a modified protocol [155]. Our experience show that manual preparation of gel plugs instead of using a digestion robot generally improves MS results.

Recently developed protein detection methods are fluorescence stains that are specific for PTMs, such as Pro-Q Diamond and Pro-Q Emerald 488 [156,157]. Pro-Q Diamond can be used to detect phosphoserine-, phosphothreonine-, and phosphotyrosine-containing proteins, whereas the Pro-Q Emerald dye stains for glycoproteins, directly in 2-DE gels. For both Pro-Q Diamond and Pro-Q Emerald it is possible to poststain with Sypro® Ruby to visualize also non-phosphorylated or non-glycosylated proteins.

DIGE

The recently introduced differential in-gel electrophoresis (DIGE) technique is a major improvement of 2-DE gel reproducibility [158]. Before IEF, two samples are labelled covalently with fluorescent cyanine dyes to 1-3% of the lysine residues (minimal labeling),i.e. Cy3and Cy5, respectively. There is also a new generation dyes that label cysteine residues to saturation [159]. A major advantage with this method is the high sensitivity which is useful for small clinical tumor samples. The labelled proteins are mixed and separated in the same 2D gel. A third cyanine dye, Cy 2, makes it possible to run an internal standard, typically a pooled mixture of all the samples in the experiment, in all gels. The CyDyes differ in their excitation and emission wavelengths and when scanned the dyes give an image of the protein patterns of each sample. In addition to the more accurately imaged protein expression, the DIGE method has several other advantages compared to 2-DE. The method is less time consuming with 50% fewer gels and a simplified comparison of protein expression patterns. However, as with all methods, there are some technical disadvantages. Proteins will be labeled with different efficiencies depending on lysine content and the technique is not applicable to proteins without lysine. The molecular weight of the CyDye molecules is between 434-464 Da, and depending on the number of labelled lysine residues in a sample, the protein patterns obtained with the CyDyes will be slightly different from those obtained with conventional systems.

Image and data analysis

Image analysis of 2D gels includes preprocessing of the gel images, background subtraction, spot detection, quantitation, normalization, and matching. There are several 2-DE image analysis softwares on the market. We used the Bio-Rad PDQuest™ software that can be used for imaging, analyzing and databasing 2-DE gels and offers a variety of analytical and statistical tools. An artificial reference (master) gel is constructed and spots are matched to the master gel. Normalization is performed in order to adjust for methodological gel-to-gel variations, such as pipetting errors and variable sample loading or staining intensity. The statistical analysis methods available within the PDQuest™ software, such as Mann-Whitney and student t-test can be used to select spots that differ in expression levels. However, all spots of interest should also be judged visually, and this is sometimes the most accurate way to determine if they are correctly matched and quantified. For multivariate statistics we have exported the match set data to other softwares.

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Image analysis softwares have been improved over the past 15 years, for more reliable and automatic spot detection, quantitation and matching, but we are still far from a completely automated system.

The protein expression varaibility between clinical samples is relatively high compared to cell lines and the image analysis requires more extensive manual editing and matching. This aspect represents additional demands on any image analysis software, which should be considered.

Mass spectrometry and bioinformatics

There are two methodological events in the history of analytical protein chemistry that have been of crucial importance for the rapid progress in proteomics. The development of matrix-assisted laser desorption/ionization (MALDI) [160] and electrospray ionization (ESI) [161] in the late 1980s allowed the ionization of peptides and proteins at high sensitivity, and they have become the methods of choice for protein identification. New developments in the mass spectrometry (MS) technology have created a complex array of instruments, but the basic components of all mass spectrometers are essentially the same.

A mass spectrometer consists of three basic parts; an ionization source, a mass analyzer, and an ion detector (Figure 4).

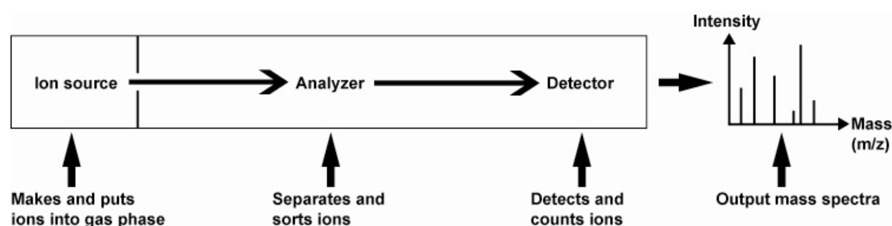

Figure 4. Diagram of a mass spectrometer. Illustration kindly provided by Simon Ekström [1].

By combining these parts, it is possible to determine the molecular weight of chemical compounds by ionizing, separating, and measuring molecular ions according to their mass-to-charge ratio (m/z) (Figure 5) [113].

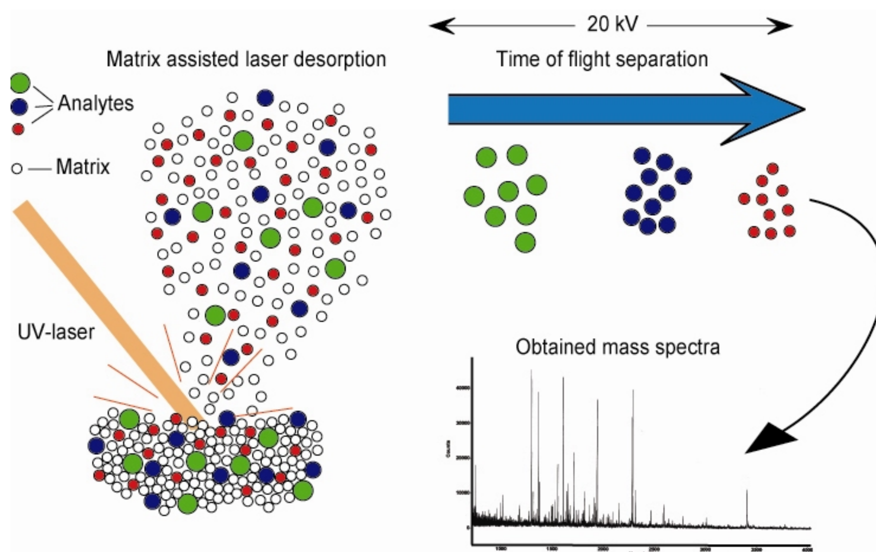

Figure 5. Laser pulses (UV radiation) on an analyte-matrix mixture results in the vaporization of the matrix and acceleration of the ions. The ions enter a field-free flight tube where they are separated according to mass. They are then detected as electrical signals at the end of the flight tube. Illustration kindly provided by Simon Ekström [1].

The first step in the MS analysis is to cleave the unknown protein with a proteolytic enzyme of known specificity (trypsin) to generate peptides. Then the peptides are ionized. ESI generates ions directly from solution. In MALDI analysis, the analyte is first co-crystallized with a large molar excess of a matrix compound, usually a UV-absorbing weak organic acid, such as a cinnamic acid or benzoic acid derivative. Laser pulses (UV radiation) of this analyte-matrix mixture results in vaporization of the matrix and acceleration of the ions. The ions are accelerated in an electric field, sorted in the mass analyzer according to their mass-to-charge (m/z) ratios and then collected by a detector. In the detector the ion flux is converted to a proportional electrical current. These electrical signals are recorded as a function of m/z and converted into mass spectra.

The mass spectra generated in an ESI or MALDI spectrometer provide masses of the analyzed peptides. The peptide mass list of a protein can be seen as a fingerprint. A list of the proteolytic (peptide) fragment masses is then matched to theoretical protein sequences in databases. This protein identification method is called peptide mass fingerprinting (PMF) [162-166].

A MS/MS spectrometer can be used in combination with ESI to further analyze selected peptides. The selected peptide ions are fragmented and MS/MS spectra are generated that basically represent the amino acid sequence of the peptide. The specificity of MS/MS based protein identification is often much higher than that of PMF. Most of the time it is not very difficult to achieve tryptic peptides and sequences from mass spectrometric analyses or to search in databases for matching theoretical peptides/sequences. It is more problematic to accurately interpret such data and to identify the best peptide or sequence match to the spectrum. Databases use different algorithms and scoring models to assess the likelihood of a match. There are mainly two categories of MS/MS search algorithms; heuristic and probabilistic [167]. Heuristic algorithms (e.g. SEQUEST [168]) calculate a score based on the similarities between the experimental and theoretical MS/MS spectrum, for example the number of peaks common to the two spectra. Probabilistic algorithms (e.g. Mascot [169]) generate a model of the peptide fragmentation process and determine the peptide identification score from this model [170]. Kapp *et al.* recently evaluated five search algorithms (databases) with respect to sensitivity, specificity and false-positive (FP) rates [167]. They calculated the true positive rate at a specified FP rate and

concluded that Mascot performs better than the other investigated algorithms. The detailed description of the algorithm that Mascot is based on is not publicly known [170].

Non 2-DE based platforms

There are other proteomic technologies, alternative or complementary to 2-DE, that are sometimes better suited for the separation and identification of low-abundance proteins, hydrophobic proteins and very small or very large proteins. In recent years many new protein profiling platforms have been developed. The multidimensional protein identification technology (MudPIT) [171] is an approach where the total protein mixture is digested by one or several proteases. The peptide mixture is then separated by chromatography and identified by MS/MS. The number of proteins that can be identified is large compared to 2-DE based methods. However, the MudPIT method gives only a list of proteins present in the sample, without any quantitative inter-sample information. Since the samples are digested prior to analysis the information about possible PTMs is also lost. Thus, the only conclusions that can be drawn is whether a certain protein is absent or present in one sample compared to another.

The liquid chromatography (LC)-based Isotope Coded Affinity Tags (ICAT), is used to separate very large or very hydrophobic proteins [172,173]. The technique is similar to MudPIT in that it has a chromatographic step coupled with MS/MS, but it can also determine relative quantities. Samples are labelled on the cystein residues with ICAT probes. However, some peptides do not contain any cystein residues and can therefore not be analyzed with this technique. Glyco-Capture Affinity Tags (GCAT) is a method that enriches for glycoproteins [174,175].

Surface-Enhanced Laser Desorption/Ionization (SELDI) is a protein chip technology, integrated with MS, that extracts and quantifies proteins with defined properties [176]. However, there is a growing scepticism toward this approach due to the difficulties to identify the proteins and validate the obtained data [177].

The MALDI MS-based *in situ* tissue profiling methodology developed by Caprioli and co-workers is an interesting approach that with further technological development may be used in the validation of potential biomarkers [178].

Array-based technologies have been developed to assess and validate potential biomarkers [179,180]. There are two classes of protein array formats, forward phase protein microarray (FPA) and reverse phase protein microarray (RPA). A FPA is comprised of immobilized bait molecules (antibodies) that are arrayed onto a surface and used to capture specific analytes from a complex sample, for example a cellular lysate or serum sample [181,182]. RPA works in the opposite way; different patient samples or cellular lysates are immobilised on a solid phase and a single analyte-specific ligand (antibody) is used to detect a defined protein or molecule [183].

Prostate cancer proteomics

The detection of PCa is currently based on one individual protein (PSA) that has shown to be rather unreliable. Proteomics technology allow for simultaneous analysis of thousands of proteins and has great potential to find a panel of biomarkers that could support early diagnosis and correlate with clinical features and tumor progression in PCa.

One of the first 2-DE studies reported was in 1985, when Guevara and colleagues reported that several proteins, tentatively identified as variants of prostatic acid phosphatase, appeared to be elevated in all BPH prostatic fluids compared to samples from men with PCa [184]. Grover *et al.* also studied prostatic fluid from PCa patients by 2-DE and found a protein occurring as several charge variants to be consistently present in samples of patients with PCa, whereas the protein was undetectable in the fluids of BPH samples [185]. Later, the same group performed a 2-DE study of urine, where a couple of markers were found to potentially distinguish BPH from PCa [185]. In another study of voided urine samples, collected from patients with PCa after DRE, a number of proteins were found to be differentially expressed between PCa and BPH samples [186]. A 2-DE analysis of nuclear matrix proteins (NMP) from prostatic tissues found 14 proteins (PC-1) that were consistently present or absent in normal, BPH, and PCa specimens [187]. The same group reported that the expression of a nuclear matrix protein, YL-1, seems to be associated with PCa of poor prognosis [188]. Several other NMP proteins have been correlated with PCa development and poor prognosis in 2-DE studies [189-191]. There is unfortunately no identity of most of the proteins that were reported for more than 7-8 years ago, due to the inaccessibility of mass spectrometry technique at the time.

There are only a few reports of 2-DE based PCa analyses of clinical tissue material, probably reflecting the sampling difficulties that comes with small tumors. Ahram *et al.* used both ethanol-fixed, paraffin-embedded and frozen tissue and performed either manual or laser capture microdissection and detected 40 quite heterogenic protein alterations in the tumors [192]. Meehan *et al.* studied the protein expression in snap-frozen tissue blocks and identified 20 proteins which were lost in malignant transformation of PCa [193]. Alaiya and colleagues from our group performed 2-DE studies of fresh tissue blocks from BPH and PCa [194 195]. They found increased levels of proliferating cell nuclear antigen (PCNA), calreticulin, heat shock protein (HSP) 90, HSP 60, oncoprotein 18(v), elongation factor 2, glutathione-S-transferase pi (GST-pi), superoxide dismutase, and triose phosphate isomerase and lower amounts of tropomyosin-1 and -2 and cytokeratin 18 in PCa than in BPH.

Several non-2-DE based technologies have been applied in the search for new biomarkers.

Griffin *et al.* reported differences in expressed proteins between benign and cancerous human prostate epithelial cells using ICAT [196].

Several studies have focused on the SELDI-TOF technique. From the same group three studies were reported; Xiao *et al.* [197] managed to discriminate between benign and malignant prostate disease by quantitation of PSA using SELDI-TOF MS. Later, Cazares *et al*. [198], detected seven not yet identified proteins that could distinguish malignant (PIN/PCa) cells from benign (normal/BPH) cells in micro dissected prostatic tissue from nine patients with 93.3% specificity and 93.8% sensitivity. Adam *et al.* built a decision tree algorithm with representative MS spectra of sera from patients and controls [199]. Using an algorithm based on nine protein peaks from serum samples, they detected PCa with 83% sensitivity and 97% specificity.

Adam *et al.* has also reported on SELDI-based studies of PCa in collaboration with two other groups; Qu *et al.* built decision tree algorithms from serum data [200]. Banez *et al.* analyzed sera from PCa patients and controls with two types of ProteinChip arrays [201]. By combining data from the two arrays they generated a decision tree algorithm that used only three peaks but achieved 85% sensitivity and 85% specificity for PCa detection.

Petricoin *et al.* found that serum MS spectrum patterns predicted the presence of cancer more precisely than serum PSA value [202].

Identification of the observed proteins would of course be valuable because of their potential as therapeutic targets. However, some of the studies mentioned above were recently questioned because of the limited reproducibility of protein patterns obtained by SELDI-TOF[203].

AIMS OF THE STUDY

The purpose of this thesis was to identify potential markers that can provide additional diagnostic and prognostic information. Specifically, the aims of the study were:

- To improve methods for tissue harvesting and processing for proteomics.
- To describe the proteome profile of the normal tissue of the three anatomical zones of the prostate.
- To identify proteins specific for PCa and potentially useful for early detection of PCa. To identify proteins with decreased or increased expression in PCa, possibly contributing to the understanding of carcinogenesis in the prostate.
- To describe the shift in the proteome as PCa progresses from low-grade to high-grade disease.

MATERIAL AND METHODS

Several technological optimizations may be required to obtain reproducible and reliable results from a 2-DE experiment. When a long-term project has started, there are limited possibilities to change parameters in the sample preparation or 2-DE protocols. Also, the limited access to clinical samples and in particular PCa samples restricts the possibility to repeat an experiment that did not succeed. In addition to this, technology access limitations, limited project time and economical restrictions have to be taken into account when planning and performing a doctoral project. At one point, the optimization of the project has to be finished and the analytical part of the project begin. The optimization of the projects in this doctoral thesis is based mainly on empirical experience from earlier 2-DE studies of PCa performed by our group. The scraping technique was quickly adopted and was first used in the study that is presented here as Paper II. The evaluation of the scraping method (Paper I) was performed simultaneously.

Samples and sample preparation

Extraction of tumor epithelial cells

Cells were scraped from the cut surface of the prostate with a scalpel and collected in 1-1.5 ml ice-cold RPMI-1640 medium containing 5% calf serum and 0.2 mM phenylmethylsulphonyl fluoride/0.83 mM benzamidine (see also detailed description in Paper I, where some samples were prepared according to the Franzén non-enzymatic extraction technique) immediately after surgery (Figure 6). Cell suspensions were washed twice in PBS at 800 × g and 4 ºC for 3 min. Finally, all samples were centrifuged for 5 min at 2700 × g (4 ºC). The wet weight (WW) of each pellet was recorded before being stored at -80ºC for later use.

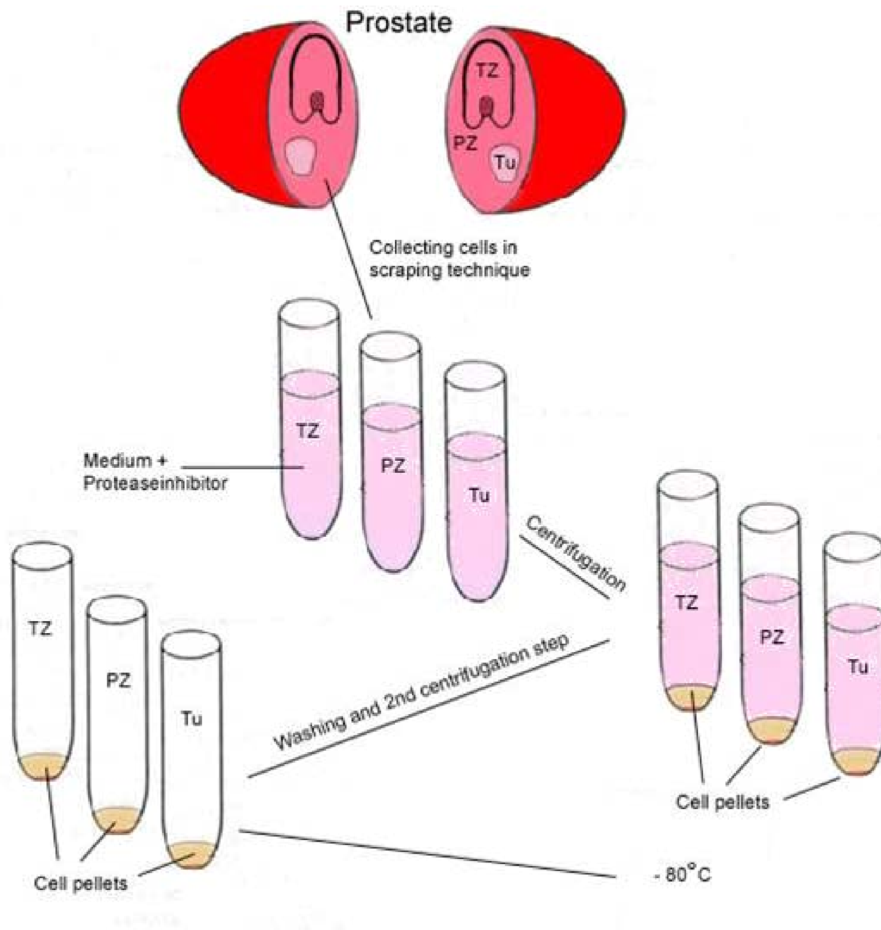

Figure 6. Schematic description of cell extraction by scraping technique.

Lysis of cells and protein determination

Each pellet was thawed on ice and resuspended in 1.89 µl mQ water per mg WW. The suspension was then frozen and thawed four times in liquid nitrogen in order to break the cells. A volume of (0.089 × WW) µl 10% SDS/33.3% mercaptoethanol was added together with (0.329 × WW)µl protease free DNase I (0.144mg/ml 20mM Tris-Hcl with 2 mM CaCl2 × 2H2O, pH 8.8) and RNase A (0.0718 mg/ml Tris) and the sample was incubated for five minutes on ice [204]. Samples were then frozen and lyophilized and sample buffer including PMSF (0.2 Mm), EDTA (1.0 mM), NP-40 (0.5%) and CHAPS (25 mM) was added (6 × WW, or 3 × WW if WW <10mg), mixed for 3h, centrifuged for 15 min at 12,000 rpm, and finally stored at -80ºC.

Protein concentration was determined using a modified Bradford method [205]. After dilution of 1µl of (duplicate or triplicate) cell lysate in 100µl mQ water, 25µl of

concentrated assay reagent (Bio-Rad, Sundbyberg, Sweden) was added to each sample on the microplate. Protein concentration was measured in a Multiscan reader (Labsystems, Ramsey, Minnesota), using different concentrations of bovine serum as reference.

2-DE

2-D gel electrophoresis was performed using precast immobilized pH-gradient (IPG) strips with a pH 4-7 linear gradient (Bio-Rad) in the first dimension of isoelectric focusing (IEF). Samples were applied via in-gel rehydration of IPG strips in a volume of 300 µl sample solution overnight. The rehydration solution contained 7 M urea, 2M thiourea, 65 mM CHAPS, 0.5% Triton X-100, 0.5 % IPG buffer (pH 4-7) and 18 mM DTT. Typically, 75 µg protein was loaded on each IPG strip and focusing was carried out during 22.5 h at 20 ºC at a total of 52000 Vhr. After IEF separation, the strips were subjected to a two-phase equilibration, each for 15 min, with 50 mM Tris, pH 8.8, 6 M urea, 30% glycerol, 69 mM SDS and a trace of bromphenol blue as tracking dye. DTT (65 mM) was included in the first equilibration solution and 135 mM iodoacetamide in the second.

The second dimension was performed in an Iso-Dalt tank (Hoefer, San Fransisco, CA USA) using 10-13% linear gradient SDS/PAGE gels (1.5 x 200 x 250 mm) with piperizine diacrylamide as cross-linker. The separation was performed in 0.25 M Tris, pH 8.6, 1.92 M glycine and 0.1% SDS at constant voltage (100 V) and temperature (12 ºC) for 18-20 hr, until the tracking dye reached 5 mm from the bottom of the gel. Ten gels were processed in parallel, stained with silver nitrate, and scanned at 106 x 106 µm resolution using a flatbed scanner GS-710 (Bio-Rad). Preparative gels were stained with Sypro® Ruby (Bio-Rad), scanned in a Molecular Imager FX (Bio-Rad) and matched to analytical gels before spot picking.

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Data analysis

Data were analyzed using the Bio-Rad PDQuest™ software (version 7.1.0 and 7.3.0) [206]. Background was subtracted, peaks were located and individual polypeptides expressed as ppm of the total integrated optical density. Each spot was given a unique

identification number. Individual quantifications of resolved proteins were normalized according to total intensity of valid spots (Paper I; total intensity of selected good quality spots matched to all gels). Spots of interest were excised, using a Proteome Works Spot Cutter robot (Bio-Rad, Hercules, CA, USA).

Mass spectrometry

The digestion of the proteins before MS analysis is an important procedure. Generally the steps are: in-gel enzymatic cleavage of the proteins into peptides (by the use of e.g. trypsin), extraction of the peptides from the gel plug, desalting and concentration of the peptides. The first two steps are most often quite easy, whereas desalting and concentration of the sample may be more difficult. There is a risk that material is lost in the desalting process, mainly due to dilution of the sample. Concentration of the peptides is necessary to achieve good crystallization with the matrix. The unpredictable ionization properties of peptides can also affect MS analysis. Some peptides do not ionize ideally and therefore give bad spectra when analyzed with MALDI but ionize better in ESI. As with gel analysis, MS identification depends on sample size, i.e. amount of protein. In clinical samples the amount of protein is limited.

Protein digestion was performed according to standard protocols (see detailed description in the articles). Identification of silver stained proteins was performed on an Ultraflex MALDI TOF/TOF (Bruker Daltonics, Bremen, Germany). Searches for protein identities were via the engine ProFound aginst the NCBInr sequence database. Sypro® Ruby (Bio-Rad) stained spots were analyzed by on-CD MALDI MS (Voyager DE-PRO, Applied Biosystems) and tryptic fragments were analyzed by electrospray ionization (ESI) quadrupole time-of-flight (Q-TOF) tandem MS (Micromass). For database searches and protein identification of on-CD MALDI MS generated peptides, the web site of ProteinProspector (http://prospector.ucsf.edu) was used. ESI derived data were analyzed using the NCBI BLAST search engine using MassLynx peptide sequence software sequences.

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RESULTS AND DISCUSSION

Paper I

Prostate cancers are often small and difficult to detect macroscopically. When harvesting research samples from the prostate, it is often difficult to obtain representative material for a 2-DE analysis without jeopardizing the histopathological diagnosis. Franzén et al. have developed a non-enzymatic sample preparation (NESP) squeezing technique (tissue block, TB) to extract cells from fresh tumor tissue blocks [128,207]. This technique has been used in studies of PCa [194,195]. A limitation with the NESP technique is that it requires a substantial amount of tissue. A less time-consuming extraction technique for prostate samples is warranted because of the rapid proteolytic degradation. We have developed a modified NESP technique, where cells are scraped from the cut surface of the prostate and directly suspended in medium. The study in Paper I evaluated the protein yield, reproducibility and overall quality of this modified NESP scraping (Scraped Sample, SS) technique. We analyzed 5 cancer specimens and 6 BPH specimens. Two of the BPH specimens were represented by duplicate samples. All specimens were processed according to both the NESP and the modified NESP cell extraction techniques, analyzed in one match set and evaluated with the PDQuest software. The preparation time for the two methods differed with approximately 25 minutes. For SS the pellet was prepared and stored in a freezer within 15 min, whereas TB processing took approximately 40 min. Protein yield from SS vs. TB were compared and the yield with SS was slightly better, although the difference was not statistically significant. Scatter plots of matched spots from two duplicate samples of benign tissue had correlation coefficients of 0.83 and 0.85 (mean 0.84) and 0.82 and 0.90 (mean 0.86) from SS and TB, respectively. The mean spot quality was similar for SS and TB (68.2% and 67.6%, respectively, $p = 0.42$). The quality of spots matched to all members (180 spots) of the match set was almost exactly the same with the two harvesting methods. There were no significant differences in average total density on gel or average valid spot quantity. The protein level was higher in 11 spots from SS compared to TB from benign tissue ($p<0.05$). Of these, six were identified by mass spectrometry as actins or tropomyosins. Three spots were found to have different protein levels in tumor material depending on sampling method; annexin, thioredoxin peroxidase B and unidentified spot SSP 3111. In a separate series of samples, where the aim was to

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identify possible autolysis sensitive proteins, one spot (unidentified spot SSP 3111) was found to decrease significantly ($p<0.05$) in samples that were harvested at five different time points, starting 15 min after surgery. Significant difference ($p<0.05$) in protein level was seen already between 15 and 30 min. The overall similarities between samples and gels of SS and TB indicate that these techniques give comparable results. However, the scraping method has advantages compared to the NESP technique, saving both time and tissue, and was therefore the method of choice for prostate tissue sampling.

Paper II

The functional roles of the anatomical zones of the prostate remain largely unknown. There are histological and pathological differences between the zones. The glandular architecture and cellular morphology of the CZ differs from the PZ and TZ, while the glandular epithelium of the PZ and TZ is almost indistinguishable (Figure 2, Physiology & Histopathology). A majority of the clinically diagnosed cancers arise in the PZ. TZ gives rise to the majority of BPH nodules and approximately 20% of the cancers. Only a few cancers arise in the CZ. Thus, it has been suggested that CZ may have a different embryonic origin than the PZ and TZ. We performed 2-DE gel electrophoresis in order to investigate the differential protein expression of the three zones. Samples were harvested by the scraping method from PZ, TZ and CZ in 18 patients. Smears from CZ contained an average of 94.8 % epithelial cells and 3.3 % smooth muscle cells, while smears from PZ and TZ contained 94.6 % epithelial cells and 1.8 % smooth muscle cells. A total of 36 gels were analyzed in two match sets and evaluated with the PDQuest software. One match set (A) contained samples from 11 patients and another match set (B) included duplicate samples from 7 patients. Mann-Whitney tests (MW) of match set A and B generated 18 spots with significantly different expression levels between the three anatomical zones. Spots were identified by peptide mass fingerprinting (PMF) and/or tandem MS sequence analysis. Two spots showed increased expression levels in CZ compared to PZ and TZ, one was identified as

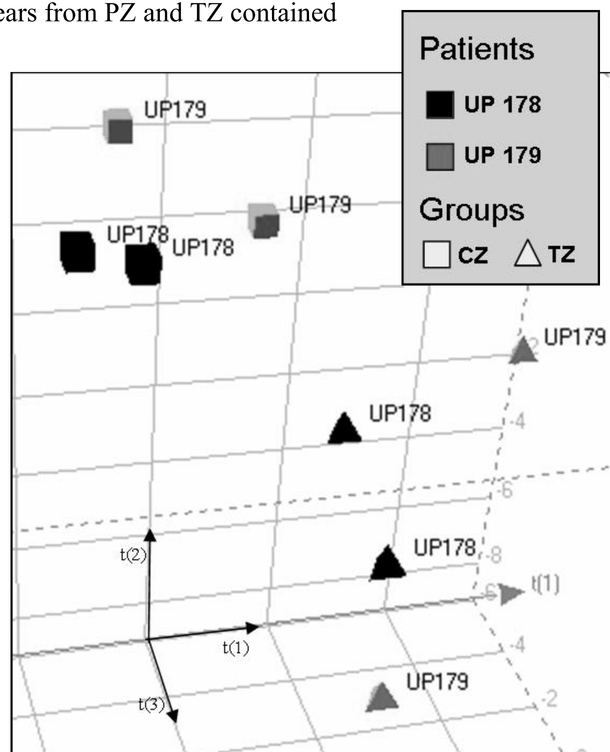

Figure 7. The three first principal components t1, t2 and t3 in a Spotfire view of a 3-D PCA plot of patients UP178 and UP179 from match set B. The picture illustrates the relations between expression profiles of paired duplicate samples from CZ (cubes) and TZ (pyramids), respectively.

peroxiredoxin 2, the other as creatine kinase B chain. Sixteen spots showed increased spot intensity in PZ and TZ. Eight of them were identified; arginase II, ATP synthase, cytokeratin 8, lamin A/C, peroxiredoxin 4, protein disulfide isomerase A3, tropomyosin, vimentin. Two patients from match set B were represented by duplicate samples from both TZ and CZ. Principal component analysis (PCA) was therefore applied exclusively to samples from these two patients (Figure 7). Two main sample patterns were recognized with CZ samples clustered on the upper left side and TZ samples clustered on the lower right side. PCA suggested that proteins responsible for the separation between TZ and CZ were the same in the two patients. Together, these findings further indicate that CZ may be functionally and possibly embryonally different from the other zones.

Paper III

In this study, we analyzed the protein expression profile of PCa. Cells from 29 PZ cancers and 10 samples of benign tissue were harvested by scraping cut surfaces of radical prostatectomy specimens. Tumor samples containing less than 90 % cancer cells were excluded. We used a pooled sample for reproducibility control of gels. Benign and malignant prostate cells from 20 patients (not included in the study) were pooled into one sample and aliquoted. Pooled controls, benign and tumor samples were processed in parallel for both the first and second dimension. 63 spots differed between cancer and benign samples ($p<0.01$), 56 were over expressed (more than 1.5 fold) in cancer and 7 under expressed (less than 0.6 fold). Two proteins that showed increased spot intensity in PZ and TZ compared to CZ in Paper I, ATP synthase and protein disulfide isomerase A3, had an even higher expression level in cancer than in normal epithelial cells of the PZ. Among over expressed proteins were also transcription factors (nucleoside diphosphate kinase 1) and enzymes involved in gene silencing (chromobox protein), protein synthesis (39S ribosomal protein L12, BiP protein, protein disulfide isomerase), degradation (cytosol aminopeptidase, endopeptidase Clp, inorganic pyrophosphatase) and energy metabolism (acyl-CoA dehydrogenase, isocitrate dehydrogenase, NADH-ubiquinone oxidoreductase, pyruvate dehydrogenase). Other overexpressed proteins were heat-shock proteins (60 and 70 kDa), structural proteins (cytokeratins) and membrane proteins (stomatin-like protein 2).

Nucleoside diphosphate kinase 1 is the product of NM-23 H1, a metastasis suppressor gene known to be upregulated in early stage PCa [208]. Lysophospholipase is related to proliferation and migration in cell lines and has been found at increased levels in ovarian cancer [209].

Paper IV

The prognosis of PCa correlates with tumor differentiation, but a majority of newly diagnosed cancers are Gleason score 6 tumors, which can be either aggressive or indolent. In Paper III we identified several proteins with differential expression in benign prostatic tissue and PCa. The aim of this study was to further analyze the data generated by correlating the protein expression with the two differentiation markers; Gleason grade and DNA ploidy. Mann-Whitney tests were performed to compare high-malignant cancers with benign samples and low-malignant cancers. Altogether we identified 39 polypeptides with expression levels associated with tumor dedifferentiation. We performed multivariate data analysis according to partial least square (PLS) discriminant analysis (DA) on all spots in the match set, using benign samples versus Gleason 7-9 samples as two classes (two samples excluded). Ninety-six variables with highest separation properties were further evaluated by principal component analysis (PCA), with the purpose to investigate the distribution of the Gleason 6 samples and proteins in the intersection of Mann Whitney and PLS analyses. The 39 polypeptides selected by Mann Whitney test were also analyzed by unsupervised hierarchical clustering. Samples with Gleason score 6 showed a wide distribution between benign and Gleason 7-9 samples in the PCA and also in the hierarchical clustering, with some Gleason 6 samples (especially the samples 2T and 6A) being close to the “high malignant” cluster. These findings indicate that our approach may be useful to discriminate potentially high malignant samples within the Gleason 6 category.

CONCLUSIONS

We have shown that the scraping method is an optimal sampling technique for prostate tissue. This technique is rapid, extracts mainly tumor cells and improves the over-all success rate of analysis. The method provides high-resolution gels and the overall quality of the samples and gels is comparable to the NESP sample preparation technique.

2-DE analysis of the anatomical zones of the prostate generated 18 spots with significantly different expression levels in CZ compared to PZ and TZ. The identified proteins give suggestions about the function of the zones, and also support the hypothesis that CZ may be of a different embryonic origin than the other zones of the prostate.

The aim of this thesis was also to define differences between benign and malignant prostatic tissue. A number of polypeptides were identified as PCa related and a set of proteins were shown to correlate with PCa progression. Some of the findings may have the potential to become diagnostic or prognostic biomarkers for PCa. We also show that multivariate analysis may be applied to discriminate potentially high malignant samples within a group of samples with unpredictable outcome.

Many of the proteins that were differentially expressed in benign prostatic tissue and PCa have a similar expression profile in other epithelial tumors. The stress proteins HSP 60 and 70 as well as many structural proteins are known to share expression patterns in a variety of epithelial tumors. This suggests a similar proteome in malignant epithelial cells. However, there are evidently some protein patterns in prostate cells that differ from other epithelial cells and may be used as PCa specific biomarkers.

It would be logical to suspect that PSA, currently the only clinically used PCa biomarker, should be detected as differentially expressed by 2-DE analysis. However, this was not the case in our study. An explanation may be that the expression of PSA does not differ very much between benign and malignant prostate cells. In fact, serum PSA elevation in patients with Pca has many other causes, such as increased leakage across the basement membrane of cancerous glands.

Future perspectives

The methodological limitations of 2-DE do not allow visualization of every single protein in a prostate cell. However, 2-DE is still a powerful method that can show the protein phenotype of a cell and downstream effects of specific gene regulations that cannot be detected on the genetic level. More and more proteins with "known" cellular functions are shown to be multifunctional, i.e. they are related not only to one pathway or protein and the function may vary with cell type. An example is α - enolase that was thought to be a cell surface plasminogen receptor on some cell types. By 2-DE Bergman *et al.* found increased levels of α -enolase in c-*jun* transformed rat fibroblasts [210]. This was shown to be a downstream effect of c-*jun* rather than an upregulation of the plasminogen receptor in rat fibroblasts.

The next step in this project will be to validate selected candidate biomarkers for PCa. We have found several proteins that are correlated to PCa and specifically to tumor differentiation. Some of the proteins may have the potential to become prognostic biomarkers for PCa such as Metaxin 2 and the degradation protein endopeptidase (ClpP). Metaxin 2 is bound to the cytosolic surface of the mitochondrial outer membrane by interaction with metaxin 1, and has been shown to be required for tumor necrosis factor-induced cell death [211]. ClpP is involved in a mitochondrial specific stress response. Accumulation of unfolded protein in the mitochondrial matrix results in up-regulation of genes encoding ClpP [212]. Lysophospholipase is another strong candidate that was found at increased levels in high-malignant tumors. This enzyme is involved in the production of lysophosphatidic acid which binds to G-protein-coupled receptors inducing proliferation and migration in cell lines and is increased in ovarian cancer [209]. The validation of a set of biomarker candidates could be performed by different approaches. The use of specific antibodies for *in situ* hybridization or immunohistochemical analysis is one possible approach. The multiplexed bead-based Luminex® assays and enzyme-linked immunosorbent assays (ELISA) are more sensitive and specific validation technologies. Napsin, a protein that was detected by our group in 2-DE of lung carcinoma was recently validated by *in situ* hybridization and showed 94.3% specificity for adenocarcinoma in non-small cell lung carcinoma [213].

More information about the nature of PCa can most likely be extracted by further 2-DE based investigations. Cup-loading could be used to increase the resolution and

also avoid excessive loss of proteins during IEF. The DIGE method is an attractive approach that is very well suited for clinical material with large patient variability. PTMs in PCa would also be an interesting target of future studies. It is now possible with the use of Pro-Q Diamond and Pro-Q Emerald to detect disease specific phosphorylations and glycosylations. The role of PTMs in biological processes and disease pathology will probably increase and the mapping of PTMs will require further development of both 2-DE and MS based technology.

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