PhD thesis

BIOMARKERS AND MODELS FOR DIFFERENTIATING PROSTATE CANCER FROM NON-CANCEROUS DISEASES

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DECLARATION

I hereby declare that I have written the thesis and performed all the work myself. Contributions to the research by others are explicitly acknowledged in the thesis.

Olomouc, October 2011 Tamar Jamaspishvili, MD

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1. INTRODUCTION

1.1. PROSTATE GLAND ANATOMY, PHYSIOLOGY, HISTOLOGY AND EMBRYONAL DEVELOPMENT

The human prostate gland is one of the male sex accessory tissues, which include the prostate, seminal vesicles and bulbourethral glands. It is a horse chestnut-shaped structure, measures 5 cm x 4 cm x 3 cm and weighs 20-30 g between ages 20 and 50. Currently used anatomical structure of the prostate is a McNeal's zonal model which defines 3 zones: central, peripheral and transition zone. The central zone is located at the base of the prostate and surrounds the ejaculatory ducts. The prostate gland is located in the pelvis, under the urinary bladder and in front of the rectus. It surrounds a part of the urethra that carries urine from the bladder during urination and semen during ejaculation. The main function of the prostate is the formation and storage of seminal plasma that constitute one half to two thirds of the 3 ml volume of the ejaculate (reviewed in Partin et al. 2002). The precise biological role of many of the biochemical substances secreted into the seminal plasma is uncertain. Though a well-defined role has been established for several enzymes that participate in the clotting and lysis of the seminal plasma clot, including prostate-specific antigen (PSA), which is a serine protease that cleaves semenogelin, a protein involved in clotting (Sokoll et al. 1997). Additional substances that are found at high concentrations in prostatic fluid include potassium, zinc, citric acid, spermine, amino acids, prostaglandins, and various enzymes. These secretory products enhance fertility by promoting sperm viability and motility. An antimicrobial role has been suggested for a few of these substances such as zinc, spermine, and proteases. The prostate is not a vital organ for life; the entire gland may be removed in radical prostatectomy (RP) without consequences in most patients. In a minority of patients, incontinence and impotence may complicate RP through damage to distal urethral sphincter and bladder neck (leading to incontinence) and periprostatic neurovascular bundles (leading to impotence) (reviewed in Humphrey 2003).

Microscopically, the normal adult prostate in men 20 to 50 years of age comprises a branching duct-acinar glandular system embedded in a dense fibromuscular stroma. Unlike in some other secretory glands in the body, the morphologic separation of ducts from acini usually is not feasible. The normal epithelium of the prostate is classically defined as having two cell layers: a

luminal or secretory cell layer requiring androgens for growth and survival and an androgen-insensitive basal cell layer. The basal cell layer separates the secretory cells from the basement membrane and is nearly continuous which is a diagnostic criterion for benign conditions. The complete absence of basal cells is an important finding in the diagnosis of invasive carcinoma. The immunophenotype of basal cells is distinctive and different from luminal secretory cells (Table 1).

Table 1. Normal prostatic epithelial cells: immunophenotype

Marker	Basal Cell	Luminal Cell	Neuroendocrine Cell
Pan-cytokeratin	+	+	+
CK 5,6,10,13, 14	+		± (focal)
CK8,18	±	+	+
PSA	-	+	+
PAP	-	+	+
Androgen receptor (AR)	-	+	+
GST-pi	+		NA
bcl-2	+		-
Chromogranin-A	-		+
MSA, SMA	-		-
S-100	-		-
Vimentin	-	+	NA
p63	+		NA
CD40, CD44H	+		NA
CD10, CD57, CDw123	-	+	NA

Abbreviations: CK, cytokeratin; PSA, prostate specific antigen; PAP, prostatic acid phosphatase; GST-pi, glutathione-S-transferase-pi; MSA, muscle-specific actin; SMA, smooth-muscle actin; NA, not available
Source: Humphrey PA. Prostate pathology. ASCP press, Chicago, 2003

The third cell type in normal prostatic epithelium are neuroendocrine cells (NE). NE cells are endocrine and sensory cells sharing structural, functional and metabolic properties with neurons normally present in human prostate, prostatic intraepithelial neoplasia and in prostate cancer (Shariff et al. 2006). These cell populations exhibit an anti-apoptotic phenotype partially explained by the observation of augmented Survivin expression (Xing et al. 2001) and further stimulate neoangiogenesis (via VEGF) (Chevalier et al. 2002). Pure NE cell prostate tumors are very rare (<0.1%) and highly aggressive (35% survival rate in 2 years) (Papandreou et al. 2002), while focal NE differentiation represents a common feature of CaP occurring in 30–100% of the

cases (Shariff et al. 2006, Cindolo et al. 2007). Because the higher number of NE cells is associated to an increased neoplastic progression rate, the neuroendocrine differentiation phenomenon is considered of general oncological interest. An increased number of overt NE cells was also observed in patients with hormone-refractory prostate cancer following long-term androgen deprivation therapy, even though the question that NE differentiation is attributable to a condition of hormone resistance or long-term hormonal therapy needs to be further studied (Hirano et al. 2004).

Stromal cells are skeletal and smooth muscle cells, fibroblasts, nerves, and endothelial cells. Occasionally, ganglion cells and paraganglia may be seen in the prostate, although they are more commonly identified exterior to the gland. Cowper's gland (a pair of pea-sized glands that lie beneath the prostate gland and produce an alkaline fluid that forms part of the semen that neutralizes the acidic environment of the urethra, thereby protecting the sperm) may also be seen on needle biopsy. Architectural and cellular features characteristic for normal as well as for cancerous epithelium are listed in Table 2.

Table 2. Features favoring and against adenocarcinoma of the prostate

	Against cancer	Favoring cancer
Architectural		
	Lobularity	Infiltrative pattern
	Larger glands	Small glands
	Branching glands	Crowded glands
Nuclear		
	Prominent nucleoli yet adjacent PIN	Prominent nucleoli
	Similar nuclear changes to adjacent benign glands	Enlargement
	Nuclear atypia with inflammation	Hyperchromasia
		Mitotic figures
		Apoptotic bodies
Cytoplasmic		
	Pale-clear cytoplasm	Amphophilic cytoplasm
	Luminal undulations or papillary infolding	Sharp luminal border
Luminal		
	Corpora amylacea	Blue-tinged mucinous secretions
		Pink amorphous secretions
		Crystalloids

Source: Epstein JI, Netto GJ. Biopsy interpretation of the prostate. Lippincott Williams & Wilkins press: Philadelphia, USA, 2008

Development and function of the prostate requires androgens, that include i) testosterone, made in the testes ii) dehydroepiandrosterone, made in the adrenal glands and iii) dihydrotestosterone, which is converted from testosterone within the prostate itself (androgens and their role in prostate cancer development is discussed in the chapter 1.3).

The prostate is derived from the urogenital sinus and is first recognizable at 9 to 10 weeks of development (McNeal 1997). At this time, testosterone from the embryonic testis stimulates ingrowth of endodermal buds into urogenital sinus mesenchyme. It has been hypothesized that there is a reciprocal interaction of epithelium and mesenchyme during development by which, under the influence of androgens, urogenital sinus mesenchyme induces urogenital sinus epithelium to undergo prostatic ductal morphogenesis and differentiation (Hayward et al. 2000). The differentiating prostatic epithelium in turn signals the urogenital mesenchyme to differentiate into smooth muscle cells that closely surround the epithelial ducts. By 13 weeks some primary ducts are present. The bud stage at 20 to 30 weeks' gestation exhibits solid cellular buds at the end of ducts, with spindled cells in the center and columnar cells at the periphery. The bud-tubule stage at 31 to 36 weeks shows small collections of cellular buds and acinar structures. The third stage is characterized by more distinct lobular organization of acinotubular clusters. After birth, there is an infantile resting period until 10 to 12 years of age and then a pubertal maturation period until age 18 years. In the resting period the size of the prostate gland remains stable but duct formation, solid budding at the periphery and branching morphogenesis continue (Timms et al. 1995). The pubertal period is marked by substantial androgen-driven increase in gland size, further branching and differentiation of immature prostatic epithelium into the adult-type basal and secretory cells. The seminal vesicles, epididymis, vas deferens and ejaculatory ducts are formed from Wolffian (mesonephric) ducts under the influence of testosterone. It has been proposed that the central zone of the prostate is also of mesodermal Wolffian duct origin (McNeal 1997). In this sense, the prostate gland is of dual embryonic derivation.

1.2. DEVELOPMENT AND PROGRESSION OF PROSTATE CANCER

In developed countries, prostate cancer (CaP) is the second most frequently diagnosed cancer and the third most common cancer causing death in men (Damber et al. 2008). It has been reported that approximately 1 in 8 men will develop CaP by 75 years of age and 1 in 5 by the age of 85 years (Tracey et al. 2006). Prostate cancer origins in the glandular epithelium and the tumor cells have generally been believed to originate from the luminal cells, since they are dependent on androgens and express luminal cell markers. However, there are increasing evidence indicating that the cancer cells are derived from less differentiated stem cells or transit amplifying cells (Collins et al. 2005, Gu et al. 2007). Most prostate tumors are heterogeneous and multifocal, suggesting that multiple neoplastic foci have emerged and evolved independently (Meiers et al. 2007). Prostate cancer development and progression is a multistep process. Through genetic and epigenetic alterations normal epithelial cells develop into malignant cells escaping from normal regulatory control. Premalignant lesions, so called prostatic intraepithelial neoplasia (PIN), are very common. PIN is considered to be an intermediate stage from benign epithelium to carcinoma that histologically is very similar to prostate cancer with the exception that the basal layer is discontinuous but still presents. Through additional alterations malignant tumors develop that initially are restricted to the prostate, but eventually penetrate the prostate capsule, invade surrounding tissues and ultimately form metastases. Transformation into a malignant phenotype is associated with a shift from paracrine to a more autocrine androgen stimulation (Gao et al. 2001). Since androgen deprivation therapy (ADT) is used to treat advanced prostate cancer, further tumor progression is associated with the loss of androgen-dependency and the development of highly aggressive castration resistant disease (see 1.3.3).

The development of prostate cancer depends on the strict balance between the rate of proliferation and programmed cell death (apoptosis). Alteration of specific chromosomes and genes has been associated with prostatic carcinoma. Definite risk factors for the development of prostate cancer include age, family history and race. Clinically, carcinoma of the prostate is most often detected in men over 60, is rare before age 40 and peaking in men older than 80 years of age (Ries et al. 1994). For men under 50, there is greater multifocality of small tumors (Deguia et al. 2001); for these patients stage and histologic grade are the independent predictors of survival. The second most important risk factor is a family history of the patient. About 25% of men with

CaP have a known positive family history (Walsh and Partin 1997). The degree of risk is related to the age of the relatives at diagnosis and the number of relatives affected. Inherited prostate cancer probably is caused by autosomal dominant transmission of rare high-risk alleles leading to early onset (Carter et al. 1992). The first discovered susceptibility locus is HPC1 (hereditary prostate cancer 1) located on chromosome 1q24-25 and is most strongly linked to large families with 5 or more affected men and with early age at diagnosis (Cooney et al. 1997; Table 3). Studies have shown that HPC1-linked hereditary prostate cancers were of higher grade and higher stage (Goode et al. 2001). Additional susceptibility loci on chromosomes 1q42.2-43 (Berthon et al. 1998), 1p36 (Gibbs et al. 1999), Xq27-28 (Xu et al. 1998) and others have been reported and are listed in Table 3.

Race is a definite risk factor for prostate cancer incidence and detection. These racial differences result from differences in genetics, diet, body size, socioeconomic status, lifestyle, physical activity, and access to medical care. For African American men <65 years of age the comparative incidence is the highest, with 79% more prostate cancer than in white men (Brawley et al. 1998). Differences in gene promoter hypermethylation may potentially underlie racial differences in prostate cancer pathogenesis (Woodson et al. 2003). Promoter regions of genes that are normally unmethylated become methylated in cancer cells. This occurs by the covalent binding of a methyl group to the 5'- cytosine of the dinucleotide pair (CpG), resulting in silencing the expression of tumor suppressor and other regulator genes. Woodson et al. (2003) studied differences in DNA methylation of three genes (GSTP1, CD44 and E-cadherin) in CaP in the population of African Americans and white people. In this study no racial difference was found in the prevalence of GSTP1 hypermethylation, CD44 hypermethylation was significantly increased in black men, and E-cadherin was not hypermethylated in any of the tumor specimens. The GSTP1 hypermethylation is specific to cancer and occurs early in carcinogenesis. E-cadherin and CD44 inactivation occurs later in prostate cancer development.

Table 3. Prostate cancer susceptibility loci

Gene	Location	Candidate Gene	Clinical Testing	Proposed Phenotype	Comments
HPC1/RNASEL Gronberg et al. 1997 Cooney et al. 1997 Xu et al. 2003 Wang et al. 2002 Rennert et al. 2005	1q25	RNASEL	Not available	Younger age at prostate cancer diagnosis (<65 years) Higher tumor grade (Gleason score) More advanced stage at diagnosis	Evidence of linkage is strongest in families with at least five affected persons, young age at diagnosis, and male-to-male transmission. RNASEL mutations have been identified in a few 1q-linked families.
PCAP Berthon et al. 1998 Xu et al. 2003	1q42.2–43	None	Not available	Younger age at prostate cancer diagnosis (<65 years)	Evidence of linkage strongest in European families.
HPCX Xu et al. 1998 Peters et al. 2001	Xq27–28	None	Not available	Unknown	May explain observation that an unaffected man with an affected brother has a higher risk than an unaffected man with an affected father.
CAPB Gibbs et al. 1999 Badzioch et al. 2000	1p36	None	Not available	Younger age at prostate cancer diagnosis (<65 years) One or more cases of brain cancer	Strongest linkage evidence was initially described in families with both prostate and brain cancer;
HPC20 Bock et al. 2001	20q13	None	Not available	Later age at prostate cancer diagnosis No male-to-male transmission	Linkage evidence strongest in families with late age at diagnosis, fewer affected family members, and no male-to-male transmission.
8p Xu et al. 2003 Sun et al. 2006	8p21–23	MSR1	Not available	Unknown	In a genomic region commonly deleted in prostate cancer.
8q Suuriniemi et al. 2007 Sun et al. 2008	8q24	None	Not available	Unknown	Population attributable risk may be higher for African American men than for men of European origin.

Abbreviations: HPC1, hereditary prostate cancer 1; RNASEL, ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent); PCAP, predisposing for prostate cancer; HPCX, hereditary prostate cancer, X-linked; CAPB, prostate cancer/brain cancer susceptibility; HPC20, hereditary prostate cancer 20; MSR1, macrophage scavenger receptor 1; Source: National Cancer Institute (NCI) http://www.cancer.gov

The ethnical differences in the two polymorphic short tandem repeat (CAG and GGC) of exon 1 in the androgen receptor gene have been reported to be an important risk factor for prostate cancer. The CAG microsatellite repeat sequence encodes a polyglutamine tract in the region of the AR associated with DNA transcription while the GGN repeat ($GGT_3GGG_1GGT_2GGC_n$) encodes a polyglycine tract (Esteban et al. 2006). In vitro studies (Ding et al. 2004) have demonstrated an inverse relationship between the length of both repeats and AR activity. Men with exceptionally long CAG repeat lengths experience clinical androgen insensitivity because of reduced transcriptional activity of the AR. Low size CAG (<19 repeats) and GGC (<15 repeats) alleles result in higher receptor activity, and have been associated with prostate cancer, earlier age of onset, and a higher grade and more advanced stage of prostate cancer at the time of diagnosis (Visakorpi et al. 2003). CAG and GGC allele length variation has been described in different ethnic groups (African, American of African descent, Asian, European and Mediterranean) (Kittles et al. 2001, Sasaki et al. 2003, Esteban et al. 2006). African-americans have the highest incidence of prostate cancer and a shorter median CAG repeat length than non-hispanic white men who have a longer median CAG repeat lengths, whereas the incidences of prostate cancer and other steroid-related cancers are remarkably low in Asia (Boyle and Ferlay 2004).

An interesting hypothesis exists about zinc and prostate cancer according to which uptake of zinc may be different in racial groups. This suggestion is based on the evidence that the normal prostate contains high amounts of free zinc ions that are secreted into the seminal fluid. In other words, the loss of the ability to retain normal intracellular levels of zinc is an important factor in the development and progression of prostate cancer. Rishi et al. (2003) have compared the relative levels of expression of the two zinc transporters (hZ1P1 and hZ1P2) in African Americans and white people. This study showed lower degree of the expression of these transporters in African American patients compared with white men. The observation that the uptake of zinc may be different in racial groups can be used as a preventive method for some people. The advantage is that dietary zinc supplements are relatively nontoxic.

Probable risk factors for development of prostate cancer include diet and steroid hormones. The strongest dietary link to prostate cancer development is high fat intake (Lophatananon et al. 2010). Dietary fat could increase CaP risk via one or more mechanisms, such as lipid peroxidation leading to the production of DNA-damaging free radicals and alteration of serum

sex hormone levels. Conversely, dietary consumption of fruits and vegetables, especially tomatoes, appears to lower the risk of prostate cancer (Giovannucci 2002). Lycopene, a carotenoid antioxidant, is an agent in tomatoes that has been associated with this diminished risk. Its levels in serum were inversely related to aggressive prostate cancer (high histological grade and stage) (Giovannucci 2002).

The steroid hormones that are likely to be involved in CaP risk include vitamin D and the sex hormones, especially testosterone and its derivates. Men with high levels of serum vitamin D are at lower risk of prostate cancer development, particularly poorly differentiated and higher-stage tumors (Schwartz et al. 2005). Androgens and their role in prostate cancer development are discussed in the following chapter.

1.3. ANDROGENS AND ANDROGEN RECEPTORS IN PROSTATE CANCER AND ANDROGEN-INDEPENDENT PROSTATE CANCER

1.3.1. Androgens and androgen receptor in the normal prostate

The secretory cells of the prostate express high levels of the androgen receptor (AR) and their survival and function is dependent on the action of androgens. The androgen receptor is a member of the steroid hormone receptor family and structurally is similar to the estrogen receptors. The release of gonadotropin releasing hormone (GnRH) from the hypothalamus results in secretion of luteinizing hormone (LH) from the pituitary gland. Circulating LH then stimulates the Leydig cells of the testes to produce and secrete testosterone. The hypothalamus also releases corticotrophin releasing hormone (CRH) that stimulates the pituitary gland to secrete adrenocorticotropic hormone (ACTH). ACTH in turn, induces the adrenal production of testosterone and other weak androgens, like androstenedione and dihydroepiandrosteron. The main androgen is testosterone. Dihydrotestosterone (DHT), a metabolite of testosterone, is a critical mediator of prostatic growth. DHT is synthesized in the prostate from circulating testosterone by the action of the enzyme 5α-reductase, type 2. This enzyme is located principally in the stromal cells; hence, these cells are the main site for the synthesis of DHT. This hormone can act in an autocrine fashion on the stromal cells or in paracrine fashion by diffusing into nearby epithelial cells. In both of these cell types, testosterone and DHT binds the AR, causing dissociation of the receptor from heat shock proteins followed by receptor dimerization and

phosphorylation. Testosterone can also bind and activate the AR, but DHT has a stronger binding affinity for the AR and is more potent than testosterone. The activated receptor complex is then translocated to the nucleus. Inside the nucleus it binds to androgen response elements (AREs) inducing the transcription of growth factors and AR-dependent gene expression (reviewed in Roy et al. 1999, Heinlein and Chang 2002, Heinlein and Chang 2004, Balk and Knudsen 2008) (Figure 1).

Removal of androgens results in loss of secretory function, decreased cell proliferation, increased apoptosis and thereby to involution of the gland. Initially this was believed to be a direct effect on the epithelium due to lack of AR activation. However, more recent studies have shown that the main function of androgens on luminal epithelial cells is to stimulate the secretory function and keep the cells in a differentiated and growth quiescent state, while androgen regulation of epithelial growth and regression mainly is mediated by the stroma (Simanainen et al. 2007). In response to androgen stimulation, stromal cells that also express AR (Iwamura et al. 1994) produce several growth factors that act in a paracrine fashion to sustain the vitality of the epithelium (Culig et al. 1996). Thus, the widespread apoptosis and loss of epithelium induced by castration is primarily due to inactivity of the AR in stromal cells rather than in epithelial cells (Simanainen et al. 2009). In addition, androgen regulation of prostate growth and regression has been suggested to be mediated by the vasculature (Lissbrant et al. 2001). In the normal prostate and in androgen-dependent tumors, castration induces endothelial cell apoptosis, vascular regression and decreased blood flow (Stewart et al. 2001). These vascular changes precede the castration induced effects on the epithelium, suggesting that the involution of the prostate gland partly is due to insufficient blood supply. Furthermore, testosterone induced re-growth of the prostate is preceded by increased angiogenesis and thereby increased blood flow. Since murine prostate endothelial cells have been reported to lack expression of AR (Prins et al. 1991), it was earlier believed that the androgen regulation of the vasculature primarily was an indirect effect mediated by epithelial and stromal cells in a paracrine fashion (Colombel et al. 2005). However, more recent studies have demonstrated that the AR indeed is expressed in human prostate endothelial cells and can mediate androgen regulated gene transcription, ultimately leading to increased proliferation of these cells (Godoy et al. 2008).

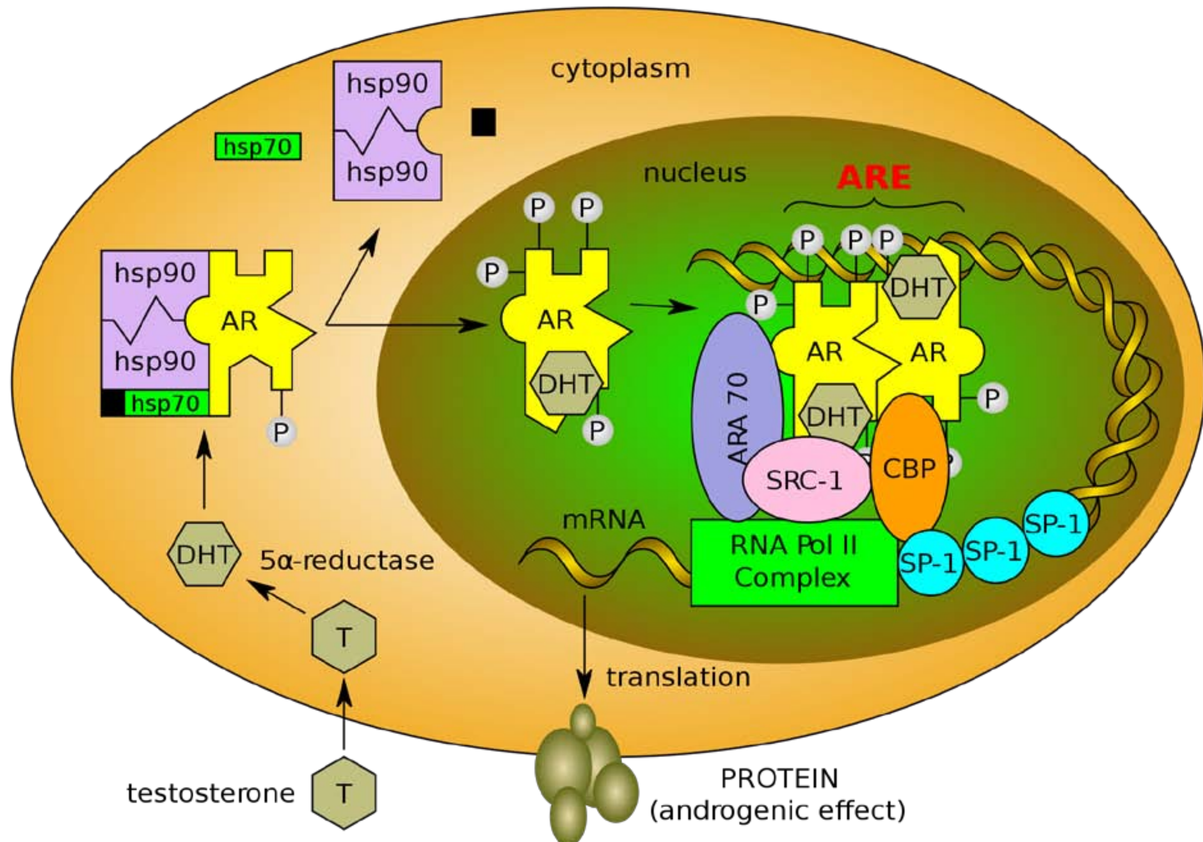

Figure 1. Androgen-AR action in the prostate. Testosterone (T) enters the cell and, if 5-alpha-reductase is present, is converted into dihydrotestone (DHT). Upon steroid binding, the androgen receptor (AR) undergoes a conformational change and releases heat-shock proteins (hsps). Phosphorylation (P) occurs before or after steroid binding. The AR translocates to the nucleus where dimerization, DNA binding, and the recruitment of coactivators occur. Target genes are transcribed (mRNA) and translated into proteins. CBP (CREB-binding protein); ARE (androgen response element); SRC-1 (steroid receptor coactivator 1).
Source: Meehan and Sadar 2003

1.3.2. AR expression and prostate carcinogenesis

Although serum androgens alone may not promote prostate carcinogenesis, androgen action and the functional status of ARs are important mediators of prostate cancer progression. Low serum testosterone levels in men with newly diagnosed and untreated prostate cancer have been found to correlate with higher AR expression, increased capillary vessel density within the tumor, and higher Gleason score (Schatzl et al. 2002).

AR expression is observed in primary prostate cancer and can be detected throughout progression in both hormone-sensitive and hormone refractory cancers (de Winter et al. 1994). Prostate cancer specimens collected from patients without preoperative treatment demonstrated that high AR expression correlated with lower recurrence-free survival and disease progression (Lee et al. 2003). Similarly, Stanbrough et al. (2001) observed that elevation of AR expression could initiate prostate cancer development, however, Lee and Chang (2003) reported that higher degree of AR positivity correlated with a greater degree of differentiation or lower Gleason score. Such controversial results might be explained by heterogeneous expression of ARs throughout the progression of the prostate cancer and might partly account for a variable response to endocrine therapy. The persistent heterogeneity of human prostate cancer suggests that increased AR expression is not generally associated with prostate cancer initiation. The cause of the loss of AR expression in some cells of tumor foci is unclear. X chromosome losses, including loss of the AR gene, are extremely rare in prostate cancer (Alers et al. 2000). Epigenetic silencing of AR expression by methylation may occur and has been observed in 8% of primary prostate cancers (Sasaki et al. 2002). Another possibility for the loss of AR expression in some tumor cells is a decrease in AR protein stability that reduces the AR protein level, difficult to detect immunohistochemically. AR is degraded by ubiquitin targeting to the proteasome (Lin et al. 2002). Ubiquitination of AR is promoted by Akt kinase-mediated phosphorylation of the receptor, suggesting that cells with increased Akt activation may have a reduced AR protein level (Lin et al. 2002).

1.3.3. Androgen-independent prostate cancer

During fetal development and puberty, the maturation of the gland is also dependent on androgens. Castration before puberty prevents maturation of the prostate and also prostate cancer. In grown-up men, likewise, removal of androgens causes an involution of the gland. Such observations prompted the idea to treat prostate cancer by androgen depletion. This can be done by surgical removal of the testes in which the cells of Leydig produce >90% of the androgen in the male body. Alternatively, androgen synthesis can be suppressed by interfering with the release of the pituitary LH which stimulates the production of androgens in Leydig cells. This is most commonly done by using drugs similar to GnRH which act on receptors in the hypothalamus inducing their down-regulation. After a short burst of LH and follicle-stimulating

hormone (FSH), no further gonadotropins are produced and the Leydig cells cease to synthesize androgens. Alternatively, binding of androgens to the androgen receptor can be inhibited by antiandrogens. Antiandrogenic treatment is used in the clinic in addition to surgical removal of the prostate (prostatectomy) or for treatment of metastases. Although the initial response to androgen ablation is encouraging, resulting in apoptotic cell death and CaP regression, prostate cancer respond no longer to continued antiandrogenic treatment followed by development of aggressive hormone resistant disease. This process may vary in the length of time, ranging from a few months to few years. The reason for this is that growth of advanced prostate cancers is dominated by cells that proliferate and survive relatively or completely independent of androgens (reviewed in Debes and Tindall 2004).

1.3.4. Mechanisms of androgen independence

It is already well-known that most of the androgen-independent prostate tumors express the AR (van der Kwast et al. 1991). It is activated in conditions of androgen deprivation by at least three mechanisms: i) amplification of the AR gene leading to an up-regulation of AR mRNA ii) activation of alternate signaling pathways leading to cancer progression (cross-activation of ARs) and iii) mutations that alter AR function that lead to receptor activation by binding of non-canonical ligands or ligand-independent activation of the receptor (Figure 2) (reviewed in Heinlein and Chang 2004, Devlin and Mudryj 2009). Besides these three mechanisms, recently proposed theory about primitive prostate cells, i.e. castration-resistant prostate cancer stem-cells represents novel insight in the development of androgen-independence and new targets of advanced disease (Goldstein et al. 2010). Goldstein and colleagues showed that primitive cells of the prostate share all properties of castration-resistant prostate cancer cells (castration-resistance, tissue-regeneration, and self-renewal) and presented evidence that such cells could serve as targets for prostate cancer-initiation.

The amplification of the AR gene has been suggested as a mechanism that enables prostate cancer cells to become sensitive to the reduced level of androgens present after androgen ablation therapy. AR amplification occurs rarely in untreated primary prostate cancers, with an observed frequency between 0 and 5% (Linja et al. 2001). However, amplification of AR is found in 20–30% of hormone refractory prostate cancers (Linja et al. 2001, Golias et al. 2009). It remains unclear whether amplification of the AR gene in hormone refractory tumors results in an increase

in AR mRNA/protein levels. Insignificant differences were found between AR expression in tumors with and without gene amplification and not all prostate tumors with AR gene amplification showed increased levels of the AR protein (Golias et al. 2009). These data suggest that other mechanisms apart from AR amplification are involved in the progression of prostate cancer. Divergent results have been obtained for the influence of AR amplification on PSA expression. Although one study found AR amplification positively correlated with an increase of serum PSA (Koivisto and Helin 1999), two subsequent studies failed to correlate the presence of AR amplification in hormone refractory carcinomas with either tumor or serum PSA levels (Palmberg et al. 2000, Linja et al. 2001). Therefore, the significance of AR amplification in prostate cancer is currently unclear.

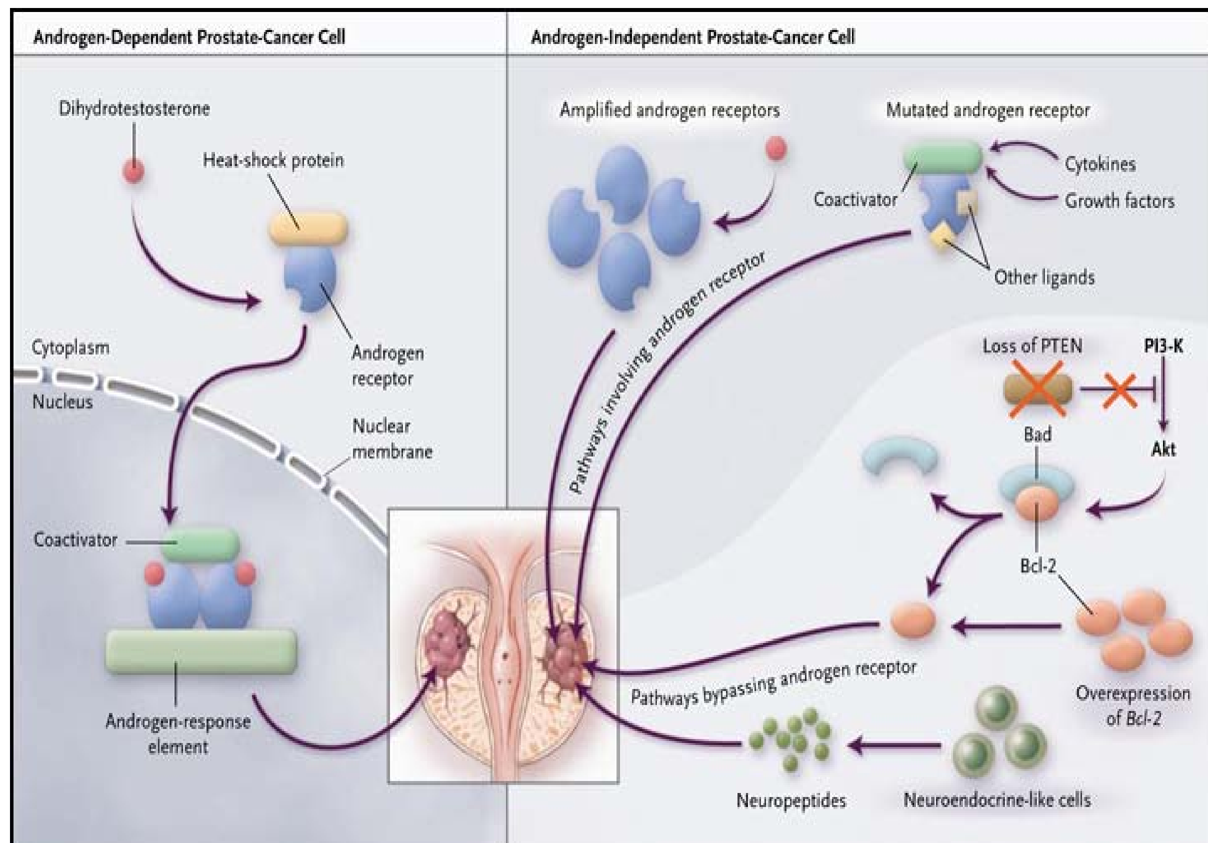

Figure 2. Mechanisms of AR-dependent and independent prostate cancer.
Source: Debes and Tindall 2004

At least five non-androgen agents have been found to cross-activate the AR in the absence of androgen, by at least three distinct signaling pathways (Figure 3). Culig and co-workers (1994) showed that insulin-like growth factor (IGF)-I activates AR in the human prostate cancer cell line DU145, although this did not occur in LNCaP cells (Ueda et al. 2002). The difference may be attributable to variations between the signaling pathways in these cell lines. Forskolin (a plant lipid that stimulates cAMP) activates AR through the protein kinase A pathway (Sadar et al. 1999, Kim et al. 2005). The canonical Wnt pathway can activate the androgen receptor through β-catenin (Terry et al. 2006, Robinson et al. 2008). Oncostatin-M may have an important role despite its reduced levels in androgen-independent cells in xenograft model as it also modulates the response to hydroxyflutamide, causing this anti-androgen to act as an AR agonist (Godoy-Tundidior et al. 2002).

The transcription factor HER2/neu activates the AR independently of androgen (Craft et al. 1999, Mao et al. 2009) and increases its response to low concentrations of androgen. HER2/neu was over-expressed in androgen-independent derivatives of an androgen-dependent human prostate cancer xenograft studied by Craft and coworkers (1999). Such alterations in gene expression during endocrine treatment may allow the AR to be reactivated by concentrations of androgen that were previously too low (Mao et al. 2009).

Several groups have found evidence that the mitogen-activated protein kinase (MAPK) pathway is involved in cross-activation of the AR (Ueda et al. 2002, Lonergan and Tindall 2011). Activation of the MAPK enzymes ERK1 and ERK2 was detected in 70% of prostate tumors with Gleason scores 8-10 and was significantly associated with increasing Gleason score. The MAPK pathway, among others, is activated by the Ras protein after its stimulation by a range of growth factors. Mutations that activate the Ras family of oncogenes frequently occur in some type of human cancer, but appear to be rare in prostate cancer (Gumerlock et al. 1991), indicating that other factors are responsible for the frequent MAPK enzyme activation in prostate tumors.

Interleukin 6 (IL-6) can also activate AR in the absence of androgen (Ueda et al. 2002, Lonergan and Tindall 2011). Multiple signalling pathways, in particular those of Janus kinases/signal transducers and activators of transcription (JAK/STAT), Ras-MAPK, and phosphatidylinositol 3-kinase (PI3-K) pathways are involved in the signal transduction by IL-6 (Smith et al. 2001a, Lonergan and Tindall 2011). It was found that the pathways of JAK/STAT and MAPK enhance AR activation by IL-6, whereas the PI3-K pathway is inhibitory (Yang et al. 2003). IL-6 has also

been shown to act through Ser/Thr kinase Pim-1 and tyrosine kinase Ekt (Kim et al. 2004) as an alternative pathway of ligand-independent activation of androgen receptor-mediated transcriptional activity.

A variety of co-activators are found to be upregulated in CaP (Heinlein and Chang. 2004). The expression of certain co-activators like SRC1, TIF2 (Gregory et al 2001), ARA70 (Hu et al. 2004), cdc25B (Ngan et al. 2003), nmt55 (Ishiguro et al. 2003) and Tip60 (Halkidou et al. 2003) is found to be increased in AR-resistant CaP. It is hypothesized that the increase in coactivator expression leads to an increase in AR-regulated gene expression. For instance, ARA70 increases AR expression, stability, and nuclear translocation (Hu et al. 2004). Thus, over-expression and recruitment of coactivator proteins to the AR-binding sites may facilitate the progression of AR-resistant CaPs.

Over-expression of anti-apoptotic proto-oncogene, bcl-2, is also found in a subset of prostate cancers and have been correlated with progression to androgen-independent status in human tumors (Kolar et al. 2000). p53 status may also influence responsiveness to androgens. In vitro experiments demonstrated that four p53 mutations common in prostate cancer each enabled the androgen-dependent prostate cancer cell line LNCaP to grow in an androgen-independent manner (Nesslinger et al. 2003). $p21^{WAF-1/CIP1}$, an effector of the p53 protein, negatively regulates the cell cycle, and its over-expression inhibits the proliferation of androgen-dependent and androgen-independent prostate cancer cells in vitro and tumorigenicity in vivo (Gotoh et al. 2003).

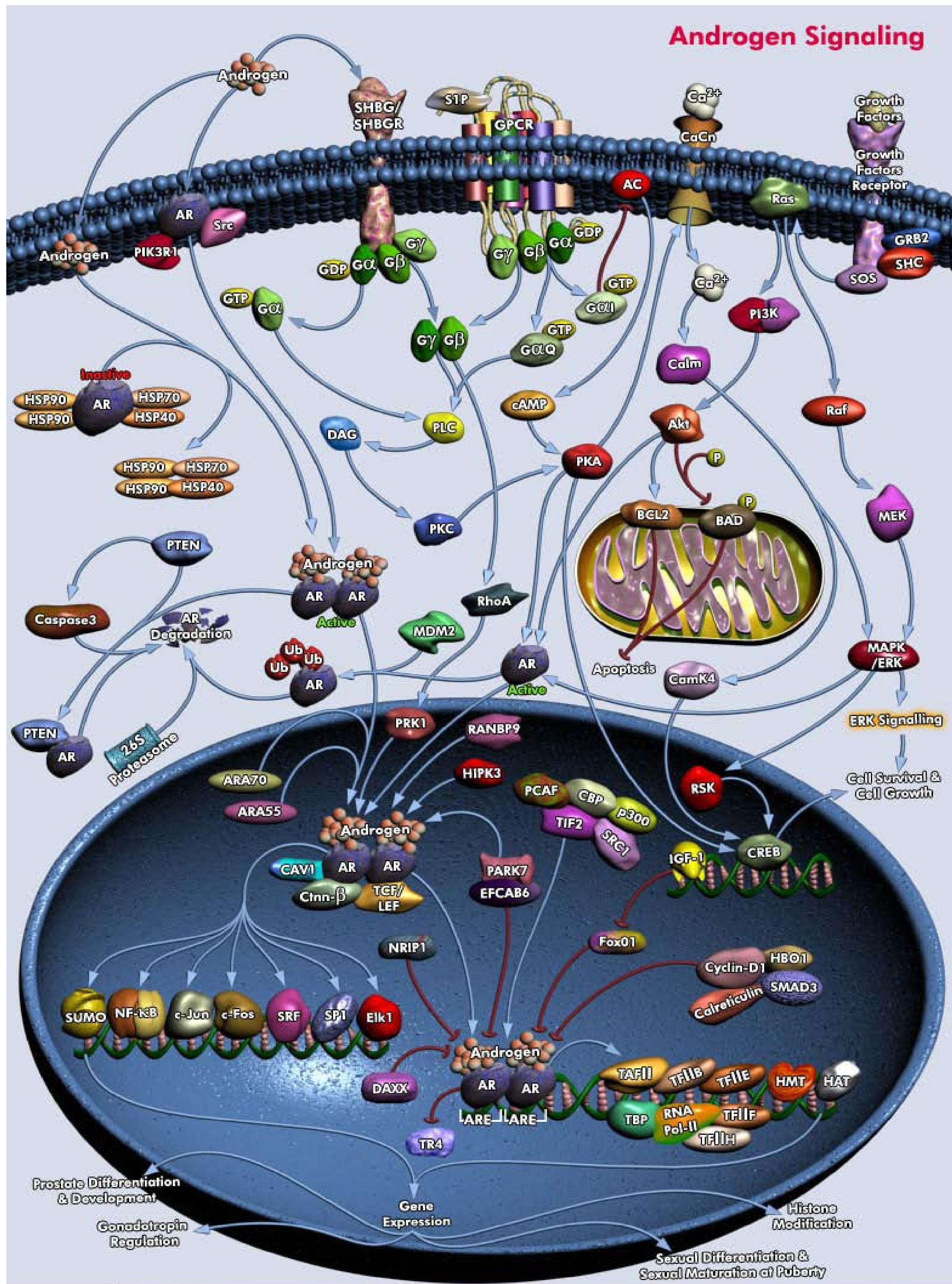

Figure 3. Complexity of androgen signaling pathway.
Source: www.qiagen.com

1.4. BIOMARKERS FOR PROSTATE CANCER EARLY DIAGNOSIS AND PROGRESSION

1.4.1. Symptoms of prostate cancer and problems associated with diagnosis

Unlike benign prostate hyperplasia or prostatitis, prostate cancer may not show symptoms in its early, curable stage and therefore it is often diagnosed in the advanced stages of the disease. Sometimesprostate cancer does cause symptoms, often similar to benign prostate hyperplasia. These include frequent urination, increased urination at night, and maintaining a steady stream of urine, blood in the urine and painful urination. Prostate cancer may also cause problems with sexual function, such as difficulty in achieving erection and or painful ejaculation. Advanced prostate cancer may cause additional symptoms as the disease spreads to other parts of the body (metastasis). The most common symptom is bone pain, often in the vertebrae, pelvis or ribs. Metastases in the spine can also compress the spinal cord, causing leg weakness and urinary and fecal incontinence.

Prostate cancer is genetically multicentric and histologically multifocal which may cause problems in its diagnosis, prognosis and treatment. Both genetic and epigenetic events occur independently in intratumor foci and hypermethylation-induced loss of gene function may be as critical as specific genetic mutations in prostate carcinogenesis. Given the poor success rate in treating advanced CaP, intervention in early stages may reduce the progression of small localized carcinoma to a large metastatic lesions, thereby reducing disease-related deaths.

The prostate specific antigen test has enhanced the detection and awareness of this malignancy. Serum PSA levels have been widely used for diagnostic purposes for more than 25 years but false-positives are still quite common (low specificity of the PSA) (Freedland et al. 2006), especially in the so-called diagnostic “grey zone” (4-10 ng/ml) which represents a dilemma for discriminating CaP from benign prostatic hyperplasia (BPH), prostatitis and urethral manipulations which often increase PSA levels (Thompson et al. 2005). Despite the use of digital rectal examination (DRE) and 12 core transrectal guided ultrasound (TRUS) biopsies by most academic centers which significantly increased the cancer detection rate, prostate cancer is still missed in a significant number of patients and repeated biopsies are needed to reveal the presence of CaP after previous negative biopsy (Djavan et al. 2005). Thus, there is still a great demand for new markers to improve the CaP diagnosis at an early stage and prevent unnecessary

prostate biopsies, especially in the serum PSA “grey zone” where these days many newly diagnosed men are.

1.4.2. Criteria for biomarker selection

The most important item regarding the selection of candidate marker is the quality of scientific and clinical data supporting its potential utility. These include scientific studies explaining the functional role of the gene/protein in the biology of the disease and clinical data linking the candidate marker with disease presence, alterations in stage, response to therapy and overall survival. New prostate cancer screening tests detecting specific biomarkers must be accurate with high sensitivity and specificity, fast, inexpensive, non-invasive and be well accepted by the population targeted for screening. Furthermore, the test should detect prostate cancer when it is still confined to the prostate and distinguish the indolent from the aggressive tumors to avoid the problem of over-diagnosis. The ideal candidate for an early detection or disease monitoring marker must be i) prostate specific ii) detectable in an easily accessible biological fluid such as human serum, urine, or prostatic fluid and iii) able to distinguish prostate cancer from non-neoplastic lesion. In addition, the marker should have sufficiently convincing clinical correlation data from several different laboratories before it is brought forward for large-scale evaluation. To provide guidance for a systematic and critical evaluation of putative biomarkers by research groups it has been proposed that biomarker development should occur in six phases: i) pre-clinical exploratory study ii) development of a second generation research assay iii) retrospective analysis iv) prospective analysis v) marker commercialization vi) FDA (Food and Drug Administration) approval (Pepe et al. 2001).

Phase 1: pre-clinical exploratory studies start with the identification of prostate cancer-specific genes. First generation research tests can be used for measurement of the putative biomarkers in tissue samples.

Phase 2: development of a second generation research assay includes protein-based tests and the molecular gene-based tests (see 1.4.3.). New biomarkers are revealed because of differences in expression patterns in malignant as well as nonmalignant prostate tissues. However, tissue specimens cannot be used for clinical screening because they are obtained through invasive and expensive procedures. In the second phase of biomarker development a second generation

research assay should be developed which is based on a specimen that does not require an invasive procedure. Such specimens are body fluids such as blood, ejaculate and urine.

Phase 3: retrospective analysis. In this phase of biomarker development the second generation research assay developed in phase two is used on stored body fluid specimens that were collected from a cohort that reflects the target population for screening.

Phase 4: prospective analysis. In this phase of biomarker development the biomarker-based research assay is applied in the screening of men for prostate cancer with the aim of early diagnosis and treatment of the disease. In these studies the true and false positive rates of the biomarker-based research assay are calculated and the stage or characteristics of the tumors are described.

Phase 5 and 6: marker commercialization and FDA approval. When the biomarker-based research assay has passed all four phases it can be commercialized and used for screening in the general population. These screening studies will be used to estimate the reduction in cancer mortality afforded by the new biomarker-based screening test. The next very important step will be clinical trials that lead to FDA approval (phase 6).

1.4.3. Current techniques for identification of CaP biomarkers

1.4.3.1. Protein-based tests

Nowadays, a proteomic study which attempts to investigate all proteins expressed by a genome has become a direct and fast way for detecting biomarkers in a variety of sample types, but most especially in biological fluids.

Common proteomic techniques used can be divided into gel-based and gel-free techniques. The typical and most commonly used gel-based technique is the two-dimensional gel electrophoresis (2DGE) which can simultaneously separate and visualize thousands of proteins in a gel on the basis of differences in their isoelectric point (pI) and molecular weight. The principle of applying this technique in biomarker seeking is to compare the final proteomic pattern shown on the gels of the normal and disease samples and to search for statistically significant differentially expressed protein spots with the help of image software. This is then followed by identification of spots of interest using mass spectrometry (MS). MS is a gel-free technique which can be coupled with 2DGE for protein identification. It is also widely used for detection of proteins or peptides that are smaller than 20 kDa and cannot be found in 2D gels. MS has two major

components: ionization and mass analysers. Electrospray ionization (ESI) and MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight) are the two major ionization techniques used in MS (Matharoo-Ball et al. 2007a). A recently invented modified version of MALDI named surface-enhanced laser desorption and ionization-Time of Flight (SELDI-TOF) (Solassol et al. 2005) and MALDI-MS in conjuction with ANNs (artificial neural networks) analysis (Matharoo-Ball et al. 2007b) has also been widely used. Four major mass analysers are TOF (Time of Flight), quadrupole mass analyzer, quadrupole ion trap (QIT) and Fourier transform ion cyclotron resonance (FTICR). Although gel electrophoresis has many advantages, there are also several disadvantages. The procedure can be time-consuming, create a heavy work load, and lack sufficient reproducibility. The insolubility of hydrophobic proteins and the difficulty in detection and separation of low-abundant proteins are additional weaknesses (Matharoo-Ball et al. 2007a). Because of these shortcomings liquid chromatography (LC) represents an alternative to 2D-PAGE for separating proteins, where peptides in solution are injected through chromatographic columns. Retained species are dependent on the chromatographic properties of the column; they are then eluted and evaluated by MS instruments thereafter. Recently, several labs have started using high performance liquid chromatography (HPLC) for separations of proteins and peptides. Two steps of HPLC (cation exchange and reverse phase) are coupled to tandem mass spectrometry and database-searching algorithms and allow rapid analysis of complex mixtures with direct identification of the generated peptide sequences. However, there are also disadvantages associated with use of the HPLC method. There is a tendency in such experiments to “re-discover” over and over again the same set of proteins (i.e. the most abundant ones). Moreover, most low-abundance proteins, which are often very important functionally, are missed by this approach. To address these problems, several groups have proposed to pre-fractionate the samples prior to reverse phase separations. There are currently two major approaches to pre-fractionate complex biological samples: (i) the chromatographic approach and (ii) the electrophoretic approach. Such MS-based assays i) can successfully compete with antibody-based methods in cost, speed, and specificity ii) represent the only viable way to characterize proteins for which no suitable antibody has yet been developed iii) are capable of simultaneously characterizing several proteins from the same sample in the same experiment.

Many publications using proteomic approaches to search for CaP biomarkers have used either tissues or cancer cell lines. This has resulted in several potential biomarkers being identified (Lexander et al. 2006, Sardana et al. 2008). However, proteomic analysis of biological fluids is challenging. This is probably due to two main reasons. Firstly, the concentration of potential biomarkers varies in different human body fluids with the highest in tissue interstitial fluid as it directly contacts tumor tissues and lower in circulating body fluids, hence it is more difficult to detect biomarkers using the easily accessible circulating fluids such as serum/plasma. Secondly, the huge dynamic range of serum proteins makes identification of proteins of low abundant extremely difficult (Moritz et al. 2004). In urine, the low concentration of proteins and high salt concentrations create problems for proteomic analysis (Norden et al. 2004). However, Rehmans et al. (2004) was able to identify calgranulin B/MRP-14 using MALDI-TOF-MS analysis on urine specimens and similarly, uromodulin and semenogelin I (SEMG1) isoform b preproprotein were proposed as biomarkers that could differentiate prostate cancer and BPH (Koma et al. 2007).

1.4.3.2. Gene-based tests

The gene-based tests can be divided into DNA-based and RNA-based tests (Table 4). The advantage of DNA is its better stability than RNA. Although there are many DNA-based techniques available, it is questionable whether they can be used as a screening tool. Currently, there are no prostate cancer susceptibility genes or loci responsible for the largest portion of prostate cancer (see Table 3). Therefore, screening men for the presence of prostate cancer-specific gene-mutations or polymorphisms is a time-consuming, expensive and ineffective approach in the detection of primary prostate cancers in the general male population. For the detection of microsatellite alterations at least 20% of the analyzed genomic DNA has to be obtained from tumor cells. Moreover, this technique is prone to artefacts using small amounts of DNA obtained from body fluids. However, among DNA-based methods methylation-specific PCR used for detection of hypermethylated GSTP1 promoter, can serve as a useful technique in the detection of cancer in body fluid specimens because it requires a tumor-to-normal ratio of only 0.1–0.001% (Hessels et al. 2004; see also below for GSTP1 as a urine marker).

Gene expression profiling is based on the identification of signatures of differentially expressed genes in prostate cancer. However, due to the multifocality and heterogeneity of prostate cancer

it may be rather difficult to establish a reliable profile pattern from biopsy specimens. Therefore, gene expression profiling can be used for predicting outcome of disease after radical surgery rather than prostate cancer diagnosis. For other RNA-based assays see chapter 1.5. below.

Table 4. Available techniques for biomarker determination in body fluids

Type of assay	Technique	Target	Disadvantage	Advantage	Specimen
Protein-based assays	ELISA	Based on a single protein		Relatively easy to establish	Body fluid
	SELDI-TOF MS	Based on a pattern of proteins	Lack of standardization	Currently validated by EDRN	
DNA-based assays	Gene expression profiling	Based on expression several genes	Due to heterogeneity of prostate cancer only applicable in tissue specimens		Radical prostatectomy tissue
	Sequence analysis	Screen for cancer-specific mutations or polymorphisms	Ineffective in detection of primary prostate cancers in general male population		Tissue, body fluid
		Mitochondrial DNA alterations	Diagnostic applicability needs to be defined		Body fluid
	LOH	Microsatellite alterations	Prone to artefacts. Requires tumor to normal ratio of >0.5%		Body fluid
	MSP	Epigenetic modifications	No FDA approved technology	Sensitive method, requires tumor to normal ratio of 0.1–0.001%	Body fluid
RNA-based assays	RT-PCR	Specific mRNA	RNA degradation	FDA approved Roche Molecular systems	Tissue, Body fluid
	NASBA			FDA approved bioMérieux	
	TMA			FDA approved Gen-Probe	

Abbreviations: ELISA, immunosorbent assay; SELDI-TOF MS, surface-enhanced laser desorption and ionization-time of flight; LOH, loss of heterozygosity; MSP, methylation-specific PCR; RT-PCR, reverse transcriptase-PCR; NASBA, nucleic acid sequence-based amplification; TMA, transciption-mediated amplification; RNA, ribonucleic acid; mRNA, messenger RNA; DNA, deoxyribonucleic acid; FDA, food and drug administration; EDRN, early detection research network

Source: Hessels et al. 2005

1.4.4. Conventional biomarkers

1.4.4.1. Serum PSA and its derivatives

PSA is a 33-kDa glycoprotein and a member of the kallikrein (KLK) family of serine proteases. It is encoded by the KLK3 gene located on chromosome 19q13.4. Its secretion is strongly dependent on androgens. PSA is mostly found in prostatic tissue, although low concentrations of the protein can be found in other tissues such as kidney and endometrium (Clements et al. 1994). It is secreted by normal, hyperplastic, and cancerous prostatic epithelia into the seminal fluid. Its function is to digest the gel that is formed in semen after ejaculation. Under normal conditions, only a minor fraction of PSA leaks into the extracellular space and into circulation. When tissue architecture is distorted in CaP, level of PSA increases in the serum. Two forms of PSA are found in serum, free (unbound) and bound PSA. Approximately 70-90% of total PSA (tPSA) forms complexes with protease inhibitors such as α_1-antichymotrypsin (PSA-ACT) and α2-macroglobulin (PSA-A2M) creating an enzymatically inactive complex, whereas 10-30% of tPSA is unbound and called free PSA (fPSA).

The PSA test measures the total amount of PSA in the blood. Increased PSA level prompts a recommendation for prostate biopsy with PSA 4 ng/ml being the traditional threshold level (Catalona et al. 1991, Antenor et al. 2004). However, nonmalignant prostatic diseases such as BPH, acute prostatitis and also prostate manipulations cause serum PSA elevation resulting in high false-positive rates of detection which is especially common in so-called “grey zone” (4-10 ng/ml) which represents a dilemma for discriminating CaP from noncancerous diseases (Thompson et al. 2005). Thus, this poor diagnostic specificity (20-44%) of the PSA test means that more than half of the men in the range of PSA 4-10 ng/ml are negative on initial biopsy (Stamey et al. 2001, Lilja et al. 2008). Moreover, numerous studies have reported that 15-33% of CaP are biopsy detectable under the PSA values <4 ng/ml (Thompson et al. 2004). The age of a person can also affect PSA levels. Moul and colleagues proposed that different PSA cut-off points should be employed for different age groups (Moul et al. 2007).

Fortunately, PSA and its introduction as a screening test has led to a sharp increase in the incidence of CaP because there has been a shift to diagnosis at earlier stages (Lilja et al. 2008), though very low diagnostic specificity still highlight the need for an improved method of testing using other derivatives of PSA as discussed in the following chapter.

Efforts have been made to increase PSA diagnostic specificity using various isoforms of PSA and PSA parameters. PSA isoforms consist of fPSA, proPSA, complexed PSA (cPSA) and benign PSA (bPSA). PSA parameters include percent free PSA (%fPSA), PSA density (PSAD), age-specific PSA ranges, PSA velocity (PSA-V) and PSA doubling time (PSA-DT). fPSA, which refers to the PSA not bound to plasma proteins, is the most studied PSA isoform so far. The ratio of fPSA/tPSA shows reliable discrimination between prostate cancer and BPH and has approved the use of %free PSA (fPSA/tPSA X 100) as an adjunct to total PSA testing in men with a serum tPSA level of 4-10 ng/ml (Catalona et al. 1998). Catalona et al. (2000) demonstrated that only fPSA (sensitivity 95%, specificity 27%) has shown consistent and significant improvements over the conventional PSA test (sensitivity 95%, specificity 15%).

Analysis of the structural composition of fPSA revealed three subfractions: proPSA, bPSA and intact PSA (iPSA). ProPSA is the precursor protein for PSA, producing four truncated forms of PSA: (−2), (−4), (−5), and (−7) proPSA. Studies have shown that proPSA defined by the sum of the four truncated proPSAs improved the specificity over tPSA and %fPSA in the PSA range of 4–10 ng/mL (Catalona et al. 2003). Recently introduced test phi (prostate health index) which is a combination of serum tPSA, % free PSA and p2PSA ([-2] proPSA isoform) had the highest specificity compared to any of the individual tests (Shariat et al. 2008, Jansen et al. 2010). However, more studies are needed to identify its specificity and sensitivity on CaP detection and its clinical significance is still under investigation.

Two multi-center studies have shown that complexed PSA (cPSA) improved the specificity of total PSA, but this was not better than f/tPSA (Djavan et al. 2002). bPSA was identified in 2000, and is an altered form of fPSA found enriched in the nodular transition zone (TZ) tissue of BPH which suggests that it could be a biomarker for BPH. When compared with PSA and fPSA, serum bPSA has been found to have a better predictive power for prostatic enlargement (Canto et al. 2004).

Other PSA parameters such as PSA velocity (rate of change of the PSA level) and PSA doubling time (the time needed for the PSA value to double) have also been introduced to improve the sensitivity and specificity. Although the diagnostic value of PSA-V is controversial, it has great potential as a prognostic marker for CaP. High PSA-V is associated with a higher risk of CaP recurrences (Patel et al. 2005) and shorter recurrence intervals after radical prostatectomy (D'Amico et al. 2004). The combination of preoperative PSA-V and PSA-DT was also shown to

have significant predictive value concerning the progression of CaP (Sengupta et al. 2005), although this finding needs to be verified by others.

Despite all the modifications and variations of PSA that have been investigated, there is still no consensus on what is the best use of PSA to obtain optimum diagnostic and prognostic information. This, together with the lack of specificity of PSA for clinically significant and biologically aggressive cancer, warrants new biomarkers to diagnose prostate cancer more accurately and decrease the number of negative biopsies.

1.4.4.2. Other conventional biomarkers

PSCA (prostate stem cell antigen) is a glycosylphosphatidylinositol (GPI)-anchored cell surface antigen that is found predominantly in prostate and play a role in stem cell functions such as proliferation or signal transduction (Reiter et al. 1998). Expression of PSCA correlates with Gleason grade and more advanced tumor stages (Hara et al. 2002, Jalkut et al. 2002). One IHC study demonstrated an association between increased PSCA expression and progression to androgen-independent prostate cancer (Ross et al. 2002). Another strength of this marker is its potential as a therapeutic target. Anti-PSCA monoclonal antibodies have been shown to inhibit tumor growth and metastasis formation in human xenografts grown in mice which opens up the possibility for therapeutic treatment of prostate cancers using immunotherapeutic regimens (Ross et al. 2002).

PSMA (prostate-specific membrane antigen) was originally found only on the membrane of prostate epithelium in 1987 (Horoszewicz et al. 1987). It is a type II transmembrane glycoprotein playing a role in folic acid utilization and metabolism (Chang et al. 2004). PSMA is highly expressed in normal prostate tissue including endometrial glands, gastrointestinal tract, brain, skeletal muscle, pancreatic islets and kidney tubuls (Mhawech-Fauceglia et al. 2007) and is negatively regulated by androgens (Murphy et al. 1998a). PSMA is highly specific (94,5%) and sensitive (65,9%) IHC marker for CaP diagnosis despite its expression in various malignant tumor types including bladder cancer (Mhawech-Fauceglia et al. 2007). PSMA is down-regulated in BPH compared to prostate cancer. Expression further increases with hPIN and is significantly over-expresses in advanced, poorly-differentiated tumors, especially in androgen-independent prostate cancer (Mhawech-Fauceglia et al. 2007, Murphy et al. 1998a, Perner et al. 2007). Also high intensity of staining is detected in tumor neovasculature in both prostatic and

non-prostatic tissues that is not observed in normal endothelial cells (Chang et al. 2004, Mhawech-Fauceglia et al. 2007). Interestingly, only tendency but no statistical correlation is observed with either Gleason score or tumor stage (Chang et al. 2004, Mannweiler et al. 2009). Noteworthy, there is significant association of high PSMA tissue expression with early PSA recurrence being independent of clinical parameters such as lymph node tumor burden, extraprostatic extension, seminal vesicle invasion and high Gleason score (Perner et al. 2007). However, its role as a serum prognostic CaP biomarker is controversial. Studies using Western blot showed that PSMA could distinguish late stage CaPs from early stage diseases (Murphy et al. 1998b) whereas other studies showed that PSMA was not more effective than PSA (Beckett et al. 1999). A number of other factors such as age, secretion from normal tissues or other tumors age are likely to contribute to the serum levels of PSMA and might affect its relation to CaP (Kinoshita et al. 2006). Overall, PSMA is regarded as a promising biomarker for CaP and further investigations and perhaps, more sensitive detection methods are needed to fully evaluate its usefulness.

EPCA (early prostate cancer antigen) is a nuclear matrix protein which could be used as a good candidate biomarker for prostate cancer detection. The association with cancer was initially established by immunohistochemical analysis which showed 84% sensitivity and 85% specificity for CaP detection (Dhir et al. 2004, Uetsuki et al. 2005). Paul et al. (2005) developed an enzyme-linked immunosorbent assay (ELISA) to detect the EPCA level in blood and showed 92% sensitivity and 94% specificity in CaP detection, however only 46 plasma samples were examined. A drawback of using EPCA alone is that not all CaP tissues express EPCA (Paul et al. 2005). Although its potential as a complement to PSA testing is promising, further research is needed to confirm its potential.

GRN-A (chromogranin A) is a member of the granin family of proteins and acts as a prohormone (Simon and Aunis 1989). GRN-A is stored in the dense core secretory granules of most endocrine and neuroendocrine cells and is a marker of neuroendocrine differentiation. Whereas serum levels of GRN-A do not accurately distinguish BPH from prostate cancer very well, they do correlate with tumor stage and grade. Studies involving GRN-A have been conducted in human serum using radioimmune assay or ELISA and on tissues using IHC. Elevated serum levels of GRN-A predict poor prognosis in patients with androgen-independent prostate cancer after endocrine therapy. Also there are statistically significant data suggesting

that when combined with PSA, elevated GRN-A levels may effectively predict a poor prognosis after endocrine therapy (Ferrero-Pous et al. 2001, Isshiki et al. 2002). Taken together, these evidences make GRN-A a good candidate for further clinical evaluation as a good prognostic and/or therapeutic marker for prostate cancer.

1.4.5. Kallikrein-related peptidases

Kallikrein-related peptidases represent a subgroup of the serine protease family consisting of 15 members located at q13.4 of chromosome 19. KLKs are tandemly arranged from centromere (KLK1, KLK15, KLK3, KLK2) to telomere (KLK4-KLK14) and are mainly under steroid hormone regulation (Yousef et al. 2001a, Diamandis et al. 2002a, Obiezu et al. 2005). The majority of kallikrein-related peptidases do not exhibit tissue specificity and are co-expressed in various organs such as CNS, skin, breast and almost all salivary glands, as proven in RT-PCR studies (Obiezu et al. 2005). Both centromeric and telomeric groups of KLKs are highly expressed in the prostate suggesting their potential as novel diagnostic/prognostic markers (Yousef et al. 2005). Serine proteases play key roles in many physiological processes such as digestion, blood coagulation and fibrinolysis, cellular and hormonal immunity, fertilization and embryonic development (Borgono et al. 2004). The function and application of kallikreins in cancer diagnosis as well as prognosis are listed in Table 5.

PSA (KLK3) is one of the human kallikreins (discussed in 1.4.4.1) and is the most useful marker for prostate cancer screening, diagnosis and monitoring.

Human kallikrein 2 (KLK2), like PSA, belongs to the serine protease family. It has 80% amino acid sequence homology with PSA. KLK2 was initially found to be over-expressed in CaP epithelial cells by immunohistochemical staining suggesting the use as a potential biomarker for CaP (Darson et al. 1997). Several groups have successfully detected KLK2 in the human serum, where it presents in free and bound forms, like PSA (Piironen et al. 1996, Grauer et al. 1998). Partin et al. (1999) using total KLK2 (tKLK2)/fPSA ratio examined the association between KLK2 serum levels and CaP and found that 40% of CaP could be detected in patients with PSA levels below 4 ng/ml. Moreover, other studies have identified potential prognostic value of KLK2. Haese et al. (2001) and Steuber et al. (2006) found that the level of KLK2 in blood was significantly associated with the risk of biochemical recurrence after radical prostatectomy in men with a serum PSA≤10 ng/ml and it can be an independent prognostic biomarker for CaP.

KLK2 showed improved accuracy (73.1% vs 69.1%) over PSA for predicting biochemical recurrence (Steuber et al. 2007). Further validation is needed to confirm its prognostic ability.

KLK4 is expressed both in normal and cancerous tissues at very low levels as assessed by immunohistochemistry and quantitatively by an KLK4-specific immunoassay (Obiezu et al. 2002). Its over-expression in prostate cancer over normal prostate tissue has been demonstrated by Western blotting (Xi et al. 2004). Interestingly, anti-KLK4 antibodies were detected solely in the serum of prostate cancer patients, suggesting that KLK4 may be not only prostatic biomarker but also a possible target in immunotherapy (Day et al. 2002).

KLK5 and its differential expression in CaP was investigated by RT-PCR. Significantly lower expression was noted in cancer with the lowest levels found in T3 stage tumors. A negative correlation between KLK5 expression and Gleason score was also observed (Yousef et al. 2002a).

KLK7 was first found in the human stratum corneum where it plays a role in desquamation processes. KLK7 is differentially expressed in ovarian and breast cancer and has been proposed as an unfavorable prognostic marker for these malignancies (Shan et al. 2006, Li et al. 2009). KLK7 is highly expressed in prostate tissues but its biological role in prostate cancer is poorly understood (Kishi et al. 2004). A single study on KLK7 expression in CaP showed its down-regulation at both mRNA (semiquantitative PCR) and protein level (Western blot analysis, IHC) with a significant negative association with Gleason grade (Xuan et al. 2008). However, recently Mo et al. (2010) observed up-regulation of KLK7 in more advanced cases of CaP suggesting that KLK7 plays an important role in mediating prostate cancer progression and that KLK7 promotes invasion and metastasis through inducing the epithelial-mesenchymal transition of prostatic carcinoma cells. Therefore, the role of KLK7 in prostate cancer needs to be further investigated.

Table 5. Kallikrein gene expression in various cancer types

Kallikrein	Cancer type	Sample type	Method	Application	Reference(s)
KLK2	Prostate	Serum; Tissue	Immunoassay; IHC	Diagnosis, prognosis	49, 303, 304
	Breast	Cytosols	Immunoassay	Uncertain	18
KLK3 (PSA)	Prostate	Serum; Tissue	Immunoassay; IHC	Diagnosis, prognosis	256, 316, 318
KLK4	Ovarian	Tissue	qPCR	Unfavorable prognostic marker	221
	Prostate	Tissue	IHC; qPCR	Diagnosis; prognosis	222, 353
KLK5	Ovarian	Tissue	qPCR	Unfavorable prognostic marker	145
	Breast	Tissue	qPCR	Poor prognosis	370
	Prostate	Tissue	qPCR	Unfavorable prognostic marker	367
	Testicular	Tissue	qPCR	Lower expression in aggressive tumors	365
KLK6	Ovarian	Serum; Tissue	qPCR; Immunoassay	Diagnosis; prognosis	60, 312
KLK7	Ovarian	Cytosols	qPCR	unfavorable prognostic marker	277
	Breast	Tissue	qPCR	unfavorable prognostic marker	168
	Prostate	Tissue	IHC; qPCR	Prognosis	200, 357
KLK8	Ovarian	Tissue	qPCR	Marker of favorable prognosis	180
KLK9	Ovarian	Tissue	qPCR	Marker of favorable prognosis	362
KLK10	Breast	Tissue	In situ hybridization	Down-regulated in breast cancer	57
	Ovarian	Serum; Tissue	qPCR; Immunoassay	Diagnosis; prognosis	178
KLK11	Prostate	Serum; Tissue	qPCR; Immunoassay	Diagnosis, prognosis	17, 59
	Ovarian	Serum; Tissue	qPCR; Immunoassay	Diagnosis, prognosis	59
KLK12	Breast	Tissue	qPCR	Down-regulated in breast cancer	363
KLK13	Breast	Tissue	qPCR	Down-regulated in breast cancer	361
KLK14	Ovarian	Tissue	qPCR	Marker of favorable prognosis	360
	Breast	Tissue	qPCR	Down-regulated in breast cancer	364
	Testicular	Tissue	qPCR	Down-regulated in cancerous tissue	364
	Prostate	Tissue	qPCR	Up-regulated in prostate cancer	372
KLK15	Ovarian	Tissue	qPCR	Marker of poor prognosis	369
	Breast	Tissue	qPCR	Marker of favorable prognosis	371
	Prostate	Tissue	qPCR	Marker of unfavorable prognosis	302, 368

Abbreviations: KLK, Kallikrein; PSA, prostate-specific antigen; qPCR, quantitative polymerase chain reaction; IHC, immunohistochemistry;

KLK11/Hippostasin also known as trypsin-like serine protease (TLSP), which was originally isolated from the human hippocampus, is under steroid hormone regulation and has three isoforms derived from alternative splicing: isoform 1 (brain-type), isoform 2 (prostate-type) and isoform 3 (Nakamura et al. 2001, Nakamura et al. 2003a). The prostate-type variant is expressed only in the prostate while the brain-type can be detected in both brain and prostate (Nakamura et al. 2001). High expression of KLK11 mRNA and protein has been demonstrated in many normal tissues, including prostate, stomach, trachea, colon, brain, skin, salivary gland (Yousef et al. 2001a, Diamandis et al. 2002a). KLK11 is a well-studied biomarker for ovarian cancer yielding high KLK11 serum levels in 70% of cancer patients (Diamandis et al. 2002b, Scorilas et al. 2006). KLK11 mRNA expression was detected in both normal and cancerous prostate tissue but with significant up-regulation in cancer (Nakamura et al. 2003b). Up-regulation of prostate-type KLK11 was significantly associated with an earlier stage, lower Gleason score and lower tumor

grade but expression was again lowered with the tumor progression (advanced stage, less-differentiated CaP) confirmed by qPCR analysis (Nakamura et al. 2003b, Bi et al. 2009). Also interesting association was found between up-regulation of TMPRSS2 (the type II transmembrane serine protease) and down-regulation of KLK11 gene in advanced and more aggressive tumors with the lowest survival rates suggesting possibility of using both biomarkers of tumor aggressiveness CaP (Bi et al. 2009). With respect to KLK11 serum levels, study by Nakamura et al. (2003c) suggested its use as a diagnostic marker of CaP. KLK11 serum levels are down-regulated in prostate cancer and better discriminate CaP from BPH than total PSA, especially KLK11:total PSA ratio in combination with % free PSA (Nakamura 2003c). Overall, KLK11 seems to be favorable prognostic as well as diagnostic marker for prostate cancer and could be useful for discrimination of advanced cancers from localized ones as well as BPH from CaP.

KLK14 expression was investigated quantitatively on samples from radical prostatectomy (Yousef et al. 2003a). This study indicated over-expression of KLK14 in 74% of cancer cases. Using qPCR Yousef and colleagues detected that KLK14 mRNA levels were significantly higher in late stage compared with earlier stage disease. The same relationship was for KLK14 expression in high grade vs low grade tumors.

KLK15 is also up-regulated in cancer vs normal prostate tissues (Yousef et al. 2001b). Another study has shown that KLK15 levels were significantly higher in patients with pT3/pT4 stage than in pT2 patients suggesting its utility in assessing aggressiveness of prostate cancer (Stephan et al. 2003).

1.4.6. Gene fusions in prostate cancer

For over 30 years, genetic rearrangements have been recognized as key events in cancer development. Many haematological malignancies and sarcomas are characterized by common, recurrent chromosomal translocations that lead to expression of fusion genes or deregulation of oncogenes. In contrast, epithelial carcinomas show many non-specific chromosomal rearrangements and until recently, recurrent translocations were not considered to play a major role (Prentice et al. 2005, Kumar-Sinha et al. 2008).

The first prostate gene fusions, untranslated region of TMPRSS2 fused to ERG or ETV1 transcription factors, were discovered by Tomlins and colleagues (2005) using a novel algorithm

Cancer Outlier Profile Analysis (COPA) to analyze DNA microarrays (Tomlins et al. 2005). TMPRSS2 is a type II transmembrane serine protease (21q22.3) expressed in normal prostate epithelium and involved in many physiological and pathological processes but its exact biological function is still unknown. TMPRSS2 is strongly androgen-regulated presumably through androgen response elements in the promoter/enhancer region (5' untranslated end) to which potential oncogenes may be transposed (with their 3' end) (Vaarala et al. 2001, Afar et al. 2001). Despite their emerging pathogenetic and clinical importance, the mechanism through which such recurrent chromosomal rearrangements occur has been elusive. As the estrogen receptor–regulated transcription involves formation of local DSBs (double-strand breaks) by TOP2B (Ju et al. 2006), Haffner and colleagues (2010) suggested existence of the same mechanism for androgen receptor-regulated genes which are frequently involved in prostate cancer-associated fusions. They have shown that androgen receptor signaling may lead to TOP2B-mediated DNA breakage and thereby entailing the formation of TMPRSS2-ERG (Haffner et al. 2010, Bartek et al. 2010).

Both ERG and ETV1 belong to the ETS (E26 Transformation–Specific) transcription factor family. Other members of the ETS transcription factor family can also be transposed to TMPRSS2 but less frequently (ERG>ETV1>ETV4>ETV5) (Tomlins et al. 2006). These and other recently described fusion genes in CaP are summarized in Table 6. Furthermore, Wang et al. (2011) reported novel gene fusions involving genes from Ras family. The gene fusion UBE2L3-KRAS which was identified in DU145 cell line, attenuates MEK/ERK signaling and instead signals via AKT and p38 MAPK pathways. A specific knockdown of the novel gene fusion decreased cell invasion and xenograft growth suggesting that this aberration may drive metastatic progression in a subset of prostate cancers.

The most common oncogene transposed to TMPRSS2 is ERG (v-ETS avian erythroblastosis virus E26 oncogene homolog), also called p55 or ERG-3 which maps to chromosome 21q22.3. ERG responds to mitogenic and/or stress signals transduced by various MAP kinases, and modulate transcription of target genes favoring tumorigenesis. Chromosomal translocations involving ERG have been found in Ewing sarcoma, myeloid leukemia and cervical carcinoma (Oikawa and Yamada 2003). Apropos CaP, the most common variants involve TMPRSS2 exon 1 or 2 fused to ERG exons 2, 3, 4 or 5 (Lapointe et al. 2007, Wang et al. 2006) with a prevalence of the exon 4 (Soller et al. 2006, Rajput et al. 2007, Demichelis et al. 2007). Less common

combinations include TMPRSS2 exon 4 or 5 fused to ERG exon 4 or 5 (Demichelis 2007) and TMPRSS2 exon 2 fused to inverted ERG exons 6-4 (Iljin et al. 2006).

TMPRSS2-ERG fusion prostate cancers appear to have a more aggressive phenotype and poor prognosis. Cases with TMPRSS2-ERG rearrangement through deletion are associated with higher tumor stage, higher PSA recurrence and metastases to the pelvic lymph nodes (Perner et al. 2006, Mehra et al. 2007). Rajput et al. (2007) found a strong association between higher Gleason pattern and TMPRSS2-ERG gene fusion. Another study found no association with Gleason score, however, the positivity of fusion status was associated with histological patterns that have been linked to more aggressive CaP (e.g. intraductal tumor spread) (Mosquera et al. 2007). Patients with gene fusions were reported to have significantly higher rates of recurrence than those lacking TMPRSS2-ERG (Nam et al. 2007, Wang 2006). Furthermore, Demichelis et al. (2007) found statistically significant association between TMPRSS2-ERG and CaP specific death in a Swedish population with up to 22 years of clinical follow up without curative treatment (watchful waiting cohort).

However, controversial results have been reported in several studies for correlations between gene fusions and prognosis. Several important positive prognosticators (longer recurrence-free survival, well and moderately differentiated stages, lower pathological stage and negative surgical margins) were surprisingly associated with TMPRSS2-ERG fusions (Petrovics et al. 2005). Similar findings have been reported in another study with a clear tendency for fusion-positive tumors to be associated with lower Gleason grade and better survival than fusion-negative tumors (Winnes et al. 2007). No significant association was found between TMPRSS2-ERG status and tumor stage, Gleason grade or recurrence-free survival in another study (Lapointe et al. 2007). The reason of such controversial results could be small sample size in almost all studies listed above. Another reason might be insufficient follow-up and the fact that patient cases and controls were not derived from the same source making it difficult to interpret correlations with specific tumor parameters.

The effect of gene fusions on CaP is still subject of investigation, however the fact that TMPRSS2:ETS gene fusions can only be found in PIN, carcinoma and metastases but not in benign prostatic hyperplasia or proliferative inflammatory atrophy (PIA) suggested that the gene fusions were likely to be the genetic trigger for the development of PIN and CaP invasion (Perner et al. 2007). TMPRSS2-ERG fusion was not sufficient for transformation from PIN to

cancer state in the absence of secondary molecular lesions (e.g. loss of NKX3-1 or PTEN) and vice versa (Tomlins et al. 2008b). Also ERG over-expression itself without fusion partners markedly increased cell invasion in benign prostate cells but it did not result in transformation, while TMPRSS2-ERG fusion mediated the PIN to CaP transition. Interestingly, a novel ER (estrogen receptor)-dependent regulation pathway was found for TMPRSS2-ERG positive prostate cancers as an alternative mechanism by which prostate cancers might develop androgen independence from an initial androgen-dependent state (Setlur et al. 2008). This should be through ERα stimulation of the TMPRSS2 promoter in castration-resistant prostate cancer which leads to CaP progression, metastasis and more aggressive phenotype. In contrast, ERβ may function as a tumor suppressor through negative regulation of TMPRSS2-ERG expression as tested in vitro by using of ERβ agonist.

TMPRSS2-ERG fusions document heterogeneity of CaP, i.e. multi-focal malignant clones in CaP may be distinguished by the presence of the TMPRSS2-ERG fusions (Rajput et al. 2007, Barry et al. 2007). Barry et al. (2007), for the first time, reported the presence of interfocal clonal heterogeneity (foci might differ for fusion status, i.e. fusion may occur both through translocation/insertion and deletion in the same patient) and confirmed intrafocal clonal homogeneity (the same fusion status throughout the entire CaP focus) as previously reported (Perner et al. 2007). Such heterogeneity should not be missed at biopsies as TMPRSS2-ERG fusion has relevance as a prognostic marker and gives opportunity to assess a patient's risk of disease progression. These findings had a great impact on the development of noninvasive diagnostic test for detection of this gene fusion. A recent study done on a cohort of prebiopsy and pre-radical prostatectomy patients showed that the detection of TMPRSS2-ERG fusion transcripts in urine was feasible (Laxman et al. 2006). Urine RNA was analyzed by qPCR after preamplification (whole transcriptome amplification) and then break-apart fluorescent in situ hybridization (FISH) was used to validate the presence or absence of the TMPRSS2-ERG gene rearrangement in the CaP tissue. Indeed, patients with high levels of ERG and detectable levels of TMPRSS2-ERG in their urine were positive for ERG rearrangement. The same research group confirmed good diagnostic ability of TMPRSS2-ERG fusion in urine for early CaPs when combined with other biomarkers (Laxman et al. 2008). Multiplexed qPCR was used to analyze a panel of 7 potential urinary CaP biomarkers including TMRSS2-ERG. This study showed improved specificity compared to the serum PSA test. Combining TMRSS2-ERG and PCA3 also

improved the predictive value of CaP compared to PCA3 test alone (Hessels et al. 2007, Salami et al. 2011).

Overall, most of the studies have suggested that TMRSS2-ERG could be a prognostic biomarker for aggressive prostate cancer. Its presence has also impacted on the ability to improve CaP detection when coupled with other biomarkers. However, studies on the clinical implications of TMRSS2-ERG have so far involved relatively small sample sizes and different experimental designs. Therefore, more studies are needed to validate its clinical significance in CaP.

Table 6. Gene fusions identified in prostate cancer

Upstream / Downstream fusion partner gene [1,2]	BRAF	ELF1	ELK4	ERG	ETS1	ETS2	ETV1	ETV4	ETV5	FLI-1	KRAS	PDEF	RAF1
ACSL3							8 ↑						
C15orf21							159, 323 ↓						
CANT1							107 ↑	114,107 ↑					
DDX5								107 ↔					
ESRP1													227↔
EST14							114 ↑						
FLJ35294							159,107 ↑	107 ↑					
FOXP1							114 ↑						
HERVK17							114,159 ↑						
HNRNPA2B1							159,323 ↔						
KLK2								114 ↑					
NDRG1				107, 242 ↑									
SLC45A3	227↑		253 ↑	107 ↑			324,107 ↑		112 ↑				
TMPRSS2		81 ↑		161, 324, 325 ↑	4 ↑	81 ↑	324,325 ↑	324, 114 ↑ ↔	112, 322 ↑	81,114 ↑ ↔		101 ↑	
UBE2L3											347 ↑		

[1] the numbers stand for references for the particular gene fusion

[2] the arrows indicate type of androgen responsiveness (up-, down-regulation, insensitive)

1.5. URINE MARKERS IN MONITORING FOR PROSTATE CANCER

More than 85% of the prostate cancers occur in the peripheral zone of the prostate, and it has been shown that exfoliated cancer cells can be found in voided urine of prostate cancer patients. Exfoliated cells and secreted products released from multiple foci of the prostate can be easily collected from the urine and used in early detection of prostate cancer (Figure 4).

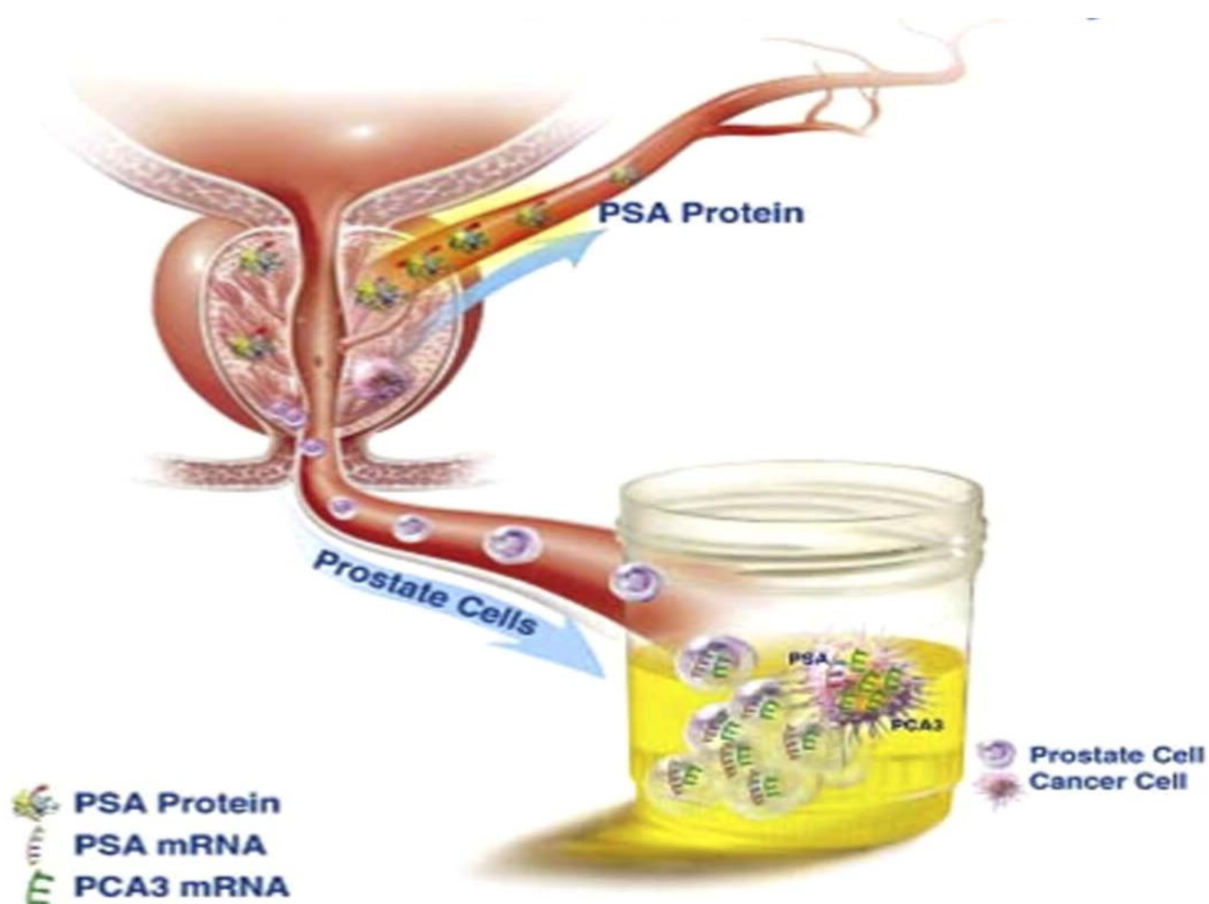

Figure 4. Diagram representing exfoliation of prostate cancer cells into urine.
Source: Reynolds et al. 2007

demonstrated to contain the highest concentration of prostatic and urethral secretions (Iwakiri et al. 1993). In case of organ confined disease, where the number of cancer cells exfoliated into the urine may be low, DRE can facilitate the detection of cancer cells in voided urine (Garret et al. 1976, Bologna et al. 1993) due to the weakening of cell-to-cell and cell-to-extracellular matrix contacts as a result of up-regulation and activation of extracellular matrix–degrading enzymes (Friedl and Brocker 2000). Importantly, for the patient it will be no extra burden since rectal examination is routinely performed in case prostate cancer is suspected. The major advantages of urine based assays are their non-invasive character and ability to monitor CaP with heterogeneous foci. Among disadvantages of urine-based assays are low amount of RNA regardless DRE and low quality of RNA which is a very common problem. During centrifugation of urine, putative extracellular nucleic acids of low molecular weight as well as

complexes containing nucleic acids with low density are lost (Menke et al. 2004). Furthermore, pelleted cells may be destroyed during the procedure, and the subsequently released nucleic acids will quickly be degraded by RNases which present in high quantities in urine. The acidic pH also leads to a decreased sensitivity of the RNA-based test giving inconsistent results (Hessels et al. 2005).

Table 7. Urine-detectable prostate cancer markers

Symbol	Description	Type of marker				References
		DNA	RNA	Protein	Metabolite	
8-OhdG [1]	8-hydroxydeoxyguanosine	+			+	130
AMACR	α-methylacyl coenzyme A racemase		+	+		224, 377
ANXA3	annexin A3			+		156, 284
BHUAE [1]	basic human urinary arginine amidase			+		188
BIRC5	baculoviral IAP repeat-containing 5 (alias survivin)		+	+		292, 344
EZH2	enhancer of zeste homolog 2		+			281, 283
F3	coagulation factor III (thromboplastin, tissue factor)			+		179
FGF1	fibroblast growth factor 1 (acidic)			+		131
FN1	bladder tumor fibronectin			+		268
GOLM1	Golgi membrane protein 1 (alias GOLPH2)		+			163, 340
GSTP1	glutathione S-transferase pi 1	+				88, 89, 92
LOH [1]	loss of heterozygosity	+				46, 319
MCM5	minichromosome maintenance complex component 5			+		306
MMP9	matrix metalloproteinases 9			+		127, 261
MSMB	microseminoprotein, beta		+	+		314, 349
PCA1 [1]	prostate cancer antigen 1			+		66
PCA3	prostate cancer antigen 3		+			27, 99, 116
PIP [1]	prostatic inhibin-like peptide			+		313
PSA	urinary prostate specific antigen			+		133
S100A9	S100 calcium binding protein A9 (alias calgranulin B)			+		249
SAR [1]	sarcosine				+	298
SPINK1	serine peptidase inhibitor, Kazal type 1		+			163, 326
SRD5A2	steroid 5-alpha-reductase type 2			+		173
TERT	telomerase reverse transcriptase		+			23, 192
TF	urinary transferrin			+		336
TFF3	trefoil factor 3		+			79
TMPRSS2-ERG	transmembrane protease, serine 2: v-ets erythroblastosis virus E26 oncogene homolog (avian)	+	+			164, 217
TMSB15A	thymosin beta 15a			+		126
TRPM8	transient receptor potential cation channel, subfamily M, member 8		+			281, 283
VEGF	vascular endothelial growth factor			+		127, 198

[1] the abbreviation is not standing for official gene symbol approved by the HUGO Gene Nomenclature Committee

Therefore, attempts are made to improve the procedure of sampling, sample storage, transportation and isolation when working with urine. If these steps are not well standardized they will have a profound negative effect on the diagnostic test result (Neumaier et al. 1998). Almost all urine-detectable prostate-specific markers have recently been reviewed (Table 7) (Muller et al. 2006, Downes et al. 2007, Jamaspishvili et al. 2010). For this reason, we focus here only on the promising urine-based markers which have been independently evaluated by several groups and markers which we used in our study (PCA3, AMACR, EZH2, GOLM1, MSMB, SPINK1, TRPM8).

1.5.1. PCA3 (DD3)

Using differential display analysis, PCA3 (DD3, differential display 3) was first described by Bussemakers et al. in 1999. The PCA3 gene maps to chromosome 9q21-22, a region that is not frequently affected in prostatic tumors. Previously it was suggested that PCA3 consists of four exons, and both alternative splicing (of exon 2) and alternative polyadenylation (at three different positions in exon 4) could produce several differently sized transcripts (Bussemakers et al. 1999). According to the recent report by Clarke et al. (2009), the PCA3 gene consists of six exons instead of four (exon 1, 2a, 2b, 2c, 3 and 4), from which exon 2a and 2b are newly identified. Exon 2 is deleted from most transcripts (present in only 5% of the transcripts) (Bussemakers et al. 1999) while exon 4 represents prostate-specific region as detected by both Gandini et al. (2003) and Clarke. et al (2009). Because of the fact that there is a high density of stop codons in all open reading frames, PCA3 belongs to the class of non-coding RNAs whose biological role in normal and diseased prostate still remains to be elucidated. The diagnostic and prognostic value of PCA3 in normal, hyperplastic and malignant prostate tissues was determined by qPCR and compared with TERT levels (de Kok et al. 2002). Interestingly, PCA3 was expressed in low levels in normal prostate but not in other normal tissues, blood or tumor samples of other origin than prostate. The median increase in mRNA expression in tumor tissues compared with nonmalignant prostate tissues was much higher for PCA3 (34-fold) than for TERT (6-fold) which is advantageous for detecting the few malignant cells shed into blood, urine, prostatic massage fluid or ejaculate suggesting the possible usefulness of the urinary PCA3 assay (de Kok et al. 2002).

PCA3 transcripts were also determined by RT-PCR both in tissues and urine sediments (Hessels et al. 2003). The amplification products (after 35 cycles of PCR) were quantified by time-resolved fluorescence based hybridization on streptavidin-coated microplates. Prostate tumors showed a 66-fold up-regulation of PCA3 in more than 95% of cancer cases compared to benign prostate tissue. For urine samples, the sensitivity of the assay was 67%, with 8 out of 24 cancer patients having low PCA3 levels. This might be in agreement with the hypothesis that false-negative samples may represent a subgroup of prostate tumors that have less tendency to invade the prostate ductal system and thus, shed less cells into the urine (Tinzl et al. 2004). Fourteen out of 84 men with negative biopsies (specificity 83%) had high PCA3 levels and their follow-up using repeated biopsies would be of great interest. This could confirm the fact that the increase in PCA3 can precede the histological diagnosis of CaP. The same research group similarly analyzed the next 583 patients undergoing biopsy. The sensitivity for the test was 65% and the specificity 66% with this cohort of patients (van Gils et al. 2007a).

Fradet et al. (2004) analyzed 517 patients undergoing biopsy at five centers and reported a sensitivity of 66% and specificity 89%. They used uPM3™ assay which comprise isothermic nucleic acid sequence based amplification (NASBA, Malek et al. 1994) and detection of the amplification products by real-time fluorescence using specific beacon probes (Tan et al. 2000). Despite good performance, the uPM3™ was withdrawn from the market after introduction of the transcription-mediated amplification method called APTIMA® assay (Groskopf et al. 2006; Gen-Probe, San Diego, CA, USA; PROGENSA™ for European countries) which measures both PCA3 mRNA and PSA mRNA in first-catch urine collected following a DRE. APTIMA® assay provides several advantages: i) The ability to use whole urine and detection of lower concentrations of RNA in clinical samples (as opposed to urine sediments used in the methods discussed above). ii) The target capture technology using magnetic particles is more effective and user-friendly than are the common RNA extraction methods. iii) The APTIMA PCA3 assay can be completed in <6 h and because of its robustness and reproducibility it can be implemented in the clinical laboratory (Groskopf et al. 2006). Several large studies have confirmed the good performance of this assay (Marks et al. 2007, Nakanishi et al. 2008, Haese et al. 2008, Sokoll et al. 2008, Deras et al. 2008) and the clinical results have recently been reviewed (Kirby et al. 2009). Nakanishi et al. (2008) have recently demonstrated that the PCA3 score is significantly associated with tumor volume and Gleason score in prostatectomy specimens, suggesting that the

urinary PCA3 score may be a novel molecular marker not only for CaP detection, but also for the classification of men diagnosed with CaP. Although the results from some institutes are promising, the diagnostic value needs to be further validated in a multicenter settings and to be followed up to show if indeed the PCA3 urine test is able to "predict" the presence of CaP.

1.5.2. AMACR

AMACR, an α-methylacyl coenzyme A racemase (also known as P504S) is involved in β-oxidation of branched-chain fatty acids and fatty acid derivates. AMACR has been demonstrated to be highly over-expressed at both protein (Zhou et al. 2002) and mRNA levels in localized prostate cancer (Jiang et al. 2001, Rubin et al. 2002) but down-regulated in metastatic androgen-independent prostate cancer suggesting a hormone-dependent expression of AMACR (Rubin et al. 2002, Kuefer et al. 2002). Currently, AMACR is used as an adjunct IHC marker in conjunction with p63 and high molecular-weight cytokeratins for diagnosis of prostate cancer (Luo et al. 2002, Rubin et al. 2002). Interestingly, Rubin et al (2005) observed association of decreased AMACR expression in localized CaP with the worse disease outcome and PSA recurrence, suggesting its possible use as a marker of prognosis.

AMACR is readily detectable in urine of prostate cancer patients and is nearly absent in urine of patients without CaP (Rogers et al. 2004). Western blot analysis for AMACR was used on voided urine after TRUS and biopsy, showing a 100% sensitivity and 58% specificity for CaP detection in the group of patients with negative biopsy findings (Rogers et al. 2004). In another study the quantification of AMACR transcripts normalized to PSA transcripts in prostate secretions was predictive of CaP yielding sensitivity of 70% and specificity of 100% (Zielie et al. 2004). Zehenter et al. (2006) used a similar design to reveal increased AMACR transcript levels in 5 of 7 patients with T1-T2 CaP. However, Laxman et al. (2008) could not confirm diagnostic power of AMACR when analyzed univariately. Attempts have been made to evaluate it in combination with other biomarkers in order to improve diagnostic accuracy for prostate cancer. Ouyang et al. (2009) reevaluated the diagnostic potential of urinary AMACR transcript with a widely tested PCA3 increasing assay sensitivity from 70-72% (AMACR-PCA3) to 81% and specificity from 59-71% (AMACR-PCA3) to 84%, when both markers were combined. Prior et al. (2010) reported that combination of methylation status of either GSTP1 or RASSF1A and

serum levels of MMP-2 and AMACR also improved significantly the diagnosis of CaP compared to the PSA test alone.

1.5.3. GSTP1 as an epigenetic marker

Hypermethylation of CpG islands, located within promoter regions of tumor suppressor genes, is an important mechanism for gene inactivation and has been described in almost every tumor type (Esteller 2002). Such epigenetic changes may result in the disruption of key regulatory pathways leading to cancer transformation and progression (Baylin and Chen 2005). The most common method of identifying hypermethylation regions involves chemical conversion of methylated cytosines to uridine residues by sodium bisulfite followed by amplification by PCR and comparison of uridine/ cytosine (unmethylated) ratios through a variety of means, including electrophoresis mini-sequencing and hybridization with mutation-sensitive oligonucleotide probes.

GSTP1 (glutathione-S-transferase P1) belongs to a family of enzymes involved in protecting DNA from free radicals. CaP is associated with the loss of GSTP1 expression due to promoter hypermethylation. To date, this DNA alteration appears in more than 90% of prostatic carcinoma tissues and several studies have also been done on urine samples (Goessl et al. 2001a, Goessl et al. 2001b, Jeronimo et al. 2002, Gonzalgo et al. 2003). In these studies the sensitivity was found to be between 19% and 76% and specificity ranged from 56% to 100%. The lowest sensitivity was found in the study in which urine was collected without previous prostatic massage (Jeronimo et al. 2002). When assessed, no significant association was found with either Gleason score (Goessl et al. 2001a, Goessl et al. 2001b) or tumor stage (Jeronimo et al. 2002, Gonzalgo et al. 2003).

1.5.4. Other potential urinary biomarkers

MMPs (matrix metalloproteinases) have been implicated in invasion and metastasis of human malignancies. Moses et al. (1998) used substrate gel electrophoresis (zymography) to determine MMPs in the urine of patients with a variety of cancers. MMP9 yielded better sensitivity (64%) than MMP2 (39%) for CaP detection while specificities were 84% and 98%, respectively. Several unidentified urinary gelatinase activities with molecular weights >125 kDa were also detected and the same group has recently used chromatography, zymography and mass

spectrometry for their identification (Roy et al. 2008). The 140, 190 and >220 kDagelatinase species were identified as MMP9/TIMP1 complex, ADAMTS7 and MMP9 dimer, respectively. MMP9 dimer and MMP9 were independent predictors for distinguishing between patients with prostate and bladder cancer ($p<0.001$ for each). Urinary MMP and VEGF (vascular endothelial growth factor) levels were also reported as predictive markers of 1-year progression-free survival in cancer patients treated with radiation therapy (Chan et al. 2004). In regard to VEGF, urinary levels have been found to be significantly higher in CaP patients than in healthy controls (Miyake et al. 2005) and were also predictive of survival of hormone refractory CaP (Bok et al. 2001).

TERT (telomerase reverse transcriptase) maintains the telomeric ends of chromosomes and if telomerase is active, cancer cells may escape cell cycle arrest and replicative senescence. Several groups have measured telomerase activity with the telomeric repeat amplification protocol assay and obtained sensitivities of 58%, 90% and 100% and specificities of 100%, 87% and 89%, respectively (Meid et al. 2001, Vicentini et al. 2004, Botchkina et al. 2005). Meid et al. (2001) also found a significant association between Gleason score and telomerase activity. Crocitto et al. (2004) measured TERT mRNA expression by reverse transcription-PCR and obtained sensitivity and specificity of 36% and 66%, respectively.

GOLM1/GOLPH2 (golgi membrane protein 1) is a cis-Golgi phosphoprotein of yet unknown function that has been described in liver disease as more sensitive serum marker of hepatocellular carcinoma than α-fetoprotein (Kladney et al. 2000, Marrero et al. 2005). GOLM1 expression has been reported to be upregulated at both mRNA (Lapointe et al. 2004) and protein level in localized prostate cancer tissues (Kristiansen et al. 2008, Varambally et al. 2008). Laxman et al. (2008) evaluated GOLM1 with other urine markers and demonstrated that its mRNA levels could serve as significant predictors of prostate cancer. GOLM1 urine detection was further validated by Varambally et al. (2008) confirming that GOLM1 transcript levels significantly predicted the presence of CaP and could outperform serum PSA.

SPINK1/PSTI/TATI (serine peptidase inhibitor, Kazal type 1) was isolated from bovine pancreas and human pancreatic juice and its normal function is thought to inhibit serine proteases such as trypsin (Paju et al. 2006). SPINK1 expression has recently been described at both mRNA and protein levels in prostate cancer (Paju et al. 2006, Paju et al. 2007). Interestingly, SPINK1 over-expression might be a late event in prostate cancer and plays important role in the

development of aggressive androgen-independent but TMPRSS2-ETS negative CaPs (10% of all cases) due to the specific genetic lesions (Tomlins et al. 2008a). SPINK1-positive tumors may constitute of unique molecular subtype of prostate cancer arising from a different prostate progenitor cell type and not attributable to known gene fusion events (Tomlins et al. 2008a). In one study SPINK1 protein expression was associated with higher Gleason grade and elevated serum levels (Paju et al. 2007). SPINK1 over-expression might represent a significant predictor of biochemical recurrence independent of various clinico-pathological parameters. For the first time, Tomlins et al. (2008a) demonstrated a feasibility of detection of SPINK1 in urine and its possible implication in urine-based analysis. Further, Laxman et al. (2008) evaluated SPINK1 in combination with other potential urine biomarkers.

EZH2 (enhancer of zeste homolog 2) is a member of the polycomb group of genes (PcG), which are important for transcriptional regulation through nucleosome modification, chromatin remodeling, and interaction with other transcription factors (Simon and Tamkun 2002). EZH2 serves as a histone methyl transferase (HMT) and disruption of EZH2 expression may lead to dysregulation of genes critical for the G2-M transition (Simon and Tamkun 2002). Increased expression of EZH2 was found in 34% of human cancers including prostate cancer (Bracken et al. 2003). EZH2 is significantly over-expressed in metastatic and hormone-refractory CaP and tumors with higher expression have worse prognosis (Varambally et al. 2002, Saramaki et al. 2006). Though Saramaki et al. (2006) found no association between EZH2 copy number and Gleason score and pT-stage. In the study of Rhodes et al. (2003) EZH2 protein expression was found to be associated with CaP recurrence in combination with E-cadherin expression. Multi-gene expression study of CaP showed that EZH2 in combination with TRPM8 and prostein outperformed PCA3 better than single marker analysis (Schmidt et al. 2006). Similarly, EZH2 was included in 4- and 5-gene model of biomarker evaluation and could successfully discriminate early prostate cancer from non-tumor patients with the highest sensitivity and specificity of the test (Schneider et al. 2008).

TRPM8/trp-p8 (transient receptor potential cation channel, subfamily M, member 8) is a member of the transient receptor potential (trp) family of Ca^{2+} channel proteins (Zhang and Barritt 2004). Tsavaler et al. (2001) found high levels of TRPM8 in both benign prostate hyperplasia and in prostate carcinoma cells, and low levels in normal prostate epithelial cells. Henshall et al. (2003) observed that with anti-androgen therapy, the expression of TRPM8 was

greatly reduced. They also showed that TRPM8 expression is decreased when prostate cancer cells become androgen-independent, suggesting that TRPM8 is regulated by androgens. Fuessel et al. (2003) have studied the expression of TRPM8 in relation to other established and potential prostate cancer markers (PSA, KLK2, PSCA). On the basis of relative amounts of mRNA, TRPM8 expression (but not PSA expression) in malignant tissue was significantly higher than that in non-malignant tissue. When only organ-confined prostate cancer tissue samples were considered, this difference for TRPM8 was greater. In malignant tissue specimens from patients with a total serum PSA concentration in the 'grey-zone' of 4–10 ng/ml, TRPM8 mRNA, but not PSA mRNA was significantly elevated (Fuessel et al. 2003). Schmidt et al. (2006) observed the highest expression of TRPM8 along with PCA3 in CaP vs tumor-free patients among other nine prostate-related genes. TRPM8 was also able to discriminate organ-confined disease from nonorgan-confined one, losing again expression in advanced tumor stages, suggesting its use as a marker of prognosis (Schmidt et al. 2006). Schneider et al. (2008) evaluated TRPM8 in multiplex model along with PCA3 and EZH2 in FFPE (formalin-fixed paraffin-embedded) tissues with promising results. Bai et al. (2010) firstly demonstrated feasibility of TRPM8 detection in body fluids such as blood and urine. Interestingly, levels of TRPM8 were elevated in metastatic CaP while there was no significant difference in its mRNA levels between localized disease and healthy men, suggesting its prognostic role for distinguishing metastatic disease from clinically localized CaP at the time of diagnosis (Bai et al. 2010).

MSMB (human β-microseminoprotein), also known as prostatic inhibin peptide (PIP), β-inhibin, or prostatic secretory protein of 94 amino acids (PSP94), is one of the major proteins secreted by prostatic epithelial cells (Green et al. 1990). MSMB accounts for 20% of the total seminal plasma protein and is reliably detectable in serum and urine (Teni et al. 1988), though its exact function remains to be fully elucidated. Interestingly, levels of MSMB mRNA expression and protein are high in normal and benign prostate tissue and lowered or lost in prostate cancer (Tsurusaki et al. 1998). Two independent studies demonstrated that MSMB status is an independent prognostic factor for survival after androgen deprivation therapy and radical prostatectomy regardless no correlation with Gleason grades (Tsurusaki et al. 1998, Sakai et al. 1999). Recently, Girvan et al. (2005) provided additional prognostic information on MSMB since its increased protein expression was associated with worse survival outcomes after RP.

Suggestion to use MSMB in patients with low serum PSA levels and its evaluation in urine as a dignostic/prognostic marker in multiplex panel was also offered by Whitaker et al. (2009).

ANXA3 (annexin A3) is one of the most recent CaP biomarkers. It belongs to a family of calcium and phospholipid binding proteins that are implicated in cell differentiation and migration, immunomodulation, bone formation and mineralization in CaP metastasis (Gerke et al. 2005). The presence of ANXA3 in urinary exosomes (Pisitkun et al. 2004) and prostasomes (Gerke et al. 2005) might be the reason for its remarkable stability in urine (Schostak et al. 2009). ANXA3 has been quantified by Western blot in the urine samples of patients with negative DRE findings and low total PSA (2 to 10 ng/ml) which is the clinically relevant group facing the biopsy dilemma (Schostak et al. 2009). Combined readouts of prostate specific antigen and urinary annexin A3 gave the best results with the area under the ROC (receiver operating characteristics) curve of 0.82 for a total prostate specific antigen range of 2 to 6 ng/ml, 0.83 for a total prostate specific antigen range of 4 to 10 ng/ml and 0.81 in all patients. Annexin A3 has an inverse relationship to cancer and, therefore, its specificity was much better than that of the prostate specific antigen. The staining pattern of ANXA3 in prostatic tissue was reported to correlate with Gleason score and was able to differentiate less and more malignant cases (Kollermann et al. 2008). Moreover, staining had also apparent correlation during the whole process of prostatic transformation, ranging from benign prostatic hyperplasia via PIN to the various stages of CaP.

Sarcosine (N-methyl derivative of the amino acid glycine) has recently been identified as a differentially expressed metabolite that is greatly elevated during CaP progression to metastasis and, importantly, can be detected non-invasively in urine (Sreekumar et al. 2009). It is worth noting that a relationship has been found between the sarcosine pathway, androgen signaling and ETS family of gene fusions. Sarcosine levels were directly increased by androgens in VCaP (ERG-positive) and LNCaP (ETV1-positive) cell lines indicating that components of the sarcosine pathway may have potential as biomarkers of CaP progression along with AR and ETS gene fusions and could also serve as a new target for therapeutic interventions.

1.5.5. Multiplex analysis of urine markers and future directions

Multiplex or combined model of urine biomarker analysis has several advantages in CaP detection. Most importantly, it does not ignore the heterogeneity of CaP, it is noninvasive and can detect prostate cancer more accurately than do single marker tests (Landers et al. 2005, Schmidt et al. 2006). Recent studies have characterized the clonality and heterogeneity of TMPRSS2-ERG fusion in multifocal CaP (Barry et al. 2007, Rajput et al. 2007). Current biopsy strategies may miss heterogenous tumor foci and urine-based assay would have a great advantage because the cells from multiple cancerous foci of whole prostate could be released and collected (Laxman et al. 2008).

Laxman et al. (2008) reported that a multiplex panel of urine transcripts outperforms PCA3 transcript and serum PSA alone in detecting CaP. Expression of seven putative prostate cancer biomarkers was measured (PCA3, PSA, GOLPH2, SPINK1, AMACR, TMPRSS2-ERG, TFF3) by qRT-PCR in urine samples. They showed that increased expression of GOLPH2, SPINK1, PCA3 and TMPRSS2-ERG fusion status were significant predictors of CaP (sensitivity 66% and specificity 76%). Improved sensitivity has also been reported for combined detection of TMPRSS2-ERG and PCA3 transcripts in urine in patients with serum PSA$\geq$ 3 ng/ml and/or an abnormal DRE (Hessels et al. 2007, Salami et al. 2011). Separate sensitivities for detecting TMPRSS2-ERG fusion and PCA3 transcripts in the urine were 37% and 62%, respectively, but by combining both markers the sensitivity increased to 73% (Hessels et al. 2007). Except Laxman et al. (2008) several scientific groups has also attempted combined evaluation of cancer-specific biomarkers in CaP (Schmidt et al. 2006, Schneider et al. 2008, Ouyang et al. 2009, Cao et al. 2010). Schmidt et al. (2006) evaluated nine-prostate-related genes by qRT-PCR and showed that inclusion of EZH2, prostein and TRPM8 significantly increased diagnostic power when evaluated along with PCA3. In the study of Schneider et al (2008) two optimized mathematical models (4- and 5-gene models) were evaluated in which EZH2, hepsin, PCA3, prostein, and TRPM8 were used. Compared to single marker analyses these models showed higher sensitivity and specificity for prostate cancer detection. As the study was done on material obtained from artificial needle core biopsies (after radical prostatectomy), Schneider and colleagues suggested that transfer of this approach to urine samples could be promising. Furthermore, Ouyang et al. (2009) showed that combined evaluation of urine AMACR and PCA3 could significantly improve sensitivity and accuracy of CaP detection, i.e. dual marker test

increased sensitivity from 70-72% (AMACR-PCA3) to 81% and specificity from 59-71% (AMACR-PCA3) to 84%. Recently, Cao et al. (2010) evaluated PCA3 along with TMPRSS2-ERG, annexin A3 and sarcosine in “grey zone” patients as well as in patients with wide range of PSA. The methods used in the study were following: qPCR (for PCA3 and TMPRSS2-ERG detection in urine samples), Western blot (for annexin A3) and liquid chromatography-mass spectrometry (for quantitative measurement of sarcosine). Using multivariate logistic regression analysis, AUCs of this panel in both cohorts were 0.840 and 0.856, respectively, being higher than that of any single biomarker (PCA3: 0.733 and 0.739; TMPRSS2: ERG: 0.720 and 0.732; Annexin A3: 0.716 and 0.728; Sarcosine: 0.659 and 0.665, respectively). Using such approach Cao and colleagues confirmed again the usefulness of combined-multiplex models in CaP detection.

Novel methods might be used for detection of multiple biomarkers in urine samples in future. Recently, simple and sensitive exon array-based assays were invented for simultaneous detection of multiple fusion genes in specimens with only a minor population of tumor cells (Lu et al. 2008, Skotheim et al. 2009). Current methods seem to have a lot of drawbacks compared to the novel array-based assays. For example, the most commonly used FISH (Rajput et al. 2007, Perner et al. 2006) has relatively low resolution, and therefore, cannot accurately determine different fusion variants in highly heterogeneous samples with small percentages of tumor cells. Quantitative PCR and sequencing are relatively easy to perform (Wang et al. 2006, Soller et al. 2006, Laxman et al. 2008) however multiple sets of primers/probes are required for assessing multiple potential fusion variants. Array CGH (comparative genome hybridization) has a high resolution but often fails when there is normal cell contamination (Perner et al. 2006, Hermans et al. 2006).

During last several decades no marker other than PSA has been used so far in the clinical routine diagnosis of prostate cancer. PCA3 as a urine marker has the potential to detect CaP in previous biopsy-negative patients and especially to detect prostate cancer at its earlier stages. The combination the novel urine and serum markers should be tested in the future in order to either increase the specificity of serum PSA or identify markers/marker combinations which could diagnose CaP more accurately. Future studies will be directed at improving the performance of the combined models by evaluating additional markers and improving risk stratification and patient counseling before treatment decision making.

2. AIMS OF THE STUDY

Prostate cancer (CaP) is the second most frequently diagnosed cancer and the third most common cancer causing death in men (Damber et al. 2008). CaP is diagnosed by histological examination of prostate tissue that is obtained by ultrasound guided transrectal biopsies which are predominantly indicated by increased serum PSA and/or an aberrant digital rectal examination. However, the increase in PSA can reflect benign as well as malignant prostatic disease, resulting in a negative biopsy rate of 70–80%. This means that a large number of patients is unnecessarily undergoing prostate biopsies. Therefore, there is a growing need for CaP markers that can increase the diagnostic specificity and differentiate CaP from non-cancerous diseases. Novel markers are urgently needed for differentiation of harmless CaP from aggressive disease and for identification of progression towards androgen independence at an early stage.

The aims of the study were as follows:

1. To identify expression differences in kallikrein-related peptidases 7 and 11 (KLK7/KLK11) between CaP and BPH using immunohistochemistry on radical prostatectomy samples and to determine their possible associations with clinicopathological parameters.
2. To validate diagnostic ability of PCA3 both for patients with wide range of PSA levels and for „PSA dilemma“ patients with PSA levels of 3-15 ng/ml.
3. To test putative urine markers in multiplex setting and identify satisfactory model which could detect prostate cancer more accurately in “PSA dilemma” patients than do other widely accepted tests like serum PSA and urinary PCA3.

3. PATIENTS, MATERIALS AND METHODS

3.1. Immunohistochemical localization and analysis of kallikrein-related peptidases 7 and 11 in paired cancer and benign foci in prostate cancer patients

3.1.1. Patients and tissue specimens selection

Seventy cases of prostate cancer were identified as clinically localized (T1-T2, n=39) or locally advanced (T3-4, N0-1, M0, n=31). The study included patients who had not received neoadjuvant treatment but who had undergone radical prostatectomy as initial treatment following positive biopsy results. The study was approved by the ethics committee of the Medical Faculty of Palacky University. Clinical (TNM, clinical stage, serum total PSA) and pathologic (pT, Gleason score and grade, pN) characteristics were defined according to the WHO classification (Table 8). Paired CaP and BPH foci were identified in each patient and tissue sections from these were then used for further IHC analysis.

Table 8. Clinical and pathologic characteristics of patients for immunohistochemical study of kallikreins 7 and 11

		Localized tumors (n)*	Advanced tumors (n)*
Age	51-59	15	8
	60-69	26	15
	70-73	1	5
Gleason Score	<7	21	8
	≥ 7	18	23
Clinical Stage	II	39	0
	III	0	25
	IV	0	6
Serum PSA	<4 ng/ml	8	3
	<8 ng/ml	14	10
	≥8 ng/ml	17	18

** Localized cases defined as pT1-pT2, advanced pT3-pT4*

3.1.2. Immunohistochemistry

IHC was performed according to our routine protocol. Seventy archived paraffin-embedded tissues were dewaxed and rehydrated with xylene and ethanol. After immersion in citrate buffer (pH 6), the sections underwent pressure cooking pretreatment for 5 minutes for optimal antigen retrieval. The slides were immunostained for KLK7 and KLK11 by rabbit polyclonal primary antibodies (Abs) provided by Prof. Diamandis (Mount Sinai Hospital, Toronto, Canada, dilution 1:200). PSA (clone ER-PR8, Dako, Denmark, dilution 1:25) and PSMA (clone YPSMA-1, Abcam, UK, dilution 1:1000) were stained by mouse monoclonal Abs and the secondary antibodies were applied for 60 minutes at room temperature. Specific binding was detected usingthe peroxidase/DAB based detection kit EnVision, Dako. Staining intensity, positive cell fraction, staining heterogeneity and cellular localization were determined for all four proteins in both BPH and CaP foci for each patient. Positivity was classified according to the percentage of all positively stained tumor cells and the intensity of the IHC staining. Staining intensity was categorized from 0 to 2 as follows, 0-0,5 no staining, 1 low, 1.5 moderate and 2 high staining, respectively. Histoscores were derived for further statistical analysis (staining intensity x percentage of cells).

3.1.3. Statistical analysis

The software Statistica 8.0 (Statsoft, CZ) was used for statistical analysis. As the distribution of variables was non Gaussian, differences in protein expressions between CaP and BPH were evaluated by nonparametric Wilcoxon Signed Ranks Test. Kruskal-Wallis one-way analysis of variance by ranks was used to compare expressions between low and high grade CaP (Gleason score <7 vs. ≥ 7), in organ-confined and advanced prostate cancers (pT1, pT2 vs. $\geq$ pT3, pT4) and in different clinical stages (II, III, IV). The correlation between different variables was assessed by the Spearman Rank correlation coefficient. $P<0.05$ was considered as the level of significance.

3.2. Urine-based detection of prostate cancer

3.2.1. Patients

Urine samples were obtained from 314 randomly chosen patients who were scheduled for needle biopsy, radical prostatectomy or other examination owing to urological complaints. The final

number of patients included in the statistical analysis was 176 due to exclusion of samples with low quality or concentration of RNA. The cohort consisted of both positive needle biopsy and radical prostatectomy patients (CaP, n=91) and noncancer patients (nonCaP, n=85) with benign prostatic hyperplasia or prostatitis. Clinicopathological characteristics were defined according to the WHO classification and are listed in Table 9.

Table 9. Patients characteristics for urine biomarkers project

	Full range of PSA		PSA (3-15 ng/ml)	
Characteristics	CaP	NonCaP	CaP	NonCaP
Age range	46-86	26-78	46-79	53-75
No. patients	91	85 [1]	62	42
serum PSA (ng/ml)				
less than 3	3	32	0	0
3-15	64	43	62	42
> 10	40	4	0	0
Gleason scores (GS)				
< 7 (low grade CaP)	22	-----	21	-----
≥ 7 (high grade CaP)	69	-----	41	-----
DRE (cT)				
positive (cT2-4)	42	5	24	4
negative (cT0-1)	49	80	38	38
Cancer stages [2]				
localized	51	-----	45	-----
locally advanced	34	-----	17	-----
metastatic	6	-----	0	-----
Risk groups [3]				
low	10	-----	8	-----
Intermediate	36	-----	33	-----
high	45	-----	21	-----

CaP, prostate cancer; NonCaP, non-cancer patients; PSA, prostate-specific antigen; GS, Gleason scores; DRE, digital rectal examination; cT, clinical tumor size

[1] serum PSA was not measured in 6 nonCaP patients

[2] localized CaP is defined as (T1-T2, N0, M0); locally advanced CaP is defined as (T3-T4, N0-N1, M0)

[3] Risk categories were determined based on both clinical and pathological data: low (T1–T2a, GS ≤ 6 and PSA ≤ 10 ng/ml), intermediate (at least one of the following: T1-T2b-c, GS ≤ 7 and 10 < PSA ≤ 20 ng/ml or T1–T2, GS = 7 and PSA ≤ 10 ng/ml), high (at least one of the following: T3–T4, GS > 7, or PSA > 20 ng/ml).

3.2.2 Urine collection, RNA isolation and preamplification

After signing an informed consent statement approved by the Ethics Committee of the Medical Faculty of Palacky University, patients were asked to provide a 20 to 50 ml urine after digital rectal examination. Urine specimens were centrifuged (250 g, 5 min), the sediment was lysed in a lysis buffer (provided by Urine Exfoliated Cell RNA Purification Kit) with β-mercaptoethanol

and stored at 4°C up to 3 days. Samples were collected twice a week and then immediately isolated at the Department of Pathology. We observed decrease neither of concentration nor of RNA quality when test samples were compared (LNCaP cells in the lysis buffer for 1, 3 and 5 days). Total RNA isolation was performed by the Urine Exfoliated Cell RNA Purification Kit (Fisher Scientific, USA), quantified by Nanodrop and 260/280nm ratio and stored at -80°C. RNA (100 ng) was then pretreated with Dnase I (Invitrogen) and reverse transcribed with SuperScript® III Reverse Transcriptase (Invitrogen). Five µl of cDNA from each sample were pre-amplified with two sets of primer pairs in order to avoid primer-dimer formation and unspecific amplification (mix.1: PCA3, PSA, AMACR, SPINK1, EZH2 and mix.2: GOLM1, TRPM8, MSMB; Table 12). After 14 cycles at 94-60-72°C preamplified cDNA products were diluted in water (1:10) and stored at -20 °C.

3.2.3. Uniformity test

cDNA preamplification was performed according to Noutsias et al. (2008) and the TaqMan® PreAmp Master Mix Kit which is intended for samples with limited amount of RNA. Uniformity test should be performed whether all amplicons are amplified uniformly without bias. Briefly, relative quantification was done for both nonamplified and preamplified cDNA. Uniformly amplified targets must produce $\Delta\Delta C_t$ values within ± 1.5. Uniformity test was first performed for PCA3/PSA with five samples when all of them passed the limit ± 1.5 $\Delta\Delta C_t$. Multiplex uniformity test was performed for eight samples, of which three were tested also for the whole transcriptome amplification (WTA) (used by Laxman et al. 2008; Sigma-Aldrich cat. number WTA1, Transplex and Omniplex technology patented by Rubicon Genomics). Nine (different transcripts) out of 49 values (some transcripts were not expressed in particular samples) preamplified by PCR were slightly out of the limit (up to ±3 $\Delta\Delta C_t$). Suprisingly, majority of the trascripts (12 out of 19 values) preamplified by WTA were out of the limit with high errors (up to ±8.5 $\Delta\Delta C_t$), however, it should be emphasized that only three samples were tested.

3.2.4. Quantitative PCR analysis

Quantitative PCR was used to validate diagnostic ability of PCA3 in the randomly selected patients with serum PSA values of 0.1-587 ng/ml. The primer/probe sequences used for PCA3 were: FW-5´GCACATTTCCAGCCCCTTTAAA-3´ (our slightly modified version shifted 7 bp

right), RV-5´-GGGCGAGGCTCATCGAT-3´ and Taqman hydrolysis probe 5´-FAM-AGAAATGCCCGGCCGCCATC-BHQ1-3´ (Schmidt et al. 2006, Table 10).

Table 10. Primers and probes used in qPCR

Gene Symbol	Gene ID	Primers and probes	Positions on exons	Product
AMACR	NM_001167595.1	FW-5´-TCAACTATTTGGCTTTGTCAGG-3´	FW-exon 2/3	67 bp
		RV-5'-GTGAGAATCCGTATGCCCC-3'	RV-exon 3	
		UPL probe #29	exon 3	
EZH2	NM_004456.3	FW-5´-GATGATGGAGACGATCCTGAA-3´	FW-exon 5	80 bp
		RV-5'-GGGCGGCTTTCTTTATCAT-3'	RV-exon 5/6	
		UPL probe #65	exon 5	
GOLM1	NM_177937.1	FW-5´-GAGATGGAGGGCCCTGAG-3´	FW-exon 8	102 bp
		RV-5'-AAACCAGCAGAAACTGAGAGGA-3'	RV-exon 9	
		UPL probe #4	exon 8	
MSMB	NM_002443.2	FW-5´-AAATTTCATGTTGCACCCTTG-3´	FW-exon 3/4	140 bp
		RV-5'-CCATTCACTGACAGAACAGGTC-3'	RV-exon 4	
		UPL probe #22	exon 4	
PCA3[1]	NR_015342.1	FW-5´-GCACATTTCCAGCCCCTTTAAA-3´	FW-exon 3	113 bp
		RV-5´-GGGCGAGGCTCATCGAT-3´	RV-exon 4	
		5´-FAM-AGAAATGCCCGGCCGCCATC-BHQ1-3´	exon 4	
PSA[1]	NM_001648	FW-5'-GTCTGCGGCGGTGTTCTG-3'	FW-exon 2	88 bp
		RV-5'-GCCGACCCAGCAAGATC-3'	RV-exon 3	
		5'-FAM-CACAGCTGCCCACTGCATCAGGA-BHQ1-3'	exon 2	
SPINK1	NM_003122.3	FW-5´-CCTTGGCCCTGTTGAGTCTA-3´	FW-exon 1	72 bp
		RV-5'-CATTGTAACATTTGGCCTCTCTT-3'	RV-exon 2/3	
		UPL probe #47	exon 2	
TRPM8	NM_024080.4	FW-5´-TCAGAATAAGAAGGAACTCTCCAAA-3´	FW-exon 13	125 bp
		RV-5'-CAGCAGCATTGATGTCGTTC-3'	RV-exon 14	
		UPL probe #32	exon 14	

FW, forward primer; RV, reverse primer; FAM, 6-carboxyfluorescein; BHQ1, black hole quencher 1
[1] FW/RV primers and probes for PCA3 and PSA are used from Schmidt et al (2006) and Specht et al (2001), respectively

Two µl of preamplified and diluted cDNA were used for qPCR (Light Cycler® 480 real-time PCR system, Roche Applied Science). Duplicate reactions were performed for each sample with both positive and negative controls (see 3.2.4). Urine PSA (housekeeping gene) was measured to confirm enough prostate epithelial material and normalize PCA3 ($\Delta C_t = C_{tPCA3}-C_{tPSA}$) as the PSA mRNA in prostate tissues is unrelated to serum PSA levels and is not changed in prostate cancer (Meng et al. 2002, Hessels et al. 2003, Kirby et al. 2009). Samples that had PSA Ct values > 30 were excluded and deemed „non-evaluable“ though we did not exclude samples with negative C_t PSA values but strong positive PCA3 values. We then selected patients with serum PSA 3-15 ng/ml (extended PSA „grey-zone“, n=104) and multiplex evaluation was performed on

seven prostate cancer-related biomarkers (AMACR, SPINK1, EZH2, GOLM1, TRPM8, MSMB and PCA3) in order to improve diagnostic accuracy for "PSA dilemma" patients (Table 9). Primers and probes used for multiplex analysis were obtained from Generi Biotech and Universal probe library set, respectively (UPL, Roche, Cat. # 04683633001). UPL human-specific sets of 90 probes provides flexibility of design real-time PCR assays at any time without having to wait for synthesis and delivery of PCR probes. We used the ProbeFinder software (Roche®, UPL assay design center) to select an optimal combination of UPL probes and primers (Table 10).

The real-time PCR rections were performed in a final volume of 25 µl, containing 2 µl preamplified and diluted cDNA, 10 µl Master Mix (Light Cycler® 480 Probes Master, Roche), 0,6 µl of each forward and reverse gene-specific primers (final concentration 300 nM) and 0,2 µl UPL probe. qPCR was as follows: denaturation by a hot start at 95°C for 15 min, followed by 50 cycles of a three-step program (denaturation, annealing and extension 95°C-60°C-72°C, each for 20 seconds). Relative quantification was performed according to ΔC_t method using a reference gene ($\Delta C_t = C_{ttarget} - C_{tPSA}$) (Pfaffl et al. 2001) and inverse values of ΔC_t ($-\Delta C_t$) were used for subsequent statistical analysis and visualization (Laxman et al. 2008).

3.2.5. Intra and inter-assay variability

Intra-assay variation of our protocol was checked by duplicate qPCR reactions for all samples (when the difference between duplicates was higher than one cycle, reaction was repeated both for the transcript of interest and reference PSA). Inter-assay variation was monitored by running the same sample on 13 different qPCR plates in the study of PCA3 and PSA only [coeficient of variance (CV) 3.5% and 4.3%, respectively]. It was possible to analyze only 5 samples (including negative controls) for eight genes on a 96 well plate. For this reason, inter-assay variation for multiplex analysis was checked for the same sample only on 6 different qPCR plates (CV AMACR 0.8%, CV EZH2 1.2%, CV GOLM1 3.0%, CV MSMB 2.5 %, CV PCA3 1.7%, CV PSA 2.4%, CV SPINK 0.5% and CV TRPM8 1.3%). Variation of the whole procedure (reverse transcription, preamplification and qPCR) was evaluated by double PSA quantification on a subset of 47 samples (these samples were first evaluated for PCA3/PSA only and then again in the multiplex analysis without PCA3. Other consecutive samples were analyzed for all genes in the multiplex setting). Mean difference between Ct PSA values ($Ct_{duplex} - Ct_{multiplex}$) was –

0.47 (95% confidence intervals -0.78 and -0.16; minimum and maximum difference -2.97 and 2.25, respectively). Even if the above mentioned variabilities are acceptable, all transcripts of interest were always analyzed together with the reference PSA on the same plate.

3.2.6. Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA). As the distribution of variables was non Gaussian, the analysis of differences in biomarker expression between CaP and nonCaP was done using the nonparametric tests. Mann–Whitney test was used for comparisons of the relative expression of PCA3, AMACR, SPINK1, EZH2, GOLM1, TRPM8, MSMB transcripts between CaP and nonCaP patients, clinical and pathological stages, low and high grade tumors and different risk group patients. The correlation between gene expressions was assessed by the Spearman Rank correlation coefficient.

Univariate and multivariate logistic regressions were used to examine associations between prostate cancer diagnostic status and test variables and determine whether the marker or marker combination could predict the presence of the cancer. Akaike information criterion (AIC)-based backward stepwise selection strategy was used in order to exclude insignificant markers, i.e. seven combination of various markers were analysed, subtracting every single marker on each step until optimal combination was found. LOOCV (leave-one-out cross-validation) strategy (Laxman et al. 2008) was used in all steps to avoid overoptimization of AUCs in regression analysis. The validity and quality of the resulting logit models were assessed by Hosmer and Lemeshow goodness-of-fit tests (Hosmer and Lemeshow 2000), by *P* values of each regression parameter, and by the estimated AUC.

Receiver operating characteristic curves were calculated in order to assess the diagnostic power of each separate variable univariately and for the multivariate analysis by the area under curve (AUC) of the ROC curve which, in turn is an estimation of the rate of correct diagnoses. The ROC curve shows sensitivity and specificity of the binary diagnostic decision for varying cut points based on a single quantitative diagnostic variable or based on a multivariate strategy. The cross-validation was performed to correct the over-fit bias and give the cross-validated estimation of the AUC. Furthermore, optimal sensitivity, specificity, accuracy and, positive and

negative predictive values were calculated for selected markers and their combinations. Statistical significance was considered when two-sided $P<0.05$.

4. RESULTS

4.1. Kallikrein-related peptidases 7 and 11 in paired cancer and benign foci in prostate cancer

4.1.1. Immunohistochemical analysis of KLK 7 and 11 in cancerous and benign cells

Staining was always cytoplasmic for both KLK7 and KLK11. Prostatic secretions were also strongly positive for KLK7/11. We also observed positive staining of urothelium and seminal vesicle epithelium for KLK7. Noteworthy was the positive staining of tumor neovasculature and muscle cells with both anti-KLK7 and anti-KLK11 antibodies in some patients. The majority of CaP foci showed decreased expression of both KLK7 and KLK11 against BPH per section per patient (*P*=0.026 and *P*=0.001, respectively) (Figure 5), though expression heterogeneity was quite common, especially for KLK7 (Figure 6).

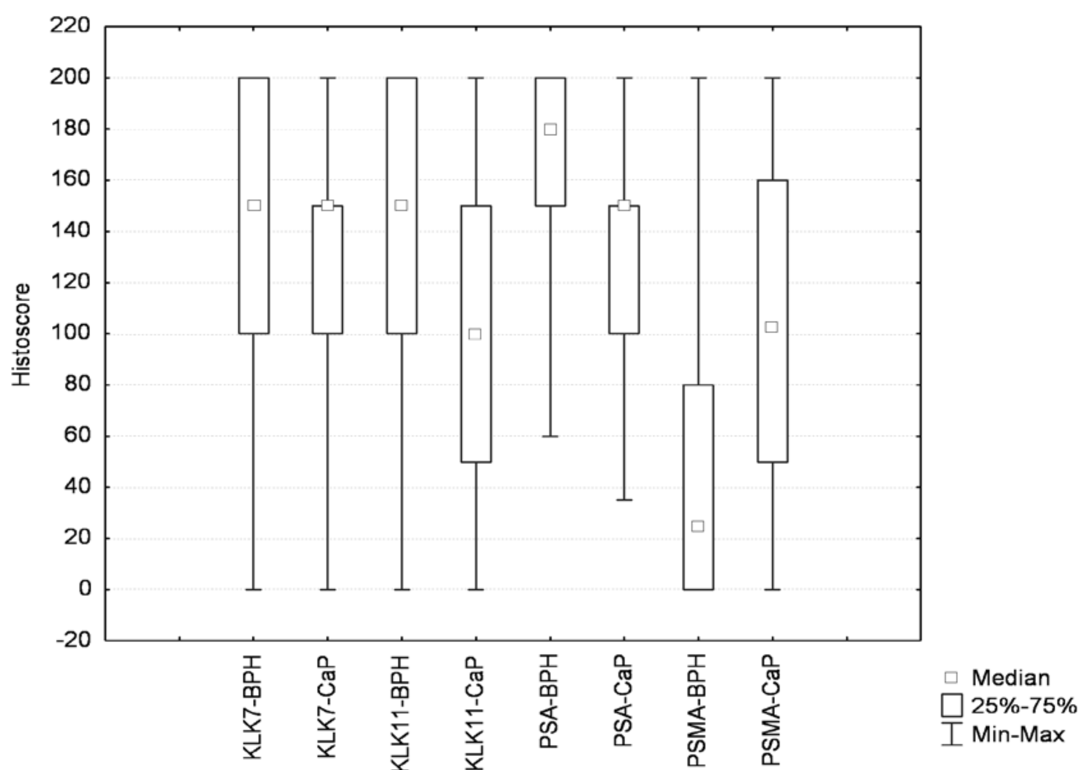

Figure 5. Differences in expression of selected proteins between BPH and CaP.

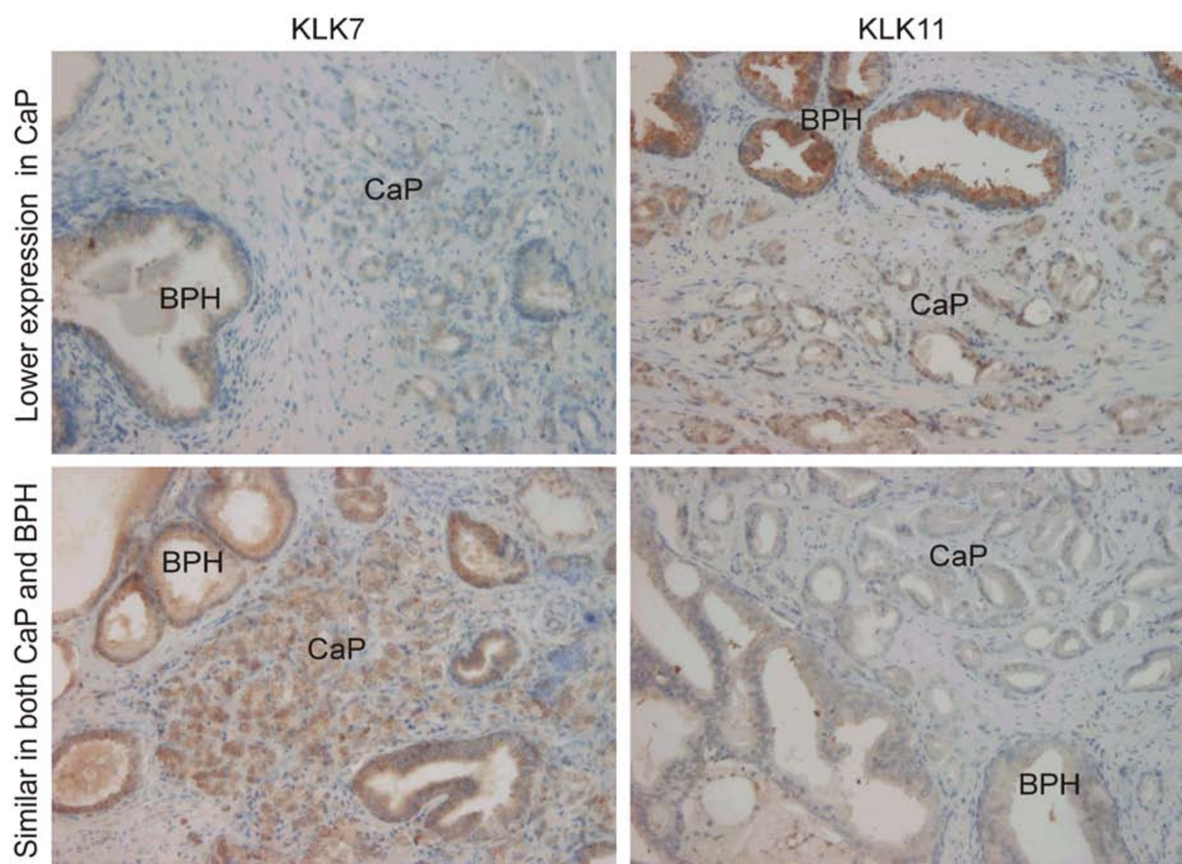

Figure 6. Heterogeneity of KLK7/11 expression in BPH and CaP.

Positive correlations were found for both KLK7 (Rs=0.74) and KLK11 (Rs=0.35) between CaP and BPH foci (Table 11). Moreover, statistically significant correlation was found between KLK7 and KLK11 (Rs=0.242, *P*=0.043) for the BPH foci. In addition, there was no significant correlation or significant differences between either Gleason score of <7 and ≥ 7 in prostate carcinoma, between pT2 and pT3-pT4 tumors or serum PSA values. However, we found a slight difference in KLK11 expression between advanced and localized cases with up-regulation in advanced ones (*P*=0.049).

Table 11. Spearman's correlation coefficient (Rs) for biomarker expressions in BPH and CaP

		BPH				CaP			
		KLK7	KLK11	PSA	PSMA	KLK11	KLK7	PSA	PSMA
KLK7-BPH	Rs	1.000	**.242**	-.224	.125	.094	**.738**	-.082	.107
	p	.	**.043**	.062	.304	.437	**.000**	.502	.378
KLK11-BPH	Rs		1.000	.039	-.117	**.348**	.202	-.126	-.080
	p		.	.751	.335	**.003**	.093	.300	.512
PSA-BPH	Rs			1.000	-.040	.068	-.140	.130	.118
	p			.	.740	.579	.247	.282	.331
PSMA-BPH	Rs				1.000	.071	.168	.036	**.240**
	p				.	.562	.165	.764	**.046**
KLK11-CaP	Rs					1.000	.155	-.107	-.020
	p					.	.200	.377	.872
KLK7-CaP	Rs						1.000	-.226	.144
	p						.	.060	.235
PSA-CaP	Rs							1.000	-.024
	p							.	.842
PSMA-CaP	Rs								1.000
	p								.

4.1.2. Tissue expression of PSA amd PSMA in CaP and BPH

We observed positive PSA staining of both non-cancerous and cancerous foci which was cytoplasmic with apical accentuation. Prostatic secretions were strongly positive in almost all patients. Staining intensity was stronger in BPH than in prostate carcinoma (P<0.001) with a trend to further decrease in less differentiated cancers (P=0.077, Figure 7). PSA expression did not correlate significantly with other proteins or clinicopathological data.

We also detected high PSMA expression at the luminal-apical surface of glandular structures and in the tumor neovasculature. Strong positive staining could also seen in the muscle cells and prostatic secretions. Cancer cells showed intense, significantly higher expression than normal/BPH glands (Figures 6 and 7), though cells lacking PSMA staining could also be identified close to the most intensively stained tumor cells indicating staining heterogeneity of tumor cells. We also found a trend to increased PSMA expression in less differentiated cancer cells in relation to Gleason score (<7 vs. ≥ 7; P=0.114) and pT stage (pT2 vs. pT3-pT4; P=0.083).

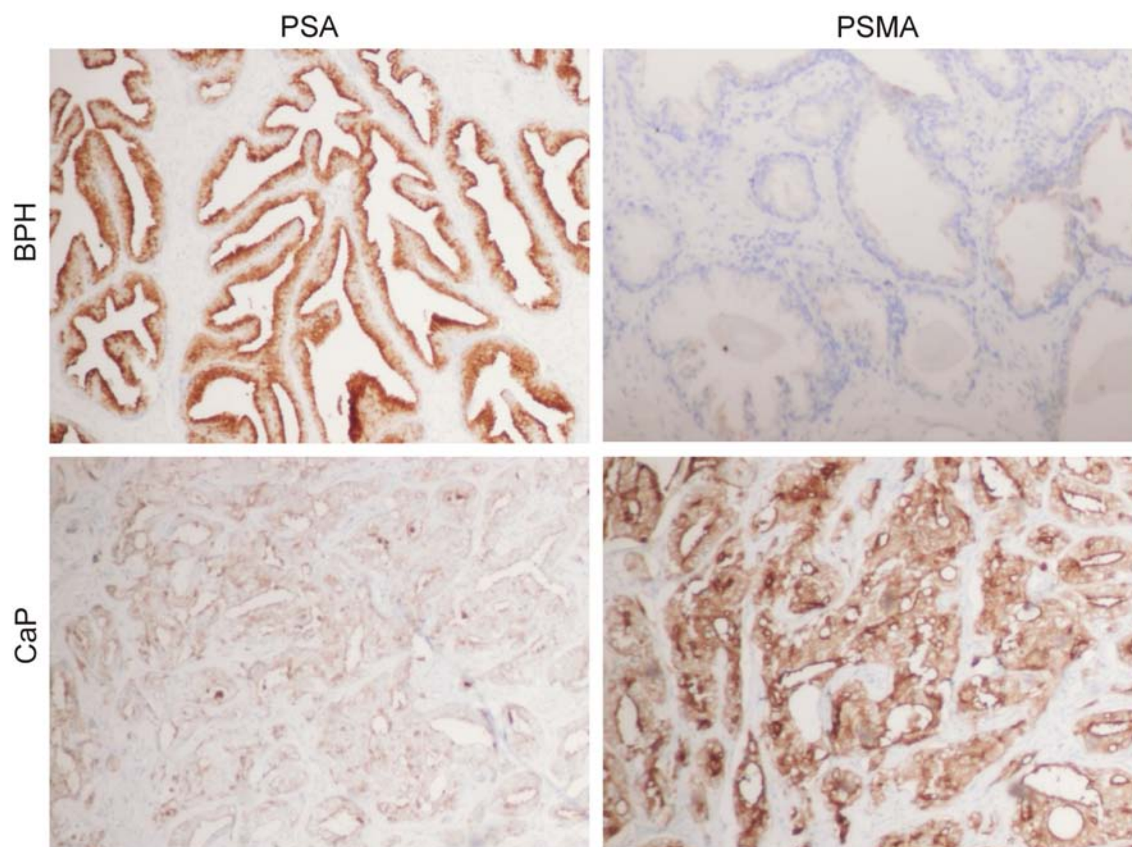

Figure 7. Expression of PSA and PSMA in BPH and CaP.

4.2. Urine-based detection of prostate cancer

4.2.1. Diagnostic power of PCA3 in patients with elevated serum PSA levels

First, we evaluated diagnostic ability of PCA3 in the randomly selected patients without restriction of serum PSA values (full range PSA=0.1-587 ng/ml). Initial qRT-PCR analysis was done on 314 patients but final cohort of 91 patients with prostate cancer (both positive needle biopsy and radical prostatectomy patients) and 85 patients without cancer was involved in statistical analysis (n=176). The reason for the reduction of the cohort was low quality RNA (C_{tPCA3}=0 and C_{tPSA}=0 or C_{tPSA}>30) and therefore such patients were deemed "non-evaluable".

Univariate logistic regression analysis showed that expression of PCA3 was an independent predictor of cancer (β coefficient=0.068, $P<0.001$) and significant discriminator of CaP from non-malignant cases in the cohort of patients without restriction of the serum PSA ($P<0.001$). The diagnostic performance of the PCA3 was evaluated using ROC analysis based on the

predicted probabilities of the logistic model (Figure 8A; AUC=0.671, 95% confidence interval: 0.592–0.751, P<0.001).

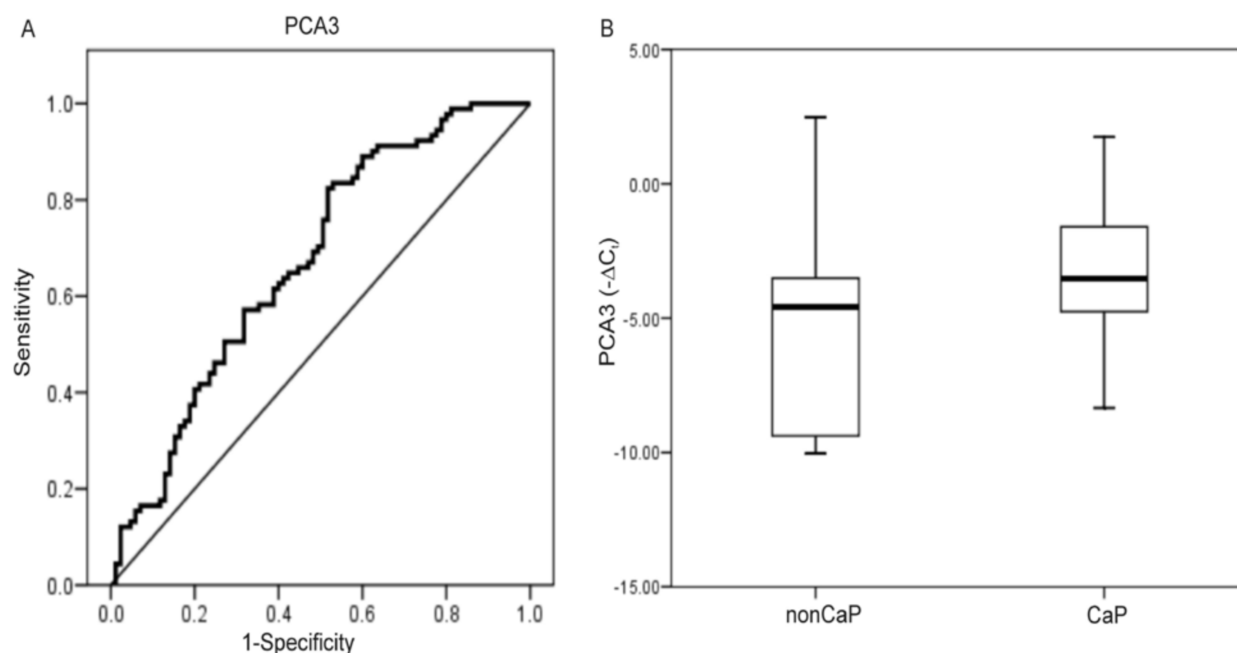

Figure 8. Prostate cancer antigen 3 (PCA3) in the full range of serum PSA (0.1–587 ng/ml). (a) Area under curve (AUC) for PCA3 was 0.671 (P=0.001, receiver-operating characteristic (ROC) analysis). (b) Expression difference of PCA3 between prostate cancer (CaP) and non-CaP patients (P<0.001, Mann–Whitney analysis). Box-and-whisker plot displays median, 25–75% percentiles and 95% confidence interval without outliers.

Mann-Whitney test did not reveal any significant differences of marker expression between low and high grade CaP (Gleason score <7 vs ≥ 7), in localized and locally advanced prostate cancers (T1-T2, N0, M0 vs T3-T4, N0-N1, M0) and in risk group patients (for definitions see Table 11). We did not find any association between clinicopathological parameters and PCA3 expression status (*P*>0.05). As expected, serum PSA had significantly higher levels in advanced stages compared to localized cancers (*P*<0.001). We also found significantly different levels of serum PSA between intermediate and high risk group patients (intermediate < high risk (*P*<0.001) but no difference was found between low and intermediate risk group patients (*P*>0.005).

4.2.2. Testing of urine-detectable prostate cancer-specific biomarkers in patients with serum PSA levels 3-15 ng/ml and generation of the combined model

As the primary aim of urine assays is to assist in identification of patients for diagnostic biopsies, we tested diagnostic ability of widely accepted PCA3 gene in the problematic group of patients with PSA levels of 3-15 ng/ml (n=104). We did not find any significant differences of PCA3 expression in this zone of PSA, i.e. PCA3 was not able to discriminate prostate cancer patients from non-cancer patient group (Figure 9f, Table 12) (potential reasons are provided in the discussion). Seven biomarkers (PCA3, AMACR, SPINK1, EZH2, GOLM1, TRPM8 and MSMB) were further analyzed in parallel. However, only AMACR differentiated CaP patients from patients with non-malignant disease (Figure 9e; Table 12). The diagnostic performance of AMACR was also evaluated by ROC analysis based on the predicted probabilities derived from univariate regression analysis (AUC=0.626, P=0.040). To further evaluate if the multiplex model could improve performance over single biomarkers, we used a multivariate logistic regression analysis plus Akaike information criterion-based backward stepwise selection strategy, which is based on excluding insignificant markers. ROC curves were derived after each step of the analysis to test diagnostic performance of the models. Indeed, generated multiplex model based on 7 biomarkers plus serum PSA was statistically significant with improved AUC (P<0.001) and yielded better specificity compared to PCA3 alone (AUC=0.744 vs AUC=0.671, specificity: 61.8% vs 47.1%, sensitivity: 81.1% vs 83.5%, P<0.001). Though we neglected the use of this model for diagnostic purposes because of several drawbacks which is are provided in the discussion part of the thesis. Thereafter, stepwise analysis derived 7 models among which the most optimal ones were quadriplex, triplex and duplex models (Figure 9 and Table 12). We then selected optimal biomarkers according to their Wald values which give the “importance” of the contribution of each variable in the multiplex regression analysis. Four markers were selected for the following statistical analysis: PCA3, AMACR, MSMB and TRPM8 which yielded the highest values (Wald: 1.643, 3.145, 6.191 and 7.421, respectively) (Table 12). As the quadriplex model had the best specificity and retained good characteristics from the model with all seven markers, it might be suggested for implementation into routine diagnostic procedures.

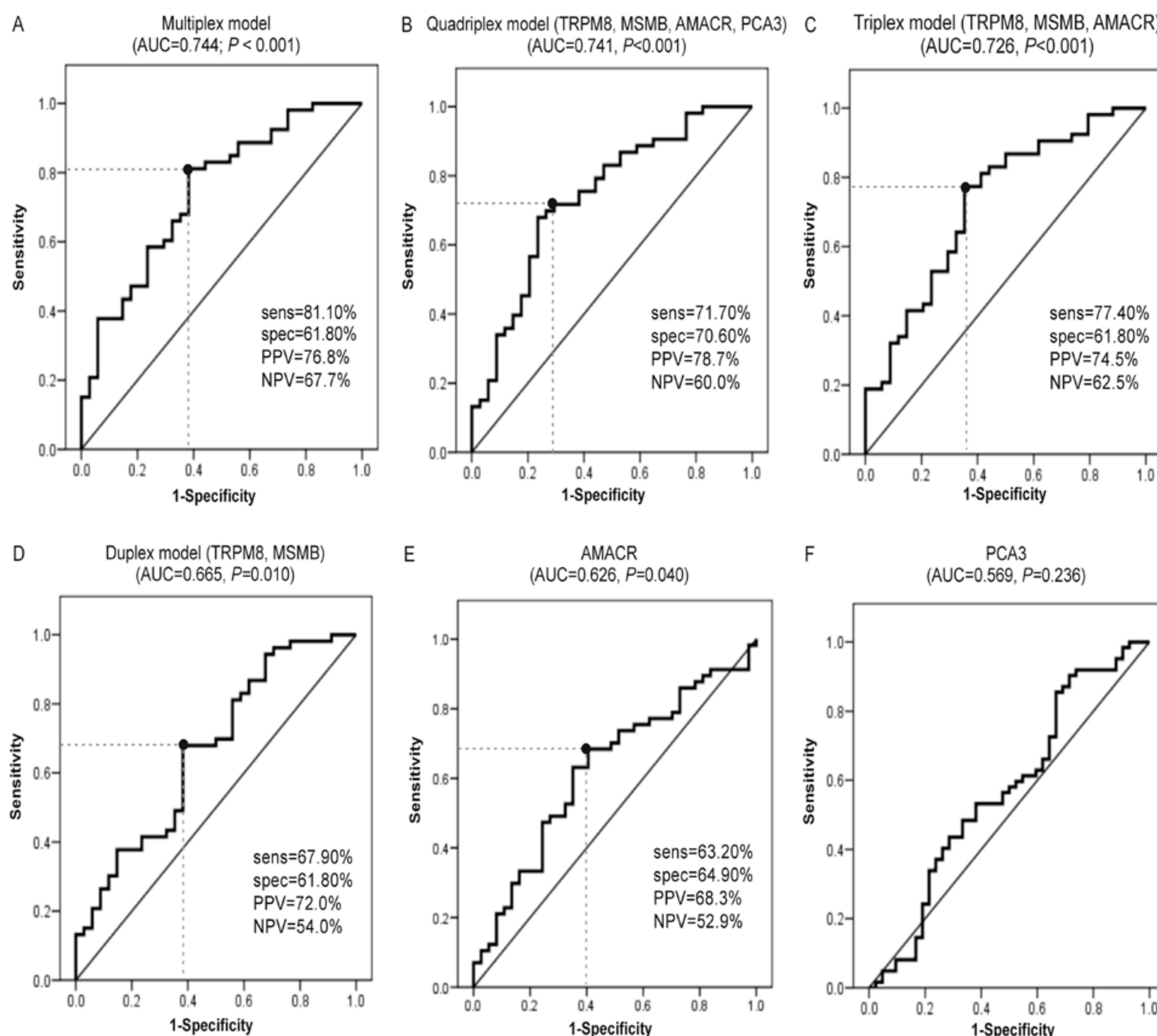

Figure 9. Receiver-operating characteristic (ROC) analysis for the early diagnosis of prostate cancer in the serum PSA zone of 3–15 ng/ml. (a) Multiplex model was composed of all biomarkers and serum PSA (N=87). (b) Quadriplex model included prostate cancer antigen 3 (PCA3); a-methylacyl-CoA racemase (AMACR); transient receptor potential cation channel, subfamily M, member 8 (TRPM8) and microseminoprotein, b (MSMB) (N=87). (c) Triplex model included AMACR, TRPM8 and MSMB (N=87). (d) Duplex model included TRPM8 and MSMB (N=87). (e) Univariately tested AMACR (N=94). (f) Univariately tested PCA3 (N=104). AUC, area under curve; NPV, negative predictive value; PPV, positive predictive value; Sens., sensitivity; Spec., specificity.

Table 12. Logistic regression and ROC analyses of the biomarkers in the range of PSA 3-15 ng/ml

Univariate logistic regression analysis				**ROC analysis**	
Variable	β coefficient	OR (95% CI)	P-value	AUC (95% CI)	P-value
PCA3	0,021	1.022 (0.973-1.072)	0,388	0.569 (0.453-0.685)	0,236
AMACR	0,047	1.048 (0.922-1.191)	0,474	0.626 (0.511-0.741)	**0,040**
SPINK1	0,010	1.010 (0.948-1.076)	0,753	0.496 (0.375-0.618)	0,954
EZH2	0,010	1.010 (0.925-1.103)	0,819	0.543 (0.426-0.661)	0,478
GOLM1	0,020	1.021 (0.974-1.070)	0,396	0.542 (0.418-0.666)	0,501
TRPM8	0,047	1.048 (0.992-1.106)	0,092	0.594 (0.474-0.714)	0,137
MSMB	-0,082	0.921 (0.824-1.030)	0,148	0.543 (0.424-0.661)	0,492
serum PSA	0,096	1.101 (0.972-1.247)	0,130	0.577 (0.465-0.690)	0,182
Multivariate logistic regression analysis					
Multiplex test				0.744(0.638-0.849)	**0,000**
PCA3	0,077	1.080 (0.960-1.216)	0,200		
AMACR	0,190	1.209 (0.980-1.491)	0,076		
SPINK1	-0,007	0.993 (0.902-1.094)	0,895		
EZH2	-0,041	0.960 (0.825-1.116)	0,594		
GOLM1	0,012	1.012 (0.952-1.074)	0,708		
TRPM8	0,120	1.128 (1.034-1.230)	**0,006**		
MSMB	-0,279	0.756 (0.607-0.942)	**0,013**		
serum PSA	0,049	1.051 (0.901-1.226)	0,530		
Quadriplex test				**0.741 (0.633-0.849)**	**0,000**
PCA3	0,172	1.083 (0.966-1.216)	0,172		
AMACR	0,071	1.194 (0.985-1.447)	0,071		
TRPM8	0,007	1.124 (1.032-1.224)	**0,007**		
MSMB	0,007	0.759 (0.621-0.928)	**0,007**		
Triplex test				0.726 (0.617-0.836)	**0,000**
AMACR	0,182	1.200 (0.987-1.459)	0,067		
TRPM8	0,122	1.130 (1.040-1.229)	**0,004**		
MSMB	-0,274	0.761 (0.620-0.933)	**0,009**		
Duplex test					
TRPM8	0,113	1.120 (1.036-1.211)	**0,004**	0.665 (0.548-0.783)	**0,010**
MSMB	-0,189	0.828 (0.707-0.970)	**0,020**		

Abbreviations: AMACR, a-methylacyl-CoA racemase; AUC, area under curve; EZH2, enhancer of zeste homolog 2; GOLM1; golgi membrane protein 1; MSMB, microseminoprotein, b; OR, odds ratio; PCA3, prostate cancer antigen 3; ROC, receiver-operating characteristic; SPINK1, serine peptidase inhibitor; TRPM8, Q8 transient receptor potential cation channel, subfamily M, member 8; 95% CI, 95% confidence interval.

4.2.3. Association of biomarkers with clinicopathological characteristics

Finally, we tested correlations between markers and any associations with clinicopathological characteristics. Several significant correlations were found with the strongest positive correlation between TRPM8 and MSMB (Rs=0.675 in the whole patient set, Rs=0.787 in CaP and Rs=0.492 in non-CaP patients; all $P<0.01$) (Table 13). Next, we found higher expression of AMACR and EZH2 in locally advanced cancer vs localized cancer ($P=0.044$ and 0.022, respectively) (Figure 10a). EZH2 expression was also gradually increased along with cancer progression (low risk < intermediate ($P=0.044$) < high risk ($P=0.067$) (Figure 10b). An inverse association was found for

PCA3 in the low- and high-risk group of patients (*P*=0.047) (Figure 10b). No differences in marker expressions were observed between low- and high-grade tumors (Gleason score <7 vs ≥7). As expected, higher levels of serum PSA (*P*=0.036) and also EZH2 (*P*=0.015) and GOLM1 (*P*=0.048) were found in patients with abnormal digital rectal examination and high probability of cancer (*P*=0.001).

Table 13. Nonparametric Spearman's rank correlation analysis between biomarkers

Biomarkers	serum PSA	PCA3	AMACR	SPINK1	EZH2	GOLM1	TRPM8
PCA3 (r)	0.003						
P-value	0.980						
AMACR	0.110	**0.327**					
P-value	0.293	**0.001**					
SPINK1 (r)	0.002	**0.327**	**0.430**				
P-value	0.984	**0.002**	**0.000**				
EZH2 (r)	0.124	0.066	**0.459**	**0.436**			
P-value	0.233	0.524	**0.000**	**0.000**			
GOLM1 (r)	0.053	**0.329**	**0.451**	**0.421**	**0.325**		
P-value	0.619	**0.001**	**0.000**	**0.000**	**0.002**		
TRPM8 (r)	-0.114	0.181	**0.224**	**0.450**	0.039	0.150	
P-value	0.289	0.089	**0.036**	**0.000**	0.716	0.165	
MSMB (r)	-0.151	**0.238**	0.090	**0.406**	-0.189	0.192	**0.675**
P-value	0.154	**0.023**	0.400	**0.000**	0.075	0.069	**0.000**

Abbreviations: AMACR, a-methylacyl-CoA racemase; EZH2, enhancer of zeste homolog 2; GOLM1; golgi membrane protein 1; MSMB, microseminoprotein, b; PCA3, prostate cancer antigen 3; r, correlation coefficient; SPINK1, serine peptidase inhibitor; TRPM8, transient receptor potential cation Q9 channel, subfamily M, member 8.

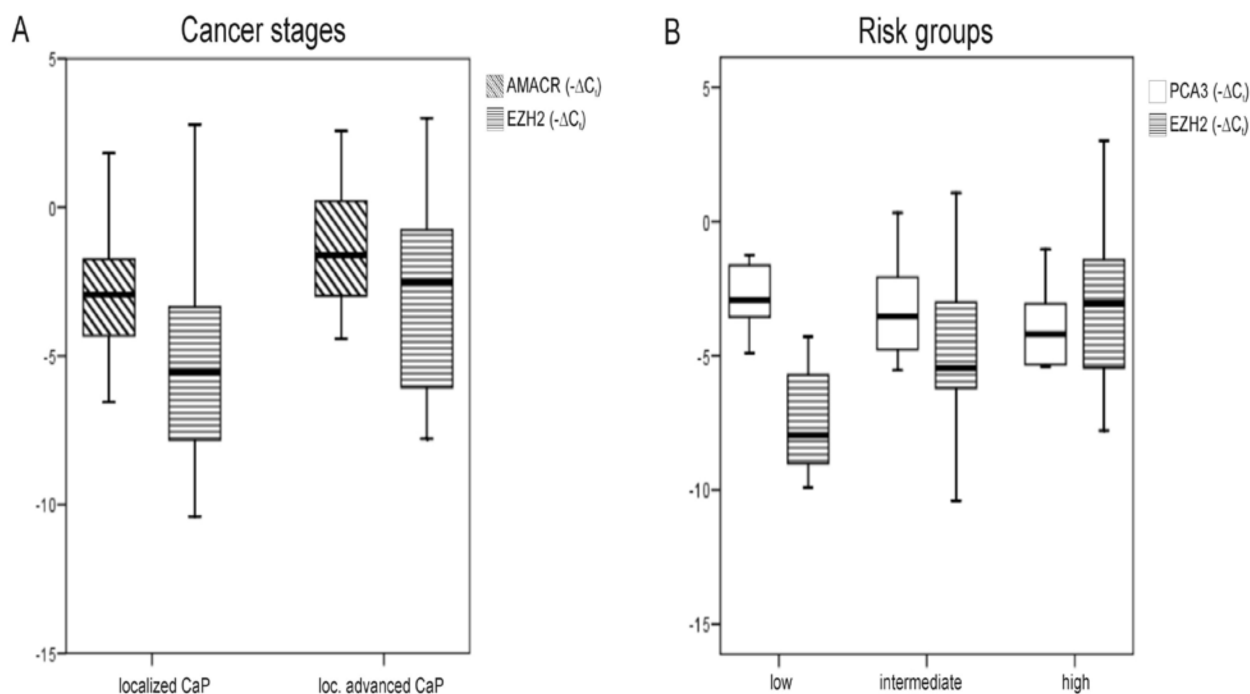

Figure 10. Expression differences between localized and locally advanced cancers and risk groups in the range of serum PSA=3–15 ng/ml. (a) a-Methylacyl-CoA racemase (AMACR) and enhancer of zeste homolog 2 (EZH2) were significantly overexpressed in locally advanced cancers compared with localized ones (P=0.044 and 0.022, respectively). (b) Significant differences were found for EZH2 expression in high- vs low-risk group patients (P=0.002), intermediate- vs low-risk group patients (P=0.044) and a trend of higher expression between high- and intermediate-risk group patients (P=0.067). Prostate cancer antigen 3 (PCA3) was inversely expressed in risk group patients, that is overexpressed in low- vs high-risk group patients (P=0.047). Box-and-whisker plots display median, 25–75% percentiles and 95% confidence interval without outliers.

5. DISCUSSION

5.1. Diagnostic potential of kallikrein-related peptidases 7 and 11 in prostate cancer

Kallikreins are a subgroup of serine proteases and are known to play important roles in different physiological processes. According to the recent studies they are involved in the development and/or progression of human malignancies by acting individually or in cascades with other KLKs or proteases and many of them already have applicability as biomarkers in various types of cancer (Yousef et al. 2001a, Diamandis et al. 2002a, Borgono et al. 2004). Human kallikrein 3 (KLK3/PSA) has been successfully used as a diagnostic and prognostic marker for prostate cancer to date (Erbersdobler et al. 2009). Other kallikreins (KLK2, KLK4, KLK6, KLK10 and KLK11) also represent potential serum biomarkers for prostate cancer detection as it was shown in the recent studies (Paliouras et al. 2007, Emami and Diamandis 2008, Schmitt and Magdolen 2009).

Our study focused on evaluation of KLK7 and KLK11 tissue expression along with PSA and PSMA. Human stratum corneum chymotrypsin gene and protein (KLK7) was very well studied in ovarian and breast cancer being unfavorable prognostic marker (Shan et al. 2006, Li et al. 2009) but little is known about its association with prostate cancer. Recently, aberrant expression of KLK7 was shown to enhance pancreatic cancer cell invasion by cleavage of E-cadherin (Johnson et al. 2007). With respect to prostate cancer there are several articles with controversial results emphasizing its heterogeneous expression in prostate cancer. Xuan et al. (2008) evaluated KLK7 expression in prostate cancer along with ALP (antileukoprotease) which is a specific inhibitor of KLK7. Both KLK7 and ALP have been shown to be down-regulated at mRNA and protein level, as assessed by semiquantitative RT-PCR, western blot analysis and IHC. Significant negative association was found between Gleason grade and both KLK7 and ALP expression. In concordance with this study we confirmed down-regulation of KLK7 in malignant prostate epithelial cells compared to benign/normal prostate epithelial cells. On the other hand, Kishi et al. (2004) reported relatively high levels of KLK7 in prostate tissue extracts and seminal fluid using an immunofluorometric assay. Mo et al. (2010) established KLK7 expressing models (22Rv1 and DU145 cells) which showed epithelial−mesenchymal transition-like changes, as evidenced by scattered cellular growth, mesenchyma-like morphology, and up-regulated expression of mesenchymal vimentin. KLK7 remarkably increased the migration and invasion of

these prostate cancer cells which suggests that KLK7 may play an important role in mediating prostate cancer progression and metastasis. Such controversial results including ours about KLK7 need to be further investigated by in-vivo experiments and large scale cohort studies in order to elucidate its role in prostate cancer. For the first time, we observed positive staining of tumor neovasculature, some muscle cells, urothelium and seminal vesicle epithelium.

Human kallikrein 11 (KLK11) was originally isolated from human hipocampus (Mitsui et al. 2000, Nakamura 2003a). It is under steroid hormone regulation and has three isoforms. KLK11 is a well-studied biomarker for ovarian cancer yielding high KLK11 serum levels in 70% of cancer patients (Scorilas et al. 2006, Diamandis et al. 2002b). Multiple studies showed that KLK11 is up-regulated at both protein and mRNA level in prostate cancer (Diamandis 2002b, Nakamura et al. 2003b, Scorilas and Gregorakis 2006, Bi et al. 2009). However, Nakamura et al. (2003c) demonstrated that compared to the BPH patients, the CaP patients tend to have significantly lower serum KLK11 concentrations and lower KLK11 to total PSA ratios in combination with % free PSA and could better discriminate CaP from PBH patients (90% sensitivity, 46.5% specificity). In concordance with this study, we have observed down-regulation of KLK11 protein at tissue level in the paired tissues of BPH and prostate carcinoma in the same patients. Expression differences for CaP and BPH foci were more significant for KLK11 than for KLK7. In other words, more heterogeneity was found for KLK7 than for KLK11 which might be related to their different regulation. Interestingly, KLK11 mRNA expression in prostate cancer does not correspond with its protein levels (Nakamura et al. 2003a, 2003b, 2003c, Stavropoulou et al. 2005, Bi et al. 2009). Such discordance has also been reported for other genes, for example lumican and decorin in breast cancer, indicating complex regulation of protein expression (Troup et al. 2003). KLK11 mRNA expression has been detected in both normal and cancerous prostate tissue but with significant up-regulation in cancer (Nakamura et al. 2003a). Up-regulation of prostate-type KLK11 was significantly associated with earlier stage, lower Gleason score and lower tumor grade but expression was again lowered with tumor progression (advanced stage, less-differentiated CaP) as confirmed by qPCR analysis (Nakamura et al. 2003b, Bi et al. 2009). Opposite trend was observed in our study with over-expression of KLK11 protein in more advanced tumors compared to localized ones. Noteworthy, Stavropoulou et al. (2005) observed down-regulation of the KLK11 gene in advanced and more aggressive tumors suggesting KLK11 useful prognostic biomarker for prostate cancer. In this study serum

total PSA concentration was found to be lower in patients with over-expression of KLK11. We did not observe either correlation or significant differences in relation to Gleason score, tumor grade, pT stage or serum PSA values.

We confirmed decreased PSA expression in CaP compared to BPH. The trend towards decreased PSA expression was also found in less differentiated cancers which is also in agreement with other IHC studies (Weir et al. 2000, Erbesdobler et al. 2009). Interestingly, protein levels of PSA in serum do not correspond with its expression at tissue level as detected immunohistochemically (Weir et al. 2000, Erbesdobler et al. 2009). Serum PSA levels in men with prostate cancer are significantly higher than levels in men without cancer (Swanson et al. 2001). The exact mechanism by which PSA gains access to the serum is unknown but possible mechanism has been suggested. When tissue architecture (i.e. basement membrane of the glands, the prostatic stroma, and the endothelial cells of the blood vessels) is distorted by diseases such as infection, inflammation, and cancer, PSA leaks from the glands into the interstitium and then into the blood instead of being confined to the ductal excretory system (Swanson et al. 2001, Hessels et al. 2003, Kirby et al. 2009). At the same time, expression of PSA in prostate tissue decreases as cancer progresses as observed in our study and by others (Weir et al. 2000, Erbesdobler et al. 2009). Regarding levels of PSA mRNA, expression has been shown to be relatively constant in normal prostate cells and is not up-regulated in cancer (Meng et al. 2002, Hessels et al. 2003, Kirby et al. 2009), therefore its use as a prostate specific housekeeping gene was suggested by multiple studies on urine biomarkers (see below).

PSMA expression in cancer was opposite to PSA expression, i.e. PSMA expression was up-regulated in CaP vs BPH, again in concordance with other IHC studies (Mhawech-Fauceglia et al. 2007, Perner et al. 2007). No significant associations with either Gleason score, tumor stage or PSA/PSMA expression were found. We detected high PSMA expression in the cancer-associated neovasculature, in some muscle cells and in prostatic secretions as reported in other studies (Chang et al. 2004, Mhawech-Fauceglia 2007). With respect to neoangiogenesis Tsui et al. (2005) reported significant positive correlation between PSMA and VEGF expression regardless of treatment modality of LNCaP xenografts. Similarly, Conway et al. (2006) identified novel role of PSMA in regulation of angiogenesis by modulating integrin signal transduction.

In conclusion, for the first time we reported lower IHC expression of KLK11 in CaP tissue compared to normal/benign prostate tissue and also slight up-regulation of KLK11 in advanced tumors compared to localized ones. In concordance with the literature, we confirm both higher expression of PSMA, lower expression of KLK7 and PSA in CaP compared to BPH. Our observations support the diagnostic potential of KLK7/KLK11 for early prostate cancers but further functional experiments and larger cohort studies are needed in order to validate the clinical value of these biomarkers and clarify their biological role in prostate development and tumorigenesis.

5.2. Novel quadriplex model for urine-based detection of early prostate cancer

Determination of markers in the urine has certain advantages: i) urine can easily be obtained ii) sampling is noninvasive in contrast to prostate tissue sampling which requires an invasive procedure (i.e. transrectal ultrasound-guided biopsy) iii) urine might be more informative specimen in contrast to core needle biopsy which may not be representative of the tumor, due to the often polyclonal nature of CaP, i.e. the tumor can be missed completely by needle-biopsy causing often a false-negative results.

In this study, we aimed to verify the association of prostate cancer with PCA3 and other urine biomarkers and to investigate how these biomarkers may be combined with serum PSA levels to develop clinically practical algorithms for predicting prostate cancer diagnosis. We confirmed that PCA3 can successfully discriminate CaP from non-CaP in randomly chosen patients with PSA levels of 0.1–587 ng/ml (i.e. wide range of PSA) consistent with other studies (Hoffman et al. 2002, Van Gils et al. 2007b, Marks et al. 2007, Haese et al. 2008). We used qPCR for detection of PCA3 gene instead of commercially available PCA3 assay. The latter is based on absolute quantification of PCA3 and PSA mRNA whereupon PCA3 score is derived. Commonly used PCA3 score cut-off is 35 [e.g. 35 copies of PCA3 mRNA] / [e.g. 1000 copies of PSA mRNA] x 1000 which matches to ΔC_t 4.8 (with optimal PCR efficiency). This well corresponds with our median ΔC_t values 4.6 and 3.5 for non-cancer and CaP patients, respectively. However, we could not confirm the diagnostic ability of PCA3 in patients with serum PSA of 3–15 ng/ml. This might be explained in part by the technologies used in commercial assays such as target capture with magnetic particles, transcription-mediated amplification and hybridization protection assay for detection of PCA3 transcripts in urine (APTIMA® assay, Gen-Probe, San

Diego, CA; PROGENSA™ for European countries) (van Gils et al. 2007b, Marks et al. 2007, Haese et al. 2008). Recently, Klecka et al. (2010) has reported only borderline significant results for PCA3 evaluated by qRT-PCR in a population of Czech men (PSA 4.1 – 580 ng/ml, P=0.045). There might be population (e.g. Slavonic, Anglo-Saxon, etc.) specific differences in PCA3 regulation which need to be further investigated. In this sense, PCA3 evaluated even by the commercial PROGENSA™ test did not outperform serum PSA in a recent South-African study (68.6% black, 25.7% white and 5.7% other race) (Adam et al. 2011). Furthermore, another recent study (Salami et al. (2011) has also reported marginal insignificance for univariatly assessed PCA3 in patients even without serum PSA restriction. According to the current call for a comprehensive biomarker study registry (Andre et al. 2011) such negative results should be always published in order to avoid false positive conclusions in biomarker studies. The publication bias is a very common problem occurring on the base of solely "positive" findings which can influence results of the particular study leading to false positive conclusions.

The main grounds for not using the commercial CaP urinary test system were the high costs of the tests and the fact that this assay uses a closed system, which does not allow the user to test additional biomarkers in parallel (Laxman et al. 2008, Thompson et al. 2007). The second part of the analysis was hence done on a multiplex model to complement PCA3 and improve diagnostic accuracy in the case of 'PSA dilemma' patients. Markers were chosen according to previous studies as being either detectable in urine or appropriate for combined models. AMACR (Ouyang et al. 2009, Rogers et al. 2004, Prior et al. 2010), SPINK1 (Laxman et al. 2008, Tomlins et al. 2008), EZH2 (Schmidt et al. 2006, Schneider et al. 2008), GOLM1 (Laxman et al. 2008, Varambally et al. 2008), TRPM8 are overexpressed in CaP (Schmidt et al. 2006, Schneider et al. 2008, Zhang and Barritt 2006, Bai et al. 2010), while MSMB is prostate specific but underexpressed in cancer (Teni et al. 1988, Tsurusaki et al. 1998, Whitaker et al. 2010). Inversed expression of MSMB does not compromise multiplex analysis since all markers equally contribute to the model, regardless of their down or up regulation (Cao et al. 2010). We have found that only AMACR was a significant predictor of cancer in the univariate analysis. The ROC curve and Mann–Whitney analysis confirmed that AMACR could complement PCA3 testing as it was shown by Ouyang et al. (2009) who achieved significantly higher sensitivity and specificity when both markers were combined. Consistent with other studies we also confirmed the idea of using AMACR as a marker of progression as its expression was increased in

advanced stages of the cancer (Figure 10a) (Kuefer et al. 2002, Rubin et al. 2005). In spite of the fact that other markers didn´t associate with cancer in univariate analysis, we performed multiplex evaluation. As expected, the multiplex model, which was composed of all seven biomarkers plus serum PSA showed significantly good AUC (AUC=0.744). However, this model would not be cost- and labor-effective because of many biomarkers used, so three additional models were generated (duplex, triplex and quadriplex). The quadriplex model retained most of the good characteristics of the multiplex model (AUC=0.741, sensitivity 71.70%, specificity 70.60%). Thus, we were able to prove the usefulness of evaluating AMACR, TRPM8 and MSMB in combined settings with PCA3. (Schmidt et al. 2006, Schneider et al. 2008, Ouyang et al. 2009, Prior et al. 2010, Cao et al. 2010, Salami et al. 2011). Similarly to our study, Schmidt et al. (2006) developed a multivariate logit model for prediction of CaP. After testing quantitatively nine prostate-related genes on prostate tissue samples, they confirmed that inclusion of EZH2, PCA3, prostein, and TRPM8 increased diagnostic power of PCA3 (AUC=0.90). Schneider et al. (2008) analyzed eleven-prostate-related genes by qRT-PCR. Relative expression levels of EZH2, hepsin, PCA3, prostein, and TRPM8 were evaluated with regard to their diagnostic potential and two optimized mathematical models were generated subsequently. Compared to single marker analyses these models yielded higher sensitivity and specificity for prostate cancer detection. Ouyang et al. (2009) showed that dual marker test, i.e. evaluation of AMACR along with PCA3 significantly increased sensitivity, specificity and accuracy of CaP detection. Hessels et al. (2007) successfully evaluated TMPRSS2-ERG (sensitivity 37%, specificity 93%) in urine samples along with PCA3 (sensitivity 62%, specificity 46%), though the combination of both markers remarkably increased the test sensitivity from 62% (PCA3 test alone) to 73%. Recently, Salami et al. (2011) re-tested the same biomarkers in combination with serum PSA. PCA3 had the highest sensitivity in predicting prostate cancer diagnosis (AUC=0.65; sensitivity=93%, specificity=37%), whereas TMPRSS2:ERG had the highest specificity (AUC=0.77; sensitivity=67%, specificity=87%). Salami and colleagues confirmed again that combining serum PSA, PCA3, and TMPRSS2:ERG in a multivariable algorithm could successfully improve cancer prediction compared to univariate testing of single biomarkers (AUC=0.88; sensitivity=80%, specificity=90%).

Biomarkers analyzed in the study of Prior et al. (2010) were AMACR and MMP-2, and GSTP1/RASSF1A methylation status, in addition to serum PSA levels. They proved that that

analysis of this biomarker combination in body fluids improves significantly the diagnosis of CaP compared to the PSA test alone. Cao et al. (2010) evaluated PCA3 along with TMPRSS2-ERG, annexin A3 and sarcosine in the two sets of patients (serum PSA 4-10 ng/ml and full range of PSA) and confirmed that the combined model improved test accuracy (AUC=0.840 and 0.856) vs single biomarkers in both cohorts (PCA3: 0.733 and 0.739; TMPRSS2-ERG: 0.720 and 0.732; annexin A3: 0.716 and 0.728; sarcosine: 0.659 and 0.665, respectively).

Patients were enrolled in our study between 2008-2010 years, so the follow-up is now up to three years. Only two patients enrolled in 2010 were reclassified as cancer positive after repeated biopsy half a year later. As a consequence, these two patients (only one in the multiplex model) were incorrectly included in the non-cancer group for statistical analysis. The patient analyzed for all eight genes had increased expression in particular of PCA3 and AMACR which might further improve the characteristics of the multiplex model.

We found no association between individual marker expression and clinicopathological parameters with the exception of AMACR (see above), EZH2 and PCA3. Expression of EZH2 was increased in advanced cancers, consistent with other studies that have suggested this as a marker of prognosis (Varambally et al. 2002, Saramaki et al. 2006). As expected, serum PSA levels were also significantly increased along with cancer progression. Surprisingly, PCA3 had a slightly decreased expression in the high-risk group of patients in contrast to EZH2, which might support the idea of being PCA3 as a marker of early diagnosis of CaP (van Gils et al. 2007b, Marks et al. 2007, Haese et al. 2008). Interestingly, we found a strong positive correlation between TRPM8 and MSMB. However, we don´t have a good explanation of this observation and future studies are needed to validate and clarify this association.

We consider additional urine markers for our future patients, in particular a novel panel of TMPRSS2–ERG fusion transcripts (Nguyen et al. 2011). Along with PCA3, recurrent gene fusion TMPRSS2–ERG has showed promising results for prostate cancer early detection in preliminary studies and can be successfully detected in post-DRE urine specimens that are suitable for possible use in clinical diagnostic strategies (Laxman et al. 2006, Hessels et al. 2007, Nguyen et al. 2011, Salami et al. 2011). The presence of TMPRSS2-ERG fusion in prostate cancers seems to predict a more aggressive course of the disease and increase in prostate cancer-related deaths has also been reported (Demichelis et al. 2007, Attard et al. 2008b).

In summary, we demonstrated that novel multiplex quantitative polymerase chain reaction assay on sedimented urine from ‘PSA dilemma’ patients could be implemented as an adjunct to the routine diagnostic analysis of CaP such as serum PSA or PCA3 and may be used to improve decision making for repeat biopsies in men with elevated PSA levels. The quadriplex urine test presented here achieves higher specificity than PCA3 for ‘PSA dilemma’ patients, which could reduce negative biopsies and identify clinically significant CaP. Future studies will be directed to further improve the performance of this test by examination of larger cohorts with other promising biomarkers.

6. SUMMARY

Prostate cancer (CaP) is the second most frequently diagnosed cancer and the third most common cancer causing death in men. The prostate specific antigen (PSA) also known as kallikrein 3 has enhanced the detection and awareness of this malignancy but still high false-positive detection occurs. Recently introduced PCA3 as a urine marker has the potential to detect CaP in previous biopsy-negative patients and especially to detect prostate cancer at its earlier stages. However, tumor biomarkers which reliably differentiate CaP from BPH and localized/curable prostate cancers from advanced ones are still a major interest of research.

The aim of this study was to study other kallikreins than PSA (KLK3), in particular to evaluate any difference of KLK7/KLK11 tissue expression in the paired cancer/benign prostate foci and to test putative urine markers in multiplex setting and generate satisfactory models with higher diagnostic accuracy compared to either serum PSA or urine PCA3.

Kallikreins are a subgroup of the serine protease enzyme family consisting of 15 members. KLK7 and KLK11 share a high degree of structural similarity with PSA suggesting potential as novel diagnostic/prognostic markers. We studied expression of KLK7/11 at a protein level on archived radical prostatectomy tissues from patients with localized and advanced prostate cancer. Seventy archived paraffin-embedded tissues were dewaxed, rehydrated, pretreated and immunostained for KLK7 and KLK11 Staining intensity, positive cell fraction, staining heterogeneity and cellular localization were determined for both BPH and CaP foci in each patient. In parallel with kallikreins, expression of PSA and PSMA was also analyzed. We found significant differences for all studied proteins between BPH and CaP foci. Both KLK7 and KLK11 expressions were decreased in prostate cancer compared with its benign counterparts. High heterogeneity of KLK7/KLK11 expression was demonstrated in CaP and BPH foci among both the same and different patients, especially for KLK7. Decreased expression in CaP was also observed for PSA while reverse one for PSMA being again in concordance with other IHC studies. Positive correlations were observed for both KLK7 and KLK11 in CaP and BPH. Neither correlation nor significant differences were found with respect to Gleason score, tumor grade, pT stage and serum PSA values. Our observations support the diagnostic potential of KLK7/KLK11 for early prostate cancers but further studies on larger cohorts are needed in order

to validate the clinical value of these biomarkers and clarify their biological role in prostate development and progression.

The major advantages of multiplex urine-based assays are their noninvasive character, ability to monitor prostate cancer with heterogeneous foci and better accuracy in detection of prostate cancer at its early stages than do single marker tests. Multiplex models might potentially supplement serum PSA test which has low specificity reducing the number of unnecessary biopsies, especially for "PSA dilemma" (PSA=4-10 ng/ml) patients and thereby obviating the complications associated with biopsy. Therefore we intended to test multiple putative prostate cancer-specific biomarkers (PCA3, AMACR, SPINK1, EZH2, GOLM1, TRPM8, MSMB and PSA as a housekeeping gene) and identify the model that could supplement widely accepted PSA and PCA3 tests. Expression of the candidate biomarkers was studied in sedimented urine using quantitative RT-PCR on two sets of patients with PSA levels of 0.1-587 ng/ml and 3-15 ng/ml. Total RNA isolation was performed by the Urine Exfoliated Cell RNA Purification Kit (Fisher Scientific, USA), quantified by Nanodrop, pretreated with Dnase I (Invitrogen) and reverse transcribed with SuperScript® III Reverse Transcriptase (Invitrogen), preamplified and tested on real-time PCR with Light Cycler® 480 (Roche). We confirmed that PCA3 is an independent predictor of cancer in the patients without restriction of serum PSA values (PSA=0.1-587 ng/ml) consistent with other studies. However we could not confirm the diagnostic ability of PCA3 in Czech patients with serum PSA of 3-15 ng/ml which includes the „grey zone“. This might be explained by different technologies used in commercial assay. Another reason might be population specific differences in PCA3 regulation which need to be further investigated. AMACR was the only parameter that differentiated CaP from nonCaP patients. Further improvement was achieved by multivariate logistic regression analysis which identified novel duplex (TRPM8 and MSMB), triplex (plus AMACR) and quadriplex (plus PCA3) models among which the most optimal one was the quadriplex one (sensitivity 72%, specificity 71%). In this study we reported novel multiplex model that could serve as an adjunct to currently used tests for early prostate cancer detection and might be successfully implemented in urological laboratories. Future studies with larger sets of patients are needed to further improve the performance of this test and verify its diagnostic power.

7. SOUHRN

Karcinom prostaty (CaP) je druhý nejčastěji diagnostikovaný druh rakoviny a třetí nejběžnější druh rakoviny, který u mužů způsobuje smrt. Prostatický specifický antigen (PSA), rovněž známý jako kallikrein 3, zlepšuje detekci a povědomí o tomto zhoubném karcinomu, ale stále dochází k vysoké míře falešně pozitivní detekce. Nedávno zavedený PCA3 jako močový marker má potenciál k detekci CaP u pacientů, u nichž byla předchozí biopsie negativní. Tento marker může zejména detekovat karcinom prostaty v raném stadiu. Nicméně biomarkery karcinomu, které spolehlivě odliší CaP od BPH a lokalizované/léčitelné karcinomy prostaty od pokročilých karcinonů, jsou stále hlavním předmětem výzkumu.

Cílem této studie bylo zkoumat jiné kallikreiny než PSA (KLK3), zejména zhodnotit rozdíl tkáňové exprese KLK7/KLK11 u párů rakovinových/benigních ložisek v prostatě a otestovat potenciální nové močové markery v multiplexním prostředí a vytvořit uspokojivé modely s vyšší diagnostickou přesností ve srovnání se sérovým PSA nebo močovým markerem PCA3.

Kallikreiny tvoří podskupinu serinových proteáz ze skupiny enzymů, která má 15 členů. KLK7 a KLK11 vykazují vysokou míru strukturální podobnosti s PSA a potenciálně se nabízí jako nové diagnostické/prognostické markery. Zkoumali jsme expresi KLK7/11 na proteinové úrovni u archivovaných tkání po radikální prostatektomii u pacientů s lokalizovaným a pokročilým karcinomem prostaty. Sedmdesát archivovaných tkání uchovaných v parafínu bylo deparafinováno, rehydratováno, přečištěno a nabarveno protilátkami na KLK7 a KLK11. Intenzita zbarvení, pozitivní frakce buněk, heterogenita zbarvení a buněčná lokalizace byly u každého pacienta zhodnoceny pro ložiska BPH a CaP. Souběžně s kallikreiny byly rovněž analyzovány exprese PSA a PSMA. U všech zkoumaných proteinů jsme zjistili významné rozdíly mezi ložisky BPH a CaP. Ve srovnání s benigními vzorky došlo u karcinomu prostaty ke snížení exprese KLK7 a KLK11. Vysoká heterogenita exprese KLK7/KLK11 byla prokázána u ložisek CaP a BPH u stejných i různých pacientů, zejména v případě KLK7. Snížení exprese u CaP bylo také zjištěno u PSA, zatímco opačný projev u PSMA byl v souladu s jinými studiemi IHC. Pozitivní korelace byly pozorovány v případě KLK7 i KLK11 u CaP i BPH.

Nebyla zjištěna ani korelace ani významné rozdíly u Gleasonova skóre, stupně nádoru, stupně pT a hodnot séra PSA. Naše výsledky podporují diagnostický potenciál KLK7/KLK11 pro rané

stádium karcinomu prostaty, ale k ověření klinického významu těchto biomarkerů a k objasnění jejich biologické role při tvorbě a progresi prostaty budou zapotřebí rozsáhlejší kohortové studie.
Ve srovnání s testováním na jednotlivé markery patří k hlavním výhodám multiplexních analýz na bázi moči jejich neinvazivní charakter, možnost monitoringu heterogenních ložisek karcinomu prostaty a zlepšení přesnosti detekce karcinomu prostaty v raném stádiu. Testování na sérové PSA, které má nízkou specificitu, by potenciálně mohlo být doplněno o multiplexní modely, které by snížily počet zbytečných biopsií, zejména u pacientů s "dilematem PSA" (PSA=4-10 ng/ml), čímž by bylo možné vyhnout se komplikacím spojeným s biopsií. Proto jsme chtěli testovat mnohočetné potenciální biomarkery, které jsou specifické pro karcinom prostaty (PCA3, AMACR, SPINK1, EZH2, GOLM1, TRPM8, MSMB a PSA jako referenční gen) a identifikovat model, který by doplnil všeobecně uznávané testy na PSA a PCA3. Exprese kandidátních biomarkerů byla analyzována v močovém sedimentu pomocí kvantitativní RT-PCR u dvou skupin pacientů s úrovněmi PSA 0,1-587 ng/ml a 3-15 ng/ml. Izolace celkové RNA byla provedena pomocí Urine Exfoliated Cell RNA Purification Kit (Fisher Scientific, USA), kvantifikována na spektrofotometru Nanodrop, ošetřena pomocí Dnase I (Invitrogen) a reverzní transkripce byla provedena pomocí SuperScript® III Reverse Transcriptase (Invitrogen), cDNA preamplifikována a kvantifikována na přístroji Light Cycler® 480 (Roche). V souladu s jinými studiemi jsme potvrdili, že PCA3 je nezávislý prediktor karcinomu u pacientů bez omezení hodnot séra PSA (PSA=0,1-587 ng/ml). Nebyli jsme však schopni potvrdit diagnostickou schopnost PCA3 u českých pacientů s hodnotou séra PSA v rozmezí 3-15 ng/ml, v němž je obsažena „šedá zóna". To je možné vysvětlit použitím různých technologií při komerční analýze. Dalším důvodem mohou být populačně specifické rozdíly v regulaci PCA3, což je nutno dále zkoumat. Jediným parametrem, který odlišil pacienty s CaP od pacientů bez CaP, byl AMACR. Dalšího zlepšení bylo dosaženo pomocí multivariační logistické regresní analýzy, která identifikovala nové duplexní (TRPM8 a MSMB), triplexní (plus AMACR) a kvadruplexní (plus PCA3) modely, z nichž nejoptimálnější byl kvadruplexní model (senzitivita 72%, specificita 71%).
Nový multiplexní model by mohl sloužit jako doplněk k aktuálně používaným testům pro zjištění raného stádia karcinomu prostaty a mohl by být takto úspěšně zaveden v urologických laboratořích. V budoucnu je nutno provést další studie s početnějšími skupinami pacientů, aby se výkon testu dále zlepšil a byla ověřena jeho diagnostická účinnost.

8. ABBREVIATIONS

ΔC_t	$C_{t(\text{target gene})}$-$C_{t(\text{reference gene})}$
2DGE	two-dimensional gel electrophoresis
8-OhdG	8-hydroxydeoxyguanosine
95% CI	95% confidence interval
A2M	α2-macroglobulin
ACT	α_1-antichymotrypsin
ACTH	adrenocorticotropic hormone
AIC	Akaike information criterion
ALP	antileukoprotease
AMACR	α-methylacyl coenzyme A racemase
ANXA3	annexin A3
AR	androgen receptor
AREs	androgen response elements
AUC	area under curve
Bcl-2	B-cell lymphoma 2
BHQ1	black hole quencher 1
BHUAE	basic human urinary arginine amidase
bPSA	benign PSA
cAMP	cyclic adenosine monophosphate
CANT1	calcium activated nucleotidase 1
cDNA	comlementary DNA
CGH	comparative genome hybridization
CK	cytokeratin
CNB	core needle biopsy
COPA	cancer outlier profile analysis
CpG	cytosine-phosphate-guanine
cPSA	complexed PSA
CRH	corticotrophin releasing hormone
C_t	threshold cycle
DD3	differential display 3
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
DRE	digital rectal examination
EPCA	early prostate cancer antigen
ER	estrogen receptor
ERG	v-ETS avian erythroblastosis virus E26 oncogene homolog
ERKs	extracellular signal-regulated kinases

ETS	E26 transformation–specific
ETV1	ets variant 1
EZH2	enhancer of zeste homolog 2
FFPE	formalin-fixed paraffin-embedded
FISH	fluorescent in situ hybridization
fPSA	free prostate-specific antigen
FSH	follicle-stimulating hormone
GnRH	gonadotropin releasing hormone
GOLM1	golgi membrane protein 1
GOLPH2	golgi phosphoprotein 2
GRN-A	Chromogranin-A
Gscore	Gleason score
GSTP1	glutathione-S-transferase P1
HPC1	hereditary prostate cancer 1
hPIN	high grade intraepithelial neoplasia
HPLC	high performance liquid chromatography
IGF-I	insulin-like growth factor-I
IHC	immunohistochemistry
IL-6	interleukin 6
KLK	kallikrein
LBD	ligand-binding domain
LC	liquid chromatography
LH	luteinizing hormone
LOH	loss of heterozygosity
LOOCV	leave-one-out cross-validation
MAPK	mitogen-activated protein kinase
MCM5	minichromosome maintenance complex component 5
MMPs	matrix metalloproteinases
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MSMB	beta-microseminoprotein
MSP	methylation-specific PCR
NASBA	nucleic acid sequence-based amplification
PAP	prostatic acid phosphatase
PCA3	prostate cancer antigen 3
PCR	polymerase chain reaction
PDEF	SAM pointed domain containing ets transcription factor
phi	prostate health index
PIA	proliferative inflammatory atrophy
PIN	intraepithelial neoplasia
PIP	prostatic inhibin-like peptide

PSA	prostate-specific antigen
PSA-DT	PSA-doubling time
PSA-V	PSA-velocity
PSCA	prostate stem cell antigen
PSMA	prostate-specific membrane antigen
PSP94	prostatic secretory protein of 94 amino acids
PTEN	phosphatase and tensin homolog
qPCR	quantitative PCR
RNA	ribonucleic acid
RNASEL	ribonuclease L
ROC	receiver operating characteristics
RP	radical prostatectomy
Rs	Spearman's rank correlation coefficient
RT-PCR	reverse transcriptase-PCR
S100A9	S100 calcium binding protein A9
Ser/Thr kinases	serine/threonine kinases
SMA	smooth-muscle actin
SPINK1	serine protease inhibitor Kazal-type 1
SPSS	statistical package for social sciences
TATI	tumor-associated trypsin inhibitor
TERT	telomerase reverse transcriptase
TF	transferrin
TFF3	trefoil factor 3
TMPRSS2	type II transmembrane serine protease
TNM	tumor, lymph node, metastasis
tPSA	total prostate-specific antigen
TRPM8	transient receptor potential cation channel subfamily M member 8
TRUS	transrectal guided ultrasound
TZ	transition zone
UPL	universal probe library
VEGF	vascular endothelial growth factor
WHO	world health organization
WNT	wingless type
WTA	whole transcriptome amplification

9. LIST OF PUBLICATIONS

Reviews and original papers

1. **Jamaspishvili T,** Kral M, Khomeriki I, Student V, Kolar Z, Bouchal J. Urine markers in monitoring for prostate cancer. Prostate Cancer Prostatic Dis. 2010 Mar;13(1):12-9. doi:10.1038/pcan.2009.31
2. **T. Jamaspishvili,** A. Scorilas, M. Kral, I. Khomeriki, D. Kurfurstova, Z. Kolar, J. Bouchal. Immunohistochemical localization and analysis of kallikrein-related peptidase 7 and 11 expression in paired cancer and benign foci in prostate cancer patients. Neoplasma 2011; 58(4):298-303. doi:10.4149/neo_2011_04_298
3. **T. Jamaspishvili,** M. Kral, I. Khomeriki, V.Vyhnankova, G. Mgebrishvili, V. Student, Z. Kolar and J. Bouchal. Quadriplex model enhances urine-based detection of prostate cancer. Prostate Cancer Prostatic Dis. 2011 Jul 26. doi: 10.1038/pcan.2011.32. [Epub ahead of print]

Presentations with abstracts

1. **Jamaspishvili T.,** Kral M., Khomeriki I., Vyhnankova V., Mgebrishvili G., Student V., Kolar Z. and Bouchal J. Novel combination of urine biomarkers for early detection of prostate cancer. VII Days of diagnostic, predictive and experimental oncology, XIX. Congress of Czech and Slovak pathologists XCVII. Diagnostic Seminar of IAP Czech division. Olomouc, November 9-11, 2011
2. **Jamaspishvili T.,** Kral M., Khomeriki I., Vyhnankova V., Mgebrishvili G., Student V., Kolar Z. and Bouchal J. Quadriplex model enhances urine-based detection of prostate cancer. The 7th Symposium & Workshop on Molecular Pathology and Histo(cyto)chemistry and the 96th Seminar of the Czech Division of the International Academy of Pathology, Olomouc, Czech Republic, April 29-30, 2011

3. Bienová M, Kučerová R, Král M, Bouchal J, Trtková K, **Jamaspishvili T,** Tovaryšová A, Reptová S, Študent V, Kolář Z. Androgenetic alopecia and its relationship to expression of the androgen-sensitive genes in benign prostatic hyperplasia and carcinoma of the prostate. Abstract book of "8th EADV Spring Symposium", Karlovy Vary, Czech Republic, April 14-17, 2011
4. **Jamaspishvili T.,** Kral M., Mgebrishvili G. and Bouchal J. Urine AMACR, MSMB and TRPM8 facilitate early prostate cancer diagnosis". Abstract book of conference "New treatments of human disesases, Kouty nad Desnou, Czech Republic, May 29-31, 2011
5. **Jamaspishvili T.,** Kral M., Khomeriki I., Kurfurstova D., Bouchal J., Scorilas A. Kallikrein 7/11 tissue expression in prostate cancer. Abstract book of congress "2nd worldwide innovative networking in personalized cancer medicine", Paris, France, July 7-9, 2010
6. Tovaryšová A., Reiterová K., Sobotka J., Řepková Z., Soumarová R., **Jamaspishvili T.,** Král M., Bouchal J., Kolář Z. Detection of fusion gene TMPRSS2-ERG on paraffin sections in prostate cancer patients by fluorescent in situ hybridization. Cytogenetic conference, Ostravice, Czech Republic, September 8-10, 2010
7. Král M, **Jamaspishvili T,** Študent V, Kolář Z, Bouchal J. The use of serum and urinary biomarkers in the diagnosis of prostate cancer. Ces Urol. 2010; 14(4):246, conference of Czech Urological Society, Prague, Czech Republic, October 6-8, 2010

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