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BIOPSIES, DIAGNOSIS AND PROGNOSIS IN PROSTATE CANCER

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Front cover: detail from histopathologic glass slide with prostate cancer;
Hematoxylin-Eosin staining (above), P504 and p63 (below).
Back cover: the old KI symbol that decorated the entrance to campus during the author's undergraduate studies.
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ABSTRACT

Prostate cancer is the third most common cancer in men worldwide, and poses a challenge both for patients and health care systems. Many aspects of prostate cancer are controversial, from screening for early detection to the concept of insignificant cancer that does not need curative treatment. This study deals with different aspects of the diagnostic process, including biopsy procedures, histopathological diagnosis and biomarkers for carcinogenesis and progression.

Biopsies of the prostate are the prerequisite for histopathological diagnosis of cancer. The standard biopsy instrument uses an inner needle with a side-notch. The tissue yield of a novel end-cutting instrument was investigated and compared with that of the standard instrument. The new instrument provided thicker biopsies, flatter embedding in paraffin blocks and an optional greater stroke length. These advantages were countered by loss of some biopsy cores. The new instrument can be recommended primarily for men with large prostates or repeat biopsies when there is a persistent serum PSA elevation and suspicion of anterior tumour.

Tumour volume of prostate cancer correlates with progression after radical prostatectomy, but its clinical utility is limited by difficulties in the preoperative assessment. The prediction of large volume prostate cancer by prostate biopsies was studied. Biopsy cancer length and percentage cancer length predicted large tumours better than number and percentage of cores positive for cancer. A cancer length of ≥30 mm predicted a tumour volume of >4 ml in 95% of cases. Gleason score and serum PSA were weaker predictors. Tumour volumes of <4 ml were found in as many as 35% of men with ≥6 biopsy cores, indicating that number of positive cores is less useful than linear cancer extent as predictor of large tumours.

DNA ploidy status correlates to stage and outcome, but studies on its clinical utility have shown divergent results. The impact of tumour heterogeneity on preoperative ploidy assessment in prostate cancer was investigated by comparing DNA ploidy of prostate biopsies with radical prostatectomy specimens that had been mapped for ploidy heterogeneity. Ploidy was both under- and overestimated in biopsies, and this finding was more pronounced in tumours with heterogeneous ploidy status. Accuracy increased with multiple biopsies.

Oxidative stress is considered to be of great importance in the development of prostate cancer. The expression of three proteins in the redox control system was investigated, in order to evaluate their involvement in prostate cancer. By immunohistochemistry on prostate tissue microarrays all three proteins showed similar expression patterns with the highest immunoreactivity in HGPIN followed by atrophy, cancer and benign tissue. No correlation with biochemical recurrence was found.

In patients with metastatic cancer it is important for treatment and prognosis to assess the site of tumour origin. The origin of certain tumour types is difficult to identify by morphology alone or with existing biomarkers. In a database search for new prostate-specific markers, GAD1 was identified. Its tissue specificity was investigated in a tissue microarray and compared with PSA and PSMA. GAD1 showed a high specificity and sensitivity to benign and malignant prostate tissue and was almost entirely negative in cancers from lung, rectum and urinary bladder, i.e. tumours that may be considered as differential diagnoses to advanced prostate cancer.

In a database search for diagnostic markers with ability to discriminate between malignant and benign prostate tissue, four proteins were identified: AMACR, CYCS, ICK and IKBKB. AMACR is a well-established diagnostic biomarker, but is negative in some prostate cancers and may also be false positive. The expression was analysed in a tissue microarray of benign prostatic tissue, precursor lesions and cancer from the same cases. All four markers showed a stronger expression in prostate cancer and HGPIN than in benign tissue. A panel of diagnostic biomarkers may serve as an adjunct tool for diagnosis of difficult cases.

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Nobody made a greater mistake than he who did nothing because he could do only a little.

Edmund Burke

This book is dedicated to the patients with prostate cancer and their families

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Diagnostic biomarkers of prostate cancer.
Scand J Urol Nephrol. 2011 Feb;45(1):60-7

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LIST OF ABBREVIATIONS

AMACR	α-methylacyl-coenzyme A-racemase
AR	androgen receptor
BPH	benign prostate hyperplasia
CYCS	somatic cytochrome c
CZ	central zone (of the prostate)
DRE	digital rectal examination
EPE	extraprostatic extension (formerly extracapsular extension)
FNAC	fine-needle aspiration cytology
GAD	glutamate decarboxylase 1
GS	Gleason score
HGPIN	high-grade prostatic intraepithelial neoplasia
ICK	intestinal cell kinase
IHC	Immunohistochemistry
IKBKB	inhibitor of nuclear factor-κB kinase subunit beta
IRP	immunoreactivity product
PAP	prostatic acid phosphatase
PIA	proliferative inflammatory atrophy
PIN	prostatic intraepithelial neoplasia
PSA	Prostate-specific antigen
PSMA	Prostate-specific membrane antigen
PZ	peripheral zone (of the prostate)
RT-PCR	real-time polymerase chain reaction
SVI	seminal vesicle invasion
TMA	tissue microarray
TRUS	transrectal ultrasound
TUR-P	transurethral resection (of the prostate)
TZ	transition zone (of the prostate)

INTRODUCTION

GENERAL BACKGROUND

The prostate - anatomical and physiological aspects

The prostate is an unpaired organ situated around the uppermost part of the urethra. It is the largest accessory gland of the male reproductive system, providing about 30% of the volume of the seminal fluid. During sexual ejaculation it helps to expel the ejaculate, thus promoting fertilization. Its size in the newborn boy is about that of a grain of wheat, but the size increases with puberty and reaches the young adult volume of a chestnut, or less than 25 ml.[12,21,77,103,107,186,187]

The internal morphology is organized in anatomical zones according to a concept introduced by McNeal in 1969.[115,116] The peripheral zone (PZ) constitutes about 70% of the gland, forming the lateral and posterior parts of the prostate. This is the zone of predilection for prostate cancer. The central zone (CZ) constitutes about 25% of the gland, and forms a wedge from the bladder neck to the verumontanum. The transition zone (TZ) constitutes only 5 to 10% of the gland in the young adult, but in benign prostatic hyperplasia (BPH) it may undergo an enlargement of great proportions during the life span of a man.[26] Lastly, the ventral fibromuscular zone is the large non-glandular part of the organ, anchoring the central and peripheral zones anterior to the urethra.

The prostate's orientation is often described as that of an inversed pyramid with the base facing upward to the bladder neck with which it is continuous, and the blunt apex resting fixed on the pelvic diaphragm. Anteriorly, the prostate surface is related to the retropubic space (cave of Retzius). Laterally, the prostate is limited by the levator prostatae fibers of the levator ani musculature. The posterior surface of the prostate is separated from the rectal ampulla by the rectoprostatic fascia (fascia of Denonvilliers), originally described by Pattison.[154] This dense septum is the reason why tumour spread of prostate cancer to the rectum is uncommon, despite its proximity.

The blood supply of the prostate derives from the inferior vesical and middle rectal arteries. The venous flow from the prostatic venous plexus drains into a larger pelvic plexus before emptying into the internal iliac veins. The pelvic plexus also communicates with the Batson veins explaining the common metastatic spread to the sacrum, ileum, and lumbar spine.[19]

The lymphatics drain into the external, internal and common iliac nodes. The innervation is both parasympathetic from S III and S IV, stimulating glandular

secretion and sympathetic from the hypogastric plexus, stimulating smooth muscle contraction.

The seminal vesicles lie above the prostate, behind the urinary bladder and lateral to the ampullae of the ductus deferentes.

The physiological role of the prostate is to produce and secrete seminal fluid emitted together with the sperms at ejaculation. The semen has several functions: primarily to provide energy support to the sperms, but also to flush away urine and bacteria from the urethra and to adjust the microenvironment including viscosity, for optimal fertilization conditions. Among the proteins at play is prostate-specific antigen (PSA), which serves to keep the seminal fluid liquid.

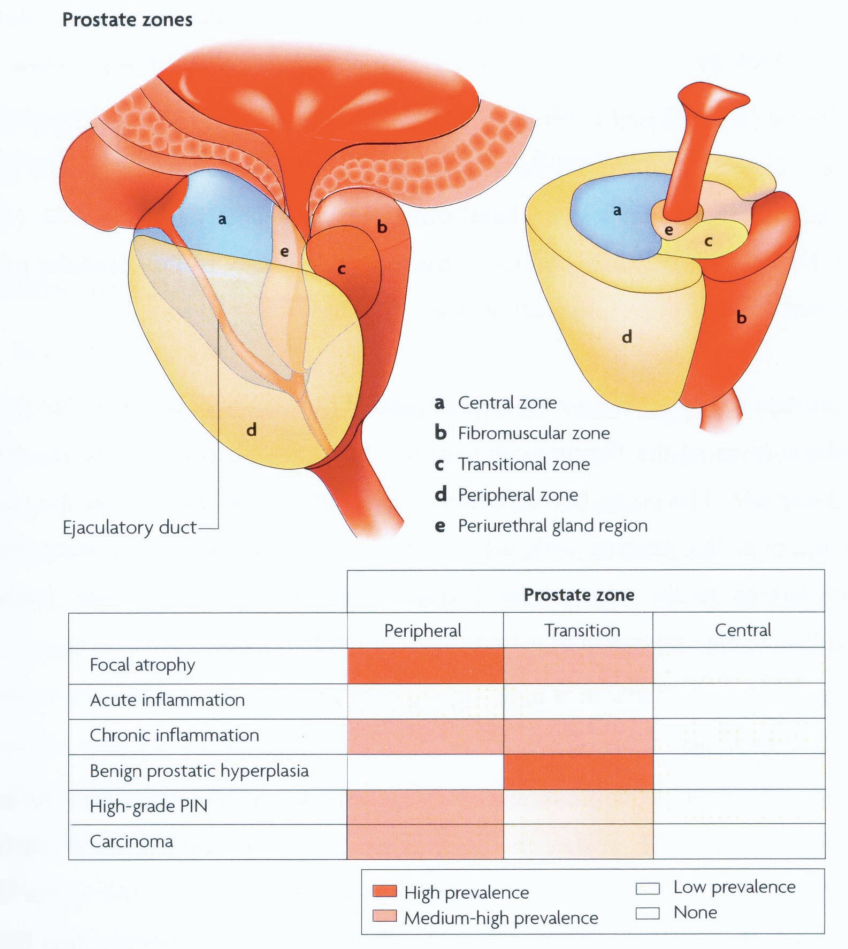

Figure 1. Anatomical prostate zones and predisposition to prostate disease[39] (Nature Publishing Group; reprinted by permission).

The function of the male reproductive system including the prostate depends on circulating androgenes, of which testosterone is the primary and most important. Androgens are crucial in the development and maintenance of sexual function, and androgen stimulation plays a central role in the development of prostate cancer.[82,185]

Historical note

The history of the medical term *prostate* and the discovery of the organ are debated. According to some authors, Herophilos (3rd century BC) was the first to discover the organ, later confirmed by Galen (129 AD – 199/217 AD). Other authors, however, argue that they may have mistaken the seminal vesicles for the prostate.[92,150,153] The discovery would then be attributed to the 16th century anatomists Nicola Massa (1536) and Andreas Vesalius (1538), the latter in his anatomical plates (*Tabulae anatomicae sex*, Basel, 1538).[178] In 1600 the anatomist du Laurens was the first to give the organ a designation in latin, and called it *prostatae* according to the misconception that it was a paired organ. It is believed that the English anatomist and surgeon William Cheselden in 1792 was the first to regard the prostate as one unpaired organ,[92] which was confirmed by studies in the following decade.

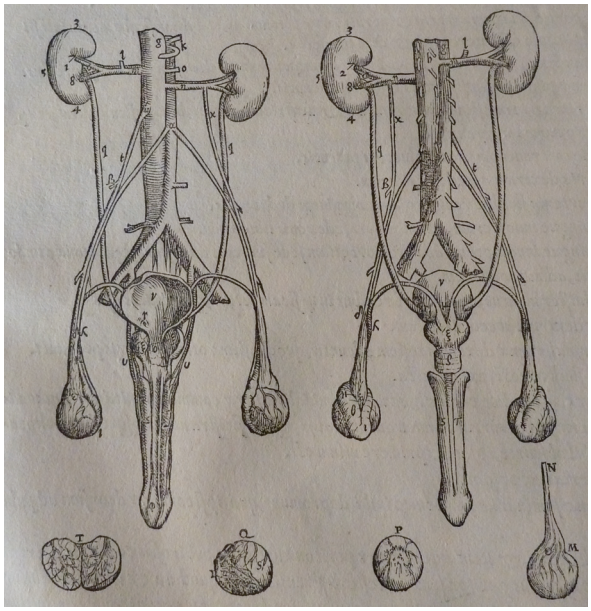

Figure 2. Detail from Vesalius: *De humani corporis fabrica libri septem,* 1st ed. Left: anterior view; right: posterior view. Prostate marked with greek letter ξ. Photo by permission of Hagströmerbiblioteket.

PROSTATE CANCER

Epidemiology

Prostate cancer is the most common non-cutaneous cancer in men in the western world, the third most common cancer in men globally and overall the sixth most common cancer. In 2000, the estimated new number of prostate cancer cases in the world was 543 000, representing slightly more than 10% of all cancers in men.[15,132,139,140] Estimated prostate cancer deaths worldwide the same year was 204 000, a little less than 6% of all cancer deaths. Incidence has increased since the 1970s, and even more since the 1980s and 1990s when new diagnostic and therapeutic tools were introduced, thus stimulating increased diagnostic activity.[40] This has become a major public health problem consuming medical and financial recources.[118,156]

In Sweden, the incidence has increased dramatically, especially in the younger population with localised disease. Mainly because of an increased incidence but also because of a slight decrease in mortality, the prevalence of prostate cancer has increased.[5,137,160] In Sweden prostate cancer accounted for almost 19% of all new cancers in 2009, while 2424 men died the same year, giving a mortality rate of 51 per 100 000, and 31 per 100 000 age-standardised.[160]

Etiology

Prostate cancer is an unpredictable disease, with no clear etiology and varying natural history.[55,72,118,184] There are several proposed risk factors but the causes are unknown. Age together with geographical, racial and hereditary factors are known risk factors, and the disease is regarded as a result of multifactorial influence. Genetic factors play an important but hitherto obscure rule, and different possible pathways for heredity are described.[31,69-71,114] Hereditary factors are known to increase the risk 2-3-fold,[27] and "Western life style" including high intake of dietary fat seems to be of importance, as observations of populations moving from low to high prevalence regions have shown an increased incidence with migration.[177] There are suggestions that traditional Japanese diet and Mediterranean diet may have protective properties.[33,86,88,121]

Pathogenesis

It is generally accepted that prostate cancer develops from a non-invasive precursor stage, which is a target for neoplastic transformation.[75] Both prostatic intraepithelial neoplasia (PIN) and proliferative inflammatory atrophy (PIA) are considered as possible steps[98,189] in this pathway, separately or together.[38,39,95,181] PIN is defined by cytological atypia in the luminal epithelial cells with morphologic signs of malignancy,

but with intact basement membrane and non-invasive architecture. PIA is described as focal atrophic lesions with high proliferative activity and with molecular expression suggestive of oxidative stress. PIA is often seen in regions of the prostate where cancer is prevalent.

Symptoms and clinical findings

There are few, if any, clinical signs in early disease stages, and no pathognomonic symptoms. The patients may present with lower urinary tract symptoms, or no symptoms at all. The first diagnostic steps in counselling are digital rectal examination (DRE) and serum PSA analysis.[112] Tumours found by palpation are often more advanced and can be detected without knowledge of serum PSA level, but a combination of these two methods gives a higher detection rate than each method alone, and they should therefore be used together.[106,127,158] Furthermore, DRE can diagnose other conditions than cancer causing serum PSA elevation; e.g. prostatitis or BPH.

Some patients have generalised cancer symptoms at diagnosis: fatigue, weight loss, triple-figure PSA elevation, hard nodular prostate. At this late-stage disease, the clinical picture is pathognomonic for metastasized prostate cancer.

Course and prognosis

The clinical course of prostate cancer is highly variable. Surprisingly, autopsy data show both high-grade prostatic intraepithelial neoplasia (HGPIN) and cancer as early as in the third decade of life, with a prevalence of infiltrative cancer of 26-29% among men in their forties.[146,161] In Mediterranean caucasians the cancer prevalence is lower.[148,165]

In Sweden 68.5% of diagnosed prostate cancers 2009 were found in men 65 years or older.[160] In many men cancer will remain clinically insignificant and asymptomatic several years.[35,90] A conservative approach to patients with low-risk disease is supported by several authors.[3,7,9,35,90,135] Prostate cancer of this stage has, particularly in men with co-morbidity, in general little effect on the health profile.

Other prostate cancers progress quickly to stages beyond cure. This is the true dilemma in uro-oncological care of prostate cancer patients; whom to treat and whom to observe. Gleason score (GS), stage and serum PSA level remain the best predictors of prognosis.[163] Studies on stratification of patient populations support that conservative treatment is beneficial for patients with low-risk cancer (serum PSA <10 ng/ml, GS <7, stage <T2b). Pre-treatment risk stratification models have been developed with a combination of prognostic factors, for prediction of individual treatment outcome.[37,133,134]

DIAGNOSIS

Digital rectal examination

Digital rectal examination (DRE) is a standard procedure for assessment of shape, texture, size and tenderness of the prostate. It is quick, simple and virtually complication-free, but dependent on the examiner and therefore subjective.[176] The positive predictive value for DRE alone is only about 20%,[76] but DRE can increase cancer detection in patients with normal serum PSA,[138] and also indicate a risk for high-grade disease.[106,127] DRE is part of the procedures that are the basis for the staging system (see below).

Prostate specific antigen

Prostate specific antigen (PSA) is an organ-specific protein produced by prostate epithelial cells and involved in the physiological fertilization process. It was first described in 1970 and further purified and characterized during the following decade.[1,67,104] It is tissue-specific but not disease-specific. Thus, serum PSA levels overlap between patients with benign enlargement or inflammation of the prostate and men with prostate cancer. Nevertheless, the discovery of this protein has had a tremendous impact in urology.[105,166] In the pre-PSA era, serum prostatic acid phosphatase was used for detection of prostate cancer, but this enzyme signals the disease at a later stage.[30] The introduction of immunoassays for PSA led to a paradigm shift in the diagnostic approach,[32] but because of the relatively low specificity for cancer, various additional assays have been used, such as percent free PSA, which improves specificity.[4,174]

Transrectal ultrasound

Transrectal ultrasound (TRUS) is the most common imaging modality for the prostate today, and the method has developed considerably since its introduction in the mid-20th century.[143] It is performed as real-time sonography, allowing for detailed imaging of the prostate and its adjacent structures.[183] TRUS is used for determination of shape, texture, volume and for biopsy guidance, and is the mainstay in the diagnostic puncture setting. The typical appearance of cancer is a hypoechoic lesion, but several authors have reported relatively high proportions of isoechoic and hyperechoic cancers.[144,147,162] Thus, both systematic mapping biopsies and biopsies directed at TRUS-detected lesions suspicious for cancer are routinely taken. The PPV for TRUS alone is not higher than for DRE in a screening population, but a combination of the methods improves PPV.[125] Quadrant and later sextant biopsy protocols used to be the standard in the 1990s, but with midlobar lateral biopsies the detection rate increased.[13] Today, 8 – 12-core protocols are most common, with 10 cores as a recommended minumum in the EAU

guidelines.[42] It is recommended nowadays to offer the patient pain relief with anaesthesia during the procedure, preferrably with a periprostatic injection of a local anaesthetic.[14,42,180]
Saturation biopsy protocols (at least 20 cores) are occasionally used and is regarded as a valuable tool by some authors.[130,142] Others, however, claim that there is little increase in detection rate,[46,73,152] and that saturation biopsies increase the risk of adverse events.[46,94] Re-biopsy strategies may therefore be preferred.[51,122]
Prostate volume estimation can be assessed according to various calculation methods,[18,100,168] of which the most commonly used in TRUS is the ellipsoid formula (height∗width∗length∗π/6).

Histopathology

Histopathology is the foundation of cancer diagnosis of the prostate. However, in late stage disease triple-figure serum PSA in a pain-ridden man with metastases on bone scan is pathognomonic for the clinical diagnosis (see above). More than 95% of prostate cancers are adenocarcinomas. There are other unusual histologic types of malignancy that develop *de novo* in the prostate, including non-glandular carcinomas, neuroendocrine tumours, stromal tumours, hematolymphoid malignancies and rare miscellaneous tumour types. Secondary tumours may also appear in the prostate, e.g. urothelial cancer. However rare, histopathological identification of these unusual tumours is crucial for appropriate therapeutic measures.[43]

Grading

The Gleason system was invented by the pathologist Donald F. Gleason, first presented in 1966 and further developed during the following decade.[16,61-65] The Gleason grading system is officially recommended by the WHO. It is based on the glandular architecture at low-power magnification with standard hematoxylin-eosin stained prostatic tissue, e.g. biopsy cores or surgical specimens. A 5-tier grading is used with increasing architectural disorganization from grades 1 to 5. The most prevalent pattern and the second most prevalent pattern are added to give a *Gleason score* or *sum*, e.g. 4+3 or 3+3. The impact of this system was immediate, probably not only owing to the fact that it proved to be a powerful tool for diagnostic and prognostic purposes,[10] but also to the striking simplicity in the drawings presented together with the descriptive texts for each grade.

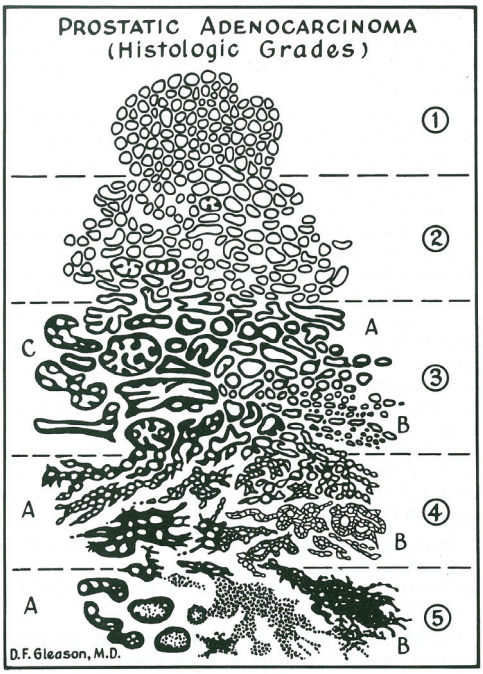

Figure 3. Histologic grades in prostatic carcinoma, depiction by DF Gleason.

Before the Gleason grading, the WHO grading was the most commonly recommended grading system. The WHO grading (originally the Mostofi grading) was based on a combination of cellular anaplasia and glandular differentiation. It was thus a cytology-based system, dividing tumours in three grades (I – III); well differentiated, moderately well differentiated and poorly differentiated or undifferentiated tumour. A major problem with the WHO grading was that there were no rules for how to combine cellular and glandular grades into a single grade. As it was designed, cellular atypia and glandular differentiation were meant to be reported separately. The European tradition was to use a 3-tier system with a single grade based on all features, often erroneously referred to as the WHO grading. It has now been replaced by the Gleason system, but has some historical importance as older literature often refer to the WHO grading system. Attempts have been made to correlate the WHO system to the Gleason system,[87,111] but there are no conversion tables between the two systems.

Staging

Tumour stage is classified according to the UICC (Union for International Cancer Control) 2009 Tumour Node Metastasis classification and is presented in the table below.

T - Primary tumour		
TX	Primary tumour cannot be assessed	
T0	No evidence of primary tumour	
T1	Clinically inapparent tumour not palpable or visible by imaging	
	T1a	Tumour incidental histological finding in 5% or less of tissue resected
	T1b	Tumour incidental histological finding in more than 5% of tissue resected
	T1c	Tumour identified by needle biopsy (e.g. because of elevated prostate-specific antigen [PSA] level)
T2	Tumour confined within the prostate[1]	
	T2a	Tumour involves one half of one lobe or less
	T2b	Tumour involves more than half of one lobe, but not both lobes
	T2c	Tumour involves both lobes
T3	Tumour extends through the prostatic capsule[2]	
	T3a	Extracapsular extension (unilateral or bilateral) including microscopic bladder neck involvement
	T3b	Tumour invades seminal vesicle(s)
T4	Tumour is fixed or invades adjacent structures other than seminal vesicles: external sphincter, rectum, levator muscles, and/or pelvic wall	
N - Regional lymph nodes[3]		
	NX	Regional lymph nodes cannot be assessed
	N0	No regional lymph node metastasis
	N1	Regional lymph node metastasis
M - Distant metastasis[4]		
	MX	Distant metastasis cannot be assessed
	M0	No distant metastasis
	M1	Distant metastasis
		M1a Non-regional lymph node(s)
		M1b Bone(s)
		M1c Other site(s)

[1] Tumour found in one or both lobes by needle biopsy, but not palpable or visible by imaging, is classified as T1c.
[2] Invasion into the prostatic apex, or into (but not beyond) the prostate capsule, is not classified as pT3, but as pT2.
[3] Metastasis no larger than 0.2 cm can be designated pN1 mi.
[4] When more than one site of metastasis is present, the most advanced category should be used.

Table 1. Tumour Node Metastasis (TNM) classification of prostate cancer.

TREATMENT

Treatment options depend on tumour stage, and consist of treatment with curative intent for localised disease and palliative treatment for generalised disease. Palliative treatment is normally instituted at the time of diagnosis in patients with symptoms related to the cancer, but can also be deferred. Radical treatment is normally considered for patients with no or relatively low comorbidity where there is hope for at least 10 years of life expectancy.

Localised disease

When the disease is localised, curative treatment by radical (retropubic, laparoscopic or robot-assisted laparoscopic) prostatectomy or radiotherapy is feasible.[22,23,80,123] An ongoing prospective swedish study, LAPPRO (LAParoscopic Prostatectomy Robot Open), is designed to study the functional and oncological outcome comparing open and robot-assisted laparoscopic techniques.[169]
Radiotherapy can be given as external treatment (EBRT), internal low or high dose rate brachytherapy (BT) or a combination of external and internal irradiation. Radiotherapy is typically preceded by neo-adjuvant total androgen blockade.

Another option in low-risk tumours is deferred treatment, especially in men who are elderly or have a significant comorbidity. This observational strategy, called watchful waiting, has recently been added with the concept of *active monitoring,* also applicable to younger patients who wish to primarily refrain from invasive therapy. With active monitoring comes a more rigid framework including repeat-biopsies and re-evaluation of chosen treatment strategy, in that way pushing an irreversible treatment forward to gain time, and in the same time avoiding unwanted treatment. The difference between the two observational strategies is fundamental. If the patient experiences progression under watchful waiting palliative treatment is instituted, while in the case of progression under active monitoring, the aim is radical treatment. In treatment protocols, the concept of active monitoring has emerged from being inferior to a rightly attractive alternative for selected patients.[171,173]

Advanced disease

When prostate cancer is locally advanced or generalised, cure is no longer feasible. The treatment is palliative, including endocrine antitumoural medication as a first step.

This can be achieved in several ways: monotherapy with antiandrogen that functions as an androgen-receptor antagonist; GnRH-antagonists (or partial agonists), which can be administered continuously or intermittently;[119] bilateral orchidectomy; combined (or total) androgen blockade, which consists of orchidectomy or GnRH-treatment together with antiandrogen; and estrogen therapy.

For patients presenting with generalised cancer symptoms and threatening fractures of the vertebral column with spinal compression, bilateral orchidectomy performed immediately is the treatment of choice, because of its rapid therapeutic effect.

When hormonal therapy is instituted, it is of great importance that the well-known metabolic side effects, e.g. osteporosis, weight gain, cardiovascular disease and mental effects can be seen to be adequately taken care of.[58,151]

With progression, cytotoxic treatment should be considered. At this stage an armoury of possible palliative treatment options have to be at hand for the control of local symptoms, e.g. deviation of the upper urinary tract with nephrostomy tube or pigtail catheter insertion in the case of symptomatic obstruction, stabilising procedures for orthopedic emergencies, and palliative measures for anemia, nausea and pain. Palliative radiotherapy also has its place during this phase of the disease, and the psychological aspects of patient care become even more important.

CONCLUDING REMARKS

There is a delicate balance between morbidity and mortality induced by disease, and induced by treatment. With minor treatment side-effects and low costs in a broad sense a treat-all intention can be justified; including instead of selecting. Without optimal selection tools, the care for the patient will always urge doctors to treat. This is especially true for prostate cancer, and has led to a debate on overdiagnosis.

While overdiagnosis is a misnomer, suggesting that some patients are diagnosed with a prostate cancer they do not have, overdetection defined as diagnosing a clinically insignificant cancer is a relevant issue. Overtreatment of insignificant, indolent cancers is a considerable concern. The medical profession is fully aware of this, and research is focused on identification of high risk groups in the population and high risk cancers. It is within this field that research for improved biopsy techniques, improved prognostication, new biomarkers, identification of risk groups and increased knowledge on prostate carcinogenesis hopefully may lead to a more accurate identification of clinically significant cancers.

AIMS OF THE STUDY

The overall purpose of this thesis was to study the diagnostic process from clinical suspicion of prostate cancer to the histopathological proof of cancer leading to a treatment recommendation, and to identify potential biomarkers that could provide additional diagnostic and prognostic information.

In particular the study aimed to:

Evaluate a novel core biopsy instrument by comparing the tissue yield with that of a standard instrument in a clinical prostate biopsy setting.

Investigate the preoperative prediction of large tumour volumes in prostate cancer utilising needle biopsy data.

Analyse the impact of tumour heterogeneity in prostate cancer on preoperative DNA ploidy assessment.

Assess the expression patterns of three redox control system proteins (thioredoxin reductase R2, thioredoxin 1 and peroxiredoxin 2) in benign and malignant prostate tissue in order to investigate their role in the development of prostate cancer, and the potential prognostic relevance of this pathway.

Investigate the role of new tissue-biomarkers for prostate cancer for identification of prostatic tumour origin.

Investigate the role of new cancer-specific biomarkers for prostate cancer.

MATERIALS AND METHODS

Clinical material

The studies included fresh frozen and paraffin-embedded tissue from prostate biopsy procedures and tumour surgery performed at the University Hospital of Uppsala (Akademiska sjukhuset) and in the Karolinska University Hospital in Stockholm, together with relevant medical records for clinical follow-up.

Study populations

In Study I the study population consisted of 60 patients referred to the Karolinska Hospital or St Göran hospital in 2001-2002 for TRUS-guided biopsies, primarily for a suspicion of cancer because of elevation of serum PSA and/or a palpable nodule on DRE.

In Study II 121 men underwent radical prostatectomy at Uppsala University Hospital because of prostate cancer between 1993 and 1999, and after exclusion of 6 men whose cancer was diagnosed through transurethral resection of the prostate (TUR-P), the preoperative biopsies and the radical prostatectomy specimens of the remaining 115 men were reviewed and further investigated.

In Study III preoperative prostate biopsies from 50 men were analysed. They were treated between 1993 and 1995 with radical retropubic prostatectomy for prostate cancer, and a previous study had mapped the DNA ploidy of these tumours. The ploidy status of the biopsies was assessed and compared with the DNA ploidy of the prostatectomy specimens.

In Study IV the study population consisted of two groups: 333 cases of prostate cancer operated with radical retropubic prostatectomy between 1998 and 2002 at the Karolinska Hospital, out of which 294 cases remained for analysis after exclusion of cases with insufficient material and/or clinical follow up; for this group a prognostic TMA was constructed. The other group consisted of 40 cases of prostate cancer operated with radical retropubic prostatectomy in 2005 at the same hospital, and here a pathogenetic TMA was constructed including different tissue from the same cases.

In Study V the study population comprised 36 men who underwent radical prostatectomy for prostate cancer in 2005, and as control, tissue retrieved from surgical specimens of bladder, rectal and lung cancers from 2001 – 2002.

In Study VI the study population consisted of two groups: 40 consecutive cases of prostate cancer operated with radical prostatectomy during 2005 used for TMA

construction, and 32 cases of fresh frozen tumour and benign prostate tissue for real-time PCR analysis.

More detailed information on the study populations is presented in the individual papers.

Transrectal ultrasound examination and biopsy protocols

The patients in Study I were biopsied at the Karolinska Hospital and St Göran hospital in Stockholm using a Bruel & Kjaer ultrasound console Leopard with an 8538 transducer and needle holder. Antibiotic prophylaxis with norfloxacin was given. Biopsies were taken according to an octant protocol (base, mid-medial, mid-lateral and apex). Ultrasound-detected lesions outside these positions were also biopsied but were excluded from the statistical analysis. The prostate volume was calculated using the ellipsoid formula. For the side-notch needle the Biopty Magnum gun was used, equipped with a single-use 18 G needle (outer diameter 1.2 mm, notch length 17 mm and stroke length 22 mm). The end-cut needle (BioPince) is a sterile single-use biopsy gun with an attached 18 G needle (outer diameter 1.2 mm and stroke length 13, 23, or 33 mm respectively). In the first 40 patients the stroke length was set to 23 mm. In the following 22 consecutive patients, 20 had a prostate volume large enough and a stroke length of 33 mm was used. In each patient the end-cut needle was used either on one side of the prostate and the side-notch needle on the other side. Laterality was randomized in each case and was not disclosed to the pathologist.

The patients in Studies II and III were biopsied at the University Hospital in Uppsala using a Bruel & Kjaer ultrasound console 3535 with an end-fired 7 MHz probe or an Acuson Sequoia 512 instrument equipped with a 6-10 MHz probe (EC10C5) and a spring-loaded core biopsy gun (Biopty) equipped with an 18 gauge needle. The notch length varied between 19 and 32 mm. Antibiotic prophylaxis with norfloxacin was given. Biopsies were taken according to a standardised protocol including apex, mid-medial, mid-lateral, base and transition zone biopsies. In case lesions were detected by ultrasound outside these standard positions additional biopsies were taken. Biopsies were also taken from the seminal vesicles in 109 patients but not included in the analysis.

Tissue collection

Biopsies from the prostate and radical prostatectomy specimens were collected according to local routines and immediately fixed in 4% buffered formaldehyde solution (Studies I – VI).

Material from macroscopically suspicious tumour and benign tissue was harvested before formalin fixation, snap frozen in liquid nitrogen and stored in -80˚ C. The

morphological diagnosis was verified microscopically by frozen sections (Studies V – VI).

Biopsy specimens

Measurements were done after fixation using a ruler for length and a Mettler A30 laboratory balance (range 0.0001-100g) for weight.

Radical prostatectomy specimens

The prostates generally arrived to the pathology laboratory unfixed, transported on ice. The prostate was cut into two halves by a horizontal section. The cut surfaces were inspected and samples taken from macroscopically visible tumour areas and from grossly normal areas. A 2 mm thick shave section was taken from each area with a scalpel, split into multiple smaller samples and snap frozen in liquid nitrogen. The samples were stored in -80 C in a dry cryotube. Sample locations were noted on a specimen map. The halves of the prostate were mounted on a cork plate. After overnight fixation in 4% buffered formalin, the prostate was inked, sliced horizontally at 4 mm and totally embedded. The slices were either cut in 2 - 6 segments or whole-mounted. The specimens were dehydrated, cut at 4 μm and stained with hematoxylin and eosin. Frozen sections from the tissue samples were also cut at 4 μm, stained with hematoxylin and eosin and reviewed.

Tissue microarrays

The tissue microrrays (TMAs) were constructed using a Beecher Manual Arrayer 1 (Beecher Instruments, Silver Spring, MD, USA) with a punch diameter of 1 mm.

Staining and immunohistochemistry

Routine staining with hematoxylin and eosin followed the normal protocol for the laboratory.

For immunohistochemistry, slides were incubated at 60˚ C for 45 min, deparaffinized in xylene (2 × 15 min) and rehydrated through graded alcohols. Endogenous peroxidase was blocked with H_2O_2 in 95% alcohol. Antigen retrieval was performed with a heat-induced method using a Decloaking chamber (Biocare Medical, Walnut Creek, CA, USA), where slides were immersed in Citrate buffer®, pH 6 (Lab Vision, Freemont,

CA, USA) and boiled for 4 min at 125˚ C. Immunostaining was performed using an automated staining instrument, Autostainer 480® (Lab Vision). Primary antibodies against GAD1, PSMA and PSA (Study V) or CYCS, ICK, IKBKB and AMACR (Study VI) and a dextran polymer visualization system (UltraVision LP HRP polymer®; Lab Vision) were incubated for 30 min each at room temperature. The slides were rinsed in wash buffer® (Lab Vision) between all steps and Diaminobenzidine® (Lab Vision) was used as chromogen for 10 min. Slides were rinsed in tap water and counterstained in Mayer's hematoxylin (Histolab, Gothenburg, Sweden).

Database searches

For Study V, a search for prostate-specific tissue markers was conducted in the Human Protein Atlas (HPA) (www.proteinatlas.org), where we had first-hand access to non-public data. A search algorithm was constructed, aiming at antibodies with strong expression in at least 10 out of 12 prostate cancer samples and negative staining in all 12 urinary bladder samples.

For Study VI, a similar search for markers of diagnostic interest was conducted but with a two-step strategy: firstly, the database was asked for proteins with moderate or strong expression in at least eight out of 12 prostate cancer samples and negative staining in benign prostatic tissue. Secondly, it was asked for proteins that were strongly expressed in at least eight out of 12 prostate cancer samples and had weak or negative staining in benign prostatic tissue. All hits were then manually surveyed. At the time of the searches (August 2007), there were approximately 3000 available proteins in the database.

Evaluation of immunohistochemistry

Evaluation of each TMA core was done visually and semiquantitatively according to increasing intensity and extent in the cytoplasm of the cells of interest. Intensity was scored according to a 4-tier scale: negative, weak, medium or strong. When a core was heterogeneously stained, the strongest intensity was graded. For extent we used a 3-tier scale, in Study V signifying 1-33%, 34-66% and >66%, respectively, and in Study VI <25%, 25-50% and >50%, respectively. Intensity and extent scores were multiplied to obtain a combined score, immunoreactivity product (IRP), from 0-9.

Real-time-PCR

For Study V, gene expression was analysed on fresh frozen tumour and benign prostate tissue derived from 10 matched patient samples. For comparison, four samples each from urothelial, lung and rectal cancer were also analysed.

For Study VI, the material consisted of fresh frozen tumour and benign prostate tissue derived from 32 radical prostatectomy cases collected in 2004– 2005 at the Karolinska University Hospital, Stockholm, Sweden.

RNA isolation was performed using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA was quantified with a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For each tissue sample a 20 mm thick section was used for RNA isolation. The average amount of extracted RNA was 1.2 mg. The cDNA was synthesized with the SuperScript III First-Strand Synthesis System (Invitrogen) using poly-dT primers, according to the manufacturer's instructions. 1 μL of cDNA was used to run the real-time polymerase chain reaction (RT-PCR) analysis using the SYBR Green Universal PCR Master Mix (Applied Biosystems, Foster City, USA). Primers were used at a concentration of 200 nM. The reaction was performed with the Applied Biosysystems 7500 Real-Time PCR system (Applied Biosystems) under conditions recommended by the manufacturer.

For Study V, the expression levels of GAD1, PSA and PSMA were normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

For Study VI, expression levels were normalized to the expression of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerokinase 1 (PGK) and hypoxanthine ribosyltransferase (HPRT).

Western blot

Western blot in Study V was performed on 15 mg of total protein lysate from normal prostate tissue on a precast 10–20% criterion sodium dodecyl sulfate-polyacrylamide gel electrophoresis gradient gel (Bio-Rad Laboratories, Hercules, CA, USA) under reducing conditions, followed by transfer to polyvinylidine fluoride membrane (Bio-Rad Laboratories). The membrane was soaked in methanol and blocked (5% dry milk, 0.5% Tween 20, 1x Tris-buffered saline; 0.1 M Tris–HCl, 0.5 M NaCl) for 1 hour at room temperature with constant shaking. The membrane was incubated with primary antibody against GAD1 for 1 hour, followed by washing (1x Tris-buffered saline Tween-20) and incubation with a secondary (Goat anti-mouse 1:7000) peroxidase-conjugated antibody. Chemilumenescence detection was carried out using a charge-coupled device (CCD) camera (Bio-Rad Laboratories) with SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA).

Statistical analyses

Differences between mean values were analysed with paired or unpaired Student's *t*-test (Studies I, II, IV, V and VI). Sensitivity, specificity, positive and negative predictive values were calculated for the identification of tumour size categories (Study

II). Differences between proportions of different categories were analysed with chi-squared test (Study I) or Fisher's exact test (Study III). Linear regression according to Pearson and multiple regression analysis was used to compare the correlation of multiple explanatory variables (Study II). The distribution of non-parametric data among different categories was compared by Mann-Whitney U-test (Study III). The correlation coefficient according to Spearman rank was used for non-continuous variables (Studies IV and V). Cox proportional hazards models were used for comparison of prognostic parameters (Study IV). Receiver operating characteristic (ROC) analysis was used for area under the curve (Study VI). A p value <0.05 was taken to indicate a significant difference.

Ethics committee decisions

The Regional Ethics Committees in Stockholm and Uppsala approved all studies according to the following decisions: Dnr 01-438 (Study I); Ups 03-081 and KI 03-091 (Study II); Dnr Ups 02-261 and KI 02-382 (Study III); 2006/4:10 (Studies IV, V and VI); and 01-353 and 2010/432-32 (Study V). Analyses performed in France were approved by the IARC Institutional Review Board Committee: IARC 06-08 (Study IV).

RESULTS AND DISCUSSION

Study 1: Evaluation of a new instrument for prostate biopsies

Tissue yield

The length and weight of biopsy specimens from a conventional side-notch biopsy needle and a new end-cut biopsy instrument were compared pairwise; pairs with one biopsy missing were omitted from the analysis. The length, weight and weight per length of the biopsies are shown in the table.

	Mean (range) specimen			
Group	*length, mm*	*weight, mg*	*weight/length, mg/mm*	*On slide length, mm*
End-cut, 23 mm	17.8 (1–36)	6.5 (0.3–14.1)	0.374 (0.05–1.0)	15.4 (1–27)
Side-notch	17.3 (6–27)	5.50 (1.7–10)	0.329 (0.13–0.6)	14.1 (5–22)
P	0.4	<0.001	<0.001	0.042
Histology				
Cancer	17.7	6.88	0.395	–
Benign	17.5	5.75	0.337	–
P	0.69	<0.001	<0.001	–
Position*				
Apex	17.0	5.55	0.326	–
Mid lateral	16.8	5.77	0.343	–
Mid medial	18.6	6.35	0.341	–
Base	17.8	6.48	0.364	–
Stroke length				
End-cut, 33 mm	19.9 (1–34)	6.53 (0.3–13.3)	–	16.0
Side-notch	14.4 (8–27)	4.91 (2.2–12.8)	–	11.8
P	<0.001	<0.001	–	<0.001

* The length and weight of the apex and mid lateral biopsies were significantly less than for the base and mid medial biopsies ($P=0.034$ and 0.002, respectively). There was a trend towards higher weight/length of the base biopsies than the other biopsies, although this was not significant ($P=0.052$).

Table 2. The length, weight and weight per length of biopsy specimens, length of biopsies as measured on the glass slide (23 mm stroke length of the end-cut needle), for those containing cancer and benign tissue in the first 40 patients, with position, and for the 33 mm stroke length.

The length of biopsy specimens from the two instruments did not differ significantly (p = 0.42) but when measured on glass slides, the biopsies from the end-cut needle were on average 1.3 mm (9.5%) longer than those from the side-notch needle (p = 0.042). Thus, by using the end-cut needle, the proportion of the specimen not seen in the histological section decreased from 18.5% (3.2/17.3) to 13.5% (2.4/17.8). The weight and the weight/length of the biopsies from the end-cut needle were 18.4% and 13.7% greater, respectively, than those from the side-notch needle (p <0.001). When the 33 mm stroke length in the end-cut needle was compared with the 22 mm stroke length needle, the length and weight of the end-cut biopsies were 38.4% and 33.0% greater, respectively, than in biopsies from the side-notch instrument.
The length of biopsies containing cancer and benign tissue, respectively, did not differ significantly. However, the weight and weight/length of biopsies positive for cancer were greater than in benign biopsies (19.8% and 17.2%, respectively, p <0.001). This

difference remained significant when the biopsies from each instrument were analysed separately.

Failure rate

In the first 40 patients biopsies were taken from 160 standard positions with each instrument. In 35 of the attempts no tissue was obtained with the end-cut needle giving a failure rate of 21.9%. In 12 of these a biopsy specimen was obtained after a second biopsy. With the side-notch needle the failure rate was three of 160 (1.9%) and two of those were successfully repeated. The difference in failure rate was highly significant ($p < 0.001$). The proportion of cancer was nearly equal between the two instruments, i.e. 24.1% (33/137) and 23.9% (38/159) from the end-cut and side-notch instrument respectively. In the last 20 patients (end-cut needle stroke length 33 mm), the biopsy specimen was lost in 22.5% of the attempts with the end-cut needle (18 out of 80 positions, necessitating a repeated biopsy attempt), compared with none of the attempts with the side-notch needle.

Discussion

Unlike other forms of cancer where radiology may play a role, the diagnosis of prostate cancer and the following therapeutic decision depends entirely on the histological evaluation of tissue samples from the prostate. Evidently, it is desirable to obtain as much tissue as possible with a minimum of trauma. While different biopsy protocols have been aiming at improving the results,[11,48,49,164] less has been done to improve the biopsy technique itself.[25,113] When a circular full core needle that pinches off the biopsies was introduced, it was anticipated that the tissue yield would increase. In the present study the new instrument was used together with the standard instrument in the same patients and biopsies were compared pairwise to ensure as equal conditions as possible. The biopsies from the end-cut instrument were heavier and thicker than those from the side-notch instrument, but there was a significant proportion of zero-yield biopsies. There was no difference in cancer detection rate between the two needle types, but these results must be interpreted with caution as the series is too small to allow conclusions regarding cancer detection. Biopsy specimens containing cancer had a greater weight and weight/length than the benign biopsies, regardless of needle type. This may reflect the different texture and tissue composition in cancer. Several studies have confirmed our results.[41,56,129,175]

To summarise, in Study I we have performed an investigation regarding the tissue yield of a new core-biopsy needle and compared it with a standard instrument. We found that the new instrument provided thicker biopsies and flatter embedding in the paraffin blocks, which together with the optional greater stroke length are features advantageous for a maximum tissue yield. However, these advantages were countered by a significant loss of biopsies because of instrument mechanism failure. We concluded that the new instrument can be recommended primarily for some specific biopsy situations such as

repeat biopsies in patients with persistent serum PSA elevation and suspicion of anterior tumours and possibly also in patients with large prostates.

Study 2: Prediction of large tumour volume in prostate cancer

In this study we found that cancer length and percentage cancer length in preoperative prostate biopsies predicted tumour volumes better than number and percentage of cores positive for cancer. Using univariate logistic regression, the number and percentage of cores positive for cancer, cancer length and percentage cancer length predicted tumour volumes of >4, >6 and >8 ml (Table 3). Tumour volumes of >4 ml had the highest positive predictive values, 83 – 100%, when using cancer length and percentage cancer length. For example, a biopsy cancer length of ≥30 mm predicted a tumour volume of >4 ml in 95% of cases. The highest Gleason score and serum PSA were weaker predictors of large tumour volume. Tumour volumes of <2 and <4 ml were found in 13% and 35%, respectively of men with as many as six positive cores, indicating that the number of positive cores was less useful as a predictor of tumour volume than the cancer length.

	No. of positive cores				Percentage of positive cores				Cancer length (mm)				Percentage cancer length				
Tumor volume (ml)	1–5	≥6	≥7	≥8	<60	≥60	≥70	≥80	<30	≥30	≥40	≥50	<20	≥20	≥25	30	Total
	Total																
	91	24	13	10	92	23	15	10	96	19	16	9	91	24	14	10	
	Positive predictive value (%)																
>4	22	67	77	80	22	70	80	80	19	95	94	89	17	83	93	100	36
>6	11	33	38	30	11	35	47	40	9	47	44	55	9	42	57	70	18
>8	4	12	23	30	4	13	20	30	3	21	25	33	1	25	28	40	7
	Sensitivity (%)																
>4	56	44	28	22	56	44	33	22	50	50	42	22	44	55	36	28	36
>6	56	44	28	17	56	44	39	22	50	50	39	28	44	55	44	39	18
>8	57	43	43	43	58	43	43	43	43	57	57	43	14	86	57	57	7

Table 3. Positive predictive values and sensitivity for tumour volumes of >4, >6 and >8 ml.

Discussion

The rational for studying tumour extent in preoperative prostate biopsies is built upon two important premises: firstly, numerous studies have shown that progression rate after prostatectomy correlates with tumour volume, and secondly, since the size of prostate cancer cannot be measured accurately by radiological techniques like in most other human malignancies, the preoperative biopsies is one of a few means at our disposal in assessing the tumour burden for the individual patient. Some authors have claimed that tumour volume is not an independent predictor of prognosis when Gleason score, extra-prostatic extension, surgical margins and seminal vesicle invasion are included in the analysis,[50,96] which may be true when the majority of tumours are small. In other studies based on series of larger tumours it was found that tumour volume is an independent prognostic factor.[83,124]

Predictive values depend on prevalence. Studies based on high-prevalence populations produce positive predictive values too high when applied in low-prevalence populations where fewer true positives are diluted by more false positives. The reverse problem may be the case in this study because of a possible bias caused by fewer large tumours among men undergoing prostatectomy than among all men who have a prostate biopsy, leading to underestimation of the actual positive predictive value of a large tumour volume in the biopsy population. However, this bias is probably counterbalanced because prostate cancers detected today are generally smaller than those included in the study.

It is however still unclear which prostate cancer patients benefit from treatment with curative intent. Hence, it is unknown at which tumour volume prostate cancers are too large to be treated with radical prostatectomy. Several authors have described the relation of measures of preoperative biopsies to tumour volume, stage and recurrence.[28,59,68,141,155]
This is to our knowledge the first study to address the issue of predicting large-volume prostate cancer by percentage of cores positive for cancer, cancer length and percentage cancer length. When Gleason score and serum PSA and either number of positive cores and cancer length or percentage of positive cores and percentage cancer length were included as explanatory variables in multiple regression models, only cancer length and percentage cancer length correlated independently with tumour volume. To summarise, our findings support the use of linear extent as a predictive factor.

The College of American Pathologists recommended 2009 in its recommendations to pathologists (Protocol for the Examination of Specimens From Patients With Carcinoma of the Prostate Gland)[164] that the pathological report should include information on number of cores, number of cores with cancer, and linear extent of cancer, either in millimetres or percentage. It is also recommended in the EAU Guidelines on Prostate Cancer,[42] and the amount of cancer in biopsies, and especially the cancer length and percentage cancer length are considered mandatory in reporting to the Oncological Centres for the Swedish Cancer Registry. Every new patient with cancer is reported to these registries and questions regarding number of biopsies and number of biopsies positive for cancer have been included since 2007, and total biopsy length as well as total cancer length since 2009.

Study 3: Tumour heterogeneity and prediction of DNA ploidy of prostate cancer

In Study III we investigated the impact of tumour heterogeneity on preoperative ploidy assessment in prostate cancer, by comparing flow cytometry results in 50 radical prostatectomy specimens with image cytometry results from preoperative biopsies in the same cases. 155 paraffin blocks out of 175 were available for DNA ploidy analysis, and after exclusion of 32 biopsy cores due to poor cell yield, no remaining cancer or low-quality ploidy histograms, a total of 123 histograms from 48 patients were analysed and compared with the results from the previous study on image cytometry.

The Gleason score was 6 or lower in 79% of the biopsies and 56% of the tumour specimens, and 7 or higher in 21% of the biopsies and 44% of the tumour specimens. Preoperative tumour stage was 10.4% T1b, 22.9% T1c and 66.6% T2 which together with the average tumour volume of 4.5 ml (range 0.3-36.2 ml) in the specimens are reflections of the clinical patient population at that time.

When ploidy was categorized as diploid or non-diploid (i.e. tetraploid or aneuploid) prostatectomy specimens were correctly predicted as either diploid (48%) or non-diploid (23%) in 34 men (71%). Ploidy was underestimated by biopsies in 19% and overestimated in 10% of the cases, respectively. The ploidy status of tumours with and without ploidy heterogeneity was correctly predicted in 55% and 82% of cases, respectively. Biopsies underestimated ploidy in 9 of 20 tumours (45%) with heterogeneous ploidy status.

These results were, however, not dependent on tumour volume: neither of the volume parameters differed significantly between the groups of correct and incorrect biopsy ploidy estimation. However, small non-diploid cancers with volumes of ≤0.9 ml were misclassified in 75% (6/8) of the cases, as compared to only 25% (3/12) of tumours with volumes >0.9 ml. There was also a significant positive correlation between correctly classified cases and number of biopsy cancer cores. Only 4/12 cases with one to two cancerous biopsies were correctly classified, as opposed to 7/8 cases with three to six cancerous biopsies.

Discussion

The impact of DNA ploidy on the prognosis for prostate cancer patients has been studied by many since the first report by Tavares[167] in 1966. Generally, a change in DNA content correlated with higher stage and worse outcome.[6,78,157,172] However, studies on the clinical utility of DNA ploidy have shown divergent results.[44,52,84,145] One explanation to this can be ploidy heterogeneity, which has been reported in 4.2% - 56% of prostate cancers.[91,126,182] In a previous study from our group[45] 42% of the tumours showed a heterogeneous ploidy, and the volume of non-diploid cancer correlated with extra-prostatic extension and seminal vesicle invasion. Our purpose was to evaluate the impact of ploidy heterogeneity on the preoperative assessment of DNA ploidy. The analysis included data on non-diploid tumour volumes, which has not been investigated previously to our knowledge. Accurate prediction was obtained in 71%. Ploidy heterogeneity was obviously a cause of inaccuracy, as ploidy was better predicted in cases without heterogeneity. There is a problem of possible over- and underestimation both for sampling and technical reasons: depending on biopsy location there will be a risk of underestimating ploidy status because of smaller proportion of non-diploid tumour. Similarly, in prostatectomy specimens the samples for flow cytometry may not contain small areas of non-diploid cancer.

The technical issues are different for image and flow cytometry, respectively: image cytometry requires only a small sample and only malignant nuclei are measured, but the limited number of cells may cause overestimation of non-diploidy when sliced or

overlapping nuclei are misinterpreted. In flow cytometry a large number of cells are measured but contaminating benign cells are included and this may cause an underestimation. Theoretically, the smaller the non-diploid component, the greater the risk it is missed. This was however not confirmed in our material, probably because of better ploidy prediction in tumours without heterogeneity. Our results support the hypothesis that increased number of preoperative biopsies improves prediction of ploidy status, and suggest that multiple biopsies are used for analysis of biomarkers with heterogeneous distribution.

Study 4: Expression of redox pathway enzymes in human prostatic tissue

The redox enzymes TxnR2, Trx1 and Prdx2 were expressed in the cytoplasm and occasionally in the nuclei of prostate epithelium. There were differences in degree of expression, with the highest immunoreactivity in HGPIN followed by atrophy, cancer and benign tissue. For TxnR2 there was an overexpression in cancer and HGPIN compared with benign tissue. For Trx1 and Prdx2 there was an overexpression in cancer compared with benign tissue and in HGPIN compared with cancer. Prdx2 expression correlated with Gleason score in the prognostic TMA, which Trx1 did not. Neither of Trx1 or Prdx2 correlated with biochemical recurrence.

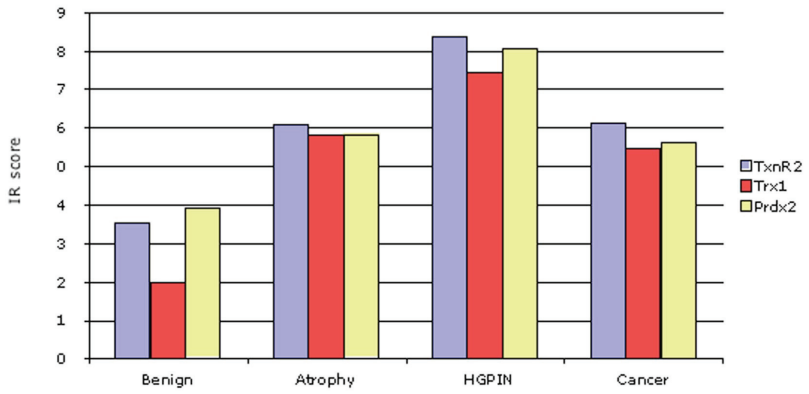

Figure 4. Expression of TxnR2, Trx1 and Prdx2 in benign prostate tissue, atrophy, HGPIN and cancer.

Discussion

It was Rudolf Virchow, the founder of cellular pathology, who already in 1863 suggested a connection between inflammation and cancer, based on observations of cancer in organs afflicted by chronic inflammation.[179] Over the years accumulating data from different research fields have shed a new light on this old hypothesis for

malignancies in the bronchi, urinary bladder and hepatocellular cancer, just to mention a few.[17] According to epidemiological data about 18% of cancer in the global perspective can be attributed to infections, with inflammation having a central role in the pathogenetic process.[131,135] For the last two decades research in molecular biology and pathology has increased the understanding of the underlying mechanisms, and the importance of protection from oxidative stress.[74]

In general, cancer develops through somatic genetic and epigenetic changes resulting in the inactivation of tumour-suppressor genes and caretaker genes, and the activation of oncogenes. The initiation, however, may be the result of chronic inflammation, whatever the infectious agent, causing a release of highly reactive substances, including superoxide, hydrogen peroxide and nitric oxide. These are released from activated inflammatory cells, causing oxidative or nitrosative damage to DNA in the epithelial cells. In the process of replacing the epithelium during these vulnerable conditions, the risk for mutation increases, thus giving way for the genetic and epigenetic changes.

The thioredoxin system is one of the major redox control systems to secure a reduced state in the microenvironment, and to protect the cells from oxidative stress. Oxidative stress is prevalent in cancer and induces the expression of redox proteins. The observed overexpression of TxnR2, Trx1 and Prdx2 may therefore reflect the level of oxidative stress. However, redox protein overexpression may be involved in the process into malignancy and can be regarded as both a plausible cause and effect of early neoplastic transformation.

The single most important tissue-based prognostic factor of prostate cancer is the Gleason score, and one of the study aims was to investigate the potential role of the redox regulators as prognostic markers. However, Prdx2, and not Trx1, reached only borderline significance for recurrence-free survival in a Kaplan-Meyer analysis.

Interestingly, the study shows that all three investigated proteins have a similar immunoexpression pattern ranging from a low level in benign prostatic epithelium to moderate immunoexpression in atrophy and cancer and high in HGPIN. Despite upregulation of all investigated proteins in prostate cancer and its precursors, no correlation with prognosis was found. Thus, these regulators correlate better with the development of prostate cancer than with its progression. This understanding of the imbalance of the redox system in malignancies is the cause for the search of new cytotoxic drugs targeted at modulating the redox system.

Study 5: GAD1 is a prostate-specific tissue biomarker

Immunohistochemistry

We found that the expression of glutamate decarboxylase 1 (GAD1) was stronger in benign and malignant prostatic tissue than in non-prostatic tissue. The expression was slightly weaker in cancer than in benign prostatic tissue, and had an inverse correlation

with Gleason score. As expected, prostate-specific antigen (PSA) also stained stronger in prostate tissue. Prostate-specific membrane antigen (PSMA) on the other hand did not stain stronger in benign prostate tissue. When comparing the staining in prostate cancer to the control cancers, GAD1 and PSA stained stronger than in urothelial cancer, rectal and lung adenocarcinomas, and PSMA had a stronger expression than in urothelial cancer and rectal cancer, but not than lung cancer. The staining results of the three antibodies are shown in figure 6.

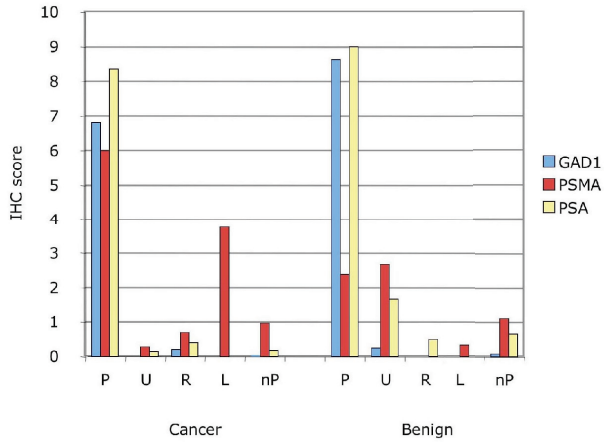

Figure 5. Immunoreactivity of glutamatedecarboxylase1 (GAD1), prostate-specific membrane antigen (PSMA) and prostate- specific antigen (PSA) in malignant (left) and benign (right) tissues from prostate (P), urinary bladder (U), rectum (R) and lung (L). Summary of expression in non-prostatic tissues shown in bars nP. All carcinomas are high-grade urothelial carcinoma (U) or adeno carcinomas (P, R and L).

The ability of GAD1, PSMA and PSA to classify cancers as prostate cancer expressed in sensitivity and specificity is shown in Table 4.

	GAD1		PSMA		PSA	
Cut-off	Sens.	Spec.	Sens.	Spec.	Sens.	Spec.
1	100.0	94.1	100.0	64.5	100.0	81.3
2	97.7	100.0	85.7	71.0	100.0	90.6
3	95.3	100.0	66.7	83.9	100.0	100.0
4	88.4	100.0	59.7	90.3	97.7	100.0

Sens. = sensitivity; Spec. = specificity.

Figure 4. Sensitivity and specificity for GAD1, PSMA, and PSA according to immunoreactivity product cut-off (IRP; product of intensity and extent of immunoreactivity).

With a cut-off point in immunoreactivity product (IRP) of 1 (i.e. a weak staining in 1–33% of cancer tissue) all three antibodies had a sensitivity of 100% but specificity was lower; 94%, 64% and 81% for GAD1, PSMA and PSA, respectively. With a cut-off point of 4 (a medium staining intensity in 34– 66% of cancer tissue) sensitivity decreased somewhat to 88%, 60% and 98% for GAD1, PSMA and PSA, respectively, but specificity rose to 100% for GAD1 and PSA and 90% for PSMA.

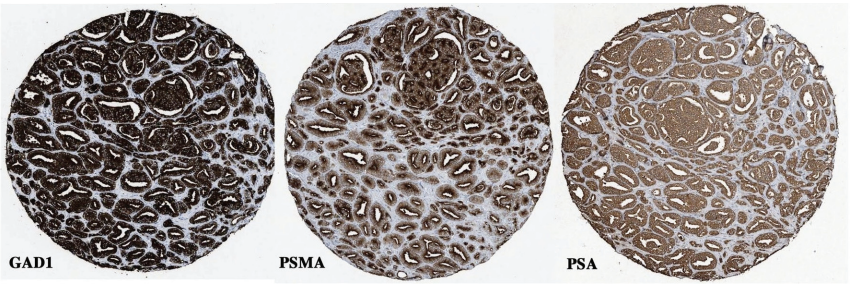

Figure 6. Three sections of the same tissue microarray core of prostate cancer stained for glutamate decarboxylase 1 (GAD1), prostatespecific membrane antigen (PSMA) and prostatespecific antigen (PSA), all with strong expression.

Real-time PCR

The RT-PCR analysis demonstrated that mRNA for all three markers was expressed in all prostate samples, but the relative expression levels of PSMA, GAD1 and PSA showed large variations between individual samples. GAD1 was also expressed in the control cancer tissue from urothelium, lung and rectum in contrast to PSMA and PSA.

Discussion

Identification of tumour origin is crucial for correct therapy descisions in patients with metastatic carcinoma. In high-grade malignancies, morphology alone cannot always determine the site of primary tumour and immunohistochemistry is an important adjunct tool. Two of the most commonly used prostate cancer markers are PSA and PSMA.[36,159] PSA is considered the best tumour marker for prostate cancer in serum analyses,[149] but the specificity of serum PSA is low. On the other hand, tissue expression of PSA is highly specific for prostatic tissue.[149] However, there is a decreased immunoreactivity with increasing Gleason score[66] and high-grade prostate cancers may be entirely negative.

PSMA is a membrane glucoprotein first discovered in prostate epithelium.[81] Intense expression has been observed in prostate epithelium, especially in cancer,[47] but PSMA is also expressed in benign and malignant non-prostatic tissue.[97,102] Consequently there is still a need for prostate-specific tissue markers for the rare cases where PSA and

PSMA show ambiguous results.

This report presents a biomarker with high specificity for prostate tissue. In our study, GAD1 showed a high specificity and sensitivity to benign and malignant prostatic tissue in IHC. GAD1 is associated with the regulation of glutamate, and has previously been described in brain tissue in association with psychiatric diagnoses.[8] GAD1 and GAD 65, another decarboxylase, are suggested to derive from a common gene.[29] Autoantibodies directed towards them have been seen in individuals who later develop type 1 diabetes,[93,188] and are also associated with autoimmune[24,136] and genetic[109] conditions. GAD has not previously been described in association with prostate cancer.

In the current study the GAD1 antibody showed high specificity and sensitivity for both malignant and benign prostate tissue, in contrast to cancer tissue from urinary bladder, rectum and lung where IHC was negative. This is of clinical importance as tumours from these organ systems are potential differential diagnoses in cases with metastatic spread from an unknown origin.

Surprisingly, RT-PCR showed expression of GAD1 in control tissue from lung, bladder and rectum, in contrast to IHC. A possible explanation may be that the IHC antibody only stained one of the three isoforms, while the detected mRNA in the control tissue may represent the other isoforms. Another explanation for discordance between IHC and RT-PCR may be that proteins remain in the tissue after mRNA is metabolised. To summarise, a GAD1 antibody showed expression with high specificity and sensitivity for prostatic tissue, and also a negative correlation between GAD1 expression and Gleason score. Further analyses are necessary to clarify the role of the GAD1 protein in prostate tissue, and to verify these data.

Study 6: Diagnostic biomarkers of prostate cancer

Immunohistochemistry

We found that all four tested biomarkers CYCS (Somatic cytochrome c), ICK (intestinal cell kinase), IKBKB (inhibitor of nuclear factor-κB kinase subunit beta), AMACR (α-methylacyl-coenzyme A-racemase) showed a stronger expression in prostate cancer and HGPIN than in benign tissue. CYCS was also overexpressed in cancer compared with HGPIN ($p = 0.001$) and benign and atrophic tissue, respectively ($p < 0.001$). Expression levels were stronger in HGPIN than in benign tissue ($p < 0.001$), and stronger in benign tissue than in atrophy ($p = 0.004$).

For ICK expression was stronger in cancer than in HGPIN, benign and atrophic tissue, and overexpressed in HGPIN compared with benign tissue ($p < 0.001$). ICK was stronger in atrophy than in benign non-atrophic glands ($p = 0.004$).

IKBKB showed a strong expression in HGPIN and cancer, and not only overexpressed in cancer compared with benign non-atrophic and atrophic tissue ($p < 0.001$), but even stronger in HGPIN than in cancer ($p < 0.003$).

For AMACR expression was stronger in cancer compared with HGPIN, benign and atrophic tissue ($p < 0.001$), and in HGPIN compared with benign non-atrophic tissue ($p < 0.001$). Mean expression of the markers is summarized in Figure 7.

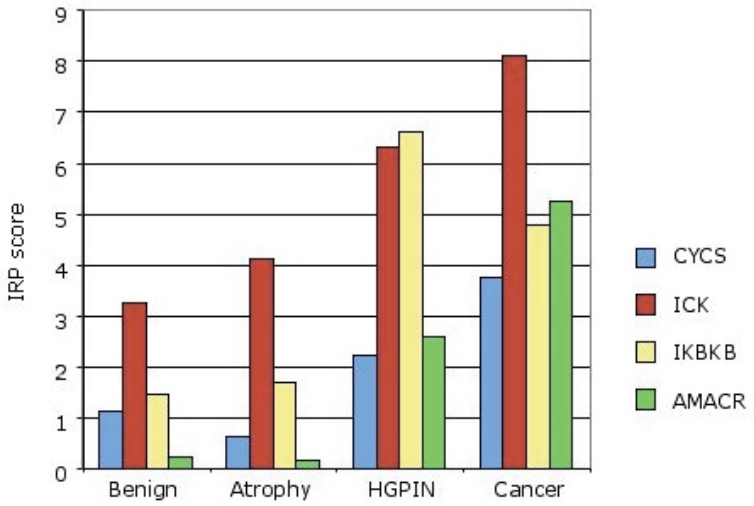

Figure 7. Expression of somatic cytochrome c (CYCS), intestinal cell kinase (ICK), inhibitor of nuclear factor-κB kinase subunit beta (IKBKB) and a-methylacyl-coenzyme A-racemase (AMACR) in benign prostatic tissue, atrophy, high-grade prostatic intraepithelial neoplasia (HGPIN) and prostate cancer. IRP score = immunoreactivity scored by the product of intensity and extent.

The best accuracy was obtained with ICK and AMACR, which reached 97% at cut-off levels of 5–6 and 2, respectively. CYCS and IKBKB had lower accuracy, at 84% and 78%, respectively.

Real-time PCR

In RT-PCR mRNA for all four biomarkers was expressed in malignant and benign prostate samples. With a definition of at least a factor 2 up- and downregulation as cut-off levels, there was however a large variation in the series when comparing cancer with benign tissue: CYCS mRNA levels were upregulated in 3% and downregulated in 10% of the cases, ICK showed upregulation in 19% and downregulation in 13% of the cases and IKBKB mRNA levels were upregulated in 19% and downregulated in 16% of the cases. AMACR alone had only upregulation in all cases.

Discussion

The established standard for diagnosis of prostate cancer is histopathological examination of transrectal ultrasound-guided needle biopsies.[79] In most cases, hematoxylin and eosin stained sections are sufficient to render a definitive diagnosis. In a meta-analysis of 24 studies including a total of 185 000 sets of biopsies, 5% of cases were diagnosed as suspicious for prostate cancer, yet not conclusive.[51] In such cases, ancillary markers are necessary to establish (or dismiss) a diagnosis of prostate cancer. Among these are the basal cell markers high-molecular weight cytokeratin (keratin 903, 34β12), cytokeratin 5 and p63, which are expected to be negative in prostate cancer.[2,191] AMACR (P504S), a marker that stains positive in prostate cancer was considered a useful adjunct in difficult cases when it was introduced in 2001.[20,89,99,108,120] However, with increasing experience it became clear that AMACR is also expressed in HGPIN and in some benign proliferations of the prostate while certain prostate cancers are negative, among these some of the most challenging for the pathologist.[53,110,190] There is therefore still a need for additional diagnostic markers with better sensitivity and specificity.

The purpose of this study was to identify new diagnostic markers for prostate cancer. Through a search algorithm four antibodies were slected from the HPA database. One of them turned out to be identical with AMACR, confirming the accuracy of the search algorithm.

CYCS is a protein found in most species functioning as a redox carrier for the electron transport chain. It can also react to apoptotic stimuli and activate nuclear apoptosis. Involvement in apoptosis may indicate an association with cancer. It has been shown that CYCS is involved in apoptosis of prostate cancer cell lines,[60] and that serum levels of CYCS are loosely associated with different malignancies.[128] The antibody used in the current study showed an accuracy of 63 – 78% in distinguishing cancer from benign tissue, but other antibodies are available with different expression pattern, warranting further investigation.

ICK is a serine-threonine kinase with a dual phosphorylation site, which is found in mitogen-activating protein (MAP) kinases. It was cloned by Togawa et al and was found to be localized in intestinal crypts, but also expressed in several cancer cell lines. However, prostate cancer was not included in the panel.[170] Mutations of the ICK coding gene are associated with multiple anomalies including endocrine, cerebral and skeletal systems, which suggests a role in the development of different organ systems.[101] The distribution of the cytoplasmatic staining, with strongly positive coarse granules present in only part of the cytoplasm, is a limitation in the assessment of IHC. Furthermore, our TMA showed a stronger postive staining in benign prostatic tissue than indicated in the limited HPA panel. Thus, the clinical utility of this biomarker for diagnostic purposes may be hampered.

IKBKB is a cytoplasmic protein that degrades the inhibitor of nuclear factor kappa enhancer binding protein (IκB), which leads to the activation of NF-κB.[117] NF-κB is a transcription factor involved in regulation of cell growth, apoptosis, angiogenesis and metastasis.[85] Expression of NF-κB in positive margin tissue from radical prostatectomy specimens correlated with biochemical recurrence,[57] indicating that NF-κB is of importance in prostate cancer development, together with its inhibitor IKBKB.

AMACR is a mitochondrial and peroxisomal oxidative enzyme involved in β-oxidation of branched-chain fatty acids in a variety of tissues.[54] AMACR is known to be overexpressed in a variety of cancers with high expression levels in prostate cancer.[190] However, despite its role as a valuable complement to basal cell markers in the diagnosis of prostate cancer,[20,110] AMACR staining is positive in only about 80% of cancers, and in 62 – 77% of the unusual morphological prostate cancers (pseudohyperplastic, atrophic and foamy types).[53,190]

The four investigated proteins are all known to be associated with cell-cycle regulation or cancer. Using IHC, the current study has shown that all proteins have stronger expression in cancer and HGPIN than in benign tissue. IKBKB had stronger expression in PIN than in cancer and the other three antibodies had stronger expression in cancer. This study confirms the diagnostic utility of AMACR, which achieved generally high values of sensitivity, specificity and accuracy. ICK had an equally high accuracy, but the granular and uneven subcellular distribution of the staining is a cause for interpretation difficulties.

Real-time PCR analysis confirmed the presence of the biomarkers in prostate cancer. For AMACR, a congruent profile to the IHC was found, i.e. upregulation in cancer. The other three biomarkers did not show this upregulation on mRNA level, which may have different possible explanations. First, it is well known that mRNA expression is not always comparable to protein expression.[34] There may be a quick metabolisation of mRNA, while the protein persists in the tissue, or vice versa. It is also known that mRNA does not always lead to protein transcription. In addition, mRNA may be translated into some other isoform or splicing variant. There may also be a difference between IHC and real-time PCR in which parts that are targeted of the investigated proteins. Furthermore, distribution differences within the tissue may add to varying results.

Although the immunohistochemical analyses indicate an association with prostate cancer development, the clinical value of CYCS, ICK and IKBKB is still unclear. Whether these proteins comprise the optimal combination for an adjunct diagnostic panel in prostate cancer warrants further investigation.

CONCLUSIONS

Study I

A novel end-cut biopsy needle provides thicker biopsies and enables flatter embedding in paraffin blocks, which together with an optional greater stroke length increases the tissue yield in prostate biopsies. This feature is countered by a higher rate of lost biopsies, but an end-cut biopsy instrument is useful in patients with large prostates or negative biopsies despite persistent elevation of serum PSA.

Study II

Large tumour volume in prostate cancer can be predicted by the linear extent of cancer in preoperative needle biopsies. Cancer length and percentage cancer length in preoperative prostate biopsies predict large tumour volumes better than number and percentage of cores positive for cancer.

Study III

Preoperative prediction of DNA ploidy status of prostate cancer by image cytometry is hampered by tumour heterogeneity, leading to a risk for underestimation of non-diploid cancer. Results improve if multiple biopsies are investigated.

Study IV

Redox pathway enzymes thioredoxin reductase 2, thioredoxin and peroxiredoxin 2 expression are upregulated in HGPIN and also to some extent in prostate cancer suggesting a role in the early development of prostatic carcinoma. There is also an overexpression in atrophy, another possible precursor lesion of cancer. However, upregulation of the redox pathway does not correlate to cancer progression.

Study V

Glutamate decarboxylase 1 is a novel tissue-specific prostate biomarker, expressed in both benign and malignant prostatic tissue. This biomarker may be useful for the identification of prostatic origin in metastatic cancer.

Study VI

Three novel diagnostic biomarkers, somatic cytochrome c (CYCS), intestinal cell kinase (ICK) and inhibitor of nuclear factor-κB kinase subunit beta (IKBKB) and the established diagnostic marker a-methylacyl-coenzyme A-racemase (AMACR) all show stronger expression in prostate cancer and HGPIN than in benign prostatic tissue. Among these, ICK and AMACR have the highest diagnostic accuracy for prostate cancer. For the histopathological diagnosis of prostate cancer in difficult cases, a panel of multiple diagnostic markers may be useful.

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