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A STUDY OF THE TRANSITION FROM PREMALIGNANCY TO CLINICAL PROSTATE CANCER

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Stockholm 2005

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Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7140-450-3

ABSTRACT

Prostate cancer remains the most commonly diagnosed life-threatening malignancy and the second leading cause of death from cancer in men. However, little is known about molecular mechanisms that underline its initiation and progression. The aim of this thesis is to characterize the involvement of three different genes and their protein products in human prostate cancer in the panel of clinical samples and established cell lines, in order to gain additional information about the development and progression of the disease. Additionally, a cytological characterization of prostatic intraepithelial neoplasia (PIN), a well established precursor of prostate cancer, has been done.

Paired tumor and constitutional DNA from prostate cancer patients has been used to perform mutational analysis of the *BRG1* gene. In total, by combining SSCP and sequence analyses, DNA from twenty one patients has been screened. The analysis of all 35 *BRG1* coding exons revealed the absence of somatic mutations, but presence of five SNPs, three of which were novel (Paper I).

In order to evaluate Pim-1 expression in human prostate cancer and prostatic intraepithelial neoplasia, immunohistochemical analysis has been done on an extended series of clinical samples. By previous studies, Pim-1 was shown to be involved in cell cycle regulation, and overexpression of Pim-1 protein was detected in prostate cancer. The results show a relative Pim-1 overexpression in HGPIN as compared to cancer. Upregulation of Pim-1 at premalignant stages suggests its involvement in the development of prostate cancer, possibly playing a role in the transition from precancerous lesions to invasive cancer. Additionally, Pim-1 expression may be a useful tool for distinguishing HGPIN from benign prostatic epithelium (Paper II).

A combination of immunohistochemistry and FISH analyses has been used to study protein expression and gene copy number of ezrin, a gene which is actively involved in the regulation of growth and metastatic capacity of cancer cells. The results show that ezrin has higher protein expression in HGPIN and prostate cancer as compared to normal prostate epithelium. However, FISH results did not reveal any copy number changes of this gene, indicating that ezrin protein overexpression cannot be explained by gene amplification. The data suggest that higher protein expression of ezrin in prostate cancer precursor lesions may indicate its involvement in the pathogenesis of the disease in its initial steps (Paper III). Paper IV assessed ezrin immunostaining patterns in benign and malignant prostatic tissue in order to investigate possible correlations between its expression and histopathological and prognostic data. The results show the correlation of ezrin immunoreactivity with adverse prognostic factors, thus strengthening the hypothesis of the role of ezrin in prostate tumorigenesis.

A sampling method for simulating fine-needle aspiration cytology (FNAC) was used to characterize cytological features of PIN. This is believed to be the first attempt to describe PIN cytologically and distinguish it from invasive cancer. For this study, cancer-free specimens containing PIN were selected. Smears with invasive prostate cancer were used for comparison. Cancer smears showed high cellularity and dissociation of atypical cells, while PIN smears only contained a few clusters of atypical cells. Furthermore, pronounced nuclear atypia, prominent and multiple nucleoli and mucin were more common in the cancer. These results indicate that PIN should not be diagnosed by FNAC alone. However, a highly cellular smear with dissociated, distinctly atypical cells seems to preclude PIN (Paper V).

Collectively, the results of this study further characterize the process of transition from premalignancy to invasive prostate cancer and suggest possible early oncogenic events in the prostate.

Keywords: prostate cancer, immunohistochemistry, prostatic intraepithelial neoplasia, aspiration cytology.

LIST OF PUBLICATIONS

I. **Valdman A**, Nordenskjöld A, Fang X, Naito A, Al-Shukri S, Larsson C, Ekman P, Li C.
Mutation analysis of the *BRG1* gene in prostate cancer clinical samples.
Int J Oncol. 2003 May;22(5):1003-7

II. **Valdman A**, Fang X, Pang ST, Ekman P, Egevad L.
Pim-1 expression in prostatic intraepithelial neoplasia and human prostate cancer.
Prostate. 2004 Sep 1;60(4):367-71

III. Pang ST, Fang X, **Valdman A**, Norstedt G, Pousette A, Egevad L, Ekman P.
Expression of ezrin in prostatic intraepithelial neoplasia.
Urology. 2004 Mar;63(3):609-12

IV. **Alexander Valdman**, Xiaolei Fang, See-Tong Pang, Bo Nilsson, Peter Ekman and Lars Egevad.
Ezrin Expression in Prostate Cancer and Benign Prostatic Tissue.
European Urology. 2005 (in press)

V. **Alexander Valdman**, Sara Jonmarker, Peter Ekman, Lars Egevad.
Cytological Features of Prostatic Intraepithelial Neoplasia.
Diagnostic Cytopathology (accepted)

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LIST OF ABBREVIATIONS

AR	Androgen receptor
BAC	Bacterial artificial chromosome
Bcl-2	B-cell lymphoma 2
BMI	Body mass index
bp	Base pair
BPH	Benign prostatic hyperplasia
BRG	Brahma-related gene
CDK	Cyclin-dependent kinase
CGH	Comparative genomic hybridization
DAPI	4', 6-diamidino-2-phenylindole
DHT	Dihydrotestosteron
DNA	Deoxyribonucleic acid
ERM	Ezrin-Raxidin-Moesin
ERSPC	European Randomized Study of Screening for Prostate Cancer
FISH	Fluorescence *in situ* hybridization
FNA	Fine-needle aspiration
FNAC	Fine-needle aspiration cytology
GG	Gleason grade
GS	Gleason score
H&E	Hematoxylin and eosin
HGPIN	High-grade prostatic intraepithelial neoplasia
HPC	Hereditary prostate cancer
HSP	Heat shock protein
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
LOH	Loss of heterozygosity
NuMA	Nuclear mitotic apoptosis protein
PBS	Phosphate buffered saline
PCa	Prostate cancer
PCR	Polymerase chain reaction
Pim-1	Premature initiation of mitosis
PLCO	Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial
PSA	Prostate specific antigen
Rb	Retinoblastoma
RNA	Ribonucleic acid
SM	Squamous metaplasia
SNP	Single nucleotide polymorphism
SSC	Standard saline citrate
SSCP	Single strand conformation polymorphism
SVI	Seminal vesicle invasion
TMA	Tissue microarray
TZ, CZ, PZ	Transition, central, and peripheral zones of the prostate
UM	Urothelial metaplasia
VDR	Vitamin D receptor

1 INTRODUCTION

1.1 THE PROSTATE

The term "prostate" was originally derived from the Greek word "prohistani", meaning "to stand in front of", and has been attributed to Herophilus of Alexandria who used the term in 335 B.C. to describe the organ located "in front of the urinary bladder" [1]. Prostate was thought to protect against urinary tract infection. However, the knowledge about gland's structure, functions, physiology and pathology has occurred only relatively recently.

The prostate is a fibromuscular and glandular organ situated in the pelvic cavity just inferior to the bladder. The normal prostate is about the size of a chestnut, weighs about 20 g. and somewhat conical in shape, and presents for examination a base, an apex, an anterior, a posterior and two lateral surfaces [2] (Fig. 1).

The early descriptions of the prostate by Lowsley suggested that the human prostate consists of 5 lobes: anterior, posterior, median, right lateral, and left lateral [3]. Subsequent studies revealed the central issue in prostate anatomy and pathology: the zonal anatomy. McNeal was the first to describe three anatomical zones: the peripheral zone, the transition zone, and the central zone [4] (Fig. 2). The peripheral zone in the normal gland comprises approximately 65% of the prostatic volume; conically shaped central zone comprises 25%, and transition zone nearly 10% of normal prostate volume.

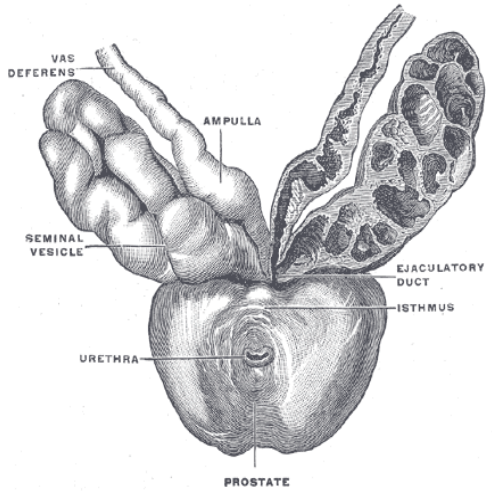


Figure 1. Prostate anatomy [2].

The clinical significance of zonal anatomy is important in terms of the development of prostate cancer.

The prostate is a male sex accessory gland. Its main function is the formation of secretions that constitute one half to two thirds of the volume of the ejaculate that participate in the clotting and lysis of the seminal plasma clot. The substances in prostatic fluid include several enzymes such as PSA, acid phosphatase, diamine oxidase, seminal proteinase, potassium, zinc, spermine, citric acid and prostaglandins.

Prostate is an androgen-dependent organ. While testosterone is the major androgen secreted by the testis, in the prostate testosterone is metabolized to DHT by the enzyme 5α-reductase [5], which has about ten-fold greater affinity for the androgen receptor (AR) than testosterone [6,7].

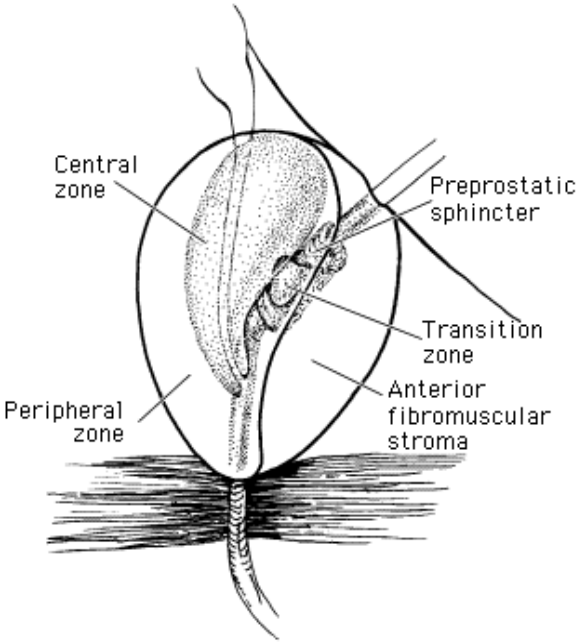


Figure 2. Zonal anatomy of the prostate [4].

1.2 PROSTATE CANCER

Back in 1853, the English surgeon J. Adams was the one who first described the clinical case of prostate cancer (PCa), which was an extremely rare disease at that time. Nowadays, the cancer of the prostate is a major and escalating international health problem.

1.2.1 Epidemiology

PCa is the most common male cancer in the Western world and the second leading cause of cancer-related death. In 2005, about 232.090 new prostate cancer cases are expected in the United States [8,9]. Approximately 9000 newly diagnosed cases were registered in Sweden the year 2004 [10]. There were 679.000 new cases and 221.002 deaths attributable to prostate cancer worldwide in 2002 [11]. The incidence of disease is increasing rapidly in many parts of the world with trends varying across populations and continents [12]. The accurate worldwide prevalence of prostate cancer is difficult to estimate due to the absence of such data from developing countries.

The variation in prostate cancer incidence between different countries is huge. It is about 100-fold worldwide, while mortality exhibits over 20-fold variation [13]. The disease is common in North America, Australia, New Zealand, and North West Europe, but rare in Asia, Africa and South America with the highest in the world incidence and mortality in Black men in the United States [14]. The age adjusted incidence rate for PCa in African men is 50% higher than in Caucasians [15]. There are also differences in lifetime specific mortality which is about twice as high in Afro-Americans [16]. Asian people have the lowest incidence and mortality rates of prostate cancer in the world [17]. It is notable, that the risk of developing the disease among Asians increases when they migrate to North America, suggesting, therefore, the importance of dietary and environmental factors in the progression of the disorder [18]. Additionally, prostate cancer has become one of the leading male cancers in some Asian countries reflecting the westernization of the lifestyle and enhanced detection rates [17].

It has been calculated that the lifetime risk for a man in a Western society of developing clinical disease is about 10% and the chances of dying from the disorder is around 3% [1]. This is the disease of elderly. Prostate cancer in patients younger than 50 years is diagnosed only in less that 0.1% of all. The

mean age of patients with PCa is 72–74 years, and about 85% of patients are diagnosed after age of 65 [19]. Autopsy studies show the presence of invasive prostate cancer in 8% men in their 20s [20] and an extremely high prevalence of PCa rising up to more than 75% in men aged older than 85 years [21], thus, postulating that most men will get prostate cancer if they live long enough.

There are three major forms of prostate cancer:

Hereditary. Men with hereditary prostate cancer (HPC) represent families that meet at least one of the following criteria: (*a*) three or more affected first-degree relatives, (*b*) prostate cancer occurring in three generations through the paternal or maternal lineage, and/or (*c*) two first-degree relatives diagnosed at an early age (≤55 years) [22]. This form accounts for 5-10% of all cases and is believed to be attributed to autosomal dominant inheritance of a rare yet highly penetrant high-risk allele [23]. Hereditary prostate cancer is diagnosed an average of approximately 6-7 years earlier [24-27] than the sporadic form of the disease, with the reported mortality rate of 75% [27,28].

Familial. Defined as two first-degree or one first-degree and two or more second-degree relatives with prostate cancer. Familial prostate cancer is estimated to account for 10% to 20% of all cases of prostate cancer [26,28]. It is likely that a combination of more common, low-penetrance alleles in genes that are components of pathways that influence prostate function are responsible for these forms of the disease [29-31].

Sporadic form account for 75-85% of all cases in the general population [22].

1.2.2 Etiology and risk factors

The etiology of prostate cancer remains unclear. It is believed that the disease has a multi-factorial origin. Estimates suggest that 58% of all prostate cancers can be attributed to environmental/lifestyle factors while 42% account for hereditable factors [32].

Ageing is still the single most significant risk factor for prostate cancer [33]. Other important factors are race and steroid hormones. It has been demonstrated that higher intakes of dairy products [34] and lower intakes of tomato products, lycopene, selenium, and vitamin E are linked to higher prostate cancer risk [35,36]. Aspirin and paracetamol have been shown to protect against prostate cancer [37], while no significant associations between fruit and vegetable consumption and prostate cancer risk were observed [38]. The role of alcohol as a risk factor for developing the disease has been ambiguous. It is now believed that greater alcohol consumption is not a strong contributor to prostate cancer risk [39].

Studies looking at the relationship between occupation and risk of PCa have not been consistent with the exception of employment in agriculture [40]. A large study revealed the association between PCa and the number of acres sprayed with herbicides [41].

An established predisposing factor for prostate cancer is the serum level of insulin growth factor (IGF) [42], which may be a connection between sedentary western lifestyle and development of the disease: consumption of large amounts of fat result in raised production of insulin, that increases production of IGF, thus explaining how IGF could be a risk factor for prostate cancer. This theory can be further supported by studies showing the relationship between PCa and body mass index (BMI). The risk of death is 2.5 times higher in overweight men [43].

Epidemiological evidence suggests there is a link between infections, subsequent inflammation and prostate cancer [44]. An increased prostate cancer risk has been linked to sexually transmitted diseases [45]. The explanation could be that the oxidative damage to cellular components and DNA may be the connection between chronic prostatitis as the consequence of sexually transmitted diseases and the development of prostate cancer.

1.2.3 Genetics

Genetic factors play a crucial role in the development of the disease. However, progression from normal prostatic epithelium, through a premalignant lesion, to invasive cancer and beyond (metastasis and androgen-independency) has an as yet incompletely characterized, genetic pathway.

There is cumulative evidence for a genetic component in predisposition to prostate cancer. **Epidemiological studies** have consistently noted the familial clustering of the disease. The data show that a family history of the disease is strongly associated with an elevated relative risk (RR). Men who have a first-degree relative with prostate cancer have a 2-8 times higher lifetime risk of the disease compared to men with no family history, varying according to the age at diagnosis, type of relative and number of relatives affected [28,46]. *Twin studies* give confirmatory results showing striking differences in concordance rates for prostate cancer in monozygotic (19-27 %) compared to dizygotic (4-7%) twins [32,47-49].

Several **segregation studies** provide evidence for an autosomal dominant Mendelian inheritance of prostate cancer [23,50,51]. However, there is also evidence for Y-linked transmission [52] and both dominant and non-dominant genetic effects [53], confirming the extreme genetic heterogeneity of the disease.

Another approach to a problem of finding prostate cancer susceptibility loci is **linkage analysis.** Several attempts to identify regions of interest have been made. Up to now, a number of different genome-wide scans have been reported [54-61]. Furthermore, based on linkage findings, lots of candidate genes have been proposed: *HPC1/RNASEL* [62], *HPC2/ELAC2* [63-66], *MSR1* [67], *CAPB* [68], *PcaP* [69], *BRCA2* [70,71].

However, the results are inconsistent when comparing several data sources, indicating the need for combined analyses of large family sets [72]. To meet these

needs, The International Consortium for Prostate Cancer Genetics (ICPCG) has been formed [73]. Their unique approach of combining the large-scale genome-wide scan with the cluster analysis of families that are more likely to segregate highly penetrant mutations led to the identification of regions that with highest probability contain prostate cancer susceptibility genes (1q25, 5q12, 8p21, 8q13, 13q14, 15q11, 16p13, 17q21, 22q12, 3p24, 5q35, 11q22, and Xq12) [74]. These results provide with the strongest data on regions of interest, which should be prioritized in order to define major prostate cancer susceptibility genes.

Low penetrance polymorphisms:

Polymorphisms (the occurrence of allelic variations) in low penetrance genes increase the risk of developing the disease only modestly, but occur with greater frequency in a population. Low penetrance polymorphisms may therefore have a greater impact on the frequency of prostate cancer in the population as a whole. Several candidates have been proposed: SRD5A2 [75], vitamin D receptor (VDR).

1.2.4 Natural history and screening

The natural history of prostate cancer is not fully established. There is a spectrum of duration and severity of the disease. It is slow-growing in many cases and has a long phase in which it remains undiscovered. This long latent phase is potentially advantageous for screening, but it appears that some tumors are very slow-growing and may never become clinically important [76]. Men with these tumors often die from another cause [77]. The relatively benign course of many tumors means that treatment might not be beneficial and could instead do harm. But, the selection of the right treatment approach is often associated with considerable uncertainty.

There are many obstacles in the way of an effective screening program for prostate cancer because of the increasing frequency of latent prostate carcinoma with increasing age and the not inappreciable morbidity and mortality of the radical procedures usually used to treat prostate cancer. Screening for prostate cancer is a controversial issue. After 1989, with the

introduction of widespread testing for PSA, there was a dramatic increase in the number of new cases. Due to PSA screening, an increasing proportion of men are detected with early-stage prostate cancer. However, it increased the risk for over-diagnosis and subsequent over-treatment [78]. Not until now it is known whether PSA testing is beneficial. To address these issues, the two large randomized trials (Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial of the National Cancer Institute and The European Randomized Study of Screening for Prostate Cancer (ERSPC) have been launched. They will provide with more definitive results, but, unfortunately only in several more years [79,80]. The preliminary data show that the screening procedure is effective, but they so far provide no evidence that prostate cancer screening decreases the mortality of the disease [81].

The conclusion is that it is necessary to establish the effectiveness of screening programs for prostate cancer by performing well-designed randomized trials, before making any recommendation for public health policy (International Prostate Screening Trial Evaluation Group, 1999).

1.2.5 Morphology and pathology

Prostate cancer and Gleason grading

Microscopically, most prostate cancers are adenocarcinomas, i.e. an epithelial neoplasia with varying degrees of glandular architecture and infiltrative growth pattern. PCa has a pronounced morphological heterogeneity and usually more that one histological pattern is present. Gleason grading system named after Donald F. Gleason is now the predominant prostate

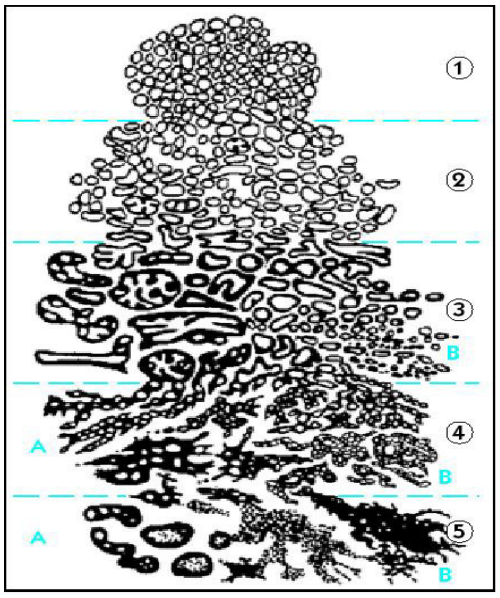


Figure 3. Gleason grading system [82].

cancer grading system. It is based on glandular architecture and evaluation of nuclear atypia. The Gleason grading system defines five histological patterns or grades with decreasing differentiation [82]. In the Gleason system, the most prevalent and the second most prevalent pattern (if at least 5% of the tumor) are added together to obtain a Gleason score (GS). Multiple studies have confirmed that Gleason score is a very powerful prognostic factor, both for the prediction of the natural history of prostatic carcinoma and for the assessment of the risk of recurrence after total prostatectomy or radiotherapy. In terms of prognosis, differentiation between GS 6 and GS 7 is of special importance, since the likelihood of having adverse findings in the prostatectomy specimen or failure following prostatectomy or radiotherapy, occurs between these two scores. In addition to behaving significantly worse than Gleason score 5-6 tumors, Gleason score 7 tumors behave significantly better than Gleason score 8-10 tumors.

1.2.6 Cytology of the prostate. Fine-needle aspiration cytology

Transrectal fine-needle aspiration (FNA) of the prostate gland was fully developed by Franzén and coworkers at Radiumhemmet [83]. This technique is cheap, easy and quick to perform, has low risk of complications [84-86]. Correlation of cytological grading with the Gleason score has been published elsewhere [87-89]. In recent years, transrectal ultrasound-guided core biopsy has become the routine method for diagnosing prostate cancer, the method, providing with the material for histological analysis. However, FNA technique still is a useful diagnostic tool. It produces clusters and sheets of epithelial cells without stroma [90]. Prostatic carcinoma can be distinguished from benign epithelium by increased cellularity, loss of cell adhesion, variation in nuclear size and shape, etc. The technique is especially applicable in elderly men who will not be considered for prostatectomy and in men with advanced disease. FNA can also serve as a diagnostic tool for comparative studies of prostate cytological and histopathological specimens. However, the inability to securely identify the aspiration site can be an obstacle. To overcome this problem, a sampling method to simulate FNA by scraping cells from cut surfaces of

radical prostatectomy specimens has been developed [91]. This technique is useful both for the study of cytological morphology of the prostate and for harvesting material for research purposes.

1.2.7 Tumor and prognostic markers in prostate cancer

Morphology-based prognostic factors such as tumor type, tumor stage, tumor grade, tumor volume, and surgical margins are the ones that are well supported by the literature and generally used in patient management [92]. A special role is assigned to prostate specific antigen (PSA), which is widely accepted and is useful in monitoring, diagnosis and staging of prostate cancer. PSA is a glycoprotein with serine protease activity and is essential for liquefaction of semen. It is found in the cytoplasm of both benign and malignant prostate cells and is more cancer specific. An elevated level above normal indicates an increased probability of prostate cancer. It is non-discriminatory and cannot differentiate between tumors that will progress and others that will not [93]. Despite its acceptance as the most useful tumor marker for PCa, there are concerns about its limited sensitivity and specificity. Thus, the incidence of significant prostate cancer has been shown to be as high as 22% in men with a total PSA in the range 2.6-4 ng/mL [94]. Furthermore, the rate of increase in serum PSA reflects tumor growth rate and prognosis but, due to substantial physiological variation in serum PSA, reliable estimation of the rate of PSA increase requires follow-up for at least 2 years [95]. Although the prognostic value of PSA is limited, measurement of the proportion of free PSA has improved the identification of patients with aggressive disease.

There is still a need for additional markers which could increase sensitivity and specificity of prostate cancer detection. All currently available molecular-based markers continue to be under evaluation to access their ability to determine initiation, development, and prognosis and guide the selection therapy for the disease.

1.2.8 Diagnosis and treatment

Prostate cancer rarely causes symptoms early in the course of the disease. The presence of symptoms as a result of prostate cancer usually suggests locally advanced or metastatic disease. The diagnostic modality in detection of prostate cancer is the triad of digital rectal examination (DRE), serum prostate specific antigen (PSA) and ultrasound-directed biopsy. The treatment strategy remains a controversial issue. There are roughly four major treatment options for localized disease: surgery, radiotherapy, hormone therapy, and watchful waiting. All of the methods have their own limitations. Therefore, defining men at high risk for prostate cancer will allow better disease prognostication and choice of treatment for the patient's best.

1.3 TRANSITION FROM PRECANCEROUS LESIONS TO MALIGNANCY

1.3.1 Precancerous lesions

The transition of normal prostatic epithelium to invasive carcinoma is a morphological continuum (Fig. 4).

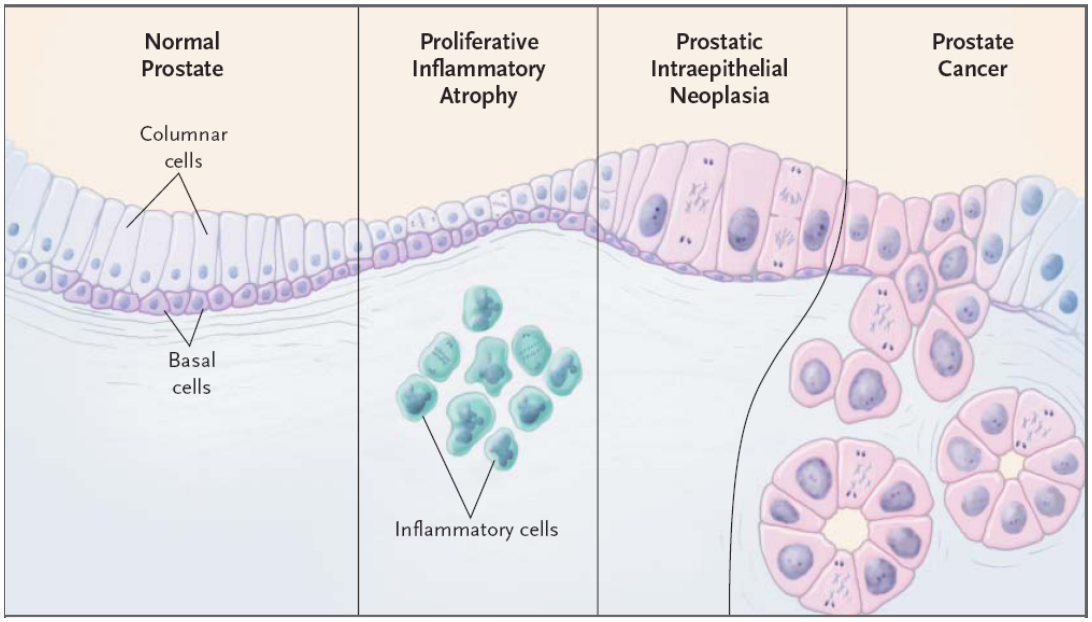


Figure 4. The model of prostate cancer development [96].

Proliferative inflammatory atrophy (PIA)

PIA is characterized as focal areas of epithelial atrophy, distinct from the diffuse atrophy seen after androgen deprivation, usually appearing in the periphery of the prostate [97]. PIA may emerge as a consequence of epithelial damage caused by infection [98], followed by epithelial regeneration and associated secondary inflammation.

PIA has been proposed as a precancerous lesion [99]. However, etiology of PIA and its possible connection to the development of prostate cancer remains unclear. There have been confusing results published on the topic [100-103]. The fact that many of PIA areas are associated with acute or chronic inflammation [99,104] indicates its possible role in the pathogenesis of prostate cancer, since the evidence of inflammation as a cause of prostate cancer is compelling [105].

Prostatic intraepithelial neoplasia (PIN)

PIN is defined as an intraductal or intra-acinar epithelial proliferation with significant nuclear atypia in the secretory cells [106]. The earliest reports on premalignant prostatic lesions date back to 1926 [107]. Later, in 1965, McNeal described different lesions with possible premalignant features in prostatic epithelium [108]. But only in 1986 the first reproducible criteria for the diagnosis of "intraductal neoplasia" were defined [109]; the lesion which was re-named to Prostatic Intraepithelial Neoplasia by Bostwick and Brawer [110] and which is now classified as a Low-Grade and High-Grade PIN (HGPIN) [111].

HGPIN is a well-defined entity, with distinctive architectural characteristics. It is classified into four major patterns: tufting, micropapillary, cribriform and flat, in which tufting is the predominant one. PIN lesions can only be diagnosed by histopathological examination of prostatic tissue. It cannot be detected by transrectal ultrasound, CT and magnetic resonance. This lesion does not elevate PSA levels either [112] and poorly correlates with PSA derivates (PSA density and PSA free/total ratio). There is a strong evidence that HGPIN is a precursor of invasive cancer [106,113-115].

1.3.2 PIN and prostate cancer. The transition.

There are several lines of evidence showing the correlation between HGPIN and prostate cancer:

- HGPIN lesions precede prostate cancer with more than a decade [116]
- HGPIN is found significantly more frequently in prostates with cancer than without cancer [117]
- The incidence and extent of PIN lesions increase with age and this increase is similar compared to the increase of the finding of PCa with age [109]
- HGPIN lesions are more pronounced in prostates with cancer than in prostates without cancer [109]
- Both PIN and PCa are mostly multifocal and preferentially found in the peripheral zone of the prostate [118]
- The basal cell layer is disrupted or fragmented in HGPIN, but completely lost in prostate carcinoma [119]
- The prevalence of HGPIN is higher in Afro-Americans [120]
- There are similarities between PIN and prostate cancer in nuclear properties, e.g. amount of DNA, chromatin texture, chromatin distribution, nuclear perimeter, nuclear diameter and nuclear abnormalities [121]. These data are supported by electron microscopic study [122] and morphometric cell image analysis [123]
- Several genetic changes can be found in both PCa and PIN cells. The most frequently found chromosomal anomalies are overexpression on chromosome 7p, 7q, 8q, and inactivation on chromosome 8p, 10q, 13q, 16q and 18q [116]
- cDNA studies showed similarities in expression between HGPIN and invasive cancer [124]
- Similar to invasive prostate adenocarcinomas, the telomere lengths of HGPIN lesions are shorter than those of normal epithelial cells [125]
- Numerous animal models support the concept of HGPIN transition to invasive carcinoma [117]

- The immunophenotype of HGPIN is close to that of invasive cancer [117]
- The proliferative activity as well as apoptotic index and PSA immunoreactivity of PIN is intermediate between benign glands and invasive cancer [126-129]
- PIN is characterized by increased microvessel density as compared to benign glands. Moreover, there is remodeling of the capillary structure in PIN [130]

All the above supports the theory of transition from HGPIN to invasive cancer in which HGPIN is considered a precursor lesion of PCa.

Biochemical markers in PIN

A number of potential biochemical markers are identified in PIN:

- p63, a p53 homologue can help to distinguish between PIN and PCa [131]
- A80, a membrane-bound glycoprotein that is related to exocrine differentiation is gradually expressed from LGPIN over HGPIN to invasive cancer [132]
- Fatty Acid Synthase (FAS), a key enzyme in the *de novo* production of fatty acids; its expression reflects earliest and most common events in the development of prostate cancer, gradually increasing from PIN to cancer [133]
- Alpha-MethylAcyl-CoA Racemase (AMACR) plays an important role in oxidation in fatty acids. The pattern of AMACR expression in prostate cancer [134] and PIN [135] suggests its role as a potential marker for discrimination between normal and cancerous tissues
- Pim-1, an oncogenic product of serine/threonine kinase, is distinctly overexpressed in HGPIN compared to adjacent invasive cancer, suggesting the role of this protein in early stages of prostatic carcinogenesis [136]
- Ezrin, a membrane-cytoskeleton linker is aberrantly expressed in HGPIN, showing higher immunoreactivity as that of prostate cancer [137].

PIN and prostate biopsy

Since PIN has been shown to be both associated with cancer at the time of its finding and predictive for the development of prostate cancer in the future, its identification is an extremely important issue. The incidence of HGPIN without concomitant cancer in needle biopsies varies from 0.7% to 16.5%, with a mean of about 4% [117]. However, with the more widespread use of extended biopsy protocols, taking sometimes up to 14 cores or more, the incidence of HGPIN can be up to 25% [115]. The presence of PIN in prostate biopsies is the most important risk factor of finding PCa in subsequent biopsies [116,138-140]. Cancer incidences in repeat biopsies have been reported in the range of 30.5% [141] to 100% of men with a previous diagnosis of HGPIN; the higher value is valid when the biopsy is taken from a palpable lesion. The risk for finding PCa in repeat biopsies seems to increase with length of biopsy interval, thus, supporting the theory of transformation of HGPIN into invasive cancer [142]. The finding of isolated HGPIN in prostate biopsies should prompt the clinician to perform repeat biopsies. The latest data show that a second prostate biopsy in all cases of a negative finding on initial biopsy appears justified [143].

1.4 BRG1, HISTONE REMODELING AND PROSTATE CANCER

The *SMARCA4/BRG1* gene product is a component of the SWI-SNF chromatin-remodeling complex and regulates gene expression by disrupting histone-DNA contacts in an ATP-dependent manner.

Histones serve a dual role in the nucleus of eukaryotic cells. First, they are assembled with DNA into nucleosomes that can form higher-order structures. Second, they establish a dynamic molecular interface and play an active role in the regulation of transcription. Modifications such as acetylation, phosphorylation and methylation modulate the nucleosome structure and the interaction with activators and repressors [144]. Enzymes that modify histones show altered activity in cancer [145]. Over the past few years, a growing number

of studies have led to the identification of additional mechanisms that regulate chromatin function in conjunction with histone covalent modifications. These involve enzymatic complexes that remodel chromatin and serve as transcriptional co-factors [146]. One class of such co-factors is represented by the SWI/SNF remodeling complexes that alter the path of DNA around the nucleosomal histone core in an ATP-dependent manner, resulting in nucleosome mobilization [147].

BRG1, as part of mammalian SWI/SNF complexes co-operates with RB to repress transcriptions of several E2F target genes that are required to entry into S phase of the cell cycle [148] (Fig. 5).

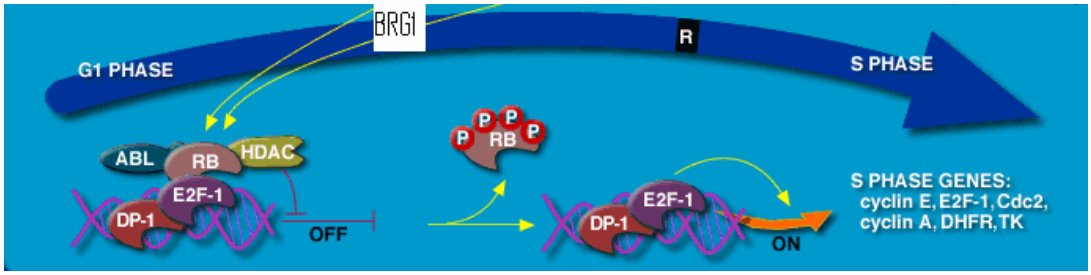


Figure 5. BRG1 and cell cycle regulation (Biocarta, modified).

Genetic alterations or dysregulated expression of genes involved in cell-cycle control and genomic integrity may be sufficient to drive malignant transformation. Thus, balanced chromatin remodeling activities are crucial to ensure accurate developmental or environmental responses, and to prevent the transition of normal cells into cancer cells.

1.5 PIM-1 AND PROSTATE

The proto-oncogene Pim-1, which is highly conserved in mammalian cells, encodes a serine/threonine kinase that is found at high levels in some carcinomas, including prostate adenocarcinoma [149]. *Pim-1* gene is located on chromosome 6p, contains six exons and five introns and produces a transcript of 2684 bp and encodes two proteins of 34 and 44 kD. Pim-1 is a downstream

effector of many cytokine-signaling pathways and the expression of the *pim-1* gene is induced by a large set of cytokines [150].

Pim-1 is associated with multiple cellular functions such as proliferation, differentiation and apoptosis. Previous studies established Pim-1 as a proto-oncogene and important player in the process of malignant transformation [151]. Expression of Pim-1 kinase can be stimulated by a variety of growth factors and regulated at four different levels: transcriptional, post-transcriptional, translational, post-translational [152]. Pim-1 is able to phosphorylate several targets and may, therefore, be responsible for the inactivation or activation of proteins involved in cell cycle progression or apoptosis. Pim-1 has been shown to phosphorylate $p21^{cip1/waf1}$, which inhibits G1/S progression [153]; CDC25A [154]; Cdc25C-associated kinase 1 (C-TAK1) [155] and NuMA (nuclear mitotic apoptosis protein), which is responsible for the organization of the spindle apparatus in the M phase [156]. Pim-1 acts as a cell survival factor and may prevent apoptosis in malignant cells [157].

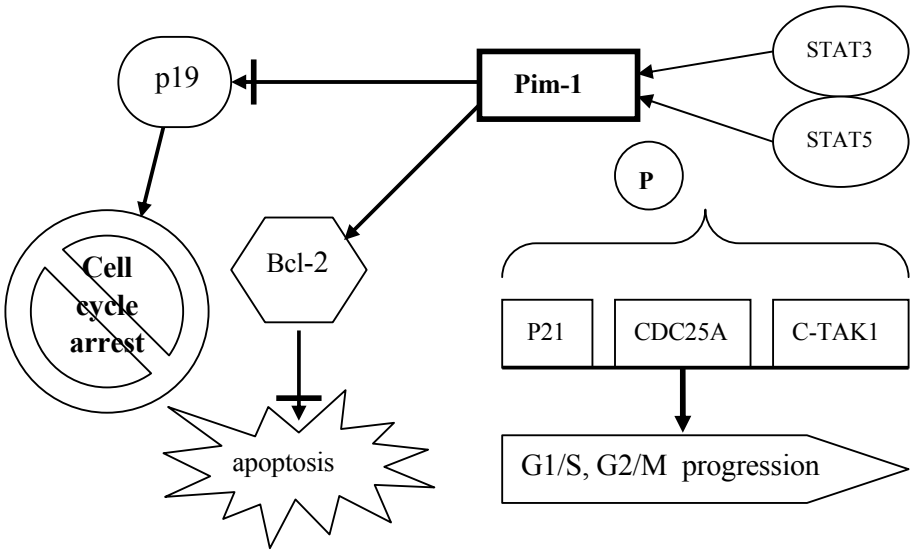


Figure 6. Pim-1 mechanisms of action.

Pim-1 levels can be modulated in cells through degradation and stabilization [157]. The half-life of Pim-1 has been found to shorten in response to the heat shock protein (Hsp) 90 inhibitor geldanamycin [158], providing evidence that increase in half-life, rather than just an increase in transcription rate, may be a

major factor in the increased levels of Pim-1 observed in tumor cells. The latest data confirm that the increased levels of Pim-1 can be a contributing factor in neoplasia [157].

Overexpression of Pim-1 in human prostate epithelial cells lead to genomic instability by subverting the mitotic spindle checkpoint, inducing centrosome amplification, abnormal mitotic spindles, and chromosome missegregation [159]. It has previously been hypothesized that Pim-1 may be involved in initiation of prostatic carcinogenesis. Pim-1 is highly expressed in HGPIN and invasive prostatic carcinoma [136], but negatively or only weakly positively in benign prostatic hyperplasia [149]. It points to an important role of Pim-1 in the initiation and progression of human prostate cancer.

1.6 THE ROLE OF EZRIN IN PROSTATE CANCER

Ezrin is a member of the ERM (Ezrin-Raxidin-Moesin) family. The gene encoding for ezrin is located on chromosome 6q25-25 and consists of 13 exons [160]. The ERM proteins (Ezrin, Radixin, and Moesin) and Merlin are closely related members of the band 4.1 superfamily of proteins that, when activated, interact with both membrane proteins and the actin cytoskeleton [161]. The function of these proteins is to link the plasma membrane to the actin cytoskeleton. By organizing membrane-cytoskeleton-associated complexes and creating specialized membrane domains, the ERM proteins regulate cellular activities such as survival, adhesion, and migration/invasion, all of which are important during tumor development and progression [162].

Cell adhesion is fundamental for the establishment and maintenance of multicellular organisms; normal development of tissues is governed by the interactions of cells with each other and with their environment, and is mediated by adhesion. Further, interactions between the cytoskeleton and adhesion molecules regulate or significantly contribute to a variety of

functions, including signal transduction, cell growth, differentiation, morphogenesis, and cell motility [12]. Reduced cell-matrix adhesion allows neoplastic cells to circumvent the control of differentiation induced by the normal extracellular environment, escape from their site of origin, and metastasize [163].

Invasive potency is one of the most important features of malignant tumors and is involved in a critical cascade of events such as extracellular matrix degradation and cell migration. The invasive phenotype formation of malignant cells requires aberrant expression of certain adhesive molecules, enzymes and related genes responsible for the interaction between cancer cells and extracellular matrix [164]. Ezrin plays a critical role in the determination of invasiveness of cancer cells [165] and has also been considered as an important molecule contributing to the malignant transformation of cells [164].

Several studies suggest an important functional relationship between the ERM proteins and the GTPase Rho, which controls actin cytoskeleton remodeling and related cellular activities. Rho-dependent phosphorylation of the ERM carboxyl-terminus constitutes the best-known mechanism of ERM activation; the ERM proteins, in turn, can regulate Rho [161,162] (Fig. 7a).

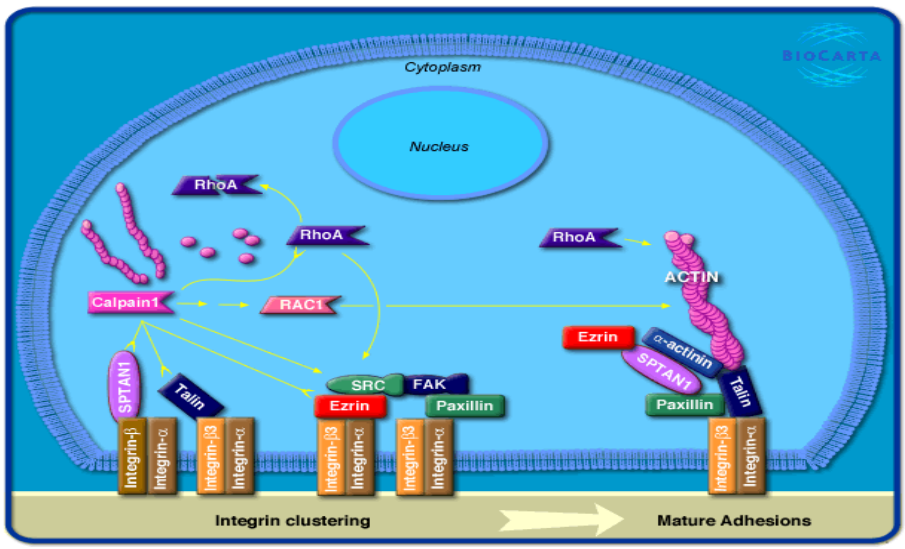


Figure 7a. Ezrin and its relations to key proteins (Biocarta).

Being the predominant ERM protein of epithelial cells, ezrin provides a linkage between membrane proteins and the cortical cytoskeleton and also participates in signal-transduction pathways [161]. It promotes survival of epithelial cells by activating the PI 3-kinase/Akt pathway and protects from induced apoptosis [166] (Fig. 7b). The expression pattern of ezrin is rather complicated: recent reports have proposed that ezrin overexpression might affect many cellular functions contributing to tumor progression and metastatic behavior [167-169]. Ezrin is overexpressed in endometrial cancer [170], metastatic ovarian carcinomas [171], osteosarcoma [172] and is required for invasion and metastasis of mammary carcinoma cells [173]. Additionally, high expression of ezrin has been detected in HGPIN, suggesting its role in promoting the transition to invasive prostate cancer [137].

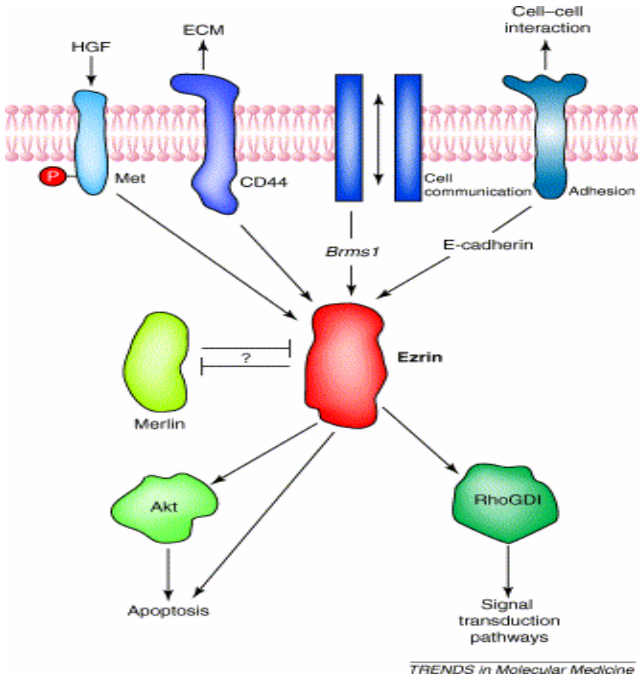


Figure 7b. Ezrin interactions with key proteins [167].

2 AIMS OF THE STUDY:

The overall purpose of this thesis is to study the process of transformation from premalignancy to clinical prostate cancer and to identify potential markers that could provide additional diagnostic and prognostic information already at early stages of tumorigenesis. Specifically the study was aimed:

I. To perform mutational analysis of the *BRG1* gene in a panel of paired tumor and constitutional DNA samples from prostate cancer patients in order to investigate possible *BRG1* involvement in the development of the disease.

II. To assess the patterns of Pim-1 expression in high-grade prostatic intraepithelial neoplasia and cancer in total prostatectomy specimens.

III. To investigate the potential role of ezrin in HGPIN and prostate cancer by studying its protein expression and gene copy number status.

IV. To correlate ezrin expression with prognostic and clinical parameters in prostate carcinomas in order to reveal its possible diagnostic utility.

V. To describe cytological features of HGPIN and correlate them to histopathological data.

3 COMMENTS ON METHODOLOGY

3.1 CLINICAL MATERIAL

The studies included fresh frozen and paraffin-embedded tissue as well as cytology specimens from radical prostatectomies performed at Karolinska Hospital.

In **paper I,** tumor and constitutional DNA samples from 21 prostate cancer patients have been used for the analysis. Peripheral leukocytes obtained from the same patients were used as source of constitutional DNA. Immediately after the tissue specimen was surgically removed, a horizontal section across the tumor lesion was made and tumor tissue was sampled inside the nodule. The tissue was snap-frozen at -70°C and used for DNA extraction. The representativeness of the tissue was estimated by histopathological examination and only samples which contained at least 50% tumor cells were included in the study. High molecular weight DNA was isolated from matched pairs of peripheral leukocytes and tumor samples using standard methods. No degradation was observed in any of the DNA samples by standard agarose gel electrophoresis.

In **paper II**, a consecutive series of 121 total prostatectomy specimens were collected from January 1999 to December 2000. Patients' mean age at surgery was 61.8 years (range 50-74). The mean preoperative PSA level was 9.9 ng/ml (range 1.3–58). None of the patients had received hormonal treatment or radiotherapy prior to prostatectomy. The average follow-up time was 30.6 months (range 3–48). The main tumor originated from the peripheral zone (PZ) in 97 (80%) and from the transition zone (TZ) in 22 (18%) of the cases. There was one cancer originating from the central zone and one with uncertain zonal origin. Twenty (17%) specimens showed seminal vesicle invasion, 77 (64%) had extraprostatic extension, and 62 (51%) had positive surgical margins. Gleason score was 6 or lower in 55 (45%) tumors and 7 or higher in 66 (55%) tumors.

Paper III included 19 radical prostatectomy specimens collected from January to December 2000 at Karolinska Hospital. Mean age of the patients at surgery was 61.3 years (range 50-74). None of the patients had received hormonal treatment or radiotherapy prior to prostatectomy. The prostatectomy specimens contained well-defined areas of HGPIN. In 13 of totally 19 specimens, foci of invasive prostate cancer were also present in the same section. In 68% and 32% of all cases, the main tumor originated from the peripheral and transition zone, respectively.

In **paper IV,** a larger consecutive series of 129 radical prostatectomy specimens was analyzed. Seventeen specimens were excluded because of the absence of cancer in the recut sections or technical problems with immunostaining and 9 due to insufficient follow-up data. The final study material included 103 radical prostatectomy specimens collected from January 1999 to December 2000 at our department. Mean age of the patients at surgery was 62 years (range 50-74). Average preoperative serum PSA was 10.4 ng/ml (range 1.3-58). The average follow-up was 28 months (range 3-60). None of the patients received hormonal treatment or radiotherapy prior to prostatectomy. The main tumor originated from the peripheral zone (PZ) in 80% and from the transition zone (TZ) in 19% of the cases. There was one cancer with uncertain zonal origin. Seminal vesicle invasion was found in 17%, extraprostatic extension in 66% and positive surgical margins in 55% of specimens. GS was 5 - 6, 7 and 8 - 10 in 43%, 46% and 12% of the tumors. Of all specimens, 17% contained urothelial metaplasia (UM) and 3% squamous metaplasia (SM). Additionally, ejaculatory duct was found in 22% and seminal vesicle in 2% of sections included in the study.

In **paper V,** cytology specimens were harvested from the peripheral zone of 177 radical prostatectomy specimens obtained between 2001 and 2004 at the Karolinska Hospital. The prostatectomy specimen was transported in a jar filled with ice and usually arrived at the department of pathology within 20 minutes. The prostate was cut in two halves by a horizontal section through the

peripheral and transition zones. Cut surfaces were scraped with a scalpel blade, and locations were noted on a specimen map. Macroscopically normal areas and lesions suspicious for cancer were sampled. The material obtained was smeared on a glass slide, air-dried and stained with May-Grünewald-Giemsa (MGG) according to standard protocols. Histological slides from the same areas were reviewed. For the PIN study, cytological smears from macroscopically normal areas were used. Cases with high-grade PIN in histological sections and atypical cells in cytological smears were selected. After exclusion of cases with microscopic foci of invasive cancer in histological sections, 17 cytological specimens from PIN areas remained for the analysis. For comparison, 17 cytological scrape specimens from invasive cancer areas were randomly selected. The Gleason scores of these tumors were 6, 7, 8 and 9 in 6, 7, 2 and 2 cases, respectively. All smears were then reviewed without knowledge of histological diagnosis.

3.2 MUTATIONAL ANALYSIS

The detection of DNA sequence variations is important for the identification of disease-causing mutations, as well as DNA polymorphisms. There are several methods which allow detection of mutations in the genomic DNA. DNA can be sequenced directly, or indirect screening methods can be applied prior to sequencing. The choice of a certain approach usually depends on the number of samples to be screened, size of the gene of interest and the sensitivity required. In this study, the combination of two methods (SSCP and direct sequencing) has been used in order to perform mutational analysis of the *BRG1* gene (paper I).

3.2.1 Single-strand conformation polymorphism analysis

Among the techniques capable of detecting a single base change, the single-strand conformation polymorphism (SSCP) analysis is one of the most commonly used due to its simplicity and effectiveness [174]. It is usually employed as a primary mutational screening combined with subsequent

sequencing. The method is based on the principle that electrophoretic mobility of a DNA molecule in a gel is sensitive to both its size and shape. Single-stranded DNA molecule has a folded structure that is determined by intramolecular interactions, and therefore, by its sequence. In SSCP analysis, a change in conformation resulting from a single base substitution can be detected as an alteration of its migration rate in a polyacrilamide gel (Fig. 8, 9) [175,176]. There are several components of SSCP analysis: PCR amplification, denaturation, electrophoresis, and detection. The optimal PCR fragment size is 150-200 bp. Over 90% of the mutations can be detected for sequences in fragments up to 200 bp by employing with SSCP under multiple conditions [177]. We have used SSCP as an indirect method for initial mutational screening of the *BRG1* gene (paper I). Figure 6 represents SNPs detected by SSCP in *BRG1* gene (paper I).

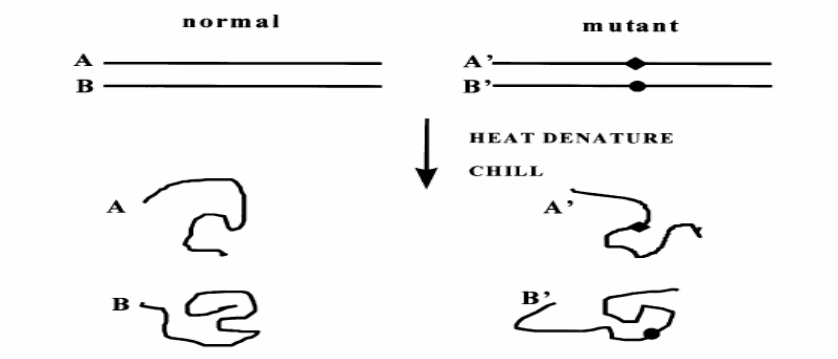


Figure 8. The principle of SSCP.

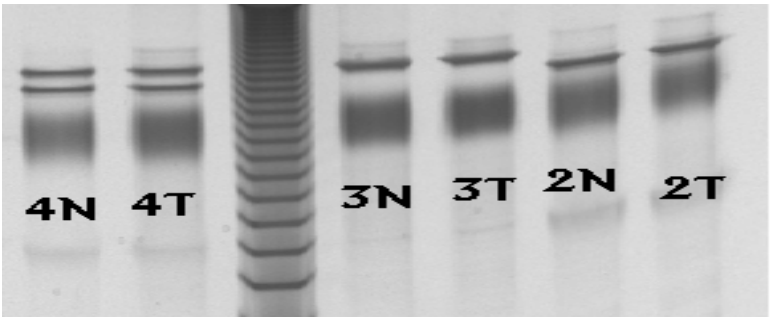


Figure 9. SNPs of the *BRG1* gene detected by SSCP.

3.2.2 Direct DNA sequencing

The process of determining the order of the nucleotide bases along a DNA strand is called sequencing. In 1977, twenty-four years after the discovery of the structure of DNA, the chain termination method for sequencing DNA was discovered by Sanger [178].

This method is based on the principle that single-stranded DNA molecules that differ in length by just a single nucleotide can be separated from one another using polyacrylamide gel electrophoresis. Nowadays, a variation of this method called cycle sequencing has been developed. It involves the synthesis of new strands of DNA complementary to a single-stranded template. The template DNA is supplied with a mixture of all four deoxynucleotides, four dideoxynucleotides, each labelled with a different colour fluorescent tag, and DNA polymerase. As all four deoxynucleotides are present, chain elongation proceeds until, by chance, DNA polymerase inserts a dideoxynucleotide. The result is a new set of DNA chains all of different lengths (Fig. 10).

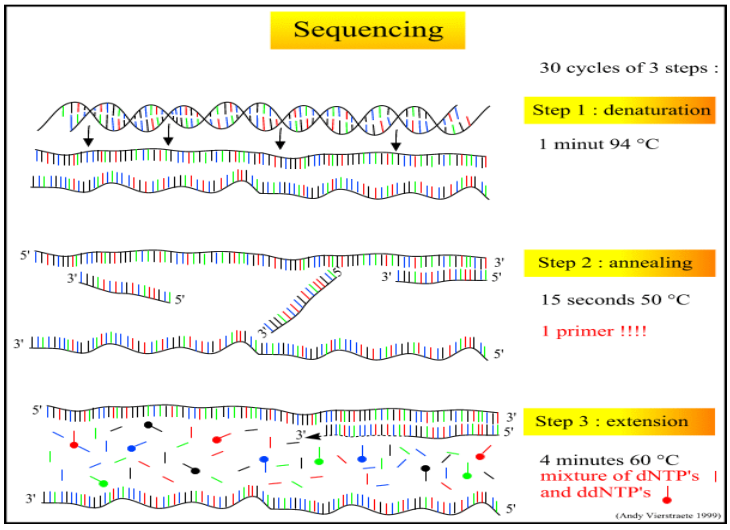


Figure 10. The principle of cycle sequencing.

The resulting products are separated by either gel electrophoresis or in capillaries, and the emission spectrum of the different dyes incorporated in the DNA is visualized as chromatograms (Fig. 11).

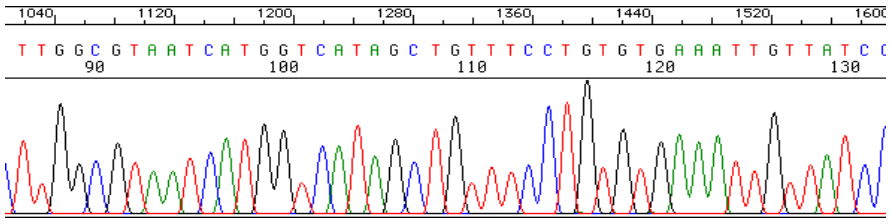


Figure 11. An example of a chromatogram.

3.3 FLUORESCENCE *IN SITU* HYBRIDIZATION

A non isotopic in situ hybridization was developed as an alternative to radioactive radiography [179]. Fluorescence *in situ* hybridization is a sensitive and rapid technique which allows detection of numerical and structural chromosomal aberrations at a single cell level [180]. Fig. 12 illustrates the principle of FISH.

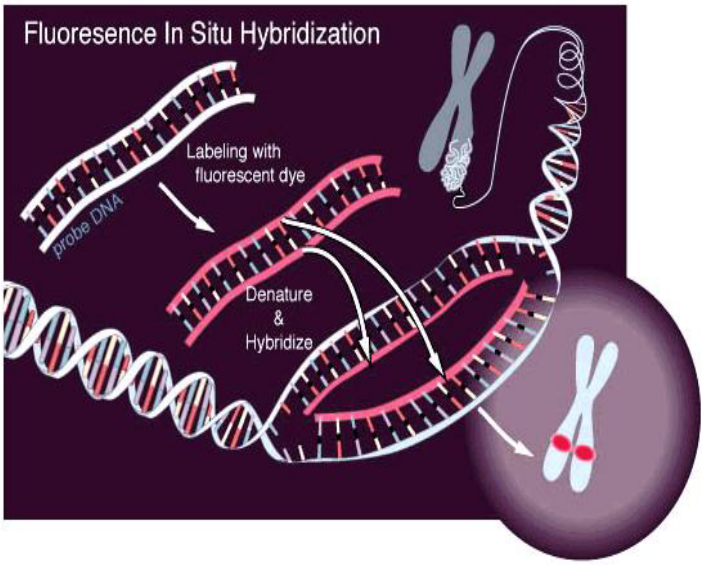


Figure 12. The principle of FISH (national human genome research institute).

A specific DNA segment is converted into a probe through the attachment of a fluorescent tag or a reporter molecule that later in the procedure will be conjugated with a fluorescent tag. The probe is denatured, exposed to a similarly denatured target DNA and, under proper hybridization conditions,

recognizes and binds to the homologous sequences in the target DNA. After hybridization, the copy number and location of the fluorescent tags, and consequently, of the target homologous regions, are recognized under fluorescence microscopy.

In the present study, locus specific *ezrin* probe (LSP) from BAC clone RP11-507C10 (Children's Hospital Oakland, CA, USA), and chromosome 6 centromeric probe (pEDZ6) (provided by Cytogenetic Unit, University of Bari, Italy) were used for detection of numerical chromosomal aberrations in prostate cancer. The probe DNA was extracted with QIAGEN Plasmid Midi Kit (QIAGEN, Hilden, Germany). All probes were labeled with SpectrumRed™ or SpectrumGreen™ fluorophore conjugated with dUTP using standard nick translation (Vysis Inc., Downers Groove, IL). The labeled probes were co-precipitated using ethanol/sodium acetate together with Cot-1 DNA (Vysis) and carrier DNA. A dual color FISH was performed on standard 4μm formalin-fixed paraffin-embedded tumor sections. After dewaxing and mild digestion with pepsin, the slides were rinsed with 2xSSC buffer, dehydrated in graded ethanol, and air dried. Twenty microliters of hybridization mixture containing 100 ng of each probe, 55% formamide, 10% dextran, and 2xSSC was applied to each slide. The slides were sealed with cover slips and denatured with probe mixture simultaneously at 92 ^{o}C for 10 minutes, then hybridized at 37 ^{o}C overnight in a humidified chamber. The slides were washed in 2xSSC buffer for 10 minutes at 72 ^{o}C, dehydrated in graded ethanol, air dried, and mounted in antifade solution containing DAPI (Vector Laboratories, Inc., CA). All samples were evaluated under a Zeiss fluorescence microscope equipped with the corresponding wavelength filter and image capturing and analyzing system. The copy number of a given gene per nucleus was recorded by two independent observers in one hundred non-overlapping nuclei per sample. For each specimen, the morphology was identified in immunostained slides and the corresponding areas on FISH slides were marked with Indian ink, which made it possible to correlate cytogenetic findings to morphological details in tissue sections.

3.4 STUDIES OF PROTEIN EXPRESSION. IMMUNOHISTOCHEMISTRY

Generally, there are two commonly used protein detection methods: immunohistochemistry and Western blotting. The choice of a method depends on the needs of the study. Western blotting is more useful in the situations when the information about the molecular size and expression level of certain protein is the primary goal. Immunohistochemistry allows studying the overall expression of a protein of interest in tissue context. The latter method has been used to detect differences in protein expression between histological structures within the same specimen.

The history of immunohistochemistry started in 1941 when Coons identified pneumococci using a direct fluorescent method. Then followed the indirect method, the addition of horseradish peroxidase, the peroxidase anti-peroxidase technique of 1979 and the use of the Avidin and Biotin complex in the early 1980s [181]. Immunohistochemical methods involve, at their core, the vitamin biotin and the protein avidin, which bind together irreversibly. By establishing a biotin link, through avidin, between the horseradish peroxidase enzyme and a secondary antibody reagent, enzyme localization can be achieved at the site of primary antibody interaction with the specimen. The biotin molecule is small and can be easily conjugated to immunoglobulin by amino substitution at alkaline pH without the loss of immunoglobulin activity. These secondary antibodies are quite inexpensive, readily available commercially, and reactive against immunoglobulin from a wide variety of species [182]. Fig. 13 schematically represents the major immunohistochemical technique.

In the present study, expression of Pim-1 and ezrin was determined by immunostaining in prostate tissue sections.

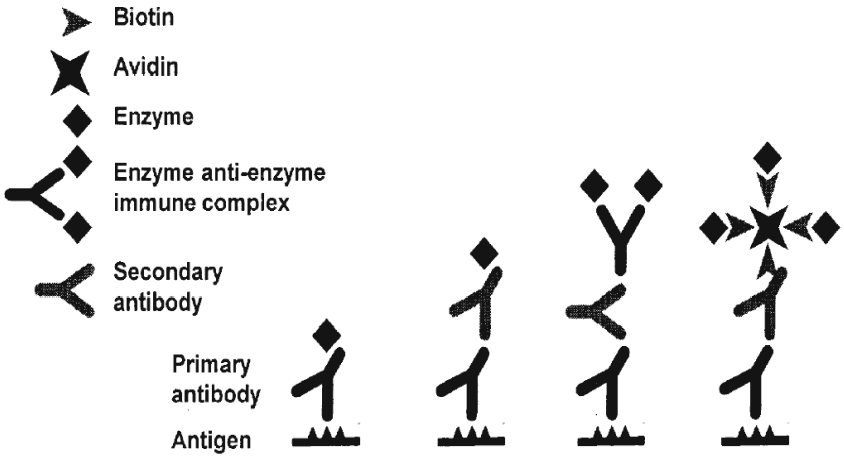


Figure 13. The principle of immunohistochemistry.

The prostates were fixed overnight in 10% buffered formalin. The specimens were inked and sliced horizontally at 4 mm intervals. Slices were cut in 2 to 6 segments (usually quadrants) and the entire prostate was subsequently blocked in standard cassettes. Specimens were dehydrated, embedded in paraffin, sectioned at 4 microns and stained with hematoxylin and eosin. Cancer and HGPIN areas were outlined on the slides. In paper IV, one representative section from the main tumor was selected from each prostatectomy specimen. In each section, five ink rings measuring 6 mm in diameter were marked on the glass slide within cancer area. The areas were selected to be representative of different intensities of immunostaining including the most intensely stained area.

Standard biotin-avidin-complex immunohistochemistry was performed using a polyclonal goat antibody against the carboxy terminus of Pim-1 (Santa Cruz Biotechnology) and a mouse monoclonal antibody against ezrin (Ab-1; Neomarker, Lab Vision Corp, CA, USA). Placenta tissue was used as positive and negative controls for the immunostaining. Assessment of the type and intensity of immunohistochemical staining was done by either two (papers II and III) or three (paper IV) observers. In papers II and III, the cytoplasmic staining intensity in cancer and HGPIN was scored as absent, weak, moderate or strong compared to adjacent normal epithelial cells. Staining in HGPIN was

evaluated in relation to cancer areas in the same slide. In paper IV, cytoplasmic IR intensity in the 5 cancer areas was scored subjectively from 0 to 3 where 0 was absent, 1 weak, 2 moderate and 3 strong staining. Average tumor IR score of each observer was calculated for each specimen. An average score of 1.5 - 2.5 was considered as moderate expression and a score > 2.5 as strong expression. Scoring was performed without knowledge of any pathological or clinical data.

3.5 CYTOLOGICAL ANALYSIS OF PROSTATIC INTRAEPITHELIAL NEOPLASIA

In order to describe cytological features of prostatic intraepithelial neoplasia, a sampling method for simulating fine-needle aspiration cytology (FNAC) has been used [91]. It overcomes the disadvantage with FNAC: the inability to identify the site of aspiration, which leads to the difficulties in comparison of cytological and histopathological specimens.

Cytological slides were evaluated by three observers and a consensus agreement was reached in an open discussion. Cellularity, size of aggregates of atypical cells, dissociation of atypical cells, nuclear atypia and prominent nucleoli were estimated according to three-tier scales. Multiple nucleoli, basal cells and presence of mucin, crystalloids, magenta-colored cytoplasmic pigment and dark cytoplasmic staining were evaluated as present or absent. Since each smear was always a mixture of different cell populations, the prevalent cellular pattern was used for scoring. Cellularity was defined as total amount of atypical cells on each smear and graded as low, medium or high. Size of atypical clusters was estimated as small, medium or large according to size of the majority of cell aggregates. Dissociation of atypical cells was defined as absent, moderate or pronounced. Nuclear atypia was graded on a three-tier scale where nuclear pleomorphism, cytoplasmic/nuclear ratio, anisonucleosis and hyperchromasia were considered. Nucleolar prominence was assessed as mild, moderate and strong.

4 RESULTS AND DISCUSSION

4.1 MUTATION ANALYSIS OF THE *BRG1* GENE IN PROSTATE CANCER CLINICAL SAMPLES (PAPER I)

In **paper I** we have performed mutational analysis of the *BRG1* gene in the panel of human prostate cancer specimens and matching constitutional controls. We have chosen to investigate the role of a given gene in prostate cancer based on the previous data, showing the presence of inactivating mutations in *BRG1* in human cancer cell lines, including the prostate cancer cell line DU145 and gene's functional properties, supporting evidence that *BRG1* act as tumor suppressor [183]. *BRG1* as being the member SWI/SNF complex is believed to be involved in tumorigenesis based on its requirement for retinoblastoma induced growth arrest [148].

BRG1 is mutated or deleted in numerous cancer cell lines, leading to the altered expression of genes that influence cell proliferation and metastasis. The latest reports show that loss of *BRG1* leads to methylation and silencing of the promoters of both CD44 and E-cadherin, thus presenting the novel mechanism of increasing DNA methylation in cancer cells [184]. Additionally, the direct recruitment of chromatin-remodeling enzymes by nuclear hormone receptors has been described for androgen receptor (AR) [185]. These data provide insight into the mechanisms underlying aberrant gene induction and repression during tumor progression. Our data, however, show the absence of somatic mutations in the panel of human prostate cancer samples involved, thus, excluding the presence of common *BRG1* mutations in prostate cancer. Additionally, we identified five single nucleotide polymorphisms (SNP), three of which were novel (Fig. 14, Table 1). Subsequent analysis revealed potential allelic associations of *BRG1* SNPs and prostate cancer clinical features.

Figure 14. *BRG1* transcript structure. Arrows indicate locations of novel SNPs.

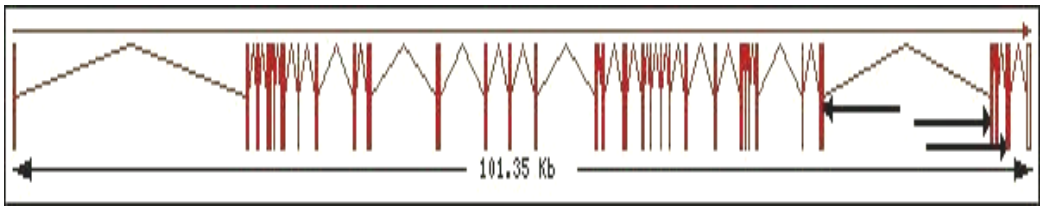


Table 1. SNPs of the *BRG1* gene in prostate cancer patients.

Location	Nucleotide position	Genotype	Amino Acid	Observed number	Observed (%)	Patients studied	Reported dbSNP	Name in present study
Exon 9	33978	T/T C/T C/C	His/His His/His His/His	12 7 2	(57) (33) (10)	21	dbSNP rs#7935	SNP33978
Intron 22	64585	C/C C/G G/G	intron intron intron	8 10 3	(38) (48) (14)	21	dbSNP rs#2075021	SNP64585
Exon 29	74061	T/T C/T C/C	Asp/Asp Asp/Asp Asp/Asp	0 1 20	(0) (5) (95)	21	Present study	SNP74061
Exon 32	97884	T/T C/T C/C	Val/Val Val/Val Val/Val	0 2 19	(0) (10) (90)	21	Present study	SNP97884
Exon 34	99209	T/T C/T C/C	Asp/Asp Asp/Asp Asp/Asp	10 9 2	(48) (43) (9)	21	Present study	SNP99209

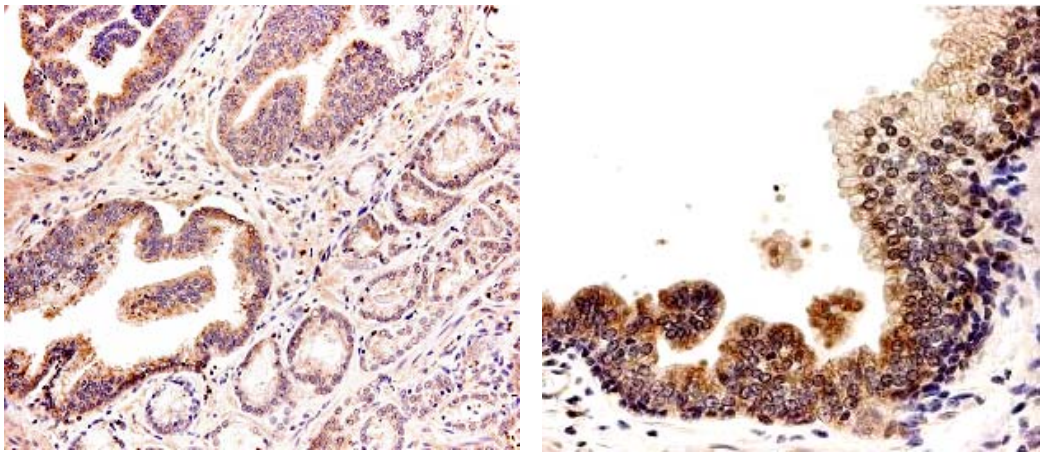
Nucleotide position is according to the reference GeneBank sequence: AF20544908. dbSNP: (http://www.ncbi.nlm.nih.gov/SNP).

Taking into account that BRG1 protein is lost in several tumor types, we hypothesize that some other mechanism, but not structural genetic alterations e.g. promoter hypermethylation could, probably, have an impact on *BRG1* expression in prostate cancer.

4.2 PIM-1 EXPRESSION IN PROSTATE CANCER AND PROSTATIC INTRAEPITHELIAL NEOPLASIA (PAPER II)

In **paper II** immunohistochemistry was used to assess the patterns of Pim-1 expression in high-grade prostatic intraepithelial neoplasia (HGPIN) and cancer in 121 radical prostatectomy specimens. The protooncogene Pim-1 is localized on chromosome 6p and encodes for a protein kinase. It was shown to

be involved in cell cycle regulation [186] and in regulation of proliferative processes [156]. Previous study with tissue microarray showed Pim-1 overexpression in prostate carcinoma [149]. In the present study we aimed to estimate Pim-1 protein expression in HGPIN and in invasive cancer separately and in relation to each other. We found strong expression in 82% of prostate carcinomas and in 97% of HGPIN cases. The protein expression in HGPIN was never lower than in invasive cancer of the same specimen. Relative overexpression in HGPIN as compared to cancer was present in 65% of specimens (Fig. 15a). Additionally, an abrupt transition from atypical cells with positive Pim-1 staining to negative benign cells was observed when HGPIN and benign epithelium were present within the same gland (Fig. 15b). The observation that Pim-1 is overexpressed in HGPIN, which has an intermediate proliferation rate between benign prostate hyperplasia and cancer, suggests that Pim-1 activation may be an early event in the development of prostate cancer, possibly playing a role in the transition from precancerous lesions to invasive cancer. Another conclusion from the study, based on the finding that expression in benign glands was either absent or only weakly positive, is that Pim-1 expression may be useful for distinguishing HGPIN from benign prostatic epithelium.



a. **b.**

Figure 15a. Pim-1 overexpression in HGPIN (upper left) compared to invasive cancer (lower right).
b. HGPIN showing an abrupt transition from atypical cells with positive Pim-1 staining (bottom left) to negative benign cells (upper right) within the same gland.

4.3 EXPRESSION OF EZRIN IN PROSTATIC INTRAEPITHELIAL NEOPLASIA (PAPER III)

In **paper III** we investigated the expression of ezrin in prostate cancer and its precursor high-grade prostatic intraepithelial neoplasia (HGPIN) in parallel with the studies of copy number status of the gene, encoding for ezrin. Ezrin has been shown to be down-regulated in androgen-withdrawal induced apoptosis and up-regulated in androgen-replacement stimulated proliferation in rat ventral prostatic epithelial cells. Additionally, several lines of evidence indicate that ezrin plays a positive role in maintaining cell shape and cell polarity, and participates in membrane trafficking pathways, cell migration, cell signaling, growth regulation and differentiation. Because of its unique functions, ezrin is actively involved in tumor biology, especially in regulating the growth and metastatic capacity of cancer cells. Our data show that ezrin has a higher expression level in HGPIN and prostate cancer as compared to normal prostate epithelium (Fig. 16). However, FISH results did not reveal any copy number changes of the given gene, suggesting that ezrin overexpression cannot be explained by gene amplification and that actual mechanisms underlying overexpression of ezrin are yet to be identified.

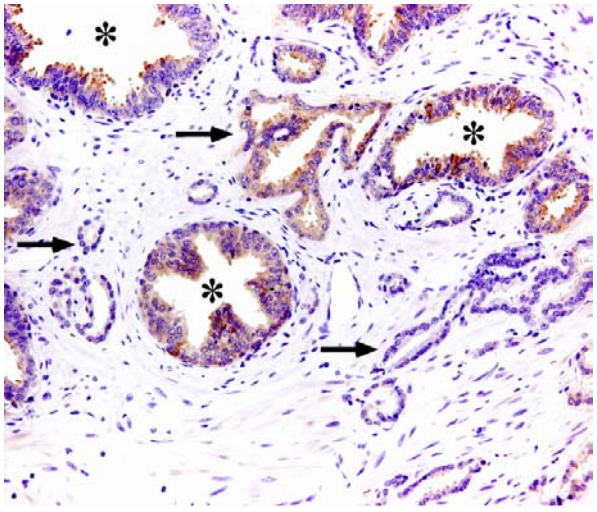
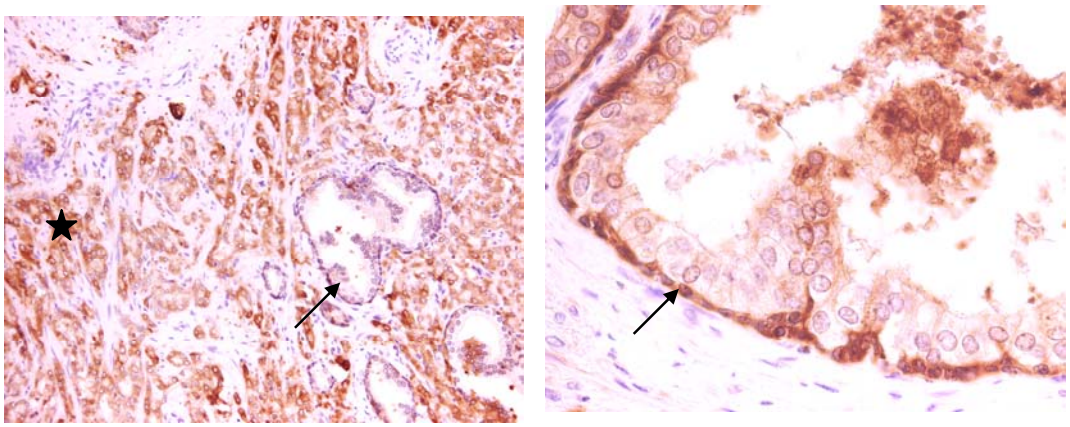


Figure 16. Expressoin of ezrin is stronger in HGPIN (asterisks) as compared to adjacent invasive prostate cancer (arrows).

4.4 EZRIN EXPRESSION IN PROSTATE CANCER AND BENIGN PROSTATIC TISSUE (PAPER IV)

In **paper IV,** immunohistochemical analysis was used to characterize patterns of ezrin expression in prostatic carcinoma and benign epithelium in 103 radical prostatectomy specimens. Our previous study (paper III) demonstrated an increased expression of ezrin in prostate cancer and high-grade PIN in comparison with normal prostatic epithelium in a limited number of specimens. This study was the first attempt to correlate the expression of ezrin with prognostic and clinical parameters in prostate carcinomas and to assess staining patterns in benign prostatic tissue. Our results show that moderate or strong expression was seen in 70% of cancer specimens while negative or only weakly positive in benign epithelium (Fig. 17a). Ezrin expression correlated with Gleason score and seminal vesicle invasion.



a. **b.**

Figure 17a. Strong cytoplasmic IR of ezrin in prostate cancer Gleason Score 4+5 (star). Ezrin is overexpressed in cancer compared to benign epithelium (arrow). b. Benign prostatic glands of central zone with prominent basal cell layer. Ezrin immunoreactivity (IR) is weak in the luminal cells and strong in basal cells (arrow).

The expression pattern in benign tissue was striking. Luminal cells of benign prostatic glands were negative or only weakly positive as opposed to basal cells which frequently expressed ezrin (Fig 17b). This observation can be linked to ezrin's function as inhibitor of apoptosis. Basal cells are known to express bcl-2, which also prevents apoptosis, and it has been postulated that they are

stem cells of luminal cells [187]. In metaplasia, similar to normal glands, the luminal cells were negative in glands involved with metaplasia, while the metaplastic cells themselves were distinctly positive. Additionally, staining of the basal cells was mainly attributable to metaplasias but not to the normal prostate glands.

Collectively, the expression pattern of ezrin is complicated. Our data suggest that the protein can be involved in prostate cancer tumorigenesis, but also plays a role in normal epithelium and benign reactive processes in the prostate. Ezrin expression in prostatic intraepithelial neoplasia is remarkably strong and it is also correlated with adverse prognostic factors in cancer, showing gradual increase in expression following tumor progression and loss of differentiation. It is likely that ezrin overexpression might affect many cellular functions contributing to tumor progression. At the initial steps of prostate cancer development, ezrin might have a role in "triggering" carcinogenesis by promoting the transition to invasive prostate cancer.

4.5 CYTOLOGICAL FEATURES OF PROSTATIC INTRAEPITHELIAL NEOPLASIA (PAPER V)

In **paper V**, an attempt has been made to describe cytological features of PIN. A sampling method for simulating fine-needle aspiration cytology (FNAC) has been used for that purpose. The resulting scrape specimens were morphologically indistinguishable from fine needle aspiration cytology specimens confirming the validity of the experimental model used in this study. The present study shows that PIN shares some cytological features with cancer, while others differ (Table 2). We found that PIN specimens had lower cellularity and less dissociation of cells compared to invasive cancer. Atypical epithelium in PIN specimens showed glandular architecture with a wide range of cluster sizes. No difference was seen between PIN and cancer with regard to cluster size. The atypical PIN clusters showed moderate nuclear atypia which

Features	Sum of ranks		p-value
	PIN	Cancer	
Cellularity	178	**417**	**p<0.001**
Size of atypical clusters	285	310	p=0.66
Dissociation	183	**411**	**p<0.001**
Nuclear atypia	175	**419**	**p<0.001**
Prominent nucleoli	239	**355**	**p=0.045**
Multiple nucleoli	238	**357**	**p=0.04**
Mucin	221	**374**	**p=0.008**
Crystalloids	263	331	p=0.24
Dark cytoplasmic staining	**382**	212	**p=0.03**
Cytoplasmic granules	314	280	p=0.55

Table 2. Comparison of PIN and invasive cancer specimens by different cytological features. Summarized results of Mann-Whitney test (significant results are marked with bold).

was less pronounced as compared to cancer. Moreover, cancer smears showed more prominent nucleoli and more often multiple nucleoli. The cytoplasm of atypical cells in PIN was darker than that of cancer cells and contained mucin more rarely. Unexpectedly, we were unable to differentiate PIN from invasive cancer based on basal cell criterion, because cells with similar features were commonly seen in atypical clusters of cancer specimens as well, which can be explained by an admixture of PIN in cancer smears. To the authors' knowledge, this is the first report on cytological morphology of PIN.

The identification of PIN has clinical importance, since this lesion is a precursor of prostate cancer. This study shows that cytological similarities of PIN and cancer make it difficult to distinguish between two lesions. Nevertheless, PIN has its own features: a few clusters of less dissociated, moderately atypical cells with darker cytoplasm should be considered as a differential diagnosis of cancer.

5 CONCLUSIONS

1. The absence of common *BRG1* mutations in prostate cancer, but presence of three novel single nucleotide polymorphisms, excludes structural genetic alterations as a mechanism having an impact on *BRG1* expression in prostate cancer.

2. Relative overexpression of Pim-1 in HGPIN as compared to cancer possibly marks the transition from precancerous lesions to invasive cancer. Upregulation of Pim-1 at premalignant stages may be an early event in the development of prostate cancer, and could be useful for distinguishing HGPIN from benign prostatic epithelium.

3. Stronger protein expression of ezrin in HGPIN compared to invasive prostate cancer. Differences in protein expression of ezrin are not due to gene copy number changes of the corresponding gene. Higher protein expression of ezrin in prostate cancer precursor lesions may be an early event in pathogenesis of the disease.

4. Ezrin expression is correlated with adverse prognostic factors in prostate cancer, showing gradual increase in expression following tumor progression and loss of differentiation. The overall expression pattern of ezrin suggests its involvement in prostate cancer development.

5. Cytologically, prostate cancer and PIN show close similarities. Therefore, FNAC should not be considered as a single diagnostic tool.

6 ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to everybody who has helped me with this work:

Lars Egevad, my supervisor, for taking care of me when it was really needed, for your encouragement and trust during these years, for radiating goodness around you and letting me take it up

Peter Ekman, my supervisor, for guidance, wisdom, and general support

Xiaolei Fang, Jacob Fang, Chunde Li, Ayako Naito, my co-workers, for your practical help

Sara Jonmarker, for being nice and helpful

Bo Nilsson, for your invaluable help with statistics

Peter Wiklund and everyone at the Department of Urology, for your kindness and supervision

Anders Zetterberg and everyone from your group, for being nice to me and letting me "breathe your air"

Sten Nilsson and everybody from your group, for being good neighbors

Klas Wiman and everyone from your group, especially **Vladimir**, for good advices

Bertha Brodin and everybody from your group, for being nice colleagues

Olle Larsson and everyone from your group, for all your help

Catharina Larsson and everybody from your group, especially Jan, Andrea, and Andrei for your friendship

Gert Auer and everybody from your group, for your support

"Tantolunden", especially Margareta and Lisa for your excellent experimental assistance

Agneta Nordenskjöld, for your guidance

Juan Castro "seniore directore", for salsa rhythms in life

Margareta Tapper-Persson, for your enthusiasm and kindness

Anders Eklöf and **Per Hertzberg**, for solving all the IT-related problems

Alicia Marchlewska and **Louise Nordfors**, for your help and support

All colleagues at **CCK** and **CMM**, for creating the nice atmosphere

Lars-Ove Farnebo, for being a wise chairman

Tina Dalianis, for you supervision, help and advices

Helena Nässén, Ann-Jeanette Fischer and **Yvonne Stridsberg,** for keeping everything under control, for your smiles and good humor

Annika Broström, for you excellent assistance

Ann-Britt Spåre, for your professionalism

Evi Gustavsson-Kadaka and **Sören Lindén**, for all your help

Joe Lawrence, for the "right here, right now" solutions to all the problems

Tommy Linné, for your never-ending enthusiasm

Salman Al-Shukri and the whole Department of Urology in

St. Petersburg, for your guidance and support

Edwin E. Zvartau, for your invaluable help

Yuri D. Ignatov, for your supervision

Nikolay A. Yaitsky, for your great support

Stefan Loening, for your good attitude

Dmitri Daniltchenko, for keeping the faith

All the colleagues at **Radiumhemmet**

Colleagues at **Danderyds Sjukhus,** especially Ulf, Peter, Kenneth and Björn

All members of the **KIRT** crew, for the great time together

Personnel of the **Department of the International Relations** at Pavlov Medical University

Maxim Pavlenko and **Tanja**, for your friendship

Gunilla Palmér, for your optimism and our "mountain-high" conversations

Axel Glaessgen and **Daria Cosaceanu,** for your kindness and hospitality

Pia and **Hilda**, for your smiles and the great time together

The family **Sviatoha**, for friendship and hospitality

Sheldon Litt, for sharing your vital force

Mariana Nordström, for being a good friend

My parents, relatives and brother, for your love and spiritual support

Olga, for being the one and only

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