AN IMMUNOHISTOLOGIC STUDY OF BIOLOGICAL PARAMETERS IN PROSTATIC INTRAEPITHELIAL NEOPLASIA AND ADENOCARCINOMA

A thesis submitted in partial fulfillment
of the requirements for the degree of

Master of Philosophy

by

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Abstract

The expressions of seven biologic parameters including p53, PCNA, Ki-67, EGFr, E-Cadherin, CD44, and nm23 were evaluated by immunohistochemical methods from 122 Hong Kong Chinese patients including 28 cases of prostatic hyperplasia, 46 cases of Prostatic Intraepithelial Neoplasia (PIN) and 48 cases of prostatic carcinoma (PCa). Their immunoreactivities were correlated with the histological grades of PIN and carcinoma, but data on the clinical stage and patient's survival were not available for correlation.

The aim of this study is to examine whether these markers may play a role in tumor development, and also to determine whether the sequence of hyperplasia, dysplasia (intr naepithelial neoplasia), and carcinoma applies to prostatic cancer, or whether PIN lesions represent a precursor that would lead to the development of carcinoma.

Overexpression of p53 and EGFr was demonstrated in carcinomas, and was correlated with increasing grade of PCa suggesting that alterations of p53 and EGFr genes may be a late event in prostatic cancer progression. The low incidence of p53 alterations also indicated that such genetic event only affects a small subset of PCa.

E-cadherin and CD44 expressions were down-regulated in PIN lesions and PCa, reflecting the loss of epithelial integrity and the acquisition of metastatic potential. Both E-cadherin and CD44 appeared to be implicated in early tumorigenesis, and CD44 appeared to be implicated in tumor progression. These observations also supported the link between PIN and PCa. Expressions of PCNA, Ki-67, and nm23 were increased in PIN lesions and PCa. Alteration of the putative anti-metastatic nm23 gene appeared to be implicated in the early stage of tumor

development, but not in the tumor progression. Immunoreactivity of PCNA, Ki-67 and nm23 demonstrated a continuity in the sequence of hyperplasia, PIN and carcinoma, supporting the concept of PIN as a lesion precursor to malignant progression. PCNA and Ki-67 may represent useful prognostic markers in PCa.

Several markers, including E-cadherin, CD44, nm23 protein, PCNA and Ki-67 demonstrated similar alterations between PIN and carcinoma, and supported the concept that PIN is a preinvasive lesion. The progressive changes from PIN to carcinoma also lend support for a link between these lesions. Although p53 and EGFr showed a marked difference between PIN and PCa, this does not exclude a relationship between them as supported by other biological markers.

ABSTRACT (Chinese translation)

用組織免疫化學檢測香港 122 位華藉病人之前列腺樣本中七種生物指標的表達，包括 p53，PCNA，Ki-67，EGFr，E-Cadherin，CD44 及 nm23。樣本中有 28 個前列腺體增生，46 個前列腺上皮內腫瘤 (PIN)，和 48 個前列腺癌 (PCa)。檢查它們的免疫反應與 PIN 以及前列腺癌的組織學分級互相關聯，但由於病人的臨床分期以及生存率未獲提供而它們的互相關聯則沒有檢查。

本研究旨在檢查這些生物指標會否與腫瘤的發展有關，並且鑑定前列腺癌會否按增生、異形增生、到癌的順序發展，或者，PIN 會否代表引致發展前列腺癌之先驅的病變。

結果發現 p53 和 EGFr 均在癌中呈過表達，並且與前列腺癌的分級呈相互關係，表示 p53 和 EGFr 基因變更可能是發生在前列腺生癌作用的後期。而 p53 變更發病數偏低亦表示這基因變更事件只會影響一小群前列腺癌。

E-cadherin 和 CD44 的表達在 PIN 與 PCa 中均下調，反影上皮性殘缺和遷徙性潛能的獲得。E-cadherin 和 CD44 均似乎與腫瘤發生早期有牽連，而 CD44 亦與腫瘤進行有牽連。這些觀察均支持 PIN 與 PCa 間的連繫。PCNA，Ki-67 和 nm23 的表達在 PIN 與 PCa

中均上升。一般公認為抗遷徙性的 nm23 基因之變更似乎與早期腫瘤發展而非腫瘤進行有牽連。PCNA，Ki-67 和 nm23 的免疫反應性證明由增生、異形增生、到癌的發展順序中的連續性，支持 PIN 作為惡性進行之前身病變的概念。此外，PCNA 和 Ki-67 在前列腺癌中可能代表有效的預後指標。

研究結果發現多個指標，包括 E-cadherin，CD44，nm23，PCNA 和 Ki-67，證明 PIN 與癌之間有類似的變更、並且支持 PIN 是侵略前病變的概念。由 PIN 以至癌之間漸進的變化亦支持成立這些病變間有連繫。雖然 p53 和 EGFr 顯示 PIN 與前列腺癌之間有明顯對比，但從其它指標的結果顯示，這並不排除它們之間有關係 。

Chapter 1. Introduction

I. Epidemiology of Prostate Cancer

Prostate cancer is a disease that affects mainly older men and is the second most common cause of cancer deaths in men after lung cancer in most countries of the Western world. In United States, it is responsible for more than 12% of all cancer deaths in men on average from 1991 to 1995 and is also the most frequently diagnosed cancer (1). It has been estimated that 184,500 new incidence cases will be diagnosed during 1998 and 39,200 man may die of it (1). As depicted in Figure 1, during the 1970s and 1980s, prostate cancer incidence and mortality increased steadily at small average rates of about 5% and 1% per year respectively. However, the rate increased rapidly during the late 1980s. The incidence is not accompanied by a correlated increase in mortality and is possibly artifactual due to increase of patient awareness, increasing life expectancy, and in particular the widespread use of the blood test for serum prostatic specific antigen (PSA) and other modalities for screening including ultrasound and organ imaging (2).

Adenocarcinoma of prostate (PCa) is common in North America and North-western Europe but is rare in the Near East, Africa, and South America. The incidence varies greatly among different racial or ethnic groups of male. The incidence and mortality rates in American blacks are distinctively higher than that of American whites. The average incidence rates of prostate cancer of American blacks and whites during the period from 1991 to 1995 were 241.2 and 157.6 per 100,000 population respectively. About 55.1 and 26.1 per 100,000 American blacks and whites respectively died each year from 1991 to 1995 on average (1).

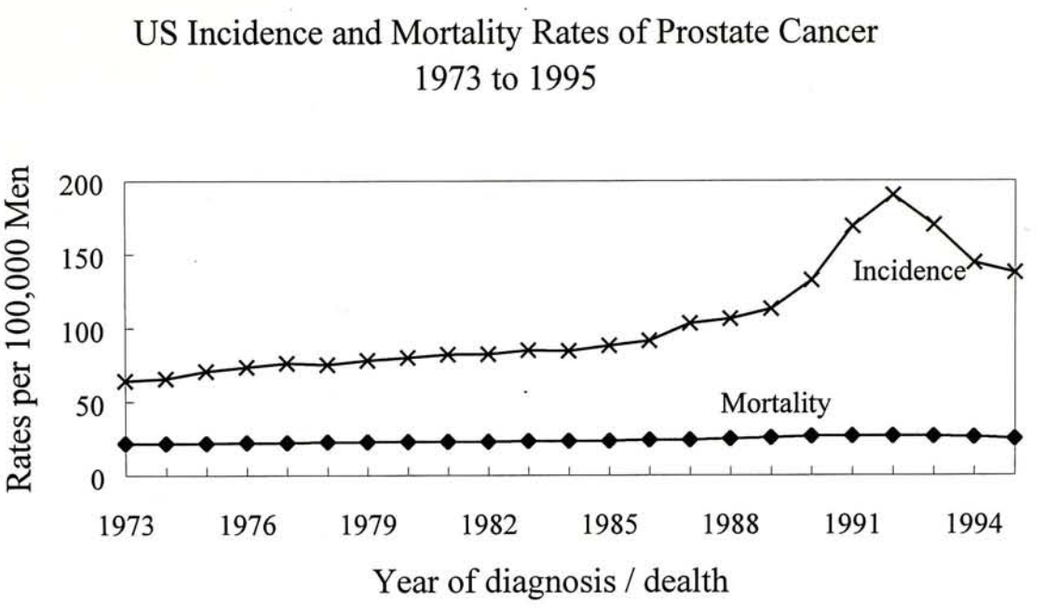


Figure 1. Incidence rate and mortality rate among the men's population in the United States from 1973 to 1995. (Data obtained from the National Cancer Institute's Surveillance, Epidemiology End Results program.) (Appendix I)

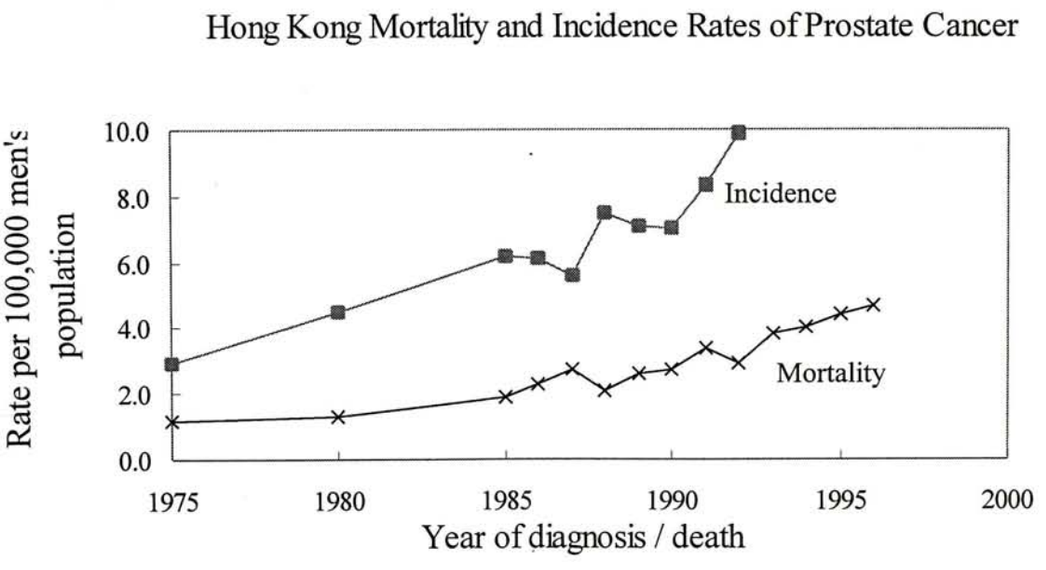


Figure 2. Incidence and mortality rates of prostate cancer among the men's population in Hong Kong from 1975 to 1996 (Appendix II).

In Hong Kong, although lung cancer is also the leading cause of cancer death, prostate cancer only contributed to 2.41% of all cancer deaths in men in 1996 and is the ninth leading cause of cancer death in men (3) (Appendix II). As depicted in Figure 2, although the mortality rate due to prostate cancer had increased 2.5 fold and 3.9 fold of the cancer death caused by prostate cancer in 1985 and 1975 respectively, it is only 4.7 per 100,000 men's population in 1996 in contrast to 26.0 cancer deaths per 100,000 men's population in the United States (Appendix III). While the occurrence of prostate cancer gradually increased from 4.45 in 1980 to 8.65 in 1994 per 100,000 men in the Hong Kong population, the incidence of prostate cancer is considerably lower in contrast with the American population which increased from 79.8 in 1980 to 190.1 in 1992 per 100,000 men although the incidence rate in American population have dropped to 137.2 per 100,000 men in 1995 (1). The increasing incidences both in Hong Kong and the United States can only partly be explained by a wider use of screening serum prostatic specific antigen levels and rectal ultrasound examination. Whether additional factors are contributing to such increase in incidence is not certain. The differences in the rate of occurrence and mortality of prostatic cancer between different racial or ethnic groups are undetermined, but have been attributed to genetic factors and environmental factors. Studies of migrants from low-incidence to high-incidence countries show an increase in clinical prostate cancers suggestive of a role of environmental factors. The incidence of prostate cancer in native Japanese is 7.7 per 100,000 men in 1990 whereas the incidence increases to 88 per 100,000 men on average during 1988 to 1992 in those living in the United States (4,5). Although there are marked differences in incidence and mortality rates between Americans and Hong Kong

Chinese, men under the age of 50 from both regions have low incidence and mortality rates of prostate cancer. Both incidence and mortality rates rise steeply thereafter.

The significance of numerous epidemiologic factors associated with the development of PCa varies, but in general none of these appear to play a determinant role. The factors regarded to be associated with increase in risk include a family history of prostate cancer, dietary intake of fat, obesity and chronic alcohol abuse, while smoking, venereal diseases and viral infection, sexual activity and vasectomy have not been conclusively associated with PCa (6,7,8,9,10). No putative gene loci have been identified to date. It has been estimated that less than 10% of prostatic cancer are associated with a family history or heredity. Countries with high average fat consumption have been correlated with higher rates of prostate cancer death (increased relative risk by 1.6 to 1.9) while studies on populations with diet rich in fiber and presumably lower in fat are associated with lower incidence of prostate cancer (8,11). The precise mechanism is not well defined. But increased hormonal bioavailabilty due to high fat diet has been implicated. Occupation in welding and electroplating industry where workers are exposed to cadmium has been shown to have increased risk of prostate cancer possibly due to antagonistic effect to zinc in a number of biological reactions. Zinc is also a crucial cofactor for several DNA repair enzymes. And prostate has been demonstrated to have a high concentration of zinc. Other occupational risks attributed to workers in rubber industries, newspapers, plumbing, sexually transmitted viruses, serum androgen levels, vasectomy, prostatic hyperplasia have been implicated as causal factors. But studies of such factors have either been inconclusive or controversial (6,7,8,9,11,12,13).

It has been suggested that, provided careful autopsy studies are carried out, finding of small, well differentiated, occult PCa is a common place in all male population although prostate cancer as a clinical disease and cause of morbidity or mortality varied remarkably in frequency all over the world and within countries, particularly among different ethnic or racial groups. It is of interest to identify factors which cause such PCa in a small fraction of men to evolve into rapid growing malignant cancer that kills (14).

II. The Normal Prostate - Prostatic Anatomy

The normal adult prostate weighs about 20 g and is shaped as an inverted cone, where the base forming part of the bladder neck and the apex forming the urethral outlet. It surrounds the urethra at the origin from the urinary bladder. The apex rests directly above the urogenital diaphragm. Its position is maintained by the puboprostatic ligaments attached to its anterior surface. There is a longitudinal midline depression called the median sulcus in the broad, flattened triangular posterior surface (15,16).

The prostate gland contains four glandular compartments, the peripheral zone, the central zone, the transition zone and the periurethral gland region. The peripheral zone is the largest glandular region, constituting approximately 70% of the glandular tissue. It forms the posterior, lateral, and apical regions of the prostate and surrounds the central zone and partially the transition zone. The peripheral zone is readily visualized by transrectal ultrasound and is the area that is primarily sampled by transrectal biopsies. The central zone is cone-shaped and comprises about 25% of the prostate. The base of the cone forms the major part of the base of

the prostate (seminal vesicle/bladder junction). The vertex of the cone is located at the verumontanum. The ejaculatory ducts pass through the central zone. The transition zone consists of two lobes that surround the proximal urethral segment laterally and anteriorly. The transition zone is surrounded posteriorly and laterally by a band of fibromuscular tissue called transition zone boundary or "surgical capsule" that separates the transition zone from the peripheral zone. The transition zone is the region sampled predominantly by transurethral resection procedures. The volume of this zone is variable due to frequent development of benign prostatic hyperplasia (BPH). The transition zone constitutes about 5 to 10% of the prostatic volume before development of prostatic hyperplasia. Benign prostatic hypertrophy may also arise in the periurethral gland region which surrounds the proximal urethral segment of the prostate and is confined by the preprostatic sphincter, constituting less than 1% of the prostatic volume (15,16).

The prostatic urethra extends from the bladder neck to the prostatic external sphincter and is divided into two segments of approximately equal lengths. The proximal urethral segment extends from the bladder neck to the verumontanum. The distal urethral segment extends from the verumontanum to the external sphincter. The two segments are joined at the level of the verumontanum at an angle of 35 degrees. The proximal urethral segment receives the ducts of the transition zone and the minute ducts from the periurethral gland region. The base of the verumontanum is located on the posterior wall of the distal urethral segment. The distal urethral segment receives the ejaculatory ducts and the ducts of the central zone at the level of the verumontanum and the ducts of the peripheral zone along the entire length of the distal urethral segment. The prostate is surrounded posteriorly and laterally by

the outer fibromuscular rim or "capsule" that separates the prostate from the surrounding tissue. At the prostatic base and along the anterior surface of the prostate, a well defined anatomic separation between the prostate and adjacent tissues and bladder neck is lacking. The prostate receives blood from a branch of the internal iliac artery via the inferior vesical artery. The venous drainage of the gland empties into the prevertebral plexus and the hypogastric venous system. Lymphatic drainage occurs through the external and internal iliac as well as obturator lymph nodes. It is innervated with both sympathetic and parasympathetic fibers.

Growth and development of the prostate are hormone dependent and need the tropic action of both oestrogens and androgens. The prostate is immature at birth and only grows to its normal size at puberty when androgen secretion commences.

Microscopically, the prostate gland is composed of branched tubuloalveolar glands forming lobules. The ducts converge to terminal tubules which open into the urethra. The tubules or alveoli are surrounded by abundant fibromuscular and fibroelastic supportive stromal tissue constituting one-third or more of the whole mass of the prostate. The epithelium shows a great variation between different glands and alveoli and even within a single alveolus. It is usually composed of the secretory or luminal cells, which consist of simple cuboidal or columnar cells with a clear cytoplasm. The cytoplasm may also contain secretion granules and lipid droplets. The epithelium and adjacent connective tissue may form folds that project into the lumen of the glands. The ducts are lined by a simple columnar secretory epithelium that changes to the transitional epithelium near the termination of the ducts. These secretory or luminal cells produce and express prostatic specific antigen and prostatic acid phosphatase (17). The ducts and acini all demonstrate the presence

of a basal cell layer, which are cuboidal or flattened and attenuated, and which contrast with secretory cells by lacking prostatic specific antigen or prostatic acid phosphatase, but expressing high molecular weight cytokeratins (18). The supporting connective tissue stroma also include bundles of smooth muscles.

III. Pathology of Prostatic Cancers

Nearly 90% of prostatic cancers exhibit some morphologic features of prostate glandular epithelium and are referred to as acinar adenocarcinoma of prostate or simply prostatic carcinoma (PCa). Ductal carcinoma or large duct adenocarcinoma constitutes less than 5% of prostate. The remaining types are rare and represent morphologic variant of prostatic acinar carcinoma. Carcinomas of prostate may be multifocal and frequently involve the peripheral zone of the prostate and less commonly the central zone and transitional zone of the prostate.

Grossly, at the early stages where carcinoma is confined within the organ, the tumor may appear as a poorly delineated, white-yellow, firm area, located at the periphery of the gland. At later stages, when the tumor is bulky and infiltrating adjacent structures, the tumor may show extensive necrosis and hemorrhages. Microscopically, PCa may exhibit a wide morphologic spectrum, ranging from well-formed small glands to anaplastic sheets, files, and single tumor cells. They are recognized by their atypical architectures and cytologic features. Infiltrative tumor alters the lobular architecture, and presents as inconspicuous small glands crowding the lobules. They are characterized by enlarged nuclei, with a high nuclear cytoplasmic ratio, coarse nuclear chromatins and membrane, and prominent nucleoli. Mucin production may become recognizable and prominent. The lumen of tumor

glands may demonstrate presence of crystalloids secretions.

The clinical behavior of PCa is remarkably variable. Patients with undifferentiated, DNA-aneuploid PCa at the time of diagnosis are biologically fated to poor outcome even when the high-grade carcinoma is confined within the prostate capsule and treated by radical retropubic prostatectomy. The low-grade (well or moderately differentiated) PCa of low volume (<4ml) and DNA-diploid are recognized by clinicians to be clinically indolent which progress slowly with little morbidity even untreated (14).

Histologic grading is one of the strongest predictors of the biologic behavior of PCa. Many grading systems have been proposed but are lacking of either reproducibility or reliability. The Gleason grading system, which is based on the architectural arrangement of the malignant epithelial cells within the prostatic stroma, is widely accepted and utilized, and has been strongly correlated with survival, tumor-free survival, metastasis-free survival, and time interval to disease recurrence (19). The system was developed by the Veterans Administration Cooperative Urological Research Group (VACURG). Nine histological patterns grouped into 5 histological grades were identified and correlated with malignancy as determined by mortality data (Table 1). While different histological grades are usually present in PCa, the behavior of PCa has been correlated with mortality rates and is proportional of the average histological grade. The ***Gleason score*** is conceived and used to account for the heterogeneity of each tumor, where often a mixture of several grades are present. The Gleason score is obtained from the sum of the dominant grade and the grade of the second most important pattern. More recently, the Gleason score is modified for prostatic biopsy, where often the score is underestimated, and the new

recommendation for assigning the Gleason score in prostate biopsies is added up from the dominant grade and the worse grade (20,21,22).

Table 1. Simplified Gleason grading system

Grade	Description
1	Simple round glands, closely packed in rounded masses with well-defined edges.
2	Simple rounded glands, loosely packed in vague, rounded masses with loosely defined edges.
3A	Medium sized single glands of irregular shape and irregular spacing with ill-defined infiltrating edges.
3B	Very similar to 3A, but small to very small glands, which must not form significant chains or cords.
3C	Papillary and cribriform epithelium in smooth, rounded cylinders and masses; no necrosis.
4A	Small, medium, or large glands fused into cords, chains, or ragged, infiltrating masses.
4B	Very similar to 4A, but with many large clear cells, sometimes resembling "hypernephroma".
5A	Papillary and cribriform epithelium in smooth, rounded masses, more solid than 3C and with central necrosis.
5B	Anaplastic adenocarcinoma in ragged sheets.

Despite the high prevalence of prostate cancer in the Western countries including U.S. and the second leading cause of cancer mortality, relatively little is known regarding the molecular mechanisms involved in prostate tumorigenesis. The etiology of prostatic cancers remains poorly understood. It is believed that malignant transformation or tumor progression involves a multistep acquisition of genetic alterations involving activation of oncogenes and repression of tumor suppression genes. The progression of tumorigenesis represents a continuum of phenotypic and

genetic changes from a premalignant state to invasive carcinoma (2,5,14).

Premalignant lesions have been clearly defined for organs such as the colon, bladder, and cervix. The recognition and the establishment of specific features of such premalignant or precursor lesions have allowed better understanding of the natural history of their related cancer, and helped to identify prognostic factors that allow prediction on the outcome of individual patient with cancer. This concept may also apply to prostatic cancer, and lead to the search and recognition of precursor lesions which can be detected and which may permit application of prevention to the development of PCa. Recently, Prostatic Intraepithelial Neoplasia (PIN) have been widely recognized as potentially representing such precursors or premalignant lesions of prostatic adenocarcinoma, similar to the lesions of Cervical Intraepithelial Neoplasia (CIN) which are closely associated with carcinoma of the uterine cervix. Hence, there have been increasing recognition of PIN lesions, as well as increasing investigations in this area, as knowledge on PIN lesions may also help better understanding of risks factors in prostate cancer (23,24,25,26,27,32,33,28).

Chapter 2. Prostatic Intraepithelial Neoplasia

I. Introduction

The concept of Prostatic Intraepithelial Neoplasia (PIN) originated from the observation of an atypical epithelial proliferation characterized by cellular atypia of the epithelium lining preformed prostatic ducts and acini (23,24,25,26,27,28). It is widely recognized as a possible biological precursor of prostatic adenocarcinoma based on its close association with PCa, and thus possibly representing the premalignant end of the morphologic continuum of cellular proliferations within prostatic ducts and acini to carcinomatous proliferation. It has been recognized as having a high predictive value as a marker for adenocarcinoma, its identification in biopsy specimens warrants further search for concurrent invasive carcinoma (23,24,25,26,27,28,29,30,31). Despite of a close relationship in anatomical distribution and in prevalence between PIN lesions and prostatic adenocarcinoma, the supporting evidence establishing a definite link between PIN and carcinoma is still lacking. But it appears to be logical that investigations focus on the relationship between PIN lesions and PCa.

II. Definition, Characteristics and Grading

A. Definition

PIN is defined as an atypical epithelial proliferation characterized by cellular atypia of the epithelium lining preformed prostatic ducts and acini (23,24,25,26,27). Earliest description of premalignant change of prostate dated back to 1926 by Oerteil (32). Subsequently, many reports referred to this notion as

premalignant changes, and it was not until 1965 that the concept of prostatic intraepithelial neoplasia, then referred to as "intraductal dysplasia", was first described by McNeal, referring to these premalignant changes in prostatic epithelium seen in patients with PCa (33). He demonstrated microscopic foci of invasive cancer arising directly from dysplastic ducts and acini. The transition was described as typically abrupt, with a strand of invasive carcinoma penetrating the duct or acinar wall at a single point. The characterization of PIN lesions was reported only much later in 1986 by McNeal and Bostwick (24).

The term "prostatic intraepithelial neoplasia" or "PIN" was proposed in 1987 by Bostwick and Brawer, and was accepted by consensus at the 1989 Workshop on Prostatic Dysplasia (Bethesda, Maryland, March, 1989) to replace previous terms as "large acinar atypical hyperplasia", "atypical primary hyperplasia", "hyperplasia with malignant change", "marked atypia", "duct-acinar dysplasia", and "intraductal dysplasia" (24,28,29,30,31,34).

B. Histologic Characteristics

PIN lesions develop from pre-existing prostatic ducts and acini, and is characterized on one hand by a normal glandular architecture which is better appreciated at low power microscopy, and on the other hand, by atypical cytologic features which are better recognized at high magnification, and are reminiscent of those seen in carcinoma.

1. <u>At low magnification - the architecture of PIN lesions</u> :

PIN is readily identified at low magnification because glandular structures involved by PIN appear darker staining than the surrounding normal ducts and acini

due to the hyperchromatism associated with crowded enlarged nuclei. The architectural patterns of cellular proliferations in PIN vary from flat epithelium to tufting and a florid cribriform pattern. Four main architectural patterns of high-grade PIN have been described: tufting, micropapillary, cribriform, and flat (29,30,31,34,35) (Figures 3 to 6). The patterns often merge with each other. Clinical significance of these patterns is not known.

- The micropapillary pattern is characterized by numerous slender, finger-like structures with bulbous tips projecting into the lumens, occasionally accompanied by delicate fibrovascular cores (Figure 3).
- The tufting pattern is characterized by stratified mounds and heaps of cells protruding into the lumens, usually no more than 5 cell layers in thickness. These tufts are evenly spaced around most involved glands, imparting an undulating appearance to the epithelium due to the convex luminal profiles (Figure 4).
- The cribriform pattern represents Gleason grade C cribriform type of PCa and is characterized by complex intraluminal proliferation of cells punctuated by multiple lumens varying from rigid, neatly punched-out rounded spaces to variably sized, ovoid to slit-like spaces (Figures 4,5).
- The flat pattern can consist of one or two layers of cells lining glands without evidence of stratification, but can also be a flat proliferation of pseudostratified epithelium (Figure 6).

Other features such as arches (gently curving, "collapsible" epithelial structures), trabecular bars (curvaceous or rigid serpentine epithelial structures that traversed the glandular lumens and inserted into opposite or adjacent walls),

"Roman" bridges (rigid, "non-collapsible" curved epithelial structures with supporting pillars) and apparent disruption of the basal cell layer with glandular outpouchings into the stroma may be found in high-grade PIN.

2. At higher magnification - the cytologic features of PIN lesions :

The epithelium with PIN lesions is broadened due to proliferation and crowding of cells exhibiting the following salient cytologic abnormalities (Figure 7):

- nuclear crowding causing overlapping or nuclear stratification
- nuclear enlargement of varying degrees
- nuclear pleomorphism
- coarsening of chromatin causing hyperchromasia
- nucleolar prominence or enlargement.

These cytologic alterations are often associated and combined in varying degree of severity, and hence a grading system has been designed to differentiate the severity of these lesions (see below). PIN is usually multifocal and involves glands at many sites of the peripheral lobes. But occasionally PIN can be limited to a single gland either partially or completely. A variety of other cytologic features may be present in high-grade PIN including apical cytoplasmic luminal blebs suggestive of apocrine secretion ("apocrine snouts"), cytoplasmic pigment varied from scant fine brown or golden-brown particles to abundant coarse granules, optically clear cytoplasm, and microcalcifications within the epithelium. Proteinaceous eosinophilic secretions, corpora amylacea and crystalloids may be present in the prostatic lumen involved by high-grade PIN. Unusual histologic types of PIN lesions that are uncommonly seen include signet-ring cell type, small cell neuroendocrine cell type, and mucinous type (36).

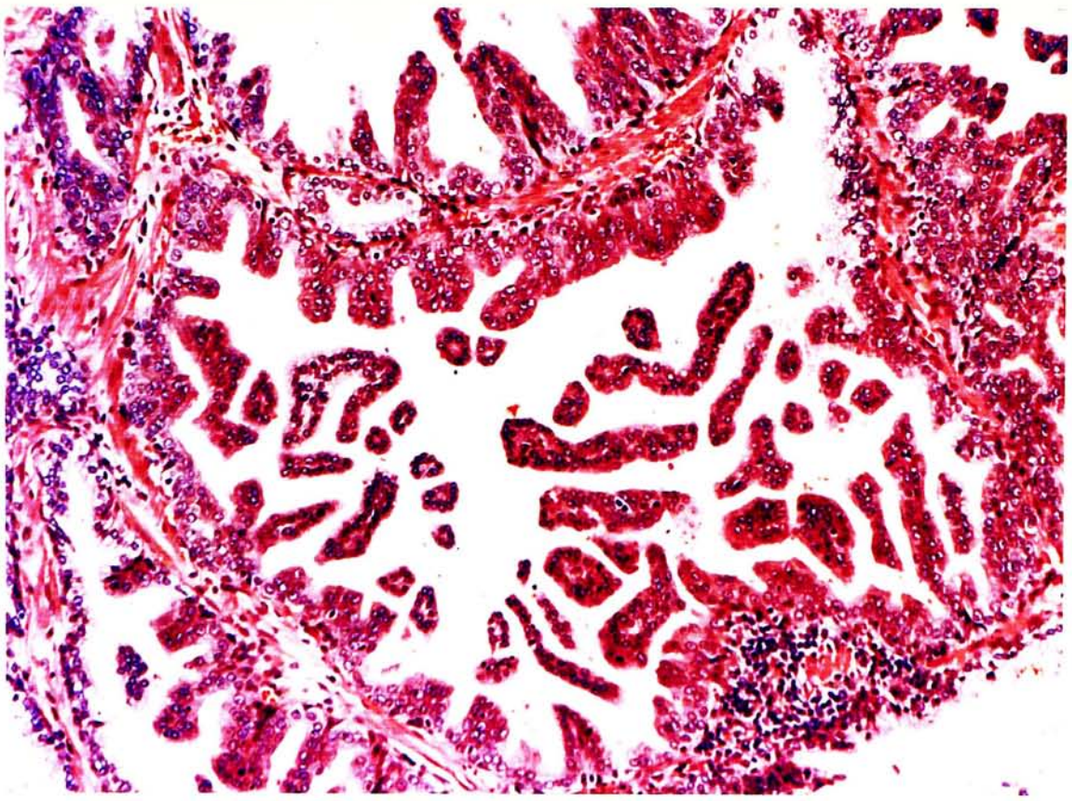


Figure 3. Micropapillary pattern of PIN showing slender finger-like projections, often with bulbous tips, with or without fibrovascular cores. (Hematoxylin-eosin stain, magnification x500)

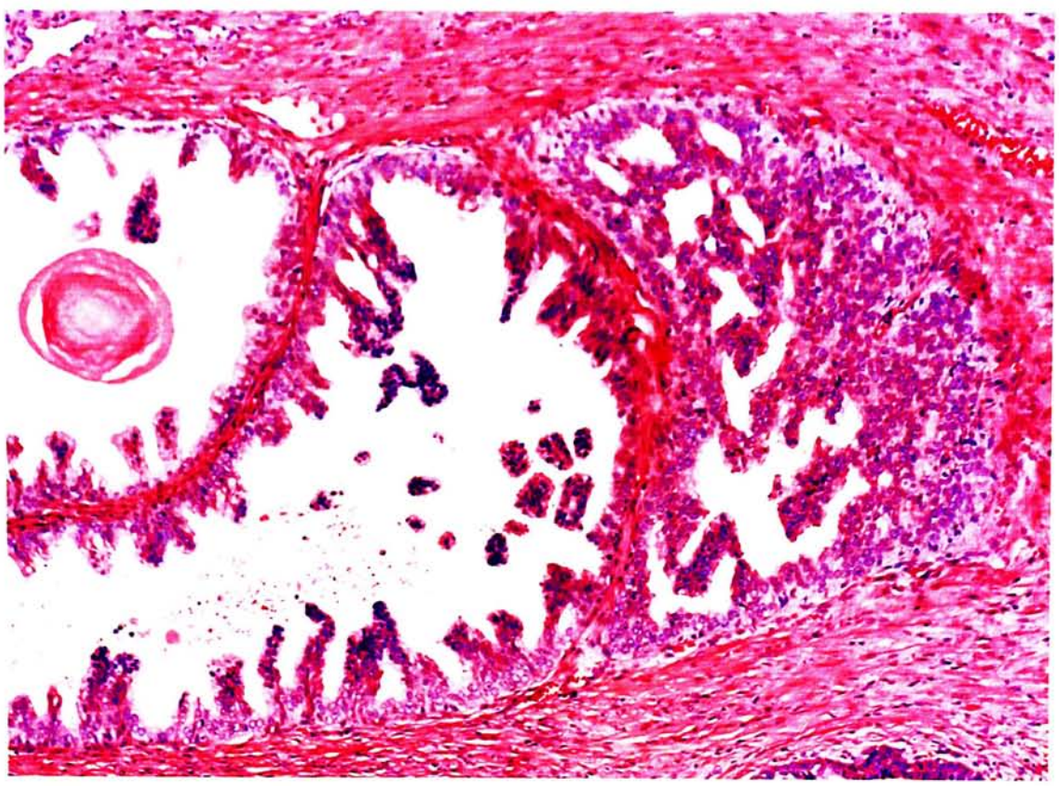


Figure 4. PIN 3 lesion showing mixed architectural pattern with cribriform pattern on the right acinus but tuft to micropapillary patterns in the center. (Hematoxylin-eosin stain, magnification x500)

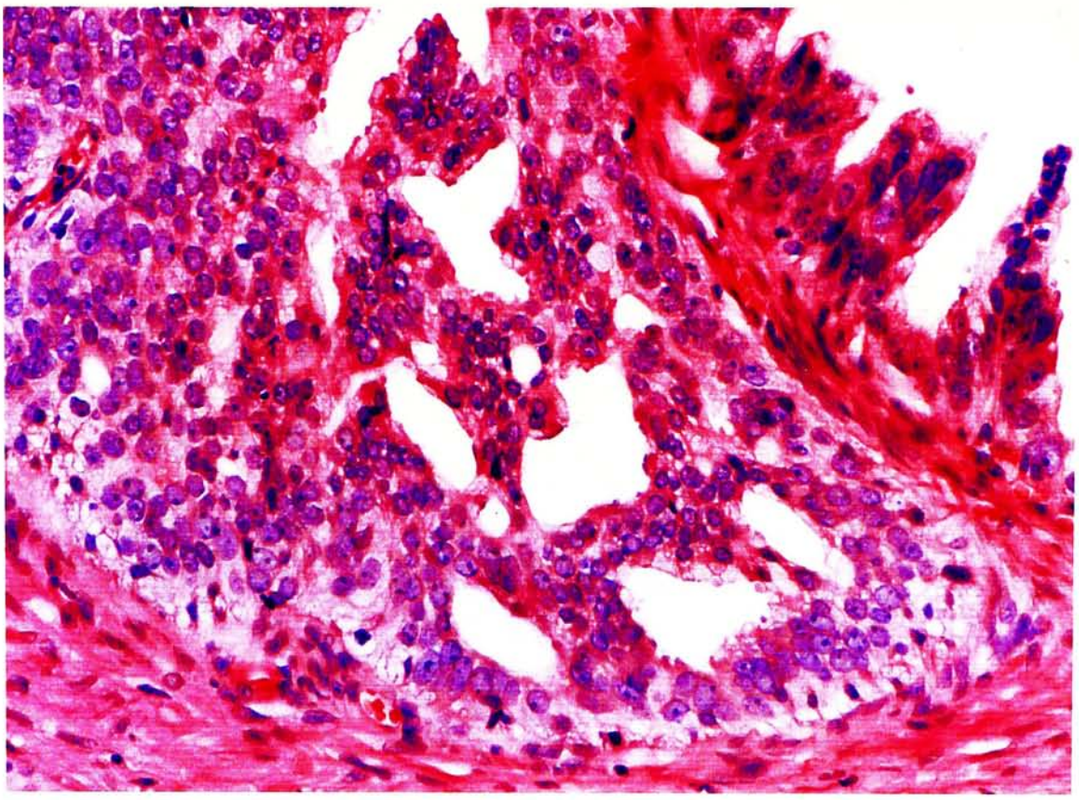


Figure 5. Cribriform pattern of PIN showing a sieve-like pattern with complex intraluminal proliferation of cells. (Hematoxylin-eosin stain, magnification x1280)

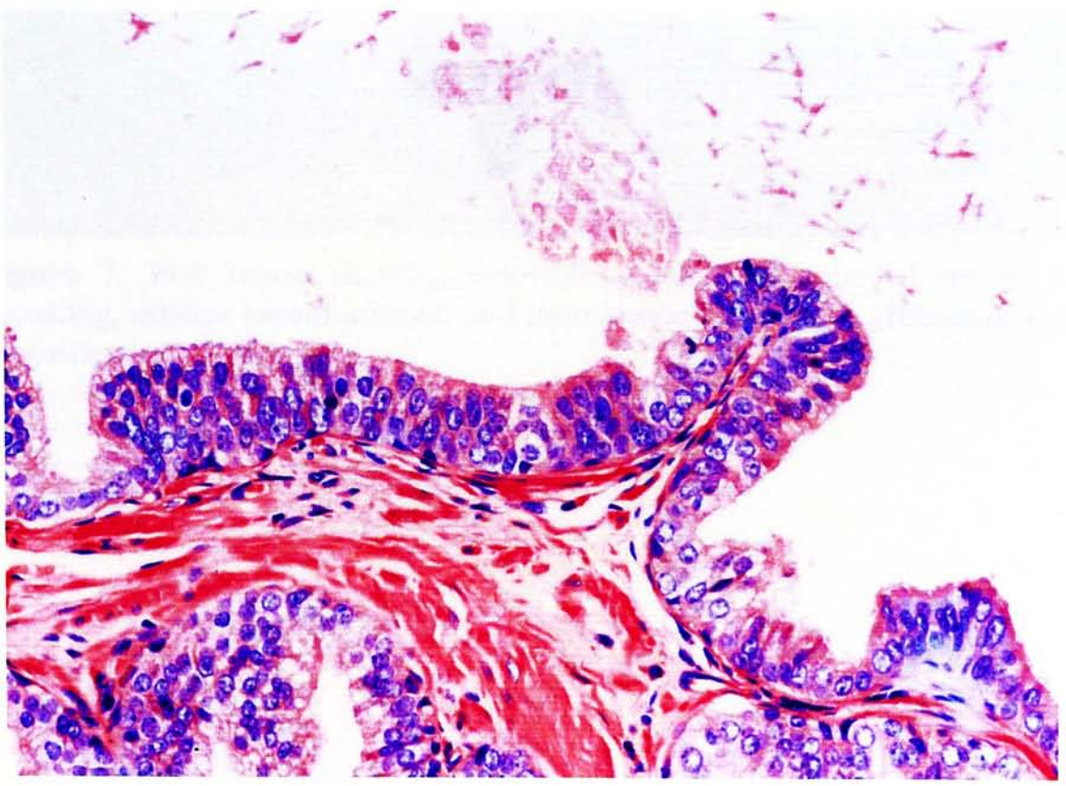


Figure 6. Flat pattern of PIN lesion with one crowding of dysplastic cells, exhibiting prominent nucleoli. (Hematoxylin-eosin stain, magnification x1000)

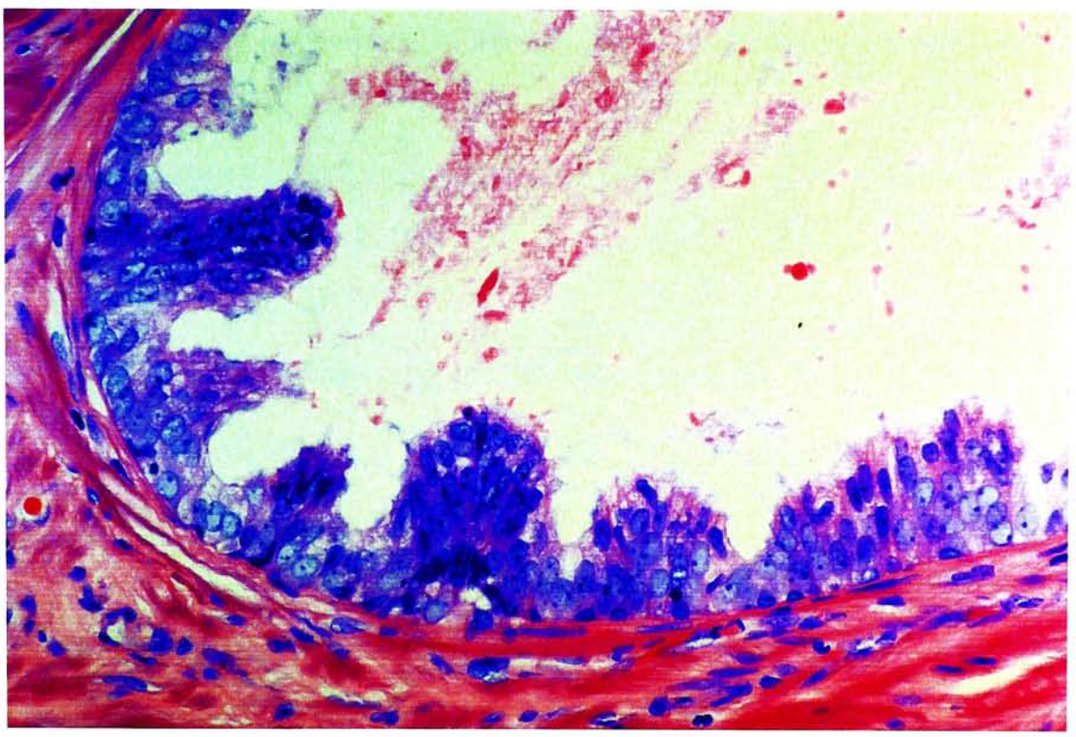


Figure 7. PIN lesion showing cytologic features of enlarged nuclei, nuclear crowding, nuclear pleomorphism, and prominence of nucleoli. (Hematoxylin-eosin, magnification x1280)

C. Grading

The grading of PIN is established at high magnification microscopy. Initially three different grades of PIN have been identified, and characterized as follows:

- PIN 1 - presence of variable nuclear enlargement, irregular cell spacing causing some nuclear stratification and generally inconspicuous nucleoli (Figure 8)
- PIN 2 - additional presence of nuclear hyperchromasia and many cells exhibiting nucleoli (Figure 9)
- PIN 3 - more extensive hyperchromasia and nearly all cell demonstrate prominent nucleoli (Figure 10)

At the 1989 Workshop on Prostatic Dysplasia, it was agreed to designate PIN 1 as low-grade PIN, and PIN 2 and PIN 3 were grouped together as high-grade PIN (29).

- Low-grade PIN - characterized by presence of variable nuclear enlargement and irregular cell spacing resulting in nuclear stratification and crowding within the epithelium. Elongated hyperchromatic nuclei and small nucleoli are also present, but are usually not prominent. The cytoplasm may stain more densely than normal.
- High-grade PIN - in addition to the above features seen in low-grade PIN, there are more pronounced nuclear stratification and nuclear crowding. The majority of nuclei are enlarged with less variability in nuclear size. The nuclei are hyperchromatic with presence of prominent nucleoli. In PIN 2 lesions, prominent nucleoli are observed only in some cells, whereas in PIN 3 lesions, almost all cells exhibit prominent nucleoli.

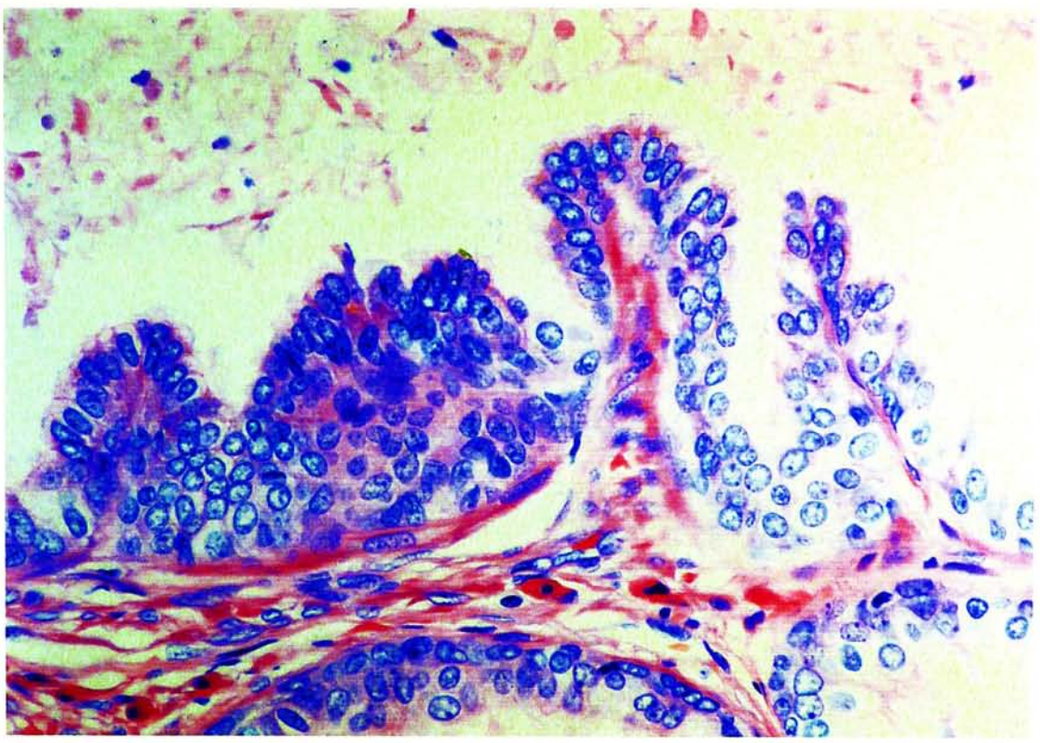


Figure 8. PIN 1 lesion characterized by enlarged, crowded nuclei showing anisokaryosis, inconspicuous nucleoli, and uneven spacing. (Hematoxylin-eosin stain, magnification x1280)

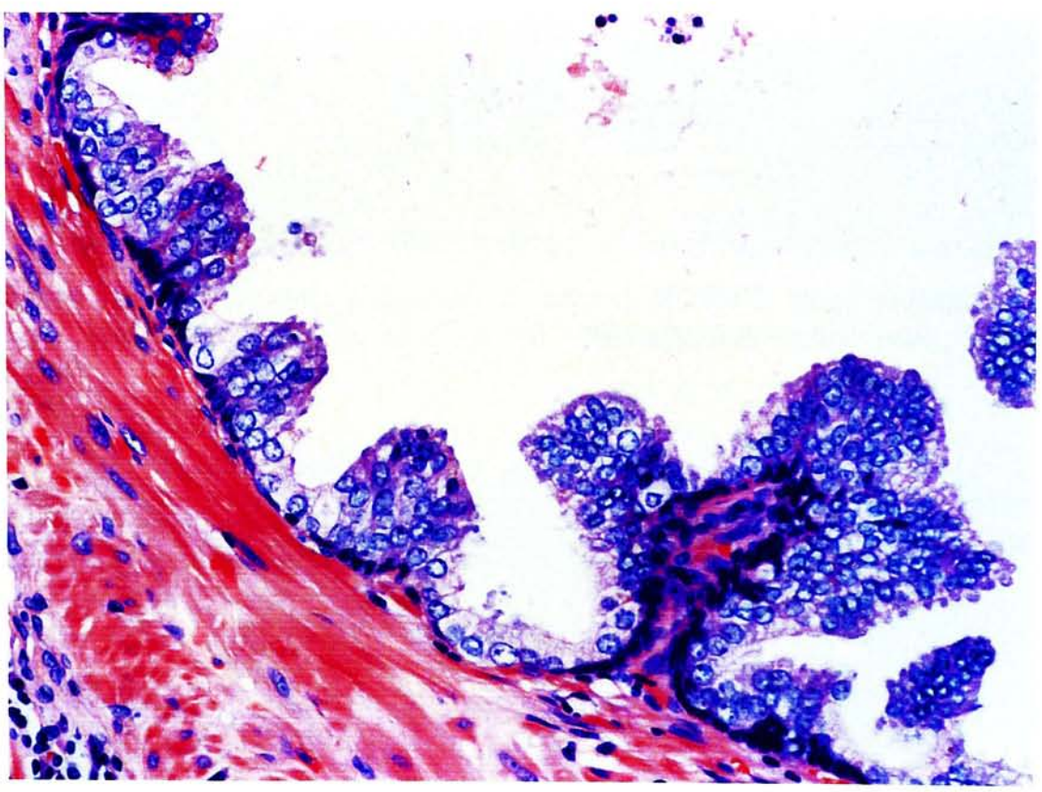


Figure 9. PIN 2 lesion. In addition to the features of PIN 1, there are hyperchromatism and a few scattered cells with prominent nucleoli. (Hematoxylin-eosin stain, magnification x1000)

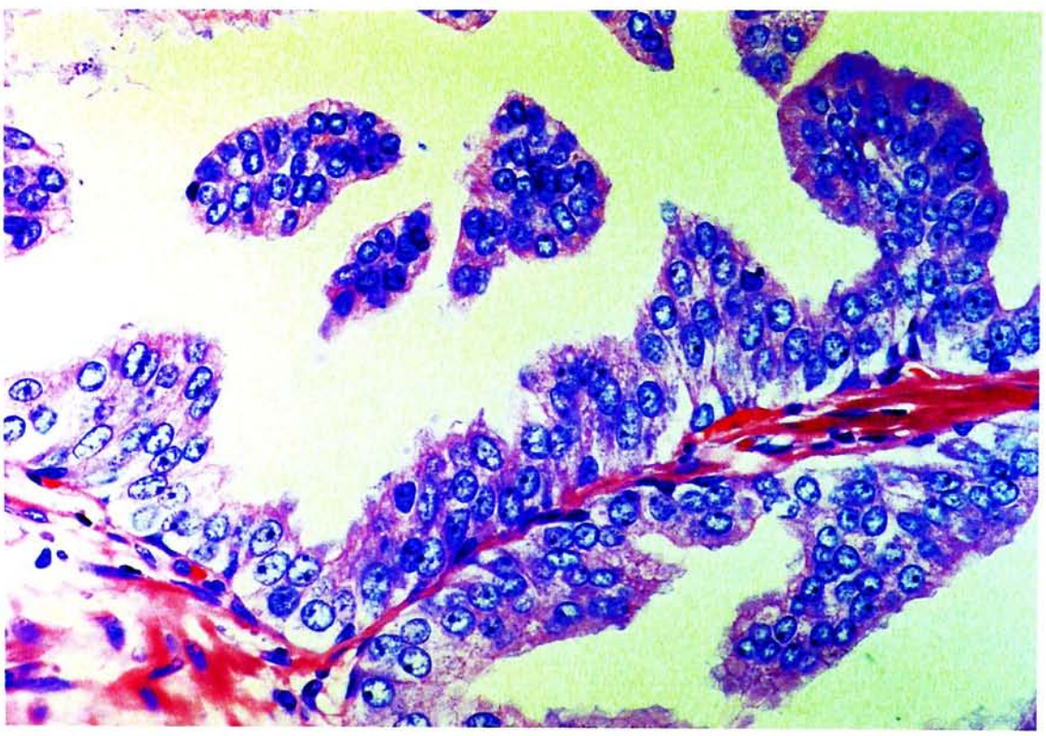


Figure 10. PIN 3 lesion. In addition to features of PIN 2, more widespread cells with large, prominent nucleoli are present. (Hematoxylin-eosin stain, magnification x1280)

III. Incidence and Prevalence of PIN

The incidence of PIN on needle biopsy varies from 7.6 to 31% in various studies depending on the population studied (37). In studies of whole prostate specimens, PIN lesions were present in most of consecutive cases of localized prostatic cancer and vary from 59 to 100%. PIN is more commonly found in the apical portion of the prostate gland and resides in the peripheral zone in most cases. PIN was also found in the central zone in 21% and in the transition zone in 18 to 31% (37). The incidence of PIN has been shown to increase with patient age in various studies. In an autopsy study with systematically step-sectioned whole prostates to study the incidence of PIN and invasive carcinoma in young men less than 50 years of age, Sakr *et al* observed presence of microscopic foci of ducts with low-grade PIN in the third decade of life with a frequency of 9% which increased to 20 and 44% in the fourth and fifth decades of life respectively. High-grade PIN occurred only in men older than 40 years and in conjunction with prostate cancer (38,39). Moreover, in the study of McNeal and Bostwick, it was noted that 45% of men aged 50 to 59 years and 52% of those aged 60 to 69 years had PIN in prostates without carcinoma (24). The frequency decreased to 37% in men aged 70 to 79 years and 38% in those aged 80 years or more. The similar trend was also noted by Kovi *et al* (40). This suggested that the peak incidence of PIN appears to antedate that of invasive carcinoma. Other studies similarly observed that the frequency of high-grade PIN was found to increase with age (39,41,44). In another series of studies of Troncoso *et al* , 70% of high-grade PIN with a mean age of 63.5 years was found associated with prostate cancer (23).

IV. Evidence Linking PIN with Prostatic Carcinoma

A. Frequency, Extent and Grade of PIN in Prostates With and Without Carcinoma

Several studies have established that the prevalence, extent and grade of PIN lesions in prostate glands harboring carcinoma are significantly higher than in prostates without invasive carcinoma, supporting the notion that PIN lesions may represent a precursor of carcinoma (23,24,30,41,42,43).(Table 2) McNeal and Bostwick compared the incidence of PIN in 100 prostates with cancer and 100 prostates without invasive carcinoma in an autopsy series and found that PIN was present in 82% of prostates with invasive carcinoma but in only 43% of prostates without cancer (24). Oyasu *et al* also reported a more than 2 fold higher incidence of PIN in prostates with invasive carcinoma (94% of 51 cases) as compared to those in prostates without invasive carcinoma (38% of 37 cases) (44).

In a study of PIN in steps-sectioning of prostates obtained at cystoprostatectomy for transitional cell carcinoma by Troncoso *et al*, the extent of PIN in prostates with carcinoma, as measured by number and size of separate foci of PIN, was significantly greater than in cases without carcinoma (23). While only 15% of the prostates without carcinoma contained more than 10 foci of PIN, 71% of the prostates with carcinoma had more than 10 foci and 28% had more than 20 foci of PIN. Moreover, large foci of PIN were also more frequent in prostates with carcinoma than those without carcinoma.

In the same study, Troncoso *et al* showed an association of higher grade of PIN with invasive carcinoma. Foci of PIN 3 were present in 72% of the prostates with carcinoma, but in only 18% of the prostates without carcinoma. Foci of PIN 1

were present in 7% and 14% of the prostates with and without carcinoma respectively. Moreover, 90% of cases with PIN 3 were found to be associated with carcinoma (23).

Table 2. Frequency of PIN (all grades) and PIN 3 in prostates with and without carcinoma observed in various studies.

Authors	PIN (all grades) (%)		PIN 3 (%)		Specimen Type
	Without PCa	With PCa	Without PCa	With PCa	
McNeal & Bostwick(24)	43	82	4	33	A
Oyasu *et al*(44)	38	94	8	82	A & RP
Kovi *et al* (30)	46	59	15	33	A & RP
Troncoso *et al* (23)	72	100	18	72	CP
Epstein *et al* (42)	-	100	-	100	RP (Stage A)
Quinn *et al* (43)	-	100	-	100	RP (Stage B)

Abbreviations: PCa - prostatic carcinoma; A - autopsy; RP - radical prostatectomy; CP - cystoprostatectomy for bladder carcinoma.

B. Zonal Distribution and Spatial Association of PIN with Carcinoma

Similar to PCa, PIN is multifocal and more commonly found in the peripheral zone of the prostate. Its incidence is much lower in the central and transition zones of the prostate (33). Troncoso *et al* noted that 81% and 86% of the foci of PIN occurred in the peripheral zone of prostates with and without invasive carcinoma respectively (23). (Table 3) Occurrence of foci of PIN in transition and

central zone of prostate was rare. In the same study, it was noted that PIN is also spatially closely associated with carcinoma. PIN was found immediately adjacent to or within the same low power field as carcinoma in 62% of the cases. A similar study by Bostwick and Brawer noted that in 1093 ducts and acini with PIN, 41% (453 acini) were found within 1 high-power microscopic field of invasive carcinoma in a radical prostatectomy series (28). Moreover, 87% of the 453 acini were involved by PIN 3 indicating a relation of grade of PIN with proximity to carcinoma. This close association of PIN and carcinoma was first described by McNeal in 1965 who noted in some cases a transition from ducts lined by highly atypical epithelium to invasive carcinoma (33). This supports the idea that invasive carcinoma may arise from PIN by budding off of small glandular structures at the sites of basement membrane and basal cell disruption.

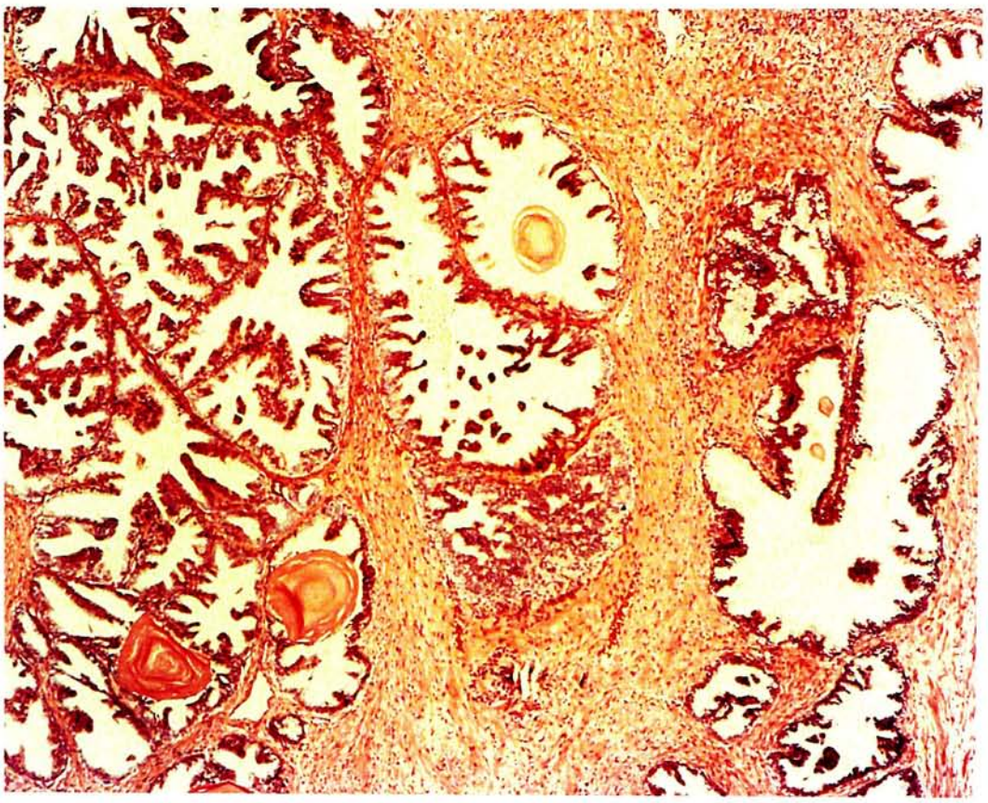
Table 3. Zonal distribution of PIN in prostates with and without invasive carcinomas (Troncoso *et al*)(23)

Zone of prostate	Foci of PIN in prostate with carcinoma (%)		Foci of PIN in prostates without carcinoma (%)	
Peripheral	147/182	(81)	916/1068	(86)
Transition	8/182	(4)	7/1068	(1)
Central	6/182	(3)	142/1068	(13)
Uncertain	21/182	(12)	3/1068	(<1)

C. Basal Cell Layer Disruption

The basal cells of the prostatic glands express high molecular weight cytokeratins which are not expressed in the luminal cells (18,28). The basal cells of the prostatic glands can be selectively labeled with monoclonal antibodies directed against these cytokeratins such as clone 34βE12. The basal cell layer is present in benign epithelial proliferations as a continuous circumferential layer but absent in carcinoma. However, the basal cell layer is disrupted in PIN. (Figures 11 and 12). Bostwick and Brawer have shown that the frequency and extent of disruption of the basal cell layer is related to the grades of PIN and is greatest in PIN 3 adjacent to foci of carcinoma (18,28). Disruption was present in 56% of cases with high-grade PIN and was more common in acini adjacent to invasive carcinoma than in distant acini. More than 30% of the cell layer was disrupted in 52% of the high-grade PIN. On the contrary, less than 1% of PIN 1 was associated with disruption of the basal cell layer. These disruptions of the basal cell layer have been interpreted by some to represent the earliest stage of invasion or progression of PIN into invasive carcinoma. In their study, foci of basal cell disruption were associated with small glandular outpouchings resembling early invasion of carcinoma or progression of PIN into invasive carcinoma (18,28). McNeal *et al* have also shown that areas of PIN apparently giving rise to invasive carcinoma (so-called "transitive glands") demonstrate fewer and more widely-spaced basal cells than adjacent PIN (34).

A.



B.

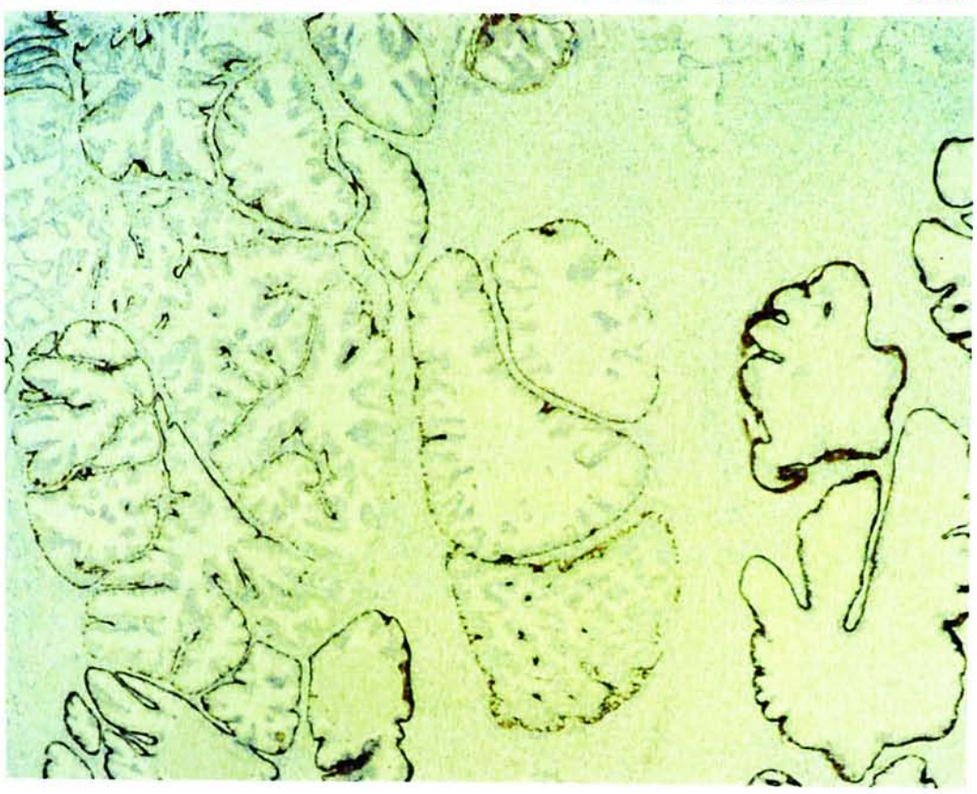


Figure 11. (**A**) PIN lesion. Epithelium linings involved by PIN are more hyperchromatic and heaped up at low magnification compared to surrounding normal glands at the top of the photomicrograph. (Hematoxylin-eosin stain, magnification x200). (**B**) Immunostaining for high molecular weight cytokeratin, clone 34βE12 showing discontinuous basal cell layer of PIN foci in contrast with normal glands at both sides of the figure. (Immunoperoxidase method, magnification x200)

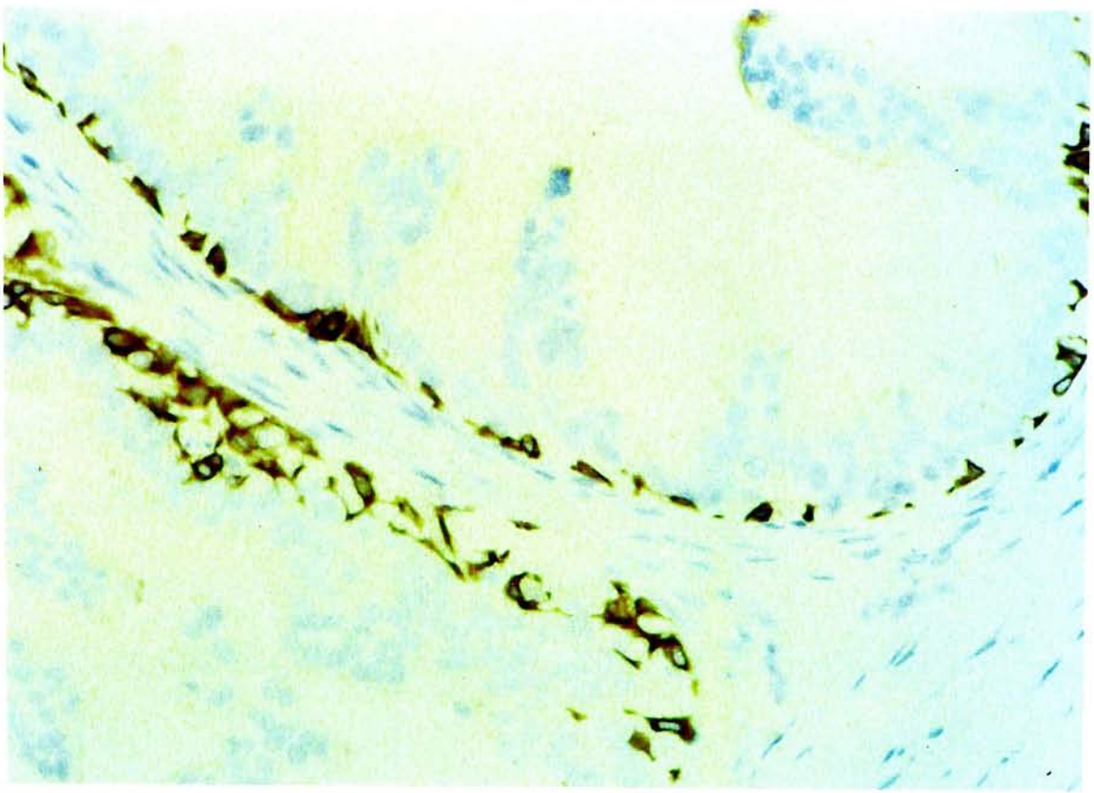


Figure 12. Higher magnification of immunostaining for high molecular weight cytokeratin in PIN lesions, demonstrating discontinuous basal cell layer. (Immunoperoxidase method, magnification x1000)

D. Morphometric Features, Proliferative Activity and DNA Content

Morphometric studies by digital image analysis of Feulgen-stained tissue sections and cytospin preparations showed progressive abnormalities of nuclei and nucleoli in the progression of low-grade PIN to high-grade PIN to invasive carcinoma. There is progressive increase in nuclear size, decrease in nuclear roundness, and increase in size and number of nucleoli and eccentrically located nucleoli (45,46,47). Studies evaluating the number of argyrophilic nucleolar organizer regions (AgNOR's) indicated that PIN has similar proliferative activity as PCa. The AgNOR count for PIN is significantly higher than benign prostatic proliferations but is similar to that of invasive carcinoma (48,49). Increasing grade of PIN have been correlated with increasing DNA proliferative activity and frequency of aneuploidy (50). The proportion of aneuploid nuclei increases from 20% in PIN 1 to approximately 50% in high-grade PIN. Moreover, aneuploid PIN tends to occur adjacent to aneuploid cancer, although diploid PIN has been found adjacent to aneuploid and diploid cancer. Aneuploid PIN next to diploid carcinoma was not observed (51).

E. Immunophenotypic Profile

Various immunohistochemical and histochemical studies showed that PIN is associated with abnormalities of phenotype and genotypic expressions, which are intermediate between normal prostatic epithelium and invasive carcinoma or similar with invasive carcinoma (Table 4). These results indicate impairment of cell differentiation and regulatory control with advancing stages of prostatic carcinogenesis. Nagle *et al* showed progressive changes of cytoskeletal proteins

similar in PIN and PCa (52). They showed that both PIN and invasive carcinoma exhibit significantly increased reactivity with a monoclonal antibody KA4 directed against cytokeratins 14, 15, 16, and 19. About 90% of cases of PIN and invasive carcinoma are immunoreactive with this antibody in contrast with only 4% of cases of prostatic hyperplasia. Vimentin expression was also shown to decrease progressively from expression in prostatic hyperplasia to reduced expression in PIN to absent in invasive carcinoma. Certain secretory proteins including PSA, PAP and Leu-7 which are secreted by the prostatic epithelium are reduced in PIN suggesting loss of biochemical differentiation early during preinvasive phase of prostate cancer (31). However, two enzymes pepsinogen II (PG-II) and tissue plasminogen activator (t-PA) which are normally identified in luminal cells of the benign glands of the central zone were detected in PIN in the peripheral zone. These findings showed presence of deregulation and impaired differentiation in PIN. Neuroendocrine differentiation was also shown to be impaired and progressively decrease in normal epithelium, high-grade PIN, and carcinoma by Bostwick *et al* (53).

Lectins bind with specific terminal sugars, some internal sugars and particular sugar linkages and to some oligomers in complex sugars present in membrane-bound and cytoplasmic glycoproteins (54). Variations in lectin binding are related to degrees of cell maturation and differentiation which may be altered in malignant transformation. In a study of lectin binding in prostatic tissue, McNeal *et al* reported decreased binding of Ulex europaeus (UEA-1) and seven other lectins in foci of dysplasia as compared to normal prostatic epithelium (55). In contrast, binding of Ulex europaeus lectin (UEA-1) was reported by Nagle *et al* to be increased in both PIN and invasive carcinoma with little or no reactivity in

hyperplastic epithelium (52). A recent study of Drachenberg and Papadimitriou also found increased lectin binding with four of nine lectins investigated, including UEA-1, in all grades of PIN and carcinoma suggesting similar aberrant expression of glycoprotein components in both PIN and invasive carcinoma (56). Aberrant expression of the ABH blood group antigens as well as the Lewis (Le) antigens commonly occur in many malignancies. The A, B, Le^a, Le^b and X antigens were found to be reduced in PIN and invasive carcinoma but present in 5 to 50% of normal cells by a study of Perlman and Epstein (57). Proliferating cell nuclear antigen, a cell-cycle proliferation marker, has been demonstrated to be increased in PIN and invasive carcinoma (58).

Other markers have been reported to show progressive increase in PIN and carcinoma, including c-erbB-2 oncoproteins (59), bcl-2 oncoprotein (60), epidermal growth factor and epidermal growth factor receptor (61), type IV collagenase (62), Lewis Y antigen (57), transforming growth factor-alpha, and apoptotic bodies (63).

F. Chromosomal and Molecular Genetic Alterations

Chromosome mapping studies by fluorescence in situ hybridization and polymerase chain reaction techniques showed allelic losses involving chromosomes 8, 10 and 16 in prostatic cancer (64,65). The short arm of chromosome 8 (8p), and the long arms of chromosomes 10 (10q) and 16 (16q) appear to be particularly affected. The 8p and 16q losses frequently occur also in early carcinomas, whereas the 10q loss appears to be a later event. Frequent 8p allelic loss has been reported in low-grade localized and in high-grade invasive tumors, as well as in PIN which suggests that 8p losses are early events in prostate tumorigenesis (37,66).

Table 4. List of various studies on immunophenotypic changes in PIN lesions and invasive carcinoma (37)

Authors	Biologic Markers	PIN	PCa
Montironi *et al* (58)	PCNA	Increase	Decrease
Nagle *et al* (52)	Cytokeratins 14, 15, 16 and 19	Increase	Increase
Perlman and Epstein (57)	Ulex	Increase	Increase
Martenson *et al* (67)	Lewis X	Increase	Increase
Bostwick and Brawer (28)	Basal cell specific cytokeratin	Decrease	Decrease
McNeal (55), Drachenberg and Papadimitriou (56)	Lectins	Decrease	Decrease
McNeal *et al* (31), Maygarden (68)	PSA	Decrease	Decrease
Nagle *et al* (52)	Vimentin	Decrease	Decrease
Hughes *et al* (69), Myers *et al* (70), Humphrey and Swanson (71)	p53	Increase	Increase
Cooney *et al* (72)	Rb	?	Increase
McDonnell *et al* (73), Stattin *et al* (74)	bcl-2	Increase	Increase
Myers *et al* (59), Bostwick (75), Ware (76)	c-erB-2	Increase	Increase
Kuczyk *et al* (77)	Cathepsin D	Increase	Increase
Iwamura *et al* (78,79)	PTHLP	Increase	Increase
Bostwick *et al* (53)	Neuroendocrine	Decrease	Decrease

Abbreviations: PCa - prostate carcinoma.

V. Conclusion

Despite the observation of anatomical, morphological, immuno-histochemical, and genetic features suggesting and attributing a malignant potential of PIN lesions, and despite the strong association of PIN lesions with PCa, the role of PIN in prostatic tumorigenesis, and the prove that PIN is the biological precursor of PCa remain to be demonstrated. The clinical observations of the progression of dysplastic lesions to development of invasive are lacking, and the current imaging modalities have not been capable to do so. It is difficult to assess the malignant potential of a histologic abnormal lesion in a solid organ, such as the prostate, due to impossibility to follow clinically the evolution of such a lesion found at biopsy. The original biopsy site cannot be located subsequently with an acceptable degree of precision, and if carcinoma develops later, the tumor is usually too large by the time of detection to allow determination of its exact point of origin.

Clinical observations also indicate that PIN lesions and PCa can be dissociated, in that PIN lesions can occur without carcinoma, and prostate with carcinoma may demonstrate complete absence of PIN lesions. In the study by Sakr *et al* of incidence of PIN in 249 younger male, they noted that the onset of high-grade PIN occurred later than the onset of carcinoma (39). While this observation does not exclude PIN as a precursor lesion to PCa, it also suggests that PIN lesions may not represent a necessary step in the development of cancer. Moreover, 70% of the prostates with early carcinomas lacked any high-grade PIN within the entirely embedded prostate gland (39). In those cases where both early cancer and high-grade PIN were present, only one-third of them clearly demonstrated the proximity of PIN lesions with carcinoma. It has also been argued that PIN lesion may be related to a

field effect, or to the intraepithelial spread of an adjacent carcinoma. It is possible that in cases where both PIN lesions and PCa are present, the two conditions may share some common steps in the process of oncogenesis. On the other hand, it may also be speculated that, while PIN lesions and carcinoma coexist, they have different pathways in proliferation and growth. Clearly, many questions on the relationship between PIN and carcinoma remain unanswered, and further studies of the development, and the biological behavior of PIN are therefore required to evaluate its significance in the progression to prostatic adenocarcinoma.

Chapter 3. Histologic Biomarkers

Although severely dysplastic cells of PIN lesions are indistinguishable from the malignant cells seen in PCa, such cytological similarities appear insufficient to attribute a role to PIN lesions. A comparison of the phenotypic and genotypic characteristics of PIN and PCa is useful in understanding the relationship of this lesion to invasive adenocarcinoma. Studies of potential biomarkers such as growth factors and their receptors, oncogene products, and other biomarkers in PIN lesions are difficult due to focal occurrence of these lesions. Other techniques involving microdissection of focal PIN lesions from tissue or tissue sections are tedious and have the disadvantage of contamination by surrounding stroma and histologically normal epithelium. Immunohistochemical techniques may be more suitable and are relatively simple to perform and can allow differentiation of biomarker expressions among the various components of the dysplastic gland or ducts (80). Expression of various biologic markers among PIN, benign epithelium and PCa were examined in this study by immunohistochemical techniques.

I. p53 Protein

Cancers are considered to result from aberrations in cellular proliferation and differentiation brought about by an accumulation of genetic alterations in or on the cells with activation of oncogenes or inactivation of tumor suppressor genes. Tumor suppressor genes are now known to play an important role in the development of a wide variety of human cancers. Various tumor suppressor genes including the retinoblastoma susceptibility gene, the Wilms' tumor (WT1) gene, the

mutated in colorectal carcinoma (MCC) gene, the adenomatous polyposis coli (APC) gene, the neurofibromatosis type I (NF1) gene, and the p53 gene have been identified (81,82,83).

Tumor suppressor genes are mainly involved in the regulation of cell growth and differentiation. The p53 gene is a well known tumor suppressor gene and its tumor suppressor function of p53 gene has been intensively studied. Alterations in the structure of p53 gene represent one of the most common genetic changes associated with human cancer, and experimental and clinical evidence have implicated p53 in the development of a wide range of malignancies. Over 2,000 mutations in the DNA encoding the p53 gene have been analyzed in human neoplasms. Mutation of p53 gene, as expected for a tumor suppressor gene, may induce either an increased proliferative activity or appearance of a new function which is usually highly oncogenic (81).

Mutations of p53 gene have been identified in colon and breast adenocarcinomas, small cell and non-small cell carcinomas of the lung, and brain tumors. The p53 immunohistochemistry is frequently employed to investigate the role of p53 gene in the tumorigenesis according to the currently accepted concepts that p53 gene mutations lead to accumulation of highly stabilized p53 mutant proteins which can be detected using monoclonal antibody immunohistochemical technique (5).

A. p53 Protein Structure

p53 protein was first identified in the late 1970s as a nuclear phosphoprotein which co-immunoprecipitated with the simian virus 40 large tumor antigen (SV40 T-

Ag) (82). The p53 protein is encoded by a gene on the short arm of human chromosome 17 (17p13 position) encoding for 11 exons. It contains 393 amino acids with a molecular weight of 53 kilodaltons (kDa) (81).

Three distinctive regions can be identified in the wild-type p53 protein. The N-terminal is acidic and manifests activity of a transcriptional transactivation domain when fused to other DNA binding domains. It encompasses the binding sites of the adenovirus E1B protein, the host MDM-2 protein, and the binding site for the TATA box binding basic transcription factor TBP. The C-terminal is more basic containing motifs involved in the regulation of DNA binding activity and oligomerization. The central region is conformationally flexible and contains the binding site for SV40 large T-antigen and sequence specific dsDNA (81).

There are multiple phosphorylation sites located at both the amino- and carboxy-termini of the protein molecule. It can be phosphorylated at different amino residues by various protein kinases which may be accounted for different immunologic phenotypes of p53 found in human tumor cells. The sequence of amino acids from residues 234 to 258 is highly susceptible to cancer-related mutations (81).

B. Biological/Physiological Function of p53 Protein

Wild-type p53 is normally expressed at low level in all normal mammalian cells and has a short half-life of 20-30 minutes (83). It has an essential role in cell growth or differentiation and is implicated in nearly all forms of cell growth stimulation and inhibition (84). Previous studies indicated that p53 may be required early in the induction of cell proliferation. p53 is a transcriptional regulatory protein, capable of both stimulating and repressing gene expression. It can bind directly with

DNA in a sequence-specific manner and also influences gene expression indirectly by interaction with other transcription factors (81,82).

A number of biological functions have been attributed to p53 protein. An important function of p53 protein is to regulate cell proliferation following DNA damage, by mediating G1 arrest or apoptosis. p53 expression is induced in cells exposed to DNA-damaging agents. As a transcriptional activator, the increased p53 expression activates the expression of a number of genes in response to the DNA damage. Among the gene expressions activated by p53 protein are the p21WAF1/CIP1, GADD45 and cyclin G genes. The p21 gene encodes for a 21 kDa. protein which is an inhibitor of the cyclin-dependent kinases. Protein product of p21 gene may regulates the transition of cell-cycle from G1 to S phase by blocking the kinase activity of cyclin/cyclin dependent kinases (Cdk) complexes which, in turn, prevent the phosphorylation of the retinoblastoma gene product (Rb). p21 also participate in DNA repair by binding with proliferating cell nuclear antigen (PCNA) which is a normal component of Cdk complexes and a regulatory subunit of DNA polymerase (84). The GADD45 protein binds with PCNA and stimulates DNA excision repair. It also inhibits entry of cells into S-phase of cell-cycle. Cyclin G may influence cell-cycle progression by modulating the activity of cyclin-dependent kinases.

p53 also plays a direct role in excision repair. It interacts with proteins implicated in excision repair, including replication factor A (RPA), and three components of the EFIIH basal transcription factor, XPB/ERCC3, XPD, and CSB (84). In cell types that undergo terminal differentiation, overexpression of p53 induces programmed cell death. Under the influence of certain cytokines, p53 has

also been associated with the induction of differentiation (81).

p53 can bind nonspecifically to regions of single-stranded DNA in regions of DNA repair or replication forks and favors in annealing of base-paired regions. The p53 protein is inactivated by binding with mdm-2 protein which is transactivated by p53 expression forming a negative feedback control of p53 activities (81).

C. p53 Protein and Neoplastic Transformation

Alterations in the structure of p53 gene are one of the most common genetic changes in human malignancies (85). Both overexpression and loss of p53 function can contribute to oncogenic transformation. Most of the p53 mutations are missense point mutations clustered in highly conserved domains of the gene spanned by four to nine exons. Several sites with frequent mutations have been described as 'hot spot' mutations and vary among cancers with different tissue types. For example, the squamous cell carcinomas of head and neck have a hot spot region at codons 238-248. Mutation at codon 175 is common in colon carcinoma but is unusual in carcinoma of the lung (81,85).

Mutant p53 proteins have poor DNA binding activity and lack function to transactivate p53 responsive genes. Most of the missense mutations of p53 are found in the central conformational domain of p53 which binds DNA directly. Mutations in the central domain abolish or weaken the DNA-binding and transactivation properties of p53. Mutant p53 proteins may also exert a "dominant negative" effect over wild-type alleles by forming oligomeric complexes with wild-type p53 protein, inhibiting the normal activity of p53 protein. p53 function may also be disrupted by

DNA tumor viruses that encode p53 binding proteins which either interfere with p53 transcriptional activity (SV40 T-Ag, adenovirus E1B 55 kDa tumor antigen) or target p53 for proteolytic degradation (human papillomavirus E6). Overexpression or amplification of mdm-2 gene is found in a number of solid tissue tumors that binds to p53 and regulates its function (81).

The loss of regulatory effect of p53 in response to DNA damage increases genomic instability and promotes gene amplification. This releases the defective cell from G1-S cell cycle control as well as p53 dependent apoptosis following DNA damage. The cell may proliferate to produce clones that are more susceptible to further genetic damage which may result in neoplastic transformation.

Mutant p53 frequently have a much longer half-life than wild-type p53 protein. It can be accumulated in the cell to high levels which can be detected by immunohistochemical methods. Immunoreactivity of anti-p53 antibodies is thus considered to be indirect evidence of p53 gene mutation (86). The increased stability of mutant p53 protein has been attributed to altered conformation which aid in stabilizing the p53 protein. Antibodies that recognize different conformational states of wild-type and mutant p53 protein products have been produced (81). Monoclonal antibody clone DO-7 which recognizes wild-type and mutant p53 proteins was used to detect accumulation of p53 protein in both benign and malignant prostatic lesions in this study.

II. Proliferating Cell Nuclear Antigen (PCNA)

It has been suggested that the metastatic potential of a tumor relies upon the random acquisition of genetic information (87,88). Higher rates of cell turnover

increase the likelihood of acquisition of such information (89). Proliferation rate may therefore be a determinant of a tumor's metastatic capability (90). Proliferation activity has been widely used as an important and independent prognostic factor in certain human cancers (91,92). Various methods have been used to determine the cellular proliferation rate including mitotic count, flow cytometry, and tritiated thymidine or bromodeoxyuridine incorporation. However, such methods have their limitations. Consistency of mitotic count is affected by variations in counting methods, technical factors, and dependence on objective identification of mitotic figures. Use of mitotic count may have problem when applied to prostate cancers which have an extremely low mitotic rates (93). Flow cytometry requires expensive equipment and the results may be affected by presence of cellular debris and non-neoplastic cells yielding imprecise counting (94). Tritiated thymidine or bromo-deoxyuridine incorporation techniques involves handling of radioactive isotopes, and can only be used with fresh specimens. Immunohistochemical assessment of cell proliferation offers the advantages of well preservation of cellular and tissue architecture during assessment and can be applied to retrospective studies with routine formalin-fixed, paraffin-embedded specimens. The technique is relatively simple and rapid. No radioactive isotopes are involved (95). Evaluations of PCNA and Ki-67 antigen immunostaining have been widely used to measure proliferative activities of various tumors.

Proliferating Cell Nuclear Antigen (PCNA) is an auxiliary protein of DNA polymerase δ and is essential for DNA synthesis (96). It is found to increase during G_1 phase of the cell-cycle and is present in highest concentrations during S. Its level decreases during G_2 phase and is lowest during mitosis (97). It was originally

detected with autoimmune serum from patients with systemic lupus erythematosus, which was found to contain an antibody against a nuclear antigen present in proliferating cells. Since then, a number of commercially available monoclonal antibodies to PCNA have been produced, including 19A2, 19F4 and PC10 (96). PCNA expression detected with these antibodies has been used as an index of cell proliferation in various studies. It has been suggested as an independent prognostic indicator in lymph node positive breast cancer (95).

A. Biochemical Characteristics and Function

PCNA is a 36 kDa non-histone acidic nuclear protein which is an auxiliary protein for DNA polymerase δ and is required for DNA synthesis or replication (96). Immunofluorescence studies showed that there are two populations of PCNA existing during S phase of cell-cycle. One of them is nucleoplasmic as in quiescent cells and is easily extracted by detergent. The other is associated to specific nuclear structures and resistant to detergent extraction. Immunoreactivity of the detergent resistant PCNA is co-localized with bromodeoxyuridine (incorporated into DNA during S phase) in replication complexes, and their order of appearance throughout the S phase are identical. This shows that PCNA is tightly associated to the sites of DNA replication and probably has a role in DNA synthesis. Moreover, use of antisense oligonucleotides which lower the levels of PCNA are associated with inhibition of DNA synthesis. In the presence of PCNA and a multi-subunit complex called replication factor C, the enzyme polymerase δ initiates leading-strand DNA synthesis by catalyzing elongation of Okazaki fragments to long DNA chains (91,94,96).

PCNA is also involved in DNA nucleotide excision-repair. PCNA can be found associated with chromatin at all phases of the cell cycle after ultraviolet irradiation *in vitro.* It is also expressed in non-cycling normal human keratinocytes *in vivo* after mild UV exposure (96).

B. Regulation of PCNA Expression

PCNA expression is regulated at both the transcriptional and post-transcriptional levels. PCNA mRNA has been well documented to be induced by growth factors. However, the regulation in quiescent cells and those which are continuously cycling is different. In the continuously cycling fraction of cells, there is very little variation in protein or mRNA levels during the cycle. The contribution of transcriptional control is not clearly understood. But homeodomain-containing proteins bind to the PCNA promoter which probably modulate gene expression as it was found the homeodomain proteins regulate gradients of gene expression in other genes (91,94,96).

Post-transcriptional mechanisms of regulation appear to be more important by *in vivo* studies. It has been observed that PCNA mRNA is stabilized in the presence of growth factors. It was therefore suggested that many cells continuously produce PCNA mRNA (at least at low levels) but rapidly degraded without translation. Consequently, in response to growth factors, cells may induce PCNA production by stabilizing PCNA mRNA. The mechanism is intriguing and involves alteration in mRNA stability as a consequence of splicing of intron 4 (96). Oncogenes may also participate in the regulation of PCNA mRNA levels by causing deregulation of PCNA expression (96).

III. Ki-67 Antigen

The Ki-67 murine monoclonal antibody was originally raised by Gerdes *et al* against a crude nuclear fraction of the Hodgkin's disease derived cell line L428 (98). The Ki-67 clone recognizes a labile epitope on a nuclear antigen which was expressed in cycling cells but absent from quiescent cells. It was named after its place of production in Kiel, West Germany and because the clone producing the antibody was grown in the sixty-seventh well of the tissue culture plate.

The Ki-67 antibody recognizes a nuclear antigen of 395 kDa encoded by a gene located on chromosome 10 at position 10q25 (99). Cell cycle analysis showed that the Ki-67 antigen is expressed in dividing cells in all stages of the cell cycle except G_o with expression appearing in mid to late G_1, rising through S phase and G_2 to reach a maximum in mitosis (91,100).

Study of topographical distribution of Ki-67 antigen by Braun *et al* showed that distribution of Ki-67 antigen in the nucleus is cell-cycle dependent and the staining patterns varied with the cell cycle (101). In the late G_1 phase, the Ki-67 antigen was present within the perinucleolar region. In S phase the antigen was also homogeneously stained in the karyoplasm. In G_2 phase, the staining within the karyoplasm existed as a mixed, finely granular and speckled pattern with perinucleolar staining still present. There was an intense perichromosomal Ki-67 staining pattern during pro- and metaphase, in addition to karyoplasmic staining in prophase and cytoplasmic staining in metaphase. The intensity of staining rapidly diminishes during anaphase and telophase. The half-life of detectable Ki-67 antigen is therefore very short and is about 1 hour or less. This is probably due to the presence of numerous PEST (proline-glutamic acid-serine-threonine) motifs in the

protein which facilitate its rapid catabolism (102).

The exact function of Ki-67 antigen is not known. It has been suggested as a timer for regulation on cell proliferation but was controversial. The Ki-67 has also been suggested as a major structural protein with DNA binding properties and a primary role in maintaining higher order structure for DNA during mitosis (102). Nevertheless, the Ki-67 antigen as a marker of cell proliferation has been used as a prognostic indicator in various malignancies, including PCa (102,105).

The original mouse monoclonal antibody to Ki-67 could be applied only to frozen sections (98). Recently, the microwave antigen retrieval technique allowed antibodies produced against recombinant portions of the Ki-67 antigen to be successfully applied on formalin fixed tissues. A monoclonal antibody (MIB-1) was later produced which can be used with formalin fixed, paraffin embedded material after microwave antigen retrieval. More recently, a polyclonal antiserum was produced by immunizing rabbits with a synthetic peptide deduced from a 62 base pair DNA region encoding for the Ki-67 antigen, and was reported to have identical immunostaining pattern with the MIB-1 antibody(103). The proliferative activity of PCNA has been correlated and implicated in many tumors (100,104,105).

IV. Epidermal Growth Factor Receptor

Epidermal Growth Factor Receptor (EGFr) is a growth factor receptor which can be activated by a family of growth factors including transforming growth factor α (TGF-α), amphiregulin, epidermal growth factor (EGF), and heparin-binding EGF (106). The receptor is a transmembrane glycoprotein with intrinsic tyrosine kinase activity which mediates proliferative effects on cells upon activation

by binding with its ligands such as the EGF or TGF-α. Many types of epithelial malignancies have been found to display increased EGFrs on their cell-surface membranes including lung cancer, glioblastoma, breast cancer, head and neck cancer, and carcinoma of the bladder (107,108,109).

A. The Protein Structure of EGFr

The EGFr is a 170 kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity (106,115). The receptor protein is composed of 1186 amino acid residues. The receptor protein can be divided into three domains. The extracellular domain comprises the aminoterminus of 621 amino acids containing the recognition site of EGF. There are 12 potential sites for amino-linked glycosylation. The membrane-spanning segment comprised of a hydrophobic domain of 23 amino acids. The intracellular carboxyterminal domain of 742 amino acids corresponds to the *erb*-B oncoprotein and contains the tyrosine kinase with binding sites for ATP and substrate and can be closely aligned with retroviral protein-tyrosine kinases and to the catalytic subunit of cAMP-dependent protein kinase. The carboxyterminal tail has three tyrosine residues which are substrates for the intrinsic kinase. Binding of ligand causes dimerization (or oligomerization) with activation of tyrosine kinase activity that mediates transphosphorylation of both of the receptors (115). The cytoplasmic domain of EGFr has a close structural similarity to other cellular protein-tyrosine phosphotransferases identified as the proto-oncogenes of several retroviral transforming proteins. This family of proteins is important in the regulation of cell proliferation and, when abnormally expressed, dictates the altered growth control of the transformed state (106).

B. Physiological Functions of EGFr

The EGFr can be activated by a family of growth factors including EGF and TGF-α. Binding of its ligands with EGFr activates the intrinsic tyrosine kinase activity which initiates second messenger pathways associated with increased cell growth. The binding of the growth factor does not permanently activate the signal transduction pathway. The EGF receptor-ligand complex is subsequently internalized in pinocytotic vesicles and degraded in lysomsomes (110,111,112).

Increased receptor expression is often associated with increased production of TGF-α by the same tumor cells, a condition known as "autocrine stimulation" (115). The concept of autocrine growth regulation was hypothesized by Sporn and Todaro that autoproduction of growth factors essential for proliferation could provide a mechanism by which a cell could escape from the requirement for those particular growth factors in its environment, resulting in unregulated cell growth (113,114). The escape from growth regulation is one of the primary characteristics of malignant cell transformation (115).

V. E-Cadherin

Cadherins are a family of transmembranous glycoproteins responsible for homotypic (to same type) and homophilic (to same molecule) calcium-dependent cell-cell adhesion, important for establishing and maintaining intercellular connections (116,117). Cadherins play a major role in mediating intercellular physical adhesion. When cadherins are functioning, the inactivation of other adhesion systems has little effect on cell-cell adhesion. Conversely, blockage of cadherins activity with anti-cadherins antibodies induces the dispersion of cell layers

(116,118,119).

Inter-cellular junctional complexes are responsible for the maintenance of epithelial structure. Weakening of which may possibly provide opportunity for cancer cells to detach from their epithelium of origin. Homotypic cell-cell adhesion molecules are found at the lateral border of most cells in normal epithelia (117).

Cadherins play an important role in embryonic development and morphogenesis and in the maintenance of the normal structure and function of adult tissues (117). They may also play an important role in formation and maintenance of cancer cell nests and may be involved in the process of metastasis (119,120).

A. Structure of Cadherins

Cadherins are encoded by a gene located in the region of chromosome 16q at position 16q22.1 where multiple genes resided in this region are known commonly deleted in prostate cancer (117,121,122). They are classified into subclasses that share a basic structure and more than 10 subclasses of cadherins have been identified (117). The molecular characteristics of four subclasses, E-cadherin (epithelial cadherin or uvomorulin), P-cadherin (placental cadherin), N-cadherin (neural cadherin), and L-CAM(liver cell adhesion molecule) have been well established (116).

Cadherins typically contain an extracellular segment with distinctive sequence motif which is tandemly repeated forming autonomously folded protein modules, a single membrane-spanning region, and a cytoplasmic region. Experiments with molecular chimeras, neutralizing antibodies and peptide inhibitors suggest that the NH_2-terminal 113-amino acid region in the extracellular segment is

essential for binding specificities of cadherins (116,121).

The intracellular carboxy-terminus interacts with a group of cytoplasmic proteins, the catenins, which function to mediate the cell-cell adhesion of cadherins by coupling the cadherin molecule to the microfilament cytoskeleton of the cell (122).

B. E-cadherin Involvement in Neoplastic Invasion and Metastasis

While Shiozaki *et al* showed that E-cadherin is expressed in the normal epithelium of various tissues, heterogeneous expression was found in primary tumors of esophagus, stomach, and breast. E-cadherin was expressed in cell cytoplasm in some tumor cells with reduced expression. These results indicated that E-cadherin-related intercellular adhesion is impaired in certain human cancer cells (119,123,124,125,127).

There is evidence from *in vitro* and *in vivo* experiments that down-regulation of E-cadherin favors the process of invasion and metastasis in some cell lines (123,124,127). Fixen *et al* showed that cancer cell lines that demonstrate an epithelioid phenotype and express E-cadherin by immunofluorescence, Western blot, and Northern blot studies are noninvasive, whereas invasive cell lines express less E-cadherin and are dedifferentiated. Moreover, transfection and expression of E-cadherin complementary DNA (cDNA) prevents invasiveness and induces differentiation. But treatment with anti-E-cadherin antibodies restores invasiveness (125,126).

Another study using Madin-Darby canine kidney (MDCK) cells, have shown a correlation between cadherin expression and cell behavior after

transformation with Harvey and Moloney sarcoma viruses. Nontransformed E-cadherin-positive MDCK cells were not invasive but transformed E-cadherin-negative MDCK cells were invasive *in vitro* (119).

Correlation have been reported between the loss of E-cadherin expression and dedifferentiation, invasiveness, and poor prognosis in gastric cancer, breast cancer, colon cancer, hepatocellular carcinoma, and head and neck cancer (123,124,126).

VI. CD44

Metastasis in tumor progression is associated with detachment of carcinomatous cells from their primary tumor, penetration through the basement membrane in the connective tissue and invade adjacent structures including lymph and blood vessels, thus attaining metastatic outgrowth. Loss of adhesive function resulting from down-regulation of adhesive molecule plays a crucial role in the initial steps of the metastatic cascade of events. Expression of invasion-related proteins is of particular clinical relevance because failure of cancer treatment results from metastases development. CD44 is a typical example of a cell adhesion protein which undergoes dramatic structural and functional changes during malignant transformation (128,129).

CD44 is a cell surface hyaluronate receptor and a cell adhesion molecule. It has been found to be widely expressed in a variety of cells of hematopoietic, epithelial, and mesothelial origins (129,130). The CD44 molecule has been associated with diverse physiological functions such as cell-cell adhesion, cell-matrix interaction, lymphocyte homing and circulation, T-lymphocyte activation,

signal transduction, hyaluran binding and internalization, and drug uptake and sensitivity.

CD44 received its name during the Third International Workshop on Leukocyte Differentiation Antigens. It was first described in the early 1980s, based on the reactivity of an antibody called F10.44.2. CD44 was previously referred to as lymphocyte homing receptor (gp90 sup Hermes), phagocytic glycoprotein (Pgp-1), extracellular matrix receptor III (ECMRIII), and hyaluronate receptors (H-CAM) and HUTCH-1 (128).

A. Molecular Structure of CD44

CD44 is a family of integral membrane glycoprotein molecules with molecular weights ranging from 80 to 250 kDa encoded by a single gene located on the short arm of human chromosome 11 (11p13 position). Genomic DNA studies have documented that CD44 is encoded by at least 20 exons. The exons 1 to 5 (s1-s5) and 16 to 20 (s6-s10) are almost invariably expressed by a large number of non-epithelial cells, including those which are hematopoietic; their product is referred to as the "standard" or "hematopoietic" form of CD44 (abbreviated to CD44s or CD44H) (6). Exons 6 to 15, more often called v1-v10, are alternatively spliced resulting in large number of possible isoforms. (Almost 100 isoforms have been documented to date. But more than 1000 combinations of isoforms are possible theoretically) (128).

The CD44 molecule consists of an extracellular domain, a transmembrane domain and a cytoplasmic tail. The extracellular domain consists of the N-terminal domain and a membrane proximal domain. The N-terminal domain is encoded by

exons s1 to s5 and is folded into globular loops with two binding sites for hyaluronic acid, also called hyaluronan. Two chondroitin sulphate chains can also be attached to the product of exon s5. The membrane proximal domain is encoded by exons s6 and s7. It contains the site for insertion of the alternative splicing products between exons s5 and s6. Up to 381 amino acids encoded by various combinations of alternatively spliced exons v1 to v10 are inserted at amino acid 223. The alternatively spliced exons do not show any significant homology with any other protein sequences and their functions are largely unknown (128).

The short hydrophobic transmembrane domain is encoded by exon s8. The cytoplasmic tail is encoded by exons s9 and s10. The cytoplasmic domain contains two putative ankyrin binding sites which associate the CD44 molecule to cytoskeletal proteins (128).

B. Physiological Functions of CD44

CD44 is a hyaluronan binding protein which confer binding of cells to extracellular matrix even without the presence of calcium ions (128,129,130,131). Hyaluronate is the main ligand of CD44. It is an abundant saccharide component of the extracellular matrix that is believed to be important in a variety of pathological processes, including inflammation and tumorigenesis (131). Standard form (CD44s) or haematopoietic form (85 to 95 kDa) which has no expression of any of the 10 variant isoforms is the most abundant form of CD44 in tissues (130).

The binding affinity of CD44 molecule is modified by presence of various sugar moieties. O-glycosylation and to a lesser extent N-glycosylation of the standard form of CD44 (CD44s) are necessary for transfected murine T lymphoma

cells to bind to hyaluronan (128,132). However, glycosylation of some of the variant domains of CD44 has the opposite effect. This indicates that variable glycosylation of the CD44 molecule can serve as a mechanism regulating lectin function of CD44 parallel to alternative splicing (128).

The hyaluronan binding capacity is reduced by attachment of chondroitin sulphate or heparan sulphate to isoforms with regions encoded by exons v3. Variant CD44 isoforms with sequences encoded by v7 to v10 are shed quite easily into the extracellular space and abolish hyaluronan binding capacity. (128). The binding affinities of variant CD44 isoforms to hyaluronan also vary depending on type of alternatively spliced variant exon in the molecule.

Apart from the hyaluronan binding capacity, CD44 can also bind with some other extracellular ligands such as chondroitin sulfate, heparin sulfate, fibronectin, serglycin and osteopontin but with lower affinity (130).

Differential response of binding of CD44 with hyaluronate and osteopontin has been reported in the study of Weber *et al* (133). They observed that binding of CD44 with osteopontin mediates chemotaxis or attachment, depending on presentation of osteopontin in soluble or immobilized form but binding of CD44 with hyaluronate mediates aggregation or attachment but not chemotaxis indicating different response of CD44 binding with separate matrix proteins.

Consensus sequences indicate that CD44 has homology with *ras* oncogene and that CD44 may be a quanine nucleotide-binding protein with GTPase (quanine triphosphatase) activity (134).

C. CD44 Involvement in Neoplastic Invasion and Metastasis

Studies on CD44 in various carcinomas and tumors showed that the expression of CD44 and its variant isoforms are associated with tumor progression, grade and aneuploidy (130). Elevated CD44 expression has been associated with systemic disease and poor outcome in non-Hodgkin's lymphoma (135). Increased expression of CD44 protein has been reported in breast carcinoma and gastric adenocarcinoma (136,137). Expression of CD44 variant isoforms has been correlated with tumor dedifferentiation and progression in stomach, lung, breast, colon and cervical cancer (138,139,140,141,142). However, down-regulation of CD44 expression has been reported in urinary bladder transitional cell carcinoma, neuroblastoma and endometrial carcinoma which appears to be correlated with increase tumor grade and aneuploidy (143,144,145).

VII. nm23

The nm23 gene is a putative anti-metastatic gene initially identified by differential hybridization of a cDNA library with total RNA extracted from weakly and strongly metastatic murine melanoma cell lines (146,147,148). Nm23 expression was reduced in highly metastatic murine melanoma cell line (K-1735). Previous studies showed that expression of nm23-H1 mRNA is inversely correlated with metastatic potential in several human malignant tumors, including primary breast carcinoma, gastric carcinoma and malignant melanoma (148,149). Moreover, mutation with deletion of the coding sequence of the nm23 gene has been reported in primary colorectal, breast, renal and lung carcinoma (149). Mutations and/or genetic alterations of nm23 have been reported to be associated with a more malignant

phenotype in neuroblastoma and colon (150). Konishi *et al* reported reduced nm23 immunoreactivity in metastatic PCa (151). Alteration of nm23 gene or gene product may cause loss of suppressor activity correlated with loss of differentiation in cell growth and possibly acquisition of metastatic phenotypes (149,152).

Two nm23 genes, nm23-H1 and nm23-H2 (or NME1 and NME2 respectively), have been identified which are 88 percent homologous to each other. Both genes are located on chromosome 17 at position 17q22, within 18 kb apart encoding for the 18.5 and 17 kDa proteins, nm23-H1 and nm23-H2 proteins respectively (152).

A. Physiological Functions of nm23 Gene Products

The specific function of nm23 gene products is not well understood. The gene products, nm23-H1 and nm23-H2 proteins, are identical to human nucleotide diphosphate kinases subunits A and B respectively purified from human erythrocytes (153). Nucleotide diphosphate (NDP) kinases are ubiquitous enzymes that catalyzes the transphosphorylation of nucleoside triphosphates to diphosphates involving transfer of the terminal phosphate of adenosine triphosphate (ATP) to a histidine residue of the enzyme and then to a nucleotide diphosphate through a *ping-pong* mechanism. They have a broad specificity for nucleotide substrates and accept both nucleotides and deoxynucleotides as substrates. Thus they are involved in the supply of most of the RNA and DNA precursors except ATP to cells (153,147). As a major source of guanosine triphosphates (GTP), NDP kinase occupies a central position in the control of cell functions and development. NDP kinase may play some roles in signal transduction by supplying GTP to GTP-binding proteins. Eukaryotic NDP

kinases have been proposed to interact with heterotrimeric G proteins which are associated with signal transduction (154) Different functions have been proposed for nm23 gene including maintenance of nucleotide triphosphate's pool, direct or indirect interaction with GTP-dependent proteins and regulation of gene transcription (147).

The regulatory function of gene transcription of nm23 gene have been implicated by the studies of Postel *et al* who identified that nm23-H2 gene is identical with the gene encoding the c-myc purine-binding factor (PuF), which is a transcription factor necessary for the expression of the c-myc proto-oncogene. The protein product of c-myc proto-oncogene functions in cellular proliferation, differentiation and tumor formation, presumably by modulating the expression of genes involved in these processes (155).

Nm23 gene has also been associated with cell growth and proliferation based on observations that anti-nm23 antibodies recognize mitotic spindle microtubules and nm23 is highly homologous to p19 protein, a neuroblastoma cell proliferation-related protein (156,157). This is supported by the studies of Caligo *et al* on nm23 gene expression in relation to growth state of the cells and observed the expression of nm23 mRNA in PHA-stimulated peripheral blood lymphocytes but undetectable in the resting counterparts (147). The level of nm23-H1 mRNA in synchronously cycling MCF-10 A cells was found to be high in the S-phase and late S-phase of the cell cycle but was absent or nearly absent in G_0 phase. The nm23-H2 gene was expressed in growth-arrested MCF-10A cells. Both genes were subject to up-regulation following serum re-addition to cell medium. The nm23 mRNA expression in late S-phase of the cell cycle have suggested that nm23 may play a role

in microtubule mitotic spindle polymerization. It has also been suggested that quantitative changes of nm23 protein on the cell surface control the cell attachment/detachment to the extracellular matrix through its shared arginine-glycine-aspartic acid (RGD) sequence, a consensus motif for binding to the integrin family (158).

B. Association of nm23 Expression in Tumors

The metastasis suppressor function of nm23 in tumor progression is controversial. Although low levels of nm23-H1 expression have been linked to increased metastatic potential of some cell lines and tumors. Yet the A chain product of nm23-H1 is overexpressed in highly proliferative cells and in a variety of invasive human tumors. Thus its role in metastasis control is disputable (154).

In colorectal cancer, conflicting results have been reported regarding nm23 expression and tumor aggressiveness. Somatic allelic deletion of the nm23 gene has been reported in advanced cases of colon cancer, although not found by others. Reduced expression of nm23-H1 mRNA has been reported in patients with colorectal cancer associated with distant and hepatic metastases while others failed to find an association between its expression and metastatic spread. These discrepancies have been attributed to cross-reactivities of the antibodies or gene probes used between nm23-H1 and nm23-H2 (152,158).

Chapter 4. Objectives of Study

1. To examine and compare the immunohistochemical expression of several biomarkers including p53, PCNA, Ki-67, EGFR, E-Cadherin, CD44, and nm23, in prostatic hyperplasia, PIN lesions, and prostatic acinar carcinoma.
2. Based on these expressions, to assess the possible correlation of these biological markers among prostatic hyperplasia, PIN lesions, and carcinoma of the prostate, and to determine any relationship between these prostatic lesions.
3. To determine from these cell proliferative biomarkers, whether there is a progression or continuum exist among prostatic hyperplasia, PIN lesions, and carcinoma of prostate.

Chapter 5. Materials and Methods

I. Materials

A. Prostatic Specimens

Prostatic specimens from the Prince of Wales Hospital (PWH) and the Hong Kong Baptist Hospital (HKBH) during the period between 1989 to 1994 were reviewed and a total of 122 cases were selected for this study. The patients were all Hong Kong Chinese with diagnosis of prostatic hyperplasia in 28 cases, of prostatic intraepithelial neoplasia (PIN) lesion alone in 46 cases, and of prostatic adenocarcinoma in 48 cases. Formalin-fixed paraffin embedded tissue blocks of 52 patients were obtained from the Department of Anatomical and Cellular Pathology, Prince of Wales Hospital and prostatic tissue blocks of 70 patients were obtained from the Pathology Laboratory of the Hong Kong Baptist Hospital. The haematoxylin and eosin (H&E) stained slides for each case was examined and a representative paraffin block was selected for each case.

Details of the types and sources of specimens, and of the pathology according to the type of specimens from these 122 patients are presented in Tables 5 and 6. Of the 48 patients with prostatic carcinoma, only 3 were from radical prostatectomy performed for the treatment of prostatic carcinoma clinically confined to the organ. The remaining 45 cases were diagnosed from transurethrral prostatic resection (TUR-P) specimens performed for obstructive symptoms or prostatism. During the period of study, radical prostatectomy performed for the treatment of prostatic carcinoma was just beginning to be introduced in the 2 institutions. Of these 3 prostatectomies, only one case was associated with PIN 3 lesions.

Table 5. Distribution of types and sources of prostatic specimens.

	PWH	HKBH	Total
TURP	49	20	69
Prostatectomy, Partial	1	16	17
Prostatectomy, Cystectomy	2	3	5
Prostatectomy, Total	0	31	31
Total No.	52	70	122

Table 6. Summary of classification of pathology according to the type of prostatic specimens studied.

Pathology	TUR-P		Total or Partial Prostatectomy		Total	Average Patients' Age (SD)
	PWH	HKBH	PWH	HKBH		
Hyperplasia	5	2	0	21	28	67.3 (7.9)
PIN 1	3	3	0	13	19	68.8 (11.0)
PIN 2	4	1	0	2	7	73.0 (11.4)
PIN 3	3	3	2	12	20	70.4 (8.2)
Prostatic carcinoma:						
Low-grade	10	3	0	0	13	72.3 (8.2)
Intermediate-grade	10	5	0	1	16	72.2 (7.6)
High-grade	14	3	1	1	19	74.9 (7.1)
Total	49	20	3	50	122	70.7 (8.8)

Of the 28 cases of prostatic hyperplasia, 21 cases were from partial or total prostatectomy, all performed as the Millin's operation at the Hong Kong Baptist Hospital, while the remaining 7 cases were from TUR-P.

Of the 46 cases of PIN lesions, 29 were from partial or total prostatectomy including 24 from the Millin's operation at the Hong Kong Baptist Hospital, and 5 with cystectomy from both institutions. Of these 29 cases, 22 were identified as from the peripheral zone, including 12 cases of PIN 3, and 2 cases of PIN 2, and 8 cases of PIN 1. The remaining cases were removed piecemeal and the zonal distribution could not be ascertained. The 17 remaining cases of PIN lesions were from TUR-P and were presumed to be from the transitional zone.

B. Histological Classification

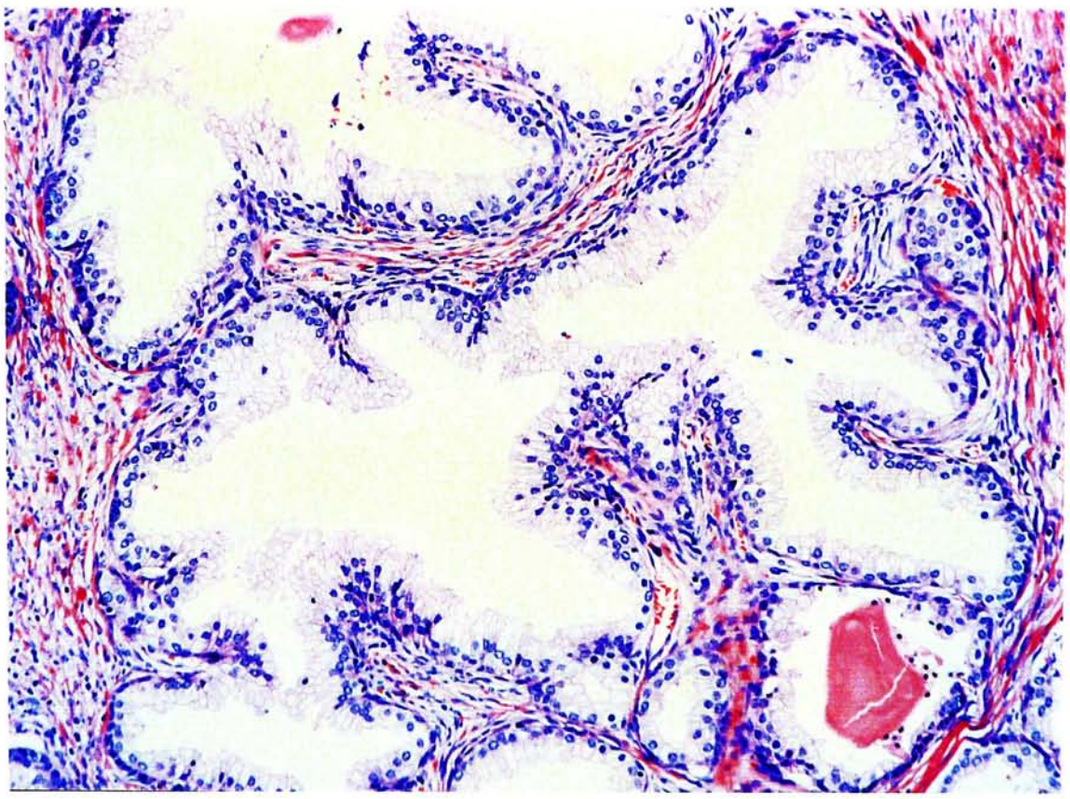
Selected cases of nodular hyperplasia may show overgrowth of glands, smooth muscle stroma or both, and no other lesions such as prostatitis or PIN. Only nodular hyperplasia of glands were examined, and for each case, areas with proliferative glands and exhibiting uniform benign cytology of the secretory cells (Figure 13) were outlined and marked with a marker pen on the slides for study. The same area in each case was examined for all biological markers.

Prostatic specimens with foci of PIN were identified and classified into PIN 1 (Figure 8), PIN 2 (Figure 9) and PIN 3 (Figure 10) according to McNeal's initial grading system as described in Chapter 3 on PIN. Similarly, selected glands or group of glands with PIN lesions were marked on the H&E slides, and on serial consecutive sections to examine the same glands or group of glands for different immunohistochemical markers. All the sections in each case with PIN were

reviewed to ensure that no coexisting PCa was present in the same patient. In a few cases of high-grade PIN lesions, the presence of basal layer was confirmed using specific antibodies for high molecular weight cytokeratin (clone 34βE12, Dako, Denmark)(159).

Prostatic adenocarcinomas were graded according to the scheme proposed by Gleason as described in Chapter 2 (III), Pathology of Prostatic Cancers. For PCa, a Gleason score was obtained in each case by combining the predominant Gleason grade with the second most extensive Gleason grade. Based on their score, the prostatic carcinomas were grouped into low-grade, intermediate-grade and high-grade carcinoma, respectively for a score ranging between 2 to 5, 6 to 7, and 8 to 10 as described by Gleason (19). Low-grade carcinomas, including grade 1 and 2, were circumscribed and relatively confined with regular borders, small glands with or without some size variation, and some intervening stroma (Figure 14). Intermediate-grade carcinomas included lesions similar to grade 2, with more complex glands to cribriform glands, and irregular invasive borders (Figure 15). High-grade tumors may present with interconnecting glands of grade 3 or large sheets (Figure 16), and poorly differentiated tumor with cords or single cells (Figure 17) The histological classification of benign and malignant lesions, as well as the number of cases studied per group are shown in Table 6.

A.



B.

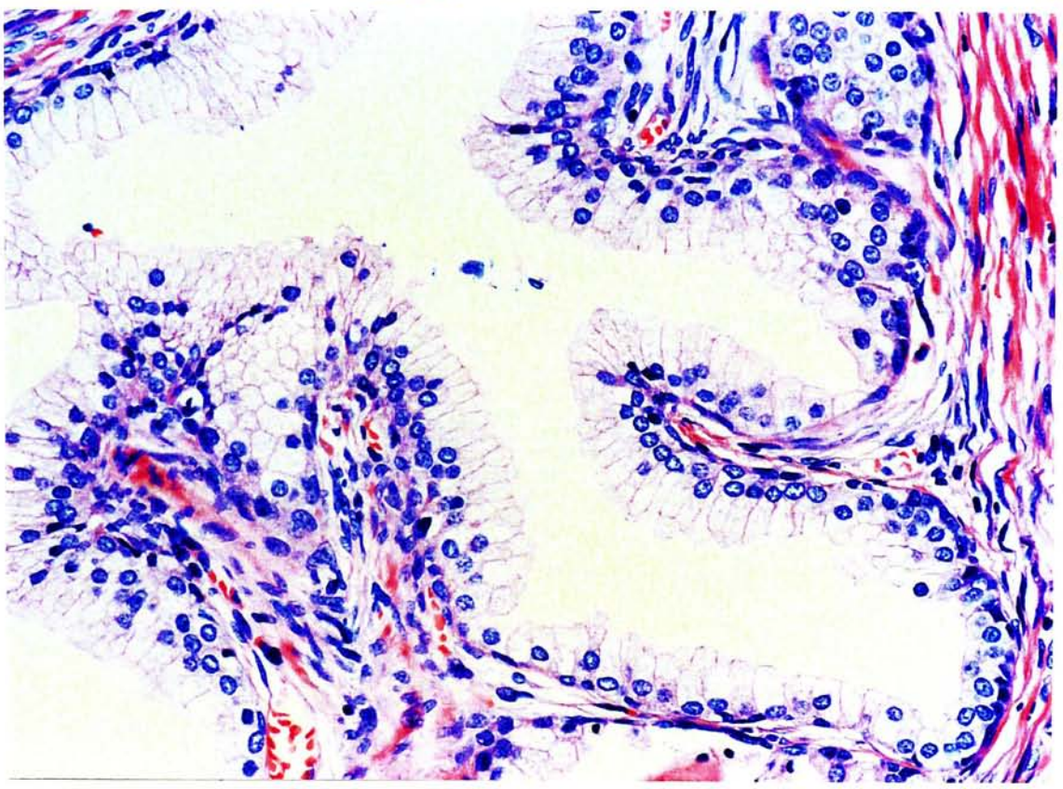
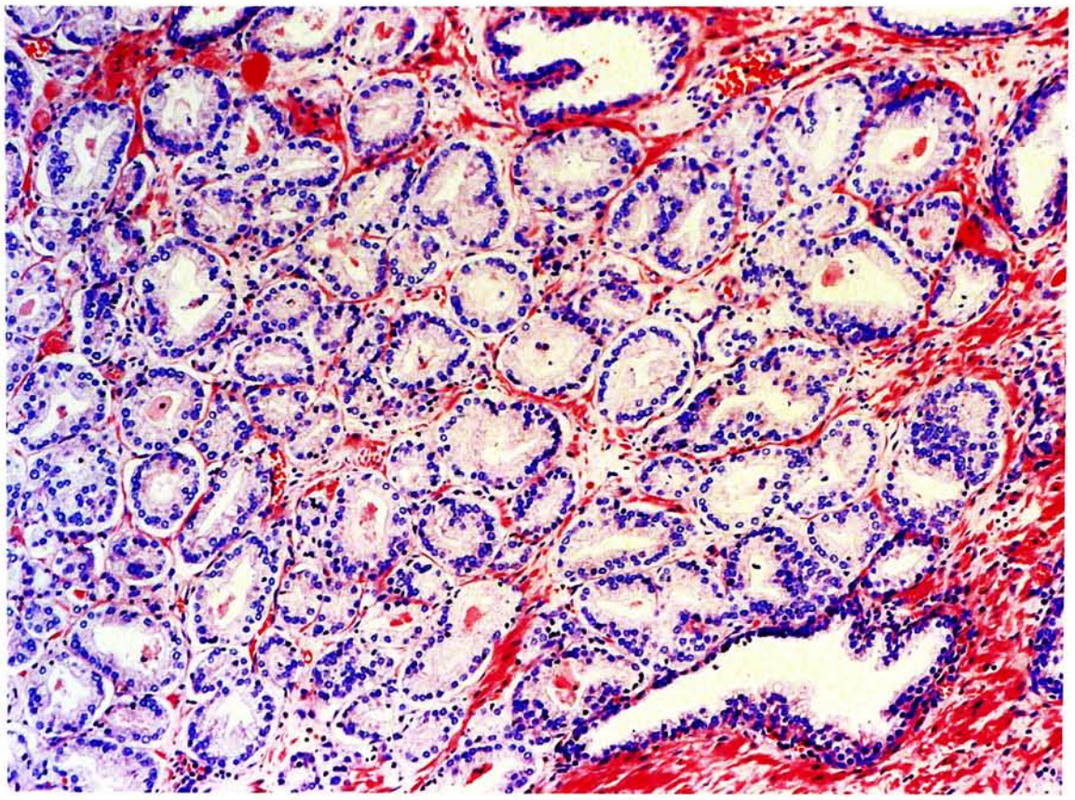


Figure 13. Prostatic hyperplasia. (**A**) The acini are enlarged tortuous with papilliform growth of epithelium into the lumens in many of them. (Hematoxylin-eosin stain; magnification x500); (**B**) The cells lining the glandular acini are tall, well-differentiated columnar cells with pale vacuolated eosinophilic cytoplasm and uniform round basal nuclei. (Hematoxylin-eosin stain; magnification x1000)

A.



B.

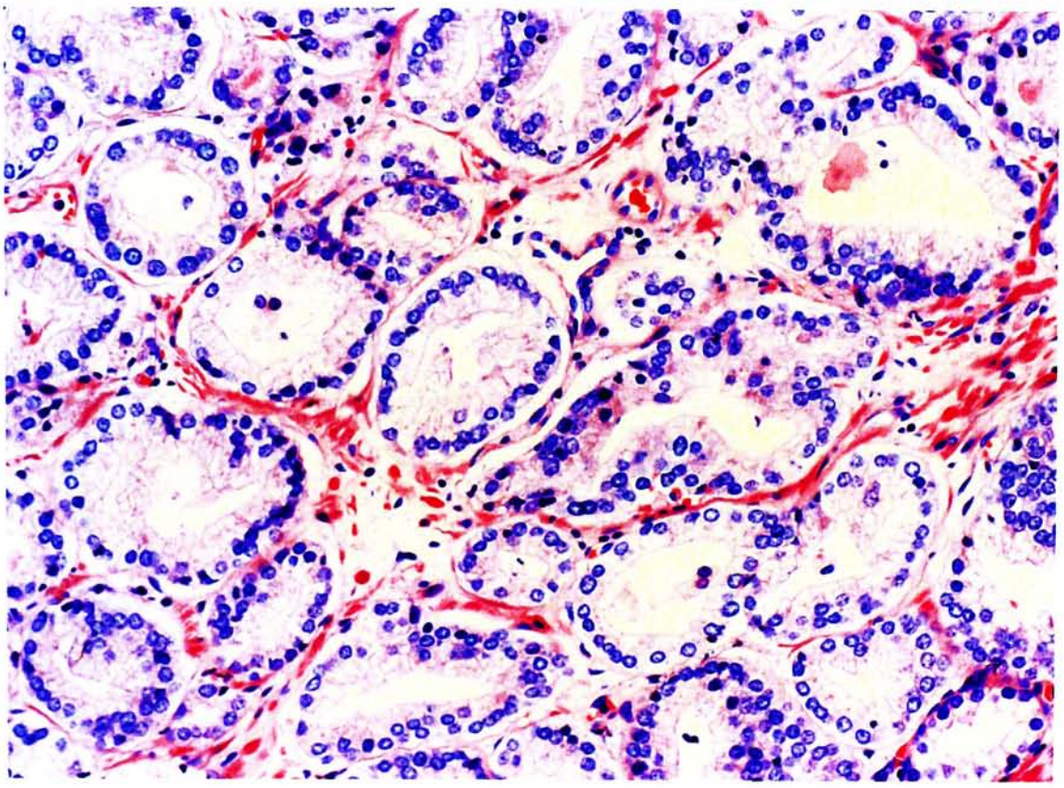


Figure 14. Low-grade prostate adenocarcinoma (Gleason grades 1,2). (**A**) Circumscribed tumor composed of simple rounded glands which are close to loosely packed. (Hematoxylin-eosin stain, magnification x500); (**B**) Glands consist of single cell layer with relatively uniform tumor cells but more conspicuous nucleoli. Basal cells are absent. (Hematoxylin-eosin stain, magnification x1000)

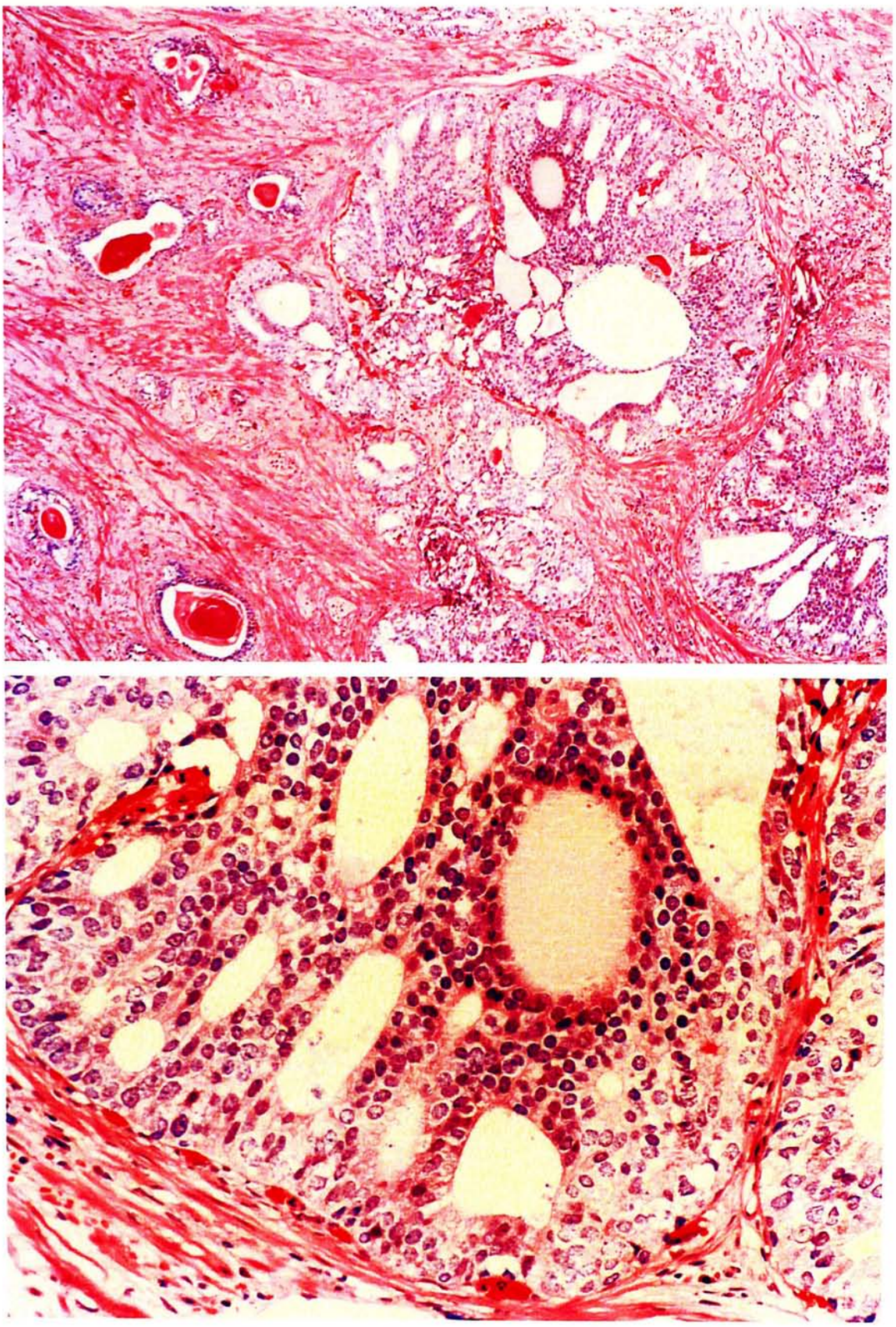


Figure 15. (**A**) Intermediate-grade PCa (Gleason grade 3,3) with cribriform epithelium in smooth, rounded cylinders and solid masses. (Hematoxylin-eosin stain, magnification x400) (**B**) Higher magnification show pleomorphic tumor cells with pale to eosinophilic cytoplasm, high nuclear cytoplasmic ratio, anisonucleosis with conspicuous to prominent nucleoli (Hematoxylin-eosin stain, magnification x1000)

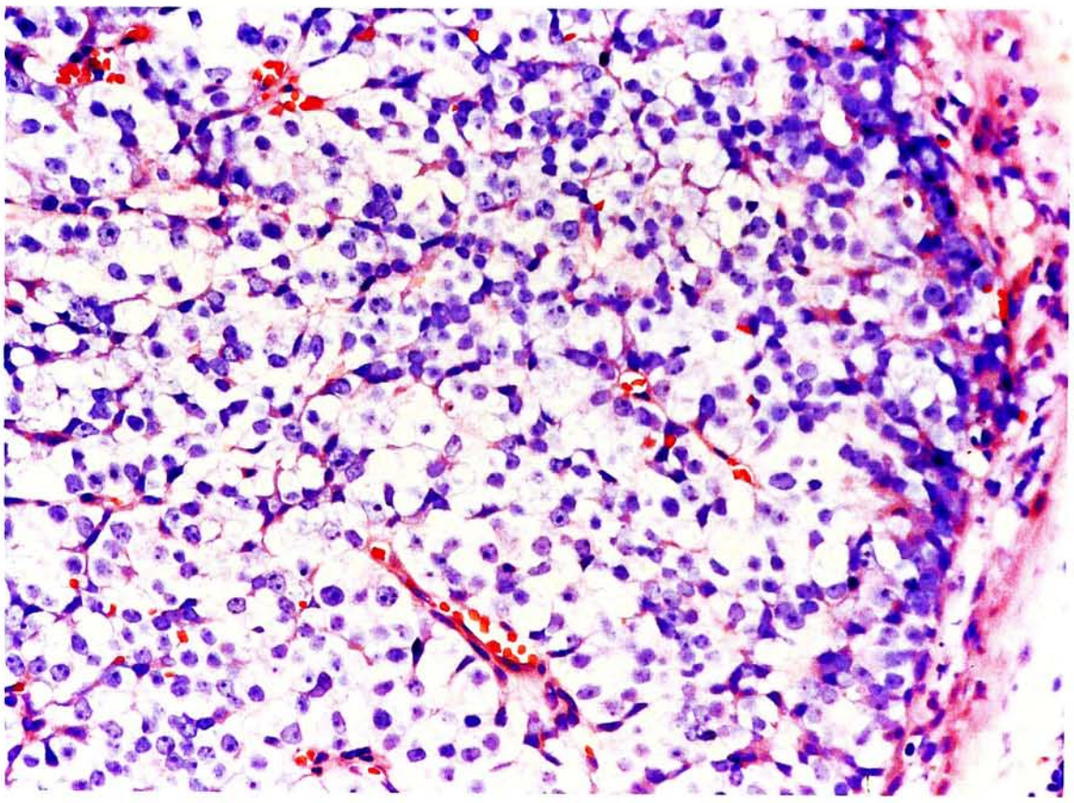


Figure 16. High-grade PCa (Gleason grade 4,5). Tumor cells are fused into cords and ragged infiltrating mass, with no glandular formation. (Hematoxylin-eosin stain; magnification x1000)

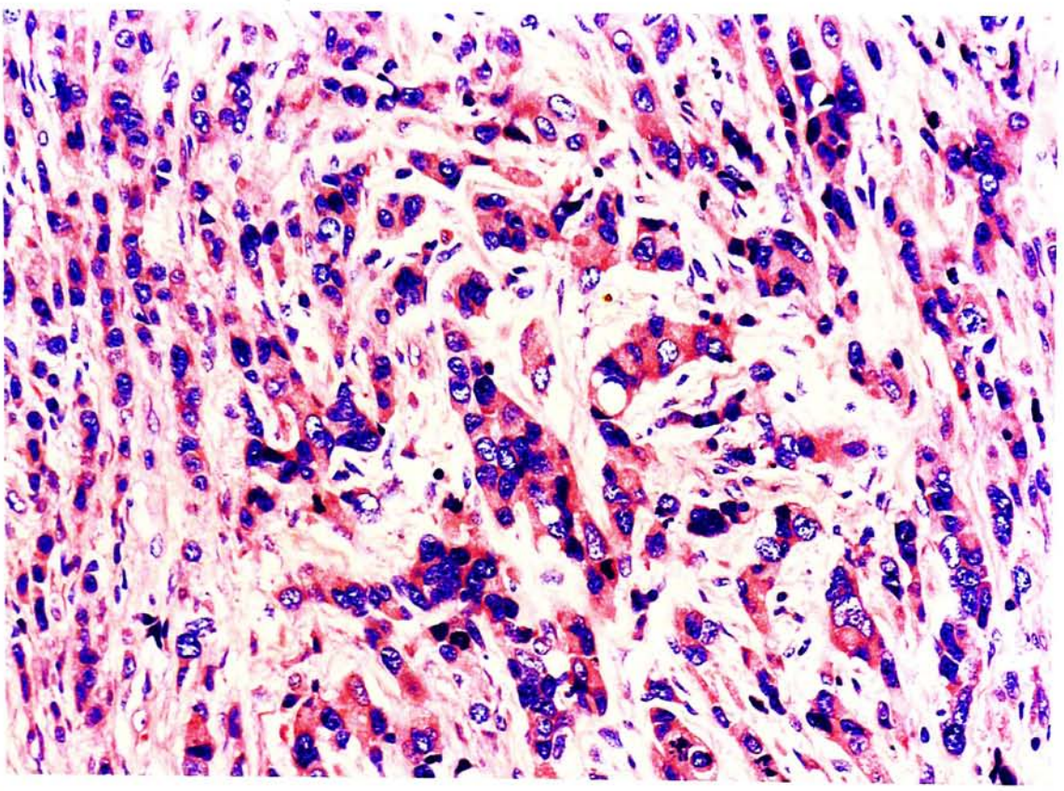


Figure 17. High-grade PCa (Gleason grade 5,5) showing anaplastic carcinoma with marked pleomorphic tumor cells arranged in cords and nests. (Hematoxylin-eosin stain; magnification x1000)

II. Methods

A. Preparation of Slides

For each case, the block selected was sectioned into twenty consecutive paraffin unstained sections of 3 μm thick using a Reichert Jung 2030 Biocut rotary microtome. The sections were mounted on 3-aminopropyltriethoxysilane-coated slides and dried in an oven at a temperature of 60°C for 1 hour. The slides were then kept in a cool dry place until further staining. For each case, a new H&E stained section was obtained and examined for 2 purposes: one was to confirm that the consecutive sections showed the desired pathological features; the second was to select and highlight with a permanent marker pen the area that would be examined in subsequent immunostainings.

B. Antibodies Used

The antibodies, the manufacturers, and the antibody dilutions used in this study are presented in Table 7. Monoclonal mouse anti-human p53 antibody, clone DO-7 was purchased from the Dako Corporation, Denmark. The antibody recognizes an epitope between amino acids 19 to 26 in the N-terminus of the human p53 protein. The antibody was previously characterized and is specific for both mutant and wild-type human p53 protein.

Monoclonal mouse anti-PCNA, clone PC10, obtained from Novocastra Laboratories, New Castle, UK, was raised with rat PCNA made with the protein A expression vector pR1T2T as immunogen. It recognizes PCNA from all vertebrate species.

The polyclonal rabbit anti-human Ki-67 was purchased from Dako

Corporation, Denmark, UK. The antibody was raised with synthetic peptide deduced from a 62 base pair region of the human Ki-67 gene as immunogen.

Monoclonal mouse anti-epidermal growth factor receptor antibody was purchased from Novocastra Laboratories, Newcastle, UK. Recombinant fusion protein to the external domain of EGFr was used as immunogen.

Monoclonal mouse anti-human E-cadherin was obtained from the Progen Biotechnik GMBH, Heidelberg. The antibody was raised with human uvomorulin as immunogen.

The monoclonal mouse anti-human CD44 protein, purchased from Novocastra Laboratories, Newcastle, UK, was raised with human T cells as immunogen.

The mouse monoclonal anti-human nm23/NDPK-A protein purchased from Novocastra Laboratories, Newcastle, UK. is specific for human nucleoside diphosphate kinase A (NDP kinase A) with low crossreactivity with nucleoside diphosphate kinase B (NDP kinase B). Nucleoside diphosphate kinase A purified from human erythrocytes was used as immunogen.

All primary antibodies were diluted with Tris-buffered saline (TBS), pH7.6[1] in this study except for the anti-E-cadherin antibody which was diluted with 0.01M Tris-HCl-$CaCl_2$ buffer, pH7.2[2].

Table 7. List of primary antibodies with dilutions and incubation time used.

Antibody	Clone	Source	Catalogue no.	Dilution and incubation time
Monoclonal anti-human p53	DO-7(IgG2b)	Dako Corp., Denmark	M7001-1	1:50 for 30 min 5 μg/ml IgG or .2 mg/mL protein
Monoclonal anti-rat PCNA	PC10 (IgG2a)	Novocastra Laboratories, Newcastle, UK	NCL-PCNA	1:50 for 30 min.
Rabbit anti-human Ki-67	Polyclonal	Dako Corp., Denmark	A047	1:75 for 30 min.
Monoclonal anti-EGFr antibody (External domain)	EGFr.113, (IgG2a)	Novocastra Laboratories, Newcastle, UK	NCL-EGFr	1:15, overnight at 4°C
Monoclonal anti-human E-cadherin	6F9 (IgG1)	Progen Biotechnik GMBH, Heidelberg	PC10704	1:50, overnight at 4°C
Monoclonal anti-human CD44: HCAM	F1044-2 (IgG2a)	Novocastra Laboratories, Newcastle, UK	NCL-CD44	1:80 for 30 min
Monoclonal anti-nm23/NDPK-A protein	37.6 (IgG2a)	Novocastra Laboratories, Newcastle, UK	NCL-nm23	1:200 for 30min

C. Determination of Conditions and Dilutions of Antibodies

The specific conditions for immunostaining with each primary antibody were determined in preliminary studies. Firstly, the effects of heat-induced epitope enhancement and protease enzyme digestion were determined using positive control tissue sections treated each with either microwave heat treatment, trypsinization, or both microwave irradiation and trypsinization or no treatment prior to immunostaining with the labeled streptavidin biotin method as described by Elias *et al* (160).

Different tissues were used as positive controls for different biomarkers. Two cases each of breast adenocarcinoma and of colonic carcinoma were used as positive controls for p53 protein, EGFr, and Ki-67 antigen detection. A case each of normal colon and normal stomach was used as positive control for E-cadherin detection. Normal prostatic glands were used as positive control for PCNA and CD44 immunostainings.

The microwave antigen retrieval technique as described by Shi *et al* was applied using a household microwave oven (SMEG, PBI International) with a maximum power output of 1000W (161). The slides were immersed in 65 ml of 10 mM citric acid buffer, pH 6.0^3 in a heat resistant plastic Hellendall staining jar with a capacity of 16 slides and heated to boiling temperature by microwaving at maximum power (1000W) for 1 minute. They were then microwaved for 2 cycles of 5 minutes each at 40% power level (400W) such that the citric acid buffer boils every 10 to 15 seconds. The buffer volume was maintained by topping up with additional buffer in between each microwave treatment. The slides were then allowed to cool for 20 minutes in the buffer and then immersed in TBS, pH 7.6.

For the study of the effect of enzyme digestion, the slides were immersed in 0.1% Trypsin solution[4] in 0.005M TRIS-HCl-$CaCl_2$ buffer, pH 7.8[5] for 30 minutes, maintained at 37°C in a water bath.

For study of the effect of both heat-induced antigen retrieval and enzyme digestion, the slides were treated with the microwave antigen retrieval method as described above, followed by enzyme digestion by immersing in 0.025% Trypsin solution[6] for 1 minute at room temperature.

Each primary antibody was tested at the dilution recommended by the manufacturer and incubated at room temperature (20 to 24°C) for 30 minutes. The immune reaction with each primary antibody was detected with the labeled streptavidin-biotin peroxidase method (LSAB). The method involved detection of specifically bound primary antibodies with species specific biotinylated secondary antibodies which were subsequently detected with streptavidin which was conjugated with the enzyme horse-radish peroxidase. The signal was then visualized with the substrate diaminobenzidene (DAB).

Immunostaining results showed that microwave treatment resulted in enhanced staining of p53 protein, PCNA, Ki-67 antigen, EGFr, CD44, and nm23 protein while trypsinization is more effective for E-cadherin detection. The effect of trypsinization was further tested by immunostaining for E-cadherin with various duration and concentrations of trypsin solution.

The optimal dilution of primary antibody was then determined by staining with the selected antigen retrieval treatment but at different antibody dilutions varying from 1 in 50 to 1 in 1600 in 2 fold increments. For antibodies such as anti-E-cadherin and anti-EGFr which showed very weak immune reaction, the primary

antibodies were incubated overnight in a refrigerator at about 4°C. The staining results varied from a spectrum of weak to strong staining with varying degree of background staining. The highest dilutions with strong staining and absence of non-specific background staining were selected for the study. But for PCNA immunostaining, a dilution was selected such that only the strongly stained positive nuclei remained positive whilst most of the secretory cells of normal prostatic epithelium were weakly to negatively stained.

D. Immunostaining

Paraffin sections were dewaxed with 2 changes of xylene for 3 minutes each and then rehydrated with graded alcohols. For detection of Ki-67, PCNA, CD44, nm23 and p53 proteins, tissue sections were pretreated with microwave antigen retrieval technique as described previously. For detection of E-cadherin, the slides were immersed in 0.1% Trypsin solution[4] for 2 hrs, maintained at 37°C in a water bath. Subsequent immunostaining for p53 protein, PCNA, Ki-67, nm23, and CD44 expressions was performed with an automatic immunostainer, Optimax immunostainer (Biogenex, San Ramon, CA). Immunostaining for EGFr and E-cadherin, which required overnight incubation of primary antibodies, was done manually.

The slides were treated with 3% hydrogen peroxide (Catalog no. H-6520, Sigma) for 10 minutes at room temperature (about 20 to 24°C) to bleach endogenous peroxidase. This was followed by treatment with 3% bovine serum albumin[7] for 10 minutes to block non-specific protein binding. The slides were then incubated with one of the primary antibodies. Species-specific biotinylated antibodies were then

applied as linking antibodies. For detection of p53 protein, PCNA, E-cadherin molecules and CD44 protein, biotinylated sheep anti-mouse whole antibody[8] was applied at a dilution of 1 in 50 and incubated for 30 minutes at room temperature. For detection of nm23 protein, the above linking antibody was applied at a dilution of 1 in 200 and incubated for 45 minutes at room temperature. For detection of Ki-67 protein, biotinylated donkey anti-rabbit whole antibody[9] was applied at a dilution of 1 in 200 and incubated for 30 minutes.

All slides were then treated with streptavidin-peroxidase conjugate (Catalog no. P397, Dako Corporation, Denmark) at a dilution of 1 in 400 in TBS, pH7.6 and incubated for 30 minutes followed by color development with diaminobenzidene solution (DAB)(Catalog no. HK153-5K, Biogenex, San Ramon, CA, USA) for 10 minutes. The slides were then counterstained with either Harris's haematoxylin[10] (p53, PCNA, CD44, nm23) or methyl green[11] solutions (Ki-67 and E-cadherin).

Dilution of anti-E-Cadherin antibody was done with 0.01 M Tris-HCl-$CaCl_2$ buffer, pH7.2. Other primary antibodies, linking antibodies and streptavidin-peroxidase conjugates were diluted with 0.05 M TBS, pH7.6. The incubation steps were performed at room temperature of 18 to 24°C except for incubation with anti-E-cadherin and CD44 antibodies which were performed in the refrigerator at a temperature of about 4°C. After each incubation step, the slides were washed 3 times with the washing buffer[12].

III. Interpretation of Immunostaining Results

A. p53 Protein

Immunostaining for p53 protein overexpression was considered positive, when brown nuclear staining was identified, regardless of its intensity. The proportion of p53-positive cells in the secretory or luminal prostatic epithelium for prostatic hyperplasia and PIN lesions or among tumor cells for carcinoma were graded semi-quantitatively as followed: 0 when all cells are negative, 1 when up to 5% of lesional cells are positive, 2 when positive cells are greater than 5% and up to 33% of positive cells, 3 when positive cells represent 33% to up to 66% of lesional cells, and 4 when more than 66% of cells to all cells are positive for p53 protein expression. Cases with expression of more than 5% or graded as 2 or above were considered to be positive for p53 mutation.

B. PCNA

Specific PCNA expression was accepted when positive brown diffuse or granular immunostaining confined to the nuclei was detected in lesional cells (162). A PCNA labeling index was obtained for prostatic hyperplasia, PIN lesions and carcinoma of the prostate, and it is expressed as the percentage of positive nuclei in these lesions when a minimum of 500 cells were examined, choosing the most positively stained area of the section, and using a 40X microscope objective.

C. Ki-67 Antigen

Immunoreactivity for specific Ki-67 expression was considered positive, when brown nuclear signal was unequivocally seen, regardless of the intensity. The

Ki-67 immunoreactivity was assessed semi-quantitatively by two light microscopic methods: one by cell counting by visual histological assessment and the other using a computer-assisted image analysis. Results from both methods were subsequently statistically compared and analyzed using Mann-Whitney U and Spearman's Rank significance methods.

1. Cell Counting by Visual Histological Immunostaining

For cell counting method, a Ki-67 labeling index was determined by observing the percentage of positive nuclei among at least 500 cells in an area of the section with highest labeling frequency with a 40X microscope objective.

2. Image Analysis of Ki-67 immunostaining

Quantitative analysis of Ki-67 protein expression was performed with the CAS200 Image Analyzer (Beckon-Dickinson, San Jose, California, USA). The percentage of area of Ki-67 immunoreactive nuclei to the total area of nuclei in tissue sections was measured with the Quantitative Proliferation Index (QPI) CAS Software Program (Cell Analysis Systems), provided by the manufacturer. The software utilizes the sum of the optical density of each nucleus to calculate the amount of nuclear immunostain present, according to the Beer-Lambert Law. The measurement of immunostaining relies on the principle of spectral separation, which allows discrimination between areas stained with two chromogens (DAB or methyl green) with complementary spectral characteristics. The CAS200 Image Analyzer is equipped with two matched image sensors which are set to capture images at wavelengths of 500 and 620 nm simultaneously. Microscopic fields of 300x200 um^2 were measured with a 40X objective and can be displayed on the computer monitor.

The antibody threshold to account for background staining was established in a field without brown reaction product in each case. During measurement, an image of the total nuclear area in a microscopic field was mapped using a red (620/10-nm band pass) filter after adjusting the nuclear threshold such that only methyl green or DAB stained nuclei were measured. Then in the same microscopic field, a map of positive nuclear staining was obtained using a green (500/10-nm band pass) filter. The areas of the positive nuclear staining and the total nuclear area of each microscopic field were accumulated by the QPI CAS200 program. The proliferation index was expressed as the ratio of the total area mapped by the brownish nuclear staining and the total nuclear area.

Before each measurement, Kohler illumination was first set up. A "Set Light" function provided by the QPI CAS program was then run to match illumination of the two image sensors and compensate for uneven illumination of the microscopic field by adjusting the light intensity and condenser position. For the assessment of Ki-67 proliferation index in PCa and benign proliferative lesions, an microscopic field with highest positivity was first selected and measured. Four adjacent fields above, below, to the left and right of it were then measured. If the total area of the measured fields was less than 10,000 um^2 which is the total area of about 100 to 200 cells, additional nearby microscopic fields together with their adjacent fields were measured in similar manner. For the measurement of Ki-67 proliferation index in prostates with PIN, due to limited number of microscopic fields with presence of PIN, as many fields as available were measured or until a total area of 10,000 um^2 was measured. During measurement, only secretory or luminal cells in prostatic hyperplasia or PIN lesions were measured by using a

special image segmentation feature of the QPI CAS software of the image analyzer such that interested areas of the image displayed on a monitor were outlined with a computer mouse before measurement.

D. E-cadherin, CD44 and EGFr

Clearly defined brown immunostaining of the cell membranes of lesional cells were considered as positive expression for either E-cadherin, CD44 or EGFr. For CD44 immunostaining, the proportion of cells with specific antigen expression on the cell membranes of luminal cells or tumor cells were graded semi-quantitatively as followed: 0 for absence of staining, 1 for up to 33% positive cells, 2 for greater than 33% and up to 66% positive cells, and 3 for greater than 66% and to 100% of positive cells, respectively.

For EGFr immunostaining, the intensity of staining of specific antigen expression on the cell membranes of luminal cells or tumor cells were graded semi-quantitatively as followed: 0 for absence of staining, 1 for weak staining, 2 for positive staining, and 3 for strong to very strong positive staining. Presence of cytoplasmic staining was noted and also recorded. For semi-quantitation of E-cadherin, the expression for such marker was scored as 0 when immunostaining was absent, as 1 when immunostaining was heterogeneous, comprising mixed positive and negative cells, and as 2 when all lesional cells were uniformly positive, as described by Umbas *et al* (117).

E. nm23

Brown immunostaining signal in the cell membrane, cytoplasm or nuclei

was considered specific for nm23 protein expression or immunoreactivity. The extent and intensity of nm23 reactive cells in the luminal layer of prostatic epithelium and foci of carcinoma were assessed semi-quantitatively. The intensity of nm23 staining was graded as followed: 0 for negative staining, 1 for weak staining, 2 for moderate staining, and 3 for strong intense staining.

IV. Statistical Analysis

Statistical analysis was performed with the SPSS statistical package, release 7 (SPSS Inc.). The Mann-Whitney U significance test was used to test for significant differences in rank-ordered measurements between groups. Kruskal-Wallis significance test was used to test for significant differences among multiple groups. Comparison of proportions was done using Fisher's exact probability and Chi-square tests. Spearman's Rank test was used for correlation between different groups of cases. A probability level of 0.05 or smaller was considered statistically significant throughout the study.

Chapter 6. Results

One hundred and twenty two archival prostatic specimens from the Anatomical and Cellular Pathology Department of the Prince of Wales of Hospital and the Pathology Laboratory of the Hong Kong Baptist Hospital in Hong Kong were examined for expression of various biological parameters by immunohistochemical technique. The biological parameters include p53 protein expression, Ki-67 antigen, Proliferative Cell Nuclear Antigen (PCNA), CD44, E-cadherin, Epidermal Growth Factor Receptor (EGFr), and the nm23-H1 polypeptide. The 122 prostatic specimens comprised of 28 prostatic hyperplasia (23%), 46 PIN lesions (38%), and 48 PCa (39%). The 46 PIN lesions were subdivided in 19, 7, and 20 cases of PIN 1, PIN 2 and PIN 3 (41%, 15% and 43%) respectively. The 48 PCa were subdivided in 13, 15 and 17 cases, respectively of low-grade, intermediate-grade and high-grade PCa. Patients' age ranged from 45 to 91 with a mean of 71. There was no significant difference in patient's age among the different sub-groups of lesions (Kruskal-Wallis, p=0.062).

I. Immunohistochemical Results for p53 Protein

The results of p53 expression for all prostatic lesions examined are summarized in Table 8. All 28 cases with prostatic hyperplasia and 46 cases of PIN lesions were negative for p53 immunostaining except for occasional equivocal weak staining accounting for less than 5% of cells. In prostatic hyperplasia, the luminal or secretory cells do not express p53, in contrast to many basal cells (Figure 18). However, not all basal cell layers demonstrated the presence of p53 positive cells,

and often both PIN lesions and their basal cell layer showed absence of p53 immunostain (Figure 19). Positive nuclear staining of p53 protein with a proportion of more than 5% of cells, graded as 2 or above, was seen in 15 of the 48 (31.2%) PCa. In most cases, cells with nuclear expression of p53 protein were scattered within the neoplastic acini or sheets, such that not all tumor cells apparently showed accumulation of p53 protein (Figure 20). Diffuse expression of p53 protein was seen in 1 case of high-grade prostatic adenocarcinoma with Gleason score 9 (Figure 21). Cytoplasmic expression of p53 was noted in some mitotic cells.

There was variation in p53 protein accumulation among the various histological types of prostatic carcinomas. While only 1 of the 13 (7.7%) low-grade PCa was immunoreactive, 31.3% and 47.4% of intermediate-grade and high-grade PCa were reactive to p53, respectively.

Table 8. p53 Protein accumulation in prostatic lesions.

Pathology	No of	Score of p53 accumulation *					p53	
	Cases	0	1	2	3	4	Pos (%)**	
BPH	28	28	0	0	0	0	0	(0.0)
PIN 1	19	19	0	0	0	0	0	(0.0)
PIN 2	7	7	0	0	0	0	0	(0.0)
PIN 3	20	18	2	0	0	0	0	(0.0)
Prostatic carcinoma								
Low-grade	13	5	7	1	0	0	1	(7.7)
Intermediate-grade	16	5	6	3	2	0	5	(31.3)
High-grade	19	4	6	4	4	1	9	(47.4.)

* p53 Score: 0: 0% positive cells; 1: 1 to 5% positive cells (interpreted as equivocal and negative); 2: 5 to 33% positive cells; 3: 34 to 66% positive cells; 4: 67 to 100% positive cells.

**p53 positive: cases with score 2 or above.

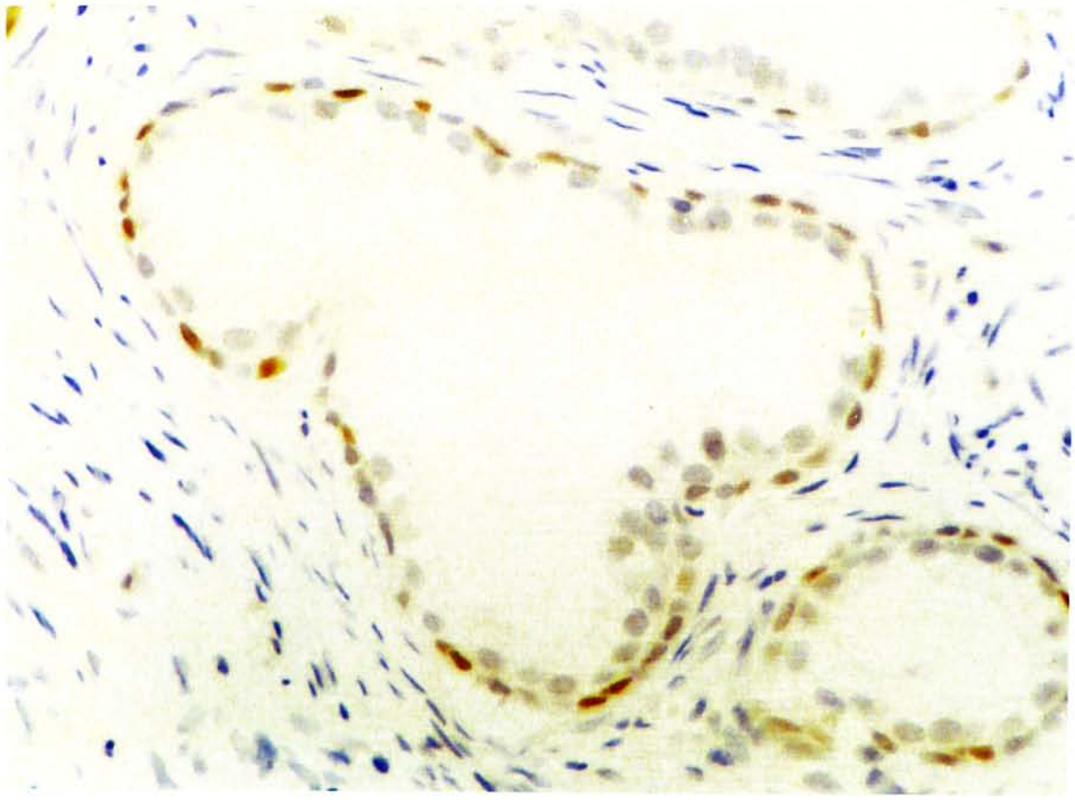


Figure 18. p53 Staining in prostatic hyperplasia with weak staining in some basal cells whereas luminal epithelial cells were negative (Immunoperoxidase method, magnification x800).

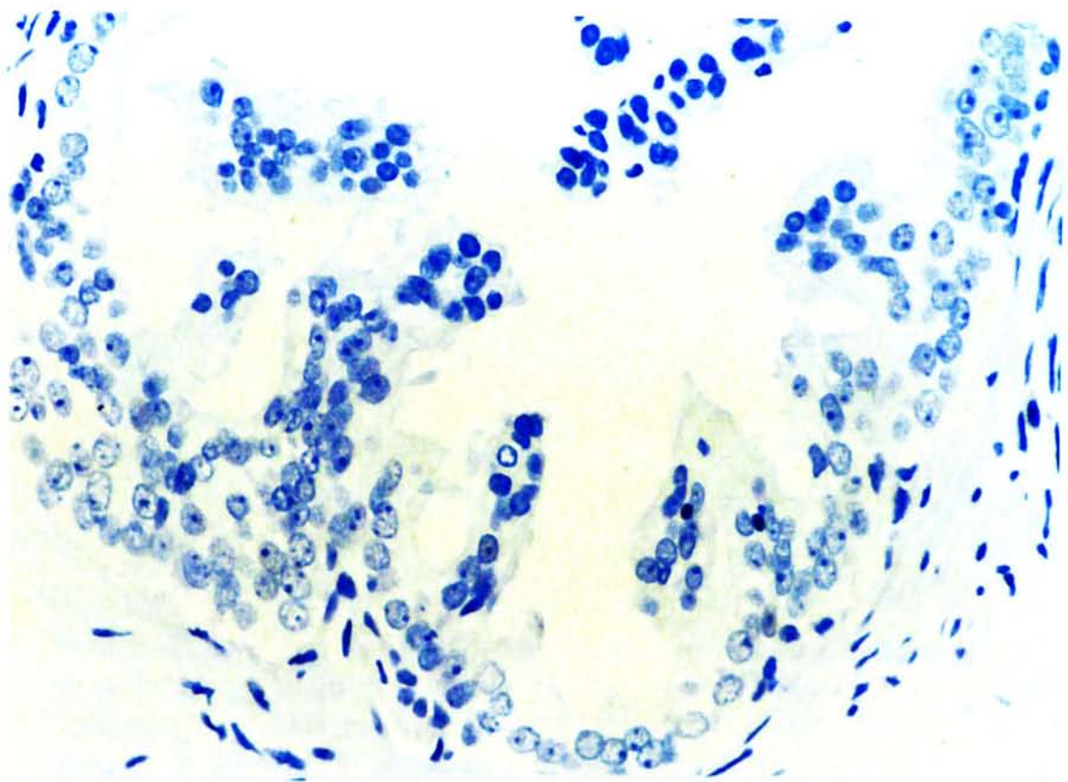


Figure 19. None of the PIN lesions demonstrate immunoreactivity for p53. (Immunoperoxidase method, magnification x800)

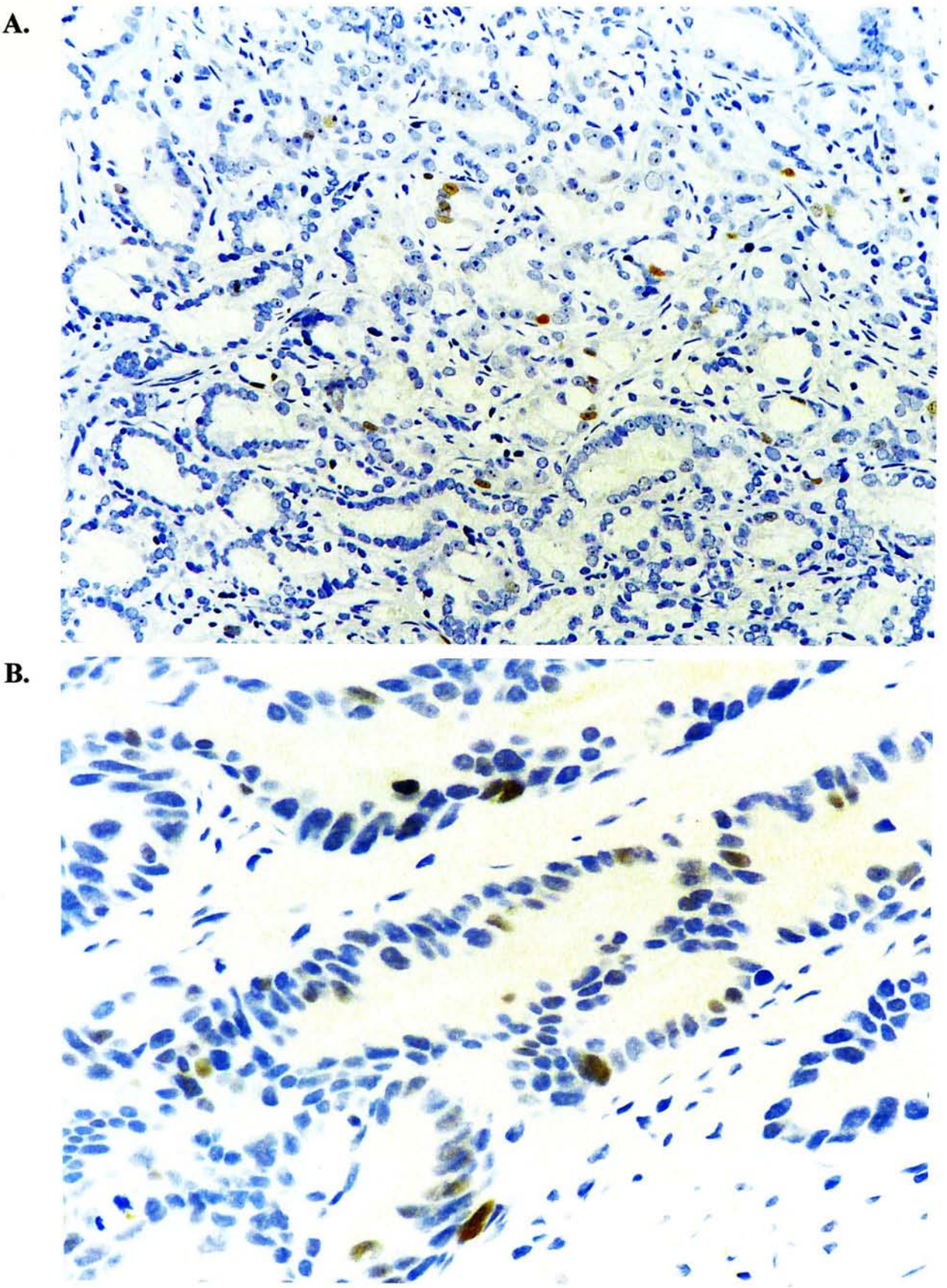


Figure 20. (**A**) Occasional p53-positive cells scattered among neoplastic glands with weak to strong staining in the neoplastic proliferations in PCa (Gleason grade 2,3). (Immunoperoxidase method, magnification x400); (**B**) More frequent p53 positive cells in neoplastic glands in PCa (Gleason grade 3,5) (Immunoperoxidase method, magnification x800)

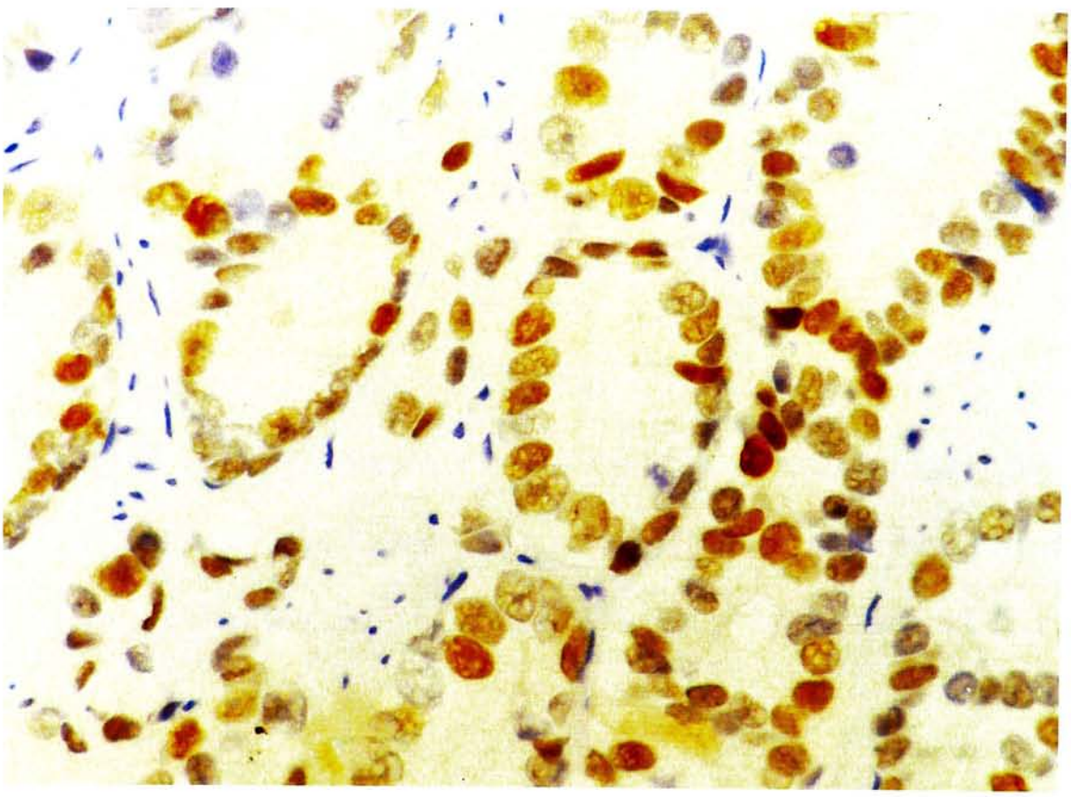


Figure 21. Diffuse staining of p53 in a case of high-grade PCa (Gleason score 9). (Immunoperoxidase method, magnification x1000)

Statistical analyses of association of p53 protein accumulation with various histological types of prostatic lesions are shown in Tables 9 to 11. p53 Protein expression was independent of patient's age. Accumulation of p53 protein in the benign lesions was negative and was, clearly and significantly lower than in the carcinomas. There was a weak association of p53 accumulation with histological grade of PCa (Spearman's rho=0.312, p<=0.031). Although there was no significant difference in p53 overexpression detected between low-grade and intermediate-grade, or intermediate-grade and high-grade PCa, p53 accumulation was significantly different between low-grade and high-grade PCa as determined by a Gleason score below or more than 6. Moreover, there was also a significant association of p53 accumulation with Gleason combined score (Gleason score less than 7 versus greater than 6, p=0.017, Mann-Whitney U). But the association of higher p53 accumulation just reached statistical significance when primary Gleason grade was considered (Gleason primary score less than 3 versus greater than 2, p=0.050, Mann-Whitney U).

Table 9. Results of univariate analysis of association of p53 overexpression in various subgroups of patients and subtypes of prostates specimens

	p53 Positive No. (%)	Mean score (SD)	Statistical Tests Results
Age			
≤ 70 (n=50)	4 (8.0)	0.30 (0.61)	Mann-Whitney U, p=0.100
> 70 (n=72)	11 (15.3)	0.61 (1.0)	Chi-square, p=0.5686
Histological Types			
Hyperplasia (n=28)	0 (0)	0 (0)	Kruskal-Wallis, p<0.001
PIN 1 (n=19)	0 (0)	0 (0)	
PIN 2 (n=7)	0 (0)	0 (0)	
PIN 3 (n=20)	0 (0)	0 (0)	
PCa (n=48)	15 (31.2)	1.19 (1.07)	
Prostatic carcinoma			
Histological grades			Kruskal-Wallis, p<0.100
Low-grade (n=13)	1 (7.7)	0.69 (0.63)	Spearman's rho 0.312, p=0.031
Intermediate-grade (n=16)	5 (31.3)	1.12 (1.02)	
High-grade (n=19)	9 (47.4)	1.58 (1.22)	
Gleason score			
≤ 6 (n=18)	2 (11.1)	0.03 (0.16)	
> 6 (n=30)	13 (43.3)	0.72 (0.83)	Mann-Whitney U, p=0.017
Primary Gleason grade			
≤ 2 (n=10)	1 (10)	1.08 (1.00)	Mann Whitney U, p=0.050
> 2 (n=37)	14 (37.8)	1.32 (1.17)	

Abbreviations: SD- standard deviation.

Table 10. Correlation of p53 accumulation in various subtypes of prostatic lesions.

Histological types	Hyperplasia	PIN1	PIN 2	PIN 3	Prostatic Carcinoma
Hyperplasia	-	n.s.	n.s.	n.s.	p<0.001
PIN 1		-	n.s.	n.s.	p<0.001
PIN 2			-	n.s.	p<0.001
PIN 3				-	p<0.001
PCa					-

Abbreviation: n.s., not statistically significant at p<0.05, 2-tailed.

Table 11. Correlation of p53 accumulation in prostatic carcinomas.

Histological types	Low-grade	Intermediate-grade	High-grade
Low-grade	-	n.s.	p=0.045
Intermediate-grade		-	n.s.
High-grade			-

Abbreviation: n.s., not statistically significant at $p<0.05$, 2-tailed.

II. Results of Immunostaining of PCNA

Intense brown nuclear staining of PCNA was seen in 99 of the 122 cases (81%) of prostatic lesions with a mean labeling index of 8% (SD 13%) positive nuclei. They were scattered among the prostatic acini or sheets of neoplastic proliferations. In general, PCNA expression was scanty in secretory cells of nodular hyperplasia, and more commonly observed in adjacent basal cells (Figure 22). PIN lesions also demonstrated variable expression from absence of staining to many immunoreactive cells (Figure 23 A,B). Carcinoma generally distinctly exhibited frequent expression of PCNA, which increased with tumor grades (Figures 24 and 25). In most cases, PCNA positive cells of varying intensity in nuclear staining were also present in the stromal tissues as well as basal epithelial cells of the prostate specimens.

The mean and standard deviations of PCNA labeling indices with strong intense nuclear staining in all prostatic lesions examined are summarized in Table 12. Positive cells with intense nuclear staining were present in 16 of the 28 cases (57%) of prostatic hyperplasia. The mean PCNA labeling index was 1.41% (SD 3.31%). Of

the 46 PIN lesions, 37 cases (80%) showed positive epithelial cells with intense nuclear staining with a mean PCNA labeling index of 2.59% (SD 3.15%). There was progressive increase in PCNA expression from PIN 1 to PIN 3 lesions. The mean PCNA labeling indices of PIN 1, PIN 2 and PIN 3 lesions were 1.31%, 1.98% and 4.03% respectively. The PCNA expression was significantly different between PIN 1 and PIN 2 ($p=0.013$), and PIN 1 and PIN 3 ($p<0.001$). But PCNA expression between PIN 2 and PIN 3 were not significantly different ($p=0.162$).

All of the 48 PCa demonstrated positive PCNA expression with a mean PCNA labeling index of 17.03% (SD 16.65%). The PCNA labeling indices increased from 6.15% (SD 3.43%) in low-grade carcinoma to 13.2% (SD 7.14%) in intermediate-grade carcinoma to 28.39% (SD 21.03%) in high-grade carcinoma.

.**Table 12.** PCNA labeling indices in various prostatic lesions

Histological Categories	No of Cases	PCNA Labeling index, % positive nuclei		
		Mean	SD	95% CI
Hyperplasia	28	1.41	3.31	-0.02 - 0.12
PIN 1	19	1.31	3.29	0
PIN 2	7	1.98	1.19	-0.53 - 2.08
PIN 3	20	4.03	3.07	-0.22 - 0.85
Prostatic carcinoma				
Low-grade	13	6.15	3.43	1.49 - 2.49
Intermediate-grade	16	13.20	7.14	1.77 - 3.88
High-grade	19	28.39	21.03	5.68 - 10.65
All groups	122	8.0	12.97	

Abbreviations: SD- standard deviation; CI- confidence interval.

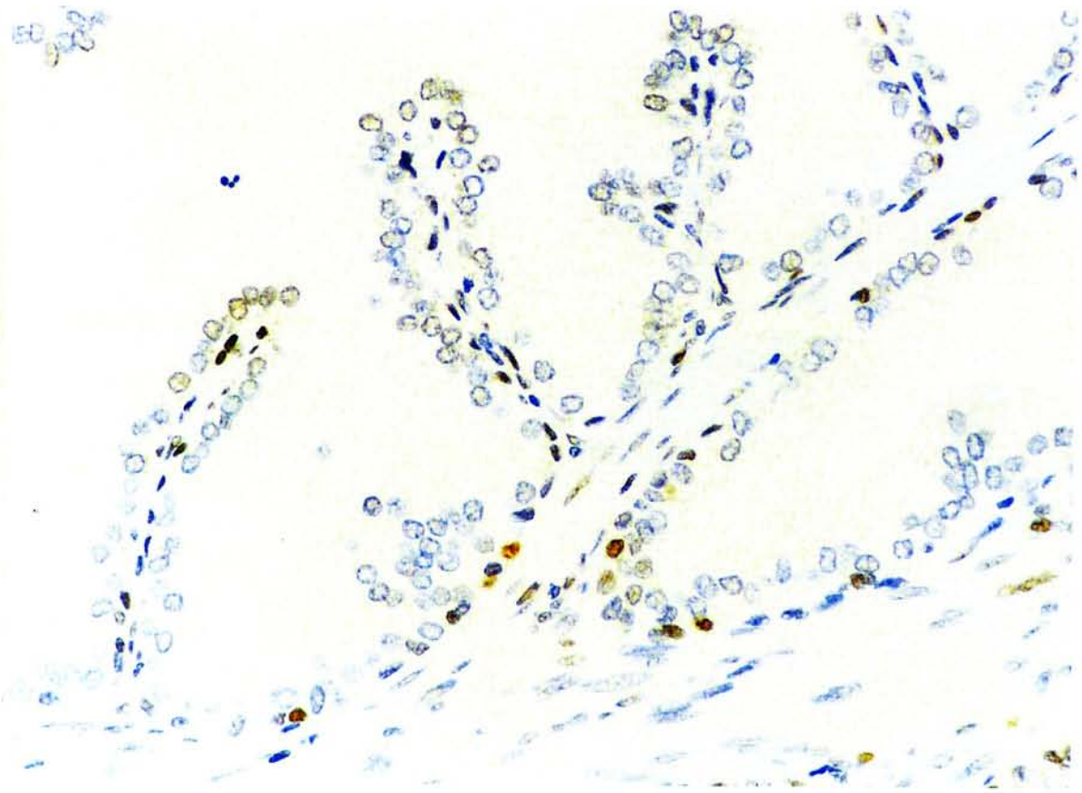
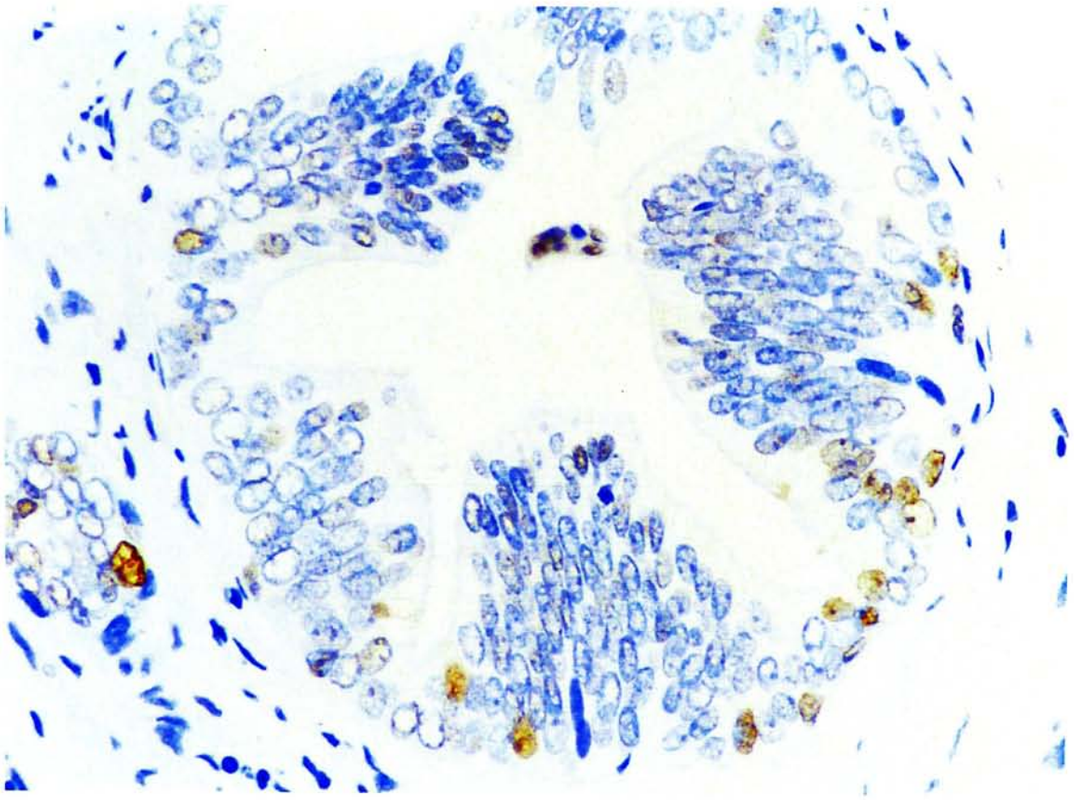


Figure 22. PCNA immunostaining in prostatic hyperplasia showing positive immunostaining in occasional basal cells but generally negative to weak staining in luminal cells (Immunoperoxidase method, magnification x600)

A.



B.

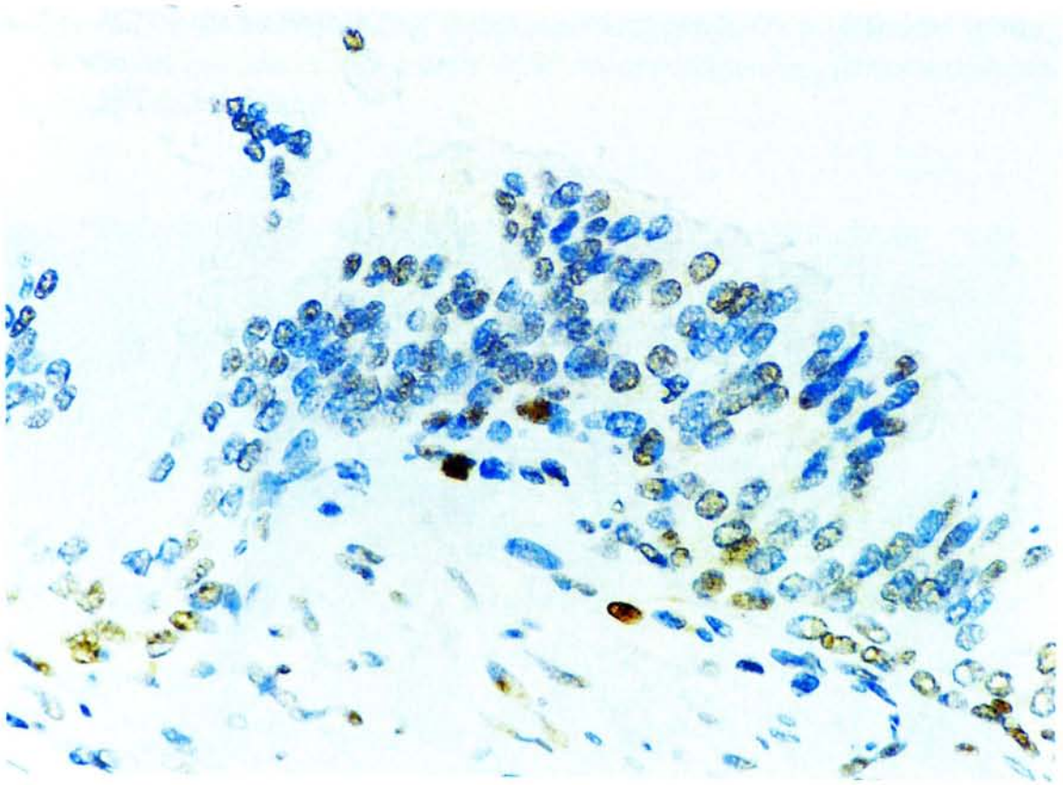


Figure 23. PCNA immunostaining in PIN 3 lesion showing occasional immunoreactive cells (**A**) to many positive cells (**B**). (Immunoperoxidase method, magnification x800)

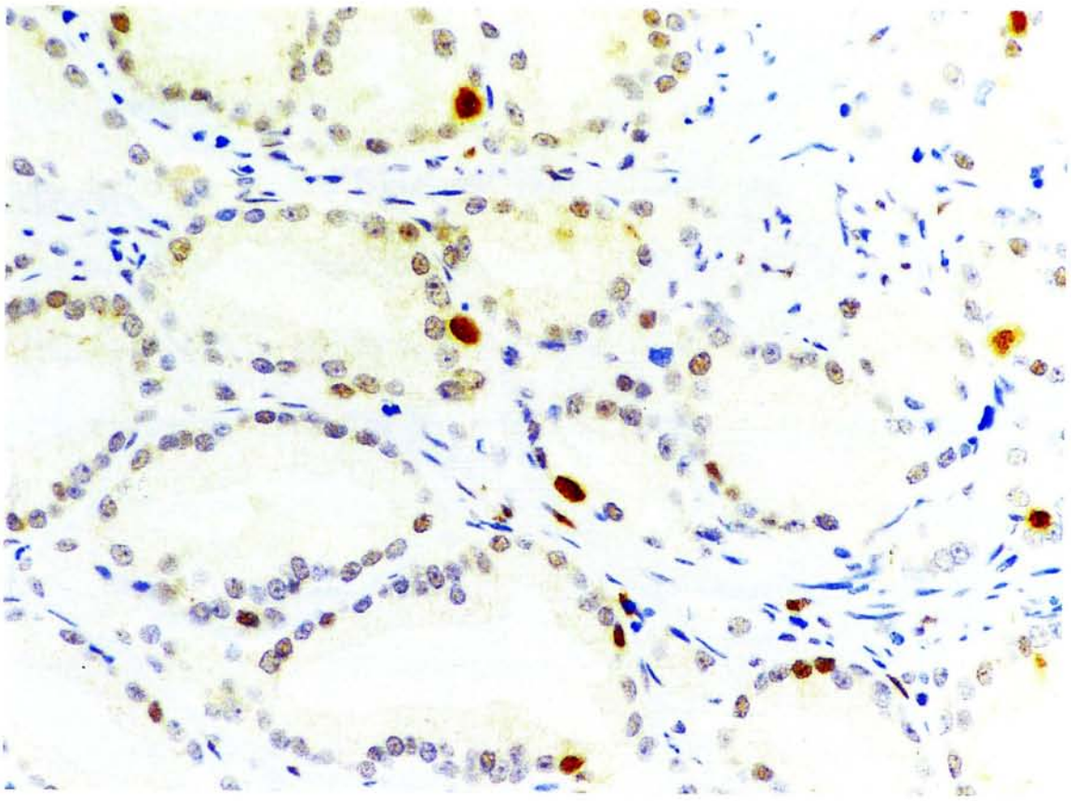


Figure 24. PCNA immunostaining in intermediate-grade PCa (Gleason grade 2,3) showing scattered immunoreactive cells with various intensities. (Immunoperoxidase method, magnification x600)

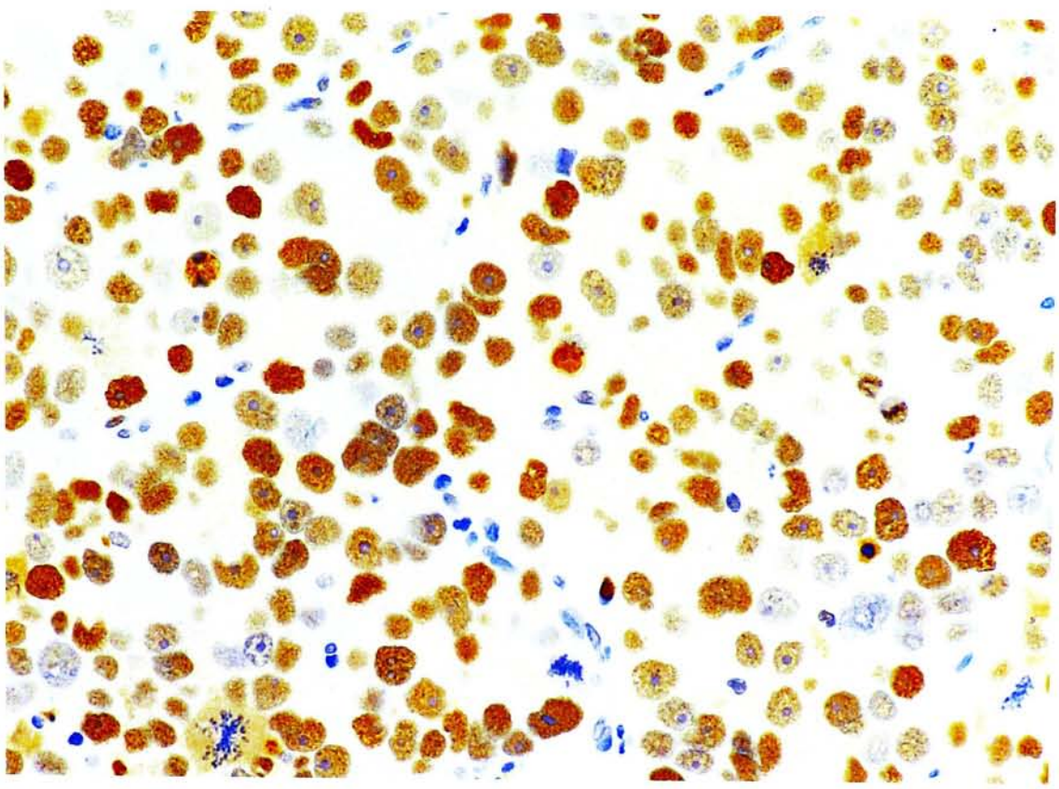


Figure 25. PCNA immunostaining in high-grade PCa (Gleason grade 4,5) showing distinctly widespread strong immunoreactivity. (Immunoperoxidase method, magnification x600)

The results of statistical tests of association of PCNA with various clinicopathological variables were summarized in Tables 13 to 15. The mean PCNA labeling indices were not correlated with patient's age. PCNA expression was strongly correlated with prostatic hyperplasia, PIN and PCa (Spearman's rho 0.786, $p<0.001$). Although no statistical difference in PCNA labeling indices was detected between hyperplasia and PIN 1 ($p=0.847$), PCNA expression was significantly different between prostatic hyperplasia and PIN 2 ($p=0.015$), or prostatic hyperplasia and PIN 3 lesions ($p<0.001$). Moreover, PCNA expression in prostatic hyperplasia or PIN lesions were significantly lower than PCa ($p<0.001$).

Among the prostatic cancers, PCNA expressions were strongly correlated with worsening of histological grade. The Spearman's rho of correlation was 0.678, $p<0.001$. PCNA expression was also associated with higher combined Gleason score (Gleason score less than 7 versus greater than 6, Mann-Whitney, $p<0.001$) and primary Gleason grade (Gleason grades 1 and 2 versus greater than 2, Mann-Whitney, $p<0.001$).

Table 13. Correlation of PCNA labeling index with subgroups of patient and histological categories and P-values generated in statistical significance tests.

	Mean PCNA Labeling index (SD)	Statistical Tests & P value
Age		
≤ 70 (n=50)	6.65 (10.51)	Mann-Whitney U, p=0.241
> 70 (n=71)	9.07 (14.58)	
Histological Types		
Hyperplasia (n=28)	1.41 (3.31)	Kruskal-Wallis, p<0.001
PIN 1 (n=19)	1.31 (3.29)	Spearman's rho 0.786, p<0.001
PIN 2 (n=7)	1.98 (1.19)	
PIN 3 (n=20)	4.03 (3.07)	
PCa (n=48)	23.16 (16.82)	
Prostatic Carcinoma		
Histological grades		Kruskal-Wallis, p<0.001
Low-grade (n=13)	6.15 (3.43)	Spearman's rho 0.678, p<0.001
Intermediate-grade (n=16)	13.20 (7.14)	
High-grade (n=19)	28.39 (21.03)	
Gleason score		
≤ 6 (n=18)	8.08 (4.73)	Mann-Whitney, p<0.001
> 6 (n=30)	23.15 (19.00)	

Abbreviations: SD- standard deviation.

Table 14. P-values of statistical significance test of association of PCNA labeling index among various histological groups of prostatic lesions (Mann-Whitney U test).

Histological types	Hyperplasia	PIN1	PIN 2	PIN 3	PCa
Hyperplasia	-	n.s.	p=0.015	p<0.001	p<0.001
PIN 1		-	p=0.013	p<0.001	p<0.001
PIN 2			-	p=0.162	p<0.001
PIN 3				-	p<0.001
PCa					-

Abbreviation: n.s., not statistically significant at p<0.05, 2-tailed.

Table 15. P-values of statistical significance test of association of PCNA labeling index in prostatic carcinomas (Mann-Whitney U test).

Histological types	Low-grade	Intermediate-grade	High-grade
Low-grade	-	$p<0.001$	$p<0.001$
Intermediate-grade		-	$p=0.010$
High-grade			-

III. Immunostaining and Quantitation of Ki-67 Expression

Ki-67 Expression was confined to the nuclei of immunoreactive cells with a focal to diffuse staining pattern. Positive cells were scattered in prostatic acini or among the neoplastic cells, but were also present in occasional basal cells of prostatic acini, stromal tissue and other tissue components. Chromosomes of mitotic cells were strongly positive for Ki-67. Immunoreactive cells for Ki-67 were generally absent in hyperplasia and PIN lesions, but were observed in high-grade PIN lesions (Figure 26) and with increasing distribution in high-grade carcinoma (Figures 27 and 28).

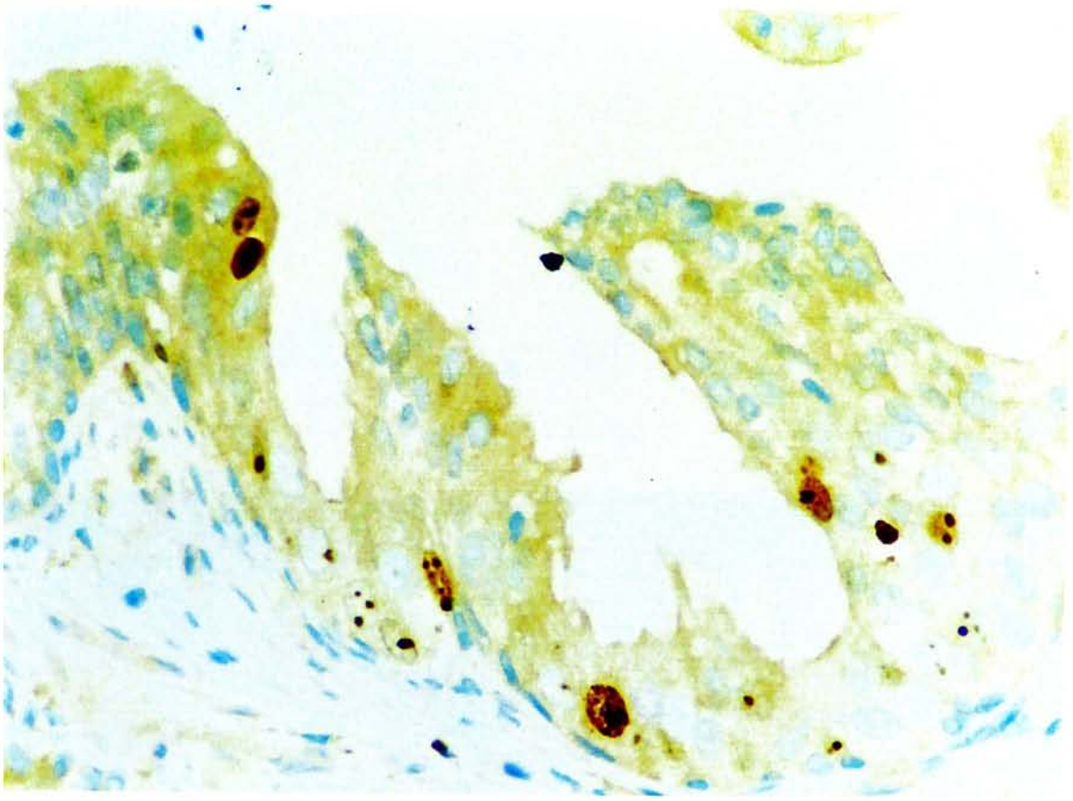


Figure 26. Ki-67 immunostaining in PIN 3 showing strong immunostaining in scattered lesional cells. (Immunoperoxidase method, magnification x800)

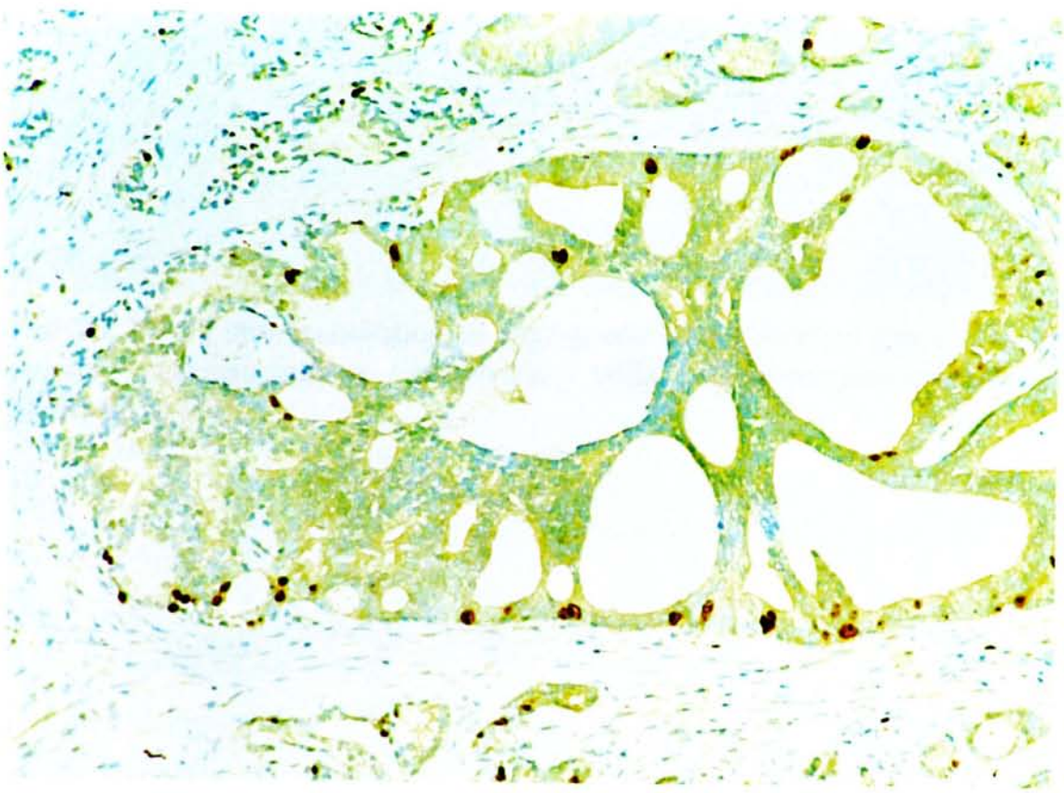


Figure 27. Ki-67 immunostaining in intermediate-grade PCa (Gleason grade 3,3) showing a few immunoreactive cells in the neoplastic cribriform glands. (Immunoperoxidase method, magnification x300)

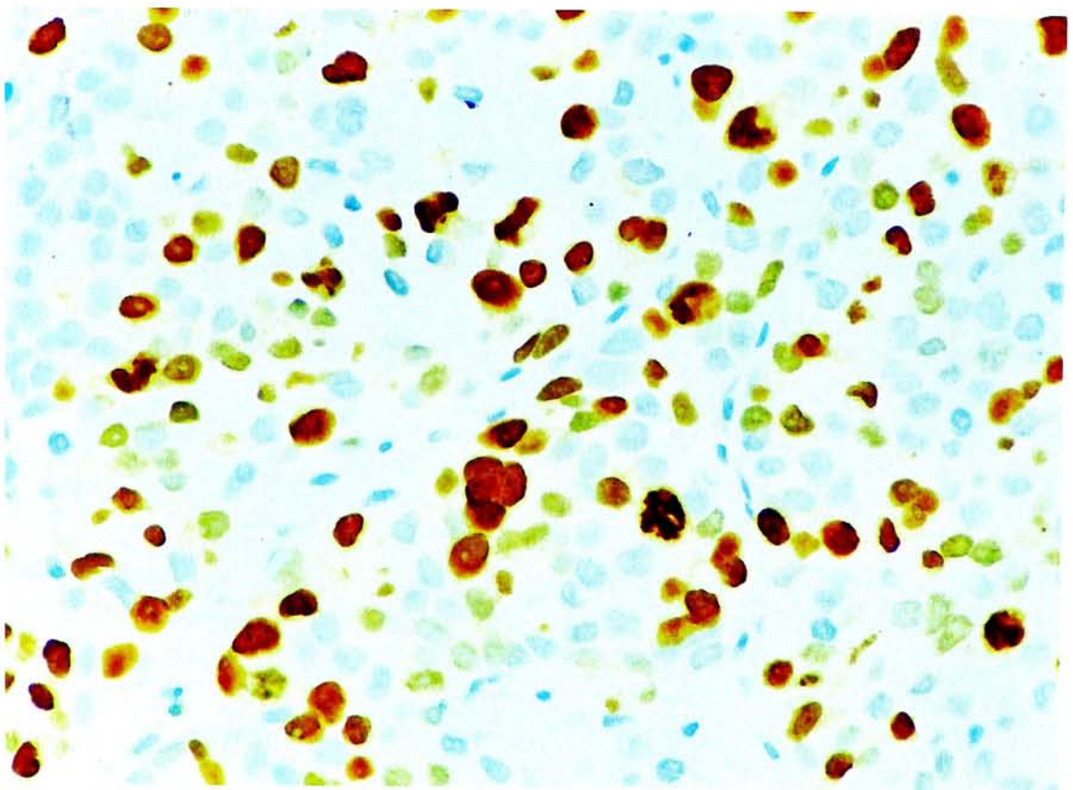


Figure 28. Ki-67 immunostaining in high-grade PCa (Gleason grade 5,4) showing numerous immunoreactive neoplastic cells. (Immunoperoxidase method, magnification x800)

The labeling indices of Ki-67 expression evaluated by manual counting and quantitative image analysis (QIA) were summarized in Table 16. The mean Ki-67 labeling indices were low in prostates with benign hyperplasia, PIN 1 and PIN 2 all with an index of less than 2%. Labeling indices of Ki-67 expression in PIN 3 lesions varied from 0 to 6.6% (QIA) and 0 to 9% (manual count). The mean labeling indices of Ki-67 expression in prostatic carcinomas as evaluated by manual method increased from 3.7% (SD 2.4%) in low-grade PCa to 6.7% (SD 5.5%) in intermediate-grade PCa to 17.4% (SD 16.0%) in high-grade PCa. A similar trend of Ki-67 expression was observed in Ki-67 labeling indices as measured by QIA. The labeling indices were 3.6% (SD 2.5%), 6.8% (SD 5.7%) and 12.9% (SD 13.6%) in low-grade, intermediate-grade, and high-grade prostatic carcinomas respectively.

Table 16. Summary of Ki-67 expression in prostate specimens.

Histological Categories	No of Cases	Manual (% positive cells)		Quantitative Image Analysis (% positive nuclear area)	
		Mean	SD	Mean	SD
Hyperplasia	28	0.03	0.10	0.37	0.59
PIN 1	19	0.14	0.39	0.25	0.40
PIN 2	7	0.27	0.41	0.99	0.86
PIN 3	20	1.16	2.07	1.50	1.68
Prostatic carcinoma					
Low-grade	13	3.66	2.40	3.55	2.51
Intermediate-grade	16	6.67	5.49	6.76	5.72
High-grade	19	17.36	16.02	12.91	13.62

Abbreviations: SD, standard deviation.

Statistical analyses of association of Ki-67 expression and various histological types of prostatic lesions are presented in Tables 17 to 21. Ki-67 expressions as measured by quantitative image analysis and manual counting were independent of patient's age. Ki-67 expression as measured with both methods was significantly associated with histological subtypes of prostate lesions with non-parametric Kruskal-Wallis test. Further evaluation showed that Ki-67 expression was strongly correlated with histological subtypes of prostate as measured by both methods. (Manual count, Spearman's rho=0.766, $p<0.001$; Quantitative image analysis, Spearman's rho=0.610, $p<0.001$). Ki-67 expression was also correlated with histological grade of PCa (Manual count, Spearman's rho=0.432, $p<0.002$; QIA, Spearman's rho=0.329, $p<0.023$). The mean Ki-67 labeling index of prostatic carcinomas with primary Gleason grades 3, 4 and 5 was significantly higher than in prostatic carcinomas with Gleason grades 1 and 2.

Moreover, Ki-67 labeling indices as measured by quantitative image analysis was strongly correlated with the results measured by manual counting. Regression analysis showed a linear relationship between the labeling indices obtained from quantitative image analysis versus manual counting with a proportional bias of 0.736 (95% confidence interval was 0.678 to 0.795; Pearson's correlation was 0.916, $p<0.001$). The proportional bias of less than 1 indicated that measurement of Ki-67 expression by quantitative image analysis always yielded a lower value than manual counting. (Figure 29)

Table 17. Expression of Ki-67 in various subgroups of patients and subtypes of prostates specimens

	Manual Counting		Quantitative Image Analysis	
	Ki-67 PI % PN (S.D)	Statistical Tests	Ki-67 PI %PNA (S.D.)	Statistical Tests
Age				
≤ 70 (n=50)	3.25 (7.22)	Mann-Whitney, p=0.269	3.47 (7.25)	Mann-Whitney, p=0.165
> 70 (n=72)	4.86 (9.99)		3.91 (7.40)	
Histological Types				
Hyperplasia (n=28)	0.03 (0.10)	Kruskal-Wallis, p<0.001	0.37 (0.59)	Kruskal-Wallis, p<0.001
PIN 1 (n=19)	0.14 (0.39)		0.25 (0.40)	
PIN 2 (n=7)	0.27 (0.41)	Spearman's rho 0.766, p<0.001	0.99 (0.86)	Spearman's rho 0.610, p<0.001
PIN 3 (n=20)	1.16 (2.07)		1.50 (1.68)	
PCa (n=48)	10.09 (12.08)		8.50 (9.97)	
Prostatic Carcinoma				
Histological grade		Kruskal-Wallis, p=0.012		Kruskal-Wallis, p=0.077
Low-grade	3.66 (2.40)		3.55 (2.51)	
Intermediate-grade	6.67 (5.49)	Spearman's rho 0.432, p=0.002	6.76 (5.72)	Spearman's rho 0.329, p=0.023
High-grade	17.36 (16.02)		12.91 (13.62)	
Gleason score				
≤ 6	4.48 (4.11)	Mann-Whitney, p=0.011	3.94 (3.17)	Mann-Whitney, p=0.024
> 6	13.45 (13.99)		10.17 (11.74)	
Primary Gleason grade				
≤ 2 (n=10)	3.20 (2.58)	Mann-Whitney, p=0.017	2.97 (2.57)	Mann-Whitney, p=0.037
> 2 (n=38)	11.9 (12.96)		10.0 (10.7)	

Abbreviations: PI- Proliferation index; PN- Positive nuclei; PNA- Positive nuclear area.

Table 18. P-values of association of Ki-67 labeling index (Manual counting) in various subtypes of prostatic lesions by Mann-Whitney U test.

Histological types	Hyperplasia	PIN1	PIN 2	PIN 3	PCa
Hyperplasia	-	n.s.	n.s.	p<0.001	p<0.001
PIN 1		-	n.s.	p=0.011	p<0.001
PIN 2			-	n.s.	p<0.001
PIN 3				-	p<0.001
PCa					-

Abbreviation: n.s., not statistically significant at p<0.05, 2-tailed.

Table 19. P-values of association of Ki-67 labeling index (QIA) in various subtypes of prostatic lesions by Mann-Whitney U test .

Histological types	Hyperplasia	PIN1	PIN 2	PIN 3	PCa
Hyperplasia	-	n.s.	n.s.	p=0.021	p<0.001
PIN 1		-	p=0.030	p=0.005	p<0.001
PIN 2			-	n.s.	p=0.005
PIN 3				-	p<0.001
PCa					-

Abbreviation: n.s., not statistically significant at p<0.05, 2-tailed.

Table 20. P-values of association of Ki-67 labeling index (Manual count) in various histological types of prostatic carcinomas by Mann-Whitney U test.

Histological types	Low-grade	Intermediate-grade	High-grade
Low-grade	-	n.s.	p=0.006
Intermediate-grade		-	n.s.
High-grade			-

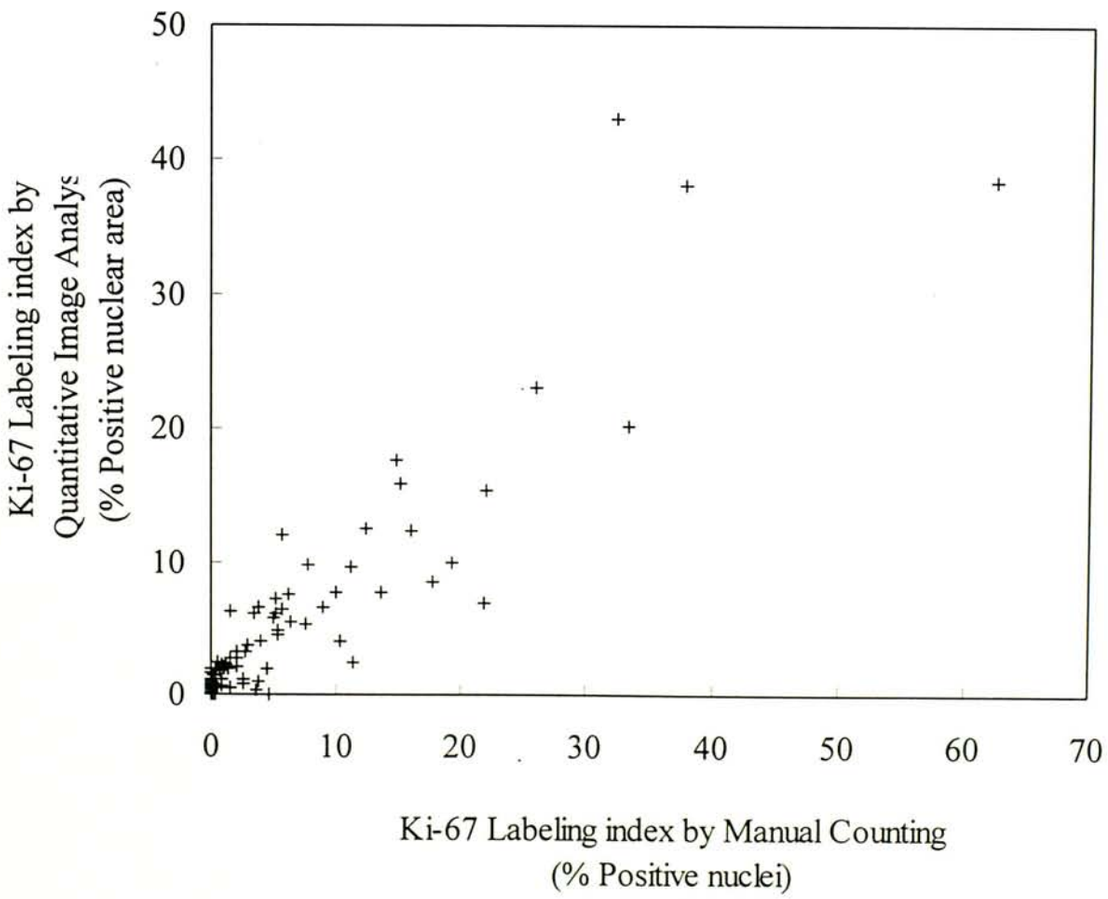
Abbreviation: n.s., not statistically significant at p<0.05, 2-tailed.

Table 21. P-values of association of Ki-67 labeling index (QIA) in various histological types of prostatic carcinomas by Mann-Whitney U test.

Histological types	Low-grade	Intermediate-grade	High-grade
Low-grade	-	n.s.	p=0.030
Intermediate-grade		-	n.s.
High-grade			-

Abbreviation: n.s., not statistically significant at $p<0.05$, 2-tailed.

Figure 29. Scatter plot of Ki-67 labeling indices by Quantitative Image Analysis versus manual counting



IV. Immunohistochemical Expression of EGFr

Positive membranous immunoreactivity for EGFr was expressed strongly in the basal cell layer of almost all hyperplastic prostatic glands as well as of glands with presence of PIN lesions. In contrast, EGFr reactivity in secretory cells in hyperplasia and PIN was much lower in staining intensity or absent. The staining of EGFr in secretory cells was membranous and confined to the basal-lateral membranes between adjacent cells, though the staining was overlapping with that of basal cells. In prostatic carcinomas, EGFr staining was identified in membranous, cytoplasmic distributions, or both, with occasional nuclear staining in some cases. EGFr was not detectable in stromal cells or other tissue elements. The results of EGFr immunostaining in all prostatic lesions examined were summarized in Table 22.

Positive staining for EGFr in the secretory cells or neoplastic cells was present in 38 of the 122 prostate specimens (31.1%). In prostatic hyperplasia, although all cases expressed strong EGFr immunoreactivity in basal cells of prostatic epithelium, only 5 out of 28 cases (17.9%) showed weak positive staining in the basal-lateral membrane of secretory cells.

Similar strong immunoreactivity of basal cells was also present in the PIN lesions. Of the 45 PIN lesions, 7 cases (15.6%) showed weak immunoreactivity in the luminal cells (Figure 30). EGFr expressions in PIN 1, PIN 2, and PIN 3 were respectively 21.1%, 28.6% and 5.0%, but no statistical difference was observed between their immunohistochemical expression of EGFr.

Table 22. EGFr Expression in prostatic glands

Pathology	No of cases	EGFr Expression *					EGFr positive **	
		0	1	2	3	Aberrant staining	+	(%)
Hyperplasia	28	23	5	0	0	0	5	(17.9)
PIN 1	19	15	4	0	0	0	4	(21.1)
PIN 2	7	5	2	0	0	0	2	(28.6)
PIN 3	20	19	1	0	0	0	1	(5.0)
Prostatic Carcinoma								
Low-grade	13	8	5	0	0	3	5	(38.5)
Intermediate-grade	16	8	7	1	0	9	8	(50.0)
High-grade	19	6	6	3	4	13	13	(68.4)
Total	122	84	30	4	4	25	38	(31.1)

* EGFr expression: 0- negative; 1- weak staining; 2- positive staining; 3- strong positive staining; Aberrant- with cytoplasmic staining.
** EGFr positive: cases with EGFr scores 1 or above.

Of the 48 PCa, EGFr immunostaining was present in 26 cases (54.2%). Although the immunostaining pattern was mainly membranous, focal cytoplasmic staining was also present in most cases (Figure 31). There was variation in staining intensity among different histological grades of PCa but the staining intensity was always lower than or similar to immunostaining of basal cells of adjacent benign epithelia. The frequency of EGFr expression increased from 38.5% (5 out of 13) low-grade carcinoma to 50% (8 out of 16) intermediate-grade carcinoma and to 68.4% (13 out of 19) high-grade carcinoma (Figures 32 and 33). A stronger intensity of EGFr staining was also noted in high-grade PCa. All 4 cases with very strong immunoreactivity were high-grade PCa.

Statistical analyses comparing various prostatic lesions, and various grade of carcinoma, are summarized in Table 23. EGFr expression in prostatic lesions was not correlated with patient's age. While there was no statistical difference in the EGFr expression between prostatic hyperplasia and PIN lesions, EGFr expression in PCa was significantly higher than both prostatic hyperplasia and PIN lesions except PIN 2 lesions. Among the PCa, EGFr expression was correlated with histological grade of prostatic carcinoma.(Spearman's rho is 0.358, $p<0.001$) and Gleason score (Mann-Whitney U, Gleason score >=6 versus >6, $p=0.008$).

Table 23. Results of univariate analysis of association of EGFr expression in various subgroups of patients and subtypes of prostates specimens

	Mean score (SD)	Statistical Tests
Age		
≤ 70 (n=50)	0.36 (0.75)	Mann-Whitney U, p=0.223
> 70 (n=72)	0.49 (0.81)	
Histological Types		
BPH (n=28)	0.18 (0.39)	Kruskal-Wallis, p<0.001
PIN 1 (n=19)	0.21 (0.42)	Spearman's rho 0.346, p<0.001
PIN 2 (n=7)	0.29 (0.49)	
PIN 3 (n=20)	0.05 (0.22)	
PCa (n=48)	0.85 (1.03)	
Prostatic carcinoma		
Histological grade		
Low-grade (n=13)	0.38 (0.51)	Kruskal-Wallis, p=0.031
Int. grade (n=16)	0.56 (0.63)	Spearman's rho 0.358, p=0.012
High-grade (n=19)	1.42 (1.31)	
Gleason Score		
≤6 (n=18)	0.33 (0.49)	Mann-Whitney U, p=0.008
>6 (n=30)	1.17 (1.15)	

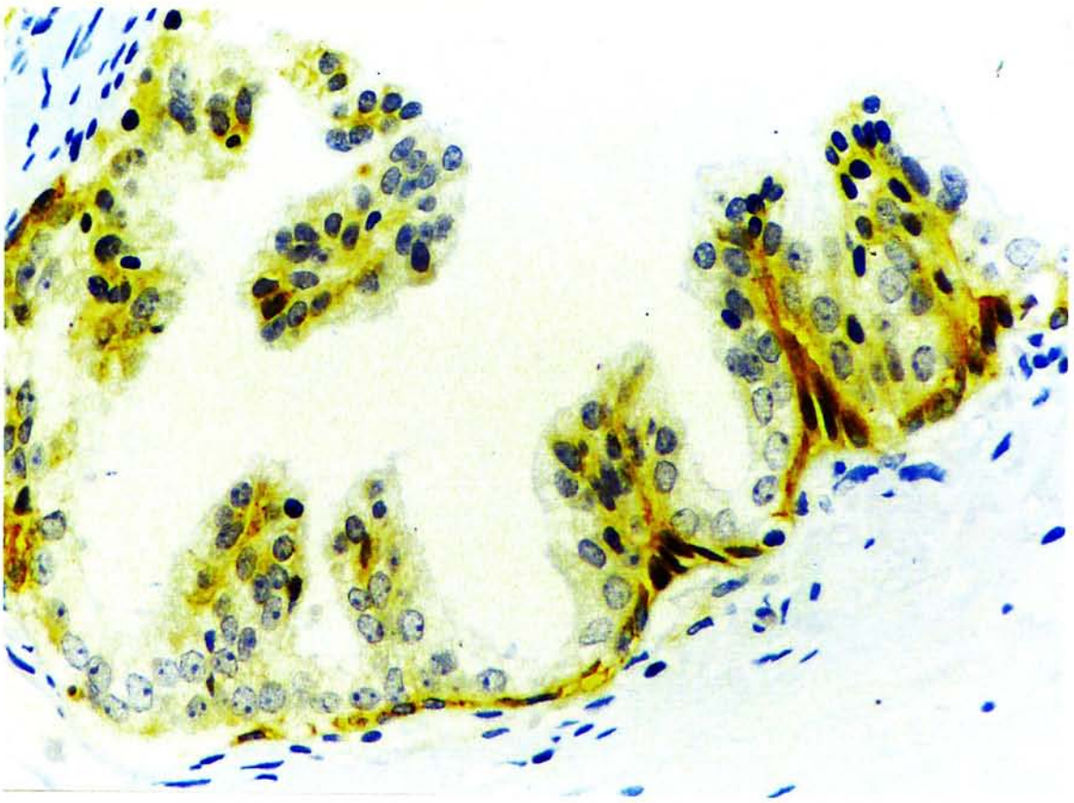


Figure 30. E-cadherin immunostaining in PIN 3 showing partial loss of membranous staining in dysplastic cells. (Immunoperoxidase method, magnification x800)

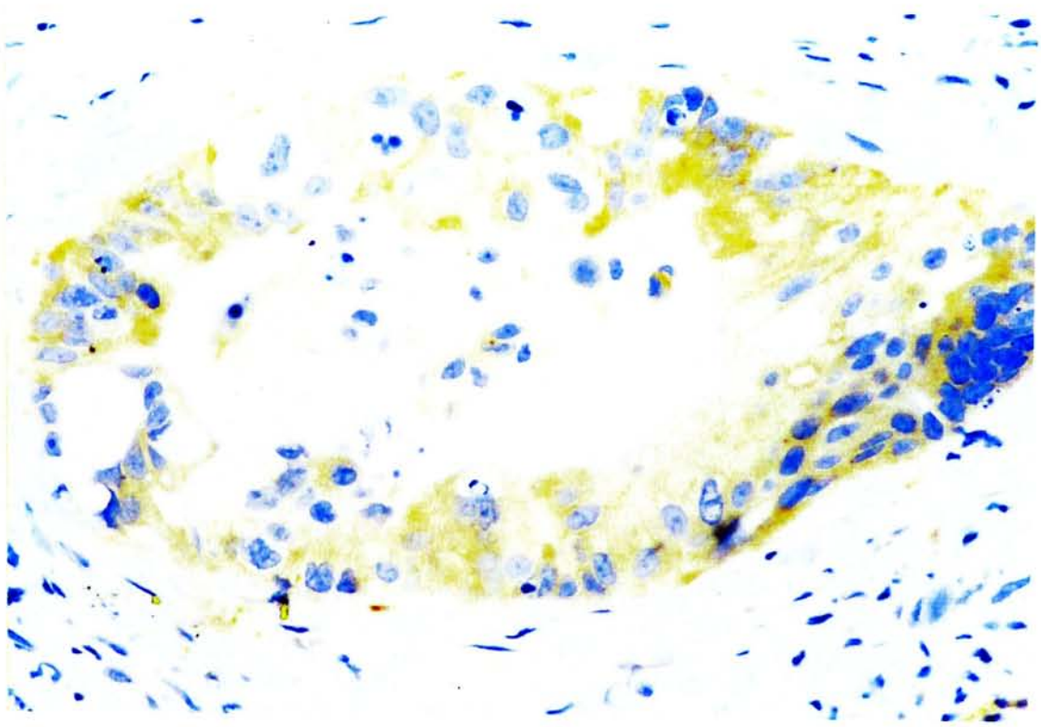


Figure 31. E-cadherin immunostaining in high-grade PCa (Gleason grade 5,4) (Immunoperoxidase method, magnification x500)

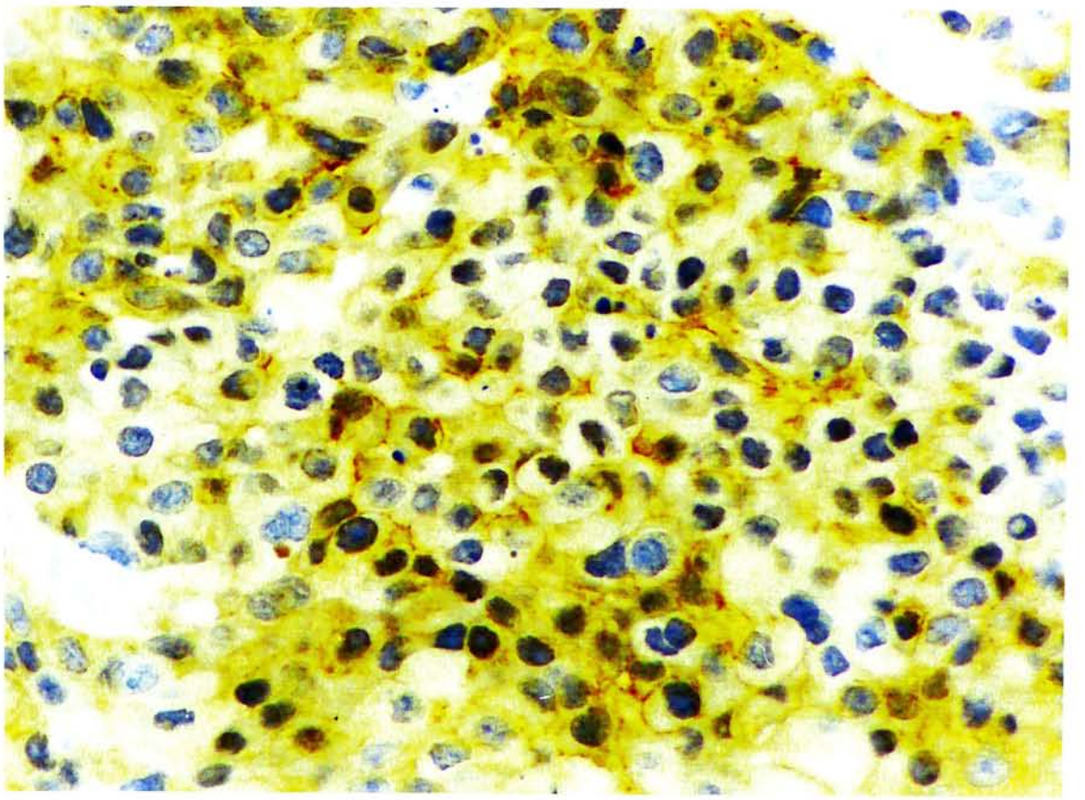


Figure 32. EGFr immunostaining in high-grade PCa (Gleason grade 4,4) showing heterogeneous expression of EGFr in neoplastic cells with both membranous and cytoplasmic staining. (Immunoperoxidase method, magnification x800)

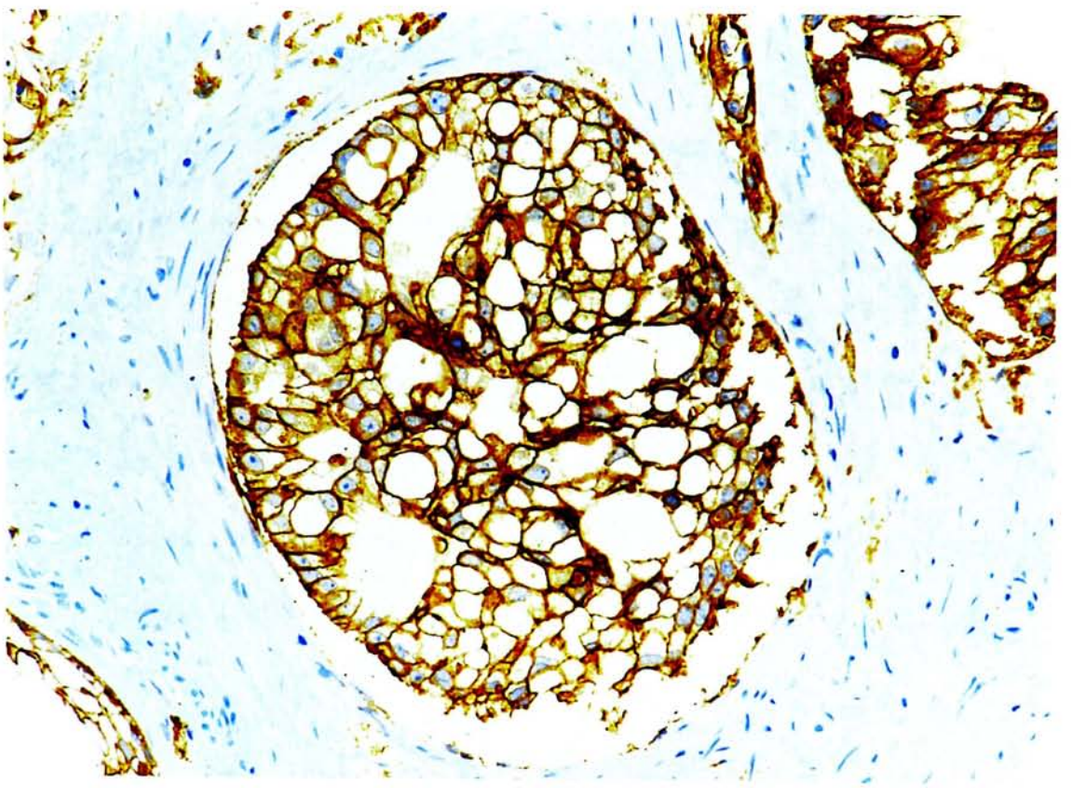


Figure 33. EGFr immunostaining in PCa (Gleason grade 5,4) showing strong membranous and focal cytoplasmic expression of EGFr in neoplastic cells. (Immunoperoxidase method, magnification x400)

V. E-Cadherin

The results of the E-cadherin immunostainings in all prostatic lesions examined are summarized in Table 24. Positive membranous immunoreactivity for E-cadherin was expressed in the secretory and basal epithelia of apparently normal glands as well as in all the proliferative glands in nodular hyperplasia. Membranous staining in luminal cells appeared to be most conspicuous in the basolateral aspects (Figure 34). However, of the 122 prostate specimens, 16 cases showed negative staining in the prostate epithelium of benign glands and were considered as a result of tissue fixation and removed from calculation of statistical analysis. Reduced expression of E-cadherin with a heterogeneous staining pattern or complete absence was present in 50 cases (47%) of the 106 remaining prostate specimens. No nuclear staining was seen but occasional cytoplasmic staining was observed in a few cases of carcinoma, representing an aberrant expression of E-cadherin. Stromal elements and vascular tissue did not show any immunoreactivity. All 25 cases of prostatic hyperplasia showed continuous membranous staining of E-cadherin in the secretory or luminal epithelium as well as basal cells of the prostatic epithelium. The prostatic epithelial cells were stained with a basolateral pattern and the luminal cell membranes were always unstained (Figure 34). Of the 36 cases of PIN lesions, 17 cases (47%) showed diffuse expression of E-cadherin (Figure 35). But the remaining 19 cases (53%) showed reduced or complete loss of expression for E-cadherin including 5 cases (42%) of PIN 1, 2 cases (33%) of PIN 2, and 12 cases (67%) of PIN 3 (Figure 36). There was no statistical difference in E-cadherin expression between PIN 1 and PIN 2, or PIN 2 and PIN 3. But PIN 1 and PIN 3 were significantly different in E-cadherin expression.

Of the 45 cases of PCa, 31 cases (69%) showed reduced or loss of E-cadherin expression including 7 cases (58%) of low-grade, 7 cases (47%) of intermediate-grade, and 17 cases (94%) of high-grade PCa (Figure 37). Correlation of loss of E-cadherin expression with tumor grade was not significant (Spearman rho=-0.270, p=0.073). Although high-grade carcinoma was associated with the lowest frequency of E-cadherin positivity and was significantly different from intermediate-grade PCa (p=0.004), its expression was not significantly different from low-grade carcinoma in E-cadherin expression. An association of loss of E-cadherin expression with high Gleason score was not observed.

Table 24. Distribution of E-cadherin protein expression in prostate lesions.

		E-cadherin Expression *			E-cadherin Loss	
Pathology	No of cases	0	1	2	Loss	(%)
Hyperplasia	25	0	0	25	0	(0.0)
PIN 1	12	0	5	7	5	(41.7)
PIN 2	6	0	2	4	2	(33.3)
PIN 3	18	5	7	6	12	(66.7)
Prostatic Carcinoma						
Low-grade	12	6	1	5	7	(58.3)
Intermediate-grade	15	3	4	8	7	(46.7)
High-grade	18	11	6	1	17	(94.4)
Total	106	25	25	56	50	(47.2)

* Extent of E-cadherin: 0 for negative or loss of immunoreactivity; 1 for heterogeneous staining or partial loss of expression; 2 for diffuse staining or normal expression.

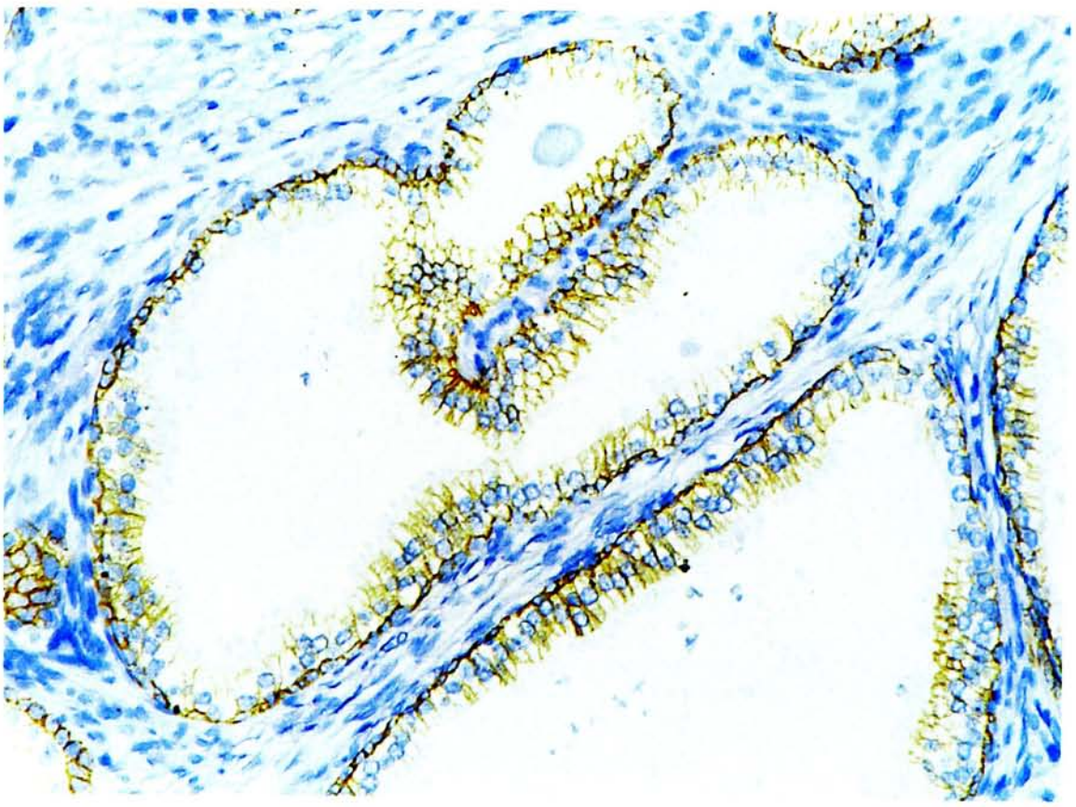


Figure 34. E-cadherin immunostaining in prostatic hyperplasia showing continuous membranous staining of basal cells, and the basolateral aspects of the luminal cells of the prostate. (Immunoperoxidase method, magnification x400)

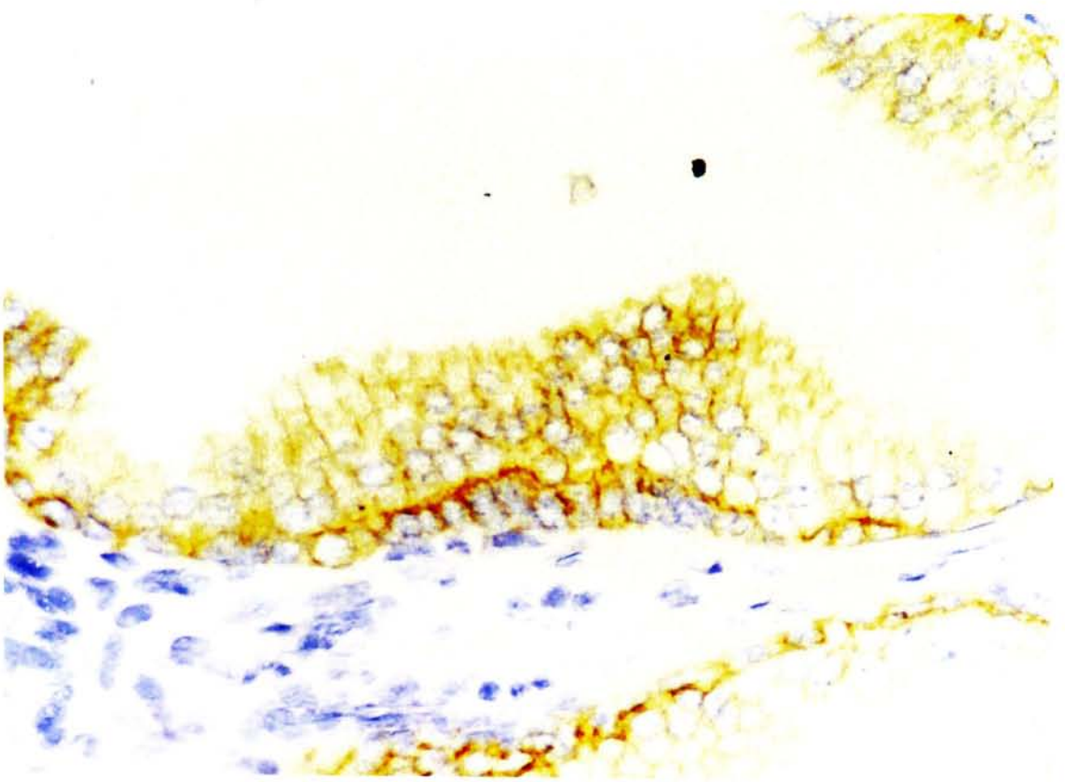


Figure 35. E-cadherin immunostaining in PIN 1 showing preserved membranous staining in basal cells and luminal cells. (Immunoperoxidase method, magnification x800)

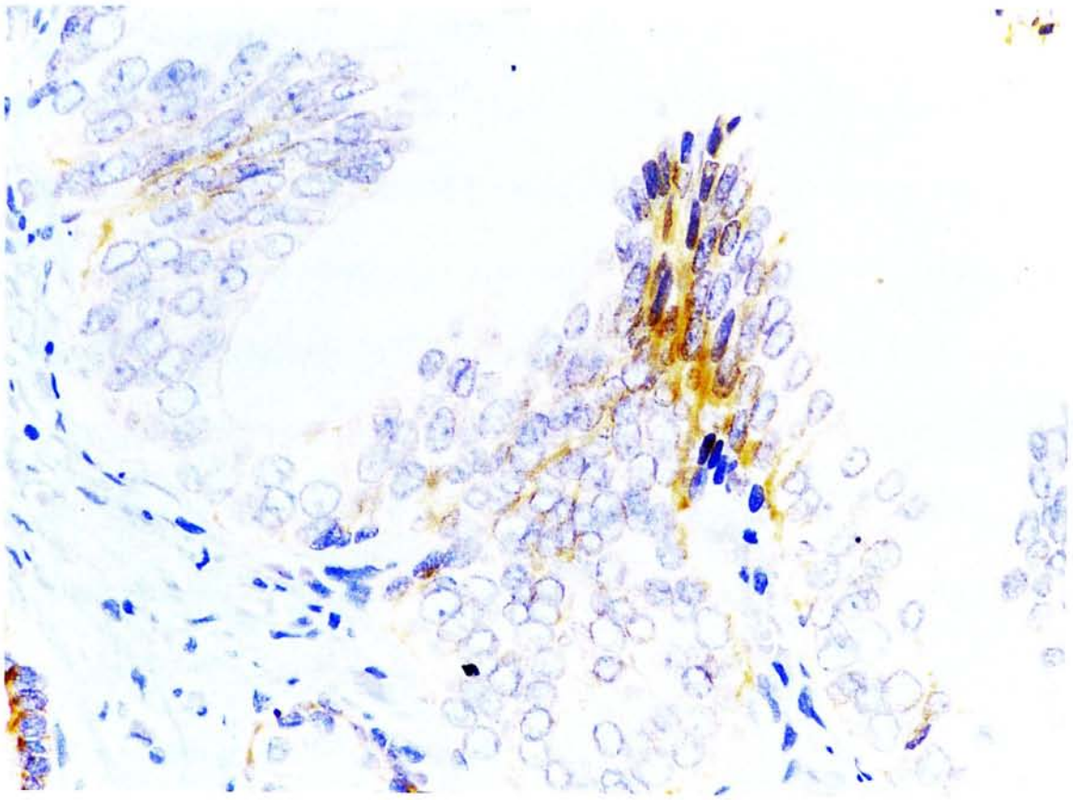


Figure 36. E-cadherin immunostaining in PIN 3 showing partial loss of membranous staining in dysplastic cells. (Immunoperoxidase method, magnification x800)

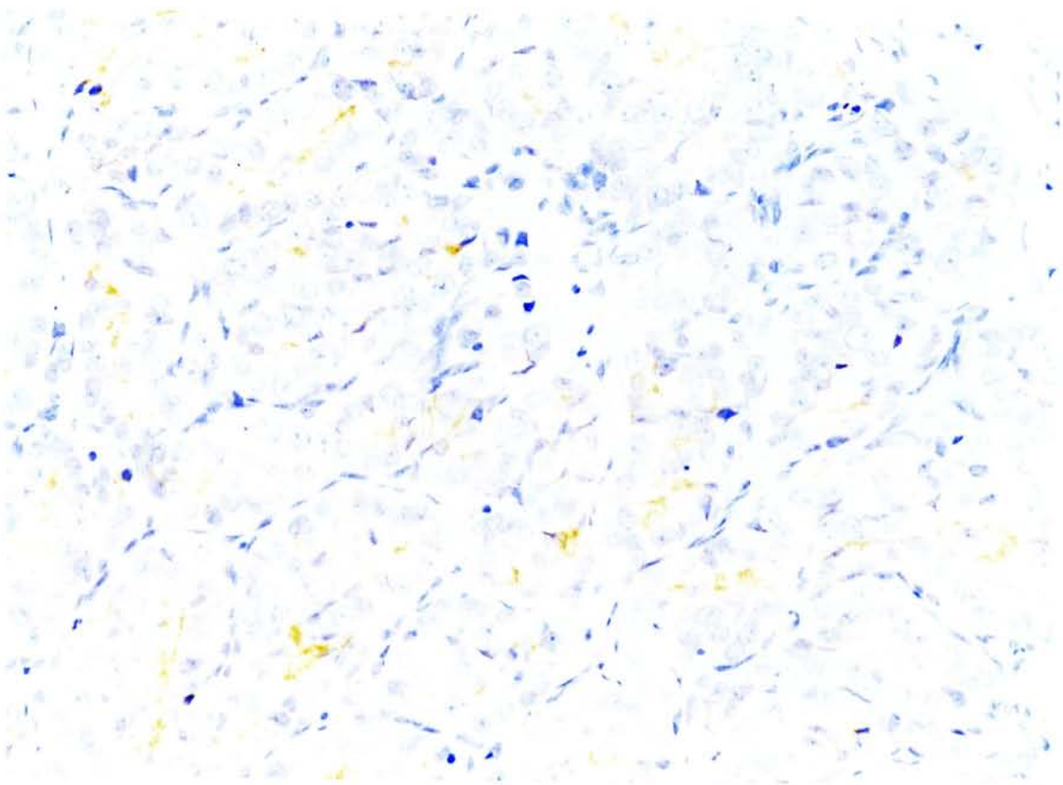


Figure 37. E-cadherin immunostaining in high-grade PCa (Gleason grade 5,4) (Immunoperoxidase method, magnification x500)

Statistical analyses comparing various prostatic lesions, and various grades of carcinoma, are summarized in Tables 26 to 28. E-cadherin expression in the prostate lesions was associated with patient's age (age >70 verses ≤ 70, p=0.004). There was a significant correlation of reduced E-cadherin expression from prostatic hyperplasia to PIN lesions to PCa (Spearman rho=-0.553, p<0.001). Although E-cadherin expression in prostatic hyperplasia was not significantly different from PIN 2 lesions, there was significant loss of E-cadherin expression in PIN 3 and PCa as compared to prostatic hyperplasia. Moreover, PIN 3 and PIN 2 were not significantly different from PCa in E-cadherin while PIN 1 showed significantly higher E-cadherin expression than PCa (p=0.001).

Table 25. Results of univariate analysis of association of E-Cadherin expression in various subgroups of patients and subtypes of prostates specimens

	Mean score (SD)	Statistical Tests
Age		
≤ 70 (n=50)	1.58(0.69)	Mann-Whitney U, p=0.002
> 70 (n=72)	1.08(0.86)	
Histological Types		
Hyperplasia (n=25)	2.00(0)	Kruskal-Wallis, p<0.001
PIN 1 (n=12)	1.58(0.51)	Spearman's rho=-0.553, p<0.001
PIN 2 (n=6)	1.67(0.52)	
PIN 3 (n=18)	1.06(0.80)	
PCa (n=45)	0.87(0.87)	
Prostatic carcinoma		
Histological grade		
Low-grade (n=12)	0.92(1.00)	Kruskal-Wallis, p=0.009
Int. grade (n=15)	1.33(0.82)	Spearman's rho= - 0.270, p=0.073
High-grade (n=18)	0.44(0.62)	
Gleason Score		
≤ 6 (n=17)	1.06(0.97)	Mann-Whitney U, p=0.171
> 6 (n=28)	0.75(0.80)	

Table 26. P-values of association of E-cadherin expression in various subtypes of prostatic lesions.(Mann-Whitney U test)

Histological types	Hyperplasia	PIN1	PIN 2	PIN 3	PCa
Hyperplasia	-	p=0.041	n.s.	p<0.001	p<0.001
PIN 1		-	n.s.	n.s.	p=0.010
PIN 2			-	n.s.	p=0.047
PIN 3				-	n.s.
PCa					-

Abbreviation: n.s., not statistically significant at p<0.05, 2-tailed.

Table 27. P-values of association of E-cadherin expression in various histological types of prostatic carcinomas. (Mann-Whitney U test)

Histological types	Low-grade	Intermediate-grade	High-grade
Low-grade	-	n.s.	n.s.
Intermediate-grade		-	p=0.004
High-grade			-

Abbreviation: n.s., not statistically significant at p<0.05, 2-tailed.

VI. CD44

Positive membranous immunostaining of CD44 protein was present in 57 of the 122 cases (47%) of all the prostatic lesions examined. In the secretory layer of prostatic epithelium, positive staining was confined to the basolateral surface of the secretory cells. The luminal apical surface was always negative for CD44 protein staining. While there was variation in distribution of CD44 staining in the secretory cells, the basal cells in prostatic glands were always strongly stained (Figure 38).

The results of immunostaining for CD44 protein expression are summarized

in Table 28. Of the 28 prostatic hyperplasia, positive CD44 staining was present in 19 cases (67.9%). Among them, 29% showed continuous membranous staining, 39% showed discontinuous membranous staining in the basolateral membranes of adjacent luminal cells (Figure 38). No staining was observed in the rest of specimens except for strong CD44 expression at the adjacent membranes between the basal cells and luminal cells.

Table 28. Distribution of CD44 protein expression in prostate lesions.

		Extent of CD44 Expression*				CD44	
Pathology	No of cases	0	1	2	3	Pos	(%)
Hyperplasia	28	9	8	3	8	19	(67.9)
PIN 1	19	5	4	4	6	14	(73.7)
PIN 2	7	2	2	2	1	5	(71.4)
PIN 3	20	12	5	1	2	8	(40.0)
Prostatic Carcinoma							
Low-grade	13	6	2	2	3	7	(53.8)
Intermediate-grade	16	13	2	0	1	3	(18.8)
High-grade	19	18	0	1	0	1	(5.6)
Total	122	65	23	13	21	57	(46.7)

* Extent of CD44: 0- negative; 1- 0 to 33% positive cells; 2- > 33% to 66% positive cells; 3- > 66% to 100% positive cells.

Of the 46 PIN lesions, 27 cases (59%) showed positive membranous staining for CD44 immunoreactivity in the luminal cells of the dysplastic glands (Figure 38). These 27 PIN lesions were distributed in 73.7%, 71.4% and 40% of respectively PIN 1, PIN 2 and PIN 3 lesions. Among them, continuous membranous staining was present in 32%, 14% and 10% of PIN 1, PIN 2 and PIN 3 lesions respectively. Although the frequency and distribution of CD44 expression was not significant different between PIN 1 and PIN 2, or PIN 2 and PIN 3, CD44 expression in PIN 3 was significantly lower than in PIN 1 ($p=0.026$).

Positive staining was present in 11 of the 48 (23%) PCa with 8% showing diffuse staining in the sheets of neoplastic cells (Figure 39). CD44 expression was inversely correlated with histological grade of the PCa (Spearman's rho=-0.433, $p=0.002$). The frequency of CD44 immunoreactivity reduced from 7 out of 13 cases (54%) of low-grade PCa, to 3 out of 16 cases (19%) of intermediate-grade PCa, to 1 out of 19 cases (5.6%) of high-grade PCa (Figure 40).

Statistical analyses comparing CD44 expression in various prostatic lesions, and various grade of carcinoma, are summarized in Tables 30 to 32. There was an inverse correlation of the CD44 protein expression with histological categories from benign hyperplasia, PIN, and PCa (Spearman's rho=-0.475, $p<0.001$). While there was no statistical difference in CD44 expression among prostatic hyperplasia, PIN.1 and PIN 2 lesions, CD44 expression in these lesions were significantly higher than in PCa (prostatic hyperplasia versus PCa, $p<0.001$; PIN 1 verses PCa, $p<0.001$; PIN 2 versus PCa, $p=0.043$). However, CD44 immunoreactivity in PIN 3 was not significantly different from PCa ($p=0.195$).

Table 29. Results of univariate analysis of association of CD44 expression in various subgroups of patients and subtypes of prostates specimens

	Mean score (SD)	Statistical Tests
Age		
≤ 70 (n=50)	0.54 (0.61)	Mann-Whitney U, p=0.423
> 70 (n=72)	0.68 (0.77)	
Histological Types		
Hyperplasia (n=28)	1.04 (0.74)	Kruskal-Wallis, p=0.001
PIN 1 (n=19)	0.95 (0.62)	Spearman's rho -0.475, p<0.001
PIN 2 (n=7)	0.86 (0.69)	
PIN 3 (n=20)	0.45 (0.60)	
PCa (n=48)	0.29 (0.58)	
Prostatic carcinoma		
Histological grade		
Low-grade (n=13)	0.69 (0.75)	Kruskal-Wallis, p=0.007
Int. grade (n=16)	0.19 (j0.40)	Spearman's rho -0.433, p=0.002
High-grade (n=19)	0.11 (0.46)	
Gleason Score		
≤ 6 (n=18)	0.61 (0.70)	Mann-Whitney U, p=0.001
> 6 (n=30)	0.10 (0.40)	

Table 30. P-values of association of CD44 expression in various subtypes of prostatic lesions. (Mann-Whitney U test)

Histological types	Hyperplasia	PIN1	PIN 2	PIN 3	PCa
Hyperplasia	-	n.s.	n.s.	p=0.005	p<0.001
PIN 1		-	n.s.	p=0.026	p<0.001
PIN 2			-	n.s.	p=0.043
PIN 3				-	n.s.
PCa					-

Abbreviation: n.s., not statistically significant at p<0.05, 2-tailed.

Table 31. P-values of association of CD44 expression in various histological types of prostatic carcinomas. (Mann-Whitney U test)

Histological types	Low-grade	Intermediate-grade	High-grade
Low-grade	-	n.s.	p=0.027
Intermediate-grade		-	n.s.
High-grade			-

Abbreviation: n.s., not statistically significant at p<0.05, 2-tailed.

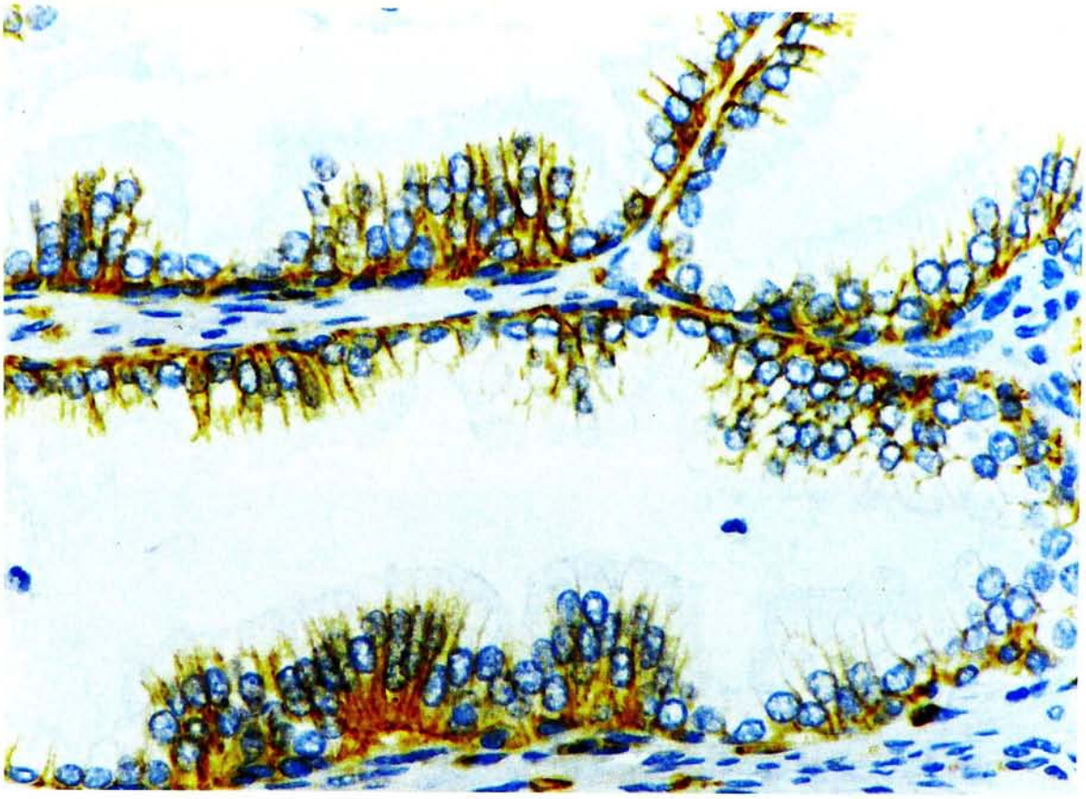


Figure 38. CD44 immunostaining in PIN 1 lesion showing strong expression of CD44 in the basolateral wall of luminal cells. Adjacent normal secretory cells also show similar expression. (Immunoperoxidase method, magnification x800)

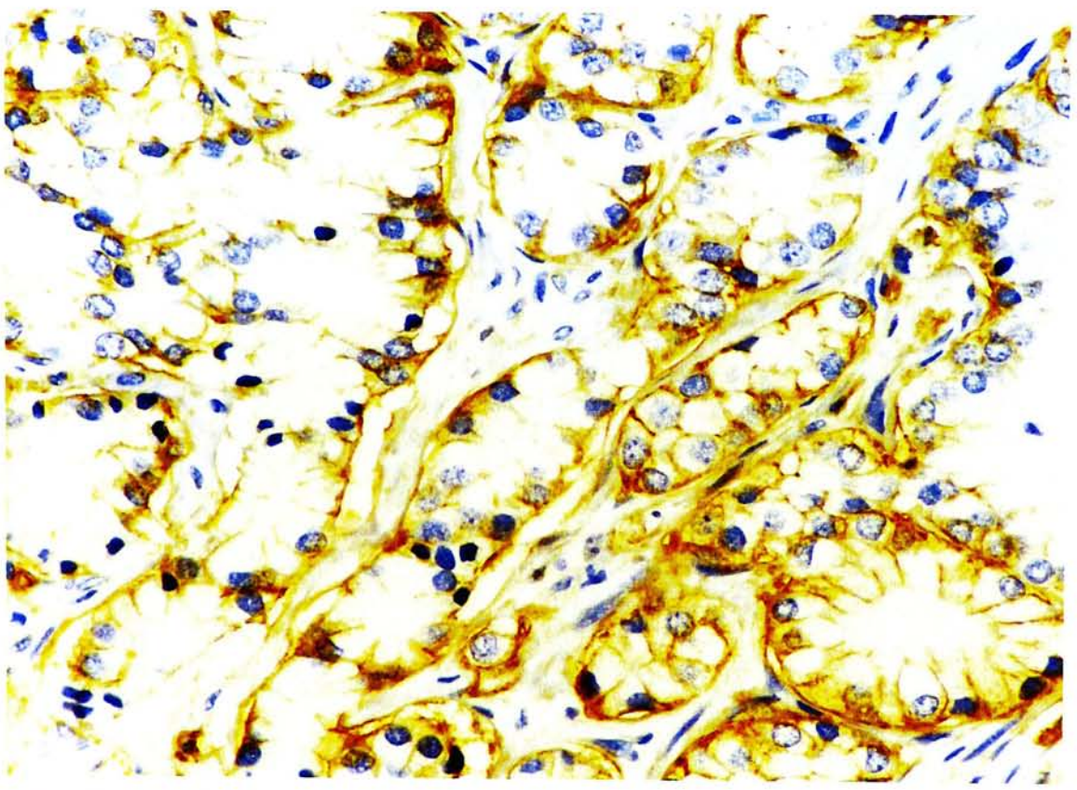


Figure 39. CD44 immunostaining in PCa (Gleason grade 2,3), showing strong expression of CD44 with preserved basolateral distribution in neoplastic cells but heterogeneous expression in the lateral borders of adjacent cells. (Immunoperoxidase method, magnification x800)

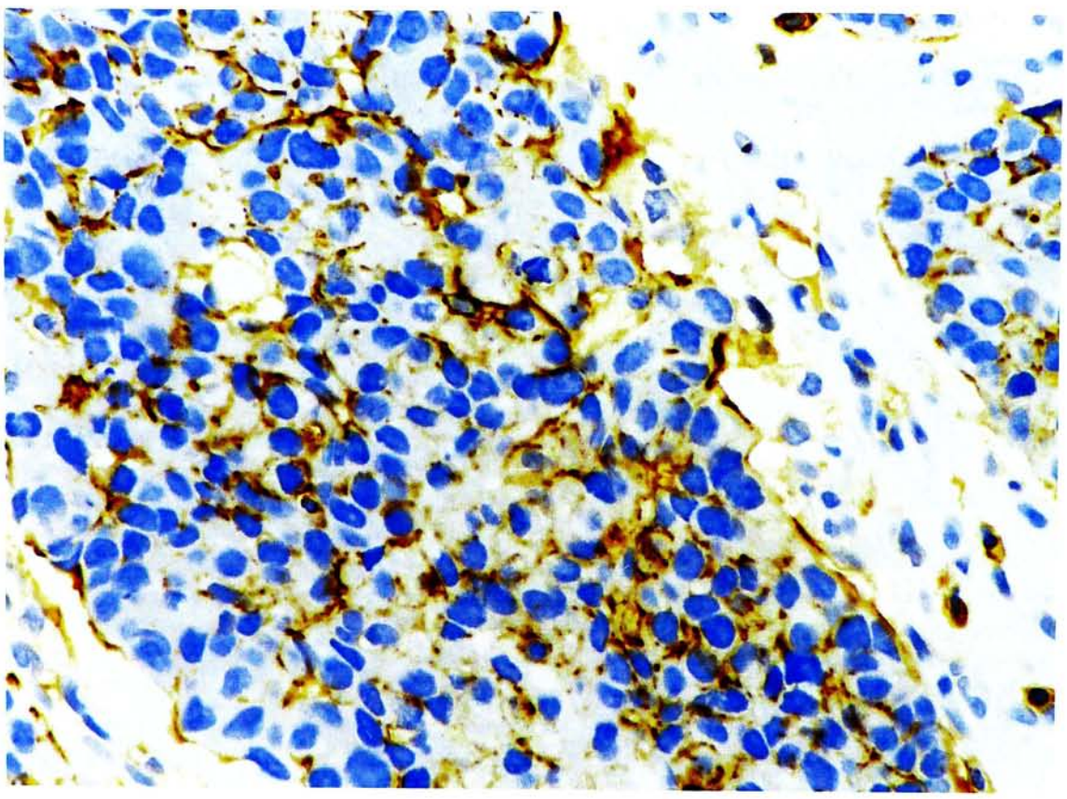


Figure 40. CD44 immunostaining in PCa (Gleason grade 4,4), showing heterogeneous or reduced expression of CD44. (Immunoperoxidase method, magnification x800)

VII. Expression of nm23 in Prostatic Lesions

The results of nm23 immunostaining of all prostatic cases examined are summarized in Table 32. The nm23 immunoreactivity was mainly expressed as cytoplasmic staining, but cell membrane might also demonstrate strong immunostain. The stromal muscle and connective tissues were consistently negative for nm23 expression. Positive nm23 immunostaining was observed in the prostatic glandular acini and neoplastic sheets of most of the prostate cases examined, though with a wide variation in staining intensity, but basal cells of benign prostatic glands might also be strongly positive. The expression of nm23 was shown to be independent of the patient's age.

Table 32. nm23 Expression in various histological types of prostatic lesions.

Pathology	No of	nm23 score *			nm23 positive**	
	Cases	1	2	3	Pos	(%)
Hyperplasia	28	17	10	1	11	(39.3)
PIN 1	19	5	5	9	14	(73.7)
PIN 2	7	1	3	3	6	(85.7)
PIN 3	20	1	5	14	19	(95.0)
Prostatic carcinoma						
Low-grade	13	0	2	11	13	(100)
Intermediate-grade	16	0	4	12	16	(100)
High-grade	19	0	8	11	19	(100)
Total	122	24	37	61	98	(80)

* nm23 Score: 1- weak, equivocal staining; 2- positive staining; 3- strong staining.
** nm23 Positive: cases with positive or strong positive staining (scores 2 and 3).

Of the 122 cases examined, 98 (80%) showed positive expression for nm23. In prostatic hyperplasia, expression of nm23 was clearly positive in the cytoplasm of 11 out of 28 cases (39%), but none exhibited a strong immunoreactivity (Figure 41). The remaining 17 cases (61%) demonstrated a weak and equivocal staining, and interpreted as background staining and negative.

Of the 46 PIN lesions, positive cytoplasmic staining were observed in 39 cases (85%), which were more or less distributed evenly among different grades of PIN lesions (74%) of PIN 1, 86% of PIN 2 (Figure 41), and 95% of PIN 3. However, among these PIN lesions expressing nm23, 26 (57%) demonstrated strong cytoplasmic immunostaining, distributed in 47%, 43%, and 70% of respectively PIN 1, PIN 2, and PIN 3 lesions (Figure 42). The frequency of such a nm23 expression was not significantly different between PIN 1 and PIN2, or between PIN 2 and PIN3.

All 48 PCa expressed nm23 but 71% of these demonstrated a strong cytoplasmic expression of nm23 (Figures 43 and 44). Furthermore, the staining pattern reflected the heterogeneous expression of nm23, with the mixture of cells with strong and weak immunoreactivity, with scattered tumor cells with strong signals intermingled with other tumor cells or sheets. However, no significant difference in nm23 expression was detected among different histological grades of PCa based on the Gleason score, and among different grades based on the Gleason grades (Table 33).

Statistical analyses comparing various prostatic lesions, and various grade of carcinoma, are summarized in Tables 34 to 36. There was a significant difference in the frequency of expression of nm23 between prostatic hyperplasia and both PIN lesions and PCa. The frequency of expression was significantly higher in PIN lesions

and PCa (Mann-Whitney U test, $p<0.001$). However, no significant difference was found in the expression of nm23 between PIN lesions and carcinoma (Mann-Whitney U, $p=0.064$). But, there was a significant correlation observed between stronger nm23 immunostaining and prostatic lesion from benign hyperplasia to PIN and to PCa. (Spearman's rho 0.585, $p<0.001$)

Table 33. Results of univariate analysis of association of nm23 expression in various subgroups of patients and subtypes of prostates specimens

	Mean score (SD)	Statistical Tests
Age		
≤ 70 (n=50)	2.16 (0.84)	Mann-Whitney U, p=0.120
> 70 (n=72)	2.40 (0.73)	
Histological Types		
Hyperplasia (n=28)	1.43 (0.57)	Kruskal-Wallis, p<0.001
PIN 1 (n=19)	2.21 (0.85)	Spearman's rho 0.585, p<0.001
PIN 2 (n=7)	2.29 (0.76)	
PIN 3 (n=20)	2.65 (0.59)	
PCa (n=48)	2.71 (0.46)	
Prostatic carcinoma		
Histological grade		
Low-grade (n=13)	2.85 (0.38)	Kruskal-Wallis, p=0.245
Int.-grade (n=16)	2.75 (0.45)	Spearman's rho -0.243, p=0.096
High-grade (n=19)	2.58 (0.51)	
Gleason Score		
≤ 6 (n=18)	2.83 (0.38)	Mann-Whitney U, p=0.144
> 6 (n=30)	2.63 (0.49)	

Table 34. P-values of association of nm23 expression in various subtypes of prostatic lesions (Mann-Whitney U test).

Histological types	Hyperplasia	PIN1	PIN 2	PIN 3	PCa
Hyperplasia	-	p=0.002	p=0.013	p<0.001	p<0.001
PIN 1		-	n.s.	n.s.	p=0.019
PIN 2			-	n.s.	n.s.
PIN 3				-	n.s.
PCa					-

Abbreviation: n.s., not statistically significant at p<0.05, 2-tailed.

Table 35. P-values of association of nm23 expression among various histological grades of prostatic carcinomas (Mann-Whitney U test).

Histological types	Low-grade	Intermediate-grade	High-grade
Low-grade	-	n.s.	n.s.
Intermediate-grade		-	n.s.
High-grade			-

Abbreviation: n.s., not statistically significant at p<0.05, 2-tailed.

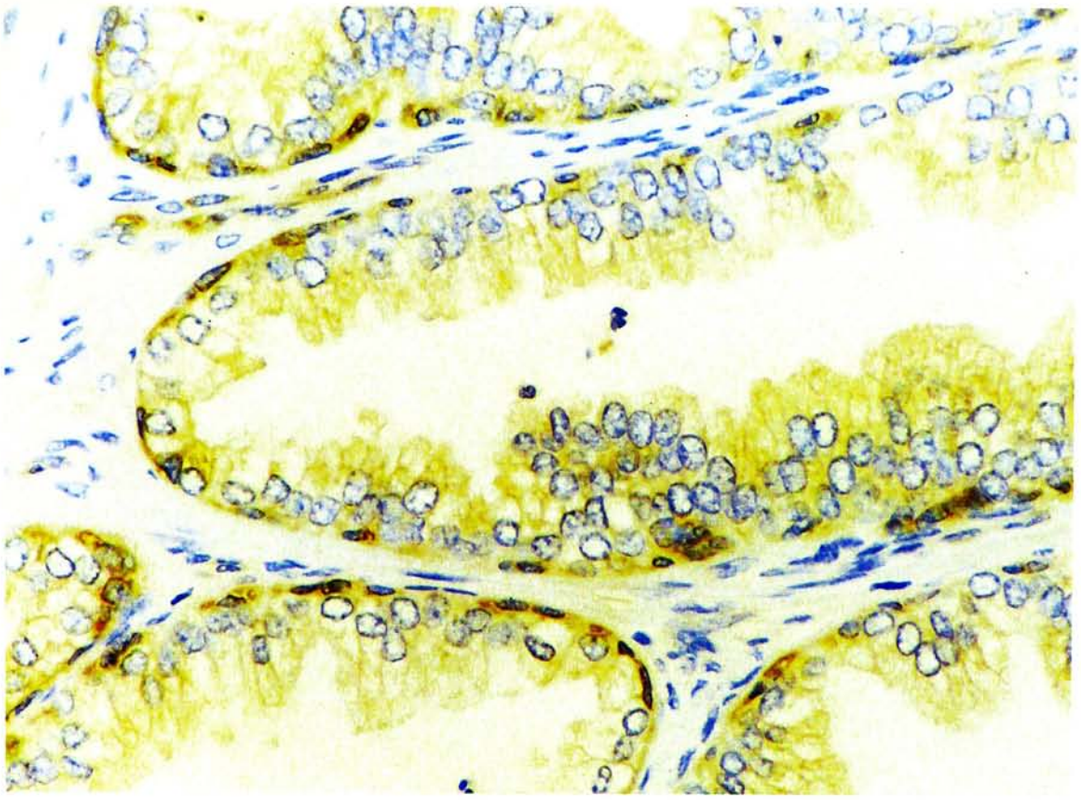


Figure 41. nm23 Staining in PIN 1 lesion showing weak staining in the dysplastic cells. Basal cells are positive stained. (Immunoperoxidase method, magnification x800)

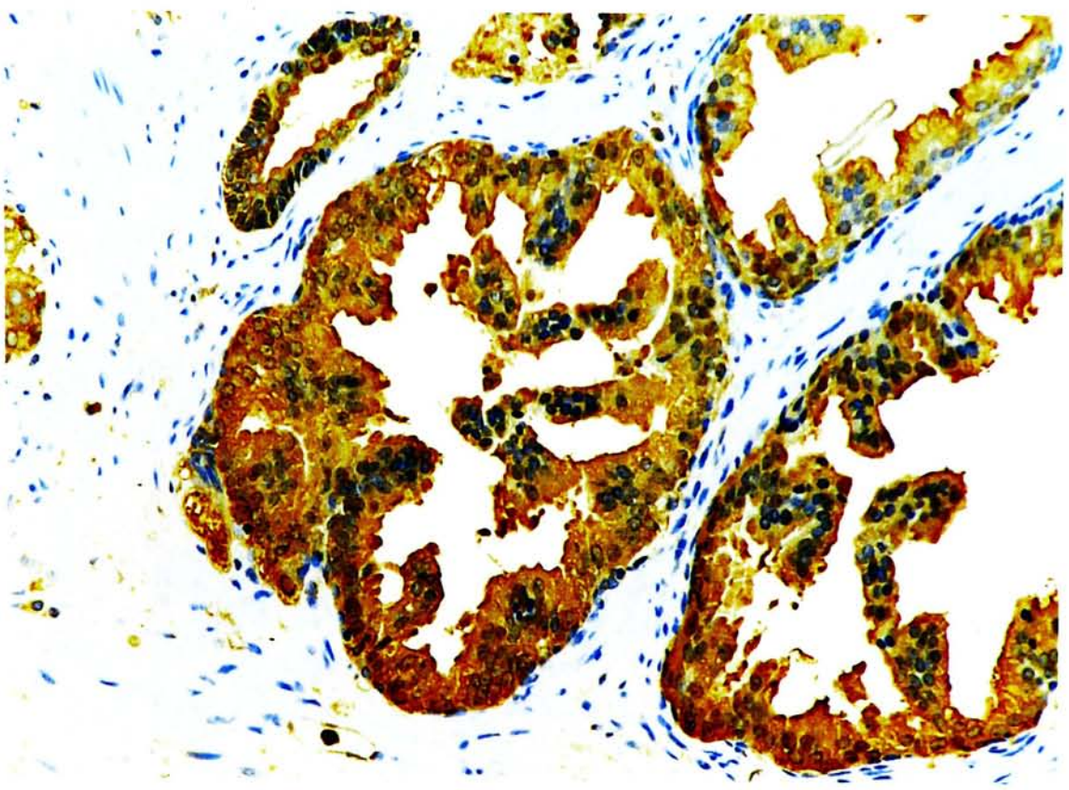


Figure 42. nm23 Staining in PIN 3 lesion showing strong cytoplasmic staining in the dysplastic cells. (Immunoperoxidase method, magnification x400)

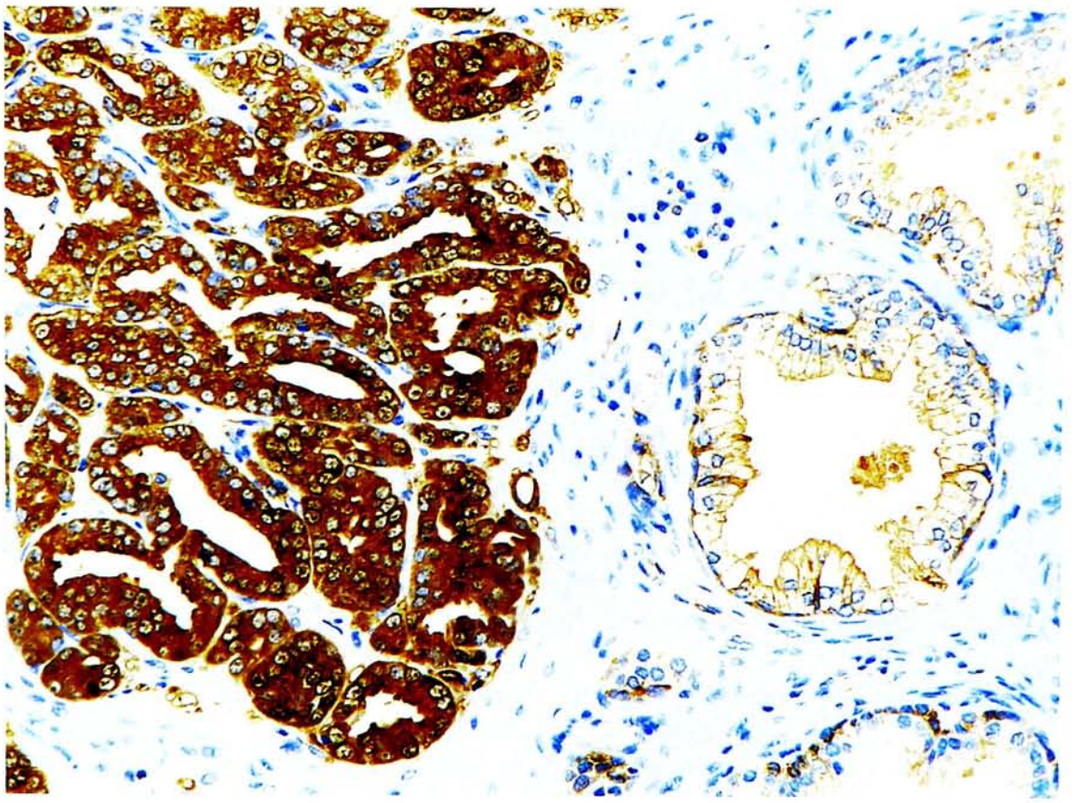


Figure 43. nm23 Staining in PCa (Gleason grade 2,2) showing strong to very strong cytoplasmic staining of neoplastic proliferations in contrast to weak staining in the adjacent benign glands. (Immunoperoxidase method, magnification x400).

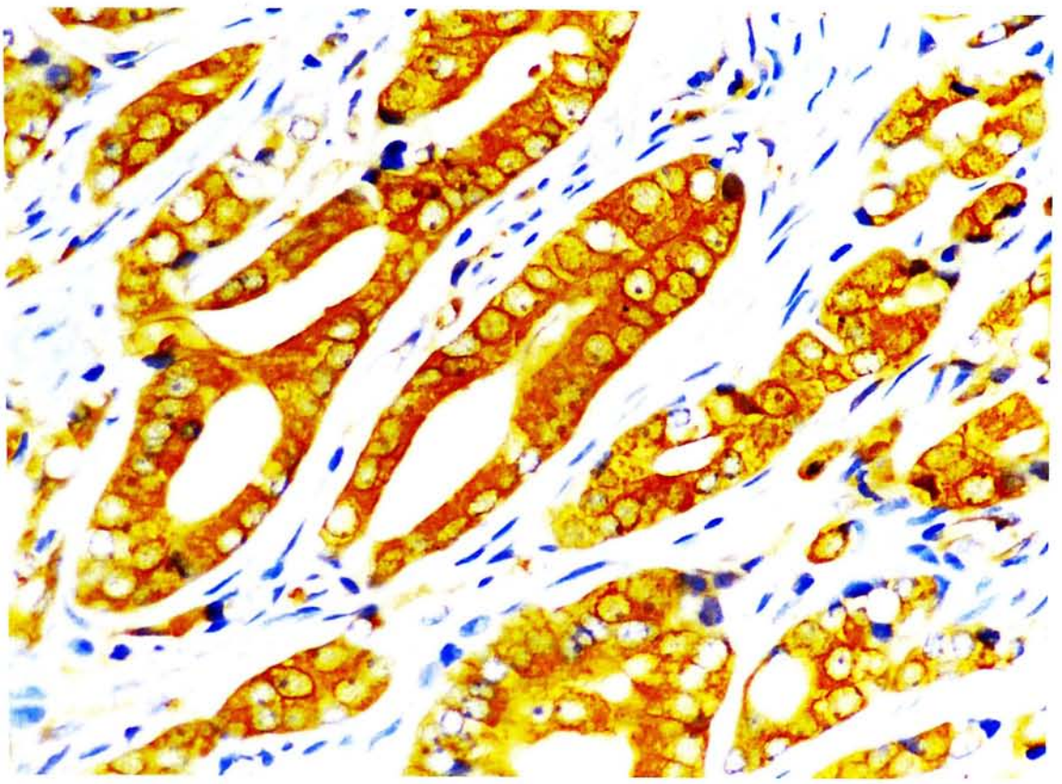


Figure 44. nm23 Staining in PCa (Gleason grade 2,2) showing positive cytoplasmic expression. (Immunoperoxidase method, magnification x800)

VIII. Correlation and Association of Expressions of All Biomarkers in Prostatic Lesions

The Spearman correlation coefficients between the biologic markers examined in all the prostatic lesions studied are presented in Table 36, and the association of changes of biomarkers expression in different histological categories of prostate lesions are summarized in Tables 38 and 39.

Table 36. Spearman correlation coefficients between the biologic markers in all prostatic lesions.

	p53	PCNA	Ki67 Q	Ki67 C	EGFR	E-Cadherin	CD44	nm23
P53	-	0.608**	0.565**	0.660**	0.431**	-0.287**	-0.269**	0.303**
PCNA		-	0.583**	0.720**	0.347**	-0.289**	-0.344**	0.467**
Ki67 Q			-	0.856**	0.376**	-0.335**	-0.267**	0.365**
Ki67 C				-	0.421**	-0.380**	-0.323**	0.431**
EGFR					-	-0.314**	n.s.	0.239**
E-Cadherin						-	n.s.	-0.191*
CD44							-	n.s.
nm23								-

Abbreviation: * - Correlation is significant at 0.05 level (2-tailed)
** - Correlation is significant at the 0.01 level (2-tailed)

There was significant positive correlation among p53, PCNA, Ki-67, EGFr, and nm23 expression, whereas inverse correlation was shown between E-cadherin expression and p53, PCNA, Ki-67, EGFr, or nm23 expressions. CD44 expression was inversely correlated with p53, PCNA, and Ki-67. CD44 expression showed no statistical correlation with EGFr, nm23 and E-cadherin expressions.

Table 37. p-Values of univariate analysis of association of all biomarkers with various histological groups of prostate specimens (Mann-Whitney U test).

		Age	p53	PCNA	Ki67 (M)	Ki-67 (QIA)	EGFr	E-cadherin	CD44	nm23
Hyperplasia vs	PIN 1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.0414	n.s.	0.0017
	HG PIN	0.0112	n.s.	<0.0001	0.0001	0.0102	n.s.	<0.0001	0.0141	<0.0001
	PCa	0.0011	<0.0001	<0.0001	<0.0001	<0.0001	0.0011	<0.0001	<0.0001	<0.0001
PIN 1 vs	HG PIN	n.s.	n.s.	0.0001	0.0067	0.0022	n.s.	n.s.	0.0390	n.s.
	PCa	n.s.	<0.0001	<0.0001	<0.0001	<0.0001	0.0092	0.0102	0.0001	0.0193
HG PIN vs	LG PCa	n.s.	0.0045	0.0106	0.0007	0.0081	n.s.	n.s.	n.s.	n.s.
	IG PCa	n.s.	<0.0001	<0.0001	<0.0001	0.0006	0.0046	n.s.	0.0487	n.s.
	HG PCa	n.s.	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0023	0.0038	n.s.
	PCa	n.s.	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	n.s.	0.0365	n.s.
LG PCa vs	IG PCa	n.s.	n.s.	0.0004	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	HG PCa	n.s.	0.0449	<0.0001	0.0059	0.0302	0.0369	n.s.	0.0272	n.s.
IG PCa vs	HG PCa	n.s.	n.s.	0.0090	n.s.	n.s.	n.s.	0.0040	n.s.	n.s.

Abbreviation: n.s.- not statistically significant at $p<0.05$, 2-tailed; LG- low-grade; IG- intermediate-grade; HG- high-grade.
M- Ki-67 index measured by manual counting; QIA- Ki-67 index measured by quantitative image analysis.

Table 38. Summary of differences of all biologic parameters examined between various histological subgroups of prostatic lesions

		Age	p53	PCNA	Ki67 (M)	Ki-67 (QIA)	EGFr	E-cadherin	CD44	nm23
Hyperplasia vs	PIN 1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	Reduced*	n.s.	Increased**
	HG-PIN	Increased*	n.s.	Increased**	Increased**	Increased*	n.s.	Reduced**	Reduced*	Increased**
	PCa	Increased**	Increased**	Increased**	Increased**	Increased**	Increased**	Reduced**	Reduced**	Increased**
PIN 1 vs	HG PIN	n.s.	n.s.	Increased**	Increased**	Increased**	n.s.	n.s.	Reduced*	n.s.
	PCa	n.s.	Increased**	Increased**	Increased**	Increased**	Increased**	Reduced*	Reduced**	Increased*
HG PIN vs	LG PCa	n.s.	Increased**	Increased*	Increased**	Increased**	n.s.	n.s.	n.s.	n.s.
	IG PCa	n.s.	Increased**	Increased**	Increased**	Increased**	Increased**	n.s.	Reduced*	n.s.
	HG PCa	n.s.	Increased**	Increased**	Increased**	Increased**	Increased**	Reduced**	Reduced**	n.s.
	PCa	n.s.	Increased**	Increased**	Increased**	Increased**	Increased**	n.s.	Reduced*	n.s.
LG PCa vs	IG PCa	n.s.	n.s.	Increased**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	HG PCa	n.s.	Increased*	Increased**	Increased**	Increased*	Increased*	n.s.	Reduced*	n.s.
IG-PCa vs	HG PCa	n.s.	n.s.	Increased**	n.s.	n.s.	n.s.	Reduced**	n.s.	n.s.

Abbreviations: * - p-values significant at $p<0.05$; ** - p-value significant at $p<0.01$, Mann-Whitney U;
n.s.- not statistically significant at $p>0.05$, 2-tailed; LG- low-grade; IG- intermediate-grade; HG- high-grade.
M- Ki-67 index measured by manual counting; QIA- Ki-67 index measured by quantitative image analysis.

Apart from being significantly correlated with each other, both p53 and EGFr were similar or not significantly different among prostatic hyperplasia and PIN lesions, but were significantly increased in PCa. Although their expressions were significantly increased in high-grade PCa in comparison with low-grade PCa, there was no significant difference in the expressions between low-grade and intermediate-grade PCa, or between intermediate-grade and high-grade PCa.

Both PCNA and Ki-67 proliferation indices showed similar pattern of results in the prostate specimens examined. Both indices were significantly increased from nodular hyperplasia to high-grade PIN to PCa. But unlike PCNA expressions, no significant difference in Ki-67 expression was observed between low-grade and intermediate-grade PCa, or between intermediate-grade and high-grade PCa.

Both expressions of cell adhesion molecules, E-cadherin and CD44, were reduced in high-grade PIN and PCa compared to nodular hyperplasia. However, there was no significant difference in E-cadherin expression between low-grade and high-grade PIN whereas CD44 expression was significantly reduced in high-grade PIN compared to low-grade PIN. Although both E-cadherin and CD44 expressions were not significantly different between high-grade PIN and low-grade PCa, there was significant difference in reduced CD44 expression and loss of E-cadherin between high-grade PIN and high-grade PCa.

There was an inverse correlation of nm23 and E-cadherin expressions. Both expressions in low-grade PIN and PCa were significantly different from nodular hyperplasia but there was no significant difference in their expressions in low-grade PIN and high-grade PIN, or high-grade PIN and PCa.

Chapter 7. Discussion

Prostatic intraepithelial neoplasia (PIN) have been the focus of medical scrutiny and investigations over a decade since McNeal and Bostwick described them under intraductal dysplasia and proposed them as the biologic precursor of invasive prostate carcinoma (24,28). PIN is characterized by cytologic atypia and proliferation of the luminal cell layer of both prostatic acini and ductules. While it is well accepted that prostatic hyperplasia is unrelated to carcinoma, the place where PIN lesions stand in relation to prostatic hyperplasia and to PCa is still undetermined. Although PIN lesions have been viewed by many as a precursor of prostate carcinoma, due to the similarity in their prevalence, anatomical distribution, cytological features and immunophenotypic expressions, certain proportion of the case observations also indicate that the two lesions may be independent and that a pathogenetic link between PIN lesion and carcinoma remains to be established (23,24,28,30,39,52,53,55,56,57).

Growth factors are important not only for the cell proliferation in normal as well as neoplastic tissues, they also have a role in cell functions, migration, differentiation and cellular adaptation to injury. Many growth factors are now known to have interrelationship with other molecular events or products, such as oncoproteins, tumor suppressor gene products (p53, TGF-beta), growth factor receptor and signal transduction, cell cyclins (intracellular complexes with cyclin-kinase) and finally the extracellular matrix. Although many growth factors have been implicated in tumorigenesis through mutations, their excess production or increased synthesis alone appear insufficient for neoplastic transformations, but they contribute the stimuli for growth. Proto-oncogenes may be transformed into oncogenes through

changes in structures of the genes with ensuing production of abnormal gene products and functions, or through changes in their regulation. These molecular events may be shared by or similar between lesions such as prostatic hyperplasia, PIN proliferations and carcinoma of the prostate.

Molecular events associated with the development and the progression of prostatic cancer remain poorly defined. Invasion and metastasis are characteristics of malignant tumors. But the metastatic potential of a given tumor is difficult to predict. The conventional assessment of the grading of a tumor is used to predict its biological behavior and metastatic potential. Many biological markers may also reflect its aggressiveness. Oncogenes c-myc, erb-b2 and tumor suppressor genes p53, Rb have been implicated in percentage varying from 10% to 35% of carcinoma. Genetic alterations on chromosomes 8p, 10p, 10q, 16q, 13q, 18q, 9p and 6q have been reported, suggesting the presence of tumor suppressor genes in these loci (64,65,66,163,164). Some of these loci are associated with cell adhesion molecules which have been implicated as tumor suppressors and in the development of carcinoma of prostate.

Development and progression of cancers appear to be associated with changes of various biologic parameters. Determination of the expression of such biomarkers in putative pre-invasive neoplastic lesions is important not only in the understanding of carcinogenesis but also to develop models of early neoplasia. Such potential characterization of early neoplastic changes may help to identify the pathway to the more aggressive subtypes of dysplastic changes. The aim of this study was to compare and characterize the expression of some of these biologic markers in nodular prostatic hyperplasia, PIN lesions and various histological grades

of prostatic acinar carcinoma. Based on these expressions, possible correlation of these biological markers between PIN lesions and carcinoma of prostate are assessed. Finally, whether there is a progression or continuum existing between PIN and carcinoma of prostate may be determined. However, correlation of the biologic markers with stage and survival has not been considered and included in this study.

While the sequence of prostatic hyperplasia, PIN and carcinoma was examined, the selection of cases has some limitations and the source of lesions needs to be clarified here. At the time of this study, prostatectomy was rarely performed for carcinoma, and this was performed as Millin's operation for hyperplasia or in the setting of radical cystectomy for urothelial carcinoma of the bladder. Thus the source of hyperplastic lesions is from nodulectomy in Millin's operation or from TUR-P. Majority of PIN lesions, in particular the high-grade lesions PIN 2 and PIN 3, are from prostatectomy without carcinoma, and from the peripheral zone. Finally, all carcinomas with the exception of 3 prostatectomies are derived from TUR-P for obstructive carcinomas, most often in patients known to have prostatic carcinoma, which generally represent advance or high stage of disease. Although it would be interesting to compare PIN lesions associated with carcinoma and PIN lesions without carcinoma, the cases of PIN associated with carcinoma were rare as prostatectomy for carcinoma was only just beginning to be practiced, therefore the PIN lesions in this study are restricted to those not associated with carcinoma, but overall they represent PIN lesions from the peripheral zone, which is the zone clinically significant for carcinoma.

I. p53 Protein

Our study demonstrated the absence of p53 accumulation in the luminal or secretory cells of glandular epithelia in prostatic hyperplasia and all grades of PIN lesions. On the other hand, up to 31% of all PCa were positive for p53 immunoreactivity. Only two cases of PIN 3 lesions demonstrated occasional p53 positive cells and several other cases with infrequent cells exhibiting weak nuclear staining. Such an infrequent p53 accumulation suggests that p53 mutation is not associated with the development of PIN lesions. However, molecular analyses of p53 mutation in PIN lesions are lacking with no information available in the literature. These are perhaps important to confirm the absence of p53 mutation in PIN lesions. Other immunohistochemical studies have demonstrated, in contrast, the presence of p53 accumulation or overexpression of p53 in PIN lesions, however these studies were made in prostatectomy associated with carcinoma and also suggested that the interpretation of the results did not discriminate secretory or luminal cells with basal cells (71,80,165,166,167). While these observations support a close relationship between high-grade PIN and carcinoma in patients with prostatic carcinomas, both expressing high-levels of p53 protein accumulation, one can argue that PIN lesions associated with carcinoma which commonly express p53 may in fact reflect Pagetoid spread of cancer cells into acini or duct interpreted as PIN lesions. In our cases, isolated PIN lesions without an underlying carcinoma demonstrate in fact that p53 mutation does not occur in these lesions. However, whether primary prostate carcinomas derived from an adjacent PIN lesion or both lesions developed independently under the influence of certain factors in the neoplastic process remains

to be determined. This study of p53 immunoreactivity on various prostatic lesions indicates that p53 does not accumulate in benign lesions, and in high-grade PIN lesions, providing that those were not associated with a carcinoma. From this perspective, PIN may not represent a precursor lesion to carcinoma. Arguably, PIN lesions associated with carcinoma, and which express p53 in the same frequency as carcinoma may represent a subpopulation of cancerous cells, which have spread within ducts or acini.

Furthermore, overexpression of p53 in immunohistochemistry does not always represent or reflect p53 mutations; binding of p53 protein by viral protein or to cellular proteins such as *mdm* 2 may lead to intracellular accumulation, and to artefactual immunoreactivity not confirmed by molecular analysis (5,168).

We found that the overexpression of p53 is correlated with histological grade of prostatic carcinomas and the association of higher frequency of p53 accumulation with higher combined Gleason score. Such observations were in keeping with the results previously reported in the literature (169,170,171,172). Although such a correlation appears weak, it may be related to the relatively small number of cases of low-grade carcinoma examined. A more clear cut correlation is demonstrated when carcinomas are subdivided into 2 groups by a Gleason score below or above 6.

We do not have follow-up information in most of our cases, and thus we have not examined the correlation of p53 with stage and prognosis. However, an association of p53 mutation with disease progression has been reported in many studies, and that higher percentage of p53 abnormalities was associated with untreated metastatic specimens as compared to untreated primary prostatic cancer (173,174).

It was interesting to note that occasional p53-reactivity was observed in the basal cells of benign prostate glands. Similar observation has been reported by Kallakury *et al* (169). They hypothesized that focal overexpression of p53 in basal cells of hyperplastic prostate glands might represent a premalignant lesion that gives growth advantage to such cells and some of them might eventually acquire further somatic mutations that allow progression to malignancy. However, the p53 overexpression may also be a normal response to DNA repair or due to presence of other stabilization factors.

P53 mutations therefore represent a late event of tumor progression in a subset of PCa and appear to be unrelated to the development of PIN as suggested by this study.

II. PCNA

A number of monoclonal antibodies to PCNA are commercially available, including 19A2, 19F4 and PC10. Immunostaining with the antibody PC10 has been reported to correlate with Ki-67 immunostaining, S-phase fraction, flow cytometry and bromodeoxyuridine labeling indices (175,176,177,178). However, other studies have shown no correlation between PC10 immunoreactivity and other proliferation indices (179,180). The discrepancies have been attributed to long half-life of PCNA which is more than 20 hours, resulting in staining persisting in cells which have left the cell cycle. Moreover, since PCNA is also involved in DNA repair, low amounts of PCNA may be present in all cells including non-cycling cells. This was demonstrated by a study which showed that it is possible to increase the

immunoreactivity to almost 100% by reducing the dilution of the anti-PCNA antibody (96).

Montironi *et al* observed different staining patterns of PCNA immunostaining in prostatic specimens. The positive nuclei showed homogenous or granular types, or a mixture of both patterns of staining. The homogenous pattern was usually more darkly stained than the granular pattern which had a wide spectrum of staining intensities (58,97). Similar observations were seen in this study. Yu *et al* reported that PCNA labeling index based only on darkly stained nuclei was strongly correlated with S-phase fraction of flow cytometry (162). Weakly stained nuclei were therefore ignored in the measurement of the PCNA labeling index.

This study showed a progressive increase of proliferative rates from benign lesions through PIN to PCa. Similar observations were reported by Montironi *et al* in their studies on expression and location of PCNA in PIN lesions (58,97). The mean values of PCNA labeling indices observed in this study are comparable to their reported values except that no PCNA positive nuclei in luminal cells of benign hyperplasia was observed by Montironi *et al*. Moreover, in our study, a significant correlation between increased PCNA expression and both combined Gleason scores or primary Gleason scores was observed, indicating progressive increase in proliferation rates associated with tumor progression.

The altered PCNA expression in PIN lesions and PCa indicated an alteration of the regulation of PCNA expression. PCNA expression is regulated at both the transcriptional and post-transcriptional level. However, the transcriptional regulation is still not well understood which may involve regulatory effects of binding of homeodomain-containing proteins to the PCNA promotor. The post-

transcriptional regulation involves stabilization of PCNA mRNA in the presence of growth factors such that the PCNA mRNA can be translated into protein with greater efficacy (96). It would appear that PCNA mRNA is produced continuously at low levels in many cells but is degraded rapidly without translation. PCNA production is induced in response to growth factors by stabilizing PCNA mRNA.

In benign glands and in PIN lesions, previous studies have reported a significantly higher PCNA immunoreactivity in the basal cell layer than the luminal cells layer, indicating a high proliferative activity of the basal cells (58,181). This supports the common concept of the role of basal cells as stem cells for the luminal cells. In a more recent study on PCNA expression in normal prostate, PIN and prostatic adenocarcinomas, Montironi *et al* reported decrease of proportion of PCNA expression in prostatic epithelial acini from periphery through intermediate layers to the luminal layer. They observed a progressive expansion of proliferation compartment to luminal cells from normal prostate to PIN to prostatic adenocarcinoma (97). Such type of expansion of the proliferative compartment had been investigated in the squamous cell epithelium of the uterine cervix by Mariuzzi *et al* that the width of the proliferative compartment is related to the degree of dysplasia, occupying the entire epithelial thickness in carcinoma-*in-situ*, and the differentiating and differentiated compartments decrease in width or even completely lost (182).

This study confirms previous observations that PCNA overexpression is positively correlated with altered proliferation from hyperplasia to PIN and carcinoma (58,97,181). The correlation with overexpression and increasing neoplastic grade supports the prognostic significance of PCNA index as determined

by PC10 on needle biopsy specimens of PCa (183). Although there appears to be a continuum from increasing grade of PIN lesions to increasing grade of carcinoma by PCNA expression, which may represent an indicator of prognosis, such marker does not reflect a specific mechanism of tumorigenesis or malignant transformation, but rather represents a simple process of cell proliferation in the presence of activated growth factors.

III. Ki-67

A frequent objection to the use of immunohistochemistry to predict clinical outcome concerns the reliability of the results, which depend on subjective assessment of the observer. Computerized image analysis systems have been developed to alleviated such problems to give objective and reproducible results (92). Measurement of Ki-67 proliferative index by computerized image analysis has been reported in other tumors including breast cancers and lung cancers (92,184). In this study, the Ki-67 labeling indices were measured by both manual microscopic counting of positive nuclei and quantitative image analysis and were highly correlated (Spearman's rho=0.916, $p<0.001$). A proportional bias of 0.736 detected in the study reflected the differences in the measured attribute of tissue components by the two methods. Percentage positive nuclear area was measured in the quantitative image analysis whereas percentage of number of positive nuclei was measured by microscopic counting method.

Both measurement methods showed a correlation of proliferative rate as expressed as Ki-67 labeling indices with histological grade of prostate carcinomas.

But quantitative image analysis yielded a lower coefficient of correlation than microscopic counting (Quantitative image analysis: Spearman's rho=0.610; manual microscopic counting: Spearman's rho=0.766). Moreover, no significant difference was detected by Kruskal-Wallis's analysis of variance of Ki-67 labeling index of different groups of PCa by quantitative image analysis whereas significant association of Ki-67 labeling index was detected by manual microscopic counting method. The lack of statistical significance in results measured by quantitative image analysis is probably due to a higher error rate during measurement. This is because, by measuring a total nuclear area of about 10,000 um^2, only about 200 nuclei were measured by quantitative image analysis and is comparatively lower than the microscopic counting method which measures at least 500 nuclei each time. The precision was also greatly affected by the nuclear and antibody threshold settings of the image analyzer.

The number of fields measured by quantitative image analysis in other previous studies varied from 2 to greater than 15 fields with a total number of cells measured varied from 100 to greater than 2000 (92,184). It appeared that a larger number of cells should be measured for prostatic specimens which are very heterogeneous in nature. However, it would take much longer time in each measurement. During this study, it took an average of 15 to 20 minutes to measure the Ki-67 labeling index for each case. From this experience, while computer assisted image analysis and quantitation may be more accurate, the careful microscopic semi-quantitation using consistent criteria appears to be more cost effective in both manpower and equipment terms.

Statistically significant differences in mean Ki-67 indices of prostatic

hyperplasia and prostate cancer have been uniformly reported (185,186,187). However, previous studies of Ki-67 antigen immunostaining in prostate cancer have shown a variable relationship with tumor stage and grade. A weak correlation of Ki-67 labeling index and tumor grade in prostatic cancer have been reported by Stattin *et al* and McLoughlin *et al* (74,88). But no significant association of Ki-67 labeling with Gleason scores was detected by Bubendorf *et al* (188). Few studies have been conducted to evaluated the Ki-67 immunoreactivity in PIN lesions.

Ki-67 and PCNA immunoreactivities in prostatic lesions were closely correlated although Ki-67 labeling index was correlated with a higher value of PCNA labeling index. PIN and PCa were correlated with a higher cellular proliferative rate as defined by the Ki-67 and PCNA labeling indices. Moreover, the proliferative rate in PCa with high Gleason scores was significantly higher than in those with low Gleason scores. Our results confirmed the findings of Tamboli *et al* who reported an intermediate proliferative rate of high-grade PIN in the continuum of tumorigenesis from benign prostate to PCa (189). The actual mean Ki-67 labeling indices were close to the values reported by Tamboli *et al* but was more than twice of those reported by Oomens *et al* and more than 5 times of those quoted by Thompson *et al* (189,190,191). However, similar to PCNA, Ki-67 antigen expressions do not reflect a specific mechanism of tumorigenesis, but rather a simple process of cell proliferation in the presence of activated growth factors. Our results indicates that PIN lesions have an intermediate proliferation rate between prostatic hyperplasia and PCa, and may reflect the biologic position of PIN lesions in the multistep process of malignant transformation.

IV. EGFr

Various non-steroidal growth factors and their receptors have been found in the human prostate and are implicated in the control of prostatic epithelial cell proliferation including the epidermal growth factor (EGF), transforming growth factor-α(TGF-α), and their common receptor, EGFr (192). EGF is required for in vitro proliferation of human prostatic epithelial cells (193). In vivo, the secretory epithelial cells are exposed to high concentrations of EGF in the prostatic fluid (194). It was suggested that some of this EGF originates from other sources and it is likely that prostatic cells themselves secrete EGF (195).

Our results showed strong expression of EGFr in the basal cells of prostatic epithelium in prostatic hyperplasia and PIN lesions but much weaker immunoreactivity in the luminal cells. Such differential expression of EGFr between the basal cells compartment and the secretory cells compartment of the normal prostatic epithelium has been described in previous reports indicating a significant regulatory role of EGF in cellular growth and proliferation of the basal cell layer of the prostatic epithelium (61,196,197). Although there was no significant difference in EGFr expression between the luminal cells of benign hyperplasia and PIN lesions, it was interesting to find that PIN 3 was associated with a much lower EGFr expression. However, the immunoreactivity of EGFr in the luminal cells among all prostatic hyperplasia and PIN lesions was weak and similar in both conditions. The significance of such finding is unknown. However, similar to our findings, Ibrahim *et al* did not find significant difference in EGFr protein expression in prostatic hyperplasia and PIN (198). It may be argued from these findings that PIN lesions,

like nodular hyperplasia, still represent a regulated proliferative process.

Our results demonstrated significantly higher EGFr expression in PCa than in prostatic hyperplasia, PIN 1 and PIN 3 lesions although significant difference in EGFr expression between PCa and PIN 2 lesion was not detected. EGFr expression was also correlated with histological differentiation of PCa, in that increasing expression was correlated with higher grades. Results on EGFr expression in PCa in previous studies were also conflicting. Both Maygarden and Ibrahim reported weak EGFr expression in PCa (61,196). Robertson *et al* also observed a low immunoreactivity of EGFr protein in PCa (197). However, using the same antibody, Ab-1, as Maygarden, Cohen *et al* observed positive immunoreactivity of EGFr in all prostatic carcinomas in a more recent study (199). Fox *et al* used another EGFr antibody, E12, and reported positivity in stage A1 PCa (200). These discrepancies in EGFr protein expression can partly be explained by differences in the antibody used and staining methods. Since frozen tissues were used in some of the studies including those of Maygarden and Ibarhim, while others including ours were examined in fixed tissue, and thus immunohistochemical results might be affected by tissue preservation. Ibrahim indicated presence of certain protease, such as prostatic specific antigen, that may destroy or alter the EGFr and interfere with recognition of the antibody (196).

The increased EGFr in PCa indicated a strong requirement of its ligand for tumor growth. Cohen *et al* reported co-expression of transforming growth factor-alpha (TGFα), a potent mitogen, and EGFr in a significantly higher frequency in prostatic carcinoma than in the prostatic epithelium of prostatic hyperplasia. This indicated a shift to an autocrine stimulatory pathway of growth in epithelial cells of

PCa which may confer growth advantage of tumor cells and promote a proliferative state (199). The contrast between PIN lesion and carcinoma, with respect to EGFr, suggests that the 2 lesions may develop according to different mechanisms, and the autocrine stimulation may be present in carcinoma, but not in PIN lesion.

Although we found no significant difference between incidence of EGFr expression and different grades of carcinoma, such results may reflect the relatively small number of cases studied, and consequently there may be overlap of tumor of different grades. More importantly, however, our findings demonstrated that overexpression of EGFr is significantly correlated with high-grade PCa (above Gleason score 6), and these findings concurred with most observations in the literature (199,201).

The observation of cytoplasmic staining of EGFr in most cases of PCa in the study was not well understood. In some cases the membranous staining was indistinct from the cytoplasmic staining. Previous studies using frozen tissues showed mainly cytoplasmic pattern of staining (61,196,199). But Fox *et al* showed predominant membranous staining using archival specimens (200). Since the antibody used in this study is specific for the extracellular domain of the EGFr protein, the presence of cytoplasmic staining of EGFr in PCa may be explained by overexpression of abnormal EGFr gene products containing the external domain. Another explanation is that such overexpression may be due to increased binding of ligand to the EGFr probably through autocrine pathway. Binding of ligand to EGFr protein normally triggers recruitment of EGFr to clathrin-coated pits, followed by internalization of the EGFr-ligand complex and its delivery to lysosomes for degradation (202). Increased binding of ligand to EGFr probably causes increased

endocytosis of EGFr-ligand complex which was detected in high amounts in the cytoplasm.

V. E-Cadherin

E-cadherin has been described as an invasion or metastasis suppressor because its expression is lost from epithelial cells as they progress to advanced or high-grade tumor. Reintroduction of E-cadherin DNA in tumor cell lines cause suppression of their invasive property or phenotype (203). Molecular studies have shown that human prostate cancer frequently show loss of heterozygosity in the long arm of chromosome 16 (16q) where the coding gene of E-cadherin resides (204).

Strong continuous membranous expression of E-cadherin in both the basal and secretory cells of the glandular epithelium in prostatic hyperplasia indicated the significant role of E-cadherin in maintaining cell adhesion among the epithelial cells. The basolateral distribution of E-cadherin molecules is probably related to maintenance of cell polarity and cellular architecture of the prostatic epithelium. Previous reports have described the loss of E-cadherin expression in PCa, and also correlated such alterations with metastasis, cancer stage, recurrence rate and patient's survival (122,205,206). Our study showed significant association of loss of E-cadherin with PCa in comparison with prostatic hyperplasia ($p<0.001$) but did not show significant correlation of loss of E-cadherin expression with histological grade.

Our results showed a higher frequency of loss of E-cadherin expression in PCa than Umbas *et al* (117). Although they observed loss of E-cadherin expression in a high proportion of high-grade PCa, they reported reduced E-cadherin expression

was present in 28% of intermediate-grade PCa but normal expression in low-grade PCa. In this study, loss of E-cadherin expression in low-grade and intermediate-grade PCa were similar in proportions and were 54% and 50% respectively. The difference may be due to differences in techniques and study group. Umbas *et al* used frozen tissues in their study while formalin fixed archival tissues were evaluated in this study. E-cadherin may be lost during fixation resulting in a lower expression which is evident as 11% of prostatic hyperplasia were also negative for E-cadherin expression. However, loss of E-cadherin expression has also been reported in well-differentiated pancreatic cancer (grade I) (207). Therefore alteration in E-cadherin expression may occur in early stages of tumor progression.

Although E-cadherin expression appeared to be suppressor of cell detachment, its expression does not necessarily indicative of progression. Normal function of E-cadherin may be affected by impaired function of catenin through which E-cadherin is anchored to the cytoskeleton. Shimoyama *et al* have suggested that loss of α-catenin expression can be causally related to dysfunction of cadherin-mediated interaction in a lung cancer cell line (PC9) (208). Morton *et al* found that impaired function of E-cadherin could be explained by homozygous deletion of the α-catenin gene in a prostate cancer cell line (PC3) (122). Moreover, increased tyrosine phosphorylation of β-catenin has been related to impaired E-cadherin function (209). Mutational inactivation of E-cadherin function was also reported for a subset of endometrial carcinoma and gastric cancers (210,211).

Another study has shown a significant correlation between reduced E-cadherin expression and tumor stage and overall survival. A significant higher survival rate for patients with normal E-cadherin expression was demonstrated

compared to patients with reduced E-cadherin expression. Moreover, reduced E-cadherin expression was also correlated with progression after radical prostatectomy (212). In our study, reduced or loss of E-cadherin expression was present in 18 (95%) out of 20 cases of high-grade carcinoma in contrast to 44% and 62% of intermediate-grade and low-grade PCa.

VI. CD44

The standard form of CD44, CD44s, was detected by immunohistochemical methods in the prostate specimens in this study. The antibody used is specific for CD44 standard form protein which is present in all hematopoietic cells and neutrophils and fibroblasts were therefore positive in all tissues examined when present. In the study, CD44s immunoreactivity was found strongly expressed in the cytoplasmic membranes of basal cells of prostatic epithelium of benign lesions. This strong expression indicated a significant role of CD44s as a cell adhesion molecule in the prostatic epithelium. The expression of CD44s in the luminal cells were usually much weaker and it was difficult to distinguish whether the CD44s immunoreactivity arose from the basal cells or luminal cells at the boundary between them. Since only the presence of CD44s expression at the lateral borders between adjacent luminal cells were considered in the study, it is possible that expression of CD44s in benign lesions may be underestimated as a result of differential distribution of CD44s in basal border and lateral borders of the luminal cells. The expression of CD44s in the basal cells of prostatic epithelium is in concordance with similar findings reported in bronchial epithelium, gastric and intestinal mucosal

epithelium (130).

Conflicting results of various studies on CD44s expression in prostatic tissue and prostate carcinoma cell lines have been reported. Several studies on CD44 expression in human prostate carcinoma cell lines reported decreased expression of CD44 in a low metastatic, androgen-sensitive cell line (LNCaP) and elevated CD44 expression in highly aggressive, androgen-independent cell lines (213,214,215). On the contrary, Kallakury *et al* reported strong membranous staining of CD44s present in epithelial cells of benign prostatic acini of 95% of 109 cases of PCa but approximately 70% of PCa showed significant loss of CD44s expression, which was correlated with high tumor grade and aneuploid status (143). Our results of CD44 expression in benign prostate lesions and prostate carcinoma were close to those reported by Kallakury *et al* (143). There was decreased expression of CD44s in PCa which correlated with tumor differentiation (Spearman's rho=-0.433, p=0.002). The conflicting results of CD44 expression from prostate carcinoma cell lines are poorly understood. The difference may be due to differences in biological behavior of the tumor cells from tumor tissues obtained from histological specimens. As pointed out by Liu in his report, the LNCaP cell line was obtained from a supraclavicular lymph node metastasis of a patient who had disseminated bone involvement with the tumor (215). Whether the two metastases differ from each other was not known. This might be related to the low CD44 expression of the tumor cell line. Kogerman *et al* suggested that platelet-derived growth factor (PDGF) regulates the level of CD44 in cultured cells (216). Hence, the finding of variations in CD44 levels in cultured cell may be related to other modulating factors. Moreover, different integrin subunits have been reported in LNCaP and other prostatic cell lines including PC-3, DU-145

and 413-P which may account for differences in metastatic potential between the cell lines other than CD44 levels (217). In more recent reports, Nagabhushan *et al* and De Marzo *et al* both found significant association of loss of CD44 expression with tumor histological differentiation (218,219). Nagabhushan *et al* also observed a significantly lower CD44 expression in metastatic prostate cancer than primary prostate cancer (218).

Although a significant difference in CD44 expression was not found between PIN 2 and PIN 3, or PIN 1 and PIN 2, there was a significant loss of CD44 expression between PIN 1 and PIN 3, or between low-grade and high-grade PIN lesions. While the number of PIN 2 lesions was small, and hampered the significance of the statistical analysis, our results support that low-grade PIN lesion or PIN 1 should be separated from high-grade lesion, because they have different biological behavior (220,221). The expression of CD44 protein in PIN lesions supports such hypothesis, and the proposal to reserve the terminology of PIN for high-grade PIN lesions, and to eliminate PIN 1 lesion from the nosology (30). Moreover, the similar frequency of CD44 expression in PIN 3 lesion and low-grade PCa is compatible with the premalignant nature of PIN lesion.

Our findings of a correlated decrease of CD44s expression with histological grade of prostate carcinoma supports the hypothesis that decreased CD44s expression may result in decreased cell-cell adhesion, causing easy detachment of the cells from the basement membrane and enabling migration to both local and distant sites. While cell-matrix interaction and increased CD44s expression may be an important invasion promoter for some cancers, it appears that the loss of CD44s mediated cell-cell adhesion may be important to tumor progression and

dissemination in prostate carcinoma.

Moreover, a recent report by Gao *et al* suggested a metastatic suppressor function of CD44s in prostate carcinoma. They showed that transfection-induced enhanced expression of CD44s in highly metastatic AT3.1 rat prostatic cells greatly suppresses their metastatic ability to the lungs without suppression of their in vivo growth rate or tumorigenicity indicating a metastatic suppressor function of CD44s (222).

VII. nm23

The nm23 gene is a putative anti-metastatic gene and previous studies have shown that expression of one of its gene products nm23-H1 mRNA was inversely correlated with metastasis in several human malignant tumors, including primary breast carcinoma, gastric carcinoma and malignant melanoma (148,149,150). Although nm23 gene products nm23-H1 and nm23-H2 have been associated with metastatic tumor and high-grade malignancy with metastatic potentials, the proteins expression in normal epithelium as well as neoplastic tissues is also well recognized (223,224). In this study, only 39% of glands in prostatic hyperplasia expressed nm23-H1, of moderate intensity. This observation is in keeping with the expression of such protein in benign epithelium as reported in colonic and breast epithelium (223,224). Furthermore, the significantly more extensive and intensible expressions of nm23 in PCa are in agreement with the notion that nm23 gene represents a metastatic suppressor gene, and overexpression in carcinoma is in keeping with NDP kinase activity, and associated with the propensity for metastasis (223,224,225).

There was a progressive increase in nm23-H1 expression from benign hyperplasia to PIN to PCa (Spearman's rho 0.589, $p<0.001$). Such a progressive expression in both extent and intensity of nm23 in prostatic lesions ranging from benign to malignant is of interest. PIN lesions which are morphologically situated between hyperplasia and carcinoma, and which have been qualified as dysplasia, inferring to its malignant potential, interestingly show significant nm23 expression.

In many cases, the expression of nm23 in PIN lesions was similar to that observed in carcinoma, in that they showed strong cytoplasmic stain, including nuclear and membranous stain, and also showed heterogeneity in the intensity of immunoreactive cells. In this respect, PIN lesions show additional features in nm23 expression, which herald those seen in PCa. One can argue that many PIN lesions may represent the intraepithelial spread of an adjacent carcinoma, thus not unexpectedly the PIN lesions showed similar features as the adjacent carcinoma. The PIN lesions we examined were not associated with carcinoma, and excluded such a possibility.

The strong nm23 immunoreactivity in all histological grades of PCa is discordant with the proposed metastasis suppressor role of nm23 gene which expression have been previously described to be inversely correlated with metastatic potential in several human malignant tumors, including primary breast carcinoma, gastric carcinoma and malignant melanoma (148,150,224,225,226). More specifically, the study of Igawa *et al* on nm23 immunoreactivity in PCa reported a positive correlation of intense nm23-H1 staining with high stage, high-grade prostatic disease (226). They concluded that nm23-H1 was related to the proliferative phase of the cell cycle as demonstrated in tissue culture studies with

flow cytometry and Northern blot analysis instead of functioning as a metastasis suppressor gene. In our study, an overexpression of nm23 in PCa is in keeping with the metastatic potential of carcinoma. Such an expression of nm23 in carcinoma is significantly different from that found in prostatic hyperplasia and low-grade PIN lesions (PIN 1), observation that is concordant with the concept and function of nm23. It is of interest to note that the high-grade PIN lesions (PIN 2 and PIN 3) show no significant difference in expression of nm23 with PCa, indicating that a subset of high-grade PIN or simply PIN lesions demonstrate a metastatic potential. Such observations support that PIN lesions may be a precursor of invasive carcinoma, at least some of the PIN lesions. This is more significant when considering that none of the cases of PIN selected in this study is associated with carcinoma. The absence of difference in nm23 expression among different grades of PCa may reflect the relatively small number of cases in each category and also possible overlap between grades. Conversely, in the study of Konishi *et al* on nm23 expressions in non-metastatic and metastatic PCa, they also reported a high frequency of positive nm23 staining in non-metastatic as well as primary tumors of metastatic prostatic carcinomas except that there was a reduced expression in the metastatic tissue (151). The discrepancies in the expression of nm23 gene or of its products, and of tumor grade or stage, can partly be explained by the presence of the 2 subunits of nm23 gene, namely nm23-H1 and nm23-H2 subunits, which expression can be imbalance or opposite giving rise to apparently contradicting results (227). Recent studies have confirmed the lack of direct relationships between nm23 expression and grade or stage of PCa, and suggested that tumor heterogeneity may be a more important factor for the metastatic potential (227). In addition, the

study of Zabrenetzky *et al* showed that the suppressive effects of nm23-H1 transfection on metastatic potential in Murine K-1735 melanoma cell lines are associated with increased thrombospondin expression, an extracellular matrix molecule involved in tumor progression (228). Furthermore, the overexpression of nm23 may be due to overlap with an identical protein called purine binding factor (PuF), transcribed through the c-myc proto-oncogene activation, and it may be such a c-myc activation that causes progression of the tumor cells, but not the nm23 gene itself (227).

VIII. Association between Biomarkers and Prostate lesions

Expressions of cell growth markers p53 and EGFr were very similar, and demonstrated two important observations. One was the lack of significant difference between nodular hyperplasia and any type of PIN lesions, and the other was a significant overexpression of p53 and EGFr in PCa of any grade as compared to PIN lesions and nodular hyperplasia. These observations suggest that while PIN lesions represent atypical proliferation of the prostatic epithelium, the growth regulation and differentiation remain within certain control, unlike carcinoma where neoplastic process appeared to have escaped growth regulation with loss of the protective function of p53. Alteration of p53 expression and upregulation of EGFr seemed to be implicated in tumorigenesis of PCa, but not in the development of PIN lesions.

Furthermore, there seemed to be poor correlation of p53 and EGFr expression with the grade of PCa. Our observation suggests that expressions of p53 and EGFr do not represent prognostic markers in PCa. Though the number of cases

studied in each category of PCa was relatively small, the prognostic values of p53 or EGFr remain to be assessed, not only in immunohistochemical study of larger series, but also using molecular analysis. In particular, the expression of p53 has been correlated with metastatic PCa and carcinoma refractory to hormonal therapy (229,230).

The results shows a strong correlation between PCNA and Ki-67 labeling indices (both manual counting and quantitative image analysis methods) in the prostate specimens examined compatible with similar role of them as markers of cell proliferation. However, there was significant difference in PCNA labeling indices among low-grade, intermediate-grade and high-grade prostate carcinomas whereas no significant difference in Ki-67 expression between low-grade and intermediate-grade, or intermediate-grade and high-grade PCa was detected, indicating that PCNA is a better classifier of PCNA than Ki-67 labeling index among various groups of PCa. However, use of PCNA as proliferation marker is limited by its long half life which results in presence of low amounts even in non-proliferative cells. Only strongly stained PCNA-positive cells should be considered during measurement and may cause difficulties in judgment of positive cells with intermediate staining intensities. Use of image analysis techniques for evaluation of PCNA labeling indices may probably eliminate such problem.

As the cell proliferation markers were closely related to DNA replication and cell mitosis, PCNA and Ki-67 both were closely correlated, and demonstrated a progressive overexpression from nodular hyperplasia, PIN lesions of increasing grades and PCa of increasing grades. These observations confirmed similar previous findings of increasing proliferative activities in the sequence of hyperplasia,

dysplasia (PIN lesions), and neoplasia in the prostate (97,181,189).

While these proliferative indices support a continuum between nodular hyperplasia, PIN lesions and carcinoma, such markers only reflect a quantitative proliferation, and may not represent a specific step in the malignant transformation (97,181,189). Thus PIN lesions remain a putative precursor of prostatic cancer, but not as an established precursor. Although PCNA and Ki-67 may be regarded as non-specific markers of active cellular proliferations, our observations of increasing expression of these markers with increasing grade of PIN lesions and PCa, support others findings that they have a prognostic value, as they have been correlated with survival data (97,181,189,231).

The results also shows a strong correlation of PCNA and Ki-67 expression with p53 protein accumulation in prostate lesions suggesting that prostate lesions with mutated p53 may be more likely to actively dividing than those negatively stained lesions. A previous study has shown that p53 gene product and the protein product of retinoblastoma gene and PCNA colocalize in cells infected with herpes simplex virus type I, whereas Ki-67 does not (232). However, since wild-type p53 functions normally in DNA repair mechanisms, the increased p53 expression may also be related to DNA damage in highly proliferative cells.

In addition, both PCNA and Ki-67 labeling indices were also significantly correlated with EGFr expression and nm23 expressions. Since EGFr is the common receptor for growth factors and mediates proliferative effects on cells upon activation by binding with its ligands, it is logical that increased EGFr expression is associated with increased proliferation of cells which in turn displays increased proliferation markers' expressions.

The strong association of nm23 expression with the proliferation markers confirms the relation of nm23 expression with the cell cycle and cell proliferation. The inverse correlation of nm23 expression with E-cadherin may imply a relation of its anti-metastatic function with cell adhesion molecules. Both nm23 and E-cadherin expressions were significantly different in prostatic hyperplasia and PIN lesions, but were not significantly different in high-grade PIN and PCa. Their expressions in PIN lesions therefore distinctly differentiate such lesions from nodular hyperplasia and reveals their similarity with PCa.

The strong association of reduced E-cadherin expression with the proliferation markers can partly be explained by acquisition of genetic loss in tumorigenesis as a result of high proliferation rate. The coding sequence of E-cadherin gene resides on chromosome 16q which have been found to be frequently deleted in prostate cancers. However, it appeared that reduced expression of adhesion molecules may be related to dedifferentiation of cells in highly proliferative cells.

Loss of normal cellular adhesion in neoplastic precursors or in tumors is an important phenomenon in the biology of neoplasms. It also reflects alteration in the growth control which favors the development of invasive behavior. Alterations in cell-cell adhesion are correlated with the loss of tumorous or lesional cells cohesion at the microscopic level, which may herald the progression into invasiveness and metastasis. The altered expressions of both CD44 and E-cadherin in PIN lesions and carcinoma of the prostate appear to be correlated with the neoplastic process, and the similar observations in PIN lesions support the neoplastic process of such lesion, and perhaps as the precursor of invasive carcinoma. Moreover, the correlation of

expression of these adhesion molecules with increasing grades of tumor reflect the increasing aggressiveness of the neoplasm. The loss of adhesion molecules in high-grade tumor indicates the progressive nature of such loss; and that perhaps in earlier stage, low-grade carcinoma and even in PIN, the partial loss or abnormality of these adhesion molecules can be remedied. It has been shown in transfected cell lines and from post-treatment tumor that partial or complete restoration of adhesion molecules are possible (233,234).

The significant alteration of the adhesion molecules, CD44 and E-cadherin in high-grade PIN lesions as compared to nodular hyperplasia reflect the altered cell growth in PIN lesions, toward loss of cell adhesion, loss of cell cohesiveness, and toward the propensity for invasion. Furthermore, despite the significant difference in the expression of these adhesion markers between PIN and high-grade PCa, there appears to be a continuity between PIN lesions and PCa as indicated by their similar expressions in high-grade PIN and low-grade PCa. From these perspectives, PIN lesions demonstrate features of loss of cell contact and invasiveness that characterize carcinoma. Adhesion molecules thus support the precursor nature of PIN lesions toward invasive carcinoma.

Chapter 8. Summary and Conclusion

The process of tumorigenesis in prostatic cancer, as well as in many other neoplasms remains largely undetermined, but an well-accepted view is that it entails a multistep development where multiple biological alterations are implicated. This study examined the immunohistochemical expression of several biological markers including p53 protein, PCNA, Ki-67, EGFr, E-cadherin, CD44 and nm23 in the luminal cells in prostatic hyperplasia and PIN lesions, and in tumor cells of PCa. One of the two main objectives was to assess the relationship of these markers between the prostatic lesions which may reflect a genetic event which may participate or may be implicated in tumor development. The other was to determine whether the sequence of hyperplasia, dysplasia or intraepithelial neoplasia, and carcinoma applies to prostatic cancer, or whether PIN lesions is a precursor of carcinoma.

Both the tumor suppressor gene p53 and EGFr markers significantly showed overexpression in carcinomas, but not in prostatic hyperplasia and PIN lesions. Their overall overexpression in PCa was respectively 29% and 52%, relatively low incidence but in line with reports from the literature. The increasing alterations of these markers were correlated with higher grade of PCa, and suggest that p53 and EGFr gene mutations represent a late event in prostatic carcinogenesis. Such genetic events did not seem to participate in the development of prostatic hyperplasia and PIN. The expression of both p53 and EGFr showed no apparent continuity between hyperplasia, PIN and PCa. While the p53 gene may be implicated in the carcinogenesis of a small subset of PCa, it is possible that other recessive oncogenes are operative in the tumorigenesis of PIN lesions and PCa, and

these remain to be discovered.

p53 and EGFr overexpression was both correlated with increasing grade in PCa, and this suggests that these markers may be of prognostic value. However, this study is limited by the lack of data to correlate with the tumor stage and the patient's survival, and therefore does not permit to draw any conclusion as prognostic factors.

This study demonstrated a down-regulation of both E-cadherin and CD44 in carcinoma whereas CD44 expression was also correlated with increasing grade of carcinoma. Such altered expression of adhesion molecules are in keeping with those observed in many malignancies, and reflect the loss of epithelial integrity and the acquisition of invasiveness and metastatic potential. Thus both E-cadherin and CD44 appear to be implicated in prostatic carcinogenesis whereas CD44 also appear to be implicated in tumor progression.

E-cadherin also showed a progressive loss of expression correlated with increasing grade in PIN, and in addition demonstrated a continuous down-regulation from PIN lesions to PCa. These results support the link between PIN and PCa, and the notion that PIN is a precursor lesion to carcinoma.

The inverse correlation between p53 and EGFr with the expression of adhesion molecules E-cadherin and CD44 reflects the acquisition of invasive growth and metastatic behavior, as PCa progresses into higher grade. This observation also suggests that p53 and EGFr alterations may contribute to the development of invasiveness or metastatic phenotypes.

An overexpression of the anti-metastatic gene products nm23 was observed in all PIN lesions and PCa, in sharp contrast to benign glands in hyperplasia, suggesting that alteration of the nm23 gene may be an early event, and that both

lesions share a similar molecular mechanism in their development. The similar nm23 expression in high-grade PIN and PCa further support the close link between PIN and carcinoma, and the precursor role of PIN to invasive carcinoma. The similarity in nm23 overexpression between high-grade PIN and various grades of PCa indicates that nm23 may not be involved in tumor progression, nor a useful marker for prognosis, a conclusion that is in line with molecular studies of both nm23-H1 and nm23-H2.

Both cellular proliferation markers PCNA and Ki-67 were closely correlated with each other and demonstrated a continuity of their overexpression in the sequence from hyperplasia, PIN lesions of increasing grade and PCa of increasing grade. Although significant overexpression of these markers is observed in neoplastic processes, and may implicate them in carcinogenesis, they reflect mostly active cell division or high proliferation rates also seen in nodular hyperplasia. The strong correlation of the overexpression with increasing grade of PIN and PCa supports of that both PCNA and Ki-67 is useful prognostic factors in carcinoma.

Measurement of Ki-67 by both traditional microscopic semi-quantitative counting and computer-assisted quantitative image analysis yielded similar results. Although computer-assisted quantitation produced more consistent and accurate results, its application in quantitation of proliferative markers in prostate tissues is hampered by the complex tissue architecture of prostatic tissues. Much more effort and time were devoted and spent in careful segmentation of luminal cells to prevent measurement of basal cells and stromal tissues during computer-assisted quantitation by image analysis. Careful microscopic semi-quantitation using consistent criteria is more cost-effective for measurement of proliferation markers in heterogeneous

prostatic tissues.

The differences in expressions of all biologic markers studied between prostatic hyperplasia and PCa supports the commonly accepted view that hyperplasia is not related to the development of carcinoma, but a benign lesion in the prostate. While both hyperplasia and PIN lesions share similar findings of negative expression or accumulation of p53 and EGFr, immunoreactivities between these two lesions for PCNA, Ki-67, E-Cadherin, CD44, and nm23 are significantly different, indicating a lack of relationship between prostatic hyperplasia and PIN lesions.

Apart from limitation by the lack of clinical information regarding the tumor stage and the patient's survival, this study of a relatively small number of PIN lesions and carcinomas may be limited by an imbalance among certain subcategories, and thus affected the power of statistical analysis. During the interpretation of results, the lack of internal positive control required exclusion of a few cases in this study, and this reflected problems inherent to immunohistochemical techniques due to variation in tissue fixation and antigen expression of archival materials.

A number of biological alterations, reflecting genetic changes have been observed in this immunohistochemical study, and some of these markers appear to be implicated in the process of prostatic tumorigenesis. Several markers, including adhesion molecules E-cadherin, CD44, nm23 cell growth protein, proliferative markers PCNA and Ki-67 demonstrated similar alterations between PIN and carcinoma, and support the concept that PIN is a preinvasive lesion, or a precursor of malignant transformation. Although immunoreactivity for p53 or EGFr is significantly different between PIN lesions and carcinoma, this does not underscore the close relationship between these lesions as supported by other biological markers.

Furthermore, while one may be tempted to argue that PIN lesions may be heterogeneous and a subset of PIN lesions may not be correlated with cancer, this is not necessarily so. These observations may benefit from translation into molecular studies, using similar microdissection approach, which can not only confirm the expression of these biological markers, but may also lead or allow to specify the type of genetic alterations.

Appendices

I. Table of incidence and mortality rates of prostate cancer in the United States from 1973 to 1995 by race

Year	Incidence Rates			Mortality Rates		
	All races	White	Black	All races	White	Black
1973	64.2	62.6	106.3	21.7	20.3	39.5
1974	65.6	64.8	100.7	21.7	20.2	39.7
1975	70.6	68.9	111.5	21.6	20.1	40.6
1976	73.5	72.6	110.1	22.1	20.6	40.8
1977	76.1	74.5	122.2	22.1	20.6	41.2
1978	75.1	73.4	116.6	22.6	21.0	42.5
1979	78.0	77.0	123.1	22.7	21.1	43.0
1980	79.8	78.7	126.6	22.8	21.1	44.6
1981	82.0	80.8	126.9	22.9	21.0	45.8
1982	82.2	81.2	130.6	23.0	21.3	44.7
1983	84.8	83.8	134.1	23.4	21.6	46.6
1984	84.5	82.9	139.6	23.4	21.5	47.1
1985	88.0	86.9	133.1	23.4	21.5	48.1
1986	91.2	90.8	131.6	24.1	22.2	48.3
1987	102.9	102.8	147.3	24.1	22.2	49.1
1988	106.0	105.8	147.8	24.7	22.8	49.5
1989	112.7	112.3	149.1	25.4	23.5	51.1
1990	132.4	133.0	173.3	26.5	24.3	54.8
1991	169.3	169.1	223.3	26.4	24.7	54.9
1992	190.9	188.3	256.9	26.7	24.5	55.6
1993	171.1	163.4	270.6	26.6	24.3	56.2
1994	147.8	140.0	245.7	25.9	23.8	55.1
1995	137.2	129.8	211.6	24.9	22.9	53.5
1991-1995	162.7	157.6	241.2	26.1	26.1	55.1

* Rates are per 100,000 and are age-adjusted to the 1970 U.S. standard population.

II. Table of leading cancer deaths in Hong Kong from 1971 to 1996

Disease Group	1971	1975	1980	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996
1. Malignant neoplasm of trachea, bronchus and lung	534	758	1223	1457	1537	1609	1500	1731	1776	1716	1807	1882	1901	1933	2009
2. Malignant neoplasm of liver and intrahepatic bile ducts	573	606	689	740	830	820	877	859	838	861	888	914	897	916	997
3. Malignant neoplasm of colon	95	123	154	217	231	267	269	300	301	275	337	368	365	421	403
4. Malignant neoplasm of stomach	233	248	295	265	325	316	317	376	354	376	342	352	351	367	363
5. Malignant neoplasm of nasopharynx	295	339	349	316	324	329	341	338	323	334	345	342	303	298	286
6. Malignant neoplasm of esophagus	150	182	287	319	316	310	317	313	318	298	308	334	303	296	298
7. Malignant neoplasm of rectosigmoid junction and anus	70	84	103	140	115	145	143	168	195	181	192	178	248	247	234
8. Malignant neoplasm of pancreas	NA	NA	NA	110	117	120	119	121	136	116	138	129	137	161	156
9. Malignant neoplasm of prostate	20	26	35	53	65	77	60	77	80	101	87	114	123	136	149
10. Non-Hodgkin's lymphoma	NA	NA	NA	78	80	109	86	99	84	78	109	120	130	135	129
11. Malignant neoplasm of bladder	NA	NA	NA	97	111	125	112	114	102	139	138	135	122	131	110
12. Leukemia	50	84	91	112	98	111	123	107	107	107	119	110	111	116	117
13. Malignant neoplasm of larynx	45	59	77	78	90	81	86	86	94	84	78	80	96	91	83
All cancer death, male	2469	3022	4001	4590	4921	5075	5040	5379	5383	5397	5569	5793	5856	5999	6195

Mortality rates are expressed per 100,000 men population.

(Data from Annual departmental reports of Hong Kong Department of Health 1971,1975,1980,1985-1996).

III. Table of incidence and mortality rate caused by prostate cancer in Hong Kong

Year	Incidence rate per 100,000 men	Mortality rate per 100,000 men
1975	2.9	1.2
1980	4.5	1.3
1985	6.2	1.9
1986	6.1	2.3
1987	5.6	2.7
1988	7.5	2.1
1989	7.1	2.6
1990	7	2.7
1991	8.3	3.4
1992	9.9	2.9
1993		3.8
1994		4.0
1995		4.4
1996		4.7

IV. Reagents

1. 0.05 M TBS, pH7.6: containing 6.055 g Trizma base (Catalog no.T-1503, Sigma) and 8.766 g anhydrous Sodium Chloride (Catalog no. 71381, Fluka) in 1 L of distilled water. pH was adjusted to 7.6 with Beckman pI-10 pH meter calibrated with buffer standard pH7.00 (Catalog no. 82596, Fluka)

2. 0.01 M Tris-HCl-$CaCl_2$ Buffer, pH 7.2: containing 1.20 g Trizma base (Catalog no.T-1503, Sigma) and 1 g Calcium Chloride (Catalog no. C-614, Fisher) in 1L of distilled water. pH was adjusted to 7.2 with Beckman pI-10 pH meter calibrated with buffer standard pH7.00 (Catalog no. 82596, Fluka).

3. 0.01 M Citrate buffer pH 6.0: contains 2.1 g Citric acid monohydrate (Catalog no. 244, Merck) in 1 L of distilled water. pH is adjusted to 6.0 with 2 M NaOH (Catalog no. 71690, Fluka).

4. 0.1% Trypsin solution: contains 0.10 g of Trypsin powder (Catalog no. T-8253, Sigma) in 100 mL of 0.005M Tris-HCl-$CaCl_2$ buffer, pH 7.8.[5]

5. 0.005 M Tris-HCl-CaCl2 Buffer, pH 7.8: containing 0.60 g Trizma base (Catalog no.T-1503, Sigma) and 1 g Calcium Chloride (Catalog no. C-614, Fisher) in 1L of distilled water. pH was adjusted to 7.8 with Beckman pI-10 pH meter calibrated with buffer standard pH7.00 (Catalog no. 82596, Fluka).

6. 0.025% Trypsin solution: contains 25 mg of Trypsin powder (Catalog no. T-8253, Sigma) in 100 mL of 0.005M Tris-HCl-$CaCl_2$ buffer, pH 7.8.

7. 3% Bovine serum albumin: original 30% BSA solution (Catalog no. A-7284, Sigma) was diluted 10 times with TBS, pH7.6.

8. Biotinylated sheep anti-mouse whole antibody (Catalog no. RPN1001, Amersham Life Science): original concentrated antibody was prepared at a dilution of 1 in 50 with TBS, pH7.6.

9. Biotinylated donkey anti-rabbit whole antibody (Catalog no. RPN1004, Amersham Life Science): original concentrated antibody was prepared at a dilution of 1 in 200 with TBS, pH7.6.

10. Harris's Hematoxylin solution: Prepared by dissolving 2.5 g of Hematoxylin powder (Catalog no. 51260, Fluka) in 25 mL of absolute Ethanol (Merck) and mixed with 50 g Potassium alum (Catalog no. 60060, Fluka) dissolved in 500 mL of warm distilled water. The mixture was rapidly brought to boil. Then 1.25 g of Mercuric oxide (Catalog no. 83373, Fluka) was added and the mixture was rapidly cooled in water bath followed by addition of 20 mL of glacial acetic acid (Catalog no. 100063, Merck).

11. Methyl green solution: 1 g of Methyl green (Catalog no. 34052, BDH) was dissolved in 200 mL of acetate buffer pH 4.1 followed by extraction with chloroform.

12. Washing buffer: containing 0.075% Brij-35 (Catalog no. 430AG-6, Sigma) in 0.05 M TBS, pH 7.6.

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