Molecular cytogenic and immunohistochemical studies for the evaluation of new prostate cancer biomarkers

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ABBREVIATIONS

Abbreviation	Full form
A	Adenin
A260	Absorption at 260 nm (for nucleic acids)
AB	Anti-body
ABC	Avidin biotin complex
Aqua dist.	Destiled water
BAC	Bacterial Artificial Chromosome
Bp	Base pair
BPH	Benign prostate hypoplasie
BSA	Bovine Serum Albiumin
BVI	Blood-vessel invasion
C	Cytosin
Ca	Circa
Cav.	Caveolin gene or antibody
Cent.	Centromer
cDNA	Complementary DNA
CGH	comparative Genomic Hybridisation
Chr.	Chromosome
Cl	Chloride
Conc.	Concentration
Cy3	Cyanin3
DAPI	4,6-Diamidin-2-phenylindol
dCTP	Dexoxycytidintriphosphat
dH_2O	distilled Water
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclase
dNTP	Deoxyribonucleotid triphosphate
ds DNA	Double strand DNA
DTT	Dithiothreitol
E	Exon

E. coli	Escherichia coli
EDTA	Ethylendiamintetraacetic acid
EtBr	Ethidium bromide
EtOH	Ethanol
FA	Formamid
FFPE	Formalin-fixed paraffin-embedded
Fig.	Figure
FISH	Flourescen in situ Hybridization
FKS	Fetal calf serum
G	Guanine
GAP	GTP- activing protein
GAPDH	Glycerin aldehyd-3-phosphate- dehydrogenase
gen./genom	Genomic
GS	Gleason score
µg	Microgram
h	hour
H_2O	Water (distilled)
H_2O_2	Hydrogen peroxide
HGPIN	High grade prostate intraepithelial neueplasia
HRP	Horse Radish Peroxidase
I	Intron
IHC	Immunhistochemistry
kb	Kilobase
kDa	Kilodalton
l	liter
LAB	Labelled Avidin-Biotin –Method
LGPIN	Low grade prostate intraepithelial neueplasia
LOH	loss of heterozygosity
LSAB	Labeled StreptoAvidin-Biotin – Method
LVI	Lymphatic-vessel invesion
Meta.	Metaphase
M	molar
Min.	minute

ml	Milliliter
MW	Moleculare weight
MOSSC	Metastatic oral squamous cell carcinoma
mRNA	Messenger RNA (Boten-RNA)
MYC	Mylocytometosis gene2, Proto-Oncogene
NaAc	Sodium acetate
NaOH	Sodium hydroxide
NCMT	Non-cancerous matched tissues
nm	nanometer
NOM	Normal oral mucosa
NP-40	Nonidet P40
non-SCLC	Non-Small cell lung cancer
OD	Optic density
Oligos	Oligonucleotide
OPL	Oral pre-cancer lesions
P-40	Protein unit 40 of the YB-1
PACs	P1 -derived artificial chromosomes
PAP	Phoshate amino phosphatase
p-Arm	Short chromosome arm (petit)
PBD	phosphate buffered detergent
PBS	phosphate buffered saline
PC	Prostate cancer
PCR	Polymerase chain reaction
PEG	Polyethylenglycol
pH	Potentia Hydrogenii
Pha	Phytohemagglutanin
PIN	Prostate intraepithelial neoplasia
PNSI	plant nitrogen spectral index
POSCC	Primary oral squamous cell carcinoma
PTEN	Phosphatase and TENsin homolog deleted on chromosome TEN
q-Arm	Long chromosome arm
RNA	Ribonucleic acid
RNase	Ribonuclease

RP11	Library description of genomic DNA-bank
rpm	Rotation per minute (round per minute)
RT	Room temperature
RZPD	Deutsches Resourcenzentrum für Genomforschung GmbH
SCLC	Small cell lung cancer
sDNA	Single strand DNA
SDS	Sodium – Dodecyl sulfate
sec.	second
SSC	Saline Sodium Citrate
STAT1; STAT5	Signal transducer and activator of transcription
T	Thymin
TAE	Tris-Acetate-EDTA-Buffer
TBE	Tris-Borat-EDTA-Buffer
TE	Tris-EDTA
tel.	telomere
TGF	transforming growth factor
Tm	Melting Temperature
Tris	Trishydroxymethyl- aminomethane
Tris-HCl	Tris (hydroxymethyl) aminomethan-Hydrochlorid
U	Unit of the enzyme
UICC	International Union Against Cancer
UV	Ultraviolet
Vol.	Volume
WHO	World health organization
X and Y	Sex chromosomes
Λ	Lambda (Bacteriophage)
YAC	Yeast artificial chromosome
YB-1	Y binding protein 1

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1. INTRODUCTION

Interest in prostate carcinoma has increased in the last few decades in the Western countries. Currently, it has become the most common non-skin cancer neoplasm amongst men in Europe and the second common in more developed countries.[1, 2] Prostate cancer is mainly found in men older than 55 with an average age of 70 at the time of diagnosis and represents a disease of older men. Early detection of prostate cancer is important for curative treatment.

1.1. Prostate

1.1.1. Function of the Prostate

The prostate is a part of the male urinary system and is a composite organ, which is made up of several glandular and non-glandular components. The different tissues are tightly fused together within a common capsule, which encloses both the urethral gland and the accessory sex gland. The prostate functions both as an accessory sex gland and as urethral gland. It is located at the bottom of the bladder and surrounds the topmost section of the urethra. As part of the male reproductive system, the prostate gland's primary function is to secrete a slightly alkaline fluid as part of the seminal vesicle's fluid to transport sperm. During male climax (orgasm), the muscular part of the prostate gland assists in transporting sperm produced in the testes into the urethra, via the secreted prostate fluid.

While the embryology, morphology, structure differentiation during prostate development, and functions of the prostate are under hormonal control**,** the clinical pathology and diagnostic differentiation between benign and malignant proliferation of the prostate epithelial gland cells is still unclear and complicated.

1.1.2. Anatomy and histology of the Prostate

As part of the urinary system, the prostate lies in the pelvic cavity of the male, underneath the urinary bladder. The prostate gland surrounds the bladder neck and proximal urethra (Fig. 1.1A).[3-4] Because the prostate is located next to the rectum, a digital rectal examination is possible for prostate cancer. The prostate gland consists of five lobes, which are separated by the urethra and ejaculatory ducts.[4, 5] Two lateral lobes and an anterior lobe enclose the urethra (Fig. 1.1B).[4] The two lateral lobes are marked by a posterior midline groove, which is palpable on a rectal examination. The middle lobe lies between the urethra and ejaculatory ducts. The posterior lobe lies solely behind the ejaculatory ducts. The normal prostate gland weighs about 20 g. It has the shape of a walnut. The prostatic urethra is divided into proximal and distal segments of approximately equal length by

an abrupt anterior angulation of its posterior wall at the midpoint between prostate apex and bladder neck. The non-glandular tissue of the prostate is concentrated antero-medially and is responsible for much of the anterior convexity of the organ.[4] The non-glandular compartment of the prostate contains the pre-prostatic sphincter, striated sphincter, anterior fibro-muscular stroma and the prostatic capsule.

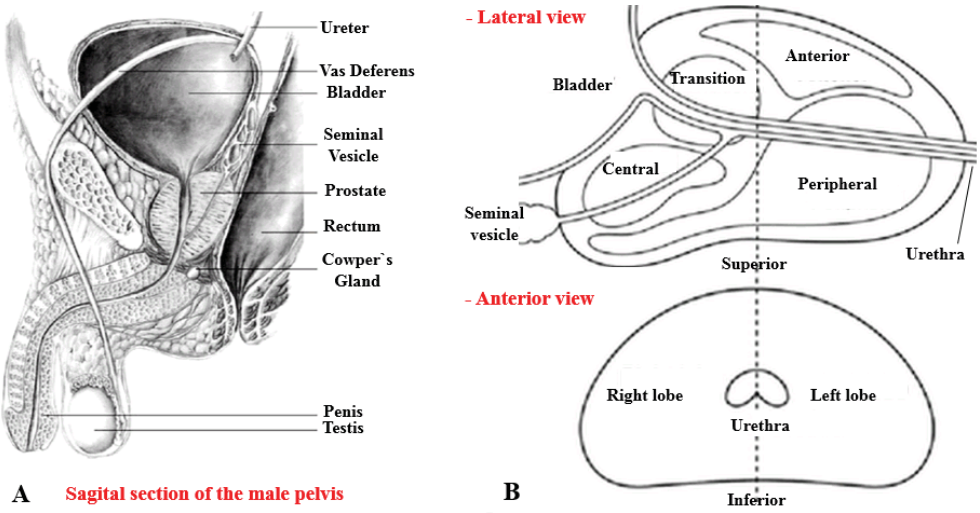


Fig. 1.1: The topography and anatomy of prostate gland. **A)** The prostate gland has a topographical relationship to the following organs in the small pelvis cavity; urinary bladder, rectum urethra, penis, and the lymph nodes in the pelvis[3]. **B)** The prostate gland is divided into: the 1 = peripheral zone, 2 = central zone, 3 = transition zone and 4 = anterior fibro muscular zone.[4, 5]

Its glandular part is composed of tubulo-alveolar glands. The contour of the glandular prostate is a disc with lateral wings that fold anteriorly and partially encircle the non-glandular tissue. The glandular compartment of the prostate contains three major glandular regions which differ histologically as well as biologically.[5] The three main regions of the prostate are the peripheral zone, the central zone and the transition zone. The transition zone surrounds the proximal prostatic urethra and is the exclusive site of origin for benign prostatic hyperplasia (BPH). Both the periurethral region and transition zone are the exclusive sites for the origin for benign nodular hyperplasia (BNH). The transition zone represents about 5% of the prostatic glandular tissue.

The peripheral zone is localized posterior and lateral to the transition zone. It surrounds the distal part of the prostatic urethra. The peripheral zone represents nearly 70% of the mass of a normal glandular prostate. Both glands of the peripheral zone and transition zone have a similar size (calibre), spacing and histological appearance but the peripheral zone is the most susceptible region to inflammations (prostatitis) and is the site of origin of most carcinoma.[6-7] The central zone comprises about 25% of the glandular prostate and little pathology is recognized in the central zone. The central zone is the only zone which is resistant to both carcinoma and inflammations. Like other glandular organs, the normal and the hyperplastic prostate gland consist of two cell layers,

namely the outer basal cell layer surrounded by the basal membrane and an inner columnar secretory cell layer lining the lumen.[8] The basement membrane separates the basal cells from the stroma which consists of smooth muscle cells and connective tissues. A third cell type has been recently recognized, the neuroendocrine cell, which is present in all areas of the prostate. However, the cells tend to be abundant in the major ducts and are more irregularly distributed throughout the acinar tissue (Fig. 1.2).[8] The secretory cells are columnar-shaped cells and consisted of about 10-20 in each acinus, secreting the enzymes PSA and PSAP. The pepsinogen II and plasminogen activator are produced only by the secretory cells of the central zone.[9-10] The basal cells are much smaller in size and contain an oval-shaped nucleus. The basal cells do not express PSA or nuclear androgen receptor and only express nuclear receptors of oestrogen and progesterone.[11]

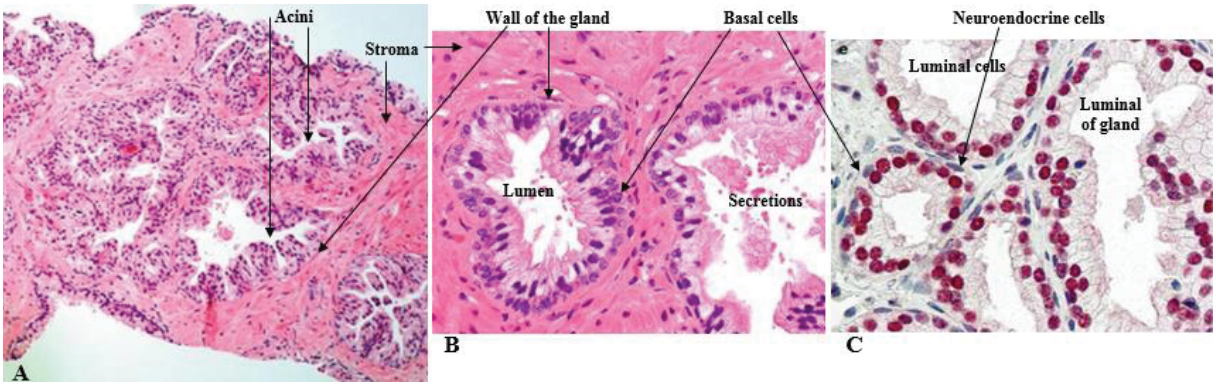


Fig. 1.2: The histology of the prostate gland. **A)** Shows the microscopic glands of the prostate. **B)** Description of the cell types within a human prostatic duct. Note: that the rare neuroendocrine cells are morphologically indistinguishable from basal cells.[8]

1.2. Prostate cancer

1.2.1. Epidemiology, incidence and prevalence

Prostate cancer is the most commonly diagnosed cancer affecting men after middle age (about age 50) in most industrialized western countries followed by lung and colorectal cancer. Prostate cancer is the second most common cause of cancer death in men after lung cancer and is responsible for approximately 13% of all cancer deaths.[12] About 30% of all men in Western countries will develop microscopic prostate cancer during their lifetime. Although most of prostate cancers tend to grow slowly, the risk of developing clinical symptoms is eight percent lifetime risk, with the risk of actually dying from prostate cancer at about three percent. Autopsy based prevalence is 80% by the age of 80 years which is why most men die with prostate cancer rather than from it. According to US data, a 50 year-old man with a life expectancy of 25 years has a 42% lifetime risk of having microscopic cancer, a 9.5 percent risk of having clinically evident cancer and a 2.9 percent risk of dying from prostate cancer. Nearly 899 000 prostate cancer cases and 258 000 prostate cancer deaths have occurred in 2008 worldwide, with 72% of the cases and 53% of the deaths in developed

countries (Europe, North America, Australia/New Zealand, and Japan) respectively.[13]

Thus the real increase in the diagnosis of prostate cancer in the last few decades has probably resulted from the widespread use of PSA testing, which allow the earlier diagnosis in men who have not yet developed symptoms.

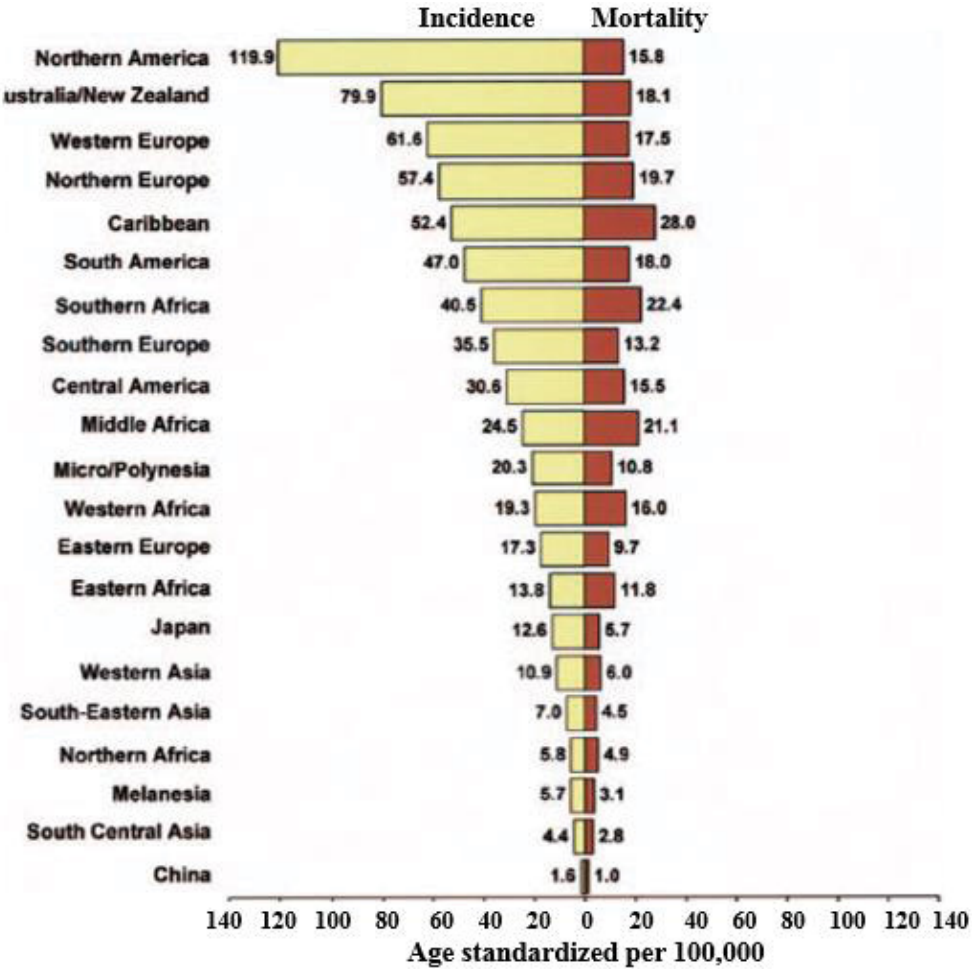


Fig. 1.3: Age-standardized Incidence and Mortality Rates for Prostate Cancer. Data shown per 100.000.[14]

The incidence and mortality of prostate cancer show variations from one continent to another, with the highest incidences in Europe and the lowest in Asia. In addition, both incidence and mortality of prostate cancer show variations from south to north as well as from east to west in Europe as shown in Fig. 1.3.[14] A large variation of incidence occurs within countries as well as populations, for example, the USA has a high incidence among black Americans followed by Caucasian men with a low incidence among men of Asian descent.[14-15] The reasons for this high degree of variability between ethnic groups are probably multi-factorial and include the availability of improved detection methods, increasing westernisation of lifestyle, and in particular, genetic risk factors. In addition to the above variation in incidence mentioned worldwide, there is a variation of stage distribution at the time of diagnosis around the world.[15]

1.2.2. Genetics

An important role in the etiology of prostate cancer is family history and genetics. Having a single first-degree relative with prostate cancer increases the risk for prostate cancer by a factor of 2.1-2.8. Having both a first-degree and a second-degree relative with prostate cancer increases the risk by a factor of 6. Familial predisposition can be due to common environmental exposures in addition to genetic factors (Table 1.1).[16] Due to the embryological development similarities in males and females between the prostate gland and mammary gland, the risk of prostate cancer may also increase in men who have a family history of breast cancer. Men with a family history of both prostate and breast/ovarian cancer among their relatives are also at an increased risk of prostate cancer.[17-18]

Table 1.1:- Relative risk related to family history of prostate cancer (modified after Zeegers et al.[16])

Risk group	Relative risk for prostate cancer
Brother with prostate cancer diagnosed at any age	3.4 (95% CI, 3.0-3.8)
Father with prostate cancer diagnosed at any age	2.2 (95% CI, 1.9-2.5)
One affected first-degree relative diagnosed at any age	2.6 (95% CI, 2.3-2.8)
One affected second-degree relative diagnosed at any age	1.7 (95% CI, 1.1-2.6)
Affected first-degree relative(s) diagnosed age < 65 years	3.3 (95% CI, 2.6-4.2)
Affected first-degree relative(s) diagnosed age > 65 years	2.4 (95% CI, 1.7-3.6)
Two or more affected first-degree relatives diagnosed at any age	5.1 (95% CI, 3.3-7.8)
Where CI is = Confidence interval	

About 9% of familiar prostate cancer is inherited with an autosomal dominant inheritance mode. Monozygotic twins have a double risk of prostate cancer in comparison to dizygotic twins, suggesting hereditary risk factors. Several loci and genes have been identified through the genome-wide linkage analyses of pedigrees. However, their function in carcinogenesis is not clear (Table.1.2). Hormonal causes have also been postulated as risk factors of prostate cancer because androgen ablation therapy causes regression of prostate cancers.

Table 1.2:- The loci and genes implicated in familial prostate cancer (Schaid DJ.[19]).

Gene or locus	Chromosomal locus	Function
HPC1	1q24-25	Contains the coding region for the RNase L gene
HPCX	Xq27-28	Function unknown
ELAC2/HPC2	17p11	Contains the coding region for the ELAC2 gene
PCAP	1q42-43	Function unknown gene
HPC20	20q13	Function unknown gene

1.3. Pathology

1.3.1. Pathology of prostate cancer

Prostate cancer is usually a slow-growing tumour. More than 95% of prostate cancers are adenocarcinomas that arise from the epithelial cells of the glandular tissue of the prostate gland and not from supporting or connective structures.[20] Other histopathological types of prostate cancer

rarely occur.

1.3.1.1. Pathological classification of Prostate cancer

Treatment decisions of prostate cancer depend on the pathological classification. Many different pathological classifications of prostate cancer exist, but only three are commonly used: TNM-Classification, WHO-Grading, and Gleason score in addition to ABCD or Storage system (Whitmore 1956)[21].

1.3.1.1.1. ABCD or Stages system (Whitmore 1956)

Prostate cancer staging includes the histology of the prostate cancer and is classified by four stages using the Roman numerals (I-IV) or the capital letters (A-D).[21] The main features of each stage are:

- Stage I or Stage A: The cancer cannot be felt during a rectal exam. It may be found by accident when surgery is done for another reason, (usually for BPH). No evidence of cancer spreading outside the prostate. (Microscopic and intra-capsular carcinoma).
- Stage II or Stage B: The tumour involves more tissue within the prostate. It can be felt during a rectal exam, or it is found with a biopsy that is done because of a high PSA level. No evidence of cancer spreading outside the prostate. (Microscopic and intra-capsular carcinoma).
- Stage III or Stage C: The cancer has spread outside the prostate to nearby tissues. (Macroscopic and extra-capsular carcinoma).
- Stage IV or Stage D: The cancer has spread to lymph nodes or to other parts of the body. (Metastasized carcinoma).

1.3.1.1.2. TNM -System (Tumour, Nodes, Metastasis)

The most common method of staging used more recently is the TNM staging system, after the UICC- Classification (Urinary International Community of Cancer): T= tumour, N= nodes and M= metastasis (Fig. 1.4).

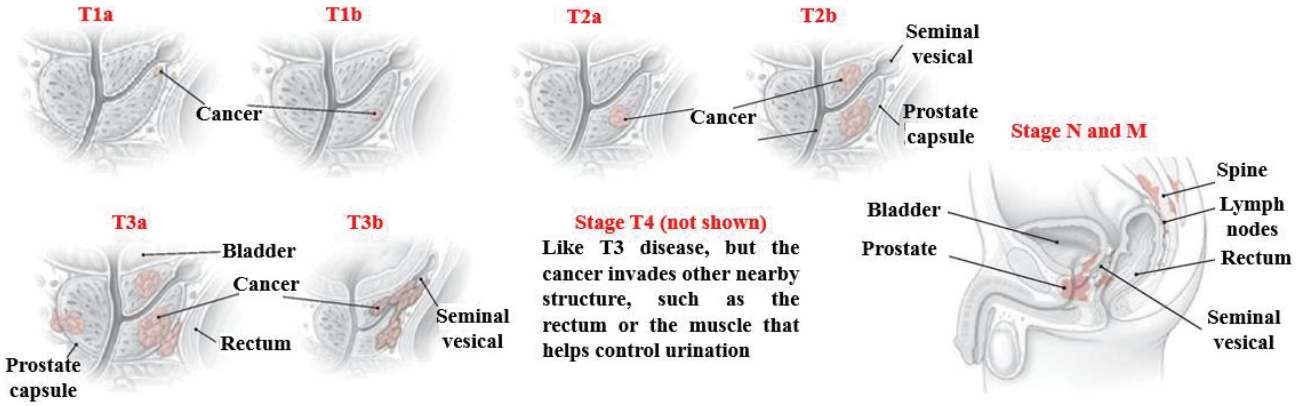


Fig. 1.4: TNM; the four stages of local prostate tumour growth.[22] 1) Tumour in pT1 is not visible by imaging. 2) Tumour pT2 is confined within prostate. 3) Tumour in pT3 extends through the prostate capsule. 4) Tumour in pT4 is fixed or invades adjacent structures other than seminal vesicles.

T: Primary tumour

- TX: Primary tumour cannot be assessed.
- T0: No evidence of primary tumour.
- T1: Primary tumour is clinically unapparent and not visible by imaging.
  - T1a: Tumour incidental histological finding in 5% or less of tissue (in prostatectomy).
  - T1b: Tumour incidental histological finding in more than 5% of tissue.
  - T1c: Tumour identified by needle biopsy (e.g., because of elevated PSA).
- T2: Tumour confined within prostate.
  - T2a: Tumour involves one-half of one lobe or less.
  - T2b: Tumour involves more than one-half of one lobe but not both lobes.
  - T2c: Tumour involves both lobes.
- T3: Tumour extends through the prostate capsule.
  - T3a: Extra-capsular extension (unilateral or bilateral).
  - T3b: Tumour invades seminal vesicle(s).
- T4: Tumour is fixed or invades adjacent structures other than seminal vesicles: bladder neck, external sphincter, rectum, lavatory muscles, and/or pelvic wall.

N: Regional lymph nodes.

- NX: Regional lymph nodes were not assessed.
- N0: No regional lymph node metastasis (lymph nodes confined to the true pelvis).
- N1: Metastasis in regional lymph node(s).

M: Distant metastasis.

- MX: Distant metastasis cannot be assessed (not evaluated by any modality).
- M0: No distant metastasis.
- M1: Distant metastasis.
  - M1a: Non-regional lymph node(s).
  - M1b: Bone(s).
  - M1c: Other site(s) with or without bone disease.

R: Residual tumour.

- RX: Residual tumour cannot be assessed.
- R0: No Residual tumour.
- R1: Microscopic Residual tumour.
- R2: Macroscopic Residual tumour.

1.3.1.1.3. Gleason system

The Gleason grading of prostate cancer is a histological evaluation of the prostate cancer tissue. It is an important part of assessing the outcome or prognosis. The Gleason grading system is one of the most widely used systems worldwide for prostate cancer. The Gleason grading system is based on the extent to which the tumour cells are arranged into recognisably glandular structures (Fig. 1.5) and the level of cell differentiation.

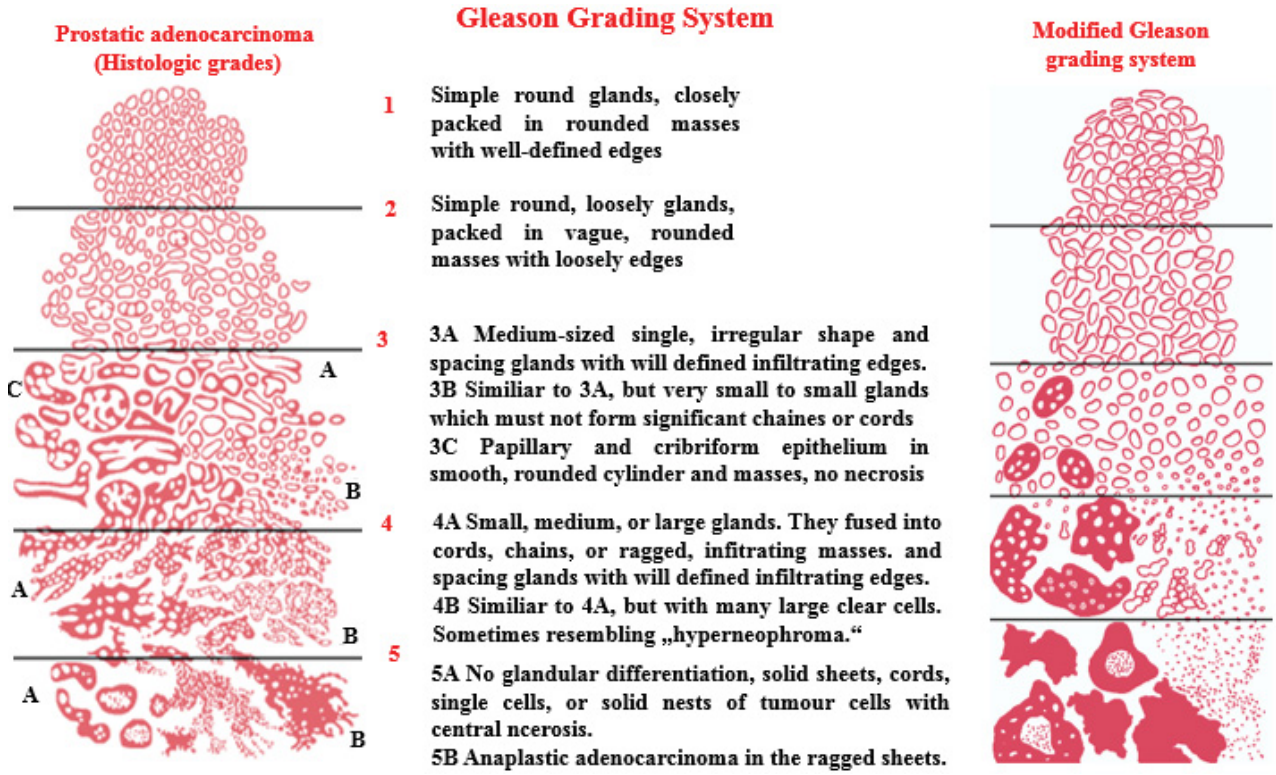


Fig. 1.5: This illustration shows Dr. Gleason's own simplified drawing of the five Gleason grades of prostate cancer.[8, 23]

The Gleason system identifies five levels of increasing disease aggressiveness with Grade 1 being the least aggressive and Grade 5 being the most aggressive cancer. Grade 3 is most common (Table 1.3). Gleason demonstrates the complexity of disease and difficulty in evaluation of prognosis.

Table 1.3:- Gleason Grading Scale.

Grade	Description
Grade 1	Small, uniform glands with minimal nuclear changes
Grade 2	Medium-sized acini still separated by stroma but more closely arranged
Grade 3	The most common finding in prostate cancer biopsies, show marked variation in glandular size and organization with infiltration of stromal and neighboring tissues
Grade 4	Markedly atypical cells with extensive infiltration into surrounding tissues
Grade 5	Sheets of undifferentiated cancer cells

Most prostate cancers are heterogeneous (have a mixture of cells at different Gleason grades). The lowest Gleason score is 2 and the highest is 10. This score provides useful prognostic

information. The Gleason score is written as the sum of the two most prominent Gleason patterns (i.e. Pattern 1 + Pattern 2 = Gleason score). The first number (or Pattern 1) corresponds to the most prominent tumour cell type. The second number (or Pattern 2) corresponds to the less prominent tumour cell type. A Gleason score of 2 + 3 = 5 has a dominant, well-differentiated pattern (pattern 1) and a less dominant, moderately differentiated pattern (pattern 2). A Gleason score of 7 or (3 + 4 = 7) is considered as a moderately differentiated tumour.[23] A score of 4 + 3 = 7 means that a poorly differentiated component (pattern 4) is dominant. A score 7 results from 3 + 4 is less malignant than that from 4 + 3. The most commonly diagnosed Gleason score is 7, while the most malignant is a Gleason score of 10.

1.3.1.1.4. WHO-grading System (Broders, 1925 and Mostofi 1975)

The third system in use is the classification of prostate carcinoma according to the WHO-Grading. Broders had studied the behaviour of the malignant cells and histological appearance of the prostate gland. He classified them into four stages.[24] Mostofi expanded the Broders – system. He studied the cytological characters of the nucleus growth and shape especially in the glandular part of the prostate.[25-26] Mostofi classified the malignancy of prostate carcinoma into three grades, which were later accepted by the UICC.[27-28] The WHO-Grading System is widely used in Germany. WHO-Grading System of the prostate after UICC and comparison to Gleason Score System is shown in table 1.4.

Table 1.4:- The WHO grading system and the equivalent Gleason score.

WHO grading system	Gleason score* and characteristics	The equivalent Gleason score	10-year probability of local progression
GX	Differentiation could not be determined		
Grade 1	Highly differentiated adenocarcinoma with lower atypia of nuclei (nuclei – atypia)	2-4	25%
Grade 2	Low differentiated adenocarcinoma with or without cribriform herds and moderate atypia of nuclei (nuclei – atypia)	5-6	50%
Grade 3	Cribriform and/or solide carcinoma with strong atypia of nuclei	7-10	75%

(Where * = Sum of the two most prominent grades)

1.3.2. Pathogenesis of the prostate cancer

Prostate cancer presumably results from a series of genetic events in the prostatic epithelial cell (Fig. 1.6).[29] These genetic events and many of the environmental factors that promote the development or progression of prostate cancer are still poorly defined. Nevertheless, in the last decades, several important studies have been made using techniques such as Fluorescence in situ hybridization on chromosome preparations or tissue sections with specific probes, flow cytometry and comparative genomic hybridization.[30-31]

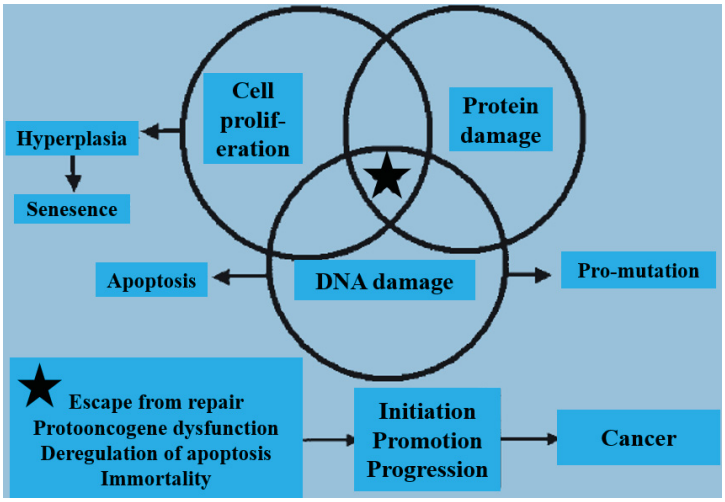


Fig. 1.6: The combination of cell proliferation, protein, and DNA damage over time in prostate cancer. The combination of cell proliferation, protein damage and DNA damage over time, results in multiple effects which ultimately result in prostate cancer.[29]

These techniques, including loss of heterozygosity (LOH) and polymorphism studies, have suggested that potential sites for genes associated with the initiation of prostate carcinoma exist on chromosomes 6q; 7q; 8p; 9p; 10q; 13q; 16q and 18q.[32-33] In one study, 74% of the primary prostatic tumours showed evidence of DNA sequence copy number changes.[34-35] Losses of genetic material were five times more frequent than gains in the case of prostate cancer. The most common abnormalities affected 8p and 13q. Furthermore, the pattern of genetic changes seen in recurrent tumours, with frequent gain of chromosomes 7, 8q and X, suggests that the progression of disease and development of hormone-independent growth may have a distinct genetic basis.[34-36]

This study is concerned mainly in sporadic factors where the patients under study were older than 50 years at the time of diagnosis.

1.3.3. Biomarkers for prostate diagnosis and prognostic value

Tumour markers may be based on the expression of genes or proteins, either structural or molecular. These markers can identify the proliferative and/or metastatic behaviour of the particular tumour as well as cellular morphology.[29] The hallmark of early malignancy of the prostate is the absence of basal cells and the loss of a basement membrane.[37-38] This process is followed with invasion of the gland and in an advanced stage leads to distant metastasis.

The first group of differentiation markers belongs to normal cell organization which characterizes many differentiated secretory cells, including tissue-specific secretions of enzymes and other secretory proteins, cell polarity, cell-cell adhesion, cell-basement membrane adhesion, cytoskeletal proteins, stromal proteins and neuroendocrine markers.[39] The loss of many of these

features is characteristic of cancer progression.[40] Such features are valuable indicators of the differentiation stage. The more differentiated state correlates with a more benign form but in aggressive tumour the tumour cells lose this feature. Thus, biochemical markers often are more appropriate indicators of a tumour presence. Cellular transformation results in the alteration of the gene expression, which consequently leads to changes in cell interactions and abnormal growth control. As a result, the cell organization is altered and gives rise to a diverse range of histologic abnormalities including prostate intraepithelial neoplasia (PIN) and cancer of the prostate.[41] The most common marker of this group is prostate specific antigen (PSA). The PSA screening test is used for early detection of prostate cancer. PSA has turned out to be organ specific but not prostate cancer specific. Although the increased level of serum PSA is not highly specific, it does suggest the possibility of cancer.[42] It is known that PSA level is increased in men with benign prostate hyperplasia (BPH) and prostatitis. This substantial overlap in serum PSA values between men with benign prostatic diseases and malignant prostate cancer is the limitation of PSA as a prostate tumour marker and the follow up test of disease progression. In addition, PSA cannot be used to differentiate the aggressive from indolent (inactive) tumour. PSA, also named human glandular kallikrein (hK-3), belongs to a human kallikrein gene family, which consists of 15 members.[43-45]

The new advanced genomic- and proteomic based studies have led to the identification of a large number of candidate biomarkers as well as signature patterns of multiple markers for prostate cancer diagnosis, progression of the disease and prediction of survival.[46-47] While these candidates include oncogenes, proliferation, differentiation markers and cytoskeletal proteins, many additional molecules were identified such as transcriptional repression and fatty acid metabolism. Also, the morphometric markers and the study of DNA ploidy belong to the group of new candidate markers for diagnosis, or prediction of survival.

The morphometric markers include nuclear size, shape, roundness, chromatin texture, size and number of the nucleoli and number of the apoptotic bodies.[48-49] These morphometric markers can be detected with image analysis (malignancy-associated changes) and provide useful predictive information in prostate cancer but are still considered investigational.[50-51]

The DNA ploidy analysis offers a good correlation between DNA ploidy, nuclear grade and histologic grade. It adds clinically useful predictive information for some patients.[52-53] Aberrant DNA ploidy indicates genomic instability and is one of the used markers. One of the hallmarks of cancer is genetic instability, which seems to generate new mutations by deletion and rearrangement. The mean proliferative index and the proportion of aneuploidy cells in high-grade PIN are similar to

those in cancer and are much greater compared with BPH and low-grade PIN.[54] Aneuploidy incidence in high-grade PIN is somewhat lower than Aneuploidy incidence in tumours.[55-56] DNA ploidy analysis of prostate cancer provides important predictive information. Patients with diploid tumours have a more favourable outcome and survive longer compared to patients with aneuoploid tumours; however, the ploidy pattern of prostate cancer is often heterogeneous which creates potential problems with sampling error.[57] The most common method of the DNA ploidy analysis is flow cytometry, which is limited by the need for large number of cells and approximately 2500-5000 nuclei.[58] Digital image analysis overcomes this limitation and is gaining popularity despite a lack of standards.[59] The third technique is molecular cytogenetic analysis such as fluorescence in situ hybridization (FISH), which can assess DNA ploidy for individual chromosome segments.

FISH analysis of interphase cells using centromere-specific and region-specific probes is useful for the detection of numeric chromosomal anomalies in solid tumours, including prostate carcinoma, which is often difficult for conventional cytogenetic analysis where growing cells in culture are needed. FISH allows the study of multiple foci of normal epithelium, PIN, and carcinoma within a single histologic specimen, and makes the evaluation of matched metastatic sites possible. Not only does prostate cancer show genetic alterations, but in 9% of atypical adenomatous hyperplasia cases genetic alterations are present, according to a FISH ploidy study using centromere specific probes for chromosomes 7, 8, 10, 12, and Y.[60] The overall frequency of numeric anomalies in PIN and carcinoma foci is remarkably similar suggesting that they have similar pathogenesis.[33, 61-62] Generally, the mean number of genetic abnormalities increases in PIN to carcinoma foci, and malignant foci contained more anomalies than paired PIN foci, suggesting that PIN is a precursor of carcinoma. According to FISH studies with centromere specific-probes for chromosomes 7, 8, 11, and 12, gains of chromosomes 7 and loss of 8 were markers of tumour aggressiveness and worse prognosis.[62-63]

Further studies are necessary to find new markers for identifying prostate cancer in the early developmental stages using prostate cancer cell lines, animal models, body fluids, biopsy materials and even the prostatectomy gland with new techniques. Thompson and his colleagues[64] (1998) identified a plasma membrane protein named Caveolin-1 in the prostate of the mouse. Caveolins are major structural proteins of caveolae, specialized plasma membrane invaginations that are abundant in smooth muscle cells, adipocytes, and endothelium and are represented in three forms (Caveolin-1, Caveolin-2, or Caveolin-3). Caveolin-1 was found to be highly expressed in 3 of 4 human prostate cancer cell lines and was shown to be over-expressed in androgen-independent clinical

prostate tumours.[65] Caveolin-1 is a caveolae integral protein known to play an important role in signal transduction and lipid transport.[66] Caveolin-1 was found to be over-expressed in primary and metastatic prostate cancer after androgen ablation therapy as well as in the primary and metastatic breast cancer.[67] It was also evaluated as an independent prognostic marker for prostate cancer progression in lymph node-negative patients who have recurrence after radical prostatectomy.[68] Caveolin-1 expression in the prostate cancer of African-American patients is more than double that of white American patients. The percentage of Caveolin-1 positive prostate cancer cells was higher in moderately differentiated (Gleason score 6) prostate cancer patients of African- American origin in comparison to white men.[69] Current studies show that Caveolin-1 expression is low in early primary tumours but becomes significantly elevated in focal areas of the primary tumour. Metastatic prostate cancer has the highest levels observed after androgen ablation therapy.[70] This increase in Caveolin-1 expression and secretion leads to autocrine/paracrine effects that further enable prostate cancer cells to survive apoptotic stimuli encountered during metastasis and following androgen ablation.[66] The anti-apoptotic activities of Caveolin-1 in prostate cancer cells could explain the apparent differences in the clinical virulence of this disease in African-American patients, where there is more Caveolin-1 expression in comparison to white American prostate cancer patients. Based on the above observations, Caveolin-1 expression has been evaluated not only as a promising novel biomarker, but also as a therapeutic target for androgen-insensitive prostate cancer.[71-72] To explore if there are further ethnic/racial differences in Caveolin-1 expression, Satoh T and colleagues (2003) evaluated Caveolin-1 expression as a predictive marker in Japanese men with prostate carcinoma.[73]

Newly identified tumour markers in other tumours could be related to, or helpful in, prostate cancer diagnosis as in the case of YB-1 found in breast cancer. This is due to the similarity in the developmental origin of the both tissues (breast and prostate) and might help to draw parallels between these two types of hormone dependent tumours.

The gene expression of Y-box binding protein (YB-1) (also named nuclease sensitive element protein-1, or p50, or DNA binding protein b (dbpb) is not expressed in normal breast tissues but was highly expressed in breast tumours.[74-75] The Y-box binding protein (YB-1) belongs to the family of Y-box transcription factors which were identified by their interactions with inverted CCAAT-boxes. The CCAAT-box is one of the most ubiquitous elements being present in 30% of eukaryotic promoters.[76-77] A limited set of elements, such as the CCAAT and GC -boxes, are present in a very high number of promoters. YB-1 is a member of the cold-shock domain (CSD) protein

"super family" that have been shown to contain a variable N-terminal domain and a C-terminal domain.[78] The structure of the C-terminal domain varies significantly in different organisms. Cold shock proteins are found in bacteria and function as RNA chaperones.[79] In the eukaryotes, the homologous region of the bacterial cold shock protein is found in the nucleic acid-binding region of the CSD protein family. CSD proteins are not found in yeast.[80] The YB-1 family includes the dbpA, dbpB, and dbpC/contrin (see Table 1.5 and Fig. 1.7).[81] The sizes of exons 2, 3, and 4 are well conserved and codon splitting within the CSD is also similar in all three genes.

Table 1.5:- Characteristics of human Y-box binding protein family (after Kohno K et al.[78]).

Accession no.	Name	Chromosome	Gene(Kbp)	Exon no.	Amino acids	Expression
NM_003651	dbpA	12p13	24	10	372	Heart, muscle
XM_059214	dbpB/YB-1	1p34	19	8	324	Ubiquitous
NM_015982	dbpC/contrin	17p11	6	9	364	Germ cell

The YB-1 gene comprises 8 exons spanning 19 kb of genomic DNA and is located on chromosome 1p34.[79] The mRNA of YB-1 is approximately 1.5 kb long and encodes a 43 kDa protein of 324 amino acids. Homology searches also show that there are five pseudo genes present in chromosomes 3, 7, 9, 14 and 15.

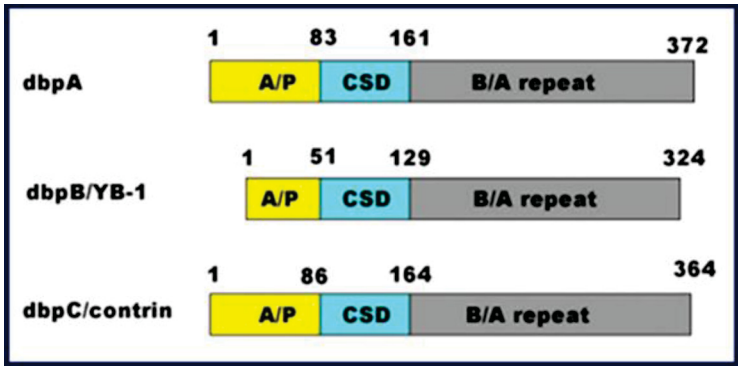


Fig. 1.7: General Domain organization of human Y-box-binding protein family members. This schematic illustration depicts the organization of domains in CSD proteins. A/P indicates the alanine- and proline rich N-terminal domain. B/A repeat indicate basic and acidic amino acid clusters.[78]

YB-1 performs a wide variety of cellular functions including transcriptional and translational regulations, DNA repair, drug resistance and stress responses to extra-cellular signals. Consequently, YB-1 expression is closely associated with cell proliferation. In all vertebrates, YB-1 protein comprises three domains: a variable N-terminal domain, a highly conserved nucleic acid-binding domain, and basic and acidic amino acid clusters.

Bargou RC and colleagues showed that YB-1 has an important role in controlling the transcription of the multi-drug resistance gene (MDR1 gene). This finding provides a basis for the

analysis of molecular mechanisms responsible for intrinsic multi-drug resistance in human breast cancer. MDR1 gene encodes P-glycoprotein and the regulated over-expression of YB-1 in drug-sensitive diploid breast epithelial cells induced MDR-1 gene expression and multi-drug resistance.[82] Nuclear localization of YB-1 protein has been reported to be associated with the intrinsic expression of P-gp in human breast cancer, which plays a major role in the development of a multi-drug-resistant tumour phenotype.[83-85] Huang J et al.[85] reported that YB-1 expression in breast cancer may be a potential marker of chemo-resistance and could possibly aid in selection of the appropriate adjuvant chemotherapy regime for breast cancers.

1.4. Aim of this study

The power of single biomarkers alone in predicting the clinical outcome of individual tumours is limited. Prostate cancer develops within and may expand out of the prostate before an initial diagnosis is made. Due to the absences of adequate therapy in advanced stages of prostate cancer, its tendency to be cured in early stages is high and showing the advantage of early diagnosis. Alternative biomarkers are therefore still needed to enable the earliest possible detection and more precise monitoring of the disease. Accumulating evidence has suggested that elevated Caveolin-1 expression is associated with a more advanced form of breast and prostate cancer. The aim of this study was to identify biomarkers that could be helpful alone, or in combination with other known markers in early detection of prostate cancer and to monitor its progression.

The first aim of this study was to employ the FISH technique to further understand correlations between Caveolin-1 expression and alterations of the Caveolin-1 gene; specifically to study the changes in the 7q31region where the gene is located. Furthermore, the study attempts to explore if the aneuploidy of chromosome 7 (7q31) and 8 could be linked to the risk of prostate cancer development as well as used for an accurate prognosis.

The second aim of this study was to explore whether there are further ethnic/racial differences in Caveolin-1 expression, which could be used as a predictive marker in German men (Caucasian peoples) with prostate carcinoma.

Prostate and breast cancers are the most commonly diagnosed cancers and are similar in that they arise in hormonally regulated secretory tissues. Etiology and epidemiology studies show growth similarities between them. YB-1 may be a helpful marker in the early stage of breast cancer. Therefore, the third aim of this study was to examine the efficiency of YB-1 as a potential biomarker in the case of prostate cancer and especially prostate adenocarcinoma.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and other Materials

2.1.1.1. Chemical Index

The chemicals used throughout the work were purchased from different companies as illustrated in table 2.1.

Table 2.1:- The chemical index.
The names of the companies and the chemicals used in the current study.

Company	Chemicals
Aldrich Chemical – Germany	Sodium Thiocyanate
Amersham Pharmacia Biotech, Freiburg – Germany	Adenine-Hemi-Sulfate, DNTPs (dATP, dCTP, dGTP, dTTP) (100 mM Solution)
Amersham Pharmacia, USA	dNTPs (dATP, dCTP, dGTP, dTTP)
B/Braun Melsungen AG – Germany	Aqua ad injectabilia Braun
Becton Dickinson, Sparks, Md, USA	Bacto ™ Agar, Yeast extract, Trypton, Trypton Peptone - Yeast extract, Trypton, Trypton Peptone
Biochrom KG, Berlin – Germany	Colcemid (Demecolcin, Seromed), Fetal Calf Serum – Phytohemagglutinin
Biozym, Gibco BRL/Life Technologies	Agarose Seakem
Roche Mannheim, Mannheim – Germany	Digoxigenin-11-dUTP
DAKO, Carpinteria, CA – USA	DAKO ® Peroxidase Blocking Reagent, DAKO ® Protein Block, DAKO ® Antibody Diluent, DAKO ® EnVision^{+TM}, Peroxidase, Mouse, DAKO ® EnVision+TM, Peroxidase, Rabbit, DAKO ® DAB Chromogen tablets, DAB Chromogen Solution,
DIFCO Laboratories, Detroit–Michigan, USA	Bacto- trypton, Casamino Acids, Yeast extract, Yeast Nitrogen Base
Fluka chemicals, Deisenhofen – Germany	Ethidium bromide
Food store	Chicken egg
Gibco BRL/Life Technology, Karlsruhe – Germany	TEMED, Dithiothreitol (DTT), Human Cot-1 DNA®, L-Glutamic acid (200mM), Trypsin-EDTA (1x)
Marabuwerke, Tamm – Germany	Fixogum Rubber cement
Merck, Darmstadt – Germany	Acetone, Aluminium calcium sulfate, Bromophenol blue, Chlorhydrat (Chloral hydrate), Citric Acid, EGTA Ethylene glycol tetra acetic acid, Ethanol, Ethidiumbromide, Formaldehyde 100%, Glycerol 100%, Glycerin water free, Hamatoxylin, Hydrochloric acid (HCl), Isopropanol 100%, Magnesium Chloride, Magnesium sulfate, Mercaptoethenol, Methanol, Phenol, Sodium Carbonate, Sodium Chloride, Sodiumdodecysulfat (DSD), Sodium Iodate, Sodium dodecyl sulfate (SDS), Tris-borate, Xylene-D(+) - Glucose (Monohydrate), Glycogen, L-Glutamine
O. Kindler GmbH & CO, Freiburg – Germany	Eukitt (mounting medium)
Promega, Madison, WI – USA	RNase free water
Oncor, Gaithersburg, MD – USA	Antifade, DAPI (200ng/ml)
Roche Diagnostic GmbH, Mannheim – Germany	NP-40 –Liquemin
Roth, Karlsruhe – Germany	Dextran Sulfate [Pharmacia LK Biotechnology AB, Uppsala, Sweden] Chloroform, Phenol/Chloroform, Phenol, HEPES, Sorbitol
Serva, Heidelberg – Germany	Sarcosyl - Xylene cyanol FF
Shandon, Pittsburg, PA – USA	Immu – Mount mounting medium

Company	Chemicals
Sigma, Taufkirchen – Germany	Aluminium calcium sulfate, Ammonium acetate, Ammonium persulfate (Ammonium peroxydisulfate), Ammonium Sulfate, Ammonium Chloride, Ascorbic acid (Vitamin C), Asparagine (anhydrous), Boric Acid, Dimethylsulphoxid (DMSO), 2 -Deoxyadenosine, N , N- Dimethyl Formamide, Deoxycholic Acid, Sodium Salt, Acid Sodium Salt dehydrate, Ethylenediaminetetraacetic, EDTA (Ethylenediaminetetra- acetic Acid), (Disodium Salt). Dihydrate, Ficoll Type 70, N Lauroyl- Sarcosine (Sodium Salt), Magnesium Chloride Hexahydrate, Magnesium Sulfate Hexahydrate, Mineral Oil, Polyethylene Glycol, Potassium Chloride (KCl), Potassium Phosphate (Monobasic anhydrous), Pyrophosphate Tetrasodium Salt (anhydrous), Potasium Phosphate (Dibasic: Trihydrate), Sodium Acetate (anhydrous), Sodium Chloride, Sodium Citrate (Tribasic dehydrate, ACS reagent), Sodium Hydroxide, Sodium Phosphate (Monobasic Monohydrate), Sodium Phosphate (Monobasic Anhydrous), Sodium Phosphate dibasic, Sodium dodecylsulfate (Lauryl/ Sulfate Sodium, SDS), Ficoll Type 70, Ficoll-400, Sodium hydrogen Carbonate, Sodium diphosphate, Sodium Hydroxide anhydrous, Sodium Hydroxide, Sodium Monophosphate, Sodium triphosphate, Tris base, Trizma Hydrochloride, Trizma ® Base, Triton X-100, Tween ® 20, Urea - Asparagine anhydrous Biotin, Bovin Serum albumin (BSA), Salmon Sperm DNA, Digoxigenin-11-dUTP, dTTP, L-Tyrosin
Vector Laboratories Inc. CA – USA	Vectashield stabiliser

2.1.1.2. DNA markers

Table 2.2:- DNA markers

DNA-marker	Company
Ready Load 1 kb-Ladder	Gibco BRL/Life Technology, Karlsruhe – Germany
pUC Mix	MBI Fermentas, Vilnius – Lithuania
Lambda/Eco 130 I-Marker	MBI Ferments, Vilnius – Lithuania

2.1.1.3. Antibodies

Table 2.3:- The antibodies used throughout the work

Antibody	Company
Avidin Cyanin 3	Dianova, Hamburg – Germany
Anti-digoxigenin	Roche, Mannheim – Germany
Caveolin-1	Transduction Laboratories, Lexington, KY – USA
YB-1	Institute for transplantation diagnostic and cell therapy - HHU-Düsseldorf – Germany

2.1.1.4. Antibiotics

Table 2.4:- The antibiotics used throughout the work

Antibiotic	Concentration	Company
Penicillin	10000 U/ml	GibcoBRL/Life Technology, Karlsruhe – Germany
Streptomycin	10000 U/ml	GibcoBRL/Life Technology, Karlsruhe – Germany
Kanamycin	10000 U/ml	GibcoBRL/Life Technology, Karlsruhe – Germany

2.1.1.5. Enzymes

Table 2.5:- The enzymes used throughout the work

Enzyme	Concentration	Company
DNase	1 U/µl	Roche Mannheim –Germany
Proteinase K		Roche Mannheim Germany

Enzyme	Concentration	Company
Taq DNA Polymerase	(5 U/µl) (recombinant)	GibcoBRL/Life Technology, Karlsruhe – Germany
Klenow (Exonuclease-free)	10 U/µl	USB, Cleveland, OH – USA
Pepsin		Sigma, Taufkirchen – Germany
RNase A		Sigma, Taufkirchen – Germany
RNase–It		Appligene Oncor
Trypsin 250		DIFCO Laboratories, Detroit – Michigan, USA
Zymolyase		Sigma, Taufkirchen – Germany
EcoRI Plus 10x buffer H		Appligene Oncor, Heidelberg – Germany

2.1.1.6. Bacteria and Yeast

BACS, PACS and YACS are shown in table 2.6a, 2.6b, and 2.6c.

Table 2.6:- PACs, BACs, and YACs clones.
Table **2.6A:-** The PACs clones**.**

Official name RP1 library	Gene	Chromosomal location
clone 162O21	Caveolin-1 (CAV1)	7q31.1
clone 233H11	C-myc	8q24
49K5	eIF3-p40 (alias EIF3S3 alias EIF3H)	8q23

Table **2.6B:-** The BACs clones library No.753 and CEP4**.**

RZPD	Official name	Chromosomal location
RPCIB753C02651Q2	RPCI-11 651C2	4cent.q
RPCIB753C19713Q2	RPCI-11 713C2	4q Tel.
RPCIB753CF1319Q2	RPCI-11 19F13	4cent.p
RPCIB753H032Q2	RPCI-11 2H3	4p Tel.
RPCIB753L1216Q2	RPCI-11 16L12	4q Tel.
RPCIB753L14440Q2	RPCI-11 440L14	4p Tel.
	SG CEP4(α satellite)	4p11-q11

Table **2.6C:-** The YACs used in the current study**.**

Gene	Official name	Chromosomal location
NELF	YAC 240 G10	8p12
	YAC 611	
	YAC 757	

2.1.1.7. Kits

- BioNick ™ Labelling System [GibcoBRL/Life Technology, Karlsruhe – Germany].
- PCR Purification Kit (250) [Qiagen, Hilden – Germany].
- Dig-Nick Translation Kit [Roche, Mannheim –Germany].

2.1.2. Consumable Supplies

The used consumable materials are listed below.

Table 2.7:- List of the consumable supplies

Consumable Materials	Company
Falcon tube (15 ml and 50 ml)	Becton Dickinson, Franklin Lakes, NJ – USA
Pasteur- pipettes (150 mm, 230 mm)	Brand, Wertheim – Germany
Pipettes (Steril; 5ml, 10ml, 25ml), Costar ® Stripette (2 ml, 5ml, 10 ml, and 25 ml)	Corning, New York, USA
Slide	Engelbrecht, Edemünde – Germany
Eppendorf tubes (0.2, 0.5 ml,1.5 m, and 2 ml), Pipettes (1µl, 2.5 µl, and 10 µl)	Eppendorf, Hamburg – Germany
pH – Papers	Fluka – Chemie, Switzerland
Pipettes: 20 µl, 50 µl, 100 µl, 200 µl, 1 ml	Gilson, Villiers-le-Bel – France
White cap tubes (10 ml), Culture bottle (6 ml and 10 ml), Cuvette, Petri dish	Greiner, Frickenhausen – Germany
Powder- Free Latex Exam. Gloves, Kimwipes® Lite paper Wipes	Kimberly-Clark, Roswell, GA 30076 – USA
Kleenex	Kimberly-Clark – Europe
Pipette Tips	Dr. Kleef, Germany
Microscope glass cover slips	Lance Proper LTD – England
Nunc cryo Tube ™ Vials	Nalge Nunc international, NY – USA
Parafilm	Peciney Plastic Packaging, Menasha, WI 54952 – USA
Syringe (1 ml, 2 ml, 5 ml, 10 ml, 20 ml, and 50 ml)	Pharma-Plast GmbH, Braunschweig Germany
Aluminium – Foil, Plastic – Foil Histokitt	ROTH -Carl Roth GmbH + Co Karlsruhe – Germany
Nylon gauze	Robert Brückmann, Mönchengladbach – Germany
Glass bottles	Schott, Mainz – Germany
Drying Block, Sterile filter (0.45 µm Diameter), Whatman-paper	Schleicher& Schüll, Dasssel – Germany, Limited, Surrey, UK
Cover Slide (Cover slip)	Shandon, Pittsburgh, PA – USA
Needle for single use	Terumo Europe N.V. Leuven – Belgium
Test – tubes Rack, Coplin Jar, Glass rods	WOLAP, Düsseldorf – Germany

2.1.3. Instruments

2.1.3.1. Centrifuges

- Cool centrifuge (Z 233MK), Table Centrifuge (Z 200 M/H) [Hermle, Wehingen – Germany].
- Eppendorf centrifuge (5415D), Eppendorf centrifuge (5436) [Eppendorf, Hamburg – Germany].
- Ultrospec 2000 [Pharmacia, Freiburg, Germany].
- Speed Vacuum Model No SVC-100 H. [SAVANT Instruments, INC, FARMINGALE, NY – USA].
- Rotor 5094A, 594 [Hettich, Tuttlingen – Germany].

2.1.3.2. PCR -Machines

- Gene Amp 2400, ABI PRISM 7700 [Applied Biosystems, Foster City, CA – USA].
- PTC-200, Pleiter Thermal Cycler PTC-200, Programmable Thermal PTC-100 Controller [MJ-Research, INC Waltham, MA – USA].
- PE 4700 PCR Cycler [PE Applied Biosystems, Minnesota – USA].
- Perkin Elmer 9600 [Perkin Elmer, Minnesota – USA].

2.1.3.3. Microscopes and Accessories

- Phase contrast microscope, Zeiss Axiolab 2 Fluorescence microscope, Filter: Aqua, Triple, Rhodamine, FITC and Oculars, Objectives [Carl Zeiss, Jena – Germany].

2.1.3.4. Incubators (Normal) and Shakers - Mixer Incubators

- Shaker No. 54121 Type REXA2 [Heidolph, Schwabach – Germany].
- Incubator [Heraeus, Hanau – German].
- Labnet Vortemp 56 EVC [National Labnet Co., Woodbridge, NJ – USA].
- Incubator oven [Biometra, Göttingen – Germany].

2.1.3.5. Other Instruments

- Vortex Mixer [Bachofer, Reutlingen – Germany].
- Accu-jet pipette, Glass Pasteur pipette [BRAND GMBH+ Co KG, Wertheim – Germany].
- Oven Mytron [Biometra, Biomedizinische Analytik GmbH, Götting – Germany].
- Hybridization oven [Biozym, Oldendorf – Germany].
- pH-Meter 766 Calimatic [Knick, GmbH&Co, Berlin – Germany].
- Eppendorf Thermomixer [Eppendorf, Hamburg – Germany].
- Water bath [Grant instruments, Cambridge – England].
- KERN 440- 47 Weighing balance [Kern & Sohn GmbH, D-72336 Balingen – Frommern, Germany].
- Cryostat Microtome [Leica Microsystems, Nussloch – Germany].
- Sartorius AC-211S Weighing balance, SARTORIUS 2354 [Sartorius AG Göttingen – Germany].
- Nano Drop® Spectrophotometer [Pharmacia Biotech, Freiburg – Germany].
- Thermometer [WOLAP, Düsseldorf – Germany].
- Tender cooker [Nordic Ware, Minneapolis – USA].

2.1.4. Mediums, Buffers and Solutions for Defined Use

2.1.4.1. Mediums, Buffers for Lymphocytes Culture

Most of the buffers, media and solutions were prepared as described by Sambrook and Russel, 2002, unless supplied with the kits. The pH was adjusted with 1M, 5M and 10M NaOH, 1M and 5M KOH or 37% (v/v) HCl. Sterilization of all solutions, buffers and media was achieved by autoclaving for thermo labile solutions, by filtration through 0.2 µm filters. Heat-sensitive components, such as antibiotics, were prepared as stock solutions and added to the medium/buffer after cooling to 50 °C.

Buffer and media used during the present work are given in table 2.8-2.12.

Table 2.8:- List of the buffers and media for lymphocytes culture.

Name	Component	Concentration
HEPES – Hypotonic (pH 7.4)	HEPES KCL EDTA Ethylene glycol tetra – acetic acid	4.8 g/l 3 g/l

2.1.4.2. Media and Buffers for Bacteria

Table 2.9:- List of the buffers and media for bacterial cultures.

Name	Component	Concentration
LB–Medium (pH 7.5) for 1L (Luria-Bertani Medium)	Tryptone Yeast extract NaCl	10 g/l 5 g/l 10 g/l
LB-Plates	LB-Medium Agar	1L 15 g
LB/Amp. Plates	LB-Medium Agar Ampicillin	1L 15 g (50 µg/ml)

2.1.4.3. Buffers and Solution for the Isolation of BAC DNA

Table 2.10:- Buffers and solutions for BAC DNA isolation.

Name	Component	Concentration
Solution – I	Glucose Tris -HCl (pH;8)	50 mM 25 mM
TE Buffer (sterile)	Tris/Cl (pH 7.5) 2 ml 0.5M EDTA	10 ml 1M 0.5M
Solution – II (Should be freshly prepared)	NaOH SDS EDTA (pH 8)	0.2 M 1 % 10 mM
Solution – III	K –Acetate (29.4 g K acetate) Acetic acid	3 M 11.5 m
Ammonium acetate (4M)	Ammonium acetate	7.708 g/ml
Buffer -H (10x)	Prepared by Roche Mannheim –Germany	
4x SSC/0.1% Triton X-100 µl in ml	4 ml SSC Triton-X	20x 0.1 ml

2.1.4.4. Media and Buffers for Yeast

The media and buffers used for yeast cultures.

Table 2.11:- list of the buffers and media for yeast cultures.

Name	Component	Concentration
2x YT -Medium (PH 7) for 1L	NaCl Yeast extract Tryptone	5 g/l 10 g/l 16 g/l
YAC -Medium for plates on (3 Litre). Notice:- The addition of Casamino-Acid should take place before pouring onto the plates	Glucose Yeast Nitrogen Base L-Tyrosin-Adenine - Hemisulfate Agar 210 ml Casamino-Acid	60 g/3l 24 g 165 mg 45 g 20%
SE buffer (in1000 ml)	15 ml NaCl 50 ml EDTA (pH 8)	(5M) (0.5 M)

Name	Component	Concentration
Zymolyase -buffer (in 100 ml)	57.5 ml Glycerin 100 µl DTT 1 ml Tris (pH 7.5) 5 ml KCl 5 ml KCl	87% 100 µl 1M 5 ml KCl 10 mg
Zymolyase -Mix (in 600 ml)	109.2 g Sorbitol 72 ml EDTA pH 7.4 Sodium citrate 4.28 ml Mercaptoethanol	1M 0.5 M 17.6 g (14 M)
Yeast lysis -Mix (in 400 ml)	20 ml Tris (pH 7.5) 20 ml EDTA (pH 8.0) 20 ml NaCl 84µl Mercaptoethanol 400 µl Non-Idet P-40 40 ml SDS	1M 0.5 M (5M) (14 M) 10%
SE buffer (in1000 ml)	15 ml NaCl 50 ml EDTA (pH 8)	(5M) (0.5 M)
ES buffer	Sarcosyl 1000 ml EDTA (pH 8.0)	10 g 0.5 M
PBS (Phosphate buffer saline solution) pH 7.5	NaCl KCl Na_2HPO_4 KH_2PO_4 $MgCl_2$ $CaCl_2$	140 mM 3 mM 8 mM 1 mM 1 mM 1 mM
PBS	NaCl KCl Na_2HPO_4 KH_2PO_4	140 mM 3 mM 8 mM 1 mM

2.1.4.5. Buffers and Solutions for FISH

- Fixative (4% Formaldehyde/1x PBS): 8 ml 37% Formaldehyde, 66 ml PBS.
- Fixative (1% Formaldehyde/1x PBS): 2 ml 37% Formaldehyde, 72 ml PBS.
- Buffered Formalin adjusts the pH value of the solution to pH 7.4.
- Acid dehydrate solution in 70% Ethanol: 100 ml Ethanol 70%, 1 ml HCl (1M).
- Acid dehydrate solution in 80% Ethanol: 100 ml Ethanol 80%, 1 ml HCl (1M).
- Acid dehydrate solution in 96% Ethanol: 100 ml Ethanol 96%, 1 ml HCl (1M).
- Acid dehydrate solution in 100% Ethanol: 100 ml Ethanol 100%, 1 ml HCl (1M).
- RNase -solution: 100 µg RNase A, 2 ml 2x SSC.
- Sodium acetate pH 5.2 for DNA precipitation: 3 Mol/l sodium acetate then sterilize.
- Sodiumthiocynate 1M: Add 0.40535 g Sodium thiocyanate to 5 ml de-ionized H_2O.
- Notice: - The Sodium thiocyanate solution should be freshly prepared before use.
- Pepsin digestion solution for deep frozen sections 10 µg Pepsin/ml H_2O: 1 mg Pepsin in 100 ml de-ionized H_2O then adjusts the pH to 2 with HCl.

- Pepsin digestion solution for paraffin sections 40 µg Pepsin/ml H_2O: 40 mg Pepsin in 100 ml de-ionized H_2O then adjusts the pH to 2 with HCl.
- Pepsin digestion solution for cell nucleus 40 µg Pepsin/ml H_2O: 4 mg Pepsin in 10 ml de-ionised H_2O then adjust the pH to 2 with HCl.
- Master -Mix (Dextran sulphate Solution): 0.5 g Dextran sulphate, 500 µl 20x SSC, then add to 2.5 ml de-ionized water, leave the mixture in a water bath at 37-42 ºC until Dextran sulphate is dissolved.
- BT buffer in 1L: 0.15 M $NaHCO_3$, 0.1 % Tween-20.
- BSA (0.5%)/BT buffer: 50 ml BT buffer, 0.25 mg BSA.
- DAPI -solution: 3 µl DAPI, 10 ml BT buffer. (Solution should be freshly prepared and kept in the dark until used).

2.1.4.6. Buffers and Solution for Nucleus Isolation

- PBS- Buffer: 1.15 g (Na_2HPO_4), 1.15 g (Na_2HPO_4), 0.20 g (KCl).
- Pepsin solution: Dilute 7 ml of 1N HCl to 100 m with sterilized distilled water. Weigh 25 mg Pepsin then add 5 ml 0.07 M HCl in 15 ml culture tube (Falcon Tube) and leave it to dissolve in a water bath at 37 ºC. (Note: Pepsin Solution should be freshly prepared).

2.1.4.7. Buffers and Solutions for Immunohistochemistry

- Citrate buffer (10x): 42 g Citric acid, 10 ml 2M NaOH.
- 3% H_2O_2 (freshly prepared).
- Avidin blocking reagent (solution): To 200 ml bidest. water add two white eggs then mix well.
- Biotin blocking reagent (solution): To 200 ml bidest. water add 2 mg Biotin then mix well. Dilution buffer.
- Hemalauna -counterstain in accordance with Mayer.
- Dissolve 1 g Hematoxylin, 0.2 g Sodium Iodide, 50 g Aluminum-potassium sulphate, 50 g chloralhydrate, and 1 g citric acid in 1000 ml Aqua dest. Mix well and through warming and stirring, leave it to dissolve. Add 10 drops of 25 % Ammonia solution (Merck). Leave the solution for one day to mature. Filter the solution. Before use, add 2 drops 25% Ammonia per 300 ml solution.

Diaminobenzidine (DAB).

A- Bulk DAB reagent (Skytek) DAB chromogen/substrate:

DAB - Substrate	DAB -chromogen
1 ml	50 µl

DAB -substrate contains H_2O_2 and prepared fresh according to the needed amount.

B- DAB-solution:

One DAB tablet is dissolved in 10 ml TBS buffer; then add 0.1 ml 30% H_2O_2 to reach the end concentration of 3% H_2O_2 (DAKO -Kit) or 1m DAB -Substrate to 50 µl DAB -chromogen.

2.1.5. Other Buffers and Stock Solutions

Table 2.12:- List of other buffers and solutions used during the current study.

Name	Components	Concentrations
RNase H buffer: Prepare fresh just before use	Tris-Cl (pH 8.3) KCl EDTA (pH 8.0) Dithiothreitol	20 mM 20 mM 0.1 mM 0.1 mM
5x Loading buffer: Mix well and store at 4 C°	Bromophenol blue Xylen cyanid FF Glycerol in water	0.25% 0.25% 0.30%
DNase I – 10x buffer	Tris pH (7.5) $MgCl_2$ $CaCl_2$	100 mM 25 mM 5 mM
Formamide loading buffer (10 ml)	9.8 ml de-ionized Formamide, 200 µl EDTA (pH 8), 10 mg Bromophenol blue (or Xylen cyanid FF)	80% (W/V) o.5 M (1mg/ml) (1mg/ml)
10x TE buffer (Tris-EDTA Buffer) in 1L	100 ml Tris/HCl (pH 7.5) l EDTA	1M 0.5 M
1 M Tris (PH 7.5) (25 C°):	Tris/HCl Tris/Base	25.4 g 4.72 g
1 M Tris (PH 8) (25 C°):	Tris/HCl Tris/Base	8.88 g 10.6 g
NP- 40 Lysis buffer	Tris (PH 8.0) NaCl NP-40	50 mM 150 mM 1.0 ml
Sodium acetate pH 5.2 for DNA	Sodium acetate	3 Mol/l
PBS-T (pH 7.5)	NaH_2PO_2, Na_2HPO_2, NaCl, Tween- 20, Tris-HCl pH 9.0,Triton X-100	80 mM 20 mM 100 mM 0.1% 200 mM 1%
PCR buffer (10x)	Tris-HCl pH 8.4 KCl	200 mM 200 mM
Proteinase K-buffer	Tris-HCl pH 7.8 EDTA SDS	10 mM 5 mM 0.5%
TE (pH 7.5):	Tris-HCl, pH 8.0 (EDTA)	10 mM
TAE (10x), pH 7.0	Tris base, Acetic acid (v/v), EDTA	3 M 5.71% 50 mM
TBE (10 x), pH 7.4	EDTA, Boric acid, Tris-HCl (pH 8.0)	0.25 M 0.7 M 0.1 M
TBS (1x), pH 7.4) for 1l	NaCl KCl	8 g 0.2 g
TNE buffer	Tris-HCl (pH 8.0) NaCl EDTA	10 mM 10 mM 0.1 mM

Name	Components	Concentrations
Ammonium persulfate (10% w/v)	1g ammonium persulfate 10 ml of bidest	10% w/v
Sodium phosphate buffer (1 M; pH 6.5)	$Na_2HPO_2xH_2O$ $Na_2HPO_2x2H_2O$	103.5 g 29.25 g
Dithiothreitol (DTT, 1M): 1 ml aliquots and store at 20 ºC	20 ml sodium acetate (pH 5.2) Dithiothreitol	0.01 M 3.09 g
Gelatin (1% w/v):	Gelatine bidest.water	g 100 ml
Sodium cyanide (I M):	NaSCN 5 ml de-ionized water	0.40535 g
Tris-Cl (1M) pH 7.4	Tris-HCl pH 7.4 70 ml HCl	(1M) (1M)
1M Tris buffer (pH 7.5) in 200 ml:	Trizma/HCl Trizma/Base	25.4 g 4.72 g
Tris-Cl (1M) pH 7.6	Tris-HCl pH 7.6 60 ml HCl	(1M) (1M)
Tris-Cl (1M) pH 8	Tris-HCl pH 8 42 ml HCl	(1M) (1M)
Magnesium chloride hexahedral in 1l	$MgCl_2.H_2O$	203.3 g
Zymolase stock solution (30 mg/ml):, keep at –20 C	Zymolyase 2 ml Zymolyase buffer	gm
SSC (20x) Stock solution (pH 6) in 1L:	NaCl Na-Citrate pH 7	175.3 g 88.2 g
SSC (1x):	NaCl Na-Citrat	0.15 M 0.015 M
Ethidiumbromide stock solution:	Ethidiumbromide stock solution	10 mg/ml
Bromphenol blue solution (saturated)	Bromphenol blue in 10 ml water	a spatula peak

2.1.6. Prostate Tumour Samples

2.1.6.1. Origin of Tissue Samples

The tissues used in this work originated mainly from the clinic of Urology, Heinrich Heine University of Düsseldorf, and were evaluated in the department of pathology. A small number of tissue samples originated from the University of Bonn and were evaluated at the department of pathology Düsseldorf.

2.1.6.2. Types of Tissue Samples

The most dominant analysed tissue type was prostate tumour. The tumour samples were obtained by transurethral prostatectomy, laparotomy with radical prostatectomy or needle biopsy (Autopsy). Other organs or tissues related to prostate cancer were also included in these samples such as urinary bladder, seminal vesicle, and lymph nodes. All paraffin blocks were fixed in 10% formaldehyde until 1999. After 1999, the samples were buffered in 10% formaldehyde and embedded in paraffin. Only one sample was deeply frozen. The total number of the samples under study was divided into three groups (A, B, and C). Group A contained (n = 69) samples, group B contained (n = 109) and group C samples. The first two groups were used to study the Caveolin-1 expression in the prostate tumour tissue (mainly prostate adenocarcinoma). Group C was used to study the YB-1 expression in the prostate tumour tissue (mainly prostate adenocarcinoma). It

contained 52 selected samples. In addition to the prostate tumour samples, other tissue samples such as normal prostate, lung, and skin (normal and ulcerated) samples were also embedded in paraffin and used as control samples. Two blocks of paraffin containing a collection of different tissues were used to show the degree/intensity of the antibodies expressions, these tissues are listed below (see Table 2.13).

Table 2.13:- list of tissues used to test the antibodies' sensitivity.

Block number	Tissue type
Tissue collection 1	Liver, colon, placenta, thymus, lymph nodes, brain, lung, mammary glands, skin (normal and ulcera).
Tissue collection 2	In addition to the above tissues: kidney, urethra, urinary bladder

2.1.7. Primers

Table 2.14:- List of primers.

Primer	Sequence
Alu1 Eco	5`-TGG AAT TCT CCC AAA GTG CTG GGA TTA CAG-3`
Alu2 Eco	5`-GTG AAT TCC AGA ATT CGC GAC AGA GCG AGA CTC CGT CT-3`
AluCl1 Eco	5`-TGG AAT TCT CCC AAA GTG CTG GGA TTA CAG-3`
AluCl2 Eco	5`-GTG AAT TCC TCC TGC ACT CCA GCC TGG G-3`

2.1.8. Software

Photoshop program (photo processing) [Adobe], ISIS program [Metasystem], Corel Paint Shop pro x [Corel], Excel Table calculation, Power point, Word Text Program [Microsoft]. R 2.4 program.[86]

2.2. Methods

2.2.1. Preparing of Paraffin Sections

From pre-cooled paraffin embedded blocks, approx. 2–5 µm sections were drafted and prepared with the help of a microtome. The first sections were used for Hematoxylin/Eosin staining; the next sections were used for immunohistochemistry. Thicker sections of about 6-8 µm were prepared for use in a FISH study. In the end, 2-3 sections were also prepared for Hematoxylin/Eosin staining to see if the tumour was still present or not.

The sections were stretched out in a water bath for 1-2 minutes at 50 ºC and ground off salinized slides or Super Plus frost slides. The slides were dried in an incubator at 37 ºC for 12-24 hours until ready to be used.

The Hematoxylin/Eosin stained sections were evaluated by a pathologist. Tumour areas

were marked and the pathological report was written. The report contains a separate evaluation of each block. The general comment for each patient, including the tumour stage, Grade, Gleason score, metastasis and other clinical data, was then recorded.

2.2.2. Slide Preparation

2.2.2.1. Slides for the Chromosome Preparations

- Place the slide in 96% ethanol for at least 48 h.
- Dry the slide by pulling it between dryer -block papers.
- Put the slide in a cuvette filled with 80% ethanol; allow it to freeze (in cold storage room) until use.
- Before use, dry the slide by pulling it between dryer -block papers.
- Drop the sediment onto the slide from the proper height to get a good spread of the chromosomes.
- Allow the slides to dry at room temperature for 24 h.
- Leave the slide in a cuvette with 70% alcohol to stand in the refrigerator for at least 24 h.

2.2.2.2. Preparation of Poly-lysine Coated Slides

- In a sterile tube, prepare a solution of 10 µg/ml poly-lysine in DEPC H_2O.
- Using a plastic Pasteur pipette, place one or two drops of poly-lysine on each slide.
- Using a clean slide, draw the poly- lysine across the slide's surface so that the slide is coated.
- Cover slides to prevent dust settling on them and leave for about 1 h to dry.
- Store slides in boxes at 4 ºC until use.

2.2.2.3. Slide Preparation for FISH

- Mark the target site on the slide with a diamond pen.
- Denature the chromosomes by immersing the slide for 5 min in denaturing solution which has been pre-warmed to 73 ºC in a water bath.
- Immediately immerse the slide in cold ethanol.
- Wash slides for 2 minutes each in 85% ethanol, 100% ethanol (dehydration series).
- Slides are to be left to dry in the air. Make sure all the ethanol is evaporated.

2.2.3. Lymphocytes Culture from Whole Blood (Cell Harvesting and Slide Preparation)

For the detection of the quality and specification of the labelled probes, lymphocytes from peripheral blood cultures are preferred for use in most laboratories due to the ease in culturing and to the high metaphase index. They were received from the cytogenetic department of the human genetic institute Heinrich Heine University.

2.2.4. BAC /PAC Culture

2.2.4.1. BAC Culture in solid Medium

- Under sterile conditions immerse the needle in the soft agar -BACs/PACs stock tube.
- Inoculate the needle in the kanamycin plate or other plate with corresponding antibiotic according to the BAC/PAC cultivation in the sterile bank.
- Place the cover on the plate.
- Incubate the plate at 37 ºC overnight.

2.2.4.2. PAC Culture in liquid Medium

- All steps should be performed under sterile conditions (sterile bank).
- In 4 sterile flasks for each PAC, add 25 ml LB-medium. Add 25 ml to each medium plus 25 µl corresponding antibiotic (for example Kanamycin in case of Caveolin PAC).
- Choose a possible single colony in the solid agar and pierce it with the needle, then inoculate the liquid medium and incubate it overnight at 37 °C.
- Sterilize the needle for next infection by heating it in the flame.
- Incubate the cultures overnight or for 24 h.

2.2.5. DNA-Isolation (BAC/PAC DNA Isolation)

A- BAC/PAC glycerin stock solution

- In a sterile 2 ml Greiner tube, add 850 µl of culture under sterile conditions.
- To each tube, add 150 µl of 100% sterile glycerine.
- Close the tubes and keep at –80 ºC for next infection of a new culture.

B- DNA isolation

- Transfer the 25 ml BACs/PACs culture from the flask to 50 ml falcon tubes.
- Spin for 10 min at 4000 rpm at 4 ºC.
- Allow solution III to be cooled on ice.
- Discard the supernatant then dissolve the pellet in 600 ml solution I (without lysozyme) with the pipette tip.
- Add 1800 µl solution II and mix it carefully by inverting the tube. Leave it on ice for 5 min.
- Add 1500 µl solution III (cold) and shake gently. Put the tubes on ice for another 5 min.
- Spin for 20 min. at 4000 rpm.
- If the supernatant is still turbid, transfer the supernatant to another 15 ml falcon tube and repeat the centrifugation for another 10 min. at 4000 rpm.
- Place the supernatant in another tube and add 2400 µl isopropanol. Mix well and leave for 15 min at room temperature. (Centrifuge again if the supernatant is not completely clear). If the

supernatant contains foam, try stirring with the needle, until you have clear supernatant before the addition of the isopropanol.

- Centrifuge for 30 min at 4000 rpm.
- Discard the supernatant and leave the pellet to dry at room temperature.
- Add 300 µl TE buffer and re-suspend the pellet with the pipette tip.
- Add 300 µl of 4 M ammonium acetate. Leave it for 10 min on ice.
- Spin for 10 min at 12000 rpm; only the supernatant will be processed.
- Add 360 µl isopropanol and mix well then leave it for 10 min on ice.
- Spin for 10 min at 12000 rpm.
- Wash the pellet with 300-400 µl 70% ethanol taking care not to disturb it. Centrifuge for 2 min.
- Discard the supernatant and leave the pellet to dry at room temperature.
- Dissolve the pellet in 30 µl TE buffer. (BAC/PAC DNA).
- Add to 3 µl Plasmid DNA 9 µl deionised water and 3 µl loading buffer. Test the DNA on a 0.8 – 1% agarose gel to estimate the amount of DNA (Fig. 2.1A).
- Keep the BAC/PAC DNA at –20 ºC.

2.2.6. RNA Digestion

- Add 3 µl RNase A (10 pg/ 1µl) to 30 µl prepared BAC/PAC DNA and shake gently. Put the tube in the shaking incubator at 37 ºC for 90 min.
- Take 5 µl RNase A -digestion product then add 3 µl deionised water plus 2 µl loading buffer (Ficoll), then in 0.8-1% agarose and estimate the digestion (Fig. 2.1B).
- If the RNA is not completely digested, add 2 µl RNace-it™ and incubate for one hour as before. (RNase-IT digests the rest of the RNA which was not digested with the normal RNase A).

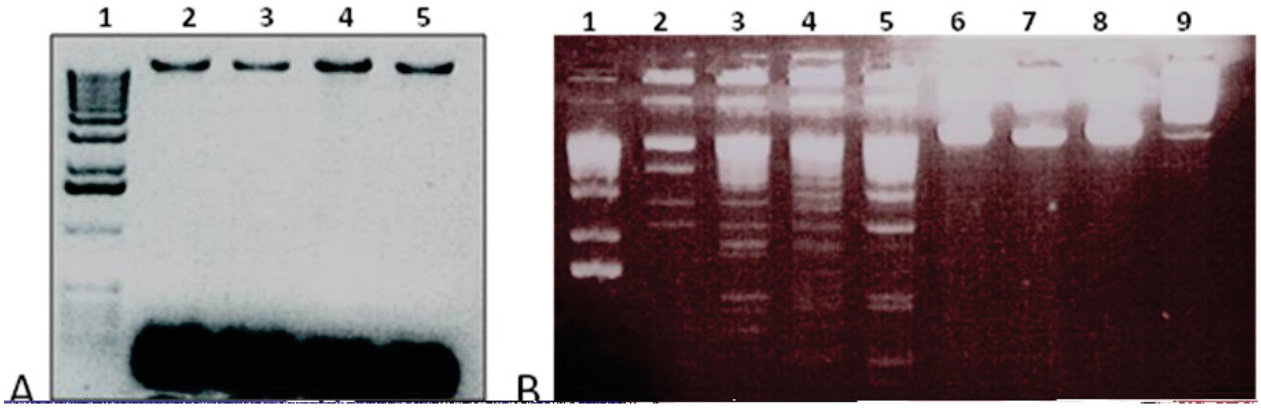


Fig. 2.1: Isolated plasmid DNA and band pattern of PACs DNA. **A)** Isolated Plasmid DNA. Lane 1, 1 kb DNA marker, lane 2-5 were Caveolin PACs after extraction from the culture without RNAse treatment. **B)** The band pattern of PACs DNA after treatment with RNase and *EcoR*I digestion. Lane 1- 1Kb ladder, where lane 2- P-40 PAC -DNA, 3- P-40 PAC -DNA , 4- Caveolin PAC -DNA, 5- P-60 PAC -DNA after incubation with RNase and *EcoR*I digestion. Lanes 6-9 contain the same PACs with RNAse treatment only.

2.2.7. Determination of DNA Concentration of the BAC/PAC Samples

2.2.7.1. Quantitative determination of DNA Concentration

- In a sterile 1.5 ml Eppendorf tube containing 3 µl of the plasmid DNA.
- Add 297 µl of ultra-pure water. In a separate tube, 300 µl of the batch of ultra-pure water to be used as a blank. Pipette the plasmid DNA (1:100 diluted) and the ultra-pure water (blank) into cuvettes.
- Use the blank to set the absorbance at 260 nm to zero. Measure the absorbance of the sample at 260 nm.
- Calculate concentration of the plasmid DNA using the following formula:
- Concentration (pmol/µl) = (A260/50) X (300/Mr) X (300/x).
- This method for quantitative determination of plasmid DNA produced different values for the same sample. Other methods therefore had to be found to determine the amount of DNA extracted from BACs, PACs and YAC samples.

2.2.7.2. Semi-quantitative determination of DNA Concentration

- In a 1.5 ml sterile Eppendorf tube, pipette 3µl PAC DNA (Caveolin, *C-myc*, or *P-40*). Add 23µl ultra-pure water and 3µl 10x buffer H, then 1µl *EcoRI* restriction enzyme and mix gently by slowly pipetting up and down.
- Incubate for 2.5 h at 37 ºC in inventor shaking incubator.
- Pipette 10 µl from the above tube into another Eppendorf tube. Add 5 µl of de-ionized water and 5µl Ficoll.
- Load all the samples on a 1% agarose gel plus 5µl 1kb Marker (1 µl = 100 ng DNA).
- Count the number and intensity of bands resulting from the restriction enzyme and compare it with the leader–bands. Notice the band pattern then estimate the amount of DNA in 1µl plasmid DNA under test (Fig. 2.1B).
- Although this method is semi-quantitative, it gives a result close to a quantitative value, if compared with DNA of known quantity (here 1 Kb marker).

2.2.8. YAC Culture

All steps should be done under sterile conditions (sterile bank).

2.2.8.1. YAC-Culture in solid Medium

- Immerse the sterile needle in the soft agar -YAC stock tube.
- Inoculate the needle in the YAC medium plate.
- Incubate the plate at 30 ºC for 24 h.

2.2.8.2. YAC culture in liquid medium

- In 4 sterile flasks for each YAC, add 50 ml YAC medium and 50 µl corresponding antibiotic (1 µl antibiotic to 1 ml medium).
- Choose a possible single colony from the agar plate on the YAC -solid agar and pierce it with needle. Inoculate the flask with liquid medium.
- Close the flask with the cover and shake it for 30 seconds.
- Put the flask in the proper position in the shaking incubator and adjust the incubator to the proper shaking speed at 30 ºC.
- Incubate the cultures for 24 h until the culture- medium colour is rose which shows the culture has grown enough.

2.2.9. DNA -isolation from YAC culture

2.2.9.1. YAC -glycerin stock solution

All steps should be carried out under sterile conditions (sterile bank).

- In a sterile 2 ml Greiner tube, add 150 µl of 100% glycerine.
- To each tube, add 850 µl of 100% culture.
- Close the tubes and keep at –80 ºC.

2.2.9.2. DNA -isolation from YAC culture

- Divide the 50 ml content of the flask into 10 ml portions in 15ml falcon tubes.
- Centrifuge the tubes at 2500 rpm for 10 min at 4 ºC then discard the supernatant.
- Re-suspend the pellet in 0.7 ml de-ionized water. Centrifuge the tubes at 2500 rpm for 10 min at 4 ºC.
- Discard the supernatant. Re-suspend the pellet in 0.7 ml Zymolyase–mix.
- Transfer into a 2 ml Eppendorf tube and add 12µl Zymolyase.
- Incubate the tubes at 37ºC for at least 2 hours (maximum 5 hours). Centrifuge for 5 min at 10000 rpm.
- Discard the supernatant. Re-suspend the Pellet in 0.7 ml Yeast Lysis -buffer and add 60 µl 10% SDS.
- Incubate for 15 min at 65 ºC in a water bath. Add 500 µl from the lower phase of 1:1 phenol/chloroform-mix.
- Invert shaking for 10 min. Centrifuge for 3 min at 12000 rpm.
- Pipette the supernatant carefully into a new tube for the next step (contains DNA).
- Add 500 µl Chloroform and invert shaking for 10 min.
- Centrifuge for 3 min at 12000 rpm. Pipette the upper phase into a new tube and add 560 µl

isopropanol. Invert shaking for 1 min.

- Centrifuge for 5 min at 10000 rpm. Discard the supernatant.
- Add 240 µl 1x TE Buffer to the pellet and dissolve at 65 ºC in a water bath.
- Add to the pellet 5 µl RNase A. Incubate overnight at 37 ºC.
- Add 280 µl of Ammonium acetate (5M) and 600 µl 100% ethanol as droplets until DNA precipitate (as visible filaments are formed).
- Centrifuge for 5 min at 10000 rpm. Discard the supernatant.
- Wash the DNA in 200 µl of 70% ethanol.
- Add 200 µl 1x TE buffer to the pellet and dissolve overnight at 4 ºC (refrigerator).
- Next day repeat the DNA precipitation process.
- Leave it again in refrigerator overnight to dissolve.
- If necessary, repeat the precipitation process as before.
- Dissolve the DNA in TE buffer.
- Load 3 µl of the samples on a 1% agarose gel plus 5µl 1kb Marker (Fig. 2.2).

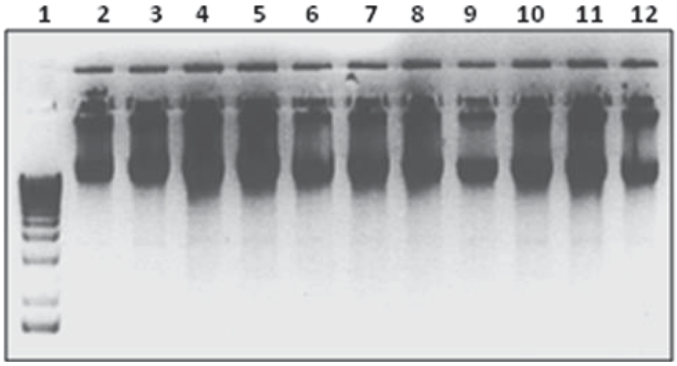


Fig. 2.2: YAC DNA directly after isolation. Lane 1 is 1Kb DNA marker and lanes 2-12 are YAC240 G10-DNA directly after the isolation.

2.2.10. FISH (Fluorescence In Situ Hybridization)

In-situ-hybridization was first introduced in 1969 independently by Pardue et al.[87] and Gall et al.[88], Buongiorno-Nardelli et al.[89], and John et al.[90] In *situ* hybridization (ISH) uses radioactively labeled probes to detect and localize specific RNA or DNA sequences in a tissue or on a chromosome. ISH relies on DNA's ability to reanneal, or hybridize, with a complementary strand. "*In situ*" means: "in the original place" in Latin. Thus, ISH involves a labelled nucleic acid probe hybridizing with a DNA or RNA sequence *in situ* (in the cells) so that the location of the sequence of interest can be detected in the cell, tissue, or chromosome. Like Northern and Southern Blots, ISH indicates the presence of a particular RNA or DNA sequence. However, ISH differs from blots in that the labelled probe reveals the actual location of the sequence in the cells (RNA or DNA).

ISH is the only procedure that allows the location of the sequence of interest to be studied.[91] The probe can either be radioactively labelled and detected by autoradiography or be non-radioactively (fluorescently) labelled (abbreviated FISH) and detected by immunocytochemistry. The specificity of the probe depends on the permeability of the cells, the type of probe, the labelling technique and the hybridization conditions, so specificity of ISH can be adjusted according to the desired results.[91] The major advantages of fluorescent probes include: safety, increased spatial resolution, reduced turn-around time for results, and the capability of simultaneous detection of multiple DNA regions of interest by using different combinations of fluorochrome labelled probes.

There are many different applications of FISH in mammalian cytogenetic. The major categories of applications include:[92]

- Mapping of genes and other DNA segments in genome research.
- Identification of species-specific chromosomes in somatic cell hybrids.
- Identification of chromosome aberrations (translocations, deletions, amplification), both numerical and structural.
- Characterization of unknown marker chromosomes.

There are two main types of probe labelling systems for FISH: direct and indirect. In the direct method, the fluorochrome molecule is bound directly to the nucleotides of the probe. This allows the probe signal to be visualized immediately following the hybridization reaction and washing steps. In the indirect labelling method, hapten molecules are attached to the nucleotides which are detected by a secondary molecule conjugated to a fluorochrome (Fig. 2.3). Two of the most popular detection systems are the digoxigenin-antidigoxygenin system and the biotin-streptavidin system.

The probes can be prepared in various ways. Two of the most common methods are referred to as Nick-Translation and polymerase chain reaction (PCR). Several companies sell Nick - translation kits that contain all the necessary reagents, which make it easy to use in this work.

Briefly, the procedure involves treating the DNA source with DNase I to generate single-stranded nicks along the double stranded DNA molecule. The 5’ to 3’ exonuclease activity of DNA polymerase I enzyme removes additional nucleotides to generate gaps. The same enzyme replaces the excised nucleotides with "labelled" nucleotides (mostly or usually fluor-dUTP or haptene-dUTP) using the other complementary intact strand as template. The "labelled" nucleotides may contain a digoxigenin or biotin conjugated base for the indirect labelling method or a fluorochrome

conjugate such as fluorescein, rhodamine, or Texas Red for direct labelling.

2.2.10.1. FISH Principle

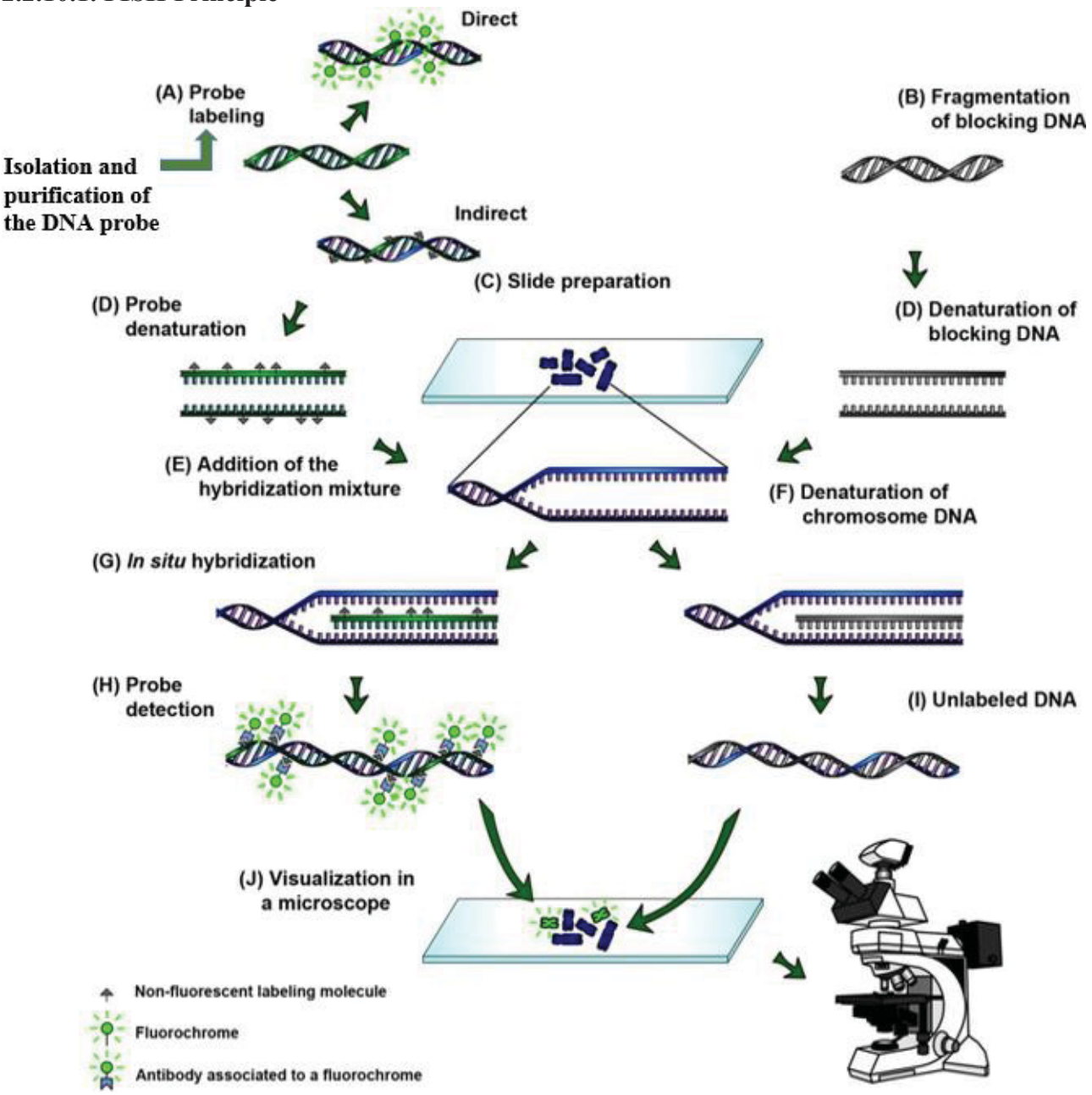


Fig. 2.3: The general FISH protocol.[93] The FISH process begins by DNA isolation, purification, followed by labelling, then denaturation and hybridization and ends with detection and examination under fluorescent microscope.

The labelled DNA is purified, concentrated, re-suspended in the hybridisation buffer (containing formamide) and is hybridised onto chromosomes and nuclei on slides (cytogenetic preparations). After overnight hybridisation, the slides are rinsed in washing solutions and, if necessary, one or several layers of fluorescent-labelled antibodies are added to detect haptene-labelled DNA. The slide is mounted with antifade solution and is visualized with the fluorescent

microscope, using appropriate filters. This procedure is illustrated in the figure above (Fig. 2.4).

2.2.10.2. DNA-labelling

Principally, the labelling process according to the manufacturer's instructions (In vitrogen BRL, Life technology) is as follows:

- µg BAC or YAC -DNA.
- 5 µl Nucleotide -buffer.
- 5 µl Enzyme -mix.
- X µl de-ionized water to reach a total volume of 50 µl in a 1.5 ml sterile eppendorf tube.

- Mix well then incubate the probe for 1h (for Bio-Nick Labelling) or 1.5 h (for Dig-Nick Labelling) at 16 ºC in a water bath.

- Stop reaction by placing tubes on ice.

- Check the size of the labelled products by running an aliquot on a 1% agarose gel as shown in Fig. 2.5A.

- If the fragment-size range is larger than 500 bp, incubate the probe for further 1 to 15 min in a water bath at 37 ºC, according to the resulting fragment size and run another aliquot on a gel to test the size again.

- Repeat the above process until reaching fragments of 100- 500 bp (optimal 300 bp).

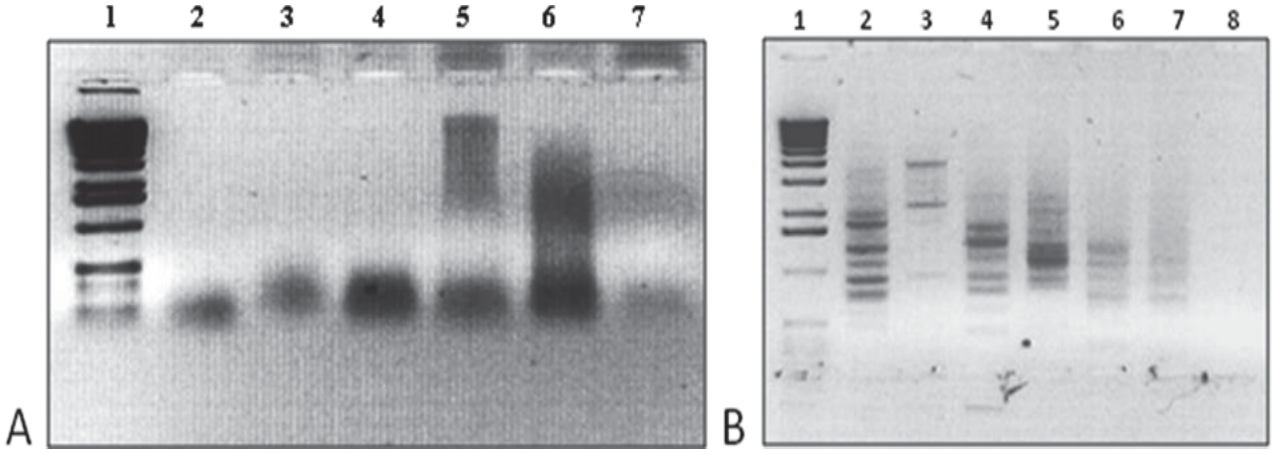


Fig. 2.4: The probes labeling and Alu-Repeat PCR. A) The labeled probes. Lane 1- 1Kb leader, 2- and 4- Dig. Nick Caveolin 3, 5 and 6- Bio-Nick YAC240G10, 7- Bio-Nick YAC240G10 Alu-Rep. B) The Alu-Repeat PCR. Lanes 1- 1Kb DNA marker, 2; Alu1x Alu1, 3; Alu2x Alu2, 4; Alu cl1x Alu cl1, 5; Alu cl2x Alu cl2, 6; Alu1x Alu2, 7: Alu cl1x Alu cl2, 8; 2x Alu 1 control.

2.2.10.3. Preparation of YAC -DNA with Alu-repeat PCR for Indirect Labelling

Isolation of the YAC from the yeast background by pulsed-field gel electrophoresis (PFGE) is laborious, with a low yield, and in many cases, difficult as the YAC may not be visible by ethidium bromide staining. Alu-repeat PCR amplification of total yeast DNA will increase the yield of YAC -DNA and therefore result in a higher hybridisation efficiency (17). YAC -DNA has at first

undergone PCR, using Alu -repeat primers, which in this work consist of Alu1, Alu2, Alu cl1, and Alu cl2 primers.

a- The PCR using Alu -repeat primers is done in 0.5 ml sterile Eppendorf tubes in the following primer combination:

1- 1 µl Alu1 X 1 µl Alu1 (i.e. 2 µl Alu1).

2- 1 µl Alu2 X 1 µl Alu2 (i.e. 2 µl Alu2).

3- 1 µl Alu1 X 1 µl Alu2.

4- 1 µl Alu cl1 X 1 µl Alu cl1 (i.e. 2 µl Alu cl1).

5- 1 µl Alu cl2 X 1 µl Alu cl2 (i.e. 2 µl Alu cl2).

6- 1 µl Alu cl1 X 1 µl Alu cl2.

7- 1 µl Alu1 X 1 µl Alu1 (i.e. 2 µl Alu1), without YAC -DNA as control probe.

b- Add to each tube the following (in order) on ice (for one probe).

- 39 µl bidest.water.
- 5 µl 10 x PCR Buffer.
- 1 µl Tag polymerase.
- 2 µl Primer or primer combination.
- 2 µl YAC -DNA.

c- Mix well. In the case of a master - mix for 8 tubes, divide it to 47 µl each, in the above seven tubes.

d- Add 2 µl YAC – DNA to the first six tubes and 2µl bidest. water to the tube No. 7 as control.

e- Put the probes in the cycler with the following PCR -program:

1- 94 ºC 4 min.

2- 94 ºC 1 min.

3- 40 ºC 1 min.

4- 72 ºC 1 min.

5- go to step 2 then repeat for 35 X.

6- 72 ºC 10 min.

7- 4 ºC ∞. Then the Alu PCR products are used later for labelling.

f- Check the size and amount of the Alu -repeat PCR products by running an aliquot (5 µl of each) on a 2% agarose gel (in TBE and containing 5µl of 5 mg/ml ethidium bromide per 100 ml) in comparison to 5 µl 1kb ladder (≈ 500 ng DNA) as follows:

5 µl Alu -repeat PCR product.

+ 10 µl sterile bidest. Water.

+ 5µl gel loading buffer (5 X Bromophenol blue).

Estimate relatively how many micro-litres of each Alu -repeat PCR product contain 1µg DNA (see Fig. 2.5B).

2.2.10.4. PAC or YAC DNA -labelling

I) Bio-Nick translation of PAC, YAC or YAC -Alu repeat DNA Labelling; (see section 2.2.10.2).

II) Dig-Nick translation of PAC or YAC DNA -labelling; (see section *2.2.10.2).*

2.2.11. Hematoxylin and Eosin staining of paraffin section

This method was used to determine the tumour areas for FISH preparation (with paraffin section as well as nucleus) and immunohistochemistry.

Rinse twice in Xylene for at least 10-15 min.

1- Rinse twice in 100% Ethanol for 30 sec.

2- Rinse once in 95% Ethanol for 30 sec.

3- Rinse once in 70% Ethanol for 30 sec.

4- Rinse in fresh water for 30 sec.

5- Rinse in Shandon's Hematoxylin -2 for 30 sec.

6- Immerse for a short time (10 sec.) in fresh water.

7- Immerse in a watery Shandon Eosin -Y for 30 sec. (either for 7 min.).

8- Rinse twice in 95% ethanol for 90 sec.

9- Rinse in 100% ethanol (fresh) for 90 sec.

10- Rinse twice in xylene (second solution fresh) for 10-15 min.

11- Leave the slide at room temperature to dry and cover.

12- Apply one drop Eukitt and cover immediately.

2.2.12. Cell nuclei isolation (Separation of cell nuclei)

1- Make 4 µm sections from a paraffin block and stain it with hematoxylin and eosin to mark tumour sites.

2- According to the tumour area, cut 4-8 sections, 10-25 µm thick and stretch each on a slide in a water bath at 50 ºC.

3- Making a Nylon-gauze -bag :- cut 6 x 4 cm from the Nylon-gauze and fold it in the middle then weld 2 edges together with help of a flame.

4- Scratch the tumour area from the slide and from the same area of the following sections (slides) and put them in the Nylon-gauze -bag. Weld the last edge together with help of a lighter.

5- Put the Nylon-gauze-bag in a chromium-plate biopsy cassette.

I- Deparaffination:

a. Rinse biopsy cassette for 30 min in xylene at room temperature.

b. Rinse biopsy cassette for another 30 min in xylene at room temperature.

II- Rehydration: (had to be done in 50 ml Falcon tube filled with solutions).

a. Rinse biopsy cassette for 30 min in 100% ethanol at room temperature.

b. Rinse biopsy cassette for 30 min in 96% ethanol at room temperature.

c. Rinse biopsy cassette for 30 min in 70% ethanol at room temperature.

d. Rinse biopsy cassette for 30 min in aqua dest. at room temperature.

e. Rinse biopsy cassette for 30 min in aqua dest. at room temperature with continuous shaking. At this step, the single cells separation can be interrupted if necessary.

III- Pepsin treatment:

- In 5 ml of 0.07 M HCl dissolve 25 mg pepsin completely in the water bath at 37 ºC. Wait until the pepsin solution reaches 37 ºC.
- Transfer the Nylon-gauze to the pepsin solution and shake well. Incubate for 30 min in the water bath at 37 ºC.
- After 30 min. stop the reaction by adding 5 ml pre-cooled PBS buffer to 4 ºC.
- VI- Production of the preparations on slides:
- Discard the Nylon-gauze where the separated cells were suspended now in PBS buffer solution.
- Centrifuge the PBS buffer solution containing the separated cells by 400 g (10000 rpm) for 10 min.
- Verify the cell concentration under a bi-ocular microscope or in a cell chamber.
- If the solution is cloudy, repeat the centrifugation, gently get rid of the upper part of the supernatant and add less or the equivalent amount of PBS.
- Repeat the control process as before.
- Add PBS if necessary to reach a proper concentration of the separated cells to be dropped on the slides.
- With the help of a Hettich Centrifuge (Rotor 5094A) and its accessories (appendages) drop an appropriate amount of the cell suspension in the column over the slide and centrifuge at 10000 rpm. Then carefully get rid of the suspension with the help of a Pasteur pipette.
- Alternatively, if the cell suspension is highly concentrated, drip one drop of the cytopreparation with the help of a Pasteur pipette, on a poly-L-Lysin covered slide or Super Frost slide (ready to use ionised coated slide).
- Let the slides dry at room temperature.

2.2.13. FISH procedures

2.2.13.1. FISH procedures on chromosome preparation from blood lymphocytes

Before FISH was performed on paraffin sections or cell nuclei, preparation probes were tested on chromosome preparations from blood lymphocytes to check the correct localization and labelling the BAC/PAC or YAC on the chromosomes.

I) DNA precipitation of the Bio-Nick or Dig-Nick labelled probe as a single probe.

- In a sterile 1.5 Eppendorf tube, pipette a volume corresponding to 100-400 ng DNA maximum (depending on the tumour area of the tumour sample under study).
- Add 2 µl salmon sperm DNA (10 µg/ 1 µl) and 4 µg human Cot-1 DNA® (1 µg/ 1 µl). Add 5 µl 3M sodium acetate (pH 5.2), up to 50 µl with water.
- Incubate the sample for 10 min on ice.
- Centrifuge the sample for 10 min at 12000 rpm.
- Dry the sample at room temperature.
- Add 5 µl 100% Formamide to the pellet and let it dissolve in the water bath at 37 ºC.
- Add 5 µl master -mix (0.5 g Dextran sulphate, 500 µl 20x SSC and add to 2.5 ml with bidest. water).

II) DNA -precipitation of the Bio-Nick and Dig-Nick labelled or α -satellite probes (Chrom.4, or X, or 10, or BAC/PAC, Or YAC DNA).

- In a sterile 1.5 ml Eppendorf tube, pipette a volume corresponding to 400 ng DNA Bio-Nick labelled sample, 1.5 µl Dig-Nick labelled α -satellite probe (Chrom.4, or X, or 10 or BAC/PAC, Or YAC), 4 µl salmon sperm DNA (10 µg/ 1 µl) and 8 µg human Cot-1 DNA® in order. Add to 100 µl with water.
- Add 1/10 the volume 3M sodium acetate (pH 5.2) (i.e. 10 µl).
- Add 2-3 volumes 100% ethanol.
- Incubate the sample for 10 minutes on ice.
- Centrifuge the sample for 10 minutes at 12000 rpm.
- Wash the sample with 100 µl 70% ethanol. Centrifuge at 12000 rpm for 2 min and discard the supernatant.
- Dry the sample at room temperature.
- Add 5 µl 100% formamide to the pellet and let it dissolve in the water bath at 37 ºC.
- Add 5 µl master-mix (0.5 g Dextran sulphate, 500 µl 20x SSC and add to 2.5 ml bidest. water).

III) Denaturation

Drop 100-150 µl 70% formamide/2x SSC (i.e. to 420 µl 100% formamide add 60 µl 20x SSC and

120 µl bidest. water) per slide and incubate at 75 ºC for 3 min.

- After addition of the master -mix to the labelled probe; incubate for 5 min at 75 ºC.
- Drop 8-12 µl of the pre-hybridisation solution (or labelled sample) per slide according.

to the section area, add glass cover slip and seal the edges with fixogum.

- Incubate the slide for more than 3 min at 75 ºC in the water bath.
- Incubate the slide over night at 37 ºC in the water bath.

IV) Demonstration

- Accurately remove the cover slip. Immerse the slide in 0.2% Tween-20 for 5 min washing at 42 ºC in a water bath.
- Wash the slide three times in 0.05 x SSC for 5 min at 42 ºC.
- Block in BSA (0.5%)/BT buffer (i.e. add 0.25 gm BSA to 50 ml BT buffer and allow. it to dissolve in a water bath at 37 ºC. Save 200 µl of BSA (0.5%)/BT buffer per slide for antibodies).
- Incubate for at least 10 min. in a water bath at 42 ºC.
- Add 0.2 µl Avidin – Cyanine -3 to the saved 200 µl BSA (0.5%)/BT buffer (i.e 1:1000) in 1.5 ml tube wrapped in aluminium (for protection from light), as well as 2µl Anti Dig- fluorescence (1:100).
- Drop the 200 µl on the slide and cover it with cover slip or parafilm. Incubate for 1h at 37 ºC in a water bath.
- Wash three times in BT buffer for 5 min each at 45 ºC and once in PBS for 5 min at RT in a dark chamber.
- Before use, add 2 µl DAPI to 5 ml BT buffer. Drop 200 µl on each slide and incubate for 5-8 min at RT in a dark chamber.
- Wash the slide with bidest. water and dry the slide at RT.
- Drop one drop of Vecta shield -stabiliser (mounting medium). Cover with a cover slip.

Evaluate the slide under fluorescence microscope.

2.2.13.2. FISH procedures in paraffin sections

- The first step is the DNA -precipitation (see section 2.2.13.1).

- The second step is the pre-treatment of the of paraffin section before the denaturation step. In the beginning we used the method below for the treatment of paraffin sections.

<u>1- Deparaffination :-</u>

a- Heat the slides overnight at 60 ºC in the oven.

b- Deparaffinize with two 15 min washes in 100% Xylene.

<u>2- Washing :-</u>

a- Immerse the slide in Isopropanol at 37 ºC for 5 min.

b- Immerse the slide in 100% ethanol at RT for 5 min.

c- Immerse the slide in 80% ethanol at RT for 5 min.

d- Immerse the slide in 50% ethanol at RT for 5 min.

e- Immerse the slide in 4x SSC/ 0.1% Triton X-100 (i.e. 4 ml 20x SSC plus 1 ml Triton X-100 then add 100 ml bidest. water). Incubate for 30 min at RT.

3- Sodium thiocyanate treatment :-

a- Drop 100-150 µl 1M NaSCN on the slide (according to the area of the section) and incubate for 25 min at 80ºC in the water bath.

b- Wash the slide in bidest. water.

4- Dehydration :- dehydrate the slide as follows:

a- Immerse the slide for 3 min in 0.01 M HCl in 70% ethanol.

b- Immerse the slide for 3 min in 0.01 M HCl in 96% ethanol.

c- Immerse the slide for 3 min in 0.01 M HCl in 100% ethanol.

d- Allow the slide to air dry.

5- Pepsin digestion :-

a- Immerse the slide in 4 mg /ml pepsin solution pH 2, pre-warmed to 37 ºC, into the water bath for 30 min (i.e. add 200 mg pepsin to 50 ml sterile water then adjust the pH with HCl to 2).

b- Wash the slide in 1x PBS. (The digestion time is variable and lies between 20-30 min according to the section area and thickness). It is possible to evaluate the degree of digestion by phase contrast microscopy before moving on to the hybridisation step. If there is a loss of cell borders, the sample is over-digested. If no individual nuclei can be seen, the sample is under digested and the incubation can be prolonged. At this stage, the nuclear image becomes grey/opaque.

c- Stretch the section by incubation at 80 ºC in the oven for 30 min.

d- Postfix in 1% formaldehyde in PBS for 5 min. at RT.

6- Dehydration :- dehydrate the slide as follows:

a- Immerse the slide for 3 min in 70% ethanol.

b- Immerse the slide for 3 min in 96% ethanol.

c- Immerse the slide for 3 min in 100% ethanol.

d- Allow the slide to dry in the air.

The preparation is now ready for the hybridisation.

After evaluation of the samples at the end of the FISH process, we found that some paraffin samples could not be evaluated due to the background and presence of coloured spots. This finding

may be related to the Haemoglobin that is present in the tissue samples which could not be removed in the above-described pre-treatment method. Latter by citrate treatment the back ground could be removed.

To avoid the loss of the labelled samples during the hybridization, if the slide had a strong background and spots, we added a new step which is the pre-hybridisation step using a pre-hybridisation solution as follows:-

7- Pre-hybridisation solution per slide

6 µl Cot-1 DNA®.
+ 4 µl Salmon sperm.
+ 38 µl bidest. water.

---

50 µl total volume.
+ 5 µl 3M Sodium Acetate pH 5.2 (1/10 of the above volume).
+ 150 µl 100 % ethanol.

- Incubate the sample for 10 min at room temperature.
- Centrifuge the sample for 10 min at 12000 rpm.
- Dry the sample at room temperature.
- Add 5 µl 100% Formamide to the pellet and allow it to dissolve in the water bath at 37 ºC.
- Add 5 µl master -mix (500 mg Dextran sulphate plus 500 µl 20x SSC and make up to 2.5 ml bidest. water).

1- Denaturation :-

a- Drop 100-150 µl 70% formamide/2x SSC (i.e. to 420 µl 100% formamide add 60 µl 20x SSC and 120 µl bidest. water) per slide and incubate at 75 ºC for 5 min.

b- After addition of the master -mix to the labelled samples; incubate for 8 min at 75 ºC.

c- Drop 8-12 µl of the pre-hybridisation solution (or labelled sample) per slide according to the section area. Place the cover slip and seal it with fixogum.

d- Incubate the slide for another 3 min at 75 ºC in the water bath.

e- Incubate the slide over night at 37 ºC in the water bath.

f- Demonstration (Visualisation).

g- Accurately get rid of the cover slip, and then immerse the slide in 2x SSC/ 0.2% Tween-20 for 5 min, then wash at 42 ºC in the water bath.

h- Wash the slide three times in 0.05x SSC for 5 min each at 42 ºC.

i- Block in BSA (0.5%)/BT buffer (i.e. add 0.25 gm BSA to 50 ml BT buffer and let it to dissolve in

a water bath at 37 ºC. Incubate for at least 10 min in water bath at 42 ºC.

j- Add 0.2 µl Avidin – Cyanine 3 to 200 µl BSA (0.5%)/BT buffer (i.e. 1:1000) in 1.5 ml tube wrapped in aluminium (for protection from light) as well as 2 µl Anti -Dig fluorescence (1:100).

k- Drop the 200 µl onto the slide and cover it with a cover slip or parafilm. Incubate for 1h at 37 ºC in water bath or incubator.

l- Wash three times in BT buffer for 5 min each at 45 ºC and 1 X in PBS for 5 min. at RT in a dark chamber.

m- Before use, add 2 µl DAPI to 5 ml BT buffer. Drop 200 µl onto each slide and incubate for 5-8 min at RT in a dark chamber.

n- Wash the slide with bidest. water and dry the slide at RT.

o- Drop one drop of vecta shield -stabilisator and cover with a cover slip.

r- Evaluate the slide under fluorescence microscope.

Note: Some of the preparations had a strong background caused by spots from red or green cells. To avoid this, a citrate buffer treatment between steps 2 and 3 was performed:

a- Immerse the slide in a heat resistant coplin jar.

b- Pre-warm pot-cooker filled to 2/3 with citrate buffer for 10 min in 750 W Microwave.

c- Put the coplin jar in the pot-cooker and cook for 15 min. Apply enough citrate buffers so that the specimen is never allowed to dry during incubation.

d- Cool by adding tap water to the coplin jar.

2.2.13.3. FISH procedures in cell nuclei

I- Washing :- Rinse the slides with cell nuclei in 1X PBS for 5 min.

II- Post-fixation :- Postfix in 1% formaldehyde in PBS for 5 min at RT.

7- Dehydration :- dehydrate the slide as follows.

a- Immerse the slide for 3 min. in 70% ethanol.

b- Immerse the slide for 3 min. in 96% ethanol.

c- Immerse the slide for 3 min. in 100% ethanol.

d- Allow the slide to air dry.

8- Pepsin digestion :-

a- Immerse the slide in 4 mg /ml pepsin solution pH 2 and pre-warmed to 37 ºC in the water bath for 5 min (i.e. add 200 mg pepsin to 50 ml sterile water then adjust the pH with HCl to 2).

b- Wash the slide in 1xPBS. (The digestion times are variable and lie between 20-30 min according to the section area and thickness).

c- Stretch the section by incubation at 80 ºC in the oven for 30 min.

d- Postfix in 1% formaldehyde in PBS for 5 min at RT.

9- Dehydration :- dehydrate the slide as follows.

a- Immerse the slide for 3 min. in 70% ethanol.

b- Immerse the slide for 3 min. in 96% ethanol.

c- Immerse the slide for 3 min. in 100% ethanol.

d- Allow the slide to air dry.

The preparation is now ready for the hybridization.

10- Denaturation :- See 2.2.12.2 page 26 and step 9.

11- Demonstration :- See 2.2.12.2 page 26 and step 10.

Evaluate the slide under the Fluorescence microscope.

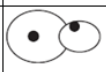
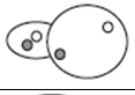
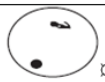
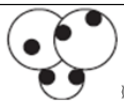
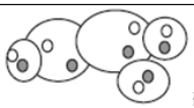
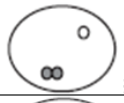
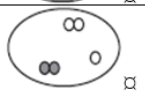
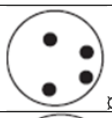
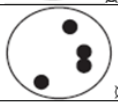
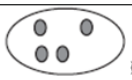
	Single colour signal counting guide		Dual colour signal counting guide	
			Key: - green probe ¶ ····· - orange probe	
1		Don`t count, skip over. This could be two cells with one signal each or one twisted nucleus.		1- Don`t count, The nuclei are overlapping and all areas of both nuclei are not visible.
2		count as 2 signals: one is very compact, the other is diffuse.		2- count as one orange and one green signal. The orange signal is diffuse.
3		Don`t count, skip over. Observer cannot determine which cellcontains the signal.		3- Don`t count, The nuclei are too close together to determine cell boundaries.
4		4- count as 2 signals: One signal is split. ¶		4- count as one orange and one green signal. The orange signal is split.
5		5- count as 3 signals. ¶		5- count as one orange signal and two green signal. One green signal is split and the orange signal is split.
6		6- count as 4 signals. ¶		6- count as two orange and one green signal.
7		7- count as 3 signals. One is split. ¶		7- count as three orange and one green signal.
8				8- count as four orange signal. ¶

Fig. 2.5: The principals of the evaluation in FISH study of the single and dual colour Signal counting guide.[94]

2.2.13.4. FISH evaluation

FISH technique was performed only on samples with prostate parenchyma. Samples with strong background and many spots of red or green colours were not evaluated. Thick samples from paraffin sections with overlapping cell nuclei or intensive (compact) cell nuclei were not analyzed. Also, slides with low number of cell nuclei were excluded from the evaluation. The evaluation principles were done according to the scheme of the Vysis protocol as shown above (Fig. 2.5).

2.2.14. Immunohistochemistry

2.2.14.1. Immunohistochemistry summary and explanation

Immunohistochemical (IHC) or immunoenzymatic staining techniques allow the visualisation of the antigens of tissue cells. The principle of this technique is based on the immunoreactivity of the antibodies and the chemical properties of enzymes or enzyme complexes which react with colourless substrate -chromogens to produce a coloured end-product. Initial immunoenzymatic stains make use of the direct method which conjugates enzymes directly to an antibody with known antigenic specification (primary antibody). The direct method lacks the sensitivity, but it allows the visualisation of tissue antigens using a standard microscope.

A significant improvement of the IHC-sensitivity was reported since the development of the indirect technique: A two-step method, in which the enzyme-labelled secondary antibodies react with the antigen-bound primary antibody. A further increase in sensitivity over the indirect technique was achieved through the application of the three step method with the introduction of the perosidase-antiperoxidase enzyme (PAP) complex. Here, the secondary antibody serves as a linking antibody between the primary antibody and the PAP complex.

The subsequent development in IHC techniques exploits the strong affinity of avidin for biotin and results in the avidin biotin complex (ABC) method of Hsu et al.[95]. ABC-Techniques employ an enzyme labelled avidin-biotin complex which is mixed prior to use and forms a complex with a biotinylated secondary antibody. ABC increases reagent sensitivity in comparison to the PAP method.

The LSAB system (Labelled Streptavidin-Biotin-Method) and HRP (Horse Radish Peroxidase) is based on modified Labelled Streptavidin-Biotin-Method or (LAB) technique in which a biotinylated secondary antibody forms a complex- conjugated streptavidin molecule. The LAB/LSAB method has been reported to be four to eight times more sensitive compared to the ABC method. The increased sensitivity is attributed to the smaller size of the enzyme-labelled (strep) avidin complex of the LAB/LSAB method, compared to the avidin-biotin enzyme complex of the ABC method (Fig. 2.6-Fig. 2.7).

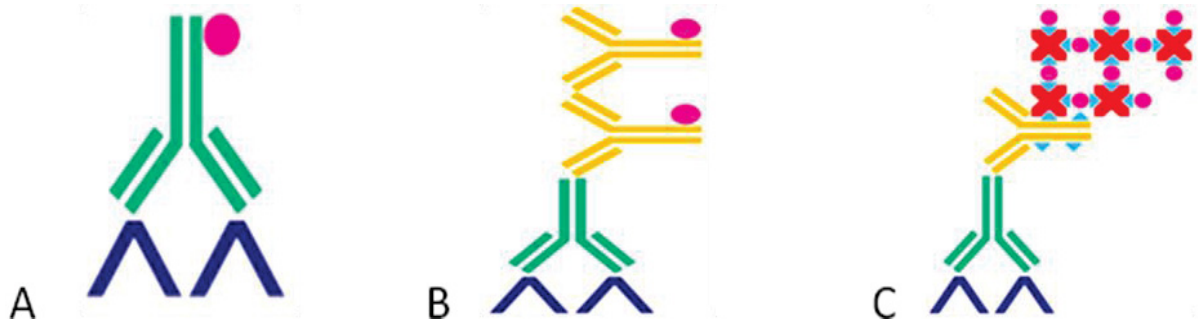


Fig. 2.6: Direct three, step labelling methods and ABC technology. **A)** Direct method: Enzyme-labelled primary antibody reacts with tissue antigen, **B)** Three step method: Enzyme-labelled tertiary antibody reacts with enzyme-labelled secondary antibody. **C)** The ABC technology. The avidin-or streptoavidin-biotin-enzyme complex reacts with the biotinylated secondary antibody (yellow).

Pseudoperoxidase or endogenous peroxidase activity can be found in haemoproteins such as hemoglobin, myoglobin, cytochrome and catalase as well as eosinophil. Their activity in formalin-fixed tissue can be inhibited by incubating specimens with 3% hydrogen peroxidase for five to ten minutes (according to the tissue) prior to application of the primary antibody. The tissue samples used in this study are formalin-fixed paraffin-embedded (FFPE), due to its superior preservation of morphology.

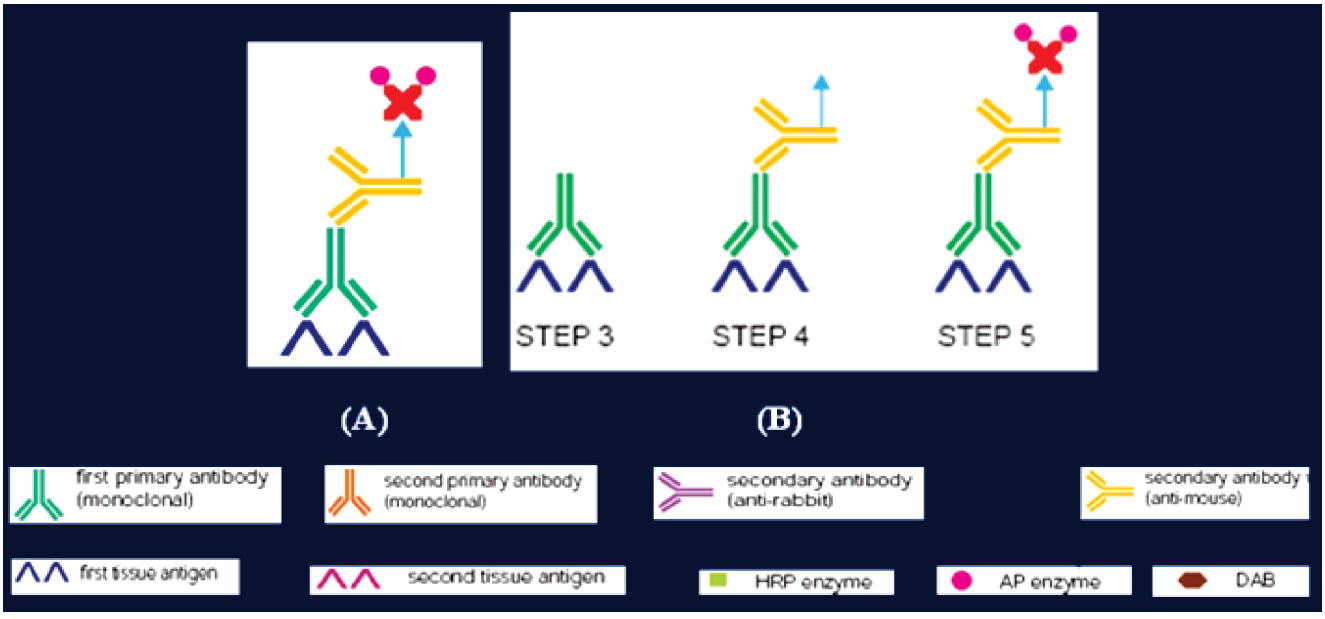


Fig. 2.7: LAB/LSAB and three step LSAB methods. A) In the LAB or LSAB technology the enzyme-labelled streptavidin reacts with a biotinylated secondary antibody. **B)** The three steps of the LSAB technology consist of the primary antibody (step3), biotinylated link antibody (step 4) and enzyme-labelled streptavidin (step 5).[96]

Formaldehyde induces conformational changes in the antigen molecules by forming intermolecular cross-linkages and masking the antigen-binding site (epitopes) to combine with the antibodies used in detection. Also, excessive formalin fixation can mask antigen sites and diminish specific staining. However, these sites may be revealed with proteolytic digestion or target retrieval

of tissue section prior to immunostaining. Well known proteolytic enzymes used for this purpose are protease, pepsin, pronase, proteinase K, chymotrypsin and trypsin with different degrees of success. Use of enzymes may also entail the risk of destroying some epitopes.

The target retrieval solutions used for the first time was reported by Shi et al.[97] and contained various metals and microwave heating for the restoration of immunoreactivity in FFPE. The term includes "antigen retrieval" (AR) and was applied for the first time. A successful improvement was reported by Cattoretti et al.[98], who also employed a citrate buffer of pH 6.0 instead of the original metal solution and heat, resulting in successful demonstration of FFPE tissue staining of a wide variety of additional markers.

The principal of antigen retrieval relies on the application of heat, for varying lengths of time, to FFPE tissue sections in an aqueous medium. Most retrieval solutions have a pH near 2, 6, 8 or 10. The above discussed techniques are not needed for the antigens, which have free epitopes for their markers.

2.2.14.2. Immunohistochemistry of Caveolin-1 antibody

A- Deparaffination and rehydration:

Prior to staining, tissue slides must be deparaffinised to remove embedding media and then re-hydrated. The incomplete removal of paraffin or residual embedding media will result in the increase of non-specific staining.

1- Place the slides with 4–5 µm thick paraffin sections in the oven overnight at 60º C.

2- Rinse the slides for 10 min 2x in xylene.

3- Tap off the excess liquid (xylene) and place slides in absolute ethanol for 2-3 minutes.

4- Rehydrate the section in alcohol series (100%, 96%, 80%, and 70%) for 1 min each. Before changing the bath, tap off excess liquid.

5- Place the slides in bidest. or de-ionised water for at least 1 minute.

6- Incubate the slide in 3% H_2O_2 2 for 10 min. Wash the slide with bidest. water.

7- Pre-warm a pressure-pot filled to 2/3 with citrate buffer for 5 min in the microwave (750 W). Rinse the slides in the citrate buffer in pressure-pot (slides should be covered with citrate buffer) and heat for 15 min in a microwave.

8- Cool the sections under tap water.

9- Incubate the sections in Avidin solution (2 egg-whites in 200 ml dist. water) for 20 min at room temperature. Wash the slide with bidest. water.

10- Incubate the sections in Biotin solution buffer (0.02% in TBS buffer) for 20 min. Wash the slide

with bidest. water.

11- Stretch out the slides on a moisture cover plate.

12- Spool the section with bidest. water with the help of a spray bottle.

13- Drip 200 µl primary antibody 1:3000 diluted Caveolin-1 antibody (i.e. add 1µl Caveolin-1 antibody to 3000 µl dilution buffer) and incubate for 2 hours at room temperature. Wash the section with bidest. water with the help of a spray bottle.

14- Drip 3 drops of secondary antibody (≈ 200 µl Yellow -link antibody) over the section to cover the specimen. Incubate for 20 min at room temperature. Wash the section with bidest. water with the help of a spray bottle.

15- Drip 3 drops tertiary antibody (≈ 200 µl Red strept avidin peroxidase reagent) over the section. Incubate for 20 min at room temperature. Wash the section with bidest. water with the help of a spray bottle.

16- Apply 500 µl Diaminobenzidin over the section and incubate for 10 min at room temperature. Wash the section with bidest. water with the help of a spray bottle.

17- Loosen the slides gently form the cover -plate. Wash quickly with bidest. water.

18- Additionally, the slide was stained in Hamalauna through incubation for 5 min at room temperature.

19- Dehydrate in alcohol series (70%, 80%, 96%, 100%, 100%) for short time (≈ 30 second), then three times in xylene.

20- Leave the slide covered in the cover-slip machine with Eukitt-mounting medium.

21- Keep the slide at 4 ºC.

B- Procedural notes :

All kit reagents, solutions and buffers should be calibrated to room temperature (20-25 ºC) prior to immunostaining. Likewise, all incubations should be performed at room temperature.

1- Tissue sections should not be allowed to dry during the staining procedure. A dried tissue section may display increased non-specific staining.

2- Slides exposed to draft should be covered.

3- If incubation time is more than 30 minutes, sections should be placed in a humid chamber or environment.

4- If the staining protocol or steps must be interrupted, slides may be kept in a buffer bath following incubation of the link antibody (step 10) for up to one hour at room temperature (20-25 ºC) or up to 18 hours at (4-8 ºC) without affecting staining performance.

5- Generally, the sensitivity of the LSAB system and HRP can be further increased by lengthening

the incubation times of the steps 13, 14, and 15 for ±5 minutes each.

2.2.14.3. Immunhistochemistry of YB-1 antibody

The immunohistochemical staining with YB-1 antibody was different from Caveolin-1 staining in three steps and the other steps were the same. In case of YB-1 staining was no citrate treatment, antibody dilution was 1:150, and primary antibody incubation was 1 hour.

2.2.14.4. Evaluation of immunhistochemistry

After the establishment of the method**,** the antibodies (Caveolin-1 or YB-1) were evaluated in different tissue samples to determine the tissues with high, middle, low expression or negative as well as their specification of expression in each tissue, in comparison with prostate tissue samples. The expression of each antibody was evaluated in the different tissue samples of prostate parenchyma in general, then in different tissue cell types such as connective tissue cells, basal cells, and glandular cells separately. The immunoscore for the evaluation was fixed and determined for each tumour sample. The immunostaining grade in parenchyma cells of prostate carcinoma was scored between 0-3 in the case of Caveolin-1. A score of 0 meant no expression or negative, a score of 1 meant weak expression, a score of 2 meant medium expression and a score of 3 meant strong expression. The immunoscore of Caveoin-1 ranged between 0-300 in parenchyma of prostate carcinoma cells according to Tan et al. as described in Caveolin immunoscore 1 (see Table 3.12 and Fig. 3.16). The immunoscore of YB-1 ranged between 0-12 in parenchyma of prostate carcinoma cells according to Remmele as described in immunoscore 2 (see Table 3.16 and Fig. 3.21).

Samples with strong background and many spots of brown colour were not evaluated. Samples which overlapped during the immunohistochemistry process or showed defects were also excluded from evaluation.

Each sample was viewed under the microscope with four different lenses (4, 10, 16, and 40 X-lenses) for more details about the expression in the cells and cell organelles. In group A, the prostate parenchyma cells (basal cells, gland cells, and transformed tumour cells) as well as metastasis cells were evaluated and listed in the table for each tumour sample (see Table 3.7-3.11 and Fig. 3.12-3.13)

Group B was evaluated according to the number of the tumour cells expressing Caveolin-1 as a percentage of the total number of tumour cells and the strength of expression in a score/grade of 0-3 (see Table 3.12 and Fig. 3.16).

A more exact semi-quantification of Caveolin-1 expression (immunoscore) was done by

evaluation of both, percentage of the expression (0-100%) and degree of staining intensity (grade 0-3) parameters. To calculate this immunoscore, two methods are mentioned in the literature. Method 1 is obtained by multiplying the staining intensity with the percentage positivity (evaluation in [%]), thereby giving immunoscores ranging from 0 to 300. The tumour samples were then categorized into four groups showing negative or low (0-50), moderate (51-100), high (101-200), and very high (201-300) expression. According to this method the quantity of Caveolin-1 expression was identified in the different stages of prostate cancer (Table 3.12).

The second method follows the principle of Remmele. The intensity of Caveolin-1 was measured on 4 intensity grades, 0 being negative, 1 weak, 2 moderate, and 3 being strong intracellular immunoreactivity. The distribution in percentage (evaluation in [%]) was measured subjectively on 4 reactive point scales as follows: 1-15% of the cells with intensity grade 1, 16-40% grade 2, 41-75% grade 3, and greater than 75% of the cells Caveolin-1 with grade 4. The result of the intensity point and reactivity point were then multiplied to get an immunosocore for each assay with a range of 0-12. This second method was used also in the evaluation study of YB-1 (see Table 3.16 and Fig. 3.21).

3. RESULTS

3.1. Proposed molecular markers and diagnostic methods

The results of this study are divided into four parts. The first part deals with the cytogenetic analysis of sub-microscopic alterations of chromosomes 7 and 8 and their correlation with clinical stages and histological pathology after Gleason score. The second part evaluates the chromosomal changes of 7q31 in relationship to the expression of Caveolin-1 protein in tumour cells of prostate cancer. The third part deals with the relationships between Caveolin-1 expression, histo-pathology of the prostate cancer tissue and organs of prostate cancer metastasis (e.g. urinary bladder, seminal vesicle, and lymph nodes). The final part is the analysis of YB-1 expression in selected tumour samples of prostate cancer and the correlation to histo-pathology. All parts aim to identify new molecular markers that can be used for a better identification and classification of prostate cancer.

Three specific regions in chromosomes 7 and 8 were chosen in this study. They were located in regions 7q31, 8p12-22, and q23-24. These regions showed imbalances in many tumours as well as prostate cancer and were mentioned in many publications. These imbalances are represented as deletion of 8p12 and gain of 8q23 resulting in the formation of isochromosomes 8q as well as gain of 7q31. They contain genes, which play an important role in tumorigenesis of prostate cancer as well as in other tumours such as breast, oesophagus, and colon cancer. The regions 7q31, 8p12-22 and 8q23-24 contain *Caveolin-1*, *NELF, c-myc*, and *p-40* (*IEF*: Initiation Elongation Factor) genes, respectively.

For achieving the results of this work, many modifications to the known methods and protocols including fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) were established and tested before the beginning of this study. The modifications of the used methods are summarized in the following section.

3.1.1. Labelling process of the probes and analysis of the correct localisation in the chromosomes (FISH in metaphase lymphocytes)

To analyze the quality of the probes and the right localisation on the chromosomes, FISH was done on normal lymphocytes (Fig. 3.1B). It shows two signals of clone 162021 (red) located in the 7q31 and two signals of YAC 240 g10 (green) located in 8p12-22. When FISH was done in lymphocytes with other probes, clone *eIF-p40* located in 8q22-23 region and *c-myc* in 8q22-24, were hybridized to the correct position as was been previously expected.

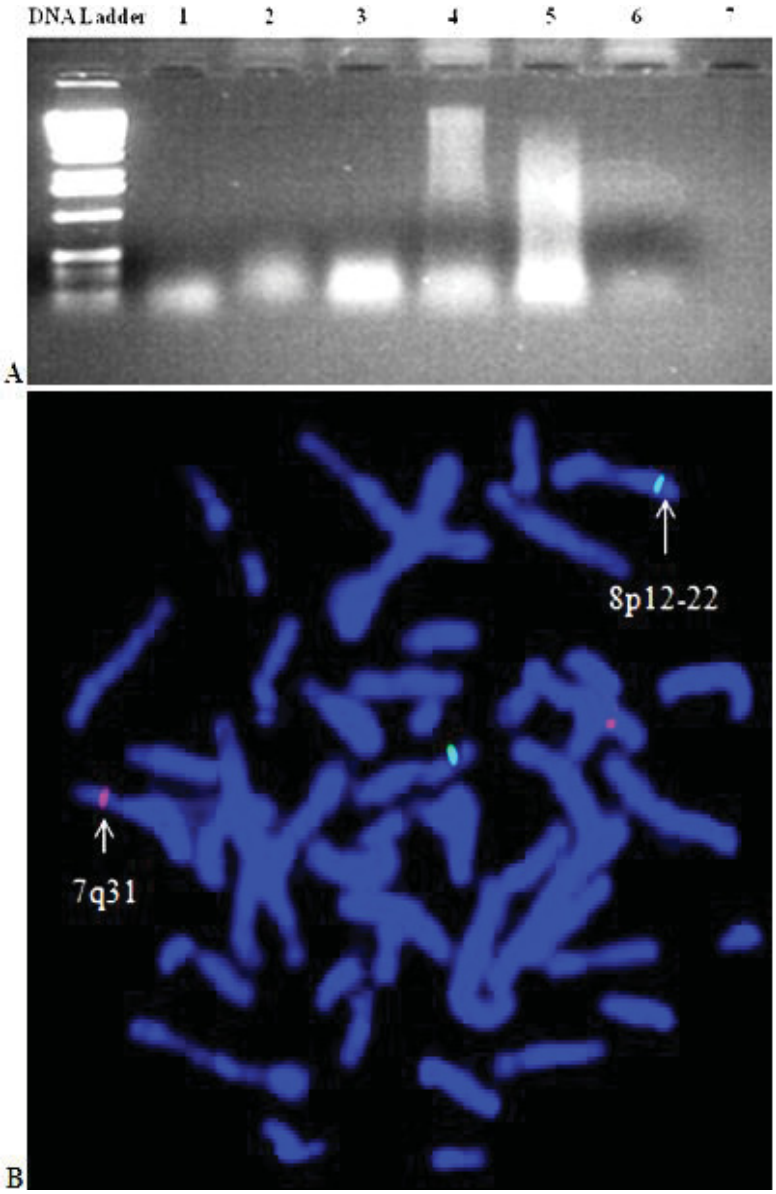


Fig. 3.1: The labelling of the DNA probes and FISH in metaphase lymphocyte. A) Shown is the size of the resulting fragments after incubation time of 60 minutes at 16 °C. Lane 1; 1Kb ladder, lane 2-5; Dig-Nick labelling of Caveolin-1 (PAC), lane 6; Bio.-Nick labelling of YAC 240 G10, and lane 7; control using bidest. water and no DNA. Lane 4 and 5 were not completely digested and labelled and should be further digested at 37 °C (using Bio-/Dig-Nick kit) until reaching 50-300 bp. **B)** Test fluorescence hybridization of normal metaphase with probes from 7q31 (red signal) in the 7q31 and 8p12-22 (green signal) regions.

3.1.2. FISH studies and applied methods

3.1.2.1. FISH with paraffin sections

For FISH with paraffin section analysis and isolated cell nuclei analysis, it was observed that the ideal thickness of the used sections should be 6-8 µm and 10-25 µm, respectively. Using neutralized formaldehyde in the fixation of prostate tissue gives better results since it reduces the spots and background. Surface coating of slides with L-lysine or using ionized slides (surface of the slide have negative ions) increases the adhesion of the tissue sections to avoid the soaking of these sections during the long FISH procedures. Overnight incubation at 60 °C instead of one hour prior

to FISH procedures melted most of the paraffin in the tissue sections and reduced also spots and background. Deparaffinization in 100% xylene for 20 minutes (two times) leads to the complete disappearance of paraffin. Using different solvent buffers like EDTA or Methanol/H_2O_2 do not reduce the unspecific spots (haemoglobin and Formaldehyde complex) and background in FISH samples. However, immersing the tissue sections completely in citrate buffer and boiled for 15 minutes in a microwave reduces the unspecific spots formed during Fixation (Fig. 3.2).

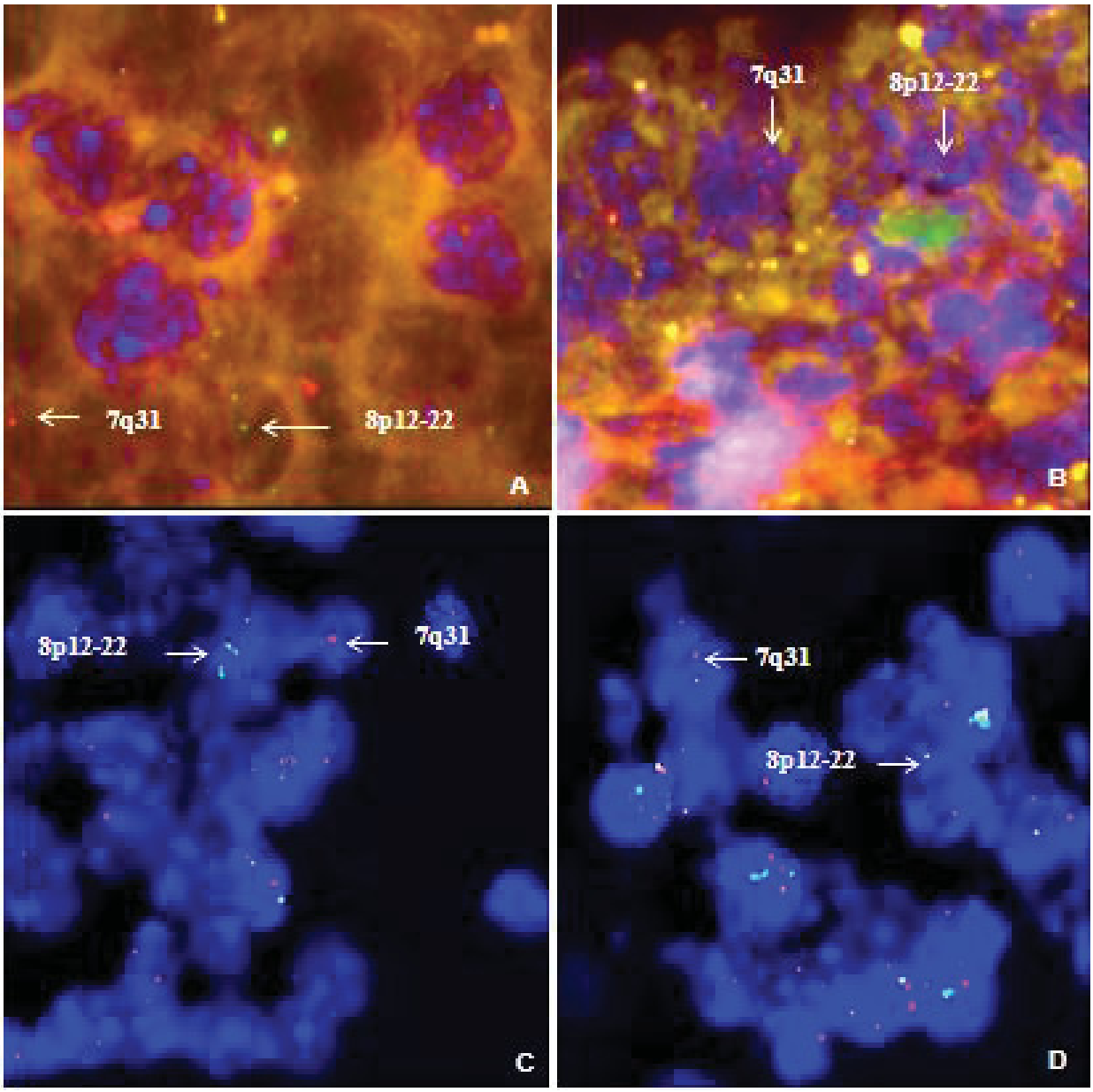


Fig. 3.2: Paraffin sections with and without citrate buffer treatment and pre-hybridization. A-B) Paraffin sections were without citrate buffer treatment in microwave and pre-hybridization (without probes in the hybridization solution), then hybridization (with two probes combination). **C-D)** Paraffin sections were with citrate buffer treatment and pre-hybridization (without probes) then hybridization with clone 162021 (7q31 red) and YAC 240 G10 (8p12-22 green).

A concentration of 40 mg/ml of pepsin and 30 minutes incubation at 37 °C and pH 2 (adjusted by HCl) showed nearly complete digestion of the cytoplasm and not the cell nuclei. The

longer the incubation time of the sections in pepsin solution leads to digestion of both cytoplasm and cell nuclei. Stretching the sections by incubation at 80 ºC in an oven for 60 minutes increased the adhesion of the sections and inhibited their detachment from the slide during the next procedures. Additionally, sections undergo post fixation for 5 minutes in buffered PBS (pH 7 adjusted with NaOH). Pre-hybridization step leads to masking the unspecific signals and checks whether the samples have lower numbers of spots. This is helpful for later evaluation of the labelled probes. In addition, it reduces the loss of the labelled probes if the tumour sample had large numbers of spots and could not be evaluated.

3.1.2.2. FISH with isolated cell nuclei

The modifications in the known protocol used in FISH with isolated cell nuclei was less than the modifications in protocol used for FISH in paraffin section. This can be summarized in two steps: The first step was the determination of the cell nuclei concentration in 1 µl using a cell chamber before dropping them onto the slides. This prevents overlapping and only one smear layer of the cell nuclei appeared. In the second step, the incubation was carried out in 40 mg/µl pepsin for 5 minutes in pre-warmed sterile water adjusted to pH 2 with HCl. This helps to get rid of the rest of the cytoplasm without affecting the cell nucleus.

3.2. Cytogenetic changes of chromosomes 7 and 8 in prostate cancer cells

FISH analysis was used to study the imbalances of chromosomes 7 and 8, specifically at regions 7q31, 8p12-22 and 8q22-24. DNA-labelling was carried out according to the manufacturer's instructions (see chapter 2.2.10.2). The DNA labelling process showed some differences between BAC, PAC, and YAC probes. Two different protocols were used for Biotin and Dig-Nick labelling. Usually, the labelling of BAC- and PAC-DNA does not require longer post-incubation times at 37 °C compared to YAC -DNA. This is due to the fact that the labelled fragments resulting from BACs and PACs are always shorter than those from YAC -DNA. The indirect-labelling of YACs always resulted in long labelled fragments even after longer incubation time. For this reason, an Alu - Repeat PCR with YAC -DNA was performed to obtain smaller fragments after the labelling process (i.e. 300-500 bp) that give better hybridisation results later (Fig. 3.1A).

Biotin labelling does not need post-incubation (i.e. digestion and labelling) to reach the size of 300-500 bp labelled DNA fragments, after the regular incubation time of 60 min at 16 °C as described in the Roche Protocol. Dig-Nick labelling often requires extended post-incubation time at 37 °C to reach 300-500 bp after the regular time stated in the protocol. There were many protocols

tested for optimization of the different FISH analyses performed with chromosome preparations (metaphase lymphocytes), paraffin sections, and isolated cell nuclei from paraffin sections.

3.2.1. FISH with paraffin section

10 samples were examined with this method (4 samples from the University of Bonn and 6 from the University of Düsseldorf, Department of Pathology). The clinical pathology record of tumour sample 2 -Paraffin and 4 -Paraffin were not available. The FISH studies performed and the clinical description of the sections are shown in Table 3.1.

Table 3.1:- Tumour samples under study and probes combination in FISH.

Tumour Sample number	FISH Combination Red 7q/Green 8p	FISH Combination Red 8p/Green 7q
Tumour 1 -Paraffin (pT1b, GS = 6)	Studied	not studied
Tumour 2 -Paraffin (Not available)	Studied	not studied
Tumour 3 -Paraffin (pT1a, GS = 6)	Studied	not studied
Tumour 4 -Paraffin (Not available)	Studied	not studied
Tumour 5 -Paraffin (pT3b, GS 3+5= 8)	Studied	not studied
Tumour 6 area 1 -Paraffin (pT3a, GS 5+3 = 8)	Studied	not studied
Tumour 6 area 2 -Paraffin (pT3a, GS 5+3 = 8)	Studied	not studied
Tumour 7 -Paraffin (PT3a, GS 2+3 = 5)	Studied	Studied
Tumour 8 -Paraffin (PT3a, GS 3+5 = 8)	Studied	Studied
Tumour 9 area 2 -Paraffin (pT3b, GS 5+3 = 8)	Studied	not studied
Normal tissue sample	Studied	not studied

GS means Gleason score

The FISH combination of the clone 162O21 (7q31 - red) and YAC 240 G10 (8p12-22 - green) was performed on all paraffin samples. In tumour sample 7 -Paraffin and 8 -Paraffin a colour swap with the combination of the YAC 240 G10 (8p12 - red) and clone 162O21 (7q31 - green) was performed to exclude the colour influence in the evaluation. The FISH combination of the clone 162O21 (7q31 - red) and centromere of chromosome 4 (green) was carried out only in tumour sample 8 -Paraffin as a control for the other signals, because chromosome 4 shows rarely aneuploidy in prostate cancer. The results of FISH were evaluated as described in Materials and Methods (see chapter 2.2.13.4) and are listed and plotted in Fig. 3.4-3.5.

The tumour cells are located close to each other in the acini (glandular/secretory cells of the prostate) if they are not able to destroy the basal membrane and invade the connective tissue. They are bigger in size than normal basal cells as well as connective tissue cells (fibroblasts), which are longer in shape with dendrites having a rod-shape. Tumour cells always have a larger cell nucleus and show different stages of mitosis. Also, the tumour cells were easily distinguished from the blood vessel cells which lined the basal membrane and were surrounded by muscle layers. Because

the normal prostate tissue was not available at the beginning of this study, the stromal and connective tissue cells of the tumour sample 6 areas 2 -Paraffin were evaluated and considered as normal control. These connective tissue cells do not take part directly in the neoplasia process. They are mesodermal in origin and show no chromosomal changes (see stroma of tumour sample 6 area 2 -Paraffin in Fig. 3.4A). The numbers of red and green signals in each cell were determined according to the instruction mentioned in chapter 2.2.13.4 (Fig. 2.6). The signals were evaluated in at least 100 cells of the FISH in paraffin section (Fig. 3.3).

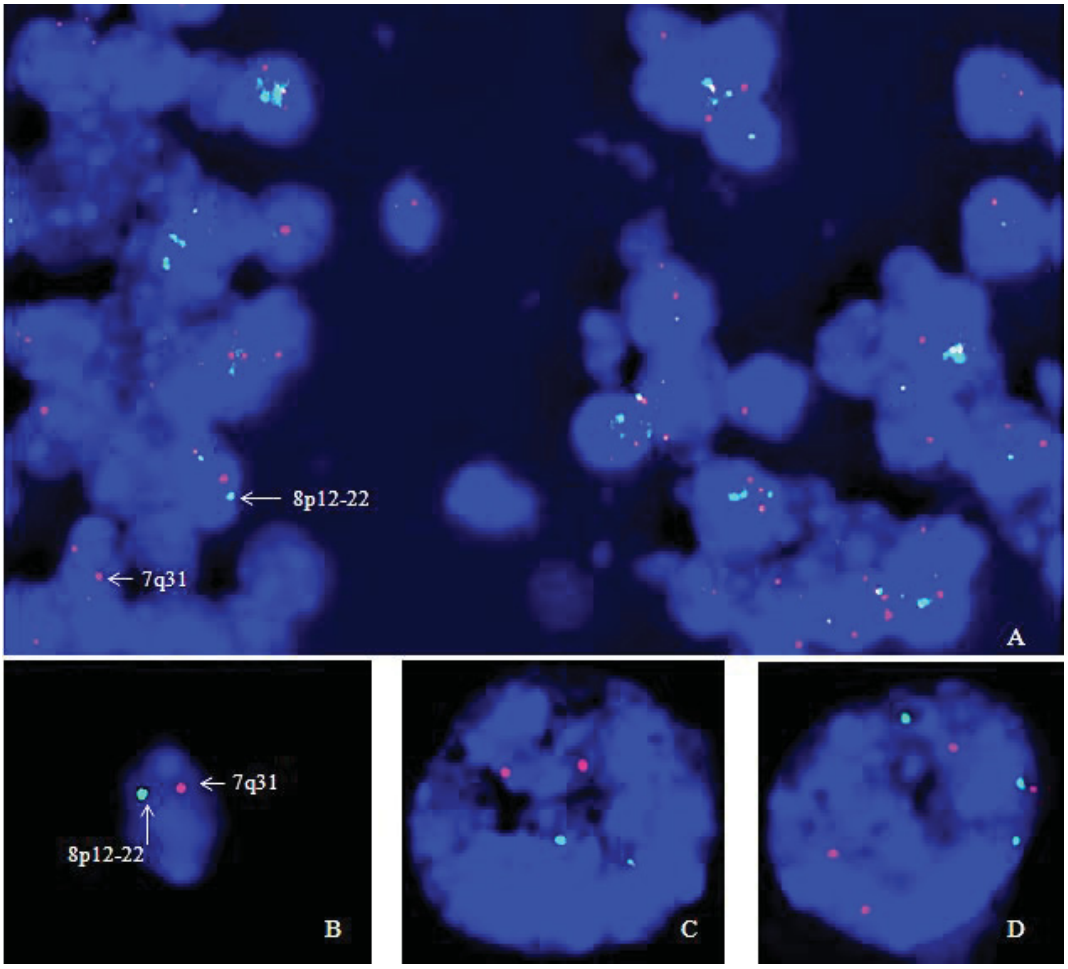


Fig. 3.3: Examples of cell heterogeneity of tumour samples in FISH with Paraffin section with various numbers of chromosomes. A) Overview of a paraffin section with many tumour cells**.** Red signal is clone 162O21 (7q31) and green signal is YAC 240 G10 (8p12-22). **B)** Loss of one 7q31 and one 8p12-22 signal. **C)** Example of normal cell with two signals of 7q31 and 8p12-22. **D)** Tumour cell with gain of 7q31 (4 signals) and 8p (3 signals).

To make sure that the control paraffin tissue is free from tumour cells, only the stroma cells of a normal area in tumour sample 6 area 2 -Paraffin were evaluated which showed similar values to the normal tissue area of the same tumour sample (data not plotted) in comparison. It showed 66.5% (red) and 69.0% (green) of cells with 2 copies of chromosome 7 and 8, respectively (Fig. 3.4A). The stroma cells of tumour sample 6 area 2 -paraffin showed 22.6% red single signal (7q31) and 24.5% green single signal (8p12-22) (Fig. 3.4A). But in isolated cell nuclei of normal

tissue, there were 5.6% (green) and 3.7% (red) single signal of 7q31 and 8p12-22, respectively (Fig. 3.4B). This shows that the cells with 1 signal each were cut in the sections and the loss is due to a technical artifact due to removal of material in the sectioning process. Because of these results, 20% of the single signals were considered as background in the evaluation of paraffin sections. The percentage of the single signal over 20.0% would be considered as loss for both 7q31 and 8p12-22 (Table 3.2). Stroma cells showed three red signals in 6.0% (7q31) and three green signals in 2.5% (8p12-22) of the paraffin sections. The percentage of the three red signals over 6.0% would be considered as gain of 7q31 and the percentage of the three green signals over 2.5% would be considered as gain of 8p12-22 in evaluation analysis of paraffin sections. In addition the stroma cells showed four red (7q31) and four green (8p12-22) signals in 0.5 and 1.0%, respectively. Due to the partial sectioning of the cells in paraffin sections, the amplification of 7q, 8p, and 8q are considered as the same in isolated cell nuclei as background in the FISH analysis. In case of FISH in paraffin section gain of more than 5.0% of the tumour cells as four signals of 7q31 means amplification, but gain of more than 6.0% as four signals of 8p12-22 and 8q22-23 means amplification of these regions or chromosome arms.

In case of FISH in isolated cell nuclei of the normal prostate tissue, the percentage of the background cells containing single, triple, and tetra signal of 7q31 were 5.6%, 8.2%, and 5.3%, in the evaluation, respectively. Also, the percentage of the back ground in normal cells of 8p12-22 containing single, triple, and tetra signal were 3.7%, 6.0%, and 4.3% in the evaluation respectively (Fig. 3.4B). The percentage of tumour cells containing one signal above 5.6% and 3.7% would refer to a loss of 7q31 and 8p12 in the evaluation, respectively. Meanwhile, the percentage of 3 signals (trisomy) above 8.2% and 6.0% would refer to the gain of 7q31 and 8p12, respectively. The percentage of 4 signals (tetrasomy) above 5.3% and 4.3% would refer to an amplification of 7q31 and 8p12-22, respectively (Table 3.2).

Table 3.2:- Signals cut off in the FISH in Paraffin section and isolated cell nuclei.

Chromosome	7q31				8p12-22				8q22-24	
	1 signal	2 signal	3 signal	4 signal	1 signal	2 signal	3 signal	4 signal	3 signal	4 signal
Paraffin section	**22.6%**	**66.5%**	**6.0%**	**0.5 %**	**24.5%**	**69.0%**	**2.5%**	**1.0%**	**2.5%**	**1.0%**
Comment (Background)	> 20% means loss	normal	> 6.0% means gain	> 0.5% means gain	> 20% means loss	Normal	> 2.5% means gain	> 1.0% means gain	> 2.5% means gain	> 1.0% means gain
Isolated cell nuclei	**5.6%**	**79.8%**	**8.2%**	**5.3%**	**3.7%**	**85.8%**	**6.0%**	**4.3%**	**6.0%**	**4.3%**
Comment (Background)	> 5.6%means gain	normal	> 8.2% means gain	> 5.3% means gain	> 3.7% means gain	Normal	> 6.0% means gain	> 4.3% means gain	> 6.0% means gain	> 4.3% means gain

The tumour sample 1 -Paraffin showed a gain of 7q31 (23.0 – 6.0% = 17.0% as trisomy- and 6.1 – 0.5 = 5.6% as tetrasomy) and loss of 8p12-22 (29.9% – 20.0% = 9.9%) as seen in Figure. 3.4C.

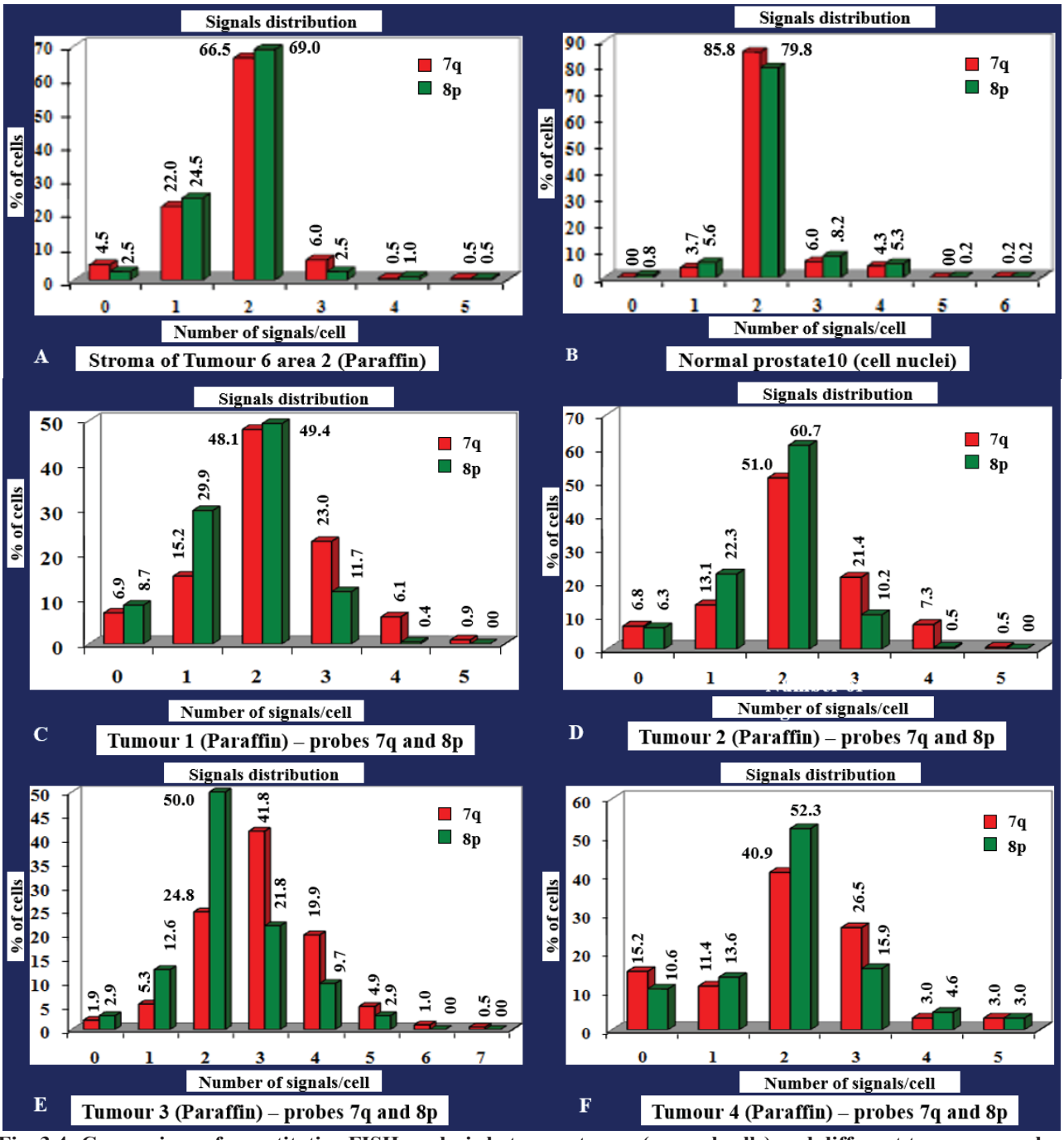


Fig. 3.4: Comparison of quantitative FISH analysis between stroma (normal cells) and different tumour samples using clone 162021 (7q31-Red) and YAC 240 G10 (8p12-22-Green) probes. A) Normal cells of the tumour sample 6 are used as a control for comparison with paraffin sections. **B)** FISH in isolated cell nuclei from normal prostate tissue to show the differences from the FISH in paraffin section. **C)** Tumour 1 showed gain of 7q31 and loss of 8p12-22 above background. **D)** Tumour 2 showed gain of 7q31 (15.4%) and low level loss of 8p12-22 (2.3) above background. **E)** Tumour 3 showed more gain of 7q31 than 8p12-22 (actually amplification). **F)** Tumour 4 showed clear gain of 7q31 and gain of 8p12-22 (13.4%) above the background.

The tumour 2 -Paraffin showed gain of 7q31 (21.4 – 6.0% = 15.4%). A low level loss of 8p12-22 (22.3% – 20.0% = 2.3%) was seen as single signal and at the same time the presence of

three green signal (8p12-22) in 7.7% (10.2% – 2.5%) referring to low level gain of 8p12-22 (Fig. 3.4D). This low level loss (2.3%) and low level gain (7.7%) of 8p12-22 in tumour sample 2 -Paraffin could only be confirmed by FISH analysis of isolated cell nuclei. This was not possible due to lack of the tumour sample 2 tissue material for cell nuclei isolation.

A gain of 7q31 and 8p12-22 was clearly seen in tumour 3 -Paraffin (Fig. 3.4E). This gain of 7q31 was 35.8% (41.8% – 6.0%) and 19.4% (19.9% – 0.5%) as trisomy and tetrasomy, respectively. Gain of 8p12-22 was lower than 7q31 represented in 19.3% (21.8% – 2.5%) and 8.7% (9.7% – 1.0%) as trisomy and tetrasomy, respectively. Both of 7q31, and 8p12-22 were amplified in tumour 3. Moreover, tumour 3 showed low level gain of 7q31 and 8p12-22 as 5 signals in 4.4% and 2.4% above the background (0.5%), respectively. These 5 signals of 7q31 and 8p12-22 confirmed the amplification of both chromosomes (chr.7 and chr.8).

Tumour sample 4 -Paraffin showed a clear gain of 7q31 (26.5% – 6.0% = 20.5%) and lower gain of 8p12-22 (15.9% – 2.5% = 13.4%). There was a low level of 4 signals (tetrasomy) of 7q31 and 8p12-22 (3.0% – 0.5% = 2.5% and 4.6% – 1.0% = 3.6%), respectively (Fig. 3.4F).

Tumour 5 -Paraffin showed clear gain of 7q31 as trisomy in 23.5% (29.5% – 6.0%) and low level gain as tetrasomy in 3.4% (3.9% – 0.5%) as shown in Figure 3.5A. The loss of 8p12-22 was 12.4% (32.4% – 20.0%) in tumour 5 -Paraffin. The area 1 of tumour 6 -Paraffin showed lower gain of 7q31 than the previous tumours (Fig. 3.5B). It showed gain of 7q31 as trisomy and tetrasomy in 11.7% (17.7% – 6.0%) and 2.5% (3.0% – 0.5%), respectively. The loss of 8p12-22 was 8.7% (28.7% – 20.0%).

Tumour sample 7 area 1 -Paraffin showed clear gain of both 7q31 and 8p12-22 regions (Fig. 3.5C). There were 29.1% (35.1% – 6.0%) and 18.2% (20.7% – 2.5%) as trisomy as well as 12.9% (14.4% – 0.5%) and 12.0% (13.0% – 1.0%) as tetrasomy of 7q31 and 8p12-22, respectively. 7q31 and 8p12-22 were clearly amplified in tumour 7 in area 1 and 2.

Gain of 7q31 was also detected in sample 8 -Paraffin in two slides of the same tumour but in different probe combinations (Fig. 3.5E-F). In the combination of the Caveolin PAC -probe (clone 162O21) (7q31-red) and YAC 240 G10 -probe (8p12-22 - green), tumour 8 -Paraffin showed clear gain of 7q31 (23.1 – 6.0% = 17.1%) and loss of 8p12-22 (30.8% – 20.0% = 10.8%). To confirm the gain of 7q31 in the tumour sample 8, a new combination of the Caveolin PAC -probe (clone 162O21) (7q31) and a probe of chromosome 4 centromere was evaluated in another slide of tumour sample 8 -Paraffin (7q31 - red /centr.4 - green). Chromosome 4 centromere probe was used because chromosome 4 shows rare changes. Comparison of the probes combination (7q31 - red/8p12-22 - green) with the other combination (7q31 - red/centromere chromosome 4 - green) in tumour 8 -

Paraffin confirmed the gain of 7q31, where the 7q31 gains were 17.1% (23.1% – 6.0%) and 24.0% (30.0% – 6.0%) as trisomy- above the background respectively in both samples (Fig. 3.5E-F).

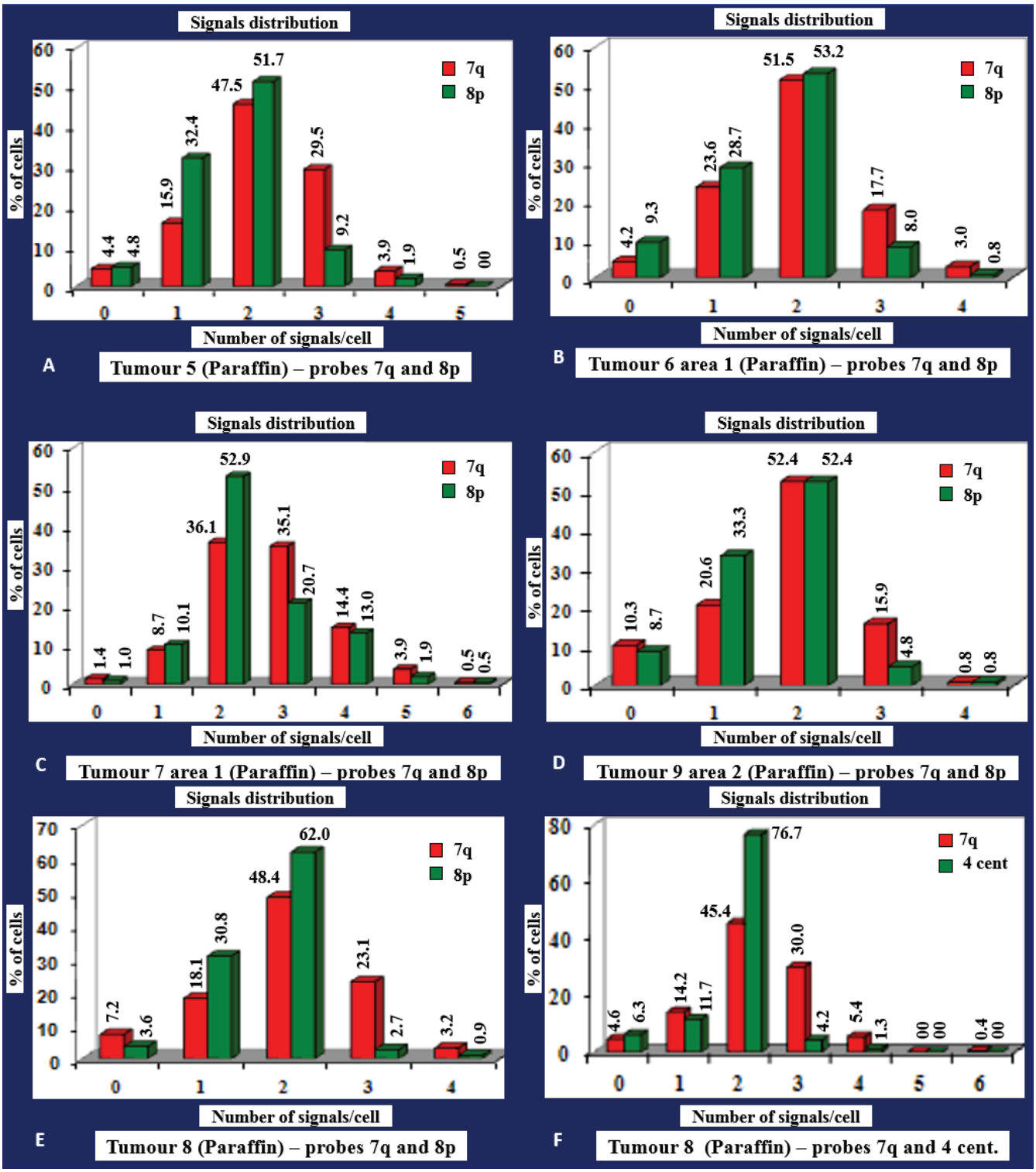


Fig. 3.5: Analysis of FISH results for paraffin sections from tumour samples number 5, 6, 7, 9, and 8. A) Tumour sample 5 showed gain of 7q31 and loss of 8p12-22. **B)** Tumour 6 area 1 showed low level gain of 7q31 and loss of 8p12-22. **C)** Tumour 7 area 1 showed clear gain of 7q31 and low level gain of 8p12-22. **D)** Tumour 9 area 2 showed low level gain of 7q31 and clear loss of 8p12-22. **E)** Tumour 8 showed gain of 7q31 and loss of 8p12-22. **F)** Tumour 8 showed gain of 7q31 and no loss or gain of chromosome 4 (normal). The result obtained from E and F confirmed the gain of 7q31.

In tumour sample 9 area 2 -Paraffin, gain of 7q31 was seen in low percentage of cells, but 8p12-22 loss was seen in more percentage of cells. Gain of 7q31 was 9.9% (15.9% – 6.0%) as 3 signals (trisomy) and loss of 8p12-22 was 13.3% (33.3% – 20.0%) (Fig. 3.5D). The loss of 8p12-22 was noticed in both tumour samples 8 -Paraffin (Red 7q31/Green 8p12-22) and tumour 9 area 2 -Paraffin (Red 7q31/Green 8p12-22) but was higher in tumour sample 9 area 2 -Paraffin (Fig. 3.5D). The percentage of the cell having double red (7q31) and green (8p12-22) signals (normal cells or euploidy) were the same in tumour 9 area 2 -Paraffin (52.4%) but double red and green signals (7q31 and 8p12-22) were 48.4% and 62.0%, in tumour 8 -Paraffin, respectively. This suggests that 8p12-22 loss in the tumour 9 area.2 -Paraffin is clear but in tumour 8 -Paraffin was lower and needs to be confirmed with other methods, e.g. isolated cell nuclei.

Taken together, there was a clear gain of 7q31 in tumour samples 1, 2, 3, 4, 5, 7, and 8 -Paraffin. Low level gain of 7q31 was seen in tumour 6 area 1 -Paraffin and tumour sample 9 areas 2 -Paraffin. Loss of 8p12-22 was clearly seen in tumour samples 1, 5, 6, and tumour 9 areas 2 -Paraffin. Low level loss of 8p12-22 was seen only in tumour 2 -Paraffin, but unclear loss in tumour 8 -Paraffin, although the loss of 8p12-22 was 10.3%. This unclear result was related to the high percentage of double signals (62.0%). In paraffin sections, amplification of 7q31 was seen in tumour 1, 2, 3, and 7. The amplification of 8p12-22 was seen in tumour sample 3, 4, and 7 of paraffin sections.

Although the studied paraffin sections showed a well preserved histology, they also displayed some disadvantages that could falsify the FISH evaluation (Fig. 3.3-Fig. 3.5) such as:
1- Overlapping of the cells.
2- Unspecific coloured spots which may be related to the presence of haemoglobin in the tissue samples.
3- Partial presence of cell nuclei due to the sectioning.

3.2.2. FISH of isolated cell nuclei

To improve the evaluation and to avoid the above mentioned disadvantages with paraffin sections as well as to clarify the suspected gains or losses, a method for the isolation of cell nuclei from paraffin sections was established.

The evaluation of the FISH-analyses of the same tumour according to the cell nucleus size in two different combinations gives confirmed and better results. The first analyses evaluated the medium and big cell nuclei representing the tumour cells. The second analyses evaluated the

medium, big and small cell nuclei. The latter are more likely normal cells. This should show if there is a difference in the signals of the probes used. For this purpose, thicker paraffin sections were cut; at least 10 µm and less than 30µm thick. To exclude colour influences in the evaluation as before, the Bio-Nick labelled probes were again Dig -Nick labelled and vice versa, and colour swap hybridisation was performed. The tumour samples analysed in this way are listed and plotted in Fig. 3.6-Fig. 3.9 as seen below.

The purpose of this study was to examine the differences in hybridisation of these probes with isolated cell nuclei compared with paraffin sections. Analysis of cell nuclei should reveal the correct numbers of signals in the cells.

The protocol used for cell nuclei isolation was provided from the Institute for Cytopathology of University of Düsseldorf. This protocol was originally established for Feulgen staining and was modified for FISH in this work, as described in chapter 2.2.12. FISH studies were performed with the samples listed below. FISH combinations under study for the isolated cell nuclei and tumour samples are illustrated there (Table 3.3).

Table 3.3:- The tumour samples analysed with isolated cell nuclei.

Tumour Sample number	FISH Combination R8p/G8q	FISH Combination R8p/G7q
Tumour 7.1 -Cell nuclei	**Studied**	**Studied**
Tumour 7 area 2 -Cell nuclei	**not studied**	**Studied**
Tumour 8 -Cell nuclei	**Studied**	**Studied**
Tumour 9 area 2 -Cell nuclei	**not studied**	**not studied**
Tumour 9 area 1 -Cell nuclei	**not studied**	**Studied**
Normal prostate 10 -Cell nuclei	**not studied**	**Studied**

R = red, G = green.

FISH combination 8p12-22 (red) and 7q31 (green) was performed in most cases of isolated cell nuclei from tumour samples. This means that the same regions or genes were studied, with only a colour change of the probes used for FISH in the paraffin sections. This proved that the colour of the probes has no influence on the evaluation. The FISH combination 8p12-22 and 8q22-24 was performed only in tumour sample 7 area 1 -Cell nuclei and tumour 8 -Cell nuclei. In the absence of histology in the case of isolated cell nuclei, the tumour samples were evaluated according to the size of their nuclei. The big and medium cell nuclei represent the tumour cells, and the small ones represent normal epithelial and connective tissue cells. For showing the quality differences in evaluation and analysis between FISH in paraffin section and cell nuclei, the same probe combinations were used in tumour sample number 7 and 8 (Fig. 3.6-3.9). Both tumour samples had paraffin section as well as isolated cell nuclei.

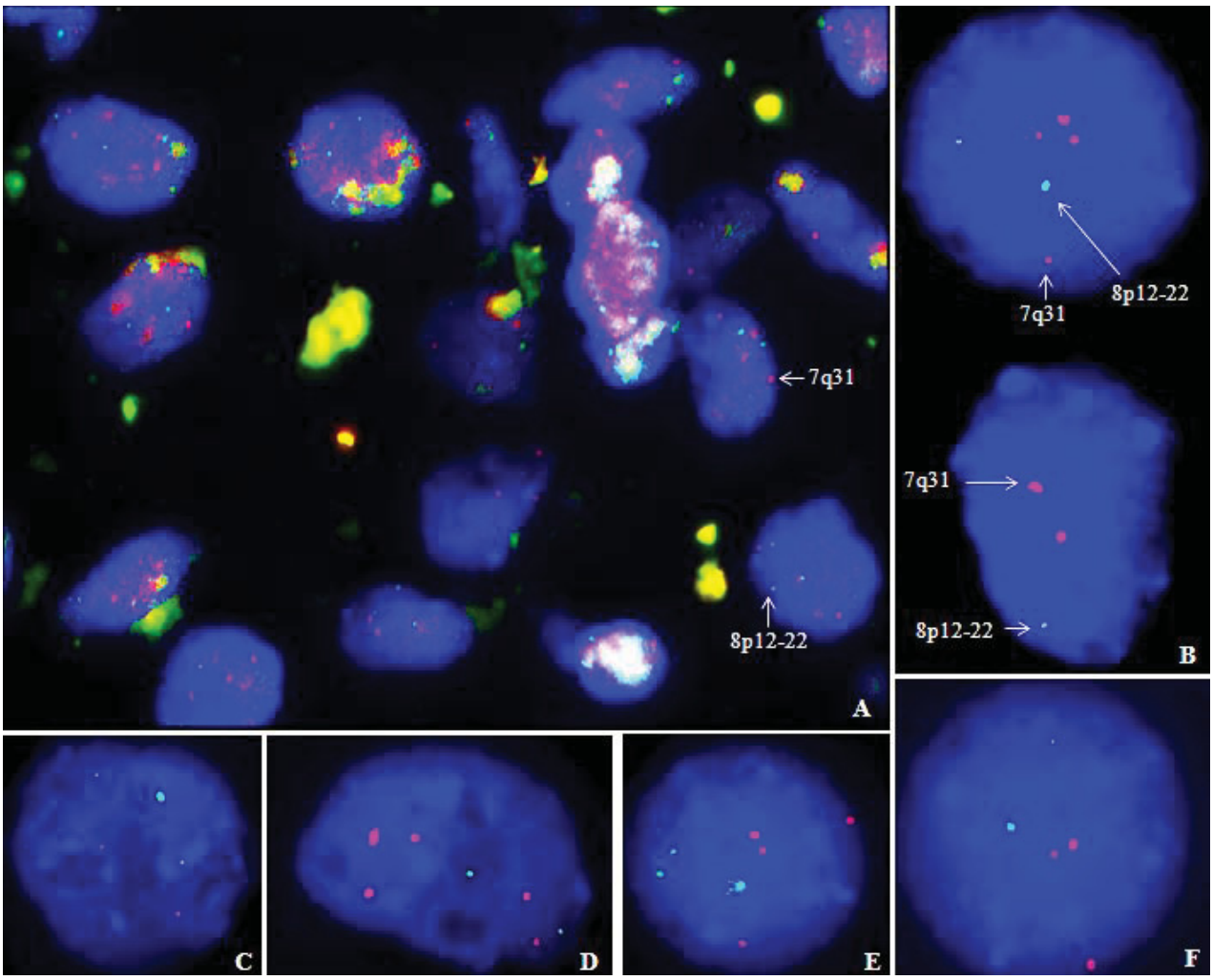


Fig. 3.6: FISH analysis of isolated cell nuclei. A) Red signal is clone 162O21 (7q31) and green signal is YAC 240 G10 (8p12-22). **B-F** Examples of nuclei with various numbers of signals for chromosome 7q (red) and 8p (green). In D amplification of 7q31 is clearly seen.

In case of FISH in isolated cell nuclei of the normal prostate tissue as mentioned before (see page 56-58 and Fig. 3.4B), the background should be taken in consideration. There were two different evaluation concepts, in one only the combination of medium (m) and big (b) cell nuclei were evaluated and in the other, the combination of small (s), medium (m) and big (b) cell nuclei. The reason for this different evaluation was the absence of tumour histology in case of isolated single cells and the medium and big cell nuclei represent tumour cells. Tumour sample 7 -Paraffin had the red colour for 7q31 probe and the green colour for 8p12-22 probe. Tumour sample 7 -Cell nuclei had the combination red colour for the 8p12-22 probe and green for the 7q31 probe. In another probe combination, tumour sample 7 -Cell nuclei and 8 -Cell nuclei had the red colour for the 8p12-22 probe and green for the 8q23 probe.

In the evaluation of small, medium, and big cell nuclei, tumour 7 area 2 -Cell nuclei showed clear gain of 7q31 (green) and 8p12-22 (red) if compared to paraffin sections of the same tumour.

Tumour sample 7 area 2 (s + m + b) showed gain of 7q31 in 10.6% (18.8% – 8.2%) as trisomy and 33.1% (38.4% – 5.3%) as tetrasomy as well as gain of 8p12-22 in 9.7% (15.7% – 6.0%) as trisomy and 18.8% (23.1% – 4.3%) as tetrasomy above the background level identified in FISH of isolated cell nuclei from normal prostate tissue.

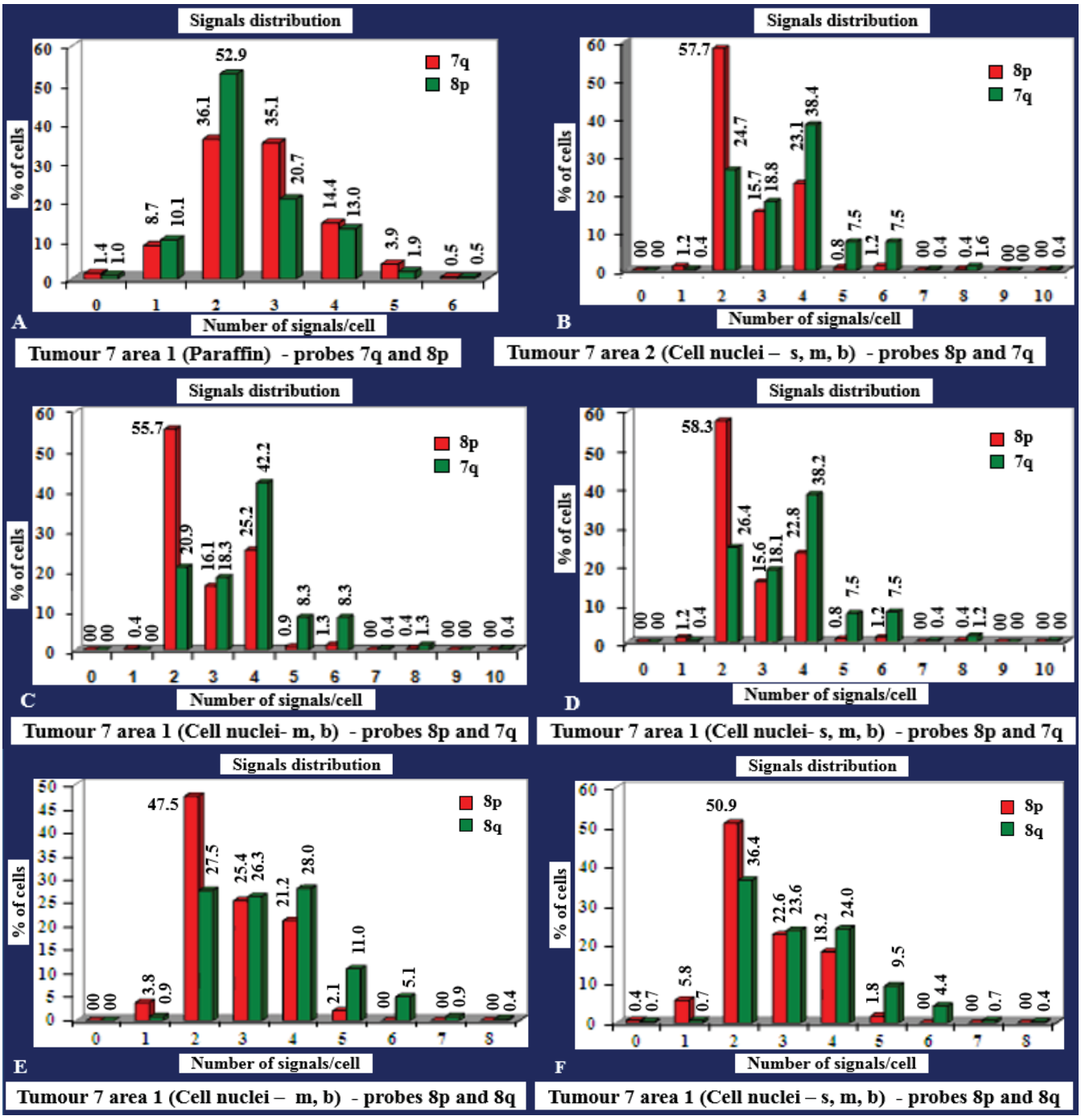


Fig. 3.7: Verification of FISH results from paraffin sections using isolated cell nuclei. Medium and big sized nuclei evaluated separately from small, median, and big. The small nuclei correspond to the normal cells. **A)** Tumour 7 area 1 (paraffin) showed gain of 7q31 and low level gain of 8p12-22. **B)** Tumour 7 area 2 (cell nuclei -s, m, b) showed clear gain of 7q31 and 8p12-22. **C)** Tumour 7 area 1 (cell nuclei -m, b) showed also clear gain of 7q31 and 8p12-22. **D)** Tumour 7 area 1 (cell nuclei -s, m, b) showed clear gain of 7q31 and 8p12-22. **E)** Tumour 7 area 1 (cell nuclei -m, b) showed clear gain of 8q22-24 and 8p12-22. **F)** Tumour 7 area 1 (cell nuclei -s, m, b) showed also gain of 8q22-24 and 8p12-22.

The tumour 7 area 1 -Cell nuclei was evaluated two times in the probe combination of 8p12-22 (red) and 7q31 (green) using the evaluation of both m + b cell nuclei combination in one (Fig. 3.7C) and s + m + b combination in another (Fig. 3.7D). The probe combination of 8p12-22 (red) and 8q22-23 (green) in tumour 7 area 1 -Cell nuclei were also evaluated using the m + b combination and s + m + b combination (Fig. 3.7E-F). The m + b combination in tumour 7 area 1 -Cell nuclei showed gain of 7q31 in 11.1% (18.3% – 8.2%) and 36.9% (42.2% – 5.3%) as tri-and tetrasomy respectively (Fig. 3.7C). Also, it showed gain of 8p12-22 in 10.1% (16.1% – 6.0%) and 20.9% (25.2% – 4.3%) as tri-and tetrasomy, respectively.

In small, medium and big cell nuclei combination, tumour 7 area 1 showed gain of 7q31 in 9.9% (18.1% – 8.2%) and 32.9% (38.2% – 5.3%) as tri-and tetrasomy respectively (Fig. 3.7D). Also, it showed gain of 8p12-22 in 9.6% (15.6% – 6.0%) and 18.5% (22.8% – 4.3%) as tri-and tetrasomy above the background respectively.

In the probes combination of 8p12-22 (red) and 8q23 (green), the medium and big cell nuclei combination showed gain of 8p12-22 in 19.4% (25.4% – 6.0%) and 16.9% (21.2% – 4.3%) as tri-and tetrasomy, respectively (Fig. 3.7E). Meanwhile the gain of 8q23 was 16.9% (26.3% – 6.0%) and 23.7% (28.0% – 4.3%) as tri-and tetrasomy, respectively. Tumour 7 area 1 showed 16.6% (22.6% – 6.0%) and 13.9% (18.2% – 4.3%) gain of 8p12-22 as tri-and tetrasomy respectively, if the small, medium and big cell nuclei combination was evaluated (Fig. 3.7F). Also in this previous combination (s + m + b), gain of 8q23 was 17.6% (23.6% – 6.0%) and 19.7% (24.0% – 4.3%) as tri- and tetrasomy respectively (Fig. 3.7F). The results obtained from the FISH analysis in Paraffin section and cell nuclei in tumour 7 confirmed not only the gain of 7q31, 8p12-22, and 8q23 but also the amplification of both chromosomes.

Tumour 8 -Cell nuclei showed gain of 7q31 (green) in 16.4% (24.6% – 8.2%) and 33.3% (38.6% – 5.3%) as tri- and tetrasomy in the combination of medium and big cell nuclei respectively (Fig. 3.8C). In combination of small, medium and big cell nuclei, tumour 8 showed gain of 7q31 (green) in 10.2% (18.4% – 8.2%) and 20.3% (25.6% – 5.3%) as tri- and tetrasomy respectively. Both FISH in paraffin sections and isolated cell nuclei confirmed the amplification of 7q31 (Fig. 3.8A-D).

Although, the evaluation of FISH in tumour sample 8 -Paraffin using the probes combination of 7q31(red) and 8p12-22 (green) showed loss of 10.3% (30.3% – 20.0%) of 8p12-22, the tumour 8 -Cell nuclei showed no loss of 8p12-22 either in the evaluation of median and big

combination or small, medium, and big combination of cell nuclei. Using the probes combination of 8p12-22 (red) and 8q23 (green), the evaluation median and big cell nuclei of tumour 8 -Cell nuclei showed clear gain of 8q23 as tri- and tetrasomy in 25.5% (31.5% – 6.0%) and 23.7% (28.0% – 5.3%) respectively (Fig. 3.8B). In addition, it showed no loss of 8p12-22. These results confirmed that there was no loss of 8p12-22 and referred to the amplification and formation of an isochromosome 8q (Fig. 3.8).

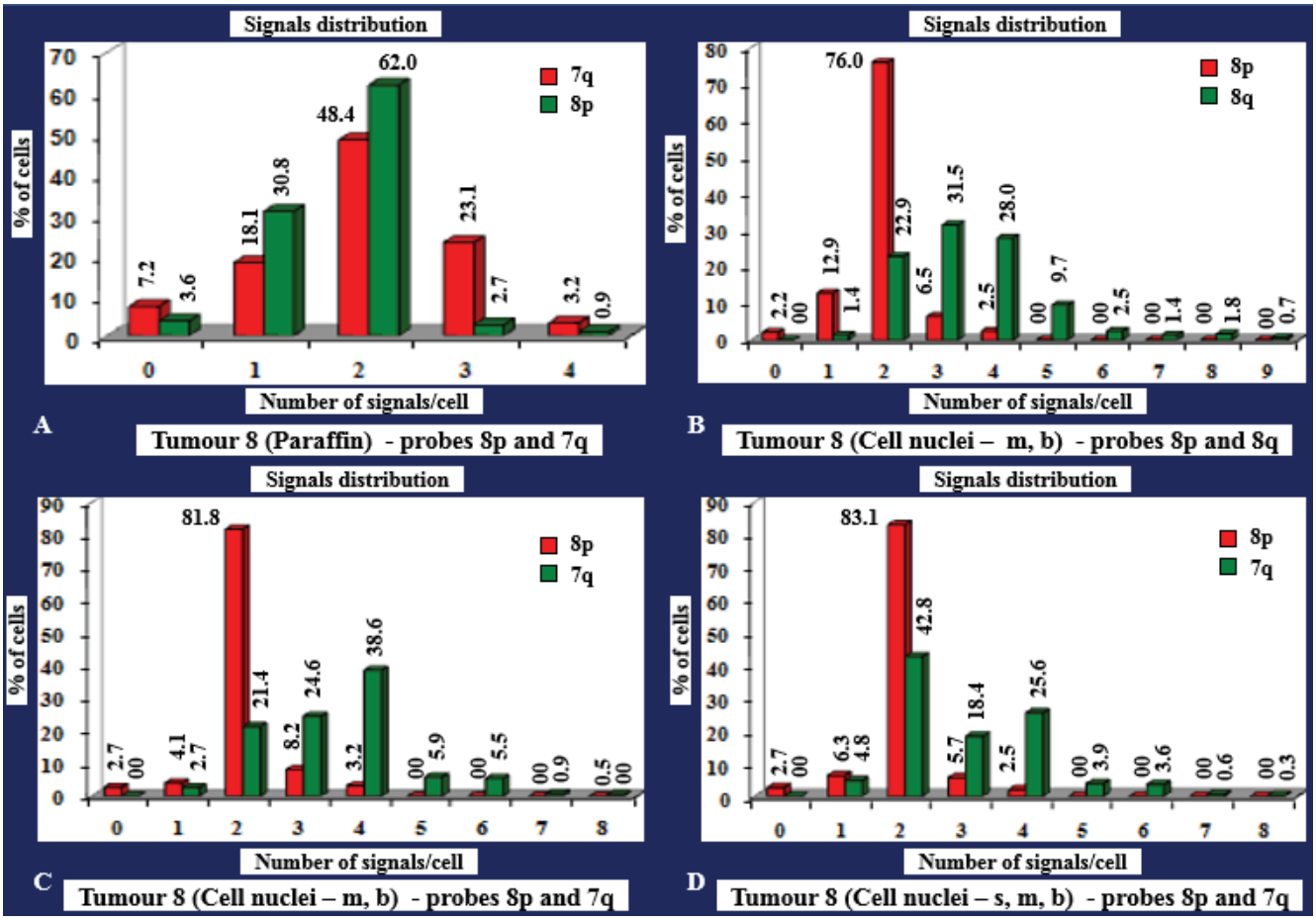


Fig. 3.8: The difference in the evaluation of FISH in Paraffin sections and isolated cell nuclei in tumour sample 8 using 7q31, 8p12-22, and 8q22-23 probes and verification of no loss of 8p12-22. A) FISH in Paraffin section showed gain of 7q31 and loss of 8p12-22. **B)** FISH in isolated cell nuclei showed the gain of 8q22-24 as trisomy, tetrasomy, and even a few cells with more than 4 copies and no loss of 8p12-22. **C)** The number of double signal is reduced in the medium and big size combination of the cell nuclei, but number of 3 and 4 signals was increased in comparison to paraffin section. This shows clear gain of 7q31 and no loss of 8p12-22. **D)** The number of double signals was more increased in the small, medium and big size combination, but number of 3 and 4 signals was slightly reduced and showed also gain of 7q31 and no loss of 8p12-22. FISH in isolated cell nuclei in tumour 8 confirmed the gain of 7q31 and no loss of 8p12-22.

Tumour 9 area 1 -Cell nuclei showed gain of 7q31 in 13.5% (21.7% – 8.2%) and 6.6% (11.9% – 5.3%) as tri- and tetrasomy in the medium and big evaluation of cell nuclei, respectively (Fig. 3.9C). It showed 12.7% (20.9% – 8.2%) and 6.1% (11.4% – 5.3%) as tri- and tetrasomy in the small, medium, and big combination of cell nuclei, respectively (Fig. 3.9D). It showed clear loss of

8p12-22 in 23.8% (27.5% – 3.7%) in the medium and big combination as well as 23.9% (27.6% – 3.7%) in the small, median and big combination of cell nuclei. The results obtained confirmed the gain of 7q31 and loss of 8p12-22 in tumour 9. Moreover, it referred to the amplification of 7q31, but in lower level than in tumour 7 and 8 (Fig. 3.9).

The evaluation of isolated cell nuclei from tumour samples showed a clear reduction of the cells with single signals in comparison to the nuclei in paraffin section from the same tumour samples (Fig. 3.6-Fig. 3.9). This is due to the thickness of the paraffin section in which cells may be cut or sectioned, as they are not present in a single layer. At the same time, the number of the cell nuclei with 2, 3 or 4 signals increased while overlapping disappeared. Thus, the evaluation of signals in FISH analysis of isolated cell nuclei reflects more exactly the number of the local gene changes of prostate cancer.

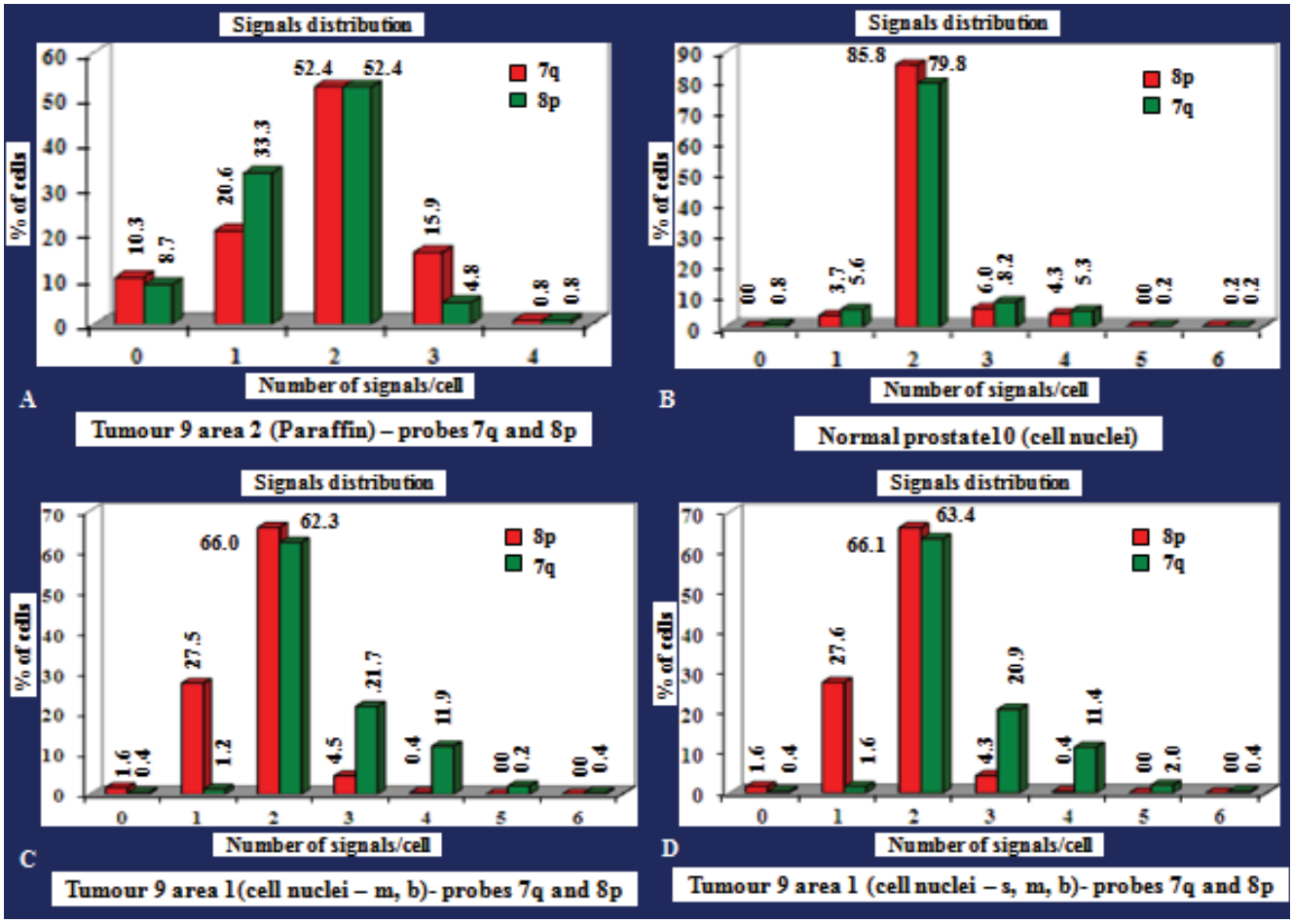


Fig. 3.9: Analyses of isolated cell nuclei from tumour sample 9 with FISH and verification of 8p12-22 loss. Medium and big sized nuclei evaluated separately from small, medium, and big. The small nuclei correspond to the normal cells. **A)** Tumour 9 area 2 paraffin showed low level gain of 7q31 and clear loss of 8p12-22. **B)** FISH in isolated cell nuclei from normal prostate tissue to show the differences from the FISH in paraffin section. **C)** Tumour 9 area 1 (cell nuclei -m, b) showed clear gain of 7q31 and loss of 8p12-22. **D)** Tumour 9 area 1 (cell nuclei -s, m, b) showed also clear gain of 7q31 and loss of 8p12-22**.**

The evaluation of the FISH analysis in tumour samples 7 and 9 was compared in paraffin and isolated nuclei in different areas of the tumour samples (Fig. 3.5-Fig. 3.9). Both of them were represented in two different areas and have been studied as paraffin sections as well as cell nuclei. Furthermore, they were analysed with different FISH probes combinations and colour swap of the signals between the probes (or chromosomes). The results of this can be summarized as follows:

- The unclear gain or loss in paraffin sections could be clarified and confirmed by the same FISH combination in the cell nuclei of the same sample.
- Identification of the probes aneuploidy in the different areas of the same tumour.
- The verification of the prostate cancer heterogeneity.
- The colour independence of the signal labelled in red or green.

The FISH analysis of the same tumour sample as paraffin section and isolated cell nuclei is useful if not necessary to clarify the unclear gain, or loss, of the chromosome segments using different probe combinations. The best example in this study is tumour sample number 8 which includes FISH in tumour sample 8 -Paraffin (7q31 - red/8p12-22 - green) combination and 8 -Paraffin (7 q31- red /cent. 4 - green) combination in the same tumour area as paraffin sections confirmed the gain of 7q31 and no change for chromosome 4 if they are studied only in FISH with paraffin. To clarify the change in 8p12-22 and 8q22-24, FISH analysis of tumour 8 -Cell nuclei (8p12 - red/ 7q31 - green) (m + b and s + m + b combination) and 8.-Cell nuclei (8p12- red/7q31 - green) (m + b and s + m + b combination) was analysed, confirming that there were no loss of 8p12-22 but gain of 8q22-24. In addition to amplification of 7q31, it showed also the formation of an isochromosome 8q.

In case of tumour sample 7, the results obtained from the FISH in isolated cell nuclei confirmed the results obtained from FISH in paraffin sections. In addition, FISH in isolated cell nuclei of tumour 7 showed amplification of 7q31, 8p12-22, and 8q22-23 (whole chromosome 8). FISH in isolated cell nuclei in tumour 9 confirmed the clear loss of 8p12-22 and gain of 7q31 in both areas of the tumour in addition to the amplification of 7q31 only.

The advantages as well as the disadvantages of the FISH hybridisation of paraffin sections and isolated cell nuclei observed during this evaluation are listed in Table 3.4. The disadvantages of the FISH in isolated cell nuclei were: the loss of the tissue histology, extensive laborious and extremely high material consumption of tissues. The prostate cancer (adenocarcinoma) is a multifocal and heterogeneous tumour which also is small in size. The tumour focus may not be

present after a small number of sections have been made. The alternative method to study more samples of prostate cancer was the immunohistochemical study for the following reasons: The previous FISH study showed obvious 7q31 gains in all tumour samples. This PAC (7q31) contains the Caveolin-1 and Caveolin-2 genes. Consequently, the Caveolin genes that were located in this region were amplified and this should be correlated with a strong Caveolin-1 and Caveolin-2 protein expression.

Table 3.4:- The differences between FISH on paraffin section and FISH in cell nuclei.

Point of differences	FISH on Paraffin section	FISH on cell nuclei
Histology	Retained	Not retained
Procedures	Extraordinarily elaborate (i.e. it needs more steps to get rid of the formaldehyde-haemoglobin complex present in the paraffin sections).	Isolation of cell nuclei needs extraordinary procedures. After isolation of the cell nuclei the FISH procedure is similar to that of FISH with lymphocytes.
Signals	Always need to be focused in different layers of sections and exact determination of the signals for each cell.	Signals can easily be focused.
Background	Always present.	Uncommon
Overlapping of cells	Usually present	Rarely present when the cell nuclei exist in only one layer.
Evaluation	- Need more attention to determine the signals for each single cell - Not always sufficient number of cells could be evaluated, laborious.	- Do not need more attention to determine the signals for each cell. - Sufficient number of cells could always be evaluated.
Confidence of the result	Less confident	More confident
Material needed	Little	Large pieces
Tumour foci	May be present after many sections with the same tumour characters (grade or Gleason score).	Due to the micro character of the prostate tumour and the thickness of the sections needed for the isolation of cell nuclei, after further sectioning the tumour foci may no longer be present. The new section may contain other tumour foci (or areas) with different histological characters or normal tissue may appear.

Due to the small size of the tumour foci found in prostate cancer, after some sections for FISH in Paraffin, the tumour may no longer be present or other tumour foci with different histological characters to the previous one appeared. This difference was extreme in the case of FISH in isolated cell nuclei. Tissue sections needed for immunohistochemical study were very thin (2-4 μm), easy to prepare without extraordinary precautions, and some of the tumour foci were still present for other studies. The immunohistochemistry (IHC) study, after establishment, is cheaper and easier to evaluate. In addition, the immunohistochemical study could clarify the relation between Caveolin-1 expression and disease development/progression as will be seen later (see chapter 3.3).

3.2.3. Specific chromosomal changes and clinical pathology

The comparison between the specific chromosomal changes and clinical pathology (TNM stages and Gleason scores) showed the following correlations:

1) Tumour samples 2 -Paraffin and 4 -Paraffin contain prostate intraepithelial neoplasia (PIN) or may be the beginning of pT1 in the pathological classification. Both tumour samples showed clear gain of 7q31. Tumour 2 showed low level loss of 8p12-22, but tumour 4 showed clear gain of 8p12-22.

2) The loss of 8p12-22 in tumour 2 could only be confirmed by FISH analysis in isolated cell nuclei, which was not possible due to insufficient material.

3) Tumour sample 1 -Paraffin and 3 -Paraffin had pT1b and pT1a stages respectively, but has the same Gleason score of 6 without known patterns. Tumour sample 1 -Paraffin showed clear gain of 7q31 and clear loss of 8p12-22, whereas tumour sample 3 -Paraffin showed a clear gain of both 7q31 and 8p12-22. Both of them showed amplification, but the amplification of 7q31 in the tumour cells was more than 8p12-22.

4) Tumour samples 7, 6, and 8 have the same stage pT3a but with different Gleason scores. Tumour sample 7 has a Gleason score of 5 (low malignant) and showed clear gain of 7q31 and 8p12-22 in both tumour areas (e.g. tumour 7 area.1 and area.2). The gain of 8p12-22 in the case of tumour sample 7 is combined with a clear gain of 8q22-24 which represents a gain of whole chromosome 8 (see 7 area 1 -Cell nuclei in Fig. 3.7). The other two samples, 6 -Paraffin and 8, have Gleason scores 8, but in a different pattern. Tumour sample 6 was 5 + 3 pattern (i.e. the first number or pattern is more dominant than the second number or pattern 2) and tumour sample 8 was contrariwise (3 + 5). Tumour sample 6 was represented in two areas (area 1 and 2) and showed gain of 7q31 and loss of 8p12-22 which could not be confirmed through FISH in cell nuclei, due to lack of the tissue sample (Fig. 3.5). A clear gain of 7q31, no loss of 8p12-22 and clear gain of 8q22-23 in the case of tumour sample 8 which has a Gleason score 3 + 5 = 8, were also observed when the FISH in paraffin and cell nuclei were analysed simultaneously (Fig. 3.8).

5) Tumour samples 5 -Paraffin and 9 -Paraffin have stage pT3b and a Gleason score of 8, but pattern 3 + 5 (was less malignant) and 5 + 3 (more malignant), respectively. The first tumour sample 5 -Paraffin showed high level gain of 7q31 and low level loss of 8p12-22 (Fig. 3.4). In contrast, tumour sample 9 showed low level gain of 7q31 and high level loss of 8p12-22 especially in area 1-Cell nuclei (Fig. 3.9).

This short molecular cytogenetic study using different labelled probes to three chromosomes areas of prostate cancer cells revealed the heterogeneity of this type of solid tumour. This was

achieved through application of FISH technique on sections derived from different tumour areas of the prostate tissue and analysing of the deferent signals in the tumour cells.

From these FISH results of a small number of samples, it became evident that cell nuclei preparations reflect more exactly the specific chromosomal changes in prostate cancer samples. The gain of 7q31 was found in all tumour samples, but with different levels of gain. 7q31 was clearly amplified in tumour samples 3, 7, and 8. The gain of 8p12-22 was found in tumours 3, 4, and 7 and loss in 1, 5, and 9. Low level loss was found in tumour 2 and no loss in tumour 8. Amplification of 8p12-22 was found only in tumour 3 and 7. Gain of 8q22-23 was found in tumour 7 and 8. In tumour 7 the whole chromosome 8 was amplified together with chromosome region 7q31 and in tumour 8 only 8q22-23 was amplified forming isochromosome 8q as well as 7q31 region and loss of 8p12-22 in two different probe combinations. Heterogeneity, multifocality and independence of prostate cancer were noted in tumour samples 6 and 7.

In the pre-cancer stage which is described by some pathologists as prostate intraepithelial neoplasia (PIN), 8p12-22 loss is clear but 7q31 is not clearly gained. In the early cancer stage, the gain (or the amplification) of 7q31, 8p12-22, and 8q22-23 were noticed. However, in the medium malignant stage of the prostate cancer, no loss of 8p12 was noticed; meanwhile the 7q31 and 8q23 were amplified. In the high malignant stage and metastasis, 8p12-22 was detected and 7q31 and 8q22-24 were gained and correlation was found with high frequencies.

Moreover a close relation was found between Gleason score (pattern 1 and pattern 2) and the chromosomal changes in regions 7q31, 8p12-22, and 8q22-33. In Gleason score 5 with 2 + 3 patterns, 7q31, 8p12-22, and 8q22-23 was amplified. In Gleason score 8 with 3 + 5 patterns 7q31 as well as 8q22-23 was amplified and no loss of 8p12-22. In Gleason score 8 with 5 + 3 patterns and metastasis 7q31 gain and 8p12-22 loss was confirmed.

The Caveolin-1 and 2 genes, which are believed to play a role in tumour development and metastasis, is located on 7q31. In at least five of the analysed tumour samples, a gain of 7q31 was found and in the others this was unclear. The same samples that were analysed by FISH were studied with immunohistochemistry of paraffin sections using Caveolin-1 antibody to analyse if there is a relationship between the number of gene copies and expression of Caveolin-1. In order to evaluate the correlation between Caveolin-1 expression and tumour stage or grade a larger number of tumour samples was analysed later in chapter 3.3.3-3.3.4.

3.3. Immunohistochemistry studies of Caveolin-1

Gain of 7q31.1 was found in samples under FISH study. The probe used contains the Caveolin-1 + 2 genes (Fig. 3.10).[99-100]

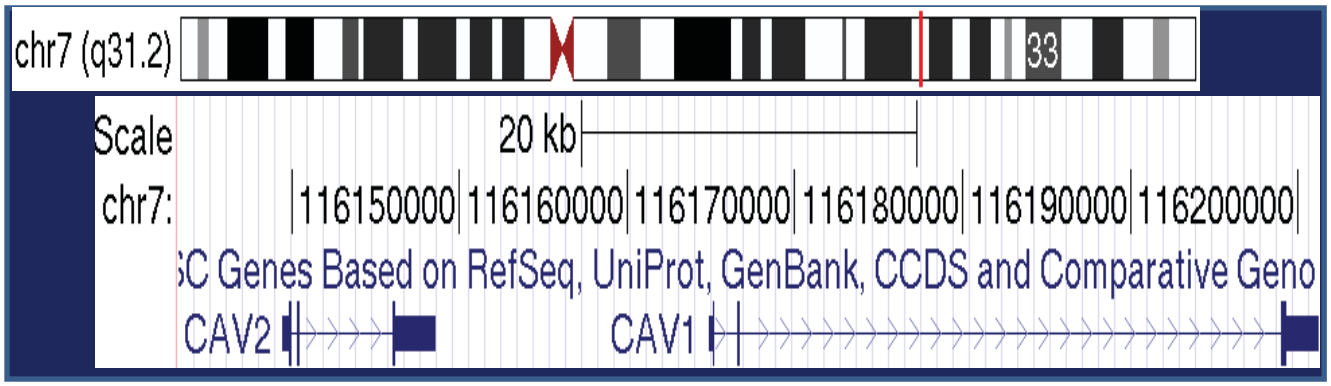


Fig. 3.10: Map of Chromosome 7q31.1 and localisation of CAV1/2. (http://www.ensembl.org/Homo_sapiens/Location/Chromosome?ph=15334;r=7:111738964-140748616 http://genome-euro.ucsc.edu/cgi-bin/hgTracks?db=hg19&position=chr7%3A115640001-116440000). [99-100]

Caveolin-1 is a known putative oncogene in many tumours. It could be considered as an interesting candidate gene to be studied in the region with copy number gain (7q31) in prostate cancer. Supposing that the Caveolin-1 gene is a target for copy number gain, then Caveolin-1 protein expression should be expressed at a higher level in the tumour samples. To study this; an immunohistological analysis was performed in a higher number of tumour samples.

First tests were performed in this work to establish an optimal protocol to study the Caveolin-1 and YB-1 protein by immunohistochemistry (IHC). This led to some modification of the methods. The previously prepared sections for FISH study were 4-6 µm thick and not suitable for this type of study. Detachment of the sections during the IHC steps, especially with a retrieval antigen and an unspecific reaction may occur. A thickness of 2-4 µm showed better adhesion and stability during the IHC-process and correct evaluation.

3.3.1. Immunohistochemistry study of Caveolin-1 anti-body

Caveolin-1 is an antigen that needs retrieval. Therefore, the application of the antibody directly on the sections can not be done. For the retrieval, slides with paraffin sections were immersed completely in a pre -warmed citrate buffer and cooked for 15 minutes in a microwave oven without dehydration of the sections during the treatment (i.e. the sections should be covered with citrate buffers). For the qualitative study, a dilution of 1:2000 was used. However, the dilution of the Caveolin-1 antibody of 1:3000 was ideal for the semi-quantitative determination of Caveolin-

1 in the prostate tissue sections. 200 µl of the primary antibody solution (Caveolin-1) was enough to cover the different sizes of the tissue sections. Overnight incubation with primary antibody (Caveolin-1) either at 4 °C or at room temperature resulted in drying of the tissue sections and causing unspecific staining. Also, the incubation for 60 minutes was not enough for staining. The ideal incubation time was 2 hours in a humidity chamber. The incubation time of the secondary antibody was increased from 15 to 20 minutes to increase the detection of the primary antibody bound to the antigen (here Caveolin-1 protein). In general, the detection with the labelled avidin-biotin (LAB)/Labeled Strept Avidin Biotin method (LSAB) were four to eight times more sensitive than the ABC (avidin-or streptavidin-biotin-enzyme complex) method.

The normal prostate tissue consists of glandular and non-glandular parts (see chapter 1.1.2 and Fig. 1.1-1.2). Prostate cancer is considered to be the most complex solid tumour. The histology of the prostate is very complicated and should be evaluated by an experienced pathologist. For this purpose many tissue blocks were prepared from different areas of the prostate gland as well as near localized tissues or organs (either topographical or functionally related organs). Each block was histologically evaluated in order to determine whether it contains tumour cells or not. Meanwhile, the size of the tumour, cell type, presence or absences of basal cells, basal cell membrane, cell cytology, malignancy grades and different Gleason grades were all taken into consideration within the classification. The main classifications used are clinical staging TNM Stage, pathological staging according to WHO Grading system (G), and Gleason score (GS). The pathological record should contain TNM classification in addition to WHO grade or Gleason score or both of them as standard in prostate cancer pathology classification. There are different types of prostate cancer according to the origin of the cell type which transformed to tumour cell. About 95% of prostate cancer is adenocarcinoma and originates from glandular tissue (Prostate secretory epithelium).

At the beginning of this study, the tumour samples were stained with haematoxylin eosin to identify the tumour area. Secondly, the tumour samples were subjected to histopathological examination to identify the tissue types that were obtained from a biopsy as well as the tumour areas in the tissue sections. Most of the tissue types obtained by biopsy were lymph nodes, prostate gland, seminal vesicles, or urinary bladder. Lymph nodes, seminal vesicles, and the urinary bladder are related anatomically and pathologically to prostate cancer and are areas for metastasis. The immunohistochemistry study was carried out in several steps and methods of analysis were based on the results obtained. It was unknown which cell types of prostate cancer expressed Caveolin-1 and in which cell compartments. To answer these questions, two different forms of evaluation were

used to study the tumours: Qualitative analyses were termed group A and semi-quantitative analyses were termed group B (Table 3.5).

Table 3.5:- The different groups of Caveolin-1 expression under study.

Group	Total number of sample	Samples with prostate Parenchyma	Antibody concentration	Type of evaluation
A	69	52	1:2000	Qualitative (single, spots, or complete tumour cells)
B	111	107	1:3000	semi-quantitative (Percentage of expression in the tumour x intensity = immunoscore)

Shown are the different groups of samples used in the immunohistochemical studies with Caveolin-1.

The staining of normal prostate tissue with Caveolin-1 is shown in the figure 3.11.

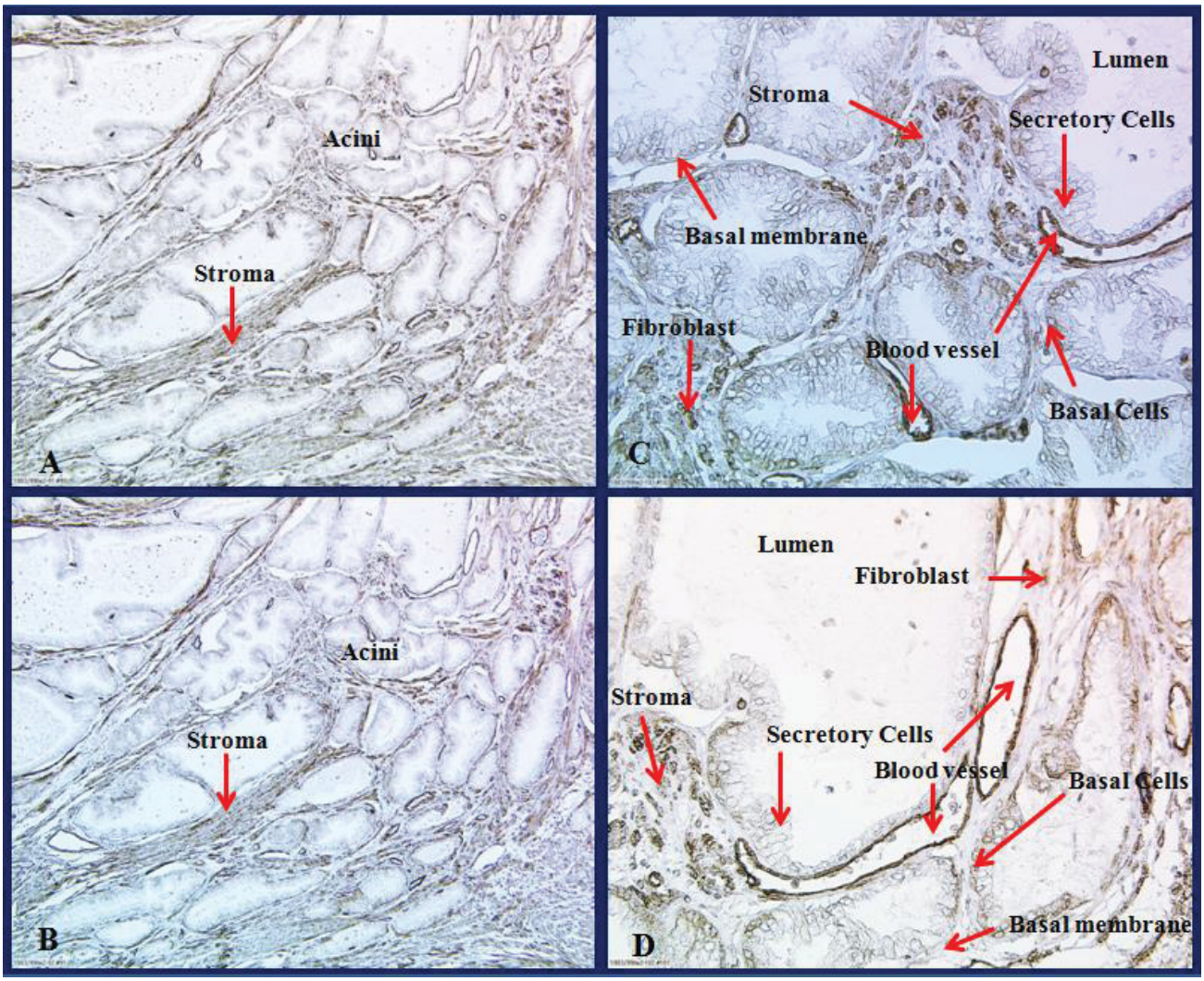


Fig. 3.11: Histology of the prostate as revealed by immunhistochemistry with Caveolin-1. Shown are the normal prostate acini surrounded by connective tissue and smooth muscles using different magnifications (**A:** 10x, **B:** 16x, **C:** 20x, and **D:** 40x). The acinus consists of the basal membrane, basal cells, and secretory or glandular cell of prostate emptying their secretion in the lumen. Caveolin-1 positive cells stained brown.

3.3.1.1. Qualitative expression of Caveolin-1 in prostate carcinoma samples (group A)

Qualitative expression was performed at first to identify the cells of the prostate which express Caveolin-1 and in which cell compartment. The sensitivity of the LSAB method was evaluated by using 12 different tissue sections including oesophagus, muscle, lymph nodes, spleen, stomach, liver, lung, prostate, and urinary bladder. These tissues are used in the pathology department of the Heinrich-Heine University (HHU) as standard to establish suitable immunohistochemical protocol for any antibody. These tissues showed varying degrees of expression, from weak in Lymph nodes, to medium in colon, and strong expression in liver and lung.

Group A contained 63 samples (Table 3.6) and consisted of urinary bladder (4 samples), lymph nodes (3 samples), prostate intraepithelial neoplasia PIN (3 samples), seminal vesicle (one sample), and tumours of prostate parenchyma (52 samples). These tissues were differentiated after haemetoxylin eosin staining. They were topographically, clinically, and anatomically related to prostate cancer metastasis. Consequently, Caveolin-1 expression in the above mentioned tissues is related with prostate cancer metastasis.

Table 3.6:- The different tissue types present in group A analyzed.

Tissue type under study	Number of sample
Urinary bladder	4
Lymph nodes	3
Prostate intraepithelial neoplasia (PIN)	3
Seminal vesicle	1
Tumours of the prostate parenchyma	52

3.3.1.1.1. Tumours of the prostate parenchyma

Tumours of the prostate parenchyma of group A (n = 52) were numbered from parenchyma – 1 to 52 as seen in table 3.7. The TNM stages were ranged from stage pT1-pT4; 2samples were stage pT1, 9 samples stage pT2, 29 samples stage pT3 and one sample was stage pT4. 11 prostate parenchyma samples were been without either clinical or histological pathological classification. In addition, the prostate parenchyma samples were evaluated according to WHO grade: G1 (n = 1), G2 (n = 11) and G3 (n = 7) and of Gleason score GS: 3 (n = 2), GS: 5 (n = 2), GS: 6 (n = 3), GS: 7 (n = 6), GS: 8 (n = 9), GS: 9 (n = 2), GS: 10 (n = 1). In 32 and 28 tumour samples were WHO grade and Gleason score unknown respectively (Table 3.7).

The immunohistochemical examination of the selected tumour samples showed different expression patterns of Caveolin-1 in the three cell types of the glandular part (acini) of the prostate

tumour samples. These three cell types; basal cells, normal glandular cells, and tumour cells expressed Caveolin-1 in different patterns in their cell compartments. The connective tissue cells, fibroblast and adipocytes expressed Caveolin-1 and were used as a control in the same sample.

Table 3.7:- Clinical pathology, qualitative immunohistochemistry evaluation and subcellular distribution of Caveolin-1 expression in the tumour samples of group A.

No.	Tissue type	TNM stage, WHO grade, and Gleason score	Expression in cell compartment	Evaluation in the cells		
				Normal	Tumour	Basal
1	par. -1	pT3b, pNX, pMX, R1; G3; GS 5+3 = 8		neg.	neg.	+++
2	par. -2			neg.	neg.	+++
3	par. -3.2	pT3b, pN1, pMX, R1; GS 4+5 = 9	Nucleus	*	++	+++
4	PIN	pT2b; GS: 3+4= 7		+++	+++	+++
5	par. -4	pT3a, pN0, pMX, R1; GS 3+5 = 8		neg.	neg.	+++
6	par. -5	pT3a, pN0, pMX; GS 2+3 = 5	Nucleus/cytoplasm	neg.	neg.	+++
7	par. -6	pT3a, pNX, pMX,R1; GS 2+3 = 5		neg.	+	+++
8	par. -7	pT1a; GS = 6	Nucleus	+	++	+++
9	par. -8			neg.	neg.	+++
10.1	par.-9.1 (Tumour)	pT1b; GS = 6	Nucleus	+	+++	+++
10.2	par. -9.2 (PIN)	pT1b; GS = 6		+	+	+++
11	par. -10	pT3b, pN1, (Micrometastasis) pMX, R1; GS 5+5 = 10		neg.	neg.	+++
12	par. -11	pT3a; GS: 3+5 = 8	Nucleus	neg.	neg.	+++
13	par. -12			*	neg.	*
14	par. -13	pT3a. pN0, pMX, R1; GS 4+3 = 7	Nucleus	neg.	neg.	+++
15	par. -14			*	neg.	*
16	Par./Urinary bladder	Metastasis		*	*	*
17	Urinary baldder	Metastasis		*	+	*
18	par. -15.2	pT2b, pN0, pMX; GS. 3+4 = 7	Nucleus/cytoplasm	+ ++	+++	+++
19	No evaluation	Prostate metastasis				
20	par. -16	pT3a, pNX, pMX; GS 4+3 = 7		neg.	neg.	+++
21	par. -17	pT3a; pN0; pMX,R1; GS 3+4 = 7		neg.	neg.	+++
22	par. -18			neg.	neg.	+++
23	par. -19	pT3a, pNO, pMX; GS 3+5 = 8	Cytoplasm	neg.	+	+++
24	par. -20	pT3a, pNX, pMX; GS: 3+5 = 8		neg.	neg.	+++
25	par. -21			neg.	neg.	+++
26	par. -22	pT3a, pN0, pMX, R1; G2		neg.	neg.	+++
27	par. -23	pT3a, pN0, pMX, R1; GS: 4+4 = 8		neg.	+++	+++
28	par. -24	PT2c, pN0, pMX; G2		neg.	neg.	+++
29	Seminal vesicle	Metastasis		*	neg.	*
30	Lymph node	Metastasis		*	neg.	*
31	par. -25			*	+	*
32	No evaluation					
33	par. -26			neg.	neg.	+++
34	Urinary bladder	Metastasis		*	neg.	*
35	par. -27	pT3a, R0; (G3),	Cytoplasm	++	neg.	+++
36	No evaluation	pT3b, GS: 3+4 = 7				
37	No evaluation	pT3b; GS: 2+3 = 5				
38	par. -28	pT4, pN1, pMX, R1; GS 5+3 = 8	Nucleus	neg.	+	+++
39	Urinary bladder	Metastasis		*	+++	*
40	par. -29	pT3c, pN0, pMX; G2		++	++	+++
41	par. -30	pT2c, pN0, pMX, R1; G2		neg.	neg.	+++
42	par. -31	pT2b, pNX, pMX,R0; G1		neg.	neg.	+++
43	par. -32	pT3a, pNX, pMX, R1; G3		neg.	neg.	+++
44	par. -33	pT2c, pNX, pMX; G2		neg.	neg.	+++
45	par. -34	pT2c, pNX, pMX; G2		neg.	neg.	+++
46	par. -35	pT3, pNX, pMX; G2		neg.	neg.	+++
47	par. -36	pT2c, pNX, pMX; G3		neg.	neg.	+++
48	par. -37	pT3b, pN0, pMX, R0; GS: 3+4 = 7		neg.	neg.	+++

No.	Tissue type	TNM stage, WHO grade, and Gleason score	Expression in cell compartment	Evaluation in the cells		
				Normal	Tumour	Basal
49	par. -38	pT3a, pN0, pMX, R1; GS 3+4 = 7		neg.	neg.	+++
50	par. -39	pT3a; G2		neg.	neg.	+++
51	par. -40	pT3c, pNx, pMX, G3		++	+++	+++
52	no Evaluation	pT1a; (G1)				
53	par. -41	pT2b, pN0, pMX, R0; G2	Cytoplasm	neg.	++	+++
54	par. -42	pT3c, pNX, pMX; G3		neg.	+	+++
55	par. -43	pT3b, pN2, pMX; GS = 3		neg.	neg.	+++
56	par. -44	pT2b, pNX, pMX, R0; G2, GS: 2+1 = 3		neg.	neg.	+++
57	par. -45	pT3b, pNX, pMX, R0; GS 3+5 = 8		neg.	neg.	+++
58	par. -46	pT3a, pN0, pMX, R1; G2		neg.	neg.	+++
59-	PIN-3			neg.	*	+++
60	par. -47			neg.	+++	+++
61	par. -48	pT3a, pN0, pMX, R1; GS 4+4 = 8		+++	+++	+++
62	no tumour					
63.1	Lymph node			neg.	neg.	neg.
63.2	Lymphe node			*	neg.	*
64	par. -49			neg.	+++	+++
65	par. -50	pT3b, pN1, pMX, R1; GS 4+5 = 9		+	+	*
66	par. -51			+++	+++	+++
67	no evaluation	pMX, R1; G3; GS 5+3 = 8				
68	par. -52	pT3, pN0, pMX; G3		neg.	neg.	+++
69	Lymph node	pT3a, pN1, pMX; G3		*	neg.	*

neg. = negative, * = no cells of this type are present, par. = prostate parenchyma, + = scattered positive cells, ++ = patches of positive cells, +++ = all cells positive.

Expression of Caveolin-1 was observed in the cytoplasm/nucleus or both of them in secretory tumour cells and this was observed in some tumour samples but not in all of them (Fig. 3.12). Subcellular distribution of Caveolin-1 expression in cell compartments showed no clear correlation with the tumour stage (TNM), WHO grading or Gleason score system. Caveolin-1 expression was negative in tumour cells of 34 tumour samples (65%), as defined if less than 10% of the total tumour cells are present in the tumour sample expressed Caveolin-1. The other tumour samples of the same group (n = 18) showed different expression patterns of Caveolin-1.

In addition to tumour samples expressing Caveolin-1 in less than 10%, tumour samples with Caveolin-1 expression in scattered cells (as defined when more than 10% and less than 40% of the tumour cells were Caveolin-1 positive) were counted as Caveolin-1 negative tumours in this type of qualitative evaluation (Fig. 3.12A-B). As single scattered positive Caveolin-1 tumour cells were found only in 6 tumour samples s. Then the total number of Caveolin-1 negative tumours was increased to 40 and represented 76.9% in this group (Table 3.8).

Table 3.8:- Immunoreactivity of Caveolin-1 expression in tumour cells of the prostate carcinoma (adenocarcinoma).

	Negative		Positive		
Type of Caveolin-1 expression	**Tumour cells negative**	**Single Scattered positive tumour cells**	**Patches of positive tumour cells**	**All tumour cells positive**	**Total**
Number of cases	34	6	4	8	52
Samples in %	65.4	11.5	7.7	15.4	100
	76.9% Negative by definition		**23.1 %+ Positive by definition**		

Patches of Caveolin-1 positive tumour cells were found in four tumour samples with more than 40% and less than 75% of the tumour cells positive (Fig. 3.12C). In eight tumour samples, Caveolin-1 expression was found in all tumour cells (i.e. > 75-100% of the cells expressing Caveolin-1) as can be seen in Fig. 3.12D. In this evaluation, samples where all tumour cells and patches of tumour cells expressed Caveolin-1 were considered as Caveolin-1 positive tumours raising the total number of Caveolin-1 positive tumours to 12 tumour samples and represented 23.1% in this group (Table 3.8).These differences in Caveolin-1 expression pattern, from a negative tumour, to scattered positive through patches (spots) of positive cells, to all cells expressing Caveolin-1, reflects the complexity of the prostate carcinoma.

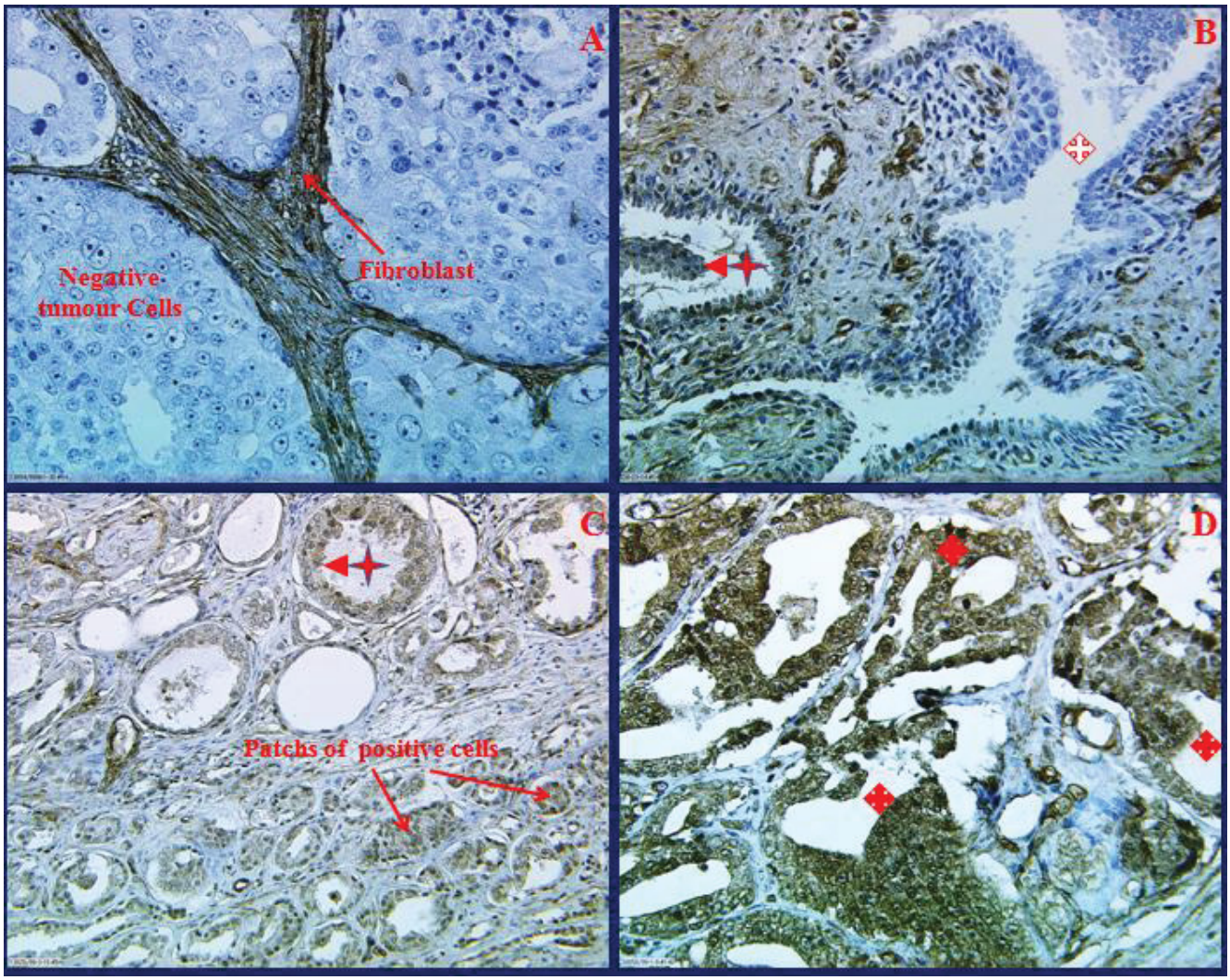


Fig. 3.12: Examples of Caveolin-1 expression in tumour samples of group A. A) The prostate cancer acini (glandular part of the prostate containing tumour cells) are all negative (✥), but the connective tissue cells are positive (fibroblast and fat cells). **B)** Caveolin-1 expression in single scattered cells either in cell nuclei (◀) or cell cytoplasm (✦) or both (◀✦) at the same time. **C)** Caveolin-1 expression in patches of cells. **D)** Caveolin-1 expression is found in all the tumour cells (✥) either in cytoplasm/nuclei or both.

The three cell types of the prostate parenchyma demonstrated differences in Caveolin-1 expression. Caveolin-1 expression was positive in all basal cells of the tumour samples (n = 48), which still contained normal acini or remains of normal tissue. Absence of basal cells in 4 tumour samples was related to the extension of the tumour cells in all foci under study and replacement of all the normal glandular tissues. In these four cases, the tumour cells displaced the basal membrane of the glands and invaded all the glandular and non-glandular parts of the prostate and may build extra capsular extension or metastasis. The normal glandular cells disappeared in 4 tumour samples (7.7%). In three samples (5.8% of the total samples in group A), all of the normal glandular cells adjacent to glandular tumour cells were Caveolin-1 positive (see no. 18, 61, and 66 in Table 3.7).

Meanwhile, in 40 samples, the normal glandular (secretory) cells did not show Caveolin-1 expression corresponding to 76.9% of the total tumour samples of group A. Caveolin-1 was expressed in single scattered cells in 3 samples of normal secretory cells. According to the evaluation criteria, the normal glandular cells expressing Caveolin-1 in single scattered positive cells were considered as Caveolin-1 negative normal cells increasing the number of these samples to 43 and representing 82.7% of the total samples (Table 3.9).

Comparing the three cell types, namely normal secretory cells, tumour, and basal cells in relation to their presence or absence in each tumour sample showed no normal secretory (glandular) cells in 4 tumour samples, as well as no basal cells in 3 tumour samples. Therefore, it can be concluded that tumour cells have replaced them completely.

Caveolin-1 expression as scattered positive cells was not found in the basal cells in any tumour sample. Scattered positive cells of Caveolin-1 expression were found in the normal glandular cells in three tumour samples. In tumour cells, scattered positive cells of Caveolin-1 were found in 6 tumour samples (Table 3.9). All normal secretory cells expressed Caveolin-1 only in three tumour samples of this group. All tumour cells expressed Caveolin-1 only in 6 tumour samples.

Table 3.9:- Analyses of Caveolin-1 expression in the different cell types present in the glandular part of prostate adenocarcinoma in group A.

	Normal cells positive				Tumour cells positive				Basal cells positive			
Manner of expression	**No normal cells**	**Scattered positive cells**	**Negative**	**positive = (all cells and patches)**	**No tumour cells**	**Scattered positive cells**	**Negative**	**Positive = (all cells and patches)**	**No tumour cells**	**Scattered positive cells**	**Negative**	**Positive = (all cells and patches)**
TSN	4	3	40	5	0	6	34	12	4	0	0	48
TS in %	7.7	5.8	76.9	9.6	0	11.5	65	23.1	7.7	0	0	92

TSN = tumour sample number, TS in % = tumour sample in %.

In summary, all basal cells present in tumour samples were Caveolin-1 positive. In 40 tumour samples, Caveolin-1 was found negative in normal secretory cells. But in tumour cells, it was found that Caveolin-1 was negative in 34 tumour samples. Consequently, the number of tumour samples expressing Caveolin-1 in their tumour cells was higher than the number of tumour samples expressing Caveolin-1 in their normal secretory (glandular) cells (Table 3.9).

3.3.1.1.2. Cells combination of Caveolin-1 expression in the glandular part of prostate (acini) during tumourigenesis

The analysis of Caveolin-1 expression in the main three cell types (basal cells, normal secretory cells, and transformed secretory to tumour cells) present in the acini of the tumour samples, revealed that Caveolin-1 was positive in all of these cells in only three tumour samples. A negative staining for Caveolin-1 was not found in the three cell types at the same time in any sample. In three tumour samples, Caveolin-1 expression was found in both basal and tumour secretory cells in combination but was negative in the normal secretory cells. One sample showed the combination of positive Caveolin-1 expression in all cells of the basal and tumour cells, and scattered positive staining in the normal glandular cells. Caveolin-1 expression was positive in the basal cells of 31 tumour samples meanwhile the normal and tumour secretory cells were negative (Table 3.7 and Table 3.10).

Table 3.10:- The combination of Caveolin-1 expression in different cell types of group A.

	Normal glandular cells	Tumour glandular cells	Basel cells	Number of tumour samples
1	Positive	Positive	Positive	3
2	Patches of positive cells	Positive	Positive	1
3	Patches of positive cells	Patches of positive cells	Positive	1
4	Scattered cells positive	Positive	Positive	1
5	No normal cells	Positive	Positive	
6	No normal cells	Patches of positive cells	Positive	1
7	Negative	Patches of positive cells	Positive	1
8	Negative	Negative	Negative	
9	Positive	Positive	Negative	
10	Positive	Negative	Positive	
11	Negative	Negative	Positive	31
12	Negative	Positive	Positive	3
13	Positive	Negative	Negative	
14	Scattered cells positive	Scattered cells positive	Positive	1
15	Scattered cells positive	Positive	Positive	1
16	Negative	Scattered cells positive	Positive	4
17	Negative	Negative	Scattered cells positive	
18	No normal cells	Positive	No basal cells	1
19	No normal cells	Negative	No basal cells	1
20	No normal cells	Scattered cells positive	No basal cells	1
21	Scattered cells positive	Scattered cells positive	No basal cells	1
22	Scattered cells positive	Scattered cells positive	Scattered cells positive	

3.3.1.2. Caveolin-1 expression in prostate intraepithelial neoplasia (PIN)

1.1.1.1 PIN is a precursor stage to prostate cancer. It has a basement membrane and basal cells. Normally, basal cells are lying between basement membranes and normal secretory cells. These glandular cells (normal secretory cells) transform later to tumour cells in prostate adenocarcinoma. In most tumour samples, these expressed Caveolin-1 (Table 3.11).

Table 3.11:- Caveolin-1 expression in PIN and metastasis cells in urinary bladder and lymph node tissues.

Expression of Caveolin-1 in PIN and metastasis organs	No tumour cells	All cells negative	Sacttered positive cells	Patches of positive cells	All cells positive	Total numbers of the samples
PIN	1	0	1	1	0	3
Urinary bladder	1	0	1	0	1	4
Lymph nodes	0	3	0	0	0	3

Shown is the Caveolin-1 expression in PIN, Urinary bladder, and Lymph nodes.

Prostate intraepithelial neoplasia **(**PIN) was identified in three tumour samples. In one sample, the tumour cells and PIN cells were present near to each other reflecting the heterogeneity of the prostate cancer. There are two types of PIN; low grade PIN (LGPIN) and high grade PIN (HGPIN). Near PIN cells (PIN foci), the normal secretory cells did not express Caveolin-1 in one tumour sample. In the second sample, Caveolin-1 was expressed as scattered positive cells; whereas, in the third sample, all the normal secretory cells expressed weakly Caveolin-1 (Table 3.7). Tumour cells were present only in two samples near PIN cells and expressed Caveolin-1 as scattered positive cells in one sample and as positive in the other (see Table 3.11 and Fig. 3.13). Tumour cells were not present in one PIN sample.

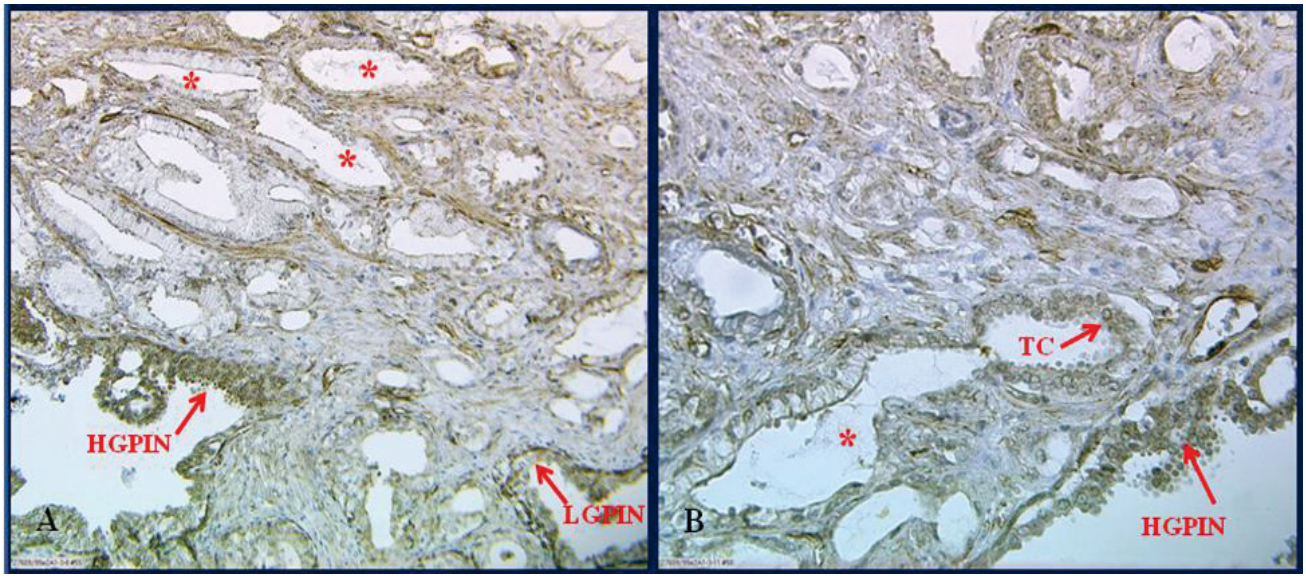


Fig. 3.13: Caveolin-1 expression in prostate intraepithelial neoplasia (PIN). A) PIN and normal acini in overview. **B)** The normal acini are negative but the basal cell and stroma cells are Caveolin-1 positive as well as the cells of the PIN. (* = Acinus, HGPIN = High grade PIN, LGPIN = Low grade PIN, TC = Tumour cell).

3.3.1.3. Preferred sites of prostate cancer metastasis

During the metastasis process, some of the prostate parenchyma cells (glandular cells) which transformed to tumour cells are transported through blood or lymph vessels to metastasize in different organs due to the destruction of the basement membrane and prostate capsule.

The other examined tissue samples have a topographical relationship to the prostate such as the urinary bladder, lymph nodes and seminal vesicle where prostate cancer cells metastasize. Caveolin-1 expression was studied in each of them separately. The presence or absence of the prostate metastasis cells (tumour cells) and their manner of expression in the metastasis tissue (urinary bladder and lymph nodes) are described in Table 3.11 above.

The four urinary bladder samples were examined according to the presence or absence of the metastasis cells of prostate cancer. Tumour cells were present in three samples of the urinary bladder tissue. For the identification of the tumour cells in the bladder, one of the known markers of the prostate cancer cells was used (e.g. 34β E12, p63, high molecular-weight cytokeratin (HMWCK) and α-methylacyl coenzyme A racemase (AMACR)). In two samples containing tumour cells, Caveolin-1 was expressed in all metastasis cells in one (No. 39) and in the other as single scattered positive cells (No. 17). In the third one (No. 34), Caveolin-1 was not expressed (negative). In the fourth urinary bladder sample (No. 16), metastasis cells of prostate cancer were absent (i.e. no metastasis cells) (Table.3.11).

Prostate cancer tumour cells may metastasize mainly through the lymphatic system to closer and distant lymph nodes which are preferred sites for metastasis in case of prostate cancer cells. Metastatic prostate cancer cells were found in the three examined samples of the lymph nodes. Two of the examined lymph node sections originated from one tumour sample and two others from two different samples (Table 3.11). Tumour cells of the prostate acini (metastasized prostate cells) were found in all three lymph node samples as a result of metastasis, but these did not express Caveolin-1. It is not clear why the metastatic cells did not express Caveolin-1 in any of the lymph node samples. It may be related to micro-environmental effects of the lymph nodes surrounding the prostate tumour cells.

3.3.2. Semi-quantitative evaluation of Caveolin-1 expression in prostate carcinoma samples (group B)

Using a 1:2000 dilution of the Caveolin-1 antibody in the qualitative method of evaluation showed differences of Caveolin-1 expression in the different cell types of prostate cancer acini and

cell compartments. However with this dilution of the Caveolin-1 antibody, the background was too high for semi-quantitative evaluation. Increasing Caveolin-1 antibody dilution to 1:3000 reduced the background and made the evaluation of tumour cell numbers more precise. Additionally, it was sensitive enough to differentiate between the degree of intensity (score 0-3) and could give more informations about the Caveolin-1 expression and its relation to clinical pathology of the prostate cancer.

Using a dilution of 1:3000 of the Caveolin-1 antibody, 111 tumour samples were studied. At first, the percentage of the tumour cells expressing Caveolin-1 was evaluated according to the total number of the tumour cells present in each area of the tumour sample (Evaluation in [%]). Secondary, the intensity of expression was evaluated in each tumour sample. The semi-quantitative value resulted from evaluation in [%] x intensity. The result value was named immunoscore of expression. The 111 tumour samples were evaluated and compared with their clinical TNM stages, and pathology WHO grades, as well as Gleason score (Table 3.12).

3.3.2.1. Clinical pathology of the tumour samples in group B

Two samples were lymph node metastases (see no. 42 and 88 in Table 3.12). Two other samples were adenoma tumours of prostate and another lymph-adenomatosis tumour (see no. 93 and 100). Adenoma (aldosteronoma) is a benign epithelial tumor arising in epithelium of mucosa (stomach, small intestine, and bowel), glands (endocrine and exocrine), and ducts. Adenoma is mostly a collection of benign tumours resulting from the growth of the endocrine glands within the same organ. Lymph-adenomatosis tumour is an abnormally enlarged lymph node. The other 107 tumour samples were prostate parenchyma samples. After histological examination, there were 20 tumour samples without pathology record. In 13 of them Caveolin-1 expression could be evaluated and in three not. In the further four samples, no more tumour tissue was present in the samples due to the previous sectioning of these microtumours for other studies (Fig. 3.14).

In 87 tumour samples, the pathology record was known. In seven tumour samples, no tumour tissues were present, due to the continuous sectioning of these microtumours characteristic in prostate cancer for other studies in the pathology department. Another four tumour samples could not be evaluated, because they were lost during staining process (Fig. 3.14).

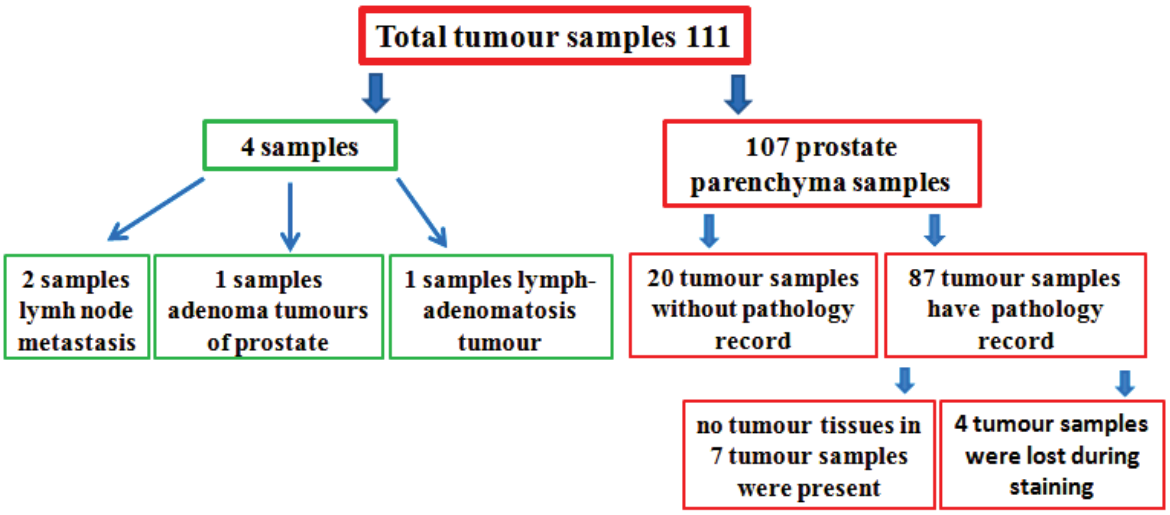


Fig. 3.14: Distribution of the tumour samples under study of Caveolin-1 expression.

For some samples in group B, the TNM classification and WHO grading was known. For others, the TNM classification and Gleason score was known, while others had the three known classifications. There was one tumour sample with known Gleason score but without TNM stage and WHO grading (see Table 3.12 no. 88). The clinical staging of them ranged from pT1-pT4 stage; 7 samples were in stage pT1, 28 samples stage pT2, 49 samples stage pT3 and two samples were in stage pT4. Samples of this group (B) were also classified according to WHO grading scale into Grade 1 (n = 2), Grade 2 (n = 21) and Grade 3 (n = 16) as well as in Gleason score into GS: 3 (n = 1), GS: 5 (n = 14), GS: 6 (n = 8), GS: 7 (n = 15), GS: 8 (n = 17), GS: 9 (n = 4), GS: 10 (n = 2) (Table 3.12). Hence the number of the tumour samples having TNM stage classification was more than those with WHO grade system and Gleason score.

Only tumour samples with pathological records (either TNM stages or WHO grades) were taken into consideration in the statistical analysis of this study. Then the numbers of the evaluated and statistically analyzed tumour samples were 4, 24, 44, and 2 from patients with pT1; pT2, pT3, and pT4 stages, respectively (Table 3.13). But the tumour sample numbers WHO grading system were 2, 21, and 16 from samples with grade 1, grade 2, and grade 3, respectively.

For each slide, all areas with well preserved prostate tumour tissue were examined using 10x to 40x objectives of a light microscope after immunostainning with Caveolin-1 antibody. The percentage of the tumour cells stained with Caveolin-1 (either in cytoplasm or cell nucleus) was calculated in comparison with the total number of the tumour cells present in each tumour sample. This manner of evaluation was referred as positive percentage of Caveolin-1 immunostainning in tumour cells (or shortly evaluation in [%]).

Table 3.12:- Clinical pathology and quantitative expression of Caveolin-1.

No	Tumour Sample areas	TNM				WHO-Grade	Gleason score	Sample number	% of the Caveolin-1 in prostate tumour cells (Evaluation in %)	Intensity grade	Immunoscore 1 (method 1)	Distribution point	Immunoscore 2 (method 2)
		T	N	M	R								
1	**1.1**	pT3a	pN0	pMX	R1		4+4 = 8	**1.1**	80	1	80	4	4
	1.2							**1.2**	80	2	160	4	8
2	**2.1**	pT3c	pN2	pMX	R1	G2		**2.1**	> 80	1	81	4	4
	2.2							**2.2**	50	1	50	3	3
3	**3**	pT4a	pNX	pMx	R1	G2		**3**	neg.	0	0	0	0
4	**4.1**	pT3a	pNX	pMX		G2		**4.1**	neg.	0	0	0	
	4.2							**4.2**	< 5	1	4	1	1
	4.3							**4.3**	neg.	0	0	0	0
5	**5**	pT3a	pN0	pMX	R0	G2		**5**	neg.	0	0	0	0
6	**6.1**	pT2b	pNX	pMX	R0	G1		**6.1**	< 25	1	24	2	2
	6.3							**6.3**	neg.	0	0	0	0
7	**7**	pT3a	pNX	pMX	R1	G3		**7**	NE	NE	NE	NE	NE
8	**8**	pT2c	pN0	pMX	R1	G2		**8**	neg.	0	0	0	0
9	**9.1**	pT3b	pNX	pMX	R0		3+5 = 8	**9.1**	neg.	0	0	0	0
	9.2							**9.2**	neg.	0	0	0	0
10	**10.1**	pT3a	pNX	pMX	R1		2+3 = 5	**10.1**	80-100 (90)	2	180	4	8
	10.3							**10.3**	100	3	300	4	12
	10.4							**10.4**	neg.	0	0	0	0
11	**11.1**	pT3b	pN2	pMX		G3		**11.1**	100	3	300	4	12
	11.2							**11.2**	100	3	300	4	
	11.2							**11.2**	50	2	100	4	
	11.4							**11.4**	25	1	25	2	
12	**12.1**	pT3a,	pN0	MX		G3	3+4 = 7	**12.1**	100	3	300	4	
	12.2	pT3a	pN1					**12.2**	50	1	50	2	
13	**13.1**							**13.1**	10	1	10	1	
	13.2							**13.2**	neg.	0	0	0	
	13.3							**13.3**	neg.	0	0	0	
	13.4							**13.4**	neg.	0	0	0	
14	**14**							**14**	NE	NE	NE	NE	NE
15	**15**							**15**	neg.	0	0	0	0
16	**16**							**16**	NE	NE	NE	NE	NE
17	**17**	pT2b					6	**17**	neg.	0	0	0	0
18	**18**	pT1a					6	**18**	no tum.	no tum.	no tum.	no tum.	no tum.
19	**19**							**19**	no tum.	no tum.	no tum.	no tum.	no tum.
20	**20**	pT1a					6	**20**	neg.	0	0	0	0
21	**21**	pT1a					6	**21**	no tum.	no tum.	no tum.	no tum.	no tum.
22	**22**							**22**	no tum.	no tum.	no tum.	no tum.	no tum.
23	**23**	pT1a					6	**23**	neg.	0	0	0	0
24	**24**	pT3a	pN1	pMX		G3		**24**	20-50 (35)	1	35	2	2
25	**25.1**	pT3a	pN0	pMX	R1	G2		**25.1**	50	1	50	2	2
	25.02							**25.02**	> 50	1	51	3	3
	25.3							**25.3**	100	1	100	4	4
26	**26**	pT2c				G2		**26**	no tum.	no tum.	no tum.	no tum.	no tum.
27	**27.1**	pT3a	pNX	pMX			5+3 = 8	**27.1**	> 50	1	51	3	3
	27.4							**27.4**	neg.	0	0	0	0
28	**28.4**	pTa3	pN0	pMX	R1		3+2 = 5	**28.4**	> 80	1	81	4	4
29	**29**	pT4	pN1	pMX	R1	G3	5+3 = 8	**29**	neg.	0	0	0	0
30	**30**							**30**	> 70	2	142	3	6
31	**31**	pT3b	pNX	pMX	R1	G3	5+3 = 8	**31**	< 5	1	4	1	4

No	Tumour Sample areas	TNM				WHO-Grade	Gleason score	Sample number	% of the Caveolin-1 in prostate.tumour cells (Evaluation in %)	Intensity grade	Immunoscore 1 (method 1)	Distribution point	Immunoscore 2 (method 2)
		T	N	M	R								
32	32.1	pT3b	pNX	pMX	R1	G3	5+3 = 8	32.1	neg.	0	0	0	0
	32.3							32.3	> 90	1	91	4	4
	32.4							32.4	neg.	0	0	0	0
	32.5							32.5	neg.	0	0	0	0
	32.6							32.6	neg.	0	0	0	0
33	33.3							33.3	100	2	200	4	8
34	34.1	pT2c	pNX	pMX		G2		34.1	neg.	0	0	0	0
	34.2							34.2	neg.	0	0	0	0
35	35.1	pT3ac	pN0	pMX		G2		35.1	10	1	10	1	1
	35.2							35.2	> 50	1	51	3	3
36	36.1	pT3a			R0	G3		36.1	30	1	30	2	2
	36.2							36.2	neg.	0	0	0	0
37	37.1							37.1	50	1	50	3	3
	37.2							37.2	neg.	0	0	0	0
38	38.1	pT3a	pN0	pMX	R1	G2		38.1	neg.	0	0	0	0
	38.2							38.2	neg.	0	0	0	0
	38.3							38.3	neg.	0	0	0	0
39	39.1	pT3c	pNX	pMX		G3		39.1	neg.	0	0	0	0
	39.2							39.2	neg.	0	0	0	0
40	40	pT2c	pN0	pMX		G2		40	neg.	0	0	0	0
41	41.1							41.1	neg.	0	0	0	0
	41.2							41.2	neg.	0	0	0	0
	41.5							41.5	neg.	0	0	0	0
42	42.2	Lymph node metastasis						42.2	neg.	0	0	0	0
	42.4							42.4	neg.	0	0	0	0
	42.5							42.5	neg.	0	0	0	0
43	43	pT2c	pNX	pMX		G3		43	neg.	0	0	0	0
44	44							44	50	1	50	3	3
45	45.1							45.1	100	3	300	4	12
	45.2							45.2	100	3	300	4	12
46	46	pT1a				G1		46	< 10	1	9	1	1
47	47.1	pT3b	pN1	pMX	R1		4+5 = 9	47.1	neg.	0	0	0	0
	47.2							47.2	100	1	100	4	4
48	48.1	pT3b	pN1	pMX	R1		4+5 = 9	48.1	> 50	1	51	3	3
	48.2							48.2	neg.	0	0	0	0
49	49.1	pT3a	pN0	pMX	R1		3+5 = 8	49.1	100	2	200	4	8
	49.2							49.2	100	1	100	4	4
50	50.1	pT2b	pN0	pMX	R0		3+5 = 8	50.1	neg.	0	0	0	0
	50.2							50.2	neg.	0	0	0	0
51	51	pT2b	pNX	pMX	R1		3+2 = 5	51	NE	NE	NE	NE	NE
52	52.1	pT3a	pN0	pMX	R1	G2		52.1	100	1	100	4	4
	52.2							52.2	50	1	50	3	3
	52.3							52.3	50	1	50	3	3
53	53.1	pT3b	pN0	pMX	R0		4+3 = 7	53.1	neg.	0	0	0	0
	53.2							53.2	10	1	10	1	1
	53.3							53.3	> 80	1	81	4	4
54	54	pT2b					2+3= 5	54	neg.	0	0	0	0
55	55	pT3a	pN0	pMX			2+3 = 5	55	no tum.	no tum.	no tum.	no tum.	no tum.
56	56.1	pT3a	pN0	pMX	R1		4+3 = 7	56.1	neg.	0	0	0	0
	56.2							56.2	neg.	0	0	0	0
57	57	pT3b	pN0	pMX	R1	G3	3+5 = 8	57	< 5	1	4	1	1

No	Tumour Sample areas	TNM				WHO-Grade	Gleason score	Sample number	% of the Caveolin-1 in prostate.tumour cells (Evaluation in %)	Intensity grade	Immunoscore 1 (method 1)	Distribution point	Immunoscore 2 (method 2)
		T	N	M	R								
58	**58**							**58**	> 90	1	91	4	4
59	**59**							**59**	neg.	0	0	0	0
60	**60**							**60**	> 90	1	91	4	4
61	**61**	pT3a	pN0	pMX	R1		3+4 = 7	**61**	> 90	2	182	4	8
62	**62**	pT3a	pN0-	pMX	R1		2+3 = 5	**62**	100	1-2	200	4	8
63	**63.1**	pT3b	pN1	pMX			4+5 = 9	**63.1**	5	1	5	1	1
	63.2							**63.2**	10	1	10	1	1
	63.4							**63.4**	neg.	0	0	0	0
64	**64**	pT3c	pN1	pMX		G3		**64**	neg.	0	0	0	0
65	**65**	pT3c	pN1	pMX	R1	G2		**65**	80	1	80	4	4
66	**66**	pT3a	pN0	pMX			2+3 = 5	**66**	100	1	100	4	4
67	**67**	pT2b	pN0	pMX		G2	3+4 = 7	**67**	100	1	100	4	4
68	**68**	pT2b	pN1	pMX	R1		4+3 = 7	**68**	> 90	2	182	4	8
69	**69.1**	pT3b	pN1	pMX	R1		4+5 = 9	**69.1**	100	2	200	4	8
	69.2							**69.2**	100	2	200	4	8
	69.3							**69.3**	100	2	200	4	8
	69.4							**69.4**	5	1	5	1	1
70	**70.1**	pT3a	pNX	pMX	R1	G3	4+3 = 7	**70.1**	80	1	80	4	4
71	**71**							**71**	100	1	100	4	4
72	**72**	pT2b	pNX	pMX		G2	3+3 = 6	**72**	10	1	10	1	1
73	**73.1**	pT3b	pNX	pMX			5+5 = 10	**73.1**	100	2	200	4	8
	73.2							**73.2**	30	1-2	60	2	4
74	**74.1**	pT2b	pNX	pMX	R0	G2	2+1 = 3	**74.1**	neg.	0	0	0	0
	74.2							**74.2**	10	1	10	1	1
	74.3							**74.3**	neg.	0	0	0	0
	74.4							**74.4**	80-100 (90)	1	90	4	4
75	**75.1**	pT3a	pN0	pMX			3+5 = 8	**75.1**	10	1	10	1	1
	75.2							**75.2**	< 5	1	4	1	4
76	**76**							**76**	neg.	0	0	0	0
77	**77.1**	pT2b	pN0	pMX			5+3 = 8	**77.1**	50; 50	1-2	150	3	6
	77.2							**77.2**	50; 50	1-2	150	3	6
	77.3							**77.3**	10	1	10	1	1
78	**78**	pT3a	pN0	pMX			3+2 = 5	**78**	NE	NE	NE	NE	NE
79	**79**	pT2b	pN0	pMX	R1		2+4 = 6	**79**	NE	NE	NE	NE	NE
80	**80.1**	pT3b,	pN1	pMX	R1		5+5 = 10	**80.1**	neg.	0	0	0	0
	80.2							**80.2**	20	1	20	2	2
81	**81.1**	pT2b	pN0	pMX	R0	G2	3+2 = 5	**81.1**	< 50	?	49	3	6
	81.2							**81.2**	< 50	1	49	3	6
82	**82.2**	Min. pT2b pT3a	pN0	pMX			2+5 = 7 3+5 = 8	**82.2**	no tum.	no tum.	no tum.	no tum.	no tum.
83	**83.1**	pT2b	pN0	pMX	R1		2+3 = 5	**83.1**	100	2	200	4	8
	83.2		pN0	pMX	R1		3+5 = 8	**83.2**	100	2	200	4	8
84	**84.1**	pT1a	pNX	pMX			2+3 = 5	**84.1**	no tum.	no tum.	no tum.	no tum.	no tum.
	84.2							**84.2**	no tum	no tum.	no tum.	no tum.	no tum.
85	**85.1**	pT1b	pNX	pMX		G3	4+4 = 8	**85.1**	neg.	0	0	0	0
	85.2							**85.2**	neg.	0	0	0	0
86	**86.1**							**86.1**	NE	NE	NE	NE	NE
	86.2							**86.2**	NE	NE	NE	NE	NE
87	**87.1**	pT3a	pNX	pMX	R1		5+3 = 8	**87.1**	neg.	0	0	0	0
	87.2							**87.2**	neg.	0	0	0	0
	87.3							**87.3**	100	2	200	4	8

No	Tumour Sample areas	TNM				WHO-Grade	Gleason score	Sample number	% of the Caveolin-1 in prostate.tumour cells (Evaluation in %)	Intensity grade	Immunoscore 1 (method 1)	Distribution point	Immunoscore 2 (method 2)
		T	N	M	R								
88	**88.1**	Lymph node metastasis					3+4 = 7	**88.1**	100	1	100?	4	4
	88.2							**88.2**	25	1	25	2	2
89	**89.1**							**89.1**	100	2	200	4	8
	89.2							**89.2**	neg.	0	0	0	0
	89.3							**89.3**	> 80	1	81	4	8
	89.4							**89.4**	> 80	2	182	4	8
90	**90**	pT2b	pNX	pMX			5+3 = 8	**90**	neg.	0	0	0	0
91	**91.1**	pT3a	pN0	pMX		G3		**91.1**	100	1	100	4	4
92	**92**							**92**	neg.	0	0	0	0
93	**93**	pT2b Adeno-matose	pN0	pMX	R 0	G 2	3+4 = 7	**93**	no tum.	no tum.	no tum.	no tum.	no tum.
94	**94**	pT3a	pNX	pMX			3+2 = 5	**94**	100	1	100	4	4
95	**95**	pT2b	pN0	pMX	R0		3+3 = 6	**95**	30-50 (40)	1	40	2	2
96	**96**	pT2b	pN0	pMX			3+2 = 5	**96**	100	2	200	4	8
97	**97.1**	pT2b	pN0	pMX	R0		2+3 = 5	**97.1**	100	3	300	4	12
	97.2							**97.2**	100	3	300	4	12
98	**98**	pT3b	pN0	pMX	R0		3+4 = 7	**98**	100	2-3	200-300	4	12
99	**99.1**	pT3b	pN0	pMX			3+4 = 7	**99.1**	neg.	0	0	0	0
100	**100**	Lymph adenomatose						**100**	no tum.	no tum.	no tum.	no tum.	no tum.
101	**101**	pT2b	pN0	pMX			4+3 = 7	**101**	neg.	0	0	0	0
102	**102**	pT2a	pN0	pMX		G2		**102**	neg.	0	0	0	0
103	**103.1**	pT2b	pN0	pMX			3+4 = 7	**103.1**	50	2	100	3	6
	103.2							**103.2**	neg.	0	0	0	0
	103.4							**103.4**	> 80	1	81	4	4
104	**104**	pT3a	pNX	pMX			3+5 = 8	**104**	neg.	0	0	0	0
105	**105**	pT2c	pN0	pMX	R1	G2	-	**105**	> 50	1	51	3	3
106	**106**	pT3b	pN0	pMX	R0		3+4 = 7	**106**	neg.	0	0	0	0
107	**107.1**	pT3a	pN0	pMX	R1		3+4 = 7	**107.1**	no tum.	no tum.	no tum.	no tum.	no tum.
	107.2							**107.2**	no tum.	no tum.	no tum.	no tum.	no tum.
	107.3							**107.3**	no tum.	no tum.	no tum.	no tum.	no tum.
108	**108.2**	pT2b	pN0	pMX	R0	G2		**108.2**	neg.	0	0	0	0
109	**1090**	pT3c	pNX	pMX		G3		**109**	> 80	1	81	4	4
110	**110.1**							**110.1**	no tum.	no tum.	no tum.	no tum.	no tum.
	110.2							**110.2**		no tum.	no tum.	no tum.	no tum.
	110.3							**110.3**		no tum.	no tum.	no tum.	no tum.
111	**111**							**111**	no tum.	no tum.	no tum.	no tum.	no tum.

NE = means no evaluation, neg. = negative, and no tum. = no tumour. Distribution point 0 = all tumour cells were negative, 1 = 1-15% of the tumour cells were positive, 2 = 16-40% of the tumour cells were positive, 3 = 41-70% of the tumour cells were positive, and 4 = 71-100% of the tumour cells were positive. Tumour sample area = more than one sample section was analyzed from the same tumour.

The evaluation was done at first without attention to the intensity of expression (intensity grade) of Caveolin-1. This evaluation was done by two pathologists namely Dr. Engers (HHU – Düsseldorf) and Dr. Naommi (RWTH – Aachen). They are specialized in the pathology of prostate cancer. They determined the percentage of the tumour cells expressing Caveolin-1 to the total tumour cells present in each sample then the intensity grade of expression.

3.3.3. Results of the analysis of Semi-quantitative evaluation of Caveolin-1 expression in prostate carcinoma samples in group B

- In tumours from patients with stage pT1 (n = 4), three tumours were negative and one had less than 10% expression of Caveolin-1 in their tumour cells.
- In stage pT2 (n = 24), 11 tumour samples (8, 17, 34, 40, 43, 50, 54, 90, 101, 102, and 108) did not express Caveolin-1. The expression of Caveolin-1 in tumour cells was 10%, 40%, and 90% in tumour samples (72, 95, and 74, respectively). In tumour samples 6, 81, 105, 103, and 68 Caveolin-1 expression was < 25%, < 50%, > 50, > 80, and > 90% of the tumour cells, respectively. Tumour samples (67, 77, 83, 96, and 97) had expression of Caveolin-1 in all their tumour cells (i.e. 100%) (Table 3.12). The Caveolin-1 expression in the tumour samples of pT2 ranged between 10% and 100%.
- In stage pT3 (n = 44) a higher number of tumour cells showed Caveolin-1 expression than in stage pT2 (Table 3.12). No expression of Caveolin-1 was found in 9 tumour samples in stage pT3 which were tumour sample number 5, 9, 38, 39, 56, 64, 99, 104, and 106. Tumour samples number 4, 31, and 57 had Caveolin-1 expression in less than 5%. The expression of Caveolin-1 in tumour cells was 10% in tumour samples number 63 and 75. In tumour samples number 80, 36, and 24 the Caveolin-1 expression was 20%, 30% and 35 % of the tumour cells, respectively. The expression of Caveolin-1 was found in > 50% of the tumour cells in tumour samples number 27, 35, and 48 as well as in > 90% in tumour samples number 32 and 61. Caveolin-1 expression in tumour cells was 80% in three tumour samples, (1, 65, and 70). Caveolin-1 expression in tumour cells was > 80% in tumour samples number 2, 28, 53, and 109. The expression of Caveolin-1 in all the tumour cells (i.e. 100%) was found in 15 tumour samples. These tumour samples were 10, 11, 12, 25, 47, 49, 52, 62, 66, 69, 73, 87,91 , 94, and 98 in Table 3.12.
- In pT3 stage the Caveolin-1 expression in 0 to less than 20% of the tumour cells was found in 14 tumour samples, 20 to less than 50% in 3 tumour samples, and in more than 50% in three tumour sample. 80-100% expression of Caveolin-1 in tumour cells was in 24 tumour samples.
- Stage pT4 is advanced stage from pT3. Only 2 tumour samples showed stage pT4 and were added to stage pT3 in the statistical data analysis. In both samples of stage pT4 present in this group, Caveolin-1 expression was negative (see tumour sample numbers 3 and 29 in Table 3.12).
- Taking in consideration that the tumour samples expressing Caveolin-1 in less than 50% of their tumour cells were negative tumours, then the negative tumour samples expressing Caveolin-1 were 100%, 62.50% (45.83 < 10% and 16.67% < 50%), and 38.63% (27.27 < 10% and 11.36 < 50%) in stage pT1, pT2, and pT3, respectively (Table 3.12 above and Table 3.13 below).

- Otherwise, the positive expression of Caveolin-1 (i.e more than 50% of the tumour cells expressing Caveolin-1) was 0.0%, 37.50% (4.17% and 33.33%), and 61.37% (6.82% and 54.55%) in stage pT1, pT2, and pT3, respectively. This shows that more than 60% (nearly 64.53%) increase of Caveolin-1 expression from stage pT2 to pT3 (Table 3.13).

Plotting the result obtained after evaluation of the tumour samples and the percentages of Caveolin-1 expression in each pT stage separately showed that the Caveolin-1 expression shifts towards increased expression from stage pT1 to pT3/pT4 tumours as shown in Fig. 3.15.

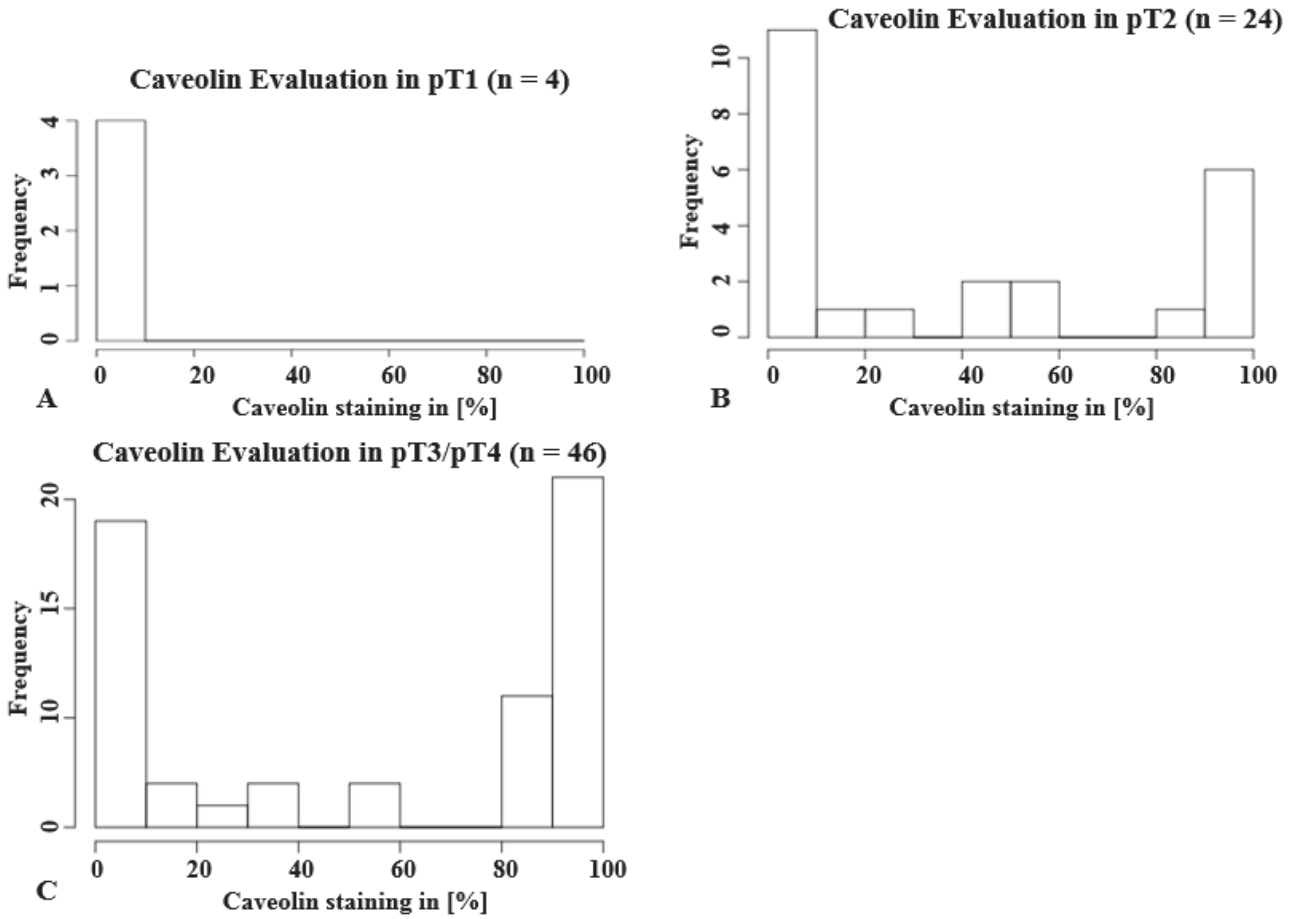


Fig. 3.15: Frequency distribution of Caveolin-1 expression in the tumours from patients with various TNM stages of prostate cancer. The histograms (A-C) show a shift towards increased expression of Caveolin-1 in higher tumour stages.

According to published studies done by Sinicrope F.A. et al.[101] and Joo H.J. et al.[102] concerning the percentage of Caveolin-1 protein expression after some modifications in tumour cells by immunohistochemistry, tumour samples were classified into four expression groups) These groups were negative (less than 10%), weak (10% - < 50%), medium (50% - 75%), and strong expression (> 75% expression) of Caveolin-1 as seen in Table 3.13. The table below shows also the percentage of positive Caveolin-1 cells in these four groups in each clinical TNM stage.

Table 3.13:- Classification of Caveolin-1 expression according to the % of expression in the tumour cells into negative, weak, moderate, and strong expression.

	The four groups of Caveolin-1 expression according to the % of expression in TNM stages			
TNM stages	**Negative (less than 10% positive) – (n)**	**Weak (10 - <50% positive) – (n)**	**Moderate (50-75% positive) – (n)**	**Strong (more than 75% positive) – (n)**
In Stage pT1 – N = 4	100.00% - (4)	0.0% - (0)	0.0% - (0)	0.0% - (0)
In Stage pT2 – N = 24	45.83% - (11)	16.67% - (4)	4.17% - (1)	33.33% - (8)
In Stage pT3 – N = 44	27.27% - (12)	11.36% - (5)	6.82% - (3)	54.55% - (24)
In Stage pT4 – N = 2	Low numbers of tumour samples for evaluation			

Where N means the total numbers in each stage and n means the numbers in subgroup of each stage.

In other evaluation process, the Caveolin-1 proportion of expression in the tumour cells in [%] was subdivided into five score categories: (0) 0-10%; (1) 11-25%; (2) 26-50%; (3) 51-75%; and (4) > 75% positive cells. This type of evaluation does not been inspected in details in this study.

Not only the tumour samples showed a variation in percentage of the tumour cells expressing Caveolin-1 between stage pT1, pT2, and pT3/pT4, but also within each stage. Additionally, the stained tumour cells showed different intensity of expression of Caveolin-1 within the same tumour. The staining intensity of Caveolin-1 expression was defined as follows: 0; no staining or negative; 1, weak; 2, moderate median; 3, strong expression. This intensity of expression increased with the increase of the percentage of tumour cells expressing Caveolin-1 (see Fig. 3.15-3.16 and Table 3.12).

Table 3.12 includes data about the tissue type, clinical pathology, and the evaluated areas of each tumour sample. At least one tumour area was evaluated from each tumour sample. Their percentage of Caveolin-1 expression (% of tumour cells stained) and intensity grade of each are listed in the table. Some tumour samples showed different intensity grade in different areas of the same tumour. In some other tumour samples the same tumour area showed different intensity grade. This demonstrated the heterogeneity character of prostate cancer not only in the gene aneuoploidy studies but also in the expression of specific proteins.

A semi-quantification of Caveolin-1 expression (immunoscore) was performed by evaluation of both, the percentage of the expression (0-100%) and degree of staining intensity (grade 0-3) parameters. For the calculation of this immunoscore, two methods are described in the literature. Method 1 (immunoscore 1) is obtained by multiplying the staining intensity grade (grade 0, 1, 2, and 3) with the percentage of positivity (Evaluation in [%]), thereby giving immunoscores ranging from 0 to 300 (Tan et al. 1953). According to this paper the tumour samples were then categorized into four groups showing negative or low (0-50), moderate (51-100), high (101-200),

and very high (201-300) expression. With this method a better semi-quantitative evaluation of Caveolin-1 expression can be performed, due to the large immunoscore range (Table 3.14).

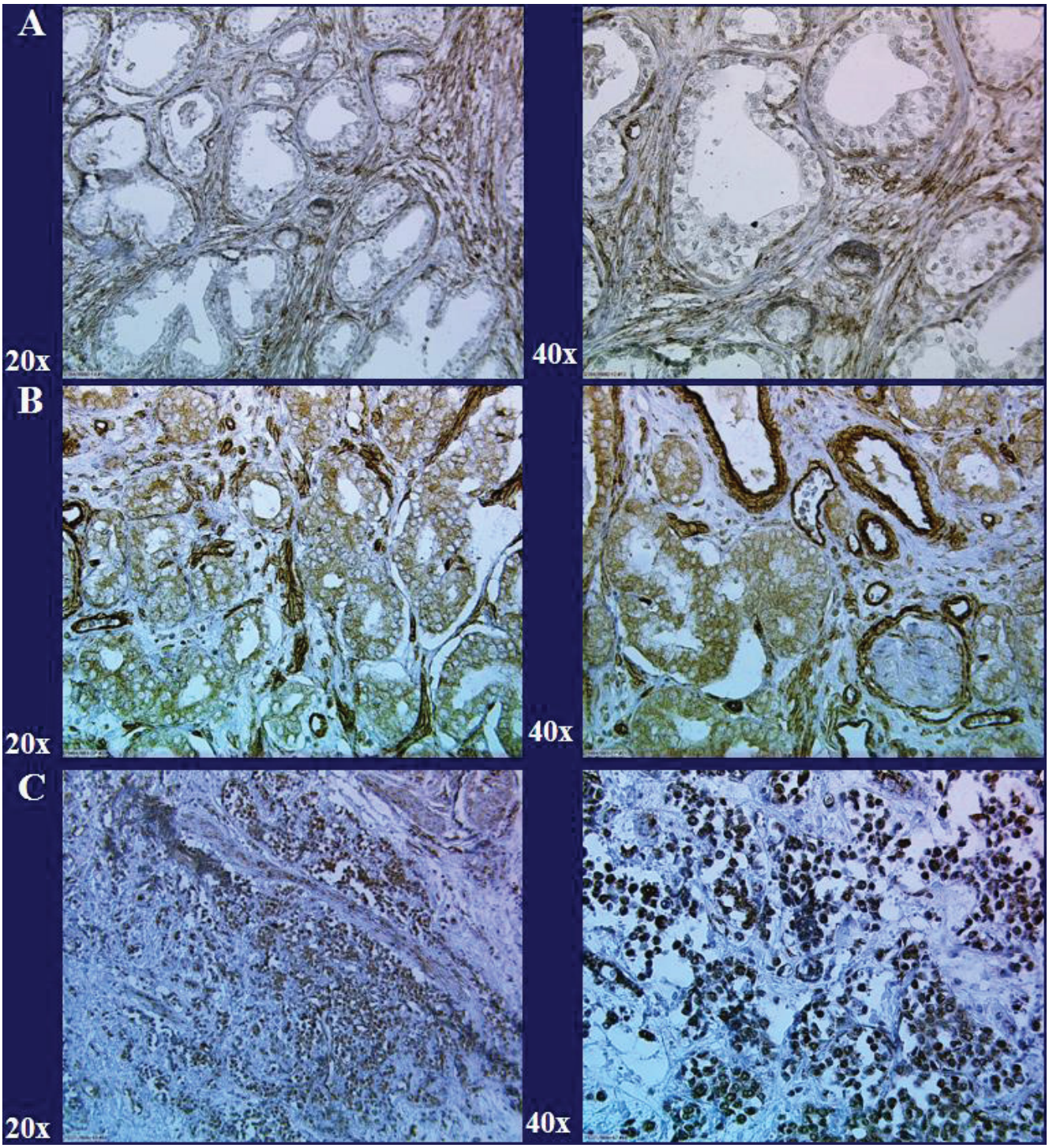


Fig. 3.16: Representative examples of Caveolin-1 Expression in group B tumours. A) 10% tumour cells expressing Caveolin-1. **B)** 50% tumour cells with weak Caveolin-1 expression (score 1) and 50% with moderate Caveolin-1 expression (score 2) in the same tumour but in different acini. **C)** Tumour cells with strong Caveolin-1 expression (score 3) were mostly nuclear. Note that the basal cells, stroma cells (fibroblasts), and blood vessels were stained with Caveolin-1 and were used as a control in each tumour sample.

Table 3.14:- The four different subgroups of Caveolin-1 expressing tumours after semi-quantification of expression (Evaluation in [%] x staining intensity = immunoscore 1) in different stages.

Semiquantitave subgroups of Caveolin-1 expression	% of the tumour sample from Stage pT1 - (n)	% of the tumour sample from Stage pT2 - (n)	% of the tumour sample from Stage pT3 - (n)
Negative or low (0-50%)	100% - (n = 4)	62.50% - (n = 15)	38.64% - (n = 17)
Moderate (51-100%)	0.0	16.67 % - (n =4)	36.36% - (n = 16)
High (101-200%)	0.0	16.67% - (n = 4)	15.91% - (n = 7)
Very high (201-300%)	0.0	4.17% - (n = 1)	9.10% - (n = 4)
Total	100% - (N = 4)	100% - (N = 24)	100% - (N = 44)

Where N means the total numbers in each stage and n means the numbers in subgroup of each stage.

The second method (immunoscore 2) follows the principle of Remmele. The intensity of Caveolin-1 was measured on four intensity grades, 0 being negative, 1 weak, 2 moderate, and 3 being strong intracellular immunoreactivity.

The distribution in percentage (Evaluation in [%]) was measured subjectively on a 4 reactive point scale as follows: 1-15% of the positive cells being 1, 16-40% being 2, 41-75% being 3 and greater than 75% of the Caveolin-1 positive cells being 4. The result of the intensity point and reactivity point were then multiplied to get an immunosocore for each assay with a range of 0-12.

3.3.4. Statistical analysis of Caveolin-1 expression in prostate carcinoma samples (group B)

A summary of the tumour samples including clinical pathology data (TNM stages, WHO grades, and Gleason score), expression of Caveolin-1 in percentage (evaluation in [%]), intensity of expression, distribution point (reactive points), and immunoscore 1 (according to Tan et al.) and 2 (according to Remmele et al.) is presented in Table 3.12. As there were only 2 tumour samples of stage pT4, these were added to the stage pT3 in the statistical analysis study. A correlation between Caveolin-1 expression and clinical TNM stages and WHO grades in prostate cancer using Kendall's rank correlation test was performed. Kendall rank correlation coefficient, which is commonly referred to as Kendall's tau (τ) coefficient test, is used to measure the association between two ordinal quantities. The level of significance in Kendall's test was set at p-value < 0.05.

The evaluation in [%] of Caveolin-1 expression was significantly positively correlated with TNM stages ($P = 0.018$), but there is no significant difference between expression (Evaluation in [%]) and WHO grades ($P = 0.561$) (Fig. 3.17). Although, there was no significant correlation between the percentage of the tumour cells expressing Caveolin-1 (Evaluation in [%]) and WHO grades (Grade 1, Grade 2, and Grade 3), the barplot showed a tendency of the Caveolin-1 expression to increase from grade 1 to grade 3.

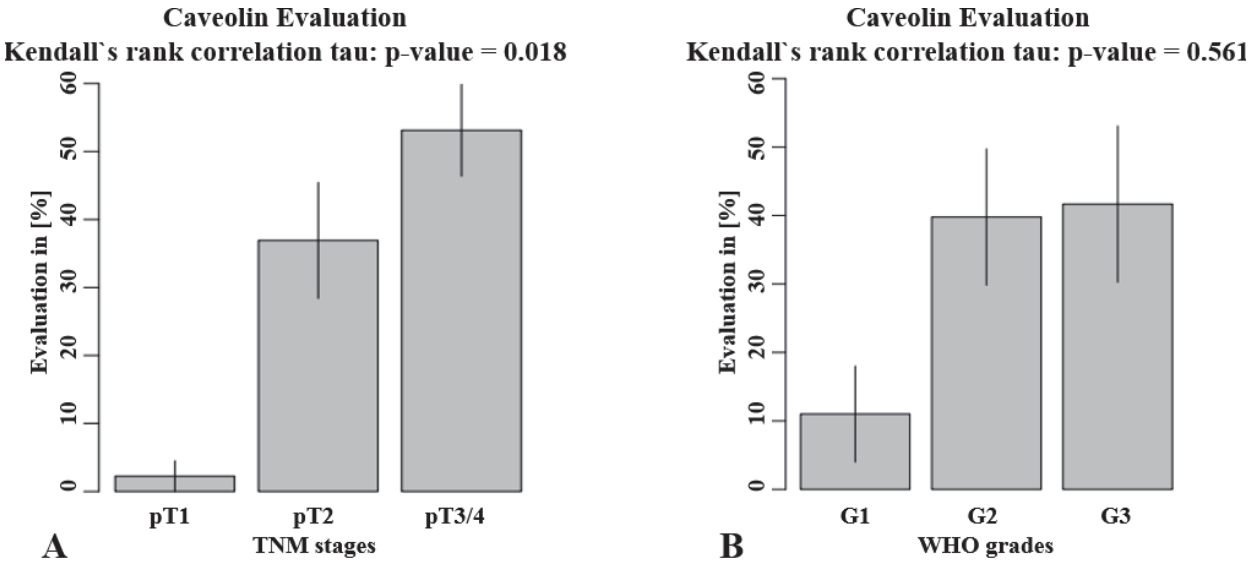


Fig. 3.17: Correlation between percent of Caveoli-1 expression (Evaluation in [%]) and TNM stages as well as WHO grades in prostate cancer. A) The correlation between percent of Caveolin-1 expression and TNM stages is positively correlated and it shows a significant P-value. **B)** The correlation between Caveolin-1 expression in percentage and WHO is not significant, probably due to the large standard deviation.

The correlation between the intensity grade of Caveolin-1 expression and TNM stages did not reach a statistical significance value (P = 0.0619) as seen in Fig. 3.18. Nevertheless, an increase of Caveolin-1 expression with the TNM stages could be observed. There was no correlation between the intensity grade of Caveolin-1 expression and WHO grades in prostate cancer (P = 0.503).

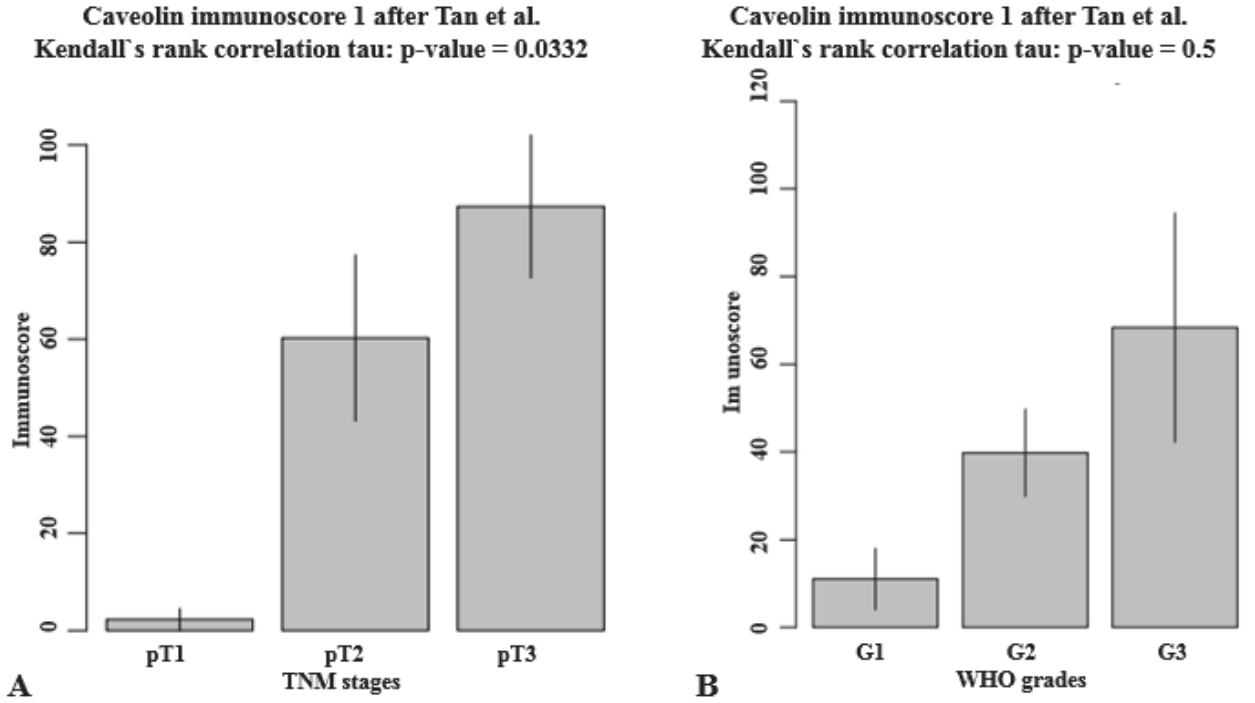


Fig. 3.18: Correlation between the intensity of Caveolin-1 expression and TNM stage as well as WHO grades in prostate cancer. A) The correlation between the intensity grade of Caveolin-1 expression (intensity grades are 0 - 3) and TNM stages is not statistically significant, but the barplot shows a gradual increase in the intensity of expression in higher stages of the tumours. **B)** There is no significant correlation between the intensity of Caveolin-1 expression and WHO grades.

Caveolin-1 expression was semi-quantified with immunoscore 1 and 2 as mentioned previously. Thereafter, the correlations between immunoscore 1 and TNM stages or immunoscore 2 and TNM stages as well as a correlation between WHO grades and immunoscore 1 or 2 were studied separately.

Caveolin-1 expression using immunoscore 1 was significantly correlated with TNM stages (P = 0.0332) using Kendall`s tau test. On the other hand, there was no significant correlation between Caveolin-1 expression and WHO grades (P = 0.5). The bar plot in Fig. 3.19 showed a gradual increase of the immunoscore 1 with WHO grades suggesting a tendency of increasing expression in higher grades.

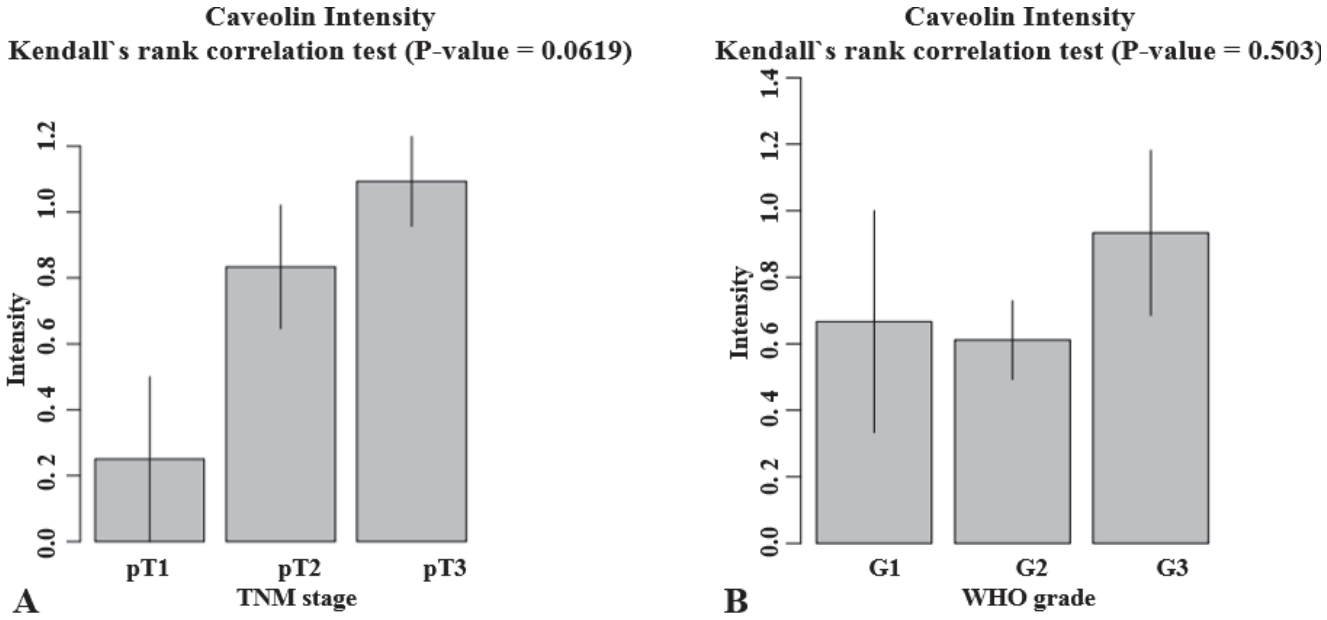


Fig. 3.19: Correlation between Caveolin immunoscore 1 and TNM stage as well as WHO grade in prostate cancer. A) Caveolin immunoscore 1 is highly correlated with TNM stages having a significant P-value. **B)** This correlation was not significant between Caveolin immunoscore 1 and WHO grades, but the barplot shows the tendency of increased expression with the higher tumour grades.

Both significant differences (P = 0.018 and 0.0332) obtained from the statistical analysis study of the correlation between percentage of Caveolin-1 expression (Evaluation in [100%]) and semi-quantitative expression of Caveolin-1 (immunoscore 1) versus the TNM stages respectively, supported the close correlation between Caveolin-1 expression and clinical TNM stages of the disease development. This correlation was not significant for the WHO grade, but a tendency of increase of Caveolin-1 expression was observed. The barplots in Fig. 3.19-3.20 showed a tendency of the increase of Caveolin-1 expression in grade 3.

There was no significant difference between immunoscore 2 of Caveolin-1 expression and

TNM stages as well as WHO grades (Fig. 3.20). The P-values were 0.132 and 0.427 in TNM stages and WHO grades respectively. Although there was no significant correlation here (both P-values were not significant), the barplot showed a gradual increase of Caveolin immunoscore 2 and TNM stages and in a less extend with WHO grades.

Due to its larger scale, the immunoscore in method 1 gives a more detailed value than the immunoscore in method 2 (scale of 0-300 versus 0-12). As an example, a tumour sample expressed Caveolin-1 in half of tumour cells (i.e. 50% expression of Caveolin-1) and had an intensity grade 1 resulting an immunoscore value of 50 (50 x 1 = 50) in immunoscore method 1. This means one sixth of the maximum value of immunoscore 1 (scale 0-300). The same tumour sample in evaluated with immunoscore method 2: 50% of their tumour cells were positive equivalent to reactive point 3 and had intensity grade 1 resulting in an immunoscore value of 3 (3 x 1 = 3). This means that the immunoscore value here is one fourth of the 0-12 scale.

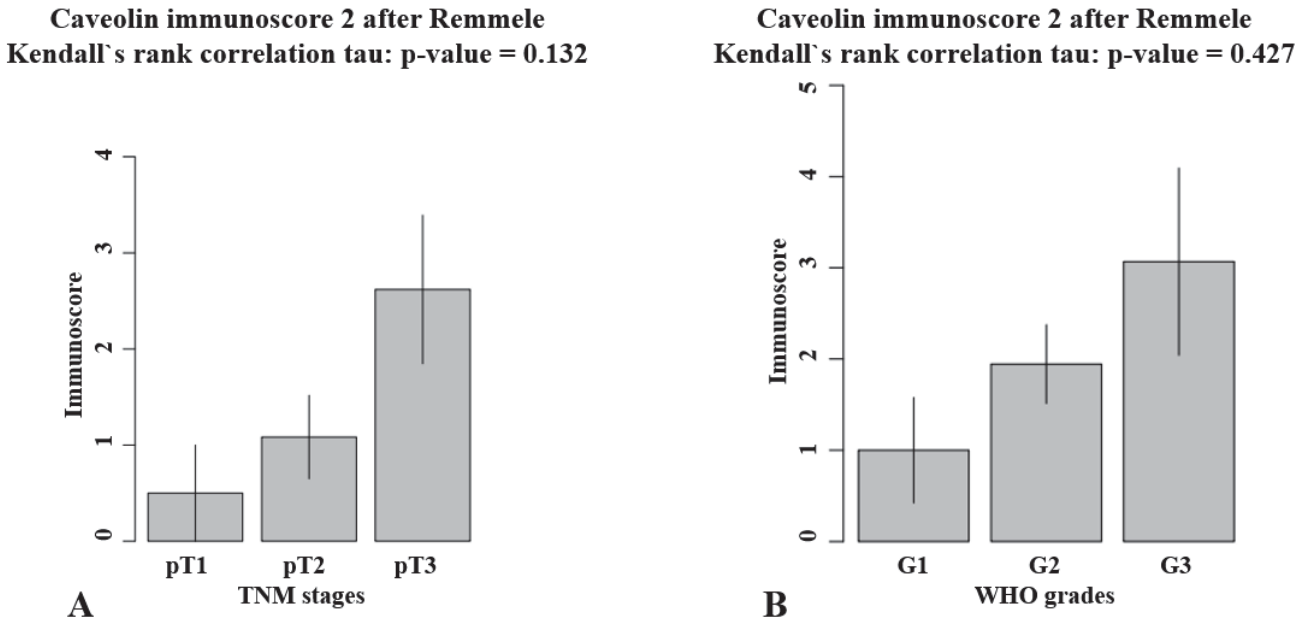


Fig. 3.20: Correlation between Caveolin immunoscore 2 and TNM stages as well as WHO grades in prostate cancer. A) Although the correlation between Caveolin immunoscore 2 and TNM stages is not significant, the parplot shows a higher tendency of increased expression with TNM stages. **B)** The correlation between Caveolin immunoscore 2 and WHO grade is not significant also, but the barplot shows a lower tendency of increased expression with WHO grades.

Using immunoscore 1 method the statistical analyses showed a significant correlation between the Caveolin-1 expression and TNM stages. Furthermore, a tendency of increase in Caveolin-1 expression was shown with WHO grades, but without reaching statistical significance. The results obtained from the histochemistry study of Caveolin-1 expression and data analyses in the different groups of TNM stages and WHO grades can be summarized in the following conclusions.

1- Basal cells were Caveolin-1 positive in all tumour samples if they were not replaced or destroyed by tumour cells. The hallmark of early malignancy of the prostate is the absence of basal cells and the loss of a basement membrane. The normal secretory cells were Caveolin-1 negative or very weak and present in a scattered pattern.

2- Tumour cells expressed Caveolin-1, but not in all tumour samples. Additionally, they expressed Caveolin-1 some times in one area and not in another of the same tumour sample. Moreover, they expressed Caveolin-1 in different intensities in the same tumour area.

3- Tumour samples expressing Caveolin-1 in less than 50% of their cells always showed weak expression. However, tumour samples with more than 50% Caveolin-1 expression in their cells were weak, moderate, or strong in expression.

4- The number of the tumour cells expressing Caveolin-1 increased from pT1 through pT2 and pT3 gradually. The increase in Caveolin-1 positive cells in the tumour samples was correlated with a higher level of expression (intensity) as seen by strong intensity in immunostaining.

5- A larger tumour area is combined with more cells expressing Caveolin-1, intensity of expression and advanced clinical stage and metastasis. Moderate and strong expressions of Caveolin-1 are clear in pT3.

6- About 35% of the studied tumour samples with high malignant grades or stages showed no expression of Caveolin-1, possibly due to the microenvironment as mentioned by Mc Keenzie S. et al.[103]. On the other hand 65% of the tumour samples with high malignant grades were positive for Caveolin-1 expression. Consequentially, Caveolin-1 expression in the tumour sample is correlated with a poor prognosis in the disease development or stage.

7- Evaluation in percentage showed a significant correlation between Caveolin-1 expression and TNM stages of prostate cancer ($P = 0.018$ according to kendall`s tau test). Although, there was no correlation between Caveolin-1 expression (Evaluation in [%]) and WHO grades, a tendency of the increase of the expression of Caveolin-1 and higher WHO grades was observed.

8- The correlation between the intensity grade of Caveolin-1 expression and TNM stages was not significant ($P = 0.0619$). But, there was a gradual increase in the intensity of expression in higher stages of the tumours (according to kendall`s tau test). There was no correlation between the intensity grade of Caveolin-1 expression and WHO grades.

9- There was a significant correlation (according to Kendall´s tau test) between immunoscore 1 of Caveolin-1 expression in the semi-quantitative analysis and TNM stage ($P = 0.0332$). There was no correlation between WHO grades and immunoscore 1 ($P = 0.5$), but the tendency of the increase of the expression was noticed.

10- There was no correlation between Caveolin immunoscore 2 and TNM stages of prostate cancer

as well as WHO grades (P = 0.132 and 0.427, respectively). Nevertheless the tendency of the increase of the immunoscore 2 was clearly seen in both TNM stages and WHO grades (Fig. 3.20). The obtained statistical results supported the correlation of Caveolin-1 expression and the clinical pathology of the prostate cancer.

3.3.5. FISH analysis of 7q31.1 and Caveolin-1 expression

The analysis of both sets of data resulting from the FISH study and the immunohistochemistry of the corresponding tumour samples can answer the question below.

3.3.5.1. Does 7q31 gain correlates with Caveolin-1 over expression?

To answer this question, the data from previous FISH and immunoreactivity studies of Caveolin-1 expression of the same tumour samples (sections) coming from the same block or area were analysed. Gain of 7q31 was clear in both FISH in paraffin section and FISH in isolated cell nuclei. Not only gain was observed but also amplification of 7q31 was observed in many tumour samples as been seen in tumour samples number 3, 7, and 8 (Table 3.15).

In contrast, Caveolin-1 expression showed variations not only between tumour samples but also in different areas of the same tumour sample. Tumour samples number 3, 5 and 8 showed a clear gain of 7q 31. Taking in consideration that Caveolin-1 expression of 5% in tumour sample 8 was also evaluated as negative according to the published studies (Table 3.15), then Caveolin-1 expression was negative in the above three samples.

Tumour sample 6 consisted of two different tissue areas (area 1 and area.2). Tissue area number 1 -Paraffin showed a slight gain of 7q31 as shown by FISH analysis, but area 2 -Paraffin did not. Caveolin-1 was moderately expressed in almost 100% of the tumour cells of sample 6.1 -Paraffin but was negative in 6.area 2 -Paraffin. Tumour sample 8 showed a clear gain of 7q31, but expressed Caveolin-1 in only 5% of their cells weak.

Table 3.15:- Clinical pathology, immunohistochemical study of Caveolin-1 expression, and specific chromosomal changes especially 7q31 in tumour samples of prostate.

Tumour sample No.	Clinical pathology	Caveolin-1 expression	7q31.1 in FISH		8p12-22 in FISH		8q22-24 in FISH	
			Change	%	Change	%	Change	%
1	pT1b; GS = 6	Negative	Gain	17.0 Tr.-5.6 Te.	Loss	9.9	Not studied	
2	Not available	tumour**	Gain	15.4 Tr.-6.8 Te.	Low level loss	2.3	Not studied	
3	pT1a; GS = 6	Negative	Gain	35.8 Tr.-19.4 Te.	Gain	19.3 Tr.-8.7 Te.	Not studied	
4	Not available	tumour**	Gain	20.5 Tr.-2.5 Te.	Gain	13.4 Tr.-3.6 Te.	Not studied	
5	pT3b,pNX,pMX,R0; GS 3+5 = 8	Negative	Gain	23.5 Tr.-3.4 Te.	Loss	12.4	Not studied	
6 area 1-Paraffin	pT3a,pNX,pMX, R1; G3; GS 5+3=8	100% positive with median expression	Gain	11.7 Tr.-2.5 Te.	Loss	8.7	Not studied	
6 area 2–Paraffin stroma		Negative	Normal		Normal		Not studied	
7 area 1-Paraffin	PT3a,pN0, pMX; - GS 2+3 = 5	100% positive with weak expression	Gain	29.1 Tr.-13.9 Te.	Gain	18.2 Tr.-12.0 Te.		
7 area 1-Cell nuclei (m+b)			Gain	11.1 Tr.-36.9 Te.	Gain	10.1 Tr.-20.9 Te.		
7 area 1-Cell nuclei (s+m+b)			Gain	9.9 Tr.-32.9 Te.	Gain	9.6 Tr.-18.5 Te.		
7 area 2-Cell nuclei (s+m+b)		100% positive with weak expression	Gain	10.6 Tr.-33.1 Te.	Gain	9.7 Tr.-18.8 Te.		
7 area 1-Cell nuclei (m+b)					Gain	19.4 Tr.-16.9 Te.	Gain	20.3 Tr.-23.7 Te.
7 area 1-Cell nuclei (s+m+b)					Gain	16.6 Tr.-13.9 Te.	Gain	17.6 Tr.-19.7 Te.
8-Paraffin	PT3a,pNO,pMX; - GS 3+5 = 8	5% positive with weak expression	Gain	17.1 Tr.-2.7 Te.	Loss	10.3		
8-Cell nuclei (m+b)			Gain	16.4 Tr - 33.3Te.	No loss			
8-Cell nuclei (s+m+b)			Gain	10.2 Tr.-20.3 Te.	No loss			
8-Cell nuclei (m+b)					No loss		Gain	25.5 Tr.-23.7 Te.
9 area 2-Paraffin	pT3b,pNX,pMX; - G3; R1-GS 5+3 = 8	Negative	Gain	9.9 Tr.-0.3 Te.	Loss	13.3	Not studied	
9 area 1- Cell nuclei (m+b)		90% positive with weak expression	Gain-	13.5 Tr.-6.6 Te.	Loss	23.8	Not studied	
9 area 1- Cell nuclei (s+m+b)			Gain	12.7 Tr.-6.1 Te.	Loss	23.9	Not studied	
10-Normal prostate		Negative or very weakly positive	Normal		Normal		Not studied	

Shown are the results of the FISH data and immunohistochemistry study of Caveolin-1 expression in the same tumour samples of prostate cancer. In the evaluation of paraffin sections, the background for 1 signal was 20% for both 7q31 and 8p12-22. Therefore, more than 20% for 1 signal would be considered as loss. The background for 3 signals (Trisomy) and 4 signals (Tetrasomy) were 6.0% and 0.5% in case of 7q31. In case of 8p12-22, the backgrounds were 2.5% and 1.0% for 3 signals and 4 signals, respectively. The percentage above the background would be considered as gain. In the evaluation of isolated cell nuclei**,** the background for 1 signal was 5.6% and 3.7% for 7q31 and 8p12-22, respectively. Then, more than 1 signal of background would be considered as loss. The background of FISH in isolated cell nuclei for 3 signals (Trisomy) and 4 signals (Tetrasomy) were 8.2% and 5.3% in case of 7q31. In case of 8p12-22 and 8q23, the background of FISH in isolated cell nuclei for 3 signals (Trisomy) and 4 signals (Tetrasomy) were 6.0% and 4.3%**,** respectively. Consequently, the percentage above the background would be considered as gain (b = big, m = medium, s = small, Tr. = Trisomy, Te. = Tetrasomy, and ** = unclear differentiated tumour).

Tumour sample 7 represented in two different tissue areas namely 7 areas 1 and.2 showed clear gains of 7q31 and weak expression of Caveolin-1 in 100% of the tumour cells in both areas. The tumour sample 9.2 showed gain of 7q31 (up to 9.9%) and Caveolin-1 expression was negative. The other tissue area of the same tumour sample 9 (area.1) showed clean gain of 7q31 (up to 13.5% and 6.6% in m + b combination as trisomy and tetrasomy respectively) and 90% of the tumour cells expressed Caveolin-1.

FISH- and immunohistochemistry- analyses of the Caveolin-1 gene and its products in the previous tumours (9 samples) gave no concrete indication of a correlation between 7q31 gain (i.e. Caveolin-1 gene amplification) and expression of Caveolin-1. However it should be kept in mind that no identical cells in the following sections could be studied and due to the heterogeneity of the tumours this could explain the discrepancy. This was the main reason to extend the scale of Caveolin-1 expression in more tumour samples to clarify whether Caveolin-1 is a putative oncogene in the case of prostate cancer.

3.4. YB-1 Expression in prostate carcinoma

3.4.1. Immunohistochemistry study of YB-1

During this study, YB-1 was identified as a biomarker for progression in breast cancer. Because of a similarity of hormone dependent tumours we explored whether YB-1 is also a useful biomarker in prostate cancer. Polyclonal specific antibodies to YB-1 were available in cooperation with Dr. Royer. The tumour samples were examined with immunohistochemical staining in order to find out whether the YB-1 protein expression could be considered as a biomarker in prostate carcinoma diagnosis. The clinical pathology of the tumour samples were analyzed in the department of pathology of HHU Düsseldorf. Here, the clinical and pathology data of all tumour samples were known.

YB-1 is not a retrieval antigen in prostate cancer and can be studied consequently without extra treatment. A dilution of 1:150 and incubation time of 2 hours of YB-1 antibody were suitable in this study. The application of 200 µl of the YB-1 antibody was enough to cover different sizes of the tissue sections. Moreover, the incubation time of the secondary antibody was increased from 15 to 20 minutes to incresase efficiency of the combination with primary antibody (see chapter 2.2.14.3).

3.4.2. Clinical pathology of the tumour samples

Tumour samples were classified according to clinical TNM stage system and the histological WHO grading system or Gleason score. In some tumour samples near the tumour areas, prostate intraepithelial neoplasia (low and high PIN grade) and prostatitis were observed which were frequently present in tumour samples.The pathology record of each sample included at least two systems (for example TNM stage and WHO grade or Gleason score). The three systems were described previously in details (see chapter 1.3). The clinical staging of this group ranged from pT2 to pT4 and WHO grading from G1 to G3. Most of the samples had TNM stage and WHO grading system (n = 51) in the pathology record. Only one sample had TNM stage and Gleason score (Table 3.16). It is important to retain the clinical as well as the pathological classification in the medical record. Table 3.17 shows the numbers of the samples in different stages.

3.4.3. Evaluation of YB-1 expression

The evaluation of the immunohistochemistry of YB-1 was done in the Institute of Pathology, Heinrich Heine University, Düsseldorf, by Dr. Engers. YB-1 immunoscore of expression was classified on a scale of 0 (= no expression) to 12 (highest expression) and evaluated

separately for normal secretory, tumour and prostate intraneoplasia cells (PIN cells). The immunoscore is a result of the multiplication of 4 reactive points representing the percentage of the cells expressing YB-1 (either normal secretory, PIN, or tumour cells) with the intensity grade of 0-3. Reactive point scale 1, 2, 3, and 4 means that the percentage of stained tumour cells was 1-15%, 16-40, 41-75, and greater than 75%, respectively. Intensity grade 0, 1, 2, and 3 means negative, weak, medium, and strong expression respectively. Through the multiplication of reactive point and intensity grade the immunoscore (immunoscre after Remmele) was obtained. The highest score of 12 was measured in one tumour sample (sample no. 20) (see Fig. 3.21 and Table 3.16). YB-1 expression is mostly localized in the cytoplasm.[104-105] The three cell types normal secretory, tumour, and PIN cells expressed YB-1 more or less differently from each other in the same tumour sample. After separate evaluation of the immunoscore in each cell type in each tumour sample, the immunoscore of the normal glandular cells was subtracted from that of the tumour cells and the immunoscore of the tumour cells was divided by that of normal cells (the quotient of immunoscore in tumour to normal cells) (see Table 3.18 in Appendix). The immunoscore could not be determined in three tumour samples under study, reducing the total number of samples under study to 49. Seven tumour samples showed prostate intraneoplasia (PIN) beside tumour foci and normal acini. The immunoscore in PIN cells was measured separately. These immunohistochemistry results were subjected to statistical analysis to investigate the existence of any correlation between YB-1 expression, tumour stage TNM and malignancy grade (WHO grades).

Malignancy grade 1 (G1) contained seven tumour samples and they were classified according to TNM stages as follow: Four tumour samples were stage pT2b; one tumour sample each was stage pT2c, pT3a and pT3c (see Table 3.19 in Appendix). Malignancy grade G1 to beginning G1-G2 contained five tumour samples, where three tumours were stage pT2a and one sample each was stage pT2b and pT3a. Eighteen tumour samples represented malignancy grade G2 and classified according to TNM stages as follows: Two tumours samples were stage pT2a, five pT2b, four stage pT2c, five stage pT3a, and only one sample each was stage pT3b and pT4a, respectively. Malignancy grade G2 beginning to G3 (G2-G3) contained five tumour samples. They were classified according to TNM stages as follows: Three tumour samples were stage pT3a and two tumour sample was stage pT2b. The high malignancy grade G3 was represented in 13 tumour samples which were subdivided according to TNM stage as follows: Eight samples were stage pT3a, two were stage pT2b, two were stage pT3b, and one was stage pT2c (see Table 3.16 and Table 3.18-3.19 in Appendix).

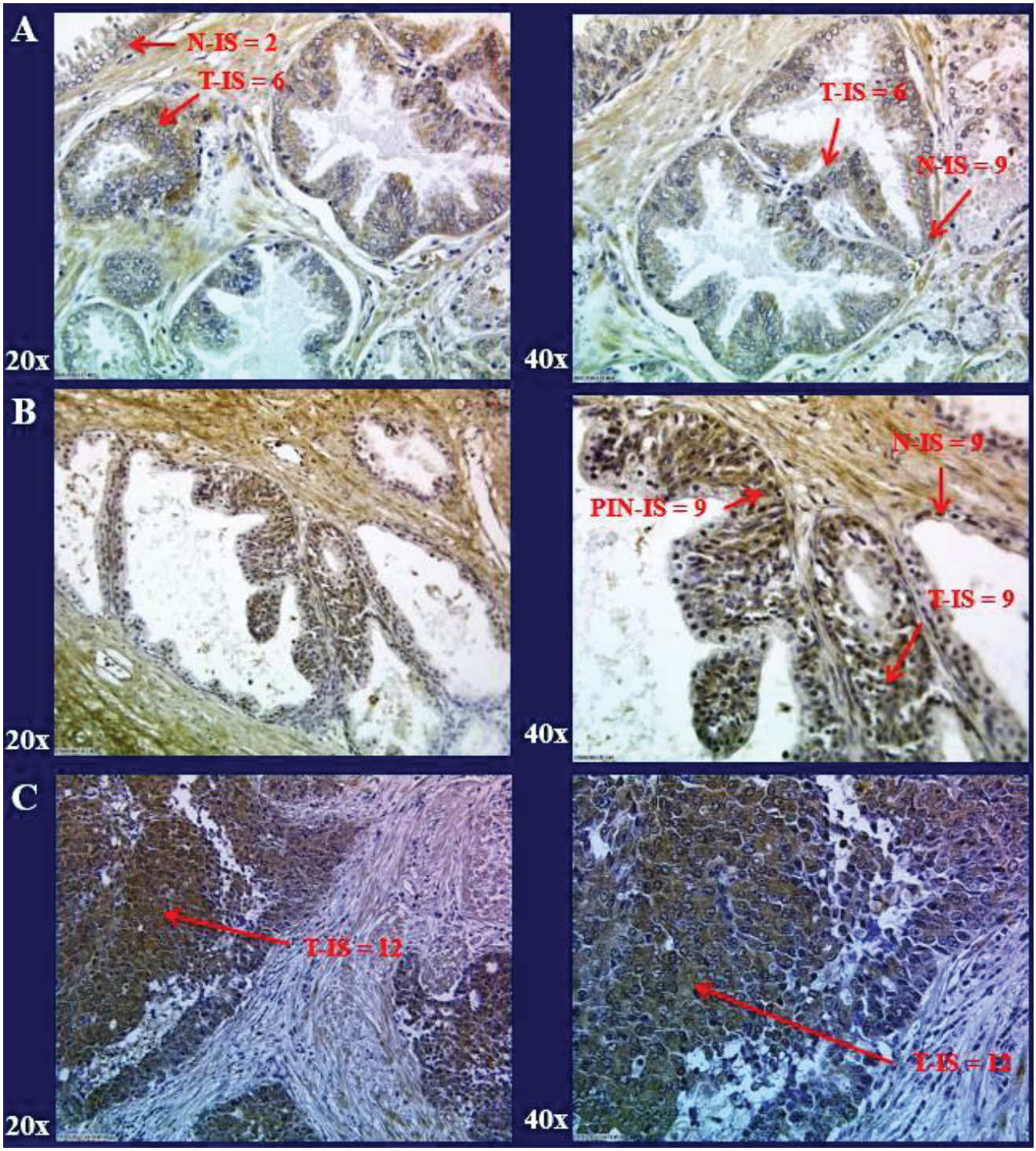


Fig. 3.21: Differences of YB-1 expression in normal, PIN, and tumour cells of prostate cancer. A) Patient 1 shows normal cells and tumour cells with immunoscore of 2 and 6 respectively. **B)** Patient 2 shows normal cells, prostate intraneoplasia cells, and tumour cells with the same immunoscore of 9. **C)** Patient 3 shows immunoscore of 12 in all tumour cells. (N = normal secretory cells, PIN = prostate intraneoplasia cells, T = tumour cells, and IS = immunoscore of expression).

To identify a possible correlation between YB-1 expression (immunoscore) and the clinical staging and histopathology grading (WHO grading) of 52 tumour samples, these were further divided according to the presence or absence of lymph nodes and distant metastasis. Also,

they were further classified according to TNM stages system and its sub-classes (pTa-c). This can show whether a correlation between YB-1 immunoscore and disease development is represented in a certain stage of the disease (pT), degree of lymph node involvement (pN), and the extent of distant metastases (pM) (Table 3.16-3.17). 25 samples belonged to stage pT2. This means that the tumour was restricted to the prostate, five samples were stage pT2a (tumour comprised half or less of one prostate lobe), 14 samples were stage pT2b (tumour involves more than one-half of 1 lobe but not both lobes) and six samples were stage pT2c (tumour involves both prostate lobes).

Table 3.16:- Clinical Pathology of the prostate cancer tumours (TNM stage (pT)), WHO grading (G), and Gleason score (GS) and YB-1 immunoscore of expression.

No. of sample	TNM stage, WHO grade, and Gleason score	YB-1 normal	YB-1 Tumour	YB-1 PIN	No. of sample	TNM stage, WHO grade, and Gleason score	YB-1 normal	YB-1 Tumour	YB-1 PIN
1	pT3a; G2-G3	2	4		**27**	pT2b, pNX, pMX; G3	1	6	
2	pT2b; G2	3	3	6	**28**	pT3a, pNX, pMX; G2a	0	4	
3	pT3a; G1-G2	2	1		**29**	pT3a; PNX, pMX, R0; G3	4	4	
4	pT2b; G1-G2	2	1		**30**	pT2b, pNX, pMX; G2	2	6	4
5	pT3a; G2-G3	0	2		**31**	pT4a, pN0, pMX; G2	1	2	
6	pT2a; G1-G2	0	6		**32**	pT3a, pN0, pMX; G2			
7	pT3a; G2-G3	2	1		**33**	pT3c, pNX, pMX; G1	2	3	
8	pT3a; G2	0	0		**34**	pT2c, pNX, pMX; G1	2	3	
9	pT3a; G2-G3	1	0		**35**	pT2c, pNX, pMX; G2.	2	6	
10	pT2a, pN0, pMX, R0; G1-G2	2	2		**36**	pT3a, pNX, pMX; G3	1	2	
11	pT2b, pNX, pMX; G2-G3	2	6		**37**	pT2c, pN0, pMX; G3	1	1	
12	pT2b, pN0, pMX; G1	2	4		**38**	pT3a, pN0, pMX, G3	0	0	
13	pT2a; G1-G2	2	2		**39**	pT2c, pNX, pMX; G2	1	2	6
14	pT3b, pNX, pMX, R0; G3	0	0		**40**	pT2c, pNX, pMX; G2	1	6	
15	pT2b, pNX, pMX; R0; G1	3	2		**41**	pT3a, pN0, pMX, R0; G3	4	6	
16	pT2b, pNX, pMX; G1	2	2		**42**	pT2a, pN2, pMX, R0; G2	2	3	
17	pT3a; G2	3	0		**43**	pT2c, pN0, pMX; G2	0	0	
18	pT3a, pN0, pMX, G1	2	6	9	**44**	pT3c; pNX, pMX, R0; G3			
19	pT2b, pN0, pMX; G3q (after Helpap)	0	2		**45**	pT3a; pNX, pMX; G2	3	4	4
20	pT3a, pNX, MX; G3	1	12		**46**	pT3b, pNX, pMX; G3	6	8	
21	pT3a, N1, pMX; Gb2	1	6		**47**	pT2b, pNX, pMX, R0; G1	2	4	
22	pT3, pN0, pMX; (IIb)				**48**	pT2b, pN0, pMX; GS: 7 (3+4)	8	8	8
23	pT3a, pN0, pMX; G3a	2	6		**49**	pT2a, pN0, pMX, R0; G2 = GS: 2+3 = 5	1	1	
24	pT3a, pN0, pMX (malignancy grade IIIa)	2	4		**50**	pT2b, pNX, pMX, R0; G2; (GS: 3+2 = 5)	2	4	
25	pT3a, pN0, pMX; G3	3	3		**51**	pT2b, pN0, pMX; G2; GS: 8 (3+5)	2	4	9
26	pT3b, pN0, pMX; G2b	1	6		**52**	pT2b, pN0, pMX, R0; G2 = GS 3+ 2 = 5	2	3	

TNM, WHO grading (G), Gleason score (GS), and the statistical analyses of YB-1 immunoscore of group C.

In 26 of the tumour samples, the cancer cells had spread out the prostate capsule, representing stage pT3. In 21 of these, cancer cells had spread out of the capsule in only one or both sides of the prostate (pT3a). Three tumour samples had infiltrated the seminal vesicle (stage pT3b) and two had infiltrated through the seminal vesicle on both sides and into the inguinal lymph nodes (stage pT3c).

Only one tumour sample showed stage pT4, indicating that the tumour infiltrated into neighboring structures (organs) other than the seminal vesicle such as the bladder neck, external sphincter muscle, rectum, and/or levator muscles and/or was fixed to the pelvis wall. Lymph node involvement could not be reviewed in 20 tumour samples (i.e. N = X) and distant metastases could not be found in other organs of the 41 tumour samples meaning that M = X. No regional lymph node metastases were registered in 19 tumour samples (i.e. N = 0). In only one tumour sample a lymph node metastasis was observed on one side and for another on both sides (i.e. N2). No tumour sample showed presence of distant metastases meaning. M = 0 according to the definationin the TNM stage system (Table 3.16-3.17).

Table 3.17:- Classification is according to TNM stage of the tumour samples.

1.2 Clinical TNM (cTNM) and pathological TNM (pTNM) characteristics from UICC	Number of patients
pT stage	
0	0
1	0
2	25
3	26
4	1
pN stage	
0	19
1	1
2	1
X	20
Missing	11
pM stage	
0	0
1	0
X	41
Missing	11
pR stage	
0	10
1	0
X	0
Missing	42

UICC: International Union against Cancer (Union Internationale Contre le Cancer).TNM: (T means tumour, N means lymph node metastasis, and M means presence or absence of distant metastasis).

The immunoscore value in normal cells was higher than that of tumour cells in five of the tumour samples (see Table 3.16 and Table 3.18 in Appendix). The quotient of the immunoscore of tumour cells and normal secretory cells in the above five samples ranged between 0 and 0.67 and did not show any correlations with tumour stage TNM or grade G of these tumour samples.

The immunoscore of the normal and tumour cells was the same in 12 tumour samples (Table 3.16). The net difference for the immunoscore of tumour cells and normal cells (i.e. tumour cells minus normal cells) was 1 in 12 tumour samples (+1 in 8 sample and -1 in 4 samples), 2 in ten

tumour samples, 4 in 6 tumour samples, 5 in 4 tumour samples, 6 and 11 in only one tumour sample. Using the above calculation, the range between normal and tumour cell immunoscore extended from minus three (-3) to plus eleven (+11) mathematically (Table 3.18-3.19 in Appendix). The minimum immunoscore of zero was registered in 8 normal cells and in 6 tumour cells samples. Four tumour samples showed an immunoscore of zero for normal glandular cells and tumour cells simultaneously. The highest immunoscore in the normal cells was 8 and in the tumour cells it was 12, represented only in one sample each. Prostate intraneoplasia (PIN) had the immunoscore of 4 (minimum), 6, and 9 (maximum) in two tumour samples each, Immunoscore of 8 was in one sample making a range of 4-9 and a mean of 6.57 (Table 3.18 in Appendix). Only one sample had the same immunoscore of 8 for normal cells, tumour cells and PIN cells. There was no tumour sample where the immunoscore of the normal secretory cells was higher than that of the PIN cells. However, it was equal in one tumour sample. The immunoscore of the PIN could be higher or lower than that of the tumour cells. However, it did not reach the highest immunoscore of the tumour cells.

The quotient of the immunoscore of the tumour cells and the normal secretory cells ranged between 0-12. The result of this quotient of tumour cells and normal secretory cells (immunoscore of tumour cells divided by immunoscore of normal cells) had the value of 1 in 9 tumour samples and 2 in 8 tumour samples; 1.5 in 5 tumour samples and 3 in 5 tumour samples; 6 in 4 tumour samples; 0.5 in 2 tumour samples and 1.33 in 2 tumour samples and in only one samples 0.67 and 12 in another. In ten tumour samples, the result of this quotient was 0.

The mean and standard deviation of the immunoscore in the normal secretory cells, tumour cells and prostate intraneoplasia cells (PIN) were calculated in 49 tumour samples and plotted (see Table 20 and Fig. 3.22).

Table 3.20:- The mean and standard deviation of the immunoscore in the normal, PIN, and tumour cells.

YB-1 and cell type	Mean	Standard deviation
YB-1 normal cells	1.81	1.5
YB-1 PIN cells	6.57	2.1
YB-1 tumour cells	3.52	2.5

YB-1 expression in the normal secretory cells was the lowest with a mean of 1.81 and standard deviation of 1.5. The mean of the YB-1 expression in Prostate intraneoplasia cells (PIN) was the highest, with a value of 6.57. This was found in 7 tumour samples. The standard deviation was 2.1. YB-1 expression in the tumour cells had a mean of 3.52 and the highest standard error of 2.5 (Fig. 3.22).

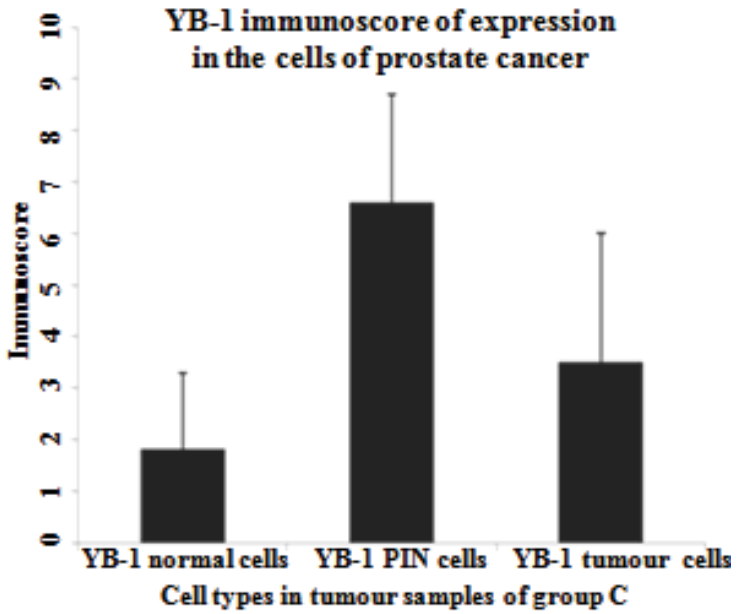


Fig. 3.22: Mean and standard deviation of YB-1 immunoscore of expression in the cells of prostate cancer. The barplot shows the immunoscore of YB-1 expression in normal, prostate intraneoplasia, and tumour cells present in prostate cancer.

1.2.1 3.4.4. Correlation between semi-quantitative expression of YB-1 (immunoscore) and clinical pathology (TNM stages and WHO grades) in prostate cancer

YB-1 expression was studied in different TNM stages and WHO grades to follow the changes with disease development. In the tumours analyzed, there were only two stages (pT2 and pT3) representing the main stages in the disease development (see Table 3.18 in Appendix) and 5 subgroups of WHO grading system available for this study (see Table 3.19 in Appendix). YB-1 was semi-quantitatively evaluated after Remmele score (immunoscore 2) by Dr. Engers.

1.2.1.1 3.4.4.1. Evaluation of semi-quantitative expression of YB-1 in different TNM stages

The stage pT2 contained 25 tumour samples for analysis. The summation values of immunoscore of YB-1 was 47 in YB-1 normal glandular cells, 87 in tumour cells, and 33 in prostate intraneoplasia cells (PIN). The mean was determined from the stage pT2 tumour samples (n = 25) and the summation of YB-1 score values in normal glandular cells, tumour cells, and prostate intraneoplasia cells which were 47, 87, and 33, respectively. The total difference (mathematicaly) of the YB-1 immunoscore between tumour and normal secretory cells was 39 (i.e. the immunoscore of YB-1 in tumour cells minus the immunoscore of YB-1 normal secretory cells) and the total quotient of YB-1 immunoscore in tumour cells to immunoscore in normal cells was 43.67 as seen in Table 3.18 in appendix (i.e. immunoscore of tumour cells divided by immunoscore of normal cells after summation of these values). In stage pT2, the mean of YB-1 was 1.88 in normal cells, 3.48 in

tumour cells, 1.32 in prostate intraneoplasia cells, 1.56 in difference between tumour cells to normal cells (i.e. tumour cells minus normal cells), and 1.75 the quotient of immunoscore in tumour to normal cells (i.e. the immunocsore of tumour cells divided by normal cells (see Table 3.18 in Appendix and Table 3.21).

Table 3.21:- The mean values of YB-1 immunoscore in YB-1 normal and YB-1 Tumour cells of group C.

TNM stage	YB-1 in normal	YB-1 in tumour	YB-1 tumour – normal	YB-1 Tumour/normal	YB-1 in PIN cells
pT2	1.88	3.48	1.56	1.75	1.32
pT3	1.74	3.57	1.83	1.92	0.57

Mean value in normal and tumour cells, the difference of mean between normal and tumour cells and the quotient of the mean of YB-1 immunoscore in tumour and normal cells in different subgroups of TNM-classification.

23 of the tumour samples of stage pT3 were evaluated. In stage pT3, The tumour samples had a total immunoscore value of 40 in normal glandular cells, 82 in tumour cells, 13 in PIN cells, 42 in tumour cells tumour cells minus normal secretory cells (the difference of YB-1 immunoscore between tumour and normal secretory cells), and the total quotient of YB-1 immunoscore was 44.16 (immunoscore of tumour cells divided by normal glandular cells). In addition, the mean of YB-1 was 1.74 in normal glandular cells, 3.57 in tumour cells, 0.57 in prostate intraneoplasia cells, 1.83 in tumour cells minus normal glandular cells, and 1.92 in tumour cells divided by normal glandular cells (the quotient of immunoscore in tumour to normal glandular cells) in stage pT3, respectively (see Table 3.18 in Appendix and Table 3.21). Only one tumour sample had pT4 was plotted and was excluded from the statistical analysis in this study due to the small sample size.

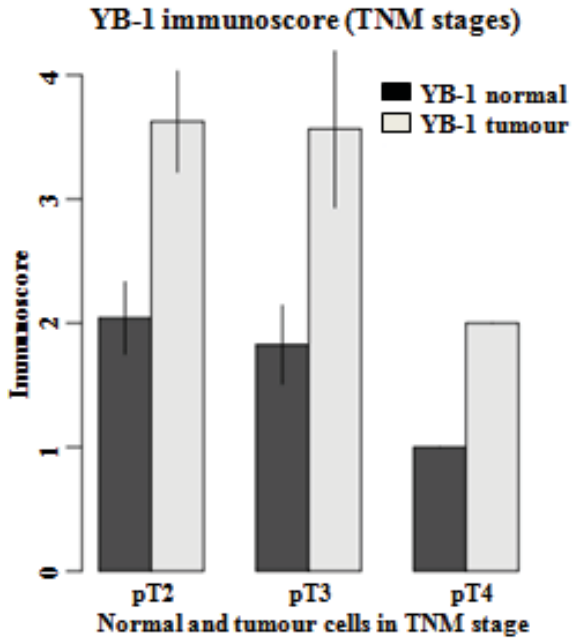


Fig. 3.23: **Correlation between YB-1 immunoscore and TNM stages in normal and tumour cells of prostate cancer**. The barplot shows no correlation between YB-1 immunoscore and TNM stages.

The barplot in Fig. 3.23 shows the comparison of the immunscore of YB-1 in normal and tumour cells in each stage. Also, Fig 3.23 shows that the YB-1 immunoscore of expression in normal cells decreased gradually from normal cells in stage pT2 to pT4, but the YB-1 immunoscore of expression in tumour cells shows no change in pT2 to pT3.

3.4.4.1.1. Statistical analysis of YB-1 expression and clinical stages (TNM stage) follow up in prostate carcinoma

A statistical analysis of the correlation between YB-1 immunoscore of expression and clinical TNM stages of prostate cancer was performed. There was no tumour sample of stage pT1 in this study. Stage pT4 was only represented in one tumour sample in this study and was excluded from this statistical analysis. There were only sufficient numbers of tumour samples of stage pT2 and pT3 for the statistical analysis. The Friedman test, kendall´s rank correlation tau (τ) test, and Wilcoxon test were used with a significance level of < 0.05. Friedman test is a non-parametric statistical test and it is used to test for the differences between groups of samples when the dependet variable/parameter being measured is ordinal. Using Wilcoxon test to study the correlation between the YB-1 immunoscore in normal secretory cells and tumour cells a significant difference ($P = 0.000014$) in the 49 tumour samples under study was found. This positive P value of significance means that the increase of the tumour stage is correlated with increase of the immunoscore correlation between YB-1 immunoscore in normal secretory cells and tumour cells (Fig. 3.24A).

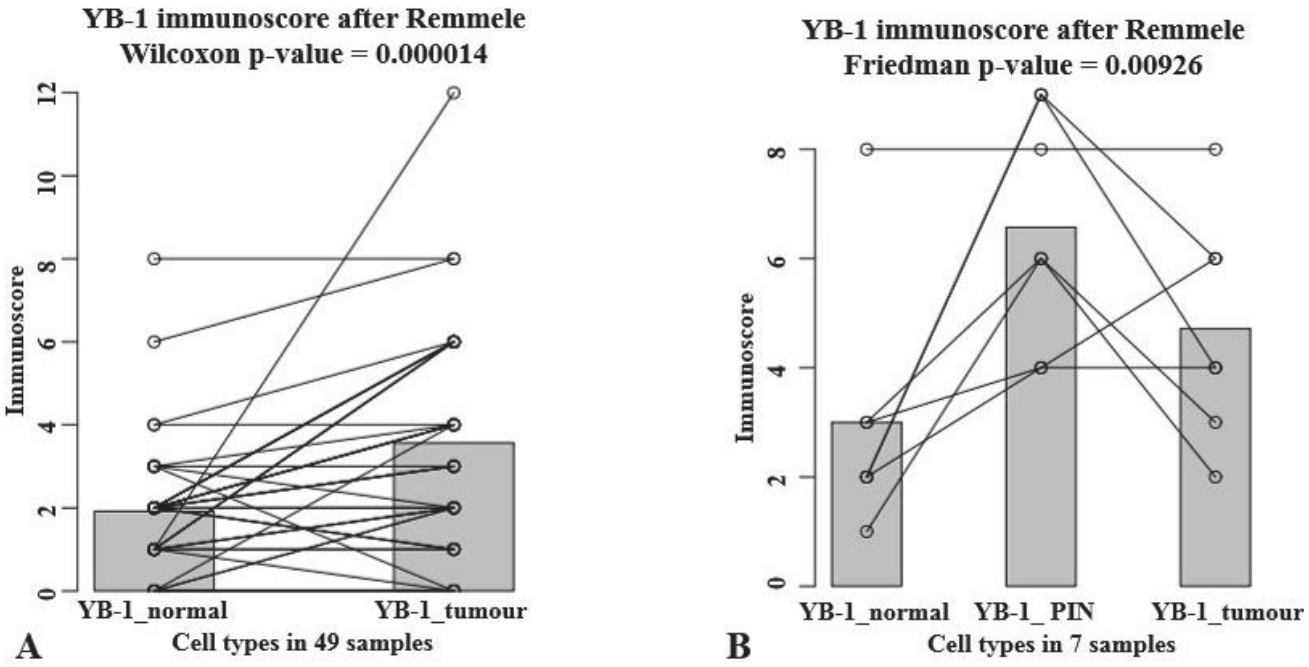


Fig. 3.24: Correlation of YB-1 immunoscore between normal, prostate intraneoplasia (PIN), and tumour cells. A) The correlation of YB-1 immunoscore between normal and tumour cells in 49 tumour samples according to Wilcoxon test is significant. **B)** The correlation of YB-1 immunoscore between normal, prostate intraneoplasia (PIN), and tumour cells in 7 tumour samples according to Friedman test is also significant.

In 7 tumour samples, the correlation of YB-1 immunoscore of expression was studied in the three cell types; precursor cells of prostate cancer (prostate intraneoplasia or PIN), tumour cells, and normal secretory cells using Friedman test. It showed a significant difference between them (P = 0.00926) as seen in Figure3.24B.

For further statistical analysis between stages, the correlation between YB-1 expression in normal secretory cells of pT2 and pT3 as well as between tumour cells of pT2 and pT3b stages is shown in the Table 3.22 using Wilcoxon test. There was no significant difference between YB-1 immunoscore of expression in normal secretory cells of stage pT2 and pT3 (P = 0.6733) as well as between tumour cells of pT2 and pT3b stages. In addition, Table 3.22 showed also the correlation between YB-1 expression in normal cells and tumour cells of stage pT2 as well as the correlation between YB-1 expression in normal cells and tumour cells of stage pT3. In contrast, a significant difference was found between YB-1 immunoscore of expression in normal secretory cells and tumour cells of stage pT2 (P = 0.000987). Moreover, a significant difference was observed between YB-1 immunoscore of expression in normal secretory cells and tumour cells of stage pT3 (P = 0.00379). Interestingly, the correlation between YB-1 immunoscore in normal secretory cells and tumour cells in stage pT2 was highly significant (P = 0.000987) in comparison to the correlation between normal cells and tumour cells of stage pT3 (P = 0.00379) (Table 3.22). The P values of significance were positive referring that these significant correlations were positive in both stages.

Table 3.22:- The P–value in normal and tumour cells of stage pT2 and pT3 using Wilcoxon test.

Variable parameters	p-value
YB-1 normal pT2 versus YB-1 normal pT3	0.6733
YB-1 tumour pT2 versus YB-1 tumour pT3	0.8835
YB-1 normal pT2 versus YB-1 tumour pT2	**0.0009873**
YB-1 normal pT3 versus YB-1 tumour pT3	**0.003792**

Studying the Correlations of YB-1 expression with the prostate cancer relapse showed no significant difference (Table 3.23).

Table 3. 23:- Correlations of YB-1 expression with the prostate cancer relapse.

	YB-1 Tumour /YB-1 Normal	YB-1 Tumour – YB-1 Normal	Relapse Yes/No +	Time until to relapse (months) ++
YB-1 Tumour/YB-1 Normal	1	0.938**	0.139	-0.160
correlation after person Significance (2-sides)		0.000	0.419	0.619
N	40	40	0.39	12
YB-1 Tumour - YB-1 Normal	0.938	1	0.239	-0.123
correlation after person Significance (2-sides)	0.000		0.113	0.662
N.	40	49	45	15
Relapse Yes/No	0.139	0.139	1	A
correlation after person Significance (2-sides)	0.419	0.113		
N	0.39	0.45	53	15
Time till relapse months	-0.160	-0.123	A	a
correlation after person Significance (2-sides)	0.619	0.662		
N	12	15	15	15

**. = The correlation is at the level of 0.01 (2 side) significant. N = Number of the tumour samples
a. = It could not be calculated because there is at least one variable constant.
+ = Relapse means recurrence of the disease after cure.
++ = Time untel to relapse means the time to recurrence of the disease after cure.

3.4.4.2. Evaluation of semi-quantitative expression of YB-1 (immunoscore) in different WHO grades

To survey the YB-1 immunoscore of expression and the subgroups of the WHO grading system, five subgroups in WHO grading system (G1, G1-G2, G2, G2-G3, and G3) with different immunoscore values in the different tumour cell samples were evaluated (see Table 3.19 in Appendix).

Table 3.24:- Number of tumour samples, highest and lowest value as well as the median of the immunoscore indifferent cells of prostate cancer in different WHO grading system.

WHO grading	1.2.1.2 o	YB-1 in normal	YB-1 in Tumour	YB-1 in PIN	YB-1 Tumour – normal	YB-1 Tumour/normal
G1	**7**	15	24	9	9	11,67
Highest value		3	6	9	4	2
Lowest value		2	2	0	−1	0.67
Mean		**2.14**	**3.43**	**1.29**	**1.29**	**1.67**
G1-G2	**5**	8	12	-	4	2,5
Highest value		2	6	0	6	1
Lowest value		0	1	0	−1	0
Mean		**1.6**	**2.4**	**0**	**1.2**	**0.5**
G2	**18**	27	60	29	33	38.33
Highest value		4	6	9	5	6
Lowest value		0	0	4	−3	0
Mean		**1.5**	**3.33**	**1.61**	**1.83**	**2.13**
G2-G3	**6**	15	21	8	6	6.5
Highest value		8	8	8	4	3
Lowest value		0	1		−1	0.5
Mean		**2.5**	**3.5**	**1.33**	**1.0**	**1.08**
G3	**13**	25	54	0	29	30.88
Highest value		6	12		11	12
Lowest value		0	0	-	0	1
Mean		**1.92**	**4.15**	**0**	**2.23**	**2.38**

The tumour samples with the same WHO grade were tabulated with their immunoscore of YB-1 expression in their normal secretory, PIN, and tumour cells. Detailed numbers of the tumour samples under study and the immunoscrores in each WHO subgrade are listed in the supplementary table. Table 3.24 summarizes the supplementary table 3.19 in appendix showing the numbers of the tumour samples, lowest, and highest value of the immunoscrore as well as the mean values in the different WHO subgrades.

3.4.4.2.1. Statistical analysis of YB-1 expression and WHO grades follow up in prostate cancer

Using Wilcoxon test, the correlation between YB-1 immunoscore of expression in normal secretory and tumour cells in each WHO sub-grade (i.e. G1, G1-2, G2, G2-3, and G3) was studied. Only the correlation of YB-1 immunoscore between normal secretory cells and tumour cells in WHO grade 2 (i.e. normal secretory cells versus tumour cells) as well as between normal secretory cells and tumour cells in WHO grade 3 showed significant differences which were P-values 0.003891 and 0.01310 respectively (Table 3.25). Otherwise, no significant differences of YB-1 immunoscore were observed between normal cells in the different grades using Wilcoxon test. Also, no significant differences of YB-1 were observed between tumour cells in the different grades were identified in this analysis (Table 3.25).

Table 3.25:- Statistical analysis of YB-1immunoscore of expression in normal and tumour cells of different WHO grading system using Wicoxon test.

Variable parameters	P-value
YB-1 normal G1 versus YB-1 normal G1-G2	0.08718
YB-1 normal G1 - G2 versus YB-1 normal G2	0.5537
YB-1 normal G1 versus YB-1 normal G2	0.1866
YB-1 normal G1 versus YB-1 normal G3	0.4026
YB-1 normal G2 versus YB-1 normal G2-G3	0.795
YB-1 normal G2 versus YB-1 normal G3	0.8029
YB-1 normal G1 versus YB-1 tumour G1	0.08898
YB-1 normal G1 - G2 versus YB-1 tumour G1-G2	0.5008
YB-1 normal G2 versus YB-1 tumour G2	**0.003891**
YB-1 normal G2 - G3 versus YB-1 tumour G2-G3	0.5876
YB-1 normal G3 versus YB-1 tumour G3	**0.01310**
YB-1 tumour G2 versus YB-1 tumour G3	0.6854

It is clear that the correlation between the expression in normal secretory cells and tumour cells of G2 is more significant than the correlation between normal secretory cells and tumour cells in G3 (Table 3.25), similar to the correlation between normal secretory and tumour cells in case of pT2 and pT3 stages. It is obvious that the positive correlation between YB-1 immunoscore of expression in both the normal secretory cells and the tumour cells of G2 and G3 were less

significant in comparison with the correlation between normal secretory cells and tumour cells of pT2 and pT3.

For this analysis the histological grade G1 and grades G1-G2 were grouped together in grade 1. Also, grade G2 and grade G2-G3 were grouped to grade 2. Consequently, three main histological grades, grade 1, grade 2, and grade 3 were used for the statisical analysis. The numbers of tumour samples in grade 1, grade 2, and grade 3 were 12, 23, and 13, respectively (Table 3.18-3.19 in Appendix). They were enough for statistical analysis study using Kendall´s tau test.

The barplot in figure 3.25 shows the comparison of the immunscore of YB-1 in normal and tumour cells in each WHO grade. It shows that the YB-1 immunoscore of expression in tumour cells increased gradually from tumour cells in grade 1 to grade 3, but the YB-1 immunoscore of expression in normal secretory cells showed little change from grade 1 to grade 3 (Fig. 3.25).

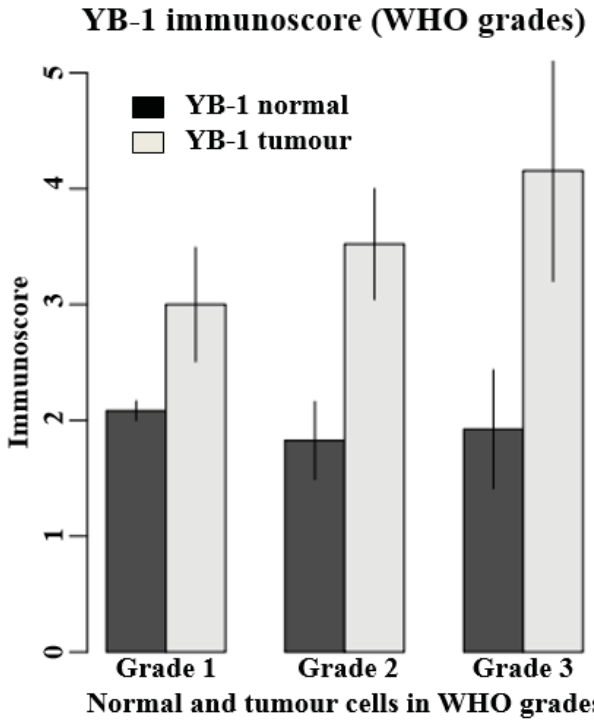


Fig. 3.25: Correlation between YB-1 immunoscore and WHO grades in normal and tumour cells of prostate cancer. The barplot shows that YB-1 immunoscore of expression in tumour cells increases with WHO grades, but no differences between the normal cells in different grads.

As in the previous study of Caveolin-1 immunohistochemistry, the distribution of YB-1 immunoscores was studied in relation to WHO grades instead of TNM stages. The distribution of YB-1 immunoscore is represented as frequency in % in the diagram below. The histograms suggest an increasing shift towards higher YB-1 immunoscores for higher WHO grades (Fig. 3.26A-C).

This gradual increase is similar to that observed for TNM stages and Caveolin-1 expression in the previous study. Kendall's tau test was used to test for a correlation between YB-1 immunoscore ratios of tumour cells over normal secretory cells and the different WHO grades (Grade 1, Grade 2, and Grade 3). Although this correlation was not significant (P = 0.206), there was a clear tendency of the YB-1 immunoscore to increase from grade 1 to grade 3 (Fig. 3.26D).

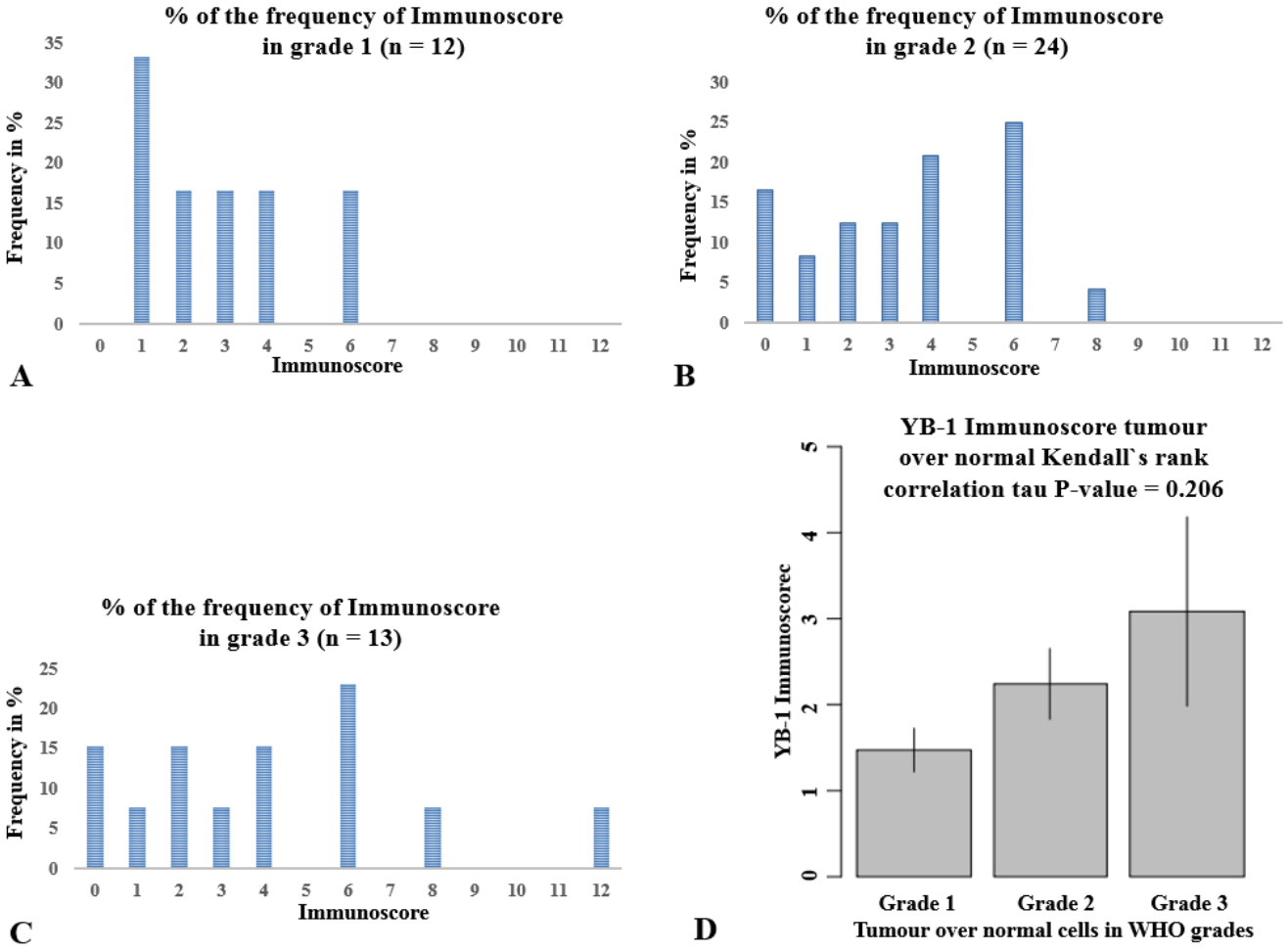


Fig. 3.26: Frequency of YB-1 immunoscore in percentage in the various WHO grades and correlation between YB-1 immunoscore ratio in tumour over normal cells and WHO grades in prostate cancer. A-C) The histograms show a shift towards increased expression in higher tumour grades. **D)** The mean YB-1 immunoscore ratios of tumour over normal cells show a tendency of being positively correlated with WHO grades.

4. DISCUSSION

4.1. Prostate cancer tissue and FISH studies

The treatment of prostate cancer in different countries varies. In many cases an approach of watchful waiting is used, which means that the patients are not operated on immediately and it is observed whether the disease progresses or not. It is very important to find markers that can identify those patients that need immediate surgery. The problem with prostate cancer is its complexity. So far no single marker has been identified which can distinguish a tumour with a high risk from a tumour with a low risk for progression. It is clear that patients with a tumour that has already penetrated the capsule and possibly invaded seminal vesicles and lymph nodes have a poor prognosis and a patient with a tumour that is entirely confined to the prostate has a much better prognosis. However, there are cases where it is difficult to predict how the tumour will develop. Therefore the aim of this work was to find markers that can help the pathologist to identify a tumour with a high risk for progression. The pathology of a prostate cancer is highly complex and only very few specialized pathologists can perform a detailed pathological review. Often different pathologists can give different Gleason scores for the same tumour. So an independent marker or markers based on molecular alterations are highly desirable. At the beginning of this thesis work several molecular alterations have been described in the literature which seemed to be correlated with a poor prognosis. Therefore the first aim was to study some of these markers which are either deleted in the tumour or are found at a higher copy number by FISH. However, it turned out that FISH is a very difficult method to use with paraffin sections. Paraffin sections were used because the histology is conserved and tumour cells can be distinguished from the normal cells after the analysis. The difficulties in those FISH-studies are discussed below separately. Another approach is to study protein markers by immunohistochemistry. Although this is an easy method to perform, it is not easy to evaluate the percentage of positive tumour cells as these cannot easily be identified by unexperienced researchers. Therefore a pathologist has to study and evaluate theses samples, and the collaboration with an experienced pathologist is very important. Unfortunately many pathologists do not have enough time to perform such a research oriented project. Therefore also in this part of the work several handicaps were encountered which needed to be addressed. The results are discussed in the following sections.

Prostate cancer is probably one of the most difficult carcinomas to be studied. Moreover, prostate cancer has many forms that make the pathology of the prostate cancer difficult to analyze.[106] The most well known type is prostate adenocarcinoma, which represent about 95% of the

prostate cancer types.[20, 106] The disease is a multi-focal, multi-factorial and heterogeneous tumor.[107-108] Genetic as well as sporadic factors are responsible for the development of this disease. However, the absence of animal models or even cell lines could be added to the difficulty of its analysis.

Prostate tissue shows continuous changes in structure, histology, and functions from the beginning of its embryonic stage through adult stage to elderly age. All of these processes are under hormonal control.[29] The structure of the prostate is complicated and consists of glandular and non-glandular parts of endodermal and mesodermal origin.[7, 109] During this study, several methods and techniques were used to establish efficient procedures allowing the best evaluation of the results. Nine tumour samples and one normal prostate tissue were selected for FISH analysis. The most effective way to cut the sections and the best method for adhering them to the slide were established. It was found that Super Frost Plus slides layered with poly-L-Lysine as well as negatively charged slides were excellent for better adhesion of the tissue sections. The histo-bond slides (another type of slides) were especially qualified for immunohistochemical analysis. The ideal thickness of the paraffin section for FISH study was 6-8 µm from a pre-cooled paraffin block. For isolation of cell nuclei the section-thickness was at least 10 µm and did not exceed 30 µm. However, the number of the sections needed for cell nuclei isolation was depended on the size of the tumour area.

There were differences between the prostate cancer tissue fixed in un-buffered formaldehyde (before 1999) and buffered formaldehyde (after 1999) (data not published). The FISH and immunohistochemistry methods (which are routinely used in pathology institutes and laboratories in many studies) using buffered formaldehyde fixed tissue blocks of prostate cancer gave better results than in un-buffered formaldehyde fixed tissue blocks. The application of currently used protocols in FISH with paraffin sections did not succeed in the paraffin tissue sections of prostate cancer. This may be due to the interaction of formaldehyde with haemoglobin and precipitates of urine which exist in the analyzed prostate tissue sections leading to formation of spots. To reduce significantly the formation of spots and precipitates, the tissue section was pretreated in warm citrate buffer and heated further in a microwave for 15 min. Also, a pre-hybridizing step was applied to reduce the background.

4.2. FISH study of chromosome 7q and 8

In solid tumours, it is known that genetic alterations often involve either the whole chromosome or specific parts of chromosomal regions.[110-111] The classical cytogenetic analysis such

as karyotyping is still very limited in case of solid tumours. This is due to the difficulties in culturing cancer cells resulting from the low mitotic activity of tumours cells and the overgrowth of non-neoplastic cells. These are the reasons for the small number of solid tumors that have been analyzed cytogenetically.[112-114] Nuclear DNA content is used to make a rough estimation of the number of numerical chromosomal aberrations. Using flow-cytometry several studies have shown changes in DNA content and provided independent prognostic information in addition to the clinical stage and histological Gleason score.[115-119]

Hereafter, DNA ploidy analysis using fluorescence in situ hybridization (FISH) with centromere-specific probes has shown greater sensitivity and specificity than flow cytometry for detecting aneuploidy in tumours.[62, 120-121] Locus specific-FISH probes recognizing a single locus were used in this study to detect both numerical alterations (amplifications and deletions) and rearrangement between chromosomes.[122-123] Locus specific-FISH can be performed on disaggregated inter-phase nuclei from both fresh and fixed tissues as well as paraffin sections. It is an accurate method for detecting gene amplification and deletion. Moreover, it allows the analysis of cell-to-cell heterogeneity. Furthermore, it can be implemented for the studies of the relationships between the aberration of small limited regions or gene constellations of the chromosomes and tumorigenesis (development, progression, or metastasis). All of these are especially important and meaningful in the case of prostate cancers that are often heterogeneous and multifocal by nature.[124-129] However, one disadvantage of FISH is that a whole genome analysis is not possible. Besides using centromere-specific probes, BAC, PAC and YAC probes were used not only for the detection of specific chromosomal gains and losses but also for the detection of a deletion or amplification of specific regions or genes. This may play a role in prostate cancer development and progression. The reason for this was that some tumours might not show aneuploidy in chromosomal number but a deletion or amplification of specific loci or genes.

Many studies have been published on the prognostic value of FISH in DNA and ploidy levels in primary, recurrent, and metastatic prostate cancer.[130] Generally, in studying a small deletion on a specific chromosome, FISH and loss of heterozygosity (LOH) analyses are prefered.[131-132] The deletion of 8p has been narrowed down using FISH and LOH analyses to the mostly deleted region encompassing 8p12-22. The used YAC 240G10 (450 Kb in size) in this study maps to the 8p12-22 region and includes the *NEFL* (Neurofilament light polypeptide or negative elongation factor) gene. With sequencing of the genome, the localization of *NEFL* gene was defined

at 8p12-22. The *NEFL* gene is adjacent to *NEX3* gene from proximal (centromeric) and to *LPL* (lipoprotein lipase) gene distal (telomeric).

Because normal prostate tissues were not available at the beginning of this study, the stroma and connective tissue cells of the tumour sample number 6 area 2 -Paraffin (stroma) shown in figure 3.4A was evaluated and considered as normal control. The choice of these cells was because they didn't take part directly in the neoplasia process and were mesodermal in origin.

The FISH analysis in this study deals with chromosomes 7 and 8 that play an important roles in the development of pre-malignant to the advanced and metastatic stage.[133] There is a widespread opinion that the short arm of chromosome 8 is often deleted in prostate intraepithelial neoplasia.[134] YAC 240G10 including the *NEFL* gene was used because it belongs to the third most deleted site after *LPL*-D8S133 and D8S87-*ANKI* in prostate cancer.[135-136] In the 8p12-21 locus, *NEFL*- D8S1731 defines a minimal region of loss in all tumours showing LOH.[136-137] Kazuo Oba and co-workers (2001) have reported that the most frequently deleted regions were 8p22 and 8p21.3 in 54.8% and 52.4% of the tumour samples respectively. The second most frequently deleted region was 8p12-p22 and was present in 38.1% of the tumours under study.[138]

Although the numbers of samples in this FISH study were small, it represented different stages of disease development, beginning from the pre-malignant stage (prostate intraepithelial neoplasia (PIN), ending with the metastases stage. The results obtained in my work reflect the changes of chromosomes 7 and 8 during the disease development. In this FISH-study, nine tumour samples plus normal prostate tissue as a control were analyzed. Two tumour samples without pathological report might represent the prostate intraepithelial neoplasia (PIN) or the possible beginning of pT1 stage. The other two samples belonged to pT1 (pTa and pTb) and had the same Gleason score of 6 without known patterns. The last five tumour samples belonged to pT3 (pTa and pTb). Four tumor samples of stage pT3a-b had Gleason score of 3 + 5 = 8 and only one had Gleason score of 2 + 3 = 5 (see Table 3.15 in page 99).

4.2.1. FISH with paraffin sections

During this study, gain of 7q31 was found in all FISH analyses in paraffin sections without exception but with different levels. Not only a simple gain of 7q31 was observed but also clear amplification in the tumour 3 -Paraffin, 7 -Paraffin, and 8 -Paraffin and low level amplification in tumour 2 -Paraffin. To study this further, FISH was performed in isolated cell nuclei. To confirm the gain of 7q31, a centromere probe of chromosome 4 was used in the tumour 8 -Paraffin that

showed gain of 7q31 and no loss of chromosome 4 (normal). It is known that chromosome 4 shows rare aberration in prostate cancer. In addition colour swap of the probes was used to prove the independence of the labeling in the results. The results showed no differences. In contrast to 7q31 region, the results from region 8p12-22 showed a loss, no loss, gain and even amplification in FISH with paraffin sections.

The results obtained in this study agreed partially with the results of Häggman et al.[139]. Häggman and co-workers have suggested that PIN and invasive cancer foci share a common "genetic history" as shown by identical allelic loss at the *NEFL* locus during development of the malignancy grades of prostate cancer. Qian et al.[33, 140] revealed that high grade PIN (HGPIN) and prostate carcinoma share the same allelic loss of 8p and have high-frequency LOH regions on chromosome 8 of prostate carcinoma located on 8p23.1-p23.2 and 8p12-p22.[140] They presumed that tumor suppressor genes located at these two regions may be potentially involved in the initiation and progression of prostate carcinoma.

Bastacky et al.[141] found that abnormalities of chromosome regions and oncogene copy number abnormalities involving chromosomes 7, 8, 17, 7q31, *c-myc*, and *HER-2*/neu gene were rare in controls. However, they are common in HGPIN, in samples without and with carcinoma, and in a limited follow-up interval of time to the disease development.[141] The small number of PIN samples (two samples) cannot give conclusive results in this study.

Two samples of FISH analyses namely 1 -Paraffin and 3 -Paraffin representing stage pT1a-pT1b had a Gleason score of 6 without knowing the Gleason score patterns of arrangement. Their pattern in the Gleason score of 6 (GS = 6) may be 2 + 4, 4 + 2, or 3 + 3. Tumours having a Gleason score between 5 and 7 (GS = Pattern 1 + Pattern 2) generally belong to moderately differentiated tumours. Gleason score 5 and 7 is the most difficult to predict the course of the disease. They showed continuous changes of the chromosome 7 and 8. The tumour sample 1 -Paraffin was from a patient with stage pT1b. This sample showed a clear loss of 8p12-22 and gain of 7q31 (Fig. 3.4C). However, tumour sample 3 -Paraffin was from stage pT1a but it showed clear gain of 8p12-22 as well as of 7q31 (Fig. 3.4E). Both 7q31 and 8p12-22 in tumour sample 3 -Paraffin were amplified with different amplification levels. These differences may be related to the clinical sub-stage (TNM stage) and malignant grade (WHO grade) of both samples where pT1b is more advanced than pT1a. In the absence of FISH in cell nuclei in both tumour samples, these chromosomal aberrations could not be confirmed because the number of the cells with one signal either red or green in tumour sample 1-Paraffin is higher than in tumour sample 3 -Paraffin. Gleason score describes the

histology of the prostate tumour cells more definitely than WHO grading system. In the future, it is necessary to study the correlation between chromosomal changes and Gleason score in prostate cancer.

Tumour samples 1 -Paraffin, 5 -Paraffin, 6 -Paraffin, and 9 -Paraffin showed clear gain of 7q31 and loss of 8p12-22 at the same time. Also, tumour sample 2 -Paraffin showed clear gain of 7q31 but low level loss of 8p12-22. This low level loss of 8p12-22 in case of tumour sample 2 -Paraffin could not be studied using FISH in isolated cell nuclei because there was no material available. The pathological record was not known for tumour 2 -Paraffin. Tumour sample 1 -Paraffin, 5 -Paraffin, 6 -Paraffin, and 9 -Paraffin had the Gleason score 6 (without known patterns), GS 8; (3 + 5), GS 8; (5 + 3), and GS 8; (5 + 3), respectively. All the above tumour samples showed a gain of 7q31as well as loss of 8p12-22.

Tumour sample 3 -Paraffin showed higher gain of both 7q31 and 8p12-22 than tumour sample 7 area 1 -Paraffin. Both tumour samples showed even amplification of both chromosomes. This was later confirmed by FISH of isolated cell nuclei using tumour sample 7 area 1 (GS = 2 + 3) and could not be confirmed in tumour sample 3 due to the lack of tissue material. But the gain in tumour sample 3 -Paraffin is more likely correct because the percent of the cells with single signal either red or green was less than in tumour sample 4 -Paraffin. Also, the gain of 7q31 and 8p12-22 in tumour sample 3 -Paraffin was more than that in tumour sample 4 -Paraffin.

Tumour sample 5 -Paraffin under FISH study had the same Gleason score 3 + 5 = 8 constellation as tumour sample 8 -Paraffin. FISH analysis of paraffin sections showed in both tumours clear gain of 7q31 and loss of 8p12-22 (Fig. 3.5A and Fig. 3.5E). The gain of 7q31 and loss of 8p12-22 in case of tumour sample 5 -Paraffin (23.5% and 12.4% respectively) was found in a higher percentage of cells as in tumour 8 -paraffin and therefore likely reflects a true alteration (17.1% and 10.3% respectively). In addition, tumour sample 5 -Paraffin may have contained more malignant cells than other sections used for the pathological evaluation. This is because the pathological record represents the average of 16 examined tissue areas of this prostate cancer.

To confirm the gain of 7q31, a combination of Caveolin PAC (7q31) probe and a probe of centromere chromosome 4 as a control was studied in the tumour sample 8 -Paraffin (Fig. 3.5F). Chromosome 4 shows neither gains nor losses in prostate cancer and was used as control. Euploidy of chromosome 4 (i.e. normal chromosome without gain or loss) agreed with the previous studies done by Nupponen NN, Visakorp T[142], and Macoska, et al.[143] They suggested that chromosome 4 is

the least changed chromosome in prostate cancer. This supports the previous results referring to 7q31 gain and shows a correlation between disease development and 7q31 gains. Otherwise, the loss or gain of 8p12-22 may be related to the heterogeneity character of prostate cancer. No loss of 8p12-22 in tumour sample 8 was confirmed by using FISH in isolated cell nuclei as can be seen later. This confirmation was not possible in case of tumour 5 due to the absence of further material (for cell nuclei isolation).

It was clear that the presence of partial cells cut by sectioning (single signals) was higher in tumour sample 1 -Paraffin than in 3 -Paraffin. Due to the absence of FISH analyses of isolated cell nuclei in both tumour 1 -Paraffin and 3 -Paraffin samples, chromosomal aberrations could not be confirmed. But, the gain of 7q31 and 8p12-22 in tumour sample 3 -Paraffin was correct because the percentage of trisomy and tetrasomy of 7q31 or 8p12-22 were clearly above background. This was not so clear in tumour 1 -Paraffin, where the loss of 8p12-22 was 9.9% above background of the single signal (20%) and at the same time a low level gain of 8p12-22 in the tumour cells was observed. These contrary results could have been verified by cell nuclei analysis, but the tissue materials were not available.

This conclusion shows that the presence of partial cells is higher in tumour 1 -Paraffin sample than in tumour 3 -Paraffin sample. The previous interpretations depended on the loss of 8p12-22 as the first chromosomal changes in the pre-cancer stage (PIN) as well as stage pT1 that followed with chromosome 7 amplifications (7q31) as reported in many studies.[144-148] Results obtained in my work did not agree completely with the previous studies due to the small sample number in pT1a-b. Actually, many authors considered that the gain of 7q31, 8q and multiple copies of *c-myc* (8q24) as well as loss of 8p, 10q, 16q, and 18q are common in both PIN and cancer.[33, 41, 124, 149-151]

The advantage of the 8p12-22 (*NEFL*) gene marker used here was that the loss of this region is observed in PIN as well as tumour cells. But, gain of c-*myc* gene (8q24) is only observed in tumours. In contrast, Fu W and co-workers[36] reported that 8q24 gains or losses were not correlated with either PIN or pT1 stage or even pT2 stage of prostate cancer. The obtained results of my work did not resolve any of the previous controversies due to the lack of sufficient tumour tissue samples needed for cell nuclei isolation.

The *NEFL* gene is located between the markers D8S2210 and D8S1739. It is located proximal from *MSR*, *LPL,* and *NKX3.1*. The four genes are the closest known markers for the

minimally deleted 8p21 region. *NEFL* co-localized with the *NKX3.1* gene and was sometimes seen slightly proximal of *NKX3.1* at the border of 8p12 and 8p21.3. *NKX3.1* and *NEFL* lay within approximately 2-3 Mb.[152] This proximal co-localization of NEFL to NKX3.1 which is regulated by androgen, strengthens the possibility that it is a tumour suppressor gene important for prostate cancer, because the entire coding region of *NKX3.1* showed no tumor specific sequence alterations in 50 specimens and total absence of the gene in 1 specimen known to have a bi-allelic deletion of 8p21.[136, 152] This means that the absence (or deletion) of *NEFL* gene is correlated with the disease development. For further analysis of 8q in the previous four tumour samples (tumour 1 -Paraffin, tumour 2 -Paraffin, tumour 3 -Paraffin, and tumour 4 -Paraffin samples) there was not enough tissue available to study FISH in cell nuclei. The importance of this analysis can be summarized as follows: It showed that the deletion of 8p12-22 is already observed in the pre- cancer stage (PIN) and there was no information about the gain of 8q24 due to the lack of tissue samples. Consequently, the data obtained in this work could not confirm the studies done by Fu W and co-workers[36] in 2000. They reported that 8q gains or loss are not correlated with prostate intraepithelial neoplasia (PIN), with pT1 stage or even with pT2 stage of prostate cancer (6.0% to 41.0%). More recently, CGH studies showed that the gain of 8q (not analyzed here) ranges between 85.0% and 89.0% in cases of metastases or hormone-refractory prostate cancers.

The other five tumour samples that were studied by FISH in paraffin sections showed clearly gain of 7q31. But, the loss of 8p12-22 was not clear in 8 -Paraffin and was clear in tumour sample 9 area 1 -Paraffin, and slight loss was shown in tumour sample 5 -Paraffin and certain loss in tumour sample 6 area 1 -Paraffin. Meanwhile, tumour sample 7 area 1 -Paraffin showed a gain of 8p12-22.

4.2.2. FISH with cell nuclei

Due to the fact that FISH with paraffin sections always gave varying hybridization signals, which may hinder the accurate evaluation (see chapter 3.2.1-3.2.2) a FISH study was started with isolated cell nuclei. This is due the fact that the cell nuclei in paraffin sections may not be completely present because the sectioning has removed parts of the nuclei. Also in FISH with paraffin sections, the overlapping of more than one cell nucleus in different layers is normal and consequently impedes the evaluation. Therefore, the uncertain gain or loss of 7q31 and 8p12 in some tumour samples demanded an alternative verification method. Therefore the differences between FISH in paraffin sections and separated cell nuclei were studied to determine the best method for identification of signals on the chromosomes giving better result of evaluations. Most

previous studies used FISH on either paraffin sections or isolated cell nuclei to study the changes of prostate cancer cytogenetics but both methods were not used simultaneously.[144, 153-154] Wu SQ et al.[144] and Reiter RE et al.[154] studied FISH in paraffin sections but Miyoshi Y et al.[153] studied FISH in isolated cell nuclei.

Due to the absence of histology in case of FISH in isolated cell nuclei, the sample evaluation was based on the size of the cell nucleus. The big and medium nuclei were considered to be tumour cells, while small ones represented normal epithelial or connective tissue cells. For more precaution during cell nuclei separations, the tumour areas were marked on the slides before they were removed in order to avoid extra separation of the normal and connective tissue cells.

A crucial difference has been noticed between the FISH- evaluation in paraffin sections and in cell nuclei. The number of cells with single signals in FISH in isolated cell nuclei was highly reduced in comparison to paraffin sections. Evaluation differences between FISH in paraffin section and isolated cell nuclei up to 4-5% were observed. On the other hand, the number of the cells with 2, 3, and 4 signals increased markedly. Otherwise, the evaluation of cell nuclei clearly simulates the chromosome aberrations in different cells. This means that the analysis of FISH with cell nuclei reflects more exactly the number of the chromosomal changes of prostate cancer cells (Fig. 3.4-3.9). The combined evaluation has confirmed the results when done simultaneously. The evaluation of the cell nuclei in each tumour sample as small, medium and big nuclei has the advantage that it did not only include the tumour cells (medium and big nuclei), but also the normal glandular cell nuclei of the acini. Consequently, the percentage of the tumour cells to the total cells present in the tumour area could be exactly determined.

Tumour sample 7 area 1 -Paraffin showed gain of 7q31, 8p12-22, and 8q23-24 in paraffin sections. These gains were confirmed after study FISH in isolated cell nuclei of two areas. There was not only gain of a certain region but amplification of both chromosomes. This means that a whole chromosome 8 was duplicated (see tumour sample 7 areas 1 and 2 -Cell nuclei in Fig. 3.7). Gain of 8p12-22 was clearly found at least in two samples (sample 3 and 7) from the 9 tumour sample studied in isolated cell nuclei (≈ 22%). Pettus JA et al.[155] and Sato K et al.[156] found that 15% and 14% cases had a gain of 8p by FISH, respectively. Brothman AR et al.[157] reported in his results about 40% gain as well as 40% loss in chromosome 8 without referring to which arm of the chromosome was affected. Brothman AR et al.[158] referred also in prostate cancer that predominant changes included gains of chromosomes 1, 7, 8, 8q sequences, 17, X and Y, and loss of chromosomes 1, 7, 8, 8p sequences, 10, 10q, 16q and 17q sequences, 17 and Y using FISH. Alers

JC et al.[159] observed also within the different pTNM groups the following aberrations (listed, within each series, in decreasing order of frequency): -Y, +8, –8, +7 in primary tumors; +8, +7, –Y, +Y, -8 in regional lymph node metastases; and +8, +7, +1, –Y, -8 in distant metastases. In primary tumors, the number of aberrant cases increased significantly with local tumor stage ($p < 0.05$). A significant increase in gain of chromosome 8 was also observed ($p < 0.02$). Gain of chromosome 7 and/or 8 showed a significant increase with progression of local tumor stage ($p < 0.02$). This variation between gain, no-loss, and loss of 8p may be related to the origin of the prostatic cancer clones. Schultze and Isaacs[160] in 1986 revealed that individual prostatic cancers are composed of multiple clones that are phenotypically heterogeneous even before therapeutic intervention.

FISH study in isolated cell nuclei of tumour sample 8 confirmed the gain of 7q31 which was observed in FISH in paraffin sections using the probe combinations Caveolin PAC (7q31)/YAC 240G10 (8p12-22) and Caveolin PAC (7q31)/centromere chromosome 4. It confirmed also that there was no loss of 8p12-22 but amplification of 8q22-24, which lead to the formation of an isochromosome 8q (i.e. duplication of the long arm of chromosome 8).

Studying FISH in isolated cell nuclei of tumour sample 9 -Cell nuclei confirmed the gain of 7q31 as observed in tumour sample 9 -Paraffin as well as loss of 8p12-22. Additionally, FISH analyses in isolated cell nuclei of tumour 9 showed higher level loss of 8p12-22 in about 10% more than in paraffin section. FISH analyses in isolated cell nuclei of tumour 9 showed also higher level amplification of 7q31 than FISH in paraffin sections. The above three tumour samples of FISH study in isolated cell nuclei demonstrated the high value of studying FISH in isolated cell nuclei.

Gains of a whole chromosome 8 agreed with the results obtained by Sato H et al.[161] in a Japanese patient. The chromosomal aberration in the tumour 7 with Gleason score = 5 representing moderately differentiated tumours is highly concomitant with the result of Sato K and co-authors[156]. They reported that the first step of the pre-carcinogenesis/tumorigenesis is an 8p12-22 deletion as well as a possible mutation or a small deletion of a gene or genes on 8p that is not detectable by the 8p12-22 FISH probe. In a second step, a gain of a whole chromosome 8 (which may have suffered a first loss or deletion of 8p12-22) occurs. The third step is the 8q gain. This may be related to the formation of an isochromosome 8q which means deletion of 8p and at the same time duplication of 8q.[34, 60, 162-164]

Alers JC et al.[159] suggested that the gain of chromosome 7 and/or 8 is implicated in prostatic tumour progression. Gain of chromosome 8 sequences is related to a local tumour growth, whereas

overrepresentation of 8q mostly by isochromosome 8q formation is involved in metastases. Several authors reported that the gain of chromosome 8 centromere or q-arm of chromosome 8 has been described simultaneously with loss of portions of the 8p-arm in PIN and carcinoma without referring to the grade.[162, 120, 165, 143] The chromosomal aberration in this case (previous studies in prostate cancer of tumour samples) does not concur with the results of Qian J et al.[125], Oba K et al.[138], Wu SQ et al.[144], Samaraki O et al.[166] and Bethel CR et al.[167, 89]. The contradiction in these results could be related to heterogeneity of prostate cancer.[133] In only one tumour sample (tumour sample 7) FISH in paraffin section as well as in isolated cell nuclei showed 8p12-22 amplification/gain (*NEFL* and *NEX3*), tumour suppressor genes (TSGs) and gain of 8q23. Both amplification of 8p and 8q means that the whole chromosome 8 was gained.

The tumour samples 5, 8, 6 and 9 had a Gleason score 8, the first two with a 3 + 5 patterns (low malignant) and last two a 5 + 3 pattern (highly malignant). These samples showed continuous increase in the percentage of 8p12-22 loss and gain of 7q31. Furthermore, tumour sample 8 -Cell nuclei showed a higher frequency of 8q22-24 gain than the tumour sample 7 area 1 -Cell nuclei. The only exception was that the tumour sample 7 showed a high frequency of the whole chromosome 8 gain in agreement with the report of Sato K et al.[156] and Brothman AR et al in their studies in (1992)[157] and (1997)[158].

Sato K and co-workers studied FISH in a group of tumour samples having the pathological stage pT3, N0, M0 and three different Gleason scores of 4-6, 7, and 8-10. They showed that 76.2 % of the studied prostate carcinoma had 8p12-22 abnormalities. Among those tumours, 62.2% had a loss of 8p22 and 14% had a gain of 8p. In this study a sample with Gleason score 5 showed a gain of 8p12-22.

The results of my thesis agreed with the results obtained by Matsuyama H and co-authors[168]. They reported that the frequency of 8p12-22 and 16q24 deletions increased significantly in parallel with the tumour grade while that of 10q deletions did not increase. They referred that, patients whose tumour samples showed 8p12-22 deletions were pT2 tumours. Wu et al showed that there was only a small increase in the number of alterations from stage pT2 to stage pT3b. Matsuyama and co-authors reported that the occurrence of 8p12 and 16q24 deletions may serve as a genetic diagnostic marker for predicting pathological staging as well as the disease progression in prostate cancer. The increase in Gleason score was correlated with the increase in the frequency of 7q31 and of 8q gains as can be seen in tumour sample 3, 5, 6, 7, 8, and 9. But the 8p12-22 loss increased with the increase in Gleason score as seen in tumour 1 and 9. The abnormalities of the gain of 8p12-22 in

some tumours may be related to the heterogeneity character of prostate cancer.

This result agreed also with the studies done in prostate cancer by Saramäki O. et al.[166] and Tørring N et al.[169]. They reported that a loss of 8p is combined with more 8q gains in advanced stages of prostate cancer.[166, 169] In comparison to other tumours, a loss of 8p and at the same time a gain of 8q was not only found in prostate cancer but also in other malignant tumours. Rummukainen J et al.[170], Kleive K et al.[171], Buerger H et al.[172], Dutrillaux B et al.[173] reported a loss of 8p, and a gain of 8q in breast cancer, Nishimura T et al.[174], Midorikawa Y et al.[175], Nishida N et al.[176], and Yang J et al.[177] in hepatocellular carcinoma, and Helou K et al.[178], Tapper J et al.[179] and Pere H et al.[180] in ovarian cancer.

In 2007, Ribera FR et al.[181] showed that the standard fluorescence protocol (FISH) can successfully be applied to diagnostic needle biopsies probes to identify relative 8q gain in prostate carcinomas. Patients having a ratio of MYC/CEP18 $\geq$ 1.5 had a significantly higher risk of dying from the prostate cancer. Also, Bubendorf L and co-worker[182] reported that *C-myc* oncogene on 8q24 was highly amplified in advanced prostate cancer. Moreover, Van Dekken H and co-workers[133] have reported that 8q is the second most common isochromosome that can be of clinical significance. Consequently, these results indicated the advantage of FISH in the delineation of numerical abnormalities. In this context, Macintosh CA et al.[183] reported that prostate cancer demonstrates the intra-tumour heterogeneity on chromosome 8p but also shows a low but significant incidence of LOH in mesenchymal tissue (non-tumorous cells of mesodermal origin).

All tumour samples of clinical stage pT3 showed a significant amplification of 7q31, except two tumour samples (Tumour sample 6 area 1 -Paraffin and Tumour sample 5 -Paraffin). Primary, this may be related to that both tumour samples were studied only in paraffin section and not in FISH in isolated cell nuclei. Secondary, this may be due to the fact that both examined areas did not represent the same histological score of the tumour areas as used for the pathological record. Furthermore the tumour foci may no longer be present in the analyzed sample by FISH due continues sectioning of the tumour blocks. 7q31 amplification is characteristic to the advanced clinical stage and metastasis. This result agreed with the recent studies which infer that 7q is significantly amplified in the later stage or higher Gleason pattern/score.[184] In other studies, in addition to 7q amplification, 7q31 was also found to be deleted.[185-187]

Three tumour samples were sectioned in two different tumour areas. Two of these samples showed similar chromosomal aberrations of chromosome 7 and 8 in both analyzed sections. The

third tumour sample showed that one of the tumour areas was without aberration of 7 or 8 but the other had a few cells with aberration of these chromosomes. This may be due to the fact that tumour foci may no longer be present in the analyzed tissue sample, or due to the heterogeneity and multifocality of prostate cancer.[125, 127, 165]

4.2.3. Specific chromosomal changes and relation to the clinical pathology (TNM stage and Gleason score)

The results obtained showed a close relation between 7q31 as well as 8q22-24 regions and the clinical pathology (TNM stage and Gleason score) especially to Gleason score, which describes the cytological changes of the tumour cells. 8p12-22 showed variation in the pre cancer stage/PIN, early malignancy stage, and moderate malignancy stage of the disease. In the high malignant stage and metastasis, 8p12-22 showed a clear loss. According to the obtained results, it can be suggested that 8p12-22 deletion especially the *NEFL* gene plays an important role in addition to others such as 16q or 10q in initiation of the prostate intra neoplasia (PIN) stage or pre-malignant stage. The frequency of this deletion increases in the advanced stages gradually. The 8p12-22 deletion may be accompanied by 7q31gain but this is not obligatory. 8p loss may combine with formation of isochromosome 8q. Both aberrations of chromosome 7 and 8 are associated with prostate cancer progression. Also, not only gain of 7q31 is increased with disease development but also amplification. The increase of 7q31 was not only found in advanced stages but also in metastasis.

This study succeeded to show that the 8p12-22 deletion simultaneously occurred with gains of 7q31 and may be the first step in the pre-cancer stage (prostate intra neoplasia (PIN)) or in very early stage of the prostate cancer. In the early stage of the disease (Gleason score 3-5), gain of 7q31 and 8q22-24 was observed and 8p12-22 gain was observed in very small numbers of the tumour samples. In moderate malignancy stages (Gleason score 6-7), tumour samples showed no loss or uncertain loss of 8p12-22, but clear gain of both 7q31 and 8q22-24 even amplification. High malignant stage (8-10) and metastatic prostate cancer showed loss of 8p12-22 and gain of both 7q31 and 8q22-24 forming isochromosome 8q without exception (Fig. 4.1).

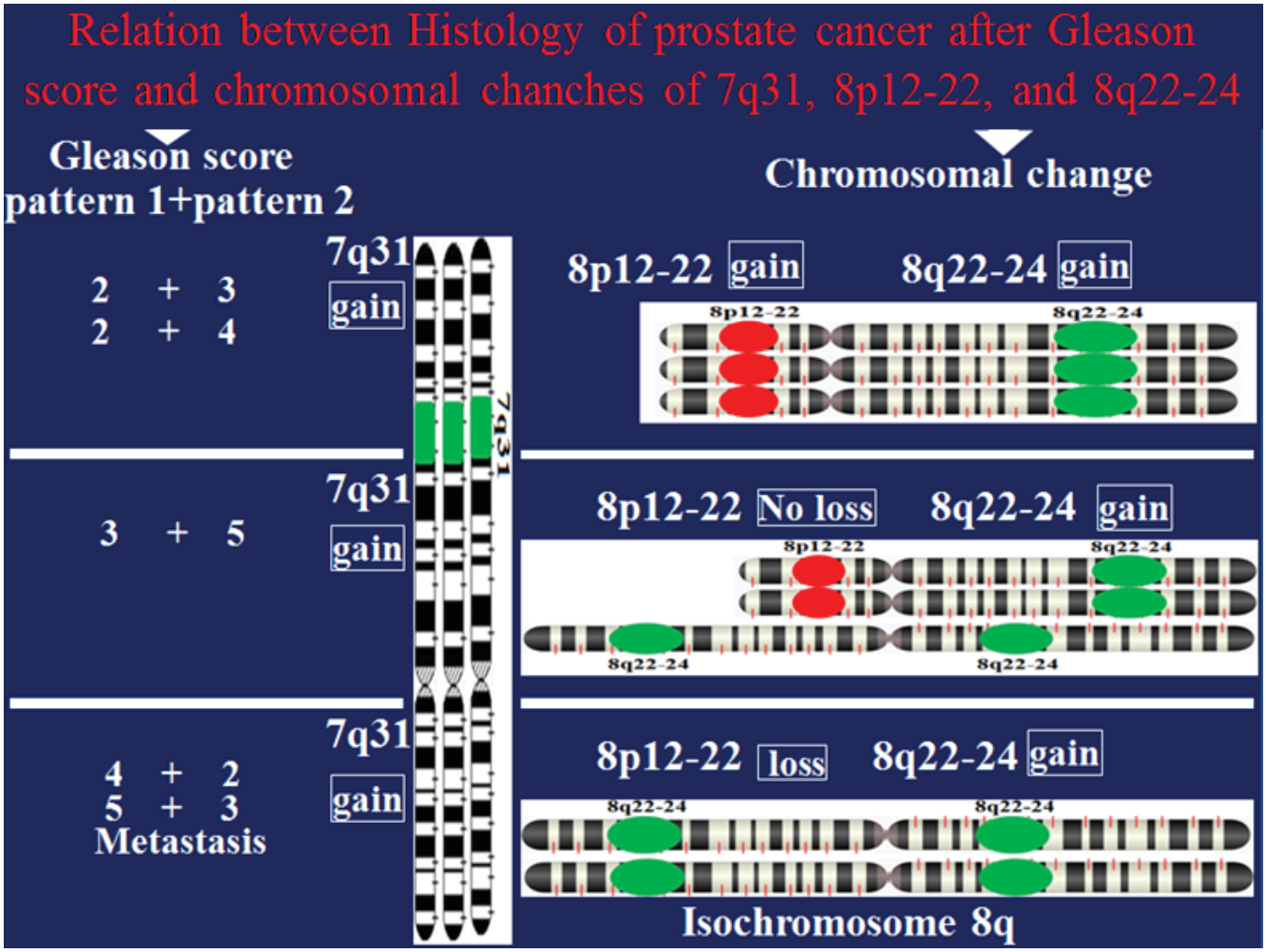


Fig. 4.1: The relationship of the disease development and the changes of chromosome 7 and 8 in prostate cancer.

In summary, FISH has been used to identify genetic markers that are frequently associated with pre-carcinogenesis (PIN)/premalignant, organ confirmed (pT1), and locally advanced or metastatic stage (pT3) of prostate cancer. FISH also represented a model for distinguishing between these three stages based on stepwise accumulation of specific genetic markers. Due to difficulties in analyzing paraffin sections and the laborious technique for isolation of cell nuclei, significant numbers of samples cannot be studied and this method is therefore not suitable for diagnostic purpose. However, even with the small number of cases analyzed similar observations were described in the literature.

4.3. Immunohistochemistry studies of Caveolin-1

4.3.1. Caveolin-1 expression in prostate cancer (prostate adenocarcinoma)

As the previous FISH study revealed a gain or amplification of the 7q31 region, containing the Caveolin-1 + 2 genes, it was of interest to study whether the encoded protein is also highly expressed in these cases. The Caveolin-1 gene codes for a 22-24 kDa integral membrane protein, which is a major structural component of the caveolae.[188-189] Caveolin-1 and Caveolin-2 show up to 60% homology in their amino acids sequences. The Caveolin-1 antibody was used because the highest levels of Caveolin-1 (also called Cav-1 and VIP21) are found in caveolae. Caveolin-1 is involved in signal transduction and is found in terminally differentiated cell types such as adipocytes, endothelia, smooth muscle cells, fibroblasts and type I pneumocytes.

The direct application of a Caveolin-1 antibody was not successful at first in immunhistochemical (IHC) studies of paraffin sections, suggesting that the Caveolin-1 protein (antigen) did not have free epitopes. Therefore it might be belong to the group of retrieval antigens: i.e. these antigens should be pretreated by heating in a proper buffer e.g. Citrate or EDTA buffers or enzymatically with pepsin, proteinase K, or trypsin. For retrieval of the Caveolin-1 antigen, the citrate buffer treatment was the best method in prostate sections. In addition to the known sensitivity of the LSAB-method, further optimization was done by testing and analyzing twelve other tissue sections using the Caveolin-1 antibody.

In the previous FISH studies, the 7q31 probe was found to be either gained or amplified in most if not all of the examined tumour samples; however these same tumours showed positive as well as negative expression of Caveolin-1. Positive Caveolin-1 expression varied in percentage of expression in tumour cells as well as intensity of expression. Caveolin-1 was not expressed in all tumours foci in this study as well as in the previous studies.[190] Because of these reasons a larger number of tumour samples were studied to obtain clear results about the relation between Caveolin-1 expression and prostate cancer. A correlation of 7q31 gain and Caveolin-1 expression was not reported in any study before.

The immunohistochemistry study of Caveolin-1 was divided into two independent groups (A and B) and the qualitative to semi-quantitative evaluation was used to answer the following questions: which cells in prostate cancer expressed Caveolin-1?, how many percent of the tumour cells expressed Caveolin-1?, and what was the intensity of expression of Caveolin-1 in these Caveolin-1 positive cells?. Answering the above questions could clarify the pattern of Caveolin-1

expression in prostate cancer. Additionally, this allowed more detailed information about Caveolin-1 expression in cell compartments, different tumour areas, and intensity of the expression not only in different tumour samples but also in the same tumour sample in comparison with the clinical and histological pathology, as has been shown in FISH study in different tumour samples and areas.

4.3.1.1. Qualitative study of Caveolin-1 expression in prostate cancer and related tissues

First, the Caveolin-1 protein expression was studied in the most common cells types of the prostate parenchyma such as the secretory (luminal/glandular) epithelial cells and the basal epithelial cells in addition to tumour cells present in the prostate acini. The third cell type of the glandular part of the prostate, namely the neuroendocrine cells, was not commonly present. Thus, they were not of special interest because this study concentrated on the adenocarcinoma, which represents about 95% of prostate cancer and originated from the secretory epithelial cells (luminal epithelial cell layer). The glandular part of the prostate is embedded in connective tissue, which consists of smooth muscle cells, fibroblast cells, adipocytes, and blood vessels.

For a new biomarker in general, it is obligatory to test the cells that express this protein in different tissue types. Next was the evaluation of the expression of this biomarker semi-quantitatively in normal or tumour cells in comparison to the other tissues.

The glandular cells of the prostate gland (acini) showed differences in expression either in numbers or intensity. The results showed a steady increase in Caveolin-1 expression from the normal secretory epithelial cells through prostate intra neoplasia (PIN) cells to tumour secretory cells as partially published during the course of this work.[67, 191] During this thesis work no study analyzed the Caveolin-1 expression in primary secretory epithelial cells, prostate intra neoplasia (PIN), and different clinical stages or malignant grades of tumour secretory cells as well as metastasis together in one complete study.[66-67, 191-192]

In the qualitative analysis, a distinction was made between 1) negative-, 2) scattered positive-, 3) patches of positive-, and 4) absolute positive cells. A Caveolin-1 negative tumour sample refers to a tumour, where all tumour cells were Caveolin-1 negative, or only a small number of the tumour cells were positive (≤ 10%). The single scattered positive tumour cells constituted up to 40% of the tumour cells, and were surrounded by Caveolin-1 negative tumour cells. The tumour cells were characterized by small cytoplasm, big nuclei, more than two nuclei, or big cell diameters. Patches of Caveolin-1 positive tumour cells contained 40-75% of the tumour cells, and the absolute Caveolin-1 positive tumour cells included about 80% or more.

These evaluations of Caveolin-1 expression into four categories (negative, scattered positive, patches of positive, and nearly all tumour cells Caveolin-1 positive) served to identify the manner of expression without referring to the number of cells stained from the total number of tumour cells (Evaluation in [%]). It is known that the hallmark of early malignancy of the prostate is the absence of basal cells and the loss of a basement membrane.[37-38] Interestingly without exception, the basal epithelial cells lining the basement membrane were always Caveolin-1 positive if not replaced or destroyed by tumour cells. Consequently, the absence of basal cell layers in prostatic epithelium of the acini is a good indicator or a key to the diagnosis of the malignancy of prostate cancer. This absence of basal cells in prostate cancer resulted from the replacement by tumour cells and spreading out the acini. Additionally, their absence in a tumour sample is an indicator of high malignancy grade or the tumour had spread out of the prostate capsule in other organs (metastasis). This is a highly significant observation useful in the analysis of prostate cancer. It helps to determine the degree of the progression and to see whether the malignancy was organ confined, extra-capsular infiltrated, or extended in addition to distant metastases. This finding about Caveolin-1 expression in basal cells was not previously described. Accordingly, Caveolin-1 could be used as a new basal cell biomarker in prostate cancer, besides the widely reported markers such as cytokeratin, Bcl-2, p63, K5, K14, and Glutathione-transferase π (GST π) proteins which were identified in the last years.[193-194]

This finding agreed with the results obtained by Rehman l and co-workers[195], who mentioned that S100A6 (Calcyclin) was intensively expressed in the basal cells of benign glands but their loss of expression in cancer could be useful as a novel diagnostic marker for prostate cancer. Also, Jarrard D F et al.[196] reported that P-cadherin represents a basal epithelial marker in normal prostate tissues that is inherently lost during the formation of the prostate cancer.[196] Independently, the absence of basal cells belongs to the major criteria and not to minor criteria for the diagnosis of prostatic adenocarcinoma.[197] Also, infiltrative glandular growth pattern and nuclear atypia belong to the major criteria for prostate cancer diagnostic.

In the current immunohistochemical analysis, Caveolin-1 was shown to be expressed not only in basal cells but also in tumour cells. This can be considered an advantage in using Caveolin-1 to identify tumours with or without loss of basal cells. Consequently, it could be used as a specific biomarker for the detection of basal cells in normal prostate tissue. Moreover, intense expression of Caveolin-1 in tumor stromal adipocytes and fibroblasts could also be used in the future as a positive internal control of the immunohistochemical technique. This control staining in each sample was

not reported previously in any other study using basal cells markers. Furthermore, this internal control supports the reliability of the result, especially in paraffin sections, which sometimes showed unspecific reaction. Therefore, the results obtained in this analysis suggest that Caveolin-1 can be used as a new component of antibody cocktails used for detection of basal cells as well as tumour cells in lesions suspected of malignancy in prostate core biopsies, paraffin section, and prostatectomy specimens.

Characteristic for Caveolin-1 in this study is their expression either in cytoplasm/nucleus or both of them and could be used alone as basal cells marker in case of prostate cancer because it was expressed in all basal cells without exemption if they were present in prostate cancer tissue. This character make Caveolin-1 expression comparable with best basal cells known markers in case of prostate cancer namely 34βE12, keratin 5/6, and p63. Boran C and co-workers suggest that the use of basal cell markers in combination was not helpful. Otherwise, they referred that the best 2 basal cell markers combination may be the 34βE12 and p63 because 34βE12 is the best cytoplasmic marker according to some studies and p63 is the only nuclear marker among basal cell markers.[198]

4.3.1.2. Caveolin-1 expression in prostate intraepithelial neoplasia (PIN)

It is normal in prostate cancer to find normal, premalignant, and malignant foci with different Gleason scores or grades near each other representing the heterogeneous nature of prostate cancer as a solid tumour. PIN has been considered as a premalignant stage in the prostate cancer by many authors and plays an important role in disease diagnosis.[199-200] PIN is either low grade (LGPIN) or high grade (HGPIN). In both PIN types, the basal cells are still present and these were always stained with Caveolin-1 (see Fig. 3.13, and Table 3.11).

The presence of scattered single Caveolin-1 positive cells in PIN stage leads to the suggestion that Caveolin-1 might play a role in disease development. This suggestion should be further studied and analyzed in a high number of prostate tumour samples containing PIN, of pT1, pT2, pT3, and pT4 stages in order to make a comparison of Caveolin-1 expression.

4.3.1.3. Preferred sites of prostate cancer metastasis

Due to topographical relationships to the urinary bladder, the prostate cancer cells could metastasize to the bladder tissue. However, this is found in a smaller number of cases (see Table 3.7 and Table 3.11), but they could unravel the manner of Caveolin-1 expression in this metastatic tissue site. The tumour cells in these metastasized foci in the urinary bladder were either negative or single scattered positive for Caveolin-1 expression. Positive Caveolin-1 expression was not found in

all of the metastatic cells (foci) in any sample of the urinary bladder. This may be related to the new microenvironment of the urinary bladder because cell interactions with extracellular matrix proteins control proliferation, differentiation and survival[201-204], as well as tumour growth, angiogenesis and metastasis.[205] Another reason may be related to the origin of metastasis cells based on their expression of Caveolin-1 before they metastize to the urinary bladder. Schultze and Isaacs[160] in 1986 revealed that individual prostatic cancer is composed of multiple clones. Therefore no conclusion can be drawn about the Caveolin-1 expression in this metastatic tissue, due to the insufficient number of urinary bladder samples. Caveolin-1 expression in lymph nodes metastasis is important because they are the first regions of metastasis after the spreading of prostate cancer and a predictor for poor prognosis or high malignancy grade. Prostate cancer cells migrate through the lymphatic system to closer and distant lymph nodes. No cells of these lymph nodes showed Caveolin-1 expression (see Table 3.11). However, some studies referred that the metastasized prostate cancer cells in lymph node express Caveolin-1.[68, 73] Thompson T[64] and Thompson et al.[206] have reported that Caveolin-1 was found to be over-expressed not only in metastatic mouse prostate cancer but also in human metastatic diseases[64, 206] as well as cell lines[207]. The negative expression of Caveolin-1 in this work in the four lymph nodes might be related to the origin of the metastasis cells. Also, the negative expression in these lymph nodes might be related to pre-treatment of the patients before radical prostactomy. Four samples of metastasis lymph nodes are not enough to give a clear statement, but we did not have more samples to study.

In conclusion, due to small sample numbers it was not possible to give a definite answer about the Caveolin-1 expression in the prostate intraepithelial neoplasia (PIN) as well as in the metastasis tissues such as urinary bladder and lymph nodes.

4.3.2. Semi-quantitative study of Caveolin-1 expression of prostate cancer

The semi-quantitative study was done to determine the variable expression in percentage and intensity of Caveolin-1 expression in prostate cancer samples.

4.3.2.1. Correlation between Caveolin-1 Evaluation in [%] and clinical pathology (TNM stage and WHO grade)

Like other prostate cancer markers, Caveolin-1 expression was not expressed uniformly in the examined cells of the tumour samples. Evaluation in [%] i.e. percentage of the tumour cells expressing Caveolin-1, showed that the percentage of tumour cells which expressed Caveolin-1 were different from one tumour sample to another. There was also variation in the expression of

Caveolin-1 in percent between different areas of the same tumour sample confirming the heterogeneity of prostate cancer. The percentage of expression ranged between 0% (negative) to 100% (positive) of the tumour cells without taking into consideration the intensity of Caveolin-1 staining.

Correlation of Caveolin-1 expression between different clinical stages showed a clear decrease in the percentage of the Caveolin-1 negative tumours and at the same time increase of the Caveolin-1 positive tumours from pT1 through pT2 to pT3 (see Table 3.13). The tumour samples were 100% negative in stage pT1, 45.83% negative in pT2, and 27.27% negative in pT3. Tumour samples showed weak expression in 0.0%, 16.67%, and 11.36% in pT1, pT2, and pT3, respectively. Tumour samples showed moderate expression in 0.0%, 4.17%, and 6.82% in pT1, pT2, and pT3, respectively. Stage pT2 and stage pT3 tumours showed strong expression in 33.33% and 54.55%, respectively. The histograms showed a suggestive correlation between the evaluation in [%] and TNM stages, pT1, pT2, and pT3/pT4 (see Table 3.13 and Fig. 3.15-3.16). This indicated a positive increase of the percentage of expression with the TNM stages of prostate cancer.

This is the first statistical analysis studying the correlation between the evaluation in [%] of Caveolin-1 expression in prostate cancer and the clinical TNM stages as well as histological WHO grades which were available in the pathology records. At the beginning of this statistical evaluation, the TNM stages were classified into three groups, pT1, pT2, and pT3. The presence of only two tumour samples in stage pT4, which is an advanced stage of pT3 were the reasons to combine stage pT3 and pT4 in one group (or pT3/pT4) in the statistical analysis study.

A noteworthy finding in this current analysis study was that the percentage of Caveolin-1 expression (Evaluation in [%]) was significantly correlated with TNM stages (pT1 to pT3/pT4) using Kendall`s tau test ($P = 0.018$) as seen in Fig. 3.17. There was no significant difference between the percentage of Caveolin-1 expression (Evaluation in [%]) and WHO grades using Kendall`s tau test ($P = 0.561$). The tendency of Caveolin-1 expression was increased from grade 1.to grade 3, but not from grade 2 to grade 3. This non-significant correlation with the WHO grade may be related to the small size of tumour samples having grade 1. Prostate cancer was mostly diagnosed in grade 2 and grade 3 and rarely identified in grade 1.

Another prostate cancer marker namely AMACR (P504S) and AMACR-p showed also heterogeneous expression in the tumour acini (cancer gland cells) like Caveolin-1. In addition,

AMCAR (P504S) and AMCAR-p showed different staining intensities in different glands within the same lesion similar to Caveolin-1.[208]

The Caveolin-1 expression ranged from 5 to 100% of the tumour gland cells. Using the same evaluation criteria that were described in previous studies, the tumour samples which expressed Caveolin-1 in less than 50% of their tumour cells were considered as Caveolin-1 negative tumours, whereas those that expressed Caveolin-1 in more than 50% of their tumour cells were considered as Caveolin-1 positive tumours.[68] Consequently, the negative tumour samples expressing Caveolin-1 were 100%, 62.5% (45.83 < 10% and 16.67% < 50%), and 38. 63% (27.27 < 10% and 11.36 < 50%) in stage pT1, pT2, and pT3, respectively (see Table 3.12 and Table 3.13). In addition in my study nearly 50% (9 tumour sample from pT2 and 27 from pT3) of the total samples (74 tumour samples) without consideration to the TNM stages were Caveolin-1 positive tumours. But 75.00% (27 from stage pT3 to 36 total positive tumour samples) of these were positive Caveolin-1 tumours coming from stage pT3 (see Table 3.12-3.13 and chapter 3.3.3). Otherwise, the expression of Caveolin-1 was positive in 0.0%, 37.50% (4.17% + 33.33%), and 61.37% (6.82% + 54.55%) in stage pT1, pT2, and pT3, respectively. Consequently, the percentage of Caveolin-1 expression in pT3 showed more than 60.00% increase than in pT2. In addition, using Kendall's tau test showed significant increase of Caveolin-1 expression in percentage (or Evaluation in [%]) from stage pT1 to stage pT3/pT4 ($P = 0.018$). The above mentioned results show the importance of Caveolin-1 as a biomarker in prostate cancer.

These statistical analysis of evaluation in [%] supported Caveolin-1 as a better prognostic biomarker than AMACR (P504S) and AMCAR-p for prostate cancer diagnostic.

4.3.2.2. Correlation between immunoscore and clinical pathology (TNM stage and WHO grade)

The second part of the Caveolin-1 expression study (group B) evaluated not only the percent of the tumour cells expressing Caveolin-1 (Evaluation in [%]) but also the intensity grade or strength of expression. Additionally, this study correlated the semi-quantitative expression of Caveolin-1 (immunoscore) and tumour progression represented in the TNM clinical stages as well as WHO histological grades and its diagnostic value as a proposed biomarker of prostate cancer.

The analysis of Caveolin-1 expression intensity showed that it increased steadily with the increased percentage of the tumour cells expressing Caveolin-1 from stage pT1 to pT3/pT4 (see Fig. 3.17A). Moreover, this increase in Caveolin-1 intensity is seen more clearly in stage

pT3/pT4 than in stage pT2. Additionally, this intensity was combined with lymph node metastasis (N) or distant metastasis (M). This means that the Caveolin-1 over-expression is correlated with the disease development as well as progression (see Table 3.12). However, some tumour samples were aggressive, but Caveolin-1 was not expressed in the tumour sample cells. This is not only the first statistical study referring to the correlation between the percentage of the tumour cells expressing Caveolin-1 (Evaluation in [%]), but also to their intensity as well as immunoscore of expression to TNM-stages as well as WHO grades.

Although the Caveolin-1 intensity of expression showed a tendency of increase with the increase of the TNM stages (pT1 to pT3/pT4), the correlation with the TNM stages using Kendall's tau test did not reach a significant P value (P = 0.0619) as seen in Fig. 3.18. There was no correlation between the Caveolin-1 intensity of expression and WHO grades (P = 0.206).

There are different ways to determine an immunoscore using the percentage of tumour cells expressing Caveolin-1 to the total tumour cells present in the tumour and intensity of expression (Evaluation in [%] x intensity = immunoscore). Two methods for determination of Caveolin-1 immunoscore of expression were used in this work, the first (immunoscore 1) was based on the method described by Tan et al. (1953); the second method (immunoscore 2) was based on the Remmele score.[209-213]

The Caveolin immunoscore 1 after Tan et al. describes four categories: negative-low (0-50), moderate (51-100), high (101-200), and very high (201-300) expression. The presence of a negative or low immunoscore was only found in stage pT1. Using immunoscore 1, tumour samples showed negative-low expression of Caveoli-1 in 100%, 62.50%, and 38.64% in stage pT1, pT2, and pT3, respectively. Moderate expression was found in 16.67% and 36.36% in stage pT2 and pT3, respectively. High expression was found in 16.67% and 15.91%, in stage pT2 and pT3, and strong (or very high) expression in 4.17% and 9 .10% in pT2 and pT3, respectively (see Table 3.14). This study of analysis showed a positive increase in the immunoscore 1 of Caveolin-1 expression with the TNM stages of prostate cancer.

A significant correlation of the immunoscore 1 with TNM stages using Kendall's tau test (P = 0.0332) was found. Both of the significant differences between the evaluation in [%] (P = 0.018) as well as immunoscore 1 of expression (P = 0.0332) and TNM stages (pT1 to pT3/pT4) obtained in this statistical analysis supported the correlation of Caveolin-1 expression with the TNM stages of the disease (see Fig. 3.17 and Fig. 3.19). Although, there was no significant difference between

Caveolin immunoscore 1 and WHO grades using Kendall's tau test (P = 0.5), the bar plot in figure 3.19 showed also a tendency of the increase of Caveolin-1 expression with the increase of WHO grades.

Using the method to determine the immunoscore 2, no significant correlation was seen with TNM stages or WHO grades using Kendall's tau test, where P values were 0.132 and 0.427, respectively (see Fig. 3.20).

Using the Caveolin immunoscore 1 was a better way to analyze the data than immunoscore 2 for the following reasons: 1) The range of the Caveolin immunoscore 1 was larger (0-300) than the Caveolin immunoscore 2 (0-12). Consequently, it describes the expression of Caveolin-1 in each tumour sample in a more detailed way. 2) Both of the Caveolin immunoscores 1 and 2 resulted from the same intensity grade (0-3) after multiplication either in the percentage of expression (0-100%) or reactive point of expression (0-4) respectively. But, the result in the Caveolin immunoscore 1 is equivalent to the exact value of the semi-quantitative expression, demonstrated in the following example: a tumour sample expressed Caveolin-1 in 50% of its cells and had intensity score 1 (weak expression), resulting in the Caveolin immunoscore 1 = 50 (i.e. one sixth of the maximal value of 300). The Caveolin immunoscore 2 in the same tumour sample would be one fourth of the maximal value (12) which results from the reactive point 3 (40%-75% of the expression in the tumour sample) multiplied by intensity score 1.

The prostate cancer disease develops very slowly and showed a slowly increase of Caveolin-1 expression in the pre-cancer to low differentiated grades. Caveolin-1 expression was highly increased in percentage and intensity of expression in the tumour cells in the late stages of prostate adenocarcinoma and showed significant correlation with TNM stages. This study showed that not only Caveolin-1 expression in cell percentage and its intensity of expression were correlated with TNM stages but also correlated with regional lymph nodes, distant metastasis and regressions of the tumours after analyzing the data obtained in table 3.12. This thesis study of Caveolin-1 expression in prostate cancer is in agreement with the recent study done by Williams TM et al.[191] in the TRAMP mice model. They demonstrated that Caveolin-1 is expressed at virtually undetectable levels in the wild-type (WT) and early transformed epithelium to prostate intraepithelial neoplasia prostate (PIN). Also, Caveolin-1 expression was significantly expressed in the poorly differentiated TRAMP tumour. Not only Caveolin-1 was intensively expressed in the late stages of prostate adenocarcinoma development, but also Caveolin-1 expression was significantly expressed in the poorly differentiated TRAMP.

Another interesting finding in this work was the staining of the tumour cells in the cytoplasm, the cell nuclei alone or in both of them. This finding was not reported previously in prostate cancer. It was not clear whether the expression of Caveolin-1 in the cell nuclei or in the cytoplasm as well as in the cytoplasm and cell nuclei at the same time has pathological or clinical importance in prostate cancer progression/development and diagnosis or not. For the investigation and answering of this question, a separate immunohistochemistry study in a large number of tumour samples from different stages or with different WHO grades and Gleason scores, including other samples from normal prostate tissue, benign prostate hyperplasia (BPH), prostate atrophy as well as premalignant tissue samples, namely prostate intraepithelial neoplasia (PIN), should be analyzed.

Yang G et al.[68] studied Caveolin-1 expression in prostate cancer of American patients in two separate studies. They reported in one study that in white Americans, Caveolin-1 was expressed in 25%, but it was expressed in 45% of African-Americans in a small sample size.[68] In the other study to prostate cancer, Caveolin-1 was expressed in 17% of white Americans patients, but it was expressed in 39% of African-Americans.[69] In addition, they reported that Caveolin-1 over-expression was significantly more frequent in African American men, whereas the differential expression of Bcl-2, C-Myc, and p53 was not different between African American and Caucasian men. Additionally, Caveolin-1 expression was 30.3% in the Japanese patients.[73] It is known that androgen expression in black people is higher than others.[214-217] It seems also that Caveolin-1 expression is different from one ethnic group to another. The results obtained in this thesis revealed details about the Caveolin-1 expression in different cell types of the prostate cancer tissue including basal cells, normal glandular, connective tissue cells, tumour cells, and metastized organs in addition to the statistical analysis to the correlation with TNM stage and WHO grade. Yang G et al.[68] showed no significant correlation between Caveolin-1 expression and clinical TNM stage in 47 tumour samples but significant association between Caveolin-1 and Gleason score in 189 tumour samples. Additionally, this increase in the Caveolin-1 expression either in percentage or intensity was more pronounced in tumour samples with lymph nodes (N) and distant metastasis (M). Satoh T et al.[73] have shown in their study of pT2N0 stage samples that patients with poorly or low differentiated tumours had a higher incidence of Caveolin-1 expression. They described that Caveolin-1 was expressed in 35.0%, 34.9%, and 20.4% in patients with Gleason score > 7, 6-7, and < 6, respectively or in patients with extra-prostatic extension versus those without extra-prostatic extension (35.4% versus 24.7%) or in patients with lymph node involvement (metastasis) compared with those without involvement, in 50% and 29% respectively. Yang et al reported also that

caveolin-1-positive cancers were significantly higher in patients who had positive lymph nodes than in those with negative nodes.

Caveolin-1 is required in the caveolae formation as demonstrated in previous studies using mice models, because virtually all tissues in Caveolin-1-null-mice completely lack these organelles.[191, 218-219] To assess the direct role of Caveolin-1 in the mouse prostate cancer cells with regard to tumorigenesis, Terence M. Williams et al.[191] investigated three prostate carcinoma cell lines derived from WT (wild type) TRAMP prostate cancer tumours-TRAMP-C1, -C2, and C3 (C1, C2, and C3 are TRAMP prostate carcinoma cell lines which represent various stages in prostate cancer progression).[191] These cell lines express cytokeratin, E-cadherin, and androgen-receptors and confirm their epithelial and prostate origin.[220] The authors found that C1 demonstrated relatively high levels of Caveolin-1. C2 possessed moderate Caveolin-1 levels, whereas C3 expressed virtually no Caveolin-1. However, Caveolin-2 expression did not change in the three cell lines.

These data suggest that the Caveolin-1 gene acts as an oncogene or tumour promoter depending on the tissue and is highly context depended. Yang G et al.[67] showed clearly the association of the increased accumulation of Caveolin-1 with the progression of human prostate cancer as well as primary and metastatic breast cancer relative to normal epithelium.[67] This accumulation of Caveolin-1 expression was extensive in metastatic disease of prostate cancer. Nasu Y et al.[221] demonstrated that suppression of Caveolin-1 levels led to re-establishment of androgen sensitivity in vitro and in vivo. Other studies in human cell lines have shown that Caveolin-1 is up-regulated in multi-drug-resistance cancer cells. In some cases, this up-regulation is independent of p-glycoprotein.[222-224] Recently, Timme and co-authors[225] showed that Caveolin-1 suppressed *c-myc*- induced apoptosis in Rat1A and LNCap cells.

At present, the TRAMP model (transgenic adenocarcinoma of mouse prostate model) provides a reasonable explanation of the Caveolin-1 role in the prostate tumorigenesis, where the intrinsic loss of the Caveolin-1 gene within prostate carcinoma cells leads dramatically to the reduction of the tumour burden and experimental metastasis. Otherwise, the cell culture studies show that Caveolin-1 has pro-survival roles in prostate cancer, as both TRAMP prostate tumours and TRAMP-cell line tumours lacking Caveolin-1 expression showed increased rates of apoptosis.[191] Furthermore, Ayala G E et al.[226] revealed that Caveolin-1 is secreted into the microenvironment (stroma) and is used by the prostate cancer cells to inhibit apoptosis. These

previous studies support the relationship between Caveolin-1 up-regulation and decreasing of apoptosis consequently increasing the cell survival and therefore tumorigenesis.[226-228]

At present, the physiological consequences of Caveolin-1 over-expression remain controversial.[229] Thus, Caveolin-1 seems to behave in a tissue-dependent manner.[230-231, 222, 232-233] It confirms that tumours often utilize multifunctional molecules to grow or to survive in the harsh environment of the host. The best example of this is the activation of the *Ras* gene.[234-235] It has been known for a long time that prostate carcinoma is androgen-dependent; and in prostate carcinoma, Caveolin-1 mediates testosterone stimulated survival/colony growth and promotes metastatic activity.[236] Caveolin-1 primary functions as a suppressor gene has been mentioned by Wiechen et al. in fibroblast cell lines.[233] There are three distinct echanisms that can serve to functionally inactivate the suppression function of Caveolin-1 protein, namely tyrosine phosphorylation, serine phosphorylation, and dominant-negative point mutation (P132L). These mechanisms could explain why Caveolin-1 has been suggested to act as a tumour suppressor or as an oncogene depending on microenvironment, signal transduction modulation, the tumour tissue type and/or tumour stage.[191, 237]

The findings of Caveolin-1 expression in this work can be summarized as follows: Evaluation in [%] and semi-quantitative (immunoscore) expression increase with the TNM stages. Both comparison of the four classes of the evaluation in [%] as well as semi-quantitative (immunoscore) of expression with the TNM stages showed a correlation (see Table 3.12-3.14 and Fig. 3.17-3.20). Additionally, there was a significant correlation between TNM stages and evaluation in [%] as well as semi-quantitative (immunoscore) expression. Moreover, it showed a gradual increase of expression with WHO grade. Caveolin-1 was found to be a good basal tumour cell marker in prostate cancer (Fig. 4.2).

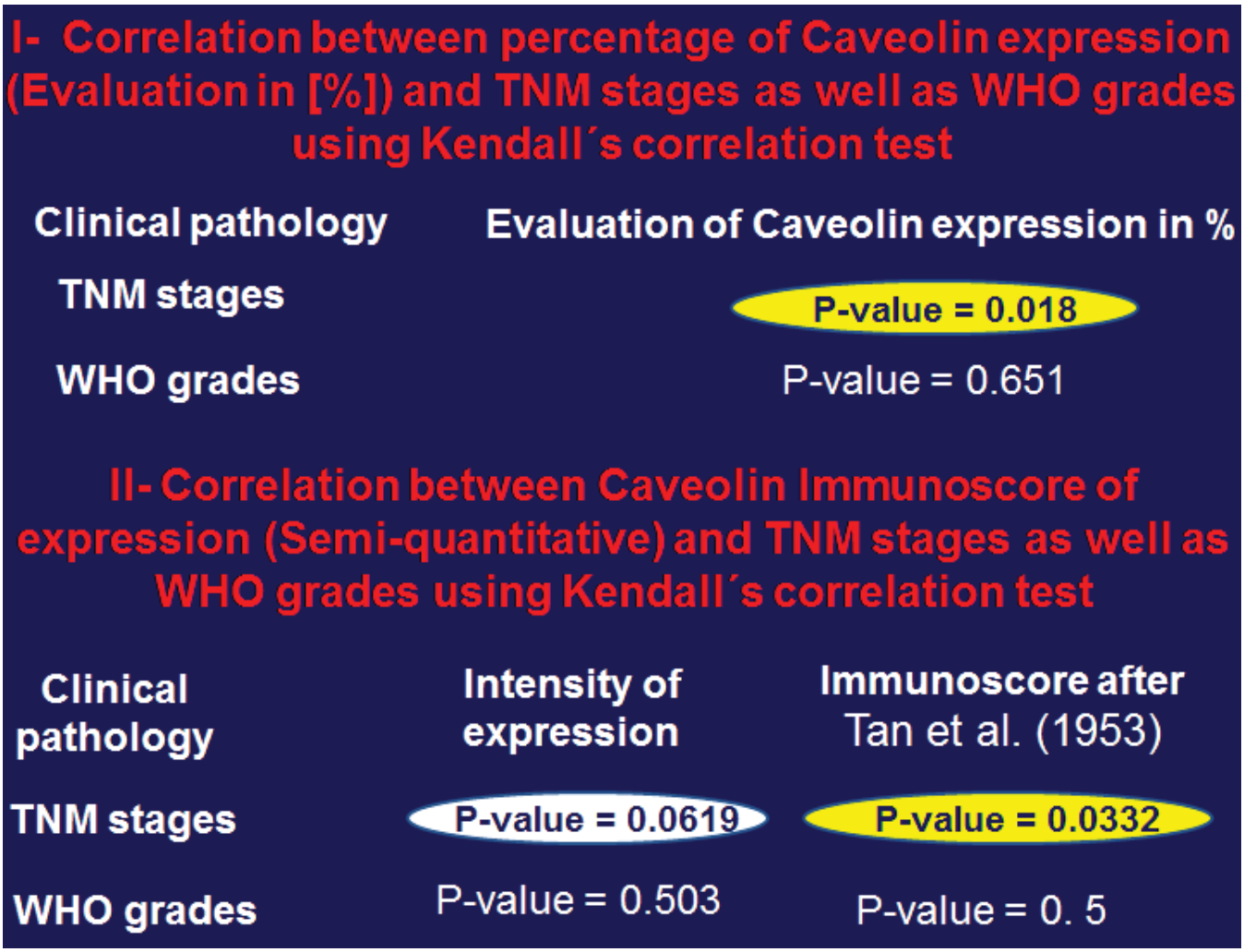


Fig. 4.2: A summary of the statistical analysis of Caveolin-1 expression and TNM stages as well as WHO grades using Kendall`s rank test in prostate cancer.

These data also show that although Caveolin-1 expression increases with clinical stage, the grades do not reflect the dignity of the tumour, most likely due to the small tumour samples size in grade 1.

4.4. YB-1 a potential biomarker for prostate cancer

YB-1 is a multifunctional DNA/RNA-binding protein that participates in reproduction, storing, and expression of genetic information. It possesses high specific and non-specific affinity for RNA and accompanies mRNA during its entire lifetime, from synthesis to decay. YB-1 regulates mRNA processing in the nucleus and is responsible for the global and selective regulation of protein synthesis and for mRNA life span in the cytoplasm. Also, YB-1 takes part in the translation, signal transduction, and RNA translation and metabolism.[238]

In addition to Caveolin-1, tumour samples also were examined here using immunohistochemistry for expression of the YB-1 protein. YB-1 has been described as a diagnostic marker for breast[85, 240], colon[239], synovial sarcoma[241], and ovarian cance[242]. In normal breast tissue cells, YB-1 is not expressed but is highly expressed in tumour cells.[74-76, 191] Both breast and prostate are controlled from the hypothalamus and pituitary glands and the two tumours are hormone dependent. During the course of my thesis work one study by Gimenez-Bonafe et al. to YB-1 described expression in prostate cancer without referring to the expression in different TNM stages as well as WHO grades, whereas many studies for YB-1 expression have been done in breast[85, 240], colon[239], and osteosarcoma cancer[84] and a positive prognostic value was established for breast cancer.[85, 240]

I could show here that YB-1 expression was negative to weak in the normal secretory prostate cells in comparison to prostate intraepithelial neoplasia (PIN) and tumour cells. A prognostic impact of YB-1 expression without attention to its distribution between the cytoplasm and the nucleus was found when tumour subgroups (according to TNM staging or WHO grading systems) were evaluated (see Table 3.16 and 3.18-3.19 in Appendix). This leads to the suggestion that YB-1 could be an independent prognostic marker of prostate cancer as it is in non-small cell lung cancer (non-SCLC).[245] The clinical stage pT1 (n = 0) and stage pT4 (n = 1) of tumour samples were not represented in a sufficient number for statistical analysis in this study of YB-1 expression. Consequently, the pT1 and pT4 stages were excluded from the current evaluation. The goal of this study was to find a correlation between YB-1 expression and clinical stages (TNM stages) as well as histology grades (WHO grades) of prostate cancer.

In this study a link between YB-1 expression in different cells of the glandular part of the prostate including normal secretory cells, PIN cells, and tumour cells as well as following their expression in different TNM stages and WHO grades was found. The number of the tumour

samples having PIN foci was decreased from pT2 to pT3, due to progression of the prostate cancer as expected. PIN foci are transformed to tumour foci in stage pT3. This can be clearly seen if the mean value of PIN in stage pT2 and pT3 was compared (1.32 and 0.57 respectively) (see Table 3.18 in Appendix). The comparison between the mean of the immunoscore of YB-1 in normal glandular cells, tumour cells, and samples with PIN foci (7 samples) showed that PIN cells had the higher mean value, followed by tumour cells and normal secretory cells which was the smallest value (see Table 20 and Fig. 3.22). The statistical analysis of the correlation between YB-1 immunoscore of expression in normal secretory cells and tumour cells in TNM stages as well as WHO grades was performed using the Wilcoxon test. For studying the correlation between YB-1 immunoscore and groups of independent variables, the Friedman test and Kendall's tau test were used. The study of the correlation between YB-1 immunoscore in normal secretory cells and tumour cells in 49 tumour samples showed a significant difference (P = 0.000014) using Wilcoxon test (see Fig. 3.24A). In 7 tumour samples, PIN foci were identified near tumour foci. The YB-1 immunoscore of expression in these foci were determined. The correlation of the YB-1 immunoscore between normal secretory cells, PIN cells, and tumour cells using Friedman test in these 7 tumour samples showed a significant difference (P = 0.00926) as seen in Fig. 3.24B.

Using the Remmele score, the YB-1 immunoscore was between 0 and 12. The mean value of YB-1 expression (immunoscore) in tumour cells was higher in the pT3 stage tumor samples than pT2 stage but lower in normal cells present in the pT3 to pT2 stage. Hence, YB-1 showed a tendency to be correlated with the disease's aggressiveness, if the expression was compared between the normal secretory cells and the tumour cells. There were no significant differences between YB-1 immunoscore in normal secretory cells in stage pT2 and pT3 as well as between tumour cells of stage pT2 and stage pT3. Interestingly, the YB-1 immunoscore in stage pT2 and pT3 of the tumour cells was higher than the corresponding normal secretory cells and showed a significant difference using Wilcoxon test with P values of 0.0009873 and 0.003792 respectively (see Table 3.22). Like clinical TNM stages, the histological WHO grades of prostate cancer showed a significant correlation with YB-1 expression between normal and tumour cells. YB-1 expression showed no significant difference between normal secretory cells (luminal epithelial cells) of grade 1 and normal secretory cells of grade 1-2, between grade G1-G2 and grade G2, between grade G2 and grade G2-G3, as well as between grade G2-G3 and grade G3 using Wilcoxon test. In addition, YB-1 immunoscore increased gradually in tumour cells of the histological grade G1 through grade G1-G2, grade G2, and reaching the highest value in grade G2-G3, and then stayed nearly constant in

grade 3. But, this increase of YB-1 immunoscore in tumour cells between the different grades did not reach a statistically significant difference. (see Table 3.25).

Not only the tumour cells showed a gradual increase in immunoscore with the increased malignant grade but also the difference of the immunoscore between tumour and normal secretory cells (immunoscore in tumour minus immunoscore in normal) showed a gradual increase in immunoscore (see Fig. 3.25). This gradual increase of YB-1 immunoscore in tumour cells of increasing grades simulates the slow developmental character of prostate cancer, which takes a long time to manifest. There was a positive correlation between YB-1 immunoscore in normal sceretory cells and tumour cells of grade G2 ($P = 0.003891$) using Wilcoxon test and between the immunoscore in normal secretory cells and tumour cells in grade G3 using Wilcoxon test ($P = 0.01310$).

The correlation between YB-1 immunoscore in normal secretory cells and tumour cells in stage pT2 and pT3 were more correlated than the comparable ones in grade G2 and G3 (Fig. 4.3). Notably, the significant correlation of YB-1 immunoscore between normal secretory cells and tumour cells in grade G2 and pT2 were more correlated than in grade G3 and stage pT3, respectively. This finding suggests that the disease progression occurred in the clinical pathology stage pT2 as well as histological grade 2 that are relevant to the midpoint of change from low malignant to poorly or low differentiated cells. The gradual development of expression of YB-1 in normal secretory and tumour cells in the tumour samples through different stages and grades indicates that these cells may be transformed slowly from normal to tumour cells may be under the influence of the YB-1 in the tumorigenesis process. Otherwise, it could act as an oncogene or tumour promoter in prostate cancer.

Using a comparable statistical analysis to the Caveolin immunoscore of expression and WHO grades, the tumour samples under study of YB-1 were sub-grouped into three main grades, namely grade 1, grade 2, and grade 3. There was no significant correlation of YB-1 immunoscore between the different WHO grades using Kendall's tau test ($P = 0.206$) as seen in Fig. 3.26A-D (where grade 1 = G1 + G1-G2, grade 2 = G2 + G2-3 and grade 3), but the tendency of the YB-1 immunoscore increased with higher grades. In contrast, there was no sign of increase in YB-1 immunoscore with the higher TNM stages. This may be due to the absence of tumour samples in stage pT1 needed for statistical analysis. To obtain sufficient tumour samples of stage pT1 for statistical analysis was difficult, because late diagnosis is characteristic for

prostate cancer. Furthermore, stage pT1 do not cause remarkable symptoms and patients are not frequently operated at that stage. Most diagnosed stages are pT2 and pT3 as seen in this study.

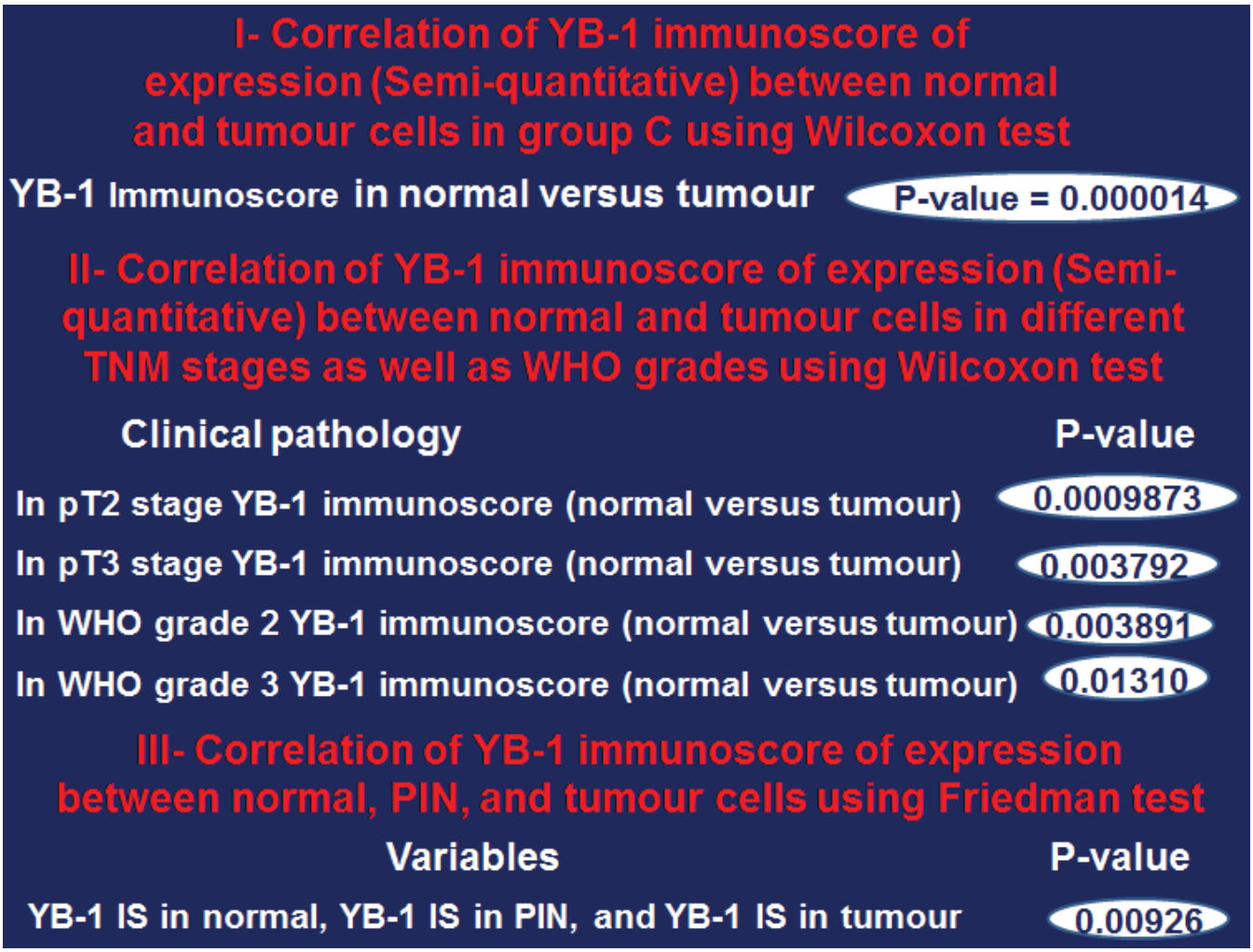


Fig. 4.3: A summary of the statistical analysis of YB-1 expression and TNM stages as well as WHO grades using Friedman test, Kendall`s rank tau (t) test, and Wilcoxon test in prostate cancer.

Although, this study showed a significant difference between YB-1 immunoscore in normal secretory and tumour cells as well as between YB-1 immunoscore in normal secretory, PIN, and tumuor cells groups, the impact of the potential use of YB-1 as a diagnostic as well as a prognostic marker needs further investigation. One important investigation could be the determination of YB-1 expression in primary tumours before and after treatment of patients with advanced disease as well as metastasis. It would be better if these studies were done in comparison with the known markers such as PSA, free-PSA, density-PSA and velocity-PSA, measured at the same time.

The localisation of YB-1 expression was found in the cytoplasm and/or cell nucleus in prostate cancer cells. This localization of YB-1 expression has not been reported so far in any study to prostate cancer cells. Immunoscore has been calculated in the whole area of each section of the

tumour tissue sample and the expression either in the cytoplasm or nucleus was considered as positive. The importance of the sub-cellular localization of YB-1 expression is related to the fact that patients with nuclear YB-1 expression in breast cancer have a poorer prognosis than those with a cytoplasmic YB-1 expression. Until now, no study has been done of prostate cancer tissues concerning this sub-cellular localisation of YB-1 expression and the correlation to the drug resistance.[243] The nuclear expression of YB-1 is associated with drug resistance and expression of *MDR* (Multi drug resistance) and *MPR* (multidrug resistance protein or multidrug resistance-associated protein) in breast cancer, synovial sarcomas, osteosarcomas, ovarian carcinomas, and NSCLC. Furthermore, the association of increased YB-1 expression in patients with drug resistance is known in many other tumours and may be a reason for less response to therapy.[74, 84, 241]

At the present time, it is not clear what triggers YB-1 to move into the nucleus of cancer cells in case of prostate cancer. But in mamma carcinoma, UV irradiation, chemotherapeutic agents, and other genotoxic stress cause YB-1 to pass into the nucleus.[246-247] Although, these stimuli lead to nuclear translocation of YB-1, there must be other signals that impart for this effect. Bargou et al.[74] reported that YB-1 is present in the nucleus of breast tumours that have been treated with irradiation. YB-1 expression was related to increase of relapse and therapy resistance in breast cancer.[240]

In contrast to other tumours such as breast, non-small cell lung cancer (NSCLC), ovarian, colon, and sarcoma cancer (a cancer that arises from transformed cells of mesenchymal origin.), YB-1 expression was found in this work mostly in the cytoplasm of prostate cancer cells and very little expression was observed in the nucleus. Not only YB-1 showed a variation in expression in cytoplasm and nucleus between tumours, but also between malignant and benign tumours like primary melanoma, melanoma metastases and benign melanocytic nevi. YB-1 expression is increased in melanoma cells in vitro and in vivo and YB-1 is translocated into the nucleus in invasive and metastatic melanoma cells.[248]

Many studies confirmed the suitability of YB-1 as a diagnostic biomarker in breast cancer with a correlation of YB-1 expression with *MDR-1* and *MPR-1* genes. YB-1 is correlated with high risk of the breast cancer disease metastasis and short survival after mammectomy. Currently, YB-1 is not only known as a biomarker in breast cancer, but also in colon, ovary, and non-small lung cancer. Over-expression of YB-1 protein was also identified during this study of human prostate cancer. My results are in agreement with data from a study described during the course of this work by Gimenez-Bonafe et al.[243] They studied YB-1 and P-glycoprotein (P-gp) expression in LNCaP

human prostate tumor model during progression to androgen independent (AI) and core biopsy samples obtained from prostate tumours.

Previous reports showed that not only in breast[244], osteosarcoma, and small cell lung cancer (SCLC) but also in LNCap tumours and in human prostate tumours increased YB-1 expression correlates with elevated P-gp. Gimenez-Bonafe et al reported that increased YB-1 expression correlates with elevated P-gp level in LNCaP tumors and in human prostate tumors. YB-1 overexpression increased efflux of DHT through P-gp upregulation and decrease androgen levels in prostate cancer cells. Androgen-regulated gene expression is decreased by over-expression of YB-1 and P-gp in prostate cancer cells. As a consequence of lower androgen level, cell survival is increased and apoptosis will be inhibited.[243]

In contrast to the previous studies concerning prostate cancer, Sullivan et al.[249] and Van Brussel et al.[250] reported that P-gp was undetectable by immunohistochemistry in any prostate carcinoma samples.[249-250] Breast and ovarian cancer represent organs in which YB-1 is known as a biomarker. These organs are under hormonal control and regulation in their function depends on the hypothalamus and pituitary glands. Bergmann et al.[238] showed that YB-1 over-expression in the mammary glands in mice is an initiating event in the process of multistage breast cancer development.[238] The authors described YB-1 as a novel breast cancer oncogene in mice with a genetic penetrance of 100%.[238] In the mammary gland of transgenic mice, YB-1 initiates a chain of events that leads to the development of chromosomal instability and consequently aneuploidy in vivo.[238]

Data obtained in this work showed that YB-1 immunoscore was positively correlated between normal secretory cells and tumour cells in tumour samples of positive cancer patients using Wilcoxon test (Fig. 4.3). There was also a positively correlation between YB-1 immunoscore in normal secretory, PIN, and tumour cells using Friedman test. Also, there was a positive correlation between immunoscore in normal and tumour cells in tumour samples of stage pT2, pT3, grade 2, and grade 3 using Wilcoxon test. Notably, the tendency of YB-1 immunoscore increased with the increased histological grades. Therefore YB-1 could be a potential new biomarker in prostate cancer.

5. SUMMARY

Prostate cancer has many different histological forms making its pathological analysis difficult. The incidence of prostate carcinoma has increased in the last few decades in the Western countries. Currently, one in six men has a lifetime risk of developing prostate cancer. Prostate cancer is mainly found in men older than 55 years with an average age of 70 at the time of diagnosis and therefore represents a disease of older men. Early detection of prostate cancer is important for curative treatment.

The aim of this study was to analyse clinical samples of prostate cancer using both molecular cytogenetic and immunohistochemical parameters in order to evaluate new molecular biomarkers for prostate cancer diagnostics.

In this study different technical methods including FISH and immunohistochemistry were used. Gain of 7q31 was found in all studied tumours even amplification in the most of the cases. FISH analyses showed that the frequency of the amplification of 7q31 and 8q22-24, and the deletion of 8p12-22 are indicators for prostate cancer development and progression of the disease. But, this method turned out to be very laborious. Therefore, it cannot be used in a routine-diagnostic setting.

A correlation between amplification of 7q31 region which contains the Caveolin-1 and -2 genes and expression could not be confirmed at the beginning of this study, due to the small number of tumour samples studied in FISH and immunohistochemistry simultaneously.

The immunohistochemical study of more prostate cancer tumour samples showed that Caveolin-1 was expressed in basal cells in both control and tumor tissues and could be assessed as a new basal cell marker. The absence of basal cells in tumour samples is a relevant indicator for advanced prostate cancer and therefore the lack of basal cells using Caveolin-1 expression can easily be identified. Caveolin-1 expression was found in tumor cells either in the cytoplasm or in the nucleus, or sometimes in both. Percentage of cells and intensity of Caveolin-1 expression increased with higher stage and metastasis. Caveolin-1 expression was found in more than 50% of the tumour cells in 51.14 % of all studied tumour samples and in 61.36 % in pT3 stage and metastasis tumours. This is the first statistical study showing a significant correlation between expression of Caveolin-1 and TNM stages in prostate cancer. A significant correlation between percentage of Caveolin-1 expression (Evaluation in %) and TNM stage (P= 0.018) was found using

the Kendall's tau test. Additionally, a significant difference between semi-quantitative expression (immunoscore) of Caveolin-1 and TNM stage (P = 0.0332) was found using Kendall's tau test. Moreover, stepwise increase of Caveolin-1 expression with WHO grade was observed.

Thus, based on the obtained data, the present study confirmed the reliability of Caveolin-1 as biomarker for the progression of prostate cancer.

The analysis of YB-1 demonstrated that it is mostly localized in the cytoplasm of examined samples and the level of its expression was more pronounced in tumor cells in comparison with normal cells. A significant correlation between YB-1 expression in tumour and normal cells was identified using Wilcoxon test (P = 0.000014). Moreover a significant difference between YB-1 expression in normal secretory cells, prostate intraneoplasia cells (PIN), and tumour cells using Friedman test (P = 0.00929) was identified. YB-1 expression in normal and tumour cells showed a significant difference in pT2 (P = 0.0009873) as well as in pT3 (0.003792) using Wilcoxon test. In addition, a significant difference between YB-1 expression in normal and tumour cells in grade 2 and grade 3 having P-values 0.003891 and 0.01310 using Wilcoxon test was found, respectively. The expression of YB-1 in the tumor tissues, increased gradually from grade 1 to grade 3, when compared with normal tissues. Thus, the application of YB-1 as a new biomarker for prostate cancer may be considered.

In conclusion, the combination of two markers such as Caveolin-1 and YB-1 in comparison to or in combination with pathological, histological, and molecular genetics parameters like alterations of 7q31, 8p12-22 and 8q22-24 can help to characterize the disease's development and progression. Consequently the combined analysis mentioned above is the preferred method. In the future this may help to establish a more individualized method of treatment.

6. ZUSAMMENFASSUNG

Das Prostatakarzinom gehört zu den soliden Tumoren und kann in verschiedenen histologischen Formen auftreten, welche die pathologische Analyse erschweren. Die Inzidenz des Prostatakarzinoms hat sich in den letzten Jahrzehnten insbesondere in den westlichen Ländern rasch erhöht. Zurzeit ist jeder sechste Mann von dem Risiko betroffen, ein Prostatakarzinom auszubilden. In der Regel wird das Prostatakarzinom bei Männern über 55 Jahren entdeckt. Das Durchschnittsalter bei Diagnose beträgt 70 Jahre, weswegen Prostatakrebs auch als Krankheit älterer Männer bezeichnet wird. Die Früherkennung des Prostatakarzinoms ist für eine kurative Therapie von großer Bedeutung.

Ziel dieser Arbeit war die Analyse klinischer Proben des Prostatakarzinoms mittels molekular-zytogenetischer und immunohistochemischer Parameter zwecks Evaluierung neuer Biomarker für die Diagnostik des Prostatakarzinoms.

In dieser Arbeit wurden verschiedene technische Methoden wie “Fluoreszenz In-Situ-Hybridisierung”, und immunohistochemische Verfahren verwendet sowie molekularbiologische Analysen. Alle Tumorproben zeigten eine Duplikation im chromosomalen Bereich 7q31, in den meisten Fällen sogar einer Amplifikation. Die Verwendung der FISH -Methode (FISH) in dieser Arbeit zeigte, dass die Frequenz der Amplifikation von 7q31, 8q22-24 und Deletion von 8p12-22 als Indikatoren für die Entwicklung eines Prostatakarzinoms und die Progression der Krankheit dienen können. Allerdings zeigte sich, dass diese Methode sehr aufwendig und deshalb für die Routine-Diagnostik ungeeignet ist.

Eine Korrelation zwischen der Amplifikation der 7q31 Region, in der sich die Caveolin-1 und -2-Gene befinden und den Grad der Caveolin-1-Expression konnte am Anfang dieser Studie nicht nachgewiesen werden, da lediglich eine kleine Anzahl von Proben gleichzeitig mit FISH und immunhistochemischer Methode untersucht wurde. Deshalb wurden weitere immunohistochemische Analysen zur Caveolin-1 und YB-1 Expression durchgeführt, dabei zeigte es sich, dass die Caveolin-1-Expression sowohl in Basalzellen der Kontrollgewebe als auch im Tumorgewebe festgestellt wurde und damit als Basalzell-Biomarker verwendet werden kann. Das Fehlen von Basalzellen in den Tumorproben ist ein relevanter Indikator für ein fortgeschrittenes Prostatakarzinom und kann mit Hilfe der Caveolin-1 Expression einfach bewertet werden. Die Caveolin-1 Expression wurde entweder im Zytoplasma, oder im Zellkern der Tumorzellen oder in beiden festgestellt. Die Zellenzahl und Intensitätsgrade der Caveolin-1 Expression in Tumorproben

stieg in höherem Stadium und Metastasen an. In 51.14% alle Tumorproben wurde Caveolin-1 Expression in mehr als 50% der Tumorzellen gefunden und in 61.36% der pT3 Stadien und Metastasen festgestellt.

Bis jetzt ist dies die erste Studie, welche eine gute Korrelation zwischen prozentualen Anteilen der Expression in den Tumorzellen (Evaluation in [%]), Intensität der Expression, sowie eine semiquantitative Auswertung der Expression von Caveolin-1 (Immunoscore) und TNM Stadien zeigte. Die statistische Analyse bestätigte eine signifikante Korrelation der Expression von Caveolin-1 zwischen Expression in % und TNM Stadium (P = 0.018) unter Verwendung von Kendall's Tau Test. Außerdem hat die statistische Analyse eine signifikante Differenz zwischen semi-quantitativer Expression von Caveolin-1 (Immunoscore) und den TNM Stadien (P = 0.0332) bewiesen und zeigte einen schrittweisen Anstieg der Expression von Caveolin-1 in Korrelation mit dem Anstieg des WHO Grades. Aufgrund der erhaltenen Daten weist die Arbeit daraufhin, dass die Caveolin-1-Expression als Biomarker für die Progression des Prostatakarzinoms, geeignet sein könnte.

Außerdem zeigte die YB-1-Analyse, dass dieses Protein hauptsächlich im Zytoplasma der untersuchten Tumorproben vorliegt, und dass die YB-1-Expression in Tumorzellen deutlich stärker ist als in Normalzellen. Die statistische Analyse zeigte eine signifikante Korrelation zwischen YB-1 Expression in Normal- und Tumorzellen unter Verwendung von Wilcoxon Test (P = 0.000014). Außerdem zeigte sich eine signifikante Differenz zwischen YB-1 Expression in normalen sekretorischen Zellen, Prostata Neoplasie Zellen (PIN) (oder Pre-Karzinoma Zellen), und Tumorzellen unter Verwendung des Friedman Tests (P = 0.00929). YB-1 Expression in Normal- und Tumorzellen zeigten eine signifikante Korrelation in pT2 Stadium (P = 0.0009873) wie auch in pT3 (0.003792) unter Verwendung des Wilcoxon Test. Zudem wurde auch eine signifikante Differenz zwischen YB-1 Expression in Normal- und Tumorzellen in Grad 2 (P = 0.003891) und Grad 3 (P = 0.01310) bestätigt. Die YB-1-Expression zeigte einen schrittweisen Anstieg in Tumorzellen von Grad 1 bis Grad 3 im Vergleich zu Normalzellen. Aus diesem Grund kommt YB-1 als neuer Biomarker für das Prostatakarzinom in Betracht.

Zusammenfassend ist die Kombination von zwei Markern wie Caveolin-1 und YB-1 im Vergleich zu oder in Kombination mit pathologischen, histologischen und molekulargenetischen Parametern wie 7q31, 8p12-22 und 8q22-24 Imbalancen geeignet, die Entwicklung und die Progression der Krankheit zu charakterisieren.

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8. APPENDIX

Table 3.18:- The immunoscore and mean of the immunoscore in different cells of tumour samples of prostate cancer in stage pT2 and pT3 of group C. UICC means Union for International Cancer Control.

A) Tumour sample number and the different measured values of immunoscore in pT2 and pT3 stages.

pT2							pT3						
No.	**UICC Classifiction and Gleason –score**	**YB-1 normal**	**YB-1 Tumour**	**YB-1 PIN**	**YB-1 Tumour – normal**	**YB-1 Tumour/normal**	**No.**	**UICC Classifiction and Gleason score**	**YB-1 normal**	**YB-1 Tumour**	**YB-1 PIN**	**YB-1 Tu-normal**	**YB-1 Tumour/ normal**
2	pT2b;.G2	3	3	6	0	1	**1**	PT3a; G2 bis beginnend G3	2	4		2	2
4	pT2b; G1-G2	2	1		–1	0.5	**3**	pT3a; G1 bis G2	2	1		1	
6	pT2a; G1-G2;	0	6		6	.	**5**	pT3a, G2-G3	0	2		2	
10	pT2a, PN0, pMX,R0; G1-G2	2	2		0	1	**7**	pT3a; G2-G3	2	1		–1	0.5
11	pT2b, pNX, pMX; G2-G3	2	6		4	3	**8**	G2; pT3a	0	0		0	
12	pT2b, G1, pN0, pMX.	2	4		2	2	**9**	pT3a; G2-beginnend G3	1	0		–1	0
13	pT2a; G1-G2	2	2		0	1	**14**	pT3b, pNX, pMX, R0; G3	0	0		0	
15	pT2b, pNX, pMX; R0; G1.	3	2		–1	0.67	**17**	pT3a; G2	3	0		–3	0
16	pT2b, pNX, pMX; G1	2	2		0	1	**18**	pT3a, pN0, pMX; G1	2	6	9	4	3
19	pT2b, pN0,pMX; G3q (nach Helpap)	0	2		2	-	**20**	pT3a, pNX, pMX; G3	1	12		11	12
27	pT2b, pNX, pMX; G3	1	6		5	6	**21**	pT3a, pN1,pMX; G2b	1	6		5	6
30	pT2b, pNX, pMX; G2	2	6	4	4	3	**22**	pT3a, pN0, pMX (IIb)					
34	pT2c, pNX, pMX, G1	2	3		1	1,5	**23**	pT3a, pN0, pMX; G3a	2	6		4	3
35	pT2c, pNX, pMX.; G2	2	6		4	3	**24**	pT3a, pN0, pMX(G3-Malignancy grade IIIa)	2	4		2	2
37	pT2c, pN0, pMX; G3	1	1		0	1	**25**	pT3a, pN0, pMx; G3	3	3		0	1
39	pT2c, pNX, pMX; G2	1	2	6	1	2	**26**	pT3b, pN0, pMX; (G2b)	1	6		5	6
40	pT2c,pNX, pMX G2	1	6		5	6	**28**	pT3a, pNX, pMX; G2a	0	4		4	-
42	pT2a; pN2; pMX; R0; G2	2	3		1	1.5	**29**	pT3a; PNX, pMX, R0; G3	4	4		0	1
43	pT2c, pN0, pMX; G2	0	0		0		**32**	pT3a, pN0, MX; G2					
47	pT2b, pNX, pMX, , R0; G1	2	4		2	2	**33**	pT3c, pNX, pMX; G1	2	3		1	1.5
48	pT2b, pN0, pMX; GS: 7(3+4)	8	8	8	0	1	**36**	pT3a, pNX, pMX; G3	1	2		1	2
49	pT2a, pN0, pMX, R0; GS: 2+3= 5 (≈G2)	1	1		0	1	**38**	pT3a, pN0, pMX; G3	0	0		0	
50	pT2b, pNX, pMX; G2, R0(GS: 3+2=5)	2	4		2	2	**41**	pT3a, pN0, pMX; G3; R0	4	6		2	1.5
51	pT2b, pN0, pMX, G2, GS: 8 (3+5)	2	4	9	2	2	**44**	pT3c; pNX, pMX, R0; G3					
52	pT2b, pN0, pMX, R0; GS 3+ 2 = 5	2	3		1	1.5	**45**	pT3a, pNX, pMX; G2	3	4	4	1	1.33
							46	pT3b, pNX, pMX; G3	6	8		2	1.33
N = 25							**N = 26 – 3 = 23**						
pT2							**pT3**						

No.	UICC Classifiction and Gleason –score	YB-1 normal	YB-1 Tumour	YB-1 PIN	YB-1 Tumour – normal	YB-1 Tumour/normal	No.	UICC Classifiction u. Gleason score	YB-1 normal	YB-1 Tumour	YB-1 PIN	YB-1 Tu-normal	YB-1 Tumour/ normal
N = 25							N = 26 – 3 = 23						
	Total value	47	87	33	39	43.67		**Total value**	40	82	13	42	44.16
	Mean	1.88	3.48	1.32	1.56	1.75		**Mean**	1.74	3.57	0.57	1.83	1.92

B) Highest and lowest values of immunoscore as well as the mean in pT2 and pT3.

NMT Stage	No	YB-1 normal	YB-1 Tumour	YB-1 PIN	YB-1 Tumour – normal	YB-1 Tumour/normal
Total value in pT2	25	47	87	33	39	43.67
Highest value		0	0	4	–1	0
Lowest value		8	8	9	6	6
Mean		1.88	3.48	1.32	1.56	1.75
Total value in pT3	23	40	82	13	42	44.16
Highest value		0	0	4	–3	0
Lowest value		6	8	9	11	12
Mean		1.74	3.57	0.57	1.83	1.92

Table 3.19:- The immunoscore and mean of the immunoscore in different cell types of tumour samples of prostate cancer in different WHO grades (Grade G1, G1-G2, G2, G2-G3, and G3) of group C.

A) Immunoscore and median of the immunoscore in G1 and G1-G2.

G1							G1-G2						
No.	UICC Classifiction and Gleason	YB-1 normal	YB-1 Tumour	YB-1 PIN	YB-1 Tu-normal	YB-1 Tu/normal	No.	UICC Classifiction and Gleason	YB-1 normal	YB-1 Tumour	YB-1 PIN	YB-1 Tu-normal	YB-1 Tu/normal
12	G1; pT2b, pN0, pMX.	2	4		2	2	**3**	G1-G2; pT3a	2	1		1	
15	G1; pT2b, pNX, pmX; R0.	3	2		–1	0,67	**4**	G1-G2; pT2b	2	1		–1	0.5
16	G1; pT2b, pNX, pMX	2	2		0	1	**6**	G1-G2; pT2a	0	6		6	
18	G1; pT3a, pN0, pMX,	2	6	9	4	3	**10**	G1-G2; pT2a, pN 0, pMX; R0	2	2		0	1
33	G1; pT3c, pNX, pMX	2	3		1	1,5	**13**	G1-G2; pT2a.	2	2		0	1
34	G1; pT2c, pNX, pMX	2	3		1	1.5							
47	G1; pT2b, pNX, pMX, R0	2	4		2	2							
	N = 7	**15**	**24**	**9**	**8**	**11.67**		**N = 5**	**8**	**12**	**-**	**6**	**2.5**

B) Immunoscore and median of the immunoscore in G2.

G2													
No.	UICC Classifiction and Gleason –score	YB-1 normal	YB-1 Tumour	YB-1 PIN	YB-1 Tu-normal	YB-1 Tu/normal	No.	UICC Classifiction and Gleason –score	YB-1 normal	YB-1 Tumour	YB-1 PIN	YB-1 Tu-normal	YB-1 Tu/normal
2	G2; pT2b.	3	3	6	0	1	**39**	G2; pT2c, pNX, pMX	1	2	6	1	2
8	G2; pT3a	0	0		0	-	**40**	G2; pT2c, pNX, pMX	1	6		5	6
17	G2; pT3a	3	0		–3	0	**42**	G2; pT2a; pN2; pMX; G2; R0	2	3		1	1,5
21	Gb2; pT3a, N1,MX	1	6		5	6	**43**	G2; pT2c, pN0, pMX	0	0		0	
26	Gb2; pT3b, pN0, pMX	1	6		5	6	**45**	G2; pT3a, pNX, pMX	3	4	4	1	1.33
28	G2a; pT3a, NX, Mx;	0	4		4		**49**	G2 = GS: 2+3= 5; pT2a, pN0, pMX, R0	1	1		0	1
30	G2; pT2b, pNX, pMX;	2	6	4	4	3	**50**	G2 = GS: 2+3= 5; pT2b, pNX, pMX, R0	2	4		2	2
31	G2; pT4a, pN0, pMx	1	2		1	2	**51**	G2; pT2b, pN0, pMX, G2, GS: 8 (3+5)	2	4	9	2	2
35	G2; pT2c, pNX, pMX.	2	6		4	3	**52**	G2 = GS: 2+3= 5; pT2b, pN0, pMX, R0	2	3		1	1.5
	N = 9	**13**	**33**	**10**	**20**	**21**		**N = 9**	**14**	**27**	**19**	**13**	**17.33**

C) Immunoscore and median of the immunoscore in G2-G3 and G3.

G2-G3							G3						
No.	**UICC Classification and Gleason –score**	**YB-1 normal**	**YB-1 Tumour**	**YB-1 PIN**	**YB-1 Tu-normal**	**YB-1 Tu/normal**	**No.**	**UICC Classification and Gleason –score**	**YB-1 normal**	**YB-1 Tumour**	**YB-1 PIN**	**YB-1 Tu-normal**	**YB-1 Tu/normal**
1	G2 bis beginnend G3; PT3	2	4		2	2	**14**	G3; pT3b, pNx, pMx, R0	0	0		0	
5	G2-G3; pT3	0	2		2		**19**	G3a: pT2b, pN0, pMX; (nach Helpap)	0	2		2	
7	G2-G3; pT3	2	1		–1	0.5	**20**	G3: pTNM: pT3, NX, MX; (G3)	1	12		11	12
9	pT3; G 2 - beginnend G 3	1	0		–1	0	**23**	G3a; pT3, pN0, pMX	2	6		4	3
11	G2-G3, pt2b, pNX, pMX.	2	6		4	3	**24**	G3, pT3, pN0, pMX ; Malignency grads IIIa	2	4		2	2
48	G2-G3≈ GS: 7(3+4), pT2b, pN0, pMX;	8	8	8	0	1	**25**	G3: pT3; pN0, pMx	3	3		0	1
		15	21	8	6	6.5	**27**	G3: pT2b, pNX, pMX	1	6		5	6
							29	G3; pT3; PNX, pMX, R0	4	4		0	1
							36	G3: pT3a; pNX, pMX	1	2		1	2
							37	G3: pT2c, pN0, pMX	1	1		0	1
							38	G3: pT3a, pN0, pMX,	0	0		0	-
							41	G3; pT3a, pN0, pMX; R0	4	6		2	1,5
							46	G3; pT3b, pNX, pMX	6	8		2	1,33
									25	54	0	29	30.83
	N = 6							**N = 13**					

- Grade 1 includes G1 (n = 7) + G1-G2 (n = 5) and it was 12 tumour samples.
- Grade 2 includes G2 (n = 18) + G2-G3 (n = 5) and it was 23 tumour samples.
- Grade 3 was represented in 13 tumour samples.

Ich versichere an Eides statt, dass die

”Molecular cytogenic and immunohistochemical studies for the evaluation of new prostate cancer biomarkers”

Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

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