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EARLY ONCOGENIC EVENTS AND DEFECTIVE APOPTOSIS IN PROSTATE CANCER

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CONTENTS

ABSTRACT

Prostate cancer is one of the most common malignancies in males in the Western world. Although the mechanisms are unclear, it is generally believed that the accumulation of genetic alterations leads to the tumor formation. My studies were aimed to define genetic events leading to the development and progression of prostate cancer, and to explore their biological and therapeutic significance.

Ezrin, a member of the Ezrin-Raxidin-Moesin(ERM) family, is involved in cell shaping and signalling. We studied its protein expression and gene copy number in 19 cases of high-grade prostatic intraepithelial neoplasia (HGPIN) with concomitant prostate cancer. We found the immunoreactivity to be absent or weak in benign prostate epithelial cells, weak or moderate in cancer, but strong in HGPIN. No alterations in the gene copy number were detected by FISH technique. The data indicate that the aberrant expression of *ezrin* may be involved in the pathogenesis of prostate cancer and be useful in the diagnosis of HGPIN.

Pim-1, an oncogene product of serine/threonine kinase, plays an important role in apoptosis, cell cycle, and transcriptional regulation. Immunohistochemical analysis was used to test pim-1 expression in HGPIN and in cancer from 121 specimens. Moderate to strong staining was observed in 68% of cancers and 97% of HGPIN, negative or weak in benign glands. Pim-1 was overexpressed in HGPIN compared to cancer in 65% of the cases. The data suggest that pim-1 overexpression in HGPIN may be an early event in the development of prostate malignancy and provides supplementary information for distinguishing HGPIN from benign epithelium.

During oncogenic processes, aberrant DNA methylation frequently occurs, thereby leading to silencing of sets of genes involved in cell cycle regulation, apoptosis, and other biological functions. In our study, prostate cancer DU145 cells were grown in the presence of the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5'-aza), followed by treatment with different doses of cisplatin. The treatment of the cells with 5'-aza followed by cisplatin induced significant cell apoptosis, which suggested that suppression of DNA methylation was capable of enhancing sensitivity of prostate cancer DU145 cells to cytotoxic agent---cisplatin.

The X-linked inhibitor of apoptosis protein (XIAP) suppresses cell apoptosis while the XIAP-associated factor 1 (XAF1) promotes apoptosis. We studied XAF1 expression in prostate cancer cells. Compared to normal tissues where a full-length of XAF1 mRNA is predominant, PC3, LNCaP, and DU145 cells expressed no or only short forms XAF1 transcripts. Inhibition of DNA methylation led to a switch to full length of XAF1 mRNA expression. Both short forms and full-length forms were detectable in primary prostate tumors, suggesting that splicing alterations of the *XAF1* transcript may occur during the development of prostate cancers due to aberrant DNA methylation.

In summary, our data suggest the activation of the *Ezrin* and *Pim-1* genes may be early oncogenic events in the development of prostate cancer, and that the alternative XAF1 mRNA splicing and aberrant DNA methylation are linked to prostate cancer cell survival. These findings have important biological and clinical implications.

2

LIST OF PUBLICATIONS

I.   Pang ST, **Fang X**, Valdman A, Norstedt G, Pousette A, Egevad L, Ekman P.

Expression of ezrin in prostatic intraepithelial neoplasia.

Urology. 2004 Mar;63(3):609-12.

II.   Valdman A, **Fang X**, Pang ST, Ekman P, Egevad L**.**

Pim-1 expression in prostatic intraepithelial neoplasia and human prostate

cancer.

Prostate. 2004 Sep 1;60(4):367-71.

III.   **Fang X**, Zheng C, Liu Z, Ekman P, Xu D.

Enhanced sensitivity of prostate cancer DU145 cells to cisplatinum by 5-aza-

2'-deoxycytidine.

Oncol Rep. 2004 Sep;12(3):523-6

IV.   **Fang X**, Liu Z, Fan Y, Zheng C, Egevad L, Ekman P, Xu D.

Switch to full-length XAF-1 mRNA expression in prostate cancer cells by

DNA methylation inhibitor

Submitted

LIST OF ABBREVIATIONS

AIF	apoptosis-inducing factor
AMACR	α-methylacryl-CoA racemase
APC	adenomatous polyposis coli
AR	androgen receptor
ARE	androgen responsive element
ATCC	American Type Culture Collection
BAC	bacterial artificial chromosomes
Bad	Bcl-xL/Bcl-2 associated death
Bak	Bcl-2 antagonist killer 1
Bax	Bcl-2 associated x protein
Bcl-2	B-cell lymphoma 2
Bid	BH3 interacting domain death agonist
Bim	Bcl-2 interacting mediator of cell death
BIR	baculovirus IAP repeat
BPH	benign prostatic hyperplasia
BRCA1	breast cancer 1
BRM	biological response modifier
cAMP	cyclic adenosine monophosphate
CCD	charge-coupled device
CDK	cyclin-dependent kinase
CGH	comparative genomic hybridization
CKI	cyclin-dependent kinase inhibitor
cM	centimorgan
CT	computed tomography
DAPI	4',6-diamidino-2-phenylindole
DAPK	death-associated protein kinase
DHT	dihydrotestosterone
DIABLO	direct IAP binding protein with low pI
DISC	death-inducible signaling complex
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase

4

EBP50	ERM-binding phosphoprotein 50
eIF4E	eukaryotic initiation factor 4E
EndoG	endonuclease G
ER	estrogen receptor
ERM	Ezrin-Raxidin-Moesin
FACS	fluorescence-activated cell sorting
FISH	fluorescence *in situ* hybridization
G-CSF	granulocyte colony-stimulating factor
GSTP	glutathione S-transferase pi
HGPIN	high grade prostatic intraepithelial neoplasia
HK2	human kallikrein 2
hMLH1	human mutL (E. coli) homolog 1
HSP	heat shock protein
HtrA2	high temperature requirement A2
IAP	inhibitor of apoptosis
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
IGF	insulin-like growth factor
ILP	IAP-like protein 2
Il-2	interleukin-2
LGPIN	low grade prostatic intraepithelial neoplasia
LOH	loss of heterozygosity
MAPK	mitogen-activated protein kinase
MDS	myelodysplastic syndromes
MGMT	O(6)-methylguanine-DNA-methyltransferase
MMLV	Moloney murine leukemia virus
MSI	microsatellite instability
MSP	methylation-specific PCR
MSR1	macrophage scavenger receptor 1
NAIP	neuronal apoptosis-inhibitory protein
NFATc	nuclear factor of activated T cell
NHE-3	sodium-hydrogen exchanger isoform 3
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline

PCD	programmed cell death
PCR	polymerase chain reaction
PGR	progesterone receptor
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PIN	prostatic intraepithelial neoplasia
PKC	protein kinase C
PMP24	peroxisomal membrane protein 24kDa
PP2A	protein phosphatase 2A
PRDX1	peroxiredoxin 1
PSA	prostate specific antigen
PSCA	prostate stem cell antigen
PTEN	phosphatase and tensin homolog
PUMA	p53-upregulated modulator of apoptosis
RARβ	retinoic acid receptor β
RASSF1A	Ras association domain family protein 1 isoform A
Rb	retinoblastoma
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SAM	S-adenosyl-methionine
Smac	second mitochondria-derived activator of caspases
SSC	standard saline citrate
STAT	signal transducers and activators of transcription
Taq	Thermus aquaticu
THBS1	thrombospondin-1
TIMP	tissue inhibitor of metalloproteinase
TMA	tissue microarray
TMS-1	target of methylation-induced silencing-1
TNF	tumor necrosis factor
TRAF 3	tumor necrosis factor receptor-associated factor 3
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
TRAMP	transgenic adenocarcinoma of mouse prostate
XAF1	X-linked inhibitor of apoptosis -associated factor 1
XIAP	X-linked inhibitor of apoptosis

1 INTRODUCTION

In 1853, J. Adams, a surgeon at the London Hospital, described the first case of prostate cancer by histological examination. At that time, he believed that it was 'a very rare disease', but nowadays, prostate cancer has become one of the most common malignancies in males in the Western world. It is generally believed that prostate cancer, like other tumors, develops and progresses through an accumulation of genetic alterations by activating oncogenes and/or inactivating tumor suppressor genes. However, the exact molecular mechanisms are still unknown.

The prostate gland epithelial cells secrete a large number of measurable compounds such as: acid phosphatases, lactic dehydrogenases, kallikrein proteases (PSA and HK2), prostaglandin, spermine, fibrogenases, aminopeptidases, zinc, citrate, cholesterol and lipids, pepsinogen II, and lactoferrin. Structurally, the prostate can be divided into a peripheral zone (70-75%), the largest anatomic subdivision of the prostate, where a majority of carcinomas originate; a central zone (20-25%), surrounding the ejaculatory ducts as they course from the base of the prostate to the verumontanum, giving rise to 5-10% of prostate cancer; transition zone (5-10%), around the prostate urethra, from which most benign prostate hyperplasia (BPH) and 20% prostate cancer arise; and non-glandular anterior fibromuscular stroma (McNeal JE, 1968). The peripheral zone is the palpable portion of the prostate on digital rectal examination.

In 2000, the number of new cases of prostate cancer was about 513,000 worldwide (Grönberg H, 2003). In Sweden the number was 9,035 in 2003, showing a tendency of increasing, and the death number of prostate cancer was 2,352 in 2002 (data from the Swedish National Board of Health and Welfare). Approximately 75% of the clinically detectable prostate cancers are diagnosed between 50-70 years of age (Brawley OW, et al, 1998). The incidence of prostate cancer varies widely among different ethnic populations and geographical regions, and the incidence rate may

differ by as high as 90-folds. The lowest rates occur in East Asia, whereas the highest are in Scandinavia and North America, especially in Afro-Americans people in the USA. The incidence of prostate cancer is increasing in both high-risk and low-risk populations (Parkin DM, et al, 2001), mainly because of a growing awareness of prostate cancer, widespread screening with PSA in men without any symptoms, and decreasing of cardiovascular diseases death. In addition to age, family history, race, steroid hormones, diet, nutrition, and oxidative stress, many other elements such as vitamins (Vit A, C, D, and E), trace elements (zinc and selenium), calcium, environmental and occupational factors may also be implicated in pathogenesis of prostate cancer (Carter BS, et al, 1990; Pienta KJ and Esper PS, 1993; Kalish LA, et al, 2000). Androgens play an important part in the development of the healthy prostate and treatment of prostate cancer as well. Withdrawal of testosterone by surgical or medical castration is a well-known treatment for prostate cancer and is effective in 75–80% of patients with prostate cancer. Insulin-like growth factor I (IGF-I), a peptide growth factor, regulates proliferation, differentiation, and apoptosis of cancer cells. A strong positive association was observed between IGF-I levels and prostate cancer risk. Men in the highest quartile of IGF-I levels had a relative risk of 4.3 compared with men in the lowest quartile (Chan JM, et al, 1998). The IGF system as risk factors likely mirrors a link between the western lifestyle and prostate cancer: consumption of large amounts of fat result in raised production of insulin that in turn increases production of IGF, thus explaining how IGF could be a risk factor for prostate cancer. Other factors such as smoking (Hickey K, et al, 2001), alcohol consumption (Dennis LK and Hayes RB, 2001), vasectomy (Peterson HB and Howards SS, 1998), physical activity (Lee IM, et al, 2001), and virus infection (Cuzick J, 1995) have been investigated in several studies, but the overall conclusion is that they do not significantly affect the incidence rates of prostate cancer (Figure 1).

More men die *with* prostate cancer rather than *from* prostate cancer (Dijkman GA and Debruyne FM 1996). The most commonly reported metastatic sites are lymph nodes, bone, liver, lung, and adrenal. Until now, there are limited treatment options available

for hormone refractory prostate cancer because chemotherapy and radiotherapy are largely ineffective.
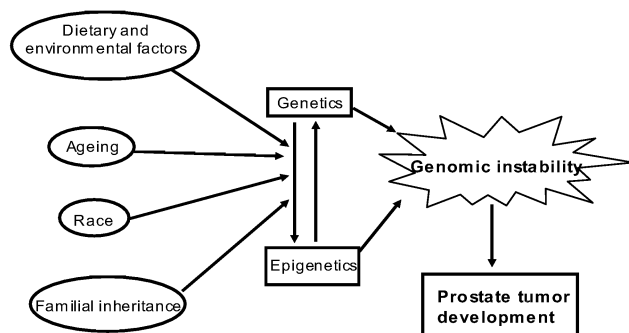

Figure 1. *Potential risk factors involved in the initiation and development of prostate cancer*

1.1 GENETIC CHANGES IN PROSTATE CANCER

Although many factors are known to contribute to the development of prostate cancer, underlying oncogenic mechanisms remain poorly understood. Accumulating evidence has suggested that cancer is a disease with impaired genomic stability characterized as chromosomal instability (markedly altered chromosomal counts and structure such as aneuploidy, deletions, and amplifications) or microsatellite instability (MSI). Various risk factors may initiate and prompt oncogenesis by triggering genomic instability.

Indeed, like all other human malignancies, prostate cancer exhibits various kinds of genetic aberrations. For instance, the *NKX3.1* gene, located at 8p21, encodes a prostate-specific homeobox gene essential for normal prostate development and loss of heterozygosity (LOH) involving the *NKX3.1* gene is perhaps the most common deletion events in prostate cancer (Bhatia-Gaur R, et al, 1999). This gene is present in androgen-dependent cells but absent in androgen-independent prostate tumors (He

9

WW, et al, 1997). In mice, targeted disruption of *NKX3.1* leads to prostatic epithelial hyperplasia and dysplasia (Kim MJ, et al, 2002). Another site of frequent allelic loss in prostate cancer is *PTEN* located at 10q23 that encodes a phosphatase active against both proteins and lipid substrates (Li J, et al, 1997). *PTEN* has been proposed to function as a general tumor suppressor gene by inhibiting the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling pathway required for cell cycle progression and/or cell survival in many cell types including prostate epithelial and cancer cells. *PTEN*, typically expressed by normal epithelial cells, is often expressed at a reduced level in human prostate cancer cells (McMenamin ME, et al, 1999). Many somatic *PTEN* alterations have been observed in prostate cancers including homozygous deletions, LOH, mutations, and suspected CpG island hypermethylation and these changes are closely associated with the disease progression and metastatic potential (Suzuki H, et al, 1998; Rubin MA, et al, 2000). The *p53* gene, encoding a well-known tumor suppression protein p53, is altered in one-third of early prostate cancer patients, and the frequency increases with the disease progression (Downing SR, et al, 2003). In addition, the tumor suppressor *Rb* gene mutation and silencing has been reported in 20% to 50% of prostate cancers (Cunningham JM, et al, 1996). Moreover, frequent somatic alterations of the *AR* (androgen receptor) are observed in prostate cancers, especially in those undergoing progression during hormonal treatment (Taplin ME, et al, 2004). *AR* belongs to a large steroid hormone receptor family, and it functions as a transcription factor. After binding to the ligand (androgens), the receptor is phosphorylated and homodimerized, and bound to the androgen responsive elements (ARE) located at the 5' regions of the genes. The *AR* gene encodes the androgen receptor and it plays critical roles in normal prostate development and function, and is also essential for the proliferation of androgen-dependent and -independent prostate cancer cells (Zegarra-Moro OL, et al, 2002). Androgen-independent prostate cancer usually continues to express *AR* and maintains *AR* dependent signaling in response to the reduced levels of circulating androgens, non-androgens or anti-androgens as agonist ligands and via ligand-independent activation of *AR* (Heinlein CA and Chang C, 2004). *P27*, a cyclin-dependent kinase

10

inhibitor encoded by CDKN1B, may also be a somatic gene target for alteration during prostate carcinogenesis. Reduced *p27* expression appears characteristic of prostate cancer cells, particularly in prostate cancer with a poor prognosis. Decreased *p27* expression has also been found in HGPIN (De Marzo AM, et al, 1998). All these genetic alterations lead to either oncogene activation or tumor suppressor inactivation, which subsequently drives formation and aggressiveness of prostate cancer by conferring a growth or survival advantage, and invasion and metastasis capabilities (Gao AC, et al, 2002; Meng MV, et al, 2002) (Figure 2).
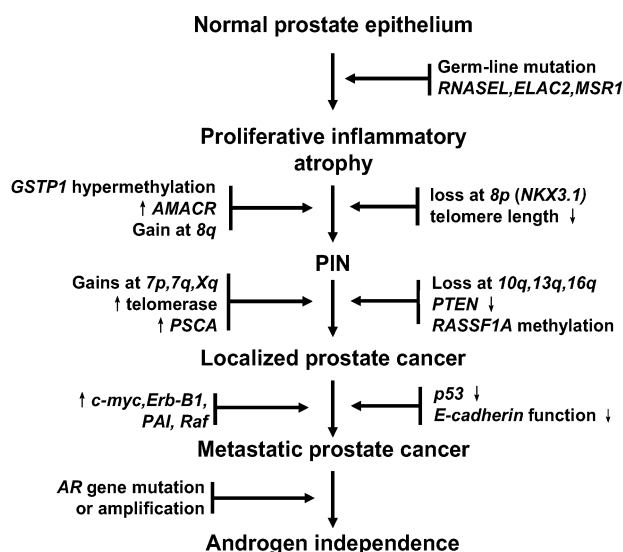
Normal prostate epithelium

Germ-line mutation
RNASEL,ELAC2,MSR1

Proliferative inflammatory atrophy

GSTP1 hypermethylation
↑ *AMACR*
Gain at *8q*

loss at *8p* (*NKX3.1*)
telomere length ↓

PIN

Gains at *7p,7q,Xq*
↑ telomerase
↑ *PSCA*

Loss at *10q,13q,16q*
PTEN ↓
RASSF1A methylation

Localized prostate cancer

↑ *c-myc,Erb-B1,*
PAI, Raf

p53 ↓
E-cadherin function ↓

Metastatic prostate cancer

AR gene mutation
or amplification

Androgen independence

Figure 2. *Summary of molecular pathogenesis and progression of prostate cancer.*

1.1.1 Ezrin and prostate cancer

During the characterization of the components of microvilli, a minor component was purified. This polypeptide was called ezrin in recognition of Ezra Cornell University where it was purified (Bretscher A, 1983). Ezrin is a member of the ERM (Ezrin-Raxidin-Moesin) family, which shares the common membrane-binding N-terminal FERM domain with Band-4.1 superfamily of protein (Bretscher A, et al, 2002). The gene consists of 13 exons and spans approximately 24kb of genomic DNA. The gene

is located on chromosome 6q25-26.

The ERM family contains: ezrin, radixin, moesin (for membrane-organizing extension spike protein), and merlin (for moesin-ezrin-radixin-like protein) members. The amino-terminal domain of ezrin is able to bind to cytoplasmic proteins such as EBP-50 (Granes F, et al, 2000). Ezrin has also been shown to anchor cAMP-dependent protein kinase and to indirectly bind to $Na^+ H^+$ exchanger 3 (NHE3) via the cytoplasmic phosphoprotein EBP50 (Tran YK, et al, 1999; Ingraffea J, et al, 2002).

Ezrin plays a role in the formation of microvilli, cell adhesion sites, lamellipodia formation and contractile rings during cytokinesis (Pawlak G and Helfman DM, 2001). It is involved in the interaction of cell cytoskeleton with plasma membrane, during signal transduction and growth control (Andreoli C, et al, 1994). Ezrin's linkage of cell membrane to actin cytoskeleton allows the cell to interact directly with its microenvironment, and provides 'intracellular scaffolding' that facilitates signal transduction through a number of growth factors and adhesion molecules (Algrain M, et al, 1993). ERM proteins exist in the cytoplasm in an inactive, 'closed' conformation, with N-terminal to C-terminal associations within the protein or with other *ERM* members (Bretscher A, et al, 2002). Ezrin especially has been detected not only in ruffles of membranes, but also at the leading edges of spreading cells, and is, therefore, associated with changes in cell motility (Hiscox S and Jiang WG, 1999). Ezrin is also necessary for several signaling pathways important for metastatic capability, including MAPK, AKT, Rho kinase, CD44 and others (Hunter KW, 2004) (Figure 3). Ezrin is found in most cell types, although it has been found to have an organ specific level of expression. It expresses at very high levels in the small intestine, stomach, lung, pancreas, prostate, and kidney; at intermediate levels in the spleen, thymus, lymph nodes and bone marrow; at very low levels in the heart, brain, testis and muscles (Akisawa N, et al, 1999). Ezrin is overexpressed in a variety of cancers including brain hemangioblastoma (Bohling T, et al, 1996), uterine endometrioid adenocarcinoma (Ohtani K, et al, 2002), and osteosarcoma (Khanna C, et al, 2001), some of which are associated with poor outcomes. It is recently found

12

that ezrin is down-regulated in androgen withdrawal-induced apoptosis and up-regulated in androgen replacement-stimulated proliferation in rat ventral prostatic epithelial cells (Pang ST, et al, 2002), suggesting the involvement of this protein in regulation of proliferation/survival of prostate cells.
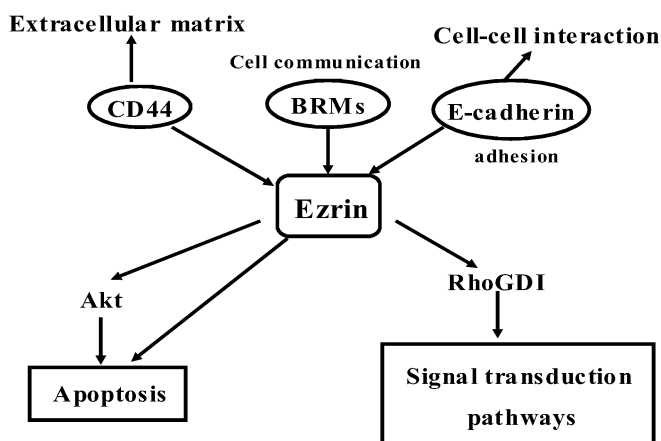

Figure 3. *Function and pathway of ezrin*

1.1.2 PIM-1 and prostate cancer/HGPIN

The *pim-1* gene was originally discovered as a preferential site for proviral integration of the Moloney murine leukemia virus (MMLV) in mice (Cuypers HT, et al, 1984). The *pim-1* gene codes for a highly conserved serine/threonine kinase whose expression is stimulated by a variety of cytokines, hormones, and mitogens at four different levels: transcriptional, post-transcriptional, translational, and post-translational. *Pim-1* is associated with multiple cellular functions such as proliferation, differentiation, apoptosis and tumorigenesis.

The human *pim-1* gene is located on chromosome 6p21.1---6p21.31, and has 6 exons and 5 introns and produces a transcript of 2684bp (NM-002648). The *pim-1* promoter is highly G+C rich and dose not contain a TATA or CAAT box, which are characteristics of promoters of housekeeping genes (Meeker TC, et al, 1987). Pim-1 protein contains

13

313 amino acids and the *pim-1* gene encodes two proteins of 34kDa and 44kDa respectively due to the use of an alternative translation initiation at an upstream CUG codon (Saris CJ, et al, 1991). There exists two other pim-1-like kinases that are termed *pim-2* and *pim-3*. *Pim-1, -2* and *-3* are well conserved in vertebrates and show high degrees of sequence and structural similarity (Mikkers H, et al, 2004). *Pim-1* is highly expressed in spleen, thymus, bone marrow, fetal liver, oral epithelia, hippocampus and prostate (Eichmann A, et al, 2000).

The pim-1 protein is found in both the cytoplasm and nucleus and is post-transcriptionally regulated by eIF-4E (Hoover DS, et al, 1997), stabilized by HSP90 (Mizuno K, et al, 2001) and degraded by PP2A (Losman JA, et al, 2003). *Pim-1* is a downstream effector of many cytokine-signaling pathways and the expression of the *pim-1* gene is induced by a large set of cytokines. Many of the *pim-1* binding partners are involved in the regulation of cell cycle progression and apoptosis. The first binding partner identified as a *pim-1* substrate was the nuclear adapter protein p100, which is an activator of the c-Myb transcription factor (Leverson JD, et al, 1998). Another identified protein is the transcription factor NFATc, which is involved in relaying signals from the T-cell receptor (Rainio EM, et al, 2002). Recently, the pro-apoptotic protein Bad was found to co-localize and physically interact with *pim-1* (Aho T, et al, 2004). This suggested a direct functional role of *pim-1* in preventing cell death since the inactivation of Bad can enhance Bcl-2 activity and thereby promotes cell survival (Figure 4).

Pim-1 protein expression was screened in benign prostatic hyperplasia (BPH), local and metastatic prostate cancer, and hormone-refractory prostate cancer. It was found that there is no or only weak expression of *pim-1* in most benign prostate samples, whereas about half of the metastatic prostate cancer samples showed moderate to strong expression, however, poor clinical outcome has been reported to be related to decreased expression of *pim-1* (Dhanasekaran SM, et al, 2001). The possible explanation of this paradox may be that *pim-1* overexpression is overriding the

14

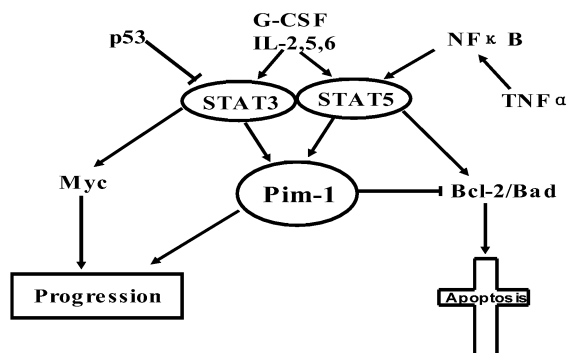mitotic spindle checkpoint, resulting in genomic instability (Roh M, et al, 2003).

Figure 4. *Pim-1 in signal transduction*

1.2 Epigenetic changes in prostate cancer

Epigenetics plays an important role in cancer biology, viral infections, activity of mobile elements, somatic gene therapy, cloning, transgenic technologies, genomic imprinting, developmental abnormalities, mental health, and X-inactivation. Somatically heritable states of gene expression can be achieved without altering the DNA sequence through the epigenetic mechanism.

One of the key components in the epigenetic control pathway is DNA methylation. DNA methylation is a postreplicative modification that occurs in a non-random fashion at cytosines that are located 5' to a guanosine in CpG dinucleotides. This procedure is catalyzed by DNA methyltransferases using S-adenosyl-methionine (SAM) as the methyl donor. Hypomethylation usually involves repeated DNA sequences, such as long interspersed nuclear element, whereas hypermethylation involves CpG islands (Ehrlich M, 2002). There are many protective mechanisms that prevent the hypermethylation of the CpG islands. These include active transcription, active demethylation, replication timing, and local chromatin structure preventing access to the DNA methyltransferase. In most of the mammalian genomes, the CpG dinucleotide

15

has been depleted during evolution while small regions of DNA, approximately 0.5 to 4.0 kb in size, harbor the expected number of CpG sites and are termed CpG islands. Most of these are associated with promoter regions of approximately half the genes in the genome. In normal cells, most CpG sites outside of CpG islands are methylated, whereas most CpG-island sites in gene promoters are unmethylated. This methylated state in the bulk of the genome may help suppress unwanted transcription, whereas the unmethylated state of the CpG islands in gene promoters permits active gene transcription (Frühwald MC, 2003). In cancer cells, however, DNA-methylation patterns are frequently shifted. Evidence has accumulated that aberrant DNA methylation affects the transcription of genes involved in the regulation of cellular growth, differentiation, apoptosis, transformation, and tumor progression (Kalebic T, 2003). Numerous genes including those involved in cell cycle regulation ($p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$, Rb), signal transduction (*RASSF1, LKB1/STK11, APC*), DNA repair (*BRCA1, MGMT, hMLH1*), apoptosis (*DAPK, TMS-1*), cell adhesion (*E-cadherin, TIMP3*), detoxification (*GSTP1, PRDX1*), hormone response (*ER, AR, PGR, and RAR β*), drug resistance, differentiation, angiogenesis (*THBS1*) and metastasis have been found to undergo hypermethylation in cancer (Das PM and Singal R, 2004). Hypomethylation has been hypothesized to contribute to oncogenesis by activation of oncogenes such as *c-Myc* and *H-Ras* or by chromosome instability (Figure 5).

Normal prostate epithelium

Genetic factors
Ageing
Dietary factors

Epigenetic changes

PIN

Methylation of *GSTP1,MGMT*

Prostate cancer

Methylation of *GSTP1, RASSF1A, RAR β 2, E-cadherin*

Advanced prostate cancer

Methylation of *APC, CD44, E-cadherin*

Metastatic prostate cancer
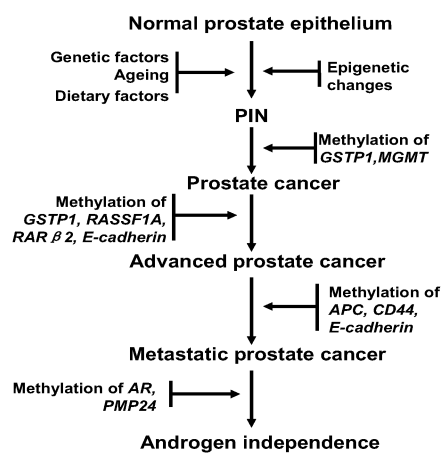
Methylation of *AR, PMP24*

Androgen independence

Figure 5. *Epigenetic contributions to prostate cancer*

16

In prostate cancer, one of the typical genes silenced through the DNA methylation is *GSTP1,* encoding the π-class glutathione *S*-transferase (GST). *GSTP1* protects prostate epithelial cells against genome damage mediated by carcinogens and oxidants, thereby preventing transformation of the cells. Immunohistochemistry has demonstrated that *GSTP1* protein is normally expressed in basal epithelial cells in the prostate. In prostate cancer cells, however, hypermethylation of *GSTP1* CpG island sequences represses *GSTP1* transcription, leading to the absence of GST protein expression (Lin X, et al, 2001). In addition, lack of *GSTP1* expression and *GSTP1* CpG island hypermethylation even commonly occurs in high-grade PIN lesions, indicating that it is an early event during carcinogenesis of prostate cells (Brook JD, et al 1998).

It is currently believed that the main mechanism of action of DNA-demethylating agents as anticancer drugs is the reactivation of dormant tumor suppressor genes by the demethylation of their aberrantly hypermethylated promoter-CpG islands. All human primary tumors feature methylation-associated silencing of tumor suppressor genes that affect all cellular pathways: from cell cycle inhibitor to inducers of apoptosis, from DNA repair genes to cell adhesion, and from hormone receptors to detoxifiers (Esteller M, 2005).

The biological effects of loss of gene function caused by promoter hypermethylation and by coding-region mutations are similar; however, the latter are irreversible and the former potentially reversible. These potentially reversible epigenetic changes provide opportunities for the clinical management of prostate cancer and other malignancies. The realization of these possibilities will require the development of strategies to reverse gene silencing for the purpose of preventing and treating tumors. In fact, inhibition of DNA methylation has been applied to hematological malignancies and certain solid tumors, and promising efficacy has been observed (Boivin AJ, et al, 2002; Sigalotti L, et al, 2003). In addition, DNA hypermethylation has been found to be associated with drug resistance acquired during cancer chemotherapy, and therefore, novel therapeutic approaches could be developed by combining epigenetic agents with

cytostatic or cytotoxic drugs (Nyce JW, 1997; Strathdee G, et al, 1999). The combination approach may lead to reduction in the dose of each drug used and reduce the side effects of each individual agent. One obstacle of all the current DNA-demethylating agents is lack of drug specificity. Demethylating agents usually cause global hypomethylation, and we cannot reactivate solely the particular gene we would wish to (Villar-Garea A and Esteller M, 2003).

1.3 Pathologic findings in the prostate

At present the preferred method of diagnosing early prostate cancer is needle biopsy (Epstein JI, 2002). Morphologically, prostate cancer is sometimes hard to diagnose because the clues to malignant disease can be subtle, increasing the risk of underdiagnosis. Although a few histological findings are specific for prostate cancer, diagnosis is generally made on the basis of architectural, cytological, and ancillary findings. Many histological benign mimickers of cancer can also lead to misdiagnosis of cancer. One distinguishing feature is that benign glands contain basal cells, which are absent in cancerous areas (DeMarzo AM, et al, 2003). Acidic mucins are often seen in acini of adenocarcinoma, appearing as amorphous or delicate, thread-like, basophilic secretions in routine sections. Acidic mucin is not specific for carcinoma: it may also be found in PIN but rarely in BPH (Goldstein NS, et al, 1995). Collagenous micronodules are a specific but infrequent and incidental finding in adenocarcinoma, consisting of microscopic nodular masses of paucicellular, eosinophilic, fibrillar stroma that impinge on acinar lumens (Bostwick DG, et al, 1995). Perineural invasion is common in adenocarcinoma, present in 20%-38% of biopsies. In some patients, perineural invasion may be the only evidence of malignancy found in a needle biopsy (Varma M, et al, 2002). Vascular/lymphatic invasion is present in 38% of radical prostatectomy specimens with cancer and is commonly associated with extraprostatic extension and lymph node metastases. It also correlated with histologic grade (Bahnson RR, et al, 1989). There is a significant increase in microvessel density in PIN and carcinoma compared with normal prostatic

tissue (Brawer MK, et al, 1994). Many studies show mean blood vessel counts are higher in tumors with metastases than in tumors without metastases, and correlate with pathologic stage (Siegal JA, et al, 1995).

1.3.1 Gleason grading system

The Gleason score is the most frequently used grading system for prostate cancer, which is based on the glandular pattern of the tumor as identified at relatively low microscopic magnification. This system was developed by Dr Donald Gleason, a pathologist in Minnesota, in 1966 (Gleason DF, 1966). The Gleason grading system is based entirely on the histologic pattern of arrangement of carcinoma cells in H&E-stained prostatic tissue sections. The system categorizes the histologic patterns at low magnification by the extent of glandular differentiation and the pattern of growth of the tumor in the prostatic stroma. The primary pattern is the one that is predominant in the area, by simple visual inspection, while the secondary pattern the second most common. Each pattern is graded from 1 (the most well differentiated tumor) to 5 (the least differentiated tumor). The two grades are added together to obtain the overall Gleason score, which ranges from 2 (for tumors uniformly of pattern 1) to 10 (for undifferentiated tumors) (Gleason DF, et al, 1974). General Gleason score 2–4 carcinoma are regarded as well-differentiated, Gleason score 5–6 as moderately differentiated, Gleason score 7 as moderately (3+4) to poorly (4+3) differentiated and Gleason score 8–10 as poorly differentiated. The Gleason score is a powerful prognostic indicator but is not absolutely accurate for the individual patient. Clinically, needle biopsy Gleason grade combined with other pretreatment factors, such as serum total and free PSA, clinical T stage, and amount of tumor in needle biopsy, can determine eligibility for clinical trials, plan for type of radiotherapy and hormonal therapy, and predict response to prostatectomy (Partin AW, et al, 1997; Humphrey PA, 2004). Men with lower Gleason scores are more likely to be recommended watchful waiting as initial management (Koppie TM, et al, 2000).

1.3.2 Prostatic intraepithelial neoplasia (PIN)

The term PIN (prostatic intraepithelial neoplasia) was first introduced by Dr. Bostwick (Bostwick DG and Brawer MK, 1987). PIN is graded from I to III. Grade I is defined as low-grade PIN (LGPIN), whereas grade II and III are currently considered together as high-grade PIN (HGPIN). In LGPIN, the nuclei are enlarged, vary in size, have normal or slightly increased chromatin content, and possess small or inconspicuous nucleoli. In HGPIN, cells have large nuclei of relatively uniform size, and possess prominent nucleoli that are similar to those of carcinoma cells. The basal cell layer, while intact or rarely interrupted in LGPIN, may have frequent interruptions in high-grade lesions. There are 4 main patterns of HGPIN: tufting, micropapillary, cribriform, and flat, in which tufting pattern is the most common, present in 97% of cases. There are no known clinically important differences between the architectural patterns of HGPIN. The pattern is only used for diagnosis.

HGPIN is the earliest accepted stage in carcinogenesis, possessing most of phenotypic and biochemical changes occurring in cancer without invasion of the basal membrane of the acini. The support for regarding HGPIN as the main premalignant lesion of prostate is based on the evidence from prostate cancer animal models, epidemiological, morphological, genetic, and molecular studies (Steiner MS, 2001). HGPIN and prostate cancer are morphometrically and phenotypically similar. They both occur primarily in the peripheral zone, and multifocally and heterogeneously. In addition, HGPIN shares many biochemical and genetic changes with cancer, including chromosomal instability or abnormality, androgen receptor expression, PSA expression, cell proliferation and apoptotic indices, high molecular weight cytokeratin expression, progressive loss of differentiation, neovascularization and microvessel density, and markers to define basement membrane proteins (Sakr WA & Partin AW, 2001; Sinha AA, et al, 2004). Recently, the genome-wide gene expression by cDNA microarray also showed the close similarity of HGPIN and invasive prostate cancer (Ashida S, et al, 2004). It was found that telomere length in

HGPIN and invasive prostate cancer is much shorter compared with normal epithelial cells (Meeker AK, et al, 2002). Telomere shortening is believed to occur at or before the development of HGPIN and it may precede the up-regulation of telomerase activity that is frequently observed in prostate cancer. These gross chromosomal alterations may lead to genomic instability and tumorigenesis. Several animal models of prostate cancer have demonstrated that HGPIN is in the direct causal pathway to prostate cancer. The transgenic mouse model of prostate cancer (TRAMP) has also been shown to mimic human prostate cancer (Gingrich JR, et al, 1997).

The incidence of HGPIN ranges between 4% and 16% in prostate biopsies (Bostwick DG, 1999). The incidence and extent of PIN seems to increase with increased age. The prevalence of PIN is similar in black and white races. The mean volume of PIN in prostates with cancer is $1.20-1.32cm^{3}$, and the volume increases with increasing pathologic stage, Gleason grade, positive surgical margins, and perineural invasion (Qian J, et al, 1997; Sakr WA, et al, 2000). The severity and frequency of HGPIN in prostates with histological cancer is substantially greater than in prostates without cancer (Helpap BG, et al, 1995). When HGPIN is found in a needle biopsy, there is a 30-50% risk of finding carcinoma on subsequent biopsies over 3-5 years, and 80% of patients with HGPIN develop carcinoma within 10 years. In immunohistochemistry, appropriate antibodies may be used to stain tissue sections for the presence of basal cells, recognizing that PIN keeps an intact or fragmented basal cell layer whereas cancer does not (Bostwick DG & Qian J, 2004).

HGPIN alone dose not increase PSA levels (Ronnett BM, et al, 1993). There is also poor correlation of HGPIN with PSA density and free/total PSA levels. An elevated serum PSA level in a patient with isolated HGPIN and no other reasons for an abnormal PSA level (eg BPH, prostatitis) suggests the possibility of a concurrent, unsampled carcinoma (Raviv G, et al, 1996). Transrectal ultrasound, CT and nuclear magnetic resonance (NMR) imaging cannot detect HGPIN. HGPIN is only a microscopic diagnosis.

Currently, any routine treatment is not recommended for patients who have HGPIN. Prophylactic radical prostatectomy or radiation is not an acceptable treatment for patients who have HGPIN only.

1.4 Apoptosis and prostate cancer

Programmed cell death (PCD) or apoptosis is a cell suicide mechanism that enables multicellular organisms to regulate cell number in tissues and to eliminate unneeded or ageing cells as an organism develops. What we call truly apoptotic cells were first discovered and described by Walther Flemming in 1885. In 1972, Dr. John Kerr first used the term apoptosis (from a Greek word meaning 'falling off') to focus attention on the yin-yan relationship of death to birth. Apoptotic cells show nuclear condensation, chromatin cleavage to 50-300kb fragment, cell shrinkage, phosphatidylserine exposure, and formation of pyknotic bodies of condensed chromatin. Apoptosis is physiologically a normal and important process for multicellular organisms (Ellis RE, et al, 1991). However, inappropriate apoptosis is implicated in many human diseases, including cancer. Investigation of the mechanisms of apoptosis is one of the presently most interesting areas of modern biology and medicine. The life and death of cells must be balanced if tissue homeostasis is to be maintained---too much growth and too little death can lead to a severe disturbance that might result in cancer (Hanahan D and Wienberg RA, 2000). There are two main pathways that can initiate apoptosis: extrinsic (receptor-mediated or death-receptor) and intrinsic (mitochondria-mediated or stress) pathway (Figure 6).

In the extrinsic pathway, ligation of death receptors is followed by the formation of the death-inducible signaling complex (DISC), which results in activation of pro-caspase-8 and pro-caspase-3.
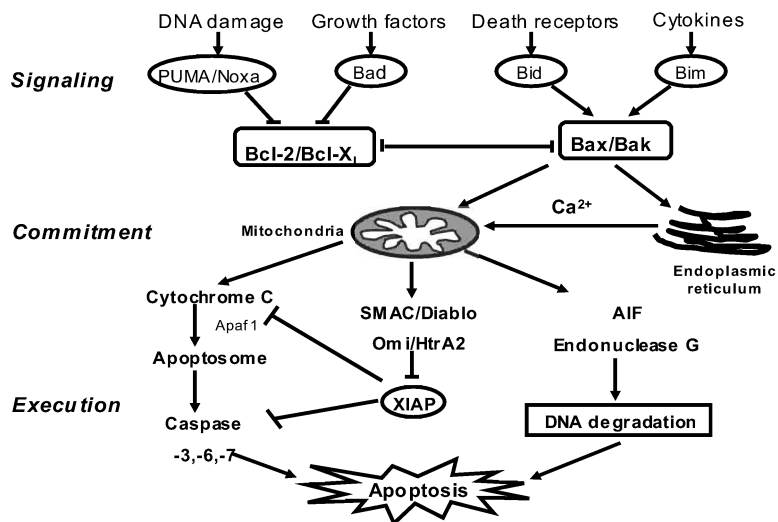
22

Figure 6. *Apoptosis pathway and mechanism*

In the intrinsic pathway, death signals function directly or indirectly on the mitochondria, resulting in the formation of the apoptosome complex. This pathway is controlled by Bcl-2 family proteins (regulation of cytochrome c release), IAPs (inhibition of caspases), and SMAC/DIABLO and HtrA2/Omi (negative regulation of IAPs). It may also operate through caspase-independent mechanisms, which involve the release from mitochondria and translocation to the nucleus of at least two proteins: apoptosis inducing factor (AIF) and endonuclease G (EndoG). The major regulators are the pro- and antideath members of the Bcl-2 family (Tsujimoto Y, 2003). IAP provides another level of control for both the extrinsic and intrinsic pathways, which often cooperate---depending on cell type and stimulus--- to kill a cell in an orderly way. P53 serves as a regulator of the apoptotic process that can modulate key control points in both pathways. There is considerable cross-talk between the extrinsic and intrinsic pathways.

The central players of apoptosis are caspases that are present in the cytosol and responsible for cellular changes that occur during apoptosis. In both pathways, caspases

are activated that cleave cellular substrates, and this leads to the biochemical and morphological changes that are characteristic of apoptosis. Understanding the mechanisms of apoptosis inhibition could be the basis for developing more effective therapeutic approaches. Because apoptosis is closely involved in the initiation and progression of human prostate cancer, many chemoprevention and therapeutic regimens attempting to manipulate the apoptosis process have been proposed to be used as treatment. It was suggested that many anticancer drugs trigger the apoptotic process through the death receptor pathway. Therefore, any deficiencies in this pathway might result in tumor cell resistance to treatment (Friesen C, et al, 1996).

1.4.1 Chemotherapy and drug resistance in prostate cancer

Although surgery and radiotherapy can cure many patients with early-stage prostate cancer, metastatic and progressive diseases are frequent problems in the management of patients. Hormone treatment is often effective in metastatic disease initially; however, eventually most patients become hormone refractory. Although chemotherapy for the treatment of cancer has been used in clinic more than half a century, the early trials using chemotherapy in prostate cancer patients were disappointing. Twenty-six single-agent chemotherapy regimens had a median objective response rate of 8.7% (Yagoda A and Petrylak D, 1993). However, by the mid-1990s, data was emerging showing a clinical benefit of chemotherapy in prostate cancer.

Cisplatin, a member of platinum family, is one of the most commonly used anticancer agents, and it is used in combination chemotherapy of advanced prostate cancer. The biological activity of cisplatin was discovered in the early 1960s, and the drug was approved in 1978 for clinical treatment (Loehrer PJ and Einhorn LH, 1984). It is generally accepted that the cytotoxic activity of the drug results from the formation of inter-and intrastrand platinum-DNA cross-links that results in blocking of DNA synthesis and induction of cell death (Fichtinger-Schepman AM, et al, 1985). By

24

binding to DNA, cisplatin inhibits DNA replication and chain elongation, influences

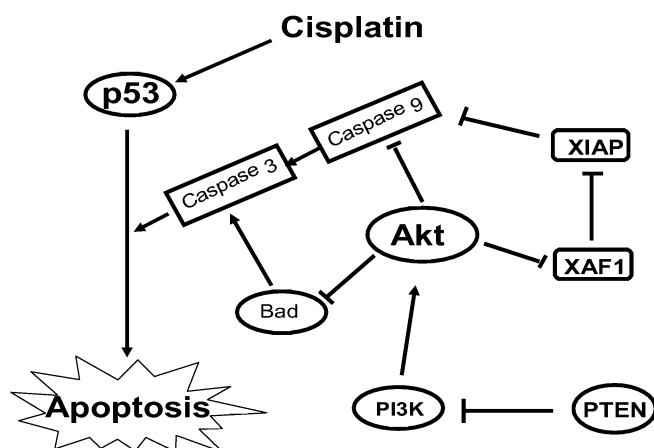DNA repair, and suppresses RNA transcription and protein translation (Figure 7).

Figure 7. *Mechanism of cisplatin to cause apoptosis and resistance*

The mechanisms involved in cisplatin-induced apoptosis are complex. Caspases cascade is activated in response to cisplatin exposure and this activation leads to an irreversible commitment to apoptotic cell death. When cisplatin induces apoptosis through caspase-3 activation, the MAPK pathway or PI3K/Akt pathway is mediated. Moreover, cisplatin also induces apoptosis mediated by the inhibition of XIAP expression (Zanke BW, et al, 1996; Asselin E, et al, 2001). The PKC signal transduction pathway acts at an early stage of cisplatin-induced apoptotic pathway that precedes caspase activation to mediate cisplatin sensitivity, and a reduction in cPKC and an increase in nPKC during the development of cellular resistance to cisplatin may be associated with drug resistance (Basu A, et al, 1996). The MAPK pathway leads to the activation of a number of transcription factors, such as c-Myc, c-Fos, c-Jun. These factors are overexpressed in cisplatin resistant cancers, and their downregulation resensitizes tumor cells to the cisplatin chemotherapy (Desbiens KM, et al, 2003). PI3K/Akt signal cascade may also play a considerable role in cisplatin resistance (Yuan ZQ, et al, 2003). cIAP-1 expression has been associated with in vitro resistance to a

variety of chemotherapeutic agents including cisplatin and DNA alkylating (Tamm I, et al, 2000).

1.4.2 XIAP-associated factor 1 (XAF1)

The inhibitor of apoptosis (IAP) gene family, containing baculoviral repeat (BIR) domains, encodes proteins that have emerged as key intrinsic inhibitors of the caspase cascade, and thus represent critical regulatory factors in apoptosis signaling. The human IAP family includes cIAP-1, cIAP-2, ML-IAP, XIAP, NAIP, survivin, apollon, ILP2, and livin. X-linked inhibitor of apoptosis (XIAP) is a particularly potent member of this family. A novel XIAP binding partner, which antagonizes its anti-apoptotic function at the level of the caspases, termed XIAP-associated factor 1 (XAF1) has been recently identified (Fong WG, et al, 2000). HtrA2/Omi and Smac/DIABLO were also found to bind XIAP and inhibit its activity (Verhagen AM, et al, 2000; Yang QH, et al, 2003).

The *XAF1* gene is located at chromosome 17p13.2, approximately 3 cM telomerically to the well-known tumor suppressor gene p53. *XAF1* encodes a 33.1kDa protein containing 7 zinc fingers and shares high amino acid similarity with the zinc finger domains of both FLN29 and TRAF3. Northern blots indicate that *XAF1* mRNA is synthesized as 4 distinct transcripts of different sizes (3.9Kb, 4.5 Kb, 6.0 Kb and 7.0 Kb) (Liston P, et al, 2000). *XAF1* mRNA is expressed ubiquitously in all normal adult and fetal tissues but is present at very low or undetectable levels in various cancer cell lines (Liston P, et al, 2000). The loss of *XAF1* expression is often coupled with a relatively high-level expression of XIAP, suggesting that dysregulation of apoptosis through the loss of *XAF1* expression might be important for malignant cell survival, and a high ratio of XIAP to *XAF1* expression in cancer cells might provide a survival advantage through the relative increase of XIAP anti-apoptotic function (Fong WG, et al, 2000). This loss of *XAF1* mRNA expression may correlate with loss of heterozygosity (LOH), a genetic phenomenon linked to many tumor suppressor genes.

The decreased expression of *XAF1* in many cancer cell lines suggests that alteration in the balance of IAP and caspase activities is a common phenomenon in the development of the transformed state. There are obvious survival benefits to cells that either upregulate IAPs or downregulate their inhibitors.

Over-expression of the IAPs has been reported to protect cells from irradiation and compounds used in chemotherapy. Loss of *XAF1* expression can also enhance resistance to apoptosis in cell lines that express appreciable levels of the protein. *XAF1* was identified as a novel IFN (interferon)-stimulated gene that contributes to IFN-β-dependent sensitization of cells to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis (Leaman DW, et al, 2002). *XAF1* mRNA was up-regulated by IFN-α and -β, and high levels of XAF1 protein were induced predominantly in cell lines sensitive to the pro-apoptotic effects of IFN-β. Loss or down-regulation of *XAF1* expression was strongly associated with aberrant CpG methylation in the promoter region. It has been found that abnormal expression of *XAF1* is correlates with advanced stages and high grades of human gastric cancer. *XAF1* expression is induced in all non-expressing cell lines and significantly elevated in low-expressing cell lines after 5'-aza-2-deoxycytine treatment, suggesting that loss or down-regulation of *XAF1* expression in cancer cells might be caused by an epigenetic mechanism, aberrant DNA methylation (Byun DS, et al, 2003).

2. AIMS OF THE STUDY

The overall purpose of the study is to define early oncogenic events and defective apoptosis in prostate cancer, and to explore novel treatment strategies. Specifically, the study was aimed:

I. To define potential roles for *ezrin* in pathogenesis of prostate cancer and HGPIN.

II. To determine changes in *pim-1* expression and clinical implications during development of prostate cancer and HGPIN.

III. To evaluate the effect of DNA methylation inhibitor in combination with chemotherapeutic drug cisplatin on inducing apoptosis of prostate cancer DU145 cells.

IV. To investigate alteration in the *XAF1* expression and relation with aberrant DNA methylation in prostate cancer cells.

3. MATERIALS AND METHODS

3.1 Human tumor cell lines and patient samples

In paper I, 19 radical prostatectomy specimens which contained well-defined areas of HGPIN were selected. The mean patient age at surgery was 61.3 years (range 50 to 74). None of the patients had received hormonal treatment or radiotherapy before prostatectomy. In 13 of them, foci of invasive prostate cancer were also present in the same section. In 13 (68%) and 6 (32%) of the cases, the main tumors originated from the peripheral and transition zone, respectively. One case showed seminal vesicle invasion, and nine cases had capsule penetration.

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

In paper III and IV, prostate cancer DU-145, PC3, and LN-CaP and renal cell carcinoma A498 cells, purchased from the American Type Culture Collection (ATCC), were maintained at $37\,^{\circ}\text{C}$ in DMEM supplemented with 10% fetal calf serum, 2mM L-glutamine and antibiotics (100 U/ml penicillin and streptomycin). For DNA demethylation, the cells were grown in the presence of $2.5\mu\text{M}$ 5-aza-2'-deoxycytidine (5'-aza) (Sigma, St Louis, MO) for 72-120 hours. Demethylation was followed by

treatment with different doses of cisplatin (Ebewe Arzneimittel Ges.m.b.H, Austria) for 24 hours.

In paper IV, 6 cases of fresh prostate cancer samples from radical prostatectomy were used. All of the samples were embedded in TissueTec OCT medium (Sakura, Tokyo) immediately after surgical section and stored at -80 ^{o}C for the future use of DNA and RNA extraction. None of the patients had any family history of prostate cancer. Patients' mean age at surgery was 58 years (range 54 to 64). The mean preoperative PSA level was 8.7 ng/ml (range 4.3 to 17.6).

All the clinical studies were approved by the local ethical committee.

3.2 Immunohistochemistry

Immunohistochemistry is a method of detecting the presence of specific proteins in cells or tissues and consists of the following steps: 1) primary antibody binds to specific antigen; 2) antibody-antigen complex is bound by a secondary, enzyme-conjugated antibody; 3) in the presence of substrate and chromogen, the enzyme forms a colored deposit at the sites of antibody-antigen binding.

The prostates were fixed overnight in 10% buffered formalin. The specimens were inked and sliced horizontally at 4 mm intervals. Slices were cut in two to six segments (usually quadrants) and the entire prostate was subsequently blocked in standard cassettes. Specimens were dehydrated, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (H&E). Cancer and HGPIN were outlined on the slides. Standard biotin-avidin-complex immunohistochemistry was performed by using a mouse monoclonal antibody against *ezrin* (Ab-1; Neomarker, Lab Vision, Calif) at a dilution of 1:300 and a goat polyclonal antibody against *pim-1* (Santa Cruz Biotechnology) at a dilution of 1:100. Placenta tissue was used as positive and negative controls for the immunostaining. Assessment of type and intensity of

immunohistochemical staining was scored by two observers in an open discussion. There was some staining heterogeneity in the majority of tumors and the score was based on the most intensively stained area within each specimen. No effort was made to assess percentage of area with positive staining because the staining intensity showed a gradual variation. Cytoplasmic immunostaining intensity in cancer areas was scored as absent, weak, moderate, and strong compared to adjacent normal epithelial cells. Nuclear staining was scored as either positive or negative. Staining in HGPIN was evaluated in relation to cancer areas in the same slide as much weaker, weaker, equal, stronger, and much stronger. Scoring was performed without knowledge of any pathological or clinical features.

3.3 Fluorescence *in situ* hybridization (FISH)

The earliest *in situ* hybridization, performed in late 1960s, was not fluorescent at all, but rather done by utilizing probes labeled with radioisotopes. The first application of fluorescent *in situ* detection was introduced in 1980, when RNA that was directly labeled on the 3' end with fluorophore was used as a probe for specific DNA sequences (Bauman JG, et al, 1980). This method consists of probe labeling, slide preparation, hybridization, and vision under fluorescence microscope. The specific *ezrin* and *xaf1* gene probes were isolated from the BAC clone RP11-507C10 (Children's Hospital, Oakland, Calif.) and RP11-609D21 (Sanger Institute, UK) and the centromere probes of chromosome 6 (pEDZ6) and chromosome 17 (pZ17-14) were kindly provided by Cytogenetic Unit, University of Bari, Italy. The probe DNA was extracted by using a QIAGEN Plasmid Midi Kit (QIAGEN, Hilden, Germany). Both probes were directly labeled with SpectrumRed or SpectrumGreen (Vysis, Downers Grove, IL) using standard nick translation. The labeled probes were co-precipitated using ethanol/sodium acetate together with Cot-1 DNA (Vysis) and carrier DNA. FISH was performed on metaphase spreads and 4-μm, formalin-fixed, parafin-embedded tumor sections. After dewaxing and a mild digestion with pepsin, the slides were rinsed in 2× SSC buffer, dehydrated in graded ethanol, and air-dried. Twenty microliters of hybridization

mixture containing 100 ng of each probe, 55% formamide, 10% dextran, and 2× SSC was applied to each slide. The slides were sealed with cover slips and denatured with probe mixture simultaneously at 92 °C, 10 min for paraffin-embedded slides and 78 °C, 5 min for metaphase spreads, then hybridized at 37 °C for overnight in a humidified chamber. The slides were washed in 2× SSC buffer for 10 minutes at 72 °C, dehydrated in graded ethanol, air dried, and mounted in antifade solution containing DAPI (Vector Laboratories, Inc., CA). All the slides were evaluated under a Zeiss fluorescence microscope equipped with the corresponding wavelength filters for DAPI/FITC/Rodamine, CCD camera, and an image capturing and analysis system.

3.4 Apoptosis assay

Cell death may occur by two mechanisms: apoptosis and necrosis. Both types of cell death have their own specific and distinct morphological and biochemical characteristics. Apoptotic cells appear smaller in size and denser in nucleus. These alterations can be detected by flow cytometry. The forward scatter, which indicates cell size, and the side scatter, which reflects the conformation of inner cellular structure, are used to monitor cells undergoing apoptosis. In our study, DU-145 cells (2×10^5) treated with 5'-aza and/or cisplatin were stained with 50μg/ml propidium iodide (PI, fluorescence) (Zamai L, et al, 2001). The flow analysis was performed by using a FACScan (Becton Dickinson). Hoechst 33342 is a cell-permeable, DNA binding fluorescence. Cells stained with Hoechst 33342 are scored as apoptotic if they display shrinkage of cytoplasm, nuclear condensation and fragmentation.

3.5 DNA extraction and PCR

Genomic DNA was isolated by Wizard® genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturers' instruction. To amplify the DNA fragment containing the alternative splicing XAF1 region, the following specific primers were used: forward: 5'-GGT TGG GTG TAC GAT GTG TCA (AC034305,

32

11740-11760), and reverse: 5'-GTG GTC AGG TGG AAA TCT CTA T (AC034305, 11881 – 11902). PCR was performed by running 35 cycles at 95°C 15 sec, 60°C 45 sec and 72°C 60 sec.

3.6 RNA extraction and RT-PCR

Total cellular RNA was extracted using Trizol® (Invitrogen, Carlsbad, CA) according to the manufacturers' instruction. cDNA was synthesized using random primers (N6) and MuLV reverse transcriptase. The PCR for XAF1 mRNA was performed by using the following primer pair: forward: 5'-ATG GAA GGA GAC TTC TCG GT-3' and reverse: 5'-TTG CTG AGC TGC ATG TCC AG-3'. RT-PCR was performed by running 35 cycles at 95°C 30 sec, 54°C 60 sec, 72°C 30 sec. β-actin expression was used as a control for RNA loading and RT efficiency and amplified with its specific primers using 25 cycles.

3.7 Methylation-specific PCR (MSP)

Methylation-specific PCR (MSP) is the most widely used technique for studying the methylation of CpG islands, which are common in the promoter regions of many genes . The differences between methylated and unmethylated alleles that arise from sodium bisulfite treatment are the basis of methylation-specific PCR. In MSP, the sequence differences are detected by amplification using primers that distinguish between the methylated and unmethylated bisulfite-modified DNA and unmodified DNA. In the design of primers specific for methylated DNA, cytosines that are conserved because of their methylation are placed in the 3′ end. In the design of primers that will only amplify unmethylated DNA after bisulfite modification, thymidines, derived from converted cytosines, are placed in the 3′ end. Stringency is increased with their positioning as close as possible to the 3′ end of the primer. A disadvantage of methylation-specific PCR is that only the few CpG sites that are situated within the

template sequence to which the primers bind can be investigated for a given primer pair.

The sodium bisulfite conversion of DNA was performed as previously described, with minor modifications. Five μg of genomic DNA was denatured using NaOH, and incubated in 3M sodium bisulfite (Sigma) containing 10 mM hydroquinine (Sigma) under the following condition: 95ºC, 1 min and 55ºC, 120 min for a total of 8 cycles. The modified DNA was then cleaned using Wizard DNA purification resin (Promega), ethanol precipitation and resuspended in 20 μl H_2O. The methylated and unmethylated fragments that span the core promoter region of the *XAF1* gene were PCR-amplified by using the specific primer sets (The unmethylated primers: GAA TGA TGG TTA AGG GTG AT, and ACA AAC TTT CAA TTA AAT TTC ATT. The methylated primers: GAA TGA CGG TTA AGG GCG AT, and ACA AAC TTT CGA TTA AAT TTC GTT). MSP was carried out in 25 μl of reaction mixture containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 μM of each primer, and 0.625 U Taq Gold DNA polymerase. Two μl of the modified DNA was added into each reaction. The PCR conditions were as follow: 95ºC, 20 sec, 56ºC, 2.5 min and 72ºC, 2.5 min with a total of 37 cycles for the methylated template; 95ºC, 20 sec, 52ºC, 2.5 min and 72ºC, 2.5 min with 37 cycles for the unmethylated template.

4. RESULTS

4.1 Dysregulation of the *ezrin* gene in HGPIN and prostate cancer (Paper I)

In paper I, we used immunohistochemistry to define the potential roles of the *ezrin* gene in 19 cases of clinical HGPIN and prostate cancer samples. The androgen-regulated *ezrin* was present in the cytoplasm of prostatic epithelial cells, and the basal cells were occasionally stained positive, especially in reactive proliferation regions of basal cell. Immunoreactivity was absent or weak in normal prostatic epithelial cells. HGPIN was positive for *ezrin* in all cases. When HGPIN and normal epithelium were found in the same gland, an abrupt transition occurred from atypical cells with positive signal to normal cells with negative staining (Figure 8A). Weak or moderate immunostaining was detected in 11 of 13 prostate cancer samples, and the signals were variable in all the cases. However, the staining signal was moderate or strong in all HGPIN. When HGPIN and prostate cancer co-existed in the same sample, the former exhibited stronger *ezrin* staining than the latter did (Figure 8B).
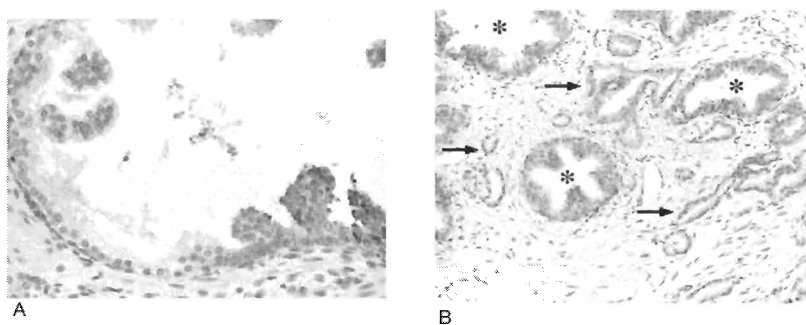
Figure 8. *(A) HGPIN with abrupt transition from atypical cells was positive for ezrin (bottom right) compared with negative benign cells (left) within the same gland. Magnification 400 ✕.*
(B) Stronger immunostaining for ezrin in HGPIN (asterisks) compared with adjacent invasive prostate cancer (arrows). Basal cells occasionally stained positive, especially in reactive basal cell proliferations. Magnification 200 ✕

Sixteen HGPIN and nine prostate cancer samples with clear FISH signals were analyzed. The overall ratio of green/red fluorescence (gene copy/chromosome) detected in both lesions was nearly 1, indicating no *ezrin* gene amplification or deletion in HGPIN and prostate cancer.

4.2 Over-expression of *pim-1* in HGPIN and prostate cancer (Paper II)

In paper II, we used immunohistochemistry to determine the expression of the *pim-1* gene in 121 cases of clinical HGPIN and prostate cancer samples. *Pim-1* staining was mostly present in the cytoplasm of cancer cells, but 15 (12%) tumors showed nuclear positivity as well. HGPIN was found in 110 slides, of which 107 (97%) showed moderate or strong staining. It is notable that the expression in HGPIN was never lower than in invasive cancer of the same specimen. In 39 (35%) cases, *pim-1* expression in HGPIN and cancer was equal. In 71 (65%) cases, there was a relative over-expression in HGPIN as compared with cancer (Figure 9A). Remarkably, an abrupt transition from atypical cells with positive *pim-1* staining to negative benign cells was observed when HGPIN and benign epithelium were present within the same gland (Figure 9B). Staining intensity in all benign glands was negative or only weakly positive. Over-expression of *pim-1* was observed in 76% of tumors with Gleason score 7 or higher compared to 58% in tumors with Gleason score 6 or lower (P=0.04).
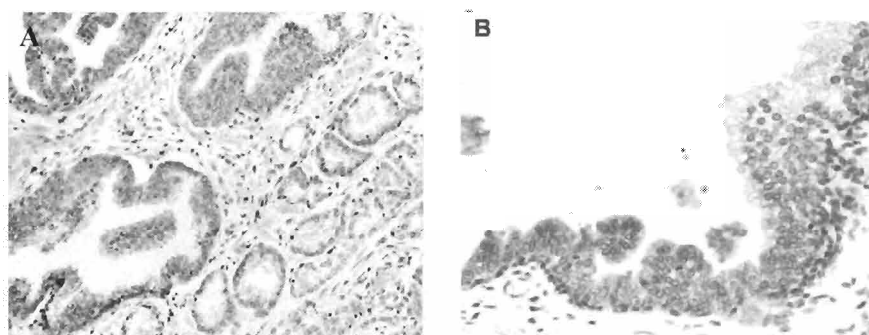

Figure 9. *(A)Pim-1 over-expression in HGPIN (upper left) compared to invasive cancer (lower right). Magnification 150✕. (B)HGPIN showing an abrupt transition from atypical cells with positive pim-1 staining (bottom left) to negative benign cells (upper right) within the same gland. Magnification 300✕*

4.3 Enhanced sensitivity of prostate cancer DU145 cells to cisplatin by methylation inhibitor 5'-aza (Paper III)

We first determined effects of 5'-aza alone on survival of DU145 cells. For this purpose, staining of the cells with PI for apoptosis assay by flow cytometry was employed. PI detects both apoptotic and necrotic cells. Up to 10% of apoptotic death occurred in those cells treated with 5'-aza at concentration between 1 to 10 μM for 96 hours, as determined using flow cytometry. Accordingly, we chose to use 2.5 μM 5'-aza in all later experiments. When DU145 cells were incubated with cisplatin alone, cellular apoptosis was induced in dose- and time-dependent manners. A detectable increase in apoptosis was seen 8 hours post-incubation of DU145 cells with cisplatin and with longer incubation time (up to 72 hours). We observed a linear increase in the number of apoptotic DU145 cells. For a 24 hour exposure, the cisplatin concentration that produced 50% of apoptotic cells (IC50) was approximately 50 μM.
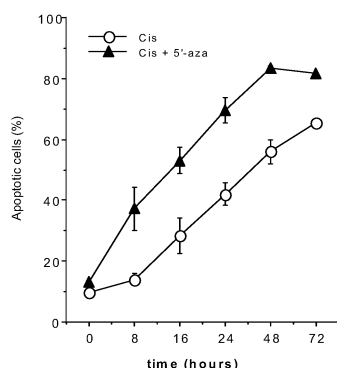
Figure 10. *Constant synergistic effects of 5'-aza on cisplatin-induced apoptosis of DU-145 cells*

To determine whether the anti-neoplastic effect of cisplatin on DU145 cells can be enhanced by 5'-aza, we incubated the cells with 2.5 μM of 5'-aza for 96 h and then added cisplatin at 50 μM into culture for various periods. The treatment of the cells with 5'-aza followed by cisplatin induced significantly increased numbers of apoptotic cells at all time points, which suggested a synergistic effect of this combination (Figure 10).

To corroborate apoptotic death of DU145 cells incubated with 5'-aza plus cisplatin, as shown by flow cytometry, we further employed Hoechst 33342 staining of those cells (Figure 11). It is known that apoptotic cells are characterised by shrinkage of cytoplasm, nuclear condensation and fragmentation. Consistent with the results obtained from flow cytometry analysis, we observed comparable percentages of apoptotic DU145 cells treated with cisplatin alone and the combination of cisplatin with 5'-aza, respectively.
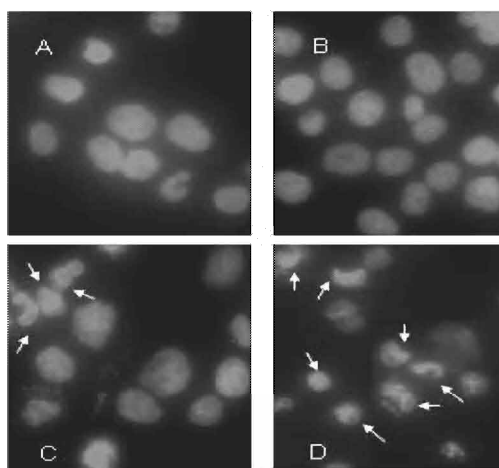
Figure 11. *Visualization of cisplatin/5'-aza-induced DU145 cells by Hoechst 33342 staining. A. control DU145 cells; B. DU-145 cells treated with 5'-aza alone; C. Cisplatin alone; D. 5'-aza plus cisplatin. Apoptotic cells were indicated by arrow*

4.4 Alteration of XAF1 mRNA expression by DNA methylation inhibitor in prostate cancer cells (Paper IV)

To determine the XAF1 mRNA expression profile in prostate cancer cell lines, we performed RT-PCR in LNCaP, DU145, and PC3. Renal cell carcinoma A498 cells were used as positive control. A full-length of XAF1 transcripts was detected in A498 cells. PC3 cells contained undetectable XAF1 mRNA, whereas LNCaP and DU145 cells exhibited the specific amplification product that was shorter than that seen in A498 cells (Figure 12B). This short form of XAF1 mRNA, recently identified as the

XAF1 transcript variant 2 (Accession: NM 199139), has a deletion of 57 nucleotides in the zinc finger region (Figure 12A).

We performed FISH analysis with the *XAF1* locus specific probe on the metaphase spreads of prostate cancer cells. We found that PC3 has normal two *XAF1* copies, while DU145 has 3 copies. In the majority of LNCaP cells, two specific *XAF1* signals are seen, whereas a small fraction of the cells have 3 *XAF1* copies among which two are localized on chromosome 17 and the third one is on different chromosomes, likely due to gains of partial chromosome 17.
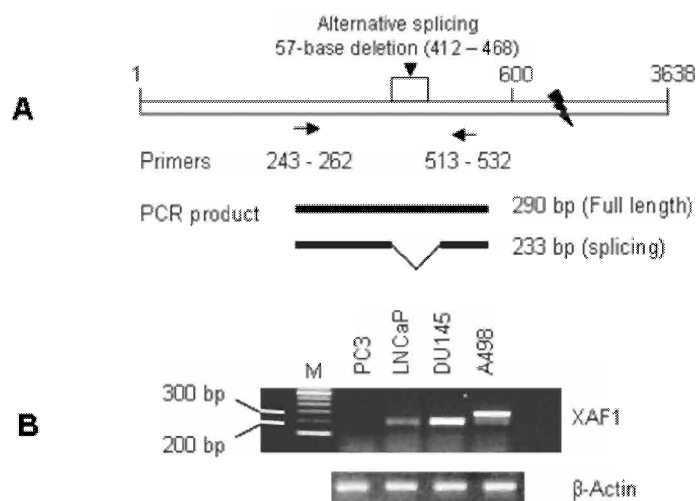
Figure 12. *Lacks of the full length XAF1 mRNA expression in prostate cancer cell lines. (A) Schematic presentation of the XAF1 transcript variants and corresponding PCR products. The alternative splicing region and PCR primer locations are indicated. The full length XAF1 (XAF1 transcript variant 1, ACCESSION: NM 017523) mRNA expression leads to the amplification of a 290 bp long fragment. An alternative splicing with 57-base deletion results in the formation of the XAF1 transcript variant 2 (ACCESSION: NM 199139), and the corresponding PCR product is 233 bps when it is present. (B) Lacks of the full length XAF1 mRNA in prostate cancer cell lines. The primers shown in (A) were used to PCR amplify the XAF1 transcripts in three prostate cell lines. A498 cells expressing full length XAF1 mRNA were included as a positive control. M: DNA molecular mark.*

To further determine whether there existed very small deletions in the *XAF1* locus that could not be detected by FISH, we designed a pair of the primers that spanned the alternative splicing region of *XAF1* mRNA and performed PCR analyses on the genomic DNA derived from LNCaP, DU145 and PC3 cells. We did not find any deletions in all the cells, concluding that lack of the full-length of *XAF1* transcripts in prostate cancer cell lines, is not attributable to the deletion of the *XAF1* allele.

It has been found that the hypermethylation at the *XAF1* promoter region is associated with silencing of *XAF1* mRNA expression in gastric cancer cells (Byun D, et al, 2003). Therefore, we sought to examine whether this same mechanism happened in prostate cancer cells. We incubated DU145 cells with 5'-aza, a potent DNA methylation inhibitor. The 5'-aza treatment switched short *XAF1* transcription to full-length transcription in DU145 cells (Figure 13A). We also examined the methylation status of the *XAF1* promoter region in DU145 cells by using MSP. In the absence of 5'-aza, the methylated primers specific for the *XAF1* promoter region led to a positive amplification signal, demonstrating hyper-methylation of the *XAF1* promoter in DU145 cells. Following the exposure of the cells to 5'-aza, the unmethylated *XAF1* promoter became detectable, which occurred concomitantly with induction of the full-length *XAF1* mRNA (Figure 13B). Because there was still substantial hyper-methylation at the *XAF1* promoter even after 5'-aza treatment of the cells, DNA methylation at the promoter region was only partially inhibited.

To determine whether the finding from the cell lines mirrors *in vivo* alterations in *XAF1* expression, we analyzed 6 pairs of primary prostate cancer samples for *XAF1* expression profile. *XAF1* mRNA was undetectable in two tumors and significantly lower in other two tumor specimens compared to their paired normal prostate tissues. A full length of *XAF1* mRNA was a predominant form in both normal and malignant prostate tissues, although presence of detectable alternatively spliced transcripts in them. Nevertheless, a complete lack or diminished expression of *XAF1* mRNA in 4 of 6

40

prostate cancer samples suggests the dysregulation of this gene during *in vivo* tumorigenesis of prostate tissues.
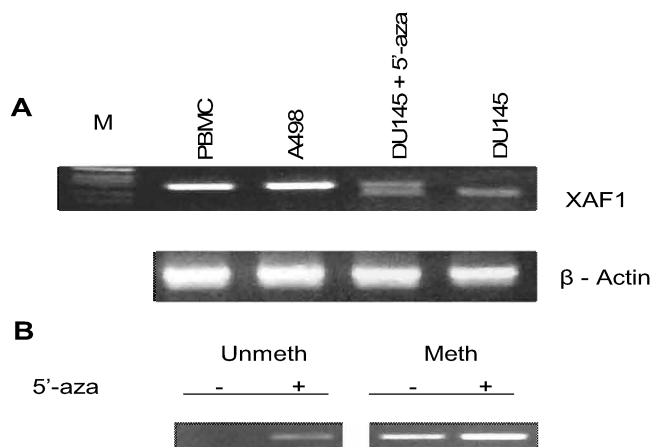

Figure 13. *The full length of XAF1 mRNA induction concomitantly occurred with de-methylation of the XAF1 promoter in DU145 cells treated with 5'-aza. (A) Up-regulation of the full length XAF1 mRNA in 5'-aza-treated DU145 cells. The cells were incubated with 2.5 μM 5'-aza for 72 hours and RT-PCR analyses for XAF1 mRNA expression were carried out. PBMC: Peripheral blood mononuclear cells. (B) Partial demethylation of the XAF1 promoter sequence in 5'-aza-treated DU145 cells. MSP was performed on the same batch of DU145 cells, as described in Materials and Methods. Unmeth and Meth: Unmethylated and methylated PCR products.*

5. GENERAL DISCUSSION

The development and progression of prostate cancer is really complex. There are still a lot of mysteries need to be solved, even though modern medicine has deepen the research to molecular level and more and more genes, proteins, small molecular agents, and chemical compounds related to cancers have been characterized and studied.

In previous studies, many androgen-regulated genes have been found to be involved in the growth regulation of the prostate gland. The *ezrin* gene is down-regulated in androgen withdrawal-induced apoptosis and up-regulated in androgen replacement-stimulated proliferation in rat ventral prostatic epithelial cells (Pang ST, et al, 2002). In our study, the *ezrin* has a greater expression level in HGPIN and prostate cancer compared with normal prostatic epithelium. Moreover, the expression of *ezrin* is always stronger in HGPIN than in the concomitant prostate cancer specimen. The aberrant expression noted in HGPIN and prostate cancer, which cannot be explained by a corresponding alteration on genomic level as shown by FISH, may be related to the androgen responsive status of *ezrin*. HGPIN is not the only precancerous lesion showing over-expression of *ezrin*. Over-expression has also been found in atypical endometrial hyperplasia, a precursor lesion of endometrial adenocarcinoma (Ohtani K, et al, 2002). Over-expression of *ezrin* has been correlated to the metastatic potential of several cancers. However, it has been shown that *ezrin* can bind to key adhesion proteins E-cadherin and beta-catenin, and removal of *ezrin* by antisense treatment increases the invasive capacity of colorectal epithelial tumor cell lines (Hiscox S, et al, 1999). The high expression level of *ezrin* in HGPIN compared with in prostate cancer is, therefore, intriguing. A possible function of *ezrin* may instead be to strengthen the cells' ability to retain their normal growth pattern. Recently, *ezrin* was shown to co-localize with CD44 in DU145 and PC3 cells, and the CD44/ezrin complex was involved in the capture and invasion of endothelial cells by these prostate cancer cells

(Harrison GM, et al, 2002). CD44 is a transmembrane protein that has been implicated in cell migration and tumor metastasis, including prostate cancer (Noordzij MA, et al 1999). Therefore, whether the expression level of *ezrin* could be a relevant factor for invasiveness of prostate cancer awaits additional studies.

Our study assesses the patterns of *pim-1* expression in HGPIN and cancer in a panel of 121 total prostatectomy specimens. Similar to previous study (Dhanasekaran SM, et al, 2001), we found a distinct *pim-1* over-expression in the majority of prostate cancers. In their study, however, there was no or weak *pim-1* expression reported in 82% of HGPIN samples. In contrast, we found that in 65% of the cases *pim-1* expression in HGPIN was even stronger than in cancer. One possible explanation for this discrepancy is that we analyzed immunoexpression of *pim-1* on conventional sections rather than tissue microarrays (TMAs). Our study was specifically designed to examine HGPIN and traditional sections are advantageous in this regard as they provide a larger amount of material for analysis than TMAs. Because our scoring was based on areas with highest staining intensity, a greater overall score can be expected with conventional sections than with TMAs. Additionally, studies of data agreement between TMAs and standard histological sections sometimes gave conflicting results. In general, variability of expression depends on tumor heterogeneity, sample representativity, and the amount of material which may explain differences between studies. Our study demonstrated distinct *pim-1* over-expression in HGPIN, taking into account the biological role of *pim-1* as gene involved in cell cycle regulation and proliferative processes. The data suggest a possible implication of *pim-1* in initiation of prostatic carcinogenesis. *Pim-1* over-expression in HGPIN may be an early event in the development of prostate malignancy, possibly marking the transition from precancerous lesions to invasive prostate cancer. *Pim-1* over-expression was indeed associated with high Gleason score. As HGPIN is not easily recognized, our data indicate that *pim-1* expression may provide additional information for distinguishing HGPIN from benign prostatic epithelium.

Chemotherapy is one of the major approaches for treatment of hormone-refractory

and advanced metastatic prostate cancers. However, systemic toxicity and clinical efficacy of chemotherapy limited its use. The early single-agent chemotherapy trials in prostate cancer patients were not satisfied. Traditionally, experimental and clinical studies focused on the combination of different kinds of chemotherapeutic drugs. Such combinations could indeed increase the response rates, but still with high toxicity. It is really necessary to test the use of rational combinations of chemotherapeutic drugs with new compound not only to lower drug doses and toxicity but also to increase the drug efficacy. To this purpose, we combined the chemotherapeutic drug cisplatin with the DNA methylation inhibitor 5'-aza to test potential synergistic effects of these two agents on prostate cancer DU145 cells. 5'-aza alone did not have significant influence on survival of DU145 cells, but considerably potentiated their apoptotic death induced by cisplatin. It is currently unclear how exactly 5'-aza enhances the sensitivity of DU145 cells to cisplatin. Given its role in inhibiting global DNA methylation, most likely 5'-aza restores functional expression of certain genes essential for controlling apoptosis through this mechanism. DNA methylation inhibitors such as azacytidine and 5'-aza have recently been used to treat several types of hematological malignancies. 5'-aza resulted in a significantly higher response rate, improved quality of life, and improved survival compared to supportive care (Issa JP, et al, 2004). Based on the present findings, it should be worthwhile trying the combination of the DNA methylation inhibitors with cisplatin in prostate cancer models, which might provide rational guidance in developing more effective strategies against advanced hormone-refractory prostate cancer.

The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of cell death. In order to survive and progress, cancer cells and their precursors must acquire efficient mechanisms to proliferate and avoid apoptosis. XAF1, a newly identified pro-apoptotic protein is ubiquitously expressed in normal human tissues. When exposed to apoptotic stimuli,

44

XAF1 expression is up-regulated. It has been shown that down-regulation of XAF1 expression is a frequent molecular event in cancer cell line (Liston P, et al, 2001). Evidence has accumulated that ectopic expression of XAF1 induces or sensitizes apoptotic death of cancer cells. Given those observations, it is likely that down-regulation of XAF1 expression is one of mechanisms by which pre-malignant or cancer cells escape from apoptosis and become invasive. In our study, we observed that all three prostate cancer cell lines lacked full-length of XAF1 transcripts but some of them expressed a short form of XAF1 mRNA. This is consistent with the finding that different forms of XAF1 transcripts are differentially expressed between normal human tissues and cancer cell lines. Because there was no detectable deletion of the entire or part of the XAF1 locus, alternative splicing of XAF1 mRNA must happen in these prostate cancer cells. Moreover, this same short form of XAF1 transcripts, together with a full-length of transcripts, was frequently expressed in primary specimens from prostate cancer patients. The finding suggests a partial or complete splicing switch from full-length to short form of XAF1 mRNA during pathogenesis and progression of prostate cancers. Such a switch leads to production of a truncated XAF1 protein with 19 amino acid deletion in its zinc finger domain that likely affects its functional interaction with XIAP, and consequently contributes to survival advantages of pre-neoplastic or malignant prostatic epithelial cells. It is also likely that the truncated XAF1 may elicit a dominant-negative action. Regulation of gene function through alternative splicing is widely present in both normal and cancer cells but underlying mechanisms are poorly understood. The finding that inhibition of DNA methylation resulted in a splicing switch of XAF1 transcripts is unexpected. In fact, it is well-established that many tumor suppressors can be inactivated through promoter hyper-methylation during carcinogenesis, which consequently promotes tumor formation and progression. However, the involvement of DNA methylation in regulation of

alternative splicing, as seen in the present study, has not yet been described. One of the possibilities is that splicing factor(s) responsible for the full-length XAF1 transcripts are silenced due to their own promoter hyper-methylation, and reactivated following the treatment of the cells with 5'-aza. Alternatively, full-length and short XAF1 mRNA transcriptions are driven by different regions of the XAF1 promoter and carcinogens may selectively target methylation of the promoter region responsible for a full-length XAF1 transcription. This later hypothesis seems to be supported by the finding that the XAF1 promoter is methylated in DU145 cells, and that the induction of the full-length of XAF1 transcripts occurs concomitantly with promoter de-methylation after 5'-aza treatment. DNA hypermethylation has been found to be associated with drug resistance acquired during cancer chemotherapy or poor outcomes (Esteller M, 2005). Our previous study demonstrated that inhibition of DNA methylation could enhance sensitivity of DU145 cells to chemotherapeutic drugs cisplatin (Fang X, et al, 2004). Given the present finding, it is likely that induction of XAF1 expression by 5'-aza is one of downstream molecules contributing to its sensitizing effects. XIAP has been suggested as a target for prostate cancer therapy (Devi GR, 2004), and manipulating XAF1 expression, an antagonist for XIAP, should provide an alternative strategy for the same purpose. Further studies will be interesting to determine whether this therapeutic strategy up-regulates XAF1 expression *in vivo*, and thereby benefit patients.

46

6. SUMMARY AND CONCLUSIONS

1. A novel androgen regulated gene, *ezrin* was found to be dysregulated in prostate cancer and HGPIN. Its over-expression may be associated with tumorigenesis and tumor metastasis potential.

2. Over-expression of *pim-1* in HGPIN may be an early event in the development of prostate cancer, and also provides supplementary information for distinguishing HGPIN from benign prostate epithelium.

3. DNA methylation inhibitor significantly increases apoptotic death of prostate cancer DU-145 cells induced by the chemotherapeutic drug cisplatin. The present finding provides a rational to evaluate therapeutic effects of the DNA methylation inhibition and chemotherapy in patients with prostate cancer.

4. Splicing alterations of the *XAF1* transcript may occur during the development of prostate cancer due to the aberrant DNA methylation. The lack of full-length of *XAF1* transcripts can confer survival advantages and progression of prostate cancer. Antagonizing XIAP function by inducing *XAF1* expression may be a novel strategy against prostate cancer.

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REFERENCES

Aho TL, Sandholm J, Peltola KJ, Mankonen HP, Lilly M, Koskinen PJ.Pim-1 kinase promotes inactivation of the pro-apoptotic Bad protein by phosphorylating it on the Ser112 gatekeeper site. *FEBS Lett.* 2004 Jul 30;571(1-3):43-9.

Akisawa N, Nishimori I, Iwamura T, Onishi S, Hollingsworth MA. High levels of ezrin expressed by human pancreatic adenocarcinoma cell lines with high metastatic potential. *Biochem Biophys Res Commun.* 1999 May 10;258(2):395-400.

Algrain M, Turunen O, Vaheri A, Louvard D, Arpin M. Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane-cytoskeletal linker. *J Cell Biol.* 1993 Jan;120(1):129-39.

Andreoli C, Martin M, Le Borgne R, Reggio H, Mangeat P. Ezrin has properties to self-associate at the plasma membrane. *J Cell Sci.* 1994 Sep;107 (Pt 9):2509-21.

Ashida S, Nakagawa H, Katagiri T, Furihata M, Iiizumi M, Anazawa Y, Tsunoda T, Takata R, Kasahara K, Miki T, Fujioka T, Shuin T, Nakamura Y. Molecular features of the transition from prostatic intraepithelial neoplasia (PIN) to prostate cancer: genome-wide gene-expression profiles of prostate cancers and PINs. *Cancer Res.* 2004 Sep 1;64(17):5963-72.

Asselin E, Mills GB, Tsang BK. XIAP regulates Akt activity and caspase-3-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells. *Cancer Res.* 2001 Mar 1;61(5):1862-8.

Bahnson RR, Dresner SM, Gooding W, Becich MJ. Incidence and prognostic significance of lymphatic and vascular invasion in radical prostatectomy specimens. *Prostate.* 1989; 15: 149-155.

Basu A, Weixel K, Saijo N. Characterization of the protein kinase C signal transduction pathway in cisplatin-sensitive and -resistant human small cell lung carcinoma cells. *Cell Growth Differ.* 1996 Nov;7(11):1507-12.

Bauman JG, Wiegant J, Borst P, van Duijn P. A new method for fluorescence microscopical localization of specific DNA sequences by in situ hybridization of fluorochromelabelled RNA. Exp Cell Res. 1980 Aug;128(2):485-90.

Bhatia-Gaur R, Donjacour AA, Sciavolino PJ, Kim M, Desai N, Young P, Norton CR, Gridley T, Cardiff RD, Cunha GR, Abate-Shen C, Shen MM. Roles for Nkx3.1 in prostate development and cancer. *Genes Dev*. 1999 Apr 15;13(8):966-77.

Bohling T, Turunen O, Jaaskelainen J, Carpen O, Sainio M, Wahlstrom T, Vaheri A, Haltia M. Ezrin expression in stromal cells of capillary hemangioblastoma. An immunohistochemical survey of brain tumors. *Am J Pathol*. 1996 Feb;148(2):367-73.

Boivin AJ, Momparler LF, Hurtubise A, Momparler RL. Antineoplastic action of 5-aza-2'-deoxycytidine and phenylbutyrate on human lung carcinoma cells. *Anticancer Drugs*. 2002 Sep;13(8):869-74.

Bostwick DG, Brawer MK. Prostatic intra-epithelial neoplasia and early invasion in prostate cancer. *Cancer*. 1987 Feb 15;59(4):788-94.

Bostwick DG, Wollan P, Adlakha K. Collagenous micronodules in prostate cancer. A specific but infrequent diagnostic finding. *Arch Pathol Lab Med*. 1995; 119: 444-447.

Bostwick DG. Prostatic intraepithelial neoplasia is a risk factor for cancer. *Semin Urol Oncol*. 1999 Nov;17(4):187-98

Bostwick DG, Qian J. High-grade prostatic intraepithelial neoplasia. *Mod Pathol*. 2004 Mar;17(3):360-79.

Brawer MK, Deering RE, Brown M, Preston SD, Bigler SA. Predictors of pathologic stage in prostatic carcinoma. The role of neovascularity. *Cancer*. 1994; 73: 678-687.

Brawley OW, Knopf K, Merrill R. The epidemiology of prostate cancer part I: descriptive epidemiology. *Semin Urol Oncol*. 1998 Nov;16(4):187-92.

Bretscher A. Purification of an 80,000-dalton protein that is a component of the isolated microvillus cytoskeleton, and its localization in nonmuscle cells. *J Cell Biol.* 1983 Aug;97(2):425-32.

Bretscher A, Edwards K, Fehon RG. ERM proteins and merlin: integrators at the cell cortex. *Nat Rev Mol Cell Biol.* 2002 Aug;3(8):586-99.

Brooks JD, Weinstein M, Lin X, Sun Y, Pin SS, Bova GS, Epstein JI, Isaacs WB, and Nelson WG. CG island methylation changes near the GSTP1 gene in prostatic intraepithelial neoplasia. *Cancer Epidemiol Biomarkers Prev* 1998 7: 531-536.

Byun DS, Cho K, Ryu BK, Lee MG, Kang MJ, Kim HR, Chi SG. Hypermethylation of XIAP-associated factor 1, a putative tumor suppressor gene from the 17p13.2 locus, in human gastric adenocarcinomas. *Cancer Res.* 2003 Nov 1;63(21):7068-75.

Carter BS, Carter HB, Isaacs JT. Epidemiologic evidence regarding predisposing factors to prostate cancer. *Prostate.* 1990; 16: 187-197.

Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, Hennekens CH, Pollak M. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science.* 1998 Jan 23;279(5350):563-6

Cunningham JM, Shan A, Wick MJ, McDonnell SK, Schaid DJ, Tester DJ, Qian J, Takahashi S, Jenkins RB, Bostwick DG, Thibodeau SN. Allelic imbalance and microsatellite instability in prostatic adenocarcinoma. *Cancer Res.* 1996 Oct 1;56(19):4475-82.

Cuypers HT, Selten G, Quint W, Zijlstra M, Maandag ER, Boelens W, van Wezenbeek P, Melief C, Berns A. Murine leukemia virus-induced T-cell lymphomagenesis: integration of proviruses in a distinct chromosomal region. *Cell.* 1984;37(1):141-50.

Cuzick J. Human papillomavirus infection of the prostate. *Cancer Surv.* 1995;23:91-5.

Das PM, Singal R. DNA methylation and cancer. *J Clin Oncol.* 2004;22(22):4632-42.

De Marzo AM, Meeker AK, Epstein JI, Coffey DS. Prostate stem cell compartments: expression of the cell cycle inhibitor p27Kip1 in normal, hyperplastic, and neoplastic cells. *Am J Pathol.* 1998 Sep;153(3):911-9.

DeMarzo AM, Nelson WG, Isaacs WB, Epstein JI. Pathological and molecular aspects of prostate cancer. *Lancet.* 2003 Mar 15;361(9361):955-64.

Dennis LK, Hayes RB. Alcohol and prostate cancer. *Epidemiol Rev.*2001;23(1):110-4.

Desbiens KM, Deschesnes RG, Labrie MM, Desfosses Y, Lambert H, Landry J, Bellmann K. c-Myc potentiates the mitochondrial pathway of apoptosis by acting upstream of apoptosis signal-regulating kinase 1 (Ask1) in the p38 signalling cascade. *Biochem J.* 2003 Jun 1;372(Pt 2):631-41.

Devi. GR. XIAP as target for therapeutic apoptosis in prostate cancer. *Drug News Perspect.* 2004;17:127-134

Dhanasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, Kurachi K, Pienta KJ, Rubin MA, Chinnaiyan AM. Delineation of prognostic biomarkers in prostate cancer. *Nature.* 2001 Aug 23;412(6849):822-6.

Dijkman GA, Debruyne FM. Epidemiology of prostate cancer. *Eur Urol.* 1996; 30: 281-295.

Downing SR, Russell PJ, Jackson P. Alterations of p53 are common in early stage prostate cancer. *Can J Urol.* 2003 Aug;10(4):1924-33.

Ehrlich M. DNA methylation in cancer: too much, but also too little. *Oncogene.* 2002 Aug 12;21(35):5400-13

Eichmann A, Yuan L, Breant C, Alitalo K, Koskinen PJ. Developmental expression of pim kinases suggests functions also outside of the hematopoietic system. *Oncogene.* 2000 Feb 24;19(9):1215-24.

Ellis RE, Yuan JY, Horvitz HR. Mechanisms and functions of cell death. *Annu Rev Cell Biol*. 1991;7:663-98

Epstein JI. Interpretation of prostate biopsies. 3rd edn. Philadelphia, PA: Lippincott Williams and Wilkins, 2002.

Esteller M. DNA methylation and cancer therapy: new developments and expectations. *Curr Opin Oncol*. 2005 Jan;17(1):55-60.

Fang X, Zheng C, Liu Z, Ekman P, Xu D. Enhanced sensitivity of prostate cancer DU145 cells to cisplatinum by 5-aza-2'-deoxycytidine. *Oncol Rep*. 2004 Sep; 12(3): 523-6

Fichtinger-Schepman AM, van der Veer JL, den Hartog JH, Lohman PH, Reedijk J. Adducts of the antitumor drug cis-diamminedichloroplatinum(II) with DNA: formation, identification, and quantitation. *Biochemistry*. 1985 Jan 29;24(3):707-13.

Fong WG, Liston P, Rajcan-Separovic E, St Jean M, Craig C, Korneluk RG.Expression and genetic analysis of XIAP-associated factor 1 (XAF1) in cancer cell lines. *Genomics*. 2000 Nov 15;70(1):113-22.

Friesen C, Herr I, Krammer PH, Debatin KM. Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. *Nat Med*. 1996 May;2(5):574-7.

Fruhwald MC. DNA methylation patterns in cancer: novel prognostic indicators? *Am J Pharmacogenomics*. 2003;3(4):245-60.

Gao AC, Isaacs JT. Molecular basis of prostate cancinogenesis. In: Coleman WB, Tsongalis GJ, eds. The molecular basis of human cancer. Totowa, NJ: Human Press, 2002: 365-379.

Gingrich JR, Barrios RJ, Kattan MW, Nahm HS, Finegold MJ, Greenberg NM. Androgen-independent prostate cancer progression in the TRAMP model. *Cancer Res*. 1997 Nov 1;57(21):4687-91.

54

Gleason DF. Classification of prostatic carcinomas. *Cancer Chemother Rep.* 1966 Mar;50(3):125-8.

Gleason DF, Mellinger GT. Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. *J Urol.* 1974 Jan;111(1):58-64

Goldstein NS, Qian J, Bostwick DG. Mucin expression in atypical adenomatous hyperplasia of the prostate. *Hum Pathol.* 1995; 26: 887-891.

Granes F, Urena JM, Rocamora N, Vilaro S. Ezrin links syndecan-2 to the cytoskeleton. *J Cell Sci.* 2000 Apr;113 (Pt 7):1267-76

Grönberg H. Prostate cancer epidemiology. *Lancet.* 2003 Mar 8;361(9360):859-64

Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell.* 2000 Jan 7;100(1):57-70.

Harrison GM, Davies G, Martin TA, Jiang WG, Mason MD. Distribution and expression of CD44 isoforms and Ezrin during prostate cancer-endothelium interaction. *Int J Oncol.* 2002 Nov;21(5):935-40.

He WW, Sciavolino PJ, Wing J, Augustus M, Hudson P, Meissner PS, Curtis RT, Shell BK, Bostwick DG, Tindall DJ, Gelmann EP, Abate-Shen C, Carter KC. A novel human prostate-specific, androgen-regulated homeobox gene (NKX3.1) that maps to 8p21, a region frequently deleted in prostate cancer. *Genomics.* 1997 Jul 1;43(1):69-77

Heinlein CA, Chang C. Androgen receptor in prostate cancer. *Endocr.* 2004 Apr;25(2): 276-308.

Helpap BG, Bostwick DG, Montironi R. The significance of atypical adenomatous hyperplasia and prostatic intraepithelial neoplasia for the development of prostate carcinoma. An update. *Virchows Arch.* 1995;426(5):425-34.

Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A.* 1996 Sep 3;93(18):9821-6.

Hickey K, Do KA, Green A. Smoking and prostate cancer. *Epidemiol Rev.* 2001; 23(1):115-25.

Hiscox S, Jiang WG. Ezrin regulates cell-cell and cell-matrix adhesion, a possible role with E-cadherin/beta-catenin. *J Cell Sci.* 1999 Sep;112 Pt 18:3081-90.

Hoover DS, Wingett DG, Zhang J, Reeves R, Magnuson NS. Pim-1 protein expression is regulated by its 5'-untranslated region and translation initiation factor elF-4E. *Cell Growth Differ.* 1997 Dec;8(12):1371-80.

Humphrey PA. Gleason grading and prognostic factors in carcinoma of the prostate. *Mod Pathol.* 2004 Mar;17(3):292-306.

Hunter KW. Ezrin, a key component in tumor metastasis. *Trends Mol Med.* 2004 May;10(5):201-4.
Ingraffea J, Reczek D, Bretscher A. Distinct cell type-specific expression of scaffolding proteins EBP50 and E3KARP: EBP50 is generally expressed with ezrin in specific epithelia, whereas E3KARP is not. *Eur J Cell Biol.* 2002 Feb;81(2):61-8.

Issa JP, Garcia-Manero G, Giles FJ, Mannari R, Thomas D, Faderl S, Bayar E, Lyons J, Rosenfeld CS, Cortes J, Kantarjian HM. Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in hematopoietic malignancies. *Blood.* 2004 Mar 1;103(5):1635-40

Kalebic T. Epigenetic changes: potential therapeutic targets. *Ann N Y Acad Sci.* 2003 Mar;983:278-85.

Kalish LA, McDougal WS, McKinlay JB. Family history and the risk of prostate cancer. *Urology.* 2000; 56: 803-806.

Khanna C, Khan J, Nguyen P, Prehn J, Caylor J, Yeung C, Trepel J, Meltzer P, Helman L. Metastasis-associated differences in gene expression in a murine model of osteosarcoma. *Cancer Res*. 2001 May 1;61(9):3750-9.

Kim MJ, Bhatia-Gaur R, Banach-Petrosky WA, Desai N, Wang Y, Hayward SW, Cunha GR, Cardiff RD, Shen MM, Abate-Shen C. Nkx3.1 mutant mice recapitulate early stages of prostate carcinogenesis. *Cancer Res*. 2002 Jun 1;62(11):2999-3004.

Koppie TM, Grossfeld GD, Miller D, Yu J, Stier D, Broering JM, Lubeck D, Henning JM, Flanders SC, Carroll PR Patterns of treatment of patients with prostate cancer initially managed with surveillance: results from The CaPSURE database. Cancer of the Prostate Strategic Urological Research Endeavor. *J Urol.* 2000 Jul;164(1):81-8.

Leaman DW, Chawla-Sarkar M, Vyas K, Reheman M, Tamai K, Toji S, Borden EC. Identification of X-linked inhibitor of apoptosis-associated factor-1 as an interferon-stimulated gene that augments TRAIL Apo2L-induced apoptosis. *J Biol Chem.* 2002 Aug 9;277(32):28504-11.

Leverson JD, Koskinen PJ, Orrico FC, Rainio EM, Jalkanen KJ, Dash AB, Eisenman RN, Ness SA. Pim-1 kinase and p100 cooperate to enhance c-Myb activity. *Mol Cell.* 1998 Oct;2(4):417-25.

Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science.* 1997 Mar 28;275(5308):1943-7.

Lin X, Tascilar M, Lee WH, Vles WJ, Lee BH, Veeraswamy R, Asgari K, Freije D, van Rees B, Gage WR, Bova GS, Isaacs WB, Brooks JD, DeWeese TL, De Marzo AM, Nelson WG. GSTP1 CpG island hypermethylation is responsible for the absence of GSTP1 expression in human prostate cancer cells. *Am J Pathol.* 2001; 159(5): 1815-26.

Liston P, Fong WG, Kelly NL, Toji S, Miyazaki T, Conte D, Tamai K, Craig CG, McBurney MW, Korneluk RG.Identification of XAF1 as an antagonist of XIAP anti-Caspase activity. *Nat Cell Biol.* 2001 Feb;3(2):128-33.

Loehrer PJ, Einhorn LH. Drugs five years later. Cisplatin. *Ann Intern Med.* 1984 May;100(5):704-13.

Losman JA, Chen XP, Vuong BQ, Fay S, Rothman PB. Protein phosphatase 2A regulates the stability of Pim protein kinases. *J Biol Chem.* 2003 Feb 14;278(7):4800-5.

McMenamin ME, Soung P, Perera S, Kaplan I, Loda M, Sellers WR. Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. *Cancer Res.* 1999 Sep 1;59(17):4291-6.

McNeal JE. Regional morphology and pathology of the prostate. *Am J Clin Pathol.* 1968 Mar;49(3):347-57.

Meeker TC, Nagarajan L, ar-Rushdi A, Croce CM. Cloning and characterization of the human PIM-1 gene: a putative oncogene related to the protein kinases. *J Cell Biochem.* 1987 Oct;35(2):105-12.

Meeker AK, Hicks JL, Platz EA, March GE, Bennett CJ, Delannoy MJ, De Marzo AM. Telomere shortening is an early somatic DNA alteration in human prostate tumorigenesis. *Cancer Res.* 2002 Nov 15;62(22):6405-9.

Meng MV, Dahiya R. Molecular genetics. In: Carroll PR, Grossfeld GD, eds. Prostate cancer. Hamilton, Ontario: B C Decker, 2002: 42-59

Mikkers H, Nawijn M, Allen J, Brouwers C, Verhoeven E, Jonkers J, Berns A. Mice deficient for all PIM kinases display reduced body size and impaired responses to hematopoietic growth factors. *Mol Cell Biol.* 2004 Jul;24(13):6104-15.

Mizuno K, Shirogane T, Shinohara A, Iwamatsu A, Hibi M, Hirano T. Regulation of Pim-1 by Hsp90. *Biochem Biophys Res Commun.* 2001 Mar 2;281(3):663-9.

Noordzij MA, van Steenbrugge GJ, Schroder FH, Van der Kwast TH. Decreased expression of CD44 in metastatic prostate cancer. *Int J Cancer.* 1999;84(5):478-83.

Nyce JW. Drug-induced DNA hypermethylation: a potential mediator of acquired drug resistance during cancer chemotherapy. *Mutat Res*. 1997 Apr;386(2):153-61.

Ohtani K, Sakamoto H, Rutherford T, Chen Z, Kikuchi A, Yamamoto T, Satoh K, Naftolin F. Ezrin, a membrane-cytoskeletal linking protein, is highly expressed in atypical endometrial hyperplasia and uterine endometrioid adenocarcinoma. *Cancer Lett*. 2002 May 8;179(1):79-86.

Pang ST, Dillner K, Wu X, Pousette A, Norstedt G, Flores-Morales A. Gene expression profiling of androgen deficiency predicts a pathway of prostate apoptosis that involves genes related to oxidative stress. *Endocrinology*. 2002 Dec;143(12):4897-906

Parkin DM, Bray FI, Devesa SS. Cancer burden in the year 2000. The global picture. *Eur J Cancer*. 2001 Oct;37 Suppl 8:S4-66.

Partin AW, Kattan MW, Subong EN, Walsh PC, Wojno KJ, Oesterling JE, Scardino PT, Pearson JD. Combination of prostate-specific antigen, clinical stage, and Gleason score to predict pathological stage of localized prostate cancer. A multi-institutional update. *JAMA*. 1997 May 14;277(18):1445-51.

Pawlak G, Helfman DM. Cytoskeletal changes in cell transformation and tumorigenesis. *Curr Opin Genet Dev*. 2001 Feb;11(1):41-7.

Peterson HB, Howards SS. Vasectomy and prostate cancer: the evidence to date. *Fertil Steril*. 1998 Aug;70(2):201-3.

Pienta KJ, Esper PS. Risk factors for prostate cancer. *Ann Intern Med.* 1993;118:793-803.

Qian J, Wollan P, Bostwick DG.The extent and multicentricity of high-grade prostatic intraepithelial neoplasia in clinically localized prostatic adenocarcinoma. *Hum Pathol*. 1997 Feb;28(2):143-8.

Rainio EM, Sandholm J, Koskinen PJ. Cutting edge: Transcriptional activity of NFATc1 is enhanced by the Pim-1 kinase. *J Immunol*. 2002 Feb 15;168(4):1524-7.

Raviv G, Zlotta AR, Janssen T, Descamps F, Vanegas JP, Verhest A, Schulman CC. Do prostate specific antigen and prostate specific antigen density enhance the detection of prostate carcinoma after initial diagnosis of prostatic intraepithelial neoplasia without concurrent carcinoma? *Cancer*. 1996 May 15;77(10):2103-8.

Roh M, Gary B, Song C, Said-Al-Naief N, Tousson A, Kraft A, Eltoum IE, Abdulkadir SA.Overexpression of the oncogenic kinase Pim-1 leads to genomic instability. *Cancer Res*. 2003 Dec 1;63(23):8079-84.

Ronnett BM, Carmichael MJ, Carter HB, Epstein JI. Does high grade prostatic intraepithelial neoplasia result in elevated serum prostate specific antigen levels? *J Urol*. 1993 Aug;150(2 Pt 1):386-9.

Rubin MA, Gerstein A, Reid K, Bostwick DG, Cheng L, Parsons R, Papadopoulos N. 10q23.3 loss of heterozygosity is higher in lymph node-positive (pT2-3,N+) versus lymph node-negative (pT2-3,N0) prostate cancer. *Hum Pathol*. 2000 Apr;31(4):504-8.

Sakr WA, Billis A, Ekman P, Wilt T, Bostwick DG. Epidemiology of high-grade prostatic intraepithelial neoplasia. *Scand J Urol Nephrol* Suppl. 2000;(205):11-8.

Sakr WA & Partin AW. Histological markers of risk and the role of high-grade prostatic intraepithelial neoplasia. *Urology*. 2001 Apr;57(4 Suppl 1):115-20.

Saris CJ, Domen J, Berns A. The pim-1 oncogene encodes two related protein-serine/threonine kinases by alternative initiation at AUG and CUG. *EMBO J*. 1991 Mar;10(3):655-64.

Siegal JA, Yu E, Brawer MK. Topography of neovascularity in human prostate carcinoma. *Cancer*. 1995; 75: 2545-2551.

Sigalotti L, Altomonte M, Colizzi F, Degan M, Rupolo M, Zagonel V, Pinto A, Gattei V, Maio M. 5-Aza-2'-deoxycytidine (decitabine) treatment of hematopoietic malignancies: a multimechanism therapeutic approach? *Blood*. 2003 Jun 1;101(11):4644-6;

60

Sinha AA, Quast BJ, Reddy PK, Lall V, Wilson MJ, Qian J, Bostwick DG. Microvessel density as a molecular marker for identifying high-grade prostatic intraepithelial neoplasia precursors to prostate cancer. *Exp Mol Pathol.* 2004 Oct;77(2):153-9.

Steiner MS. High grade prostatic intraepithelial neoplasia is a disease. *Curr Urol Rep.* 2001;2(3):195-8.

Strathdee G, MacKean MJ, Illand M, Brown R. A role for methylation of the hMLH1 promoter in loss of hMLH1 expression and drug resistance in ovarian cancer. *Oncogene.* 1999 Apr 8;18(14):2335-41.

Suzuki H, Freije D, Nusskern DR, Okami K, Cairns P, Sidransky D, Isaacs WB, Bova GS. Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues. *Cancer Res.* 1998 Jan 15;58(2):204-9.

Tamm I, Kornblau SM, Segall H, Krajewski S, Welsh K, Kitada S, Scudiero DA, Tudor G, Qui YH, Monks A, Andreeff M, Reed JC. Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. *Clin Cancer Res.* 2000 May;6(5):1796-803.

Taplin ME, Balk SP. Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. *J Cell Biochem.* 2004 Feb 15;91(3):483-90.

Tran YK, Bogler O, Gorse KM, Wieland I, Green MR, Newsham IF. A novel member of the NF2/ERM/4.1 superfamily with growth suppressing properties in lung cancer. *Cancer Res.* 1999 Jan 1;59(1):35-43.

Tsujimoto Y. Cell death regulation by the Bcl-2 protein family in the mitochondria. *J Cell Physiol.* 2003 May;195(2):158-67.

Varma M, Lee MW, Tamboli P, Zarbo RJ, Jimenez RE, Salles PG, Amin MB. Morphologic criteria for the diagnosis of prostatic adenocarcinoma in needle biopsy specimens. A study of 250 consecutive cases in a routine surgical pathology practice. *Arch Pathol Lab Med.* 2002; 126: 554-561.

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell*. 2000 Jul 7;102(1):43-53.

Villar-Garea A, Esteller M. DNA demethylating agents and chromatin-remodelling drugs: which, how and why? *Curr Drug Metab*. 2003 Feb;4(1):11-31.

Yagoda A, Petrylak D. Cytotoxic chemotherapy for advanced hormone-resistant prostate cancer. *Cancer*. 1993 Feb 1;71(3 Suppl):1098-109.

Yang QH, Church-Hajduk R, Ren J, Newton ML, Du C. Omi/HtrA2 catalytic cleavage of inhibitor of apoptosis (IAP) irreversibly inactivates IAPs and facilitates caspase activity in apoptosis. *Genes Dev*. 2003 Jun 15;17(12):1487-96.

Yuan ZQ, Feldman RI, Sussman GE, Coppola D, Nicosia SV, Cheng JQ. AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: implication of AKT2 in chemoresistance. *J Biol Chem*. 2003 Jun 27; 278(26) : 23432-40.

Zamai L, Canonico B, Luchetti F, Ferri P, Melloni E, Guidotti L, Cappellini A, Cutroneo G, Vitale M, Papa S. Supravital exposure to propidium iodide identifies apoptosis on adherent cells. *Cytometry*. 2001 May 1;44(1):57-64.

Zanke BW, Boudreau K, Rubie E, Winnett E, Tibbles LA, Zon L, Kyriakis J, Liu FF, Woodgett JR. The stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum, UV irradiation or heat. *Curr Biol*. 1996 May 1;6(5):606-13.

Zegarra-Moro OL, Schmidt LJ, Huang H, Tindall DJ. Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. *Cancer Res*. 2002 Feb 15;62(4):1008-13.