17β-HYDROXYSTEROID DEHYDROGENASES/17-KETOSTEROID REDUCTASES (17HSD/KSRS) IN PROSTATE CANCER

The role of 17HSD/KSR types 2, 5, and 7 in steroid hormone action and loss of heterozygosity at chromosome region 16q

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Abstract

Prostate cancer is the most frequently diagnosed cancer in men in industrialized countries. Despite the substantial clinical importance of the disease, the mechanisms underlying the development and progression of prostate cancer are poorly understood.

In the present study, fragment analysis of chromosome arm 16q was carried out with the aim of searching for sites of consistent chromosomal deletion, possibly uncovering the location of target genes that become inactivated in prostate carcinogenesis. The highest percentage of loss of heterozygosity (LOH) was found at chromosomal region 16q24.1-q24.2, including the gene for 17β-hydroxysteroid dehydrogenase/17-ketosteroid reductase (17HSD/KSR) type 2, *HSD17B2*. The data further indicated an association between loss of the most commonly deleted region and clinically aggressive features of the disease. A fragment analysis performed using sequential primary and locally recurrent prostate cancer specimens suggested the location of the genes related to prostate cancer progression to be at 16q24.3 and, further, gave rise to a hypothesis of the potential role of locus *HSD17B2* as a prognostic marker for prostate cancer progression. Quantitative real-time polymerase chain reaction (PCR) revealed a decreased *HSD17B2* gene copy number in prostate cancer specimens compared to their normal counterparts. A diminished *HSD17B2* gene copy number was significantly associated with poor differentiation of the tumor.

The progression of prostate cancer during androgen deprivation is a serious clinical problem, the molecular mechanisms of which largely remain to be clarified. The present data of enzyme activity measurements performed using high-performance liquid chromatography (HPLC) provided evidence of a substantial decrease in oxidative and an increase in reductive 17HSD/KSR activity during the transition of prostate cancer LNCaP cells into an androgen-independent state. Further, the changes detected in the activities largely coincided with the changes in the relative expression levels of genes for the potential 17HSD/KSR isoenzymes; 17HSD/KSR types 2, 5, and 7, as evidenced by relative quantitative reverse transcription PCR (RT-PCR). The data on the expression analysis of mRNA for 17HSD/KSR types 5 and 7 in prostate tissue specimens performed using *in situ* hybridization showed a moderately low but constitutive level for 17HSD/KSR7 mRNA in tissues of cancerous as well as hyperplastic origin. The expression of mRNA for 17HSD/KSR type 5, instead, varied considerably between different specimens, the highest expressions being strongly associated with aggressive and metastatic prostate cancer. Interestingly, furthermore, the intense expression of 17HSD/KSR5 was significantly associated with the androgen deprivation achieved either surgically or medically.

Since 17HSD/KSRs critically contribute to the control of the bioavailability of active sex steroid hormones locally in the prostate, the variation in intraprostatic 17HSD/KSR activity might be crucially involved in the regulation of the growth and function of the organ.

Keywords: 17-hydroxysteroid dehydrogenases, androgens, estrogens, loss of heterozygosity, prostatic neoplasms

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Oulu, November 2005

Päivi Härkönen

Abbreviations

Hormones

A-diol	androstenediol, 5-androstene-3β,17β-diol
3αA-diol	5α-androstane-3α,17β-diol
A-dione	androstenedione, 4-androsten-3,17-dione
5αA-dione	androstanedione, 5α-androstan-3,17-dione
ADT	androsterone
3βA-diol	5α-androstane-3β,17β-diol
DDX	dihydrodiol dehydrogenase X
DHEA	dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one
DHEA-S	dehydroepiandrosterone sulfate
DHT	dihydrotestosterone, 5α-dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one
E1	estrone, 3-hydroxy-1,3,5 (10)-estratriene-17-one
E2	estradiol, 1,3,5 (10)-estratriene-3,17β-diol
GnRH	gonadotropin hormone-releasing hormone
P	progesterone, 4-pregnene-3,20-dione
PG	prostaglandin
T	testosterone, 17β-hydroxy-4-androsten-3-one

Others

AKR	aldo-keto reductase
AR	androgen receptor
BNP	brain natriuretic peptide
BPH	benign prostatic hyperplasia
CAR / CMAR	cell adhesion regulator / cellular adhesion regulatory molecule
CDH13	H-cadherin
CDH15	M-cadherin
cDNA	complementary DNA
CFE	colony-forming efficiency
CGH	comparative genomic hybridization

Da	dalton
dNTP	deoxynucleoside triphosphate
E-cad	E-cadherin
FCS	fetal calf serum
GAS11	growth arrest-specific 11
H-cad	H-cadherin
HPLC	high-performance liquid chromatography
3α-HSD	3α-hydroxysteroid dehydrogenase
20α-HSD	20α-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
17HSD/KSR	17β-hydroxysteroid dehydrogenase/17-ketosteroid reductase
HSD17B2/5/7	gene for 17HSD/KSR type 2/5/7
HZD	homozygous deletion
IGF	insulin-like growth factor
IGF-IR	IGF type I receptor
LNCaP	lymph node carcinoma of the prostate
LOH	loss of heterozygosity
M-cad	M-cadherin
NAD^+	nicotinamide-adenine dinucleotide
$NADP^+$	nicotinamide-adenine dinucleotide phosphate
P	progesterone, 4-pregnene-3,20-dione
PAP	prostatic acid phosphatase
PCR	polymerase chain reaction
PIA	prostate inflammatory atrophy
PIN	prostatic intraepithelial neoplasia
PISSLRE	putative cyclin-dependent kinase
PSA	prostate-specific antigen
RT-PCR	reverse transcription polymerase chain reaction
SDR	short-chain dehydrogenase/ reductase
SHBG	sex hormone-binding globulin
TNM	tumor-node-metastasis (classification)
TSG	tumor suppressor gene
UGT	uridine diphosphoglucuronosyltransferase

List of original publications

The thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Elo JP, Härkönen P, Kyllönen AP, Lukkarinen O, Poutanen M, Vihko R & Vihko P (1997) Loss of heterozygosity at 16q24.1-q24.2 is significantly associated with metastatic and aggressive behavior of prostate cancer. Cancer Res 57: 3356–3359.

II Härkönen P, Kyllönen AP, Nordling S & Vihko P (2005) Loss of heterozygosity in chromosomal region 16q24.3 associated with progression of prostate cancer. The Prostate 62: 267–274.

III Härkönen P, Törn S, Kurkela R, Porvari K, Pulkka A, Lindfors, A, Isomaa V & Vihko P (2003) Sex hormone metabolism in prostate cancer cells during transition to an androgen-independent state. J Clin Endocrinol Metab 88: 705–712.

IV Härkönen P, Soini Y, Hirvikoski P, Ilves M, Porvari K, Pulkka A & Vihko P (2005) High 17β-hydroxysteroid dehydrogenase type 5 (17HSD5, AKR1C3) expression is significantly associated with aggressive prostate cancer and androgen ablation therapy. Submitted.

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1 Introduction

The most frequently diagnosed malignancy among men in Western industrialized countries, prostate cancer (Parkin *et al.* 2005, Jemal *et al.* 2004), is a complex, multifactorial disease with genetic and environmental components involved in its etiology. Prostate cancer displays a wide range of clinical behaviors from relatively indolent to highly aggressive metastatic disease. Despite its high prevalence and mortality rate, the molecular mechanisms underlying the initiation and progression of prostate cancer are poorly understood. Hereditary factors are thought to account for approximately 10% of all instances of prostate cancer and up to 40% of early-onset disease cases, *i.e.* cases diagnosed before the age of 55 years (Carter *et al.* 1992). The multistage process of tumorigenesis and cancer progression in general is believed to be driven by progressive acquisition of mutations and other genetic alterations affecting the expression and function of critical genes (Fearon & Vogelstein 1990, Solomon *et al.* 1991). Multiple chromosomal loci of susceptibility have been identified but, unlike such diseases as breast cancer and colorectal cancer, no major genes predisposing to prostate cancer have been defined (Visakorpi 2003 and refs. therein). The etiology of prostate cancer involves several genetic loci, with no major gene accounting for a large proportion of susceptibility to the disease (Nwosu *et al.* 2001 and refs. therein).

Despite the heterogenous characteristics of prostate cancer in terms of its causes and progression, androgen signaling has long been considered as a common element in its development and progression. Androgens are needed for the normal growth and function of the prostatic gland, but androgen action is also associated with the initiation of benign and malignant prostatic neoplasms (Coffey 1988). Under normal conditions, local androgen metabolism maintains a balance between the synthesis of active androgens and the inactivation of these steroids. Intraprostatic concentrations of active androgens maintain organ homeostasis by regulating the balance between proliferation and apoptotic cell death by prostatic epithelial cells (Isaacs *et al.* 1992). With regard to prostatic carcinogenesis, the majority of prostate tumors arise from the secretory, androgen-dependent epithelial cells (Ware 1994). In addition to androgens, estrogens have also been suggested to play a role in prostate cancer induction and progression (Bosland 2000, Härkönen & Mäkelä 2004 and refs. therein).

The mainstay treatment of advanced prostate cancer, endocrine therapy, aims to eliminate androgenic activity from the circulation and the prostate tissue (Labrie *et al.* 1993). Low androgen concentrations are usually achieved by surgical or chemical castration. Initially, most prostate carcinomas are responsive to androgen withdrawal, but eventually, a large number of them begin to grow androgen-independently, thus also becoming refractory to androgen deprivation therapy (Crawford *et al.* 1989). Androgen withdrawal has been used in the treatment of prostate cancer patients since 1941 (Huggins & Hodges 1941), but the failure of this therapy still represents a serious clinical problem. The molecular mechanisms which lead to hormonal unresponsiveness largely remain to be clarified.

The biological activity of steroid hormones is regulated at the pre-receptor level by several enzymes, including 17β-hydroxysteroid dehydrogenases/17ketosteroid reductases (17HSD/KSRs), which are responsible for catalyzing the reactions between the active 17β-hydroxysteroids and the less active 17-ketosteroids. Generally, the reduction step is essential for the formation of active estrogens as well as active androgens, while the oxidative reaction is required for the inactivation of potent sex steroids into compounds having only low biological activity or no activity at all (Peltoketo *et al.* 1999b). At present, ten different human 17HSD/KSRs, which are encoded by independent genes with relatively low similarity to the genes encoding the other isoenzymes, are known. In intact cells, the activity catalyzed by each type of 17HSD/KSR enzymes is almost exclusively unidirectional: for example, the 17HSD/KSR types 1, 3, 5, and 7 catalyze the reductive reaction, while the types 2, 4, 8, 10, and 11 catalyze the reaction into the oxidative direction (Peltoketo *et al.* 1999b, Luu-The 2001, Mindnich *et al.* 2004, Vihko *et al.* 2004). 17β-hydroxysteroid dehydrogenase/17-ketosteroid reductase activity is not only present in classical steroidogenic tissues, such as human placenta, ovary, and testis, but also in several peripheral tissues, including breast, prostate, gastrointestinal tract, liver, and kidney (Peltoketo *et al.* 1999a). Thus, 17HSD/KSRs are of crucial importance in the regulation of the intracellular levels of biologically active steroid hormones in a variety of tissues.

In the present study, loss of heterozygosity (LOH) at chromosome arm 16q and especially the potential involvement of the gene for 17HSD/KSR type 2, *HSD17B2,* in the regions of most common loss were characterized. Specifically, further, involvement of *HSD17B2* as a target gene for chromosome arm 16q losses was aimed to be confirmed using quantitative real-time PCR. In order to better understand the biology of the disease, changes in the 17HSD/KSR enzyme activities critically contributing to prostatic steroid metabolism were explored regarding the transition of prostate cancer LNCaP (lymph node carcinoma of the prostate) cells (Horoszewicz *et al.* 1983) into an androgen-independent state. Related to this, the relative expression levels of genes for the potential 17HSD/KSR isoenzymes; 17HSD/KSR types 2, 5, and 7, were assessed by relative quantitative RT-PCR. Finally, the expression levels of different types of 17HSD/ KSRs were further characterized in a series of prostate cancer patient specimens representing various clinical features.

2 Review of the literature

2.1 Histological and clinical progression of prostate cancer

Detailed understanding of the histological and clinical characteristics of tumor progression is the basis on which studies on the genetic background of cancer progression can be built. The multistep genetic progression of colorectal cancer, a model for studies of neoplasias in general, has been revealed based on careful correlation of genetic changes with clinicopathological cancer progression (Fearon & Vogelstein 1990). In the case of prostate cancer, however, there exists extensive variability in the disease spectrum, ranging from early preinvasive lesions to symptomatic clinical cancer as well as to recurrent, metastatic, and treatment-refractory aggressive cancer types (Gittes 1991, Nagle *et al.* 1991). Furthermore, the sequence of phenotypic progression events in prostatic cancer is less clear than that in colorectal cancer, for example, and the histological and clinical hallmarks as well as the boundaries between the different stages of progression are often difficult to define. Moreover, prostate cancer and its precursor lesions may often be multifocal, and individual cancer foci in a given section of prostate cancer tissue may display different genetic changes, thus suggesting that multiple genetically distinct neoplastic foci may emerge and evolve independently (Greene *et al.* 1991, Sakr *et al.* 1994, Macintosh *et al.* 1998). Consequently, it is not inevitable that prostate cancer would conform to simple linear progression models.

Prostate cancer is believed to arise from the secretory epithelial cells lining the luminal surface of the prostatic ducts and acini (Ware 1994). Most prostatic carcinomas arise in the peripheral zones of the organ, where putative precursor lesions, prostate inflammatory atrophy (PIA), and prostatic intraepithelial neoplasia (PIN) are also typically found. Albeit the reproducible histopathological diagnosis of PIN is considered difficult, there is a consensus of opinion emerging that at least a certain proportion of prostate carcinomas originate from PIN lesions (Nagle *et al.* 1991, Ware 1994, Haggman *et al.* 1997). Furthermore, a prostate cancer progression model has been proposed, in which PIA is a precursor to prostate cancer via an intermediate stage of high-grade PIN (De Marzo *et al.* 1999, Putzi & De Marzo 2000, Nelson *et al.* 2004). The likelihood that an individual PIN lesion evolves into clinical cancer is, however, probably quite low (Epstein 1994), and the

same tendency can also be seen with another very common early lesion, indolent microscopic prostate cancer. The formation of histological cancer can be regarded as almost inevitable by the age of 80, but in most cases, these lesions never progress to clinical cancer. It has been estimated that as many as 9 out of 10 histological prostate carcinomas never become clinically detectable during the individuals' lifetime (Gittes 1991).

Clinically detected primary prostate carcinomas display a wide range of phenotypic features and malignant potential. Conventionally, most prostatic carcinomas represent typical adenocarcinomas, which can be further categorized into different tumor grades (Gleason 1992). The morphology of a poorly differentiated tumor may differ dramatically from that of a well differentiated one, but there is only a gradual morphological transition between the adjacent grades. The tumor stage, based on tumor size, as well as the presence of lymph node and distant metastases, is used along with grade to assess the prognosis of patients (Gittes 1991). However, there may be considerable heterogeneity in biological aggressiveness and prognosis within a given grade or stage, thus suggesting the need for improved tools for the prognostic assessment of clinical outcome for individual patients. The influence of neuroendocrine differentiation in adenocarcinoma of the prostate on poor prognosis, tumor progression and androgen independence has been extensively studied and reported in the literature, but attempts to correlate neuroendocrine cell populations with clinical variables such as stage, grade, or disease-free survival have remained controversial (Vashchenko & Abrahamsson 2005 and refs. therein, Yang *et al.* 2005). Clinically relevant subtypes of prostate cancer have recently been studied for identification by way of complementary DNA (cDNA) microarray-based gene expression profiling (Lapointe *et al.* 2004), which is currently considered an objective supplementary approach to the histopathological work-up of precancerous or cancerous lesions of the prostate. There have also been efforts to identify distinct gene expression signatures between primary prostate tumors and metastases (Dhanasekaran *et al.* 2001, LaTulippe *et al.* 2002). The acquisition of invasive and metastatic potential is one of the key events in prostate cancer progression, and distant metastases are, along with the androgen-independent tumor growth, the leading cause of mortality among prostate cancer patients. Understanding of the molecular mechanisms of metastasis and identification of the high-risk cases likely to metastasize is, consequently, especially important goal in prostate cancer research.

Prostate cancer is a heterogeneous disease in terms of its causes and progression, but androgen signaling has long been assumed to play a pivotal role in prostate tumorigenesis, with prostate cancer being considered as perhaps the most hormone-dependent of all tumor types. In the treatment of advanced disease, the androgenic activity from the circulation as well as from the prostate tissue is reduced by means of endocrine therapy, a treatment to which most prostate carcinomas initially respond favorably (Labrie *et al.* 1993). However, when androgen deprivation is continued, the response deteriorates, and a recurrent, hormone-refractory tumor emerges. The transition from an androgen-responsive to an androgen-unresponsive stage also signals substantial worsening of prognosis. After recurrence, the average survival of patients is only 4–15 months, reflecting the especially aggressive characteristics of hormone-refractory tumors (Gittes 1991, Labrie *et al.* 1993). Thus, particularly when

developing improved therapies, it would be essential to understand the molecular mechanisms underlying the failure of endocrine therapy and the tumor recurrence.

2.2 Multistep progression, genetic instability, and clonal evolution in human prostate cancer

Prostate cancer displays a broad range of clinical behaviors from relative indolent to aggressive metastatic disease. The observed clinical heterogeneity is believed to reflect the underlying molecular heterogeneity of prostatic tumors (Lapointe *et al.* 2004). A particularly important question with respect to prostate cancer is: what are the molecular events responsible for the progression of the disease or, in other words, why and how does prostate cancer evolve from an indolent to a life-threatening disease. Furthermore, is this evolution inevitable or are some prostate cancers destined never to progress to advanced disease, regardless of the time frame? Or conversely, are some prostate cancers capable of metastasis very early in their natural history? Application of the multistep concept to carcinogenesis in the human prostate would suggest that incidental or latent cancers as well as putative precursor lesions, such as PIN, will have undergone only a subset of the steps, hits, or mutations required for full outcome of the malignant phenotype. Furthermore, this hypothesis would suggest that specific and discrete genetic alterations are possibly associated with different stages and even grades of prostate cancer. However, despite the attractiveness of such a hypothesis and, further, regardless of the fact that certain specific mutations, e.g. in the p53 gene, have been suggested to be closely associated with the progression of prostate cancer (Visakorpi *et al.* 1992, Bookstein *et al.* 1993, Navone *et al.* 1993, Aprikian *et al.* 1994), no definitive proof has been provided to show that this is the case.

The molecular genetics of the progression of prostate cancer is poorly understood, but it is generally believed that cancer development and progression, including the development of hormone-refractory prostate cancer, are consequent to accumulation of genetic alterations targeting critical genes and molecular mechanisms. In addition to classical oncogenes and tumor suppressor genes (TSGs), the genes that are dysregulated in the progression of normal cells to aggressive, highly metastatic, and treatment-refractory cancer may include other genes critically involved in cell proliferation, adhesion, differentiation, and death as well as the repair or surveillance mechanisms for damaged DNA (Fearon & Vogelstein 1990, Kinzler & Vogelstein 1997). The majority of these genetic changes arise in somatic cells, but occasionally, an inherited germline genetic defect may predispose to tumor development. The rate of accumulation of genetic damage is accelerated by the genetic instability of most cancer cells. Genetic instability leads to intratumor heterogeneity, *i.e.* the coexistence of multiple genetically related cell clones, each with their unique biological properties. Additionally, genetic instability results in the continuing acquisition of further genetic changes in the progeny of the originally initiated cells. If these additional genetic changes induced in the novel clones provide a proliferative advantage to the cells, these clones eventually become the predominant cell types present within the primary tumor (Isaacs 1994). Changes that do not provide an advantage for cell growth, instead, are eliminated as clones of that kind are overgrown by other cells (Nowell 1976). When tumors encounter a strong selective force,

such as endocrine therapy in prostate cancer, most tumor cells may die. As a consequence of the genetic instability and intratumor heterogeneity, however, there may be a small pre-existing cell clone which is able to withstand therapy and subsequently continues to proliferate and evolve further (Koivisto *et al.* 1995). The expansion of such a clone eventually leads to recurrence and therapy failure. Such genetic diversity is also likely to underlie the formation of a cell clone that has an increased propensity to metastasize. A variety of experimental analyses have indicated that genetic instability producing novel transformed clones, coupled with the selection of clones with a growth advantage, is the driving force behind the progression of prostatic cancer to a highly metastatic state (Isaacs *et al.* 1982). Schematic representation of the evolution of prostate cancer, according to current knowledge, is illustrated in Fig. 1.

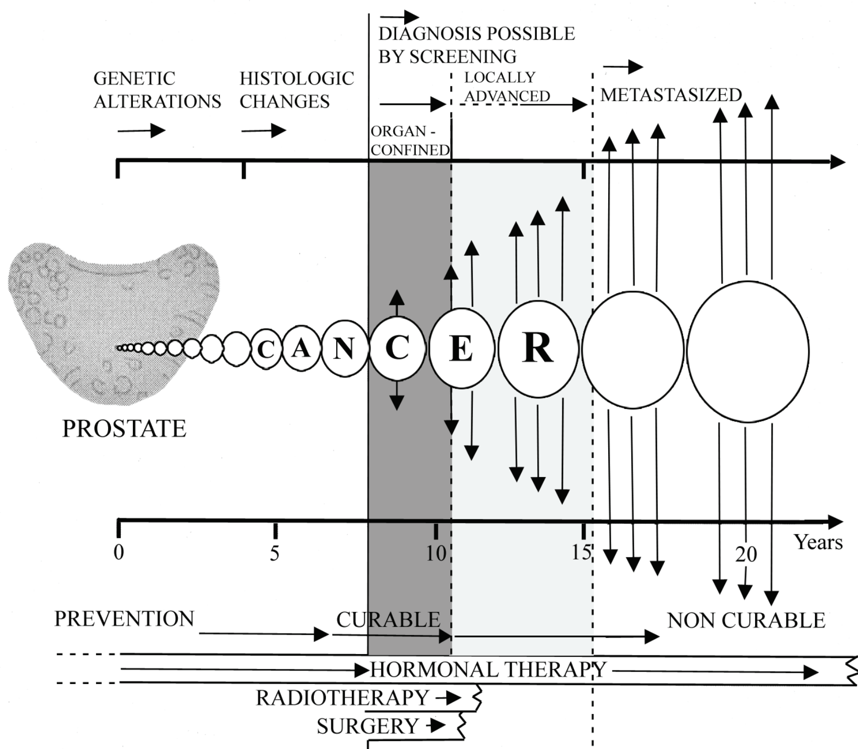

Fig. 1. Schematic representation of the evolution of prostate cancer. The rate of cancer growth is variable between individuals, and the scale represented is an estimated average. It is important to note that, at the time of diagnosis possible by screening with serum prostate-specific antigen (PSA), digital rectal examination, and / or transrectal ultrasonography of the prostate, approximately 50% of prostate cancers are no longer organ-confined. Radical prostatectomy is able to cure the disease at the organ-confined stage, and radiotherapy and brachytherapy (seed implants) are believed to have an efficacy comparable to that of surgery. Hormonal therapy is highly efficient (although not curative) when started before the cancer metastasizes. At the advanced stage of bone metastases, no curative treatment is available, but castration achieved by orchiectomy or gonadotropin hormone-releasing hormone (GnRH) agonist is used to prolong the survival of men with metastasized prostate cancer. Modified from Labrie (2002) and Labrie *et al.* (2004).

2.3 Inherited predisposition to prostate cancer

Familial clustering of prostate cancer has been well documented; male relatives of prostate cancer patients have been observed to have a significantly increased risk of developing prostate cancer. For example, Steinberg *et al.* (1990) reported that men with a first-degree relative with prostate cancer have a two-fold risk and those with two first-degree relatives have a five-fold risk of developing prostate cancer compared to men with no family history. Generally, the genes predisposing to familial prostate cancer do not seem to be mutated in sporadic cancers, and the clinical course also differs between the familial and sporadic variants. Men with an inherited predisposition are prone to develop cancer at an early age. Familial types of prostate cancer have been estimated to account for up to 40% of early-onset prostate cancer cases, *i.e.* cases diagnosed before the age of 55 years, whereas the estimated percentage of all prostate carcinomas attributable to an inherited predisposition is only 5–10% (Carter *et al.* 1992). Genome-wide linkage analyses have identified several loci that are likely to contain dominant susceptibility genes for familial prostate cancer, including HPC1 (1q24-q25), PCAP (1q42-q43), HPCX (Xq27-28), CAPB (1p36), HPC20 (20q13), HPC2/ELAC2 (17p11), and 16q23 (Smith *et al.* 1996, Berthon *et al.* 1998, Xu *et al.* 1998, Gibbs *et al.* 1999, Berry *et al.* 2000, Suarez *et al.* 2000, Tavtigian *et al.* 2001).

2.4 On the genetics of sporadic prostate cancer

Prostate cancer, like other solid tumors, is belived to be the result of an accumulation of mutations and other genetic changes that confer a malignant phenotype to healthy cells. It has been reported in various studies regarding somatic genetic changes in the cells of the prostate gland, that during prostatic carcinogenesis, specific genomic regions are frequently deleted and certain regions are frequently amplified, while other regions appear essentially stable as regards genetic aberrations (Bova & Isaacs 1996, Visakorpi 2003 and refs. therein). The modified function of genes contained within the most frequently altered regions is believed to be largely responsible for the malignant behavior of prostate cancer. Regarding the ability of a tumor to metastasize, it seems certain that a combination of genetic changes is critical to the acquisition of metastatic potential (Cher *et al.* 1996, Isaacs 1997 and refs. therein, Ramaswamy *et al.* 2003).

The accumulation of somatic genetic changes in the cells of the prostate gland has been investigated through the use of a variety of techniques, including cytogenetic, molecular genetic, and, more recently, molecular cytogenetic methods. Analyses concerned with the discovery of somatic genetic alterations involved in prostate cancer have led to the identification of a large number of changes affecting multiple chromosomal loci (Visakorpi 2003 and refs. therein). Recently, cDNA microarray-based gene expression profiling analyses have been able to identify a selective set of genes that define a molecular signature for prostate cancer, as evidenced by the significantly differential gene expression patterns between benign and malignant prostate tissues (Dhanasekaran *et al.* 2001, Luo *et al.* 2001, Welsh *et al.* 2001, Ernst *et al.* 2002). Gene expression profiling has, furthermore, been used in efforts to identify biologically and

clinically relevant tumor subtypes of prostate cancer as well as for the identification of molecular signatures exclusively typical for metastatic disease (LaTulippe *et al.* 2002, Lapointe *et al.* 2004). The data support the existence of distinct gene expression subtypes in prostate cancer, thus possibly providing a basis for improved prognostication and treatment stratification of the disease.

Hitherto, allelic loss events are among the most consistent changes detected in prostate cancer (Isaacs 1995, Visakorpi 2003 and refs. therein). In addition, several other recurring genetic aberrations, including point mutations and changes in DNA methylation patterns, alterations at the level of specific cell-to-cell adhesion molecules, as well as aneuploidy and aneusomy of specific chromosomes, have been identified (Van Den Berg *et al.* 1995 and refs. therein). The stabilization of chromosome telomeres, most probably mainly via increased telomerase activity, has also been described as an intriguing mechanism of tumorigenesis (Hanahan and Weinberg 2000 and refs. therein). In the present review, interest is focused on DNA sequence copy number changes, more specifically on the loss of specific regions of the genome associated with prostate cancer. A characteristic feature of prostate cancer is the finding that, in primary tumors, losses and deletions are up to five times more common than DNA gains and amplification, giving rise to the suggestion that inactivation of recessive tumor suppressor genes is more important in early prostate cancer than activation of oncogenes. Hormone-refractory tumors, instead, often contain gains and amplifications, indicating that the late progression of prostate cancer is also characterized by the activation of oncogenes (Visakorpi *et al.* 1995b).

2.4.1 Loss of specific chromosomal regions

In tumor cells, two types of deletion of genomic DNA have been identified; first, loss of one of two alleles at a particular locus, commonly detected as loss of heterozygosity (LOH), and second, loss of both alleles in a particular region, known as homozygous deletion (HZD). Loss of heterozygosity or relative loss of copy number has proved useful in the attempt to identify important chromosomal regions as well as in narrowing down the regions of interest in particular chromosomes. Loss of specific genomic sequences has especially been associated with loss of TSG function in cancer cells (Bova & Isaacs 1996). In the search for critical TSGs in prostate cancer, a variety of candidate chromosomal regions harboring TSGs associated with prostatic cancer have been identified by way of LOH (Isaacs & Carter 1991). In addition to the specific involvement of TSGs located within regions of loss, allelic deletions may reflect the generalized instability of tumor cell genomes (Uchida *et al.* 1995).

Chromosomal regions demonstrating a high rate of loss of genetic material are frequently found to harbor putative TSGs. Tumor suppressor genes are normal cellular genes, the products of which regulate cellular growth and differentiation and, through this action, have an important role as inhibitors of the uncontrolled cellular proliferation characteristic of prostate cancer (Carter *et al.* 1990b). The manner in which these TSGs function to suppress the malignant phenotype has not been established definitively, but regardless of the mechanism of tumor suppression, the physical loss or mutation of TSGs results in a loss of ability to affect the proliferative cell cycle (Isaacs & Carter 1991). The

classic model of TSG inactivation is described as a two-hit process which stipulates that inactivation of both alleles of a TSG is required to promote tumor progression (Knudson 1985). This recessive nature of TSGs has, however, been challenged by a growing number of reports showing that the mutation or loss of a single allele may be sufficient to exert a cellular phenotype that leads to tumorigenesis without inactivation of the second allele (Goss *et al.* 2002, Gruber *et al.* 2002, Spring *et al.* 2002). This gene-dosage effect is called haploinsufficiency (Quon & Berns 2001, Fodde & Smiths 2002, Santarosa & Ashworth 2004). While the recurrent observation of LOH in the same chromosomal region of a given tumor type has been regarded as a signal of the presence of a TSG specific for the tumor in question in the deleted chromosomal segment (Carter *et al.* 1990b, Canzian *et al.* 1996), chromosomal regions with a low frequency of alterations have been presumed to occur as a result of random genomic instability (Cher *et al.* 1996). Generalized genomic instability has been regarded as a very clear indication of neoplastic transformation, and alteration in genomic instability has also been suggested to be significantly involved in the onset of prostate cancer (Uchida *et al.* 1995).

Probably the most exciting aspect as regards the demonstration of specific allelic loss in human cancers, at least from a clinical point of view, is the ability of various aspects of these deletions to correlate with and be predictive of tumor progression (Isaacs & Carter 1991). Based on the possible prognostic implications of LOH, it has been suggested that tumors of certain types might be worth analyzing routinely for LOH at specific loci (Canzian *et al.* 1996). The ability to assess tumor aggressiveness on the basis of molecular markers would have particular relevance to prostate cancer, since this cancer type is characteristically clinically silent in a large number of men who have lesions within the prostate that are histologically identifiable as prostate cancer (Isaacs & Carter 1991). Definition of the full spectrum of common allelic changes in prostate cancer could lead to the identification of associations between specific changes and clinical outcome, as indicated in studies on colon cancer and Wilms` tumor (Jen *et al.* 1994, Grundy *et al.* 1994). Allelic losses in adenocarcinoma of the prostate have been observed at numerous chromosomal regions, with LOH of the chromosome arms 1p, 6q, 8p, 10q, 13q, 16q, 17p, 17q, and 18q being among the most consistently identified (Kallioniemi & Visakorpi 1996 and refs. therein, Dumur *et al.* 2003, Lieberfarb *et al.* 2003).

2.4.2 LOH at chromosome arm 16q

Molecular analyses have shown that one of the most recurrent aberrations observed in prostate tumors involves chromosome arm 16q (Carter *et al.* 1990b, Bergerheim *et al.* 1991, Kunimi *et al.* 1991, Phillips *et al.* 1994, Visakorpi *et al.* 1995b, Cunningham *et al.* 1996, Dumur *et al.* 2003, Lieberfarb *et al.* 2003). Frequent LOH in chromosome arm 16q has also been reported in other carcinomas, such as breast and ovarian cancers (Sato *et al.* 1991a,b, Cleton-Jansen *et al*. 1994, Tsuda *et al*. 1994, Dorion-Bonnet *et al.* 1995, Kerangueven *et al.* 1997, Osborne & Hamshere 2000, Shen *et al.* 2000, Miller *et al.* 2003, Wang *et al.* 2004), hepatocellular carcinomas (Tsuda *et al.* 1990, Jou *et al.* 2004), primitive neuroectodermal tumors of the central nervous system (Thomas & Raffel 1991), and Wilms´ tumors (Maw *et al.* 1992, Klamt *et al.* 1998, Yeh *et al.* 2002). Recently,

furthermore, the high frequency of allelic loss at 16q23.2 has been detected to be striking in the epithelial component of a rare neoplasm composed of epithelium-lined cysts and channels embedded in a variably cellular stroma, called phyllodes tumor of the prostate (McCarthy *et al.* 2004).

Regarding the losses on chromosome arm 16q in human prostate cancer and the frequencies at which these losses have been detected, Carter and colleagues (1990b) reported LOH of markers on arm 16q in 30% of clinically localized tumors, whereas both Bergerheim *et al.* (1991) and Kunimi *et al.* (1991) found higher rates of loss, 56% and 60%, respectively, in a series of both metastatic and localized tumors. The observed rates are in good agreement with those of two more recently published analyses performed using comparative genomic hybridization (CGH), a molecular cytogenetic method that provides an overview of DNA sequence copy number changes of the entire tumor genome in a single hybridization. The study by Joos *et al.* (1995), particularly concerning primary tumors, revealed frequent losses on 16q in 30% of cases, and the study published by Visakorpi *et al.* (1995b), concerning recurrent prostate cancers, in 56% of cases. Similar proportions were earlier reported by Isaacs *et al.* (1994), who observed 32% of primary tumors to exhibit chromosome arm 16q deletions, while the respective proportion among metastatic tumors was as high as 60%.

On the basis of a deletion mapping study presented by Bergerheim *et al.* (1991), the critical region of loss was suggested to be located between D16S4 and 16qter. More recently, Cher *et al.* (1995) bracketed the critical region of loss within the smallest deleted region earlier found by Bergerheim and colleagues, thus indicating a more distal location of a TSG between 16q23.1 and 16qter. Chromosome region 16q23.2 has been demonstrated to contain a high frequency of allelic imbalance in prostate tumors and, furthermore, to exhibit the strongest linkage signal in the genome screen of prostate cancer sibling pairs (Paris *et al.* 2000, Suarez *et al.* 2000), thus suggesting that a region of 16q23.2 may harbor a prostate cancer TSG implicated in the development of non-familial and possibly familial forms of prostate cancer. Evidence of loss at 16q24 correlating with late-stage prostate tumors and higher serum prostate-specific antigen (PSA) levels has been reported (Strup *et al.* 1999). Furthermore, data indicating the presence of three distinct commonly deleted regions at 16q in prostate cancer have been presented. Suzuki and co-workers (1996) observed deleted regions at 16q22.1-q22.3, q23.2-q24.1, and q24.3-qter in human primary and metastatic prostate cancers. The correlation between losses at 16q and progression of the disease was also reported; allelic losses found on the long arm of chromosome 16 were significantly more frequent in cancer-death cases than in early-stage tumors. In addition to that study, data providing evidence of three different target regions possibly involved in the pathogenesis of prostate cancer have also been presented by Latil *et al.* (1997), who located the most centromeric region of loss to chromosome band 16q22.1, the intermediate one to 16q23.2, and the most telomeric region to 16q24.3, the data thus being consistent with that previously reported. The rate of LOH at 16q24.3 was found to be significantly higher in metastatic than in localized forms, 80% versus 32%, respectively, pointing to involvement of a gene related to the invasiveness in prostate cancer. Furthermore, the presence of three independent regions on chromosome arm 16q in human prostate cancer was reported by Elo *et al.* (1999), who localized the most common region at 16q24.1-q24.2, and the others at 16q21.1 and 16q24.3-qter. Regarding the clinicopathological parameters of prostate cancer, the LOH at

16q24.1-q24.2 was found to be significantly associated with aggressive behavior of the disease, recurrent growth, poor differentiation of the tumor, and poor prognosis for the patient. Evidence of more than one deleted region on 16q has also been found by Godfrey *et al.* (1997) and Li *et al.* (1999). The latter of these reports suggested four commonly deleted regions at 16q, two of which at 16q23-24 were strongly associated with metastatic disease. Godfrey and co-workers indicated that prostate cancer genomes frequently exhibit two common regions of 16q allelic imbalance, one at 16q21-22 and the other at 16q24.2-qter. Neither of those regions showed a correlation between allelic imbalance and the clinical parameters of prostate cancer (Gleason grade, tumor stage or metastasis). Interestingly, however, these regions coincided with those found by other researchers working on breast cancer (Sato *et al.* 1991a, Callen *et al*. 1992, Cleton-Jansen *et al*. 1994, Tsuda *et al*. 1994), raising the possibility that the same TSGs might be important in both of these diseases.

Of possible candidate TSGs residing at 16q, the gene for E-cadherin (E-cad), located at chromosomal region 16q22.1, is one of the most thoroughly studied (Berx *et al.* 1995). E-cadherin is a cell surface glycoprotein adhesion molecule important in cell-cell interactions, and reduced E-cadherin function has been postulated to lead to an increasingly invasive phenotype arising from decreased cellular cohesiveness. Indeed, decreased E-cadherin expression is associated with high-grade prostate cancer (Umbas *et al.* 1992) as well as with poor prognosis in patients with the disease (Umbas *et al.* 1994, Richmond *et al.* 1997). The mechanism by which E-cadherin expression is silenced is not clearly undersood; mutation, LOH, as well as DNA hypermethylation of the promoter region have been implicated in the inactivation of the gene for E-cadherin (Graff *et al.* 1995, Yoshiura *et al.* 1995, Hirohashi 1998). Based on the finding of E-cadherin gene losses and reduced expression, interest has also been focused on the other cell adhesion molecules possibly playing a role in prostate tumorigenesis. The genes for two other members of the cadherin superfamily, M-cadherin (CDH15, M-cad) and H-cadherin (CDH13, H-cad), are also located on the long arm of chromosome 16, at region 16q24 (Kaupmann *et al.* 1992, Lee 1996). Cadherins are a family of transmembrane glycoproteins that mediate selective Ca^{2+}-dependent intercellular adhesion by way of homophilic interactions (Kaupmann *et al*. 1992). The down-regulation of H-cadherin is considered to be a frequent event in the progression of breast cancer, and, moreover, based on the broad expression pattern of H-cadherin, it has been suggested to play an important role in many cell types (Lee 1996). *CDH13* expression is frequently silenced by aberrant methylation of the ´5 region of the *CDH13* gene in breast and lung cancers as well as in colorectal cancers and adenomas (Toyooka *et al*. 2001, Toyooka *et al.* 2002). The combination of deletion and aberrant methylation has been reported to inactivate *CDH13* in human lung cancers, in ovarian tumors, and in invasive cutaneous squamos cell carcinomas (Sato *et al.* 1998, Kawakami *et al.* 1999, Takeuchi *et al.* 2002). Another potential candidate, a cell adhesion regulator (*CAR*) gene (Koyama *et al.* 1993), also maps to 16q24 and could conceivably have a TSG function. In the same way as the loss of E-cadherin is suggested to lead to increased invasiveness (Cher *et al.* 1995), the loss of CAR activity is proposed to be a key early step in tumor invasion and metastasis, and hence a decrease in the expression of CAR is assumed to be involved in decreased tumor invasion suppression (Pullmann & Bodmer 1992, Yamamoto *et al.* 1996, 1997). However, despite the identification of a few genes at frequently deleted regions of chromosome arm

16q, none of the candidates has been confirmed to be the main target of the losses observed. Generally, in prostate cancer, multiple chromosomal loci of susceptibility have been detected, but the specific susceptibility genes for most of these regions remain to be identified.

2.5 Androgen action in normal and cancerous prostate tissue

2.5.1 Androgens in normal prostate physiology

It is well recognized that androgens in men arise from two main sources: first, the endocrine testis, which releases the hormones into systemic circulation; and second, the local intracrine synthesis from the adrenal precursors dehydroepiandrosterone (DHEA), its sulfate DHEA-S, and 4-androsten-3,17-dione (A-dione). In human, elevated levels of DHEA and DHEA-S are present throughout adult life, thus providing the high level of substrate required for conversion into potent androgens in peripheral tissues (Labrie 1991). Androgens, being steroid hormones, are derivatives of cholesterol, which is either hydrolyzed from the cholesterol esters stored in lipid droplets, synthesized from acetate *de novo*, or transferred into cells from blood plasma (Gwynne & Strauss 1982 and references therein).

The general role of androgens is to promote normal male sex differentiation and development as well as pubertal masculinization, initiation of spermatogenesis, and maintenance of male sexual function. Steroid hormones also play an important role in prostate biology, the formation and growth of the prostate as well as of other male reproductive organs being dependent on androgens, a class of C_{19} steroids that act through androgen receptors (ARs) to regulate gene expression (Biswas & Russell 1997). The predominant androgens in this regard are testosterone (T), which is synthesized principally in the Leydig cells of the testis from A-dione by way of the action of a reductive 17HSD type 3 enzyme (Geissler *et al.* 1994), and dihydrotestosterone (DHT), the major prostatic androgen formed from T by way of steroid 5α-reductase isozymes (Russel & Wilson 1994). Suggesting the importance of androgen action in the normal biology of the prostate, both AR mutations and 5α-reductase deficiency as well as castration prior to puberty have been reported to prevent the growth of the gland (Imperato-McGinley *et al.* 1974, McPhaul *et al.* 1993).

The prostate gland is composed of stromal and epithelial compartments, the interaction between these two being important not only for prostatic growth but also for the maintenance of normal prostatic functions. The stromal compartment of the gland has been suggested to act as a mediator of several of the effects of androgens during the development of the prostate. The growth of epithelial cells *in vitro* can be stimulated only when the cells are co-cultured with stromal cells, or alternatively, when growth factors are added (Chang & Chung 1989, Kusama *et al.* 1989). In addition to the function of androgens as regulators of the expression of growth-controlling genes, androgens also influence the expression of prostatic secretory proteins. In prostate cancer LNCaP cells,

androgens have been shown to increase the secretion of PSA, while the secretion of prostatic acid phosphatase (PAP) into the culture medium has been observed to decrease as a result of androgen action (Henttu *et al.* 1992).

Under normal conditions, there exists an equilibrium between androgen synthesis and breakdown, a steady-state level of active hormone thus being maintained in the target tissues (Biswas & Russell 1997). In prostatic glandular cells, a balance between cell death and proliferation is regulated by the supply of androgens, so that neither overgrowth nor involution of the gland normally occurs (Isaacs 1996). Organ homeostasis is achieved by the balancing of two distinct processes, one responsible for initiating DNA synthesis and cell proliferation, *i.e.*, an agonist effect of androgen, and the other responsible for inhibiting cell death, *i.e.*, an antagonist effect, with both processes being under androgenic control. If an adequate level of androgen is not maintained, e.g., following surgical castration or medical androgen ablation therapy, androgen-dependent glandular cells of the prostate undergo a process of active cellular suicide called apoptosis or programmed cell death, which subsequently eliminates those cells and results in involution of the gland (Isaacs *et al.* 1992). Alternatively, an imbalance between androgen input and output can lead to an excess of androgen and, through this action, to certain disease states, the most important of which are benign prostatic hyperplasia (BPH) and prostate cancer (Biswas & Russell 1997).

2.5.2 Androgen metabolism in the prostate

The predominant androgen in the blood circulation is T, either unbound or complexed with albumin or a specific steroid hormone-binding globulin (SHBG). The fraction of T that remains unbound can diffuse from the circulation into prostatic cells. Within the prostate, T is rapidly and irreversibly converted to DHT by way of membrane-bound NADPH-dependent Δ^4-3-ketosteroid 5α-oxidoreductase, *i.e* 5α-reductase (Isaacs 1994). The human prostate is also able to synthesize its androgens, T and DHT, from circulating adrenal precursors DHEA, DHEA-S, and A-dione, with all the enzymes required for the transformation of these into DHT having been shown to be expressed in the human prostate (Labrie 1991). In addition, some DHT is formed via reductive 3α-hydroxysteroid dehydrogenase (3α-HSD) activity, converting 5α-androstane-3α,17β-diol (3αA-diol) into DHT (Penning 1997). It has been demonstrated in several independent studies that the major mediator of androgen action in the prostate is DHT rather than T, and that the 5α-reductase conversion of T to DHT functions as a means of amplifying androgenic stimulation in the prostate (Isaacs 1994). It is important to realize, however, that although T has an affinity for prostatic AR that is approximately five to ten times lower than that of DHT, the affinity of the former is also still sufficient to cause stimulation (Nicholson & Waxman 1997).

The androgenic stimulation of prostatic target cells is mainly mediated through the binding of DHT to the AR as well as through the intranuclear activities subsequently exerted by these steroid-receptor complexes. Under the conditions of a physiological hormonal environment, that is, normal function of the gonads and corresponding high plasma concentrations of T, the intraprostatic level of DHT results from the equilibrium

of three biochemical functions. The first of these is the above-mentioned formation of DHT from T by the enzyme 5α-reductase, the second is the subsequent deactivation of DHT into 3αA-diol and 5α-androstane-3β,17β-diol (3βA-diol) by 3α/β-HSDs, and the third is the transient sequestration of DHT by high-affinity binding to ARs, this function temporarily protecting this active androgen from immediate enzymatic deactivation (Klein *et al.* 1991). However, in the prostate, T is not only metabolized to DHT, but also to A-dione, which can subsequently be 5α-reduced to 5α-androstan-3,17-dione (5αA-dione) and androsterone (ADT). These conversions are under the control of the enzymes 17HSD/KSR, 5α-reductase, and 3α-HSD, respectively (Negri-Cesi & Motta 1994). Additionally, in the prostate, T can also be converted into estradiol (E2) by the catalytic activity of aromatase (Voigt & Bartsch 1986).

Androgen action predominates until the active steroids are converted into metabolites having little or no affinity for the AR. The major catabolic pathway involves the sequential conversion of DHT to 5α-reduced C_{19} metabolites such as 3αA-diol and, further, to ADT by the action of reductive 3α-HSD and oxidative 17HSD/KSR, respectively (Fieser & Fieser 1959). However, according to Beaulieu *et al.* (1996), conversion of DHT by 17HSD/KSR, 3α-HSD, and 3β-HSD does not irreversibly metabolize DHT, but glucuronidation is also involved in the deactivation of androgens in the human prostate. Uridine diphosphoglucuronosyltransferase (UGT) enzymes are capable of inactivating DHT, 3αA-diol, T, and ADT into their respective glucuronides. The presence of an irreversible enzymatic step, such as glucuronidation of androgens by specific UGT enzymes, may be a means by which the level of active androgens is regulated in prostate tissue (Beaulieu *et al.* 1996).

2.5.3 Androgens and prostate cancer

The majority of prostate cancers arise from the secretory, androgen-dependent glandular epithelial cells that line the luminal surface of the prostatic ducts and acini (Ware 1994). Prolonged presence of androgens is suggested to be an important factor in the development of prostate cancer, and the stimulation of tumor growth by androgens is well established. Among all hormone-sensitive cancers, prostate cancer is even considered to be the most hormone-dependent (Labrie *et al.* 1993). In prostate carcinoma tissue, the concentration of DHT, the major mediator of androgen action in the prostate, has been reported to be higher than in the surrounding benign areas. In contrast, 5α-reductase activity has been reported to be lower in carcinoma tissue. This suggests that decreased inactivation of active androgens, primarily via 17β-oxidation by 17HSD/KSR or via the activities of 3α/β-HSDs, could be one factor resulting in the entrapment of DHT in carcinoma cells in the prostate (Klein *et al*. 1991). In a recent prostate cancer prevention trial, the 5α-reductase inhibitor finasteride was found to prevent or delay the appearance of prostate cancer with a relative risk reduction of 25 percent (Thompson *et al.* 2003), this also being a distinct indication of the steroid dependence of the disease.

Regarding the steroid dependence of prostate cancer, it should be possible to reduce the risk of prostatic glandular cells undergoing both the initiation and the promotional stages of carcinogenesis by diminishing the androgenic influences on the cells at risk and

thus decreasing the proliferative drive in these cells (Isaacs 1996). Androgen withdrawal, the mainstay treatment in the clinical management of advanced prostate cancer, aims at eliminating androgen activity from the circulation as well as from the prostate tissue (Labrie *et al.* 1993). Androgen deprivation may be achieved by a multitude of means, including orchiectomy, gonadotropin hormone-releasing hormone (GnRH) agonists, GnRH antagonists, and androgen antagonists (Tammela 2004 and refs. therein). Initially, eighty to ninety percent of prostate carcinomas respond favorably to androgen withdrawal, but when the treatment is continued, the response deteriorates and a recurrent, hormone-refractory tumor emerges (Crawford *et al.* 1989). A transition from an androgen-responsive to an androgen-unresponsive stage is seen during the clinical course of almost all patients with prostate cancer. The transition also signals a substantial worsening of the prognosis, since effective therapeutic regimens for this patient group are not yet available (Deutsch *et al.* 2004 and refs. therein). Androgen withdrawal has, for decades, been used in the treatment of prostate cancer patients (Huggins & Hodges 1941), but the failure of this therapy still represents a serious clinical problem. The initially non-responding tumors as well as the recurrences after endocrine treatment are called androgen-independent diseases.

The precise cellular mechanisms regarding androgen independence are poorly understood. It has been speculated that the cancer within an individual patient is heterogeneously composed of clones of both androgen-dependent and -independent prostatic cancer cells even before the institution of therapy. In that case, androgen ablation alone does not affect the pre-existing androgen-independent cancer cells that keep proliferating despite the androgen blockade, thus allowing an androgen-independent disease to progress (Kyprianou *et al*. 1990 and refs. therein). Another theory suggests that the suppression of androgenic stimuli brought about by endocrine treatments would be incomplete, and that the remaining androgens metabolized from adrenal steroids could result in tumor growth despite androgen withdrawal, subsequently leading to failure of the therapy (Crawford *et al*. 1989, Schellhammer *et al.* 1996). The role of anti-apoptotic genes blocking programmed cell death normally induced by androgen deprivation has also been proposed as a possible mechanism through which hormone-refractory prostate cancer may arise (Koivisto *et al.* 1997). Furthermore, peptide growth factors, such as insulin-like growth factors (IGFs) I and II, have been suggested to be involved in the androgen-independent proliferation of prostatic cells (Culig *et al.* 1996 and refs. therein, Ritchie *et al*. 1997). In recent studies, elevated IGF type I receptor (IGF-IR) expression and enhanced IGF responsiveness have been associated with androgen-independent progression of prostate cancer (Nickerson *et al.* 2001, Krueckl *et al.* 2004). Peptide growth factors influence not only the proliferation of androgen-independent prostate cancer cells but also that of androgen-dependent cells, the regulation of some growth factor receptors being affected by androgens (Schuurmans *et al.* 1991, Zuck *et al*. 1992, Liu *et al*. 1993, Brass *et al*. 1995, Pandini *et al.* 2005).

The results of a number of studies have indicated the recurrence of prostate cancer to be possibly caused by a variety of mechanisms affecting the *AR* gene. While *AR* gene amplification has been proposed to lead to hypersensitization of tumor cells to the residual androgen activity, *AR* gene mutations have been suggested to be likely to alter receptor specificity and thereby to drive cell proliferation via an androgen-independent mechanism (Kallioniemi & Visakorpi 1996 and refs. therein). In a recent study performed

using microarray-based profiling of isogenic prostate cancer xenograft models, a modest increase in AR mRNA was detected to be consistently associated with the development of resistance to antiandrogen therapy. This increase in AR mRNA and protein was found to be both necessary and sufficient to convert prostate cancer growth from a hormone-sensitive to a hormone-refractory stage (Chen *et al.* 2004). Auto-activation of AR protein, with protein kinase activity taking place even in the absence of androgens, has also been reported (Koivisto *et al*. 1997). In one-third of hormone-refractory tumors, high-level overexpression of AR is caused by amplification of the gene (Visakorpi *et al.* 1995a, Koivisto *et al.* 1997). At present, however, it is of note that no single biomarker or group of biomarkers has yet been identified that could successfully predict disease recurrence (Shah *et al.* 2004).

The fact that metastatic prostate cancers, similarly to the normal prostates from which they arise, may be sensitive to androgenic stimulation of their growth is due to the presence of androgen-dependent prostatic cancer cells within such metastases (Isaacs 1996). However, a major reason why metastatic prostate cancer has been a fatal disease for which no therapy has been available to effectively increase survival, has been the inability of androgen ablation monotherapy to increase survival in patients with cancer heterogeneously composed of both androgen-dependent and androgen-independent cells (Kyprianou *et al.* 1990). A striking degree of heterogeneity in the genotype and phenotype of end-stage hormone-refractory metastatic prostate cancer has been indicated between patients as well as within an individual patient (Roudier *et al.* 2003, Shah *et al.* 2004).

2.6 17β-Hydroxysteroid dehydrogenase/17-ketosteroid reductase (17HSD/KSR) enzymes

The biological activity of steroid hormones is regulated at the pre-receptor level by several enzymes, including 17HSD/KSRs. The 17HSD/KSRs are nicotinamide-adenine dinucleotide [NAD(H)]- and/or its phosphate form [NADP(H)]-dependent enzymes that are responsible for the interconversion of 17-ketosteroids with the corresponding 17β-hydroxysteroids. The 17HSD/KSR isoenzymes are essential for both the synthesis and the metabolism/inactivation of C_{19} and C_{18} steroid hormones; androgens and estrogens (Fig. 2.). Generally, the reduction step is essential for the formation of active estrogens as well as active androgens, while the oxidative reaction is required for the inactivation of potent sex steroids into compounds having only low biological activity or no activity at all (Peltoketo *et al.* 1999b). Primarily, the 17HSD/KSR enzymes convert the relatively active steroid hormones estrone (E1), A-dione, and 5αA-dione to their more potent forms E2, T, and DHT and *vice versa* (Peltoketo *et al.* 1999a). Hitherto, ten different human 17HSD/KSRs, which are encoded by independent genes with relatively low similarity to the genes encoding the other isoenzymes, have been characterized (Peltoketo *et al.* 1988, Luu-The *et al*. 1989, Wu *et al.* 1993, Geissler *et al.* 1994, Adamski *et al.* 1995, Lin *et al.* 1997, Dufort *et al.* 1999, Krazeisen *et al.* 1999, Ando *et al.* 1996, He *et al.* 1999, Brereton *et al.* 2001, Moon & Horton 2003). With the exception of 17HSD/KSR5, all 17HSD/KSRs cloned so far belong to a protein family of short-chain dehydrogenases/reductases (SDRs) (Krozowski 1994, Jörnvall *et al.* 1995).

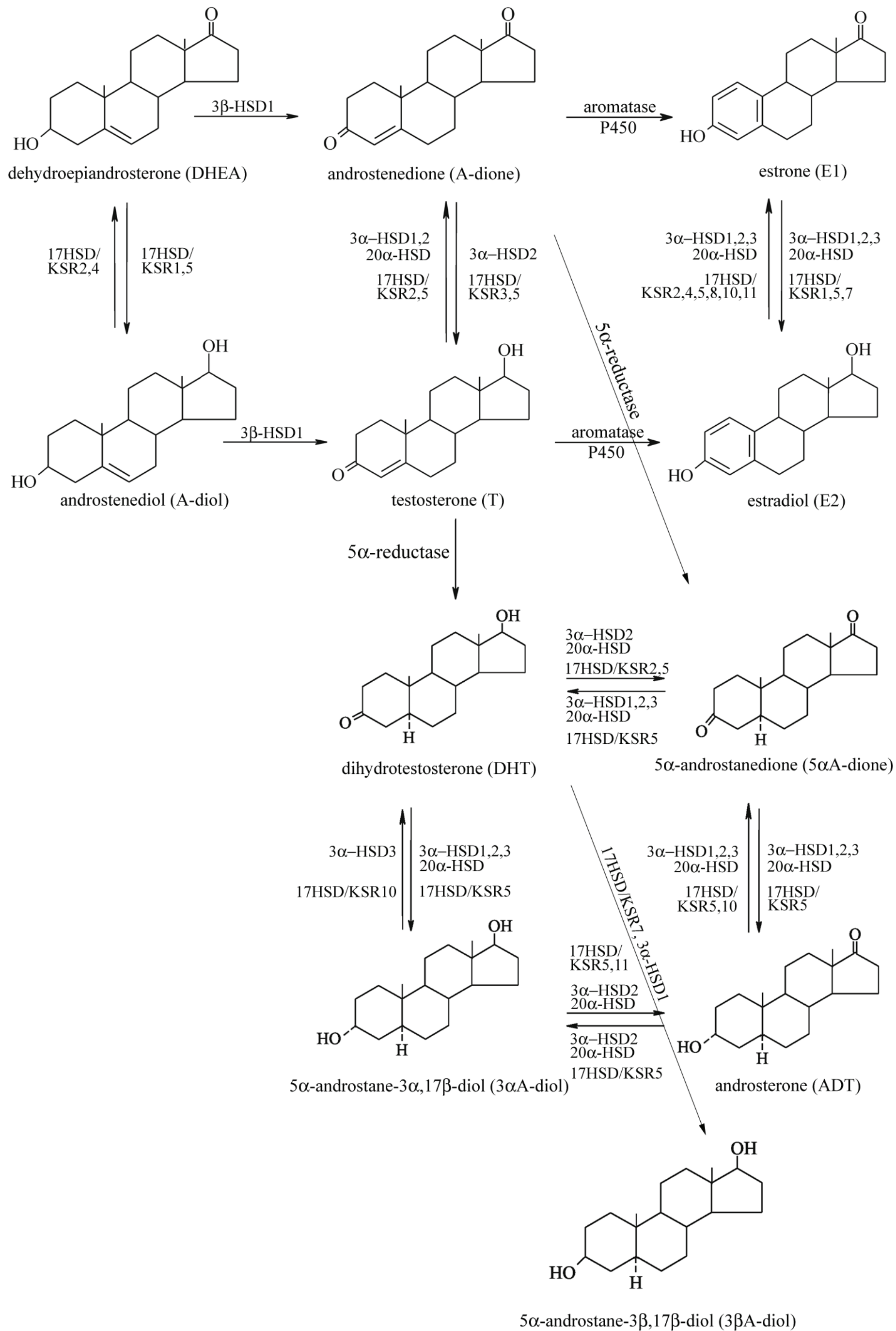

Fig. 2. The main reactions in androgen and estrogen metabolism that can be catalyzed by different types of 17HSD/KSR enzymes.

The 17HSD/KSR isoenzymes differ markedly in their tissue distribution as well as in their substrate specificity (Peltoketo *et al.* 1999b and refs. therein, Labrie *et al.* 2000 and refs. therein, Mindnich *et al.* 2004 and refs. therein). In intact cells, the activity catalyzed by each type of 17HSD/KSR isoenzymes is almost exclusively unidirectional: for example, the 17HSD/KSR types 1, 3, 5, and 7 catalyze the reductive reaction, while the types 2, 4, 8, 10, and 11 catalyze the reaction into the oxidative direction (Peltoketo *et al.* 1999b and refs. therein, Mindnich *et al.* 2004 and refs. therein). 17β-Hydroxysteroid dehydrogenase/17-ketosteroid reductase activity is not only present in classical steroidogenic tissues, such as human placenta, ovary, and testis, but also in several peripheral tissues, including breast, prostate, gastrointestinal tract, liver, and kidney (Peltoketo *et al.* 1999a). Thus, 17HSD/KSRs are of crucial importance in the regulation of the intracellular levels of biologically active steroid hormones in a variety of tissues. In addition to their 17HSD/KSR activity involved in steroid hormone metabolism, a few of the 17HSD/KSR enzymes have also been reported to have 20α-hydroxysteroid dehydrogenase (20α-HSD) and 3α/3β-HSD activities (Wu *et al.* 1993, Lin *et al.* 1997, Dufort *et al.* 1999, Suzuki *et al.* 2000, Penning *et al.* 2001, Törn *et al.* 2003) as well as to catalyze several other reactions, such as peroxisomal and mitochondrial fatty acid -oxidation (Leenders *et al.* 1996b, He *et al.* 1999), the oxidation of xenobiotics (Dufort *et al.* 1999), and the metabolism of bile acids (Novikov *et al.* 1997, Shafqat *et al.* 2003) and neuroactive steroids (He *et al.* 2005). The characteristic properties of distinct types of human 17HSD/KSR enzymes are summarized in Table 1.

Table 1. Human 17HSD/KSR isoenzymes.

Type/ protein family	Chromosomal localization	Subcellular localization	Tissue distribution**	Substrate specificity	Type of activity	References
1/SDR	17q12-q21	cytosol	ovary, placenta, mammary gland	estrogens	reductive	(Peltoketo *et al*. 1988*, Luu-The *et al.* 1989*, Poutanen *et al*. 1992, Puranen *et al.* 1997)
2/SDR	16q24.1-q24.2	endoplasmic reticulum	placenta, liver, kidney, gastrointestinal tract, endometrium, prostate	estrogens, androgens, (progestins)	oxidative	(Wu *et al.* 1993*, Casey *et al.* 1994, Labrie *et al.* 1995, Elo *et al.* 1996, Mustonen *et al.* 1998)
3/SDR	9q22	microsomes	testis	androgens	reductive	(Geissler *et al.* 1994*)
4/SDR	5q2.3	peroxisomes	widely distributed	D-3-hydroxyacyl-CoA, (estrogens)	oxidative	(Adamski *et al.* 1995*, Markus *et al.* 1995, Leenders *et al.* 1996a, Möller *et al.* 1999)
5/AKR	10p14-15	cytosol	liver, prostate, testis, adrenal, endometrium, mammary gland, corpus luteum	androgens, (estrogens), progestins	reductive	(Qin *et al.* 1993*, Khanna *et al.* 1995b*, Lin *et al.*1997*, Dufort *et al.* 1999, El-Alfy *et al.* 1999, Pelletier *et al.* 1999)
7/SDR	1q23	endoplasmic reticulum	ovary, mammary gland, placenta, testis, prostate, liver, adrenal, lung, thymus	estrogens, androgens, progestins	reductive	(Krazeisen *et al.* 1999*, Breitling *et al.* 2001, Törn *et al.* 2003)
8/SDR	6p21.3	unknown	liver, pancreas, kidney, skeletal muscle	estrogens, (androgens)	oxidative	(Ando *et al.* 1996*, Kikuti *et al.* 1997)
10/SDR	Xp11.2	mitochondria	liver, gonads, brain	L-3-hydroxyacyl-CoA, androgens, estrogens, neurosteroids, bile acids	oxidative	(He *et al.* 1998*, 1999*, 2001, Shafqat *et al.* 2003, He *et al.* 2005)
11/SDR	4q22.1	unknown	pancreas, kidney, liver, lung, adrenal, ovary, heart, small intestine	androgens, (estrogens)	oxidative	(Li *et al.* 1998*, Brereton *et al.* 2001, Chai *et al.* 2003)
12/SDR	11p11.2	microsomes	skeletal muscle, heart, liver, kidney, placenta, lung, spleen, small intestine, brain, colon	3-ketoacyl-CoA	reductive	(Moon & Horton 2003*)

* references regarding cloning

** not all-inclusive: the tissues in which each enzyme is most commonly expressed are mentioned

2.6.1 17HSD/KSR activity in the prostate

Regarding the regulation of androgen action locally in the prostate, the conversion of T into its more potent form, DHT, a reaction catalyzed by 5α-reductase, is considered to be a crucial step (Enderle-Schmitt *et al.* 1986). Furthermore, the activities of several other enzymes involved in steroid metabolism, including both the reductive and the oxidative activities of 17HSD/KSRs, are present in the prostate (Abalain *et al.* 1989). Measurements of 17HSD/KSR activities have been performed *in vitro* in prostate tissue homogenates as well as in the stromal and epithelial compartments of the gland (Bartsch *et al.* 1987, Martel *et al.* 1992**,** Tunn *et al.* 1993). In the prostate, the function of 17HSD/KSRs is to catalyze the interconversion between the active 17β-hydroxysteroids (DHT, T) and the less active 17-ketosteroids (5αA-dione and A-dione). The results published by Tunn *et al.* (1993) suggest that particularly in the stromal compartment of the prostate, the balance of the reversible conversion between T and A-dione is shifted toward T, thus favoring the reductive reaction. Furthermore, Bartsch and colleagues (1987) have shown the reversibility of the reactions catalyzed by 17HSD/KSRs in separated stromal and epithelial compartments and suggested further that 17HSD/KSR action takes place principally in the epithelial compartment of the prostate. In addition, prostatic 17HSD/KSR activity has been found to be among the most abundant when compared with that of other tissue homogenates, and the activities detected have favored the formation of T rather than that of A-dione (Martel *et al.* 1992).

Experiments carried out using various prostate cell lines have shown relatively strong 17HSD/KSR activity in most cell lines studied. In cultured prostate epithelial cells as well as in prostate cancer PC-3 cells (Kaighn *et al.* 1979), the formation of the 17-keto metabolites 5αA-dione and A-dione has been the predominant metabolic reaction of the active androgens DHT and T (Castagnetta *et al.* 1994, Délos *et al.* 1995, Tsugaya *et al.* 1996). On the other hand, the results concerning androgen-responsive LNCaP cells have been more controversial. In a study carried out by Castagnetta *et al*. (1994), LNCaP cells mostly retained high levels of unconverted T, with the limited production of A-dione and its 17-keto derivatives. In this cell line, the activities of 17HSD/KSR and 5α-reductase are directed in such a way that both T and DHT accumulate (Castagnetta *et al*. 1994). On the other hand, the formation of A-dione has been shown to predominate over that of DHT when T is the substrate (Negri-Cesi & Motta 1994, Limonta *et al.* 1995), owing to decreased activity of 5α-reductase coupled with increased activity of 17HSD/KSR (Negri-Cesi & Motta 1994). Thus far, the expressions of only 17HSD/KSR types 2, 4, 5, and 7 have been located in human prostate (Casey *et al.* 1994, Adamski *et al.* 1995, Elo *et al.* 1996, Dufort *et al*. 1999, Krazeisen *et al.* 1999). With the exception of 17HSD/KSR4, which has been suggested to have its main function in the peroxisomal fatty acid -oxidation and metabolism of bile acids and only a minor role in steroid metabolism (Dieuaide-Noubhani *et al.* 1996, Leenders *et al.* 1996b, Novikov *et al.* 1997, Qin *et al.* 1997, van Grunsven *et al.* 1998, Baes *et al.* 2000), the human 17HSD/KSR types hitherto detected to be prostatic are presented in more detail below.

2.6.1.1 17HSD/KSR type 2

The gene for 17HSD/KSR type 2, *HSD17B2*, has been localized to the chromosome region 16q24 (Casey *et al.* 1994), more exactly to 16q24.1-q24.2 (Durocher *et al.* 1995). The *HSD17B2* gene encodes two alternatively spliced mRNAs, giving rise to the functional type 2A 17HSD/KSR protein of 387 amino acids as well as to a related 291-amino acid type 2B 17HSD/KSR protein of unknown function. The gene comprises seven exons, the exons 1–3 and 6–7 encoding type 2A 17HSD/KSR, and the exons 4 and 5 being only found in type 2B mRNA. Both proteins are identical from the first methionine to glycine 222. In contrast to the type 2A 17HSD/KSR protein, type 2B is devoid of significant enzymatic activity toward androgens and estrogens (Labrie *et al*. 1995).

A cDNA encoding a polypeptide of 387 amino acids with a predicted molecular mass of 42,782 dalton (Da), 17HSD/KSR type 2, was isolated and characterized from a human prostate cDNA library using an expression cloning technique. The presence of an amino-terminal type 2 signal-anchor motif and a carboxy-terminal endoplasmic retention motif in the structure of 17HSD/KSR type 2 gave rise to the suggestion that the enzyme is associated with the membranes of the endoplasmic reticulum (Wu *et al.* 1993). This assumption was supported by experiments in which fractionation of cell extracts resulted in recovery of recombinant enzyme in the membrane fraction (Andersson *et al.* 1995). More recently, full-length human 17HSD/KSR type 2 was localized in the endoplasmic reticulum using a double immunofluorescence labeling technique (Puranen *et al.* 1999).

The expression of 17HSD/KSR type 2 mRNA has been detected in human placenta, endometrium, liver, small intestine, kidney, pancreas, colon, and prostate as well as in several breast, endometrial, and prostate cancer cell lines (Casey *et al.* 1994, Elo *et al.* 1996, Miettinen *et al.* 1996). In benign prostatic hyperplasia (BPH) and prostate cancer tissues, 17HSD/KSR type 2 appears to be the principal 17HSD/KSR isoenzyme expressed (Elo *et al.* 1996). Kinetic data from experiments carried out *in vitro* have revealed that the enzyme preferentially catalyzes the oxidative reactions of both active androgens and active estrogens, *i.e.*, catabolism of androgens and estrogens by converting DHT, T, and androstenediol (A-diol) to 5αA-dione, A-dione, and DHEA, respectively, as well as by converting E2 to E1. In addition, 17HSD/KSR type 2 possesses 20α-HSD activity toward 20α-dihydroprogesterone, as it converts this into progesterone (P) (Wu *et al.* 1993, Puranen *et al.* 1999). The 17HSD/KSR type 2 enzyme possibly plays a significant role in the inactivation of sex steroids from the blood circulation as well as in diminishing sex hormone action in the target tissues, consequently having a protective role as regards excessive androgen action in peripheral steroid target tissues. Additionally, 17HSD/KSR type 2 is suggested to be important in maintaining high P concentrations during pregnancy (Wu *et al.* 1993).

2.6.1.2 17HSD/KSR type 5

Human 17HSD/KSR5 is unique among the 17HSD/KSRs as it belongs to the aldo-keto reductase (AKR) family (Bruce *et al.* 1994), while the others are members of the SDRs

(Krozowski 1994, Jörnvall *et al.* 1995). Human type 5 17HSD/KSR, also known as AKR1C3 (Penning *et al.* 2000), dihydrodiol dehydrogenase X (DDX) (Burczynski *et al.* 1998), and prostaglandin D_2 11-ketoreductase (prostaglandin F_2 synthase) (Matsuura *et al.* 1998), has been cloned and characterized as 3α-HSD type 2 (Khanna *et al*. 1995b, Lin *et al.* 1997), and it is highly homologous with type 1 (84%) and type 3 (86%) 3α-HSD as well as with 20α-HSD (88%) (Dufort *et al.* 1999). The sequence reported for type 2 3α-HSD (Khanna *et al.* 1995b) shows a two-amino acid difference compared to the one for 17HSD/KSR5 (Dufort *et al.* 1999), but these substitutions were determined not to significantly affect the 17HSD activity of the enzyme (Dufort *et al.* 1999). Human type 5 17HSD/KSR protein is encoded by the *HSD17B5* gene located at chromosome region 10p14-15 (Khanna *et al.* 1995a, Rheault *et al.* 1999). The enzyme 17HSD/KSR type 5 has broad tissue distribution and, at least *in vitro*, recognizes diverse substrates.

Ribonuclease protection assays have demonstrated abundant expression of human 17HSD/KSR5 in the liver as well as in prostate cancer (DU-145, LNCaP) and osteosarcoma (MG-63) cells, in addition to which 17HSD/KSR5 is modestly expressed in normal prostate and in the adrenal (Dufort *et al*. 1999). Real-time PCR quantification has illustrated 17HSD/KSR5 expression in female subcutaneous and omental adipose tissue as well as in preadipocyte primary cultures (Blouin *et al.* 2003). Furthermore, type 5 17HSD/KSR has been detected in human kidney using RT-PCR (Quinkler *et al.* 2003). An immunocytochemical analysis has shown that human 17HSD/KSR5 is also expressed in corpus luteum, in the epithelial cells of endometrium and mammary gland, in Leydig cells, and occasionally in Sertoli and germ cells (Pelletier *et al.* 1999). In human prostate, type 5 17HSD/KSR is highly expressed in basal epithelial cells and stromal fibroblasts, and also in the endothelial cells and fibroblasts of blood vessels (El-Alfy *et al*. 1999).

Although human 17HSD/KSR5 has originally been characterized as 3α-HSD2 (Khanna *et al*. 1995b, Lin *et al.* 1997), it has been reported that, in intact cells, type 5 17HSD/KSR catalyzes 17HSD reactions several times more efficiently than 3α-HSD reactions (Dufort *et al*. 1999). The type 5 17HSD/KSR is able to function as bi-directional 3α-, 17β-, and 20α-HSD (Penning *et al.* 2000), but studies in a cellular context have suggested that this enzyme could prefer a reductive reaction (Dufort *et al.* 1999). *In vitro* characterization of k_{cat}/K_m for the reactions has indicated that, as a reductase, it prefers DHT and 5αA-dione as substrates to A-dione, but this has not been established within a cellular context (Penning *et al.* 2001). On the contrary, the enzyme has been reported to be involved in the transformation of DHEA into DHT in prostatic basal cells by concerted action with 3β-HSD type 1 and 5α-reductase type 2 (Dufort *et al.* 1999, El-Alfy *et al.* 1999, Qiu *et al.* 2004) (Fig. 3.). The testosterone formation by this enzyme has been suggested to be an important factor, particularly in patients who have undergone surgical or medical castration (Qiu *et al.* 2004). Lately, in addition, 17HSD/KSR type 5 has been detected to possess 11-ketoreductase activity, being able to convert prostaglandin (PG) D_2 to PGF_2 (Desmond *et al.* 2003).

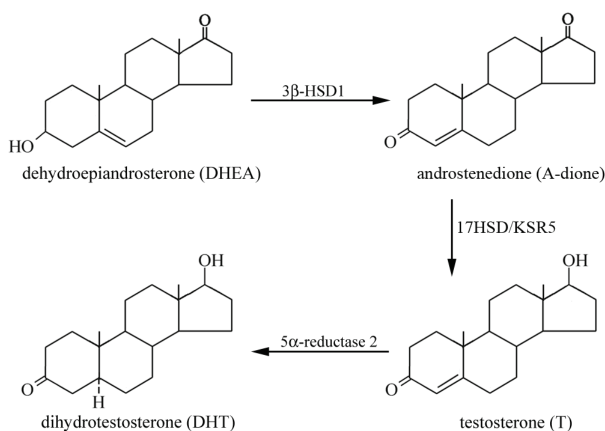

Fig. 3. The role of 17HSD/KSR type 5 in the androgen formation locally in the peripheral tissues. Androstenedione is synthesized from DHEA by 3β-HSD1 in prostate basal cells, 17HSD/KSR5 then carries out the local conversion of A-dione into T, and DHT is synthesized from T by 5α-reductase type 2 in either the basal or the luminal cells of the prostate (Qiu *et al.* 2004).

2.6.1.3 17HSD/KSR type 7

17β-Hydroxysteroid dehydrogenase/17-ketosteroid reductase type 7 (17HSD/KSR7) is a 37 kDa membrane-associated enzyme encoded by the *HSD17B7* gene (Krazeisen *et al.* 1999). *In silico*, the gene corresponding to the cDNA has been localized on chromosome 1q23 (Törn *et al.* 2003). Purified human 17HSD/KSR type 7 has been determined to predominantly catalyze the reductive reactions converting DHT to 3βA-diol and E1 to E2. A weaker reductive activity converting P to 4-pregnen-3β-ol-20-one has also been detected (Törn *et al.* 2003). Further, enzymatic measurements have demonstrated 3-ketosteroid reductase activity for 17HSD/KSR7, thus suggesting that type 7 17HSD/KSR plays another role in cholesterol biosynthesis (Marijanovic *et al.* 2003). Using RT-PCR and *in silico* Northern blotting, expression of 17HSD/KSR7 has been detected in several human tissues, including ovary, breast, placenta, testis, prostate, and liver (Krazeisen *et al.* 1999, Breitling *et al.* 2001, Törn *et al.* 2003).

3 Outlines of the present study

Regulation of the intraprostatic concentrations of active androgens is suggested to maintain organ homeostasis by modulating the balance between the proliferation and apoptotic death of prostatic epithelial cells. In addition to androgens, estrogens have been suggested to be involved in the abnormal growth of the prostate, even though the precise role of hormones still remains undefined. Changes in the activities of 17HSD/KSR enzymes that critically impact on steroid hormone activation and inactivation may lead to considerable changes in the bioavailability of sex steroid hormones locally in prostate cells during the progression of prostate cancer. This, consequently, may have a significant role in the biological behavior of prostate cancer and, further, be of importance for the therapeutic management of the disease.

The present work was focused on the following topics:

1. Definition of the most common regions of genomic loss at chromosome arm 16q in human prostate cancer and, especially, assessment of the possible involvement of the gene for 17HSD/KSR type 2, *HSD17B2*, as a target for chromosome arm 16q losses. Examination of the clinicopathological correlation as regards the allelic losses detected at 16q.
2. Identification of the variations in chromosome arm 16q deletion patterns taking place sequentially in the course of the disease, in order to further assess the significance of the specific genetic alterations in the progression of prostate cancer. Detection of the possible chromosomal alterations exclusively typical for metastases.
3. Detection of the variation in the enzyme activities critically contributing to the intraprostatic steroid metabolism related to the transition of prostate cancer cells into an androgen-independent state. Assessment of the involvement of different types of 17HSD/KSRs into the changes in the enzyme activities.
4. Characterization of the alterations in the gene copy number of *HSD17B2* as well as in the expression of 17HSD/KSR types 5 and 7 in prostate cancer patient specimens representing a broad range of clinical behaviors.

4 Materials and Methods

Detailed descriptions of the materials and methods are presented in the original articles, I–IV.

4.1 Tissue specimens (I, II, IV)

Tissue specimens for fragment analysis and quantitative real-time PCR were collected from prostate cancer patients undergoing radical prostatectomy or transurethral resection of the prostate (Department of Pathology, University of Oulu and Department of Pathology, Helsinki University Central Hospital), and the Tumor, Node, Metastasis (TNM) classification system (Chisholm 1988) was used to categorize the tumor specimens. The T-stage was determined by digital rectal examination, transrectal ultrasonography, bimanual palpation and cystoscopy, while the M-stage was determined by bone scanning, thorax x-ray and ultrasonographic examination of the abdomen. Staging pelvic lymphadenectomy was carried out only in connection with a radical operation. The tumor specimens were analyzed histopathologically to confirm that at least 60% of the cells were malignant.

The prostate specimens for *in situ* hybridization were retrieved from the archives of the Department of Pathology, University of Oulu. The specimens were catecorized according to the World Health Organization classification for tumors (Eble *et al.* 2004). The permission to use the material was obtained from The National Authority for Medicolegal Affairs (Helsinki, Finland).

4.2 DNA extraction (I, II, IV)

Approximately 5 µm sections of tumor and benign prostate specimens were dissected from fixed, paraffin-embedded tissues, and DNA was extracted using a standard protocol for sample preparation from paraffin-embedded tissues (Wright & Manos 1990).

4.3 Detection of LOH (I, II)

4.3.1 PCR

Polymerase chain reaction amplification was performed with a Perkin Elmer GeneAmp PCR System 9600 in a reaction volume of 50 µl (I) / 25 µl (II) containing 250 ng (I) / 125 ng (II) of genomic DNA, 50 pM (I) / 20 pM (II) each of two primers (one of which was labeled with fluorescein amitide, Pharmacia LKB Biotechnology AB), 0.16 mmol/l (I) / 0.08 mmol/l (II) of each deoxynucleotide triphosphate (dNTP, Advanced Biotechnologies Ltd), 1 mM (I) / 0.5 mM (II) $MgCl_2$ (Advanced Biotechnologies Ltd (I) / Applied Biosystems (II)), 0.5 units of Red Hot DNA polymerase (Advanced Biotechnologies Ltd) (I) / 0.9 units of AmpliTaq Gold DNA polymerase (Applied Biosystems) (II), and 1× Reaction Buffer IV (20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl, pH 9.0, 0.01 % (w/v) Tween and 1.5 mM $MgCl_2$, Advanced Biotechnologies Ltd) (I) / 1× GeneAmp Buffer II (100 mM Tris-HCl, pH 8.3; 500 mM KCl, Applied Biosystems) (II). After an initial denaturation step, 5 min (I) / 10 min (II) at 95 °C, the samples were subjected to 35 cycles of amplification consisting of 1 min of denaturation at 95 °C, 1 min of annealing at 55–65 °C (I) / 55–63 °C (II) (optimized for each primer pair) and 2 min of extension at 72 °C. After the cycles, the final extension step at 72 °C was extended to 7 min.

4.3.2 Fragment analysis

The prostate cancer specimens as well as their autologous control specimens were screened with nine (I) or seventeen (II) polymorphic microsatellite markers spanning chromosome arm 16q13-qter. With the exception of the pentanucleotide repeat D16S476, all the microsatellite markers used in the study were dinucleotide repeats. The probable order of microsatellite loci was assessed by reference to the Genethon Human Linkage Map (Dib *et al.* 1996) and the maps of human chromosome 16 published in the Genomic Data Base (http://gdbwww.gdb.org/).

The sizes of the alleles and loss of heterozygosity were determined by separating the fluorescein-labeled PCR products on a 6% denaturing acrylamide gel (Amresco) in the presence of fluorescein amitide-labeled allelic size markers (Pharmacia LKB Biotechnology), using an A.L.F. sequencer (Pharmacia LKB Biotechnology). Alternatively, the labeled PCR products were separated on a 5% Long Ranger gel (FMC BioProducts) in the presence of PRISM^{TM} Genescan-500 Tamra allelic size markers (Applied Biosystems), using an ABI 377 sequencer running GeneScan software (Applied Biosystems). The data obtained from the A.L.F. sequencer were analyzed by using the Fragment Manager 2.0 software (Pharmacia LKB Biotechnology AB) and the data obtained from the ABI 377 sequencer by using the Genotyper software (Applied Biosystems). Tumor specimens were considered to show allelic loss when signal

reduction compared with the corresponding control signal was 40% or greater, a method described by Canzian *et al*. (1996).

4.4 Cell culture (III)

The human prostate carcinoma cell line LNCaP (CRL-1740) was obtained from American Type Culture Collection (ATCC; Manassas, VA). First, cells were cultured in cell culture flasks in RPMI-1640 medium (ATCC) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin. When the transformation of the cells was started and the cells were able to grow anchorage-independently, they were transferred to grow in large-scale suspension cultures. LNCaP cells, from 6–8 cell culture flasks with a culture area of 175 cm^2, were trypsinated with trypsin/EDTA solution (Clonetics, BioWhittaker, Inc., Walkersville, MD) and transferred into a spinner flask to a culture volume of 300 ml. In this culture, cell density was 6–8 × 10^5/ml. For spinner flasks, 0.1% Synperonic F68 (Serva Electrophoresis, Heidelberg, Germany) was added into the culture medium to reduce the shear force effects. Production of PSA (µg/ liter) was determined using the DELFIA PSA kit (Wallac, Inc., Turku, Finland).

4.5 Cell growth (III)

Both PSA-producing and non-PSA-producing LNCaP cells were seeded in RPMI-1640 medium (ATCC) containing 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin, in cell culture flasks with a culture area of 25 cm^2, at a density of 2 x 10^4 cells/cm^2. The cells were cultured for 8 days, during which the medium was replaced at the days 4 and 6. The cells were trypsinated daily. Trypan blue (Sigma, St. Louis, MO) was added to detect cell viability, and the cell number was determined using Bürker´s cell-counting chamber. The doubling time of the cells was determined from the logarithmic phase of the growth curve. The assays were performed in triplicate.

4.6 Colony-forming assay (III)

A single cell suspension (10^2, 5 x 10^2, 10^3, 5 x 10^3 cells/ml) in growth medium was plated on 35-mm tissue-culture dishes. After incubation for 1 week at 37 ^oC in a 5% CO_2 atmosphere, the colonies were fixed with 80% ethanol for 30 minutes and stained with 1% crystal violet in a 10% ethanol solution for 5 minutes. Colony-forming efficiency (CFE) was measured as follows: (number of colonies formed / number of cells plated) × 100%.

4.7 Enzyme activity measurements (III)

For enzyme activity measurements, the LNCaP cells were transferred into five spinner flasks. At this stage, the culture medium of the cells was replaced by charcoal-dextran-treated 5% FCS. The cell density used was 2×10^6/ml and the volume of the medium in each spinner was 50 ml. The metabolism of the steroids was followed by adding ^{3}H-labeled substrates ([2,4,6,7-^{3}H]oestrone at 94–95 Ci/mmol; [2,4,6,7-^{3}H]-estradiol at 88 Ci/mmol; [1,2,6,7-^{3}H]testosterone at 95 Ci/mmol; [1,2,6,7-^{3}H]androst-4-ene-3,17-dione at 99 Ci/mmol; or 5α-dihydro[1,2,4,5,6,7-^{3}H]-testosterone at 125 Ci/mmol); 200,000 cpm/ml; Amersham Pharmacia Biotech, Little Chalfont, UK) and corresponding unlabeled steroids E1, E2, T, A-dione, or DHT (Steraloids, Inc., Newport, RI) to a final concentration of 1 nM. The cell suspensions were incubated for the indicated times (1, 2, 4, 6, 10 and 20 h) at 37 °C in a 5% CO_2 atmosphere, after which the reactions were stopped by immediately freezing the medium samples in an ethanol-dry ice bath. The steroids were extracted into an organic phase (diethyl ether:ethyl acetate, 9:1) from 1-ml aliquots. Enzyme activities were analyzed by determining the conversion of substrates to specific products using an HPLC system (Waters Corp., Milford, MA). The substrates and products were separated in a Symmetry C18 reverse-phase chromatography column (3.9 × 150 mm) using an acetonitrile/water (48:52, vol/vol) solution as a mobile phase, and radioactivity was measured by an on-line β-counter (150TR, FLO-ONE Radiomatic, Packard, Meriden, CT) connected to the HPLC system. Ecoscint A (National Diagnostics, Atlanta, GA) was used as a scintillation solution.

To identify substrate specificity, human 17HSD/KSR type 7 was produced as a recombinant protein in *Spodoptera fugiperda* cells according to the method described in earlier studies (Vihko *et al.* 1993). The activity of purified 17HSD/KSR7 was, with minor modifications, measured *in vitro* as previously described (Puranen *et al.* 1997). Briefly, ^{3}H-labeled substrates (5α-dihydro[1,2-^{3}H]testosterone (125 Ci/mmol), [2,4,6,7-^{3}H]oestrone (94–95 Ci/mmol), [1,2,6,7-^{3}H]progesterone (86 Ci/mmol), or [1,2,6,7-^{3}H]androst-4-ene-3,17-dione (99 Ci/mmol)); 300,000 cpm/ml; Amersham Pharmacia Biotech, Little Chalfont, UK) were mixed with the corresponding unlabeled substrates (Steraloids, Inc.), the final concentration of which were 0.5 μM, and the cofactor NADPH (Boehringer Ingelheim GmbH, Mannheim, Germany) was added to a final concentration of 1 mM. The reactions were initiated by adding the samples of purified recombinant protein, which were diluted in 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, and 0.01% BSA, and the mixtures were incubated at 37 ºC for 10 min. The stopping of the reactions and all the stages after that were performed following the procedure described above.

4.8 Isolation of RNA and RT-PCR analysis (III)

Total RNA from LNCaP cells was extracted using the TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD), and poly(A)-enriched RNA was extracted using oligo(dT)-cellulose (Amersham Pharmacia Biotech, Uppsala, Sweden) according to

standard protocols. For relative quantitative RT-PCR, the GeneAmp RNA PCR Kit and GeneAmp 9600 thermal cycler (Perkin-Elmer Corp., Foster City, CA) were used, starting with 200 ng of poly(A)-RNA and random hexamers. Reverse transcription was as follows: 22 ^{o}C, 10 min; 42 ^{o}C, 15 min; 99 ^{o}C, 5 min; and 5 ^{o}C, 5 min. The gene-specific primers (4 μM) used in PCR were 5'-AGTTGCTTCCATCCAACCTGGA-3' and 5'-TTCCATTGCCTAGGTGGCCTTT-3' for *HSD17B2*; 5'-GGTGTCAAACTTCAAC-CGCA-3' and 5'-GGATAATTAGGGTGGCTAGCA-3' for *HSD17B5*; and 5'-CCACCAAAAGCCTGAATCTCTCA-3' and 5'-GAGTGTCATTGTCAGCTCTG-GTT-3' for *HSD17B7*. PCR consisted of denaturation (95 ^{o}C, 15 sec), annealing (62 ^{o}C, 64 ^{o}C, and 62 ^{o}C for *HSD17B2, -B5* and *-B7,* respectively; 30 sec), and elongation (72 ^{o}C, 30 sec). The linear range for each gene / sample was determined by removing aliquots at various PCR cycles (27, 23, and 25 cycles in the linear range for *HSD17B2, -B5* and *-B7,* respectively*)*, and the optimal ratio of the 18S primers:competimers (Classic II 18S Internal Standards, Ambion, Inc., Austin, TX) was adjusted for each gene-specific primer pair (1:7, 1,5:9, and 2:7 for *HSD17B2, -B5* and *-B7,* respectively). Finally, the volume of 40 μl from each of the PCR reactions was run in 1.5% agarose gel containing 0.1% ethidium bromide (w/v). After the gel had been photographed, the optical densities for the PCR products were determined using a laser scanner (Molecular Dynamics, Inc., Sunnyvale, CA). The ratios of optical densities for gene-specific and 18S PCR products were counted for each specimen.

4.9 cDNA microarray (III)

For the cDNA microarray, total RNA from LNCaP cells was extracted using TRIzol (Life Technologies, Inc.), and poly(A)-enriched RNA was extracted using a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). mRNA from the cells was used for Human UniGEM V v1.0 Custom Screening (GenomeSystems, St. Louis, MO). mRNAs were labeled with different fluorescent labels, and average signals for the elements in the array were achieved for both probes. For additional information, see http: / / www.incyte.com.

4.10 Quantitative real-time PCR (IV)

The alterations of the gene copy numbers for *HSD17B2* in prostate cancer were determined by quantitative real-time PCR using the Perkin Elmer/Applied Biosystems Division 7700 Sequence Detector with TaqMan chemistry according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Shortly, 10 ng of genomic DNA was used for the PCR reactions consisting of 5 min at 95 °C and 40 cycles of 1 min at 95 °C and 45 s at 60 °C. The *HSD17B2* gene-specific sense and antisense primers used in the reactions were TTGCGAAGAACCTGCTCTCC and TCTGCACTGGCTTCGTGATG, respectively. The fluorogenic dual-labeled probe 5'-FAM-CGCCTCTCGGTGCTCCAAATGG-TAMRA-3' was used for the detection of

HSD17B2 amplicon. In each sample, the acquired signal was normalized to the corresponding signal of the reference single-copy gene *BNP,* brain natriuretic peptide. The gene-specific sense and antisense primers for *BNP* were CAGGAGCAG-CGCAACCAT and CAGGGATGTCTGCTCCACCT, respectively, and a fluorogenic probe 5'-FAM-TGCAGGGCAAACTGTCGGAGCTG-TAMRA-3'. Normalized gene doses for each of the tumor specimens were determined by the following ratio, and specimens with the ratio < 0.75 were considered to have a decreased gene copy number of *HSD17B2* in cancer tissue:

(Gene copy number of target gene (*HSD17B2*) : Gene copy number of reference gene (BNP))$_{Ca}$ /
(Gene copy number of target gene (*HSD17B2*) : Gene copy number of reference gene (BNP))$_{control}$.

In this analysis, 48 prostate cancer specimens representing various clinical features were studied with their corresponding controls from benign prostate tissue. All the specimens were analyzed as triplicates, and the corresponding means were used for the calculations.

4.11 *In situ* hybridization (IV)

A 594-bp fragment (nucleotides 407–1000) of human 17HSD/KSR type 5 cDNA (Dufort *et al.* 1999) and a 832-bp fragment (nucleotides 39–870) of human 17HSD/KSR type 7 cDNA (Krazeisen *et al*. 1999) were constructed in the pCRII-TOPO vector (Invirtogen, Carlsbad, CA). The fragments were used as templates for *in vitro* transcription. Sense and antisense [α-^{35}S]CTP-labeled (PerkinElmer, Boston, MA) RNA probes were transcribed from linearized plasmids with T7 or SP6 RNA polymerases. The *in situ* hybridization reactions were performed otherwise as previously described (Mustonen *et al.* 1997), but a different staining method was used. In the present study, the specimens were stained with hematoxylin-eosin (HE), and Pertex (Histolab, Göteborg, Sweden) was used for the mounting of slides. The exposure times were 15 days for 17HSD/KSR5 and 30 days for 17HSD/KSR7.

4.12 Statistical analyses (I, II, IV)

Two-tailed Fisher´s exact tests and backward stepwise logistic regression analysis (SPSS for Windows, SPSS inc.) were used to analyze the associations between LOH and the clinicopathological parameters of prostate cancer (I). Evaluation of the development of LOH during disease progression with respect to individual-locus allelic losses was performed using Sign test (SPSS for Windows, SPSS Inc.) (II). Further, logistic regression analysis (SPSS for Windows; SPSS Inc.) was used to analyze the associations between the altered *HSD17B2* gene copy number and the clinicopathological parameters of prostate cancer (IV). Statistical analyses concerning the associations between the expression of 17HSD/KSR5 / 17HSD/KSR7 and the clinico-pathological parameters of prostate cancer were carried out using Pearson Chi-Square test and Fisher`s exact test (SPSS for Windows; SPSS Inc.) (IV).

5 Results

5.1 LOH at chromosome region 16q in prostate cancer (I, II)

In the loss of heterozygosity analysis performed to search for sites of consistent chromosomal deletion, LOH on at least one locus was found in 32 of the 50 tumor DNAs (64%). The most common area exhibiting LOH was located to the chromosomal region 16q23-q24.2 between the loci D16S504 and D16S422. The highest percentage of tumors exhibiting LOH was found within this area at the loci HSD17B2 (63%) and D16S422 (60%), located at 16q24.1-q24.2. Further, our data indicated the frequency of LOH at 16q24.1-q24.2 to be significantly associated with metastatic ($p < 0.05$) and clinically aggressive behavior ($p < 0.01$) of prostate cancer. Higher tumor grade (grade 3) was also significantly associated ($p < 0.01$) with LOH at 16q24.1-q24.2 (I).

To discriminate potential biological markers for tumor progression, the chromosomal deletions at 16q were also analyzed in locally recurrent and metastatic tumors compared to their matched primary counterparts (II). In this analysis, the most commonly deleted individual marker regarding the whole specimen material was HSD17B2, exhibiting LOH in 47% (28/60) of the specimens. The highest percentage of primary tumors showing LOH was also found at locus HSD17B2 (38%), while among locally recurrent tumors, the occurrence of LOH was most frequently observed at locus D16S520 (61%). When comparing the existence of LOH individually in each of the primary – recurrent tumor pairs, a statistically significant association ($p < 0.01$) as regards the development of LOH during local disease recurrence was found with one of the 17 markers spanning 16q13-q24.3. The marker associated with disease progression was D16S520, locating at chromosomal region 16q24.3. With the marker D16S520, 38% of informative cases showed the appearance of LOH during local recurrence of the disease.

There was no significant difference in the overall rate of allelic loss between the prostate cancer tumors of primary tissue and the tumors of metastatic origin. When the LOH frequencies observed on 16q were 68% for primary prostate tumors and 90% for locally recurrent tumors, the respective proportion for tumors of metastatic origin was 75%. Regarding individual markers, the highest frequency of metastatic tumors showing LOH was found at locus HSD17B2 within the region 16q24.1-q24.2 (50%) and at locus

D16S503 (50%) within 16q21.1. In most of these cases (80% for HSD17B2 and 100% for D16S503), LOH was also observed in the primary and/or the locally recurrent counterpart. Overall, distant metastases contained chiefly the same chromosomal alterations as their counterparts of prostatic origin. In 67% of the cases in which both the metastatic and the corresponding locally recurrent prostate tumors were available, the LOH pattern observed in the metastasis was identical to that seen in the locally recurrent counterpart. On the basis of the data, there do not seem to be allelic loss events exclusively typical of metastatic tumors at 16q.

5.2 Gene copy number of *HSD17B2* (IV)

Quantitative real-time PCR analysis was used to analyze the gene copy number of *HSD17B2*. In this analysis, 14 of the 48 prostate cancer patients (29%) showed a decreased *HSD17B2* gene copy number in cancer specimens compared to their normal counterparts.

The data indicate that the frequency of decreased *HSD17B2* gene copy number is significantly ($p < 0.05$) associated with higher Gleason scores. While a decreased gene copy number was found in 41% of Gleason 7–10 tumors, only 7% of Gleason 2–6 tumors had decreased *HSD17B2* gene dosage. Correspondingly, a decrease of the *HSD17B2* gene copy number was seen in 39% of aggressive tumors, while the respective proportion among non-aggressive local tumors was only 20%. The tendency towards an increasing incidence of lowered *HSD17B2* gene copy numbers in more advanced prostate cancer was also seen in the case of metastasis stage. The gene copy number for *HSD17B2* was decreased in 40% of metastasized tumors, while only 24% of non-metastasized tumors had altered gene dosage. Regarding tumor stage, a lowered gene copy number was detected in 28% of stage 3–4 tumors and in 18% of stage 1–2 tumors.

5.3 17HSD/KSR activity in prostate cancer LNCaP cells during transition into an androgen-independent state (III)

To identify mechanisms related to the progression of prostate cancer into an androgen-independent state, the variations in estrogen and androgen metabolism during the malignant transformation of prostate cancer LNCaP cells were investigated. In the LNCaP cell model used for these studies, PSA-producing cells represent well-differentiated prostate cancer, while non-PSA-producing LNCaP cell variants serve as a model of progressive prostate cancer. The enzyme activity measurements performed using HPLC indicate that androgen-dependent LNCaP cells possess predominant oxidative 17HSD/KSR activity converting active 17β-hydroxysteroids E2, T, and DHT into their less active 17-keto derivatives E1, A-dione, and 5αA-dione, respectively. Oxidative activity decreases the potency of estrogens and androgens, thus possibly protecting tissues from excessive steroid hormone action (Mustonen *et al.* 1998). At a

PSA-producing stage, after 1 week of growth in a suspension culture, E2 was totally converted to E1 in 2 h, whereas, in transformed cells, after 5 weeks of growth in a suspension culture, it took 10 h to convert 9% of E2 to E1. At the same time points during the cellular transformation, the oxidative activity converting T to A-dione decreased from 96% to 9% within a 10-h reaction time. With the DHT substrate, the formation of 5αA-dione in 10 h decreased from 72% to 6% during the transformation process.

At the transformed stage, LNCaP cells possess remarkable reductive 17HSD/KSR activity, leading to the formation of active estrogen E2 and, to a lesser extent, active androgen T, from their less active 17-keto metabolites E1 and A-dione, respectively. During the transformation process, the biosynthesis of E2 was activated so that, within the 10 h, a maximum of 62% of E1 was converted to E2; while, at the PSA-producing stage of the same cell lot, there was no detectable production of E2 (0%). Regarding the androgenic substrate A-dione, the formation of T at the transformed stage reached a maximum of 26% in 10 h and 37% in 20 h versus only 3% at the PSA-producing stage. With the DHT substrate, the formation of 3αA-diol in 10 h increased from 7% to 90% during the transformation process. The formation of 3βA-diol reached its peak value in a 2-h reaction; and during the period in question, the production of this metabolite, which has been reported to act as an estrogen in the prostate (Voigt & Bartsch 1986, Weihua *et al.* 2001), increased from 0 to 11%.

5.4 Expression of 17HSD/KSR types 2, 5, and 7 in prostate cancer LNCaP cells during transition into an androgen-independent state (III)

The present data show a remarkable decrease in oxidative 17HSD/KSR type 2 activity during the malignant cellular transformation of prostate cancer LNCaP cells. At the time point of maximum decrease in the relative expression of *HSD17B2*, the inactivating reactions converting E2 to E1, T to A-dione, and DHT to 5αA-dione decreased from 100% to 14%, from 96% to 5%, and from 72% to 5%, respectively. During this period, the relative expression of *HSD17B2* decreased from 100% to 18%.

The results of relative quantitative RT-PCR revealed that, maximally, *HSD17B5* reached the level of 3.9-fold expression, compared with the level at the PSA-producing stage of the same LNCaP cell lot. Regarding simultaneous variation in the potential reactions for the 17HSD/KSR type 5 enzyme, the conversion of DHT to 3αA-diol in 10-h reactions increased from 7% to 90%. In addition, the conversion of A-dione to T reached a value of 26%, compared with 3% at the PSA-producing state.

At the time point of maximal *HSD17B5* expression, the expression of *HSD17B7* also reached its peak value, showing a 1.7-fold level compared to that at the starting point. At the same time, the conversion of E1 to E2, in 10 h, increased from 0 to 62%; and the conversion of DHT to 3βA-diol, in 2 h, from 0 to 11%. Purified human 17HSD/KSR type 7 was determined to predominantly catalyze the reductive reactions converting DHT to 3βA-diol and E1 to E2. A weaker reductive activity converting P to 4-pregnen-3β-ol-

20-one was also detected, but no conversion was observed when A-dione was used as a substrate.

5.5 Expression of 17HSD/KSR types 5 and 7 in prostate tissue specimens (IV)

In expression studies performed using *in situ* hybridization, moderately low but constitutive expression of mRNA for 17HSD/KSR type 7 was detected in benign prostate hyperplasia and malignant prostate tissues. Of the 28 prostate cancer specimens studied, 89% showed expression of 17HSD/KSR7. In most of the cases, the level of expression was low or moderate (43% for each), while only 4% of the specimens exhibited strong expression of 17HSD/KSR7. The staining for 17HSD/KSR7 mRNA was localized to glandular epithelial cells of the organ. The expression level had no correlation with the differentiation state of the cancer, nor was it significantly different in cancer specimens compared to hyperplasia specimens.

The expression of mRNA for 17HSD/KSR type 5 varied considerably between different specimens. Most of the 76 prostate cancer patient specimens studied, 86%, exhibited at least low or moderate 17HSD/KSR5 expression. Regarding the clinicopathological features of prostate cancer, the most intense 17HSD/KSR5 expressions had a statistically significant association with aggressive ($p < 0.0005$) and metastasized ($p < 0.005$) prostate cancer as well as with higher Gleason scores ($p < 0.005$). Intense expression for 17HSD/KSR5 mRNA was detected in 41% of aggressive disease cases, while only 4% of non-aggressive diseases showed high 17HSD/KSR5 expression. Of the metastasized tumors, 55% exhibited intense 17HSD/KSR5 expression, while high expression of 17HSD/KSR5 was only observed in 14% of non-metastatic tumors. Interestingly, furthermore, the intense expression of 17HSD/KSR5 was significantly associated with the androgen deprivation therapy performed either surgically or medically ($p < 0.05$). Among the patients treated with androgen deprivation, high expression of 17HSD/KSR5 was detected in 43% of the cases, while only 5% of the non-treated patients exhibited a respective level of expression.

6 Discussion

6.1 LOH at chromosome 16q in prostate cancer and involvement of *HSD17B2* as a potential target gene in losses (I, II, IV)

Allelic loss is often combined with a mutation altering the expression of the remaining allele or the function of the target gene (Vogelstein & Kinzler 1992, Isaacs 1994). However, it has also been suggested that mutation or loss of a single allele may be sufficient to exert a cellular phenotype that leads to tumorigenesis without inactivation of the second allele. This gene dosage effect is called haploinsufficiency (Quon & Berns 2001, Fodde & Smiths 2002, Santarosa & Ashworth 2004).

The present data indicate the most common region of LOH at chromosome 16q to be located at region 16q24.1-q24.2. Regarding the clinicopathological parameters of prostate cancer, allelic loss at the most frequently deleted area was found to be significantly associated with aggressive ($p < 0.01$) and metastatic ($p < 0.05$) behavior of the disease. Furthermore, an increased occurrence of LOH was significantly associated with poor differentiation of the tumor ($p < 0.01$). The observed association between LOH at 16q24.1-q24.2 and clinically aggressive features of prostate cancer suggests that the deletion of this chromosome region is possibly associated with a mechanism by which the proliferative pressure of malignant prostatic epithelial cells is increased. (I)

Despite the identification of a few genes located at the frequently deleted region, none have been confirmed to be the main target gene for the losses observed. One of the interesting genes is *HSD17B2*, which is located at 16q24.1-q24.2 (Durocher *et al.* 1995) and thus included into the region of the most common LOH. The gene encodes the 17HSD/KSR type 2 enzyme, which inactivates the active androgens T and DHT into their less active metabolites in several peripheral tissues, including the prostate. In our recent studies, the possible involvement of *HSD17B2* as a target gene for losses was further tested by sequencing the exon regions of the gene. Since no mutations were observed in the remaining allele, the data provide no evidence of *HSD17B2* to be a target gene for a classical two-hit model of tumorigenesis (Elo *et al.* 1999). Quantitative real-time PCR, however, specifically revealed a lowered gene-copy number for *HSD17B2* related to prostate cancer (IV), thus giving evidence of the position of *HSD17B2* as one of the hit

genes for LOH at 16q24.1-q24.2 and, further, also giving rise to the speculation concerning the possible role of haploinsufficiency as an inactivating mechanism for 17HSD/KSR type 2.

The chromosomal deletions at 16q were, furthermore, analyzed sequentially in locally recurrent and metastatic tumors compared to their matched primary counterparts. In this analysis, the most common locus exhibiting LOH in the whole specimen material, and even among primary tumors, was HSD17B2 (II). The observation that allelic loss at the marker locus HSD17B2 is associated with aggressive disease behavior (I) but, on the other hand, seems to be a relatively early event in prostatic tumorigenesis (II) could possibly warrant speculation concerning the potential role of this locus as a prognostic marker for prostate cancer progression. When identifying the individual-locus allelic losses, a statistically significant increase was noted in the appearance of LOH during prostate cancer recurrence with the marker D16S520 ($p < 0.01$), locating at 16q24.3 (II). Thus, the data suggest that allelic loss events taking place at 16q24.3 may be among the potential factors underlying the progression of prostate cancer, pointing to the location of a gene or genes having a role in disease recurrence in the chromosomal region in question. In previous studies carried out using only one specimen per patient, the distal deletions at 16q have shown clinical associations in prostate cancer (Carter *et al.* 1990b, Visakorpi *et al.* 1995b, Suzuki *et al.* 1996, Latil *et al.* 1997). Regarding the putative target genes, H-cadherin (*CDH13*), which has shown associations with several types of human cancers, including breast, lung, and colorectal cancers as well as ovarian tumors and cutaneous squamos cell carcinoma (Lee 1996, Sato *et al.* 1998, Toyooka *et al.* 2001, Maruyama *et al.* 2002*,* Takeuchi *et al.* 2002, Toyooka *et al.* 2002), has been mapped to the region 16q24.2-q24.3 (Kremmidiotis *et al.* 1998). Further, M-cadherin (*CDH15*), another cell adhesion molecule, has been localized to the region that exhibits LOH in a number of sporadic breast cancer tumors at 16q24.3 (Kremmidiotis *et al.* 1998). The other plausible candidate genes located at 16q24.3 include the growth arrest-specific 11 (*GAS11*) gene (Whitmore *et al.* 1998) as well as the gene for CMAR, the cellular adhesion regulatory molecule (Pullman & Bodmer 1992), and that for PISSLRE, a putative cyclin-dependent kinase likely to be involved in the regulation of cell cycle (Grana *et al.* 1994, Brambilla & Draetta 1994).

Due to the limited availability of samples, very few studies regarding chromosomal aberrations in prostate cancer metastatic lesions have been published. In a few reports, however, distant metastases have, concordantly with the present data, been assumed to contain mostly the same chromosomal alterations as locally recurrent hormone-refractory prostate carcinomas (Visakorpi *et al.* 1995b, Cher *et al.* 1996, Nupponen *et al*. 1998). In the present study, the LOH pattern observed in the metastasis was identical with that detected in the locally recurrent hormone-refractory counterpart in 67% of the available cases. The highest frequency of distant metastases showing LOH (50%) was detected with the marker HSD17B2, and in 80% of these cases, LOH was initially observed in the primary or the locally recurrent counterpart. Thus, in the light of the results, there do not seem to be chromosomal deletions exclusively typical of metastases at 16q. Instead, the data reflect an accumulation of multiple genetic alterations during disease progression as well as a close clonal relationship between the primary and metastatic tumors, as evidenced by a time-dependent increase in the total number of losses and a high proportion of shared genetic alterations, respectively.

6.2 17HSD/KSR isoenzyme types 2, 5, and 7 in human prostate cancer (III, IV)

The progression of prostate cancer during endocrine therapy is a serious clinical problem, the molecular mechanisms of which largely remain to be clarified. To gain some insight into the mechanisms related to the transition of prostate cancer into an androgen-independent state, the variations in the enzyme activities critically contributing to intraprostatic steroid metabolism during the malignant transformation of prostate cancer LNCaP cells were identified. In this analysis, the decreased inactivation of E2 to E1 observed concomitantly with the cellular transformation is most probably due to the decrease in the oxidative 17HSD/KSR2 activity, which was also shown as a reduced relative expression of the *HSD17B2* gene (III). The observation of a remarkable decrease in oxidative 17HSD/KSR type 2 activity during the cellular transformation is in concordance with our data on an association between a chromosomal deletion at 16q24.1-q24.2, including the *HSD17B2* gene, and clinically aggressive features of prostate cancer (I, II). In earlier studies, reduced expression of 17HSD/KSR type 2 mRNA has also been detected in prostate cancer specimens (Elo *et al.* 1996). Furthermore, downregulated *HSD17B2* expression has been observed to be related to other forms of cancer, including the development of colon cancer (Oduwole *et al.* 2002). Since 17HSD/KSR type 2 is known to have a function in the inactivation of the active androgens T and DHT (Wu *et al.* 1993), decreased or even absent expression of 17HSD/ KSR2 could possibly be involved in prostate carcinogenesis through decreased inactivation and, consequently, increased local bioavailability of androgens. On the other hand, 17HSD/KSR type 2 also has a role in estrogen metabolism by inactivating active estrogen E2 to E1 (Wu *et al.* 1993, Miettinen *et al.* 1996). The variable balance between the concentrations of biologically active estrogens and androgens might also be of importance for prostate cancer progression.

The present data showed that, maximally, 17HSD/KSR type 5 reached a 3.9-fold gene expression level and simultaneous enhancement of the androgenic reactions catalyzed by the enzyme in non-PSA-producing LNCaP cell variants when compared to those in PSA-producing cells (III), which results suggest an increase in 17HSD/KSR5 activity related to the progression of prostate cancer. Type 5 17HSD/KSR is able to function as bi-directional 3α-, 17β-, and 20α-HSD (Penning *et al.* 2001), but studies in a cellular context have suggested that this enzyme could function as a reductase (Dufort *et al.* 1999). *In vitro* characterization of k_{cat}/K_m for the reactions has indicated that, as a reductase, it prefers DHT and 5αA-dione as substrates to A-dione, and on the basis of this, the presence of 17HSD/KSR5 in the prostate has been hypothesized to favor the formation of inactive androgens (Penning *et al.* 2001). However, this has not been established within the cellular context, but 17HSD/KSR5 enzyme has, contrariwise, been reported to be involved in the transformation of DHEA into DHT in prostate basal cells by concerted action with 3β-HSD type 1 and 5α-reductase type 2 (Dufort *et al.* 1999, El-Alfy *et al.* 1999, Qiu *et al* 2004). Testosterone formation by this enzyme has recently been hypothesized to be an important factor, particularly in patients who have undergone surgical or medical castration (Qiu *et al* 2004), but no direct evidence has been provided regarding the matter. The data of the present *in situ* hybridization analysis gives evidence

of the significant association between intense expression of 17HSD/KSR5 and clinically aggressive behavior of prostate cancer. Furthermore, a significant association was noticed between high 17HSD/KSR5 expression and androgen deprivation performed either surgically or medically (IV). The results give rise to speculation concerning the possibility that, during androgen ablation therapy, there may occur a process of compensating for the deprived testicular androgen levels peripherally in prostatic cells by way of intensifying intraprostatic T production. Thus, this result could partially explain the recent findings suggesting that the evolution of prostate cancer to androgen-independence would be, at least in part, due to reactivation of the androgen-response pathway and restoration of androgen responsive genes in the absence of androgens following androgen deprivation (Amler *et al.* 2000, Mousses *et al.* 2001). The issue needs further clarification, but if this really turns out to be a case, the blockage of 17HSD/KSR5 activity could interrupt the androgen formation chain in human peripheral tissues, and thus, inhibitors of the enzyme might even contribute to the treatment of prostate cancer.

At the time point of maximal 17HSD/KSR5 expression during the malignant transformation of prostate cancer LNCaP cells, the expression of 17HSD/KSR type 7 also exhibited a slight increase, showing a 1.7-fold level compared to that at the starting point (III). In further studies, *in situ* hybridization indicated a relatively low but constitutive expression of mRNA for 17HSD/KSR7 in prostate tissue specimens (IV). The expression level was not significantly different in cancer specimens compared to specimens of hyperplastic origin, nor was it associated with the clinicopathological parameters of prostate cancer. However, consistency in the expression of 17HSD/KSR7, being able to catalyze an activation of E1 to E2 as well as an inactivation of DHT into its estrogenic metabolite 3βA-diol, in prostate tissue gives rise to further speculations concerning the significance of estrogens, suggesting that there is at least enzymatic potentiality for the production of active estrogens locally in the prostate.

In peripheral tissues, the various levels of expression of different types of 17HSD/KSRs play a critical role in the control of the formation and inactivation of active androgens and estrogens. Regulation of the intraprostatic concentrations of active androgens is suggested to maintain organ homeostasis by modulating the balance between proliferation and apoptotic death of prostatic epithelial cells (Isaacs *et al.* 1992). In addition to androgens, estrogens have been suggested to be involved in the regulation of prostate growth (Weihua *et al*. 2001, Weihua *et al.* 2002, Härkönen & Mäkelä 2004 and refs. therein), even though the precise role of hormones still remains undefined. The observed remarkable changes in the expression and activities of 17HSD/KSR enzymes that critically impact on steroid hormone activation and inactivation may lead to considerably changing bioavailability of active sex steroid hormones locally in prostatic cells during the progression of prostate cancer. Through this action, the 17HSD/KSR enzymes may play a significant role in the biological behavior of prostate cancer and, further, even be of importance for the therapeutic management of the disease.

7 Conclusions

Hitherto, multiple chromosomal loci of importance for susceptibility to prostate cancer have been identified. Very few specific genes, however, have been conclusively implicated in prostate cancer initiation, and even less is known about the late progression and hormone resistance of the disease. Androgen withdrawal has, for decades, been used as the mainstay treatment of prostate cancer, but the failure of this therapy still represents a serious clinical problem, progression to the androgen-refractory stage being the main event responsible for prostate cancer -related mortality. Up till now, no definitive molecular pathway has been conclusively correlated with the initiation and progression of prostate cancer, and the mechanisms underlying the transition to androgen-independence remain largely unsolved.

The present findings demonstrate a remarkable decrease in the expression and activity of 17HSD/KSR type 2 related to the progression of prostate cancer. Since the enzyme 17HSD/KSR2 is believed to have a protective role as regards excessive steroid hormone action in peripheral steroid target tissues, decreased or even absent 17HSD/KSR2 expression could possibly be involved in prostate carcinogenesis through decreased inactivation and, thus, increased local bioavailability of active androgens and estrogens. Enhanced availability of active androgens may lead to increased proliferative pressure of malignant prostatic epithelial cells and, thus, to uncontrolled growth of the organ. The exact effects of estrogens on prostate epithelium still primarily remain to be clarified, but both epidemiological and experimental studies have suggested a role for estrogens in prostate cancer. The observed consistency in the expression of 17HSD/KSR type 7, being able to catalyze the formation of E2 and 3βA-diol, in prostate tissue gives rise to the assumption that there is at least enzymatic potentiality for the production of active estrogens locally in the prostate. The variable balance between the concentrations of biologically active estrogens and androgens driven by the altered 17HSD/KSR activity may also be of importance for prostate cancer progression.

The present findings, for the first time, give evidence of a significant association between high-level expression of 17HSD/KSR type 5 and clinically aggressive behavior of prostate cancer. This association was seen in a prostate cancer LNCaP cell line that served as a model of progressive prostate cancer, and in concordance with that, the finding was further confirmed in prostate cancer patient specimens representing various

clinical features. Furthermore, intense intraprostatic 17HSD/KSR5 expression was indicated to be significantly associated with androgen deprivation therapy achieved either surgically or medically. The enzyme 17HSD/KSR5 is considered to play an important role in T formation in peripheral tissues, and on that basis, enhanced 17HSD/KSR5 expression related to androgen deprivation gives rise to the suggestion that the role of the enzyme in T production may be of greater importance in circumstances where testicular sources of androgens are absent. Thus, the present data could partially explain the recent findings suggesting that the evolution of prostate cancer into an androgen-independent state would be, at least in part, due to reactivation of the androgen-response pathway and restoration of androgen responsive genes in the absence of androgens. On that basis, the findings could even propose a potential contribution of 17HSD/KSR5 to the failure of androgen ablation therapy, a major clinical problem. The issue needs further clarification, but on the basis of the hypothesis, the blockage of 17HSD/KSR5 enzyme could interrupt the peripheral androgen formation chain, and consequently, the inhibitors for the enzyme might even contribute to the treatment of prostate cancer. Without any speculation, the present data give evidence of the association of both a decrease in 17HSD/KSR2 activity and an increase in 17HSD/KSR5 activity with the mechanisms related to the progression of prostate cancer into a clinically aggressive stage.

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